
Redox-responsive nanogels for targeted nucleic acid delivery: a potential approach for metabolically reprogramming prostate cancer

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Statement of Originality

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Abbreviations

AAV	Adeno-associated virus
ACC	Acetyl-Coenzyme A carboxylase
ADT	Androgen deprivation therapy
AETC	[2-(Acryloyloxy)ethyl]trimethylammonium chloride
Akt/PKB	Protein kinase B
AM	Acrylamide
AMPK	AMP-activated protein kinase
AP1	Activator protein 1
APS	Ammonium persulphate
AR	Androgen receptor
ARE	Androgen response element
ASRI	AR signalling inhibitors
ATP	Adenosine triphosphate
BiTE	Bispecific T-cell engagers
BPH	Benign prostatic hyperplasia
CAF	Cancer associated fibroblasts
CAMMK2	Calcium/Calmodulin-dependent protein kinase kinase 2

CAR	Chimeric antigen receptor
CCK-8	Cell counting kit-8
CPT	Carnitine palmitoyltransferase
CRPC	Castration resistant prostate cancer
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
ECAR	Extracellular acidification rate
EE	Encapsulating efficiency
ERRalpha	Oestrogen-related receptor alpha
EPR	Enhanced permeation and retention
ETC	Electron transport chain
FABP	Fatty acid binding protein
FASH	Fatty acid synthase
FATP	Fatty acid transport protein
FBS	Foetal bovine serum
FDA	Federal Drug Administration
FRET	Förster Resonance Energy Transfer
FTIR	Fourier-transform infrared spectroscopy
G6PD	Glucose 6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good manufacturing practice
GSH	Glutathione

GR	Glucocorticoid receptor
HBP	Hexosamine biosynthesis
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNF4ALPHA	Hepatocyte nuclear factor 4 alpha
ICI	Immune checkpoint inhibitor
IUPAC	International union of pure and applied sciences
jetM	jetMESSENGER [®] mRNA transfection reagent
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
LE	Loading efficiency
LHRH	Luteinising hormone-release hormone
LKB1	Liver kinase B1
LNP	Lipid nanoparticle
LXR	Liver X-receptor
MUC1	Mucin 1
mRNA	Messenger ribonucleic acid
MFI	Mean fluorescence intensity
MAA	Maleic acid amide
MPS	Mononuclear phagocytic system
MTG	Mitotracker green
mTORC1	Mammalian target of rapamycin complex 1
MYC	Myelocytomatosis proto-oncogene
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)

NEPC	Neuroendocrine prostate cancer
NG	Nanogels
NMR	Nuclear magnetic resonance
NP	Nanoparticles
NRF	Nuclear respiratory factor
NT-PGC1alpha	N terminal Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PAP	Prostatic acid phosphatase
PARP	Poly(ADP-ribose) polymerase
PCa	Prostate cancer
PCK1	Polo-like kinase 1
PDSA	Polydisulfide amide
PEI	Polyethylenimine
PGC1alpha	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PEG	Poly(ethylene glycol)
PI	Polydispersity index
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIN	Prostatic intraepithelial neoplasia
PPP	Pentose phosphate pathway
PRC	PGC1alpha-related coactivator
PSA	Prostate specific antigen

PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
PTEN	Phosphate and tensin homolog
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RHEB	Ras homolog enriched in brain
RB1	Retinoblastoma 1
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RXRalpha	Retinoid X receptor alpha
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SNAPL	Stable nucleic acid lipid particle
SPARTA	Single particle automated raman trapping analysis
SREBP	Sterol regulating element binding protein
STEAP	Six transmembrane epithelial antigen of the prostate
TCA	Tricarboxylic acid cycle
TCEA1	Transcription elongation factor A1
TEMED	Tetramethylethylenediamine
TME	Tumour microenvironment
TMU	Thiol-mediated uptake
TMPRSS2-ERG	Transmembrane protease serine 2-ETS related gene

TMRM	Tetramethylrhodamine methyl ester
TP53	Tumour protein p53

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Abstract

Prostate cancer (PCa) is now the most commonly diagnosed cancer among men in the UK and US. Increasing evidence implicates metabolic dysregulation as a critical driver of disease progression. AMP-activated protein kinase (AMPK), a central regulator of cellular energy homeostasis, has been shown to suppress PCa growth *in vivo* through induction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α). PGC1 α , acting via oestrogen-related receptor alpha (ERR α), promotes oxidative metabolism and mitochondrial biogenesis while inhibiting metastatic programs. This work investigates the therapeutic potential of PGC1 α overexpression via mRNA delivery.

To this end, a library of redox-responsive polymeric nanogels (NGs) were synthesised through combinatorial free-radical polymerisation to entrap and deliver RNA cargo. Following systematic optimisation, a lead formulation was identified, exhibiting superior RNA encapsulation, cytosolic release, and transfection efficiency relative to commercial transfection agents. Functionalisation of these NGs with a peptide targeting prostate-specific membrane antigen (PSMA) enabled selective delivery of PGC1 α and its N-terminal isoform (NT-PGC1 α) to PCa cells. Various bioassays were used to confirm sustained PGC1 α expression, and increased mitochondrial protein content, indicative of enhanced mitochondrial biogenesis. Transcriptomic analysis demonstrated activation of tumour suppressor pathways and suppression of proliferative markers, consistent with metabolic rewiring and growth inhibition. *In vivo*, systemic administration of NT-PGC1 α -loaded PSMA-NGs resulted in tumour-selective accumulation and significant suppression of xenograft growth, with no overt systemic toxicity. This study presents the first example of a prostate-targeted,

disulfide-crosslinked nanogel system for mRNA-mediated metabolic reprogramming in PCa, and highlights its promise as a platform for future RNA-based cancer therapies.

Chapter 1

Introduction

1.1 Prostate cancer: a metabolic malignancy

Prostate cancer (PCa) is a highly prevalent and biologically heterogeneous malignancy primarily affecting older men, particularly in the western world, where it accounts for a significant proportion of cancer-related deaths.[1, 2] 1 in 8 men will be diagnosed with PCa, making it the second leading cause of cancer mortality.[2] Despite its prevalence, a comprehensive understanding of the molecular and cellular underpinnings of PCa remains limited, in part due to the complex biology of the human prostate. PCa is typically detected via a prostate biopsy, which is performed following elevated prostate-specific antigen (PSA) levels in blood and/or abnormalities identified during a digital rectal examination.[3] The disease typically progresses over several decades, with early histological changes such as prostatic intraepithelial neoplasia (PIN) sometimes observed in men as young as their twenties, while clinically evident PCa most often emerges after the age of 50.[4]

PCa can be identified as either androgen sensitive or insensitive, which defines the treatment options proposed. Standard treatment includes active surveillance, surgical prostatectomy, radiation therapy, chemotherapy, and hormone-based interventions such as androgen deprivation therapy (ADT).[3] Active surveillance is typically recommended for older patients with early-stage, indolent tumours, especially when the potential risks of intervention outweigh expected benefits.[5, 6] Most patients initially respond well to ADT, experiencing tumour regression; however, resistance inevitably develops within one–three years, resulting in the emergence of castration-resistant prostate cancer (CRPC). CRPC represents an aggressive, treatment-refractory stage of the disease that significantly reduces both quality of life and overall survival. Furthermore, each therapeutic intervention is accompanied by significant adverse effects, including systemic toxicity and a decline in both white and red blood cell counts, which can manifest as fatigue, hair loss, peripheral nerve damage, urinary incontinence, sexual dysfunction, and, in advanced cases, increased metastatic spread and the development of treatment resistance.[4]

Importantly, however, the study of CRPC has catalysed a deeper investigation into the metabolic reprogramming that underlies PCa progression and metastasis. Understanding these pathways offers the potential to identify new vulnerabilities that can be exploited therapeutically. As traditional androgen deprivation strategies lose efficacy, targeting the altered metabolic networks that sustain tumour survival, proliferation, and resistance becomes increasingly critical, thereby highlighting a critical unmet medical need. Metabolic rewiring in PCa not only supports bioenergetic and biosynthetic demands, but also intersects with transcriptional regulation, redox homeostasis, and immune evasion.[7] By dissecting these interconnected processes, it becomes possible to design targeted interventions that disrupt the tumour’s metabolic plasticity and adaptability.

1.1.1 PCa formation, progression and therapeutic landscape

The prostate is a male accessory reproductive organ located underneath the bladder, encircling the urethra.[3] Its primary role is to generate seminal fluid components that support sperm function and viability. Anatomically speaking, the adult prostate comprises of several distinct zones: central, transition and peripheral, along with fibromuscular and periurethral regions.[8] In young adult males, the peripheral zone accounts for more than 70 % of the gland’s epithelial tissue and is the principal contributor to normal secretory function.[9] However, it is also the predominant site of tumourigenesis in older men, with approximately 80 % of tumours originating here.

Histologically, the prostate is composed of glandular structures (ducts and *acini*) embedded within stromal tissue (Figure 1.1).[8] The epithelial lining of these structures consists of a luminal layer of simple columnar cells, underlaid by a basal epithelial layer responsible for basement membrane formation. This membrane is connected to the surrounding stroma, largely composed of smooth muscle cells facilitating fluid movement through contractile activity.[10] Additionally, fibroblasts populate the stromal compartment, particularly around ducts. While their primary role in the adult prostate is structural support, fibroblast-derived signalling during development helps shape ductal architecture.

In the context of cancer, stromal fibroblasts are believed to play a significant role in tumour progression. Within the tumour microenvironment (TME), these fibroblasts, referred to as cancer-associated fibroblasts (CAFs), can promote malignant transformation of epithelial cells and activate survival pathways, potentially sustaining cancer cell proliferation even after therapeutic intervention.[3]

A key feature of both healthy and malignant prostate epithelial cells is their high expression of the androgen receptor (AR).[11] Dependence on androgen signalling is central to disease development and progression. These same epithelial cells also produce PSA, a serine protease whose expression is regulated by AR activity.[12] Elevated PSA levels are commonly observed in men with PCa and are widely used as a clinical biomarker for screening, diagnosis, and monitoring of the disease.

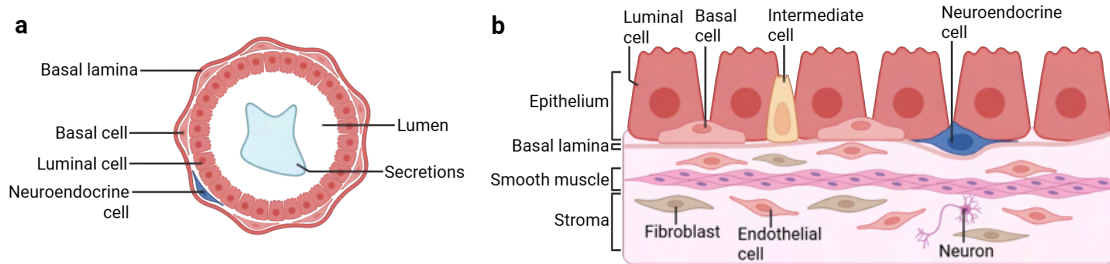


Figure 1.1: **Schematic representation of prostate tissue architecture.** a) Transversal and b) longitudinal cross-sections of the prostate gland, showing key cell types. Each region of the prostate consists of ducts and *acini* embedded within the stroma, which contains various cell types, primarily smooth muscle cells and fibroblasts involved in prostate development. The ducts and *acini* are lined by a single layer of luminal epithelial cells and an underlying layer of basal epithelial cells responsible for producing the basement membrane. This extracellular matrix layer connects to the surrounding stromal cells. Neuroendocrine cells are also present. Figure adapted from [13].

PCa arises through a well-characterised sequence of pathological stages, beginning with PIN, advancing to organ-confined adenocarcinoma, and in some cases culminating in metastatic disease, which is ultimately responsible for lethality.[3] While benign prostatic hyperplasia (BPH) is a common, non-malignant enlargement of the prostate typically occurring in the transition zone, it is not considered a direct precursor of PCa (Figure 1.2).[14] In contrast, PIN – particularly high-grade PIN – is widely regarded as a premalignant lesion. This is supported by its frequent

co-localisation with PCa in biopsies and its shared molecular alterations.[15, 16]. PIN lesions represent proliferative abnormalities within the prostatic epithelium that disrupt glandular architecture but retain an intact basal cell layer, thus lacking stromal invasion. High-grade PIN is characterised by greater epithelial crowding, nuclear atypia with prominent nucleoli, and basal layer fragmentation.[14]. The transition to adenocarcinoma is marked by the loss of the basal layer and the infiltration of these atypical cells into the surrounding stroma.[16]

At the cellular level, prostate adenocarcinomas have been thought to originate from either basal or luminal epithelial cells within the gland, with genetic mutations driving malignant transformation. One of the most frequently observed genetic alterations involves the fusion of the AR-responsive promoter regions with sequences coding for members of the ETS (erythroblast transformation-specific) transcription factor family.[11] The most prevalent of these is the fusion between transmembrane protease serine 2 (TMPRSS2) and ETS-related gene (ERG).[17, 18] Acquisition of this mutation is thought to disrupt normal prostate epithelial differentiation and promote oncogenic processes. Indeed, PCa progression is then shaped by further genetic and epigenetic modifications that promote survival, proliferation and eventual resistance to therapy.

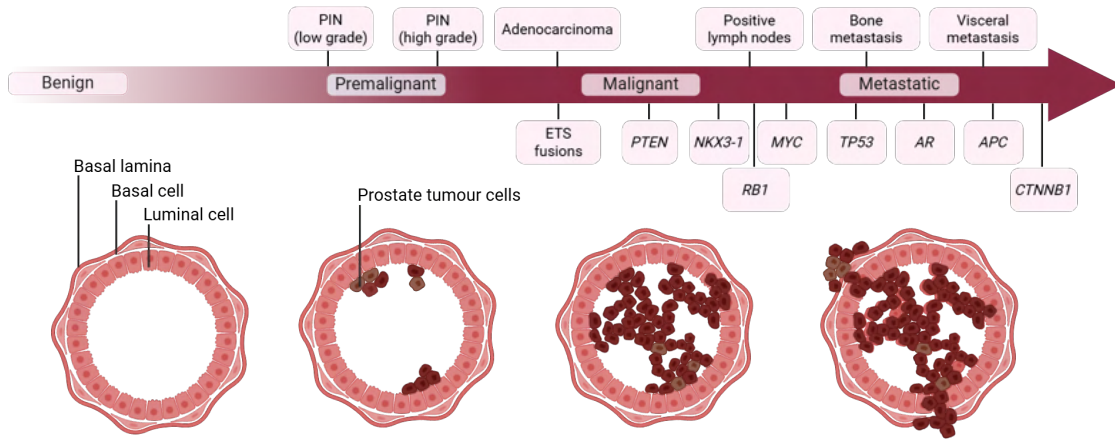


Figure 1.2: **Genetic modifications during PCa progression.** Genetic alterations are displayed based on their prevalence at different stages of disease progression. Common events across all disease stages include *TMPRSS2-ERG* fusions, *PTEN* and retinoblastoma 1 (*RB1*) deletions, tumour protein p53 (*TP53*) mutations, and myelocytomatosis proto-oncogene (*MYC*) overexpression. In contrast, some mutations show stage-specific enrichment; AR amplification is predominantly observed in metastatic castration-resistant prostate cancer (mCRPC). Figure adapted from [19].

Tumour heterogeneity – both between different tumour *foci* (intertumoural) and within a single tumour (intratumoural) – poses a major challenge to treatment.[3] Distinct clonal populations may coexist within the prostate, some of which harbour mutations that confer resistance to standard therapies such as ADT and AR signalling inhibitors (ARSI)s.[20]

As the disease advances, additional genetic changes drive progression to more aggressive phenotypes (Figure 1.2). Loss of the tumour suppressor gene phosphate and tensin homolog (*PTEN*), through deletion or mutation, is seen in up to 17% of localised tumours but becomes significantly more prevalent in metastatic CRPC (mCRPC), affecting over 40% of cases.[21] This loss activates the phosphatidylinositol-4,5-bisphosphate 3-kinase-protein kinase B (PI3K–AKT) pathway, a key driver of cell survival and growth. Gain-of-function mutations in pathway intermediates such as *PIK3CA*, *PIK3CB*, and *AKT1*, observed in PCa and model systems, can further amplify signalling.[22] In preclinical models, such alterations promote resistance to androgen deprivation, and are currently being investigated as therapeutic targets.[23, 24]

Another pathway implicated in advanced disease is Wntless-related integration site (Wnt) signalling, which is relatively quiescent in localised cancer but becomes dysregulated in 18% of mCRPC cases, often due to mutations in adenomatous polyposis coli(*APC*) or catenin beta 1 (*CTNNB1*). [22, 25] Concurrently, chromosomal instability, particularly involving chromosome 8, is a frequent event. Gain of function mutations on 8q, where the oncogene myelocytomatosis(*MYC*) resides, and losses on 8p, where the tumour suppressor NK homeobox 1 (*NKX3.1*) is located, are common in advanced cancers.[22, 25] *MYC*, in particular, is implicated across all stages of tumour evolution and may be upregulated by diverse transcriptional networks, supporting proliferation and treatment resistance.[26, 27]

Further, a subset of tumour cells may evolve mechanisms to escape hormonal control, entering the castration-resistant phase, characterised by sustained AR signalling despite low systemic androgen levels.[11] This can occur through AR gene amplification, point mutations, splice variants, or intracrine androgen synthesis, well studied in the literature. In some cases, tumour cells lose AR dependency altogether, often accompanied by inactivation of tumour suppressors like retinoblastoma 1 (*RB1*) and *TP53*, and a shift toward a neuroendocrine phenotype, which is associated with particularly poor prognosis.[28, 29]

The most life-threatening aspect of PCa is its potential to metastasise. Metastasis is a complex, multistep process involving the detachment of tumour cells from the primary site, their invasion into surrounding tissues, intravasation into the bloodstream, survival during systemic circulation, extravasation at distant sites, and eventual colonisation.[30] For PCa, skeletal metastases, particularly to the axial skeleton, are the most common and occur in over 80% of patients with advanced disease.[31] These lesions severely compromise quality of life and reduce five-year survival rates to 25%, with a median survival of approximately 40 months.[32]. The bone microenvironment offers a supportive niche for metastatic PCa cells, facilitated by chemokine signalling (e.g. CXCR4–CXCL12) and lipid-rich marrow adipocytes. In the instance that the cancer is castration sensitive, the predominant treatment option at this advanced stage is medical castration using lutenising hormone-release

hormone (LHRH) agonists, including leuprolide, goserelin and triptorelin.[5] These desensitise the pituitary LHRH receptors and decrease testosterone levels. Further, combination therapies alongside ADT include ARSIs, bone-targeted radionuclides, and poly (ADP-ribose) polymerase (PARP) inhibitors. ARSIs such as the CYP17 inhibitor abiraterone acetate suppress androgen synthesis, while AR antagonists (e.g., enzalutamide, apalutamide) block AR activity by preventing ligand binding and nuclear translocation.[5] However, the prolonged use of these agents has been associated with the development of CRPC. Radionuclides effectively target bone metastases but may compromise healthy bone density. PARP inhibitors, which impair DNA repair in tumour cells, show promise but are typically restricted to patients with homologous recombination repair defects, limiting their broad applicability.

If however, the cancer progresses to the castration-resistant stage, treatments include chemotherapy, immunotherapy, PARP inhibitors, bone-targeted agents, and radionuclide therapies.[33] Taxane-based chemotherapies such as docetaxel (first-line) and cabazitaxel (second-line) remain standard, though resistance and toxicity are common. Immunotherapies like sipuleucel-T (a dendritic cell-based vaccine) and immune check-point inhibitors (e.g., pembrolizumab) offer limited benefit, with response largely confined to patients with specific genomic alterations.[34] PARP inhibitors (e.g., olaparib, rucaparib) exploit synthetic lethality in breast cancer (BRCA) 1/2-mutated tumours but are only effective in a genetically defined subset, necessitating prior genomic testing.[35] Bone-targeting agents such as bisphosphonates and denosumab help manage skeletal complications but do not improve overall survival.[36] Radium-223, an alpha-emitting radiopharmaceutical, and prostate-specific membrane antigen (PSMA)-targeted radioligand therapies (e.g., Lu-177-PSMA-617) show promise in bone and PSMA-positive metastatic lesions, respectively, but access and efficacy depend on imaging-based patient selection.[37]

New therapies for PCa

Antibody-drug conjugates (ADCs) are emerging as a promising approach to selectively deliver cytotoxic agents to PCa cells, with PSMA serving as a well-validated target.[38] These combine the specificity of monoclonal antibodies with the potent cytotoxicity of small-molecule drugs. Several PSMA-targeted ADCs have entered clinical trials, including MLN2704 (an anti-PSMA antibody linked to mertansine (DM1)), PSMA-MMAE (using the microtubule inhibitor (monomethyl auristatin E (MMAE))), and MEDI3726 (linking the monoclonal antibody J591 to a DNA-damaging payload).[38–40] While these agents demonstrated some antitumour activity, their clinical development has been hindered by significant toxicities, such as neurotoxicity and myelosuppression, often related to linker instability or off-target effects. In parallel, cellular immunotherapy approaches, including PSMA-directed chimeric antigen receptor (CAR)-T and CAR-NK cells, are being explored to enhance tumour-specific killing.[38, 41] Among various modalities, CAR-T cell therapy has received particular attention. CARs redirect T cells toward tumour-specific antigens, such as PSMA, through synthetic receptors that initiate cytotoxic activity upon antigen engagement. CAR-T cells incorporating modifications like dominant-negative transforming growth factor (TGF)- β receptors and interleukin-2 supplementation aim to overcome the immunosuppressive TME, while CAR-NK cells have shown strong PSMA-specific cytotoxicity in preclinical models, despite challenges such as limited persistence and the need for irradiation prior to infusion.[42] More recently, bispecific T cell engagers (BiTEs) are emerging as a promising anti-PSMA PCa treatment. These are engineered antibody fragments that simultaneously bind a tumour-associated antigen and CD3 on T cells, bringing T cells into close proximity with cancer cells to induce their killing. [42] AMG 160, a second-generation, half-life-extended BiTE, has demonstrated antitumour activity in preclinical models and is being evaluated in ongoing clinical trials, both as monotherapy and in combination with AR pathway inhibitors or immune checkpoint blockade.[43]

1.1.2 Metabolic reprogramming in PCa

Understanding the metabolic state of benign prostate cells is crucial for understanding the consequences of metabolic alterations in PCa cells (Figure 1.2).[44] Among healthy prostate epithelial cells, specialised acinar cells in the peripheral zone of the prostate accumulate high levels of zinc. This inhibits mitochondrial aconitase (ACO2), preventing citrate oxidation and preventing its entry into the tricarboxylic acid (TCA) cycle. Instead, citrate is secreted into prostatic fluid. To compensate for disrupted citrate metabolism, it is likely that these cells rely on alternative energy pathways and could have elevated aerobic glycolysis.[45] However, our understanding of such metabolic adaptations is limited and warrants further study.

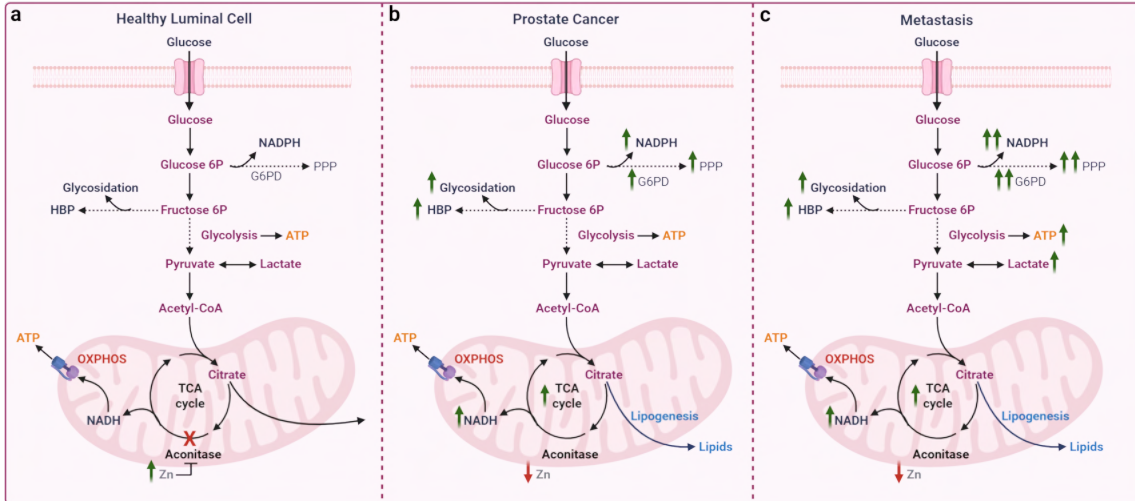


Figure 1.3: **Metabolic rewiring occurring in epithelial cells during PCa progression.** a) Normal prostate epithelial cells accumulate zinc, which inhibits mitochondrial aconitase, leading to citrate secretion and truncated tricarboxylic acid (TCA) cycle activity, resulting in inefficient oxidative phosphorylation (OXPHOS) b) In PCa cells, reduced zinc levels restore aconitase activity, reactivating the TCA cycle and OXPHOS, alongside increased flux through the hexosamine biosynthesis (HBP) and pentose phosphate pathways (PPP). c) Metastatic PCa cells display the Warburg effect while retaining active TCA cycle, OXPHOS, and PPP metabolism. Figure adapted from [7].

1.1.2.1 Glycolysis and the Warburg Effect

Given the unique, citrate-influenced metabolism of normal prostate tissue, it is likely that tumours arising from the epithelium may display specific metabolic

properties.[46] Indeed, where primary prostate tumours prefer OXPHOS, and favour lipogenesis in the form of fatty acid synthesis, increased glycolysis is typically observed in advanced stages of the disease.[47] This reliance on the Warburg effect, although less efficient than OXPHOS, is thought to provide metabolic intermediates and regenerates NAD⁺, which is critical when its demand exceeds ATP production needs.[48, 49] The AR plays a central role in promoting glycolysis by regulating glucose transporters (GLUT1, GLUT2) and glycolytic enzymes. In advanced or neuroendocrine prostate cancer (NEPC), AR-independent drivers like *MYC* and *SOX2* further sustain glycolysis and therapy resistance. PTEN loss, common in aggressive PCa, activates the PI3K–AKT–mTOR pathway, promoting glycolysis and this correlates with high lactate levels in patients. Inhibitors targeting lactate transport (e.g. MCT4) or glycolytic enzymes (e.g. hexokinase 2 via BKIDC-1553) are under investigation, although clinical translation remains limited.[50, 51]

Despite the relevance of glycolysis for tumours, many studies report that OXPHOS also remains active to a certain extent in metastatic PCa, which can complicate the use of aforementioned lactate inhibitors.[52] Indeed, TCA cycle intermediates are maintained through alternative inputs such as pyruvate, aspartate, and glutamine metabolism. AR signalling also downregulates zinc transport (e.g. via hZIP1 zinc transporter downregulation), restoring TCA cycle function and sensitising cells to OXPHOS inhibitors.[53] Therapies targeting mitochondrial metabolism, such as complex I inhibitors (e.g. metformin, rotenone), show promise, particularly in PTEN-deficient tumours.[54, 55]

1.1.2.2 Amino acid metabolism

Metabolic reprogramming in PCa extends beyond glucose metabolism, involving the upregulation of the pentose phosphate pathway (PPP) and hexosamine biosynthesis pathway (HBP).[49, 56] This supports redox balance and nucleotide biosynthesis, that are crucial for managing oxidative stress prolific in a cancerous environment. A central enzyme in this pathway, glucose-6-phosphate dehydrogenase (G6PD), is often upregulated in various cancers, including PCa.[57] This upregulation is driven partly by AR signalling via mTORC1 activation. Increased G6PD activity

fuels nucleotide and lipid biosynthesis, while simultaneously enhancing antioxidant capacity via NADPH production.[58] In PCa models, genetic or pharmacological inhibition of G6PD impairs proliferation, increases oxidative stress, and sensitises tumours to chemotherapy. Importantly, G6PD inhibition also reduces bone metastatic burden *in vivo*, indicating therapeutic potential.[59, 60] Another key PPP enzyme, 6-phosphogluconate dehydrogenase (6PGD), supports cell survival by mitigating oxidative stress and exhibits a feedback loop with AR signalling. Inhibition of 6PGD not only activates AMPK, a tumour suppressor, but also destabilises AR through enhanced ubiquitylation.[60] Dual targeting of AR and 6PGD shows greater anti-tumour efficacy than either alone, positioning the PPP as a promising metabolic vulnerability in PCa, especially in bone metastasis and castration-resistant disease.[59]

In parallel, the HBP diverts a small fraction of glycolytic flux to convert fructose-6-phosphate to UDP-N-acetylglucosamine (UDP-GlcNAc), a substrate for glycosylation processes that modify proteins and lipids[49] In PCa, HBP flux is enhanced by increased glucose and glutamine uptake and by oncogenic signals including Ras, mTORC2, and TGF- β . Elevated levels of UDP-GlcNAc result in increased O-GlcNAcylation, a post-translational modification implicated in tumour growth and survival.

Key enzymes such as UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1) and the polypeptide n-acetylgalactosaminyltransferase 7 (GALNT7) are upregulated in PCa, promoting resistance to endoplasmic reticulum stress and enhancing tumour proliferation.[61] Therapeutically, targeting O-GlcNAc transferase (OGT), the enzyme that catalyses protein O-GlcNAcylation, reduces PCa cell proliferation and impairs expression of invasion-related proteins (matrix metalloproteinases-2/9, vascular endothelial growth factor (VEGF)) and oncogenes like c-MYC and forkhead box M1 (FoxM1).[62] However, the impact of HBP inhibition may be stage-specific. In more advanced PCa, HBP activity and metabolite levels appear reduced, suggesting that the utility of targeting this pathway could vary with disease progression.

1.1.2.3 Lipid metabolism

Lipid metabolism is profoundly dysregulated in PCa, contributing to tumour initiation, progression, and therapeutic resistance. Alterations encompass all major facets of lipid homeostasis—*de novo* synthesis, uptake, storage, and catabolism—and are largely driven by oncogenic pathways and AR signalling.[63] This metabolic reprogramming enables PCa cells to fulfil the increased bioenergetic and biosynthetic demands of rapid proliferation, supports intratumoural androgen synthesis, and promotes aggressive phenotypes.

Lipogenesis and fatty acid metabolism *De novo* lipogenesis (DNL) is significantly upregulated in PCa and generates fatty acids from non-lipid precursors, primarily via ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN).[64] These enzymes are transcriptionally activated by sterol regulatory element-binding proteins (SREBPs), particularly SREBP-1, whose expression increases along PCa progression in an AR-dependent manner. Fatty acids synthesised through DNL are used for membrane biogenesis, energy production via β -oxidation, and the generation of lipid-derived signalling molecules. Importantly, the oncogenic PI3K/AKT and human epidermal growth factor receptor (HER)-2 pathways further enhance lipogenic enzyme expression, often correlating with nuclear localisation of AKT. Transcriptomic analyses confirm upregulation of lipogenic genes such as *ACLY*, *acetyl-CoA carboxylase alpha (ACACA)*, *FASN*, and acetyl-CoA synthetase long-chain *ACSL* family members in prostate tumours.[65]

Functional studies highlight that pharmacological inhibition of FASN, using agents like IP-9119 and C75, leads to reduced AR expression and transcriptional activity, thereby impairing tumour growth.[66] Another FASN inhibitor, dutasteride, indirectly reduces FASN mRNA by blocking the conversion of testosterone to dihydrotestosterone, underscoring the link between lipid metabolism and androgen signalling.[66]

Fatty acid uptake and oxidation In addition to endogenous synthesis, PCa cells increase their uptake of exogenous fatty acids by upregulating the expression of

transporters including CD36 and fatty acid transport proteins (FATPs).[67–69] Once inside the cell, fatty acid binding proteins (FABPs), particularly FABP5, facilitate intracellular trafficking. Targeting these proteins has shown therapeutic potential; for instance, SBFI-103, a FABP5 inhibitor, is effective in AR-resistant PCa models. Similarly, anti-CD36 antibodies (FA6.152 and JC63.1) block fatty acid uptake and have been shown to suppress metastasis in related tumour types.[70]

Fatty acids are also oxidised via mitochondrial β -oxidation to generate ATP and NADH, with carnitine palmitoyltransferase 1 (CPT1) being the key transporter for mitochondrial entry.[71] CPT1 is upregulated in PCa, supporting proliferation and potentially sustaining castration resistance. Recent studies have implicated other enzymes such as 2,4-dienoyl-CoA reductase 1 (DECR1) and the fatty acid elongase ELOVL5 (elongation of ery long chain fatty acids protein 5) in this metabolic rewiring.[72] Notably, ELOVL5-mediated production of polyunsaturated fatty acids (PUFAs) may promote enzalutamide resistance by activating AKT–mTOR signalling.[73]

Lipolysis, the breakdown of triglycerides into free fatty acids and glycerol, is also elevated. Enzymes like monoacylglycerol lipase and adipose triglyceride lipase (ATGL) are overexpressed and correlate with poor prognosis. Inhibition of ATGL shifts metabolism towards glycolysis and suppresses tumour growth, suggesting a dependency on lipid-derived energy sources in CRPC.

Cholesterol metabolism Cholesterol plays a central role in PCa progression.[74, 75] It serves as a precursor for steroidogenesis, including intratumoural androgen synthesis, and is a major component of lipid rafts that organise signalling complexes such as AKT–AR. Elevated levels of cholesterol esters stored in lipid droplets, especially in PTEN-deficient tumours, are associated with aggressiveness and metastatic potential.

AR signalling upregulates genes involved in cholesterol biosynthesis, including 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, and cooperates with PI3K–AKT–mTOR signalling to enhance SREBP-2 activity.[76, 77] This coordinated action promotes cholesterol accumulation, partly by suppressing liver X receptor

(LXR) activity.[78] Pharmacological inactivation of cholesterol acyltransferase impairs cell proliferation, migration, and survival.[64] Further, statins, inhibitors of HMG-CoA reductase, have been investigated extensively for their anticancer potential.[64] At high concentrations, they inhibit small GTPases and disrupt oncogenic signalling, leading to reduced PCa growth and PSA levels. Fatostatin, which targets the SREBP–SCAP (SREBP cleavage-activating protein) complex, has shown efficacy in inhibiting both fatty acid and cholesterol biosynthesis, reducing tumour burden in preclinical models.[79] Furthermore, the TME contributes to cholesterol availability under androgen deprivation.[80] Macrophages have been shown to supply cholesterol to PCa cells, facilitating progression to CRPC.[81] Inhibiting cholesterol biosynthesis enzymes like squalene epoxidase using terbinafine or FR194738 attenuates tumour growth and reduces PSA levels in both preclinical and limited clinical studies.[82, 83]

In sum, these findings underscore the complex, stage-specific metabolic adaptations in PCa, highlighting both AR-dependent and independent mechanism. Understanding these shifts is essential for developing effective therapeutic strategies across the disease states.

1.1.3 The role of AMPK: The Guardian of Cellular Energy Homeostasis

5′-AMP-activated protein kinase (AMPK) is a serine/threonine kinase that functions as a cellular energy sensor and plays a central role in linking metabolism with cancer development. It forms a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits, and responds to an increased cellular AMP/ATP ratio, by promoting energy-producing (catabolic) processes while inhibiting energy-consuming (anabolic) ones (Figure 1.4).[84] AMPK mediates this metabolic switch by both phosphorylating key targets and modulating gene expression.[85]

In the context of cancer, cells display rapid growth, division, and movement, which demands significant reprogramming of their metabolism, particularly boosting lipid synthesis and protein production. While these metabolic pathways present promising targets for PCa therapy, current efforts have largely failed, highlighting the urgent need for new approaches. In this instance, AMPK plays a central role in maintaining

energy balance and serves as a critical link between cellular metabolism and oncogenic processes. While AMPK's role in basic metabolism is well understood, its role in the pathophysiology of PCa is more contentious and seems to be dependent on context, tumour stage and cellular environment.

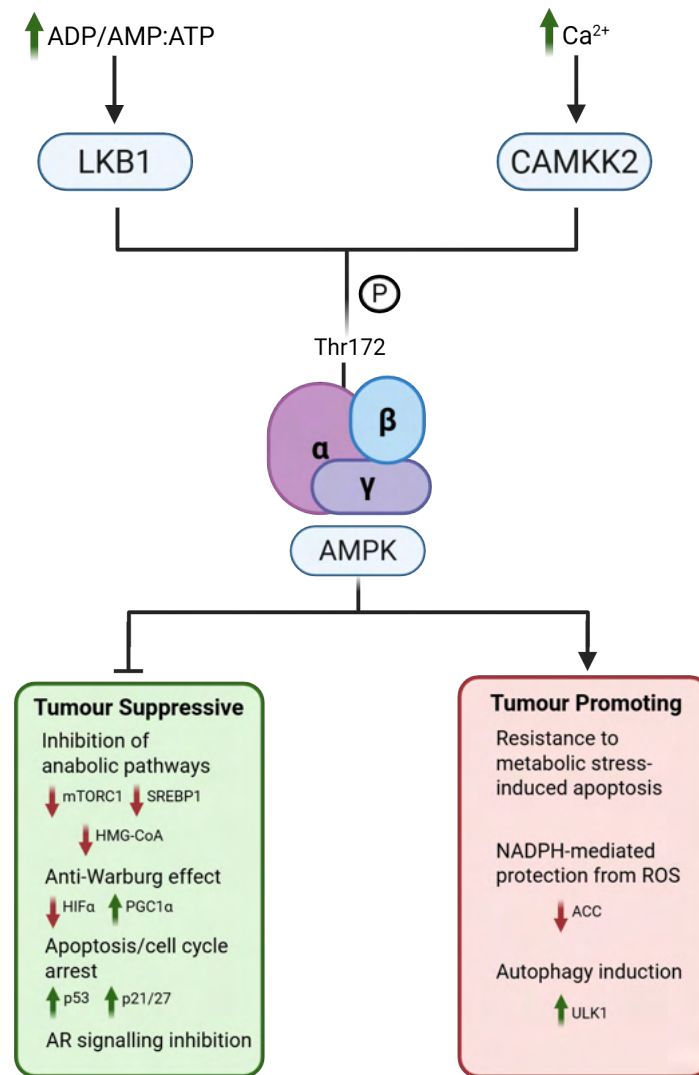


Figure 1.4: Activation and context-dependent roles of AMPK in cancer progression. AMPK regulation and its context-dependent role in cancer. AMPK is activated by phosphorylation at Thr172 on its α -subunit, primarily by the upstream kinases LKB1 and CaMKK β . Once activated, AMPK can either suppress or promote tumour progression in a context-dependent manner by modulating various downstream targets. Proteins activated by AMPK are shown in green, while those inhibited are in red; arrow represents activation and blunt-ended line indicates inhibition. Figure adapted from [86]

Evidence for tumour-suppressive role of AMPK

Activation of AMPK is primarily dependent on phosphorylation at threonine 172 of its α subunit by upstream kinases such as liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2).[87] Notably, LKB1 is a well-established tumour suppressor, and its ability to activate AMPK links AMPK to anti-cancer functions (Figure 1.4). Early studies suggested that AMPK activation plays a tumour-suppressive role, and this has been substantiated by multiple *in vitro* and *in vivo* studies.[88, 89] Once activated, AMPK serves as a metabolic checkpoint, inhibiting pathways that are essential for cancer cell proliferation and survival.

One of the key ways AMPK exerts its tumour-suppressive effects is through inhibition of the mechanistic target of rapamycin complex 1 (mTORC1), a central driver of anabolic processes such as protein and lipid synthesis. AMPK downregulates mTORC1 by phosphorylating tuberous sclerosis complex 2 (TSC2), which enhances its GTPase-activating activity and suppresses the upstream activator Ras Homolog Enriched in Brain (RHEB). Additionally, AMPK phosphorylates Regulatory-associated protein of mTOR (RAPTOR), a positive regulator of mTORC1, promoting its inactivation.[90, 91] This dual regulation ultimately reduces mTORC1 activity and curbs cell growth.

Beyond mTORC1, AMPK also inhibits other metabolic pathways critical for tumour growth. It suppresses *de novo* lipogenesis by directly inhibiting the SREBP1, a master transcriptional regulator of fatty acid and cholesterol synthesis.[65] AMPK also phosphorylates and inactivates ACC and HMG-CoA reductase, key enzymes in lipid and cholesterol biosynthesis, thereby limiting the production of essential building blocks required for membrane synthesis in rapidly dividing cancer cells.[86, 92]

AMPK further contributes to its tumour-suppressive role by reducing aerobic glycolysis (the Warburg effect), partly through the inhibition of mTORC1-mediated hypoxia-inducible factor 1-alpha (HIF1 α) translation.[93] HIF1 α is frequently upregulated in cancers and promotes glycolysis and angiogenesis. AMPK deficiency has

been associated with elevated HIF1 α levels, increased glycolytic flux, and enhanced tumour progression, particularly in Myc-driven cancers.

In addition to metabolic regulation, AMPK influences cell cycle control and survival. It has been shown to activate the tumour suppressor p53 by promoting its phosphorylation, leading to transcriptional activation of p53 target genes involved in cell cycle arrest and apoptosis, such as p21 and Bax.[94, 95] Moreover, AMPK can phosphorylate p27 at Thr198, a modification that contributes to cell cycle inhibition independent of p53 status.[96]

Taken together, these findings highlight AMPK as a multifaceted tumour suppressor. It integrates cellular energy status with control over anabolic growth signals, metabolic flux, and proliferation, making it an attractive therapeutic target in cancer, including PCa. Evidence from both preclinical models and clinical studies using agents such as metformin and AICAR supports the concept that pharmacological activation of AMPK can impair tumour growth.[97–99]

Specifically for PCa, several studies have demonstrated that AMPK activation suppresses cell proliferation and survival. For instance, Motoshima et al., showed that inhibition of AMPK in PCa C4-2 cells led to enhanced proliferation, increased migration, and anchorage-independent growth.[97] This was associated with downregulation of tumour suppressors like p53 and p21 and activation of growth-promoting pathways including insulin-like growth factor (IGF)-1/1R and S6K (ribosomal protein S6 kinase). In contrast, AMPK activation by AICAR reversed these phenotypes.

Similarly, Jurmeister et al., and Shen et al., demonstrated that AMPK negatively regulates AR signalling, a key oncogenic driver in PCa.[100, 101] Activation of AMPK using AICAR or metformin decreased AR transcriptional activity, reduced AR target gene expression, and diminished nuclear localisation of AR, thereby inhibiting androgen-driven tumour growth. Zadra et al., further supported this notion with a novel AMPK activator (MT63-78) that reduced tumour growth in both androgen-sensitive and CRPC cells by blocking *de novo* lipogenesis and synergising with AR inhibitors like abiraterone.[86]

In terms of cell migration and invasion, Scott et al., showed that AMPK activation reduced PC3 cell motility via inhibition of ACC, leading to decreased fatty acid synthesis and invadopodia formation.[102] Exogenous phospholipid supplementation rescued this effect, linking AMPK’s anti-migratory function to lipid metabolism.

Evidence for tumour-promoting roles of AMPK Conversely, several studies have revealed an oncogenic link to AMPK activation in PCa (Figure 1.4).[29, 103–107]. Some of these discrepancies may arise from the promiscuous effects of widely used pharmacological agents such as metformin or AICAR, which impact multiple molecular targets beyond AMPK. This complicates interpretation, as it remains unclear whether AMPK activation alone accounts for the observed anti-tumour effects.[86, 92, 108–111]

Importantly, analyses of clinical PCa samples reveal elevated levels of phosphorylated AMPK (pAMPK) and ACC (at Ser80, a canonical AMPK target) compared to benign tissue. Higher pAMPK levels have also been correlated with disease progression, including recurrence and higher Gleason scores, suggesting a role for AMPK activation in aggressive disease phenotypes.[112, 113]

A key mechanistic pathway underlying this tumour-promoting role involves AR-driven expression of CAMKK2, a major upstream kinase of AMPK in prostate cells. Frigo et al. and Massie et al. identified a strong androgen response element (ARE) in the CaMKK promoter, establishing a feed-forward loop wherein AR induces CAMKK2, which then activates AMPK.[114, 115] Notably, this was among the most prominent AR-binding sites identified in CRPC tissues, thereby highlighting its pathological relevance.[116] Subsequent studies confirmed that CAMKK2-dependent AMPK activation enhances PCa cell migration and invasion. Supporting this, Fu et al. showed that silencing CAMKK2 using miR-224 suppressed PCa proliferation, underscoring CAMKK2 as the dominant AMPK kinase in this context.[117]

In addition to promoting migration and invasion, AMPK activation has been shown to facilitate tumour cell survival under metabolic stress. In LNCaP cells, Chhipa et al. observed that combined androgen deprivation and hypoxia strongly activated

AMPK, which promoted autophagy via Beclin-1, thereby enhancing cell viability.[118] Laderoute et al., similarly observed AMPK activation in hypoxic regions of prostate tumours, where it supported tumour growth, potentially through enhancement of HIF1 α transcriptional activity.[106]

Jeon et al., further demonstrated that AMPK activation maintains NADPH homeostasis during glucose deprivation and matrix detachment by inhibiting ACC and promoting fatty acid oxidation.[119] This redox support enables tumour cells to resist ROS-induced death in metabolically stressed microenvironments. Similarly, AMPK-induced autophagy may aid tumour dormancy and survival during therapy or nutrient deprivation.[120, 121]

Collectively, these molecular and clinical studies suggest that AMPK has a Janus-like role in PCa, warranting further *in vivo* studies to fully understand its value as a potential therapeutic target. While it can restrain tumour growth by inhibiting anabolic processes and AR signalling, AMPK may also act as a metabolic and survival checkpoint that enables cancer cells to adapt to therapeutic or environmental stress, thereby facilitating progression and recurrence.

1.1.4 PGC1 α : The master regulator of mitochondrial biogenesis

Despite the accumulating evidence that AMPK can exert tumour-promoting effects in PCa through pathways, such as CAMKK2-mediated migration and invasion, it is important to note that not all downstream targets of AMPK contribute to disease progression. One such example is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α), a well-established AMPK effector best known for its role as the master regulator of mitochondrial biogenesis and oxidative metabolism.[122, 123] Unlike other AMPK-mediated pathways that may enhance cancer cell survival and adaptability, emerging evidence suggests that PGC1 α plays a tumour-suppressive role in PCa, with its expression limiting androgen-driven proliferation and metabolic plasticity.[88, 124]



Figure 1.5: **Schematic representation of PGC1 α isoforms.** The N-terminal region of all PGC-1 coactivators contains a conserved activation domain. Adjacent to this, PGC-1 α and PGC-1 β possess a repression domain (RD) that negatively regulates their activity. The C-terminal region includes a conserved RNA recognition motif (RRM) involved in alternative RNA splicing. Within the RRM, the RS domain, important for mRNA splicing, is present in PGC-1 α and PRC, but absent in PGC-1 β . Multiple promoter regions and alternative splicing events within the PGC-1 α gene give rise to several isoforms, including NT-PGC-1 α . Figure adapted from [125]

The PGC1 family comprises three transcriptional coactivators – PGC1 α , PGC1 β , and PGC1-related coactivator (PRC) – that regulate genes involved in energy metabolism (Figure 1.5).[125] These coactivators share conserved structural domains, including an N-terminal activation domain and a C-terminal RNA-binding domain, but differ in expression patterns and regulatory roles. PGC1 α , the first identified and most extensively studied member, was discovered in brown adipose tissue as a coactivator of peroxisome proliferator-activated receptor (PPAR) γ and is induced by cold exposure.[123] It enhances mitochondrial biogenesis and oxidative metabolism, especially in thermogenic and metabolically active tissues like brown fat, skeletal muscle, heart, and brain.[126]

PGC1 α acts as a transcriptional coactivator, lacking intrinsic DNA-binding ability but interacting with a range of transcription factors such as PPAR α , estrogen receptor (ER), glucocorticoid receptor (GR), retinoid X receptor α (RXR α), hepatocyte nuclear factor 4 α (HNF4 α), and nuclear respiratory factors 1 and 2 (NRF1/2).[125] Indeed, the N-terminal domain of PGC1 α contains conserved leucine-rich LXXLL motifs that facilitate the recruitment of histone acetyltransferases that subsequently enable

chromatin remodelling.[123] Through these interactions, PGC1 α regulates genes involved in mitochondrial respiration, lipid metabolism, and adaptive thermogenesis. Importantly, PGC1 α is subject to alternative splicing, which expands its functional diversity. While the specific expression pattern of every isoform has not been fully detailed in the context of prostate or PCa, PGC1 α -b and PGC1 α -c are full length isoforms that retain the canonical functional domains of PGC1 α and are believed to have roles similar to the canonical PGC1 α -1 isoform in regulating mitochondrial biogenesis and oxidative metabolism. Nonetheless, one particularly well-characterised splice variant is N-terminal PGC1 α (NT-PGC1 α), a truncated isoform lacking the C-terminal RNA-binding domain (Figure 1.5).[127] NT-PGC1 α retains the N-terminal activation domain and nuclear receptor interaction motifs, enabling it to coactivate transcription, but it may have distinct stability, subcellular localisation, or regulatory functions compared to the full-length protein. Unlike full-length PGC1 α , NT-PGC1 α appears to have increased protein stability, and its activity is more acutely inducible, making it well-suited to mediate rapid metabolic adaptations. It drives the expression of a wide array of genes related to ubiquitin-dependent protein degradation, ribonucleoprotein complex biosynthesis, phospholipid biosynthesis, angiogenesis, glycogen metabolism and autophagy.[127] Its expression is also responsive to physiological cues such as exercise, suggesting a potential role in adaptive metabolic responses. While the functional role of NT-PGC1 α in cancer remains underexplored, its regulation of mitochondrial capacity and metabolic reprogramming could be particularly relevant in the context of tumours with altered energy demands or redox states. Recent evidence also suggests that NT-PGC1 α may interact with a distinct set of transcriptional partners compared to full-length PGC1 α , contributing to its non-redundant biological functions.[125]

Together, the PGC1 family plays a central role in orchestrating metabolic gene programs, particularly those supporting mitochondrial oxidative metabolism, and their dysregulation has been implicated in metabolic disorders and cancers, including PCa.

PGC1 α 's role in oxidative metabolism

A central mechanism through which PGC1 α supports oxidative metabolism is its interaction with oestrogen-related receptor alpha (ERR α), an orphan nuclear receptor highly expressed in prostate tissue (Figure 1.6).[128, 129] PGC1 α coactivates ERR α to induce genes involved in mitochondrial biogenesis, OXPHOS, and fatty acid oxidation.[129] This interaction underlies a shift toward enhanced mitochondrial respiration and metabolic efficiency, which can support the energy demands of tumour cells, particularly in androgen-independent or castration-resistant stages of PCa. The PGC1 α -ERR α interaction enhances oxidative capacity and may counterbalance glycolytic reprogramming, contributing to a more metabolically flexible tumour phenotype.

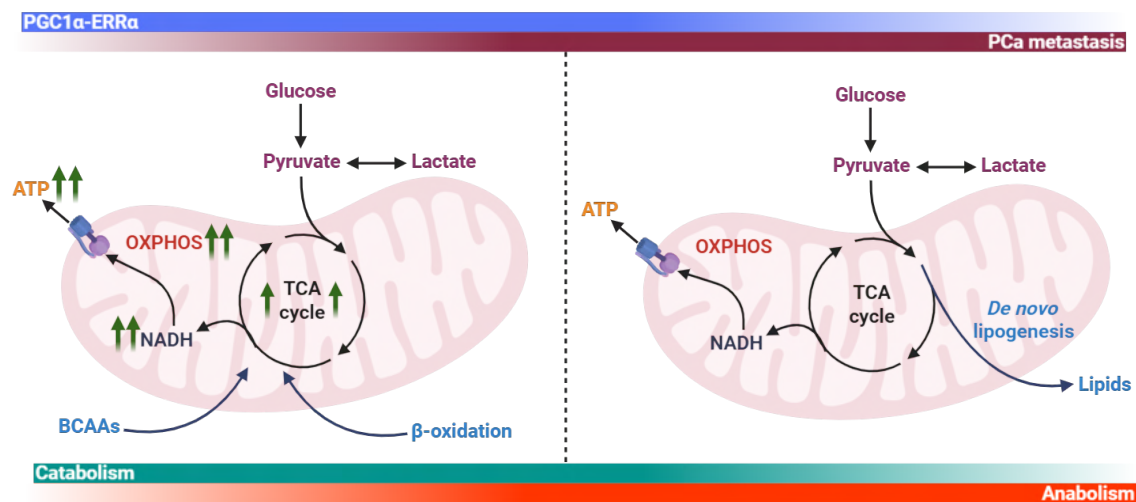


Figure 1.6: **PGC1 α -ERR α axis suppresses PCa metastasis via catabolic reprogramming.** Activation of PGC1 α and its interaction with ERR α suppresses metastasis, although this expression pattern is diminished in human PCa. PGC1 α enhances glucose and fatty acid oxidation, reduces lipid synthesis, and upregulates branched-chain amino acid (BCAA) catabolism. Through these effects, PGC1 α modulates the balance between anabolic and catabolic metabolism, thereby influencing PCa progression. Figure adapted from [130]

Moreover, PGC1 α directly influences antioxidant defence systems. Under metabolic or oxidative stress, it coactivates ERG, a PCa-relevant fusion oncogene, leading to the transcriptional induction of antioxidant enzymes such as superoxide dismutase 1 and thioredoxin, thereby mitigating ROS accumulation and apoptosis.[131] This protective function is especially significant in prostate tumours experiencing heightened mitochondrial respiration, where ROS generation is elevated.

Tissue-specific metabolic effects of PGC1 α also suggest nuanced roles in cancer progression. In skeletal muscle, PGC1 α facilitates glucose uptake via GLUT4, promotes fatty acid oxidation (FAO), and inhibits glycolysis, aligning with a catabolic oxidative program.[132, 133] In contrast, in hepatocytes, PGC1 α activation enhances gluconeogenesis and glucose output, highlighting the context dependency of its metabolic effects.[134] Notably, in PCa, increased PGC1 α expression has been associated with reduced metastasis and a less aggressive phenotype, potentially due to its role in maintaining oxidative metabolism and redox homeostasis.

In lipid metabolism, PGC1 α enhances mitochondrial and peroxisomal β -oxidation, particularly during energy stress or fasting.[135] This is achieved through ERR α coactivation, further reinforcing the oxidative phenotype. However, PGC1 α also promotes lipogenesis and lipid droplet formation in skeletal muscle, suggesting that its role in lipid handling may vary with cellular context and transcription factor availability.[78]

Additionally, PGC1 α contributes to branched-chain amino acid (BCAA) catabolism, by upregulating key enzymes, thereby reducing circulating BCAA levels and linking amino acid flux to mitochondrial metabolism.[136] Intriguingly, this cross-talk between amino acid and fatty acid pathways can promote endothelial fatty acid uptake, potentially influencing nutrient exchange within the TME.[137]

Taken together, PGC1 α orchestrates a complex oxidative metabolic program in PCa cells, involving enhanced mitochondrial function, antioxidant defences, and substrate flexibility.

Table 1.1: Predominant transcription factors interacting with PGC1 α .

Transcription Factor	Function
NRF1/2	Mitochondrial biogenesis
PPAR α / β / γ	Mitochondrial biogenesis, Fatty acid oxidation
ERR α / β	Mitochondrial biogenesis
TR β	CPT1 expression
FXR	Triglyceride metabolism
GR; HNF4 α ; FOXO1	Gluconeogenesis
SREPB1/2; LXR α / β	Lipogenesis, lipoprotein secretion

Given PGC1 α 's well-established role as a master regulator of oxidative metabolism and mitochondrial biogenesis, its functional modulation presents a compelling node of metabolic vulnerability in PCa. In the study by Torrano et al., ectopic expression of PGC1 α in PCa cells resulted in a profound shift in metabolic flux, characterised by enhanced mitochondrial respiration, glucose oxidation, and fatty acid oxidation.[124] This reprogramming was accompanied by a concurrent downregulation of *de novo* lipogenesis, suggesting a transition from an anabolic to a catabolic metabolic phenotype. Mechanistically, transcriptional analysis revealed that this shift was dependent on the co-activation of nuclear receptors, particularly oestrogen-related receptor alpha (ERR α /ESRRA), a known PGC1 α -binding partner. Importantly, genetic suppression of ERR α in PGC1 α -expressing cells abrogated the anti-metastatic effects, highlighting a critical role for the PGC1 α -ERR α axis in constraining PCa progression (Figure 1.6).

Despite these findings, the specific mechanisms through which suppression of PGC1 α confers a growth advantage in PCa remain incompletely understood. One proposed mechanism is that the loss of oxidative metabolism, especially glucose and fatty acid oxidation, frees up carbon substrates for nucleotide, non-essential amino acid, and lipid biosynthesis, thereby supporting the biosynthetic demands of proliferating tumour cells.[138] However, mitochondrial metabolism is also essential for generating NADH, FADH₂, and intermediates such as aspartate, α -ketoglutarate, and citrate,

all of which are critical for both bioenergetic and biosynthetic functions. Thus, suppression of PGC1 α may represent a context-dependent trade-off that enables tumour cells to optimise nutrient allocation based on microenvironmental constraints.

Further supporting this model, Torrano et al. observed alterations in the BCAA degradation pathway in PGC1 α -overexpressing PCa cells. Given that BCAA catabolism (mediated by BCAT2 and the branched-chain α -keto acid dehydrogenase (BCKDH) complex) feeds into the TCA cycle for energy production, its suppression in proliferating cancer cells may serve to preserve BCAA pools for anabolic processes, including protein synthesis and activation of mTORC1. Therefore, downregulation of PGC1 α may facilitate the accumulation of BCAAs, enhancing proliferative and pro-survival signalling. While the precise role of this pathway in the PGC1 α phenotype is not yet established, its disruption presents a candidate mechanism for further investigation.

Notably, overexpression of PGC1 α did not increase intracellular ROS levels despite enhanced oxidative metabolism. Measurements of ROS using fluorescent reporters, along with assessments of lipid peroxidation *in vivo*, revealed no significant differences between PGC1 α -expressing and control cells. This finding suggests that the anti-metastatic phenotype associated with PGC1 α is not attributable to redox imbalance or oxidative stress-mediated cell death. However, considering the complex interplay between ROS, mitochondrial function, and metastatic stress—particularly during detachment and colonisation—future studies should examine redox buffering systems such as NADPH-dependent pathways (e.g., the pentose phosphate pathway) under conditions mimicking the metastatic niche.

Importantly, the role of PGC1 α in cancer appears to be highly context- and tissue-dependent.[130, 138, 139] In some cancer types, such as colorectal and renal cell carcinoma, high PGC1 α levels are associated with reduced tumour growth, potentially due to elevated oxidative stress. Conversely, in subsets of melanoma and pancreatic cancer, PGC1 α expression correlates with enhanced survival under metabolic stress and sensitivity to OXPHOS inhibition. These findings contrast with breast cancer, where PGC1 α has been implicated in promoting both primary tumour growth and metastasis via enhanced mitochondrial function. Such divergence likely reflects the

influence of tumour-specific metabolic phenotypes and microenvironments. In the prostate, normal epithelial cells exhibit a unique metabolic profile characterised by zinc accumulation and citrate secretion; traits that are lost during transformation but may leave a residual metabolic scaffold that modulates the response to PGC1 α re-expression.[88] As such, strategies aimed at reactivating PGC1 α signalling in the context of PCa, either through the overexpression of full-length PGC1 α or its splice variants with preserved transcriptional coactivator function via mRNA delivery, may offer a novel approach to limit PCa progression and metastasis.

1.2 The world of RNA therapeutics

RNA-based therapeutics have revolutionised the biomedical research field by transforming the treatment outcomes of diverse diseases, and have gained increased traction owing to their unique physicochemical and physiological properties.[140–142] These therapeutics offer precise control over gene transcription and protein expression, thereby reducing the likelihood of off-target effects. Compared to traditional small-molecule drugs, RNA therapeutics present invaluable advantages, including the ability to be quickly designed and synthesised in response to newly discovered targets. Their adaptable dosing schedules and modes of administration further support their clinical utility.

Recently, several RNA-based therapies have been approved for clinical use by the U.S. Food and Drug Administration (FDA).[143] Notable examples include avacincaptad pegol, an RNA aptamer used to treat age-related macular degeneration, and patisiran, a small interfering RNA (siRNA) therapeutic for hereditary transthyretin-mediated amyloidosis.[144, 145] Moreover, mRNA vaccines have been widely deployed to combat SARS-CoV-2.[146, 147] Beyond these approved treatments, a wide range of RNA-based modalities, including vaccines, gene therapies, and protein therapeutics, are also undergoing clinical trials.[140] Various RNA types have now been identified or engineered for therapeutic and research applications. There are three broad RNA categories, namely small RNA, translatable RNA and CRISPR guide RNA (Figure 1.7).

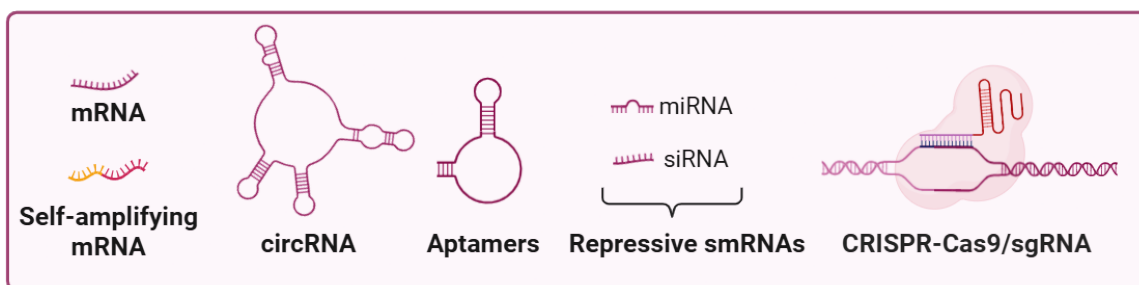


Figure 1.7: **Classes of RNA therapeutics.** RNA-based drugs include messenger RNA (mRNA) therapies, small interfering RNAs (siRNAs), microRNAs (miRNAs), aptamers, circular RNA (circRNA), self-amplifying mRNA (saRNA) and guide RNAs used in clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing. These therapeutics function through diverse mechanisms such as protein replacement (mRNA, saRNA, circRNA), gene silencing (siRNA, miRNA), inhibition of protein function (aptamers), or targeted gene editing (CRISPR guide RNAs).

Small RNAs include siRNA, which silences gene expression at the post-translational level via the RNA interference (RNAi) pathway; microRNA (miRNA), which fine-tunes gene expression by binding to target mRNAs; and aptamers, which selectively bind to specific molecules to modulate their activity.[148–150] In the context of PCa, the (antisense oligonucleotide) ASO miR-375, packaged with exosomes, suppressed mCRPC growth in preclinical models, whilst sensitising the tumours to enzalutamide.[151] Further, Kim et al., characterised the anti-PCa potential of the ASO miR-21 in preclinical metastatic PCa models.[152] An ASO against AR, delivered *in vitro* and *in vivo* using tumour-targeting liposomes showed considerable accumulation in tumour tissue and decreased AR expression relative to free ASOs in PCa xenografts.[153] In a similar vein, the cell penetrating peptide-mediated delivery of an anti-AR ASO, AR-V7, downregulated AR-V7 expression in several PCa cell lines, thereby reducing the androgen-independent cell proliferation of the PCa cells.[154] While the ASOs IONIS-AR-2.5Rx (AZD5312) is currently under Phase I trials for mCRPC in combination with enzalutamide, it has showed limited success due to limited bioavailability.[155] In other work, silencing synaptotagmin 4 (upregulated in enzalutamide-resistant PCa cell lines) using exosome-delivered siRNA, sensitised cells to enzalutamide treatment.[156]

Second-generation RNA aptamers, for instance truncated forms of the aptamers A10 and A9, including A10–3, A10–3.2, and A9g, that have been selected through a

systematic evolution of ligands by exponential enrichment process to bind specifically to the extracellular domain of PSMA. Indeed, these aptamers have demonstrated specific binding to PSMA-positive cells, with A9g uniquely retaining the ability to inhibit PSMA enzymatic activity, thereby suppressing metastatic progression *in vivo*.[\[157, 158\]](#) Aptamer–siRNA chimeras have been also developed, with studies showing that A10 and A9 aptamers can effectively deliver siRNAs to PSMA-positive cells and inhibit tumour growth.[\[159, 160\]](#) These chimeras benefit from aptamers’ targeting specificity and the gene-regulatory potential of RNAi, though limitations such as rapid renal clearance, nuclease degradation, and endosomal entrapment have restricted their therapeutic efficacy. Various strategies have addressed these challenges, including covalent aptamer–siRNA conjugation, structural modifications to the siRNA duplex, and incorporation of cell-penetrating peptides such as TAT to enhance intracellular delivery.[\[157, 161, 162\]](#) Additionally, aptamer-functionalised nanoparticles (NPs), especially PLGA-based and lipid-polymer hybrid systems, offer a protective and tunable platform for siRNA delivery. Farokhzad et al., demonstrated a 77-fold increase in cellular uptake of A10–3-conjugated PLGA NPs in PSMA-positive LNCaP cells, with enhanced intra-tumoural accumulation shown in follow-up studies.[\[163, 164\]](#) These platforms also enable dual-therapy designs, such as radiosensitizing aptamer–shRNA chimeras targeting DNA-PK to enhance radiotherapy efficacy while minimizing collateral tissue damage.[\[165\]](#) Dual-aptamer approaches have also been introduced, combining PSMA-specific A10 variants with DUP-1 peptides targeting PSMA-negative PCa, thereby improving diagnostic and therapeutic coverage.[\[166\]](#) Several studies have explored the therapeutic potential of small interfering RNA (siRNA) in PCa, particularly in the context of AR targeting for CRPC.[\[167\]](#) Zhang et al., developed a PSMA-targeted polymeric micelle system capable of co-delivering docetaxel and AR-directed siRNA.[\[168\]](#) In this construct, docetaxel was encapsulated within the hydrophobic core, while AR siRNA was electrostatically condensed onto the hydrophilic shell composed of polyethyleneimine–poly(lactic-co-glycolic acid) (PEI–PLGA) micelles. The surface of the micelles was modified with a PSMA-targeted anionic polyethylene glycol–polyaspartic acid coating, resulting in a nanoparticle formulation with prolonged systemic circulation, enhanced tumour

selectivity, and synergistic anticancer activity. In other work, Diao et al. showed that engineered extracellular vesicles (EVs) could deliver a mixture of siRNAs targeting AR and induce apoptosis in LNCaP-AI cells, a model of androgen-independent PCa.[161] Similarly, Chen et al. employed mesoporous silica NPs to deliver siRNA against AKR1C3, an enzyme involved in intratumoural androgen synthesis.[169] This led to decreased AKR1C3 expression, suppression of AR signaling, and inhibition of CRPC growth both *in vitro* and *in vivo*. Compagno et al. confirmed that AR-dependent mechanisms persist in androgen-resistant tumours and that siRNA-mediated knockdown of AR substantially reduced tumour growth.[170] Similarly, Haag et al., demonstrated growth suppression in both castration-sensitive and castration-resistant models following AR downregulation with siRNA.[171] *In vivo* delivery approaches are also being refined; lipid nanoparticle (LNP)-mediated siRNA delivery has shown efficacy in preclinical models, while adeno-associated virus (AAV)-based expression of AR-targeting shRNA resulted in robust AR silencing, tumour cell apoptosis, and regression in PCa xenografts.[172, 173] Collectively, these findings highlight the therapeutic potential of AR-targeted siRNA delivery systems for CRPC, though challenges remain in translating these approaches into clinical settings.

Additionally, messenger RNA (mRNA), circular RNA (circRNA), and self-amplifying RNA (saRNA) can be translated into therapeutic proteins.[174] However, these represent a relatively nascent therapeutic approach in the PCa context. Clinical evidence remains limited, with only a few trials having reported outcomes to date. An mRNA vaccine, CV9103 initially exhibited promising patient outcomes in a Phase I/II CRPC clinical study, extending patient survival by 31.4 months.[175]. However, the second generation of this vaccine, formulation with six mRNA encoding for six PCa-associated antigens (PSMA, PSA, prostate stem cell antigen (PSCA), six transmembrane epithelial antigen of the prostate (STEAP), prostatic acid phosphatase (PAP) and mucin 1 (MUC1)), CV9104, showed minimal cancer ameliorating effects, likely due to the instability of the mRNA-based formulation, which drastically reduced its bioavailability.[176] A Phase I/II clinical trial evaluated an unmodified RNA vaccine encoding kallikrein-2, kallikrein-3, PAP, HOXB13, and NKX3.1 (PCa

antigens), with promising preliminary immune responses and PSA reduction in a subset of patients.[177] Another emerging mRNA vaccine, BNT112, is currently undergoing early-phase testing in patients with CRPC or high-risk localized PCa. This vaccine encodes five tumour-associated antigens and is being evaluated alone and in combination with the PD-1 inhibitor cemiplimab (NCT04382898). Although the trial has been terminated, results are pending, and the potential synergistic effects of combining mRNA vaccination with immune checkpoint inhibition (ICI) remain an area of significant interest (NCT03815942).

Contrary to the growing success of the mRNA vaccines, circRNA-based therapies are still in preclinical stages of development. This is largely due to the ongoing challenges associated with identifying optimal therapeutic targets and designing functional circRNA constructs, which are inherently more structurally complex than linear, single-stranded mRNAs. Pan et al, recently identified the tumour-suppressive circBNC2, loaded these within polyacrylic acid and polydopamine-composed polymeric NPs in combination with docetaxel and noted prominent anti-tumour effects in xenograft and metastatic models of PCa.[178] While several other potential circRNA targets have been reported (circ_0006156, and circTENM3), these have yet to be validated in an *in vivo* setting.[179, 180]

CRISPR/Cas9-mediated gene editing has become an indispensable tool for probing the molecular underpinnings of PCa, particularly in the context of target identification for therapeutic intervention.[181] Multiple studies have used CRISPR-mediated knockouts to explore the roles of the PI3K/AKT/mTOR pathway components such as mTOR, *DEPTOR*, and *NKX3.1*. [182] CRISPR depletion of *DEPTOR*, a negative regulator of mTORC1 and mTORC2, resulted in increased proliferation, migration, and invasiveness of PCa cells, along with EMT induction and β -catenin nuclear translocation. These changes were associated with accelerated tumour growth *in vivo*, suggesting that restoring *DEPTOR* function may offer a therapeutic benefit. Another study developed a liposome-aptamer-CRISPR/Cas9 chimera to target polo-like kinase 1 (*PLK-1*), a pro-survival gene overexpressed in most human PCa tumours.[183] They reported highly specific targeting and gene silencing *in vitro*, demonstrating

the potential of such an approach. Park et al., used CRISPR to knockout *NKX3.1*, an androgen-regulated homeobox gene with tumour-suppressive properties.[184] Loss of exon 1 led to increased prostate size in mice, hyperproliferation as indicated by dense Ki-67 staining, and suppression of p53 levels. Interestingly, this also resulted in inhibition of PI3K/AKT/mTOR signaling, suggesting a complex feedback interaction between *NKX3.1* and PI3K activity.

In efforts to understand transcriptional regulation in PCa progression, researchers used multiplex CRISPR editing in mouse prostate cells to target the activator protein-1 (AP-1) complex, consisting of *JUN* (Jun proto-oncogene) and *FOS* (FBJ murine osteosarcoma viral oncogene homolog) family members.[185] Simultaneous loss of *JunB* or *Fos* alongside *PTEN* promoted invasive disease, while loss of *Jun* suppressed proliferation. These findings highlight the context-dependent duality of AP-1 members, with *Jun* acting as an oncogene and *JunB/Fos* behaving as tumour suppressors.

Further studies investigated genes associated with racial disparities in PCa. GPRC6A, a G-protein-coupled receptor bearing the human-specific “Y.K” polymorphism in its third intracellular loop, was deleted in PC-3 cells via CRISPR.[186] The knockout inhibited cell proliferation and migration *in vitro* and significantly reduced tumour growth *in vivo*, suggesting GPRC6A as a therapeutic and biomarker candidate, particularly for aggressive, human-derived PCa subtypes.

Beyond individual gene studies, genome-wide CRISPR/Cas9 screening has been applied to uncover synthetic vulnerabilities in combination with standard therapies. A notable example is the identification of transcription elongation factor A-like 1 (*TCEAL1*) as a modulator of docetaxel sensitivity.[187] Knocking out *TCEAL1* in human PCa cell lines enhanced docetaxel-induced apoptosis and disrupted normal cell cycle progression. While *TCEAL1*'s mechanistic role remains to be fully elucidated, its inhibition alters the transcriptional landscape in a promoter-dependent manner, suggesting that combining gene knockout with chemotherapy may uncover new therapeutic synergies.

In sum, these studies exemplify the role of CRISPR/Cas9 not as a direct therapy, but rather as a functional genomics platform to reveal dependencies in PCa. While delivery innovations, such as aptamer-conjugated liposomes, represent steps toward clinical translation, most current efforts remain preclinical and are aimed at validating genetic targets that may be modulated more precisely or reversibly in therapeutic contexts.[183]

1.2.1 Challenges in development of RNA-based therapeutics

Despite the significant progress, the clinical translation of RNA-based therapeutics remains fettered by numerous challenges. Extending delivery of these agents to anatomically or physiologically less accessible tissues remains a prominent challenge, alongside the need to enhance therapeutic efficacy whilst mitigating hepatotoxicity and off-target effects.[141] Structurally, therapeutic RNAs tend to be composed of a 5' cap, untranslated regions (UTRs), a poly(A) tail, and coding sequences, each of which is amenable to chemical modification.[188] However, the optimisation of these modifications is still in its infancy, and the influence of specific alterations on RNA stability, translational efficiency, and innate immune recognition remains incompletely characterised, thus far. Further, for CRISPR-based therapeutic systems, the impact of chemical modifications on gRNA stability, target recognition fidelity, and editing efficiency has yet to be fully elucidated. As new Cas variants continue to be discovered, careful optimisation of gRNA chemistry is critical for achieving favourable pharmacokinetic and pharmacodynamic profiles.[189] Additionally, the risk of off-target effects, both from the editing machinery and the delivery vehicles, requires rigorous evaluation as more *in vivo* and clinical data become available.

