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**Synthesis and characterisation of bio-
inspired polymers for pH-mediated
intracellular delivery and therapeutic
applications**

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Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Imperial College London. The research reported herein was carried out under the supervision of Prof. Rongjun Chen between October 2019 and August 2024.

I can confirm that the research presented within this thesis was the result of my own work. Any results obtained in collaboration have been specifically indicated and acknowledged within the text. This thesis does not contain the material that has been previously accepted for the award of any qualification at Imperial College London or any other educational institutions.

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To my beloved family

“There is only one true heroism in the world: to see the world as it is, and to love it.”

— Roman Rolland

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Abstract

Effective intracellular delivery remains a significant challenge in the development of non-viral delivery systems. Endosomal entrapment, which results in enzymatic degradation of bioactive compounds in lysosomes, is one of the main barriers. This thesis outlines endeavours to develop biomimetic nanoscale systems tailored for pH-triggered intracellular delivery.

A library of innovative pH-responsive polymers was developed to mimic fusogenic viral peptides. They were synthesized by grafting fatty acids to the pendant carboxyl groups along the backbone of the pseudopeptide poly (L-lysine iso-phthalamide) (PLP) at varying degrees of grafting. The effects of hydrophobic chains on pH-responsive properties in aqueous solution, cell membrane destabilization activity, and in vitro cytotoxicity were examined. The synthesized and characterized 12-aminododecanoic acid-grafted PLP (PLP-ADA) exhibited minimal cytotoxicity toward different mammalian cell lines. The polymer-mediated translocation of model payloads into cells was demonstrated. The mechanism behind membrane destabilization was elucidated, showing the superior endosomal-escape ability of the polymer to release endocytosed materials into the cytoplasm. Under mildly acidic extracellular conditions, its intracellular delivery was significantly enhanced. The thesis systematically explores the effects of side chain, polymer concentration, treatment time, extracellular pH, and payload size on delivery efficiency across various cell types, demonstrating its broad applicability in drug delivery. Furthermore, PLP-ADA has shown the capability to enhance the internalization of the ribosome-inactivating protein Saporin, inducing cell death in cancer cells, suggesting its potential for cancer therapy applications.

In addition, the optimal pH-responsive polymer was utilized to facilitate intracellular loading of membrane-impermeable trehalose for cryoprotection of mammalian cells. This could avoid the use of toxic dimethyl sulfoxide (DMSO). With the assistance of PLP-ADA, efficient delivery of trehalose into red blood cells (RBCs) and an immortalized line Jurkat cells was achieved. The successful cryopreservation of cells was demonstrated, which addresses a crucial aspect in emerging fields of cell therapy and biobanking.

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List of Abbreviations

A

ADA Amino dodecanoic acid

ANOVA Analysis of variance

C

CAR Chimeric antigen receptor

CHO Chinese hamster ovary derived cell

CME Clathrin-mediated endocytosis

CPA Cryoprotective agents

Cy5 Cyanine5

D

DCC *N,N'*-dicyclohexylcarbodiimide

DCU Dicyclohexylurea

DDS Drug delivery system

DI water Deionised water

DIC Differential interference contrast

DLS Dynamic Light Scattering

DMAP 4-Dimethylaminopyridine

DMEM Dulbecco's modified Eagle's medium

DMF Dimethylformamide

DMSO Dimethyl sulfoxide

D-PBS Dulbecco's phosphate-buffered saline

DSC Differential scanning calorimetry

E

EDTA Ethylenediaminetetraacetic acid

F

FBS Fetal bovine serum

FT-IR	Fourier Transform Infrared
FITC	Fluorescein isothiocyanate
FITC-Dextran	Fluorescein isothiocyanate–dextran

H

HCl	Hydrochloric acid
HeLa	Henrietta Lacks cell line

K

kDa	Kilodalton
-----	------------

M

M β CD	Methyl- β -cyclodextrin
MFI	Mean fluorescence intensity
MW	Molecular weight
MWCO	Molecular weight cutoff

N

NaCO ₃	Sodium carbonate
Na ₂ HPO ₃	Disodium hydrogen phosphite
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
NDA	Decylamine
NMR	Nuclear magnetic resonance

P

PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
Phe	L-Phenylalanine
pH _p	pH value at the onset of precipitation
pH _h	pH at the onset of hydrophobic association
pK _a	Logarithmic acid disassociation constant

PLP	Poly(L-lysine <i>iso</i> -phthalamide)
PLP-NDA	poly(L-lysine <i>iso</i> -phthalamide) grafted with decylamine
PLP-ADA	poly(L-lysine <i>iso</i> -phthalamide) grafted with amino dodecanoic acid
PP75	PLP grafted with L-phenylalanine at a stoichiometric ratio of 75%
PVA	Polyvinyl alcohols

R

RBCs	Red blood cells
Rt	Retention time

T

T _g	Glass transition temperature
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Chapter 1

Introduction

This chapter presents an overview of the fundamental concepts and the current status of intracellular delivery systems based on polymers, with a focus on pH-responsive polymers. The challenges associated with traditional chemotherapy and the limitations to achieving efficient intracellular drug delivery are discussed. The chapter then summarizes the published work on pH-responsive polymers, especially synthetic polyanions for drug delivery applications. Furthermore, this chapter presents an introduction of the basic concepts of cell cryopreservation, cryoprotective agents with a focus on trehalose, and different intracellular trehalose delivery methods, particularly the polymer-based method. The research gaps are then summarized, followed by an introduction of the project objectives, novelties, and thesis outline.

1.1 Intracellular delivery

1.1.1 The historical development of drug delivery systems

Traditional forms of drug delivery, such as tablets, capsules, syrups, and ointments, often encounter challenges like low bioavailability and variable drug levels in the bloodstream, making it difficult to maintain a consistent release (Adepu and Ramakrishna, 2021). An ineffective delivery mechanism can undermine the entire treatment regimen. Furthermore, for optimal effectiveness and safety, it is critical that the drug is dispensed at a regulated rate and directed as accurately as possible to the intended site of action (Adepu and Ramakrishna, 2021). Drug delivery systems (DDSs) are required to deliver and release active pharmaceutical agents to achieve the intended therapeutic effects. Researchers across various fields persist in their pursuit of enhanced and safer methods for localized drug delivery to targeted areas, striving to augment precision and effectiveness (Wells et al., 2019). Over the years, extensive research on

biomaterials has yielded successful advancements in the creation of controlled DDSs (Ganta et al., 2008, Li et al., 2014). Delivering substances into cells is a crucial step in a wide range of areas, from medical treatments (e.g., nanomedicine and cell-based therapies) to basic scientific research (e.g., genome editing) and industrial processes (e.g., biomanufacturing).

In the 20th century, there was an introduction of advanced coating technologies using materials like keratin, shellac, sugar, enteric, and pearl coatings. However, the use of keratin and shellac proved ineffective due to their instability during storage and inadequate dissolution at the high pH levels found in the small intestine (Ezike et al., 2023). Addressing this issue, Malm and colleagues developed an enteric-coating material using polymeric cellulose acetate phthalate (Malm et al., 1951). This material was designed to dissolve at the slightly alkaline pH of the small intestine, making it well-suited for enteric controlled release applications.

The first generation of DDSs was highly productive, focusing on the development of a wide array of oral and transdermal controlled-release formulations for clinical use, as well as the establishment of controlled drug-release mechanisms. In 1951, Lipowski pioneered a patented oral sustained-release formulation. This involved coating pills with enteric polymers in a bead-like fashion, arranging the drug and coating in alternate layers to ensure a consistent and slow release of the medication (Hillery and Park, 2016). This technology was further refined in 1952 by Smith, Klein Beecham, and French (SKF) with the development of Spansule technology (Lee and Li, 2010). This oral formulation was designed for predetermined drug release, controlling the gradual release of the medication. It consisted of hundreds of drug-loaded micro pellets, each coated with varying thicknesses of natural water-soluble wax. Upon ingestion, the outer capsule quickly disintegrated, allowing the waxy coating to gradually dissolve in the gastrointestinal tract and release the drug-loaded beads (Yun et al., 2014). This innovation significantly improved patient compliance and convenience by reducing the frequency of dosage, leading to widespread popularity. Later advancements replaced the wax with more reproducible synthetic polymers (Zhang et al., 2017).

In 1955, Jatzkewitz reported the first nanoparticle therapeutic, preparing the inaugural polymer-drug conjugate. The 1960s saw the discovery of the first nanotechnology in the form

of liposomes, or lipid vesicles. These developments marked the beginning of nanocarriers, with polymer-drug conjugates and liposomes being key innovations (Bangham and Horne, 1964, Bangham et al., 1965). During this decade, the ALZA Corporation specialized in the targeted and controlled release of drugs, focusing on precise delivery rather than drug creation (Shen and Burgess, 2011). In 1972, Scheffel and his team introduced the first protein-based microspheres. By 1976, Peter Paul Speiser's research group utilized micelle and emulsion polymerization techniques to create drug-loaded nanoparticles and microcapsules (Birrenbach and Speiser, 1976). In 1977, Couvreur and colleagues reported on the lysosomotropic effects of nanoparticles and produced the first rapidly biodegradable acrylic nanoparticles (Couvreur et al., 1977).

The second generation of DDSs, though impressive, did not yield the anticipated clinical results (Park, 2016). Researchers during this era focused on developing DDSs with consistent drug release rates, self-regulating long-term depot formulations, and nanotechnology-based formulations, particularly those involving nanoparticles (Ezike et al., 2023). This period saw the creation of long-term depot-sustained drug-release formulations for peptide/protein drugs, alongside the development of smart polymers and hydrogels that responded to physiological changes like pH, temperature, electric fields, and glucose levels (Ye et al., 2010). There were also efforts to develop targeted nanotechnology-based DDS for cancer treatment, using biodegradable polymers in nanoparticle structures such as polymeric micelles, chitosan, lipids, and dendrimers. These nanoparticles were tailored for direct body administration, aiming to enhance drug accumulation at a targeted site of action. Although these DDSs showed high efficacy in controlling or inhibiting tumour growth in animal models, only a few received Food and Drug Administration (FDA) approval (Zhang et al., 2017, Park, 2014).

1.1.2 Drug delivery systems for controlled release

The controlled release technology marks the advent of the third generation of DDSs. To address the limitations of traditional drugs, the controlled DDSs aiming to enhance the effectiveness of therapeutic agents (Park, 2014, Safari and Zarnegar, 2014). To be effective and successful, these systems must overcome both physicochemical and biological barriers, which were

challenges associated with earlier drug delivery systems. Physicochemical challenges arise from issues such as poor water solubility, the high molecular weight of therapeutic proteins and peptides, and the complexity of achieving targeted and controlled drug release. These systems play a crucial role in improving drug solubility by creating hydrophobic/hydrophilic environments and shielding drugs from degradation prior to reaching the targeted tissue (Allen and Cullis, 2004). On the other hand, biological barrier challenges are linked to problems in systemic drug distribution (Zhang et al., 2017, Yun et al., 2015). However, DDSs enable controlled release of drugs at predetermined rates and intervals, modify the pKa of drugs, and significantly reduce rapid renal clearance, ultimately necessitating lower dosages and minimizing undesirable side effects (Shi et al., 2017a). These modern DDSs have been developed with enhanced features, including reduced particle size, greater permeability and solubility, improved efficacy, targeted delivery to specific sites, enhanced stability, reduced toxicity, and extended drug release. These improvements offer a substantial enhancement in the performance of therapeutic agents compared to traditional dosage forms (Faheem and Abdelkader, 2020, Rahman et al., 2020). In recent years, a multitude of DDSs has been developed, divided into two primary categories as illustrated in Figure 1-1: viral and non-viral vectors (Allen & Cullis, 2004b; Park, 2014; Safari & Zarnegar, 2014).

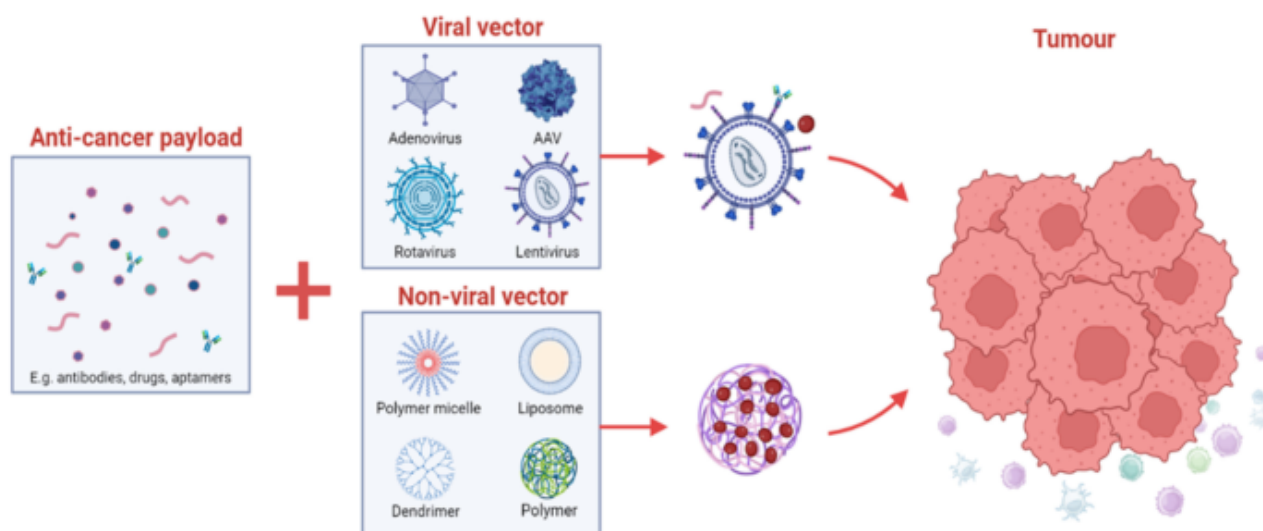


Figure 1-1. Viral and non-viral vectors for drug delivery. AAV stands for adeno-associated virus (Dogbey et al., 2023). Copyright 2023, with permission from Springer Nature International.

1.1.2.1 Viral vectors

Engineered viral vectors have varied applications in medical biotechnology and biomedical sciences, serving purposes such as drug and gene delivery, and vaccines/nanocarriers. (Bruckman et al., 2016, Zabaleta et al., 2021, Qian et al., 2020, Varanda et al., 2021, Ylösmäki and Cerullo, 2020). Viruses are recognized as inherent carriers of nucleic acids due to their ability to efficiently invade intracellular organelles and infect host cells, making this characteristic a pivotal aspect utilized for drug delivery (Elzoghby et al., 2012). To escape from endocytic vesicles post-internalization, viruses have evolved fusogenic peptides located at the amino terminus of fusogenic proteins. These peptides typically undergo a pH-dependent conformational change in response to the low pH within endosomes, enabling them to achieve a fusogenic state (Epan, 2003). Viral fusogenic peptides play a crucial role in facilitating endosomal escape by either fusing with endosomal membranes in the case of enveloped viruses or lysing and forming pores on the endosomal membrane for non-enveloped viruses, depending on the viral type (White et al., 1990). The drug payload can be incorporated, enclosed, assimilated, or linked to the inner and outer interfaces of the coat protein through diverse chemical methods and by connecting to different functional groups inherent in the protein structure (Elzoghby et al., 2012).

Commonly employed virus vectors for nucleic acid delivery include lentivirus, poxvirus, adenovirus, adeno-associated virus, retrovirus, human foamy virus (HFV) and herpes virus (Lundstrom, 2003). Being a variant of simple retroviruses, lentiviruses derived from HIV-1 possess the ability to hijack host-mediated machinery to facilitate an effective nuclear import across the intact nuclear membrane (Lewis and Emerman, 1994). Despite their popularity, recombinant retroviruses, face limitations in *in vivo* applications due to their relatively low transfection efficiency (McTaggart and Al-Rubeai, 2002). Adenovirus as the most effective gene delivery system across diverse cell and tissue types among the currently available viral vectors, with a notable increase in both the number and efficiency of its applications in gene delivery since its initial development (Kaliberov and Buchsbaum, 2012). However, the use of viral vectors for drug delivery encounters challenges such as high immunogenicity, inflammatory responses, toxicity, and difficulties in large-scale production, even when viruses are modified or inactivated (Dogbey et al., 2023)

1.1.2.2 Non-viral delivery systems

The non-viral vector stands out due to its minimal immunogenicity and cost-effectiveness in reproduction (Lee and Kim, 2005), and it lacks restrictions in packing DNA size, allowing for easy modification with ligands for precise cell targeting (Jones et al., 2014). Comprising synthetic organic and inorganic materials with specially engineered characteristics, non-viral vectors aim to efficiently transport genetic materials or biotherapeutics into various cells, tissues, or even whole organs to enhance or treat the health conditions of patients (Pandya et al., 2014) (Yin et al., 2014). The rapid advancement of non-viral delivery platforms towards clinical applications is evident, tailored to address diverse diseases such as COVID-19, genetic disorders, and malignant neoplasms. This includes the delivery of therapeutic oligonucleotides and aptamers like RNA, DNA, siRNA, mRNA, and ribozymes, with increased research investments and developments in this subfield observed in recent years (Dzuovor et al., 2022, Pan et al., 2021, Santana-Armas and de Ilarduya, 2021).

1.1.2.2.1 Lipid-based systems

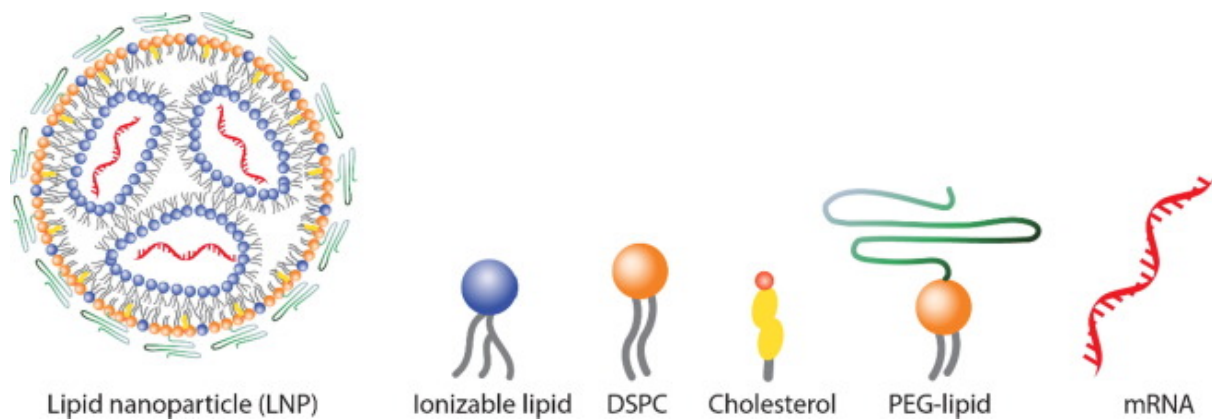


Figure 1-2. Basic depiction of a lipid nanoparticle and its constituent elements. The lipid nanoparticle has the capability to encapsulate various nucleic acid cargo types, including, but not restricted to, DNA, ASOs, siRNA, microRNA, and mRNA (Albertsen et al., 2022). Copyright 2022, with permission from Elsevier.

Lipid nanoparticles (LNPs) comprise multi-component lipid systems, typically consisting of phospholipids, cholesterol, ionizable lipids, and PEGylated lipids as shown in Figure 1-2. There are two primary types of carriers: liposomes and lipid nanoparticles. Although both lipid nanoformulations share a similar design, they exhibit slight variations in composition and function. Liposomes, also known as "spherules," discovered and defined by Alec Bangham in 1963, are spherical or multi-layered vesicles with a lipid bilayer structure formed by the self-assembly of diacyl-chain phospholipids in aqueous conditions (Bangham, 1963, Düzgüneş and Gregoriadis, 2005). Their amphipathic nature allows liposomes to trap both hydrophilic and hydrophobic compounds, capturing hydrophilic molecules in the aqueous core and entrapping hydrophobic compounds in the bilayer membrane (Bozzuto and Molinari, 2015). Liposomes can be zwitterionic, containing an equal number of positively and negatively charged functional groups (Nsairat et al., 2022). Preparation of liposomes involves using naturally occurring or synthesized double-chain lipids and sterols, resulting in a size range of 50–450 nm for medicinal applications (Olusanya et al., 2018) .

In contrast to liposomes, LNPs may not possess a continuous bilayer some exhibit intricate micelle-like structures of ionizable lipids enclosing molecules in a non-aqueous core. LNPs typically comprise phospholipids, cholesterol, ionizable lipids, and a PEGylated lipid for surface functionalization (Thi et al., 2021). Cationic lipid-based structure enables the encapsulation of various nucleic acids like DNA and RNA due to the positive charge acquired at acidic pH, making them widely utilized as non-viral gene delivery systems. Despite presenting a neutral charge at physiological pH, reducing the risk of toxic effects compared to positively charged lipids like liposomes (Editorial, 2021), LNPs are efficiently taken up by cells through endocytosis. Their ability to form destabilizing non-bilayer structures at acidic pH is crucial for endosomal escape, facilitating the release of cargo such as RNA into the cytoplasm (Schlich et al., 2021). Additionally, LNPs often incorporate a helper lipid for improved cell adhesion, cholesterol to bridge lipid gaps, and polyethylene glycol (PEG) to prevent opsonization and subsequent phagocytosis in circulation (Suk et al., 2016).

Years of dedicated research have led to the development of contemporary LNP technology, now standing as the most clinically advanced non-viral gene delivery system. Building on the success of Onpattro®, the pioneering RNA interference therapeutic approved by the FDA in

2018, LNP technology has evolved to facilitate gene editing, protein replacement, and vaccine applications (Adams et al., 2018).