Large-scale production of RNA agents introduces further complexity. While both solid-phase and enzymatic synthesis are widely employed to generate chemically modified RNAs of various lengths and sequences, these methods are difficult to scale due to the unique synthetic demands of each modification, which often necessitate specialised reagents and reaction conditions.[190] Maintaining precise control over reaction parameters, such as temperature, pH, and reagent concentrations, becomes increasingly challenging at industrial scales. Furthermore, consistency in product

quality and purity must be ensured through stringent quality control and advanced analytical characterisation techniques.

The stability of chemically modified RNAs during long-term storage poses another major bottleneck.^[148] Modified RNAs often exhibit distinct degradation profiles compared to their unmodified counterparts, with stability being highly sensitive to the nature of the chemical modifications and environmental conditions. Establishing optimal storage parameters, such as temperature, humidity, and light exposure, is therefore critical to preserving the therapeutic integrity and bioactivity of these molecules. Moreover, the high cost of synthesising modified RNAs underlines the urgent need for more scalable and cost-efficient production strategies to enable broader clinical use.

Safety remains a paramount concern for RNA-based therapeutics, particularly with respect to immunogenicity and unintended off-target effects. LNP systems, while effective at protecting RNA from nuclease degradation and facilitating cellular uptake, can themselves provoke innate immune responses.^[191] Similarly, although certain chemical modifications (e.g., pseudouridine or N1-methylpseudouridine) are designed to reduce immunogenic stimulation, some alterations may inadvertently interfere with endogenous RNA sensing and processing, thereby enhancing the risk of immunotoxicity or misregulation of host pathways.^[181] Chronic or repeated administration, as required for treatment of persistent or relapsing conditions, may exacerbate these risks, with concerns around cumulative toxicity and potential delayed adverse effects.

Given the incomplete understanding of how various RNA modifications are metabolised and regulated *in vivo*, further investigation is necessary to assess long-term safety and therapeutic tolerance. Finally, the route of administration plays a pivotal role in directing RNA therapeutics to target tissues efficiently, influencing biodistribution, cellular uptake, and off-target effects. The rational design of delivery and dosing strategies will therefore be essential for maximising therapeutic outcomes while minimising systemic toxicity.

1.3 Nanoparticle-mediated RNA delivery

While the International Union of Pure and Applied Chemistry (IUPAC) identifies NPs as particles with dimensions between 1–100 nm, where unique nanoscale properties distinct from bulk materials typically emerge, less rigid definitions are often adopted in biomedical contexts.[192] In drug delivery, for example, particles exceeding 100 nm are commonly used to ensure sufficient therapeutic loading and functional performance. This work adopts the more inclusive definition of NPs as colloidal entities within the 10–1000 nm size range.

As drug delivery systems, NPs can encapsulate, adsorb, or entrap therapeutic agents, enhancing solubility, stability, and bioavailability.[193] By improving pharmacokinetics and protecting labile drugs from degradation, NPs can revive compounds with otherwise poor pharmaceutical profiles and enable new therapeutic strategies.

Chemically, NPs can be classified into three broad categories: lipid-based, inorganic, and polymeric.[194] Lipid-based NPs dominate clinical nanomedicine due to their biocompatibility, ease of formulation, and capacity to deliver large payloads. Inorganic NPs, by contrast, are well-suited for diagnostic and photothermal applications due to their magnetic and optical properties.[195] Polymeric NPs, however, offer unmatched structural and functional flexibility, as their properties can be precisely tuned through the use of a wide variety of synthetic and natural polymers, as well as functional monomers.[196]

1.3.1 Rationale for RNA delivery

Irrespective of their mode of action, the susceptibility of RNA-based agents to RNAses present in circulation and tissues, and their anionic charge make it difficult to realise their therapeutic potential by efficiently entering cells and exerting their function. To overcome this barrier to therapeutic efficacy, many viral-vector-based and non-viral delivery systems have been developed to protect the RNA from being degraded, improve delivery to target tissues, and minimise off-target effects.[197] Viral vectors have yielded promising clinical outcomes in gene therapy applications.[198–203]

However, their scope is inherently limited due to pre-existing host immunity, vector-induced immunogenicity, risks of unintended genomic integration, limited payload capacity, inability to perform repeat dosing, challenges in large-scale manufacturing, and the high costs associated with vector production.[204, 205] While some of these barriers are being addressed through ongoing innovation, these have motivated the exploration of alternative, non-viral delivery strategies.

Significant progress has been made in the design of synthetic carriers, including polymers, lipids, and lipid nanoparticles (LNPs), has accelerated the development of RNA-based therapeutics delivered via non-viral platforms.[205] This includes the FDA approval of subcutaneously administered N-acetylgalactosamine (GalNAc)–siRNA conjugates for targeted hepatocyte delivery, as well as intravenously administered LNP-based siRNA therapeutics for hepatic indications.[145, 206–209] Furthermore, the emergency use authorisation and subsequent FDA approval of intramuscularly delivered LNP-based mRNA vaccines against COVID-19 has provided critical clinical validation for this delivery modality.[210–212] These regulatory milestones underscore the potential of nanoparticle systems to enable RNA delivery beyond hepatic tissues, as well as site-specific applications targeting the central nervous system, ocular tissues, and inner ear. In addition to RNA delivery, nanoparticle-based platforms are being explored for non-viral DNA delivery, offering an alternative to viral vectors for gene transfer, as detailed in other reviews.[206, 213]

1.3.2 Lipids and lipid nanoparticles for RNA delivery

Liposomes were first proposed for RNA delivery in the late 1970s. Since then the use of lipids for nucleic acid delivery has evolved dramatically.[214, 215] The concept expanded in late 1980s with the development of cationic liposomes for DNA and RNA transfection, laying the foundation for clinical applications to come.[216] The first clinical trial employing cationic liposomal formulation for gene delivery targeting melanoma was initiated in 1993.[217, 218] A major turning point in the field came with the introduction of stabilized antisense–lipid complexes in 2001, which were later termed Stable nucleic acid lipid particles (SNALPs) and are now widely known

as LNPs.[219] These systems significantly enhanced the stability, efficiency, and therapeutic potential of RNA delivery.

The architecture of these particles is influenced by the balance between the hydrophilic head group and hydrophobic tail(s) of the lipid molecules, enabling the formation of diverse nanostructures.[220, 221] FDA-approved formulations typically consist of four core components: a cationic or ionisable lipid to complex with the anionic RNA, cholesterol to enhance stability and membrane fusion, a helper lipid (such as DSPC), and a polyethylene glycol (PEG)-lipid to improve circulation and colloidal stability.[220]

Extensive work has been undertaken to refine LNP composition through both high-throughput lipid screening and rational design approaches. Early examples include lipids such as C12-200, synthesised via epoxide–amine chemistry, and cKK-E12, a peptide-mimetic lipid, which demonstrated efficient siRNA delivery to hepatocytes in mice and non-human primates.[222, 223] Innovations in lipid chemistry led to a significant drop in effective dose for gene silencing—from 1.0 mg/kg to as low as 0.002 mg/kg—highlighting the impact of lipid structure on delivery potency.[224] DLin-MC3-DMA, the ionizable lipid used in Onpattro®, is a prime example of this optimisation.[225]

These platforms have also proven effective for mRNA delivery. Multiple LNPs originally designed for siRNA, including those containing C12-200, cKK-E12, and DLin-MC3-DMA, have successfully delivered mRNA to the liver in animal models.[226–228] More recent lipid formulations, such as LP0177 (Intellia), Lipid H128 (Moderna), and FTT5103 (Ohio State/Beam Therapeutics), have expanded the chemical diversity of RNA-delivery vehicles.[189, 229] Notably, preclinical studies have shown that a single intravenous administration of LNPs carrying base editor mRNA and sgRNA targeting *PCSK9* results in months-long gene silencing in non-human primates – an approach with strong therapeutic potential for cardiovascular diseases.[208]

Despite these advances, several limitations constrain the broader application of LNP-based systems. One of the most critical is organ tropism, LNPs predominantly

accumulate in the liver, making extrahepatic targeting a major challenge.[230, 231] While modification of LNP components (e.g., PEG-lipids, cholesterol analogues, or helper lipids) has enabled redirection of delivery to organs such as the lungs, spleen, or bone marrow, such approaches often lack consistent translatability across species or administration routes.[232] Indeed, these strategies still require comprehensive optimisation and mechanistic elucidation. While emerging work from Intellia and Beam Therapeutics has demonstrated that LNPs can be engineered to target hematopoietic stem and progenitor cells *in vivo*, however, achieving targeted delivery to these cell populations without off-target accumulation in the liver or spleen continues to be a major technical hurdle.[233] Further, the delivery efficiency and pharmacokinetics of LNPs can also be highly sensitive to seemingly minor changes in component structure or ratio. For instance, the PEG-lipid modulates circulation time and immune recognition but can also inhibit cellular uptake, especially at higher molar percentages.

Immunogenicity remains another key concern. Although ionisable lipids minimise systemic toxicity by remaining neutral at physiological pH, cationic intermediates generated during formulation or endosomal escape can trigger innate immune responses. Repeated dosing is often limited by immune memory against the PEG component or other lipid elements, compromising the feasibility of chronic treatment regimens. Furthermore, anti-PEG antibodies have been detected in a significant portion of the human population, either pre-existing or induced upon exposure to PEGylated drugs, which can lead to accelerated blood clearance, reduced efficacy, or hypersensitivity reactions.[191] To offset the immunogenicity associated with PEGylated NPs, alternatives such as polysarcosine, an uncharged, hydrophilic polypeptoid, are currently being explored; it offers stealth-like properties similar to PEG but with a lower risk of eliciting anti-polymer immune responses.[234]

1.3.3 Polymeric nanoparticles for RNA delivery

A crucial advantage of polymeric NPs over lipid-based systems lies in their chemical engineerability. Chemists can systematically tailor polymer properties, including charge density, hydrophobicity, degradability, and molecular weight, to optimise RNA

complexation, protection, cellular uptake, and intracellular release. This level of control is more difficult to achieve with LNPs, which rely on a smaller set of chemically similar lipid components and offer limited flexibility in structure-function design. In particular, the chemical versatility of polymers enables the facile installation of chemical motifs that can respond to specific biological stimuli ([Figure 1.8](#)).^[235] For example, polymers can be engineered to degrade or change conformation in response to triggers such as acidic pH, extracellular enzymes, elevated glutathione levels, hypoxia or reactive oxygen species, conditions commonly found in the TME. This stimulus-responsiveness allows for spatially and temporally controlled release of RNA payloads, improving therapeutic precision and minimising off-target effects. Three key biological stimuli most relevant to this work are discussed below. For further information regarding the other stimuli that could influence NP structure in the TME, more detailed reviews are available.^[236]

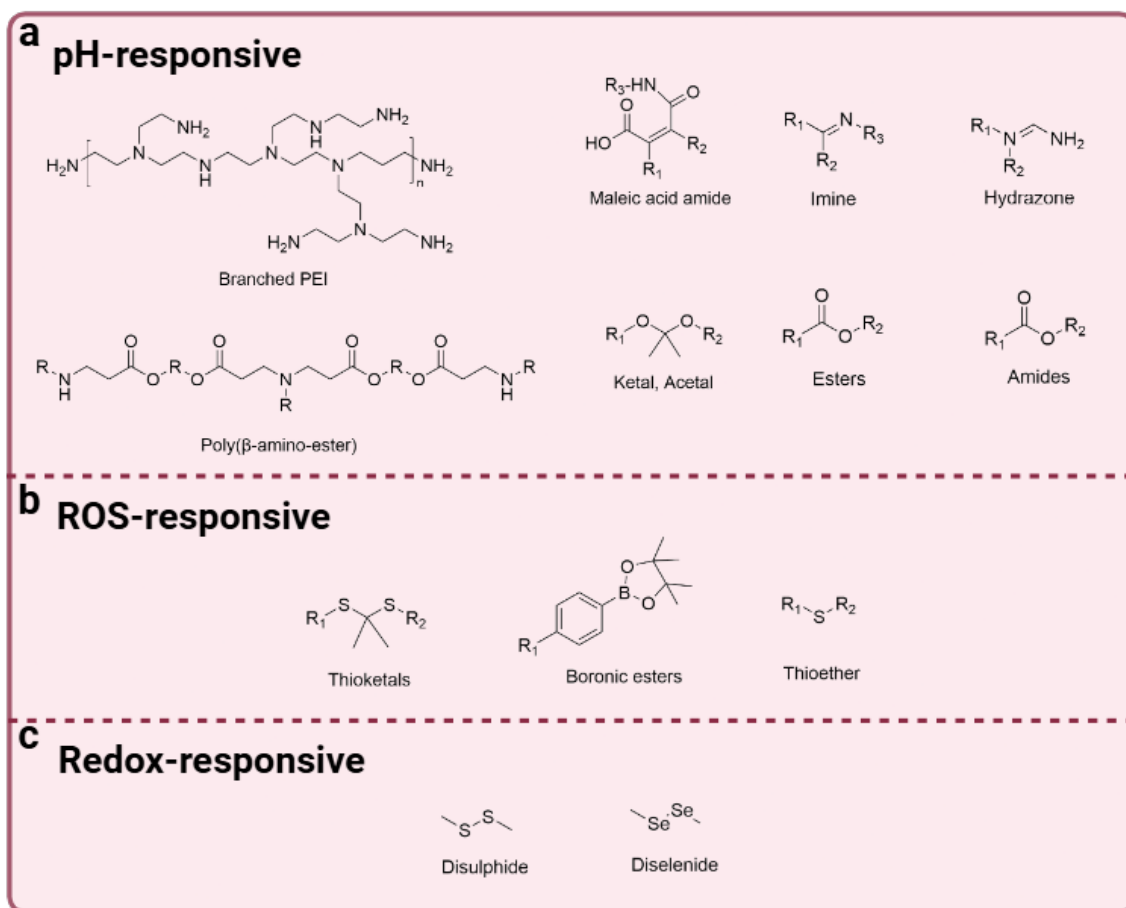


Figure 1.8: **Key functional groups used in stimuli-responsive NPs.** a) pH-responsive NPs often incorporate acid-labile linkages such as hydrazones, imines, esters, and acetal groups, which degrade or change conformation under acidic tumour or endosomal environments. b) ROS-responsive NPs use thioketals, boronic esters, or selenium-containing moieties that undergo cleavage in the presence of elevated ROS typical of inflammatory or tumour tissues. c) Redox-responsive NPs are engineered with disulfide or diselenide bonds that are reduced in the intracellular environment rich in glutathione, enabling controlled cargo release.

1.3.3.1 pH-responsive nanomaterials

An altered metabolic profile is a standard hallmark of solid tumours, particularly the reliance on aerobic glycolysis (the Warburg effect), which generates a mildly acidic extracellular TME with pH levels ranging from 6.5 to 7.[80] This is significantly lower than the physiological pH of 7.2-7.4. This acidification fosters an environment that permits tumour progression and the development of resistance to conventional therapies, but also provides a key biochemical trigger that can be used to activate pH-responsive RNA delivery systems.

Many nanocarriers have been developed that possess acid-labile linkers or pH-sensitive protonation mechanisms. Common acid-sensitive bonds employed include esters, hydrazone, imine, phenylmethyline, amide, and maleic acid amide (MAA) derivatives, among others (Figure 1.8a).[237] NPs constructed using these chemistries can undergo structural rearrangements, including charge reversal or shedding of polyethylene glycol (PEG) layers, in mildly acidic environments, thereby enhancing tumour penetration and cellular uptake. For instance, a MAA-containing copolymer, meo-PEG-DlinKm-PLGA, was used for *PTEN* mRNA delivery.[238] *In vivo* imaging showed that the NPs selectively accumulated in tumour tissues, leading to significant tumour growth inhibition via downregulation of the PI3K–Akt pathway. Another mechanism of pH responsiveness is based on protonatable groups, such as tertiary amines or imidazoles, which gain positive charges under acidic conditions.[237] Polyethylenimine (PEI) is a classic example; its high density of amines enables proton sponge-mediated endosomal escape.[239] However, its non-biodegradability and associated cytotoxicity necessitate chemical modifications. For instance, PEI has been conjugated with chitosan (CS) and linked to doxorubicin via a pH-sensitive hydrazinobenzoic acid linker to form a multifunctional micelle that co-delivers siRNA and chemotherapy.[240] This system achieved 60% apoptosis *in vitro* and ~80% tumour inhibition *in vivo*.

To address PEI's cytotoxicity, biodegradable alternatives such as poly(β -amino esters) (PBAE), polycaprolactone-block-poly(2-(dimethylamino)ethyl methacrylate), and disulfide-containing polymers have been developed. These designs combine pH sensitivity with reduced immunogenicity and improved biodegradability. Dastgerdi et al., developed a PBAE-based saRNA delivery system co-formulated with the polyanion γ -polyglutamic acid (γ -PGA), which significantly improved transfection efficiency and reduced cytotoxicity by lowering surface charge without compromising colloidal stability or RNA condensation.[241] While the formulation was not explicitly tested for pH-responsiveness, the PBAE backbone has the chemical potential to respond to acidic environments, suggesting that this platform could be further optimised for stimuli-responsive RNA release. Joubert et al., prepared novel lysine and poly(amidoamine) assembled dendrimers to complex RNA, providing

well-encapsulated, positively charged dendriplexes with tailored surface modifications, including fusogenic groups that exploit the acidic endosomal pH to promote endosomal escape and enhance mRNA delivery efficiency.[242] Gurnani et al. recently developed self-hydrolysing, charge-reversible polycation poly((dimethylaminoethyl acrylate) for RNA complexation.[243] Interestingly, while it was not designed as a pH-responsive carrier, the polymers' hydrolysis-driven charge reversal and disassembly likely occurred under mildly acidic conditions, suggesting an inadvertent sensitivity to pH that could be further exploited in future designs.

Lipid-based carriers have also been adapted for pH-mediated charge conversion. Notable examples include the first synthesis of 1,2-distearoyloxy-N,N-dimethyl-3- amino-propane and ethylenediamine-based polycarboxybetaine-modified cationic lipids.[244, 245] These materials change charge state in acidic environments, promoting endosomal escape and RNA release. Furthermore, pH-responsive inorganic NPs and protein-mimetic polymer nanorods have emerged, expanding the landscape of pH-sensitive RNA delivery vehicles.[246, 247]

1.3.3.2 Reactive oxygen species (ROS)-responsive nanomaterials

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are byproducts of cellular metabolism, particularly during mitochondrial respiration.[248] Elevated ROS levels are commonly observed in tumour tissues and can be exploited to trigger the release of therapeutic cargo from responsive delivery systems.[249] To harness this, chemists have developed nanocarriers incorporating oxidation-sensitive chemical groups such as boronic esters, thioethers, and thioketals, each capable of undergoing cleavage or structural disruption in ROS-rich environments (Figure 1.8b). For instance, a triple-interaction polymeric siRNA nanomedicine was designed by combining electrostatic, hydrogen bonding, and hydrophobic interactions using phenylboronic ester-based polymers.[250] This served not only to stabilize the complex but also to confer ROS responsiveness. Upon encountering elevated ROS levels, the boronic ester was cleaved, exposing a carboxyl group that disrupted the remaining interactions and promoted siRNA release intracellularly, resulting in effective tumour growth inhibition.

In another study, a polycationic nanocarrier incorporating thioketal and cis-aconitate linkages for co-delivery of siRNAs targeting the immune checkpoints FGL1 (fibrinogen-like protein 1) and PDL1 (programmed death-ligand 1), was developed.[251] These ROS-sensitive bonds underwent degradation in the TME, facilitating siRNA release and endosomal escape, resulting in >50% knockdown of target protein expression. The formulation significantly also slowed tumour progression in mice with negligible systemic toxicity.

1.3.3.3 Redox-responsive nanomaterials

TMEs are characterized by a redox gradient, featuring oxidative extracellular conditions and highly reductive intracellular compartments. This redox gradient is largely driven by elevated levels of the tripeptide glutathione (GSH). In a typical intracellular environment, GSH levels vary between ($\sim 1\text{--}10\text{ mM}$), which is significantly higher compared to extracellular levels ($\sim 2\text{--}20\text{ }\mu\text{M}$).[252, 253] Tumour cells exhibit even higher GSH levels, up to four-fold higher than normal cells, to neutralise ROS and support cellular survival.[254] This redox imbalance has been widely exploited for stimuli-responsive RNA delivery, particularly through the incorporation of reduction-sensitive moieties such as disulfide bonds (-SS-) and diselenide bonds (-SeSe-) (Figure 1.8c).

Among these, disulfide bonds are the most widely explored, likely owing to their facile synthesis. Poly(disulfide amide) (PDSA)-based NPs composed of cationic lipids and DSPE-PEG were shown to efficiently encapsulate and release siRNA upon intracellular GSH-mediated bond cleavage.[255] Similarly, amphiphilic polymers like PEG-b-P(TMC-DTC)-PEI and CNGQ-PEG-b-P(TMC-DTC), featuring disulfide-linked cyclic dithiocarbonates (DTC), were engineered for targeted siPLK1 delivery to lung cancer cells, where the high cytosolic GSH levels triggered disassembly and effective gene silencing.[256] Additional systems such as poly(disulfide amines), disulfide-containing poly(amido amines), and PEG-shedding micelles have demonstrated enhanced transfection efficiency and RNA release *in vivo* by enabling rapid intracellular unpacking.[257] Disulfide linkages have also been used in siRNA-grafted polymers and multimerised RNA systems, as well as in poly(disulfide) vectors capable

of co-delivering CRISPR–Cas9 components, including Cas9 plasmids, mRNA, and sgRNA, with high genome editing efficiency.[258]

More recently, a study incorporating disulfide-based, poly(CBA-co-4-amino-1-butanol) (pABOL) exhibited significant saRNA loading, forming monodisperse polyplexes that achieved potent *in vivo* transfection and protein expression following intramuscular or intradermal delivery.[243, 259] In an influenza vaccine model, pABOL outperformed polyethylenimine (PEI), demonstrating strong immunogenicity and protection, thereby highlighting its translational potential.

Crosslinked polymeric NPs formed via PEG-based disulfide linkers represent a promising redox-responsive platform for mRNA delivery. By tuning cationic components such as polyarginine, polyhistidine, and acrylated guanidine, these systems achieve high RNA encapsulation (up to 89.3%) and glutathione-triggered release under intracellular reducing conditions. They demonstrated superior transfection efficiency across multiple cell types, including traditionally hard-to-transfect lines.[260] These crosslinked polymeric NPs also enabled efficient mRNA delivery in *C. elegans*, in later work by the same authors, achieving robust GFP expression *in vivo* and underscoring their versatility for both therapeutic and research applications.[261]

Diselenide bonds, which exhibit even greater redox sensitivity and improved membrane permeability than disulfides, are also gaining attention. Mitochondrial-targeting poly(diselenide)s (Mito-CPDs), for instance, have enabled delivery of siRNA and antisense oligonucleotides directly to mitochondria, effectively downregulating target proteins and modulating mitochondrial function.[262]

In sum, these findings illustrate the versatility and growing sophistication of redox-responsive RNA delivery platforms tailored to the TME, offering enhanced targeting, controlled release, and functional gene modulation. In general, however, the advantages offered by a polymeric nanoplatform, including their ease of functionalisation and tunability, chemical modularity, and precise control over degradation and cargo release, are most impactful when integrated into a highly customisable architecture. Despite progress in lipidic systems, a major unmet need remains for delivery vehicles that combine the stability and biocompatibility of lipids, the functional versatility

of polymers, and the structural precision necessary for targeted, cytosolic mRNA delivery. Crosslinked, multi-monomer, and therefore multi-functional, polymeric NPs emerge as a promising solution to this challenge. Their functionalisable matrix enables the incorporation of diverse functional monomers, allows fine-tuning of size, charge, and crosslinking density, and supports triggered disassembly in response to intracellular cues. Together, these features offer a remarkably flexible platform for engineering next-generation RNA delivery systems.

1.4 Nanogels: an engineerable platform for RNA delivery

Polymer-based drug delivery has a long and well-established history, with foundational contributions dating back to the 1960s from pioneers such as Folkman, Langer, Peppas, and others.[263–275] This field has since expanded to include a diverse array of polymeric platforms to transport therapeutic agents, ranging from implants and sutures to microparticles and NPs.[276] Polymeric NPs, composed of either natural or synthetic polymers, can be formed through physical or chemical modification of pre-existing polymers or by direct polymerisation of monomers.

These NPs are capable of encapsulating therapeutic agents within their core, embedding them within the polymer matrix, attaching them to the surface, or conjugating them covalently to the polymer chains. Compared to other delivery systems, polymeric NPs are considered especially versatile due to their structural stability, capacity for high drug loading, tunable release profiles, and ease of surface functionalisation.[277] In contrast to lipid-based delivery systems, polymeric NPs typically offer enhanced *in vivo* stability, higher drug-loading efficiency, and controlled or stimulus-responsive release mechanisms.

Functionally, they are designed to:

1. provide sustained and programmable release of payloads,
2. achieve site-specific delivery with controlled kinetics,
3. maintain therapeutic concentrations over time,

4. and shield payloads from degradation and premature clearance.

Importantly, polymeric platforms can be engineered to respond to environmental stimuli enabling precise, on-demand drug release. These characteristics make them well suited for applications in cancer therapy and other areas of precision medicine, with several formulations already advancing through clinical development.[278, 279]

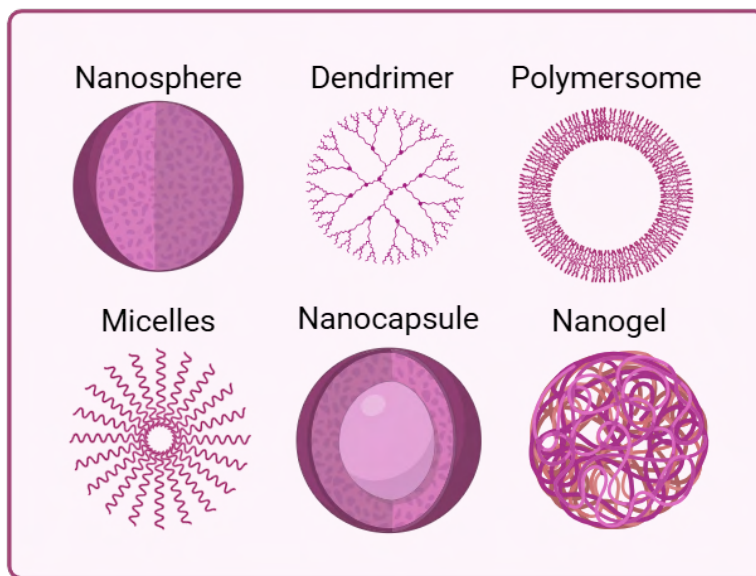


Figure 1.9: **Categorisation of polymeric NPs.**

Polymeric NPs can be broadly divided into two main morphological types: nanospheres, which are solid matrix particles, and nanocapsules, which consist of a central cavity enclosed by a polymeric shell or membrane (Figure 1.9). These primary categories encompass several subtypes, including polymeric micelles, structures with a hydrophobic core and hydrophilic shell; polymersomes, which are vesicle-like assemblies with aqueous cores similar to liposomes; dendrimers, highly branched polymers with intricate three-dimensional architectures; and nanogels (NGs), which are nanoscale hydrogels with a soft, hydrated network structure.[280]

NG, by definition, are crosslinked polymeric NPs, and are formed by either physical crosslinker or covalent interactions.[281] Covalently crosslinked NGs are thought to offer greater *in vivo* stability and reduced premature release of their therapeutic cargo compared to their non-covalently crosslinked counterparts. Their spheroidal structure

permits the facile encapsulation of various drug types, thereby protecting them from degradation in circulation or long-term storage.[282] Furthermore, the extent of crosslinking within these systems has been shown to significantly impact the level of payload leakage.[283] Their physicochemical properties can be precisely modified by controlling not only the monomers added during their synthesis but also the synthetic methodology used. Finally, NGs can be tailored to be multi-functional and targeted, by the covalent attachment of targeting modalities, thereby prolonging their circulation time and allowing active targeting of payload delivery to suit particular applications.

1.4.1 Stimuli-responsive nanogels

Stimuli-responsive NGs offer the potential for precise spatial and temporal control over therapeutic payload release by undergoing structural or chemical changes in response to specific triggers.[284] These changes may include polymer backbone degradation, surface layer shedding, or charge reversal upon exposure to particular stimuli, resulting in an "switchable" release of the encapsulated therapeutic agents. The chemical versatility of polymers enables the integration of a wide range of stimuli-sensitive motifs, which can be synthetically modified to finely tune their reactivity and optimise release profiles.

To date, NGs have been engineered to respond to external stimuli such as temperature, ultrasound, and light.[285, 286] In parallel, there has been significant progress in developing systems responsive to endogenous cues, including acidic pH, redox gradients, oxidative stress, and disease-specific enzymes. These features allow NGs to release their contents selectively within particular biological environments, such as diseased tumour tissues. For example, the acidic extracellular pH characteristic of many tumours (pH 6.2–6.9) can be leveraged by pH-sensitive NGs to preferentially discharge their payloads at the tumour site. Similarly, the acidic interiors of endosomes and lysosomes (pH $\sim$ 4.0–6.0) facilitate intracellular delivery through triggered release in these compartments.

Enzymes offer another layer of selectivity. Since enzyme expression is often altered in diseases like cancer or inflammation, NGs incorporating enzyme-cleavable linkages (e.g., Gly-Pro-Leu-Gly-Val-Arg-Gly) can be specifically degraded by overexpressed enzymes such as matrix metalloproteinase-2, thereby releasing the drug only in targeted cells.[287]

Redox-responsive NGs are particularly promising for intracellular delivery. They remain stable during circulation and in normal tissues but degrade in the reducing environments typical of tumour cells or inflamed tissues.[254] Polymers containing redox-sensitive elements such as sulphur or selenium are especially attractive for this purpose, as they respond to intracellular agents like GSH or ROS, enabling selective drug release where it is most needed[284].

1.4.1.1 Disulfide-based NGs

As outlined in Section 1.3.3.3, by taking advantage of the natural redox differential between intracellular and extracellular environments, disulfide-based NGs can be used as very efficient intracellular RNA delivery agents, as shown in recent work that forms the foundation of this thesis.[260, 261, 288, 289] Disulfides are frequently employed as redox-responsive motifs in polymeric NPs due to their ease of synthesis, natural occurrence in biological environments, and well-characterised sensitivity to physiological levels of GSH (Figure 1.10a). In essence, the thiol–disulfide exchange reaction follows an S_N2 -type mechanism, where a thiolate anion serves as the nucleophile and attacks the sulphur atom of a disulfide bond, resulting in the formation of a new sulphur–sulphur linkage (Figure 1.10b).

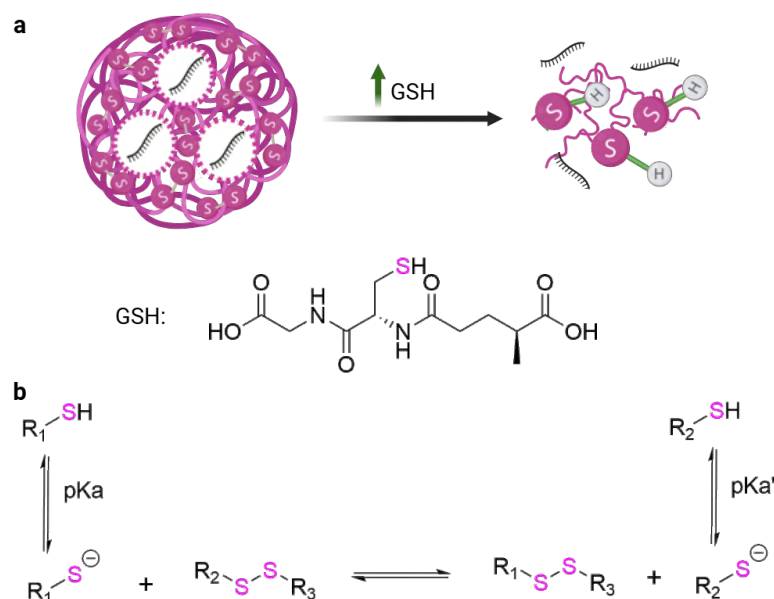


Figure 1.10: **Disulfide-crosslinked NGs for drug delivery: mechanism of degradation.** a) Disulfide-NGs degrade at high GSH concentration present in tumour cells. b) Chemical structure of GSH and mechanism of disulfide bond cleavage.

Beyond GSH-triggered cleavage, disulfide groups incorporated into polymeric NPs are also thought to promote cellular uptake and aid endosomal escape, potentially by forming covalent bonds with membrane thiols and disrupting endosomal membrane structure.[290] As such, disulfide-based crosslinkers are widely used in the design of redox-responsive polymeric NPs. Common reagents such as cystamine, cystine, and 3,3'-dithiopropionic acid serve as adaptable precursors for synthesising a variety of polymerisable disulfide monomers, which can then be polymerised into nanoparticle systems.

1.4.1.2 Radical polymerisation synthesis of NGs

Radical polymerisation of functionalised monomers and concurrent NG formation allows precise control over NG architecture, enabling the fine-tuning of their physical and chemical properties.[291] Crosslinked polymeric NGs can be generated by simultaneous radical crosslinking copolymerisation of alkene-functionalised monomers with multi-functional divinyl crosslinkers. Various synthetic strategies have been reported for producing polymeric NPs directly from monomers, including both

conventional and controlled/living radical polymerisation techniques. These can be performed in different environments such as organic solution, miniemulsions, microemulsions, or confined within liposomal nanoreactors.[291]

Aqueous radical polymerisations are preferred for biomedical use due to their biocompatibility. Fully aqueous systems for synthesising crosslinked NPs have been reported using monomers including acrylamide and biomolecular templates, such as proteins or mRNA.[260, 261, 287, 289] These methods rely either on non-covalent interactions between monomers and biomolecules, resulting in local enrichment of polymerisable monomers around the biomolecular surface, or on covalent modification of biomolecules with vinyl groups that serve as anchoring sites for directed polymerisation. These biomolecule-templated polymerisations are typically initiated by free radical-based systems, such as ammonium persulphate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED), at room temperature, leading to the formation of crosslinked NPs that encapsulate biologically active cargo. Indeed, Basak et al. demonstrated the synthesis of crosslinked polymeric NPs encapsulating the enzyme paraoxonase-1 via electrostatic interaction between the negatively charged enzyme and a cationic monomer (2-((acryloyloxy) ethyl)trimethylammonium chloride: AETC).[287] This process allowed for simultaneous encapsulation and crosslinking, while preserving the functional activity of the biological payload. In a similar vein, Dabas et al. showed the synthesis of mRNA-loaded disulfide-based NGs, in a one-pot free radical polymerisation strategy triggered by the APS/TEMED initiating system that have since shown functionality in numerous *in vitro* and *in vivo* models (Figure 1.11).[260, 261]

In addition to redox initiation, aqueous-compatible photoinitiators such as Ir-gacure2959 and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) have been employed to trigger radical polymerisation via light exposure, offering spatiotemporal control over NP formation.[292] However, the use of photoinitiation presents challenges in reproducibility due to factors such as the power, wavelength, and distance of the light source from the reaction vessel, which must be carefully standardised to achieve consistent results.

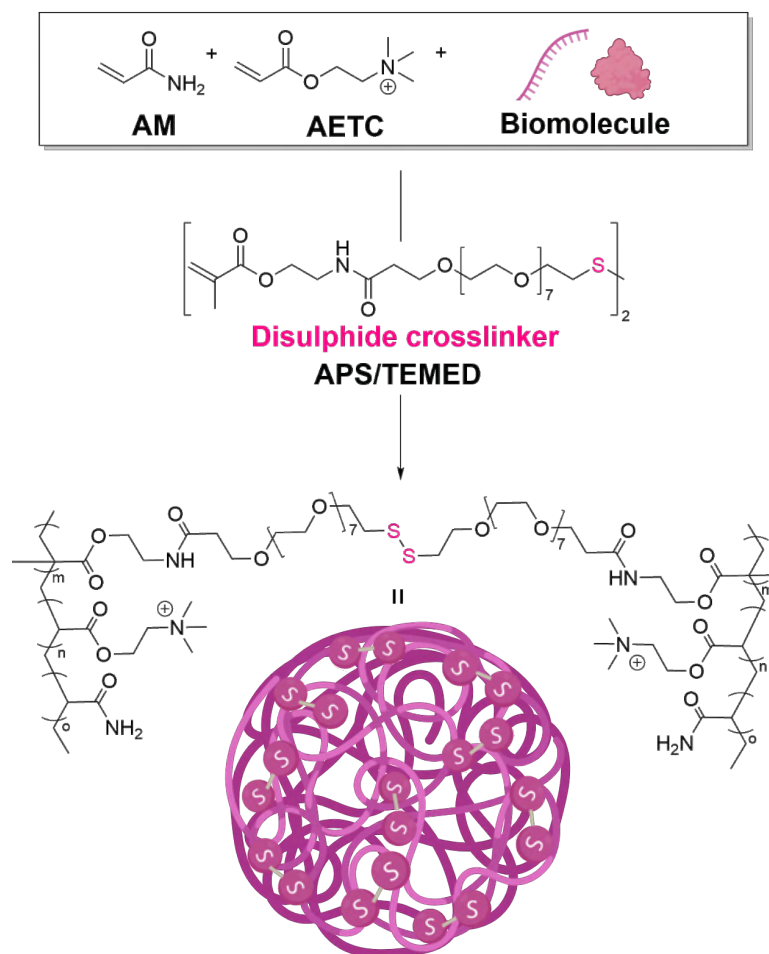


Figure 1.11: **Synthesis of redox-responsive NGs via free radical polymerisation.** The reaction involves the copolymerization of acrylamide (AM) with a cationic monomer, 2-((acryloyloxy)ethyl)trimethylammonium chloride (AETC), in the presence of a disulfide-based crosslinker. Ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) initiate the polymerisation under mild aqueous conditions, leading to the formation of a crosslinked NG network.

1.4.1.3 Active vs passive targeting mechanisms

Passive targeting relies on the enhanced permeability and retention (EPR) effect, a phenomenon whereby NPs preferentially accumulate in certain tissues, particularly tumours, due to their leaky vasculature and poor lymphatic drainage.^[293] The effectiveness of this approach is dependent on the circulation time of the NPs within the bloodstream. In contrast, by decorating the nanoparticle surface with targeting ligands, such as antibodies, antibody fragments (Fab), peptides, aptamers, or nanobodies, the delivery system can bind selectively to receptors overexpressed

on the target tissue, actively enhancing both specificity and uptake.[163, 294, 295] When appropriately engineered, active targeting strategies offer greater therapeutic precision and are particularly valuable for reaching less accessible or heterogeneous tumour sites. Among various biomedical applications, cancer therapy remains the most prominent area for exploiting both passive and active targeting strategies to improve treatment outcomes. There are many possible bioconjugation techniques that permit the surface functionalisation of the NGs, including using activated esters, thiol-ene/yne or thiol-maleimide reactions, imine/oximes formation, cycloadditions, among others.[296] For instance, by co-assembling an azide-terminated polymer, Huynh et al., prepared NGs with controlled surface presentation of a clickable azide motif, which was then conjugated to a dibenzotriazacyclooctyne-functionalised-anti-HER2 antibody.[297] These targeted NGs demonstrated uncompromised and preferential uptake in HER2-presenting cells. In other work, an amine coupling strategy was used to conjugate NGs to aptamers specific to white adipocyte to induce a "browning" phenotype by targeted delivery of docosahexaenoic acid, an anti-obesity agent.[298] In an interesting approach, Zhang et al., used non-covalent hybridisation to conjugate a DNA-nanobody to a miRNA-based nucleic acid NG to enable highly specific anticancer therapy that exhibited a promising targeting ability *in vitro* and *in vivo*. [299] In sum, NGs offer a modular and adaptable platform with virtually limitless potential in therapeutic delivery. Their tunable physicochemical properties, capacity for precise stimulus responsiveness, and compatibility with a wide range of therapeutic cargos and targeting motifs position them as a powerful and flexible toolkit for modern medicine. Whether applied in oncology, infectious disease, regenerative medicine, or gene therapy, NGs offer a rational design framework to meet diverse clinical challenges. This inherent versatility enables the construction of delivery systems tailored not only to the therapeutic agent, but also to the biological environment and disease context, making NGs a universal scaffold for next-generation drug delivery solutions.

1.5 Hallmarks of clinically relevant delivery systems

To date, there are 100 NP-based formulations on the market, with an additional 563 undergoing clinical trials, targeting a wide spectrum of diseases.[278, 279, 300] Despite this breadth of potential, the pace of nanomedicine research is not matched by successful clinical translation. Progress is largely fettered by batch-to-batch variability, poor long-term stability, manufacturing complexities, and misalignments between academic innovation and regulatory expectations. In part, the difficulty arises from our limited understanding of the biophysicochemical interactions of the NPs with biological interfaces. Further, a lack of standardised screening strategies to investigate their interactions in a comprehensive manner, thereby leading to conflicting conclusions even with NPs with similar structural properties.[301] Furthermore, many current NP studies tend to be single-parametric, rendering a thorough prediction of their *in vivo* potential difficult. NP physicochemical properties also have significant influence on their fates in the *in vivo* settings.[239, 302, 303] Recent studies have focused on establishing and exercising precise control on such size, shapes, chemical compositions, surface charges and functionalisations, rigidities and hydrophilicity.[304, 305] The interdependency and interrelatedness of these factors and parameters defines a narrow yet sophisticated window for achieving optimal NP design to maximise transfection efficiency. Indeed, clinical approved RNA delivery systems tend to share a set of defining characteristics that future therapeutic platforms may benefit from emulating:

- **Scalable and biodegradable synthesis:** Built from chemistries that support large-scale production and incorporate biodegradable elements (e.g., ester or disulfide linkages) to enhance safety.
- **Chemically simple and manufacturable:** Composed of minimal, well-defined components that can be reliably produced under Good Manufacturing Practice (GMP) conditions at human scale.
- **Favourable on-target vs. off-target activity:** Demonstrate efficient biodistribution and functional delivery, ensuring that RNA reaches and acts within the intended cells while minimising off-target effects.
- **Wide therapeutic window:** The effective dose remains significantly below toxic levels, ideally confirmed in non-human primates for better translational relevance.
- **Batch-to-batch and storage stability:** Show consistent performance across production batches and retain functional integrity during storage and shipping, including under lyophilised conditions when necessary.
- **Re-dosable without loss of efficacy or safety:** Capable of being administered multiple times without triggering immune responses or diminishing therapeutic effectiveness.

While the clinical translation of NGs remains in its infancy, the design principles demonstrated by successful NP platforms offer a clear and inspiring benchmark. These hallmarks have informed the standards and priorities of this work, guiding efforts to design NGs that are not only functionally effective but also align with key translational criteria. Despite the persistent challenges in formulation consistency, regulatory alignment, and *in vivo* validation, these clinical precedents provide a concrete framework to strive toward. Real progress in the field will require a collective effort to harmonise nanoparticle development and evaluation methods. Still, by anchoring preclinical research in these translational hallmarks, it is possible to close the gap between innovation and implementation, and move NGs closer to clinical reality.

As such, this project will investigate the following aims:

Hypothesis

Overexpression of PGC1 α and its N-terminal isoform (NT-PGC1 α) in PCa cells, delivered selectively via PSMA-targeted redox-responsive polymeric nanogels, can reprogram cellular metabolism towards oxidative phosphorylation, enhance mitochondrial biogenesis, and suppress tumour proliferation and metastatic potential, thereby providing a novel therapeutic strategy for prostate cancer.

- **Design and optimise NG delivery system**
 - Synthesise a library of redox-responsive polymeric NGs capable of RNA encapsulation.
 - Optimise NG formulations for efficient cytosolic release, RNA protection, and transfection efficiency.
 - Functionalise NGs with PSMA-targeting peptides for PCa-specific delivery.
- **Evaluate PGC1 α and NT-PGC1 α expression and function *in vitro***
 - Confirm sustained expression of full-length and NT-PGC1 α in PCa cells post-delivery.
 - Assess mitochondrial biogenesis and oxidative metabolism via mitochondrial protein content, respiration assays, and metabolic profiling.
 - Perform transcriptomic analyses to identify activation of tumour-suppressive pathways and inhibition of proliferative/metastatic markers.
- **Assess Therapeutic Efficacy In Vivo**
 - Determine tumour-targeted delivery and accumulation of NT-PGC1 α -loaded PSMA-NGs in PCa xenograft models.
 - Evaluate antitumour efficacy by measuring tumour growth suppression, metabolic rewiring, and metastasis inhibition.
 - Assess systemic safety and toxicity to ensure translational potential of the NG delivery platform.

Chapter 2

Experimental section

2.1 Standard characterisation methods

Commercial reagents were used as supplied, without any further purification. All non-aqueous reactions were conducted under a nitrogen atmosphere in flame-dried glassware using conventional inert-atmosphere techniques. Flash column chromatography was carried out on 230–400 mesh silica gel with the specified solvent systems, following standard procedures. Analytical TLC was performed on pre-coated aluminium silica gel sheets.

2.1.1 Nuclear magnetic resonance (NMR)

^1H and ^{13}C NMR spectra were recorded using a Bruker AV 400 MHz spectrometer at room temperature. ^1H NMR spectra were recorded and chemical shifts are expressed in parts per million (ppm) relative to tetramethylsilane (TMS), using the residual solvent signal as an internal reference. The following solvent peaks were used: CDCl_3 at $\delta = 7.27$ ppm, D_2O at $\delta = 4.79$ ppm, and DMSO-d_6 at $\delta = 2.50$ ppm. Spectral data are presented as chemical shift values, followed by signal multiplicity [s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad], coupling constants (J, in Hz), and the number of protons represented (integration). ^{13}C NMR spectra were acquired under complete proton decoupling conditions. Chemical shifts are reported in ppm and referenced to the solvent peak of CDCl_3 at $\delta = 77.0$ ppm.

2.1.2 Fourier–Transform infrared spectra (FT-IR)

FT-IR spectra were collected using Agilent technologies Cary 630 FTIR. Measurements were performed at room temperature in the wavenumber range of 650 cm^{-1} to 4000 cm^{-1} . Air was used as the background reference.

2.1.3 Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry data were collected using a Waters® Micromass® MALDI microMX™ MALDI Q-ToF instrument. Samples were prepared by mixing the analyte solution

with an appropriate MALDI matrix (α -cyano-4-hydroxycinnamic acid, CHCA) at a defined ratio, and 1–2 μ L of the mixture was spotted onto a stainless-steel MALDI target plate and allowed to air dry. The instrument was calibrated using a standard peptide or protein calibration mixture to ensure accurate mass assignment. Data acquisition was performed in positive-ion mode, with the laser intensity, the number of laser shots per spectrum (on average 200), and acquisition mass range optimised for the analytes of interest. Spectra were collected and averaged from multiple laser shots to improve signal-to-noise ratio. Mass spectra were processed using the instrument’s software for baseline subtraction, smoothing, and peak picking. Molecular weights were assigned based on comparison with the calibration standards, and the isotopic distribution of the detected peaks was examined to confirm analyte identity.

2.1.4 Dynamic light scattering (DLS) and electrophoretic light scattering (ELS)

DLS was used to measure NG size, polydispersity index, whereas *zeta*-potential was determined via ELS. For DLS, the Malvern Zetasizer Ultra (Imperial College London) instrument with backscatter detection at 173° ($n = 3$) was used. Each run measured 25 subscanning cycles. Before loading the sample, it was filtered using MF-Millipore™ membrane filter (0.45 μ m pore size, Merck Millipore, UK). The size measurements were taken at 22 °C for each NG synthesised. ζ -potential measurements were carried out using a Zetasizer Nano-ZS instrument with a folded capillary cell in RNase-free water (pH 7.4). A total of 50 subscans were acquired per sample at 22 °C, and the applied voltage was set to 100 V, following recommended conditions to minimise Joule heating and improve stability.

Recalculations of ζ -potential values were performed based on the measured electrophoretic mobility μ_e using Henry’s equation:

$$\zeta = \frac{3\eta\mu_e}{2\varepsilon_0\varepsilon_r f(\kappa a)}$$

where:

- ζ is the zeta potential,
- μ_e is the electrophoretic mobility,
- η is the dynamic viscosity of the solvent,
- ε_0 is the vacuum permittivity,
- ε_r is the relative permittivity (dielectric constant),
- $f(\kappa a)$ is Henry's function, dependent on Debye length and particle size.

The solvent parameters used for these calculations were:

- Relative permittivity (dielectric constant), $\varepsilon_r = 78.5$
- Dynamic viscosity, $\eta = 0.8870 \text{ mPa s}$
- Refractive index, $n = 1.3300$

The ionic strength I was determined based on the composition of the measurement solution. Where needed, $f(\kappa a)$ was approximated using the Smoluchowski model ($f(\kappa a) = 1.5$) for high ionic strength aqueous conditions. In cases requiring refinement, the Debye length and ionic strength were computed using:

$$\kappa = \sqrt{\frac{2N_A e^2 I}{\varepsilon_0 \varepsilon_r k_B T}} \quad \text{and} \quad I = \frac{1}{2} \sum_i c_i z_i^2$$

where:

- N_A is Avogadro's number,
- e is the elementary charge,
- k_B is the Boltzmann constant,
- T is the absolute temperature,
- c_i and z_i are the concentration and valence of ion i .

2.1.5 Transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) NG suspensions were prepared in ultrapure water (pH 7.4) at a concentration of 1 mg mL^{-1} . A volume of $4 \mu\text{L}$ was pipetted directly onto a glow-discharged lacey carbon film grid (300 mesh copper, Agar Scientific, UK). Glow discharge was performed under vacuum for 30 s at 15 mA to render the carbon surface hydrophilic, improving sample adherence. The suspension was allowed to adsorb to the grid for 1 min, after which excess solution was gently removed using filter paper. The grid was then stained with $4 \mu\text{L}$ of 2% (w/w) uranyl acetate for 1 min to provide negative contrast, enhancing the visibility of the NG structures. Excess stain was removed gently with filter paper, and the grids were air-dried at room temperature for 5 min before imaging. Samples were imaged using a Talos F200X G2 Transmission Electron Microscope (Thermo Fisher Scientific) operating at 200 kV. Images were acquired at a nominal magnification of $45,000\times$, using a Ceta CMOS camera. Multiple fields of view were recorded for each sample to ensure representative imaging of particle size, morphology, and distribution. Image processing and analysis, including particle size measurement, were performed using ImageJ (NIH, USA).

2.1.6 UV-vis absorption spectroscopy and UV-vis fluorescence spectrophotometry

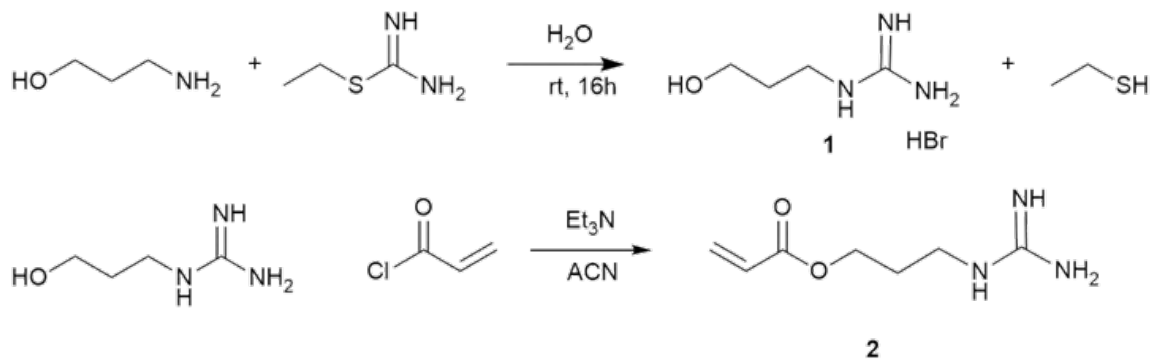
UV-vis absorption spectra of the NGs (0.1 mg mL^{-1}) were recorded using an Agilent, Cary 60 UV-vis spectrophotometer over the wavelength range of 200 nm to 800 nm. Fluorescence spectra were acquired using an Agilent, Cary Eclipse spectrofluorometer. Samples were excited at the respective excitation wavelength for the each fluorophore used in the study, and emission spectra were collected in the range of 360 nm to 700 nm. All measurements were conducted at room temperature. NG samples were dispersed in deionised water and measured in a quartz cuvette with a 1 cm path length. Instrument parameters such as excitation/emission slit widths, scan speed, and PMT voltage were optimised as required. Baseline correction was performed using the corresponding solvent or buffer as a blank.

2.1.7 SPARTA Raman Micro-Spectroscopy Analysis

Raman spectra were acquired using the SPARTA (Single Particle Automated Raman Trapping Analysis) platform on an alpha300R+ confocal Raman micro-spectroscope (WITec, Ulm, Germany), equipped with a 785 nm Toptica XTRA II laser (SPARTA Biodiscovery). A 63 $\times$ /1.0 NA water immersion objective lens (W Plan-Apochromat, Zeiss, Oberkochen, Germany) was employed for sample interrogation. Raman-scattered light was directed through a 100 μ m optical fibre to a UHTS 300 spectrograph (600 grooves/mm, WITec) and detected using a thermoelectrically cooled, back-illuminated CCD camera (iDus DU401-DD, Andor, Belfast, UK), offering a spectral resolution of 3 cm^{-1} . The laser power at the sample was maintained at 85 mW, and remote control of the laser was performed via serial interface using custom MATLAB scripts (version 2016b, MathWorks, MA, USA). For sample preparation, 200 μ L of NG solution was used. Ideal particle concentrations ranged from 1×10^{10} to 1×10^{12} particles/mL. Samples were mounted by pipetting onto a 22 mm coverslip placed on a standard microscope slide and secured with a droplet of phosphate-buffered saline (PBS). Measurements were conducted under the water immersion objective. Spectral preprocessing included truncation of the raw spectra to 350–1825 cm^{-1} to eliminate the excitation peak, with the spectral centre set to 1000 cm^{-1} . Cosmic ray spikes were removed using an automated algorithm based on peak intensity and second derivative analysis, followed by manual inspection. Background correction was performed via Whittaker smoothing ($\lambda = 100,000$), and spectral noise was reduced using a 3-point, first-order Savitzky–Golay filter. Spectra were normalised to the total area under the curve, except for sizing analyses, which relied on perchlorate peak ratio normalisation. Subsequent multivariate analyses, including hierarchical cluster analysis (HCA), principal component analysis (PCA), multivariate curve resolution (MCR), and partial least squares discriminant analysis (PLS-DA), were carried out using the software on the instrument.

2.2 Synthesis of cationic monomers

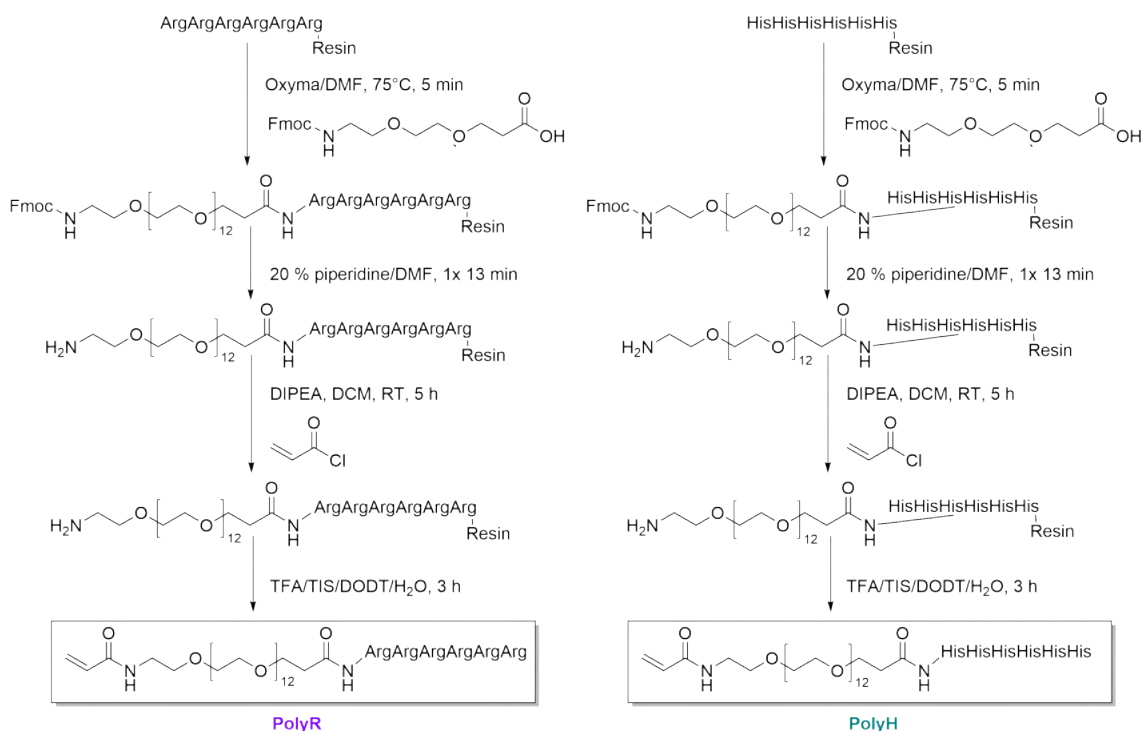
2.2.1 Acrylated guanidinium (AC-GUA)



Scheme 2.2.1: Schematic representation of AC-GUA synthesis.

3-aminopropanol (1 equiv., 0.65 g, 8.54 mmol) was slowly added to 2-ethyl-2-thiopseudourea hydrobromide (1 equiv., 1.58 g, 8.54 mmol) in a 25 mL flask, to which 2 mL of deionised water was added. The flask was sealed and left to stir overnight at room temperature. After lyophilising the reaction mixture, a viscous transparent oil was obtained (1). To this, 5 mL of acetonitrile was added and this was allowed to dissolve at room temperature for 1 h. Freshly activated molecular sieves were used to remove any residual water. Triethylamine (1 equiv., 0.85 mL, 8.43 mmol) was added dropwise and allowed to stir for 10 min. The flask was then kept at 0 °C and acryloyl chloride (1 equiv., 0.76 mL, 8.43 mmol) was added dropwise. The solution was stirred for 24 h at 0 °C. Acetonitrile was then removed *in vacuo* and crude product washed with ethyl acetate/ethanol (1:1) and filtered to remove molecular sieves. Yield 72.1% $R_f = 0.35$ (20% methanol/ethyl acetate). IR (film)/cm⁻¹ N-H stretch: 3700–3500 cm⁻¹; alkyl sp³ C-H stretch: 2950–2800 cm⁻¹ (strong), carbonyl C=O stretch: 1655 cm⁻¹ (strong); C-O-C stretch: 1095 cm⁻¹ (sharp), ¹H NMR (400 MHz, D₂O): δ6.35 (d), δ6.11 (dd), δ5.86 (d), δ4.18 (t), δ3.22 (t), δ2.25 (s), δ1.89 (q). APCI-MS m/z M = (C₇H₁₃N₃O₂) [M + H]⁺ = 172.1064 m/z.

2.2.2 Polyarginine (R6) and Polyhistidine (H6)



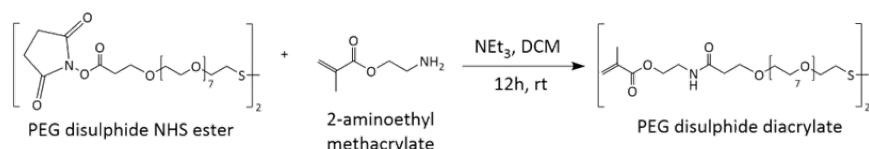
Scheme 2.2.2: Schematic representation of H6 and R6 synthesis.

Both peptides were synthesised using standard Fmoc-based solid-phase peptide synthesis (SPPS) protocols on a Rink amide resin (0.60 mmol/g, 100 to 200 mesh, Merck). For the synthesis of polyarginine (R6) and polyhistidine (H6), 100 mg of Rink amide resin was pre-swollen in dichloromethane (DCM) for 30 min, followed by washing with *N,N*-dimethylformamide (DMF, 3 × 5 mL). Fmoc deprotection was carried out using 20% piperidine in DMF (v/v) for 2 × 10 min, followed by DMF washes (3 × 5 mL). Coupling of each amino acid was performed using 4 equiv. (1.24 g) of Fmoc-Arg(Pbf)-OH or Fmoc-His(Trt)-OH (0.84 g), 4 equiv of Oxyma Pure, and 4 equiv of diisopropylcarbodiimide (DIC) in DMF (final concentration ~0.2 M). The reaction was allowed to proceed for 30 min under gentle agitation. This coupling-deprotection cycle was repeated six times to construct the hexameric peptide (Arg or His). After the final amino acid coupling and Fmoc deprotection, the resin was washed thoroughly with DMF and DCM and dried under vacuum. Peptides were cleaved from the resin using a cleavage cocktail composed of trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water (95:2.5:2.5, v/v/v) for 2 h at room temperature.

The cleavage mixture was filtered, and the filtrate was concentrated under a stream of nitrogen. Crude peptides were precipitated in cold diethyl ether (-20°C), centrifuged (4000 rpm, 10 min), washed twice with cold ether, and dried under vacuum. Yield: 54.4%, 67.8% for R6 and H6, respectively. R6: ^1H NMR (400 MHz, D_2O): δ 7.15 (d), 6.87 (d), 4.23 (t), 3.85 (t), 3.77 (t), 3.58 (tt), 3.48 (t), 3.34 (t), 3.11 (t), 2.50 (m), 1.70 (q), 1.11 (t). Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS): $m/z = 1666.9$ ($[\text{M} + \text{H}]^+$); calculated for $\text{C}_{69}\text{H}_{135}\text{N}_{25}\text{O}_{22}$. H6: ^1H NMR (400 MHz, D_2O): δ 8.54 (s), 7.20 (m), 5.58 (d), 5.34 (d), 3.69 (t), 3.58 (tt), 3.48 (t), 3.09 (t), 1.15 (t). MALDI-TOF MS: $m/z = 1552.8$ ($[\text{M} + \text{H}]^+$); calculated for $\text{C}_{69}\text{H}_{105}\text{N}_{19}\text{O}_{22}$.

2.3 Synthesis of disulfide crosslinker

2.3.1 Procedure and characterisation



Scheme 2.3.1: Schematic representation of SS-DiA crosslinker synthesis.

2-aminoethyl methacrylatehydrochloride (2 equiv., 0.04 g, 0.22 mmol) (Sigma Aldrich, UK), 0.06 mL, 0.16 of triethylamine (Fluorochem, UK) and 3 mL of anhydrous dichloromethane (DCM) (Sigma Aldrich, UK), were allowed to mix for 20 min under an inert atmosphere, in a 25 mL round bottomed flask. After dissolving the 2-aminoethylmethacrylatehydrochloride, 4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-Hexadeca-28,29-dithiahexapenta-contanedioic acid di-*N*-succinimidyl-ester (1 equiv., 0.05 g, 0.05 mmol) (Sigma Aldrich, UK), in 1 mL anhydrous DCM and adding to the flask, and 4 mL of anhydrous DCM were added and the reaction flask was left to stir under nitrogen at room temperature overnight. Subsequently, the mixture was concentrated *in vacuo* and purified via flash chromatography (5% methanol: DCM). The purified fractions were concentrated to attain a pale-yellow oil. Yield: 68%. R_f : 0.26 IR cm^{-1} 3700–3200 (broad), 2950–2850 (sharp), 1650 (sharp). ^1H NMR (400

MHz, (CD₃)₂SO): δ 8.05 (t, 2H), 6.07 (s, 2H), 5.69-76 (m, 2H), 4.05-4.09 (m, 8H), 3.61-3.65 (t, 4H), 3.57-3.59 (t, 4H), 3.48-3.53 (m, 56H), 2.88-20.91 (t, 4H), 2.31-2.34 (t, 4H), 1.88 (s, 6H). ¹³C NMR (400 MHz, (CD₃)₂SO): δ 171.04, 166.89, 135.18, 125.36, 69.98-70.24, 69.20, 64.38, 64.54, 38.23, 36.79, 35.58, 18.49. MALDI-ToF (C₅₀H₉₂N₂O₂₂S₂⁺): calc: 1137.5656 m/z, actual: 1137.5660 m/z.

2.3.2 Confirmation of cleavage of disulfide crosslinker with GSH.

Disulfide crosslinker was incubated with GSH at 20 mM for 24 h. MALDI-ToF (in ultrapure water) was used to detect the presence of the cleaved disulfide product. APCI-MS [M+H]⁺ (*m/z*): 577.7885, where M = CH₃C(CH₂)COOCH₂CH₂NHOOCCH₂CH₂O [CH₂CH₂O]₇CH₂CH₂SH.

2.4 NG synthesis

2.4.1 mRNA-loaded NG synthesis

RNA-loaded nanogels were synthesized with slight modifications to a previously reported protocol, aimed at minimising RNA degradation. Prior to the procedure, all equipment was UV-sterilized and oven-dried overnight to ensure RNase-free conditions. Surfaces were thoroughly cleaned with RNaseZap (ThermoFisher), and RNase-free, nitrogen-degassed water was used throughout the synthesis. Briefly, 6 μ g of RNA was added to a 1 mL reaction solution containing acrylamide (AM; 2 mg, 0.029 mmol), a disulfide-based crosslinker (4.5 mg, 0.004 mmol), 2-[(acryloyloxy)ethyl]trimethylammonium chloride (AETC) or an alternative cationic monomer (2 μ L, 0.01 mmol) and mPEG(2kDa)-acrylate (100 μ g, 0.05 μ mol). For targeted NG, acrylate-PEG(1kDa)-maleimide (0.1 %mol) was added to the monomer mixture. The mixture was stirred on ice for 30 minutes under a nitrogen atmosphere. For fluorescently labeled NGs, fluorescein o-acrylate or Nile blue acrylamide (0.2 mg, 0.005 mmol) was added, and the reaction was protected from light. Micelle formation was induced by the addition of sodium dodecyl sulphate (SDS; 8 mg, 0.028 mmol), followed by a further 30-minute stirring period. Polymerisation was initiated by adding ammonium persulphate (APS; 2.28 mg, 0.01 mmol) and N,N,N',N'-

tetramethylethylenediamine (TEMED; 2.5 μL , 0.022 mmol) in 1 mL of RNase-free, deoxygenated water. The reaction mixture developed a pale blue opalescence within 30 minutes, indicating successful polymerisation. To monitor monomer conversion, parallel reactions were conducted in deoxygenated D_2O , and aliquots were withdrawn at defined time points for ^1H NMR analysis. Polymerization was terminated by exposure to air once opalescence appeared. The resulting NGs were purified via centrifugation at 1200 x g for 10 minutes using 15 mL Amicon[®] Ultra-15 centrifugal filter units with a 300 kDa MWCO (Sigma Aldrich, UK). The samples were washed five times with RNase-free water and stored at 2 °C–8 °C until further use.

2.4.2 PSMA-targeted NG synthesis

Lyophilised PSMA-peptide was reconstituted in DMSO and various concentrations were added to maleimide-functionalised nanogels (10 mg mL⁻¹) in aqueous buffer (pH 6.5–7.5). The conjugation reaction was performed overnight at room temperature under gentle rotation to allow for efficient thiol–maleimide coupling. Following incubation, the reaction mixture was purified by centrifugal filtration using 2 mL Amicon[®] Ultra-2 centrifugal filter units (50 kDa MWCO, Merck Millipore, UK). Samples were washed three times with Milli-Q water at 4000 x g, 4 °C for 10 minutes per wash to remove unreacted peptide and byproducts. To assess the extent of conjugation, the residual maleimide content on the nanogels was quantified post-coupling using a colorimetric maleimide quantification assay (MAK162, Sigma-Aldrich, UK). Based on this data, as well as the nanogel concentration (approximated as 1 mg mL⁻¹) and particle size, the surface density of maleimide groups was calculated using Equations 2.1 and 2.2.

$$\#_{\text{mal/NG}} = \frac{4}{3}\pi r^3 \times [\text{mal}] \times \rho \times N_A \quad (2.1)$$

$$\#_{\text{mal/nm}^2} = \frac{\#_{\text{mal/NG}}}{4\pi r^2} \quad (2.2)$$

where r is the nanogel radius (in metres), $[\text{mal}]$ is the maleimide concentration (mol/m^3), ρ is the nanogel density (assumed as $1.0 \text{ g}/\text{cm}^3$ or $1.0 \times 10^9 \text{ mg}/\text{m}^3$), and N_A is Avogadro's number ($6.022 \times 10^{23} \text{ mol}^{-1}$). To estimate the number and surface density of PSMA-peptides on the nanogels, absorbance measurements at 280 nm were taken before and after peptide conjugation. The differential absorbance (ΔA_{280}), attributable to the naphthylalanine residue, was used to determine peptide concentration via the Beer–Lambert law:

$$[\text{PSMA-peptide}] = \frac{\Delta A_{280}}{\varepsilon_{280} \cdot l} \quad (2.3)$$

where $\varepsilon_{280} = 4395.44 \text{ M}^{-1}\text{cm}^{-1}$ is the molar extinction coefficient of the peptide and $l = 0.1 \text{ cm}$ is the path length. Using the peptide concentration $[\text{PSMA-peptide}]$ in place of $[\text{mal}]$, the number of PSMA-peptides per nanogel and their surface density were calculated using analogous equations:

$$\#_{\text{pep/NG}} = \frac{4}{3}\pi r^3 \cdot [\text{PSMA-peptide}] \cdot \rho \cdot N_A \quad (2.4)$$

$$\#_{\text{pep/nm}^2} = \frac{\#_{\text{pep/NG}}}{4\pi r^2} \quad (2.5)$$

2.5 Biological-focused NG and mRNA characterisation routines

2.5.1 *In vitro* transcription of mRNA

mRNA encoding all transcripts used in the work was transcribed *in vitro* from PCR-amplified DNA templates. Templates for GFP, RNA Mango aptamers, $\beta 2$, mCardinal, PGC1 α , NT-PGC1 α and GFP were generated by PCR from the following plasmids: mCherry–Mango II $\times$ 24 (Addgene 127587), Mango II $\times$ 9, AMPK- $\beta 2$ -FLAG (Addgene 40603) mCardinal (Addgene 51311), PGC1 α (Addgene 10974) and GFP (Addgene 13031). The DNA templates for GFP, $\beta 2$, PGC1 α and NT-PGC1 α were produced by polymerase chain reaction of the respective plasmids and adding T7 promoter sequence through PCR extension wherever necessary

(BioMix™ Red, Bioline). After each PCR step, purification and gel extraction were performed (QIAquick Gel Extraction Kit, Qiagen), according to the manufacturer's instructions. T7 promoter sequences were introduced during PCR amplification using BioMix™ Red polymerase (Bioline). Following each PCR reaction, the products were purified and gel-extracted using the QIAquick Gel Extraction Kit (Qiagen), following the manufacturer's instructions. *In vitro* transcription was performed using the mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen) according to the manufacturer's protocol. The resulting mRNA was subsequently polyadenylated using the Poly(A) Tailing Kit (Invitrogen). Final purification of the mRNA was carried out using the RNeasy Mini Kit (Qiagen). RNA concentration and purity were assessed using a NanoDrop One spectrophotometer (ThermoFisher, UK), and samples were stored at -80°C until use. Each plasmid DNA was transformed into *E. coli* 5 α (New England BioLabs, UK) using standard heat-shock treatment. Following a 1 hour recovery at 37°C with agitation, the bacteria were plated on Luria broth (LB) agar plates containing ampicillin ($100\text{ }\mu\text{g mL}^{-1}$) (Sigma-Aldrich, UK) and incubated overnight at 37°C . Single colonies were picked the next day and used to inoculate 10 mL of liquid LB medium containing $100\text{ }\mu\text{g mL}^{-1}$ ampicillin. Cultures were grown at 37°C for 5 h, after which 200 μL of the suspension was transferred to 200 mL of fresh LB medium supplemented with ampicillin and cultured to saturation. Plasmid DNA was extracted using the Plasmid Plus MaxiPrep Kit (Qiagen, UK) following the manufacturer's protocol. The concentration and purity of the purified plasmid DNA were assessed using a NanoDrop One spectrophotometer (ThermoFisher, UK), and samples were stored at -20°C .

2.5.2 Characterisation of mRNA-NG complexation ability and mRNA stability by agarose gel electrophoresis

To identify the optimal amount of cationic monomer needed to enable efficient mRNA complexation and loading, gel electrophoresis was used. Twenty microliters of the complex solution or 200 ng of naked mRNA as control was loaded on to Reliant™ Precast RNA Gels (1X MOPS buffer) and gel electrophoresis was performed. The gel was visualised using the iBright™ CL750 Imaging System (Invitrogen). Stability of

mRNA inside the NGs was investigated by incubation with RNase A. NG formulation or naked mRNA was incubated with RNase A (Invitrogen) at 37 °C for 1 h, followed by mixing with RNA-loaded dye (Thermo Fisher Scientific) and gel electrophoresis as described.

2.5.3 Cell culture

C4-2, C4-2b, bEND.3, RAW264.7, Caco-2, and HeLa cell lines were sourced from the American Type Culture Collection (ATCC). β_2 -knockout HEK293T cells were generated in-house in the Carling Lab. C4-2 cells were cultured on flasks and plates coated with poly-*L*-lysine (0.1 mg/mL; Thermo, UK) in RPMI 1640 medium (Thermo, UK), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Sigma-Aldrich, UK). bEND.3, RAW264.7, Caco-2, and HeLa cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; GibcoTM), also supplemented with 10% FBS and 1% penicillin–streptomycin. For all experiments, C4-2, bEND.3, and RAW264.7 cells were used at passage numbers below 25. All cell lines were maintained at 37 °C in a humidified incubator with 5% CO₂. C4-2, C4-2b, Caco-2, HEK293T and HeLa cell lines were split twice a week (ratio 1:10) while maintaining cultures. RAW264.7 and bEND.3 cells were split at a ratio of 1:5 once a week to maintain the cultures.

2.5.4 Protocols for *in vitro* transfection studies

C4-2, HEK293T, bEND.3, RAW264.7, Caco-2, and HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well, in triplicate for each condition. Cells were allowed to adhere overnight prior to the experiment. The plates were placed in the IncuCyte instrument, housed within a standard humidified incubator maintained at 37 °C and 5% CO₂. Cells were treated with various concentrations of AETC_NGs suspended in complete culture medium containing 10% FBS. Where applicable, transfection efficiency was benchmarked against two commercial reagents—Polyplus® jetMESSENGER® and LipofectamineTM MessengerMaxTM—each prepared following the manufacturer’s protocol using 3.25 μ g of RNA. Automated phase-contrast and green fluorescence imaging was performed using the IncuCyte system. Images were

acquired every 1 hour over a total period of 86 hours, with four images captured per well (image resolution: 1.22 μm /pixel). Green fluorescence was imaged using the default exposure time of 800 ms. Initial image analysis was optimised using untreated control wells to establish a threshold for background fluorescence. Processing definitions, including confluence masks for both fluorescence and phase-contrast images, were adjusted to minimise background signal and exclude cellular debris. Once optimal settings were determined, they were applied uniformly across all wells and cell types. GFP fluorescence was automatically normalised to cell density by the IncuCyte software to account for differences in cell number between wells. To quantify total cellular protein content, cells were lysed in RIPA buffer and centrifuged at 14 000 rpm for 10 minutes at 4 °C. The resulting supernatant was used to determine protein concentration via the BCA protein assay (ThermoFisher, UK), following the manufacturer's instructions.

2.5.5 Cellular uptake studies via confocal microscopy

HeLa cells (5×10^4 cells/well) were seeded into μ -Slide 8-well chambered slides (Ibidi, GmbH) and incubated overnight. The following day, the medium was replaced with fresh culture medium containing 20 $\mu\text{g}/\text{mL}$ of fluorescent nanoparticles (NGs; fluorescein-labelled: 494/521 nm; Nile blue-labelled: 635/670 nm), and the cells were incubated for 2–4 hours. After treatment, cells were washed three times with PBS and fixed using 4% paraformaldehyde for 10 minutes on ice. Subsequent washes were followed by nuclear staining with Hoechst 33342 (ex/em: 350/461 nm) (100 ng/mL in PBS) for 15 minutes. Cells were again washed thrice with PBS and imaged using a Leica TCS SP8 DLS inverted confocal microscope (Leica, Germany) with LAS X software. Image analysis was performed using ImageJ (NIH, USA).

2.5.6 Endosomal escape analysis via confocal microscopy

HeLa cells (5×10^4 cells/well) were seeded into μ -Slide 8-well chambered slides (Ibidi, GmbH) and allowed to adhere overnight. On the next day, cells were treated with 20 $\mu\text{g}/\text{mL}$ of fluorescein-labelled NGs (FLNG and FLSSNG: ex/em: 494/521 nm) for 4 hours. Following treatment, cells were washed three times with PBS and

stained with 75 nM LysoTracker Red (577/590 nm) for 30 minutes at 37 °C to label late endosomes and lysosomes. Cells were then fixed in 4% paraformaldehyde for 10 minutes on ice, washed, and stained with Hoechst 33342 (ex/em: 350/461 nm) (100 ng/mL) in PBS for 15 minutes. Imaging was conducted using the Leica TCS SP8 DLS microscope. Image analysis was performed using ImageJ (NIH, USA).

2.5.7 Quantification of cellular uptake via flow cytometry

HeLa cells (1×10^5 cells/well) were seeded into 24-well plates and incubated overnight. Cells were then treated with 20 µg/mL of fluorescein-labelled disulfide and non-disulfide NGs (ex/em: 494/521 nm) for 8 hours. Post-incubation, cells were washed with PBS, trypsinised, and collected by centrifugation at 1200 rpm for 3 minutes. The pellet was washed with 500 µL PBS, centrifuged again, and resuspended in FACS buffer (1% BSA in PBS). Flow cytometry was performed using the FACSymphony™ A3 instrument (BD Biosciences). Single-cell suspensions were prepared and stained with appropriate fluorophore-conjugated dyes. During acquisition, at least 10,000 events per sample were collected. Gating was performed sequentially using FlowJo software (vX, BD Biosciences): live, single cells were selected based on forward scatter (FSC) vs side scatter (SSC), doublets were excluded using FSC-A vs FSC-H, and dead cells were excluded using a viability dye where applicable. Fluorescence gates were defined using unstained, single-stained, and fluorescence-minus-one (FMO) controls. Mean fluorescence intensities (MFI, %) of the gated populations were calculated to assess marker expression and functional readouts.

2.5.8 Thiol-mediated uptake inhibition assay

HeLa cells (1×10^5 cells/well) were seeded into 12-well plates and incubated overnight. After 24 hours, the culture medium was replaced with media containing thiol-blocking agents provided by the Matile Lab (University of Geneva). The inhibitors were used at the following concentrations: DTNB (1.2 mM), Iodoacetic Acid (IA, 10 mM), AspA (500 µM), BI (10 µM), CTO (100 µM), EBS (100 µM), ETP (25 µM), MAC (100 µM), and SS (10 µM) in DMEM for 1 hour at 37 °C. All inhibitor solutions were prepared in media with final DMSO concentration below 0.1%. [306–309] Following

treatment, cells were washed with PBS ($3 \times 500 \mu\text{L}$), and subsequently incubated with $50 \mu\text{g/mL}$ NGs in DMEM ($400 \mu\text{L}$) for 4 h. Cells were harvested and analysed as described before.

2.5.9 Immunofluorescence studies

C4-2 or HeLa cells used for imaging were seeded in poly-L-lysine-treated Ibidi glass-bottomed 8-well chamber slides (Ibidi, GmbH) at a seeding density of 50,000 cells/well and were left to adhere overnight prior to the transfection and imaging experiments. The cells were either left untreated or treated with NGs (FLSSNG or FLNG (ex/em: 494/521 nm) or PGC1 α RNA-loaded NGs ($40 \mu\text{g/mL}$) for a period of 24 h and in the case of the experiment relating to PGC1 α , the cells were treated with or without BI ($10 \mu\text{M}$)) for an additional 24 h. The cells were then fixed in 4% paraformaldehyde for 10 min on ice and washed thrice with PBS. Cells were permeabilised in 0.1% Triton X-100 in PBS for 3 min and washed thrice with PBS. Blocking was performed in 1% BSA in PBS for 1 h, followed by PBS washes and overnight incubation in primary antibody (PGC1 α (rabbit mAb, 1:100 NBP1-04676); Rab5 (rabbit mAb, 1:200, CST 3547S); Clathrin (rabbit mAb, 1:50, CST 4796S), Caveolin-1 (rabbit pAb, 1:400, CST 3238S)). After washing, secondary antibody Goat anti-Rabbit IgG (H+L) Alexa Fluor™ 568 (1:500)(ex/em: 577/590 nm) was added for 1 h. The cells were counterstained with Hoechst 33342 (ex/em: 350/461 nm) ($1 \mu\text{g/mL}$) for 10 min, washed, and imaged on a Leica TCS SP8 DLS inverted microscope (Leica, Germany) using LASX software. Image analysis was performed using ImageJ (NIH, USA), and colocalisation was quantified using JACoP, reporting Pearson's correlation coefficient.

2.5.10 Transfection efficiency quantification by western blot analysis

$\beta 2$ -knockout HEK293T and C4-2 cells (1×10^6) were seeded into 6 cm collagen-coated plates and incubated for 24 h. After replacing with fresh media, cells were treated with various concentrations of $\beta 2$ or PGC1 α -loaded NGs for specific durations. For protein collection, cells were washed twice with ice-cold PBS and lysed using RIPA buffer. Lysates were centrifuged at 14 000 rpm for 10 min at 4 °C, and supernatants were collected. Protein content was quantified using a BCA assay kit (ThermoFisher,

UK). Samples were resolved by NuPAGE™ 4–12% Bis-Tris gels and transferred to PVDF membranes. Primary antibodies were used at 1:1000 dilution: PGC1 α (Novus, NP1-04676), total OXPHOS (Abcam, ab110413), AMPK β 1 β 2 (CST, 4150), vinculin (Sigma, V9131), phospho-CAMKK2 (Ser155) (CST, 12818), cleaved caspase (CST, 18C8), and NT-PGC1 α (Santa Cruz, sc-518025), mCardinal (STJ140229). Secondary antibodies used were LI-COR IRDye (1:10,000; ex/em: 680/694 nm and/or 780/794 nm) or HRP-conjugated (1:3000), with detection via Odyssey Infrared Imager or Amersham ImageQuant 800. Quantification was done using Odyssey software or ImageJ, and results were expressed as the signal ratio relative to control antibodies.

2.5.11 Transfection efficiency quantification by RT-qPCR

RNA was extracted from human C4-2 cells using TRIzol reagent (Invitrogen) and purified using an RNeasy column (Qiagen). cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™), followed by qPCR using PowerUp™ SYBR™ Green Master Mix. Gene expression was normalised to GAPDH. RT-qPCR was conducted using a CFX Connect system (Bio-Rad, UK), with technical triplicates for each reaction. C_t values were averaged and analysed using the calibration curve method. All primer sequences used in this study are detailed in Tables 2.1 and 2.2.

Table 2.1: RT-qPCR primer sequences (F labelled for forward, R labelled for Reverse)

Target Gene	Direction	Sequence (5'–3')
PGC1 α	F	AGCCTCTTTGCCCAGATCTT
	R	GGCAATCCGTCTTCATCCAC
mCardinal	F	ACAACGTCAAGCTCAGAGGG
	R	TTCTTTCCAGTCTGCGGTCC
GAPDH	F	ACTCCACTCACGGCAAATTC
	R	TCTCCATGGTGGTGAAGACA
NT-PGC1 α	F	TCACACCAAACCCACAGAGA
	R	CTGGAAGATATGGCACAT

Table 2.2: PCR primer sequences used in this study (F labelled for forward, R labelled for Reverse). T7 promoter sequences are included at the 5' ends.

Target Gene	Direction	Sequence (5'-3')
GFP	F	TAATACGACTCACTATAGGATGCCCGCCATGAAGATCGAGTGCCGC
	R	TTTTTTGGCGAAGCGGATGGGGTCTTTGAAGGCGTGTGG
mCardinal	F	TAATACGACTCACTATAGGAGAGACCCAAAGCTTGGTACCGAGCTC
	R	CTTACTTGTACAGCTCGTCCATGCCATTAAAGTTTGTGCCCC
Mango II 24	F	ATTCTAATACGACTCACTATAGCGCCACCATTGGTGAGCAAGGGCGAGGA
	R	TCAGTTGTGGTTTGTCCAAACTCATCGGATCTGATGAACCCCTGG
Mango II 9	F	TAATACGACTCACTATAGGG
	R	CTAGTTGTGGTTTGTCCAAACTCATCGGAT
U6-MangoIV	F	TAATACGACTCACTATAGCGCCACC
	R	AAAAAATATGGAACGCTTCACG
T75S-F30-MangoIV	F	TAATACGACTCACTATAGCGCCACCCTCTACGG
	R	TTGCCATGAATGATCCCCGAAGG
NT-PGC1 α	F	TAATACGACTCACTATAGGATGGCGTGGGACATGTGCAACC
	R	GTTACTTGTTCATCGTCGTCCCTTGTAGTCTAAAAAC

2.5.12 Mitochondrial biogenesis assessment using flow cytometry

C4-2 cells (2×10^5 cells per well) were seeded in 24-well plates and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were treated with PGC1 α _NGs (40 μ g/mL), PGC1 α _jetM, and/or BI9774 (10 μ M) in complete growth medium. After 24 h, NG-treated cells received a second dose of PGC1 α _NGs with or without BI9774 for an additional 24 h. At 48 h, cells were incubated with MitoTracker® Green (MTG; ex/em 494/521 nm) to assess mitochondrial mass, and TMRM (Ex/Em 548/574 nm) to measure mitochondrial membrane potential, for 30 min at 37 °C. Following staining, cells were washed twice with PBS, detached using trypsin-EDTA, pelleted, and resuspended in FACS buffer (PBS containing 2% FBS and 1 mM EDTA). Flow cytometry acquisition was performed using a FACSymphony™ A3 instrument, collecting at least 10,000 events per sample. Compensation controls, unstained samples, and fluorescence-minus-one (FMO) controls were included to set voltages and gates. Gating strategy involved sequential selection of live single cells using FSC vs SSC, exclusion of doublets (FSC-A vs FSC-H), and analysis of MTG and TMRM fluorescence. Mean fluorescence intensities (MFI) were calculated using FlowJo software to quantify mitochondrial mass and membrane potential across treatment conditions.

2.5.13 Protein corona evaluation

AETC_NGs were incubated with human serum (1%, 5%, 20%, 55%) at different time points (4, 12, 24, 48 h). NGs were diluted in PBS (pH 7.4) and used at a constant concentration of 2 mg/mL. To isolate the hard corona, samples were centrifuged at 16 000 rpm for 10 min at 4 °C. Pellets were washed thrice with PBS to remove loosely bound proteins. The final pellet was resuspended in protein loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.04 M DTT, 0.01% bromophenol blue), boiled at 95 °C for 5 min, and run on NuPAGE™ 4–12% Bis-Tris gels. Gels were stained with InstantBlue® Coomassie and scanned using the Odyssey LI-COR imaging system to quantify band intensities.

2.5.14 Endocytosis inhibition studies

HeLa or C4-2 cells (1×10^5) were seeded in 24-well plates and incubated overnight. Cells were washed with cold PBS ($2 \times 500 \mu\text{L}$) and preincubated with the following inhibitors in serum-free media at 37°C for 1 h:

- Hyperosmolar sucrose (45 mM, clathrin-mediated endocytosis)
- EIPA (100 μM , macropinocytosis)
- Nystatin (30 μM , caveolin pathway)
- Dyngo (30 μM , clathrin-independent/dynamin-dependent)
- 7-ketocholesterol (30 μM , clathrin-independent/dynamin-independent)
- Cytochalasin D (5 μM , phagocytosis/macropinocytosis)
- NaN_3 /2-deoxy-D-glucose (affects all pathways)

A control set was kept at 4°C to inhibit all energy-dependent processes. Following inhibitor treatment, cells were incubated with 20 $\mu\text{g}/\text{mL}$ NGs and the same inhibitors. After incubation, cells were washed with PBS, detached using 0.25% trypsin-EDTA for 3 min at 37°C , and prepared for flow cytometry as described above.

2.5.15 Cytotoxicity assessment

Various cell lines including bEND.3, RAW264.7, Caco-2, HEK293T, C4-2, and HeLa cells (1×10^4 cells/well) were seeded in 96-well plates and incubated overnight. The following day, cells were treated with jetMESSENGER® (0.25 μL , as per manufacturer's instructions for 96-well format) and increasing concentrations of NGs (0–500 $\mu\text{g}/\text{mL}$). After 24 hours, cytotoxicity was measured using the Cell Counting Kit-8 (Sigma-Aldrich, UK) following the manufacturer's protocol.

2.5.16 Mitochondrial respiration analysis using the seahorse XF mito stress test

Mitochondrial function in C4-2 cells was evaluated using the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies) on a Seahorse XF96 Extracellular Flux

Analyzer. Cells were seeded at a density of 20,000 cells per well in Seahorse XF DMEM assay medium (Agilent), supplemented with 10 mM Seahorse XF glucose, 2 mM Seahorse XF L-glutamine, and 5% fetal bovine serum (Gibco, 12676), and incubated overnight at 37 °C in a CO₂-free incubator. On the day of the assay, the medium was replaced with fresh assay medium, and cells were equilibrated for 1 hour at 37 °C in a non-CO₂ incubator. Mitochondrial stress was induced by sequential injection of the following compounds through the Seahorse cartridge ports to reach final concentrations in each well: oligomycin (1.5 µM, port A), FCCP (0.5 µM, port B), and a combination of rotenone and antimycin A (1.0 µM each, port C). This allowed for the measurement of key respiratory parameters including basal respiration, ATP production, maximal respiration, and non-mitochondrial respiration. Each measurement cycle consisted of 3 min mixing, 0 min wait, and 3 min measurement, with three cycles run per condition. Data were collected and analysed using Seahorse Wave software (version 2.6.1, Agilent Technologies). After the assay, cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Following washing and drying, the dye was solubilised using 10% acetic acid, and absorbance at 590 nm was measured to confirm consistent seeding. Oxygen consumption rate (OCR) values were normalised to crystal violet absorbance.

2.5.17 Haemolysis assay

To assess the potential membrane-disruptive effects of NGs, haemolysis assays were performed using red blood cells (RBCs) isolated from animal blood. Whole blood (6 mL) was diluted with 12 mL PBS and centrifuged at 3000 rpm for 10 minutes. The pellet was washed repeatedly with 10 mL PBS until the supernatant became clear. A 2% RBC suspension was prepared in sterile PBS (pH 7.4), and incubated with various concentrations of AETC_NGs (0–500 µg/mL) at 37 °C. PBS and 10% SDS were used as negative and positive controls, respectively. At 2, 4, 8, 12, and 24 hours, samples were centrifuged at 2500 rpm for 5 minutes, and 100 µL of supernatant was transferred to a 96-well plate. The absorbance of released haemoglobin was measured

at 570 nm. Percentage haemolysis was calculated using:

$$\% \text{Haemolysis} = \frac{\text{Abs}_{\text{of sample}} - \text{Abs}_{\text{of negative control}}}{\text{Abs}_{\text{of positive control}} - \text{Abs}_{\text{of negative control}}} \cdot 100\% \quad (2.6)$$

Percent haemolysis values were calculated from three separate experiments.

2.5.18 RNA encapsulation stability and release kinetics in response to various physiologically mimetic stimuli.

RNA-loaded NGs were incubated with varying concentrations of GSH, or resuspended in buffers of various pH (4.5, 5.5, 6.5, 7.4), or exposed to various ROS species (as generated below) at 37°C, and the solution was centrifuged using 0.5 mL Amicon® Ultra-0.5 Centrifugal Filter Units (100 kDa Sigma Aldrich, UK) at 5000 x g for 3 min at specific time intervals. The amount of RNA in the filtrates was quantified using the Quant-iT® RNA assay kit, according to the manufacturer's instructions. For structural studies, the NGs were incubated as before and DLS and ELS measurements were conducted at the same time intervals.^[260]

2.5.19 Generation of reactive oxygen species.

H₂O₂ was purchased from Sigma Aldrich.

Hydroxyl radicals (HO·): Generated using the classical Fenton reaction by reacting hydrogen peroxide (H₂O₂) with ferrous sulphate (FeSO₄ · 7H₂O). Stock solutions were prepared at concentrations of 4.5 M H₂O₂ and 0.15 M FeSO₄ · 7H₂O. A constant molar ratio of [H₂O₂]:[Fe²⁺] = 10:1 was maintained across all experiments, based on previously established optimal conditions.^[310] All reagents were diluted with ultrapure water (pH adjusted to 2.8 ± 0.1 using concentrated sulfuric acid) to reach the desired working concentrations, which ranged from 1–100 mM for H₂O₂ and 0.1–10 mM for Fe²⁺. Reactions were initiated in a total volume of 2 mL by sequential addition of: (1) acidified water, (2) hydrogen peroxide, and (3) ferrous sulphate. Samples were maintained at room temperature throughout the reaction period. Hydroxyl radical formation was indirectly quantified by measuring H₂O₂ consumption using the Amplex Red assay, which produces a fluorescent resorufin

product in the presence of remaining H_2O_2 and horseradish peroxidase (HRP). A decrease in fluorescence was interpreted as increased H_2O_2 consumption and, by extension, greater hydroxyl radical generation.

Superoxide ($\text{O}_2^{\cdot-}$): Generated by enzymatic reaction of xanthine (300 μM) and xanthine oxidase (0.01 U/mL) in potassium phosphate buffer (0.1 M, pH 7.4, 0.1% DMF) at 25 °C.[311, 312] $\text{O}_2^{\cdot-}$ formation was quantified by quantified stoichiometrically by monitoring the reduction of 10 μM partially acetylated cytochrome *c* at 550 nm using a UV–Vis spectrophotometer. Absorbance changes over time (dA/dt) were recorded and used to calculate the concentration of reduced cytochrome *c* using Beer-Lambert’s law:

$$\frac{dA}{dt} = \varepsilon \cdot l \cdot \frac{dc}{dt} \quad (2.7)$$

where ε is the molar extinction coefficient of cytochrome *c* ($18.6 \text{ mM}^{-1} \text{ cm}^{-1}$), l is the path length (1 cm), and dc/dt is the rate of reduction. Based on an observed change in absorbance of 0.002 695 AU/min, the superoxide generation rate was calculated to be 0.01 U/mL, equivalent to 20.42 nmol/min per mg protein. This value reflects the enzymatic activity of the system used to generate superoxide radicals. Superoxide dismutase (SOD) was used as a control to confirm the specificity of cytochrome *c* reduction by superoxide.

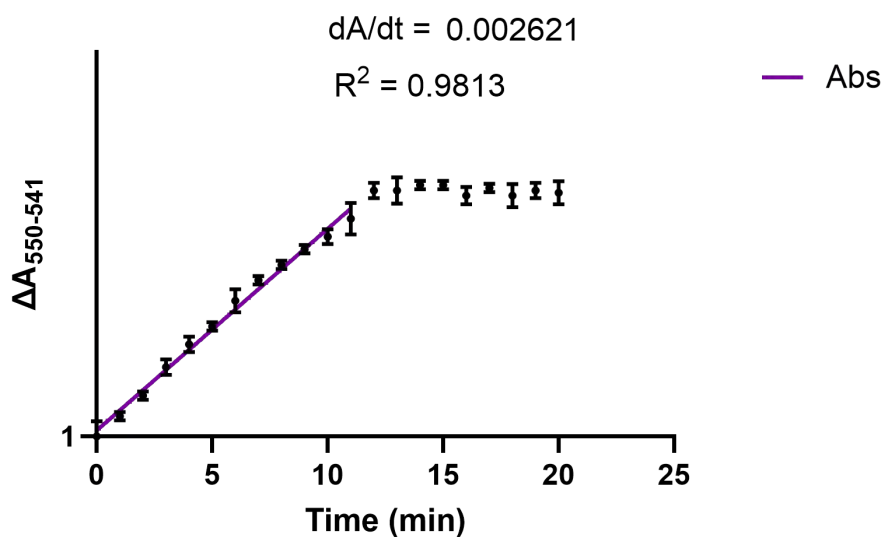


Figure 2.1: Standard curve showing the time-dependent increase in absorbance of cytochrome *c* at 550 nm upon enzymatic generation of superoxide ($O_2^{\bullet-}$) using xanthine and xanthine oxidase. The rate of absorbance change ($\Delta A_{550}/\Delta t$) was used to quantify superoxide production in experimental samples by comparison with this curve. Reduction of cytochrome *c* was monitored spectrophotometrically.

2.5.20 Potentiometric titration to determine pKa values of NGs

Stock solutions of NGs were prepared at a concentration of 1 mg/mL in distilled water. Potentiometric titrations were performed using a Mettler Toledo titrator (DL-25, Mettler-Toledo, Zurich, Switzerland) at 298 K. The NG solution was titrated with 0.01 N HCl containing 10 mM NaCl, and the titrant was added in increments of 0.05 mL at intervals of 30 s, allowing the solution to equilibrate between additions. The pH was continuously monitored using a calibrated glass electrode connected to the titrator. The titration curve (pH vs volume of titrant) was recorded, and the first derivative of the curve ($d(\text{pH})/dV$) was calculated to identify the inflection points corresponding to protonation events. The pKa of the NGs was determined from the volume at which the inflection point occurred, corresponding to the midpoint of the protonation/deprotonation transition.^[313] All measurements were performed in triplicate to ensure reproducibility.^[313]

2.5.21 Immunohistochemical and immunofluorescence staining of tumour sections

Whole tumours were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, embedded in paraffin, and sectioned at a thickness of 5 µm. For immunohistochemical analysis, tissue sections were deparaffinised in 100% xylene, rehydrated through a graded ethanol series (100% to 70%), and subjected to antigen retrieval by boiling in sodium citrate buffer (pH 6.0) for 5 min using a pressure cooker. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide (H₂O₂) in distilled water. After washing in Tris-buffered saline (TBS), non-specific binding was blocked by incubating the sections in 10% normal goat serum in TBS for 1 hour at room temperature. Primary antibodies against Ki-67 (Abcam, ab16667; 1:200 dilution) and TOMM20 (Abcam, ab186735; 1:100 dilution) were applied overnight at 4 °C. The following day, sections were washed in TBS containing 0.1% Tween-20 (TBS-T) and incubated for 1 hour at room temperature with a biotinylated goat secondary antibody (1: 500, Invitrogen, B2770). After further washing in TBS-T, sections were incubated with an avidin–biotin complex (VECTASTAIN® Elite ABC Kit, Vector Laboratories) for 30 min according to the manufacturer’s instructions. Staining was visualised using the DAB Substrate Kit (Vector Laboratories), followed by counterstaining with Gill’s hematoxylin (Sigma-Aldrich). Sections were then dehydrated, cleared, and mounted using DPX mountant (Sigma-Aldrich) and imaged using the AxioScan Slidescanner, Zeiss. For quantification, Fiji (ImageJ) software was used. Automated cell detection was performed on manually annotated regions using the colour-deconvolved haematoxylin channel. In parallel, immunofluorescence staining was performed on 5 µm frozen tumour sections to validate mCardinal expression. Sections were incubated overnight at 4 °C with primary antibody against mCardinal (St John’s Laboratory, STJ140229; 1:500 dilution, ex/em: 604/646 nm), counterstained with DAPI (ex/em: 350/461 nm), and imaged to assess expression, as before.

2.5.22 Spheroid formation, imaging, and nanoparticle uptake

C4-2 cells were seeded into Nunclon™ Sphera 96-well U-bottom plates (Thermo Fisher Scientific) at densities ranging from 100 to 500 cells per well in 200 μ L of complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and 24.8 μ g/mL rat tail collagen (Sigma-Aldrich). Cultures were maintained at 37 °C in a humidified 5% CO₂ incubator. Media were replenished every 72 h by removing 100 μ L of medium from each well and replacing it with an equal volume of fresh growth medium using a multichannel pipette. Spheroid growth and morphology were assessed using the Incucyte® Spheroid Analysis Software Module. For live imaging, mature spheroids were embedded in an agarose matrix within 8-well Ibidi chamber slides, counterstained with Hoechst 33342 (1 μ g/mL) (ex/em: 350/461 nm), and visualised using a Leica TCS SP8 DLS confocal microscope. Image analysis was performed using Fiji (ImageJ, NIH). For nanoparticle uptake studies, spheroids were cultured for 7 days prior to treatment with NGs at a final concentration of 40 μ g/mL (ex/em: 494/521 nm). NG penetration was evaluated by acquiring z-stack images across the spheroid depth using confocal microscopy. To quantify fluorescence signal across the spheroid, the integrated density method was employed: for each z-slice, a region of interest (ROI) encompassing the entire spheroid cross-section was manually drawn in Fiji, and the integrated density was measured as the product of the ROI area and the mean gray value. This approach provided a depth-resolved measure of nanoparticle fluorescence, enabling the assessment of NG distribution throughout the spheroidal mass.

2.5.23 Bulk RNA sequencing

Total RNA was extracted from C4-2b cells treated with NT-PGC1 α -loaded NGs or Empty NGs and cultured in 6-well plates (n=5), using the Qiagen™ RNeasy Mini Kit. RNA was subsequently cleaned using the RNA Clean and Concentrator Kit (Zymo Research, Germany) and quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). A total of 2 μ g RNA per sample was diluted in 20 μ L of nuclease-free water and analysed on a Bioanalyzer 2100 system (Agilent Technologies,

Santa Clara, CA, USA) to assess RNA integrity. All samples exhibited RNA Integrity Numbers (RIN) greater than 9. Single-end sequencing (100 bp reads) was performed using the Illumina NextSeq 2000 platform (Illumina, San Diego, CA, USA) by Mr Ivan Andrew at the MRC LMS Genomics Facility. RNA-seq reads were processed using cutadapt v4.7 [314] to remove Illumina adapters, to quality trim at Q20, and to filter read pairs containing N bases or where either read <31 bps. Processed reads were quantified with Salmon v1.9.0 [315] using transcripts from the GRCh38 Ensembl v107 annotations. Salmon’s expectation maximisation procedures were set to enable modelling of sequencing and GC biases. Within R, transcript-level counts from Salmon were imported and aggregated to the gene level using tximport v1.34.0 [316]. Sample quality assessments were performed in relation to collected RNA integrity (RIN) values, sample concentrations used in library preparation (ng/µl), read GC content, and percentage read mapping. Normalisation, PCA-based and clustering-based quality control based on normalised values, and differential expression analyses were performed using DESeq2 v1.46.0 [317]. Independent hypothesis weighting was applied to optimise the power of p-value filtering using IHW v1.34.0 [318], and log₂ fold change shrinkage was performed with ashr v2.2-63 [319] to reduce the impact of low expression on fold change estimation. Multiple testing correction was applied by passing thresholds for both false discovery rate and log₂ fold change within DESeq2. Significance was assessed using a log₂ fold change threshold of 1 (i.e., 2-fold change) and a false discovery rate of $q < 0.01$. Downstream gene ontology (GO) enrichment analysis was performed using clusterProfiler v4.14.4 [320], targeting the Biological Process (BP) GO terms. Benjamini–Hochberg correction was applied, with GO terms considered significant at $q < 0.05$.

2.5.24 *In vivo* biodistribution and therapy assessment

2.5.24.1 mCardinal_NG biodistribution in healthy mice

All animal experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the internal ethics committee and UK Home Office under project license PP0283859 and personal license I77452048. Animals were provided food and water *ad libitum*. To evaluate the biodistribution of

mCardinal_NGs, C57BL/6 mice received 200 μ L injections of NGs via either the tail vein or subcutaneous fat in adult male mice. The formulation contained 0.25 mg/kg mCardinal mRNA and 60 mg/kg polymer. Mice were euthanised at predefined time points following administration. Major organs—including brain, heart, liver, spleen, lungs, and kidneys—were harvested, along with tumour tissues when applicable. Fluorescence biodistribution was assessed using an In Vivo Imaging System (IVIS; excitation: 600 nm; emission: 640 nm) to quantify mean fluorescence intensity (MFI). To do so, first the machine was cleaned and a black cardboard paper was placed on the imaging platform. An image of the background was taken to ensure that no background fluorescence was present. Then, the organs were placed on to the same black cardboard platform and the pictures were taken. Background subtraction is carried out by the software and the fluorescent signal is captured as radiant efficiency. In essence, radiant efficiency is a measure of the emitted photons from the fluorescent signal, normalised to the area of the region of interest. This metric enables quantitative comparison of fluorescent signal intensity across different samples and time points, independent of acquisition settings or background variations, and is considered to be a robust method for signal detection. The field of view can be altered in the imaging software depending on the area to be imaged and was adjusted accordingly for each image presented in this work.

2.5.24.2 C4-2b xenograft model for therapeutic assessment

Male NOD-scid gamma (NSG)-614 mice (6–8 weeks old) were purchased from Charles River (UK) and acclimatized for one week before experimentation. Mice were subcutaneously inoculated on the right flank with 2.5×10^6 C4-2b cells in 100 μ L of serum-free medium mixed with 100 μ L of Matrigel Basement Membrane Matrix (High Concentration, Corning). Tumours were allowed to grow to approximately 50 mm³ before initiating treatment. Mice were randomly assigned to five treatment groups: (1) untreated, (2) NT_PGC1 α _NG, (3) mCardinal_NG, (4) PSMA_NT-PGC1 α _NG short-term, and (5) PSMA_NT-PGC1 α _NG long-term. In all groups, the mRNA dose was 0.25 mg/kg, and the polymer was administered at 60 mg/kg. NGs were injected subcutaneously three times per week for 30–60 days. Tumour

volume was monitored twice weekly using digital calipers and calculated using:

$$\text{Volume} = \frac{\pi}{6} \cdot \text{length} \cdot \text{width} \cdot \text{height} \quad (2.8)$$

At the end of the study, blood samples were collected for serum analysis to assess potential systemic toxicity.

2.5.24.3 Serum Biochemistry for *in vivo* cytotoxicity

To evaluate systemic toxicity, serum samples obtained from tumour-bearing mice at study endpoint were analysed for liver enzyme levels. Alanine transaminase (ALT) and aspartate aminotransferase (AST) concentrations were measured by the Core Biochemical Assay Laboratory, Cambridge, UK. This analysis was supported by the MRC MDU Mouse Biochemistry Laboratory (grant reference: MC_UU_00014/5).

Chapter 3

Optimisation of RNA-loaded nanogel synthesis

3.1 Introduction

There is considerable interest in understanding the relationship between the chemical structure and the physiochemical properties of nanogels (NGs). Given the random nature of a radical polymerisation, careful monitoring of monomer conversions is required to gain a better insight into the final chemical composition of the NG. As such, detailed studies of monomer reactivities and reaction conditions are essential to ensure that the isolated polymer possesses a formulation that is as close to the initial combination of the starting materials (feed composition) as possible. Despite its significance, literature data on radical polymerisation rarely investigate the actual polymer composition and instead limit the description to the initial formulation. In this chapter, the significance of each component involved in the NG reaction is discussed. The effect of monomer reactivities on conversion rates and the final composition of NGs is described. Following this, the morphological changes resulting from variations in chemical structural of functional monomers and crosslinker content will be highlighted. The knowledge obtained from this analysis was used to derive optimal reaction conditions and synthetic steps which permitted efficient entrapment of viable RNA payload.

The NG reaction had been previously optimised for the encapsulation of protein-based payloads[287], and the reaction took four hours to complete, at room temperature. However, for nucleic acid-based payloads, that are more prone to degradation at longer reaction times and higher reaction temperatures, there was a need to minimise both without compromising on any desirable structural features of the NGs. These included NG sizes that were below 200 nm with a polydispersity index (PI) of < 0.1 and ζ -potential (ζ) in the range $|\zeta| \geq 30$ mV in order to enhance cellular uptake.[321] In addition, an aqueous-based synthetic strategy was preferred to avoid the use of harsh organic solvents in order to minimise damage to the sensitive nucleic acid payload.

Finally, the NGs obtained were tested for their *in vitro* transfection efficiency in various cell lines to obtain the best-performing platform for RNA delivery, in comparison to commercial standards used for transfection.

3.2 Results & Discussion

3.2.1 Influence of surfactant concentration on NG synthesis

The methodology employed for NG synthesis involved the dispersion of the monomers in water, with the addition of the classical anionic surfactant sodium dodecyl sulfate (SDS), which is surmised to aid micelle formation and thereby lead to the accumulation of added monomers within the micellar core.[287] The surfactant concentration is one of the most critical factors affecting emulsion polymerisation.[322] Upon addition of the surfactant, the surfactant molecules adsorb on the solvent/aqueous surface, decreasing the surface tension until the critical micelle concentration (CMC) is reached. This leads to micelle formation in the solution. Any further increase in the surfactant concentration will continue to decrease the interfacial tension between the monomer/surfactant surface, whereby the hydrophobic parts of the surfactant molecules will orient themselves inside the micelle to lower interaction with surrounding water molecules.[323, 324] In particular, for SDS, continued addition of the surfactant to a certain threshold increases micelle formation, which in turn reduces the size of the micelles due to electronic repulsion.[322, 325] The increase in the number of micelles affords a high number of active centers in the polymerisation initiation step, resulting in higher monomer conversion rates whilst decreasing nanoparticle size.

In the case of SDS, increasing the concentration from 2 mM to 20 mM had a dramatic effect on NG size and PI, with the size decreasing from 445.6 nm to 50.7 nm (Figure 3.1a). Although using 20 mM of SDS provides the smallest NG size, it becomes increasingly difficult to purify the NGs, which required ten or more washes with water using centrifugation. This also increased the total time required for nanoparticle isolation which would be detrimental for any nucleic acid payloads, that can be prone to degradation.[326] Further, *in vitro* toxicity assays (CCK-8) in HeLa cells indicated

higher toxicity for NGs made using 20 mM SDS suggesting the presence of residual SDS (Figure 3.1b). As the complete removal of SDS is essential for downstream biological applications, a lower concentration is needed. Notably, at the CMC of SDS (8 mM)[322, 325] a satisfactory NG size (125.6 ± 1.4 nm) and PI (0.02 ± 0.01) was achieved with minimal cytotoxicity. As such, this concentration of SDS was employed for all further NGs discussed in this work.

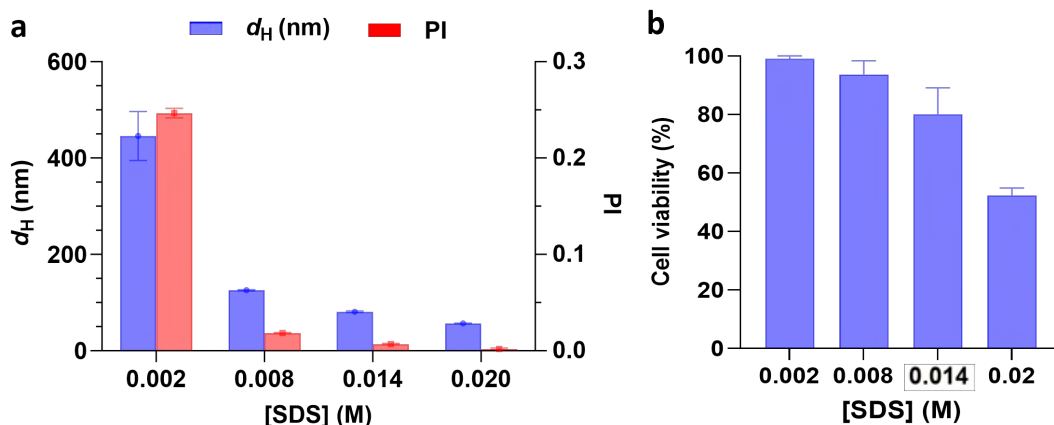


Figure 3.1: **Effect of SDS concentration on NG size and cytotoxicity.** a) Physicochemical characterisation of size (hydrodynamic diameter) and polydispersity (PI) as obtained from dynamic light scattering measurements. b) Cell viability determined for NGs synthesised using varying SDS concentrations. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$.

3.2.2 Influence of initiator content on NG synthesis

The previously optimised NG reaction used ammonium persulfate and N,N,N',N' -tetramethylethylenediamine as the initiating system.[287, 288], In this work, two water soluble photoinitiators, namely Irgacure2959 (IG2959) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) were also investigated in an attempt to decrease the overall reaction time. These were chosen as they are water soluble and have demonstrated promising reaction dynamics in other similar reaction conditions.[292, 327, 328] Reaction completion was measured as a function of total monomer conversion as determined by ^1H NMR analyses of initial and final polymerisation solutions.[329] Conversion was determined by measuring the vinyl olefin signals in the respective ^1H NMR spectra (using sodium acetate as an internal standard) (Figure A1).

As shown in Figure 3.2a, using 5 mol% (mol% refers to the moles of initiator used relative to the total moles of monomers used) of IG2959 provided 99% monomer conversion with a 30 min reaction time, and reasonable NG sizes (113.4 ± 2.2 nm; PI: 0.01 ± 0.01) (Figure 3.2a,b). Likewise, 15 mol% of APS provided modest monomer conversion of 71% and 30 min reaction time with NG sizes of 81.2 ± 1.8 nm and PI of 0.07 ± 0.05 (Figure 3.2c,d). The use of LAP, however, did not provide any significant improvement in NG size/PI, despite having the fastest reaction time (~ 10 min) (Figure 3.2e,f). For all initiators investigated, increasing the initiator content, although increasing the monomer conversion and reducing the reaction time, led to the formation of large NGs (> 500 nm) with poor PIs (> 0.2). Faster polymerisation kinetics can result in uncontrolled radical coupling, thereby relinquishing control over particle size.[330] Despite the short reaction times provided by IG2959, its use was precluded due to reliance on a custom UV-lamp reaction set-up. Reproducing the synthesis using the photoinitiator would require maintaining the same lamp, distance and power which is impractical due to variability among UV lamps. Thus, APS/TEMED was preferred as the initiating system for all subsequent reactions, due to the reproducibility it offers.

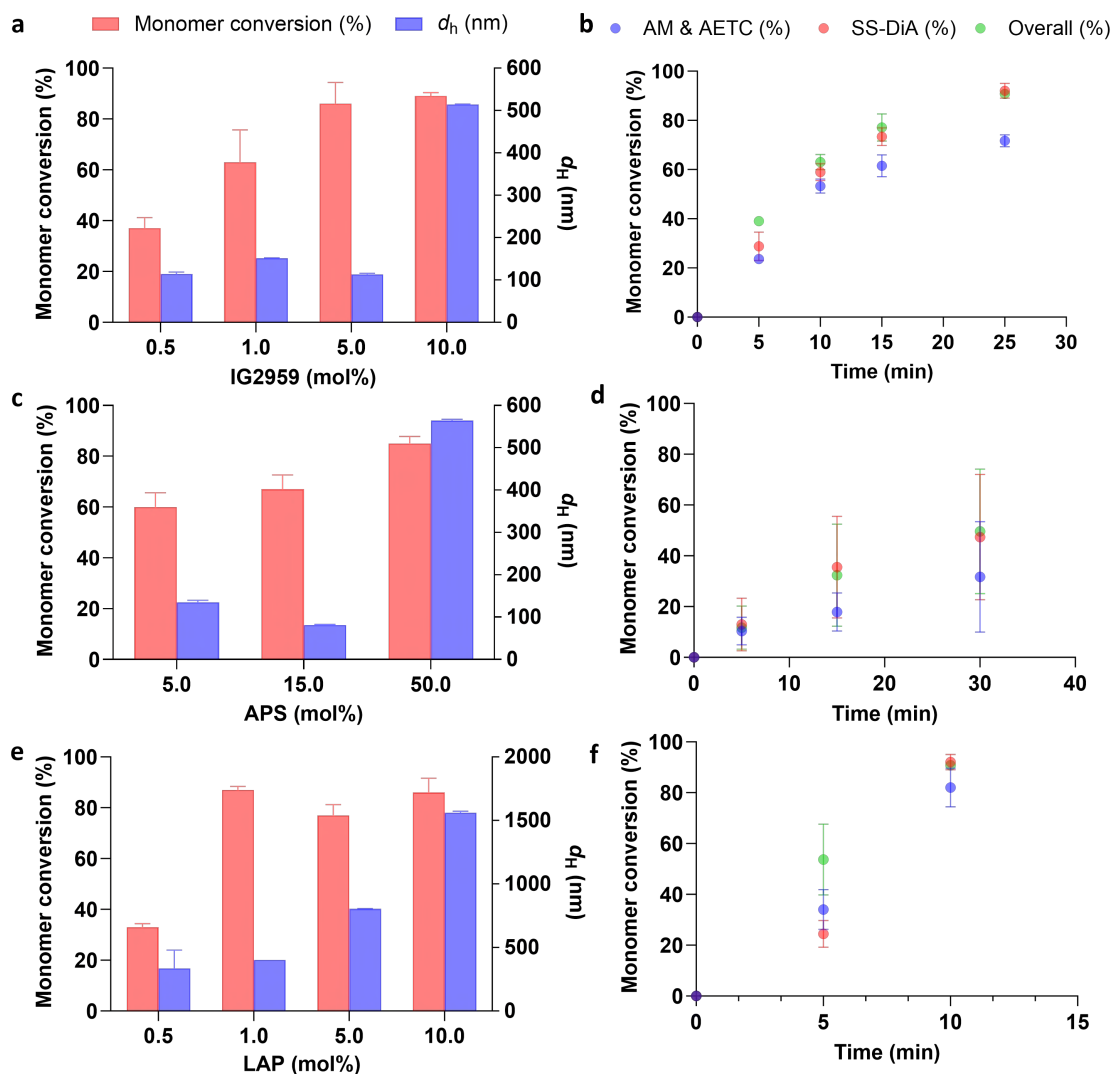


Figure 3.2: **Effect of initiator type and amount on NG structure and polymerisation dynamics.** Graphs comparing monomer conversions, as determined ^1H NMR analyses, and associated NG sizes for a) Igracure2959, c) APS, e) LAP. Polymerisation dynamics as determined by assessing monomer conversion over time using ^1H NMR for b) Igracure2959, d) APS and f) LAP. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$.

3.2.3 Optimising crosslinker ratio

To optimise the crosslinking of the covalent NG matrix, disulfide-based, diacrylate crosslinker (SS-DiA) conversion was measured at varying concentrations using ^1H NMR. This information was then compared with the obtained physicochemical properties of the synthesised NGs as measured by dynamic light scattering (DLS), electrophoretic light scattering (ELS) and transmission electron microscopy (TEM) (Figure 3.3a,b,c). Evidently, increasing crosslinker feed concentration from 1 up to 7 mol% demonstrated a decrease in NG size and PI, as monitored by DLS and TEM. Interestingly, increasing the crosslinker feed ratio higher than 7 mol% resulted in the formation of large aggregates $\sim 600 - 800$ nm, that demonstrated compromised colloidal stability. This indicates that the synthesis is highly sensitive to higher concentrations of crosslinkers and works best in high-dilution conditions. Notably, NGs with the lowest sizes and PI demonstrated increased colloidal stability over longer periods of time, as indicated by the maintenance of the size and PI over the time period of the experiment (Figure 3.3d). NGs with the largest sizes however demonstrated a steady increase in size and PI, which could be indicative of aggregation (Figure 3.3e).

NG RNA encapsulation (EE %) and loading efficiencies (LE %) as a function of the crosslinker ratio, ranging from 1 to 10 mol% SS-DiA, were then evaluated. Evidently, the extent of crosslinking greatly impacts the RNA entrapment within the NGs, where increasing crosslinker concentration led to improved RNA encapsulation (Figure 3.3f). Notably, 7 mol% SS-DiA incorporating NGs exhibited a high EE and LE of 91% and 10%, respectively. As a significant drop in the EE % and LE % were observed at 10 mol% crosslinker feed ratios, the 7 mol% feed ratio was used for subsequent experiments and comparisons. As no significant improvement in EE % and LE % was observed upon increasing the SS-DiA crosslinker ratio any further, it would be interesting to investigate whether the lack of formation of a tightly crosslinked NG core may be more prone to degradation and payload leakage.

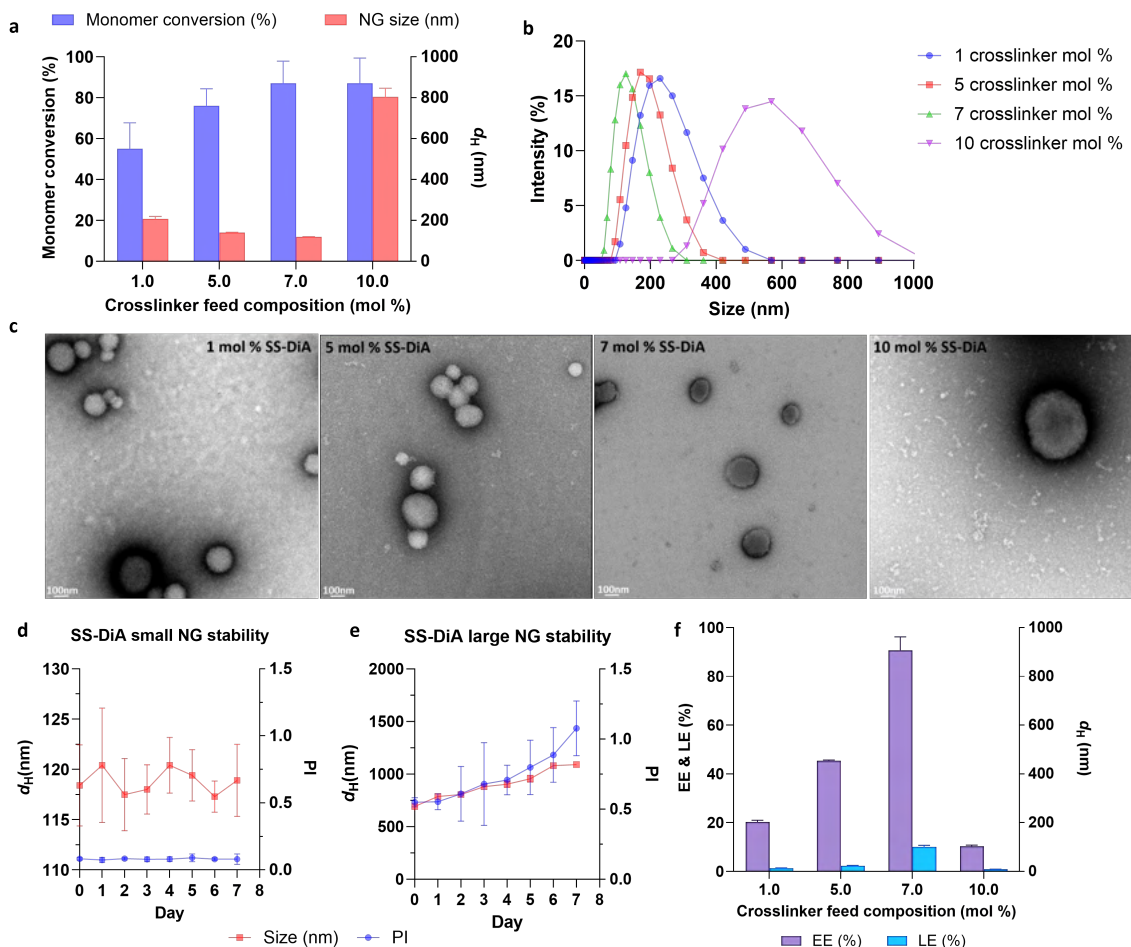


Figure 3.3: **Effect of varying crosslinker concentration on NG size, stability and payload entrapment efficiency.** a) Monomer conversions, as determined by ^1H NMR analyses, compared against NG size for varying crosslinker concentrations. b) DLS traces for various crosslinker concentrations. c) Negative stain transmission electron micrographs of NGs with varying crosslinker concentrations. Scale bar at 100 nm. Nanogel stability (DLS) analysis recorded over seven days for d) small SS-NG and e) larger SS-NG. f) Graph comparing the effect of varying crosslinker feed composition of NG loading and encapsulation efficiency. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$.

3.2.4 Enhancing RNA entrapment efficiency by identifying optimal cationic monomer ratio

In order to further optimise the encapsulation of RNA within the NGs, the feed ratios of the monomers 2-(acryloyloxy)ethyl]trimethylammonium chloride (AETC) and acrylamide (AM) were also systematically varied and the resultant effects of NG physicochemical properties were measured. It is well known that in copolymerisation

reactions, rates are not only dependent on individual intrinsic reactivities of each monomer but also on the presence of other monomers and their relative concentrations in the mixture.[329, 331, 332] In this study, the molar concentrations of AETC were inversely proportional to the one for AM to ensure that the total monomer amount was below the threshold for micro-sized polymer formation.[287] Selected ratios of AETC ranged from 0-50 mol% and the monomer conversions, as estimated from the ^1H NMR traces, are summarised in Table A1. The changes in NG structural features upon varying AETC content were determined by DLS, ELS and TEM, as before.

The NG composition lacking AETC had a large size and PI (186.2 nm and 0.28, respectively) and an effective surface charge close to neutral (-8.5 ± 0.8 mV) which is indicative of compromised colloidal stability (Figure 3.4a,b,d).[321] Unsurprisingly, these NGs also exhibited the lowest encapsulation (EE; 21.1%) and loading efficiencies (LE; 0.9%) (Figure 3.4c). The most physiologically relevant size distributions were observed for NGs synthesised using 7-24 mol% of AETC. Further, the effective surface charge of the NGs became more positive with increasing AETC content, as consistent with greater incorporation of the cationic monomer within the NG framework, which also led to increased RNA EE% and LE%. These measurements also indicate that RNA present within the core of the NGs minimally affects the effective surface charge. This is further evidenced by the minimal changes in the effective surface of the empty NGs for the AETC monomer (Figure 3.5a,b). Notably, however, the increase in ζ -potential was only observed up to 49 mol% AETC, at which point the compromised stability of the NG framework would prevent the incorporation of any further AETC molecules. Increasing the AETC content only improved the EE % and LE % up to 49 mol% AETC. As the NG composition with 93 mol% AETC, lacked AM, the NGs demonstrated undesirably large size and size distribution (312.8 ± 0.2 nm; 0.35 ± 0.01) (Figure 3.4a,d). AM is known to improve the adhesion and crosslinking of polymers,[333] it provides mechanical strength to the crosslinked NG network. As such, it is a critical parameter for obtaining robust and durable nanoparticles. Further, due to its bioadhesive properties,[334] AM can contribute towards an increase RNA EE % and LE %. Indeed, NGs synthesised without AM demonstrate compromised RNA EE % (74%) and LE % (3%). Despite

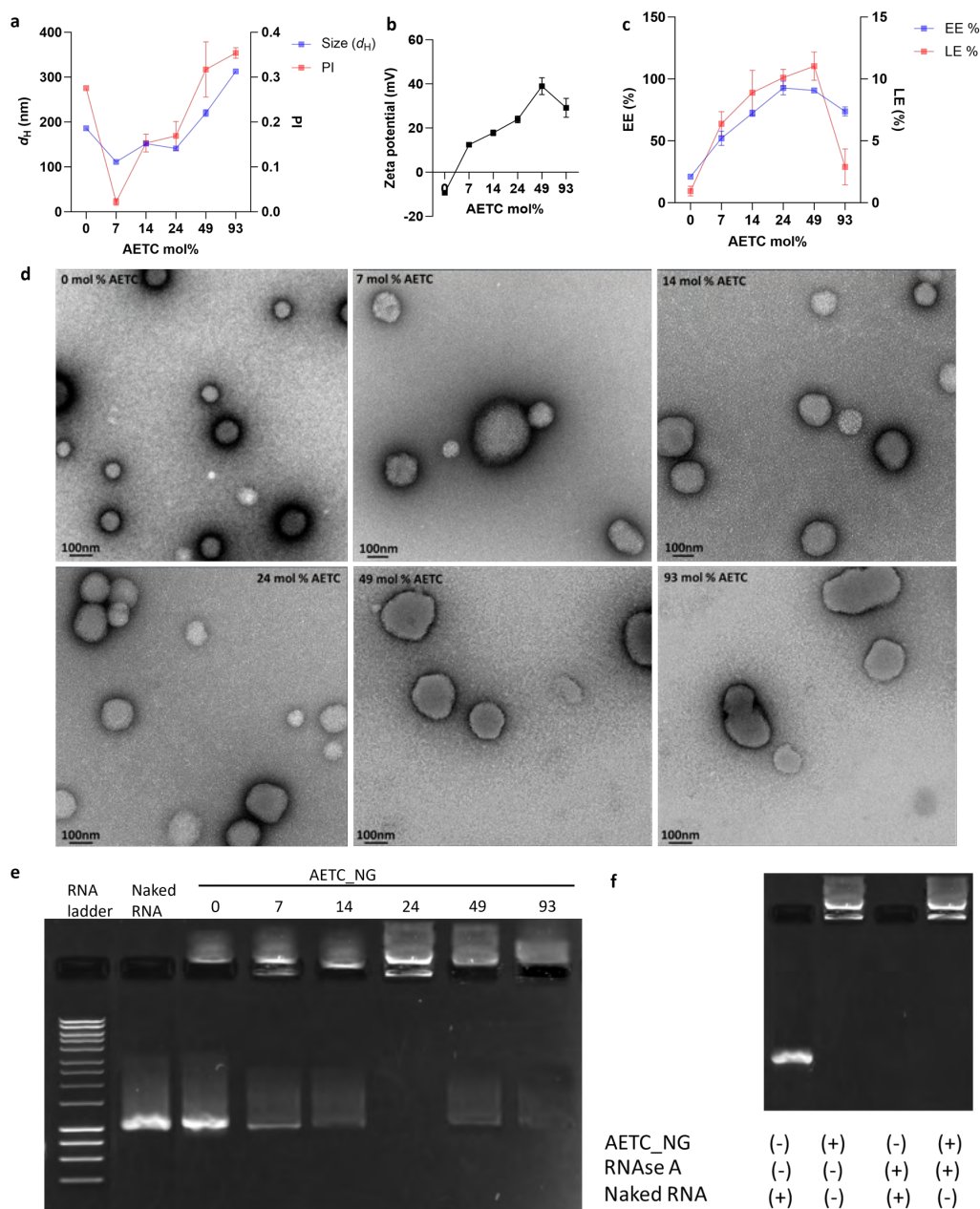


Figure 3.4: Defining optimal AETC concentration for RNA-loaded NG synthesis. Effect of varying AETC concentration on a) NG size, PI, b) ζ -potential of different NG types, as measured by DLS and ELS, respectively and c) encapsulation and loading efficiencies. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. d) Representative negative stain TEMs of NGs. Scale bar at 100 nm. e) Agarose gel electrophoresis assay to determine free RNA. Amount of monomer added to NG is indicated by numbers at the top of respective lanes, alongside the RNA ladder and naked RNA. f) Determination of RNA stability upon NG treatment with RNase A (100 U/mg) for 2 h at 37 °C.

the high effective surface charge of these NGs (29.2 ± 4.3 mV), lack of AM could have led to RNA leakage, thereby offsetting the advantage of the increased AETC content.

Agarose gel measurements mirrored the EE % and LE % analyses and highlighted minimal free RNA for NGs synthesised using 24 mol% of AETC (Figure 3.4e). Upon treatment of these NGs with RNase A, minimal RNA degradation was observed (Figure 3.4f). Having identified the optimal molar % range of the cationic monomer, various monomers with different head groups were screened to further improve the mRNA packaging of the NGs.

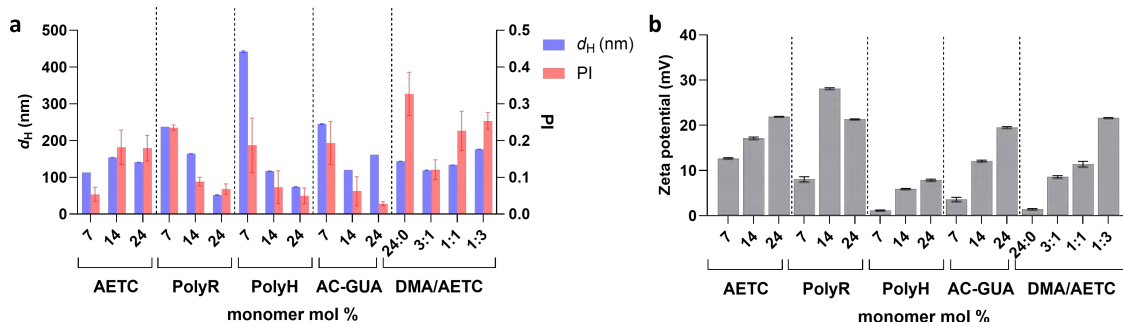


Figure 3.5: **Physicochemical characterisation of empty NGs with different concentrations of different cationic monomers.** a) Size, PI and b) ζ -potential of different NG types, as measured by DLS and ELS, respectively. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$.

3.2.5 Assessing best cationic monomer type for optimal RNA binding

While the cationic monomer, AETC, provided promising NGs that met the requirements outlined previously, various cationic monomers with different head groups were screened in order to further improve the mRNA packaging of the NGs (Figure 3.6). First, a monomer with a guanidinium moiety, called acrylated guanidinium (AC-GUA), was synthesised via a novel two-step reaction involving nucleophilic displacement of ethyl carbaminidithioate by the amine group in 3-aminopropan-1-ol, followed by esterification of the primary alcohol using acryloyl chloride to give AC-GUA.

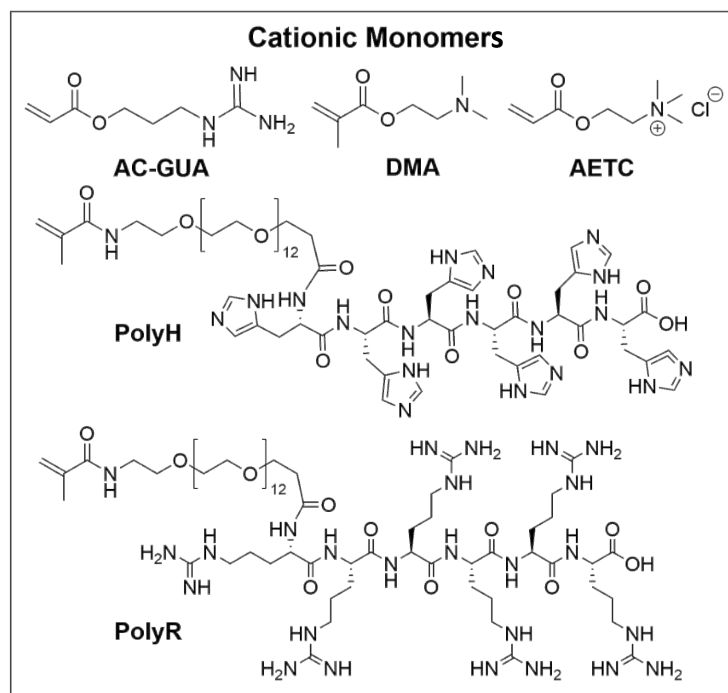


Figure 3.6: **Schematic presentation of cationic monomers used in NG synthesis.**

Prior to its incorporation within NGs, AC-GUA was characterised by ^1H NMR (Figure A2a), atmospheric-pressure chemical ionisation mass spectrometry (Figure A2b), and FT-IR (Figure A2c). The near-neutral pH (~ 7.4) at which the NG reaction is conducted, was surmised to be sufficient in allowing the protonation of guanidine moiety and the deprotonation of the RNA phosphodiester moieties.[335, 336] This would be conducive to the formation of intermolecular salt bridges between AC-GUA and the added RNA. Experiments with the AETC monomer revealed an optimal molar ratio range (7-24 mol%) within which NGs could be synthesised reproducibly, without the risk of microgel formation. Increasing AC-GUA content to 24 mol% improved NG size and more positive effective surface charge, thereby improving the RNA encapsulation (82%) and loading efficiencies (10%) (Figure 3.7a,b,c,d). However, agarose gel measurements, while reflecting the EE % and LE % analyses, highlighted considerable RNA leakage from the NGs (Figure 3.7e). This was mirrored in the RNase A treatment assay for the 24 mol% AC-GUA NGs where degraded RNA was visible in lanes loaded with NGs (Figure 3.7f). Despite these

results, the high RNA entrapment efficiency obtained using AC-GUA merited further *in vitro* testing.

The second set of cationic monomers were inspired by the structures of cationic cell-penetrating peptides that have been used successfully for mRNA delivery as they combine lower charge densities with excellent membrane disruptive abilities.[337–340] In particular, the polyarginine motif was considered due to its promising endosomal escape capabilities and previous success with the guanidinium motif (Scheme 2.2.2).[341, 342] First, solid-phase peptide synthesis was used to generate a polyarginine peptide incorporating six arginines, which was then coupled to a bifunctional PEG with an Fmoc protected amine, that was deprotected and acrylated using acryloyl chloride. This provided an acrylated, PEGylated polyarginine (PolyR) that was characterised by ^1H NMR (Figure A3a) and atmospheric-pressure chemical ionisation mass spectrometry (Figure A3b), before incorporation into the NG reaction. Six amino acids were used to ensure an optimal balance between cellular uptake efficiency, biocompatibility and RNA complexation ability. Polyarginine-based cell penetrating peptides have been widely studied, with numerous reports identifying oligomers comprising of six to eight residues as best for facilitating transport across cell membranes without mediating excessive cellular toxicity.[343] Further, six units were thought to provide sufficient cationic charge density to promote strong electrostatic interactions with the nucleic acid payload, without introducing excessive steric hindrance, thereby preventing undue increases in NG size.

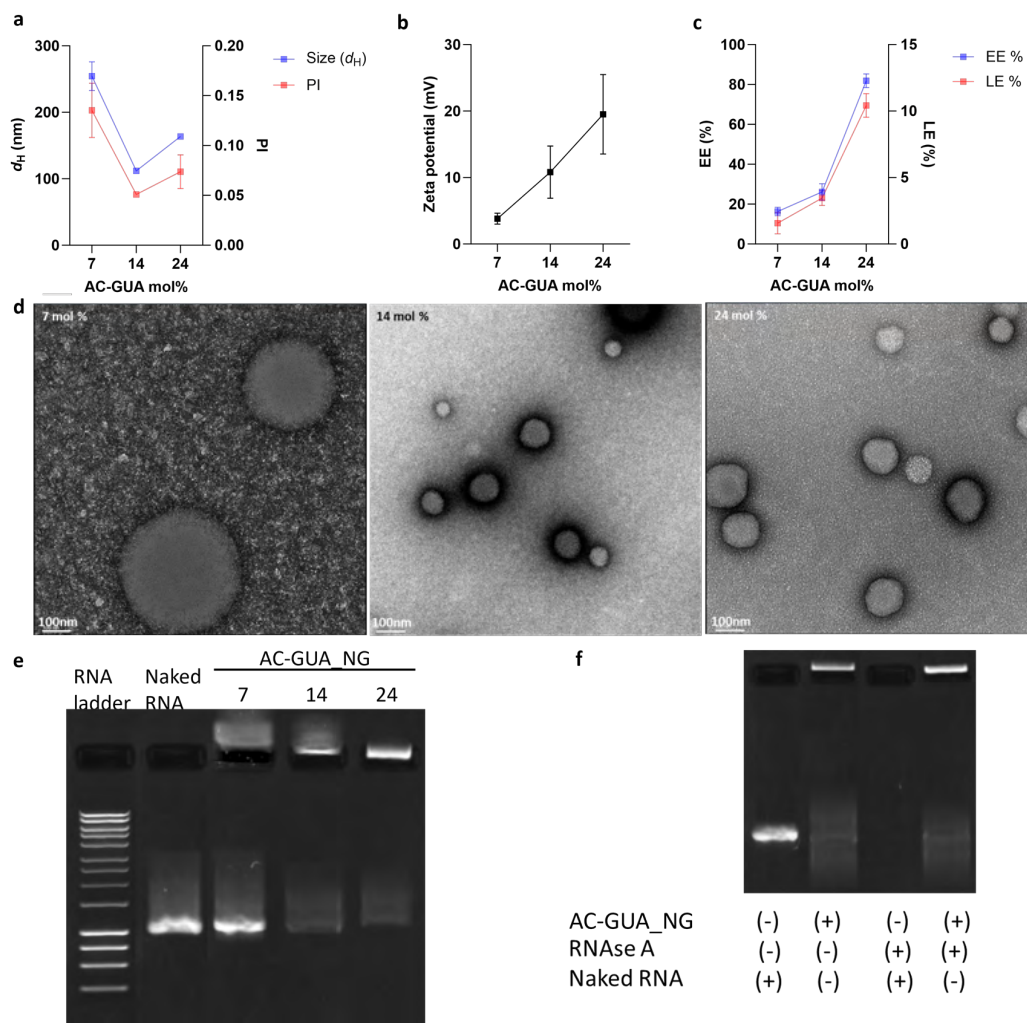


Figure 3.7: Defining optimal AC-GUA concentration for RNA-loaded NG synthesis. Effect of varying AC-GUA concentration on a) NG size, PI, b) ζ -potential of different NG types, as measured by DLS and ELS, respectively and c) encapsulation and loading efficiencies. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. d) Representative negative stain TEMs of NGs. Scale bar at 100 nm. e) Agarose gel electrophoresis assay to determine free RNA. Amount of monomer added to NG is indicated by numbers at the top of respective lanes, alongside the RNA ladder and naked RNA. f) Determination of RNA stability upon NG treatment with RNase A (100 U/mg) for 2 h at 37 °C.

Although increasing PolyR molar ratio decreased NG size, the PI was higher (0.28 ± 0.01) than previously obtained with AETC-incorporating compositions (Figure 3.8a,d). This heterogeneity could stem from inefficient packaging of the bulky arginine residues, likely due to inherent repulsive intermolecular interactions. This effect could also explain the decrease in effective surface charge as PolyR content was increased from 14 to 24 mol%, which led to a resultant decrease in RNA EE % and LE %

(Figure 3.8b,c). As before, the agarose gel electrophoresis mirrored the EE % and LE % analyses (Figure 3.8e). It is notable that 14 mol% PolyR provided optimal NG size (168.9 ± 2.1 nm), PI (0.09 ± 0.02) and EE % (82%) and LE % (11%). Indeed, the RNase treatment assay revealed minimal RNA degradation for this composition, explicating its potential for *in vitro* RNA delivery (Figure 3.8f).

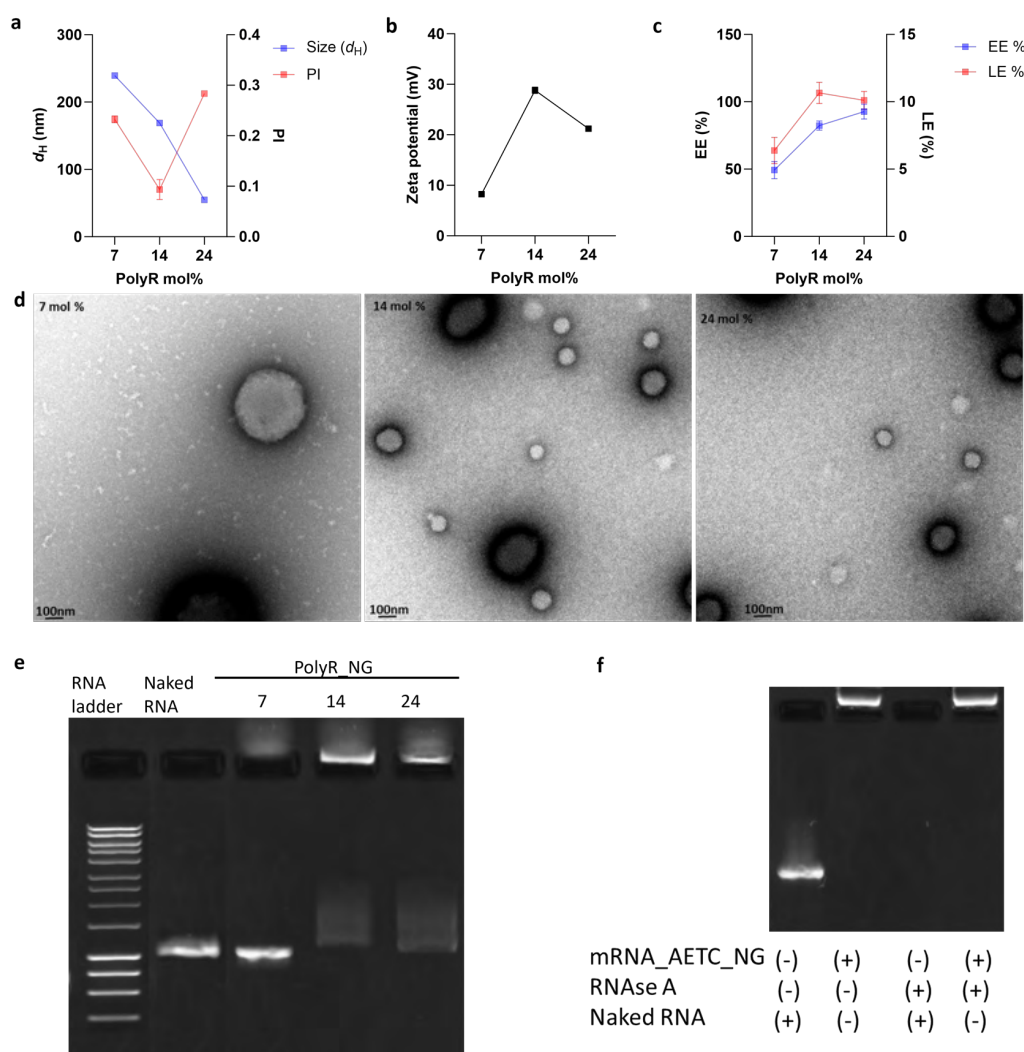


Figure 3.8: Defining optimal PolyR concentration for RNA-loaded NG synthesis. Effect of varying PolyR concentration on a) NG size, PI, b) ζ -potential of different NG types, as measured by DLS and ELS, respectively and c) encapsulation and loading efficiencies. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. d) Representative negative stain TEMs of NGs. Scale bar at 100 nm. e) Agarose gel electrophoresis assay to determine free RNA. Amount of monomer added to NG is indicated by numbers at the top of respective lanes, alongside the RNA ladder and naked RNA. f) Determination of RNA stability upon NG treatment with RNase A (100 U/mg) for 2 h at 37°C.

For the second peptide-based monomer, the arginine residues were replaced by six histidine residues (PolyH). The imidazole group in histidine permits the stabilisation of nucleic acids via hydrogen bonding and augments endosomal lysis by osmotic swelling.[344] In addition, histidine-containing carriers have shown improved serum stability.[345] The acrylated and PEGylated polyhistidine was synthesised using the same techniques as aforementioned and characterised by ^1H NMR (Figure A4a) and APCI-MS (Figure A4b) prior to incorporation in the NG reaction. Increasing PolyH content led to decreased NG size, however the PI for 24 mol% PolyH was noticeably higher, presumably due to inefficient packaging efficiency of the bulky imidazole groups (Figure 3.9a,d). The modest increase in effective surface charge (7.8 ± 0.7 mV for 24 mol% PolyH) improved EE % (57%) and LE % (5%), however not to comparable levels achieved with AETC or PolyR (Figure 3.9c). The increase in RNA retention was mirrored in the agarose gel electrophoresis assay, however, RNA leakage from these NGs are evident in the lanes for 14 and 24 mol% PolyH (Figure 3.9e). Where 7 mol% of AETC demonstrated minimal RNA leakage, the same amount in PolyH showed almost complete RNA loss. Furthermore, RNA leakage and degradation is similarly visible in the RNase treatment assay (Figure 3.9f). Despite these results, the potential endosomal escape characteristic of this monomer merited further *in vitro* testing.