1.1.2.2.2 Polymer-based systems

Polyplexes are created through electrostatic interactions, where the negative charges from nucleic acids interact with the positive charges of cationic polymers, enabling the effective delivery of DNA or drugs to cells (Santana-Armas and de Ilarduya, 2021). Polyethyleneimine (PEI), a synthetic cationic polymer characterized by its elevated amine density and substantial buffer capacity, is considered a promising approach for gene delivery (Boussif et al., 1995, Rehman et al., 2013).

Micelles and polymersomes are both nanocarriers formed from amphiphilic copolymers in aqueous media. Their distinct structures and properties make them suitable for various applications. Polymeric micelles are self-assembling structures with a core-shell configuration, created by various copolymers in the presence of an aqueous system. These micelles can encapsulate medications within their hydrophobic core, supported and stabilized by the hydrophilic shell, which also facilitates the carriage of molecules like DNA or siRNA (Tiwari and Rohiwal, 2019, Ghosh and Biswas, 2021). With a compact size, typically under 100 nm in diameter, they promote blood circulation, tissue penetration through the enhanced permeability and retention (EPR) effect, and cellular uptake. Additionally, the hydrophilic surface enhances water solubility, contributing to the effective administration of the drug (Rana and Sharma, 2019). Polymersomes are nanocarriers with an aqueous core and a polymeric bilayer membrane, making them suitable for next-generation drug delivery systems (Thambi et al., 2012). They can transport both hydrophilic and hydrophobic molecules and can be targeted to specific cells through surface functionalization with ligands. With enhanced toughness, reduced membrane permeability (Discher et al., 1999), and low immunogenicity (Anajafi and Mallik, 2015), polymersomes are extensively researched for drug delivery (Anajafi and Mallik, 2015, Hu et al., 2017), gene and protein delivery (Onaca et al., 2009), imaging (Tanner et al., 2011), and diagnostics (Brinkhuis et al., 2011).

Dendrimers are synthetic macromolecules characterized by a spherical, tree-like branched structure, uniquely constructed around a linear polymeric core, exhibiting biomolecule-like properties, high physicochemical customizability, and low polydispersity (Tiwari and Rohiwal, 2019). These dendrimers consist of three distinct topological components: a central core, multiple interior layers with repeated units, and numerous peripheral functional groups (Thakore et al., 2019). Synthesized algorithmically in a stepwise fashion, each chemical reaction repetition results in a "higher generation (G) molecule," progressively developing branching layers of "generations" with increased molecular weight and distinct functional end-groups. PAMAM dendrimers, widely employed in drug delivery systems, were initially introduced by Tomalia et al. in 1985 (Tomalia et al., 1985). They described dendrimers as possessing "reactive end groups," including amines and amides, enabling controlled molecular weight construction, regulated topology or branching, and flexibility in design through modifications to the terminal end (Tomalia et al., 1985). The outer branches of these dendrimers have the capability to end with diverse functional groups, including amines, hydroxides, aldehydes, methoxycarbonyl, tert-butyloxycarbonyl, and various other groups (Abedi-Gaballu et al., 2018).

Despite the development of various viral and nonviral gene delivery systems over the last three decades, their inherent disadvantages, such as complex production process and limited transgene capacity of the particles, immunogenicity, and toxicity have imposed limitations on their clinical application (Tiwari et al., 2012). A universal delivery system capable of gene therapy across diverse cell types in vitro and in vivo without constraints and side effects has yet to be realized. While certain delivery systems show promise for specific cells or tissues, the overall development of successful nonviral delivery systems for in vivo applications is still in its early stages, requiring further efforts. Challenges and barriers persist in this area, which will be explored in the next section.

1.1.3 Challenges for intracellular delivery

Examining the perspective of drug delivery barriers, the stages outline the movement of a therapeutic agent from the extracellular to intracellular: (a) binding to the cell membrane,

facilitated by non-specific or specific interactions, promoting subsequent internalization; (b) internalization occurring through endocytosis, with subsequent release from endosomes, membrane fusion, or diffusion; and (c) transportation within the cytoplasm towards intracellular organelles or the nucleus. A fraction may be released back into the extracellular space through exocytosis. These processes are intricately regulated by various factors, with nanoparticles having additional and distinctive determinants (Au et al., 2016). As shown in Figure 1-3, the internalization mechanisms encompass (a) non-endocytic pathways, involving the diffusion/active transport of small molecule therapeutics across cell membranes and the fusion of nanoparticles with the cell membrane, and (b) endocytic pathways, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin-independent and caveolae-independent endocytosis, and macro-pinocytosis. In endocytic pathways, the cargo is presented to early endosomes, subsequently sorted into recycling endosomes, either directly recycled back to the cell membrane or indirectly through the endosome recycling center, or late endosomes that fuse with lysosomes. This sorting process may lead to the budding of early endosomes, forming intraluminal vesicles, a component of multivesicular bodies that either mature into late endosomes or fuse with the cell membrane. This fusion results in the release of intraluminal vesicles and their contents as exosomes (Au et al., 2016).

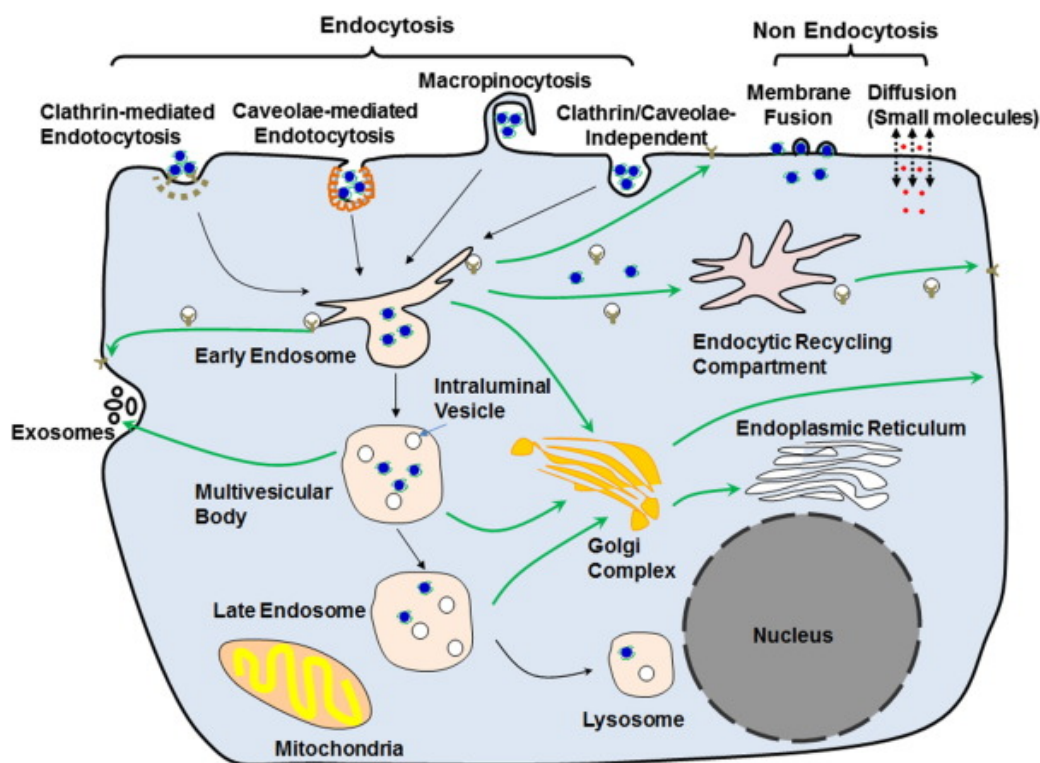


Figure 1-3. Internalization and intracellular trafficking of therapeutics and nanoparticles involve diverse mechanisms (Au et al., 2016). Copyright 2015, with permission from Elsevier.

1.1.3.1 Intracellular barriers

The cell membrane functions as a barrier to block external environments from infiltrating cells and also impedes the treatment of cells with drugs from outside the cell. These modern DDSs are engineered with enhanced characteristics such as smaller particle size, improved permeability and solubility, increased efficacy, precise targeting to specific sites, better stability, lower toxicity, and sustained delivery capabilities. Recent years have seen remarkable advancements in the development of DDSs, particularly those utilizing organic, inorganic, and hybrid nanoparticles for targeted drug delivery. Most macromolecular drugs and genes exert their effects intracellularly, emphasizing the crucial requirement for their carriers to traverse the cell membrane. The internalization of NPs relies heavily on their interaction with the cell membrane, with surface charge, hydrophobicity, and size identified as significant factors in various studies (Chen and Bothun, 2014). Charged particles tend to exhibit increased interactions with the membrane, while uncharged ones, like PEGylated NPs, experience reduced interactions due to electrostatic interactions and steric hindrance, potentially leading to NP clustering around the membrane and impeding the entry of extracellular barrier (Verma and Stellacci, 2010, Cahn and Duncan, 2022). Proteins adsorbed onto the NP surface based on their charge may influence their entry into the cell through receptor-mediated endocytosis. Size plays a crucial role in intracellular entry, with particles smaller than 200 nm preferentially internalized via clathrin-mediated endocytosis, while larger particles beyond 200 nm shift toward caveolin-mediated endocytosis (Fleischer et al., 2013). The internalization process is energy-dependent, and cholesterol depletion has been observed to inhibit particle uptake. Active targeting of NPs to cancer cells, which often overexpress various cell-surface receptors, is a promising approach for enhanced internalization. However, simply increasing the density of targeting ligands may not always result in better internalization, as high-affinity binding can lead to a "binding barrier" on peripheral cancer cells, hindering subsequent NP penetration. Careful consideration of receptor recycling kinetics is essential when designing ligand density and NP dosing regimens to avoid receptor oversaturation (Ciechanover et al., 1983, Zorko and Langel, 2005, Paulos et al., 2004, Verdurmen et al., 2013).

1.1.3.2 Endosomal escape

Although various methods have been identified for intracellular drug delivery, overcoming endosomal escape remains a significant hurdle for therapeutic molecules to access the cytosol. Upon internalization, the NP must navigate intracellular trafficking to reach its target and release its cargo. Endosomal vesicles play a crucial role in transporting macromolecules to various intracellular sites such as the Golgi apparatus, endoplasmic reticulum, nucleus, mitochondria, and lysosomes. The predetermined trafficking of cargo is signal-dependent and forms the foundation of intracellular drug delivery (Trowbridge et al., 1993). Various pathways and processes, including clathrin-dependent endocytosis, caveolin-dependent endocytosis, macropinocytosis, and phagocytosis, caveolae- and clathrin-independent endocytosis, with different NPs being internalized through distinct pathways (Sahay et al., 2010). However, many of these pathways may lead to trafficking to non-target organelles or the lysosome, where the NP is subsequently degraded, posing a significant challenge for the delivery of more labile drugs like genes and peptides. Cationic lipids and polymers, such as 1,2-di-O-octadecenyl-3-trimethylammonium propane chloride salt (DOTMA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), polyethyleneimine, and Polyamidoamine (PAMAM) dendrimers, are extensively used for intracellular drug delivery by promoting endosomal escape (Ting and Wang, 2011). These agents can interact with the endosomal membrane, releasing their contents into the cytoplasm. As the endosome matures and acidifies internally, a variety of NPs exploit this property to facilitate endosomal escape into the cytoplasm. The use of fusogenic lipids like DOPE is popular, as they transition from a bilayer to a hexagonal phase based on pH changes, facilitating fusion with the endosomal membrane. The fusogenic lipids, when conjugated to low molecular weight polyethyleneimine, enhance endosomal escape for the delivery of DNA and siRNA (Sawant et al., 2012, Navarro et al., 2011). Receptor-mediated endocytosis through active targeting of NPs is another approach to improve intracellular drug delivery. For instance, transferrin-targeting of liposomal ceramide enhances lysosomal compartmentalization, while folic acid targeting of liposomal doxorubicin allows for nuclear accumulation, inducing apoptosis through DNA intercalation (Whitehead et al., 2009).

1.2 pH-responsive polymers

In the past two decades, there has been a growing interest in using stimuli-responsive smart nanomaterials as effective carriers for drugs and genes (Karimi et al., 2016). The development of polymeric biomaterials that respond to stimuli has proven to be an efficient method for delivering medications. In line with continuous efforts to reduce side effects, these materials are engineered to release therapeutic agents in the presence of specific triggers, which may be chemical, physical, or biological. The responsive nature of these advanced biomaterials can enhance the precision of drug release in terms of both location and timing. These smart nanoparticles are designed to respond to various environmental triggers such as changes in temperature (Ding et al., 2013), pH (Tomar et al., 2014, Dong et al., 2014), light (Chiang and Chu, 2015), electric and magnetic fields (Indermun et al., 2014, Karthik et al., 2013), ultrasound (Cavalieri et al., 2013, Rapoport et al., 2009), mechanical stress, and biochemical stimuli (Lai et al., 2010). Some are even designed to react to a combination of stimuli (Wells et al., 2019). Due to their ability to react nonlinearly to these external factors, they are often referred to as 'smart' or 'intelligent' materials (Ballauff and Lu, 2007). Their unique properties have led to their widespread use in various fields, including drug delivery (Liu et al., 2014), catalysts (Bergbreiter et al., 1998), and biosensors (Kim et al., 2011).

Different areas of the gastrointestinal tract, various organs, tissues, and cellular compartments exhibit distinct pH values. For instance, the stomach has a pH range of approximately (1.5–3.5), the small intestine falls within the range of approximately (5.5–6.8), and the colon maintains a pH range of approximately (6.4–7.0) (Balamuralidhara et al., 2011). In normal tissues and blood, the extracellular pH is typically around 7.4, while many tumors exhibit a mildly acidic extracellular environment (6.3–6.9) due to elevated glycolysis rates (Bogdanov et al., 2022, Gatenby et al., 2006). For acidic cellular compartments, the pH can decrease to 6.0–6.8 in early endosomes, 5.0–6.0 in late endosomes, and 4.5–5.0 in lysosomes (Matsuzaki et al., 1997, Scott et al., 2014). The diverse pH environments across cellular compartments, organs, and tissues lead to tailored physiological responses for pH-dependent drug delivery.

pH-sensitive polymers that can exchange protons from their molecules with aqueous solution in abnormal pH conditions undergo structural changes, making them suitable for such applications. For instance, poly(ϵ -caprolactone) (PCL) nanoparticles have been adapted to enhance tamoxifen delivery to estrogen receptor-positive breast cancer (Chawla and Amiji, 2002). Copolymeric systems that are pH-responsive can be created by integrating acid-functionalized groups into the polymer backbone, which alters their conformation and solubility with pH shifts, or by using acid-labile bonds that disintegrate to release chemotherapy agents. For example, chitosan-based systems have been modified to release tumor necrosis factor alpha (TNF α) at tumor sites upon amino group protonation (Ding and Guo, 2022). There has been a surge in research on nanoformulations made from natural polysaccharides and modified for pH-sensitive drug delivery. Chen et al. crafted cellulose-based hydrogels with incorporated pH-sensitive diblock copolymer micelles for sustained drug delivery (Chen et al., 2019). Luo et al. generated nanoparticles from amphiphilic stearic acid and carboxymethyl chitosan that carried paclitaxel, using pH to induce effective cancer cell apoptosis (Luo et al., 2019). Saha et al. devised a pH-activated auto-fluorescent polymeric nanoplatfrom to transport the non-fluorescent aromatic nitrogen mustard chlorambucil (CBL) to cancerous tumors (Das et al., 2020, Saha et al., 2018). Research conducted by Mishra has demonstrated that HPMC-AS (a mixture of acetic acid and monosuccinic acid esters of hydroxypropylmethyl cellulose) and Eudragit-P4135 F (Methacrylic acid) can be developed into nanoparticles exhibiting traits such as acidic resistance or enteric coating, ensuring targeted drug delivery to the colon (Ashish, 2018, Mishra, 2013). Mishra explored the formulation of Metoprolol succinate with HPMC-AS and Eudragit-P4135 F, resulting in pH-sensitive nanoparticles suitable for a colon-specific targeted drug delivery system (Gupta and Damodharan, 2019). This approach is particularly beneficial for pharmacological substances that face degradation or have minimal absorption in the stomach acidic medium and the intestinal pH. Hence, pH variations are key to developing advanced DDSs. While pH is a common factor in intelligent DDSs, combining it with other stimuli such as temperature or redox reactions can ensure more precise targeting (Das et al., 2020).

pH-responsive polymers undergo structural and property alterations such as changes in surface activity, chain structure, conformation, solubility, and configuration in response to variations in environmental pH. These pH-sensitive polymers typically incorporate acidic or basic groups capable of accepting or donating protons, reacting to fluctuations in the surrounding pH. In

particular, pH-responsive polymers have garnered considerable attention (Deirram et al., 2019) due to their ability to facilitate endosomal escape, activated by the pH disparities between the extracellular physiological environment at 7.4 and the acidic endosomal milieu, 6.0–6.8 in early endosomes, 5.0–6.0 in late endosomes, and 4.5–5.0 in lysosomes (Matsuzaki et al., 1997, Scott et al., 2014). Based on their ionizable groups, pH-responsive polymers are broadly categorized into two main types: polycations and polyanions (Bazban-Shotorbani et al., 2017).

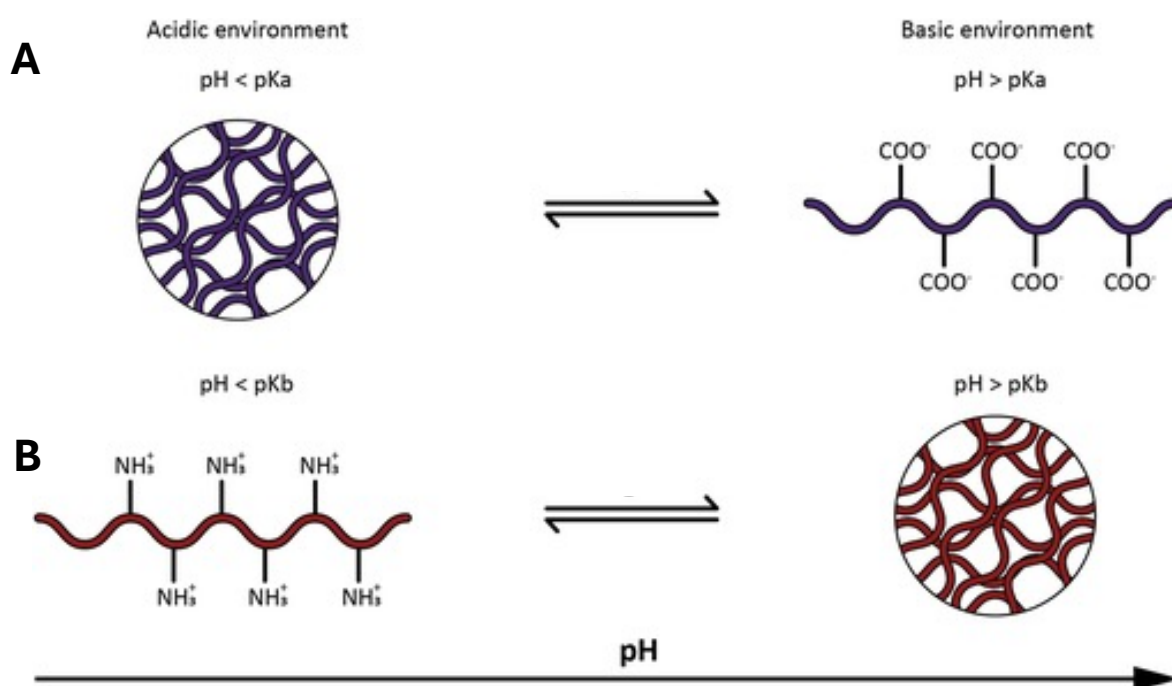


Figure 1-4. Polymeric chain states of (A) a polyacid and (B) a polybase depending on the ionization degree (Ofriam et al., 2021). Copyright 2021, with permission from John Wiley & Sons Ltd.

Figure 1-4 illustrates how a polyacid (polyanion) and a polybase (polycation) changes their conformation in response to the ionization degree of their ionizable groups. When the pH is above the pK_a ($\text{pH} > \text{pK}_a$), a polyacid is in an expanded state, while the polymeric chains collapse when the pH falls below the pK_a . On the other hand, for a polybase, the polymeric chains tend to collapse when the pH exceeds its pK_b value ($\text{pH} > \text{pK}_b$), while they expand in conditions where the pH is less than the pK_b (Ofriam et al., 2021).