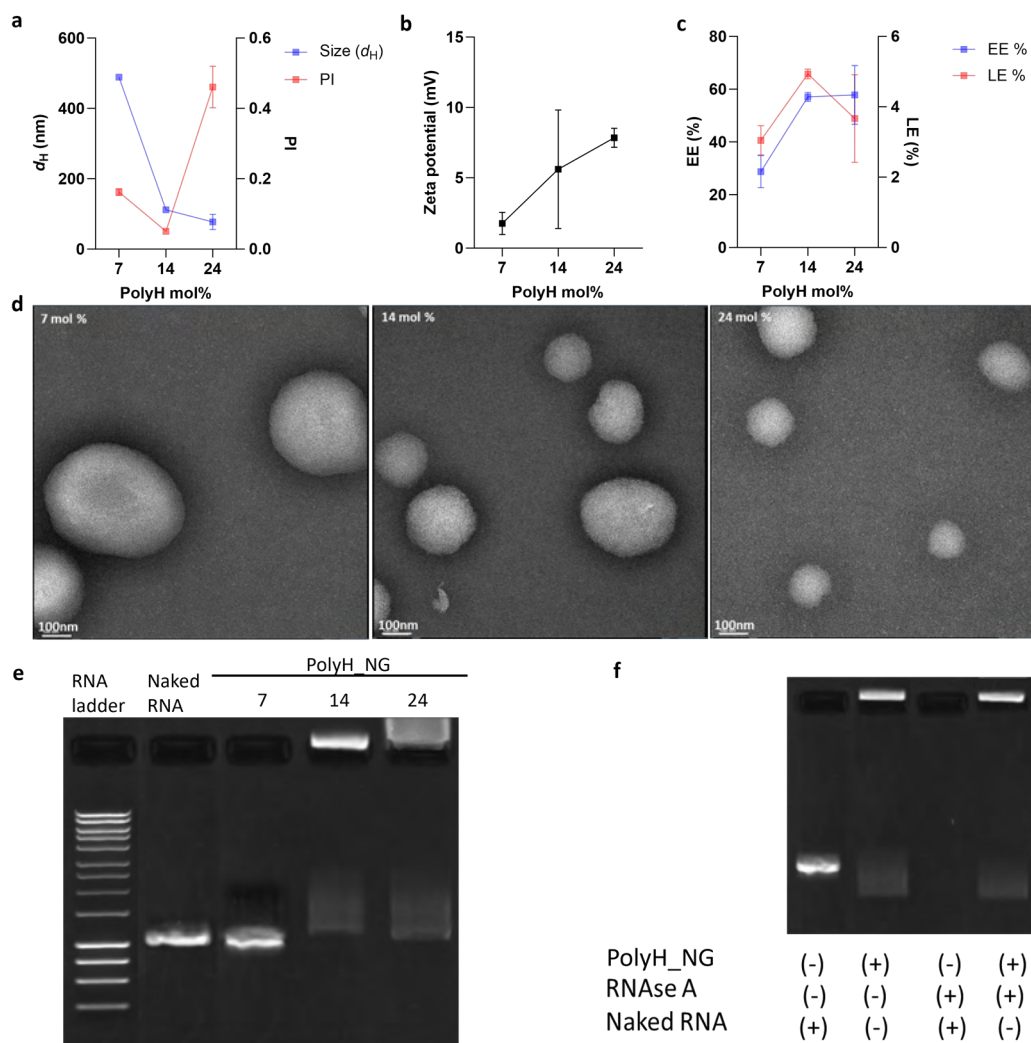


Figure 3.9: Defining optimal PolyH concentration for RNA-loaded NG synthesis. Effect of varying PolyH concentration on a) NG size, PI, b) ζ -potential of different NG types, as measured by DLS and ELS, respectively and c) encapsulation and loading efficiencies. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. d) Representative negative stain TEMs of NGs. Scale bar at 100 nm. e) Agarose gel electrophoresis assay to determine free RNA. Amount of monomer added to NG is indicated by numbers at the top of respective lanes, alongside the RNA ladder and naked RNA. f) Determination of RNA stability upon NG treatment with RNase A (100 U/mg) for 2 h at 37 °C.

The final monomer examined was 2-(dimethylamino)ethyl methacrylate (DMA) which was commercially available and was used as provided. The ionisable tertiary amine in DMA could potentially buffer the acidic endosomal environment via the proton sponge effect, thereby promoting endosomal membrane disruption and subsequent NG release into the cytoplasm. NGs synthesised with 24 mol% DMA had reasonable

sizes and PI, but demonstrated minimal effective surface charge, EE % and LE % (Figure 3.10a,c,d). As such, DMA was examined alongside the inclusion of AETC, which offers permanent positive charges for stable RNA complexation. Using this dual monomer system, it was surmised that the NGs could benefit from RNA complexation stability and enhanced endosomal escape ability. Indeed, the RNA entrapment efficiency improved as the AETC content in the NGs increased, presumably due to the congruent increase in ζ -potential (Figure 3.10b). This is reflected in the agarose gel electrophoresis assay where no RNA leakage/degradation was observed in the lane with 1:3 DMA/AETC ratio, thereby emphasising the negligible effect of DMA on RNA entrapment efficiency (Figure 3.10e). Furthermore, the RNase protection assay revealed some RNA leakage and degradation (Figure 3.10f). Despite this, the 1:3 DMA/AETC NG composition was screened for its *in vitro* transfection efficiency to test whether it exhibited enhanced endosomal escape.

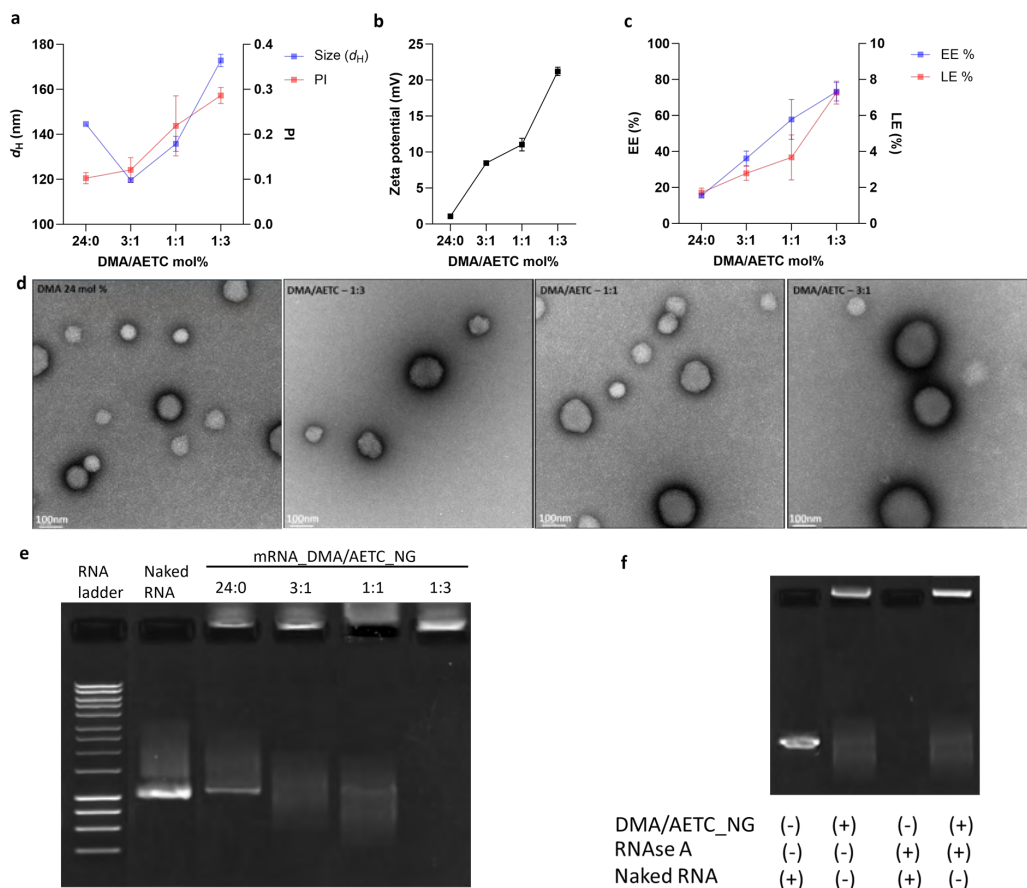
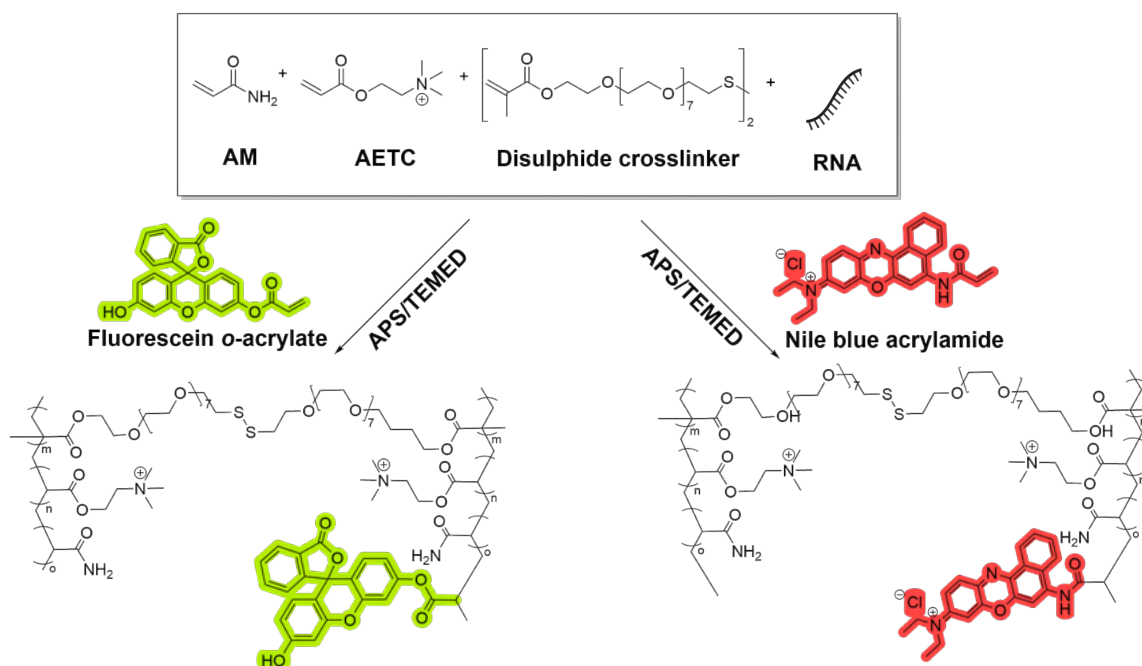


Figure 3.10: Defining optimal DMA/AETC ratio for RNA-loaded NG synthesis. Effect of varying DMA/AETC ratio on a) NG size, PI, b) ζ -potential of different NG types, as measured by DLS and ELS, respectively and c) encapsulation and loading efficiencies. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. d) Representative negative stain TEMs of NGs. Scale bar at 100 nm. e) Agarose gel electrophoresis assay to determine free RNA. Amount of monomer added to NG is indicated by numbers at the top of respective lanes, alongside the RNA ladder and naked RNA. f) Determination of RNA stability upon NG treatment with RNase A (100 U/mg) for 2 h at 37 °C.

Taken together, results indicate that AETC and PolyR were the most efficient at complexing and entrapping the RNA payload. Further, these structural studies show that maintaining a delicate balance between the monomer, crosslinker, surfactant and initiator concentrations is crucial for maximising the benefits accrued from the tuneable NG platform.

3.2.6 Fluorescent nanogel synthesis and optimisation

To facilitate monitoring of cellular trafficking, NGs were synthesised incorporating varying concentrations of the fluorescently labelled monomers, fluorescein-*o*-acrylate (FL: ex/em: 495/517 nm) and nile blue acrylamide (NBA: ex/em: 635/674 nm) (Scheme 3.2.1).



Scheme 3.2.1: Schematic representation of fluorescent NG synthesis.

Fluorescein and nile blue were used to facilitate NG monitoring *in vitro* and *in vivo*, respectively. NG size did not increase significantly until 0.005 mmol (5 mol%) of dyes were included (Figure 3.11a,b,c,d,e,f). Presumably, the steric hindrance caused by increasing dye amount could influence the NG packaging density. Intriguingly, while for FL-NGs, increasing concentration of the FL monomer led to improved NG fluorescence intensity, in the case of NBA it is likely that closer packaging of the dye quenched the fluorescence signal by increased π - π stacking (Figure 3.11g,h).[346] It is also equally likely that the FL-NG system remains within the regime where increasing FL content still enhances fluorescence intensity. A decrease may only have become apparent at even higher concentrations, once quenching effects began to dominate. Nonetheless, 0.005 mmol (5 mol%) dye concentration was chosen for both dyes in

all subsequent studies. Furthermore, the longevity of the NG fluorescent signal was evaluated by incubating the NGs in serum where FL-NGs demonstrated a slight decrease in fluorescence intensity by 24 h, whereas for NBA-NGs complete depletion of NBA signal was observed (Figure 3.11i,j). This could be attributed to NBA-salt-induced aggregation which causes quenching of the fluorescent signal.[347, 348] Lastly, by conducting confocal microscopy, the fluorescence of the NGs was validated in HeLa cells, where the punctate fluorescence in the cytosol is typical for nanoparticles (Figure 3.11k,l). Taken together, these results highlight the tunability of the NG platform for fluorescence-based applications. By optimising dye concentration, NG signal intensity and stability was enhanced to mediate effective cellular uptake monitoring.

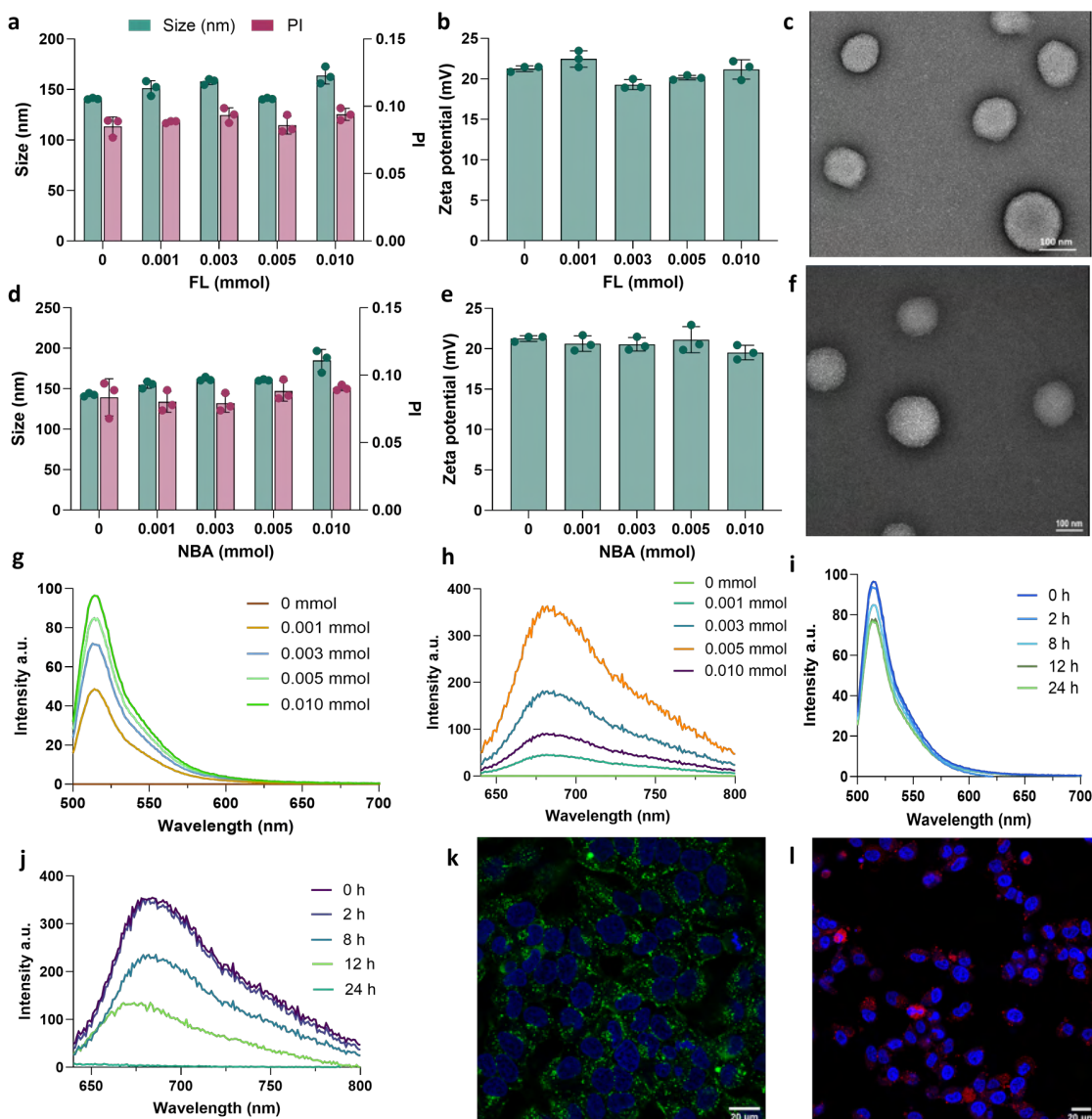
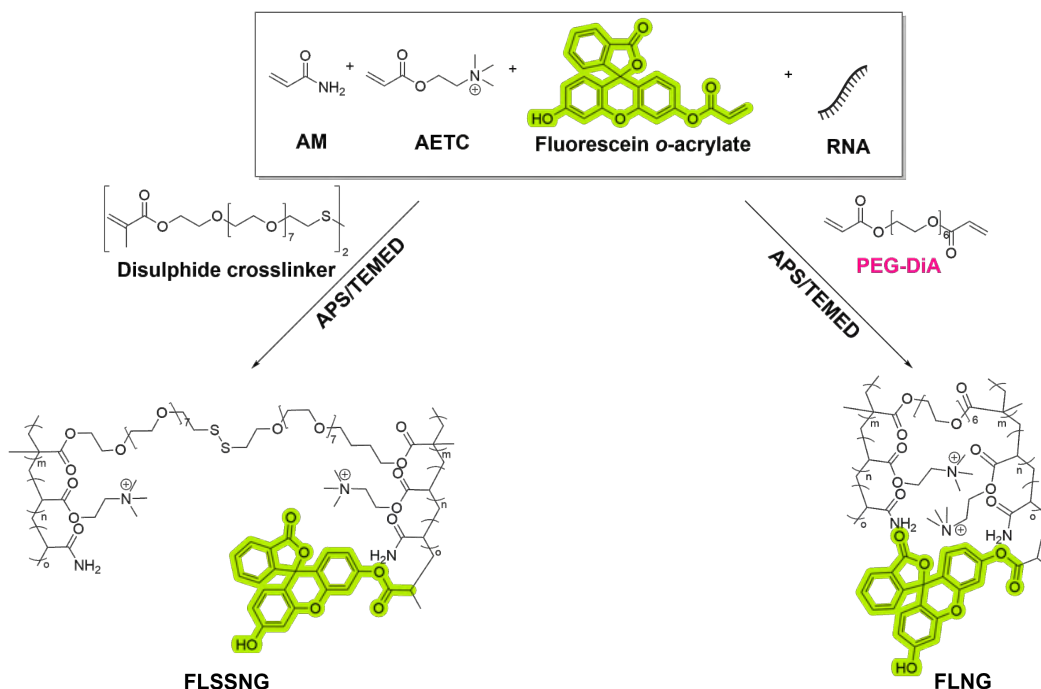


Figure 3.11: Optimisation of RNA-loaded fluorescently-tagged NGs. Effect of varying fluorescein *o*-acrylate (FL) mol% on a) NG size, PI, b) ζ -potential of different NG types, as measured by DLS and ELS, respectively. c) Representative negative stain TEMs of optimised FLNGs. Scale bar at 200 nm. Effect of varying Nile blue acrylamide (NBA) concentrations on d) NG size, PI, e) ζ -potential of different NG types, as measured by DLS and ELS, respectively. f) Representative negative stain TEMs of optimised NBA-NGs. Scale bar at 100 nm. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. Emission spectra for varying molar amounts of g) FL and h) NBA demonstrate concentration dependent fluorescence intensity. Excitation at 488 and 633 nm, respectively. Emission spectra for NG degradation in serum over 24 h for i) FL-NG and j) NBA-NG. Degrading NBA-NGs demonstrate loss of Nile blue fluorescence over time. Confocal microscopy images showing the intracellular uptake of k) FL-NGs and l) NBA-NGs in HeLa cells. Cells were stained with 1 μ g/mL Hoechst 33342 (blue). All images were taken with 40 x oil immersion objective. Scale bar at 30 μ m.

3.2.7 Endosomal escape evaluation of optimised NGs

Endocytosis is the predominant route of cellular entry for nanoparticles, where upon interaction with the plasma membrane, they are engulfed into vesicular endosomes that are then transported to specialised intracellular trafficking compartments.[349] There are five major classes of endocytosis mechanisms, including, phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin or caveolae-independent endocytosis and macropinocytosis.[350] Following this endocytotic vesicles then fuse with early endosomes, that then mature into recycling endosomes or late endosomes, that transform into lysosomes.[349, 351, 352] Critically, this transformation is accompanied by a pH change from pH 7 to around 4.5-5 in lysosomes.[349] The lysosomal lumen is a degradative environment comprising of various hydrolytic enzymes including nucleases, proteases and lipases.[349] Consequently, to mediate effective RNA delivery, it is imperative for RNA-loaded NGs to escape endosomes before the lysosomal stage. Keeping this in mind, it was crucial to investigate the cellular internalisation and endosomal escape capacities of the NGs. Given the recent success of disulfide-containing molecules and polymers in enhancing cellular uptake and cytosolic localisation, the influence of the disulfide-based crosslinker used in the current NG platform was evaluated and the work has been recently published.[260] NGs with (FLSSNG) and without (FLNG) the disulfide-based crosslinker were synthesised (Scheme 3.2.2).



Scheme 3.2.2: Schematic representation of fluorescent disulfide (FLSSNG) and control non-disulfide (FLNG) NG synthesis.

Flow cytometry was used to compare cellular internalisations. Despite their similarity in size and zeta potential (FLSSNG: 200.9 nm and 22.1 mV; FLNG: 219.7 nm and 22.0 mV) (Figure 3.12a,b,c), a ~2.5-fold increase in uptake in HeLa cells was observed for the FLSSNG, compared to the FLNG, as confirmed by confocal microscopy (Figure 3.12d,e).

First to determine the pathway of cellular internalisation, NG uptake was studied in the presence of the following inhibitors comprising all uptake pathways, using flow cytometry. [353]

- Hyperosmolar sucrose (45 mM, clathrin-mediated endocytosis)
- EIPA (100 μ M, macropinocytosis)
- Nystatin (30 μ M, caveolin pathway)
- Dynngo (30 μ M, clathrin-independent/dynamin-dependent)
- 7-ketocholesterol (30 μ M, clathrin-independent/dynamin-independent)
- Cytochalasin D (5 μ M, phagocytosis/macropinocytosis)

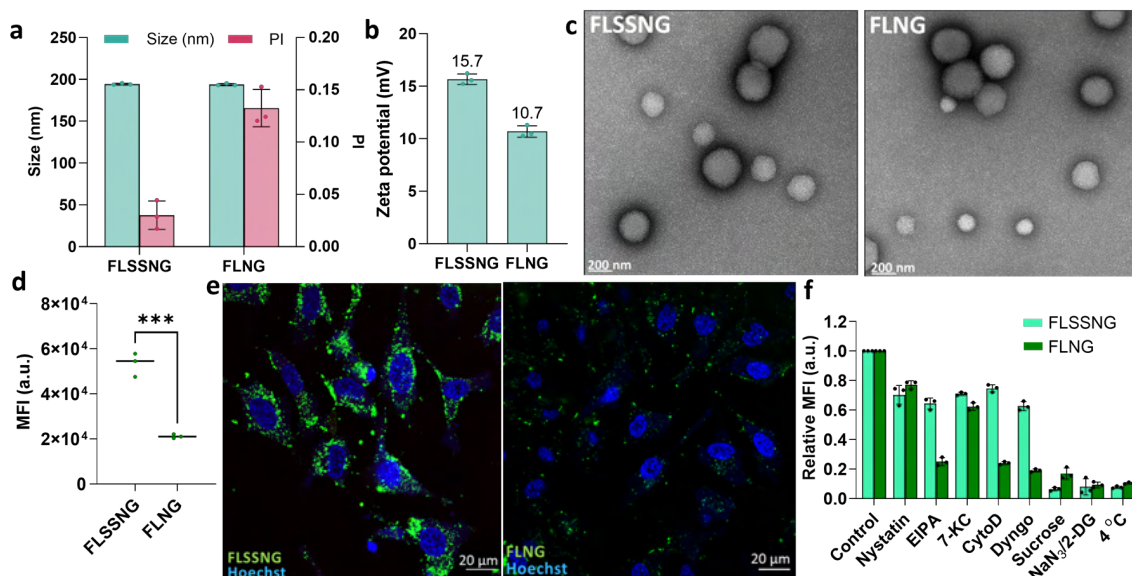


Figure 3.12: Optimisation of fluorescently-tagged FLSSNG and FLNGs. Comparing physicochemical properties of fluorescent NG, including a) size, PI, b) ζ -potential, as measured by DLS and ELS, respectively. c) Representative negative stain TEM of the NGs. Scale bar at 200 nm. d) Quantification of the intracellular uptake of the two different NGs by flow cytometry. Mean fluorescence intensities (MFI %) of the two differently treated cell populations are shown (n=6). Data shown are mean $\pm$ SD and statistical significant was determined using an unpaired t-test, *** $p < 0.001$. Confocal microscopy images showing the intracellular uptake of FLSSNGs and FLNGs in HeLa cells fixed 8 h post-treatment, stained with 1 μ g/mL Hoechst 33342. Images were taken with 63 $\times$ oil immersion objective. Scale bar at 25 μ m. f) Effect of endocytosis inhibitors on FLSSNG and FLNG (n = 3).

- NaN₃/2-deoxy-D-glucose (affects all pathways)

NaN₃/2-deoxy-D-glucose treatments caused significant inhibition in the cellular uptake of both NGs, which was consistent with previous reports and confirms that NG uptake occurs through an energy-dependent mechanism (Figure 3.12f).[290] Furthermore, the most dramatic decrease in the uptake of FLSSNGs was observed after hyperosmolar sucrose treatment (decreased FLSSNG uptake to 12%), suggesting the clathrin-mediated pathway to be a preferred mode of internalisation. Similarly, the non-significant uptake of FLSSNGs at 4°C is indicative of endocytosis over direct uptake. Notably, however, FLSSNG uptake was also reduced by 20-30% upon treatment with EIPA, cytochalasin D, dyngo and 7-KC, suggesting that they may enter cells via alternative pathways in addition to clathrin-mediated pathways. FLNG internalisation was affected by EIPA, cytochalasin D, dyngo and sucrose, also

suggesting the use of a combination of uptake pathways. Overall, the non-disulfide NGs were comparatively inefficient at entering the cells. While this data highlights the mode of internalisation to be primarily endosomal, it is well known that most endocytotic inhibitors tend to be promiscuous in their mechanism of action, with each inhibitor having off-target effects on other internalisation pathways.[353]

To confirm these results, immunofluorescent labelling was used to assess potential NG colocalisation with clathrin-coated vesicles, caveolae (caveolin-1) and early endosomes (Rab5) in HeLa cells, using confocal microscopy (Figure 3.13a,b). To quantify the extent of colocalisation, the Pearson's coefficient was determined. A high Pearson's coefficient number indicates colocalisation, which makes the data points in the plot fall along the diagonal. Divergence of the points from the diagonal indicates disparate locations of the two fluorescent signals, corresponding to a lower Pearson's coefficient value.

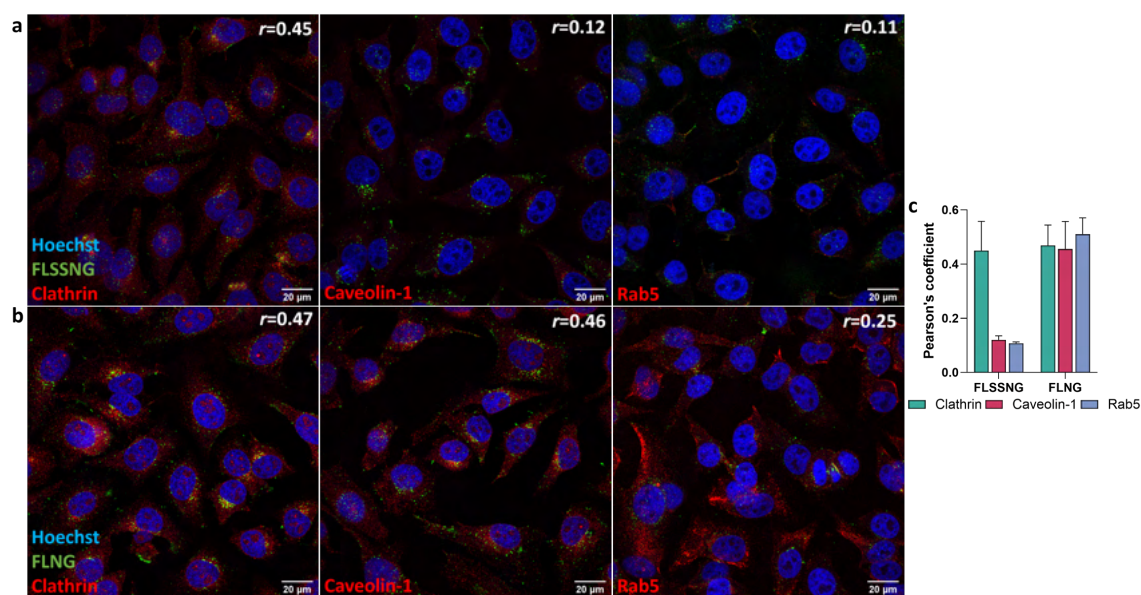


Figure 3.13: **Investigating mechanism of FLSSNG and FLNGs endocytosis using immunofluorescence.** Confocal microscopy images showing a) FLSSNG and b) FLNG colocalisation with clathrin, caveolin-1, rab5 antibodies. Scale bar at 20 μ m. c) Pearson's correlation plot for all antibodies with the two NG types (n = 3).

FLSSNGs were primarily colocalised with clathrin-coated pits ($r = 0.45$), whereas low colocalisation was observed for caveolin-1 ($r = 0.12$) and Rab5 ($r=0.11$) (Figure

3.13c). However, FLNGs exhibited colocalisation with clathrin-coated pits ($r = 0.47$) and caveolin-1 ($r = 0.46$), and greater colocalisation with Rab5-stained early endosomes ($r = 0.25$) than the FLSSNGs. While the data reiterates that the disulfide NGs seem to be taken up via the clathrin-mediated endocytosis route, the low to moderate immunofluorescence observed could also be due to the rapidity with which the NGs are internalised. As the disulfide NGs are uptaken quicker than their non-disulfide counterparts and subsequently trafficked intracellularly, the fluorescent signal at the membrane or early endosomes might be transient or diminished by the time of imaging, thereby leading to a lower detectable signal. Ideally, a more robust investigation would involve a precise knockout/down approach to target particular internalisation pathways.

Although a knockdown strategy was not possible within the scope of this study, another approach was taken to specifically investigate the influence of the disulfide moiety on potential thiol-mediated uptake (TMU). To test whether potential covalent contacts between the FLSSNGs and cell surface-exposed (exofacial) thiols could engender internalisation by potential membrane destabilisation, a library of exofacial thiol blockers was screened, of which asparagusic acid (AspA), was noted to be the most potent TMU inhibitor (Figure 3.14a).[306–309, 354] This was confirmed by both flow cytometry and confocal microscopy (Figure 3.14b,c).

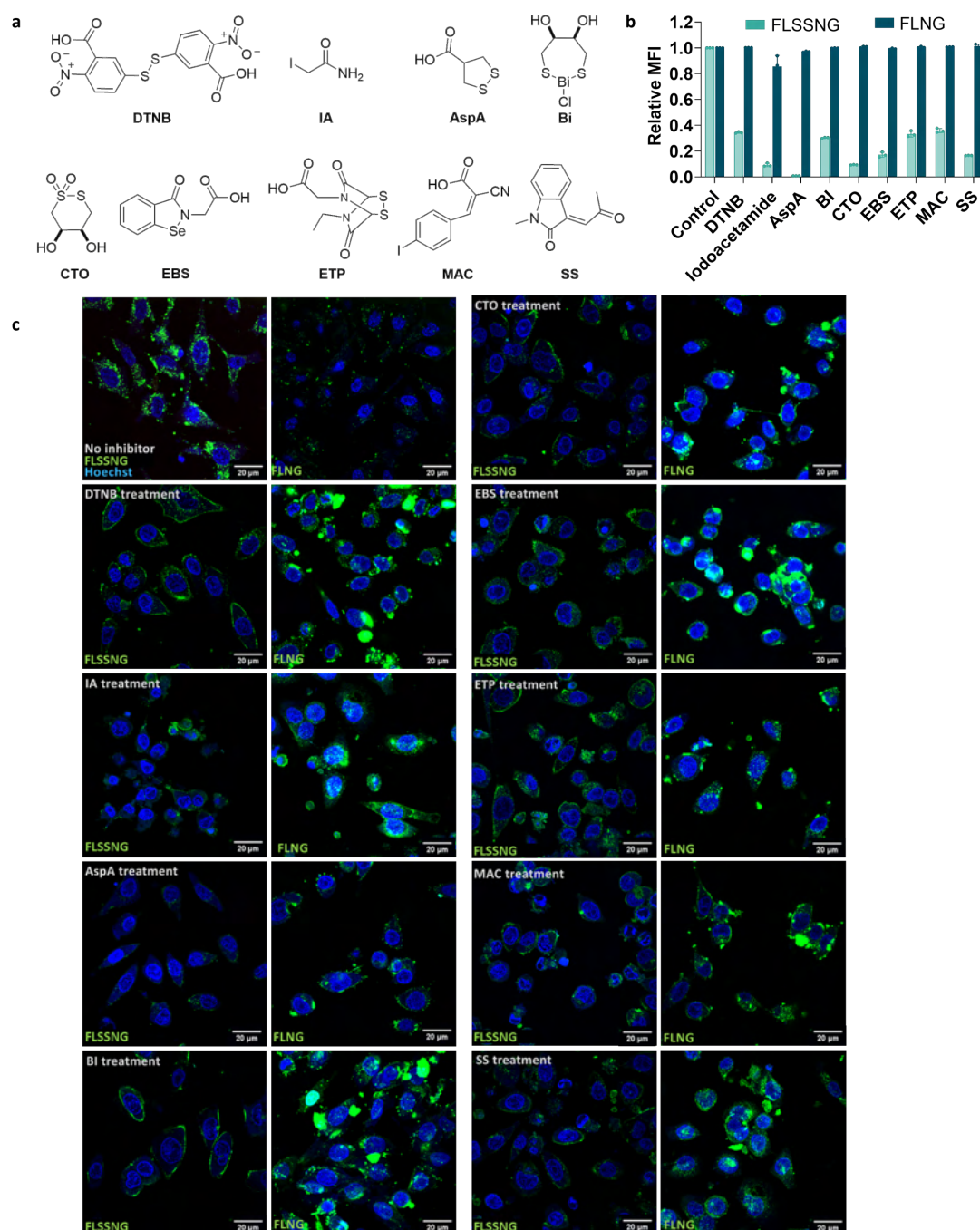


Figure 3.14: **Investigating inhibition of thiol-mediated uptake (TMU).** a) Chemical structures of TMU inhibitors used. b) Cellular uptake of FLSSNG and FLNG post-TMU inhibitor treatment, as measured by flow cytometry ($n = 3$). c) Confocal microscopy images showing the intracellular uptake of FLSSNG and FLNG (green channel) in HeLa cells treated with TMU inhibitors (1 h), fixed 4 h post-NG treatment and stained with 1 $\mu\text{g}/\text{mL}$ Hoechst 33342. Scale bar at 20 μm .

Indeed, a significant reduction in FLSSNG uptake was observed with AspA treatment, whereas FLNGs were unaffected. It is interesting that DTNB (Ellman's reagent)

and sodium iodoacetate (IA), that have been widely used for TMU inhibition [290], did not have a significant effect on the NGs, suggesting the need for better TMU inhibitors, such as those used in this work. Notably, DTNB is positively charged, and it is possible that electrostatic repulsion between DTNB and the positively charged NGs hinders its interaction with target thiols, thereby reducing its inhibitory efficiency.

Having confirmed TMU to be the preferred internalisation mechanism, the relative propensity of the two NGs to undergo endosomal escape was examined, by monitoring the colocalisation of the fluorescent NGs with LysoTracker Red™ over 24 h, using confocal microscopy.[260] FLSSNGs demonstrated significant endosomal escape, as evidenced by the independent fluorescent colour from the LysoTracker Red™ and FLSSNG (Figure 3.15a). Indeed, even a few yellow, fluorescent *foci*, indicating endosomal entrapment, were resolved by 24 h. However, FLNGs were largely colocalised with Lysotracker even at the 24 h time point, indicating considerable endosomal entrapment (Figure 3.15b). Where the FLNGs exhibited a Pearson's value of 0.79 at 12 h (Figure 3.15c,d), a bifurcated distribution of fluorescent signals was observed for the FLSSNGs by 12 h, giving a Pearson's value of 0.49 (Figure 3.15e,f). Pearson's coefficient for FLNGs did not undergo any significant change over the entire 24 h time period, whereas, for the FLSSNGs, the coefficient continuously decreased from an already lower 0.62 at 1 h to 0.41 by 24 h (Figure 3.15d,f). Despite the low Pearson's coefficient, the FLSSNGs also exhibit punctate fluorescence in the cytosol, which can be attributed to endosomal entrapment.[260, 290] While this may indicate cytosolic aggregation of the NGs, as the fluorescein fluorophore is covalently conjugated within the NG framework, causing localised signals upon NG clustering or degradation. However, a similar punctate pattern could also arise simply from the accumulation of multiple intact NGs in close proximity. Therefore, while aggregation is a possibility, further investigation is required to definitively distinguish between these scenarios.

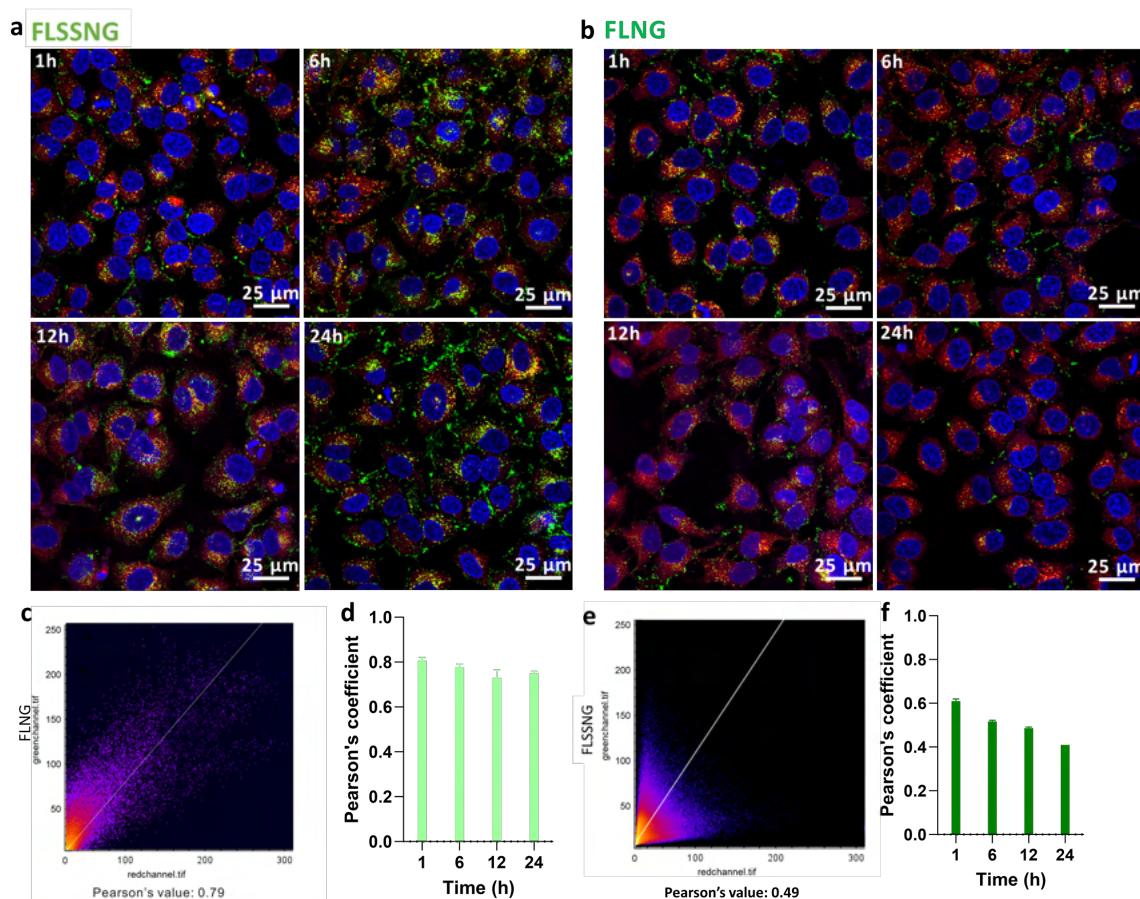


Figure 3.15: Endosomal escape evaluation of fluorescent disulfide and non-disulfide NGs. Confocal microscopy images showing the intracellular uptake of a) disulfide (FLSSNG) and b) non-disulfide (FLNG) fluorescent NGs at 12 h and 24 h. Cell stained with 75 nM Lysotracker Red and 1 μ g/mL Hoechst 33342. All images were taken with 63 $\times$ oil immersion objective. Scale bar at 25 μ m. c) Pearson's correlation plot for FLNGs at 12 h and d) temporal evaluation of Pearson's coefficient for FLNGs; e) Pearson's correlation plot for FLSSNGs at 12 h and f) the time dependent Pearson's coefficient evaluation for FLSSNGs.

To take this a step further, the effect of various cationic monomers on NG endosomal escape efficiency was examined. Notably, the guanidinium moiety present in AC-GUA and PolyR can reportedly trigger endosomal escape via the proton sponge effect.[335, 355] Further, the imidazole ring present in PolyH can augment endosomal lysis by osmotic swelling.[344] As before, the colocalisation of fluorescently-labelled NGs with Lysotracker RedTM was monitored to assess endosomal escape. While the five types of NGs were similarly efficient at mediating cellular uptake, their endosomal escape capabilities differed considerably. As shown in Figure 3.16a,b,

AETC_NG and PolyR_NG demonstrate significant endosomal escape, as evident by the independent fluorescent colour from the fluorescein-labelled NGs and Lysotracker Red™. On the contrary, PolyH_NG, AC-GUA_NG and DMA/AETC_NG were largely colocalised with Lysotracker Red™ indicating endosomal entrapment (Figure 3.16c-e). To further quantify the extent of colocalisation, the Pearson's coefficient for colocalisation was calculated for all NGs (Figure 3.16f). AETC_NG and PolyR_NG had Pearson's values of 0.38 and 0.32, respectively, whereas the other NGs had higher Pearson's values. Overall, these results highlight AETC_NG and PolyR_NG as the most promising candidates for mediating efficient endosomal escape, likely via the osmotic effect facilitated by the permanent positive charge.[355–357]

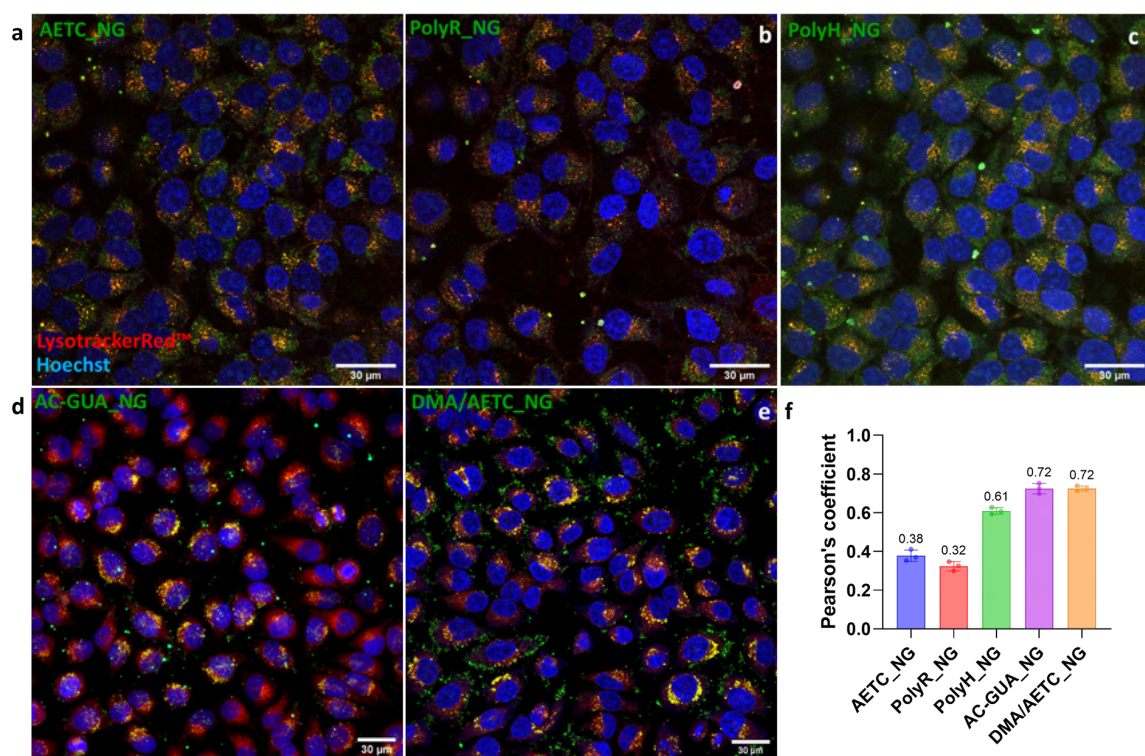


Figure 3.16: **Evaluation of endosomal escape in HeLa cells.** Confocal microscopy images showing the intracellular uptake of fluorescent (green) a) AETC_NG, b) PolyR_NG, c) PolyH_NG, d) AC-GUA_NG and e) DMA/AETC_NG. Cells were stained with 75 nM LysotrackerRed™ (red) and 1 µg/mL Hoechst 33342 (blue). All images were taken with 40× oil immersion objective. Scale bar at 30 µm. f) Pearson's correlation plot for all NGs (n=3).

3.2.8 *In vitro* mRNA transfection efficiency screening of cationic monomers

The mRNA transfection efficiency of the optimised NG compositions was examined by delivering mRNA encoding for green fluorescent protein (GFP) to HeLa cells *in vitro*. Three different doses of GFP mRNA-loaded NGs (20, 40 and 80 $\mu\text{g}/\text{mL}$: 0.55, 1.45, 3.24 μg RNA) were administered and the expression of GFP mRNA was monitored using the Incucyte® Live-Cell Analysis System for 86 h. JetMESSENGER® (jetM: 3.25 μg RNA) was used as a positive control following its demonstration of effective mRNA delivery in different cell models.[358] Figure 3.17a-e shows the green fluorescence signal obtained per image for the different NG types. In all cases, NGs demonstrated concentration-dependent GFP expression, whereby increasing the NG concentration increased the GFP signal, as observed. Notably, where the commercial reagent jetM enabled rapid transfection and maximum GFP expression within 12 h, NG-mediated transfection exhibited a gradual increase in GFP signal with maximal GFP expression ~ 24 h post-transfection. This delayed and sustained protein expression was concordant with the controlled release profile of the NGs as observed *ex vivo*, thereby highlighting the stimuli-responsiveness of the platform. Evidently, AETC_NG and PolyR_NGs performed best, providing higher and more sustained GFP expression across all concentrations. In absolute numbers, the commercial reagent provided higher and faster GFP expression before up to 24 h. However, after normalising the fluorescence with total protein content, 40 and 80 $\mu\text{g}/\text{mL}$ AETC_NG and 80 $\mu\text{g}/\text{mL}$ PolyR provided a significant increase in GFP expression compared to the commercial reagent (Figure 3.17f). Although increasing the AETC_NG concentration from 20-40 $\mu\text{g}/\text{mL}$ provided a ~ 3 -fold increase in GFP signal, any further increase in concentration did not provide a significant increase in GFP expression, indicating that the effective NG concentration should be around 40 $\mu\text{g}/\text{mL}$ or higher.

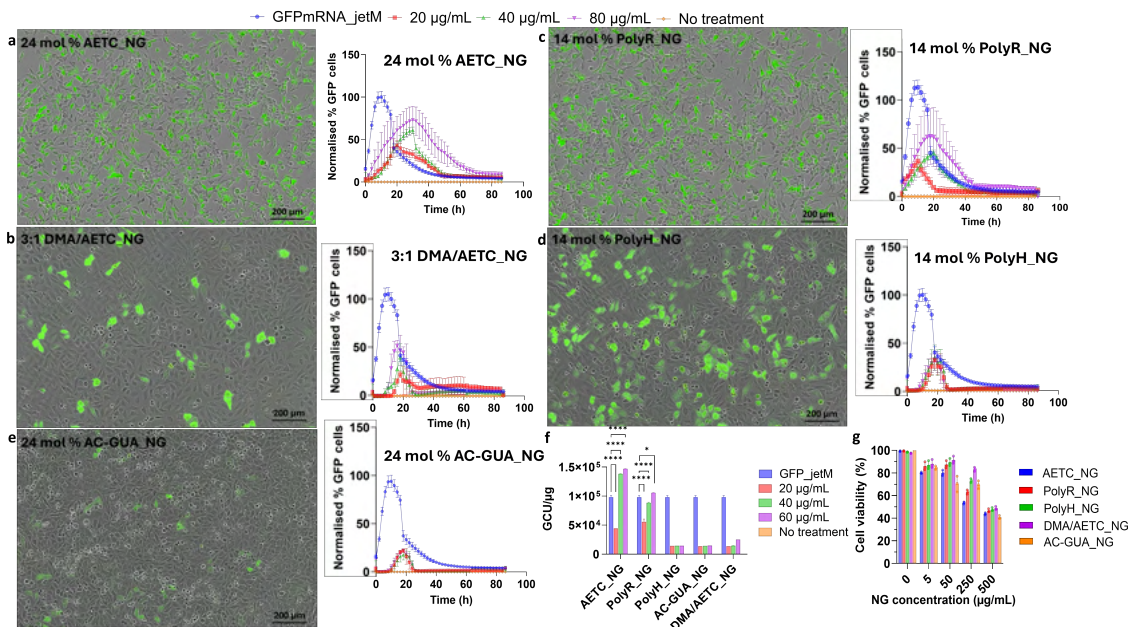


Figure 3.17: *In vitro* NG GFP mRNA expression profiles with associated IncuCyte images of GFP expression in HeLa cells. Cells treated with 60 $\mu\text{g/mL}$ (3.24 μg RNA) of NGs at 20 h for a) AETC_NG, b) PolyR_NG, c) DMA/AETC_NG, d) PolyH_NG, e) AC-GUA_NG. Each expression profile shows the fluorescence expression (normalised % of GFP positive cells) as a function of time. In all cases, transfection efficiency was compared with the commercial reagent Polyplus® jetMESSENGER® (GFPmRNA_jetM; 3.25 μg). Scale bar at 200 μm . f) Fluorescent protein expression normalised by total protein quantification (BCA assay) for each NG dose, compared against untreated cells and cells treated with the commercial reagent Polyplus jetMESSENGER (jetM). Data analysed and evaluated for statistical significance by employing a two-way ANOVA with Tukey's multiple comparisons test. Statistical significance was determined using an unpaired t-test. ($ns < 0.1234$, $*p < 0.0332$, $**p < 0.0021$, $***p < 0.0002$, $****p < 0.0001$).

To then confirm the optimal concentration for *in vivo* delivery, cell viability was measured using the CCK-8 assay in HeLa cells for each NG composition. Figure 3.17g, shows that treatment with AETC_NG, PolyR_NG and PolyH_NG upto 50 $\mu\text{g/mL}$ did not cause any significant toxicity. However, at concentrations above 250 $\mu\text{g/mL}$, compositions with a high cationic charge, including AC-GUA_NG, induced significant cytotoxicity, resulting in approximately $\sim 55\%$ decrease in cell viability. Further, DMA/AETC_NG demonstrated lowest cytotoxicity among the NGs investigated, presumably due to reduced cationic charge of the composition.

AETC_NGs demonstrated improved transfection efficiency compared to other cationic NGs, likely due to their superior cellular uptake and enhanced endosomal escape capabilities. As the transfection efficiency can vary across different cell lines, owing to the differences in uptake and intracellular transport mechanisms, the transfection potency of the AETC_NG platform was evaluated in six different cell lines.[359, 360] These included several “easy to transfect” (HEK293T, HeLa, Caco-2, C4-2) and “difficult to transfect” (bEND.3, RAW264.7) cell lines.[361, 362] As before, GFP expression for three different doses of the GFP mRNA-loaded AETC_NGs (20, 40 and 60 $\mu\text{g}/\text{mL}$, containing 0.55 μg , 1.45 μg and 3.24 μg mRNA, respectively) was monitored using the IncuCyte® for 86 h. Figure 3.18a shows the representative images and green fluorescence signal obtained per image of the different cell types. Despite the variation in GFP expression across the different cell lines, AETC_NGs demonstrated a concentration-dependent increase in GFP expression, as expected. As before, the NG demonstrated controlled release of the GFP mRNA, thereby allowing delayed and sustained GFP expression. While jetM and Lipofectamine™ MessengerMax™ displayed the highest and fastest GFP expression, when the normalised fluorescence was compared (to account for different cell counts across the cell lines), 40 and 60 $\mu\text{g}/\text{mL}$ AETC_NG exhibited improved transfection efficiency compared to the commercial reagents (Figure 3.18b). As anticipated, RAW264.7 and bEND.3 cells showed the lowest transfection efficiency.

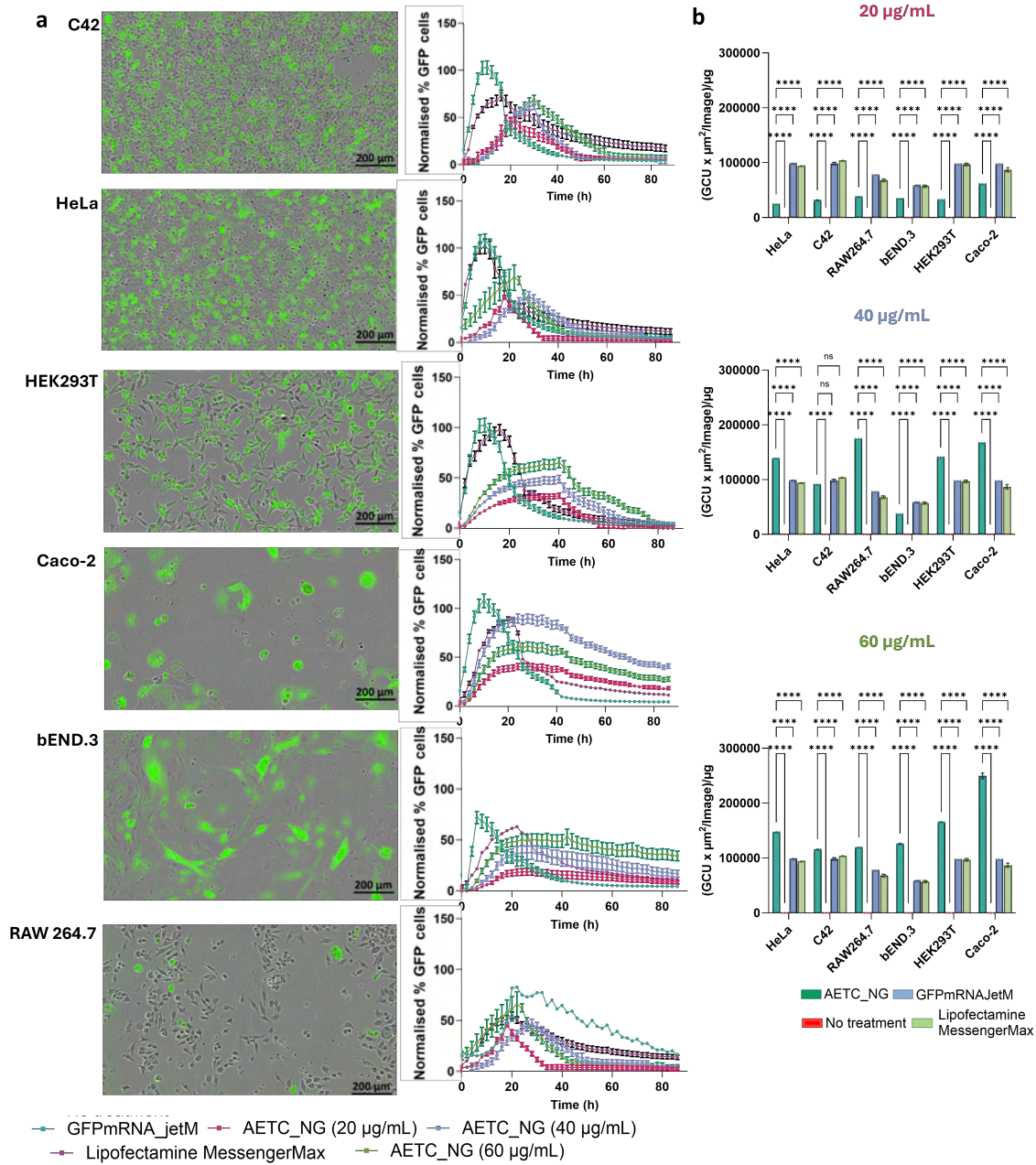


Figure 3.18: **Comparing transfection efficiency of varying doses of AETC_NG with Polyplus® jetMESSENGER® and Lipofectamine™ MessengerMax.** a) The leftmost panel shows representative Incucyte images of each cell line transfected with 60 $\mu\text{g/mL}$ AETC_NGs, at the 20 h time point. Scale bar is at 200 μm . Magnification at 10 $\times$. The next panel shows the fluorescence expression (normalised % of GFP positive cells) as a function of time for each cell line. b) Fluorescent protein expression normalised by total protein quantification AETC_NG dose, compared against untreated cells and cells treated with the commercial reagent Polyplus® jetMESSENGER® and Lipofectamine™ MessengerMax™. Data analysed and evaluated for statistical significance by employing a two-way ANOVA with Tukey's multiple comparisons test ($ns < 0.1234$, $*p < 0.0332$, $**p < 0.0021$, $***p < 0.0002$, $****p < 0.0001$).

Finally, the cytotoxicity of AETC_NG was evaluated in all six cell lines (Figure 3.19a) and was found to be minimal up to 250 $\mu\text{g/mL}$, whereas the commercial reagents demonstrated higher cytotoxicity in RAW264.7, Caco-2 and bEND.3 cells. Furthermore, the AETC_NGs demonstrated minimal haemolysis even at the highest concentration at 24 h, where only $\sim 19.3\%$ of RBCs were haemolysed upon incubation with 500 $\mu\text{g/mL}$ NGs by 24 h (Figure 3.19b).

Following this, NGs were evaluated for their ability to deliver functional mRNA *in vivo* in *Caenorhabditis elegans* via feeding.[288] As a genetically tractable multicellular model organism, *C. elegans* provided a useful preliminary system to investigate the *in vivo* trafficking dynamics of the NGs. The NGs demonstrated remarkable stability, surviving the acidic environment of the digestive tract and enabling successful mRNA transfection in the gut. Therefore, this small, proof-of-concept study highlighted the potential of NGs as a non-invasive platform for functional mRNA delivery in whole organisms.

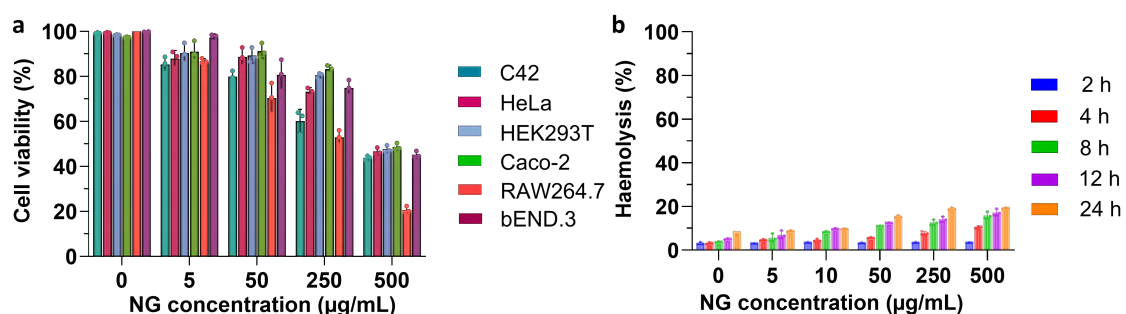


Figure 3.19: **Cellular viability and haemolysis reveal minimal detrimental effects on cell health.** a) Cell viability for different AETC_NG concentrations in various cell lines, after 24 h of incubation. b) Haemolysis assay conducted on red blood cells isolated from mouse blood, over various timepoints and AETC_NG concentrations. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$.

3.2.9 Evaluating transfection efficiency of AETC_NG using fluorogenic Mango II RNA aptamers: a case study.

As a proof of concept, fluorogenic RNA aptamers were delivered using AETC_NGs to demonstrate their potential for precise, spatiotemporal cytoplasmic RNA detection. RNA aptamers are single-stranded oligonucleotides with well-defined three-

dimensional structures that allow them to bind with high affinity and specificity to a diverse range of molecular targets, including proteins, small molecules or other nucleic acids.[363, 364] Owing to these properties, they are widely employed in diagnostics, therapeutics and in biosensing applications.[365–367]

Fluorogenic RNA aptamers — such as the Mango II (MII) RNA aptamer — become fluorescent upon binding specific small-molecule fluorophores (e.g. TOI-Biotin), allowing for highly sensitive and precise subcellular visualisation and quantification of RNA molecules (Figure 3.20a).[368–370] This study was conducted in collaboration with the Single Molecule Imaging Group at the LMS, leveraging MII RNA aptamer templates previously optimised in Professor David Rueda’s lab. DNA templates encoding the MII aptamers were subsequently transcribed *in vitro* to generate the RNA aptamers that were encapsulated within AETC_NGs (hereafter known as MII-RNA_NGs) (Figure 3.20a).[260]

To determine the RNA loading capacity and structural compatibility of the NGs [371], they were loaded with MII-RNA transcripts of increasing lengths. Although these NGs shared structural features with prior compositions, EE% and LE% decreased with increasing RNA size, likely due to increased steric hindrance (Figure 3.20b,c,d). As larger RNA molecules are more structurally complex and occupy more volume, their extended secondary and tertiary structures can physically obstruct tight packing, thereby reducing the packaging efficiency within the NG matrix (Figure 3.20d). This result was confirmed by total internal reflection fluorescence (TIRF)-based photobleaching analysis to estimate the number of RNA transcripts within the NGs (Figure: 3.20e,f). TIRF microscopy enables selective excitation of fluorophores within ~100-200 nm of the coverslip surface, thus reducing background fluorescence and allowing high-contrast single-molecule imaging of the NGs injected into a flow cell. The sample was then subjected to constant illumination and *foci* with adequate intensities were selected and their photobleaching traces analysed. A maximum likelihood estimate analysis of the photobleaching trajectory revealed that on average as the RNA size increased, the number of transcripts inside the NGs decreased.[372, 373] On average, NGs encapsulated ~six molecules of the smallest RNA (5S-MIV; 131

nt) but only one of the largest (MII x24; 2097 nt). Notably, the photobleaching step size distributions exhibited at least two populations corresponding to either one or two molecules bleaching within the time resolution used (Figure 3.20e). This observation is common, as Mango dyes tend to bleach quickly under constant illumination. This is likely due to dye isomerisation and fluorescence quenching effects, which may result from homo-Förster resonance energy transfer (FRET) between closely spaced fluorophores in RNA *foci* with high aptamer density.

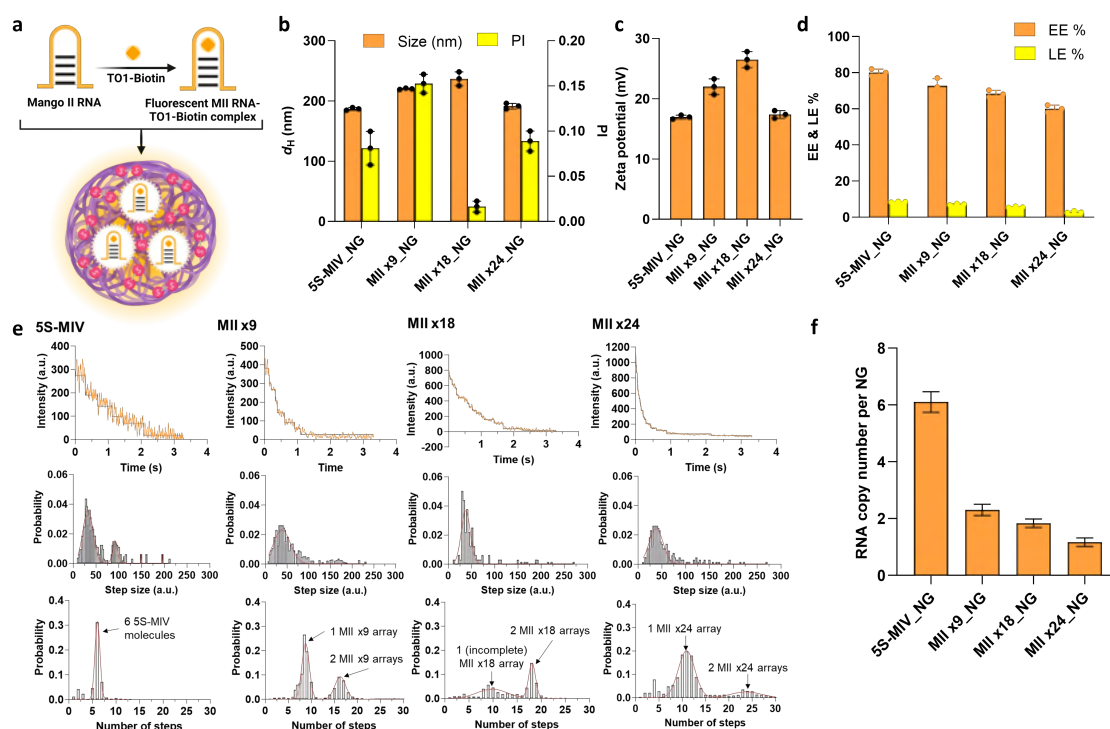


Figure 3.20: Physicochemical characterisation and single-molecule quantification of Mango II RNA-loaded NGs. a) Schematic representation of Mango II RNA complexing with TO1-Biotin. Graphs comparing b) sizes and PIs, c) ζ potentials, d) encapsulation and loading efficiencies of NGs loaded with various Mango RNA payloads. e) Representative bleaching curves for individual Mango RNA *foci* (yellow) fitted with a maximum likelihood step finding algorithm (black) with associated distributions of photobleaching step sizes and number of steps fitted with gaussian curves. Note: the incomplete MII x18 array could have resulted from incomplete transcription of the aptamer repeat sequence, which can be difficult to transcribe via *in vitro* methods. f) RNA copy number per NG as estimated from the step-wise photobleaching analysis.

Importantly, the fluorescence emission spectra of NG-encapsulated and free RNA were nearly identical, indicating preservation of RNA structure and function (Figure

3.21a. Following this, to evaluate the transfection efficiency, NGs were added to HeLa cells and compared against commercial reagents by counting the Mango II RNA *foci* (Image J FociPicker3D plugin). MII-RNA_NGs demonstrated a 3.5-fold increase in transfection efficiency relative to the commercial reagent (Figure 3.21b, Figure A7). The kinetics of MII-RNA_NG uptake were subsequently assessed to determine whether changing the RNA payload would affect NG-mediated release and degradation. Time-course analysis revealed ~36% cellular uptake by 8 h, followed by RNA release peaking at 10 h. Fluorescence persisted for > 24, indicating protection from degradation and extended intracellular retention, and closely aligning with previous observations for GFP mRNA delivery (Figure 3.21c,d). Remarkably, the persistence of MII-RNA fluorescence even at 24 h post-transfection suggests that the NGs effectively protect the RNA cargo from degradation, enabling prolonged intracellular retention.

The true power of the MII aptamer platform lies in its application for visualising the subcellular localisation of functional RNA transcripts. Therefore, mRNA encoding for the mCherry protein tagged with 24 MII repeats was loaded within the NGs (mChMII-RNA_NG).[369] HeLa cells were transfected with mChMII-RNA-NG and time-dependent evolution of MII-RNA and mCherry signal intensities were tracked over time using confocal microscopy (Figure 3.21e,f). While the RNA signal increases over the initial 12 h, as seen previously, the mCherry signal only increases 6 h post-transfection. This result is consistent with delayed RNA release, followed by sustained protein expression for > 24. The distinct, punctate MII *foci* contrasted with the diffuse mCherry protein signal, confirming successful RNA delivery, translation, and preservation of structural integrity.

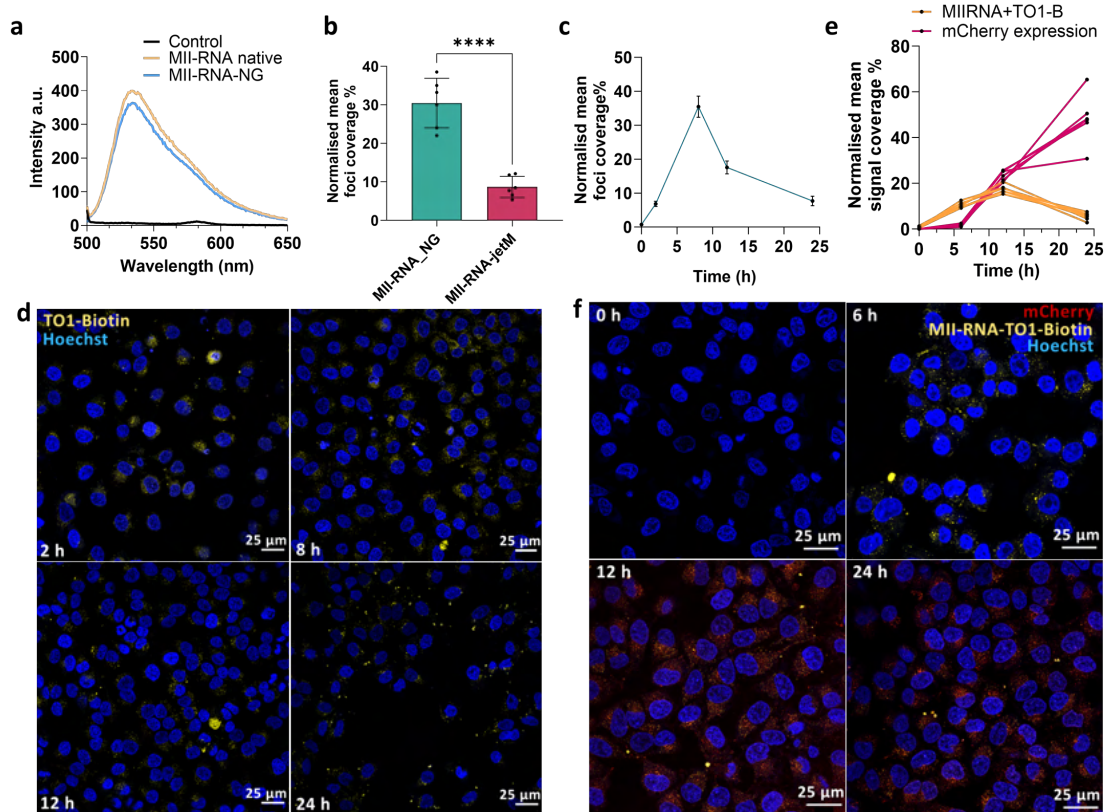


Figure 3.21: **Evaluation of the transfection efficiency of MII-RNA-loaded NGs.** a) Emission spectra for 125 nM MII-RNA encapsulated within NGs and unencapsulated native RNA, in the presence of 200 nM TO1-Biotin. PKM buffer is shown as a control. b) Comparison of transfection efficiency against commercial reagent jetMESSENGER by determining Mango *foci* (n = 6). In all cases, data shown are mean $\pm$ SD and statistical significance determined using a one-way ANOVA with Tukey's multiple comparisons test, ***p < 0.0002, *p < 0.0332, ns < 0.1234. c) Comparison of Mango *foci* signal coverage across different timepoints (n = 6). d) Confocal microscopy images of HeLa cells treated with MII-RNA_NGs and fixed at various time points. Cells were stained with 200 nM TO1-B (yellow) and 1 μ g/mL Hoechst 33342 (blue). All images were taken with 40 x oil immersion objective. e) Characterisation of mChMII-RNA-loaded NGs and transfection - kinetics of Mango *foci* and mCherry signals (n = 6). f) Maximum projection of HeLa cells treated with mChMII-RNA-SS-NG, stained with 200 nM TO1-B (yellow) and 1 μ g/mL Hoechst 33342. Images taken with 63 x oil immersion objective.

3.3 Conclusions and limitations

In sum, after careful optimisation of each reaction component, an optimised NG composition for RNA encapsulation and delivery was developed. The results above emphasise the biocompatibility of this composition whilst defining optimal concen-

trations to be used in subsequent investigations. By testing a variety of monomers to electrostatically entrap RNA within the NGs, the tunability of the NG platform was elucidated. However, the loading efficiency of the optimised NGs was moderate, likely due to the comparatively high molecular weight of the polymer relative to the encapsulated RNA payload. Future work could investigate the impact of increasing the amount of RNA during synthesis on loading efficiency. Nevertheless, the AETC_NG system demonstrated the greatest proficiency at cellular internalisation and transfection, when compared to commercial standards, and was therefore carried forward for functional studies.

These functional studies, particularly those involving Mango II (MII) RNA aptamers, convincingly demonstrate the potential of AETC_NGs as a diagnostic RNA delivery platform. However, although the encapsulated RNA remained functional, the overall encapsulation and loading efficiencies were inversely correlated with RNA size, potentially restricting the platform’s use for long or structurally complex transcripts. Further, the use of TIRF-based photobleaching to estimate loading provides single-molecule sensitivity, the method may underestimate the total RNA content due to rapid photobleaching or incomplete dye activation.

While the current work focused on RNA entrapment, the modularity of the NG platform lends itself to a multitude of potential investigations for broader applications. For instance, a deeper understanding of the initially formed emulsion and polymerisation processes could yield further control over NG reaction dynamics and architecture. By applying deep learning models to the data collected from such optimisation studies, it would be possible to predict reaction outcomes and guide NG structure to suit specific delivery needs. Ultimately, such insights could make it possible to employ strategies for automating the synthesis, a key step towards scaling and translating the NG composition for clinical use.

Chapter 4

Assessing the Stimuli-Responsive Properties of Disulfide-Based Nanogels

4.1 Introduction

In the era of precision medicine, there is an increasing emphasis on developing RNA delivery systems with the capability to deliver therapeutic agents to specific sites.[374] The non-specific distribution and uncontrolled drug release profiles of conventional delivery systems have led to the emergence of stimuli-responsive delivery platforms.[285] These can respond to endogenous stimuli including biological signals or pathological environments,[375] enabling highly precise spatiotemporal control over payload delivery, thereby improving the therapeutic efficacy. Such biological stimuli include elevated temperatures,[376] pH,[329] high reduction potential,[377] enzyme concentration[378] and reactive oxygen species (ROS)-rich environments.[379] Among the plethora of smart materials that have been fabricated by integrating bioreceptive features, disulfide bonds have emerged as a particularly promising stimuli-responsive motif.[380] Indeed, the reversibility of the dynamic covalent disulfide chemistry has attracted extensive attention in biomedical fields due to its susceptibility to multiple intracellular redox environments.[381]

Cellular redox homeostasis refers to the dynamic equilibrium between oxidising and reducing species within cells. In cancer cells, this balance is often disrupted, resulting in elevated levels of both intracellular glutathione (GSH) and ROS, which further accentuates differences from non-cancerous tissues.[380] GSH can act as a potent exchange partner in the intracellular environments, where its concentration ranges between 1-10 mM, that is substantially higher than its extracellular concentration (2-20 μ M).[382] Although this concentration gradient in reduction potential offers a potent strategy for exerting spatiotemporal control over payload delivery, the potential of RNA-loaded disulfide-based NGs remains underassessed. As such, this chapter describes and evaluates the GSH-responsiveness of the disulfide NGs.

Furthermore, oxidative stress is a hallmark of cancer cells, where ROS are overproduced due to oncogene activation, mitochondrial malfunction, and chronic inflammation.[379, 383] While the [ROS] in normal cells is maintained in the range of 0.001–0.7 μ M, ROS levels in diseased cancerous cells can reach up to 100 μ M.[384,

[385] The mitochondrial electron transport chain (ETC) is the primary source of endogenous ROS, with complexes I and III being key sites where redox reactions can lead to electron leakage to the cytoplasm, resulting in ROS formation.[248, 386] These free electrons combine with O_2 to form superoxide ($O_2^{\cdot-}$), which is then converted to hydrogen peroxide (H_2O_2) by superoxide dismutase.[248] Typically, H_2O_2 is reduced to H_2O and O_2 by catalase. However, in tumour cells, the elevated ROS levels can cause H_2O_2 to undergo Fenton-like reactions, generating cytotoxic hydroxyl radicals ($HO^{\cdot}$).[248] Due to their reductive nature, sulphur-based carriers can also be oxidised in the presence of diverse ROS species including H_2O_2 , $O_2^{\cdot-}$, and $HO^{\cdot}$. [249, 380] Although many polysulphide-based, ROS-responsive nanomaterials have been tested to date,[387] few exhibit cargo release under the relevant ROS concentrations present in tumours. As such, this chapter also examines the extent to which the disulfide NGs demonstrate susceptibility to various ROS species.

Aside from the redox imbalances observed in the tumour microenvironment, owing to the innate heterogeneity and hypoxia, cancer cells can lack a consistent nutrient supply.[387] This encourages metabolic rewiring to favour glycolytic pathways for energy production, resulting in increased lactic acid accumulation and a consequent decrease in the pH around the solid tumour.[80, 388] Such differences in pH offer a biological rationale for tailoring polymeric delivery vehicles to degrade selectively at the tumour microenvironment. Prior reports have indicated that polymers with acidic or basic side chains undergo conformational changes over the physiological relevant pH range ($\sim 5-8$). [240, 389–392] Similarly, pH-sensitive NGs prepared using natural and synthetic polymers or employing various release mechanisms (pH-triggered swelling or degradation) have also gained traction.[240, 388, 390–395] These systems are synthesised by incorporating various pH-sensitive motifs chemical motifs, including esters, acetals, ketals, oximes, hydrazones and maleic anhydride derivatives that are hydrolysed in acidic conditions.[395, 396] However, in addition to extracellular pH effects, intracellular pH gradients – particularly within endosomal and lysosomal compartments (pH $\sim 4.5-6$) – also present an opportunity to enhance cytoplasmic nucleic acid delivery.[356] For successful nucleic acid delivery, the NGs must undergo endosomal escape. pH-responsive motifs can facilitate this by disrupting

the endosomal membranes under acidic conditions.[397] Cationic polymeric systems with high buffering capacities (e.g. polyamines) [398] can trigger endosomal escape via the proton sponge effect, or alternatively by insertion of free cationic polymer chains (e.g. membrane disrupting peptides[399]) into the endosomal membrane to increase membrane permeability.[260, 397] Therefore, owing to the presence of pH-sensitive chemical architectures within the NGs, their morphological changes and cargo release profiles were also assessed in response to various physiological pH changes.

4.2 Results & Discussion

4.2.1 Evaluating GSH-responsiveness of disulfide NGs

To install GSH-responsiveness in the NGs, an aminoethyl methacrylate-incorporating disulfide diacrylate (AEM-SS-DiA) crosslinker was synthesised using a standard NHS coupling strategy and characterised using ^1H NMR (Figure A5a), ^{13}C NMR (Figure A5b), FT-IR (Figure A5c), and APCI-MS (Figure A6a).[288] The crosslinker was initially optimised to encapsulate protein-based payloads, and was then adapted for the synthesis of RNA-loaded NGs, as required by the aims of the current project.[260, 288]

The crosslinker incorporated a disulfide motif flanked by poly(ethylene glycol) (PEG) units (to improve serum stability and biocompatibility) and NHS ester handles (to enable amide bond coupling to the acrylate-functionalised amine). As such, the crosslinker was endowed with a unique multifunctionality: once the acrylated reactive handles were covalently polymerised to form the NG framework, this enabled both redox-responsive release of the entrapped payload and provided critical structural stability (Figure 4.1).

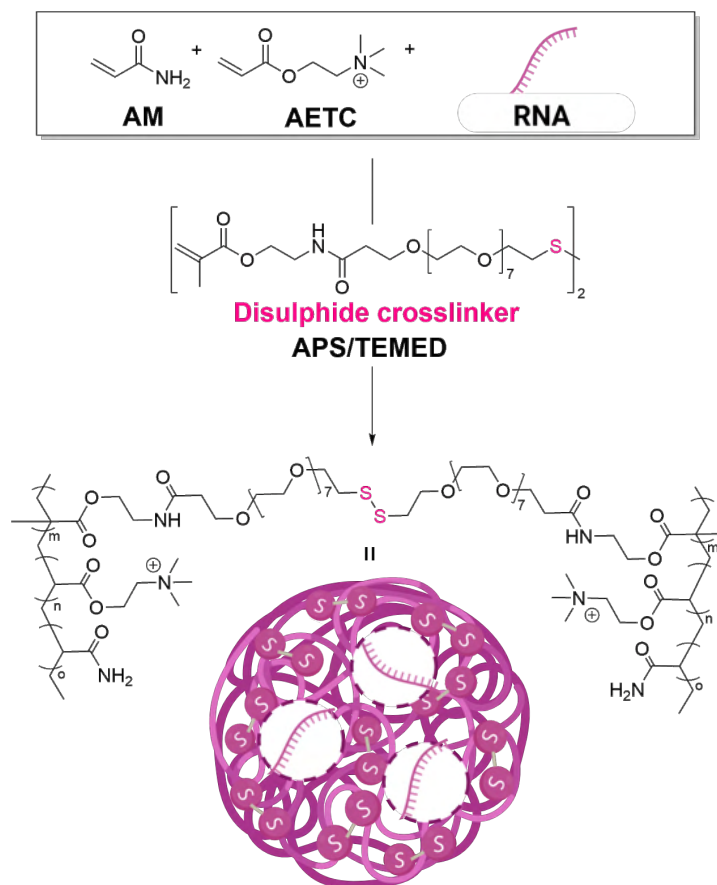


Figure 4.1: Representation of NG synthesis with disulfide-based crosslinker.

Crosslinker cleavability was assessed by incubation with 20 mM GSH for 24 h at 37 °C. The resultant products were then examined using APCI-MS (Figure b), where the cleaved fragment peak was present at 586.3 m/z $[M + H]^+$, where $M = (C_{26}H_{49}NO_{11}S)^+$, thereby confirming that the crosslinker was GSH-responsive (Figure A6b).

To confirm NG stability in the extracellular environment during circulation, both disulfide (SS-NGs) and non-disulfide NGs (n-NGs - synthesised with a commercially available peg-diacrylate (PEG-DiA) crosslinker were treated with 20 μ M GSH at 37 °C (Figure 4.2a). Similar amount of RNA (11%) was released from the n-NGs comparable to the non-GSH-treated NGs (11%). This could be attributed to release of surface-bound RNA. Furthermore, SS-NGs treated with 10 mM GSH (corresponding to intracellular levels), led to 83% release of RNA within 24 h (Figure 4.2b). The GSH-responsiveness was also evaluated by examining changes in NG architecture by

DLS and TEM (Figure 4.2c,d,e). Non-disulfide and untreated NGs demonstrated constant hydrodynamic diameters and PIs. However, the disulfide NGs decreased in size for up to six hours post-incubation with 10 mM GSH, which can be attributed to the rapid cleavage of the disulfide bonds at the NG surface.[400] This exposes the inner disulfide crosslinks to the surrounding environment, leading to an initial swelling and aggregation, as evidenced by the gradual increase in size and PI.[377] In contrast, at 20 μ M GSH there were no significant changes in size or PI of the disulfide-bearing NGs, thereby reinforcing the specificity of this nanoplatform.

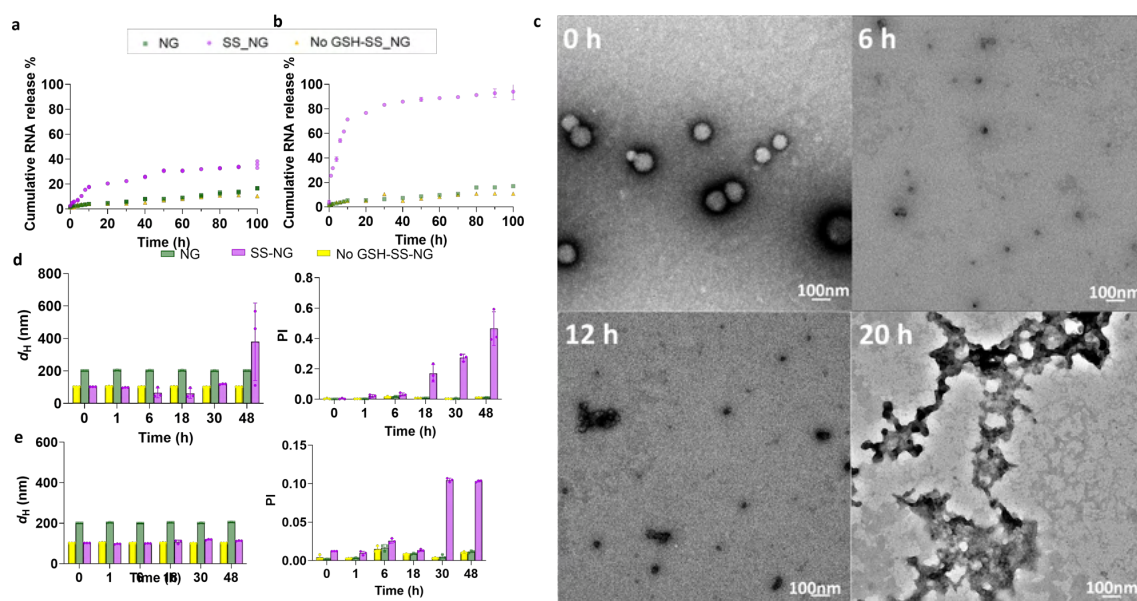


Figure 4.2: **GSH-responsiveness of disulfide NGs.** Encapsulation stability of the disulfide and non-disulfide NGs at a) 20 μ M and b) 10 mM GSH concentration over 100 h, determined by cumulative RNA release. ($n = 3$). c) Negative stain TEM characterisation of SS-NG morphological changes upon incubation with 10 mM GSH at various time points. d) NG morphological changes upon incubation with 10 mM GSH, monitored by DLS ($n = 3$). E) NG morphological changes upon incubation with 20 μ M, monitored by DLS ($n = 3$).

In Section 3.2.3, the structural features of the SS-NGs were optimised using the crosslinker feed ratio. Herein, the extent to which varying the crosslinker feed ratio could impact payload release and NG architecture was investigated for both SS-NGs and n-NGs. The cumulative RNA release was measured using the Quant-iT RNA quantification assay. RNA-loaded NGs with various crosslinking densities were initially incubated with a low GSH concentration (20 μ M). SS-NGs demonstrated

considerable stability, where the RNA leakage was minimal and comparable to that lost from the n-NGs over four days (Figure 4.3a,d). Next, both NGs were exposed to 10 mM GSH to evaluate their redox-responsiveness. Indeed, SS-NGs demonstrate a rapid and complete release, as compared to the n-NGs (Figure 4.3b,e). Notably, the SS-NGs demonstrate negligible RNA leakage without GSH treatment to comparable levels to n-NGs (Figure 4.3c,f).

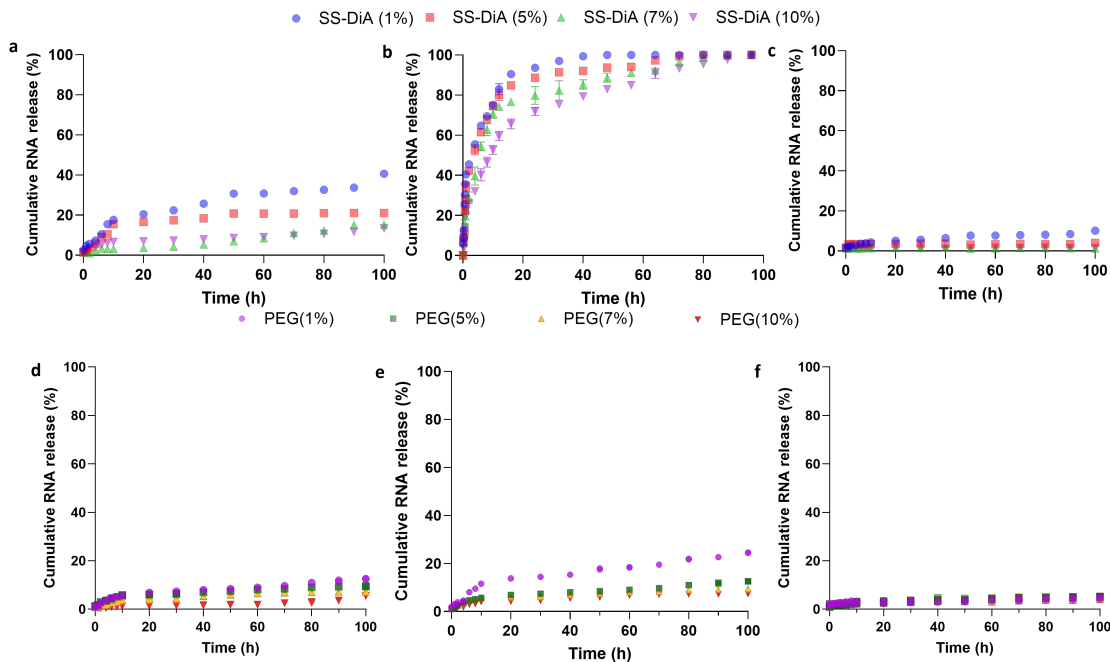


Figure 4.3: GSH-triggered RNA release profiles of NGs with varying crosslinker concentrations. Cumulative RNA release from NGs synthesised with varying [SS-DiA] at a) [GSH]: 20 μ M, b) 10 mM and c) no GSH, over 100 h. Cumulative RNA release from NGs synthesised with varying [PEG-DiA] at d) [GSH]: 20 μ M, e) 10 mM and f) no GSH, over 100 h.

Interestingly, the release kinetics of the SS-NGs vary as the degree of crosslinking changes. This indicates that the release kinetics are not merely determined by the relative amounts of disulfide crosslinking exposed on NG surface, but also is sensitive to the polymer packing in the NGs, which is determined by polymer architecture. Therefore, the release profile can be fine-tuned by altering the crosslinking density, where a higher degree of crosslinking results in more stable encapsulation and slower release kinetics. This is mirrored in the structural changes observed at 20 μ M and 10 mM GSH where SS-NGs undergo more dramatic structural changes at higher

GSH concentration, as expected (Figure 4.4a,b,d,e). Interestingly, increasing the crosslinker feed ratio higher than 7 mol% resulted in the formation of large aggregates ~600-800 nm in size that with low colloidal stability, as evident in the low potential (Figure 4.4c,f). This also highlights the sensitivity of the polymerisation reaction to higher crosslinker concentration as optimal NG structures are generated under high-dilution conditions.

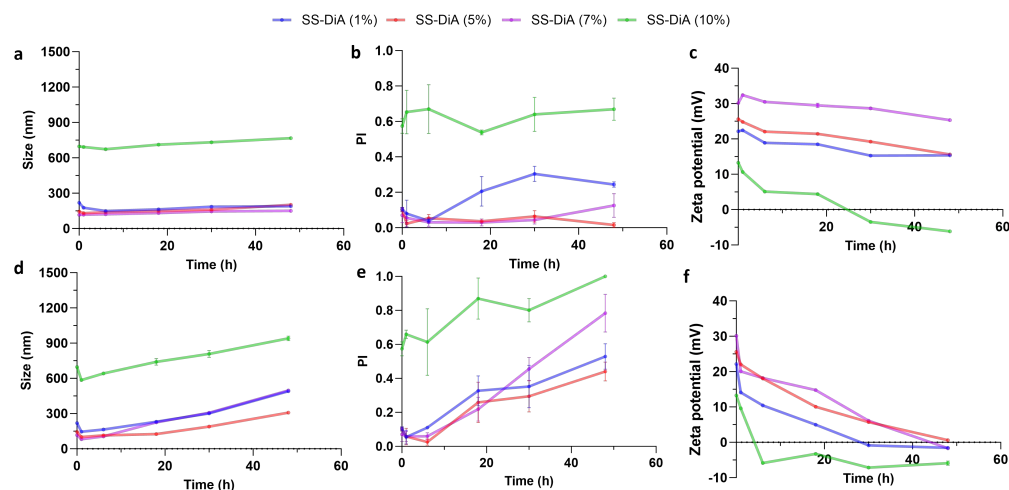


Figure 4.4: **NG physicochemical changes upon GSH exposure at varying [SS-DiA].** Graphs showing effect of varying [SS-DiA] on a) size, b) PI and c) ζ -potential at 20 μ M GSH. Graphs showing effect of varying [SS-DiA] on d) size, e) PI and f) ζ -potential at 10 mM GSH.

Notably, for both crosslinkers, NGs with lower sizes and PIs demonstrated increased colloidal stability over extended time periods, as indicated by the maintenance of the size and PI over the course of seven days (Figure 4.5a,c). In contrary, NGs with larger sizes exhibited a steady increase in size and PI, suggesting aggregation (Figure 4.5b,d).

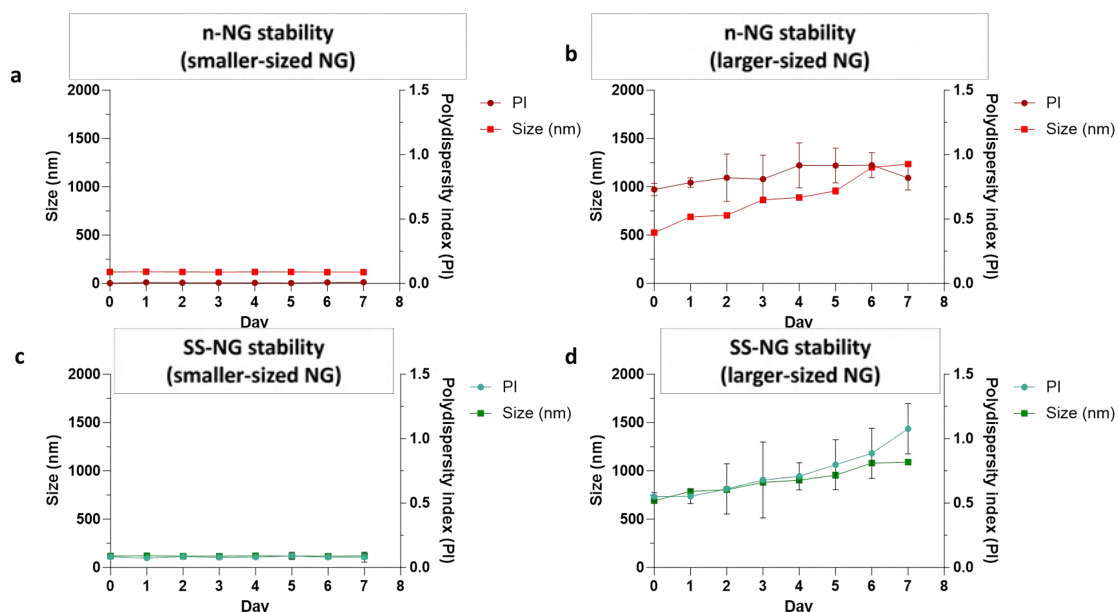


Figure 4.5: NG stability (DLS) analysis recorded over seven days. Graphs showing relative stability of a) small vs b) large n-NG and c) small vs d) large SS-NG, as measured by DLS.

In Section 3.2.5, various monomers were evaluated for their mRNA complexing ability. It is therefore of interest to investigate whether these monomers also influence NG degradation or RNA release kinetics. As such, NGs incorporating the AETC monomer (AETC NGs) were compared against the optimised compositions: AC-GUA_NG, PolyR_NG, PolyH_NG, and DMA/AETC_NGs by incubating them with extracellular and intracellular GSH concentrations. As expected, all NGs demonstrated minimal size changes when left untreated or exposed to 20 μ M GSH (Figure 4.6a–f). However, the PI for PolyH_NG increased dramatically in comparison to the other NGs at [GSH] = 20 μ M, which indicates its instability (Figure 4.6e). Indeed, PolyH had the lowest ζ -potential compared to other NGs, which decreased over the course of the experiment, as expected for NGs with poor colloidal stability prone to aggregation.[377]

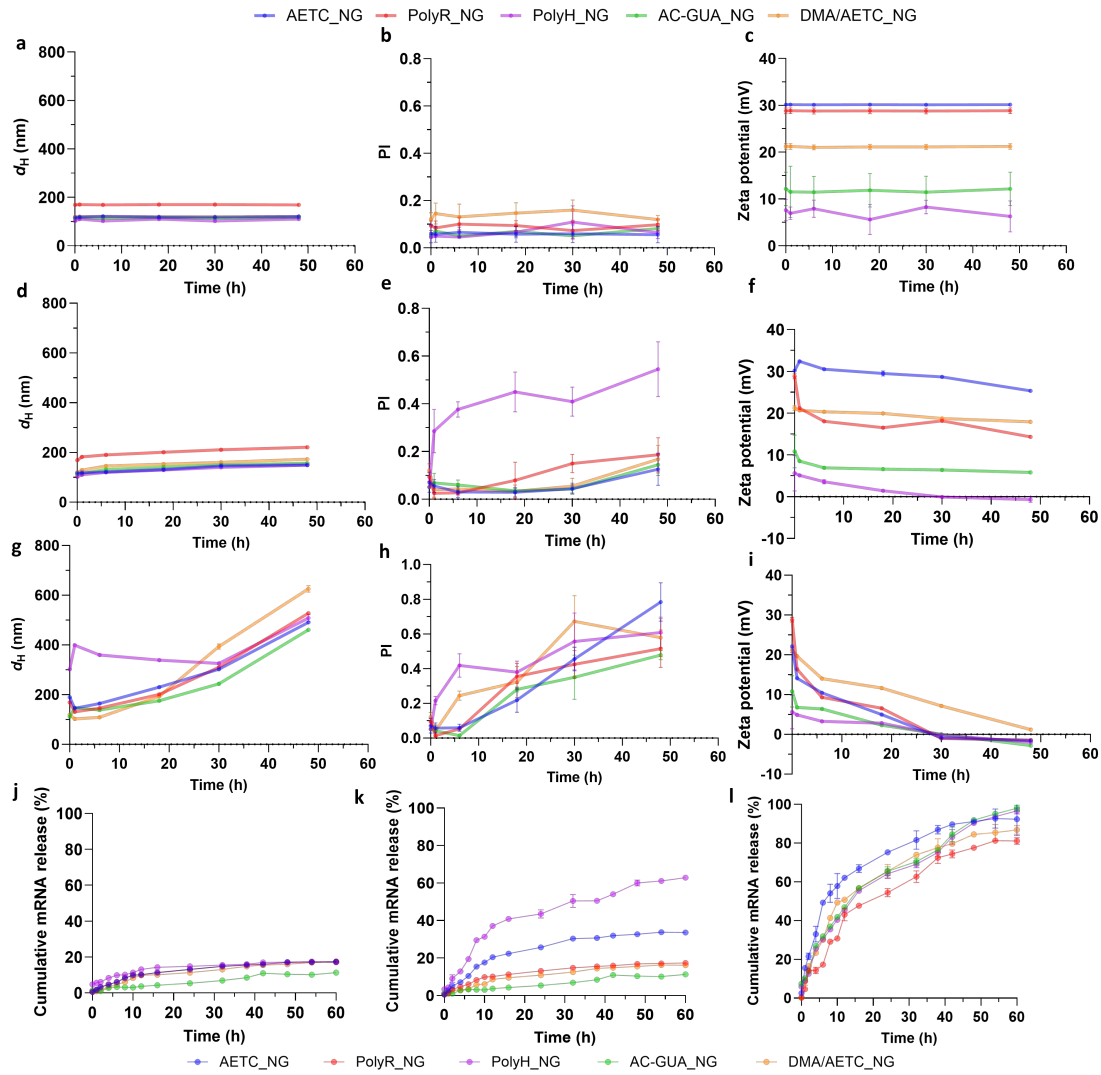


Figure 4.6: **Characterisation of GSH-mediated degradation of different SS-NG types.** NGs were incubated with (a–c) 0 μ M, (d–f) 20 μ M, or (g–i) 10 mM GSH, and changes in (a, d, g) hydrodynamic size, (b, e, h) PI, and (c, f, i) ζ -potential were measured. Cumulative RNA release profiles shown for j) 0 μ M, (k) 20 μ M, and (l) 10 mM GSH. Data represent mean $\pm$ SD ($n = 3$).

Interestingly, while PolyR_NG demonstrated minimal changes in size, the PI increased to 0.18 and the ζ -potential decreased to ~ 14 mV by 48 h, implying some degradation of the NG (Figure 4.6e,f). It is possible that sterically hindering peptide motifs prevent efficient packaging of the NG matrix, allowing GSH to penetrate the structure and trigger untimely degradation of PolyH_NG and PolyR_NG.

AETC_NG showed the best structural stability, with minimal changes in size, PI, or ζ -potential, highlighting the robustness of this composition. Upon treatment

with 10 mM GSH, all NGs exhibited controlled degradation over 48 h (Figure 4.6g–i). Notably, all NGs—except PolyH—initially decreased in size over the first 6 h post-incubation, likely due to the rapid cleavage of disulfide bonds at the NG surface (Figure 4.6g,h).[401] This exposes internal disulfide crosslinks, leading to swelling and aggregation, evidenced by the gradual increase in size and PI. Due to the inherent instability of PolyH_NG, it continued to swell and aggregate, leading to the structural changes observed. As expected, all NGs showed a dramatic decrease in ζ -potential, consistent with NG matrix degradation (Figure 4.6i).

To determine whether these structural changes could translate into functional consequences, the RNA release profiles of these NGs upon GSH incubation were subsequently evaluated. Reassuringly, only ~18% RNA was released without GSH treatment, which highlights the encapsulation stability offered by the NGs (Figure 4.6j). At 20 μ M, all NGs except PolyH_NG (67.2%) demonstrated minimal RNA release, which was consistent with previously characterised structural instability of this composition (Figure 4.6k). While AETC_NG did demonstrate 22% RNA leakage, this could be due to the release of any RNA molecules entrapped closer to the outer surface of the NGs. On the contrary, all NGs treated with 10 mM GSH demonstrated gradual, yet complete release of the encapsulated RNA over the course of the experiment, as expected (Figure 4.6l). Interestingly, while AETC_NG exhibited the fastest RNA release (75.3% within 24 h), PolyR_NG had the slowest (54.4% within 24 h). This elucidates bioreducibility of the platform, while also highlighting the tunability of the NG system, as different monomers can be used to tailor degradation to suit particular applications.

To further confirm the specificity of GSH-responsiveness, HeLa cells were pre-treated with a known GSH inhibitor, buthionine sulfoximine (BSO), and transfected with NGs loaded with mRNA encoding for GFP.[401] GFP expression was measured using the Incucyte® Live-Cell analysis system over five days. This was used to assess whether the SS-NGs had the same transfection with normal or reduced intracellular levels of GSH. SS-NGs showed a concentration dependent decrease in transfection efficiency following BSO pre-treatment (Figure 4.7a,b), whereas n-NGs

demonstrated low and unchanged GFP expression (Figure 4.7c,d). This indicates that BSO pretreatment did not impact the ability of the cells to express GFP and that GSH is integral in SS-NG degradation, thereby exemplifying the specificity of the platform.

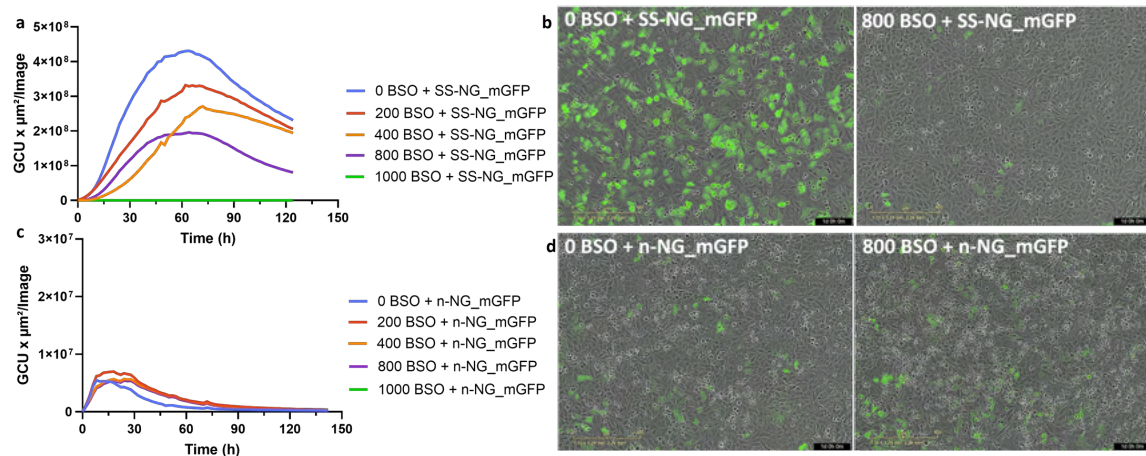


Figure 4.7: **Effect of GSH inhibition on SS-NG and n-NG GFP mRNA transfection efficiency.** Representative *in vitro* NG GFP mRNA expression profiles with associated IncuCyte images of GFP expression in HeLa cells pre-treated with BSO and treated with a), b) SS-NG and c), d) n-NG. Each expression profile shows the fluorescence intensity (green calibrated units $\times \mu\text{m}^2/\text{image}$) as a function of time. Data represent mean $\pm$ SD ($n = 3$).

4.2.2 Evaluating ROS-responsiveness of disulfide NGs

The ROS-responsiveness was studied by exposing SS-NGs and n-NGs to different ROS environments and observing subsequent morphological changes using DLS, ELS and TEM (Figure 4.8). In particular, H_2O_2 is one of the most stable forms of ROS and is highly abundant in the preinflammatory phase.[402] The concentration of H_2O_2 in the blood and normal cells has been estimated to be between 1 and 10 μM , while in tumour cellular milieu it increases to 100 μM . [385, 403]

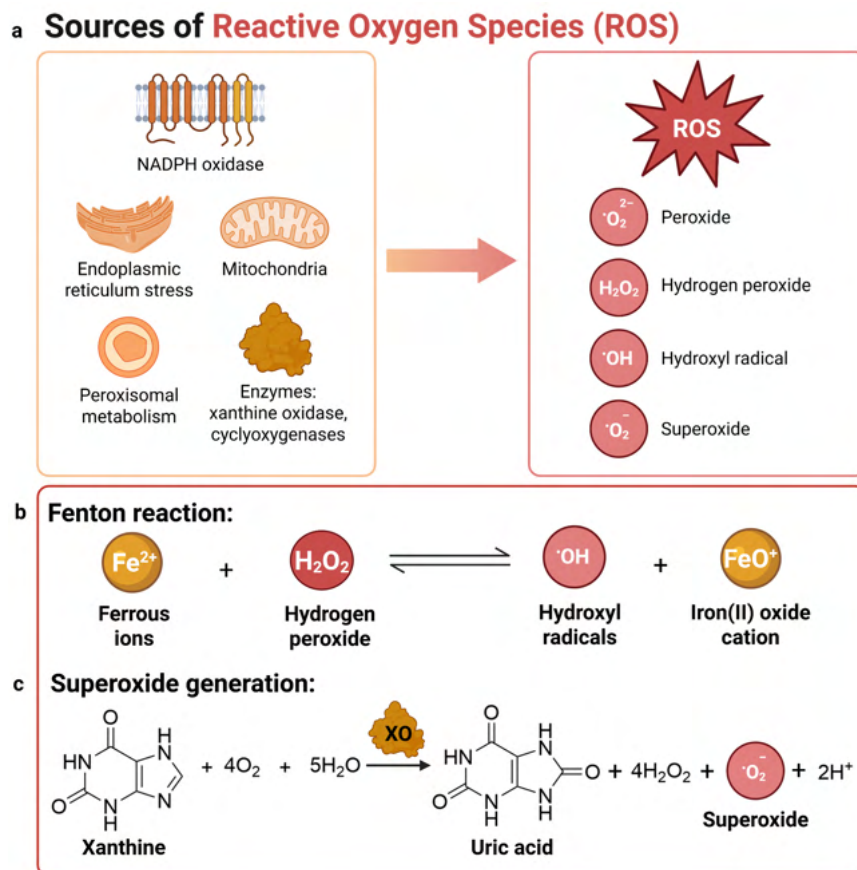


Figure 4.8: **Schematic representation of key ROS species in tumour microenvironments (TME).** a) Common sources of ROS species in the TME. b) The Fenton reaction involves a reaction between ferrous iron (Fe^{2+}) and hydrogen peroxide (H_2O_2) to generate hydroxyl radicals ($\text{HO}\cdot$). c) Oxidation of xanthine to uric acid by xanthine oxidase (XO) produces superoxide anion radicals ($\text{O}_2^{\cdot-}$) as byproducts.

At all $[\text{H}_2\text{O}_2]$, n-NGs demonstrated minimal size changes (Figure 4.9a,e,i). At the lowest $[\text{H}_2\text{O}_2]$, SS-NGs demonstrated a slight increase in size (by 10 nm) and PI (by 0.02) by 24 h (Figure 4.9b,c). A similar negligible change in ζ -potential was observed (Figure 4.9d). At higher $[\text{H}_2\text{O}_2]$ values (10 μM) incubation of SS-NG caused a greater increase in NG size (by 52 nm), PI (by 0.02) and a slight decrease in ζ -potential (6.1 mV) (Figure 4.9e,g,h). This is likely due to SS-NG swelling as opposed to degradation, thereby indicating NG stability in simulated blood and normal cell surroundings. Indeed, a well-designed RNA carrier must maintain a consistent size and morphology in these conditions, only releasing encapsulated payloads at the target site.

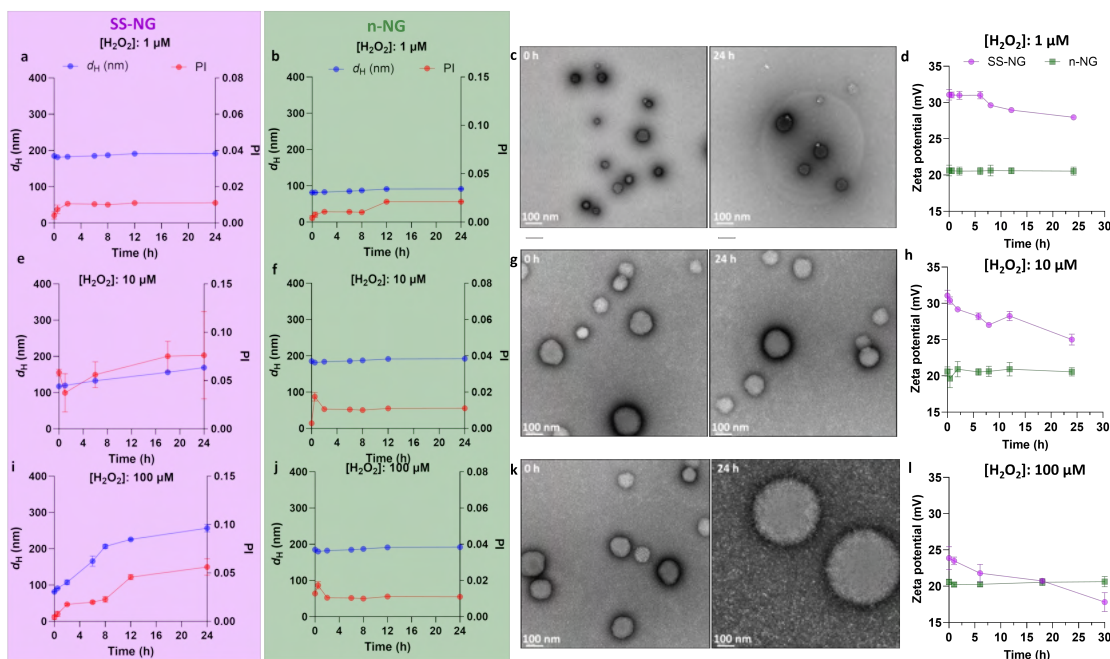


Figure 4.9: **Effect of increasing $[\text{H}_2\text{O}_2]$ concentrations on N-NG and SS-NGs.** Each row corresponds to a different concentration of $[\text{H}_2\text{O}_2]$: 1 μM (top row: a–d), 10 μM (middle row: e–h), and 100 μM (bottom row: i–l). For each condition: (a, e, i) show hydrodynamic size of n-NG; (b, f, j) show hydrodynamic size of SS-NG; (c, g, k) display representative negative-stain TEM images of SS-NG; and (d, h, l) show ζ -potential measurements for both NGs. Data represent mean $\pm$ SD ($n = 3$).

At $[\text{H}_2\text{O}_2]$ of 100 μM , reflecting the tumour environments, SS-NG size increased dramatically from 81.5 to 256.8 nm over a period of 24 h (Figure 4.9i,k). Interestingly, although a slight increase in PI (~ 0.05) and a decrease in ζ -potential (~ 6.1 mV) were observed, this was not significant enough to indicate complete NG degradation (Figure 4.9i,l). This is expected as disulfide crosslinks are more susceptible to GSH-mediated thiol-disulfide exchange, as demonstrated in Section 4.2.1, than the swelling effect mediated by H_2O_2 oxidation.[404, 405] This is evident in the minimal release of RNA from the disulfide-NGs even at 100 μM $[\text{H}_2\text{O}_2]$, where a mere $11.6 \pm 0.2\%$ of encapsulated RNA was released (Figure 4.10).

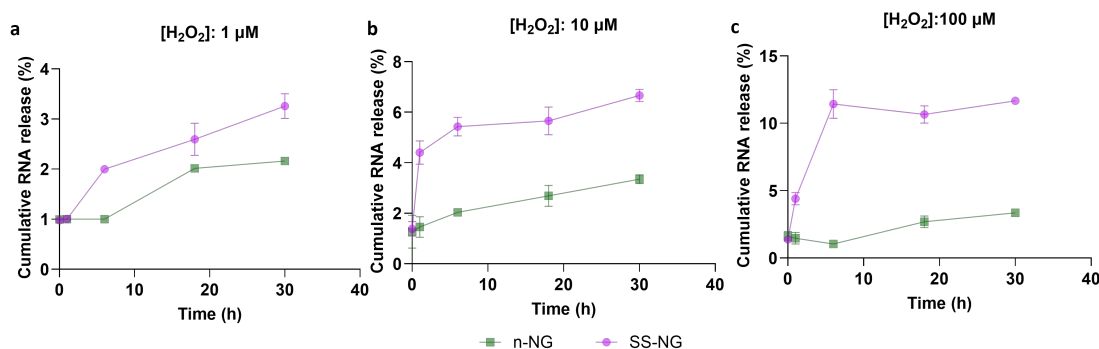


Figure 4.10: **RNA release in response to H₂O₂-mediated NG swelling for SS-NG and n-NG.** Cumulative RNA release profiles shown for a) 1 μM, b) 10 μM, and (c) 100 μM H₂O₂. Data represent mean ± SD (n = 3).

As the disulfide NGs demonstrated minimal structural changes upon exposure to H₂O₂, they were exposed to more aggressive radical species, namely HO[•] and O₂^{•-}. Although such ROS are highly reactive and consequently short-lived, their presence in tumour environments has been widely established.[248, 406, 407] Further, their effect on disulfide bond cleavage has been described.[387] However, as there is little consensus in the literature regarding the intratumoural concentrations of these species, the NGs were exposed to various [HO[•]]. HO[•] radicals were generated using the Fenton reaction and radical formation was measured indirectly by detecting the decreasing [H₂O₂], using the Amplex Red assay (Figure 4.8b).[408] As the [H₂O₂] showed a concentration-dependent decrease, this method was concluded to reliably generate HO[•] radicals and measure their subsequent effects on NP stability within the same time frame (Figure 4.11). From this, [H₂O₂]: 100 μM and 500 μM were used for further measurements as these afforded sufficiently distinct levels of HO[•], unlike the minimal difference observed between 500 μM and 1 mM. This enabled effective assessment of potential structural changes linked to HO[•] radicals.

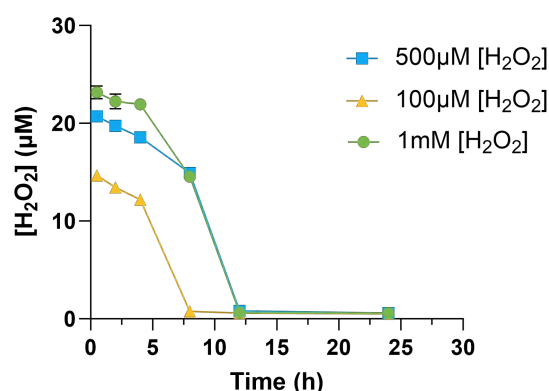


Figure 4.11: **Hydroxyl radical ($\text{HO}^\cdot$) generation and detection.** Quantification of $\text{HO}^\cdot$ generation by indirect measurement of decreasing H_2O_2 concentration using the Amplex Red assay. Data represent mean $\pm$ SD ($n = 3$).

At both concentrations, the NGs demonstrate similar structural changes with a gradual increase in size and PI, consistent with the swelling behaviour observed upon ROS exposure (Figure 4.12c,d,f). [153, 409] While n-NGs showed slight increase in size and PI (100 μM H_2O_2 : by 51.1 ± 16.1 nm, PI 0.15 ± 0.01 ; 500 μM H_2O_2 : by 173.6 ± 15.4 nm, PI 0.15 ± 0.01), these changes were not as dramatic as those seen for SS-NGs (100 μM H_2O_2 : by 233.1 ± 41.3 nm, PI 0.29 ± 0.04 ; 500 μM H_2O_2 : by 210.9 ± 34.7 nm, PI 0.26 ± 0.01). It is likely that the aggressive nature of $\text{HO}^\cdot$ disrupted the disulfide bonds, thereby decreasing NG molecular network density, increasing particle size and making the PI unstable. Interestingly, for both NG types and at both $[\text{HO}^\cdot]$, the ζ potential exhibits an initial increase followed by a gradual decrease indicating NG degradation and aggregation (Figure 4.12c,f). This can be visually confirmed in the electron micrographs shown for SS-NG over varying time points (Figure 4.12g,h). The initial spike in ζ -potential for SS-NGs could be caused by increased surface exposure of concealed cationic quaternary ammonium head groups prior to NG degradation (Figure 4.12c,f). By contrast, n-NGs showed greater colloidal stability of the n-NGs, as shown by the nearly constant ζ -potential. This comparison offers preliminary insight into the specific responsiveness of disulfide crosslinks to hydroxyl radical exposure. Given the extensive swelling observed, SS-NGs also demonstrated proportionally increased RNA release (100 μM H_2O_2 : $22.5 \pm$

1.3% and 500 μM H_2O_2 : $32.2 \pm 1.2\%$) by 24 h, with increasing $[\text{HO}^\cdot]$, as compared to the n-NGs (Figure 4.13).

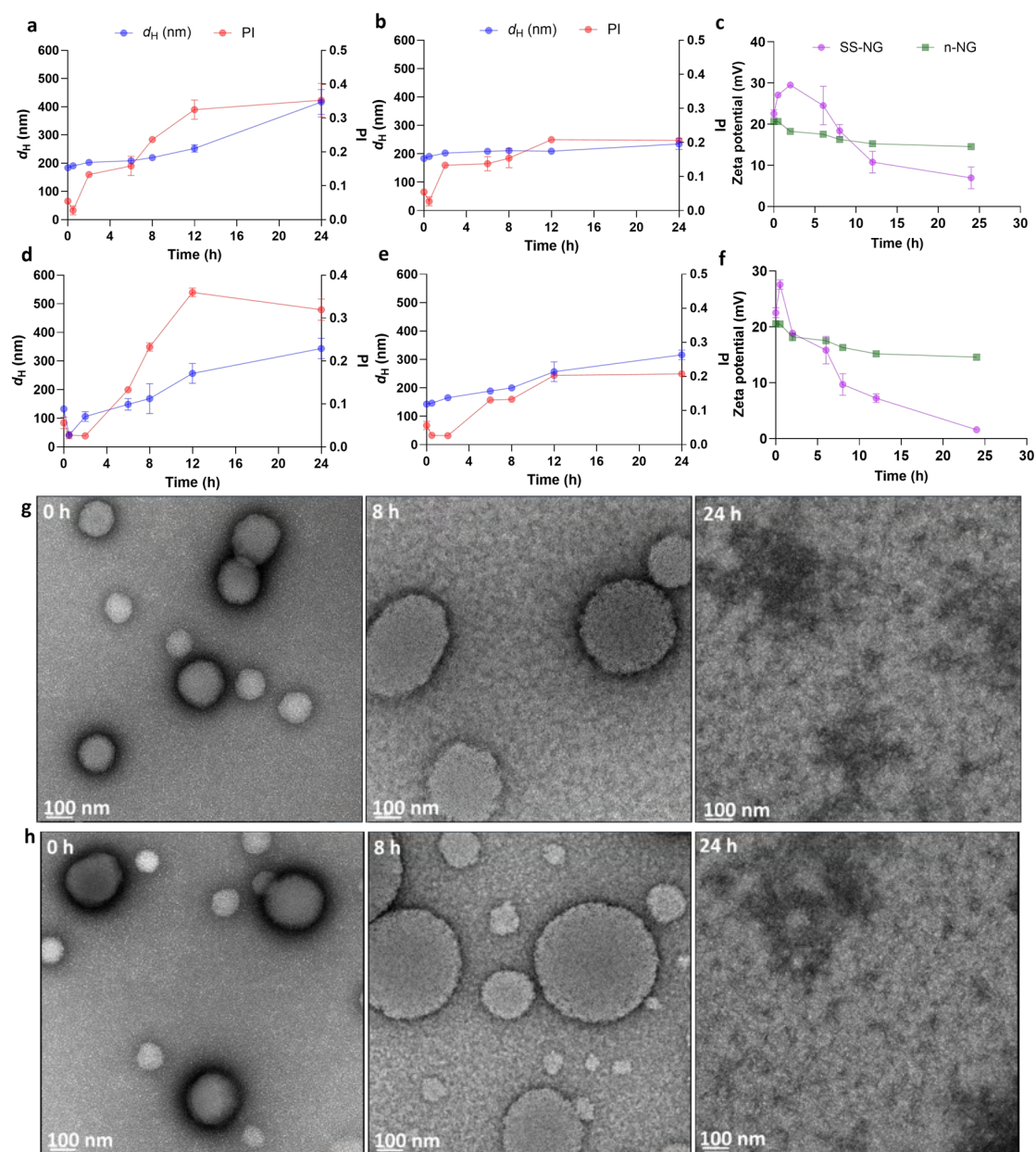


Figure 4.12: **Effect of varying $(\text{HO}^\cdot)$ concentrations on n-NG and SS-NGs.** Each row corresponds to a different $[\text{H}_2\text{O}_2]$: 100 μM (top row: a–c) and 500 μM (middle row: d–f). For each condition: (a and d) show hydrodynamic size of SS-NG, (b and e) show hydrodynamic size of n-NG. ζ -potential measurements for both NGs at (c) 100 μM and (f) 500 μM . Negative stain TEM images of SS-NG at (g) 100 μM and (h) 500 μM $[\text{H}_2\text{O}_2]$, respectively. Data represent mean $\pm$ SD ($n = 3$).

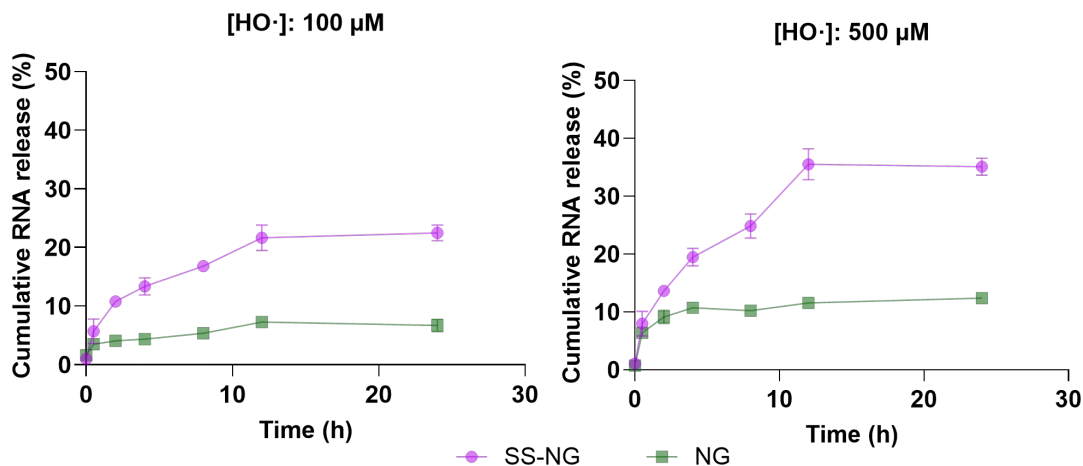


Figure 4.13: **RNA release in response to HO $\cdot$ -mediated NG swelling for SS-NG and n-NGs.** Cumulative RNA release profiles shown for 100 μ M and 500 μ M HO $\cdot$. Data represent mean $\pm$ SD ($n = 3$).

The NGs were then exposed to O $_2^{\cdot-}$ radicals, generated enzymatically through the reaction of xanthine with xanthine oxidase.[410] SS-NGs exhibited a dramatic increase in size (235.1 ± 1.3 nm to 1468.7 ± 102.7 nm) and PI (0.05 ± 0.02 to 0.67 ± 0.07) within 2 h of superoxide exposure (Figure 4.14a). TEM images confirmed rapid swelling and subsequent degradation of the disulfide framework (Figure 4.14d). This was confirmed by rapid decrease in colloidal stability, as indicated by the ζ -potential which dropped by 15.8 mV (Figure 4.14c). In contrast, n-NGs demonstrated a gradual increase in size and PI more reminiscent of swelling behaviour, as observed previously (Figure 4.14b). This indicates the aggressive nature of the ROS species that likely target any labile bonds (i.e. ester bonds) within the NG framework. Indeed, this is reflected in the rapid mRNA release profiles for both NGs, with SS-NGs reaching $79.3 \pm 4.4\%$ within 2 h (Figure 4.14d).

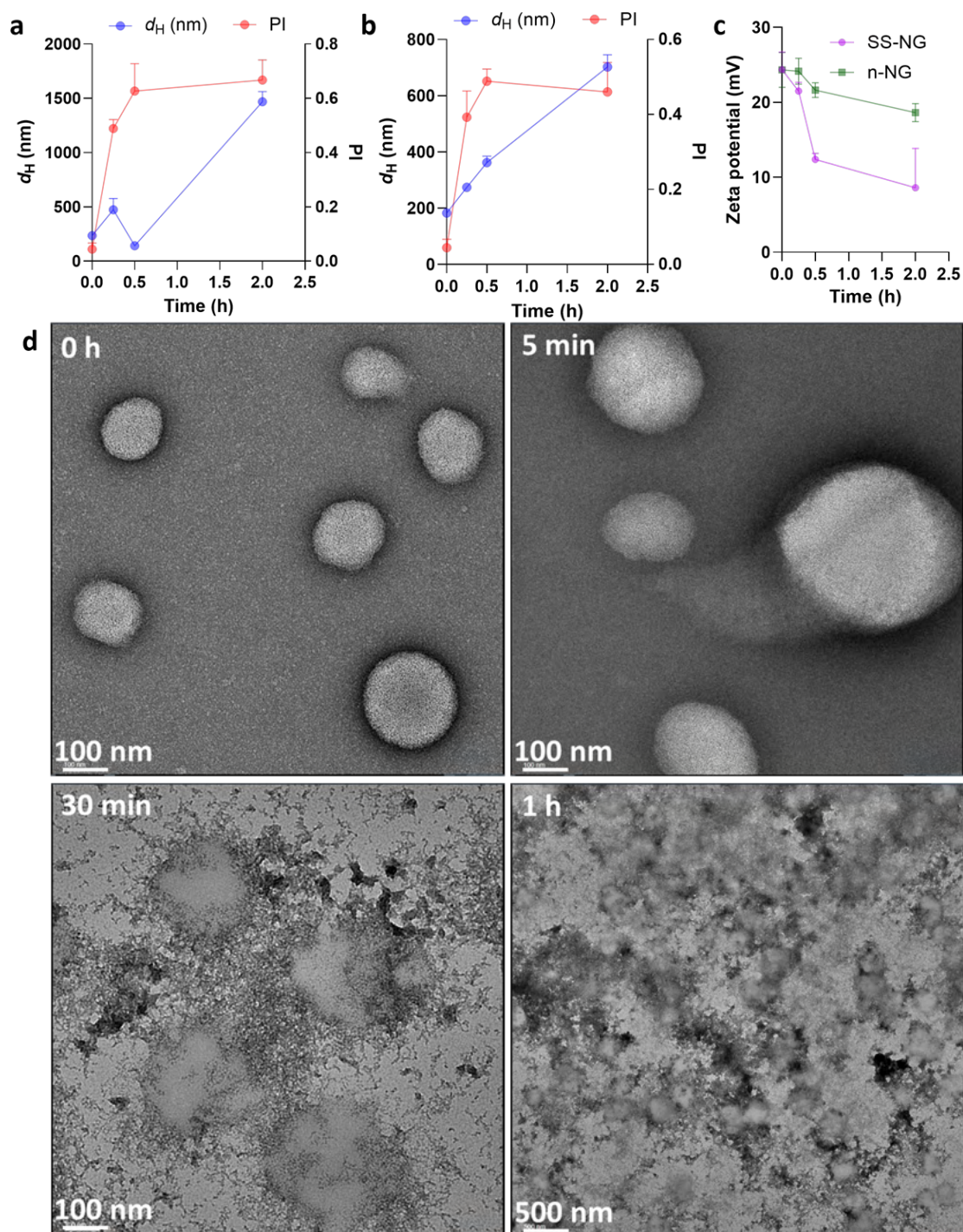


Figure 4.14: **Effect of varying superoxide concentrations on n-NG and SS-NGs.** Changes in size and PI for a) SS-NG and b) n-NG. c) ζ -potential measurements for both NGs. d) Representative negative stain TEM images of SS-NG. Scale bar at 100 nm for all except for bottom right where scale bar is at 500 nm. Final timepoint shown at lower magnification (500 nm) as this broader view allows visualisation of the complete breakdown and distribution of the NG fragments. Data represent mean $\pm$ SD ($n = 3$).

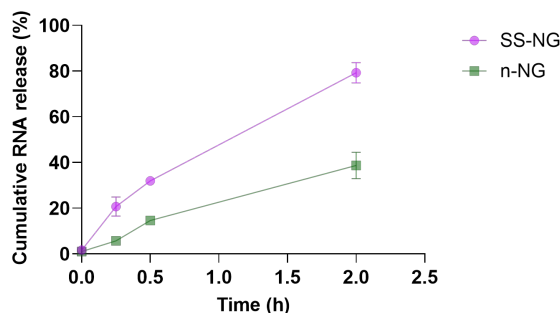


Figure 4.15: **RNA release in response to superoxide-mediated NG swelling for SS-NG and n-NGs.** Cumulative RNA release profiles shown. Data represent mean $\pm$ SD ($n = 3$).

In sum, these experiments highlight the ROS-responsive nature of the disulfide NGs. Interestingly, both types of NGs exhibited varying degrees of structural change upon exposure to different types of ROS. Both showed greatest structural sensitivity to hydroxyl radicals, as evidenced by dramatic increases in size and PI (n-NG: by 136.0 ± 4.1 nm and PI by 0.23 ± 0.02 ; SS-NG: by 586.5 ± 14.9 nm and PI by 0.22 ± 0.04) (Figure 4.16a,b,d,e). However, overall SS-NGs were more susceptible to ROS-mediated damage, as indicated by the significant decrease in ζ -potential, suggestive of a greatly reduced colloidal stability, where n-NGs maintained their ζ -potential (Figure 4.16c,f).

Although both NG types suffered considerable damage upon exposure to superoxide radicals, SS-NGs again showed greater sensitivity, reinforcing that the disulfide motif could be considered ROS-responsive. Furthermore, it is possible that depending on the type of ROS, different chemical motifs in the NGs are attacked. Future studies investigating the mechanisms of ROS attack, supported by downstream mass spectrometry analysis, could help to identify the degradation products formed. It is important to note, however, that the high concentrations used in the above experiments may not accurately reflect intracellular conditions. Rather, the aim was to explore the potential of SS-NGs to be engineered for ROS-responsiveness in future applications.

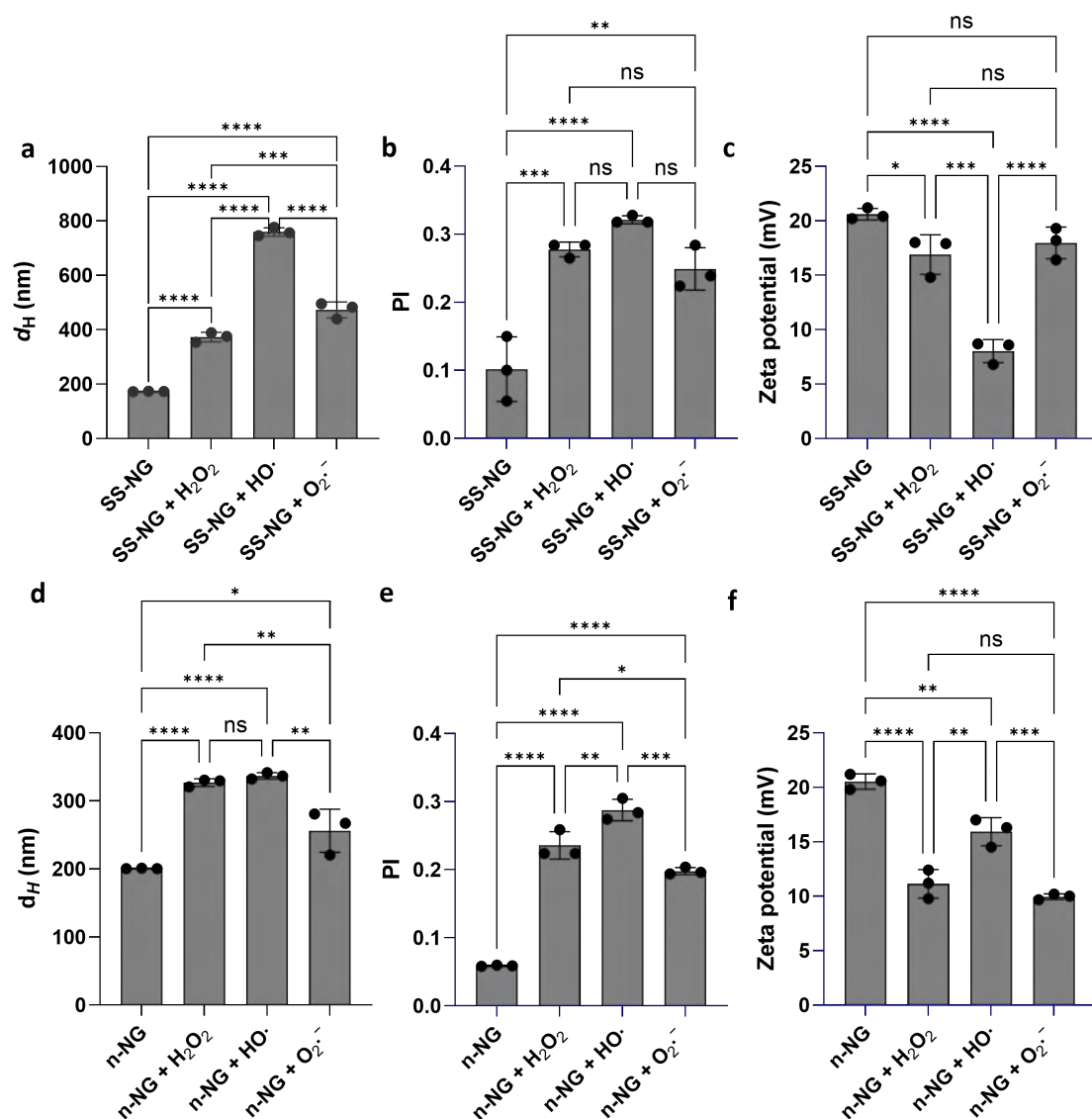


Figure 4.16: **Comparison of SS-NG and n-NG stability under oxidative stress.** (a–c) Size, PI, and ζ -potential of SS-NG following treatment with various ROS species. (d–f) Corresponding measurements for n-NG under identical conditions as above. Data represent mean $\pm$ SD ($n = 3$).

4.2.3 Evaluating pH-responsiveness of disulfide NGs

Among various intracellular stimuli, pH is of particular interest as a drastic change in pH is known to undergo in the TME as well as in organelles such as endosomes/lysosomes, where pH becomes lower than that of normal tissue and the blood stream (pH $\sim$ 7.4).[313, 411–413] As many of the monomers employed during NG synthesis possess ester bonds (AETC, disulfide crosslinker) and ionisable head groups (pol-

yarginine, polyhistidine, guanidium, DMA), the effect of pH change on NG structural features was investigated to preliminarily forecast NG stability under physiological conditions.

For all NGs, the hydrodynamic diameter increases upon a change in pH from 7.4 to 4.5, although the increase for AC-GUA_NG is smaller than that of the other NGs; this, supported by the increase in PI, indicates that the core of the PEGylated NGs is swollen at low pH (Figure 4.17a,b). Such structural changes can cause increased aggregation and NG instability which is echoed in the way the zeta potential decreases upon decreasing pH (Figure 4.17c). Notably, among the other NGs, DMA/AETC_NG showed the greatest increase in size (by 33.3%), as the pH decreased from 6.5 to 5.5 (Figure 4.17a). According to the titration measurement, it is likely that the nitrogen atoms of DMA in the core were not protonated under alkaline conditions (pH 9), but became protonated under acidic conditions (pH 4.5) by the influx of H^+ ions; the ions can attach themselves to an amine group of DMA (Figure 4.17d). This leads to increasing electrostatic repulsion between the protonated amine head groups, causing swelling of the NG core.

While they were not as pronounced as DMA/AETC_NGs, AC-GUA_NGs, PolyR_NGs and PolyH_NG exhibited a similar increase in NG size and PI, and these changes were supported by concordant changes in the pH titration curves. Moreover, the pKa values derived from the pH titration curves can give an indication of the potential endosomal escape capacity of the NGs. AC-GUA_NGs, PolyH and PolyR_NGs had pKas within the range $\sim 6-7$, supposedly ideal for enabling escape from the early endosomes via the proton sponge effect.[260, 355, 356] While PolyR_NG did demonstrate endosomal escape capability, as seen in Section 3.2.7, the other NGs were less efficient in this regard. Interestingly, despite the high pKa, AETC_NGs exhibited substantial endosomal escape, likely due to endosomal membrane disruption driven by their high cationic charge. These results suggest that although pKa can influence the endosomal escape properties of polymers, other structural features may play a more dominant role in mediating intracellular NG degradation.

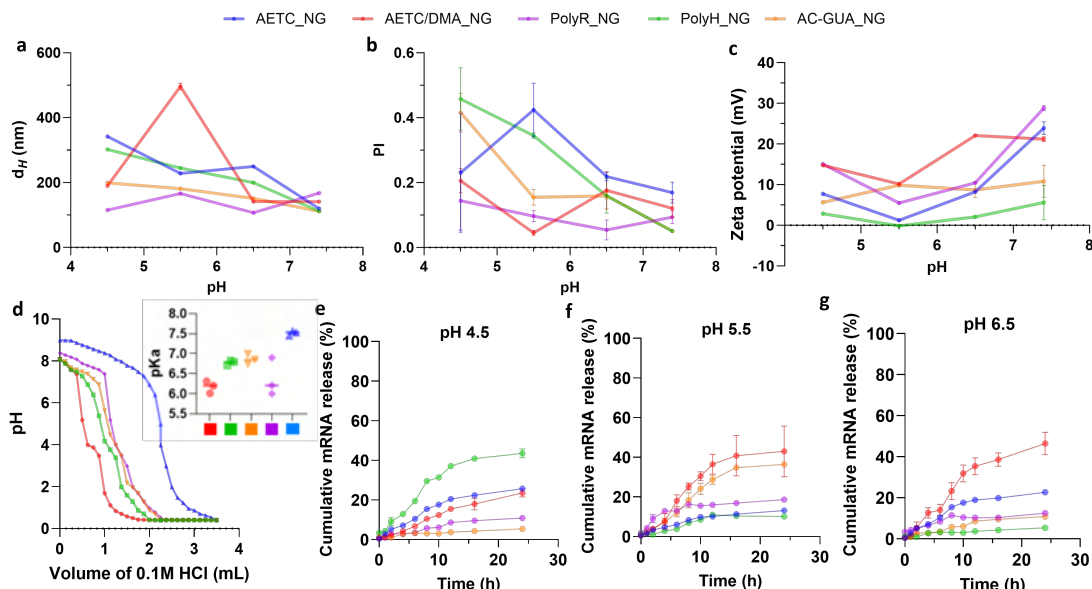


Figure 4.17: **Summary of pH-responsive behaviour of NGs synthesised using various cationic monomers.** (a–c) Changes in hydrodynamic size, PI, and ζ -potential of NGs upon incubation in buffers of varying pH. (d) pH titration curves with inset highlighting the apparent pK_a for each monomer-based NG composition. (e–g) RNA release profiles at pH 4.5, 5.5, and 6.5, respectively, for all NGs. Data represent mean $\pm$ SD ($n = 3$).

In the context of the slightly acidic tumour microenvironment, the mild pH responsiveness of AETC_NGs suggests a desirable level of structural stability for preserving RNA integrity in the extracellular space prior to cellular uptake. This is supported by the limited RNA release observed at the pH values investigated (Figure 4.17f,g). Notably, increased RNA release was detected at pH 4.5 and 6.5, where NG swelling was more pronounced, resulting in $25.7 \pm 0.3\%$ and $22.7 \pm 1.1\%$ release, respectively (Figure 4.17e,g). This likely reflects reduced crosslinking density, allowing the RNA to diffuse out. DMA/AETC_NGs consistently exhibited highest RNA leakage, with over $43.0 \pm 12.7\%$ and $46.4 \pm 5.4\%$ mRNA released at pH 5.5 and 6.5 over 24 h, respectively, in concordance with the observed increase in NG size (Figure 4.17f,g). Across all NGs tested, a clear relationship between NG size increase and RNA leakage was observed, further highlighting their pH-responsive nature.

4.2.4 Physiological ionic strength as an environmental cue: impact on NG architecture

While the NGs are synthesised in water, as therapeutic agents they must maintain their structural stability when reconstituted in physiologically compatible media, for instance, medical-grade saline and phosphate-buffered saline (PBS). The hydrodynamic diameter of particles in suspension is determined by their core size and surface organisation, which is influenced by electrostatic charge density that, in turn, depends on the ionic strength of the surrounding solution.[414] This occurs through changes in the thickness of the electric double layer (Debye length: κ^{-1}). When particles are suspended in a low conductivity media, the Debye length will be extended, which slows particle diffusion and causes a larger measured hydrodynamic diameter. Conversely, higher conductivity media reduces the electrical double layer and leads to a smaller hydrodynamic diameter.

As such, the effect of these buffers on the morphological characteristics of the optimised AETC_NG composition was quantified. When fully hydrated in 7.70 mmol/L saline (173.6 ± 2.1 nm) and 0.07X PBS (117.4 ± 16.5 nm) at room temperature the hydrodynamic diameter did not change, presumably because this is a low ionic strength buffer (Figure 4.18a,d). However, when suspended in increasingly concentrated salt solutions, NGs demonstrated signs of aggregation and compromised RNA encapsulation and loading capacity. At the highest saline concentration, the hydrodynamic diameter increased to 468.4 ± 35.7 nm, with PI increasing from 0.09 ± 0.01 to 0.25 ± 0.04 (Figure 4.18a). This was also observed in the TEM images of the NGs (Figure 4.18c). Further, the ζ -potential decreased from 21.5 ± 1.1 mV to 1.3 ± 2.1 mV and a similar decrease was observed in RNA EE% and LE% (from 95.1% to 18.9% and 9.60 to 0.37%, respectively) upon immersion in 77 mmol/L saline (Figure 4.18b,g). Similarly, exposure to the highest concentration of PBS, led to increased NG size 269.6 ± 1.4 nm and PI 0.22 ± 0.02 , and decreased ζ -potential (13.1 ± 0.4 mV) (Figure 4.18d,e,f). As before, RNA EE- and LE-efficiency decreased to 21.7% and 1.31%, respectively, upon resuspension (Figure 4.18h). Aggregation is observed because the high ionic strength of the NaCl solution causes charge screening

of the cationic quaternary amine functional groups present on the polymer backbone. This causes complete collapse of the NGs as the matrix is no longer charged, as evident in the decreasing ζ -potential. Furthermore, the reduced RNA entrapment efficiency and increased leakage could be attributed to the disruption of electrostatic interactions between RNA and the NG's cationic moieties by anions in the solution, as well as the degradation of the NG framework.

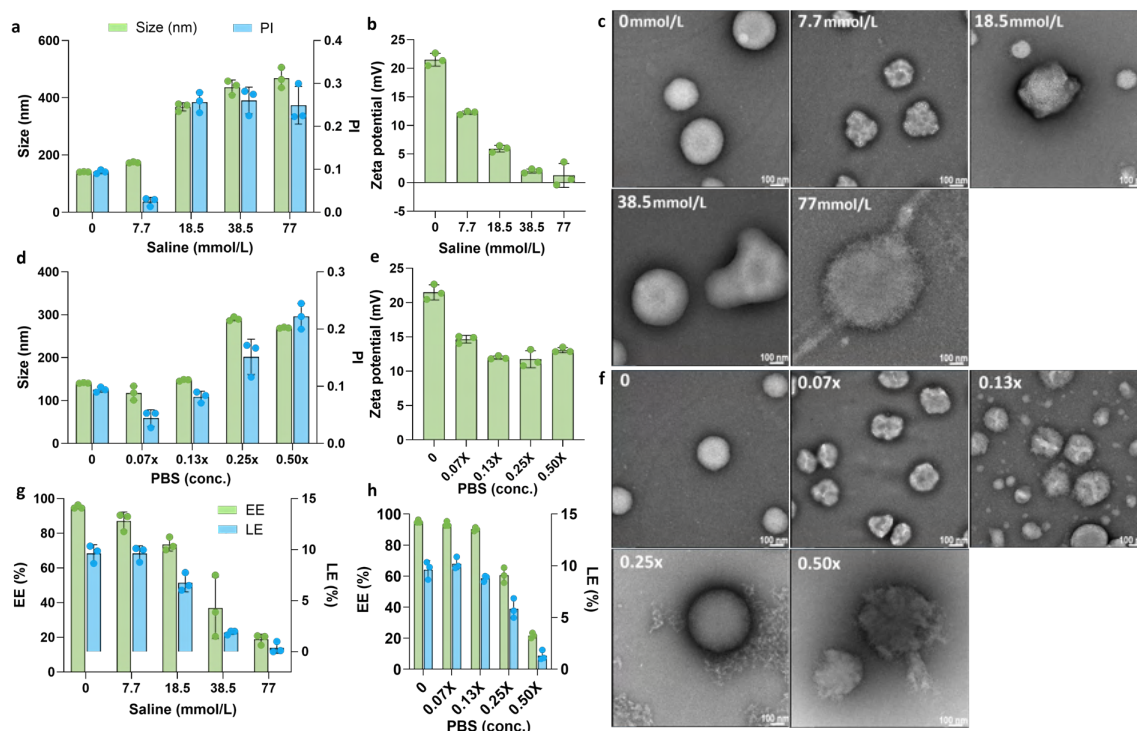


Figure 4.18: Effects of physiologically relevant buffers on NG physicochemical features. Graphs summarising effects of NG resuspension in varying saline concentrations on a) NG size, PI, and b) ζ -potential, as determined by DLS and ELS. c) Negative stain TEMs of NGs resuspended in varying saline concentrations. Scale bar at 100 nm. Graphs summarising effects of NG resuspension in varying PBS concentrations on d) NG size, PI, and e) ζ -potential, as determined by DLS and ELS. f) Negative stain TEMs of NGs resuspended in varying PBS concentrations. Scale bar at 100 nm. Encapsulation and loading efficiencies of NGs resuspended in varying concentrations of g) saline and h) PBS. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$.