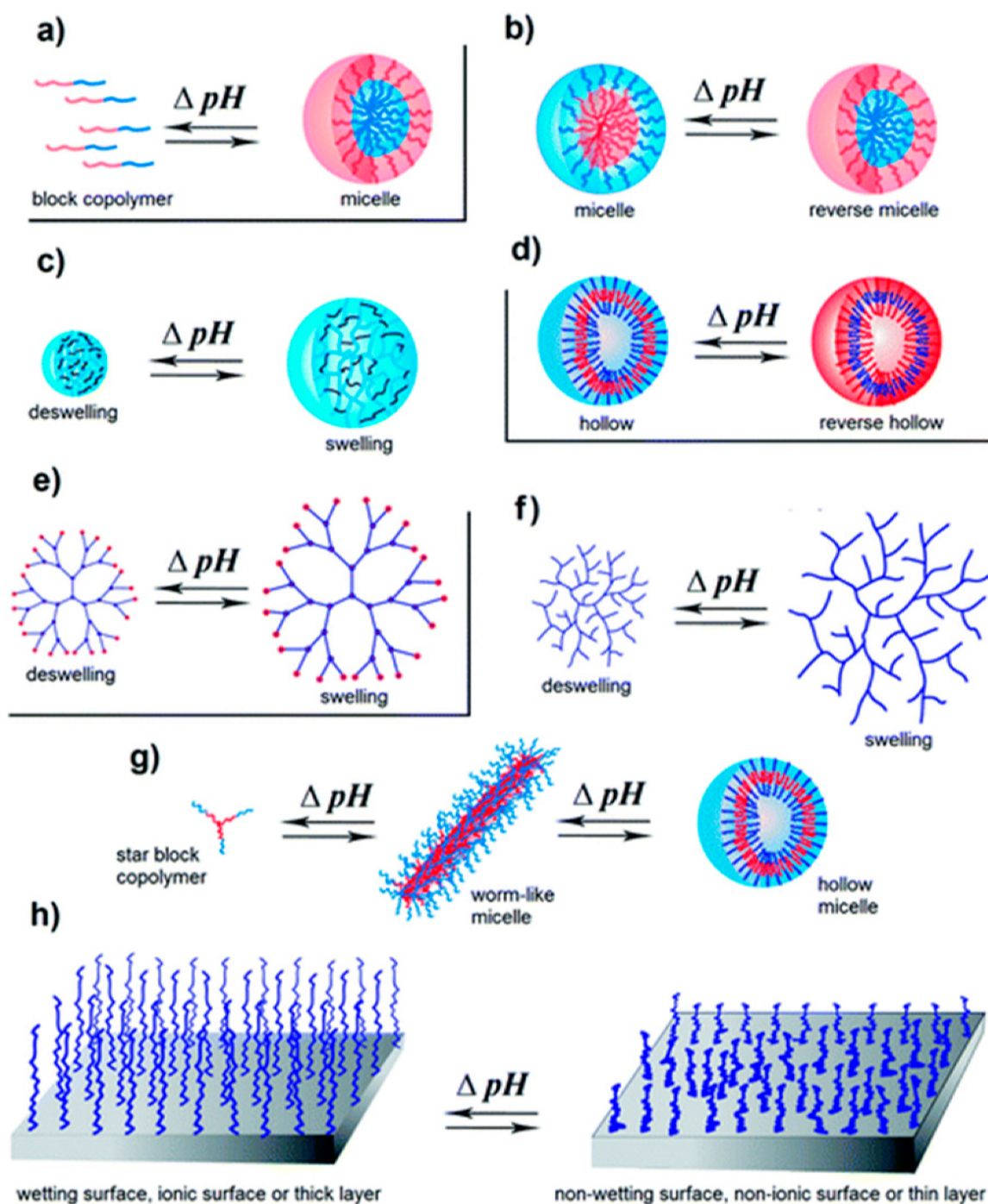


Figure 1-5. pH-responsive polymers of different architectures: (a) unimer–micelle, (b) micelle–reverse micelle, (c) nanogels or microgels, (d) hollow–reverse hollow, (e) dendrimer, (f) hyper-branched, (g) micelle morphology changes (from worm-like to hollow), and (h) polymer brushes (Kocak et al., 2017). Copyright 2017, with permission from Royal Society of Chemistry.

Figure 1-5 illustrates the variety of behaviors displayed by polymers with different architectures when subjected to pH changes. Polymers that are sensitive to pH levels can possess various structural forms, such as linear, branched, or cross-linked networks (Kocak et al., 2017). Their reactions to the environment of a solution and their self-organizing tendencies can vary with their structure. For instance, alterations in pH can lead to the (de)protonation of active groups within the polymer chains, which, in homopolymers, can result in phenomena like flocculation, the contraction or elongation of chains, and precipitation. Such pH shifts can also trigger the self-assembly of structures, including micelles, unimers, gels, vesicles, and processes like swelling or shrinking. Polymers with pH-responsive segments, including block copolymers, branched copolymers, and star copolymers, exhibit surfactant-like behavior when the pH is altered. Structures resembling hydrogels and dendrimers exhibit swelling or deswelling in response to pH changes. Polymer-modified surfaces provide the opportunity to create ionic interfaces and form layers of varying thickness in response to pH shifts.

1.2.1 Polycations as pH-responsive polymers

Polycations incorporate weak basic functional groups like amines, imidazole, and pyridine, undergoing a positive charge transition when the pH falls below their pK_a (the negative base -10 logarithm of the acid dissociation constant of a solution) (Hu et al., 2014, Han et al., 2013, Yu et al., 2009, Mitsukami et al., 2006, Song et al., 2016, Dan et al., 2013). They may be linear, dendritic or hyperbranched. These polymers undergo deprotonation at pH values above their pK_b (the negative base -10 logarithm of a base solution dissociation constant), while become positively charged at pH values below their pK_b . These polymers, including polyethylenimine (PEI), Poly (N, N' -dimethyl aminoethyl methacrylate) (PDMAEMA) and poly[(2-diethylamino)ethyl methacrylate] (PDEA) can mitigate endosomal acidification through protonation, inducing osmotic swelling of endosomes and ultimately leading to endosomal rupture. Figure 1-6 shows the well-documented "proton sponge" effect induced by polycations (Bus et al., 2018; Vermeulen et al., 2018) in three steps. (1) Upon the entry of polyplexes into cells through endocytosis, they localize within endosomal vesicles. (2) As these vesicles mature, V-ATPase proton pumps on the membrane actively transport protons into the endosomal lumen. Given the high buffering capacity of the polymers involved in the proton sponge hypothesis, they effectively bind these protons, preventing excessive acidification of the endosome. (3)

Consequently, the proton pumps intensify proton translocation in an effort to reduce the endosomal pH. This process triggers the entry of chloride ions to maintain charge balance, resulting in a higher ionic concentration and an influx of water to maintain osmolarity (the number of particles or the concentration of a specific solute or solutes per liter of solvent.). The influx of water generates osmotic pressure, causing the endosome to swell. Combined with polymer swelling due to internal charge repulsion, this swelling eventually leads to endosomal rupture, releasing its contents into the cytosol.

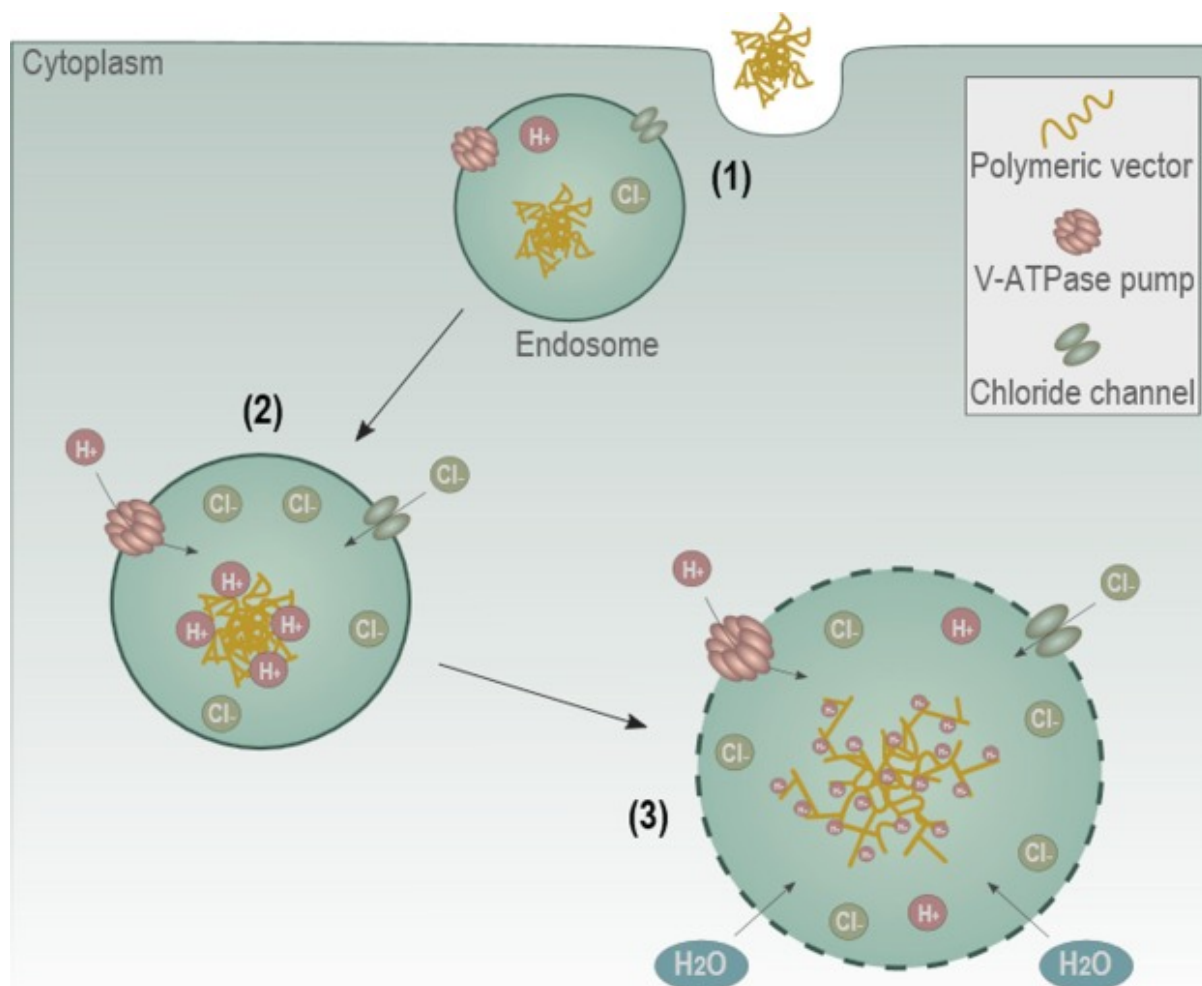


Figure 1-6. The ‘proton sponge hypothesis proposed by Behr and colleagues (Vermeulen et al., 2018). Copyright 2018, with permission from Elsevier.

In 1995, Boussif and colleagues first documented the efficacy of the cationic polymer PEI, attributing its success to a significantly high charge density. This is due to the placement of ionized groups directly along the chain backbone (Boussif et al., 1995). PEI demonstrated notable success in achieving high transfection efficiencies and served as a gene delivery vector

in both in vitro and in vivo settings (Boussif et al., 1995). Since then, it has been widely utilized in crafting a diverse array of more advanced delivery vectors (Neu et al., 2005, Lungwitz et al., 2005). Linear chain PEI is a polyamine featuring alternating NH groups and short aliphatic spacers (CH_2CH_2). Despite a smaller number of amine groups, these can efficiently accept protons in acidic environments. At lower pH levels, the conformation of linear polyethylenimine (PEI) undergoes elongation, whereas at higher pH levels (non-protonated states), it assumes a coiled structure (Sharma et al., 2011). Considering the pKa values of PEI (4.5 for primary and 6.7 for secondary amine groups), this polymer emerges as a suitable candidate for gene delivery due to its proton buffering capacity (Choudhury and Roy, 2013, Ziebarth and Wang, 2010).

Poly(2-(diethylamino)ethyl methacrylate) (PDEAEMA), a well-known charge-shifting cationic polymer with a pKa around 7, is frequently used in drug delivery systems due to its ability to change charge state under physiological conditions (Su et al., 2011, Hu et al., 2007, Chen et al., 2016, Yang et al., 2017). The transition in pH aligns with the shift from the extracellular environment to endosomes, offering a suitable range for intracellular release. This pH transition can be fine-tuned by the substituents on the amine group. When applied to nanoparticles, this transition from hydrophobic to hydrophilic conditions has been demonstrated to efficiently induce nanoparticle disassembly and the subsequent release of therapeutic payloads (Lomas et al., 2007).

PEI is regarded as the gold standard due to its buffering properties (with only 25% of amine groups protonated at physiological pH). However, PEI is cytotoxic, exhibiting an IC₅₀ (half-maximal inhibitory concentration) of approximately 30 $\mu\text{g/ml}$ (Neu et al., 2005), and formulate PEI for systematic studies on gene transfection is a non-trivial task. Recently, attention has shifted towards poly(N, N'-dimethyl aminoethyl methacrylate) (PDMAEMA) as a non-viral gene delivery system. It possesses buffering capacity and lower cytotoxicity, with an IC₅₀ of around 40 $\mu\text{g/ml}$ and a pKa of 7.5 (Agarwal et al., 2012). The preparation of this polymer is straightforward, involving the radical polymerization of the corresponding vinyl monomer (Agarwal et al., 2012). It features functional tertiary amine groups, refer to Figure 1-7. These amine groups undergo positive ionization by accepting protons when the pH is below their

respective pKa values. Conversely, they deprotonate and release these protons when the pH exceeds their pKa values (Orakdogan, 2011).

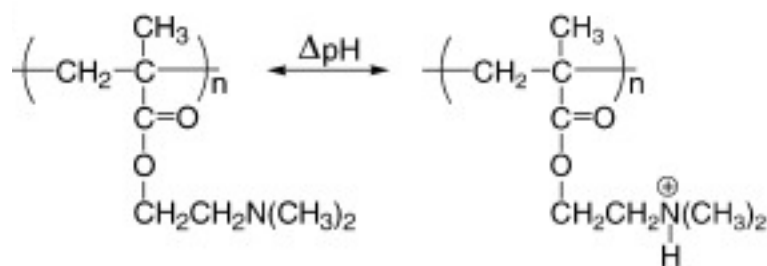


Figure 1-7. pH-Responsive protonation/deprotonation of Poly (N,N'-dimethyl aminoethyl methacrylate) (PDMAEMA) (Bazban-Shotorbani et al., 2017). Copyright 2017, with permission from Elsevier.

In vitro, these polycationic delivery systems demonstrate significant potential, surpassing the effectiveness of other systems such as gene delivery system. However, polycationic delivery systems encounter obstacles prior to reaching their intended target site, or when studied in vivo, their performance proves to be markedly disappointing. This comprehensive polyanionic barrier, as shown in Figure 1-8, offers excellent protection against toxic xenobiotics with a polycationic nature. However, it simultaneously hinders polycationic delivery systems presenting a challenge known as the polycation dilemma. The skin, at physiological pH, serves as a negatively charged membrane due to a higher prevalence of protein amino acid residues bearing negative charges, such as carboxylic groups, in contrast to positive charges like amine moieties. This results in a greater ease of movement for anionic carrier systems across the skin compared to cationic ones (Lee et al., 2013, Gupta and Rai, 2017).

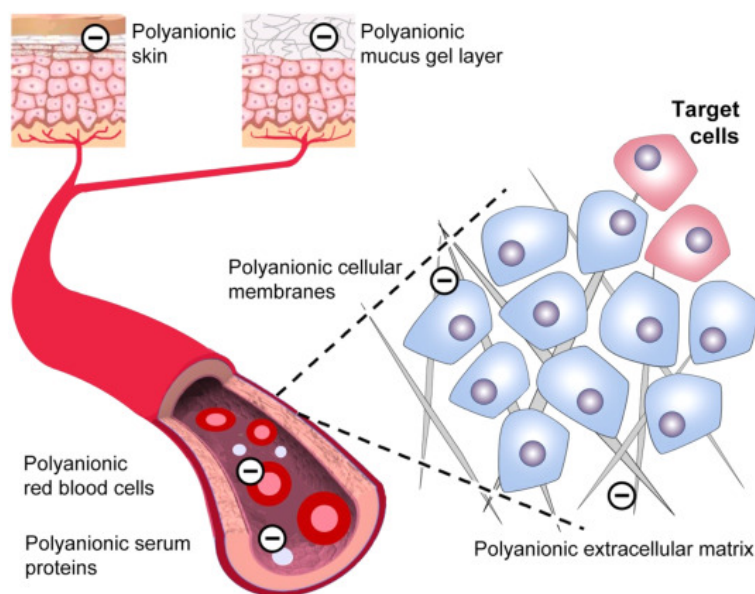


Figure 1-8. Summary of the polyanionic barriers encountered by polycationic drug delivery systems following dermal (polyanionic skin), mucosal (polyanionic mucus gel layer), or parenteral administration (polyanionic extracellular matrix, polyanionic cellular membranes, polyanionic red blood cells, polyanionic serum proteins) route to their target cells (Bernkop-Schnürch, 2018). Copyright 2018, with permission from Elsevier.

1.2.2 Polyanions as pH-responsive polymers

Polyanions that demonstrate pH-sensitive behaviours are characterized by their acidic side chains or groups, such as carboxylic acid, sulfonic acid, phosphoric acid, and boronic acid. They employ a distinct strategy for achieving endosomal escape. Typically, these polymers carry a negative charge at physiological pH, rendering them repulsive to negatively charged plasma membranes and exhibiting non-membrane lytic properties via their functional groups. The specific pKa values of these polyacids, which indicate the threshold at which pH-responsive polymers change their charge status, are influenced by their composition and molecular weight, and they vary from the pKa values of single acids. When the pH decreases below their pKa within the endosomes, they lose their charge and become hydrophobic. The resultant pH-induced changes in overall charges, amphiphilicity, and conformation enhance their interaction with endosomal membranes, ultimately leading to membrane disruption and the release of the payload into the cytoplasm as shown in Figure 1-9A. In comparison to

polycationic polymeric carriers, anionic pH-responsive membrane permeabilizing polymers are less toxic due to their repulsive charges against plasma membranes (Wang, 2018, Evans et al., 2019). While not as widely recognized, recent studies indicate their emerging potential in the delivery of proteins, genes, and vaccines (Mukalel et al., 2018, Qiu et al., 2018) (Evans et al., 2019, Jacobson et al., 2019, Kopytynski et al., 2020).

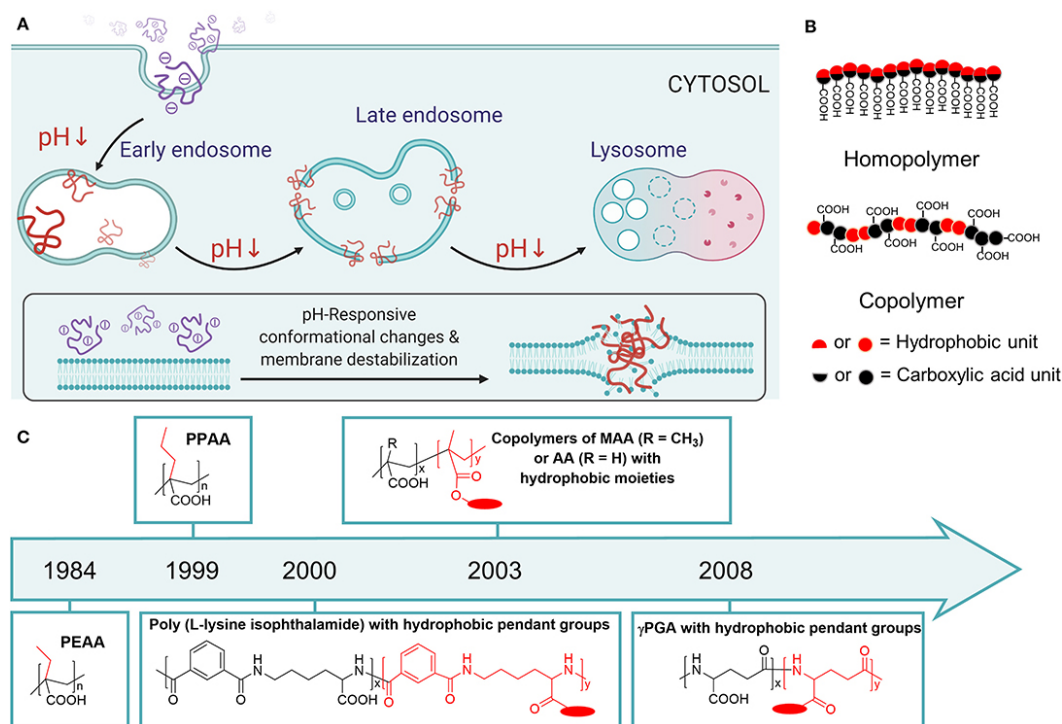


Figure 1-9. (A) Illustration pH-responsive membrane permeabilizing polymers and their role in facilitating endosomal escape. (B) General design principles of carboxylated polyanions for endosomal escape applications. (C) Timeline highlighting the chronological development of carboxylated polymers for endosomal escape applications. The time plotted signifies the initial application for pH-dependent membrane permeabilization rather than the first reported synthesis of the polymer (Wang, 2021). Copyright 2021, with permission from Frontiers Media.

Carboxylic groups are weak acids that shed their protons at high pH levels, resulting in the formation of anionic polyelectrolytes. Conversely, at lower pH levels, these groups accept protons, rendering the macromolecule neutral. Typically, these polymers consist of two essential units: a carboxylic acid unit for pH responsiveness and a hydrophobic unit to enhance interactions with lipid membranes (Wang, 2021) as shown in Figure 1-9B. These units may

either be integrated into a single monomer (homopolymer) or distributed across two different monomers (copolymer). Homopolymers, such as poly(ethylacrylic acid) (PEAA) and poly(propylacrylic acid) (PPAA) as shown in Figure 1-9C, have been extensively studied over the past two decades for gene and protein delivery. Copolymers, on the other hand, can be formed by copolymerizing methacrylic acid (MAA) or acrylic acid (AA) as the carboxylic acid unit with hydrophobic methacrylates. MAA and AA are easily polymerized through various methods and are cost-effective. Sometimes, protective groups are employed during polymerization, followed by deprotection to yield polymers with carboxylic groups. Leroux et al. have compiled a comprehensive review on polycarboxylates that form pH-responsive structures like vesicles, polymeric micelles, and nanospheres (Felber et al., 2012). pH-sensitive dendrimers with carboxyl terminal groups have also been developed (Dong et al., 2007).