4.2.5 Protein corona formation as an environmental cue: impact on NG architecture

NG surfaces can be modified by the deposition of biomacromolecules in the biological environment, leading to a biomolecule interface organisation loosely divided into

“hard” and “soft” coronas, with “long” and “short” exchange times, respectively.[415, 416] In the physiological context, this corona defines the biological identity of the NP, and directly impacts cell-particle interactions, thereby affecting cellular uptake, *in vivo* biodistribution and NP half-life.[417, 418] Many factors affect the nature of the NP-biomolecule corona, including size, surface charge, shape and solubility.[415, 419] It is well known that NPs studied at low serum concentrations (*in vitro*) may have little relation to those in the *in vivo* setting, with higher serum concentrations.[350] This suggests a need to investigate the changes in NG structure, in order to optimise a given delivery platform for *in vivo* studies. Indeed, there is a lack of understanding of the extent to which NG-protein complexes can hinder *in vivo* delivery. PEGylation of NP surface has reportedly decreased protein adsorption, thereby reducing recognition and subsequent clearance by the mononuclear phagocytic system.[420–422] Indeed, the recent success of the PEGylated lipid NP-mRNA COVID-19 vaccines has established the potency of this technique.[421] As such, a preliminary comparison of the structural changes that PEGylated and non-PEGylated NGs undergo upon exposure to human serum is provided to investigate whether inclusion of mPEG enables reduced protein adsorption on the NG surface. To synthesise PEGylated NGs (mPEG_NG), an acrylate-functionalised-mPEG (2 kDa) monomer was used. NGs lacking this monomer (non-mPEG_NG) were also made as a comparison (Figure 4.19).

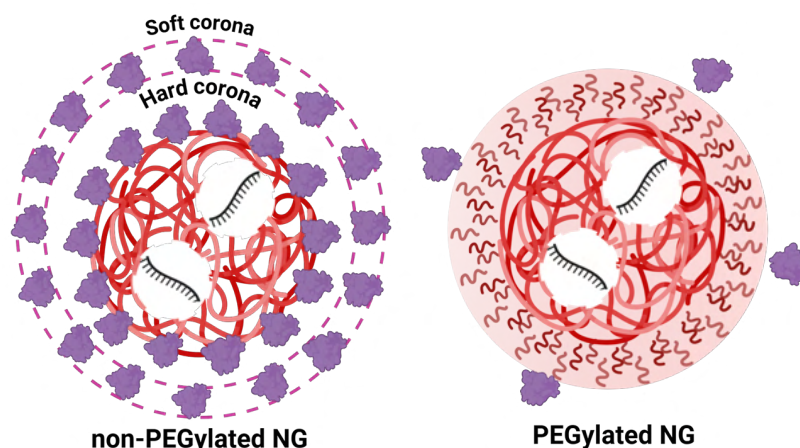


Figure 4.19: Representation of protein corona formation on non-PEGylated and PEGylated NGs.

NGs were incubated in human serum for varying timepoints (4, 12, 24 and 48 h) at different serum concentrations upto 55 % (human *in vivo* serum concentration) (1, 5, 20 and 55%).^[419] The resulting NG-protein complexes were separated from excess plasma by centrifugation and extensive washing to remove the unbound, “soft” corona. The complexes were subjected to DLS, ELS and TEM, and the hard corona assessed by SDS-PAGE. Representative data for the 48 h timepoint are shown in [Figure 4.20](#). We observed a constant increase in size and PI of the non-mPEG_NGs upon increasing serum exposure, as well as considerable decrease in the ζ potential which was consistent with significant protein adsorption ([Figure 4.20a](#)). In contrast, the mPEG_NGs did not exhibit as much increase in sizes and polydispersities as the non-mPEG_NGs ([Figure 4.20b](#)). Similarly, the ζ potential decreased slightly upon increased serum concentration, but not to the extent noted for the non-mPEG_NGs. [Figure 4.21](#) illustrates the SDS-PAGE results in which both NGs were incubated in plasma concentrations from 1% to 55%, exhibiting a clear difference in the protein corona composition and evolution trend for the two materials. With increasing serum concentration, the intensity of the bands in [Figure 4.21a](#) increases, as reflected in the respective densitometry analyses shown below each gel. This mirrors the prior structural characterisation for the non-mPEG_NGs, thereby confirming increased protein deposition. While the gels for the mPEG_NG demonstrate a similar increase, the overall protein deposited is still lower in comparison to the non-mPEG_NGs ([Figure 4.21b](#)). This indicates that PEGylation of the NG surface can mitigate protein corona formation to some extent. Accordingly, this composition was used in all subsequent experiments throughout this study.

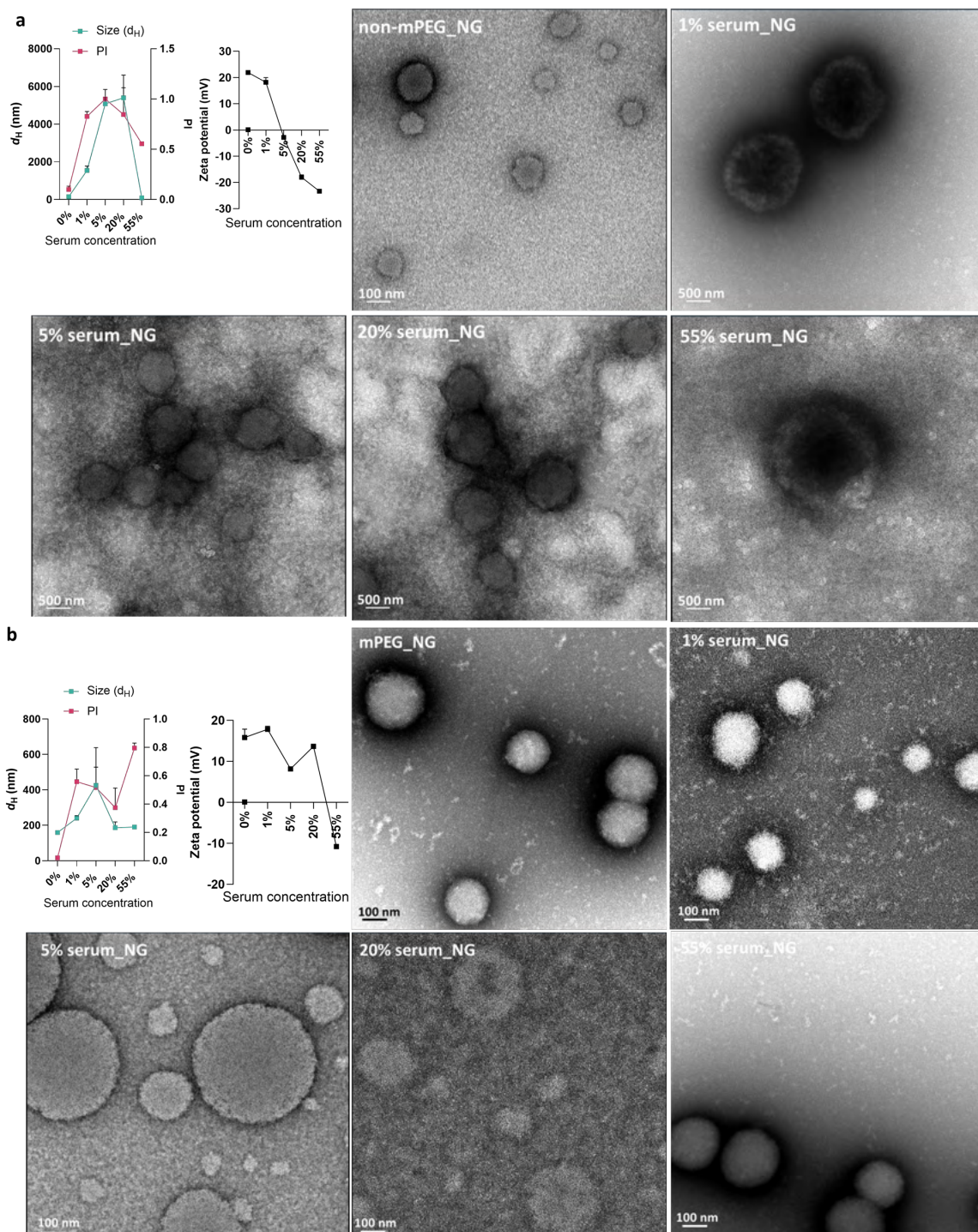


Figure 4.20: Physicochemical characterisation of NGs upon incubation with serum. Representative example for NGs incubated with varying serum concentrations for 48 h is shown. a) From left to right, DLS, ELS and TEM characterisation of NG without PEG brush border exposed to varying concentrations of human serum. Scale bar at 100 nm for mPEG_NG and at 500 nm for the rest of the images. b) DLS, ELS and TEM characterisation of NG comprising of a PEG brush border exposed to varying concentrations of human serum. Scale bar at 100 nm. Data represent mean $\pm$ SD ($n = 3$).

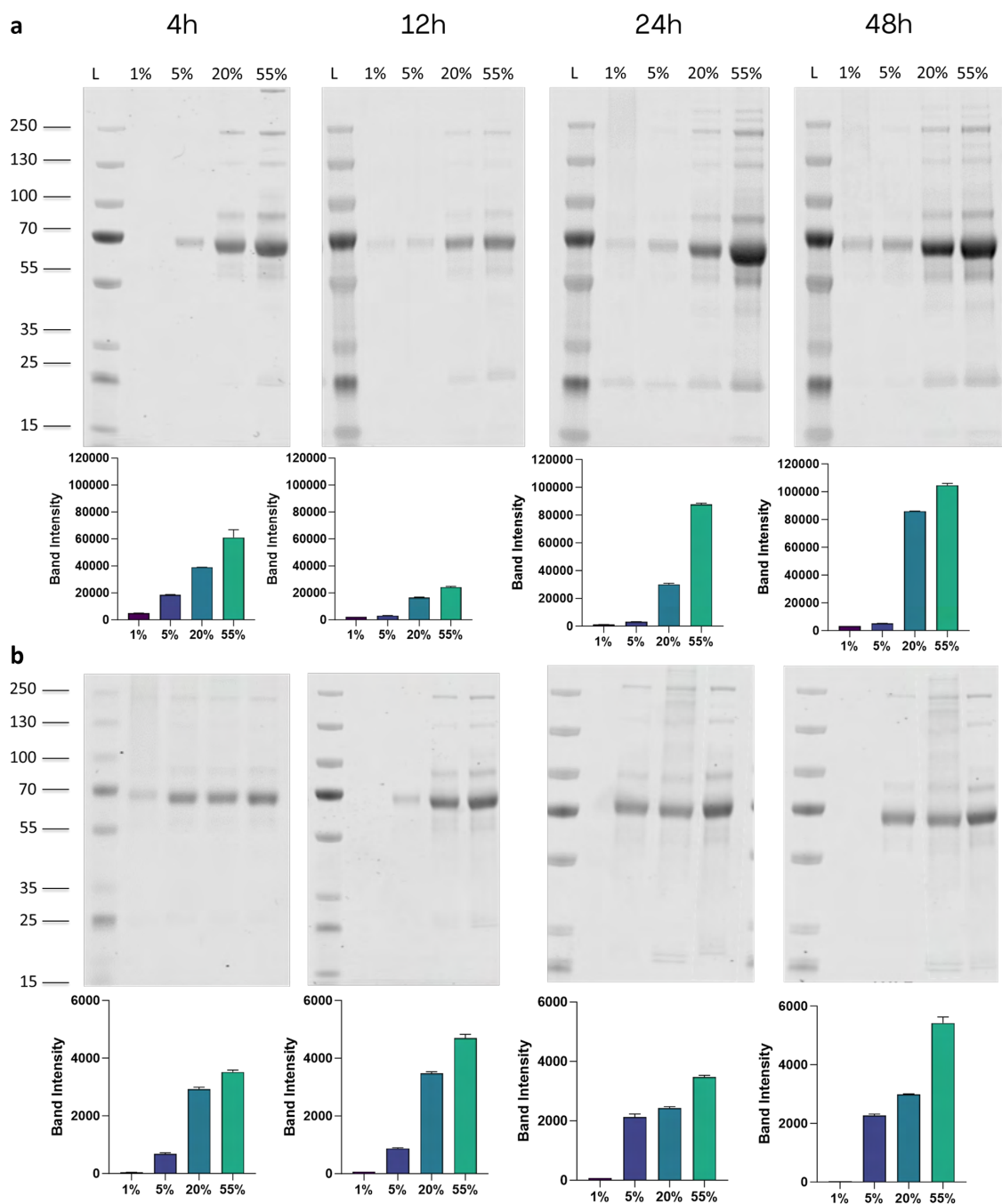


Figure 4.21: SDS-PAGE gel of human plasma proteins obtained from a) non-mPEG_NG-protein complexes and b) mPEG_NG-protein complexes following incubation for different timepoints at varying plasma concentrations. The molecular weights of the proteins in the standard ladder are reported on the left for reference. Histograms representing total band intensity of proteins are shown below each gel. Data represent mean $\pm$ SD ($n = 3$).

4.3 Conclusions and Limitations

By assessing changes in NG physicochemical characteristics alongside RNA release profiles, the platform’s responsiveness to multiple physiologically relevant stimuli was evaluated. Although the disulfide NGs have been systematically tailored for maximal GSH-responsiveness, their additional sensitivity to ROS and the pH-dependent swelling behaviour highlights the potential versatility of the platform. In particular, SS-NGs demonstrated swelling and matrix degradation upon exposure to different ROS species. However, it is important to note that the concentrations of ROS used in aforementioned experiments may not be entirely reflective of physiological conditions where many mechanisms exist to ameliorate their detrimental effects. While it was not covered in this work, of particular interest is the potential interplay between GSH and ROS species, which coexist in the TME and may exert combined effects on NG structure. While studying these stimuli individually simplifies understanding their distinct impacts on NG architecture, it does not fully capture the complexity of the *in vivo* environment.

In an effort to emulate this complexity, the influence of circulating proteins and salt concentrations (two key physicochemical factors present in plasma) was considered in relation to NG behaviour. However, it remains difficult to predict precisely how such interactions unfold *in vivo*, especially given the dynamic and often synergistic interplay between ionic strength and protein corona formation, as well as other biological stimuli aforementioned. These components rarely act in isolation, and it is likely their combined effects exert a more significant influence on NG behaviour than either factor alone. It is possible that more robust assays investigating the synergistic or antagonistic nature of these nano–bio interactions could be developed based on the fundamental experiments presented in this work.

Nevertheless, examining the responsiveness of different types of optimised NGs (PolyR_NG, PolyH_NG, DMA/AETC_NG, AC-GUA_NG, AETC_NG) reveals that each monomer subtly influences polymer architecture, imparting distinct degrees of stimuli-responsiveness. This was especially prominent in the differences in pH-

responsiveness that each monomer imparted to the NG structure – a feature that not only dictated their stability under acidic conditions but also influenced their capacity for endosomal escape. The results also indicate that while pKa of the respective NGs provides a useful indication of protonation behaviour, it is the interplay of multiple structural features that ultimately governs NG responsiveness - a finding that merits further systematic investigation. Ultimately, the tunability of the NGs expands and enhances their behavioural repertoire, thereby paving the way towards highly tailored nanoplatforms for therapeutic applications.

Chapter 5

Design, development and validation of PCa targeting redox-responsive nanogels

5.1 Introduction

Metabolic reprogramming and adaptation are key hallmarks of cancer, allowing tumours to maintain cell survival, rapid proliferation, resist apoptosis and adapt to environmental stressors such as hypoxia and nutrient deprivation.[423] Indeed, since Otto Warburg’s pioneering discovery, that cancer cells preferentially use glycolysis to meet their energetic demands, many studies have reported the altered metabolic state of cancer cells relative to normal cells.[63, 424] While oncogenic signalling pathways trigger many of these changes, their full implementation requires coordinated regulation of complex metabolic networks. The transcriptional co-activator PGC1 α , a master regulator of mitochondrial biogenesis and oxidative metabolism, has emerged as a crucial node in this regulatory landscape.[425]

In the context of PCa, where metabolic flexibility plays a key role in tumour progression and therapy resistance, it is unsurprising that the dysregulation of PGC1 α activity can contribute to the altered energetic and biosynthetic demands of malignant cells.[124, 426] Given its unique capacity to orchestrate broad transcriptional changes through interactions with a vast array of transcription factors, PGC1 α can pose as an ideal therapeutic candidate to oppose oncogenic metabolic programs. Accordingly, this chapter examines whether PGC1 α overexpression could serve as a viable anti-PCa therapy.

While encapsulating PGC1 α mRNA within the NGs represents a promising first step, the NG composition presented thus far remains untargeted. While PGC1 α overexpression in normal tissues is unlikely to have adverse effects, in order to realise its maximum therapeutic benefit, it is desirable to achieve expression precisely in the malignant tissues. From a clinical perspective, it would be ideal to be able to inject such a therapy intravenously, as opposed to locally, as this would be more comfortable for the patients. As such, a potential PCa targeting strategy is proposed, exploiting the prostate-specific membrane antigen (PSMA) receptor that is ubiquitously overexpressed on PCa cells. Indeed, several PSMA-targeted therapies have been developed including radionuclide-labelled small molecule PSMA

inhibitors (^{68}Ga -PSMA and ^{177}Lu -PSMA-617), antibody drug conjugates (MLN2704, PSMA-MMAE, MEDI3726) and cellular immunotherapy (PSMA-targeted bispecific T-cell engager).[38, 427] These have been extensively used in clinical settings for PCa imaging and therapy, thereby highlighting the potential of PSMA as an active target.

Of the wide variety of PSMA-targeting modalities available, a targeting peptide was surmised to be the most optimal and least likely to cause physicochemical modifications to the optimised NG, as opposed to a sterically bulky monoclonal antibody.[428] Further, the chemical flexibility offered by the NG platform facilitated facile exploration of different chemical bioconjugation strategies, of which the thiol-maleimide was considered to be the most practical. Therefore, first the physicochemical properties of the targeted NGs are assessed. Targeting specificity was rigorously validated *in vitro* using PSMA-positive and -negative cell lines, to confirm preferential internalisation of the PSMA-targeted NGs into the target cells. Furthermore, transcriptomic profiling analysis was conducted to gain mechanistic insights into the metabolic pathways affected by PGC1 α overexpression and their subsequent effects on PCa progression. In addition, to further bridge the gap between *in vitro* and *in vivo* settings, 3D culture models were also employed to more accurately recapitulate tumour architecture and nano–bio interactions, thereby providing a closer approximation of *in vivo* trafficking dynamics and tumour penetration.

To investigate NG targeting efficiency *in vivo*, an mRNA encoding for a far-red fluorescent protein, mCardinal, was used to monitor the biodistribution. A PCa xenograft model was subsequently developed to determine the effects of systemic PGC1 α _NG administration on tumour progression, thereby exploring the clinical potential of such a nanotherapeutic.

5.2 Results & Discussion

5.2.1 Design and characterisation of NGs targeting the prostate specific membrane antigen (PSMA)

To achieve precise delivery into PCa cells, PSMA, encoded by the folate hydrolase gene (also known as glutamate carboxypeptidase II), was selected as the molecular target due to its abundant and consistent expression in PCa.[423] Small molecule PSMA inhibitors labelled with radionuclides (^{68}Ga -PSMA and ^{177}Lu -PSMA-617) have been widely used for PCa imaging and therapy, demonstrating their high specificity and favourable safety profile.[428, 429] The effectiveness of these inhibitors arises from a lysine-urea-glutamate motif which binds to the active binding site with high affinity.[430] ^{177}Lu -PSMA-617 also includes 2-naphththyl-L-alanine and tranexamic acid, of which the naphthyl amino acid is crucial for improving interactions with the lipophilic binding pocket.[427, 430–433] Inspired by this structure, a targeting peptide incorporating the key binding motifs was synthesised by Pepmic Co., Ltd. (Suzhou, China). To enable installation of the peptide onto the NG surface via thiol–maleimide click chemistry, which was chosen for its high specificity and yield, a free thiol was introduced at the N-terminus (Figure A8). Subsequently, functionalisable NGs were synthesised by incorporating a bifunctional monomer, acrylate-PEG-maleimide (PEG-mal), during the polymerisation process, thereby allowing post-polymerisation conjugation of the targeting ligand onto the NG surface (Figure 5.1a, Figure A9).

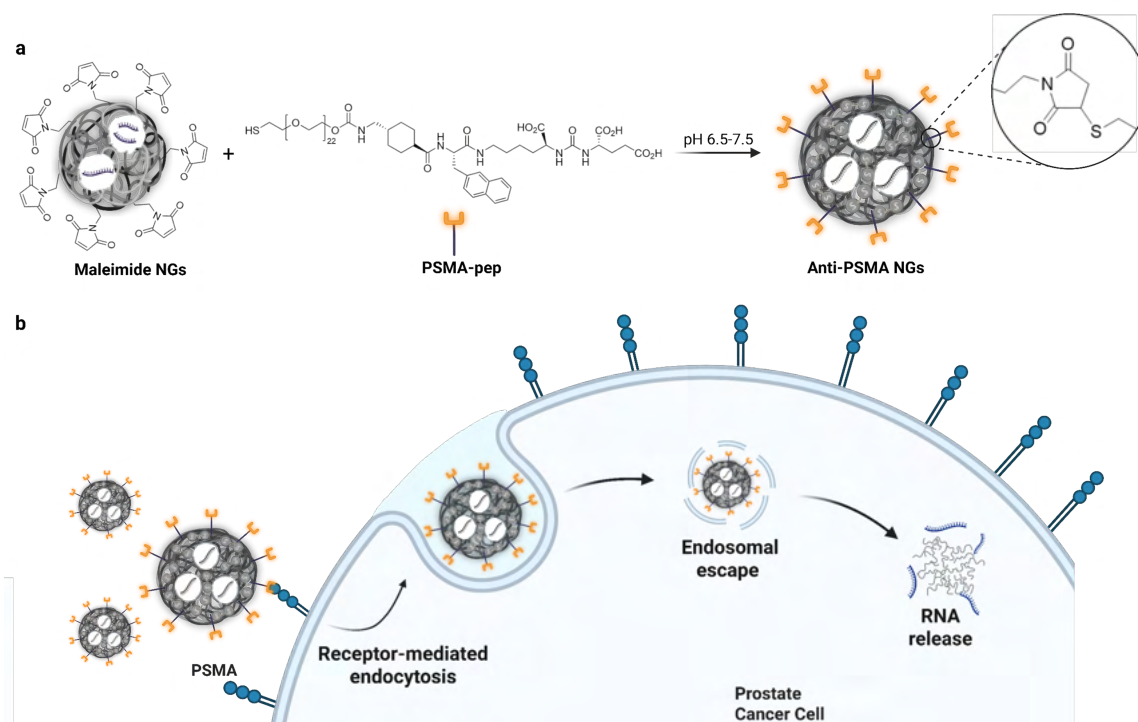


Figure 5.1: Schematic presentation of a) synthesis of PSMA-targeted NGs and b) proposed mechanism of cellular interaction and internalisation following interaction with PSMA.

Prior to conjugation with the targeting peptide, the amount of PEG-mal incorporated during NG synthesis was optimised to achieve favourable structural features and an appropriate maleimide surface density. Interestingly, the highest PEG-mal mol% yielded the smallest NG sizes (145.3 ± 1.6 nm), lowest PI (0.07 ± 0.03), a favourable ζ -potential (21.8 ± 0.9 mV) and uncompromised EE% and LE% (Figure 5.2a,b,c,e). This effect is likely due to the higher monomer concentration increasing the number of crosslinkable sites per unit volume, thereby promoting the formation of a denser polymer network and resulting in more compact nanogels.[434]

To quantify maleimide incorporation, a maleimide colorimetric assay was used. The results showed that increasing the PEG-mal mol% led to a corresponding increase in maleimide concentration within the NGs (Figure 5.2d). Given the known maleimide concentration, NG density (approximated to 1 mg/mL) and NG size, the surface density of the maleimide groups (#mal/nm²) was also determined, and reflected the trend in increasing maleimide concentration, reiterating the successful conjugation

of maleimide moieties onto the NG surface (Figure 5.2d). These calculations have been summarised in Section 2.4.2.

It is important to note, however, that the maleimide quantification assay may not distinguish between surface-exposed and internally embedded maleimide groups. It is plausible that internal maleimide groups are less accessible due to the dense crosslinking of the NG core. In future work, this could be investigated by performing the assay before and after glutathione (GSH)-mediated NG degradation to assess whether any difference in maleimide quantification is observed. Based on these optimisation results, 0.1 mol% PEG-mal was selected for subsequent PSMA-labelling studies.

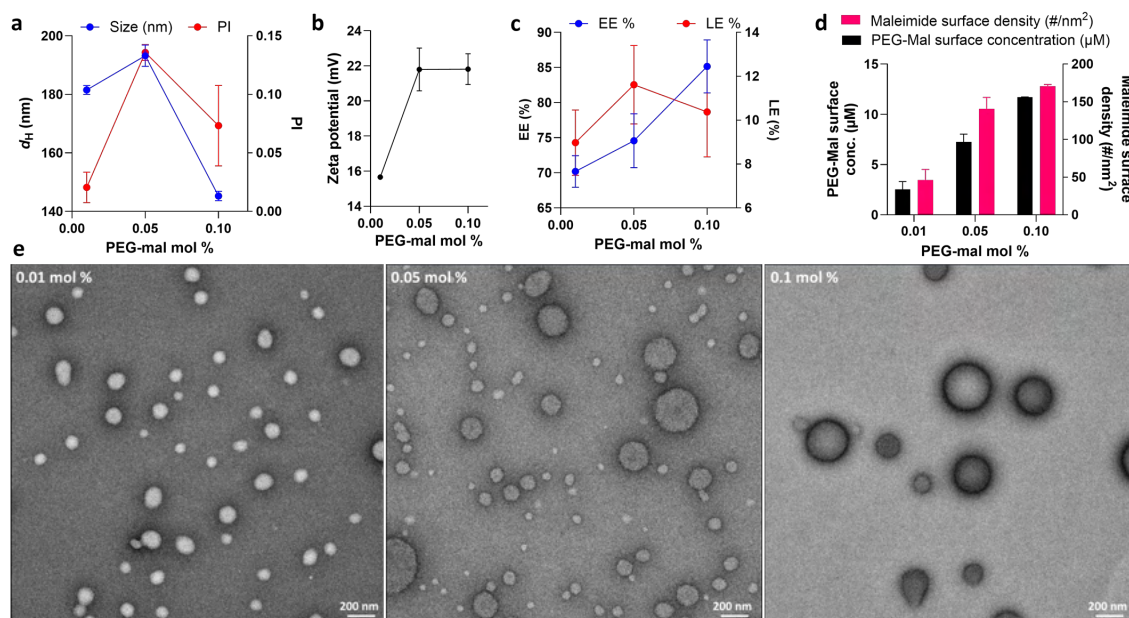


Figure 5.2: Physicochemical characterisation of maleimide-incorporating NGs. Effect of varying PEG-maleimide monomer mol% on a) NG size, PI, b) ζ -potential, as measured by DLS and ELS, respectively, c) encapsulation and loading efficiencies, and e) maleimide surface concentration and coverage. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. f) Representative negative stain TEMs of NGs. Scale bar at 200 nm.

Following this, the PSMA-pep amount conjugated on the NG surface was systematically optimised. At pH 6.5-7.4, the thiol-maleimide reaction yielded via a Michael addition a stable thioether bond between PSMA-pep and the maleimide-coated NGs. As the peptide doesn't possess any other exposed free thiols, this ensured

site-specific conjugation, without compromising its bioactivity. This work presents the first attempt at conjugating such a peptide to the NGs. Notably, increasing PSMA-pep amount to 0.052 μmol resulted in larger NG size ($256.9 \pm 4.1 \text{ nm}$) and a higher PI (0.10 ± 0.02) as well as a less positive ζ -potential ($8.9 \pm 0.9 \text{ mV}$) (Figure 5.3a,b,e). While the low ζ -potential could indicate compromised colloidal stability, in this instance, it is also likely that the free carboxylic acid motifs decorating the NG surface could be deprotonated, leading to a decreased NG effective surface charge. However, the large NG size achieved using the highest amount of PSMA-pep was likely to prevent cellular internalisation, as it no longer conforms to the stipulations proposed earlier for successful cellular delivery.[321] Further, as is evident in the TEM images corresponding to 0.052 μmol PSMA-pep, NG morphology seems to be distorted more so than for lower concentrations of the peptide (Figure 5.3e). After peptide conjugation, the surface PSMA-pep surface coverage was calculated by referring to the difference of absorbance at 280 nm between the targeted and untargeted NGs. The naphthyl alanine moiety provides strong absorbance at 280 nm which was used to determine the approximate measure of peptide surface concentration and density. The calculations used are summarised in Section 2.4.2. It was hypothesised that increasing the PSMA-pep concentration would lead to a corresponding decrease in maleimide surface concentration—a trend that was indeed observed (Figure 5.3d). Notably, 0.052 μmol PSMA-pep did not result in a substantial reduction in NG surface maleimide coverage compared to 0.026 μmol , and was therefore excluded from further optimisation due to its limited added benefit. Given the high cost of the peptide, the limited available quantity, and time constraints, optimisation efforts were focused on lower, more practical concentrations.

Indeed, 0.026 μmol PSMA-pep provided the most promising size ($202.0 \pm 1.0 \text{ nm}$) and polydispersity index (PI) (0.04 ± 0.02). Although the ζ -potential ($12.0 \pm 0.5 \text{ mV}$) was lower than the desirable range [321], the increased PSMA-pep surface concentration (0.02 μmol) and density ($19.1 \pm 1.9 \text{ \#mal/nm}^2$) compensated for this decrease without any adverse effects on NG morphology. As such, this concentration of PSMA-pep was chosen for investigating the *in vitro* targeting efficiency of the labelled NGs.

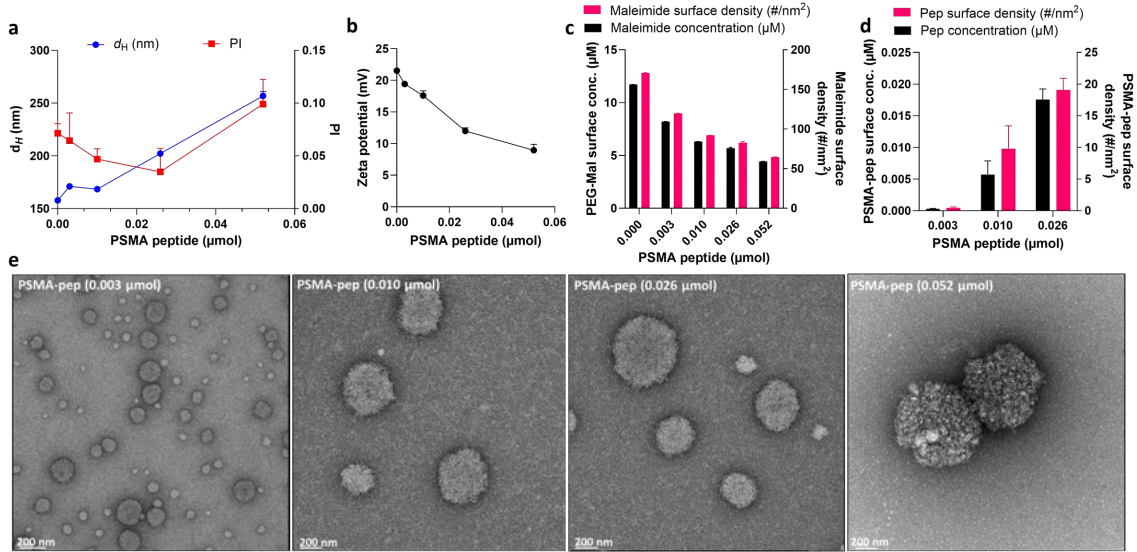


Figure 5.3: Physicochemical characterisation of PSMA-pep incorporating NGs. Effect of varying PSMA-pep amount on a) NG size, PI, b) ζ -potential as measured by DLS and ELS, respectively, c) maleimide surface concentration and coverage, and f) calculated values for NG surface concentration and density of PSMA-pep. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$. e) Representative negative stain TEMs of NGs. Scale bar at 200 nm.

In parallel, the optimal time frame for the conjugation reaction was also investigated. Due to the sensitive nature of the RNA payload, which is prone to degradation at higher temperatures, the conjugation reaction was allowed to occur at 4 °C for varying time points and targeted NG formation was tracked using DLS and ELS. As the size, PI and ζ -potential observably plateaued 24 h post conjugation for all PSMA-pep concentrations, this indicated a potential endpoint for the reaction (Figure 5.4). However, for the PSMA-pep concentration chosen 0.026 μmol , this time point yielded large, unstable NGs (268.3 ± 17.6 nm; $\text{PI } 0.11 \pm 0.03$) with extremely low ζ -potential (3.3 ± 0.1 mV), indicating collapsing colloidal stability (Figure 5.4e,f). Therefore, a 12 h timepoint was selected for the conjugation as it provided NGs of desirable sizes (201.3 ± 22.5 nm; $\text{PI } 0.14 \pm 0.04$) and consistent ζ -potential (12.0 ± 0.2 mV).

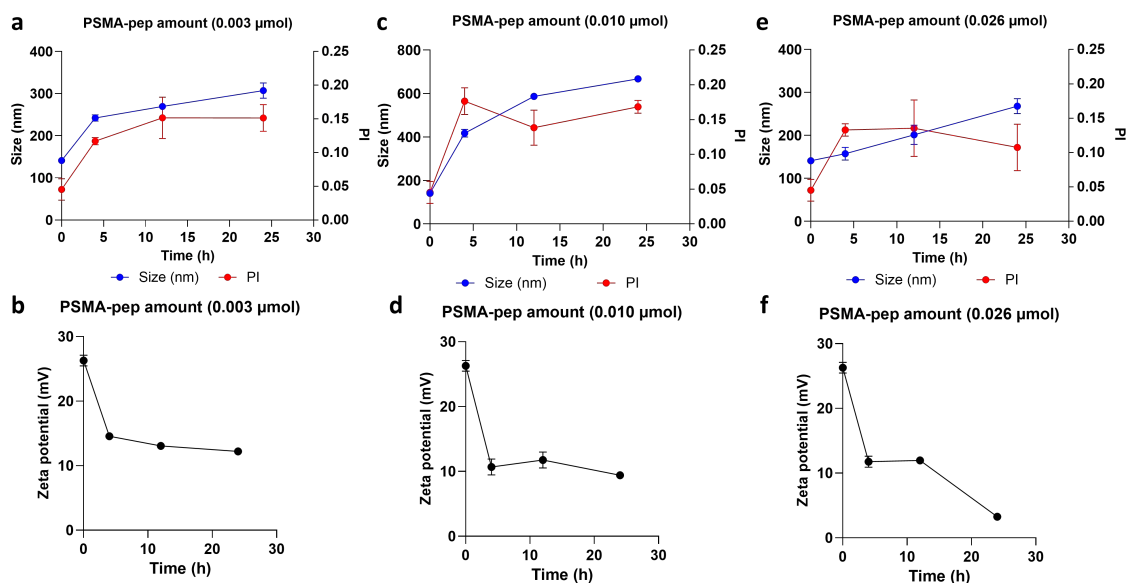


Figure 5.4: **Time dependent physicochemical characterisation of PSMA-pep incorporating NGs.** a,c,e) NG size, PI, b,d,f) ζ -potential as measured by DLS and ELS, for various PSMA-pep concentrations, respectively. In all cases, data are presented as mean $\pm$ standard deviation for $n = 3$.

As a further validation of PSMA conjugation on NG surface, single particle automated Raman trapping analysis (SPARTA) was used.[435] Conventionally, DLS and NP tracking analysis are used to understand NP population size distribution. These techniques can be complemented by mass spectrometry and (Fourier-transform)-Infrared (IR) spectroscopy or other such bulk approaches to gain an insight into NP composition.[436, 437] However, there is a general disharmony between these techniques, with each requiring varying dilutions, sample preparation as well as differences in detection sensitivity. This can lead to discrepancies and complicate direct comparisons across datasets. Furthermore, it is crucial to understand NP population heterogeneity for downstream biological applications, however this cannot be easily resolved using such bulk analysis techniques.[437] SPARTA, on the other hand, offers simultaneous analysis of size and composition at a single particle resolution. By integrating optical trapping with Raman acquisition, it provides a molecular fingerprint of each particle, such that subtle surface modifications can be detected.[435] Compared to IR spectroscopy, Raman analysis via SPARTA offers distinct advantages, particularly for NPs dispersed in aqueous media, since

Raman signals are minimally affected by water absorption. Additionally, Raman’s sensitivity to non-polar bonds and certain functional groups enhances its suitability for detecting surface chemistry modifications such as PSMA conjugation. Crucially, as SPARTA analyses each particle individually, it also enables quantitative assessment of heterogeneity in surface modification across a population, which would be masked in bulk measurements.

As the final NG construct is a complex, multi-monomer-based NP system, NGs were synthesised using each monomer in isolation as well as in various binary and ternary combinations to serve as controls. This approach enabled direct comparison and identification of the individual monomer contributions within the final NG composition. Single monomer systems included AETC only NG (AETC_NG), acrylate-PEG-maleimide only NG (MAL_NG), and disulfide-crosslinker only NG (PEGSS_NG) (Figure 5.5a,b,c). Notably, NGs could not be synthesised with acrylamide only, likely because the concentration used was too low to form a three dimensionally crosslinked network.

For the single monomer systems, common regions of interest pertain to $\nu(\text{C-N})$ from the C-C-O backbone at $\sim 1450\text{ cm}^{-1}$, and bending modes from NH_2 moieties ($\gamma(\text{NH}_2)$) at $\sim 1295\text{ cm}^{-1}$ as well as CH_2 moieties ($\gamma(\text{CH}_2)$) at $\sim 1060\text{ cm}^{-1}$. Notably, maleimide-incorporating NGs demonstrate a distinct peak at in the region $\sim 1600 - 1640\text{ cm}^{-1}$ (Figure 5.5b). This peak only appears in the spectra of the NG synthesised with all monomers combined, suggesting it corresponds to the $\nu(\text{C=O})$ amide I band of the maleimide functional group exposed on the NG surface (Figure 5.5f,g). The spectrum for the complete NG composition, which includes all monomers, is presented in Figure 5.5h, revealing the cumulative vibrational features. In the complete NG composition containing all monomers, this broad maleimide peak was notably absent, suggesting successful surface conjugation with the PSMA peptide. Instead, a pronounced amide I band at $\sim 1606\text{ cm}^{-1}$ appeared, consistent with amide bonds in the peptide backbone, further supporting PSMA conjugation. Furthermore, smaller peaks in the regions $\sim 800 - 880\text{ cm}^{-1}$ (PO_2^- symmetric stretch), $\sim 1090\text{ cm}^{-1}$ (PO_2^-

asymmetric stretch), $\sim 1480 - 1500 \text{ cm}^{-1}$, could be indicative of RNA entrapment within the NGs.[438, 439]

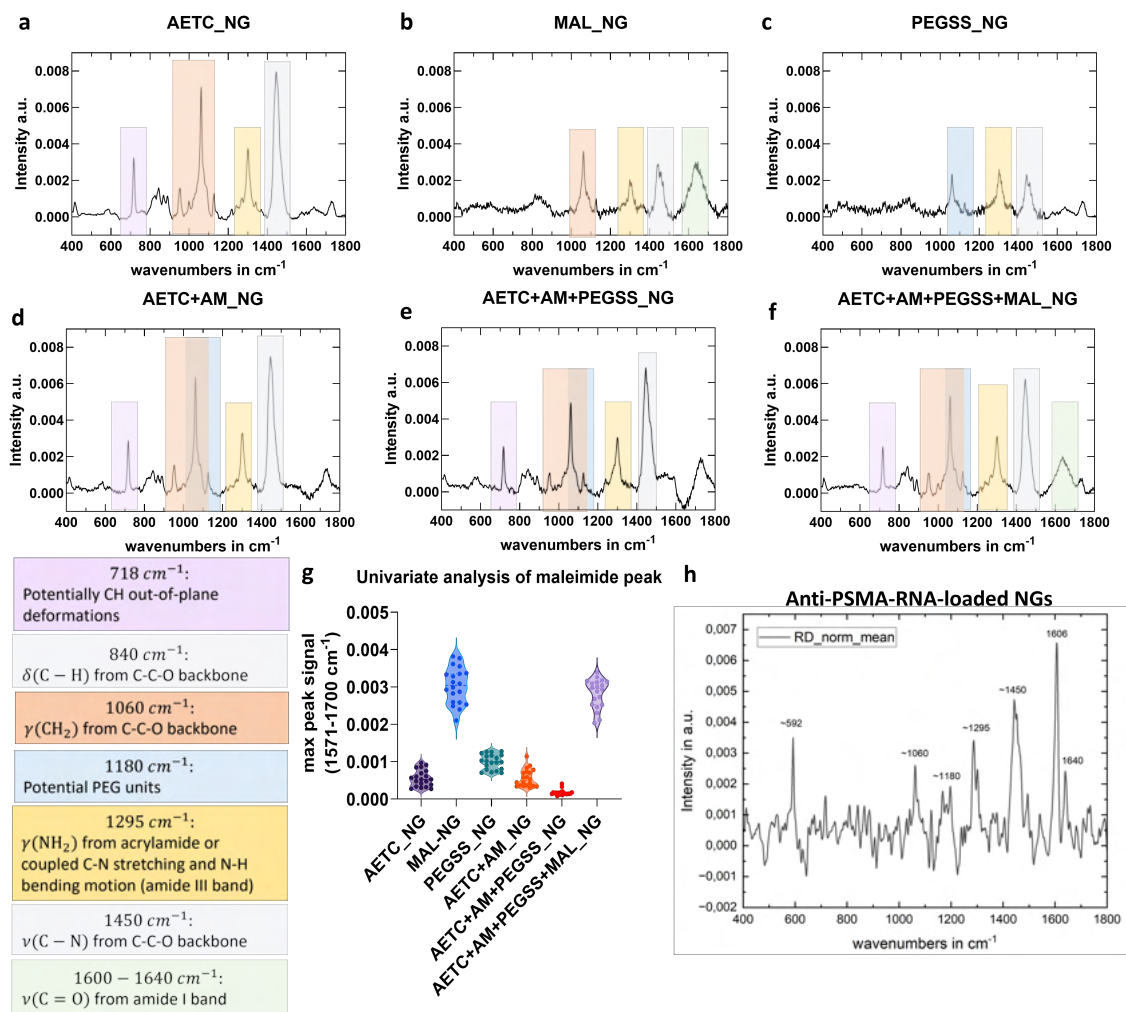


Figure 5.5: **SPARTA composition analysis of NGs.** Offset averaged Raman spectra of a) AETC_NG, b) Mal_NG, c) SS_NG, d) AETC+AM_NG, e) AETC+AM+PEGSS_NG, f) AETC+AM+PEGSS+MAL_NG with key regions of interest highlighted in various colours as explicated below the graphs in the coloured panels. g) Univariate analysis in the region 1600 – 1740 cm^{-1} corresponding to the maleimide functional group exposed on NG surface. h) Offset Raman spectra of the complete PSMA-targeted, RNA-loaded NG.

Despite the promising trends observed, it is important to acknowledge that the SPARTA system was originally optimised for simpler NP formulations than the complex NG constructs studied here. The intrinsic structural complexity of the NGs and overlapping vibrational modes may lead to some loss of spectral resolution or incomplete peak assignment. Furthermore, factors such as particle aggregation,

laser-induced heating, and photobleaching could affect also measurement accuracy. Therefore, while SPARTA provides valuable compositional and heterogeneity insights at the single particle level, its results are best interpreted in conjunction with complementary characterisation techniques aforementioned.

5.2.2 *In vitro* specificity assessment of PSMA-targeted NGs.

After optimising the conjugation protocols, it was imperative to determine the cellular targeting ability of the PSMA_NGs. To establish a suitable model system for evaluating relative uptake efficiency, various PCa cell lines were first screened for their PSMA expression levels. As reported in the literature, C4-2 cells demonstrate high levels of PSMA expression, whereas PC3 demonstrate negligible expression (Figure 5.6a).[440, 441] As these cell lines demonstrated the greatest differential PSMA expression among those tested, these were chosen for subsequent cellular uptake investigations.

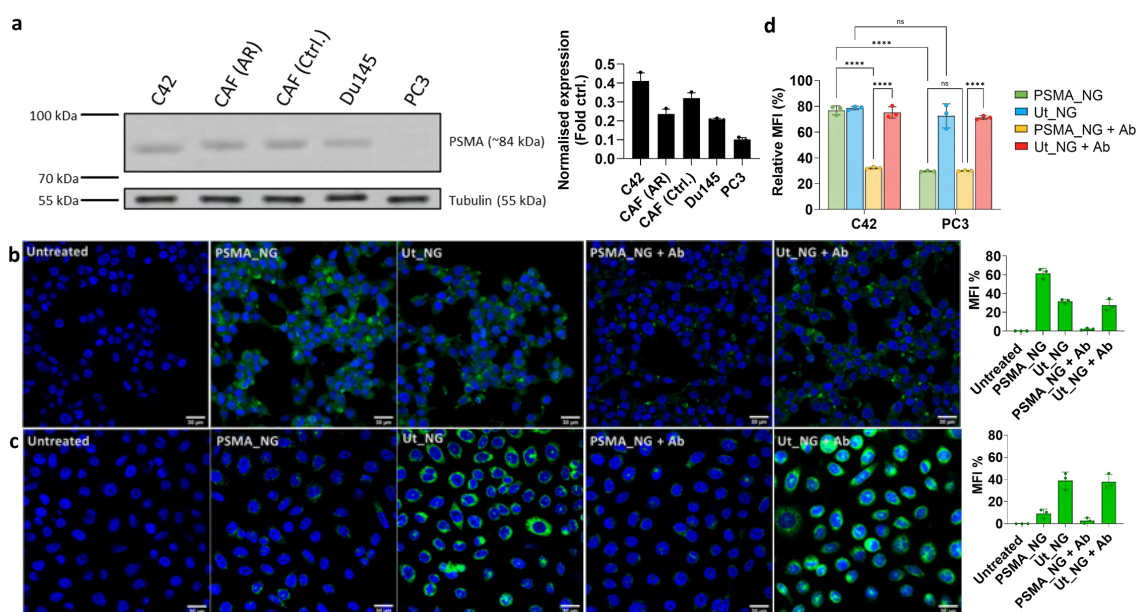


Figure 5.6: Investigating cellular uptake efficiency of targeted and untargeted NGs. a) Western blot analysis of PSMA expression across PCa cell lines. Cell lysates from C4-2, CAF AR (androgen receptor positive), CAF Ctrl. (androgen receptor negative), Du145 and PC3 cells were probed for PSMA. Tubulin served as a loading control. Densitometric quantification of PSMA signal intensity, normalised to tubulin, is shown on the right. Confocal microscopy images of b) C4-2 and c) PC3 cells showing the uptake of fluorescein-labelled (ex/em: 494/521 nm) PSMA_NGs or Ut_NGs with or without PSMA antibody pre-treatment. Scale bar at 30 μ m. Quantification of green mean fluorescence intensity (MFI) from multiple fields of view is shown in the bar graph on the right. d) Flow cytometry analysis of fluorescent NG uptake in PSMA-positive (C4-2) and PSMA-negative (PC3) PCa lines. Cells were incubated with fluorescein-labelled (ex/em: 494/521 nm) PSMA_NGs or Ut_NGs, with or without pre-treatment using an anti-PSMA antibody. Mean fluorescence intensity (MFI) reflects the extent of NG internalisation. Data represent mean $\pm$ SD from 3 independent experiments.

It was hypothesised that PSMA_NG uptake would be reduced in both PSMA-low cells and in cells pre-treated with an anti-PSMA antibody, the latter expected to competitively block receptor binding and thereby limit internalisation. Using confocal microscopy in tandem with flow cytometry, PSMA_NG uptake was reduced in PC3 cells (microscopy: $9 \pm 4\%$, cytometry: $30 \pm 1\%$) overall, compared to C4-2 cells (microscopy: $62 \pm 5\%$, cytometry: $77 \pm 4\%$) (Figure 5.6b,c,d, Figure A10). Notably, the uptake of (untargeted NGs) Ut_NGs in PC3 cells was comparatively unaffected (microscopy: $39 \pm 8\%$, cytometry: $32 \pm 2\%$) (Figure 5.6c,d). Strikingly,

C4-2 cells pre-treated with the anti-PSMA antibody prior to PSMA_NGs incubation showed a 97% reduction in fluorescence signal in microscopy images and a 59% decrease in FITC-positive signal by flow cytometry (Figure 5.6b.d). In contrast, the uptake of Ut_NGs following anti-PSMA treatment remained largely unaffected in the both cell lines. As further confirmation of the specificity of the PSMA_NGs for the PSMA receptor, immunofluorescence studies were performed to investigate the colocalisation of the PSMA signal (red) with the fluorescent NG (green) signal using confocal microscopy. A higher Pearson's correlation coefficient was observed in PSMA_NG-treated cells ($r=0.65$) as opposed to those treated with Ut_NGs ($r=0.29$) (Figure 5.7).

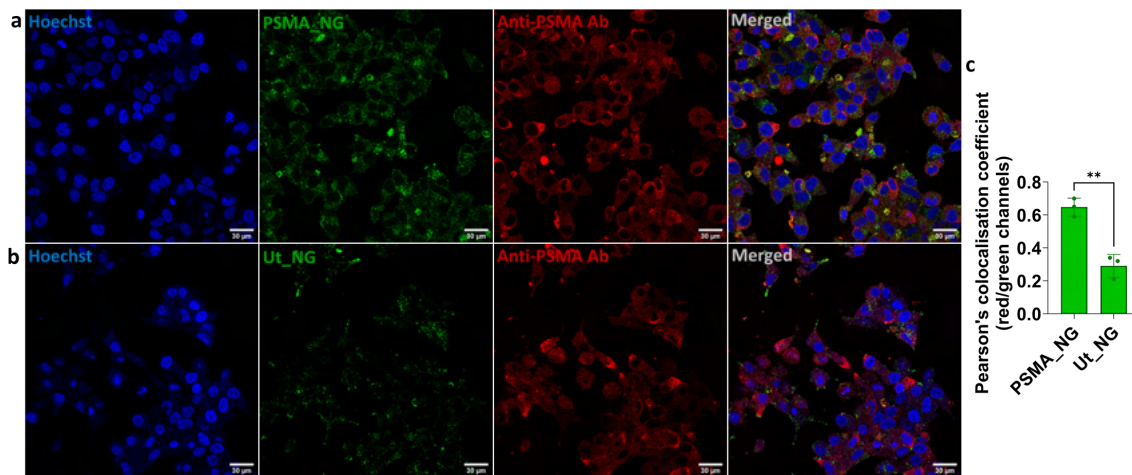


Figure 5.7: Maximum intensity projection confocal microscopy images showing uptake of fluorescein-labelled (green: ex/em: 494/521 nm) PSMA-targeted (PSMA_NG) and untargeted NGs (Ut_NG) in C4-2 cells. Immunofluorescence staining of PSMA expression (anti-PSMA antibody (abcam, EPR6253) red: ex/em 570/590 nm) demonstrates differential uptake of a) PSMA_NGs vs b) Ut_NGs. Nuclei are counterstained with DAPI (blue). Scale bars = 30 μ m. c) Quantification of colocalisation between NGs (green) and PSMA (anti-PSMA antibody (abcam, EPR6253) red: ex/em: 570/590 nm) channels using Pearson's correlation coefficient, indicating significantly higher colocalisation for targeted vs untargeted NGs. Data are mean $\pm$ SD from three independent experiments. Statistical significance determined by an unpaired t-test with Welch's correction. ** $p < 0.01$

While the mode of cellular internalisation was studied in Section 3.2.7 by studying fluorescein-labelled NG uptake in the presence of inhibitors comprising all known uptake pathways, this was repeated owing to the significant structural modifications

made to the original NG.[353] Although, C4-2 and PC3 cell lines internalise NPs through endocytosis, in the case of C4-2 cells, it is likely that the anti-PSMA peptide interacts with the PSMA receptor to undergo receptor-mediated endocytosis via clathrin-coated vesicles.[442–444] Indeed, a threefold increase in PSMA internalisation was observed following the binding of specific ligands.[445, 446] As before, treatment with hyperosmolar sucrose and NaN_3 /2-deoxy-D-glucose inhibited the uptake of both NG types, suggesting clathrin-mediated endocytosis remains the predominant pathway (Figure A11). Interestingly, despite the morphological changes that the NGs undergo, the mode of internalisation appears to be the same. It is possible that the difference lies in the specific receptors they interact with in order to undergo cellular uptake. Future studies can investigate the colocalisation of the NGs with common cell receptors to exemplify their internalisation pathway more precisely.

Conventional NP delivery studies tend to focus on the accumulation of NPs in the tumour tissue, as opposed to the penetration profile and dynamics of the NPs into the tumour. Indeed, penetration ability of delivery vehicles is a crucial parameter as the therapeutic efficiency could be limited when the drug is delivered to the periphery of solid tumours. As such, C4-2 spheroids were cultured to investigate the NG trafficking and penetration in a 3D system. First, the growth conditions were systematically optimised by evaluating spheroidal growth using the IncuCyte Spheroid Analysis software module. Different collagen concentrations were tested to identify the formulation that consistently produced well-defined, tightly packed spheroids. As adequately-shaped spheroids developed within five days, they were treated with the fluorescein-labelled NGs on the sixth day of culture, and the uptake monitored using the fluorescence detection feature of the software (Figure 5.8a). Interestingly, PSMA_NGs (top panel in Figure 5.8b) entered the spheroids more rapidly and demonstrated a homogeneous distribution both at the periphery and within the interior. Conversely, Ut_NGs (bottom panel in Figure 5.8b) exhibit poorer distribution and slower uptake, as confirmed visually and through fluorescence quantification (Figure 5.8c). However, since the imaging capacity of the Incucyte is limited to spheroid periphery, this provides limited information on the depth of NG penetration. As such, confocal microscopy was used to image the spheroids to

complement the IncuCyte data. Z-stack images were acquired and analysed using Fiji software, where fluorescence intensity was quantified as integrated density (total fluorescence signal within a defined area) across multiple slices, allowing for a more accurate assessment of NG distribution throughout the entire spheroid volume.

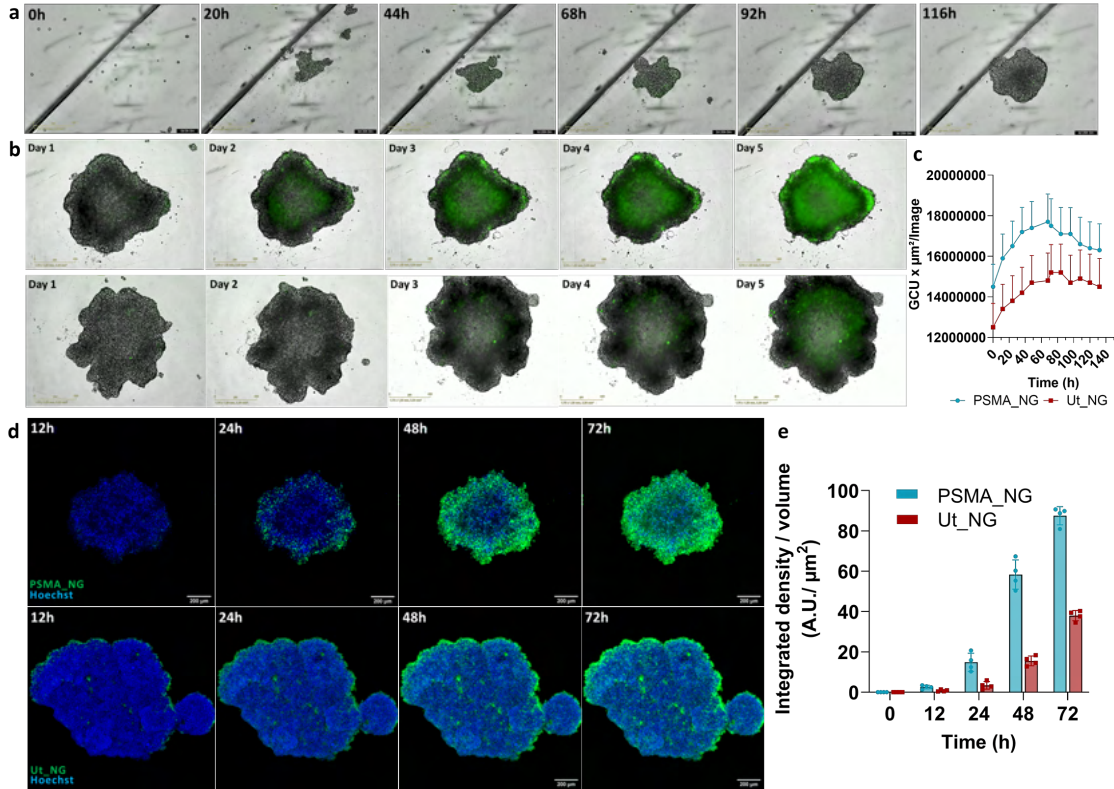


Figure 5.8: **Investigating spheroid penetration capacity of fluorescein-labelled PSMA_NG and Ut_NGs.** a) Timeline schematic showing how IncuCyte was used to track spheroid growth by capturing images every 12 h; representative images are shown. b) Top panel: Uptake of PSMA_NGs over five days monitored by IncuCyte fluorescence imaging; bottom panel: Uptake of Ut_NGs under the same conditions. c) Quantification of the fluorescence expression (green calibrated unit $\times \mu\text{m}^2/\text{image}$) as a function of time, corresponding to (b). d) Uptake of fluorescent NGs (ex/em: 494/521 nm) within 72 h shown by maximum intensity projections of confocal microscopy images of live spheroids embedded in agarose. e) Quantification of NG uptake by integrated fluorescence density (sum of pixel intensities across all z-cross sections) normalised to spheroid volume.

Interestingly, even within a 24 h period, a marked increase in the uptake of PSMA_NGs was observed compared to the Ut_NGs, underscoring both the efficiency of targeted delivery and the importance of employing complementary imaging techniques to confirm results (5.8d,e). The lack of detectable NG fluorescence at

earlier timepoints in the IncuCyte could be attributed to its limited resolution and signal interference caused by plate-based light scattering. Although extended confocal imaging over five days would have provided a more comprehensive temporal profile, this was not feasible due to the phototoxicity and the viability constraints associated with prolonged laser exposure on live spheroids. Nonetheless, the rapid uptake efficiency and homogeneous spheroid distribution of the PSMA_NGs highlights their promise as targeted RNA delivery vehicles. Given the comparatively larger size of these NGs (~ 200 nm), these results are particularly remarkable, as previous studies have noted a distinct NP size constraint on organoid penetration efficiency. This highlights a unique advantage conferred by the likely PSMA-mediated internalisation mechanism of the fluorescent PSMA_NGs.[321, 447]

5.2.3 Monitoring *in vivo* biodistribution and targeting efficiency assessment in C57BL/6 mice

There is a significant disconnect between the *in vitro* vs the *in vivo* efficacy of delivery vehicles, where many systems that transfect successfully *in vitro* fail to function or cause serious toxicity *in vivo*. [304, 448, 449] The limited success of NPs in the clinic emphasises the need to better understand their pharmacology, as well as the need to adopt a more refined approach for their discovery, development and validation. Understanding how various NP types interact with biological interfaces *in vivo*, along with the physicochemical properties that govern these interactions, is essential for accurately predicting their pharmacological and clinical behaviour.[448] In light of this, the *in vivo* biodistribution and targeting capacity of the PSMA_NGs was examined. In order to expedite the screening process for a proof of concept approach, an mRNA encoding for a far-red fluorescent protein, mCardinal (ex/em: 604/659 nm), was encapsulated and administered at a dose of 0.25 mg/kg (polymer dose: 15 mg/mL; 60 mg/kg), based on doses reported in previous studies using similar formulations.[450–452] Following intravenous injection, the radiant efficiency of the main organs were measured using the *in vivo* imaging system at 24 h (Figure 5.9b,c). In essence, radiant efficiency is a measure of the emitted photons from the fluorescent signal, normalised to the area of the region of interest. This metric enables

quantitative comparison of fluorescent signal intensity across different samples and time points, independent of acquisition settings or background variations, and is considered to be a robust method for signal detection. As shown in [Figure 5.9b,c](#), the distribution of both NG types was evident in the liver, spleen and kidneys after 24 h, as expected for NPs that are injected intravenously.^[453] Furthermore, the results from *ex vivo* imaging were supported by western blot analysis and RT-qPCR analyses, which showed increased protein expression and mRNA levels were observed in the same organs as before ([Figure 5.9d,e,f](#)). The NGs are surmised to reach the liver via the hepatic portal vein, and are then eliminated via the hepatobiliary route, which could offer an explanation for expression in the kidney tissues.^[453] Further, NG presence in the spleen could be explained by uptake by the mononuclear phagocytic system (MPS). Comprising of macrophages, monocytes and dendritic cells, the MPS is actively involved in NP distribution and clearance.^[453] On entering the vascular compartment, NGs likely interact with opsonins present in the blood, leading to bio-corona formation.^[454] Despite efforts to minimise protein adsorption by engineering the NG surface to reduce corona formation, the observed accumulation in the spleen suggests that some degree of opsonisation is still present. This highlights the limitations of relying solely on *in vitro* assays to predict *in vivo* behaviour, as the biological complexity of the systemic environment can significantly alter NP fate.

Notably, the targeted NGs (PSMA_mC_NGs) exhibited expression in the prostate, thereby exemplifying the targeting capability of our platform ([Figure 5.9b,c](#)).¹ This result was echoed in the mRNA levels and protein expression analysis, where a significant increase in mCardinal expression was noted for the targeted NGs as compared to the untargeted NGs in the prostate ([Figure 5.9d,e,f](#)). Despite the non-specific expression in other organs, this work represents the first example of a multi-functional NG-based composition specifically targeting the prostate, marking a crucial step towards the development of a more efficient and targeted delivery platform for PCa therapy.

¹The images show both the seminiferous tubules and the prostate. While the technique for isolating the prostate was refined during the course of the project, this image still effectively conveys the intended anatomical context.

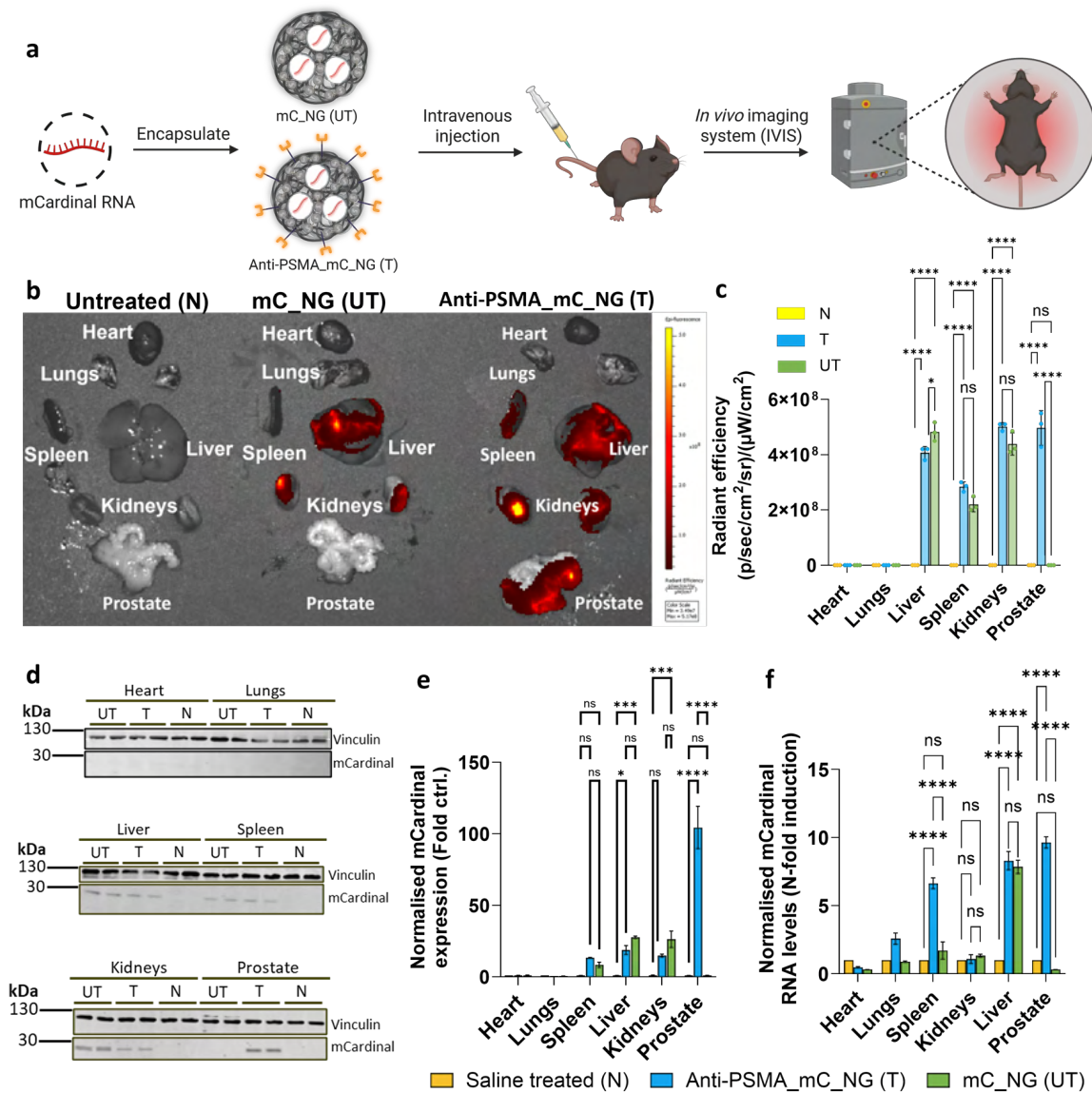


Figure 5.9: **Assessment of *in vivo* biodistribution of NGs in C57BL/6 mice.** a) Schematic presentation of the experimental set up where mCardinal mRNA, encoding for a far-red fluorescent protein mCardinal, was encapsulated inside the targeted (Anti-PSMA_mC_NG; T) and untargeted (mC_NG; UT) NGs, that were injected intravenously and the expression monitored using the *In vivo* Imaging System (IVIS) 24 h post-administration. b) *Ex vivo* fluorescence imaging and c) radiant efficiency quantification of main organs extracted from the mice at 24 h after injection of NGs (n = 3). d) mCardinal protein expression in major organs determined by western blotting and e) associated quantification. f) Quantification of mCardinal mRNA expression using RT-qPCR. Expression was normalised to GAPDH expression and shown as fold change relative to saline-treated mice. In all cases, data are presented as means $\pm$ SD, and statistical significance was determined using a 2-way ANOVA. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

The time-dependent expression profile of the targeted NGs was then assessed by harvesting organs of interest at 6, 24 and 48 h (Figure 5.10a,b). The elevated mRNA levels observed up to 6 h post-injection correlate with increased protein expression at later timepoints. As mRNA levels decline, protein expression also decreases, albeit with a delay, reflecting the time required for translation and protein turnover (Figure 5.10c,d,e). While protein expression was already evident at 24 h, as seen previously, it is notably sustained for up to 48 h post-administration which equals to a certain extent the expression dynamics observed for LNP-based formulations.[455]

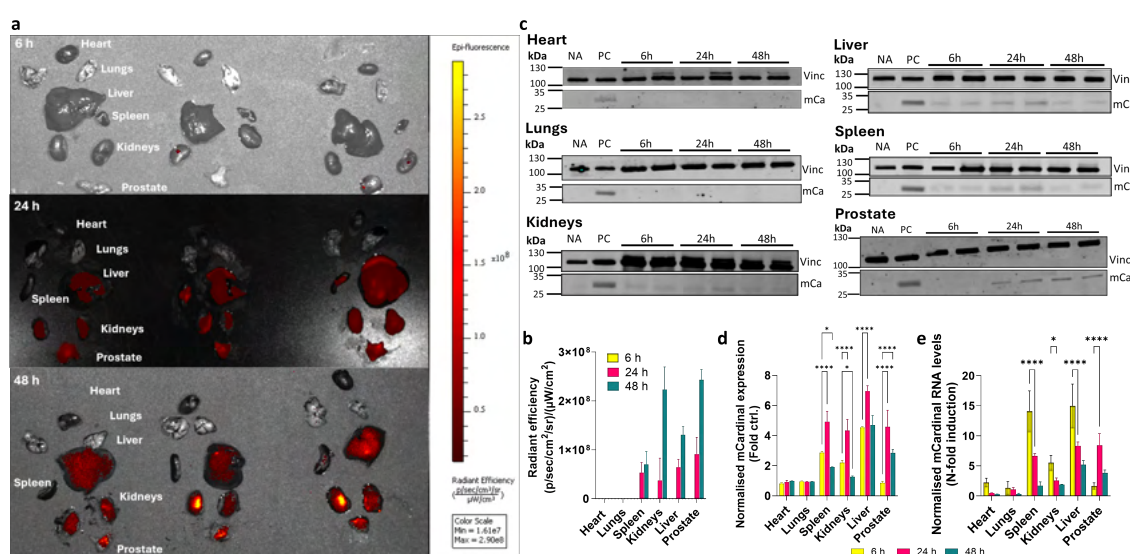


Figure 5.10: Time dependent evolution of mCardinal expression in C57BL/6 mice. a) *Ex vivo* fluorescence imaging and b) radiant efficiency quantification of main organs extracted from the mice at 6, 24 and 48 h after injection of NGs (n = 3). c) mCardinal protein expression in major organs determined by western blotting and d) associated quantification. In the blots, NA refers to untreated samples and PC refers to a positive control sample based on cell lysates transfected with mCardinal RNA-loaded NGs. e) Quantification of mCardinal mRNA expression using RT-qPCR. Expression was normalised to GAPDH expression and shown as fold change relative to saline-treated mice. In all cases, data are presented as means $\pm$ SD, and statistical significance was determined using a 2-way ANOVA. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

A key point of divergence from the LNPs lies in the delayed RNA release observed with the disulfide-based NGs. Whereas LNPs often mediate *in vivo* transfection within 6 h, likely due to a burst release profile, the disulfide NGs demonstrate a more gradual, biologically triggered release. This feature provides greater control over

RNA release kinetics, offering opportunities for fine-tuning delivery timing to suit specific therapeutic or controlled release applications.

Prior to functional *in vivo* assays, a pilot study was conducted to: 1) determine the optimal number of cells required to establish a controlled xenograft model of PCa, 2) to assess the difference in mCardinal expression in tumours between mice treated with mC_NGs or Anti-PSMA_mC_NGs and 3) to evaluate whether the mode of administration (subcutaneous vs intravenous injection) influenced the expression profile and biodistribution of the NGs (Figure 5.11a). Since 2.5×10^6 cells produced an optimal tumour volume growth profile – reaching the target volume of 50 mm³ within seven days – this cell number was selected for subsequent experiments (Figure 5.11b). For both modes of administration, PSMA_mC_NGs demonstrated uptake in the prostate and tumour, as well as some non-specific accumulation in the liver, spleen and kidneys, as before. Untargeted mC_NGs also exhibited tumour uptake, most likely driven by the enhanced permeation and retention (EPR) effect, which is primarily governed by the leaky vasculature in the tumour tissue that allows NPs to extravasate from circulation (Figure 5.11c,d).[456] Notably, in the case of the targeted NGs, an overall increase in accumulation in tumour tissue was confirmed by immunofluorescence analysis on frozen tissue sections.

Two modes of administration were investigated to explore the potential for repeated dosing of the animals with the functional PGC1 α mRNA. Intravenous injections can be challenging to administer multiple times per week, as this may jeopardise animal health and welfare. In contrast, subcutaneous injections are comparatively easier to perform repeatedly and are less invasive. While tumour uptake was comparable between both routes, a slight reduction in uptake was observed in the liver, spleen and kidneys for subcutaneous injections, as shown in the western blot analysis (Figure 5.11e). These findings suggest that the route of administration evidently does not compromise on the efficacy of transfection *in vivo* - representing a key advantage when considering optimal routes for repeated dosing.

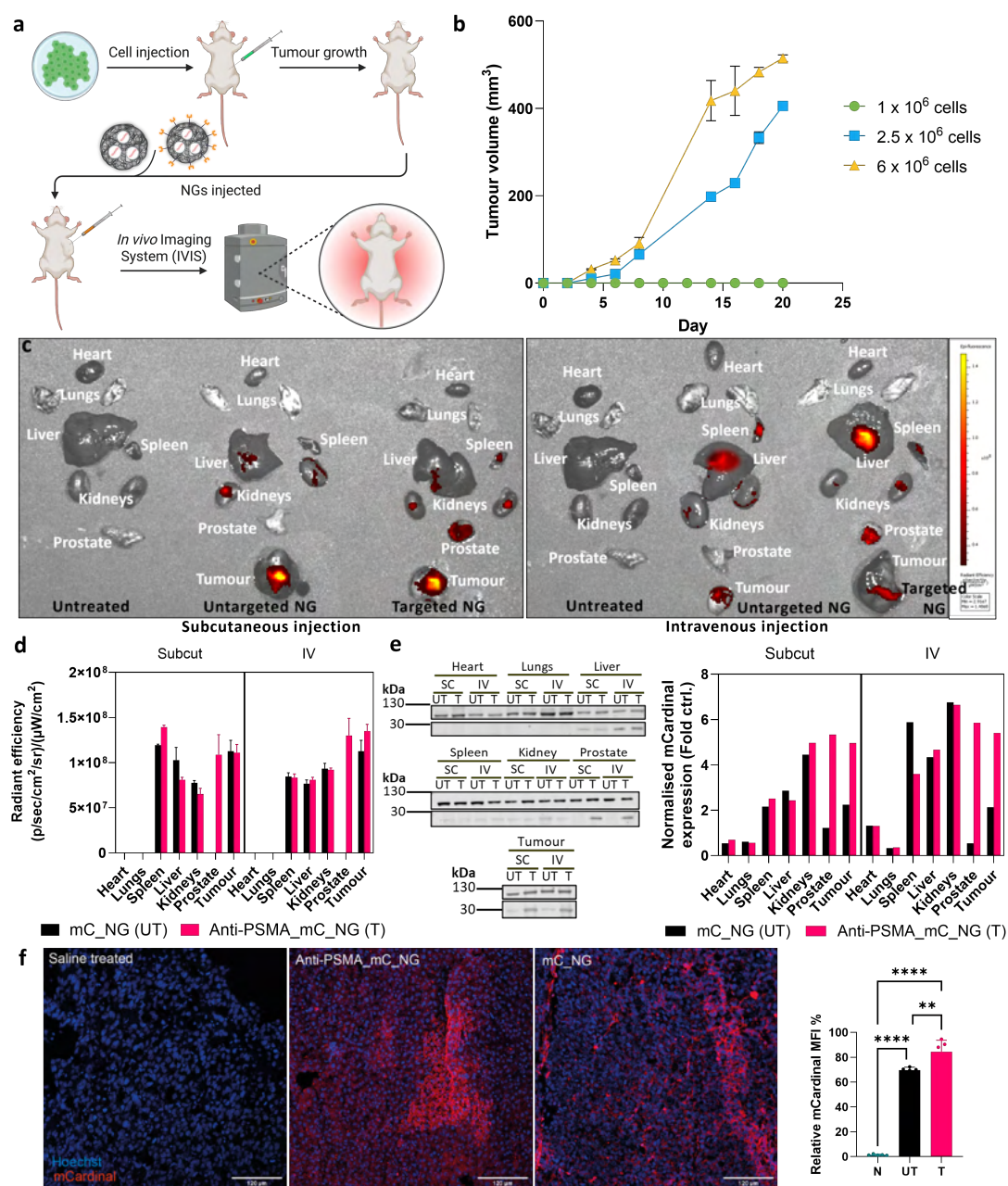


Figure 5.11: Time dependent evolution of mCardinal expression in C57BL/6 mice. a) Schematic presentation of PCa xenograft generation via implantation of C4-2b cells on the flanks of immunocompromised NSG-614 mice. b) Monitoring average tumour volume over a period of 20-days. c) *Ex vivo* fluorescence imaging and d) radiant efficiency quantification of main organs extracted from the mice at 24h after injection of NGs (n = 3). e) mCardinal protein expression in major organs determined by western blotting and associated quantification. f) Representative immunofluorescence images of frozen tumour sections showing nuclear staining with Hoechst (blue) and mCardinal expression (red), for various treatments with quantification.

5.2.4 Validating PGC1 α & NT-PGC1 α mRNA overexpression and downstream effects on mitochondrial biogenesis

Owing to PGC1 α 's encouraging role in PCa suppression[88, 124], the overexpression of PGC1 α was investigated in PCa cells via untargeted PGC1 α mRNA-loaded NGs (PGC1 α _NG) (Figure 5.12a).

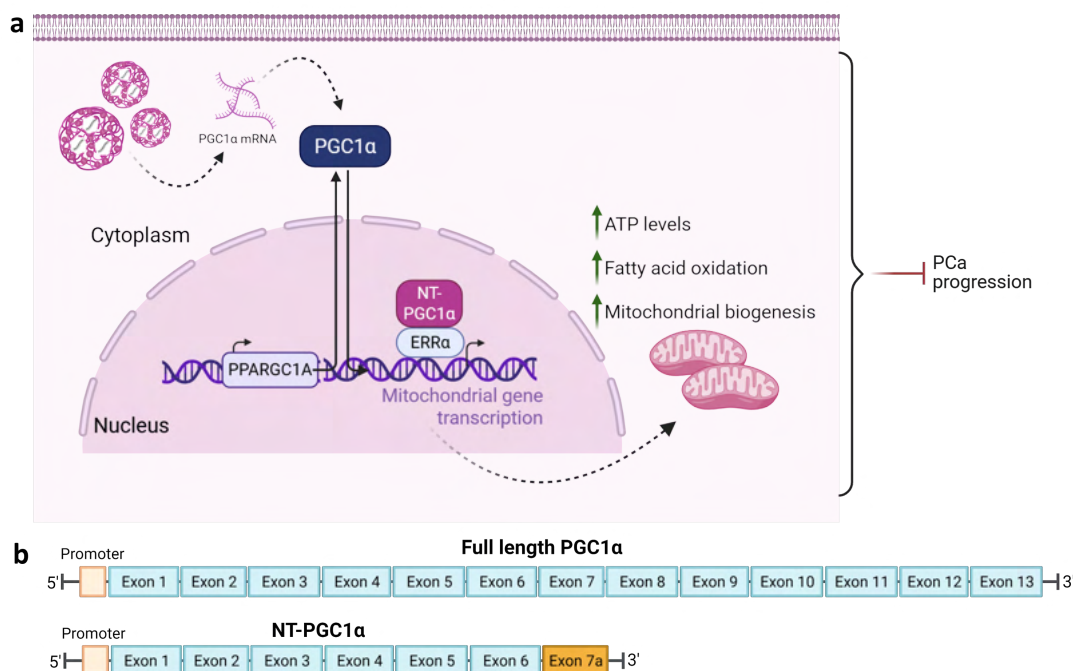


Figure 5.12: **AMPK-mediated activation of PGC1 α suppresses PCa progression through metabolic and transcriptional reprogramming.** a) Schematic presentation a) of the way in which delivery of PGC1 α mRNA within untargeted NGs leads to overexpression of PGC1 α , a key regulator of mitochondrial biogenesis and oxidative metabolism. Elevated PGC1 α promotes mitochondrial biogenesis, fatty acid oxidation, and ATP production, contributing to a metabolic shift away from lipid accumulation; b) of the two predominant splice isoforms of *PPARGC1A*.

The transient expression of PGC1 α mRNA in castrate-resistant PCa C4-2 cells was tracked by western blot analysis and RT-qPCR.¹⁸ PGC1 α overexpression was straightforward to measure in this particular cell line as the basal expression of PGC1 α is very low.[88] To maintain consistency in comparison, the cells were treated with equivalent doses of PGC1 α mRNA in the untargeted NGs and commercial transfection reagent (PGC1 α _JetMESSENGER (jetM)). First, the dose dependency of PGC1 α expression was confirmed by treating the cells with varying concentrations

of PGC1 α _NGs, which was evident from the positive correlation observed between the NG dose and subsequent protein expression (Figure 5.13a). This was mirrored in the PGC1 α RNA levels that were measured by qPCR from the RNA extracted from cell lysates (Figure 5.13b).

To then determine the longevity of expression of the transfected PGC1 α mRNA, a time course experiment was conducted where cells were lysed at set intervals, the protein was extracted and blotted for comparison (Figure 5.13c). Evidently, RNA NG-treated cells not only presented significantly improved expression for all time points in comparison to the untreated cells, they also demonstrated a sustained protein expression until 72 h post-transfection in comparison to the PGC1 α _jetM-treated cells, that exhibited reduced PGC1 α overexpression by 48 h (Figure ??a). Further, the NG-treated cells demonstrated increased protein expression relative to PGC1 α _jetM-treated cells at all time points except for the 6 h time point, which showcases the controlled release of the mRNA from the NGs, due to their gradual degradation. A significant release of the entrapped PGC1 α RNA was also observed over 24 h, followed by an eventual decrease, presumably related to the degradation of the RNA in the C4-2 cells (Figure 5.13d). Thus, RNA release predominantly took place within the 12-24 h-time frame, with higher transfection efficiency relative to the commercial transfection reagent 12 h post-transfection (Figure ??b). These results are promising when considering that other RNA-delivery platforms demonstrate maximal expression within 6-12 h post-transfection, with a gradual decrease in RNA and corresponding decrease in protein expression at later time points.[337, 455, 457]

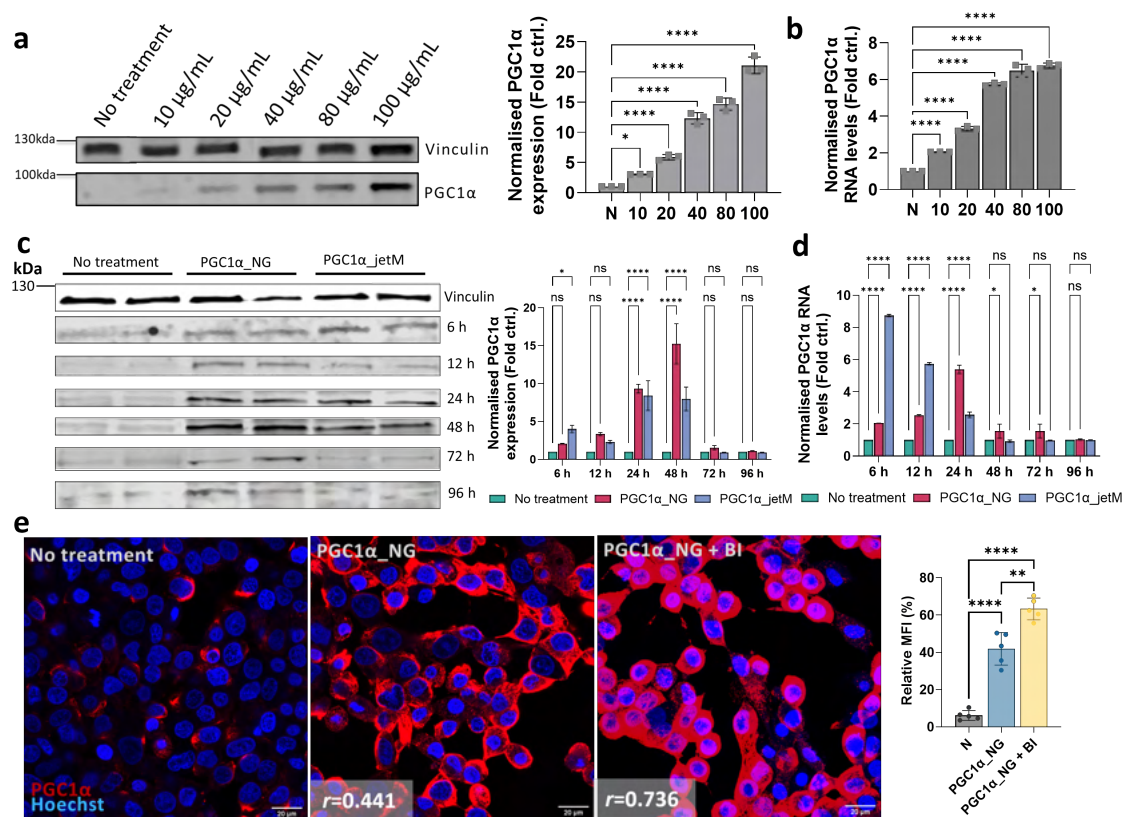


Figure 5.13: **Evaluating transfection efficiency of untargeted PGC1 α RNA-loaded NGs (PGC1 α _NG) by monitoring overexpression in C4-2 cells.** a) C4-2 cells were treated with increasing concentrations of untargeted PGC1 α _NG for 24 h, lysed and blotted for PGC1 α expression, as detected by anti-PGC1 α mAb (NBP1-04676). Quantification of the normalised protein expression is also shown on the right ($n = 3$). b) Comparison of PGC1 α mRNA levels following the administration of PGC1 α _NG, as quantified by RT-qPCR. Absolute expression was determined by the standard curve method by interpolating unknown C_t values against known concentrations of PGC1 α cDNA. RNA levels normalised to GAPDH expression and shown as fold change relative to untreated cells. c) Comparison of PGC1 α expression over different time points, as determined by western blotting ($n = 3$). d) Comparison of PGC1 α mRNA levels at various time points after treatment with PGC1 α _NG, as quantified by RT-qPCR ($n = 3$). e) Maximum projection immunofluorescence images of C4-2 cells either left untreated or treated with PGC1 α _NG and/or PGC1 α _NG + BI9774 for 48 h, fixed and stained with 1 µg/mL Hoechst 33342. Cells were treated with rabbit anti-PGC1 α mAb (NBP1-04676, 1:100) and then treated with Goat anti-Rabbit IgG (H+L) Alexa FluorTM 568 secondary antibody (1:500). Quantification of the red fluorescence (MFI) is shown on the left for all conditions ($n = 5$). All images were taken with 40 x oil immersion objective. Scale bar at 20 µm. Colocalisation analysis conducted using JACoP revealed Pearson's correlation coefficient values as labelled on the relevant images. In all cases, data are presented as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test, where RNA/protein levels of treated cells were compared against those of untreated cells: **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.0011$, * $p < 0.0322$, ns < 0.1234 .

Further, immunofluorescence was used to confirm the overexpression and nuclear localisation of PGC1 α , as the transcriptional activity of PGC1 α requires its nuclear localisation (Figure 5.13e).[425, 458, 459] While PGC1 α overexpression was observed in NG treated cells relative to the untreated cells, nuclear localisation remained low, as is evident in the low value of the Pearson's correlation coefficient (0.441). However, upon treatment with a small molecule AMPK activator, BI9774 [88], we observed considerable nuclear localisation, as indicated by the increase in Pearson's coefficient value (0.736). Indeed, AMPK activators have been shown to enhance the transcriptional activation of PGC1 α . [88]

While investigating a combined NG and small molecule therapeutic approach may be promising, the inherent complexity of determining dosing regimes, given the need for different delivery methods for both agents, could extend the development time for this strategy beyond the scope of this work. As such, a way of enhancing the transcriptional activity of PGC1 α without the need for BI9774, was needed. In humans, PGC1 α is encoded by the *PPARGC1A* gene, and this gene undergoes extensive alternative splicing.[127] Splicing between exons 6 and 7 of the *PPARGC1A* gene generates a transcript encoding a truncated, N-terminal isoform of PGC1 α (NT-PGC1 α), that comprises of 270 AAs (Figure 5.12b).[127, 460] NT-PGC1 α presents a persistent transcriptional coactivator comprising of the transcription activator and nuclear receptor interaction domains of full length PGC1 α . However, it is no longer subject to phosphorylation-mediated turnover, thereby resulting in the generation of a PGC1 α variant that has constitutive transcriptional activity with an extended protein half-life.[127, 460] Therefore, the transcript for the spliced version of PGC1 α was synthesised and entrapped within the NGs (NT-PGC1 α _NG). This study is, to our knowledge, the first to attempt the therapeutic delivery of truncated PGC1 α encapsulated in NGs.

As before the untargeted NGs mediated improved NT-PGC1 α expression when compared to the commercial reagents, as evident in the western blot and RT-qPCR analyses (Figure 5.14a,b). This transfection efficiency was mirrored in two other PCa cell lines tested, including PC3 and Du145, where NT-PGC1 α _NGs

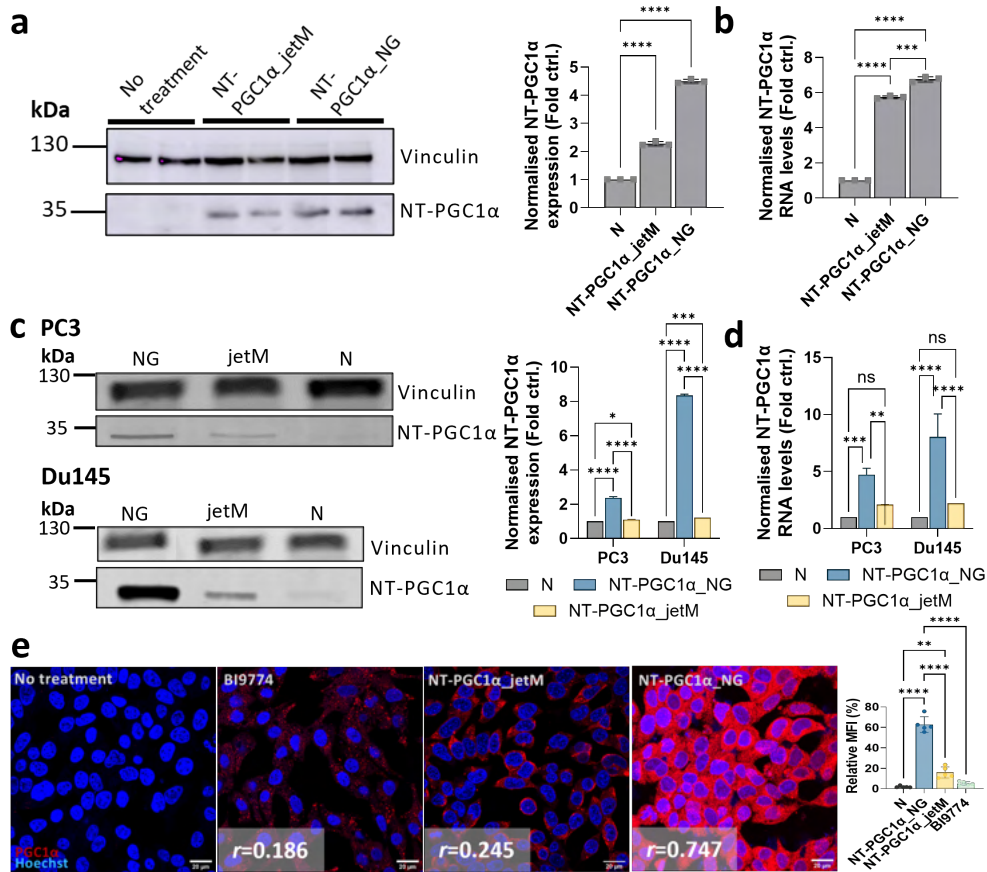


Figure 5.14: Investigating effect of NT-PGC1 α overexpression in C4-2 cells. a) C4-2 cells were treated with increasing concentrations of untargeted NT-PGC1 α _NG, lysed and blotted for NT-PGC1 α expression, as detected by anti-NT-PGC1 α mAb (sc-5816). Quantification of the normalised protein expression is also shown on the right ($n = 3$). b) Comparison of NT-PGC1 α mRNA levels following untargeted NT-PGC1 α _NG administration, as quantified by RT-qPCR. RNA levels normalised to GAPDH expression and shown as fold change relative to untreated cells. c) Comparison of NT-PGC1 α expression in different PCa cell lines, as determined by western blotting and d) RT-qPCR ($n = 3$) Note: expression observed in PSMA- cells as untargeted NGs were used to conserve the PSMA peptide for *in vivo* experiments. e) Maximum projection immunofluorescence images of C4-2 cells either left untreated or treated with BI9774, NT-PGC1 α _NG and NT-PGC1 α _NG + BI9774 for 48 h, fixed and stained with 1 μ g/mL Hoechst 33342. Cells were treated with rabbit anti-NT-PGC1 α mAb (sc-5816, 1:100) and then treated with Goat anti-Rabbit IgG (H+L) Alexa FluorTM 568 secondary antibody (1:500). Quantification of the red fluorescence (MFI) is shown on the left for all conditions ($n = 5$). All images were taken with 40 x oil immersion objective. Scale bar at 20 μ m. Colocalisation analysis conducted using JACoP revealed Pearson's correlation coefficient values as labelled on the relevant images. In all cases, data are presented as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test, where RNA/protein levels of treated cells were compared against those of untreated cells: **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.0011$, * $p < 0.0322$, ns < 0.1234 .

demonstrated increased protein and mRNA levels ([Figure 5.14c,d](#)). In addition, while BI9774 treatment led to a modest but significant increase in NT-PGC1 α expression relative to untreated cells, delivery via NT-PGC1 α _NG resulted in a substantially greater upregulation of the protein ([Figure 5.15a](#)). Furthermore, immunofluorescence was used to confirm the overexpression and nuclear localisation NT-PGC1 α as before ([Figure 5.14e](#)). Increased NT-PGC1 α expression was observed in NG-treated cells relative to all other conditions. More notable is the observation that NT-PGC1 α _NG treated cells showed greater nuclear localisation (0.747), relative to the other conditions.

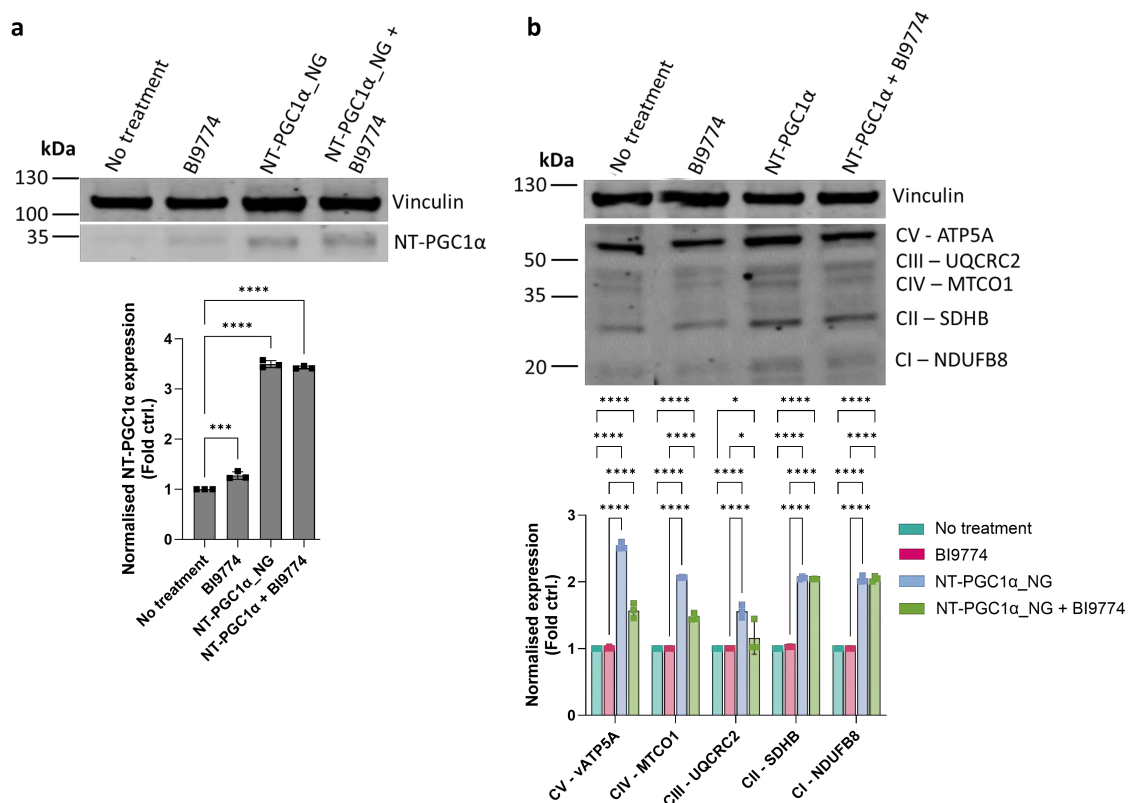


Figure 5.15: **Effect of NT-PGC1α overexpression on mitochondrial function in C4-2 cells.** a) Western blot confirming the overexpression of the NT-PGC1α. The graph below displays the quantification of the normalised band intensities and their comparison to the untreated cells ($n = 3$). Vinculin was used as a loading control. b) Western blot analysis of mitochondrial electron transport chain proteins and quantification of normalised protein expression in C4-2 cells treated with NT-PGC1α_NG and/or BI9774 and lysed after 48 h ($n = 3$). Quantification of the normalised protein expression is shown for each protein type and the treated cells compared against untreated cells. In all cases, data are presented as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test, where RNA levels of treated cells were compared against those of untreated cells: **** $p < 0.0001$, *** $p < 0.0002$, ns $p > 0.1234$.

Having confirmed that both transcripts can be successfully overexpressed using the NGs in C4-2 cells, the downstream effects of PGC1α and NT-PGC1α were compared to ensure that the therapeutic efficacy of the payload was not compromised. As PGC1α is the master regulator of mitochondrial biogenesis, the impact of its overexpression on mitochondrial abundance and activity was assessed. First, upregulation of downstream mitochondrial electron transport chain (ETC) proteins in C4-2 cells treated with untargeted PGC1α_NG or NT-PGC1α_NG was confirmed (Figure

5.16a,b).[425] Evidently, NG treatment resulted in increased ETC protein levels relative to untreated and jetM-treated cells. Although some ETC proteins seem to demonstrate increased expression in jetM- treated cells relative to the NG-treated cells, it is important to note that selective post-transcriptional regulation may prevent increases in certain ETC proteins, particularly if their expression depends on additional coactivators or specific cellular conditions. More interestingly, the increase in ETC protein expression was more pronounced in cells treated with NT-PGC1 α _NG compared to those treated with BI9774, indicating that direct delivery of NT-PGC1 α induces a stronger mitochondrial response (Figure 5.15b). These findings suggest that NT-PGC1 α delivery alone can recapitulate the combined efficacy of BI9774 and full-length PGC1 α , to potentially provide an enhanced therapeutic benefit.

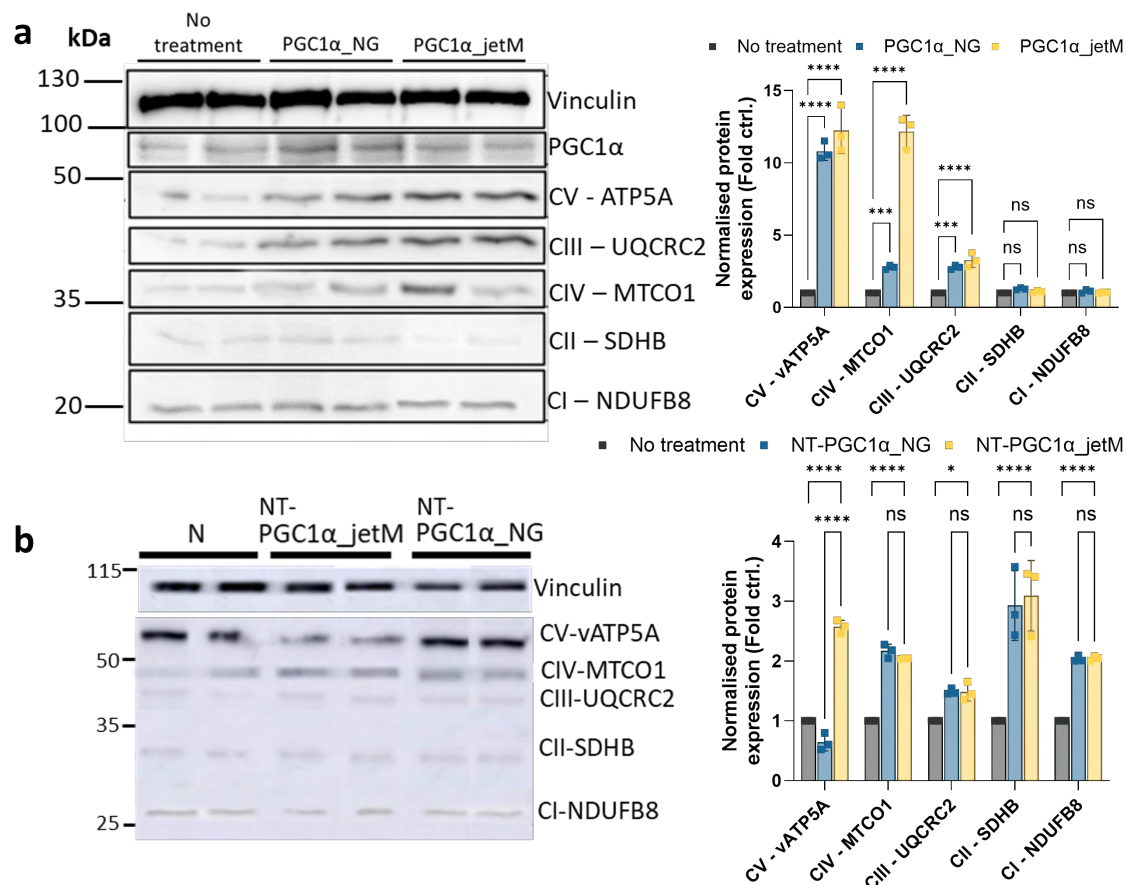


Figure 5.16: Investigating effect of NT-PGC1 α and PGC1 α overexpression on mitochondrial electron transport chain proteins in C4-2 cells. a) Representative western blot analysis of mitochondrial electron transport chain proteins and quantification of normalised protein expression in C4-2 cells treated every 24h with untargeted a) PGC1 α _NG and b) NT-PGC1 α _NG as well as other conditions and lysed after 48h (n = 3). Quantification of the normalised protein expression is shown for each protein type and the treated cells compared against untreated cells on the left. Data is shown as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test, where protein levels of treated cells were compared against those of untreated cells: **** p < 0.0001, *** p < 0.0002, ** p < 0.0011, * p < 0.0322, ns < 0.1234.

To further assess the effects of PGC1 α and NT-PGC1 α on mitochondrial abundance and activity, C4-2 cells were treated with the respective RNA constructs delivered either via untargeted NGs or JetMESSENGER (jetM). Cells were stained with MitoTracker™ Green (MTG), which labels mitochondria regardless of membrane potential and serves as a marker for mitochondrial abundance, and with tetram-

ethylrhodamine methyl ester (TMRM), a potential-sensitive dye that accumulates in active mitochondria to reflect mitochondrial membrane.[461]

Flow cytometry was used to generate bivariate dot plots of TMRM versus MTG staining intensity (Figure 5.17a,e). These plots revealed distinct shifts in the populations of cells treated with PGC1 α _NG or NT-PGC1 α _NG toward higher intensities of both dyes, suggesting increases in both mitochondrial content and activity compared to untreated cells, scrambledRNA_NG controls, and jetM-delivered groups. Quantitative analysis of the mean fluorescence intensity (MFI) for MTG (Figure 5.17b,f) confirmed a significant increase in mitochondrial abundance in NG-treated cells compared to controls. Similarly, TMRM MFI values (Figure 5.17c,g) indicated enhanced mitochondrial membrane potential, with the NT-PGC1 α _NG group showing the highest signal among all conditions. To integrate both abundance and functional capacity, the ratio of MTG to TMRM MFI was calculated (Figure 5.17d,h). This ratio further confirmed that NG-mediated delivery of both PGC1 α and NT-PGC1 α resulted in the most pronounced enhancement in mitochondrial biogenesis and activity. Most notably, it is evident that NT-PGC1 α delivery alone is sufficient to induce an increase in mitochondrial activity. Seahorse experiments were conducted to measure oxygen consumption rate (OCR) as a measure of mitochondrial function in NG-treated cells (Figure A12a). NT-PGC1 α _NG treatment markedly enhanced mitochondrial respiration, with elevated basal and maximal respiration, as well as spare respiratory capacity, thereby indicating greater mitochondrial fitness compared to other control groups. Extracellular acidification rate (ECAR) profiles were largely unchanged across conditions, though a modest reduction in glycolysis was noted in NT-PGC1 α _NG-treated cells, consistent with a metabolic shift towards oxidative phosphorylation. These results indicate that NG-mediated NT-PGC1 α expression enhanced mitochondrial oxidative capacity without promoting compensatory glycolytic activity. Accordingly, subsequent studies focus on investigating the potential therapeutic benefits of the NT-PGC1 α transcript encapsulated within the NGs.

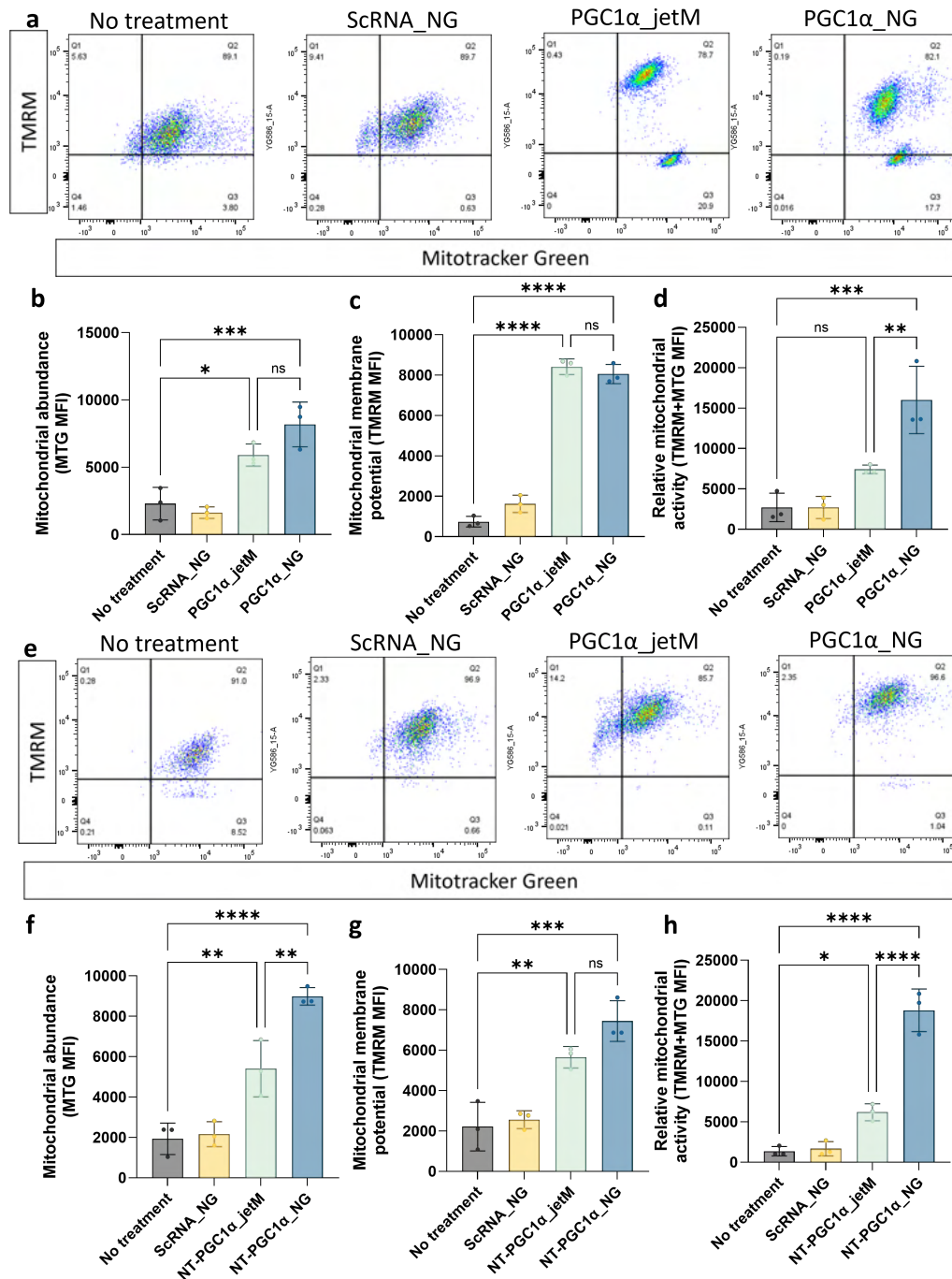


Figure 5.17: Investigating effect of NT-PGC1 α and PGC1 α overexpression on mitochondrial function. a, e) Representative bivariate flow cytometry plots of TMRM versus MTG staining following treatment with (a) PGC1 α or (e) NT-PGC1 α RNA. Conditions include untreated cells, NG- or jetMESSENGER-mediated delivery of PGC1 α or NT-PGC1 α (PGC1 α _NG, NT-PGC1 α _NG, PGC1 α _jetM, NT-PGC1 α _jetM), and a NG-encapsulated scrambled RNA control (scramble-dRNA_NG). (b, f) Bar graphs quantify the mean fluorescence intensity (MFI) of MTG; (c, g) MFI of TMRM; and (d, h) the MTG/TMRM ratio, providing a comparative measure of mitochondrial abundance, membrane potential, and relative efficiency (n = 3). Data is shown as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test: **** p < 0.0001, *** p < 0.0002, ** p < 0.0011, * p < 0.0322, ns < 0.1234.

Previously, Torrano *et al.*, examined the role of PGC1 α in suppressing PCa metastasis.[124] In this work, gene expression profiling and metabolomic analyses showed that PGC1 α significantly modulates both gene expression and metabolite levels, particularly those associated with mitochondrial catabolic pathways and oxidative processes, such as fatty acid β -oxidation. Collectively, these findings suggested that in PCa, PGC1 α functions as a key metabolic regulator, tipping the balance between a catabolic, tumour-suppressive state (characterised by high PGC1 α expression) and an anabolic, tumour-promoting state (associated with low PGC1 α expression). Inspired by this study, the effect of NT-PGC1 α overexpression on C4-2 cell proliferation was examined. Evidently, treatment with NT-PGC1 α _NGs decreased cell proliferation considerably, whereas treatment with the AMPK activator had negligible effects over the same time period (Figure 5.18a).

To gain an improved mechanistic understanding of the transcriptome influenced by NT-PGC1 α _NG treatment, C4-2b cells were treated with the loaded and empty NGs every 24 h for three days and subjected to RNA sequencing analysis. Although the transcriptional changes observed upon NT-PGC1 α overexpression in the current RNA-seq dataset did not show strong concordance with the gene expression profile reported by Torrano *et al.*, likely reflecting both biological and technical differences between the studies, including the use of RNA-seq versus microarray platforms, the data nonetheless demonstrate that NT-PGC1 α overexpression resulted in widespread transcriptional and biological reprogramming in the PCa cell line investigated. Transcriptomic profiling and gene sequence enrichment analysis (GSEA) reveal a coordinated suppression of proliferative pathways as well as the concurrent activation of potential metabolic stress adaptation mechanisms. Indeed, NT-PGC1 α overexpression downregulated several genes involved in cell cycle regulation, including *CCND1*, *CCNE1/2*, *CCNA1/2*, *CCNB2/3*, *CDCA4/7/8*, and *CDC25A/C*[101, 255, 462–466], as well as mitotic regulators including, *PLK1*, *BUB1B* and *TOP2A*. [467–470] This transcriptional program likely halts both G1/S and G2/M transitions, reinforced by the upregulation of CDKN1A (p21), CDKN1B (p27), and TP53 (Figure 5.18b). [471–473]. To complement these gene-level changes, GSEA identified significant suppression of key processes involved in genomic maintenance, such as

homologous recombination and DNA damage repair (chromosome segregation and mitotic transitions, DNA replication and double-strand break repair, sister chromatid segregation and cilium organisation and glycoprotein biosynthesis) (Figure 5.19a). Suppression of glycosylation pathways could potentially compromise protein folding and receptor maturation, further limiting the proliferative capacity of the treated cancer cells. Given these results, it would be possible to hypothesise that NT-PGC1 α overexpression can induce a cell cycle arrest phenotype while predisposing cells to a senescent or apoptotic state due to genomic instability.

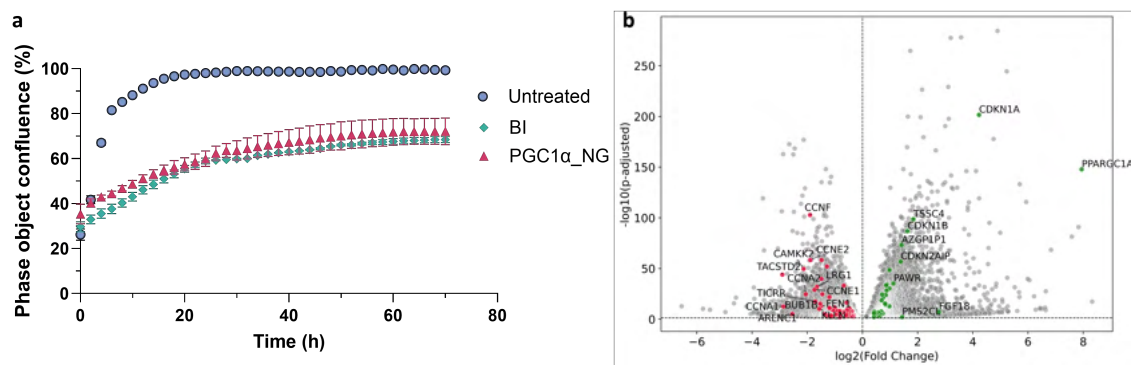


Figure 5.18: **Transcriptional changes following NT-PGC1 α overexpression in a PCa cell line (C4-2b).** a) Proliferation of C4-2 cells left untreated or treated with BI9774 or NT-PGC1 α _NGs, monitored using IncuCyte over a period of three days ($n = 6$). b) Volcano plot summarising the effect of NT-PGC1 α _NG treatment and NT-PGC1 α overexpression in C4-2b cells ($\log_2$ fold change < 2 , adjusted $p < 0.05$).

Notably, multiple tumour suppressor genes were upregulated in the NT-PGC1 α _NG-treated cells, including *TASC1*, *TSC2*, *SMAD4*, *LATS1*, and *APC*. These genes are crucial negative regulators of pathways frequently hyperactivated in PCa. Their increased expression may suggest a reactivation of tumour suppressive signalling – specifically via the mTOR, TGF- β , and Hippo pathways – that are involved in regulation of cellular proliferation, promoting differentiation and inducing cell cycle arrest under stress conditions.[474–476] For instance, *TSC1/2* are upstream repressors of mTORC1, whose inhibition leads to reduced anabolic metabolism and protein synthesis, consistent with the observed suppression of growth-promoting processes.[474] Further, the induction of *SMAD4* and *LATS1* suggests that the TGF- β and Hippo pathways, respectively, may be re-engaged to impose growth control and reinforce apoptotic checkpoints. *SMAD4* is a central mediator of canonical

TGF- β signaling, which in the early stages of cancer can function as a potent growth suppressor. Meanwhile, *LATS1* inhibits the activity of YAP/TAZ transcriptional co-activators, which promote proliferation and stemness when active in the nucleus.[475, 476]

Complementing this tumour-suppressive gene expression profile, a panel of pro-apoptotic markers including *BAX*, *CASP8*, *PAWR*, *TRIAP1*, *TP53BP2* were also upregulated.[477–480] These genes are typically activated in response to intracellular stress, DNA damage, or oncogenic deregulation. For instance, *BAX* promotes the destabilisation of the outer mitochondrial membrane, thereby driving apoptosis, while *CASP8* is a key initiator of extrinsic apoptotic signalling.[481, 482] Together their upregulation supports the hypothesis that NT-PGC1 α overexpression drives the convergence of both intrinsic and extrinsic death pathways. Further, many of these pathways are also responsive to metabolic and oxidative stress, linking the transcriptional changes to the metabolic rewiring imposed by NT-PGC1 α . Indeed, *TP53BP2* (ASPP2) can sensitise cells to apoptosis in response to energetic stress and is known to cooperate with p53.[483] Importantly, these changes were in parallel to the suppression of a panel of oncogenic drivers, including *MYC*, *KRAS*, *AKT1*, *AURKA* and *CTNNB1*, as well as the androgen receptor (AR) signalling axis (e.g *AR*, *ARLNC1*, *NKX3-1*). As *MYC* is a master regulator of ribosomal biogenesis, nucleotide synthesis, and glycolysis; its suppression likely contributes to the reduced proliferative and biosynthetic capacity observed in treated cells.[484, 485] *KRAS* and *AKT1* are pivotal mediators of mitogenic and metabolic signaling through the MAPK and PI3K/AKT pathways, respectively – both of which are frequently activated in advanced PCa.[486] Their downregulation would therefore deprive cancer cells of essential prosurvival and growth signals. Similarly, the repression of *AURKA* (Aurora kinase A), which governs centrosome maturation and mitotic entry, may further reinforce the mitotic arrest phenotype seen in these cells.[485] *CTNNB1*, encoding β -catenin, is a central effector of the WNT signaling pathway and plays a dual role in transcriptional regulation and cell adhesion.[487] Its downregulation may limit the expression of WNT-responsive genes involved in stemness, migration, and therapy resistance, while potentially affecting cell–cell adhesion dynamics.

Crucially, NT-PGC1 α also suppresses the androgen receptor (AR) signaling axis, including *AR* itself, as well as associated factors such as *ARLNC1* (an AR-regulated long non-coding RNA) and *NKX3-1* (a prostate-specific tumour suppressor that is paradoxically AR-regulated).[488, 489] Given the central role of AR signalling in PCa pathogenesis and progression, this repression is particularly significant. AR drives the transcription of genes involved in cell cycle progression, metabolism, and survival, and its activity is tightly linked to both the development of PCa and resistance to androgen deprivation therapy.[112] The observed downregulation of AR and its co-factors suggests that NT-PGC1 α may disrupt the androgen-dependent transcriptional program that prostate tumours rely on for sustained growth. Since AR signalling promotes glycolysis, lipogenesis, and mitochondrial biogenesis in PCa cells, its inhibition may help shift the cells away from anabolic metabolism toward a more catabolic, oxidative state – consistent with the known functions of PGC1 α family members in promoting mitochondrial oxidative phosphorylation.[489, 490] Thus, NT-PGC1 α may function as a transcriptional antagonist to AR in this context, reshaping the metabolic landscape and weakening the hormone-dependence of the tumour.

More crucially, the GSEA of upregulated transcripts reveals a remarkable activation of processes associated with cellular homeostasis and metabolic adaptation (ribosome biogenesis and rRNA processing, RNA splicing and transcription elongation, autophagy and mitochondrial autophagy, protein–RNA complex assembly and endosomal transport) (Figure 5.19b). These point towards a reprogramming of proteostasis and organelle quality control, likely in response to metabolic stress induced by the NT-PGC1 α activity. The upregulation of autophagy-related genes suggests activation of cellular survival pathways, enables the clearance of damaged organelles and protein aggregates. Similarly, increased ribosomal and RNA processing capacity may serve to maintain essential transcriptional and translational functions despite the suppression of cell division. Key metabolic regulators such as *PPARGC1A* (endogenous full-length PGC1 α), *ACACB*, *MPC1*, and *GOT1* are also upregulated, consistent with NT-PGC1 α 's role in promoting mitochondrial oxidative metabolism and fatty acid oxidation. This metabolic shift away from glycolysis may

support long-term cellular fitness under nutrient or energy stress, even in a quiescent or pre-apoptotic state.

Overall, the overexpression of NT-PGC1 α in PCa cells leads to a transcriptional landscape characteristic of a tumour-suppressive state. The simultaneous suppression of proliferation, mitotic progression, and DNA repair – combined with the upregulation of autophagy, mitochondrial turnover, and metabolic homeostasis – suggests a reprogramming from a proliferative phenotype to a stress-adapted, survival-oriented state. However, the paradoxical upregulation of biosynthetic and translational pathways in the context of growth arrest implies that NT-PGC1 α may prepare cells for either adaptive survival or regulated cell death, depending on context. This dual capacity warrants further investigation and validation by protein expression analysis of key genes of interest.

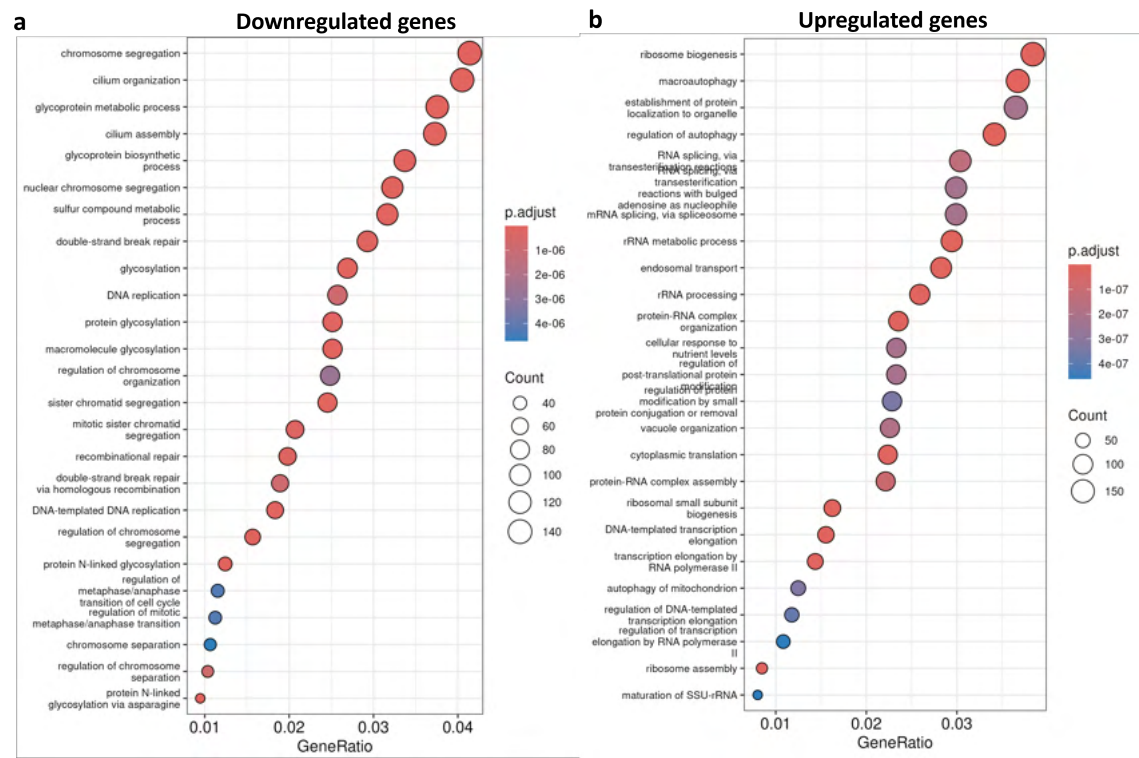


Figure 5.19: RNA sequencing analysis was performed on PCa (C4-2b) cells treated with NT-PGC1 α _NG and empty NGs. Gene set enrichment analysis (GSEA) was performed on genes that were a) downregulated or b) upregulated, and significant hits are shown.

5.2.5 Validating effects of NG-mediated NT-PGC1 α overexpression on PCa progression in a xenograft model of PCa

Driven by the promising results from the *in vitro* and *in vivo* experiments, a xenograft model of PCa was developed, with the intention of assessing the extent to which NT-PGC1 α treatment could inhibit tumour progression (Figure 5.20). A xenograft model was chosen as these provide a physiologically relevant environment to evaluate tumour growth kinetics, drug delivery efficiency and preliminary treatment responses. In this instance, human PCa-derived C4-2b cells, which represent a castration-resistant and metastatic subtype of PCa, were chosen for tumour induction due to their high PSMA expression and relevance to advanced disease. For this experiment, immunocompromised male mice were subcutaneously injected with C4-2b cells to establish the tumours.

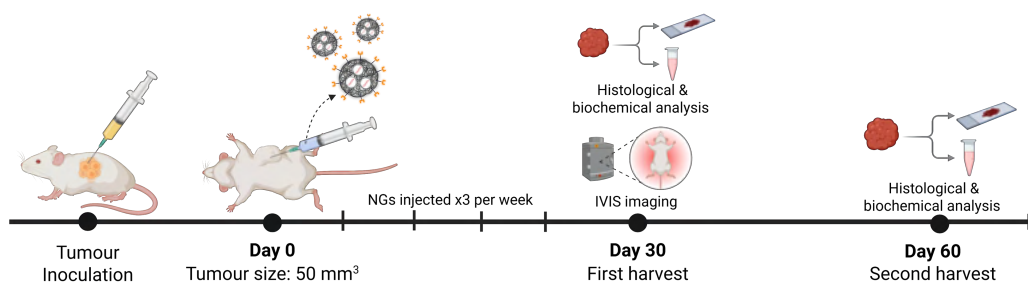


Figure 5.20: **Schematic presentation of timeline for PCa xenograft generation, NG treatment regime and downstream analysis using harvested animal tissue.**

Once the tumours reached a predetermined size (50 mm³), the mice were treated with the following conditions:

1. Untreated: to assess baseline tumour progression
2. Untargeted NT-PGC1 α _NG (Ut-NT-PGC1 α _NG): to assess the therapeutic efficacy offered by targeting PSMA
3. mCardinal-loaded NGs (mC_NGs): as an NP control to account for delivery vehicle effects.
4. PSMA_NT-PGC1 α _NG: the targeted treatment.

To further investigate the impact of sustained PGC1 α delivery on tumour progression and potential tumour adaptation, an additional group of mice were treated with PSMA_NT-PGC1 α _NG (so called long term, and abbreviated to LT) for an extended period of 60 days. In each case, the polymer dose used was 15 mg/mL (60 mg/kg) and the mRNA dose was 0.25 mg/kg per mouse, administered thrice per week.

As shown in [Figure 5.21a,b,d](#), treatment with NT-PGC1 α _NGs, irrespective of their targeting ability, reduced tumour growth significantly in comparison to the untreated or the vehicle-control treated xenografts. Evidently, targeting the NGs provided a considerable therapeutic benefit as the tumour progression was remarkably slower. By day 32, where the untreated and vehicle-treated tumours had reached volumes of 641 mm³ and 667 mm³, respectively, those in the targeted NGs group measured only 171 mm³, showing nearly a fourfold reduction. Notably, even after 60 days of treatment, the tumours in the long-term targeted-NG treatment group had only grown to 450 mm³. To further confirm that NT-PGC1 α was indeed being expressed in the tumours, the biodistribution of this protein was determined by measuring expression levels across key organs including the heart, lungs, liver, spleen, kidneys, prostate and tumour tissue, by western blotting and RT-qPCR analysis ([Figure 5.22a,b](#)). NT-PGC1 α transcripts were primarily detected in the liver, spleen, kidneys, tumours and in the case of the targeted NG-treated animals, in the prostate at significantly increased levels compared to the untreated and vehicle-treated controls. This was consistent with PSMA-mediated delivery of the RNA payload, as observed previously.

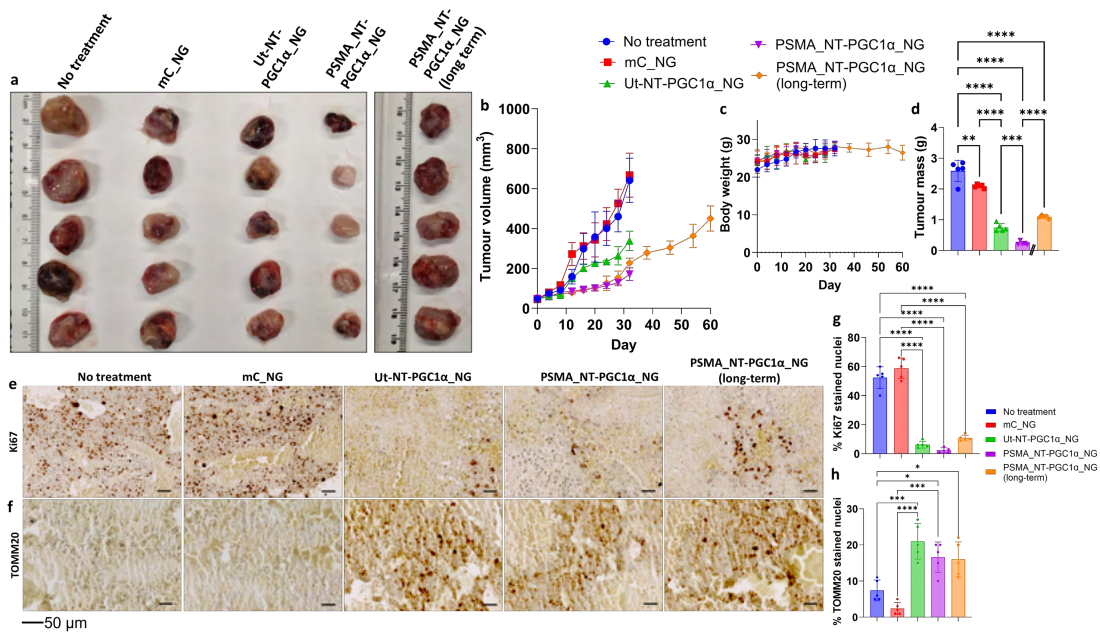


Figure 5.21: NT-PGC1 α overexpression shows enhanced therapeutic efficacy in subcutaneous PCa tumour. a) Images of dissected tumours from conditions including untreated, mC_NG, Ut_NT-PGC1 α _NG, PSMA_NT-PGC1 α _NG after day 30 and PSMA_NT-PGC1 α _NG (LT) after day 60. NGs were injected thrice weekly subcutaneously ($n = 5$). b) Tumour growth curves and c) changes in body weight of the treated mice. d) Bar graph comparing mass of tumours dissected on day 30 and day 60 for different conditions. e,f) Representative images of Ki-67 and TOMM20-stained sections taken from xenografts isolated from mice with various treatments highlighted above are shown, respectively. Scale bar at 50 μ m. g,h) Quantification of the percentage of Ki-67- and TOMM20-positive cells in stained sections. Data is shown as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test: **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.0011$, * $p < 0.0322$, ns < 0.1234 .

To further assess the biological impact of NT-PGC1 α expression in the tumour tissues, histological analysis was performed on tumour sections stained for Ki-67, a proliferation markers and TOMM20, a mitochondrial outer membrane protein indicative of mitochondrial mass (Figure 5.21e,f). Tumours from NT-PGC1 α -treated mice exhibited a striking 85% reduction in Ki-67-positive nuclei, reflecting a strong anti-proliferative effect (Figure 5.21e,g). In parallel, TOMM20 staining intensity increased by approximately 300%, indicating a substantial improvement in mitochondrial content, likely driven by NT-PGC1 α activity (Figure 5.21f,h).

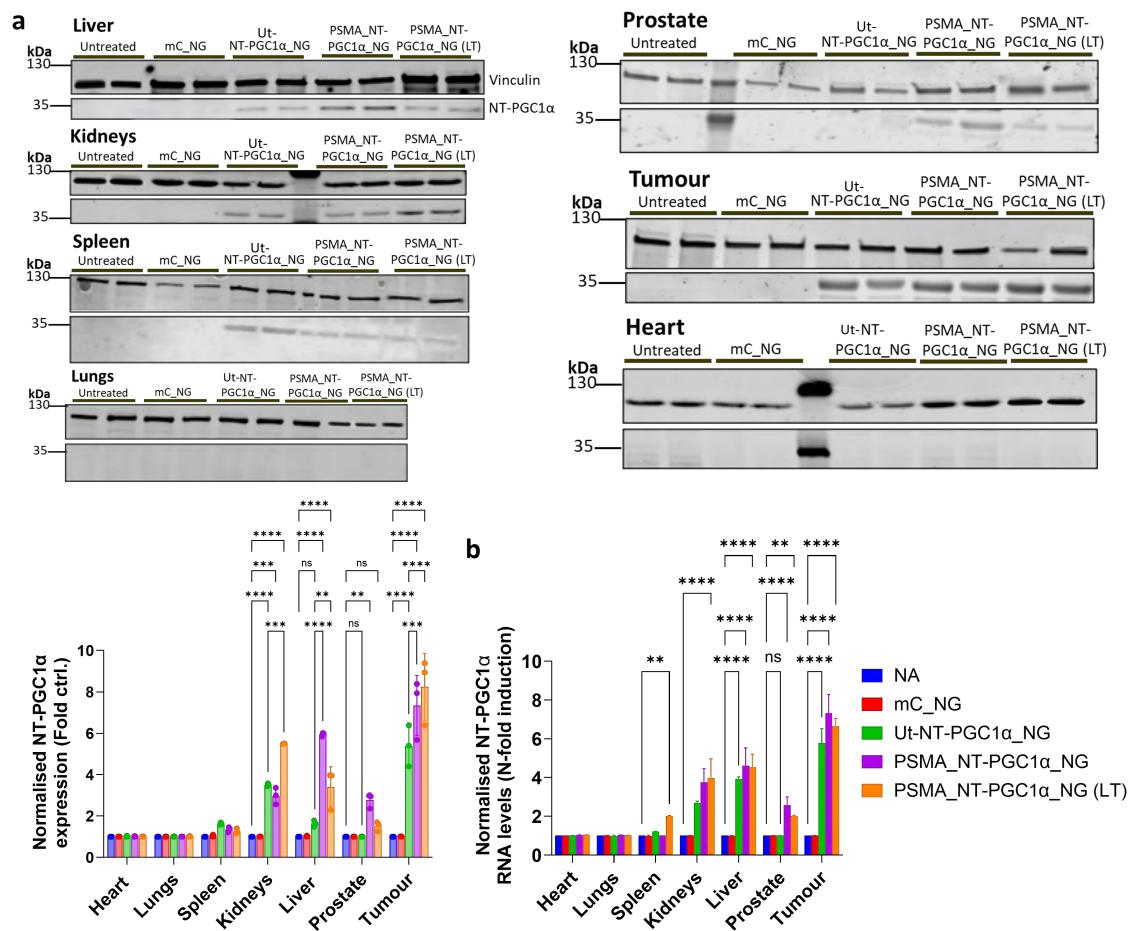


Figure 5.22: Tracking biodistribution of NT-PGC1α expression in xenograft-bearing immunocompromised (NSG) mice. a) Western blot showing NT-PGC1α expression profiles in all main organs and tumours for each condition tested including untreated, mC_NG, NT-PGC1α_NG, PSMA_NT-PGC1α_NG and the long-term targeted NG treatment. The graph below displays the quantification of the normalised band intensities and their comparison to the untreated cells ($n = 3$). Vinculin was used as a loading control. b) Comparison of NT-PGC1α mRNA levels in all main organs and tumours, as quantified by RT-qPCR. RNA levels normalised to GAPDH expression and shown as fold change relative to untreated cells. In all cases, data are presented as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test, where RNA levels of treated cells were compared against those of untreated cells: **** $p < 0.0001$, *** $p < 0.0002$, ns < 0.1234 .

To visually confirm RNA expression and PSMA-mediated targeting efficiency, xenograft-bearing mice were injected with mCardinal RNA-loaded NGs, as the mCardinal protein would act as a fluorescent reporter. Strong mCardinal signal was observed in the tumours and in the prostate, consistent with PSMA expression in

both tissues, as seen in the *ex vivo* imaging (Figure A13a,b). This was corroborated by western blotting and RT-qPCR analysis, which also confirmed residual expression in the liver, spleen and kidneys (Figure A13c,d,e). This indicated that while the targeting was efficient, there was a degree of off-target uptake in clearance-associated organs.

Based on prominent transcriptional changes observed in the RNA-seq dataset, selected targets were further examined at the protein level. Notably, *CAMKK2*, which was strongly downregulated, and cleaved caspase-8 (a marker of apoptosis) were investigated by western blot analysis to confirm and extend the transcriptomic observations (Figure 5.18b, Figure 5.23). *CAMKK2* is a key metabolic regulator, an established AR target and known AMPK activator.[89, 490, 491] Paradoxically, in PCa, it is a known tumour promoter and supports anabolic processes such as mitochondrial biogenesis and lipogenesis in PCa cells.[88, 89] Western blot analysis showed a pronounced loss of *CAMKK2* protein expression post-treatment (Figure 5.23a,b), thereby suggesting a reduced metabolic drive in the tumour cells. Given *CAMKK2*'s role in integrating androgen signalling with energy metabolism, its repression by NT-PGC1 α also provides a direct mechanistic link between metabolic rewiring and growth suppression in this model. Furthermore, cleaved caspase-8 levels were elevated in NT-PGC1 α -treated tumours, suggesting that the treated cells may be committing to apoptotic pathways (Figure 5.23b,c).[492] This protein-level evidence complements the upregulation of pro-apoptotic transcripts such as *BAX*, *CASP8*, and *TP53BP2*. Taken together, these results support the hypothesis that NT-PGC1 α drives an anti-proliferative and pro-apoptotic phenotype.

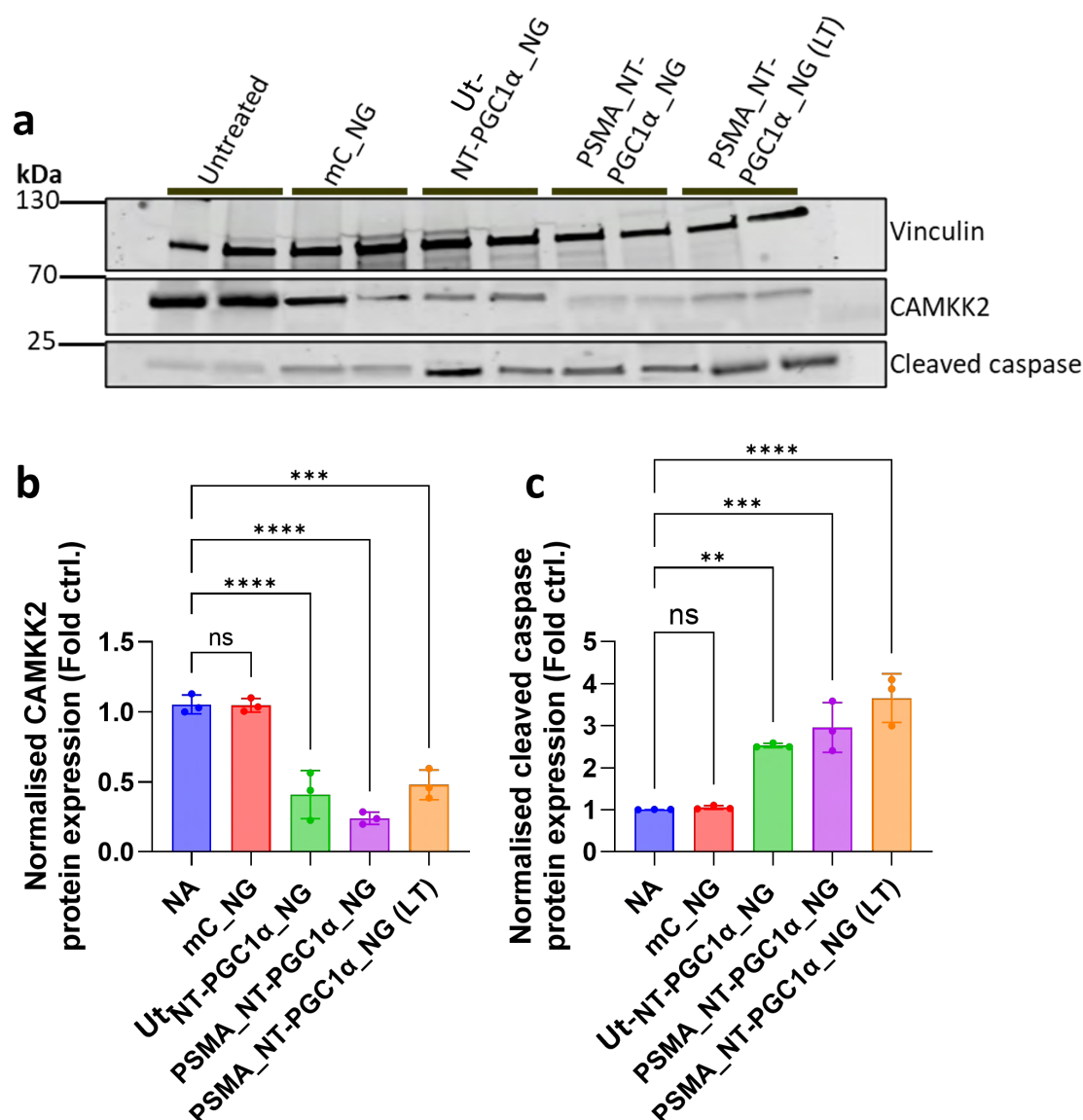


Figure 5.23: **Validation of transcriptomic hits at the protein level.** a) Western blot analysis of prostate cancer cells treated with NT-PGC1 α RNA-loaded NGs shows a marked reduction in CAMKK2 (12818) protein levels, consistent with its strong downregulation at the transcript level in RNA-seq data. b) Cleaved caspase-8 (18C8) levels are elevated, supporting activation of apoptotic pathways following treatment. Vinculin was used as a loading control. In all cases, data are presented as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test, where RNA levels of treated cells were compared against those of untreated cells: **** $p < 0.0001$, *** $p < 0.0002$, ns < 0.1234 .

Finally, to ascertain whether the animals demonstrated any NG-related systemic toxicity, varying concentrations of the NGs were incubated with red blood cells for 2 h to 24 h (left panel). At shorter incubation periods, haemolysis remained

minimal across all NG concentrations, with only $3.5 \pm 0.1\%$ haemolysis observed at the highest concentration after 2 h. However, a modest increase was detected following 24 h exposure, reaching $19.3 \pm 0.2\%$ haemolysis at the highest NG concentration. These findings indicate that while prolonged static incubation can lead to increased membrane disruption, this likely overestimates *in vivo* toxicity, as NGs in circulation are under continuous flow and thus unlikely to interact with erythrocytes for extended periods. Furthermore, the concentrations tested here were substantially higher than those used therapeutically *in vivo*. Therefore, these results suggest that clinically relevant NG doses are unlikely to cause significant haemolytic toxicity (Figure 5.24a). Furthermore, serum levels of hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured following treatment (Figure 5.24b). [493] While ALT levels remained comparably low across all groups, a modest elevation in AST was observed in the cohort treated with the PSMA_NT-PGC1 α _NGs. Although this doesn't reach the levels associated with acute hepatotoxicity (> 1000 U/L), it could reflect mild hepatic stress in response to NT-PGC1 α overexpression. Given the role of PGC1 α in mitochondrial biogenesis and oxidative metabolism, it is plausible that elevated systemic expression could transiently impact liver mitochondrial dynamics, leading to slight perturbations in transaminase activity. More importantly, no overt signs of liver damage or behavioural toxicity were observed in treated animals. Nevertheless, these findings suggest that liver enzyme monitoring may be warranted in future translational applications involving systemic PGC1 α -based therapies.

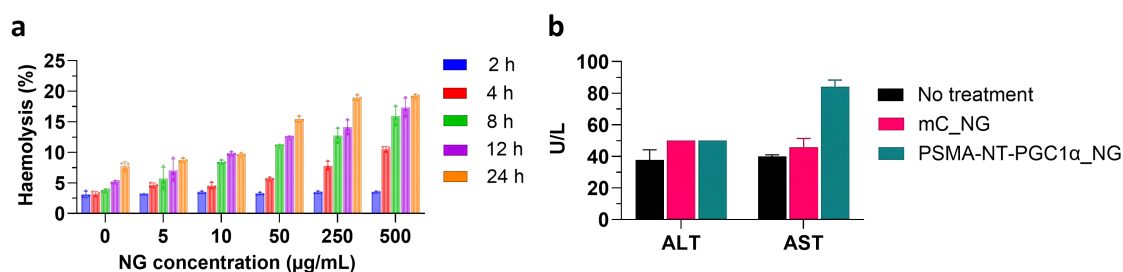


Figure 5.24: ***In vivo* cytotoxicity assessment** a) The haemolysis results of red blood cells incubated with NPs ($n = 3$). b) Quantification of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in mouse serum is shown. Data is shown as mean $\pm$ SD.

5.3 Conclusions & Limitations

Herein the development and validation of PSMA-targeted NGs was presented for the selective delivery of mRNA therapeutics to PCa cells. Initially, the rational design and characterisation of the NG system was presented. While the NGs were largely homogeneous in their physicochemical properties and their *in vitro* effects, it is worth noticing that the peptide conjugation is likely to be heterogeneous owing to the inability to ensure consistency in the degree of maleimide incorporation within each individual NG during synthesis. To address this limitation, size exclusion chromatography could be employed to isolate a more uniform population of conjugated NGs prior to downstream bioassays. This approach would also yield a precise estimate of the bioconjugate's molecular weight, which is essential for determining pharmaceutically relevant dosing concentrations. Alternatively, modifying targeted NG synthesis process by incorporating antibody conjugation *prior* to polymerisation may help reduce batch-to-batch variability. Post-synthesis attachment of targeting ligands often demands large quantities of reagents to achieve efficient conjugation and necessitates additional purification steps to eliminate unbound components. In contrast, using pre-functionalised antibody-conjugated copolymers would enable more controlled and reproducible fabrication of targeted NPs, as the stoichiometry of each component can be finely tuned during synthesis.

With regards to the conjugation method used, while thiol-maleimide coupling provided a high reaction success rate, the potential effects of thiol-disulfide exchange between the exposed thiol on PSMA-pep and the disulfide crosslinks within the NG structure were not considered. While this is largely an equilibrium-based process and it is likely that the disulfide crosslinks are deeply enmeshed within the NG structure, the surface exposed disulfide linkages could be susceptible to this reaction. This could explain the increase in NG size observed due to unravelling of the outer disulfide matrix at higher concentrations of the peptide.

While a robust examination of the targeted NG uptake dynamics was conducted using the 3D PCa spheroids, it is important to note that these systems cannot

mimic the effects of blood flow, complex stroma and vascularisation. Further, while the NP uptake was visually assessed using basic microscopy techniques, a far more advanced analysis could have been conducted using light-sheet microscopy to screen the penetration efficiency of the NGs. For instance, work by Niora *et al.*, uses confocal imaging of nuclear-stained spheroids, coupled with radial intensity profiling at half-depth intensity decline ($d_{1/2}$), to quantitatively understand NP distribution.[447] Moreover, defining a penetration efficiency ratio (δ) based on fluorescence intensity at shallow (0–20 μm) versus deeper (60–80 μm) spheroid regions allows for direct comparisons of delivery performance across formulations. These methodologies would be particularly valuable for evaluating the intratumoural diffusion and retention of PSMA-targeted NT-PGC1 α _NGs in a controlled, reproducible setting.