Initially, it was observed that amphiphilic carboxylate polymers exhibited pH-dependent membrane permeabilization on liposomal membranes (Seki and Tirrell, 1984). The impact of this phenomenon was initially investigated using PEAA, revealing pronounced membrane disruptive effects at its pKa (6.5), while maintaining membrane integrity at physiological pH (Thomas and Tirrell, 1992). The disturbance in the membrane was attributed to the pH-dependent transition from coil to globule conformation, as indicated by variations in hydrodynamic size and the desolvation of the hydrophobic fluorescent probe pyrene (Eum et al., 1989). The uncharged polymer in its globule conformation could interact with the lipid membrane, leading to complete membrane lysis at elevated polymer/lipid ratios (Thomas et al., 1994). In the late 1990s, PPAA was developed by Murthy et al. (1999) (Murthy et al., 1999). In comparison to PEAA, PPAA features a slightly longer pendant alkyl group on the monomer, imparting greater hydrophobicity. Murthy et al. chose red blood cells, as opposed to simple liposomes, for assessing the pH-dependent membrane permeability of PPAA. Red blood cell membranes are more intricate, comprising not only lipids but also proteins and polysaccharides, making them a more representative model for biological membranes (Evans et al., 2013). PPAA exhibited heightened hemolytic activity at acidic pH compared to PEAA, displaying no hemolytic activity at physiological pH at equivalent concentrations. The varying pH sensitivity of PPAA is likely attributed to its greater hydrophobicity compared to PEAA. At pH 6.0, free PEAA is 12 to 15% ionized, while PPAA is only 4% ionized (Murthy et al., 1999, Joyce and Kurucsev, 1981). This observation led to the speculation that PPAA could form pores on red blood cell membranes only under acidic conditions, resulting in hemolysis (Murthy et al., 1999).

1.2.3 Biodegradable pH-responsive anionic polymers

Despite notable advancements in polymer synthesis tools and the exploration of endosomal escape mechanisms, several fundamental issues persist in this field. One such issue is regarding whether polymeric carriers undergo degradation. This aspect is crucial for their biomedical applications due to long-term biosafety concerns (Wang, 2021). For non-biodegradable polymers used in drug delivery, maintaining molecular weights below the renal excretion threshold is essential to prevent unwanted bioaccumulation, which may restrict their clinical use. Consequently, the creation of biodegradable anionic polymers that respond to pH changes and exhibit optimal membrane activity has gained importance. These polymers can incorporate hydrolytic or enzymatic cleavable bonds such as amides, esters, and anhydrides within their structure. For example, biodegradable anionic poly(amino acid)s like poly(aspartic acid) and poly(glutamic acid) and their derivatives have been employed as delivery vehicles (Ohya et al., 2011). These anionic polymers, made of repeating amino acid units with ionizable side groups, can disrupt lipid membranes under acidic conditions and are used in DDSs due to their biocompatibility and minimal cytotoxicity.

1.2.3.1 Poly(L-lysine isophthalamide) (PLP)

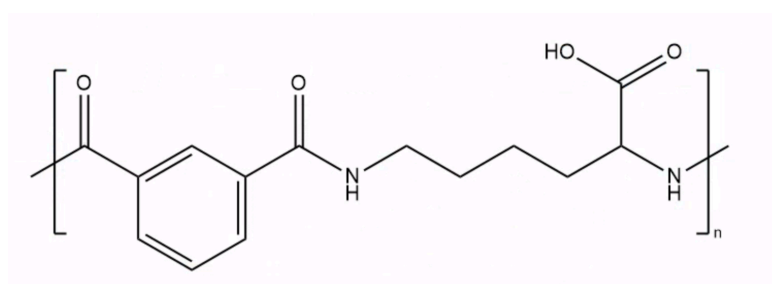


Figure 1-10. The unit structure of PLP.

Eccleston et al. have synthesized a pH-responsive, biodegradable amphiphilic pseudopeptide, poly(L-lysine isophthalamide) (PLP) (Figure 1-10), through the polycondensation of L-lysine with diacyl chlorides, forming amide linkages. PLP, featuring a hydrophobic backbone with

ionizable carboxyl groups, undergoes a pH-dependent conformational transition from coil to globule (Eccleston et al., 2000, Eccleston et al., 1999). However, its clinical application is limited by its low membrane-disruptive capability and the fact that effective disruption occurs at a pH lower than that of endosomes.

1.2.3.2 Grafted pseudopeptides

To address the limitations of PLP, modifications with various hydrophobic amino acids like valine, leucine, and phenylalanine have been explored to develop the grafted pseudopeptides. The aim is to enhance the polymer's membrane-disruptive activity within the endosomal pH range, particularly in early endosomes. PP polymers (Figure 1-11) are a class of derivatives of PLP prepared through grafting phenylalanine (Phe) onto the carboxyl groups pendant to the PLP backbone at various degrees of grafting (Chen et al., 2009a). PP75 and PP50, the superior grafted pseudopeptides at the stoichiometric grafting percentage of 75 and 50 mol%, respectively, demonstrated the most effective membrane-destabilizing activity at early endosomal pH values due to the enhanced hydrophobic interactions and π - π interactions between aromatic rings in phenylalanine and PLP backbone. Upon protonation at mildly acidic pH, the enhanced hydrophobic and π - π interactions enable them to undertake the compact structure with hydrophobic microdomains, facilitating cell membrane interactions. At physiological pH, they displayed the expanded coil structure due to charged repulsion and remains non-lytic (Chen et al., 2009c).

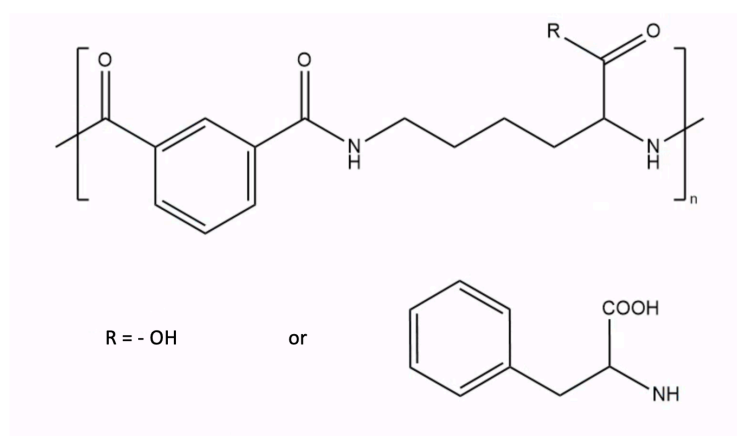


Figure 1-11. The unit structure of PP polymers.

For delivering small interfering RNA (siRNA), PP75 demonstrates effective cellular uptake, safely permeabilizes endolysosomes in a pH-dependent manner, and enables the direct transfer of co-endocytosed materials into the cytoplasm (Khormaei et al., 2013). The covalent attachment of siRNAs to PP75 using disulfide linkages results in conjugates that effectively transport siRNAs to the cytoplasm of target cells both in vitro and in vivo (Khormaei et al., 2013). During interaction between polymer and dioleoylphosphatidylethanolamine (DOPE, a zwitterionic lipid) tested by electrochemical impedance spectroscopy, the polymers at the investigated concentrations do not induce the solubilization of phospholipids through micellization or irreversible monolayer replacement (Zhang et al., 2011a). Rather, a complex conformation is formed during the interaction between the amphiphilic polymer and the phospholipid, likely involving the hydrophobic domains of the polymers and the hydrophobic tails of the phospholipid (Zhang et al., 2011a). Additionally, hydrogen bonding between the carboxyl groups of the polymer and the lipid phosphodiester group contributes to the formation of this complex conformation (Chen et al., 2009b). To understand the mechanism of trehalose delivery facilitated by PP-50, Lynch and colleagues employed molecules with sizes ranging from sucrose to 10 kDa poly(ethylene glycol) (Lynch et al., 2011). The findings revealed a consistent linear decrease in the logarithm of relative diffusant membrane permeability correlated with the diffusant molecular volume. This observation suggests that the mode of transport operates through non-Stokesian diffusion (Lynch et al., 2011). Unlike Stokesian diffusion, where the medium flows freely around diffusing molecules, non-Stokesian diffusion occurs when diffusing molecules jump into gaps between anchored solvent molecules. The process of trehalose diffusion could undergo alterations with temperature variations, potentially influenced by phase transitions occurring in the erythrocyte membrane.

These innovative pseudopeptidic polymers have demonstrated significant promise for drug delivery. PP75 and PP50 has been effective for intracellular delivery of small molecules and proteins, traversing cellular barriers in tumor models, and facilitating endosomal release of cargo into the cytoplasm (Lynch et al., 2011, Chen et al., 2009a, Kopytynski et al., 2020, Liechty et al., 2009, Ho et al., 2011). Additionally, PP75 has been used for the cytoplasmic delivery of siRNA to targeted cells both in vitro and in vivo, contributing to gene silencing and inhibition of tumor growth in a subcutaneous malignant glioma tumor model (Khormaei et al., 2015). Moreover, PP50 has been applied for cell cryopreservation and desiccation by enabling the direct penetration of trehalose into cells without causing notable cell damage (Lynch et al.,

2010, Lynch and Slater, 2011). PP75-coated liposomal system, mimicking viruses, in facilitating endosomal escape into the cell cytoplasm, coupled with the effective pH-responsive release of doxorubicin from liposomes, significantly boosted its potency against HeLa cells compared to doxorubicin-loaded uncoated liposomes (Chen and Chen, 2016). This indicates promising potential for applications in intracellular drug delivery. PP polymers (PP25, PP50, and PP75) in conjunction with polyethylenimine (PEI) using instant nanoformulation strategies to generate coacervate-like nanosystems with doxorubicin (Zhu et al., 2023). The PP-PEI-Dox system displays notable encapsulation efficiency for doxorubicin and can release it in response to pH stimuli (Zhu et al., 2023).

1.2.3.3 Comb-like and hyperbranched pseudopeptides

PLP-NDA pseudopeptides (Figure 1-12) are another class of derivatives of PLP with the comb-like structure. Like membrane proteins, these pH-responsive polymers contain long hydrophobic chains that serve as membrane anchors. They have shown enhanced membrane binding and destabilizing capabilities at endosomal pH values, whilst remain non-membranolytic at physiological pH. The PLP-NDA polymers were synthesized by attaching long hydrophobic decylamine (NDA) chains to the carboxylic acid groups along the PLP backbone. Chen et al. examined the effect of varying decylamine grafting densities (3 mol%, 10 mol%, and 18 mol%) on the hemolytic activity of these polymers. They discovered that a higher decylamine grafting percentage improved the polymer hemolytic activity, following the order: PLP < PLP-NDA3 < PLP-NDA10 < PLP-NDA18. The most effective polymer, PLP-NDA18, exhibited exceptional membrane-destabilizing ability even at dilute concentrations (e.g., 0.025 mg mL⁻¹), only at pH values characteristic of endosomes at 5.5. The higher concentrations (> 5 mg mL⁻¹) were associated with increased cytotoxicity.

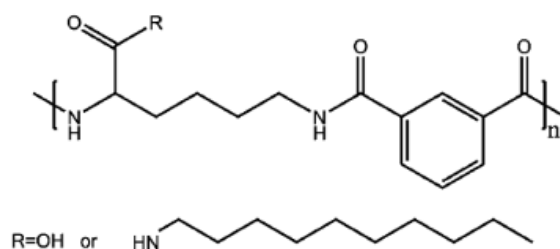


Figure 1-12. The unit structure of PLP-NDA polymers.

The effective entry of FITC-dextran (average Mw of 4, 10, 70, 150, and 2000 kDa, respectively) into RBCs under endosomal pH conditions suggests that the newly developed comb-like polymer has the capability to destabilize the endosomal membrane (Chen et al., 2017). This facilitates the efficient release of endocytosed macromolecules into the cytoplasm without causing lysis of vesicular compartments (Chen et al., 2017). After treatment with PLP-NDA18 and membrane-impermeable Calcein, diffused staining was noted in different cell lines, including HeLa human cervical cancer cells, A549 human lung cancer cells, and Chinese Hamster Ovary (CHO) cells, indicative of successful cytoplasmic localization. In contrast, cells treated with free Calcein, or Calcein+PLP, displayed only green punctate spots, indicative of entrapment within intracellular compartments after endocytosis. The design incorporated PLP-NDA18 emulating fusogenic peptides in viral spikes, a self-assembled liposomal bilayer structure resembling the viral envelope, and an encapsulated drug payload mimicking the viral genome (Chen et al., 2022). The pH-responsive release characteristics of these multifunctional liposomes were controllable by manipulating the structure and concentration of the adsorbed polymers. While PLP-NDA18 is structurally distinct from PP75 or PP50, it too demonstrated the ability to reversibly increase the membrane permeability without causing permanent cell damage. PLP-NDA nanogels are physically cross-linked by a simple nanoprecipitation method (Wang et al., 2018). This is a new approach for orally delivering hydrophobic and poorly intestinal epithelium-permeable drugs through the utilization of pH-responsive nanogels with membrane-active properties (Wang et al., 2018). Additionally, PLP-NDA18 has been shown to be effective in rapidly and efficiently delivering trehalose into cells and reduce the duration for loading from 9 hours (using PP50) to 15 minutes. under mildly acidic conditions (Chen et al., 2017). The increased speed and effectiveness in the intracellular delivery of PLP-NDA18 can be attributed to the heightened membrane curvature resulting from the robust membrane insertion of the comb-like polymer, characterized by relatively lengthy alkyl side chains (Chen et al., 2020). This innovative approach not only paves the way for the successful cryopreservation of erythrocytes and potentially other mammalian cells but also establishes a basis for the efficient intracellular delivery of various membrane-impermeable molecules.

In addition to those linear, grafted and comb-like polymers, the hyperbranched structures were shown to efficiently enhance their interaction with cell membranes (Wang and Chen, 2017). This resulted in a significantly increased membrane-lytic activity compared to their linear counterpart. The distinctive structures and pH-responsive cell-penetrating capabilities position

the novel hyperbranched polymers as promising candidates for the cytoplasmic delivery of biomacromolecular payloads. Unfortunately, scientists face numerous challenges in implementing this idea. These challenges include the accumulation of vehicles in off-target tissues, their immunogenicity, the complexity of delivering drugs to intracellular targets, and the difficulties in fabricating carriers that meet all the required specifications. There are also limited effectiveness of the permeability and retention effect currently poses the greatest challenge for the pH-responsive drug delivery concept, as the penetration of carriers into the tumor interstitium is a crucial step. The continuous process of identifying new challenges and finding solutions drives the development of drug delivery through pH-responsive DDSs.

1.3 Cell cryopreservation

Cell preservation, essential for cell therapy and blood transfusion (Hanna and Hubel, 2009), enables the storage of cells for extended periods under specific conditions. Methods of cell preservation encompass hypothermic storage, lyophilization, and cryopreservation. However, hypothermic storage offers a limited duration for cell storage, and lyophilization faces challenges in implementation due to the lack of suitable protective agents. Cryopreservation, which involves preserving cells, tissues, organs or any other biological materials by cooling samples to cryogenic temperatures to extend their shelf life, is a crucial technique in the fields such as fundamental research, clinical, biomedical, and food sciences (Giwa et al., 2017) (Fowler, 2008). The field of cell cryopreservation originated over 60 years ago with the widespread use of glycerol and dimethyl sulfoxide (DMSO) as good standard cryoprotectants (Murray and Gibson, 2022). However, DMSO has toxicity both in vitro and in vivo, impacting numerous cellular processes through alterations in DNA methylation and disruption of gene expression regulation (Erol et al., 2021). Development of glycerol/DMSO-free methods for cryopreservation of therapeutically valuable cells is vital for driving sustained innovation in cell therapy (Weng and Beauchesne, 2020b).

1.3.1 Cell cryopreservation technologies

Cryopreservation is crucial in the maintenance of cells, reproductive medicine, genetic technologies, and cell transplantation (Matsumura et al., 2010, Liu et al., 2008, Eroglu et al., 2000, Zieger and Gupta, 2006). In the context of cell-based therapies, cryopreservation consists of a range of procedures, including tissue and cell isolation, cryoprotection during the cooling and freezing processes, cryostorage and transport of frozen samples, followed by thawing and the subsequent recovery of cells. (Meneghel et al., 2020).

1.3.1.1 The importance of cell cryopreservation

Cryopreservation presents significant prospects for both scientific research and medical treatments. It enables blood banks to maintain sufficient supplies, facilitates the use of stem cell therapies for various diseases, advances the assisted reproductive technology, and is utilized in a range of settings from small-scale laboratory experiments to large-scale therapeutic applications (Fuller et al., 2004a). In the realm of regenerative medicine and cancer immunotherapy, these cells play a pivotal role (Crowe and Crowe, 2000). Notably, T-cells can be genetically modified to express chimeric antigen receptors, thereby enabling them to specifically target tumor cells that present certain antigens. This approach has shown clinical success in treating hematological cancers (Crowe and Crowe, 2000). Furthermore, stem cell therapy is instrumental in enhancing the healing process of diseased, injured, or dysfunctional tissues. An example of this is cardiac regenerative therapy, where stem cells are injected into cardiac muscle post-myocardial infarction, effectively reducing the size of the infarct (Weng and Beauchesne, 2020a). Cell therapy is particularly beneficial for patients who are unable to produce enough healthy cells or are undergoing cancer immunotherapy. Moreover, cryopreservation is instrumental in the long-term preservation of seeds (Walters et al., 2004), endangered plant species (Kaczmarczyk et al., 2013), and animals (Ballou, 1992). Cryopreservation is a critical technology for a multitude of applications, from bolstering medical to conserving biodiversity, while ongoing innovations aim to enhance its efficacy and reduce associated cellular damage.

1.3.1.2 Mechanisms of cryopreservation

Cryopreservation is a process to preserve cells in a frozen state at ultra-low temperatures. Under cryopreserved conditions, the chemical and biological activities within living cells are markedly diminished or halted altogether, providing the fundamental mechanism for the extended preservation of diverse biological specimens (Mazur, 1984, Steponkus et al., 1990). During cryopreservation, the primary sources of damage to biological samples arise from chemical and physical stresses encountered in the freezing and thawing stages (Gao and Critser, 2000, Wolkers et al., 2018). The formation, growth, and recrystallization of ice crystals represent significant hazards to cryopreserved cells, leading to the primary challenges and barriers to achieving successful cryopreservation. (Pegg, 2010, Bissoyi et al., 2014). Liquid water is vital for preserving cellular structure and function, but the transition from water to ice during freezing often results in mechanical damage from ice within and outside cells (Chang and Zhao, 2021). Additionally, increased solute concentrations from ice formation can cause irreversible cell damage through freeze-thaw cycles (Gao and Critser, 2000). Current cryopreservation approaches are categorized into slow freezing and vitrification. While traditional slow-freezing allows for lower concentrations of cryoprotective agents (CPAs), it does not adequately prevent ice-related cell damage (Zhao and Fu, 2017, Mazur et al., 1972). Researchers are, therefore, optimizing cryopreservation conditions such as CPA concentrations, cooling and warming rates, and cooling temperatures to minimize ice damage (Weng and Beauchesne, 2020b, Stoll et al., 2012, Oldenhof et al., 2017). Vitrification, which prevents ice formation during freezing, is emerging as a promising method for organ preservation (Brockbank et al., 2010). Nevertheless, ice nucleation and devitrification during warming remain significant obstacles, often leading to fatal damage (Bischof and Diller, 2018). Clearly, the genesis and proliferation of ice crystals, along with the resultant mechanical harm, are the principal factors undermining the structural integrity and functionality of cryopreserved biological specimens (He et al., 2018, Biggs et al., 2017, Shi et al., 2017b). Thus, controlling and eliminating ice crystal formation is a fundamental and critical challenge in advancing the science of cryopreservation.

The process of ice formation in water involves nucleation, which is initiated by hydrophilic sites on particles or surfaces that resemble the pattern of water molecules on an ice crystal

surface (Turnbull, 1950). Typically, there is no ice nucleators inside cells (Meryman, 2007), leading to the initial formation of extracellular ice. This process depletes water from the extracellular space, causing an increase in intracellular solute concentration, higher osmotic pressure, and cellular dehydration. However, the viscosity of the extracellular solution increases due to ice and solute presence, slowing down the diffusion of water to the ice crystals (Meryman, 2007). As a result, the cellular solution becomes vitrified, hindering further water diffusion and ice crystal formation (Hunt, 2011).

Intracellular ice usually forms under three circumstances: at temperatures low enough to induce spontaneous freezing within the cells (Mazur, 1984), when the ice crystals are small enough to penetrate the cell membrane (Pegg, 2015), or when the membrane is damaged (Meryman, 2007), allowing ice to grow through the defects. Such ice formation can mechanically harm cells, leading to dehydration, shrinkage, or rupture. The use of penetrating cryoprotectants in most cryopreservation protocols is crucial to lower the freezing point, reduce ice formation, and increase the temperature at which vitrification occurs.

1.3.1.3 Damage of cryopreservation

Cryopreservation can lead to various types of cellular damage, potentially causing harmful alterations in cell morphology, characteristics like adhesion and cell surface markers, metabolic activities such as proliferation and potency, functionality including immunomodulation, and even cell death (Fuller et al., 2004b, Bahsoun et al., 2019). A decrease in temperature during cryopreservation can initiate specific stress response pathways (Gao and Critser, 2000, Woods et al., 2016), and may activate apoptotic and necrotic processes post-thawing (Bissoyi et al., 2014). Even though the damage caused by cryopreservation is not directly due to ultralow temperatures, the processes of freezing and thawing required to reach such temperatures cause harm to the biological material in various ways, such as mechanical damage and cell lysis (Murray and Gibson, 2022).