While the targeted NGs demonstrated a clear uptake in the desired tissues, their off-target uptake in clearance organs presents a significant area for future optimisation. Given the significance of NG physicochemical characteristics in dictating uptake by specific organs, it would be interesting to investigate the influence of NG size and charge and peptide surface coverage on the biodistribution. Alternatively, coupling an anti-PSMA nanobody to the NG surface would be a promising, highly precise targeting approach that could be explored further.[294]. Furthermore, while intratumoural injection could have improved tumour localisation of the NT-PGC1 α -loaded NGs, systemic administration was favoured due to the clinical relevance of this route, particularly for therapies requiring repeated dosing, where local administration is less practical and more invasive.

Functional studies using PGC1 α and its N-terminal isoform, NT-PGC1 α , revealed potent anti-proliferative effects *in vitro*, with transcriptomic analysis highlighting cell cycle arrest, metabolic reprogramming, and induction of apoptotic and tumour suppressor pathways. These findings were supported by protein-level validation of key targets in tumour lysates, including cleaved caspase-8 and CAMKK2. However, the precise mechanism by which NT-PGC1 α overexpression leads to amelioration of PCa progression has not been elucidated in this work and should be explored further to exemplify the therapeutic value of this agent.

Although systemic administration of NT-PGC1 α -loaded NGs in a xenograft model led to a measurable reduction in tumour burden, with minimal systemic toxicity, the tumours were observed to increase in size more rapidly 30 days after treatment, even after continued administration of the targeted NT-PGC1 α -loaded NGs. It is possible that after the tumours reach a certain size, they are no longer as susceptible to the subtle effects of metabolic rewiring offered by NT-PGC1 α overexpression. Exploring the effect of increasing NG dose or the therapeutic benefit achieved using combinatorial approaches (e.g. BI9774 or enzalutamide) could be a key area for future work.[88] Furthermore, it is likely that increasing NG dose could reduce the frequency of the dosing regime, however, this was not possible within the scope of this work due to the limitations imposed by mRNA availability. Finally, although no evident organ toxicity was observed over the two-month treatment period, further studies are needed to investigate the metabolic fate of the NGs and their potential immunological effects.

While the current study was conducted in immunocompromised male mice to allow for human xenograft tumour growth, this model inherently limits the evaluation of immune-related mechanisms that could influence therapeutic outcomes. Given the known roles of PGC1 α in cellular metabolism and inflammation, it would be particularly valuable to investigate how systemic immunity—especially interactions with dendritic cells, T cells, and the TME—modulates or enhances the efficacy of PGC1 α -based nanotherapeutics. Future studies in immunocompetent or humanised mouse models could offer critical insight into the immunomodulatory effects of the therapy and its potential for long-term tumour control or recurrence prevention.

Chapter 6

Conclusions and Outlook

This work presents the systematic development, optimisation and functional validation of a modular NG platform for targeted RNA delivery, culminating in a PSMA-targeted NG capable of selectively delivering therapeutic mRNA to PCa cells. Initial efforts focused on optimising the NG composition parameters—including monomer ratios, reaction stoichiometry, and RNA:polymer charge ratios, ultimately yielding AETC_NG that demonstrated efficient RNA encapsulation, superior cellular uptake and biocompatibility compared to commercial lipid-based and polymeric systems. While encapsulation efficiencies remained suboptimal for larger or structurally complex RNA transcripts, the NG reliably maintained RNA functionality post-encapsulation. While this reduced encapsulation is likely attributable to the relatively high molecular weight of the polymer network and steric hindrance within the NG matrix, further optimisation of the NG architecture is warranted. Specifically, tuning the crosslinking density or increasing the RNA-to-polymer ratio during synthesis may enhance entrapment efficiency. Alternatively, post-synthetic loading strategies that leverage the inherent swelling behaviour of the NGs, an aspect not explored in this study, could provide a promising route to improve payload incorporation without compromising structural integrity.

Beyond loading capacity, the physicochemical properties of the NGs were extensively characterised, revealing high colloidal stability and a favourable size range for cellular uptake. Detailed investigation into the monomer composition, including the comparison of PolyR_NG, PolyH_NG, DMA/AETC_NG, AC-GUA_NG, and AETC_NG variants, underscored the modularity and tunability of the NG system. Each monomer imparted distinct electrostatic and hydrophilic/hydrophobic properties, directly influencing the NG's responsiveness to these stimuli, such as pH, ROS and GSH. While this study demonstrated independent responsiveness to these stimuli, it is clear that the interplay between such cues, particularly in the heterogeneous TME, requires more sophisticated modelling and experimental investigation.

The diagnostic utility of the platform was demonstrated through the successful delivery and fluorescence recovery of Mango II (MII) aptamers. Using TIRF-based photobleaching assays, the NGs were shown to maintain single-molecule sensitivity

in detecting aptamer function post-release. However, limitations of this method, including rapid fluorophore photobleaching and incomplete dye activation, may have led to underestimation of loading capacity. Development of alternative quantification strategies, such as FRET-based readouts or molecular beacons, may provide a more accurate measure of RNA integrity and release kinetics.

In terms of therapeutic delivery, NGs functionalised with a PSMA-targeting peptide enabled selective mRNA delivery to PCa cells, both in monolayer and 3D spheroid models. While thiol–maleimide coupling was effective for peptide conjugation, heterogeneity in maleimide distribution across NG populations likely contributed to batch-to-batch variability in targeting efficiency. These findings point to the need for more controlled conjugation strategies, such as pre-functionalised copolymers or site-specific click chemistry, to ensure reproducibility and scalable synthesis.

Functionally, NT-PGC1 α -loaded NGs induced pronounced anti-proliferative effects in PCa cells, with transcriptomic profiling revealing downregulation of proliferative signalling, metabolic reprogramming, and upregulation of pro-apoptotic pathways. Despite these promising results, the mechanistic basis of NT-PGC1 α -mediated tumour suppression remains incompletely understood. Given the emerging roles of PGC1 α in cellular metabolism, mitochondrial biogenesis, and inflammation, further dissection of its downstream interactome and metabolic rewiring effects is warranted. In particular, integration of metabolomic and phosphoproteomic analyses could provide deeper insights into how PGC1 α isoforms influence oncogenic signalling cascades and cellular energetics.

In vivo studies in xenograft-bearing immunocompromised mice confirmed the systemic tolerability of the NG and its tumour-selective accumulation. However, the therapeutic effect, while initially strong, diminished beyond 30 days, suggesting that tumour progression may eventually override the metabolic blockade imposed by NT-PGC1 α overexpression. This underscores the potential need for combinatorial therapeutic strategies, such as co-delivery with AMPK agonists (e.g., BI9774), to reinforce tumour suppression and prevent resistance. Furthermore, increasing NG dosage or incorporating sustained-release architectures may enhance therapeutic durability.

However, such modifications must be balanced against safety and immunogenicity concerns, which were not exhaustively evaluated in this study.

A significant limitation of this work lies in the use of immunocompromised models, which precludes analysis of immune-mediated effects. Considering that NGs may interface with innate immune cells (e.g., macrophages, dendritic cells), and given the immunomodulatory potential of PGC1 α , future studies in immunocompetent or humanised models are imperative. These investigations could elucidate the extent to which NG-mediated gene delivery reshapes immune cell metabolism, antigen presentation, and anti-tumour immunity. Such knowledge would not only inform NG design but also open up possibilities for synergistic immuno-nanotherapy combinations. Indeed, the versatility of NG platforms offers the potential for multi-modal delivery approaches, in which the therapeutic RNA cargo could be co-delivered alongside small-molecule drugs, proteins or immunomodulatory agents. Such strategies are particularly attractive for synergistic therapies, such as combining siRNA or mRNA with immune checkpoint inhibitors, metabolic modulators, or chemotherapeutic agents. By enabling spatiotemporally controlled co-delivery, NGs could potentiate both immune and tumour-intrinsic effects, allowing for more comprehensive modulation of disease pathways.

Formulation design for co-encapsulation would need to consider potential cargo–cargo interactions, differential release kinetics, and loading efficiency limitations. Strategies such as compartmentalised nanogel cores or sequential layering via electrostatic interactions may offer pathways to mitigate these challenges. Moreover, orthogonal release triggers (e.g., pH-sensitive vs. redox-sensitive linkages) could be engineered to allow staged release of payloads in response to specific cues within the tumour microenvironment. This could enable, for instance, a burst release of immunomodulatory agents followed by sustained mRNA translation. Such functionality would provide a high degree of programmability and could be especially useful in personalised medicine or combination immunotherapy contexts.

Another area for future exploration is the pharmacokinetics, biodistribution, and metabolic fate of NGs post-administration. Although minimal toxicity was observed

over the two-month treatment window, long-term accumulation in clearance organs, NG degradation kinetics, and potential immunogenicity remain unresolved. Moreover, the observed off-target uptake in liver and spleen indicates a need to optimise NG surface properties, such as size, zeta potential, and ligand density, to minimise MPS clearance. Employing nanobody conjugation may improve circulation time and tumour-specific delivery. While the inclusion of PEG was explored in this work to extend the circulation time and limit protein-corona formation, recent clinical and preclinical findings have highlighted a growing incidence of anti-PEG antibodies in patients, particularly those receiving repeated doses of PEGylated therapeutics or mRNA vaccines. These immune responses can accelerate blood clearance, reduce therapeutic efficacy, and, in some cases, provoke adverse events. Given the potential immunogenicity and the increasing scrutiny of PEG in clinical applications, the substitution of PEG with alternative stealth polymers warrants serious consideration.

Polysarcosine (pSar), a non-ionic, hydrophilic polypeptoid, has emerged as a promising PEG alternative due to its excellent biocompatibility, biodegradability, and lack of detectable immunogenicity.^[234] Like PEG, polysarcosine confers colloidal stability and resists opsonisation, thereby prolonging NP circulation half-life. Unlike PEG, however, polysarcosine is less prone to eliciting immune responses, making it particularly attractive for chronic or repeated administration regimens. Incorporating polysarcosine into the NG architecture—either as a grafted surface polymer or as a block copolymer integrated into the network—could enhance the platform’s clinical translation by mitigating immunological risks while maintaining the pharmacokinetic benefits traditionally associated with PEGylation. Future studies should therefore evaluate the impact of pSar-modification on NG pharmacokinetics, biodistribution, and bio-nano interactions, particularly in the context of systemic administration where stealth characteristics are critical for therapeutic efficacy.

While preliminary efforts were made to understand NG penetration of solid tumours using the 3D spheroid model, the NG platform would benefit from testing in more advanced 3D *in vitro* systems including, patient-derived organoids and organ-on-chip microfluidic devices. These models offer the ability to simulate tissue-specific

architecture, extracellular matrix stiffness, cell-cell and cell-matrix interactions, as well as dynamic perfusion that better mimics human physiology. For instance, tumour organoids derived from patient biopsies can provide insight into how NGs penetrate, distribute, and activate within heterogeneous tumour niches. Similarly, vascularised organ-on-chip models incorporating endothelial barriers, flow, and immune cells can be used to study NG biodistribution, transcytosis, and clearance in a more realistic context. These systems can also be leveraged to study NG-induced immunogenicity, cytokine responses, and cellular uptake mechanisms in real-time using live imaging and multiplexed analysis.

While efforts were made to develop a system to match the stringent requirements proposed by the FDA-approved NP formulations in the clinic, there are many aspects of the NG platform that remain underdeveloped and insufficiently validated, which limit the translatability of this platform. These criteria—ranging from chemical simplicity and large-scale manufacturability to biodegradability, biodistribution precision, batch-to-batch consistency, and redosability—set a high bar for clinical viability. Although the NGs developed herein were rationally designed using customisable, facile, biodegradable chemistries (e.g., disulfide linkages) and demonstrated favourable *in vitro* performance, key limitations persist in areas such as synthesis scalability, structural heterogeneity, off-target accumulation, and long-term storage stability. As such, this platform requires significant optimisation both *in vitro* and *in vivo* prior to successful clinical translation. Furthermore, comparison against clinically relevant LNP platforms is essential to contextualise the performance and the translational potential of the NGs. Head-to-head benchmarking will determine whether the advantages offered by the NG chemistry translate into superior or comparable payload retention, delivery efficiency, safety and manufacturability.

One of the major barriers to clinical translation of nanomedicine platforms lies in their alignment with regulatory and manufacturing standards, particularly GMP. In particular, to enable systematic optimisation of NG compositions, more comprehensive screening of monomer types, their relative incorporation ratios, and their influence on NG architecture is essential. However, a key limiting factor in this work

was the synthetic strategy itself, which constrained the breadth and throughput of formulation screening. While free-radical polymerisation offers several advantages—most notably, its compatibility with aqueous reaction conditions and minimal reliance on harsh organic solvents—it inherently suffers from a lack of control over key reaction parameters. This results in significant variability in polymer network formation and contributes to batch-to-batch inconsistencies, ultimately impeding the reproducibility and scalability of the platform. Moreover, the stochastic nature of this synthetic methodology limits the ability to draw clear structure–activity relationships (SARs) across formulations. To address these challenges, alternative synthetic approaches such as nanoprecipitation or emulsion-based polymerisation could be explored. These methods offer improved control over particle formation and network uniformity, and may facilitate higher RNA loading efficiencies through better encapsulation mechanics, without requiring increased RNA input. Implementing such controlled or semi-controlled synthetic methods would also open the door to high-throughput formulation screening, thereby enabling a more robust evaluation of formulation–performance relationships and accelerating translational development. Once a robust and scalable synthetic framework is established, the integration of AI-driven analysis pipelines could markedly accelerate the design process. By applying machine learning models to datasets derived from physicochemical characterisation, cellular uptake, transfection efficiency, and stimuli-responsiveness, it would be possible to predict formulation behaviour and identify optimal NG compositions tailored to specific therapeutic applications. This data-driven approach could enable adaptive formulation development, wherein iterative experimental cycles are guided by computational outputs, thereby significantly enhancing the efficiency, precision, and customisability of RNA delivery platform design. Furthermore, to ensure regulatory compliance, future protocols must be GMP-compatible, which includes the use of pharmaceutically acceptable monomers, catalysts and solvents to limit any residual toxicity. This will ultimately suppose the reproducibility and safety required for clinical trials and market authorisation.

A particular factor left underexplored in this work was the storage and long-term stability of the mRNA-loaded NGs. This is especially important in global contexts

where cold-chain infrastructure is limited. Although this work did not assess the lyophilisation compatibility of the NG platform, future studies should explore the feasibility of freeze-drying NGs to enhance storage stability, improve shelf-life, and potentially enable room-temperature transport and administration. Key considerations include identifying suitable cryoprotectants and lyoprotectants (e.g., trehalose, sucrose, mannitol) that can prevent particle aggregation or morphological collapse during the freezing and drying process. Further, the effect of lyophilisation on RNA stability and NG integrity under accelerated aging and stress conditions (temperature/humidity cycling) could be evaluated to model real-world transport scenarios.

Taken together, this body of work demonstrates the feasibility of using disulfide-crosslinked, stimuli-responsive NGs as a flexible and effective platform for RNA delivery. While challenges remain in achieving consistent targeting, efficient RNA loading, and durable therapeutic responses, the fundamental principles established herein provide a robust foundation for continued innovation. By integrating advanced characterisation tools, systems biology approaches, and clinically relevant models, the NG platform can be further refined to meet the growing demand for precise, personalised RNA-based nanomedicines.

Chapter 7

Appendix

Table A1: Summary of NG feed compositions and physicochemical properties.

Cationic monomer type	Nanogel	Feed composition			Initiating system	Nanogel structural features		
		AM	Cationic monomer	SS-DIA		Size (nm)	PI	Zeta potential (mV; pH 7.4, water)
	no.	mol%			mol%			
AETC	1	90.7	0	7.0	15	126.4 $\pm$ 4.0	0.033 $\pm$ 0.009	-8.5
	2	83.56	7.14	7.0	15	198.8 $\pm$ 17.7	0.010 $\pm$ 0.056	6.6
	3	77.2	13.5	7.0	15	144.3 $\pm$ 1.3	0.042 $\pm$ 0.021	10.5
	4	66.9	23.8	7.0	15	118.1 $\pm$ 11.7	0.072 $\pm$ 0.062	22.5
	5	41.5	49.2	7.0	15	247.2 $\pm$ 2.8	0.336 $\pm$ 0.018	30.5
PolyR	7	85.86	7.14	7.0	15	239.4 $\pm$ 2.7	0.232 $\pm$ 0.007	8.3 $\pm$ 0.6
	8	79.5	13.5	7.0	15	168.9 $\pm$ 2.1	0.094 $\pm$ 0.020	28.8 $\pm$ 0.6
	9	69.2	23.8	7.0	15	54.8 $\pm$ 0.9	0.283 $\pm$ 0.004	21.2 $\pm$ 0.6
PolyH	10	85.86	7.14	7.0	15	488.5 $\pm$ 6.1	0.162 $\pm$ 0.010	1.8 $\pm$ 0.8
	11	79.5	13.5	7.0	15	111.8 $\pm$ 0.5	0.051 $\pm$ 0.003	5.6 $\pm$ 4.2
	12	69.2	23.8	7.0	15	77.3 $\pm$ 21.8	0.461 $\pm$ 0.059	7.8 $\pm$ 0.7
AC-GUA	13	85.86	7.14	7.0	15	254.3 $\pm$ 21.5	0.135 $\pm$ 0.027	3.8 $\pm$ 0.8
	14	79.5	13.5	7.0	15	111.8 $\pm$ 0.5	0.051 $\pm$ 0.003	10.8 $\pm$ 3.9
	15	69.2	23.8	7.0	15	163.7 $\pm$ 0.9	0.074 $\pm$ 0.017	19.5 $\pm$ 6.0
AETC/DMA	16	69.2	23.8	7.0	15	144.6 $\pm$ 0.4	0.102 $\pm$ 0.012	1.1 $\pm$ 0.1
	17	69.2	5.95	7.0	15	119.6 $\pm$ 1.2	0.121 $\pm$ 0.027	21.2 $\pm$ 0.6
	18	69.2	11.9	7.0	15	135.8 $\pm$ 3.4	0.219 $\pm$ 0.067	11.0 $\pm$ 0.9
	19	69.2	17.85	7.0	15	172.8 $\pm$ 2.8	0.286 $\pm$ 0.018	8.5 $\pm$ 0.3
			AETC DMA					

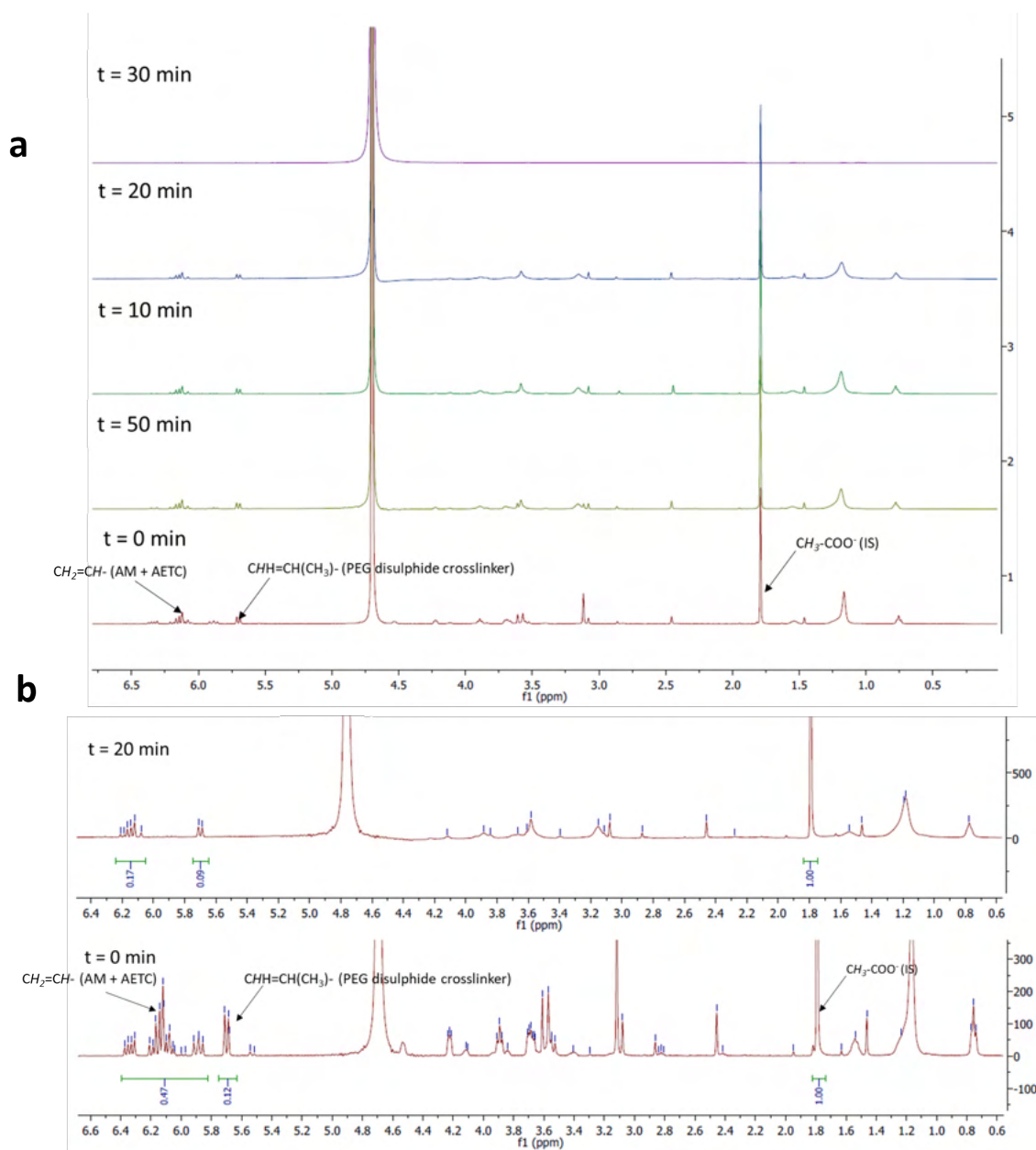


Figure A1: **Quantification of monomer conversion by ^1H NMR analysis.** a) Reaction kinetics of polymerization monitored by ^1H NMR by the gradual disappearance of the vinyl olefin signals. b) Partial ^1H NMR spectra of the polymerisation mixtures for the preparation of nanogels included in the study as a representative example. Peaks integrated to calculate the monomer conversions are indicated. All NMR were conducted in D_2O . Sodium acetate was used as internal standard (IS). Intensities have been scaled for clarity.

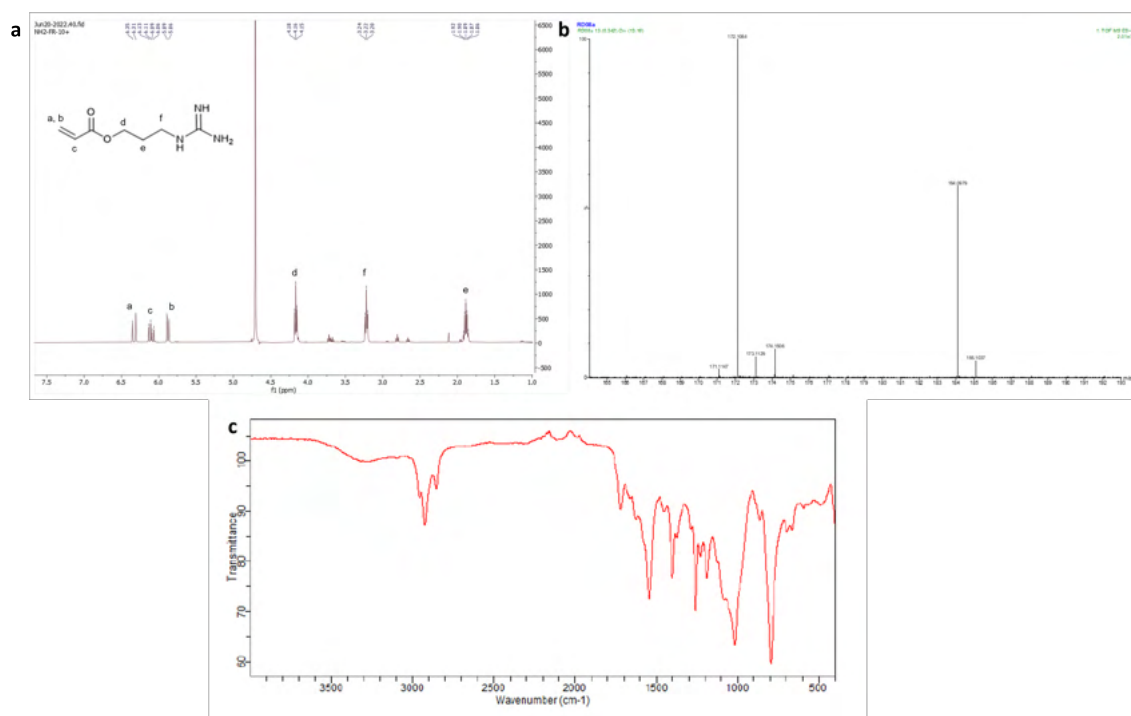


Figure A2: **Characterisation of AC-GUA monomer.** a) ^1H NMR spectrum of acrylated guanidine (AC-GUA). ^1H NMR (400 MHz, D_2O): δ 6.35 (d), δ 6.11 (dd), δ 5.86 (d), δ 4.18 (t), δ 3.22 (t), δ 2.25 (s), δ 1.89 (q). b) Atmospheric pressure chemical ionization – mass spectra of acrylated guanidine. $\text{M} = (\text{C}_7\text{H}_{13}\text{N}_3\text{O}_2) [\text{M} + \text{H}]^+ = 172.1064 \text{ m/z}$. c) Fourier transform infrared spectroscopy (FT-IR) of acrylated guanidine (AC-GUA) N-H stretch: $3700\text{--}3500 \text{ cm}^{-1}$; alkyl sp^3 C-H stretch: $2950\text{--}2850 \text{ cm}^{-1}$ (strong), carbonyl C=O stretch: 1655 cm^{-1} (strong); C-O-C stretch: 1095 cm^{-1} (sharp).

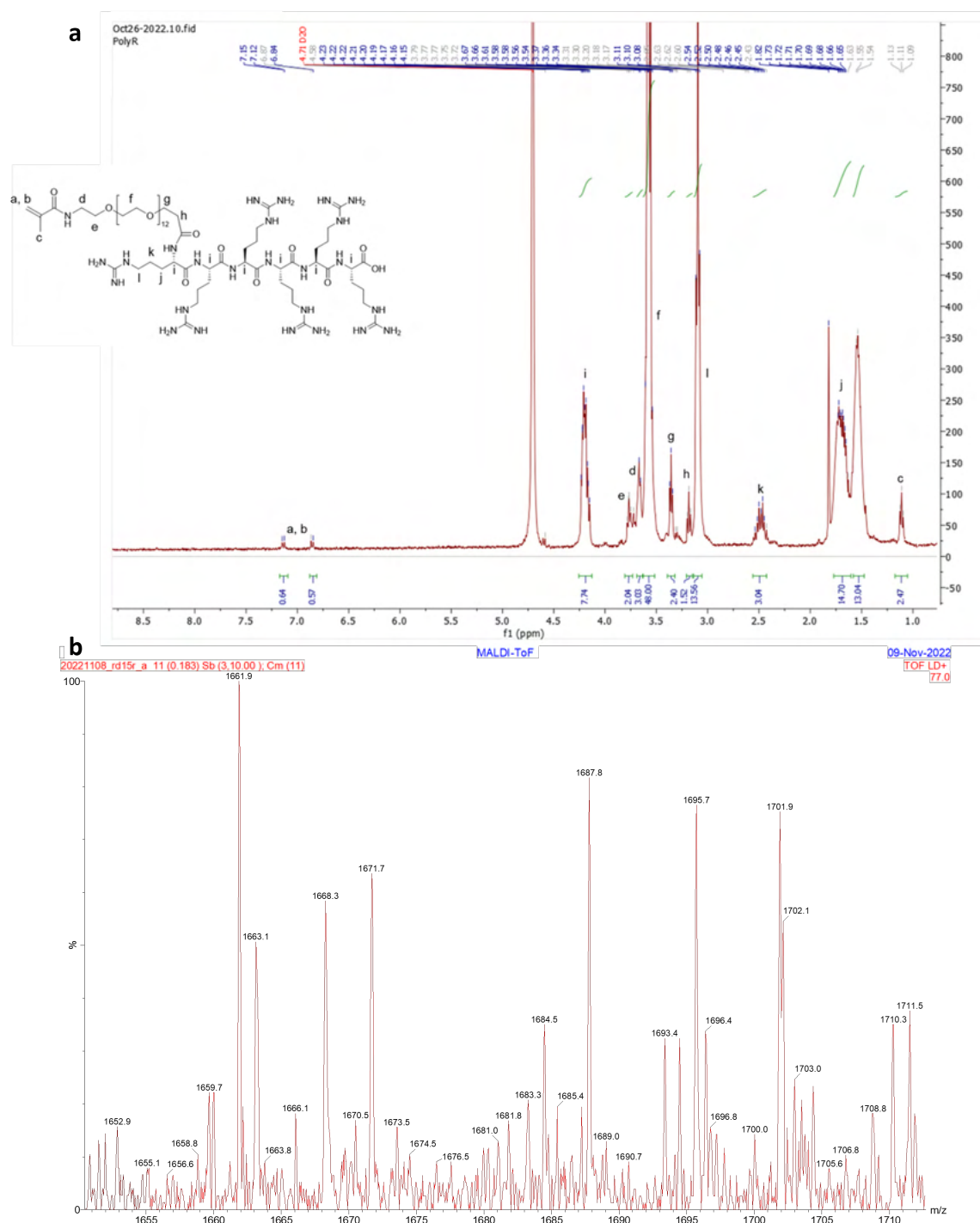


Figure A3: **Characterisation of PolyR monomer.** a) ^1H NMR spectrum of PolyR. ^1H NMR (400 MHz, D_2O): δ 7.15 (d), δ 6.87 (d), δ 4.23 (t), δ 3.85 (t), δ 3.77 (t), δ 3.58 (tt), δ 3.48 (t), δ 3.34 (t), δ 3.11 (t), δ 2.50 (m), δ 1.70 (q) δ 1.11 (t). b) Matrix assisted laser desorption ionization-time of flight – mass spectra (MALDI-ToF MS) of PolyR. $\text{M} = (\text{C}_{69}\text{H}_{135}\text{N}_{25}\text{O}_{22}) [\text{M} + \text{H}]^+ = 1666.9 \text{ m/z}$.

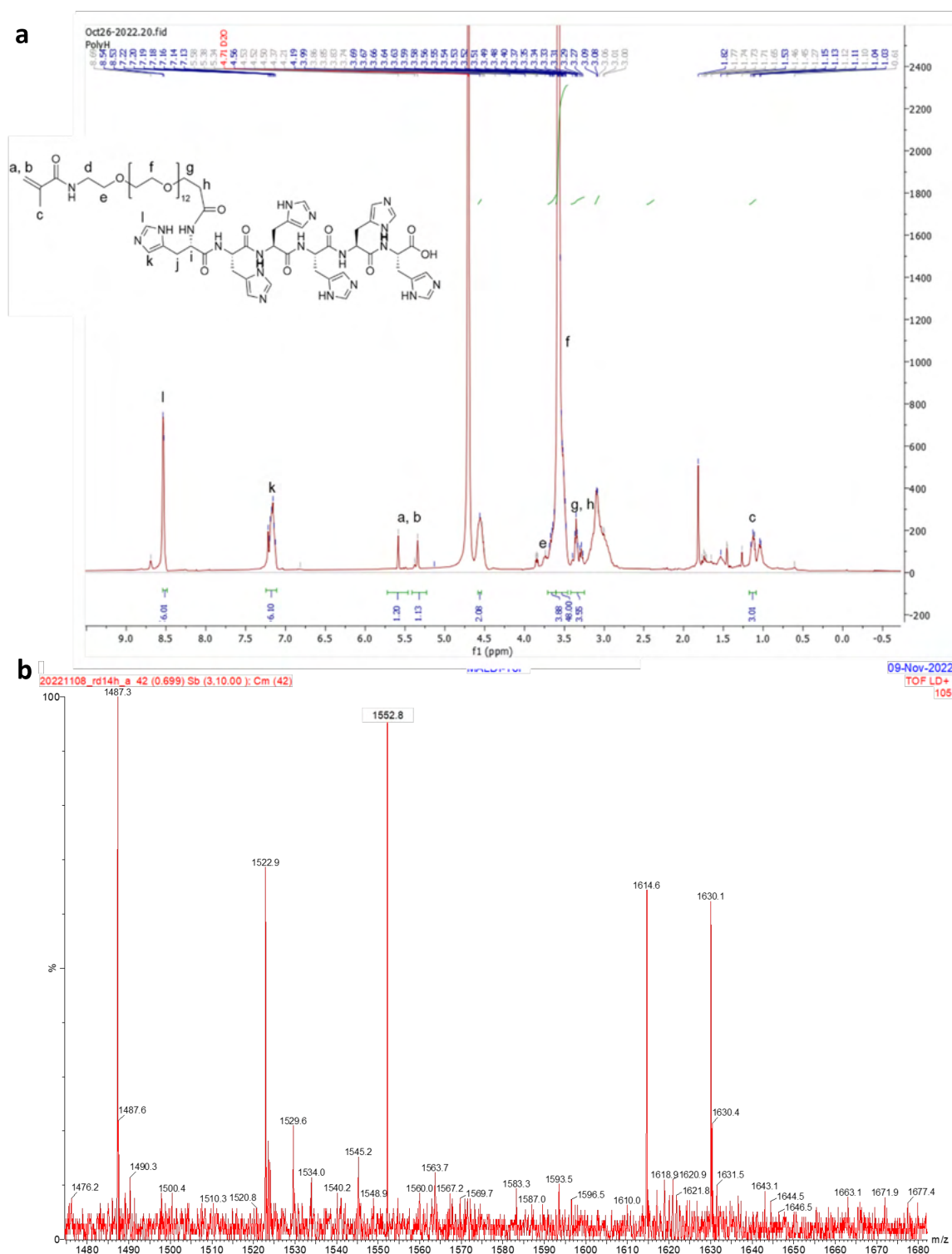


Figure A4: **Characterisation of PolyH monomer.** a) ¹H NMR spectrum of PolyH. ¹H NMR (400 MHz, D₂O): δ8.54 (s), δ7.20 (m), δ5.58 (d), δ5.34 (d), δ3.69 (t), δ3.58 (tt), δ3.48 (t), δ3.09 (t), δ1.15 (t). b) Matrix assisted laser desorption ionization-time of flight – mass spectra (MALDI-ToF MS) of PolyH. M = (C₆₉H₁₀₅N₁₉O₂₂) [M + H]⁺ = 1552.8 m/z.

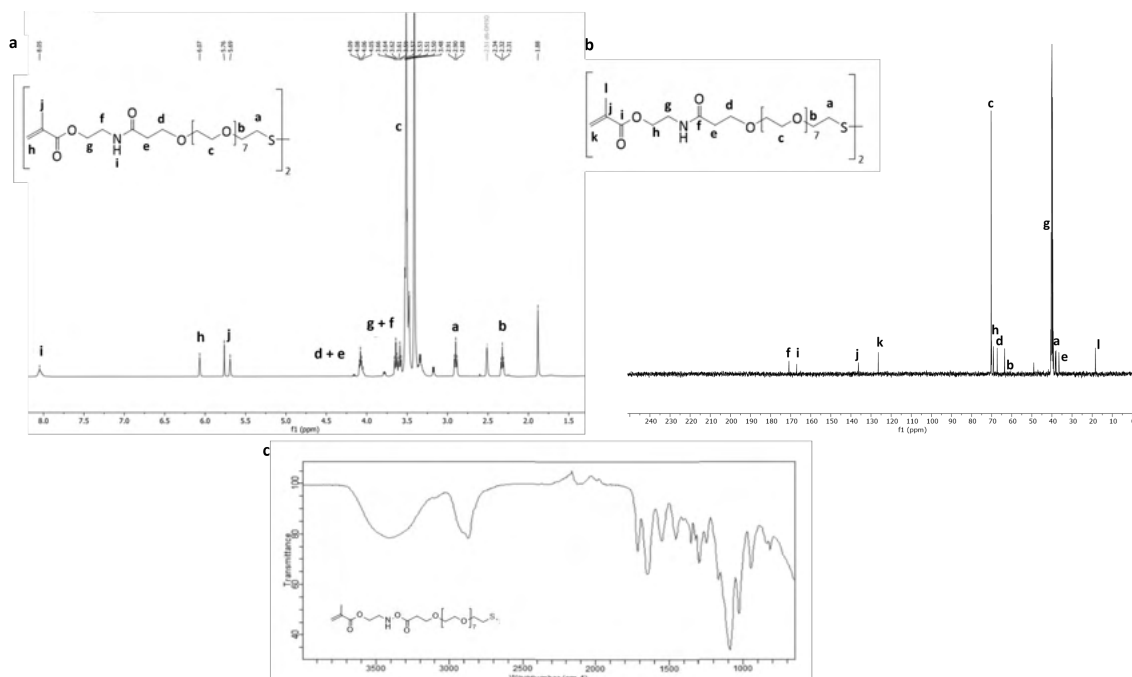
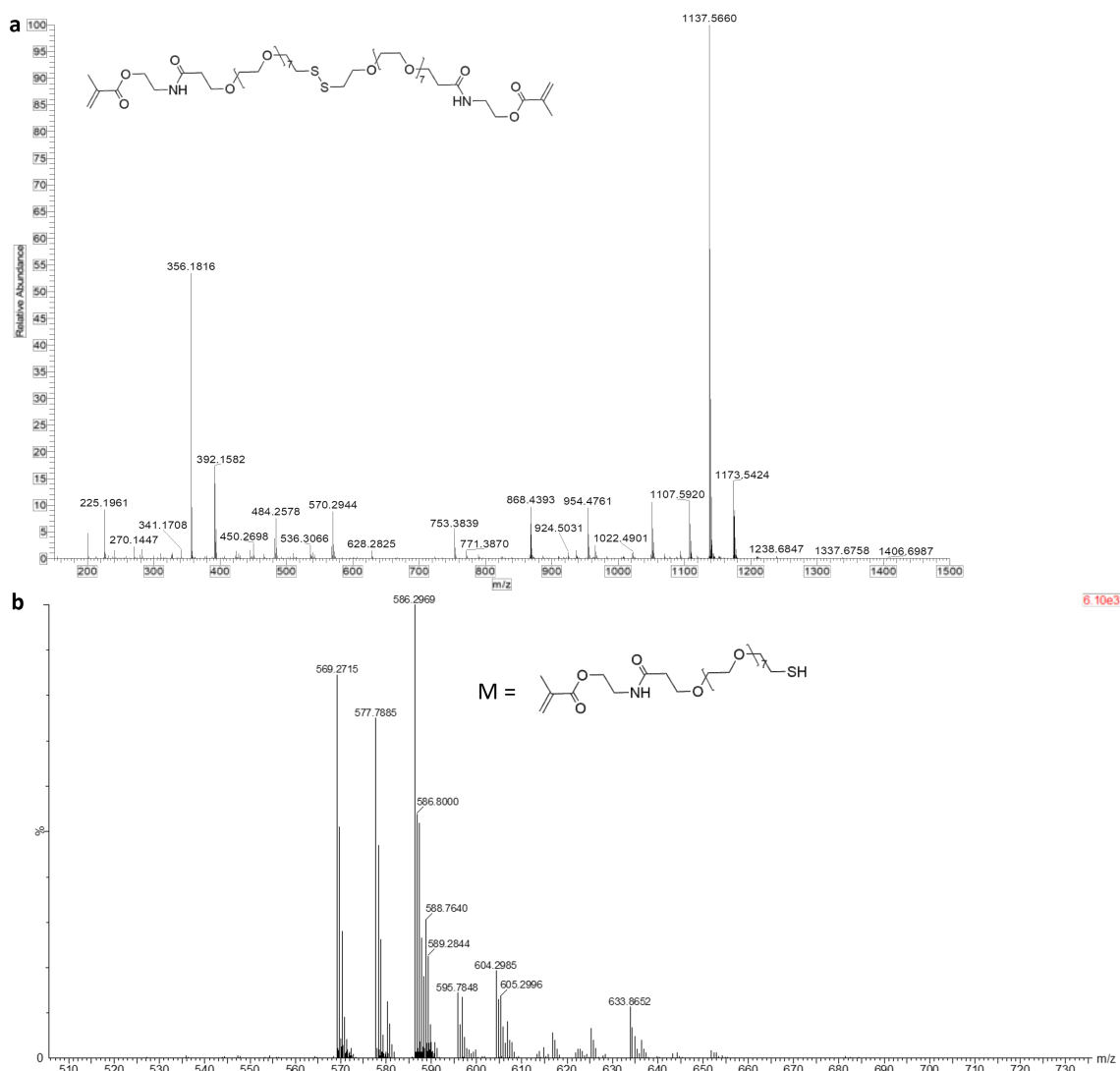


Figure A5: **Characterisation of SS-DiA crosslinker.** a) ^1H NMR spectrum of the disulfide crosslinker (SS-DiA). ^1H NMR (400 MHz, $(\text{CD}_3)_2\text{SO}$): δ 8.05 (t, 2H), 6.07 (s, 2H), 5.69–76 (m, 2H), 4.05–4.09 (m, 8H), 3.61–3.65 (t, 4H), 3.57–3.59 (t, 4H), 3.48–3.53 (m, 56H), 2.88–2.91 (t, 4H), 2.31–2.34 (t, 4H), 1.88 (s, 6H) *Residual solvent $(\text{CD}_3)_2\text{SO}$ (δ 2.50). b) ^{13}C nuclear magnetic resonance (NMR) spectrum of SS-DiA disulfide-based crosslinker: ^{13}C NMR (400 MHz, $(\text{CD}_3)_2\text{SO}$): δ 171.04, 166.89, 135.18, 125.36, 69.98–70.24, 69.20, 64.38, 64.54, 38.23, 36.79, 35.58, 18.49. c) Fourier transform infrared spectroscopy (FT-IR) of SS-DiA disulfide-based crosslinker: N-Amine C(O)N-H stretch: 3700–3200 cm^{-1} (broad); alkyl sp^3 C-H stretch: 2950–2850 cm^{-1} (sharp), carbonyl C=O stretch: 1650 cm^{-1} (sharp).



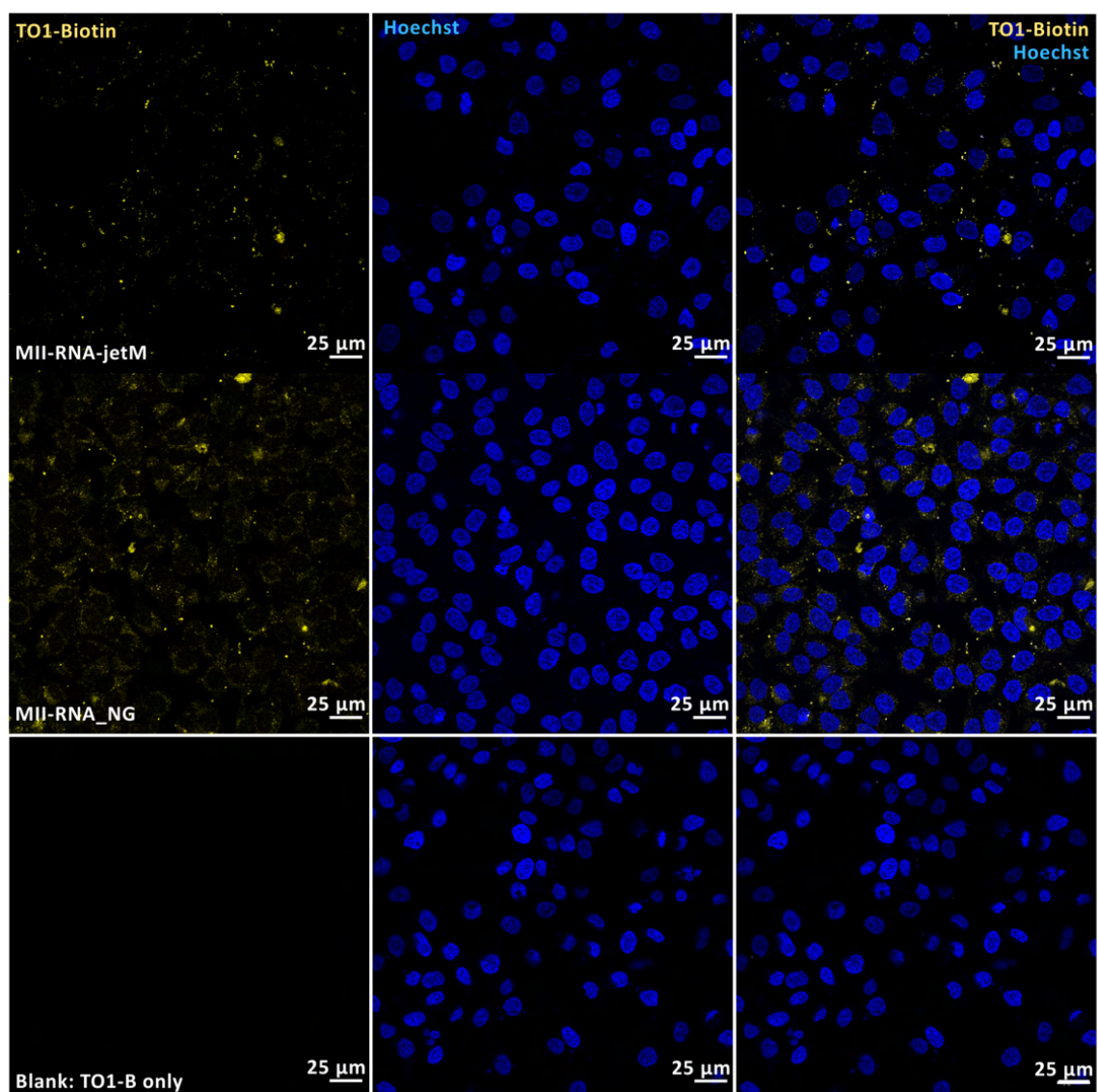


Figure A7: **Imaging Mango II RNA transfection.** Maximum projection of HeLa cells treated with MII-RNA-jetM, MII-RNA_NG and fixed at 12 h post-transfection, stained with 200 nM TO1-B (yellow) and 1 μ g/mL Hoechst 33342. Control cells treated with only 200 nM TO1-B and no Mango II RNA also shown. All images were taken with 40 x oil immersion objective.

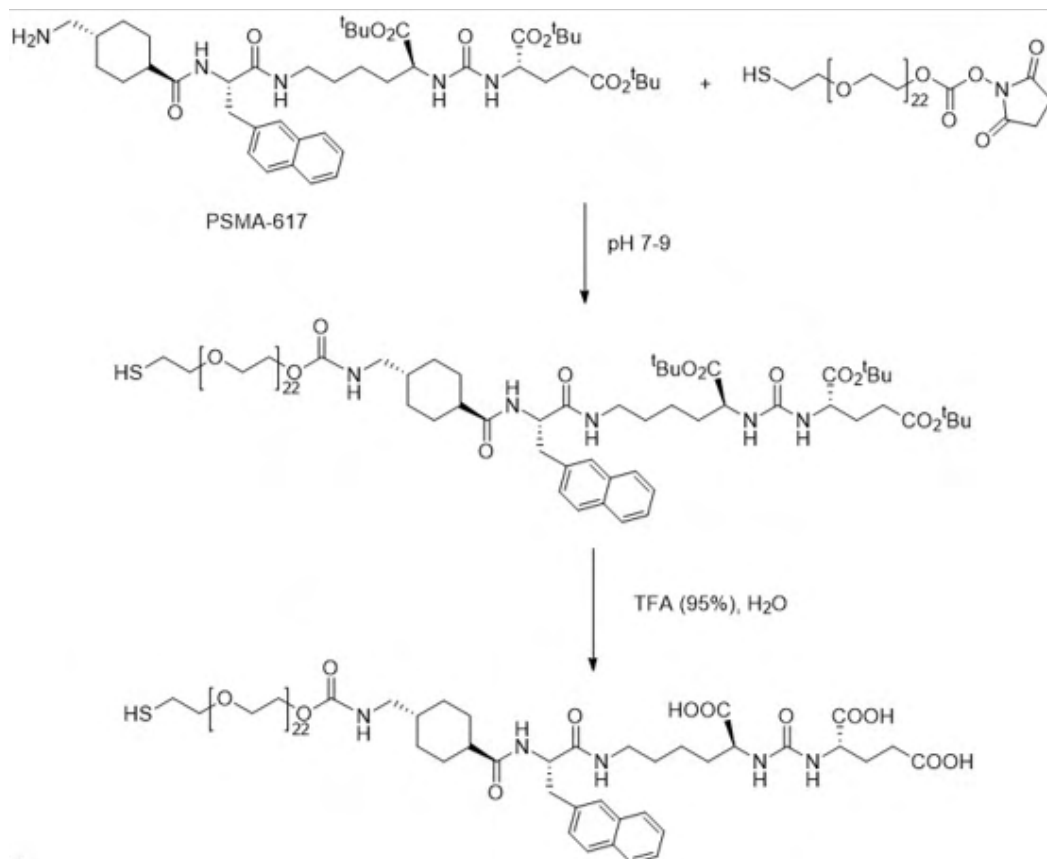


Figure A8: **Schematic presentation of PSMA-pep synthesis.** The terminal amine of the PSMA-617-inspired peptide reacts with the NHS ester of a bifunctional PEG-based linker bearing a free thiol, resulting in a thiolated, PEGylated PSMA peptide (PSMA-pep). Subsequent deprotection yielded the final targeting construct.

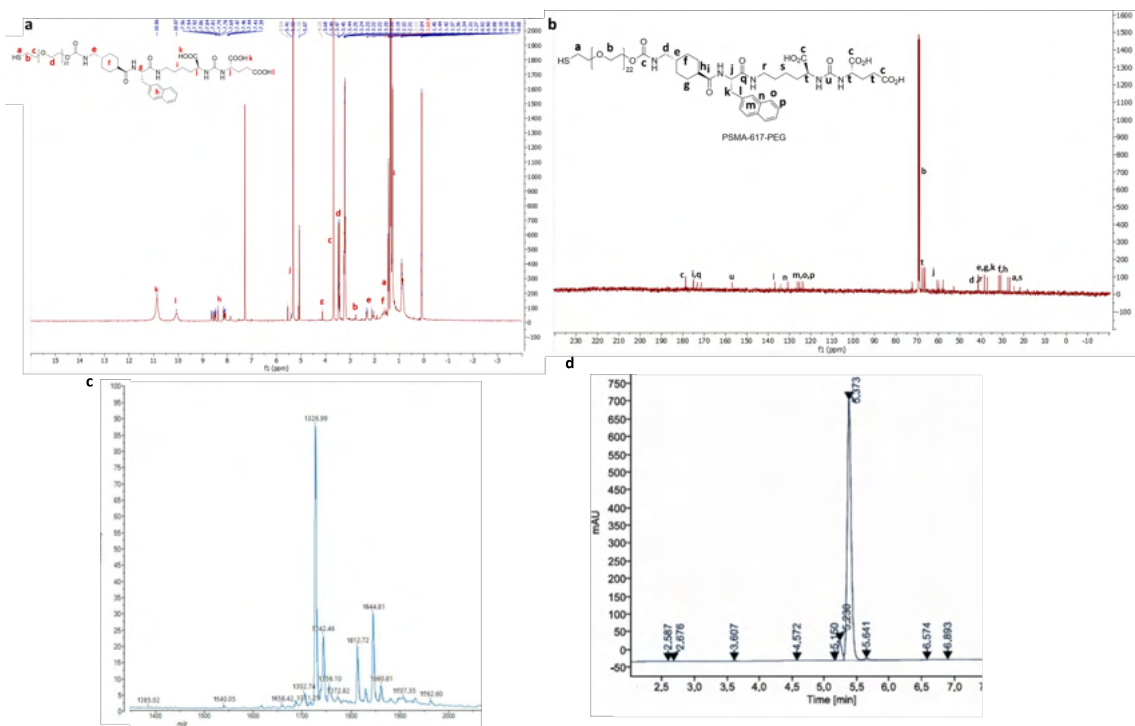


Figure A9: **Characterisation of SS-DiA crosslinker.** a) ^1H NMR spectrum of the thiolated, PEGylated PSMA-peptide (PSMA-pep). ^1H NMR (400 MHz, CDCl_3): δ 10.86 (s, 2H), 10.07 (s, 1H), 7.34-8.10 (m, 7H), 5.32-5.54 (t, 2H), 4.13 (t, 1H), 3.22-3.25 (t, 88H), 2.89 (t, 2H), 2.10 (d, 2H), 1.54-1.78 (m, 8H), 1.45 (t, 1H) *Residual solvent (CDCl_3 (δ 7.29)). b) ^{13}C NMR of thiolated, PEGylated PSMA-pep (PSMA-pep). ^{13}C NMR (400 MHz, CDCl_3): δ 179.04, 175.78-174.5, 157.56, 136.50, 130.01, 122.34-126.57, 68.04-69.98, 60.34, 42.28, 37.56-38.94, 31.28-32.94, 27.67-28.98. c) Matrix-Assisted Laser Desorption/Ionization mass spectrometry: calculated mass ($\text{C}_{89}\text{H}_{139}\text{N}_5\text{O}_{33}\text{S}_1^+$): 1741.91, actual mass : 1726.99 m/z. d) HPLC chromatogram of the purified PSMA-pep. A single peak at 5.37 min indicates >91% purity. Conditions: Phenomenex-C18(2) luna 3 μm 100 $\text{\AA}$ reverse-phase column, gradient elution using water (0.1% TFA) and acetonitrile (0.09% TFA), flow rate 1.0 mL/min, detection at 220 nm.

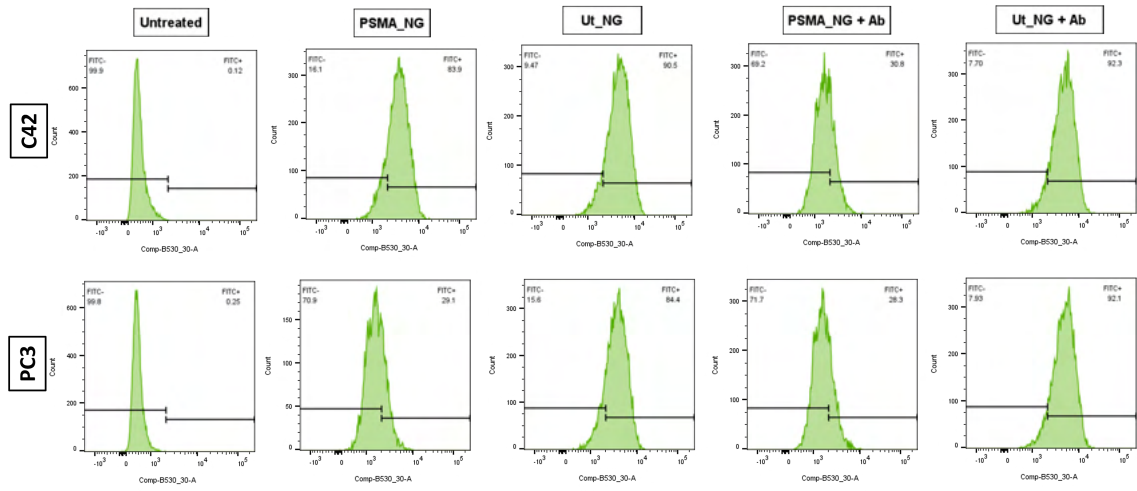


Figure A10: Flow cytometry histograms showing fluorescent NG uptake in C4-2 and PC3 cells following incubation with PSMA-targeted nanogels (PSMA_NG) or untargeted nanogels (Ut_NG), with and without prior blocking using anti-PSMA antibody. Untreated cells served as a negative control. Each panel shows representative histograms from three independent biological replicates (top and bottom rows). Percentages of FITC+ cells are indicated in each plot.

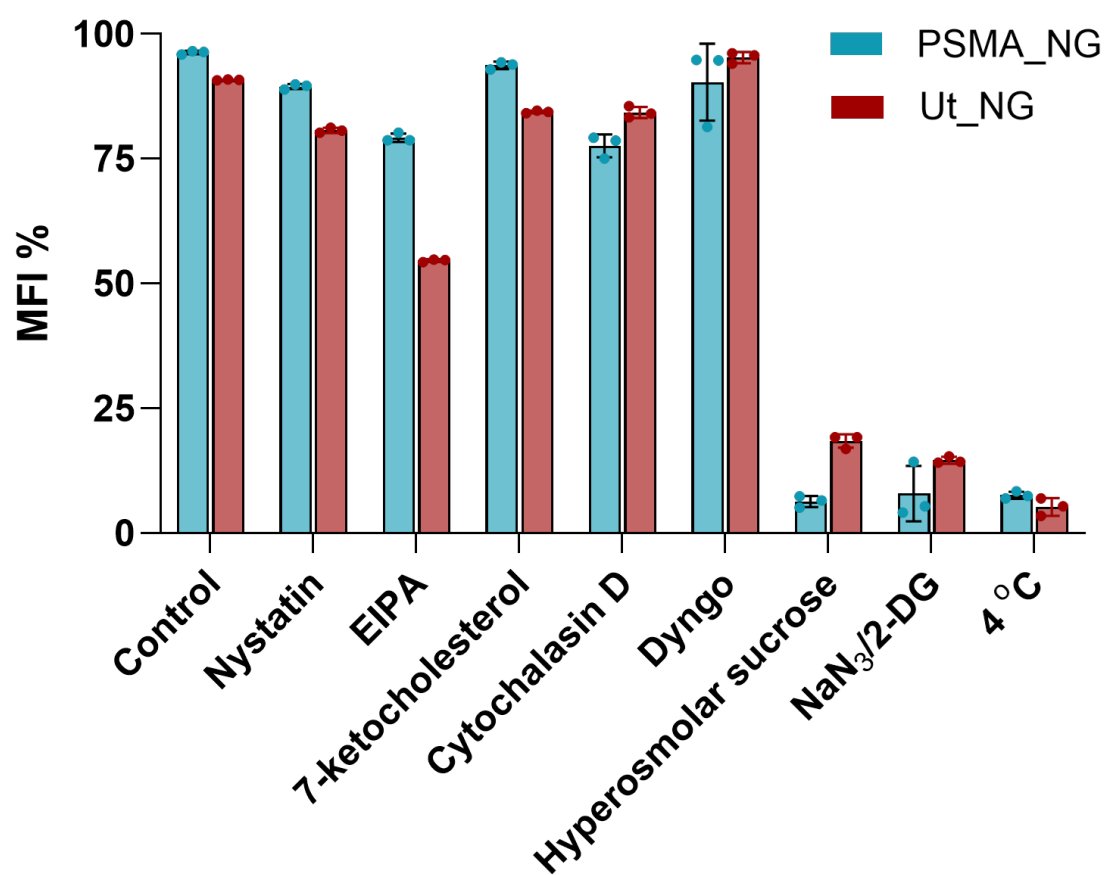


Figure A11: Quantification of the intracellular uptake of the targeted and untargeted NGs by flow cytometry in C4-2 cells, after pre-treatment with various endocytotic inhibitors. Mean fluorescence intensities (MFI %) of the two differently treated cell populations are shown (n=3).

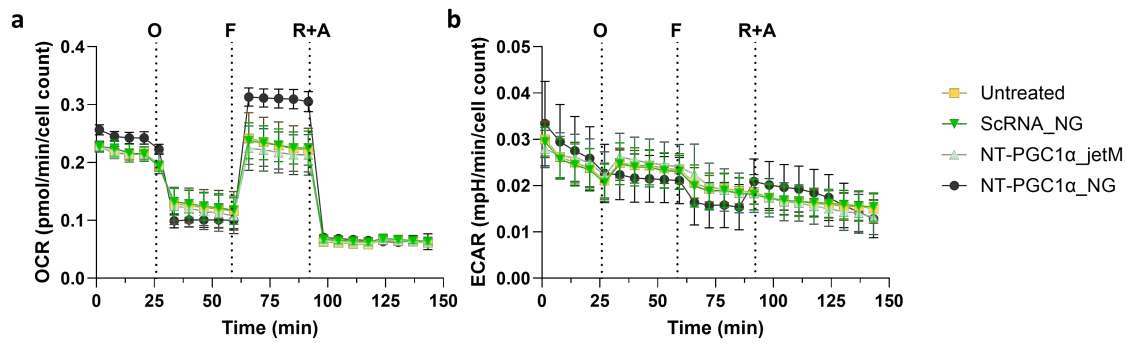


Figure A12: Mitochondrial and glycolytic function analysis following NT-PGC1 α delivery. a) Oxygen consumption rate (OCR) measurements indicate mitochondrial respiration profiles of C4-2 cells treated with NT-PGC1 α encapsulated in NGs (NT-PGC1 α _NG, black), JetMESSENGER-mediated NT-PGC1 α (light grey), scrambled RNA in nanogels (scrRNA_NG, green), or untreated (yellow). NT-PGC1 α _NG treatment leads to the highest spare respiratory capacity, suggesting enhanced mitochondrial functionality. b) Extracellular acidification rate (ECAR) traces show glycolytic activity under the same treatment conditions. NT-PGC1 α _NG-treated cells display the lowest compensatory glycolysis following oligomycin injection, indicating reduced reliance on glycolysis and a metabolic shift toward oxidative phosphorylation. (n = 6).

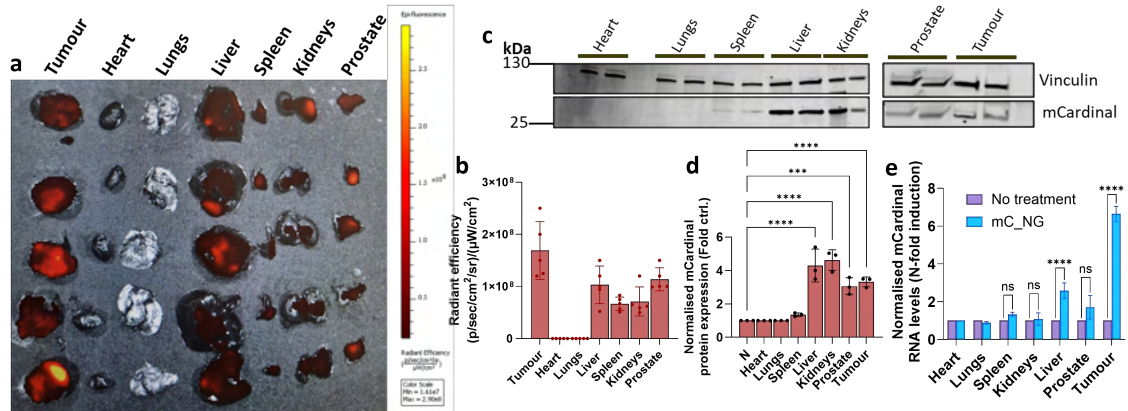


Figure A13: Tracking biodistribution of NT-PGC1 α expression in xenograft-bearing male immunocompromised (NSG) mice. a) *Ex vivo* fluorescence imaging and b) radiant efficiency quantification of main organs extracted from the mice at 24 h after injection of NGs (n = 5). c) mCardinal protein expression in major organs determined by western blotting and d) associated quantification. Vinculin was used as a loading control. e) Comparison of mCardinal mRNA levels in all main organs and tumours, as quantified by RT-qPCR. RNA levels normalised to GAPDH expression and shown as fold change relative to untreated cells. In all cases, data are presented as mean $\pm$ SD, and statistical significance was determined using an ordinary one-way ANOVA with Dunnett's multiple comparisons test, where RNA levels of treated cells were compared against those of untreated cells: **** $p < 0.0001$, *** $p < 0.0002$, ns < 0.1234 .

Table A2: RNA-seq results including gene ID, fold change, standard error, adjusted p-value, symbol, and full name.

Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG00000110931	-2.130155	0.142734	2.41e-50	CAMKK2	calcium/calmodulin dependent protein kinase 2
ENSG00000205835	-0.812562	0.365933	8.43e-03	GMNC	geminin coiled-coil domain containing
ENSG00000168496	-1.500977	0.207207	1.41e-13	FEN1	flap structure-specific endonuclease 1
ENSG00000105173	-1.737972	0.154733	1.01e-29	CCNE1	cyclin E1
ENSG00000110092	-1.285692	0.083452	1.13e-52	CCND1	cyclin D1
ENSG00000145386	-2.055204	0.199568	2.54e-25	CCNA2	cyclin A2
ENSG00000163249	-0.440921	0.116798	3.50e-04	CCNYL1	cyclin Y like 1
ENSG00000112237	-0.512942	0.090743	4.58e-08	CCNC	cyclin C
ENSG00000157456	-0.531118	0.125624	4.41e-05	CCNB2	cyclin B2
ENSG00000290445	-0.951795	0.232529	2.10e-05	CCNYL2	cyclin Y like 2 (pseudogene)
ENSG00000135083	-1.131349	0.349001	2.83e-04	CCNJL	cyclin J like
ENSG00000110092	-1.285692	0.083452	1.13e-52	CCND1	cyclin D1

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Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG000000147082	-1.370073	0.343101	1.23e-05	CCNB3	cyclin B3
ENSG000000105173	-1.737972	0.154733	1.01e-29	CCNE1	cyclin E1
ENSG000000175305	-1.888932	0.116234	4.96e-59	CCNE2	cyclin E2
ENSG000000162063	-1.899096	0.087436	1.58e-103	CCNF	cyclin F
ENSG000000145386	-2.055204	0.199568	2.54e-25	CCNA2	cyclin A2
ENSG000000133101	-2.862001	0.426034	3.40e-13	CCNA1	cyclin A1
ENSG000000156970	-1.528796	0.192194	6.44e-16	BUB1B	BUB1 mitotic checkpoint ser- ine/threonine kinase B
ENSG000000166851	-0.961654	0.156496	9.37e-10	PLK1	polo like kinase 1
ENSG000000151465	-0.315585	0.079324	1.18e-04	CDC123	cell division cycle 123
ENSG000000060339	-0.474326	0.069069	2.40e-11	CCAR1	cell division cycle and apoptosis regulator 1
ENSG000000081377	-0.494692	0.106779	8.36e-06	CDC14B	cell division cycle 14B
ENSG000000170779	-0.561737	0.089030	8.81e-10	CDCA4	cell division cycle associated 4
ENSG000000122783	-0.673257	0.054598	7.27e-34	CYREN	cell cycle regulator of NHEJ
ENSG000000144354	-0.699305	0.127824	9.35e-08	CDCA7	cell division cycle associated 7

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Table A2 – continued from previous page

Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG000000164045	-0.798859	0.138684	1.00e-08	CDC25A	cell division cycle 25A
ENSG000000203995	-0.818930	0.196868	2.72e-05	ZYG11A	zyg-11 family member A, cell cycle regulator
ENSG000000134690	-0.849342	0.144590	7.01e-09	CDC48	cell division cycle associated 8
ENSG000000134371	0.860694	0.069722	3.78e-34	CDC73	cell division cycle 73
ENSG000000158402	-1.007692	0.142970	2.61e-12	CDC25C	cell division cycle 25C
ENSG000000184661	-1.193469	0.175889	7.42e-12	CDCA2	cell division cycle associated 2
ENSG000000136982	-1.487110	0.111447	1.92e-40	DSCC1	DNA replication and sister chromatid cohesion 1
ENSG000000122692	-0.384598	0.066351	1.61e-08	SMU1	SMU1 DNA replication regulator and spliceosomal factor
ENSG000000227268	-1.564095	0.248390	6.02e-11	KLLN	killin, p53 regulated DNA replication inhibitor
ENSG000000148773	-1.115534	0.359410	6.68e-04	MKI67	marker of proliferation Ki-67
ENSG000000132646	-1.481250	0.090992	3.97e-59	PCNA	proliferating cell nuclear antigen

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Table A2 – continued from previous page

Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG000000260896	-2.538199	0.771316	8.34e-06	ARLNC1	androgen receptor regulated long noncoding RNA 1
ENSG000000121211	-0.329228	0.108929	3.02e-03	MND1	meiotic nuclear divisions 1
ENSG000000163781	-0.391839	0.108241	4.50e-04	TOPBP1	DNA topoisomerase II binding protein 1
ENSG000000133703	-0.360793	0.108396	1.18e-03	KRAS	KRAS proto-oncogene, GTPase
ENSG000000125952	-0.320572	0.084330	3.49e-04	MAX	MYC associated factor X
ENSG000000136997	-0.447698	0.078154	5.70e-08	MYC	MYC proto-oncogene, bHLH transcription factor
ENSG000000214114	-1.075113	0.177774	1.20e-09	MYCBP	MYC binding protein
ENSG000000131747	-0.573301	0.168125	9.57e-04	TOP2A	DNA topoisomerase II alpha
ENSG000000142208	-0.585347	0.119196	3.41e-06	AKT1	AKT serine/threonine kinase 1
ENSG000000168036	-0.567244	0.065979	4.47e-17	CTNNB1	catenin beta 1
ENSG000000087586	-1.177350	0.177017	1.59e-11	AURKA	aurora kinase A
ENSG000000078900	-0.595830	0.148486	9.02e-05	TP73	tumour protein p73

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Table A2 – continued from previous page

Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG000000184292	-2.900372	0.209353	1.15e-44	TACSTD2	tumour associated calcium signal transducer 2
ENSG000000167034	-1.285965	0.362267	1.65e-04	NKX3-1	NK3 homeobox 1
ENSG000000151491	-0.815315	0.146603	3.69e-08	EPS8	EGFR pathway substrate 8, signaling adaptor
ENSG000000146416	0.429979	0.076001	4.90e-08	AIG1	androgen induced 1
ENSG000000169083	-1.450187	0.139072	2.51e-25	AR	(AR) androgen receptor
ENSG000000140534	-1.707940	0.199479	3.80e-18	TICRR	TOPBP1 interacting checkpoint and replication regulator
ENSG000000123983	-1.195940	0.121172	1.48e-22	ACSL3	acyl-CoA synthetase long chain family member 3
ENSG000000171236	-1.646205	0.138257	1.15e-32	LRG1	leucine rich alpha-2-glycoprotein 1
ENSG000000124762	4.223427	0.138625	2.34e-202	CDKN1A	cyclin dependent kinase inhibitor 1A

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Table A2 – continued from previous page

Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG000000111276	1.631122	0.081842	1.43e-87	CDKN1B	cyclin dependent kinase inhibitor 1B
ENSG000000141510	0.835634	0.104475	3.74e-15	TP53	tumour protein p53
ENSG000000171862	0.694890	0.075167	2.22e-19	PTEN	phosphatase and tensin homolog
ENSG000000121879	0.664806	0.145223	8.90e-06	PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
ENSG000000173334	0.736740	0.157285	2.73e-06	TRIB1	tribbles pseudokinase 1
ENSG000000168564	1.387163	0.086350	1.50e-57	CDKN2AIP	CDKN2A interacting protein
ENSG000000087088	0.627359	0.189230	1.21e-03	BAX	BCL2 associated X, apoptosis regulator
ENSG000000064012	0.877330	0.077369	4.53e-29	CASP8	caspase 8
ENSG000000103197	0.416142	0.082528	1.30e-06	TSC2	TSC complex subunit 2
ENSG000000165699	0.979395	0.065657	2.86e-49	TSC1	TSC complex subunit 1
ENSG000000144589	0.683484	0.119491	2.65e-08	STK11IP	serine/threonine kinase 11 interacting protein

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Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG000000109819	7.942138	0.307032	1.48e-148	PPARGC1A	PPARG coactivator 1 alpha
ENSG000000177425	1.125073	0.089384	6.15e-36	PAWR	pro-apoptotic WT1 regulator
ENSG000000146457	0.498347	0.100948	1.54e-06	WTAP	WT1 associated protein
ENSG000000141646	0.746383	0.076126	5.74e-22	SMAD4	SMAD family member 4
ENSG000000131023	0.977974	0.083537	5.55e-31	LATS1	large tumour suppressor kinase 1
ENSG000000187953	1.436446	0.811389	7.09e-03	PMS2CL	PMS2 C-terminal like (pseudo-gene)
ENSG000000122512	0.416783	0.152727	7.55e-03	PMS2	PMS1 homolog 2, mismatch repair system component
ENSG000000003402	0.712126	0.067676	4.84e-25	CFLAR	CASP8 and FADD like apoptosis regulator
ENSG000000134982	0.625026	0.128837	2.46e-06	APC	APC regulator of WNT signaling pathway
ENSG000000103126	0.847708	0.105691	3.04e-15	AXIN1	axin 1
ENSG000000168646	0.975029	0.131043	1.76e-13	AXIN2	axin 2

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Table A2 – continued from previous page

Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG000000184281	1.844764	0.086904	2.84e-99	TSSC4	tumour suppressing subtransfer- able candidate 4
ENSG000000143514	0.820243	0.099733	4.20e-16	TP53BP2	tumour protein p53 binding pro- tein 2
ENSG000000180953	0.556704	0.159980	6.95e-04	ST20	suppressor of tumourigenicity 20
ENSG000000107816	0.433945	0.094758	1.13e-05	LZTS2	leucine zipper tumour suppressor 2
ENSG000000007341	-0.352469	0.091115	2.59e-04	ST7L	suppression of tumourigenicity 7 like
ENSG000000175274	-0.476562	0.101592	5.09e-06	TP53I11	tumour protein p53 inducible pro- tein 11
ENSG000000156427	2.770734	0.612832	5.21e-08	FGF18	fibroblast growth factor 18
ENSG000000170855	0.847339	0.081565	1.46e-24	TRIAPI	TP53 regulated inhibitor of apop- tosis 1
ENSG000000003402	0.712126	0.067676	4.84e-25	CFLAR	CASP8 and FADD like apoptosis regulator

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Table A2 – continued from previous page

Gene ID	log2FoldChange	lfcSE	padj	Gene Symbol	Gene Name
ENSG00000060762	0.501522	0.107309	9.25e-06	MPC1	mitochondrial pyruvate carrier 1
ENSG00000120053	0.884321	0.070487	5.89e-35	GOT1	glutamic-oxaloacetic transami- nase 1
ENSG00000076555	0.572981	0.108906	2.14e-07	ACACB	acetyl-CoA carboxylase beta
ENSG00000291148	1.418012	0.077548	7.36e-74	AZGP1P1	AZGP1 pseudogene 1

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