When aqueous solutions are cooled below their equilibrium freezing point, ice crystals inevitably form in the extracellular medium, and water concentration in the sample decreases.

This leads to two major effects. One of the primary causes of cell injury during cryopreservation is the formation of ice crystals in the frozen sample. Initially, ice forms in the extracellular medium, with its crystalline structure leading to solute exclusion and a resultant increase in the concentration of solutes in the remaining liquid (Jang et al., 2017, Körber et al., 1991, Mazur et al., 1972, Fuller et al., 2004b). This process leads to heightened osmolarity in the suspending medium, subjecting the cells to osmotic stress, depicted in Figure 1-13 (Murray and Gibson, 2022). As the freezing continues and the ice fraction expands, the extracellular osmolarity rises further, exacerbating the osmotic stress (the pressure exerted on cells due to imbalances in water and solute concentrations, and is a common factor in various abiotic stresses such as salt, drought, and cold) and significantly contributing to viability loss during cryopreservation (Gao and Critser, 2000, Mazur, 2004, Morris et al., 2006). As samples cool, the creation of ice crystals outside cells causes an osmotic imbalance and leads to cell dehydration. An osmotic gradient is created across the cell membrane. A certain level of cellular dehydration is beneficial for cryopreservation, as it lowers the risk of excessive intracellular ice formation, which is usually fatal, the precise mechanisms of this are still under investigation. Without extracellular ice, samples may supercool beneath the equilibrium freezing point, increasing the likelihood of lethal intracellular ice formation. Yet, excessive dehydration can be irreversible and is a primary reason for cryopreservation-induced damage to biological materials. Secondly, as ice crystals grow in the extracellular medium, solutes that were evenly distributed in the solution become concentrated in the remaining water channels between the ice crystals. Cells in these channels are exposed to much higher solute concentrations than in the original solution without ice, leading to osmotic shock and heightened toxicity.

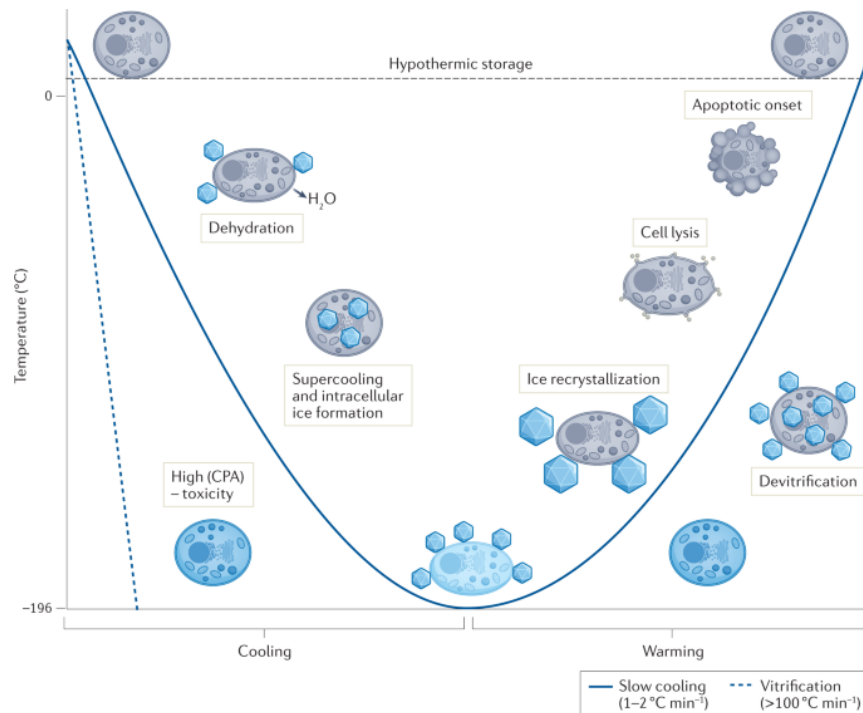


Figure 1-13. Potential mechanisms of cellular damage during cryopreservation (Murray and Gibson, 2022). Copyright 2022, with permission from Springer Nature Limited.

A further obstacle is cooling rate. Slow cooling rates (less than 1°C per minute) give cells enough time to dehydrate, thereby preventing intracellular ice formation (IIF). However, this exposes cells to high concentrations of solutes and any added cryoprotective agents, which might be toxic, for longer durations. Rapid cooling rates (such as more than 100°C per minute) reduce exposure to high-solute environments but can cause IIF since cells cannot dehydrate quickly enough, leading to irreversible harm. Mazur's two-factor hypothesis proposes that for optimal cell survival during cooling, there must be a balance between two key factors: the rate of ice formation and the extent of cellular dehydration. If the cooling rate is too slow, cells may suffer from excessive dehydration as water moves out of the cells and forms ice extracellularly. On the other hand, if the cooling rate is too fast, intracellular ice formation can occur, which can be lethal to cells. Therefore, an intermediate cooling rate is ideal as it minimizes both excessive dehydration and intracellular ice formation, leading to higher cell survival rates. It is crucial to note that different cell types have varying optimal cooling rates (Mazur, 1970). One exception to Mazur's hypothesis is that at extremely high cooling rates, cells can bypass the ice phase altogether, resulting in a highly viscous glass-like state known as vitrification. This state avoids the severe cellular damage that ice nucleation and growth can inflict during

cryopreservation. Nonetheless, vitrification often necessitates the use of very high levels of cryoprotectants, which can cause osmotic stress when the agents are introduced and later removed from the cells.

In summary, freezing is detrimental to living cells since the chemical component changed by the ice formation inside and outside cells (Karlsson and Toner, 1996). The phase transition from water to ice at low temperatures is the major difficulty for cells to overcome. Cooling rate is important for cryopreservation. Fast cooling rates is harmful due to the intracellular ice formation, and slow cooling causes cell dehydration, shrinkage or rupture due to the highly concentrated extracellular solutions resulting in osmotic changes (Jang et al., 2017). To address these issues, a cryoprotectant that is not intended to be washed out or removed prior to a patient transfusion must adhere to stringent regulatory and safety standards. In contrast, cryoprotectants used in earlier stages or for basic research may be implemented and utilized with greater ease. In this advanced realm, reliance on serendipitous discoveries is not sufficient. Consequently, a methodical approach to discovery science that systematically addresses specific biochemical and biophysical mechanisms is critical.

1.3.2 Cryoprotective agents (CPAs)

Cryoprotectants are vital for both storing cells at deep cryogenic temperatures and enhancing the recovery and functionality of cells post-thawing. To reduce cellular damage primarily caused by solute effects, it is crucial to incorporate cryoprotective agents. Cryoprotectant agents are employed to inhibit ice formation, which is known to cause damage to biological tissue during the cooling of organs. When CPAs are dissolved in water, they have the capacity to lower the melting point of ice (Mazur et al., 1972, Mazur, 1963) helping to maintain a larger proportion of the extracellular fraction in an unfrozen state at any given temperature. By elevating the overall concentration of solutes in the system, they minimize the formation of ice at various temperatures. This action helps to diminish the osmotic impact of concentrating solutes, thereby substantially lessening osmotic damage to the cells (Kilbride and Morris, 2017, Gao and Critser, 2000). Without CPAs, the prevention of intracellular ice formation is largely left to salts and dissolved molecules, leading to nearly all cellular water freezing during the

cooling process. However, when CPAs are present, CPAs' concentration increases alongside ice formation. Ice growth ceases once the concentration of CPA is high enough to balance the melting temperature with the current temperature. At this concentration, the CPA reaches a state where it cannot freeze. As the temperature drops further, no additional ice forms, and cells find survivable spaces within the pre-existing ice crystals. When the temperature falls below approximately -100°C , the remaining solution that is unfrozen transitions into a glassy state, which can indefinitely preserve cell membranes. Cellular survival during freezing is facilitated by their residence within this glassy matrix amidst the ice crystals. The initial concentration of CPAs determines the magnitude of the unfrozen volume at the end of the freezing process, with higher concentrations resulting in a larger unfrozen volume. Their effectiveness and potential in supporting cell biology are attributed to the biological and metabolic impacts of the cryoprotectant solutes. These compounds, which do not ionize in aqueous solutions, exhibit relatively low toxicity to cells at low temperatures (Elliott et al., 2017).

It is essential for these agents to be able to permeate into cells while being non-toxic or minimally toxic. There are two kinds of CPAs, which can be classified depending on their cell membrane permeability. Penetrating CPAs, such as DMSO and glycerol, have been commonly used in most cryopreservation protocols (Matsumura et al., 2010). However, these CPAs are limited by their toxicity for medical usage. A concentration ranging from 5% to 15% is generally adequate for ensuring the survival of isolated cells through the processes of freezing and thawing at liquid nitrogen temperatures. This concentration effectively reduces the melting point of water and prevents various phenomena such as precipitation, hydration, and eutectic formation (Pegg, 2009). Another kind of CPAs is carbohydrate sugars, e.g., trehalose and sucrose, which are capable of stabilizing and protecting proteins (Carpenter et al., 1987, Crowe et al., 1985, Crowe et al., 1984, Sun and Davidson, 1998, Green and Angell, 1989, Israeli et al., 1993, Rossi et al., 1997, Read et al., 1995, Miller et al., 1997). However, these CPAs cannot easily penetrate through the cell membrane during cryopreservation, thus limiting their effectiveness. In contexts outside cryobiology, these substances are often referred to as "antifreeze."

1.3.2.1 Dimethyl sulfoxide (DMSO)

It's essential to verify that any cryoprotectant under consideration has been clinically validated, such as being approved for transfusion or patient injection. DMSO, for instance, is highly effective as a cryoprotectant, and many alternative compounds are comparatively less effective, particularly for cells that are sensitive to freezing and thawing (Miyamoto et al., 2012). DMSO has a long history of use in injection carrier solutions and in bone marrow stem cell transplants (Bahsoun et al., 2019, Hunt, 2011). The most common concentration of DMSO used is 10% v/v, especially with stem cells (Bahsoun et al., 2019, Hunt, 2011). However, there is a noticeable shift towards reducing this concentration to 5% v/v, either alone or combined with non-permeable CPAs. This reduction aims to enhance cell recovery after thawing and to decrease the amount of DMSO administered to patients.

DMSO prevents ice formation at sub-zero temperatures, providing the environment for osmotic cell shrinkage to retard cell damage (McGANN, 1979). The low cost of DMSO make it an ideal candidate for cryopreservation (Fahy, 1986). DMSO is capable of reducing the electrolytic concentration in the residual chilled contents in and around of a biological cell, during cryopreservation (Oh et al., 2006). DMSO has a typical property that it transformed into solids below room temperature within 18.5°C. This property makes it most suitable as a CPA. The cryoprotective effect of DMSO is generally attributed to its ability to inhibit ice formation and mitigate the negative impacts of the freeze-concentrated solution (Klbik et al., 2022).

However, DMSO affects the differentiation of various kinds of cells, hence after thawing, it has to be washed out immediately (Weng and Beauchesne, 2020b, Bakken, 2006, Liseth et al., 2009, McCullough et al., 2010, Worsham et al., 2017, Li et al., 2020). Safety concerns related to DMSO have been extensively documented (Wang et al., 2012). Questions have arisen about its suitability for use in cellular therapy products intended for localized injections into tissues like the brain (Meneghel et al., 2020). Its usage has been linked to significant clinical issues in patients receiving infusions of cryopreserved cell-therapy products, leading to allergic reactions, gastrointestinal problems, cardiac complications, neurological issues, and seizures (Shu et al., 2014, Martino et al., 1996, Ruiz-Delgado et al., 2009, Horacek et al., 2009, Donmez et al., 2007, Windrum and Morris, 2003, Bauwens et al., 2005, Abdelkefi et al., 2009). In vitro,

the presence of DMSO has been demonstrated to induce cell death (Da Violante et al., 2002) and compromise cell permeability (Gurtovenko and Anwar, 2007). Additionally, it has been reported to adversely affect the function of immune cells, leading to the suppression of pro-inflammatory cytokine and chemokine production and a reduction in immune cell viability (Elisia et al., 2016). Specifically, the presence of DMSO in cryopreservation media has been shown to alter physiological characteristics of various cell types (Yi et al., 2017, Chinnadurai et al., 2014) and induce undesired epigenetic changes (Iwatani et al., 2006, Tunçer et al., 2018). In the context of CAR-T cell infusions, DMSO has been observed to cause symptoms such as nausea, vomiting, and hypotension (Cruz et al., 2010), as well as triggering anaphylaxis and severe infusion reactions (Yáñez et al., 2019).

1.3.2.2 Glycerol

Glycerol, a compact solute with multiple hydroxyl groups, is highly soluble in water and exhibits low toxicity to living cells over brief exposure periods (Fuller, 2004). When water molecules transform to ice crystal, the functions of glycerol are to form a hydrogen bond between biomacromolecules (Lopes et al., 2009) and to replace the position of water molecules. While it can diffuse through the plasma membranes of various cell types, this occurs at a comparatively leisurely pace. Therefore, cells can live with their native physiological structure and biological function even without aqueous environment (Jeyendran et al., 1985). Depending on the specific cell variety and the conditions of exposure, cells can withstand glycerol concentrations ranging from 1 to 5 mol L⁻¹. Lovelock took into account of these various characteristics and formulated his theory on the colligative effects of CPA (Lovelock, 1954). He suggested that due to the well-established molar freezing point depression found in solute mixtures, the addition of glycerol could counteract the rise in salt concentration, particularly sodium chloride, which is prevalent in most cellular media. By doing so, glycerol helps to avoid reaching harmful concentrations of salt while the system is cooled down to form a 'glassy matrix' state. Furthermore, the increasing viscosity of glycerol with the decrease in temperature could also play a role in kinetically hindering or slowing the expansion of ice crystals.

Nevertheless, deglycerolization needs to be conducted post-thaw to prevent post-transfusion osmotic hemolysis (Klein et al., 2007). The addition and removal of glycerol or DMSO can induce osmotic stress on cells (Thomas and Bell, 1995). This process, while labor-intensive and technically challenging, also leads to a notable decrease in cell viability by 15–20% (Mitchell et al., 2016).

1.3.2.3 Sugars

As depicted in Figure 1-14 there are two primary approaches to replace DMSO in the cryopreservation of therapeutic cells: 1) the discovery of a single CPA or a combination of CPAs that can replicate the protective roles of DMSO, such as preventing ice formation or maintaining membrane stability. 2) the adoption of innovative technologies to develop new cryopreservation techniques that do not rely on DMSO (Weng and Beauchesne, 2020b).

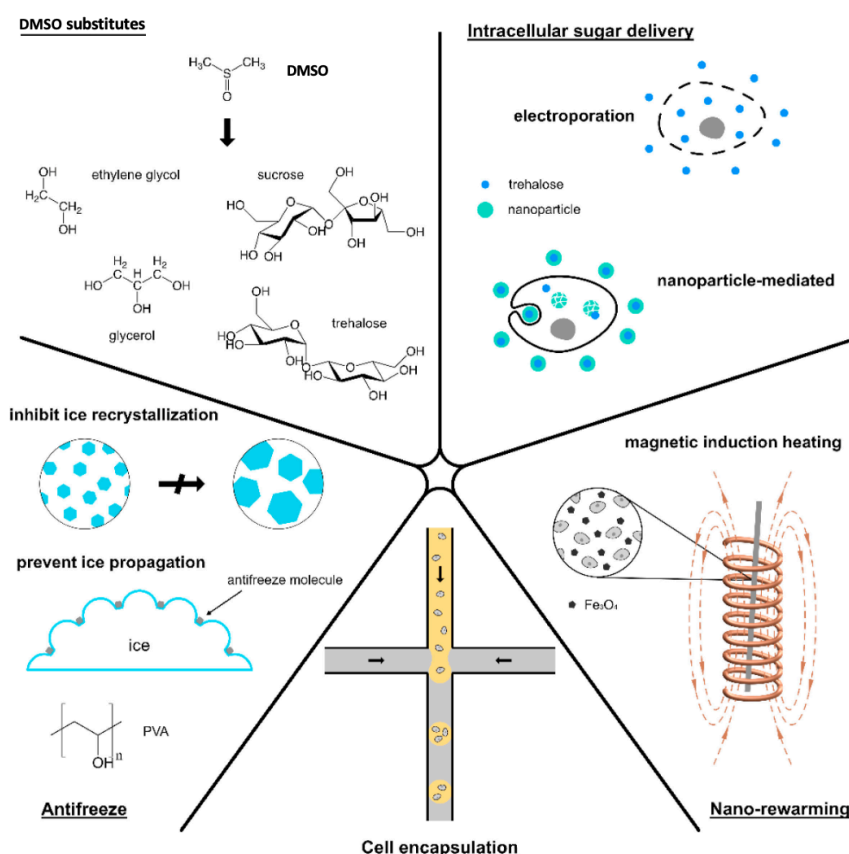


Figure 1-14. Overview of key approaches for substituting DMSO in the cryopreservation of therapeutic cells (Weng and Beauchesne, 2020b). Copyright 2020, with permission from Elsevier.

However, toxicity of DMSO is the limitation of cryopreservation as they cause damage during the procedure. DMSO-free cryopreservation emerges as a preferable method, aiming to eradicate the potential toxicity and side effects of DMSO, standardize practices across transplant centers, and unify cryopreservation procedures in cell-based therapies (Aijaz et al., 2018). Intracellular sugars, in particular trehalose and sucrose, are commonly used as stabilizing agents for cryo-storage and anhydrobiotic preservation, but trehalose is often superior for biological preservation, such as stabilization against thermal stress and during lyophilization (Crowe and Crowe, 2000, Eroglu et al., 2000, Rothschild and Mancinelli, 2001, Jain and Roy, 2009, Cordone et al., 2015). Key differences include higher solubility of sucrose in water compared to trehalose (Lammert et al., 1998). However, trehalose has a higher glass transition temperature (T_g), which allows it to form a more stable amorphous network, slowing water dynamics and preventing water crystallization (Bellavia et al., 2009, Green and Angell, 1989, Crowe et al., 1996). Trehalose also maintains a more rigid vitrified environment, even with small amounts of water, making it more effective for biological stabilization (Magazu et al., 2008). A recent study found trehalose acts as a non-toxic bioprotectant to protect cells from injury during cryopreservation (Crowe, 2007, Nakamura et al., 2008). Accordingly, trehalose is needed on both sides of the cell to achieve optimal protection (Eroglu et al., 2000, Wolkers et al., 2002). However, trehalose is membrane impermeable. Therefore, intracellular trehalose delivery is crucial. Trehalose is one of the most commonly used intracellular sugars. However, mammalian cells cannot synthesize or import trehalose, and their membrane is impermeable to these sugar-based CPAs (Acker et al., 2004, Bhowmick et al., 2002). Accordingly, effective intracellular delivery of trehalose is of great importance in achieving the cryopreservation of mammalian cells. Similar to water, trehalose can form hydrogen bonds and preferential hydration of biomacromolecules. The function of biomacromolecules can still be retained during cell cryopreservation (He, 2011), and the form of a steady matrix with deficient molecular mobility halt any degradative and metabolic activities in the dehydrated state (He, 2011, He et al., 2006, Crowe et al., 2001). As a result, sugars have been explored as non-toxic CPAs to protect cells from freezing induced damage (Sun et al., 1996, Crowe et al., 2003, Satpathy et al., 2004, Sun and Davidson, 1998, Wolkers et al., 2001).

For optimal bioprotection during preservation, it is essential for trehalose to be present both inside and outside the cell. To address the challenge of cell membrane impermeability to sugars, a genetically engineered mutant of wild-type *Staphylococcus aureus* hemolysin was utilized,

which forms 2 nm homoheptameric transmembrane pores when introduced into lipid bilayers. This engineered pore, designated H5, can be toggled between open and closed states by the addition or removal of micromolar concentrations of Zn^{2+} ions (Eroglu et al., 2000). While the precise mechanism behind trehalose protective effects during freezing requires additional investigation, several potential mechanisms could be operating synergistically. Intracellular trehalose might enhance cell survival by elevating cytosolic viscosity, impeding the growth of harmful intracellular ice crystals. Additionally, trehalose could contribute to the stabilization of cell membranes and proteins during freezing, preventing membrane damage and promoting the preservation of native protein conformations. Furthermore, the ability of trehalose to enable a higher temperature glass transition in the unfrozen fraction may enhance sample stability (Green and Angell, 1989). The presence of extracellular trehalose induces cell dehydration, thereby reducing the cellular water content before freezing. This dehydration process also assists in preventing excessive swelling and osmotic shock when removing permeating cryoprotectants. Moreover, trehalose plays a role in stabilizing membranes and proteins both during freezing and drying (Honadel and Killian, 1988, Rudolph and Crowe, 1985).

1.3.2.4 Antifreeze (glycol) proteins

Polymers are not limited to the delivery of CPAs but can also act as an agent to promote vitrification and preserve ice formation. Specialized polymer, as shown in Figure 1-15, namely poly(vinyl alcohol) (PVA) (Mitchell et al., 2016), was invented to imitate natural antifreeze (glycol) proteins (AF(G)Ps) (Weng et al., 2019). It is also essential to prevent recrystallization during the thawing process, and studies have indicated that the incorporation of PVA has resulted in high survival rates of cells, even when the thawing occurs at a gradual pace (Biggs et al., 2017). Incorporating PVA into erythrocyte suspensions curtailed the expansion of ice crystals through the freeze-thaw cycle, leading to a suppressed hemolysis relative compared to samples where no PVA was present (Figure 1-15A buffer alone) (Matsumura et al., 2023). PVA also enhanced the cryosurvival rate of nucleated cells during cryopreservation mediated by DMSO (Figure 1-15B) (Deller et al., 2016, Biggs et al., 2017). The function of AF(G)Ps as an agent is to modulate the effect of ice on living cells by ice recrystallization inhibition, thermal hysteresis, and dynamic ice shaping (Deller et al., 2014). It is shown that 0.1 wt% of

the polymeric AF(G)Ps is sufficient to achieve remarkable cell recovery after freezing, compared with more than 20 wt% needed for solvent-based approaches (Deller et al., 2015).

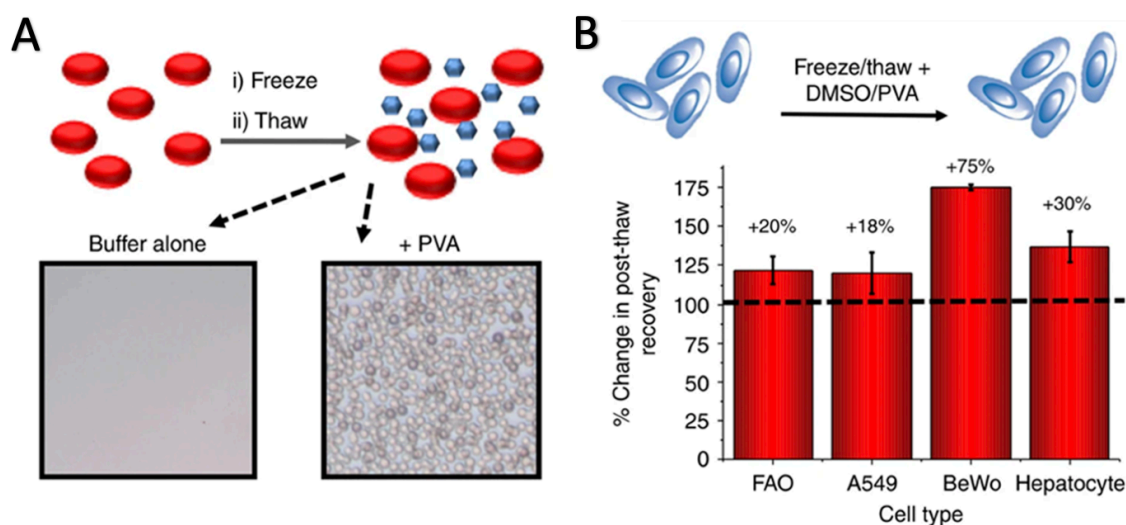


Figure 1-15. Cell cryopreservation employing poly(vinyl alcohol) (PVA). (A) RBCs could be cryopreserved without a solvent by adding 1 mg mL⁻¹ PVA, with micrographs revealing the preservation of intact RBCs. (B) Recovery of somatic cells post-thaw was successful in mixtures of PVA and dimethyl sulfoxide (DMSO) (Biggs et al., 2017). Copyright 2017, with permission from Springer Nature Limited.

Recently, a growing number of compounds have been identified or synthesized, exhibiting AF(G)P functions, such as polyampholyte (Matsumura et al., 2016, Rajan et al., 2016), and polyproline (Graham et al., 2017). Polyampholyte achieves maximumly 88% post-thaw cell viability for adherent cell monolayers. As a comparison, the conventional DMSO cryopreservation only gets 24% cell viability (Bailey et al., 2019). By applying poly(ampholyte), the consumption of DMSO decreases from 10 wt% to 2.5 wt% with comparable outcomes, thus permitting to preserve DMSO-intolerant cell lines (Bailey et al., 2019). Furthermore, this polymer can improve post-thaw growth rates as well as minimize potential membrane damage (Bailey et al., 2019). Nevertheless, it has been noted in certain studies that antifreeze proteins (AFP) do not always exhibit cryoprotective properties, suggesting that the cryoprotective action cannot be solely attributed to the suppression of ice crystal nucleation and growth (Sun et al., 2022).

1.3.3 Intracellular trehalose delivery methods

As mentioned above, trehalose, as a nontoxic CPA, has a better performance in cryopreservation but cannot easily penetrate through the cell membrane. To offer cryoprotection, trehalose must be present inside of the cellular membrane. However, mammalian cells neither synthesize trehalose nor possess the ability to diffuse it through their plasma membranes (Stewart and He, 2018). Hence, achieving intracellular delivery of trehalose is crucial for cryopreservation.

1.3.3.1 Physical Methods

1.3.3.1.1 Electroporation

Electroporation, also known as electroporation or electroinjection, is employed to increase membrane permeability and facilitate the entry of extracellular molecules into the cytoplasm (Shirakashi et al., 2002). It has been shown that when short, intense electric pulses are applied to cells, the positions of hydrophilic molecules on both sides of the cell membrane can be switched. Correspondingly, the permeability of the cell membrane reversibly increases (Rols, 2006). Based on this mechanism, Zhou et al. successfully loaded intracellular trehalose (64 mM) into human erythrocytes (Zhou et al., 2008). However, as a side effect of the enhanced permeability, the other undesirable compounds can also be delivered through the cell membrane, which may result in cytotoxicity. Dovgan and colleagues utilized this technique to cryopreserve human adipose-derived stem cells (hADSCs) effectively by pairing reversible electroporation with trehalose, enabling long-term storage without the need for DMSO (Dovgan et al., 2017). The benefit of this approach lies in the ability to adjust the concentration gradient to regulate the uptake of trehalose while enhancing membrane permeability. Nonetheless, this increased permeability is nonselective, allowing not only trehalose but also other substances to penetrate the cell and concerns arise regarding cytotoxic effects and the stability of the cell membrane due to thermal, osmotic, and electrical stresses imposed on the cells. This approach is also considerably invasive and can inflict significant

harm to the cells, potentially exacerbating damage during the cryopreservation process (Wang et al., 2019, Zhang et al., 2019).

1.3.3.1.2 Osmotic shock

Satpathy and colleagues introduced a technique to infuse red blood cells (RBCs) with trehalose (Satpathy et al., 2004). Extracellular trehalose solution of high concentration has been applied to RBCs. Accordingly, trehalose is permitted to diffuse into the cells because of the concentration gradient by inducing osmotic pressure differences and leveraging the transition in the phospholipid phases. It is observed that osmotic force drives extracellular trehalose to go through the cell membrane at 37°C incubation temperature for 7 hours, resulting in the highest uptake of trehalose about 40 mM (Satpathy et al., 2004). Notably, trehalose tends to remain stable within RBCs without breaking down. It is important to acknowledge, however, that during the infusion process, the RBCs are subjected to a hypertonic trehalose solution, which could lead to alterations in cell shape and potential damage (Zhou et al., 2008).

Electropermeabilization and osmotic shock fail to achieve the targeted 100-200 mM intracellular trehalose. Earlier research has indicated that intracellular trehalose provides cellular osmoprotection to human erythrocytes and nucleated mammalian cells under low osmolarity conditions (de Castro and Tunnacliffe, 2000, Satpathy et al., 2004).

1.3.3.2 Biological methods

1.3.3.2.1 Mutant bacterial toxins

Pores created in cell membranes can facilitate the introduction of trehalose into the mammalian cell cytoplasm. Eroglu and colleagues utilized a mutant form of *Staphylococcus aureus* α -hemolysin as a toxin to generate membrane pores, enabling the infusion of trehalose into 3T3 fibroblasts and human keratinocytes to reach an intracellular trehalose concentration of 200

mM (Eroglu et al., 2000); (Buchanan et al., 2004). This technique substantially enhanced cell survival rates during cryopreservation, resulting in post-thaw survival more than 80% of 3T3 fibroblasts and 70% of human keratinocytes (Eroglu et al., 2000). This finding demonstrated that intracellular trehalose of a low concentration is already sufficient to notably enhance the cryopreservation (Chen et al., 2001). An improved method has been proposed by using extracellular Zn^{2+} as a switch to manipulate the 2 nm pore hence the loading process can be controlled (Acker et al., 2003). Pores close in the presence of micromolar concentrations of Zn^{2+} due to the formation of a coordination complex with the added histidine residues (Russo et al., 1997). These pores can be reopened by adding a chelating agent such as ethylenedinitrilotetraacetic acid disodium salt (EDTA) or by rapidly washing in a Zn^{2+} -free solution (Bashford et al., 1996, Sunzel et al., 1995). By adjusting the concentrations of the pore former and Zn^{2+} , the permeability of the lipid bilayer can be effectively controlled (Russo et al., 1997).

The pores exhibit non-selectivity towards various materials, and the use of this method involving the toxin may potentially result in the loss of cell components (Wei et al., 2014). Using mutant bacterial toxins in the manufacturing process of cellular products poses a significant safety risk, as these toxins are potent poisons capable of inducing food poisoning, causing tissue damage, and leading to severe complications, potentially resulting in death (Popoff, 2020). Therefore, other improved techniques should be developed.

1.3.3.2.2 Mitochondrial permeability transition pore

Moore and associates demonstrated that trehalose could be inserted into the mammalian mitochondrial matrix via the temporary opening of the mitochondrial permeability transition pore (MPTP), a large channel within the inner membrane built from adenosine triphosphate (ATP) synthase dimers (Moore and Hand, 2016). Although these methods are effective (~190 mM) in 5 min, their invasive nature can significantly harm the cells, potentially leading to additional damage when cells are frozen (Zhang et al., 2019).

1.3.3.3 Chemical methods

1.3.3.3.1 Liposomes

Liposomes are considered promising carriers for transporting trehalose into mammalian cells (Holovati et al., 2009). Typically, a phospholipid includes one hydrophilic head and two hydrophobic tails. The liposome, a microscopic vesicle that can be either unilamellar or multilamellar, is self-assembled by phospholipids in an aqueous environment (De Leeuw et al., 2009). Besides being utilized as a cell membrane model, liposomes also turn to be a system for intracellular delivery (Schmid and Korting, 1996). Since liposomes and cell membranes have an analogous composition, liposomes may reduce the cell membrane injury from the exchange of phospholipids during rehydration (Kheirrolomoom et al., 2005). The hypothesis is that liposomes can modify the cell membranes through the transfer of lipids and cholesterol, thus altering their physical characteristics and affecting their stability at reduced temperatures (Hu et al., 2023). Stoll and colleagues observed that liposomes could contribute to the cryopreservation process of RBCs (Stoll et al., 2012). Unsaturated phosphatidylcholine lipids like 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) demonstrated the effectiveness in minimizing haemoglobin leakage during freeze-drying (Kheirrolomoom et al., 2005). However, this method is limited to introducing only micromolar levels of trehalose into the cells, and the discharge of trehalose from these vesicles into the cells is not particularly efficient (Stewart and He, 2018, Zhang et al., 2019).

1.3.3.3.2 Polymers

A polymeric nanocapsule synthesized from Pluronic F127 and polyethylenimine (PEI) has been created for the delivery of trehalose into mammalian cells (Choi et al., 2006). The characteristics of this nanocapsule, including its size, surface charge, and permeability, are dependent on temperature (Zhang et al., 2009). These thermally responsive properties were leveraged to encapsulate trehalose, allowing for its effective delivery into cells and controlled release (Zhang et al., 2009). The successful intracellular delivery of a substantial quantity of trehalose into mammalian cells, enough for the biostabilization of mammalian cells (100mM–

300 mM), can be accomplished with the assistance of Pluronic F127–PEI nanocapsules (Zhang et al., 2009). However, it is essential to regulate the concentrations of extracellular trehalose and nanocapsules. Furthermore, the nanocapsules exhibit negligible cytotoxicity (Zhang, Rong et al. 2009), making them a viable option for successfully introducing trehalose into mammalian cells. The attachment efficiency of human adipose-derived stem cells after cryopreservation was $59.2\% \pm 4.1\%$ for 6.2 mM nanoparticle-encapsulated trehalose in the preincubation medium, respectively (Rao et al., 2015). While this attachment efficiency is lower compared to cells cryopreserved with DMSO ($75.2\% \pm 5.8\%$).

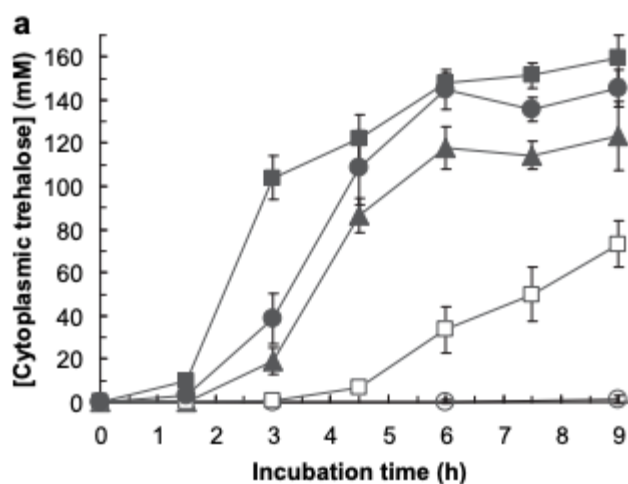


Figure 1-16. Trehalose loading of erythrocytes in 360 mM M trehalose solution (□) and with the addition of 50 mg mL⁻¹ of polymer PP-50 (▲), 100 mg mL⁻¹ PP-50 (●), 150 mg mL⁻¹ PP-50 (■), and 300 mg mL⁻¹. Temperature at 37 °C; pH at 7.05. Error bars represent standard deviations (Lynch et al., 2010). Copyright 2020, with permission from Elsevier.

pH-responsive, membrane-active polymers were designed for more efficient intercellular delivery of trehalose. The pH-responsive polymer has a better performance in loading trehalose, through reversibly increasing the permeability of the membrane to trehalose without causing significant cell damage (Lynch et al., 2010). L-phenylalanine, one of the hydrophobic amino acids, was grafted onto the carboxyl groups pendant to the PLP backbone at different degree of grafting to improve the membrane permeabilising activity of the pseudopeptidic polymer. The amphipathic PP50 polymer (grafted with L-phenylalanine at a stoichiometric ratio of 50 mol%) displays a pH-responsive change of conformation from an expanded coil at physiological pH to a collapsed, compact globular structure at acidic pH due to protonation of pendant carboxyl

groups. The resulting hydrophobic microdomains at acidic exhibit enhanced the membrane association, resulting in a thinner bilayer membrane and consequently increasing the trehalose uptake (Chen et al., 2009b). PP50 was shown to increase the intracellular concentration of trehalose to 123 ± 16 mM (Lynch et al., 2010). The erythrocyte survival rate reaches $82.6 \pm 3.4\%$ after loaded with trehalose, $20.4 \pm 5.6\%$ higher compared with the erythrocytes incubated with extracellular trehalose only (Lynch et al., 2010). PP50-mediated trehalose delivery method has also been utilized in DMSO-free cryopreservation of nucleated human cell line SAOS-2. The number of metabolically active cells at 24 hours post-thaw has been significantly improved to the range of 103 ± 4 to $91 \pm 5\%$, which was comparable to the results using DMSO (Chen et al., 2009c). However, the delivery of trehalose by PP50 needs a long time for loading (Lynch et al., 2010). It can take approximately 9 hours to reach intracellular trehalose threshold concentrations (100-200 mM) as demanded for optimal bioprotection of cells during cryopreservation as Figure 1-16 shows (Lynch et al., 2010). The long incubation time needed for intracellular trehalose delivery is unsuitable for cell therapies, which usually consists of many time-consuming processes. On the other hand, the intracellular trehalose delivery efficiency, namely the ratio of intracellular trehalose concentration to the concentration of the trehalose loading solution, was no more than 35%. Consequently, high concentrations of loaded extracellular trehalose must be used.

Recently, in order to overcome the drawbacks of PP50, new comb-like PLP-NDA biopolymers were introduced to mimic membrane proteins containing relatively long hydrophobic chains acting as membrane anchors as Figure 1-16 shows (Chen et al., 2017). The pH-responsive, membrane-anchoring PLP-NDA polymers were synthesized by grafting hydrophobic relatively long decylamine (NDA) alkyl chains onto the carboxyl groups pendant to the PLP backbone at different degrees of substitution (Chen et al., 2017). The significantly enhanced hydrophobicity of PLP-NDA18 (at a degree of grafting of 18 mol% with NDA) resulted in the exceptional membrane-disruptive ability at endosomal pH compared to PLP. When cells were incubated with a PBS buffer (pH 6.1) containing 0.8 mg mL^{-1} PLP-NDA18 and 360 mM trehalose for 60 minutes, the concentration of trehalose inside the cells reached 300 ± 23 mM. In contrast, when using PP50 under the same conditions but for a prolonged period (9 times the duration), the intracellular trehalose concentration achieved was 2.4 times lower than that attained with PLP-NDA18 (Chen et al., 2017). Consequently, the erythrocyte survival rate achieved $90.3 \pm 0.7\%$ after freeze-thaw, 20.9% higher than the results in the absence of PLP-NDA18. Both the

intracellular trehalose delivery of efficiency and erythrocyte cryosurvival were much higher than the results with PP50. Furthermore, the trehalose loading time was significantly reduced from 9 h to 15 min compared with PP50 (Chen et al., 2017). Subject to optimized conditions, 178 ± 9 mM trehalose was loaded into erythrocytes by PLP-NDA18 after only 15 min of incubation (Chen et al., 2017). The limitation of the study is that the loading of trehalose into erythrocytes using PLP-NDA18 need to be done under slightly acidic conditions, which can be physiologically challenging. Such conditions could potentially affect the stability and function of erythrocytes. Ensuring that the process can occur efficiently under more neutral pH levels would be crucial for its practical and clinical applications.

1.4 Research gaps

This section evaluates the current research gaps in the field of pH-responsive intracellular delivery systems, a rapidly evolving area in drug delivery and biomedical engineering. Despite notable progress, there are still several areas requiring further exploration to enhance the efficacy and applicability of these systems in clinical settings. Consequently, pH-responsive polymers have potential as drug carriers, with the capability to target environments with marginally lower pH levels, such as the extracellular surroundings of cancer cells (Zhou et al., 2023). These systems exploit the differential pH levels in various body compartments to trigger drug release, offering a promising approach for targeted therapy. However, challenges remain in optimizing these pH-sensitive drug delivery systems for clinical use include ensuring precise control over drug release to prevent premature or delayed release, which could reduce therapeutic efficacy or cause side effects (Smith et al., 2019).

Another area needing attention is the range of pH sensitivity. Most current systems are optimized for the acidic environments of endosomes or tumor tissues, but they might not respond adequately to subtle pH variations present in other physiological or pathological conditions (Wang, 2021). This calls for the development of systems with a broader or more finely tuned pH-responsive range. Moreover, the response of these systems to subtle pH changes in various diseases could lead to early detection and more effective treatment strategies (Kim et al., 2014). However, this area is still underexplored.

Understanding the mechanisms of endosomal escape is also crucial. While some progress has been made, a more detailed understanding could lead to the development of more efficient delivery systems (Deng et al., 2016). Additionally, ensuring the biocompatibility and reducing the toxicity of these materials is paramount for their safe long-term use in humans. Nonetheless, there is an urgent and significant need to develop innovative nano-DDSs that offer high payload capacity, minimal toxicity, accurate targeting, multifunctionality, outstanding biocompatibility, and robust stability (Chen et al., 2023).

Research gaps in the field of trehalose-based cell cryopreservation are crucial areas where further studies could lead to significant advancements (Motta et al., 2014). Disaccharides like sucrose and trehalose have been extensively utilized as natural cryoprotectants. Trehalose has been shown to be a more suitable extracellular cryoprotectant medium for vitrification, based on its lower apoptotic level compared to sucrose (Lestari et al., 2018). Trehalose known for its remarkable ability to protect organisms and cells against stress conditions like desiccation and freezing. Despite its potential, there are several areas that need more exploration: determining the optimal concentration of trehalose for different cell types remains a challenge. Different cells may respond uniquely to trehalose, and finding the right balance to maximize protection without causing cellular damage is crucial.

Effectiveness largely depends on its ability to penetrate cell membranes, a process not fully understood in many cell types. Research into enhancing trehalose cellular uptake or developing methods to facilitate its membrane penetration could significantly improve cryopreservation outcomes. Making trehalose-based cell cryopreservation methods cost-effective and accessible, particularly in resource-limited settings, is another area that requires attention. By addressing these research gaps, the field of cryopreservation can be advanced, potentially leading to ground-breaking developments in medical science and biotechnology. For instance, PP50 is shown to load trehalose into erythrocytes, reaching high intracellular trehalose concentrations in approximately 9 hours (Lynch et al., 2010, Lynch et al., 2011). PLP-NDA18 displayed decent erythrocyte membrane permeabilizing activity with high concentrations of intracellular trehalose at mildly acidic pH within 15 min (Chen et al., 2017), but the low solubility limits its usage. Therefore, a new improved polymer is required to overcome this problem. Nevertheless, due to the hydrophobic alkyl chains grafted on the PLP backbone, it reduces solubility and pH-responsiveness. Its hydrophilic carboxyl groups were partially substituted (18 mol%) by the hydrophobic alkyl side chain, resulting in a reduced solubility of PLP-NDA.

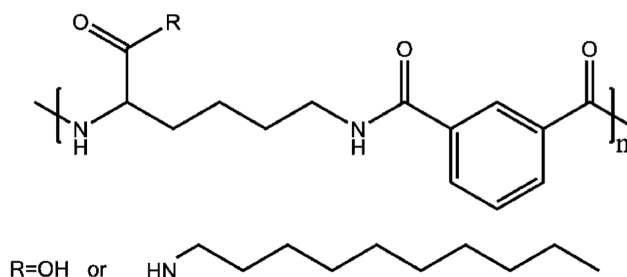


Figure 1-17. Schematic of poly(L-lysine iso-phthalamide)s grafted with NDA (Chen et al., 2017). Copyright 2017, with permission from American Chemical Society.

Addressing these gaps could significantly enhance the development of pH-responsive intracellular delivery systems, leading to more effective, safe, and targeted treatments. This would not only advance the fields of drug delivery and cell cryopreservation but also have a profound impact on patient care and treatment outcomes.

1.5 Aim and objectives of the project

This Ph.D. project aims to develop novel, biocompatible, pH-responsive, membrane-permeabilizing polymers for efficient delivery of different payloads into cells and spheroids. The versatile polymer method is applied for intracellular drug delivery for cancer therapy and intracellular trehalose loading for DMSO-free cell cryopreservation. Key objectives include:

1) Synthesis and characterization of pH-responsive pseudopeptidic polymers: This involves modifying PLP by attaching long fatty acid side chains to its pendant carboxyl groups at varying degrees of substitution to manipulate the pH-dependent membrane-permeabilizing activity. The presence of one carboxyl group at the end of each fatty acid is intended to boost the hydrophilicity and its pH-sensitive membrane interaction, offering an improvement over the PLP-NDA polymers grafted with mere alkyl side chains.

2) Investigation of physicochemical properties and cell membrane activity: The study examines how these fatty acid side chains affect the pH-responsive properties of the polymers.

Using a haemolysis model, the pH-dependent membrane destabilization capabilities and the underlying polymer-membrane interaction mechanisms are investigated.

3) Characterization of the pH-responsive polymers for intracellular delivery: This entails identifying the most effective endosomolytic pseudopeptidic polymer for cytoplasmic delivery. Its delivery efficiency is evaluated under different conditions including pH levels, polymer concentrations, and treatment durations. The ability of the polymer to deliver various payloads into multiple cell types (including spheroids) is assessed, alongside the in vitro cytotoxicity, and the intracellular delivery mechanisms are elucidated. Successful intracellular delivery of a therapeutic protein is demonstrated for cancer therapy.

4) Development of the polymer-based intracellular trehalose delivery method for DMSO-free cell cryopreservation: This section aims to advance current cryopreservation techniques and make a significant impact on cell therapy. It achieves this by optimizing the intracellular delivery of trehalose to mammalian cells (erythrocytes and Jurkat cells) under physiological pH conditions. This enhancement in cell viability has the potential to revolutionize cell preservation and therapeutic strategies.

1.6 Novelties and impact of the research

The research introduces several novel aspects:

- **Novel bio-inspired pseudopeptidic polymers:** Drawing inspiration from membrane proteins with long fatty chains, this project is to develop pH-responsive, membrane-active polymers by grafting long fatty acid side chains to the PLP backbone. This modification improves the pH-mediated membrane binding and destabilization.
- **A novel method for non-toxic, rapid intracellular delivery:** The project introduces a unique, efficient method for polymer-mediated delivery a range of payloads with different sizes into various cell types at slightly acidic pHs. This method is applied for

intracellular delivery of therapeutic agents for cancer therapy and intracellular trehalose loading for cell cryopreservation.

The research outcomes enhance our understanding of polymer-membrane interactions and intracellular delivery mechanisms. The findings will inform the design of novel polymers for delivering diverse therapeutic agents to target sites within cells. These biomimetic delivery systems, applicable across various cell types, hold promise for advancing disease therapy.

Furthermore, this research lays the groundwork for developing other delivery platforms and biomedical applications. For instance, the polymers are applied for cell preservation and cell therapy. These advancements have significant implications for the biopharmaceutical and healthcare industries, potentially enhancing treatment efficacy and improving patient outcomes.

1.7 Thesis outline

This thesis is structured into six chapters, each focusing on various aspects of intracellular delivery, particularly using pH-responsive polymers. The chapters are outlined as follows.

Chapter 1 provides a contextual foundation by delving into the domain of DDSs, tracing their historical development, examining the challenges encountered, and discussing the present status of the field. It emphasizes pH-responsive polymers, a critical element of this thesis, and outlines the significance of these polymers in intracellular drug delivery for disease treatment and intracellular trehalose loading for cell cryopreservation.

Chapter 2 outlines the methodologies employed in the research, encompassing the synthesis and characterization of pseudo-peptidic polymers, the investigation of their physicochemical

properties and membrane activity, and the cell assays used for evaluating cytotoxicity, intracellular delivery effectiveness and mechanisms, and cell cryopreservation.

Chapter 3 describes the synthesis and characterization of the novel pH-responsive pseudopeptidic polymers. It discusses the modifications of PLP with various fatty acid side chains and investigates how these modifications influence the physiochemical properties of the polymers.

Chapter 4 describes the pH-responsive membrane activity of the pseudopeptidic polymers and their use for delivering a diverse range of payloads into various cell types, alongside their *in vitro* cytotoxic effects. Additionally, it investigates the cellular pathways of endocytosis and endosomal escape. The emphasis is placed on the ability of the polymer to enhance the toxic effect of Saporin on A549 cells and its ability in delivering to three-dimensional multicellular spheroids.

Chapter 5 provides an in-depth examination of the polymer-mediated intracellular delivery of trehalose, detailing the optimization of various conditions such as polymer concentration, intracellular pH, and incubation time. It further discusses how the optimized polymer-mediated trehalose loading improves the cryosurvival of erythrocytes and Jurkat cells.

Chapter 6 summarizes the research presented in the thesis, drawing conclusions from the research findings and outlining recommendations for future research in this field.

Chapter 2

Materials and Methods

This chapter describes detailed information about the materials and methods used in the thesis research. The materials contain chemicals, solvents, cell lines, culture media, assay kits, and analytical instruments. The methods include synthesis and characterization of various polymers, polymeric delivery protocols, and cell-based assays for investigating efficacy and cytotoxicity.

2.1 Materials

iso-phthaloyl chloride, Dulbecco's modified Eagle's medium (DMEM), MEM non-essential amino acid solution (100×), fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (D-PBS), RPMI-1640 medium, Saporin from *Saponaria officinalis* seeds, amiloride hydrochloride hydrate, ammonium chloride, nystatin, chlorpromazine hydrochloride, Methyl beta-cyclodextrin (M β CD), Calcein, pyrene, and fluorescein isothiocyanate-dextran (FITC-Dextran, average Mw = 4 k, 10 k, 70 k, 150 k and 2000 kDa) were purchased from Sigma-Aldrich (Dorset, UK).

Dimethyl sulfoxide (DMSO), triethylamine, *N,N*-dimethylformamide (DMF), 4-dimethylaminopyridine (DMAP), sodium chloride, hoechst 33342, alamar blue, Texas Red® hydrazide, and LysoTracker® red DND-99 were purchased from Fisher Scientific (Loughborough, UK).

L-Glutamine (200mM), Penicillin-Streptomycin (10,000 U/mL), HEPES, Nonessential amino acids, Trypsin-EDTA (0.25%), Dead Cell Apoptosis Kit with PI were purchased from ThermoFisher.

Anhydrous ethanol, acetone, hydrochloric acid, d_6 -DMSO, potassium carbonate, sodium hydroxide, methanol, diethyl ether, chloroform, triethylamine, sodium hydrogen carbonate, sodium citrate dehydrate and sodium phosphate were obtained from VWR (Lutterworth, UK).

L-lysine methyl ester dihydrochloride, *N,N'*-dicyclohexylcarbodiimide (DCC), and triton® X-100 were purchased from Alfa Aesar (Heysham, UK).

CellTiter-Glo® 2.0 Assay were purchased from Promega (Southampton, UK). Cyanine5 amine (Cy5, cat. no. ab146463) were purchased from Abcam (Cambridge, UK).

12-amino-1-dodecanoic acid methyl ester hydrochloride salt were purchased from Toronto Research Chemicals (Toronto, Canada).

Trehalose Assay Kit were purchased from Abbexa (Cambridge, UK)

Defibrinated sheep blood (cat. no. SB054) were obtained from TCS Biosciences Ltd (Buckingham, UK).

Glass-bottom cell culture dishes (35 mm, cat. no. P35G-1.5-14-C) were purchased from MatTek, (Ashland, MA, USA).

Corning® spheroid microplates (cat. no. CLS4515-5EA), Corning™ Falcon™ Test Tube with Cell Strainer Snap Cap were purchased from Corning (Flintshire, UK)

Eppendorf 96-Well Cell Imaging Plates (cat. no. 0030741013) was purchased from Eppendorf (Stevenage, UK).

2.2 Methods

2.2.1 Polymer synthesis

2.2.1.1 PLP synthesis

Poly(L-lysine isophthalamide) (PLP) was synthesized by single phase polymerization method as described by Eccleston (Eccleston et al., 1999, Eccleston et al., 2000) in Figure 2-1.

2.2.1.1.1 Polycondensation

0.2 molar L-lysine methyl ester·2 HCl (0.15 mole, 35 g) and 0.8 molar potassium carbonate (0.6 mole, 83 g) were dissolved in precooled deionized water (750 mL, 0 °C) and stirred with a Teflon-coated magnetic stir bar in ice bath. 0.2 molar anhydrous iso-phthaloyl chloride (0.15 mole, 30.5g) was dissolved in pre-cooled dried acetone (750 mL, -20 °C) and rapidly poured into the aqueous solution and stirred at the maximum speed. The reaction was allowed to proceed around 30 minutes. The precipitation of poly(L-lysine methyl ester iso-phthalamide) (PLP methyl ester) was collected with a spatula from reaction mixture. The polymer was repeatedly washed with deionized water to remove any residual solvent and salt, and then dried in an oven at 90 °C overnight. Once completely dry, the polymer was blended into a fine white powder.

2.2.1.1.2 Ester hydrolysis

The PLP methyl ester powder was dissolved in DMSO. 5 wt% NaOH (1.25 M, 2.5 molar equivalents to PLP methyl ester) was prepared in anhydrous ethanol and then added in PLP methyl ester in DMSO. The hydrolyzed product precipitated out in 10 minutes and was collected by vacuum filtration and redissolved in deionized water.

2.2.1.1.3 Purification

HCl solution (2 M) was added to polymer solution to precipitate the polymer completely. The precipitated polymer was collected by vacuum filtration and redissolved in NaOH (0.2 M). The process of precipitation, filtration, and redissolution was repeated at least three times. To purify and remove inorganic salts, residual organic solvents, and low molecular weight oligomers, dialysis was performed. The crude polymer solution was dialyzed using a Visking tubing membrane (Medicell, MWCO 12-14 kDa) in deionized water for 5 days.

The deionized water was changed 2-3 times a day to maintain the concentration gradient. After 5 days, the clear polymer solution was adjusted using NaOH (0.2 M) to the final pH at 7.4. It was then lyophilized to produce PLP in the sodium salt form. For structural characterization and side chain modification, the PLP was prepared in its acidic form by acidifying the dialyzed polymer solution to a pH of approximately 3.0 using a HCl (2 M). The precipitate was collected by vacuum filtration, washed with deionized water to remove HCl, and lyophilized to fine white powder.

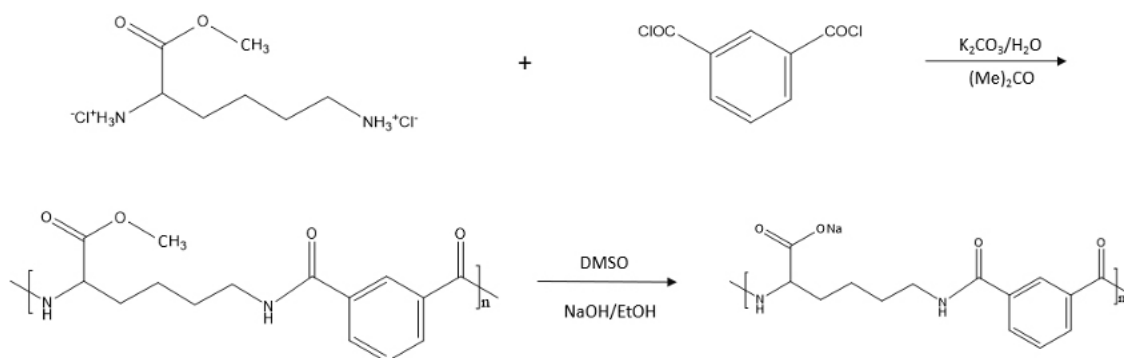


Figure 2-1. Schematic of synthesis of poly(L-lysine iso-phthalamide).

2.2.1.2 Synthesis of PLP-ADA polymers

12-amino-1-dodecanoic acid methyl ester (ADA methyl ester) was conjugated onto the pendant carboxylic acid moieties along the PLP backbone at various degrees of substitution via DCC/DMAP mediated amide coupling chemistry following the method reported by Chen et al. (Chen et al., 2009a). The numbers (30, 45, or 60) represent the stoichiometric percentages of ADA relative to the pendant carboxylic acid groups.

PLP (3.0 g), 12-amino-1-dodecanoic acid methyl ester hydrochloride salt (0.87 g for PLP-ADA30, 1.30 g for PLP-ADA45 and 1.73 g for PLP-ADA60, triethylamine (2.4 molar equivalents of ADA) and DMAP (0.6 g) were dissolved in 60 mL anhydrous DMSO/DMF (1:3 v:v). DCC (3 molar equivalents of ADA) was dissolved in 20 mL anhydrous DMF and added dropwise to the previous solution. The reaction was proceeded at room temperature for around 60 h.

Solid impurities were removed by vacuum filtration. The reaction mixture was added with 5 wt % NaOH (200 mL) in anhydrous ethanol and hydrolysed for a 12-hour reaction. After that, five volumes of diethyl ether rapidly poured into the hydrolysed solution to precipitate PLP-ADA. The precipitate was collected by vacuum filtration and redissolved in deionized water. HCl solution (0.2 M) was then used to precipitate the polymer. The precipitate was collected by vacuum filtration and redissolved in deionized water with NaOH (0.2 M). The precipitation–filtration–redissolution process was repeated twice to remove inorganic salts and residual organic solvents. The polymer was further purified by dialysis against deionized water in a Visking dialysis tubing for a week. The acidic and salt forms are produced by lyophilization into a fine white powder as described above.

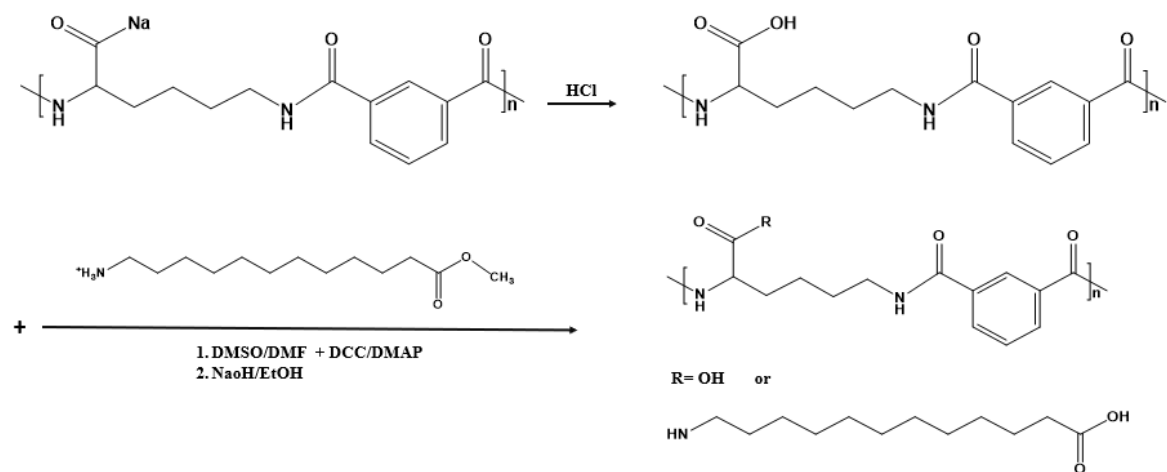


Figure 2-2. Schematic of synthesis of PLP-ADA polymers.

2.2.2 Polymer characterization

The properties of the polymers, including their compositions, structures, pH-responsive conformational changes, critical association concentrations (CACs), hydrodynamic diameters, and pH-responsive membrane-destabilizing activities, were analysed by Fourier Transform Infrared (FT-IR) spectroscopy, nuclear magnetic resonance spectroscopy (NMR), transmittance measurement, fluorescence spectroscopy, dynamic light scattering (DLS), zeta potential measurement, and haemolysis assay. Details for these measurements are provided below.

2.2.2.1 Fourier transform infrared (FT-IR) spectroscopy

Fine acidic powdered polymer was pressed on the platform and FT-IR spectra were recorded on a Spectrum 100 FT-IR Spectrometer (Perkin Elmer, USA) to provide details on the functional groups of polymer samples.

2.2.2.2 Nuclear magnetic resonance (NMR) spectroscopy

Powdered acidic polymers were dissolved in d_6 -DMSO, and their ^1H -NMR spectra were recorded using a 400 MHz NMR spectrometer (Bruker, Germany) at ambient temperature. The ratio between the integral peaks at 2.10-2.29 ppm and 7.38-8.42 ppm in the spectrum of each PLP-ADA polymer was utilized to determine the actual degree of grafting, as the number of ADA grafts per 100 carboxylic acid groups on the PLP backbone, measured in mol%. Subsequently, the molecular weight of a PLP-ADA was calculated using the actual degree of substitution and the molecular weight (M_w) of the parent PLP.

2.2.2.3 Gel permeation chromatography (GPC)

Gel Permeation Chromatography (GPC) is a common method for analysing both the molecular weight and polydispersity. This chromatographic technique separates polymers based on their hydrodynamic volume, using a stationary column densely filled with polymer beads of varying pore sizes. Larger molecules are unable to enter the pores and are thus eluted more quickly

from the column. Calibration of GPC systems is typically done using standards with known molecular weights to ascertain the sizes of the target macromolecules.

The aqueous Agilent Technologies Infinity 1260 MDS instrument (Department of Chemistry, University of Warwick) utilized a differential refractive index, light scattering, UV and viscometry detectors. The system was equipped with 2 x PLgel Mixed D columns (300 x 7.5 mm). The mobile phase consisted of DMF with 5 mmol NH_4BF_4 additive with a flow rate of 1 mL min^{-1} . Calibration was performed using poly(methyl methacrylate) standards (Agilent EasiVials) within the range of 955,000 to 550 gmol^{-1} . Analyte polymer samples were dissolved in a 0.1 M NaNO_3 aqueous solution and filtered through a nylon membrane 0.22 μm pore size prior to injection. The experimental molecular weights and polydispersities of the synthesized polymers were recorded on Varian 390-LC system.

2.2.2.4 Thermogravimetric analysis (TGA)

Polymer powder samples were weighed and carefully placed into Tzero Aluminium Hermetic pans, with each sample ranging from 4 to 5 mg. The pans were subsequently sealed and subjected to testing using a TA Q200 differential scanning calorimeter. During the testing process, the samples underwent controlled cooling, ranging from 0 $^{\circ}\text{C}$ to 800 $^{\circ}\text{C}$, with a gradual ramp rate of 5 $^{\circ}\text{C}$ per minute. The resulting data, including the glass transition temperatures (T_g) of the polymer samples, were then meticulously analyzed using the TA DSC software.

2.2.2.5 Transmittance

Polymer solutions were prepared in 100 mM phosphate or citrate buffer, with specific pH values. These solutions were left to reach equilibrium over a period of 48 hours. The transmittance of the polymer solutions at different pH levels was determined by measuring the absorbance using a Genesys 10S UV-vis spectrophotometer from Thermo Scientific (UK) at a wavelength of 480 nm. The optical densities of polymer solutions at different pHs were investigated on a UV-Vis spectrophotometer (Genesys 10s UV-Vis, Thermo Scientific, UK) at 480 nm. solutions were prepared with buffers at different pHs and equilibrated for 48h before measurement.

2.2.2.6 Fluorescence spectroscopy

Pyrene, a hydrophobic compound, was employed to study the conformational attributes of polymers in aqueous solutions (Chen et al., 2009a, Chen et al., 2009c, Aguiar et al., 2003). A fresh 1.0 mM pyrene solution was freshly prepared in absolute methanol. This solution was then added to each polymer solution at a specific pH, resulting in a final pyrene concentration of 600 nM. The critical association concentration (CAC) was also determined using the fluorescence excitation spectra of pyrene. Here again, pyrene was added to the polymer PB buffer solutions to achieve a final concentration of 600 nM. To shield them from light, the samples were covered with foil and allowed to equilibrate for 48 hours prior to any measurements. Fluorescence intensities at the emission ($\lambda_{em} = 390$ nm) and excitation ($\lambda_{ex} = 333$ nm, 338 nm) spectra of pyrene were recorded using a FluoroMax spectrofluorometer (HORIBA, Japan). The ratio of fluorescence excitation intensities, denoted as I_{338}/I_{333} , was calculated. By studying the concentration-dependent variations of I_{338}/I_{333} at pH 7.4, the CACs of the polymers were determined.

2.2.2.7 Dynamic light scattering (DLS) and zeta potential

The hydrodynamic diameters and zeta potential of the polymers were examined using a Zetasizer Nano ZS (Malvern, UK). Aqueous solutions of the polymers were formulated in different buffers with designated pH levels, filtered through a 0.45 μ m Milipore filter, and allowed to equilibrate for 48 hours before any measurements were taken. Hydrodynamic diameters were measured in 10 mm-wide disposable cuvettes at a scattering angle of 137°. Each run was replicated 11 times to ensure accuracy. Zeta potential for each sample was evaluated using disposable capillary cells and each measurement was conducted three times for consistency.

2.2.2.8 Haemolysis assay

The membrane-disrupting capabilities of the polymers were assessed through a haemolysis assay (Chen et al., 2009b). Polymer stock solutions were mixed into either 0.1 M phosphate buffered saline (PBS) or 0.1 M citric buffer at various concentrations and pH. Sheep Red Blood

Cells (RBCs) were washed three times with a PBS (pH 7.4) solution, and then resuspended in the prepared polymer buffer solution. The final concentration of cells was maintained within a range of $1 - 2 \times 10^8$ RBCs mL^{-1} , to ensure the linear relationship between the RBC density and haemoglobin absorbance (see the standard curve in Figure A-1 in Appendix A). Two control samples were set up, one with RBCs resuspended solely in buffer at the same pH (negative control), and the other with RBCs resuspended in deionized water (positive control) at the same cell density. Each test was performed in triplicate. Samples were incubated at 37°C in a shaking water bath at 1000 rpm for different time periods, followed by centrifugation at 3500 rpm for 3 minutes. They were transferred to corresponding cuvette, and its absorbance at 540 nm was measured using a UV-Vis spectrophotometer to determine the release of haemoglobin. The relative percentage of haemolysis was subsequently calculated by Equation 2-1:

$$\text{Relative hemolysis (\%)} = \frac{\text{Sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance}} \times 100$$

Equation 2 - 1

2.2.3 Cell study

2.2.3.1 Cell culture

Cell culture was maintained in a humidified incubator at 37°C with 5% CO_2 . All cell culture work was conducted under aseptic conditions. For long term storage, cells were cryopreserved in liquid nitrogen. Briefly, cells were harvested, and the density was determined using a cell counter (CytoSMART, Corning). The cells were centrifuged at 1000 rpm or 500 g for 5 minutes and resuspended in a cryopreservation medium containing 10% DMSO (v/v) and 90% FBS (v/v). The final density of cells in the cryopreservation medium was kept between 1×10^6 to 1×10^7 cells per mL. Cryogenic storage vials containing 1 mL of cells were transferred to a Mr Frosty™ and placed in a -80°C freezer overnight. Afterwards, the vials were transferred to the liquid nitrogen tank. Cells were thawed from the liquid nitrogen by placing the cryogenic storage vial in a preheated water bath at 37°C . Once the ice had melted, 1 mL culture medium was added to the cell suspension

and centrifuged at 1000 rpm or 500 g for 5 minutes. The medium was removed, and the cell pellet was resuspended in fresh culture medium and transferred into a cell culture flask for incubation.

HeLa adherent epithelial cells (human cervical cells) and A549 adherent epithelial cells (human lung cells) were grown in DMEM supplemented with 10% (v/v) FBS and 100 U mL⁻¹ penicillin unless specified otherwise. CHO adherent epithelial cells (Chinese hamster ovary cells) were cultured in DMEM supplemented with 1% (v/v) non-essential amino acids, 10% (v/v) FBS and 100 U mL⁻¹ penicillin unless specified otherwise. The HeLa, A549 and CHO cells were maintained in a humidified incubator with 5% CO₂ at 37 °C. The initial cell seeding density was approximately 1×10⁵ mL⁻¹, ensuring that cell numbers could double within 2-3 days. The morphology, density, and confluence were daily observed using an optical microscope (Inverso TC100 Inverted Biological Microscope, Medline Scientific, UK). Upon reaching approximately 70% confluence, 2 mL of trypsin-EDTA (ethylenediaminetetraacetic acid) was introduced to the flask for cell trypsinization. The flask was then placed in a 37 °C incubator for about 3 minutes until more than 70% of the cells became detached. Subsequently, 8 mL of cell culture medium was added to neutralize trypsin, and the cell solution underwent centrifugation at 1000 rpm for 5 minutes. The resulting cell pellets were resuspended in fresh culture medium for the subsequent cell passage.

Jurkat cells (human T lymphocyte) was generously provided by Professor Xiao-Ning Xu (Department of Infectious Disease, Faculty of Medicine, Imperial College London UK) and were grown in RPMI media supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) HEPES and 1% (v/v) sodium pyruvate. The cell culture flasks were placed in a humidified incubator with 5% CO₂ at 37 °C. The morphology and density were examined daily using an optical microscope (Inverso TC100 Inverted Biological Microscope, Medline Scientific, UK). When the cells formed small, loosely associated clusters, cell solutions underwent centrifugation at 500 g for 5 minutes. The resulting cell pellets were then resuspended in fresh culture medium for subsequent cell passaging.

2.2.3.2 Laser scanning confocal microscopy

2.2.3.2.1 Laser scanning confocal microscopy of RBCs

To investigate the mechanism of cell membrane destabilization, laser scanning confocal microscopy of RBC was performed. RBCs were washed three times with PBS (pH 7.4), and then resuspended with buffers at specific pHs with 10 μ M FITC-Dextran and 1 μ M Texas Red® hydrazide with or without the presence of polymer at a cell concentration of 10^7 RBCs mL⁻¹. The RBCs were then imaged using a laser scanning confocal microscope (LSM-510 inverted, Zeiss, Germany) at 37 °C. FITC and Texas red were excited at 488 and 543 nm respectively.

2.2.3.2.2 Endosomal escape of Calcein co-treated with polymer

In order to verify whether the polymer can induce endosomal escape, Calcein, a membrane-impermeable dye was used as a tracer molecule. 2 mL of HeLa cells (1×10^5 cells mL⁻¹) were cultured in a glass-bottom dish (35 mm, MatTek, USA) for overnight followed by the treatment with 2 mL of the serum free DMEM containing Calcein (2 mg mL⁻¹) and with or without polymer. After incubation at 37 °C for different time periods, the cells were washed with DPBS for three times and replaced with fresh DMEM serum free medium for 2 h of further incubation. Hoechst 33342 (1 μ g mL⁻¹) and LysoTracker (50 nM) were used to stain nuclei and endosomes/lysosomes respectively. The cells were then imaged using a laser scanning confocal microscope as described above. Calcein was excited at 488 nm and the emission at 535 nm was collected.

2.2.3.2.3 PLP-ADA mediated intracellular delivery of Calcein in the presence of endocytosis inhibitors

To elucidate the mechanism of PLP-ADA entry, HeLa cells (1 mL, 1×10^5 cells) were seeded in a 35 mm glass-bottom Mattek dish overnight. The cells were pre-treated with chlorpromazine (3 μ g mL⁻¹), amiloride (30 μ M), nystatin (10 μ g mL⁻¹) or M β CD (1.5 mg mL⁻¹)

¹), in serum-free media for 30 minutes. The medium was removed and replaced with PBS containing Calcein (2 mg mL⁻¹) and PLP-ADA (0.5 mg mL⁻¹) with inhibitors for 30 minutes. The cells were washed three times with PBS and replaced with fresh media containing inhibitors and Hoechst 33342 (1 µg mL⁻¹). Cells were excited using the 405 nm, 488 nm, and 633 nm lasers.

2.2.3.2.4 Staining procedure for live multicellular cancer spheroids

A549 cell spheroids were prepared by seeding 0.1 mL of a 2.5x10³ cell suspension into 96-well ultra-low attachment spheroid plates from Corning and incubating them for 72 hours. Post-incubation, these spheroids were treated with a PBS mixture containing 0.2 mg mL⁻¹ of PLP-ADA60 for 2 hours. Subsequently, the spheroids underwent three PBS washes and were then placed in new media for an additional incubation period of up to 72 hours. Staining was performed using 2 mg mL⁻¹ Calcein AM, and the spheroids were excited with a 488 nm laser at various intervals. Visualization of the spheroids was achieved through Z-stack imaging using the FIJI software.

2.2.3.3 Flow cytometry

All samples were filtered into a 5 mL falcon polystyrene test tube with a cell strainer cap for flow cytometry analysis. Flow cytometry data collection was performed on a BD LSRFortessa and analysed using FlowJo v10.

PLP-ADA-mediated cellular uptake of FITC-Dextran. Flow cytometry was employed to quantitatively evaluate the cellular uptake of FITC-Dextran. A549 cells (1 mL, 3 x 10⁵ cells/mL) were seeded in a 6-well plate overnight and then treated with a PBS solution containing FITC-Dextran 150 kDa (10 µM) with or without PLP-ADA (0.5 mg mL⁻¹) for 30 minutes. The cells were then washed with PBS three times, detached using Trypsin-EDTA, centrifuged (1000 rpm, 5 minutes) and replaced with 0.5 mL fresh PBS. Jurkat cells (1 mL, 5x10⁵ cells/mL) were treated with a PBS solution containing FITC-Dextran 150 kDa (10 µM) with or without PLP-ADA (0.5 mg mL⁻¹) for 30 minutes. The cells were then washed with PBS three times and replaced with 0.5 mL fresh PBS. All samples were excited at 488 nm, and the emission was

collected in the 530/30 nm band. FlowJo v10 was employed for the analysis of the obtained results.

PLP-ADA mediated intracellular delivery of FITC-Dextran in the presence of endocytosis inhibitors. Flow cytometry was employed to quantitatively evaluate the cellular uptake of FITC-Dextran and PLP-ADA in the presence of different endocytosis inhibitors. A549 cells (1mL, 3.0×10^5 cells) were seeded in a 35 mm glass-bottom Mattek dish overnight. The cells were pre-treated with chlorpromazine ($3 \mu\text{g mL}^{-1}$), amiloride ($30 \mu\text{M}$), nystatin ($10 \mu\text{g mL}^{-1}$) or M β CD (1.5 mg mL^{-1}), in serum-free media for 30 minutes. The medium was removed and replaced with sterile-filtered PBS containing FITC dextran 150 kDa ($10 \mu\text{M}$) and PLP-ADA (0.5 mg mL^{-1}) with inhibitors for 30 minutes. The cells were washed three times with PBS, detached using trypsin-EDTA, centrifuged (1000 rpm, 5 minutes) and replaced with fresh PBS containing inhibitors. Cells were excited at 488 nm, and the emission was collected at 530/30 and 670/30 bands. FlowJo v10 was employed for the analysis of the obtained results.

Jurkat cells ($5 \times 10^6 \text{ mL}^{-1}$) were incubated $10 \mu\text{M}$ FITC-Dextran 150 kDa in the absence and presence of PLP-ADA60 at different pH in a humidified incubator with 5% CO_2 at 37°C for 30 min. After the incubation, the cell solution was washed with D-PBS until the supernatants were colourless. After the final centrifugation, the cell pellets were resuspended in D-PBS at the cell density of around $1 \times 10^6 \text{ mL}^{-1}$. Jurkat cells samples were transferred to 5 mL Falcon™ round-bottom polystyrene test tubes. Subsequently, each sample in preparation for analysis using a BD LSRFortessa cytometer (BD Biosciences, USA). Excitation at 488 nm was applied, with FITC emission collected in the 530/30 nm band. FlowJo v10 was employed for the analysis of the obtained results.

2.2.3.4 CellTiter-Glo® 2.0 cell viability assay

To assess the cell viability of cells after treatment with PLP-ADA and payloads, the CellTiter-Glo® 2.0 cell viability assay was employed, which detects the amount of ATP present correlating to the number of metabolically active cells. Cells (0.1 mL , 5×10^3 cells) were treated with a PBS solution containing PLP-ADA at varying concentrations for 30 minutes. The cells were washed three times with PBS, replaced with fresh media, and incubated for 24 hours.

After 24 hours, the medium was removed and replaced with serum-free media (30 μ L) plus CellTiter-Glo® 2.0 Cell reagent (30 μ L) per well. The plate was incubated for 10 minutes at room temperature to stabilise the luminescent signal before the luminescence was read using a spectrofluorometer. Values of viability of treated cells were expressed as a percentage of that from corresponding control cells. For the cell survival of A549 cells after Saporin (1 nM) delivery, A549 cells (0.1 mL, 5×10^3 cells) were seeded in a white flat-bottom opaque 96-well plate and incubated overnight. The cells were either treated with PBS containing free Saporin, polymer or polymer and Saporin combinations at varying concentrations and incubated for varying times. The cells were then washed three times with PBS, replaced with media, and incubated for 72 hours. After incubation, the growth medium was removed and treated with the CellTiter-Glo® 2.0 Cell Viability Assay as described above. Values of viability of treated cells were expressed as a percentage of that from corresponding control cells.

2.2.3.5 Alamar Blue cell viability assay

The cytotoxicity of the pseudopeptidic polymers was studied by Alamar Blue assay in PBS buffer. HeLa cells (0.1 mL, 1×10^4 cells) were seeded in a black, flat transparent bottom 96-well plate and incubated overnight. The medium was replaced with PBS containing PLP-ADA alone at specific concentrations. After a certain period of time, the cells were washed three times with D-PBS and incubated with DMEM with 10% (v/v) Alamar Blue for 4 h. The fluorescence was measured using a spectrofluorometer at emission wavelength 590 - 640 nm and excitation wavelength 525 nm. The fluorescence was determined, and cell viability was calculated as described below.

$$\text{Cell viability (\%)} = \frac{F_t - F_0}{F_{max} - F_0} \times 100$$

Equation 2 - 2

F_t is the fluorescence intensity of the cells treated with sample, F_{max} is the fluorescence intensity of the cells treated with culture medium without sample and F_0 is the fluorescence intensity of the cells lysed with triton® X-100.

2.2.3.6 Trypan Blue assay

Trypan Blue is a dye that is impermeable to cell membranes and thus can stain dead cells, whilst live cells are excluded from staining. 10 μ L cell solution was mixed well with 10 μ L 0.4% Trypan Blue solution, after which 10 μ L of the mixed solution was transferred to a haematocytometer. The live cell number and dead cell number were measured manually under an optical⁴⁹ microscope (Inverso TC100 Inverted Biological Microscope, Medline Scientific, UK). or automatically by an automatic cell counter (Corning, UK). The viability of the cell sample was then calculated by dividing the live cell number by the sum of the live cell number and the dead cell number.

2.2.4 Trehalose loading

2.2.4.1 Preparation of red blood cells and trehalose solution

Sheep RBCs need to be washed by 306 mOsm PBS buffer (pH 7.4) to remove the free haemoglobin before any use. RBCs were first centrifuged by Eppendorf centrifuge 5424 (Hamburg, Germany) at 3500 rpm for 3 minutes. Supernatants were then discarded and then the PBS buffer was added. This process needs to be repeated for three times to ensure that supernatant was colourless. Trehalose solutions were prepared by dissolving trehalose in 306 mOsm phosphate-buffered saline (PBS) at various concentrations and pHs. The loading solutions underwent sterilization using 0.2 μ m Sartorius MinisartTM Plus syringe filters. Erythrocytes (0.5 mL) were washed three times with 1 mL of 306 mOsm PBS (pH 7.4) and then resuspended in 1 mL of trehalose solution in the presence or absence of the polymers. The resulting erythrocyte samples with a final packed cell volume of 15% were incubated in a shaking water bath (100 rpm) at 37 °C to allow trehalose loading.

2.2.4.2 Intracellular Trehalose Loading

2.2.4.2.1 Loading trehalose into RBCs

Approximately 30mL RBCs ($1-2 \times 10^{10} \text{ mL}^{-1}$) were washed with PBS (306 mOsm, pH 7.4) followed by centrifugation at 3500 rpm for 3 min. The washing steps were repeated multiple times to ensure that the supernatant became colorless. After the final centrifugation, the cell pellets were resuspended in twice the volume of the prepared loading solution in the absence or presence of polymer. The cell solutions were then placed in the 37 °C shaking water bath at 120 rpm for a certain period to allow intracellular saccharide delivery. After loading, the cell solutions were centrifuged to remove the unloaded trehalose and free polymer.

2.2.4.2.2 Loading trehalose into Jurkat cells

Jurkat cells were washed with D-PBS once (500 g, 5 min) and the cell pellets were resuspended in the loading solutions in the absence or presence of polymer and at a certain cell density. Typically, the cell density during loading was $1 \times 10^7 \text{ mL}^{-1}$. The cell solutions were then left in a humidified incubator with 5% CO₂ at 37 °C for a certain period. After loading, the cell solutions were centrifuged to remove the unloaded trehalose and free polymer.

2.2.4.3 Intracellular Trehalose Quantification

2.2.4.3.1 Anthrone method

Anthrone reaction was applied for intracellular trehalose quantitation. After trehalose loading, the erythrocyte samples were centrifuged at room temperature and washed twice with 1.5 mL of PBS (pH 7.4) iso-osmotic to the incubation mixture to remove extracellular trehalose. The erythrocytes were then mixed with 5 mL of 80% (v/v) methanol and incubated in a water bath at 90 °C for 1 h. The samples were centrifuged at 10,500 rpm for 5 min. Their supernatants were collected and then evaporated overnight in an oven at a preset temperature of 100 °C.

Each dry residue was dissolved in 2 mL of deionized water. The resulting trehalose solution (0.5 mL) was mixed with 5 mL of 66% (v/v) sulfuric acid containing 0.05% (w/v) anthrone, followed by incubation at 100 °C for 15 min. The absorbance at 620 nm was measured after sample cooling. Negative control (erythrocytes incubated in trehalose for less than 1 min to yield negligible trehalose loading) was prepared to determine the anthrone absorbance of residual extracellular trehalose after washing. Intracellular trehalose concentrations were obtained by comparing to a trehalose concentration versus absorbance standard curve. The trehalose delivery efficiency was then calculated according to the following equation:

$$\text{Delivery efficiency (\%)} = \frac{C_i}{C_t} \times 100$$

Equation 2 - 3

where C_i is the intracellular trehalose concentration after the polymer mediated delivery and C_t is the total trehalose loading concentration.

Standard curve

Trehalose solutions in PBS at different concentrations were prepared to generate the standard curve using the anthrone method. The relationship between the concentration of the trehalose and the absorbance of the trehalose solutions treated by the anthrone method at 620 nm was modelled by linear regression, as shown in Figure A-2 in Appendix B.

2.2.4.3.2 Trehalose assay

The quantification of trehalose uptake by Jurkat cells post-freezing and thawing was conducted using the Abbexa trehalose assay (Abbexa Ltd, Cambridge, United Kingdom), following the manufacturer's instructions. Following the freezing-thawing process of Jurkat cells, the cell samples were prepared by centrifuging at 1000g for 5 minutes, and the supernatant was discarded. Subsequently, the cells underwent washing with 660 mosm PBS, followed by another centrifugation at 1000g for 5 minutes, and the supernatant was again discarded. To lyse the cells, 1 mL DI water was added, and three freeze-thaw cycles were performed. The resulting cell solution was then collected in glass vials. Using a 0.2 µm filter, pre-wetted with 1 mL DI

water, the cells were filtered with a syringe. The filter was then cleaned with an additional 1 mL DI water to ensure the absence of trehalose residue. The glass vial containing the solution was placed in an oven at approximately 60 °C to facilitate the drying of trehalose. Once dry, the trehalose was re-dissolved in 300 uL D-PBS buffer and transferred into glass vials, preparing for measurement using the assay kit.

Before the assay procedure, reagents were brought to room temperature. The prepared Jurkat cells samples and reagents included the creation of assay buffer 2 solution, enzyme working solution, and standard solution in the assay kit. 0.1 ml of the sample and 0.1 ml of assay buffer 2 solution were added to a new tube, vortexed, and incubated at 40°C for 30 minutes. Following this, 0.1 ml of assay buffer 3 was introduced, observing vigorous effervescence. The mixture was vortexed thoroughly, and the assay was conducted immediately. Sample, standard, control, and blank wells were set up, and their positions were recorded. Samples and standards were added to wells, and enzyme working solution was introduced. The plate was gently tapped, incubated, and subjected to subsequent steps involving dye reagent and temperature variations. The absorbance at 540 nm was then read and recorded.

Standard Curve

Four tubes were labeled with concentrations (2.5 mmol L⁻¹, 1.25 mmol L⁻¹, 0.625 mmol L⁻¹, and 0.312 mmol L⁻¹), and 0.5 ml of distilled water was aliquoted into each tube. The first tube (2.5 mmol L⁻¹) received 0.5 ml of 5 mmol L⁻¹ standard Solution, and subsequent tubes were mixed accordingly. The correlation between trehalose concentration and the absorbance of trehalose solutions at 540 nm was represented through linear regression, as depicted in Figure A-3 in Appendix C. The final trehalose concentration was subsequently computed using the following formula:

$$\text{Trehalose (mmol L}^{-1}\text{)} = n \times \frac{C_{\text{Standard}} \times V_{\text{Standard}}}{V_{\text{Sample}}} \times \frac{OD_{\text{Sample}} - OD_{\text{Control}}}{OD_{\text{Standard}} - OD_{\text{Blank}}}$$

Equation 2 - 4

where n is the dilution factor, is the concentration of the highest standard at 5 mmol L⁻¹, V_{standard} and V_{sample} are the volume of standard and samples which both set at 0.05 ml. OD corresponds

to optical density, assessed by testing samples, controls, and blanks at an absorbance wavelength of 540 nm.

2.2.14 Cryopreservation

2.2.14.1 Cryopreservation of RBCs

RBCs loaded with trehalose were washed with PBS and resuspended at a certain cell density in the pre-prepared trehalose cryo-solution. 1 mL cell solution was then transferred to a 2 mL cryovial and put in a 5×5 Nalgene™ cryogenic storage box which was then stored in a liquid nitrogen tank. After a certain period of storage, the cell samples were taken out from the liquid nitrogen tank and were thawed in the 37 °C water bath for 5 min. After thawing, 1 mL cell solution was transferred to a 2 mL centrifuge tube and centrifuged at 3500 rpm for 3 min. The supernatants were transferred to another tube with the volume recorded. A certain volume of DI water was added to lyse the cell pellet till the total volume was 1 mL. The absorbance at 540 nm of the supernatants and the lysed cell pellets was measured to calculate the number of lysed cells and the number of live cells after the freezing and thawing processes, respectively, based on the haemoglobin standard curves as shown in Figure A - 1 in Appendix A. It was assumed that the total cell number in the sample was unchanged during freezing and thawing. The cryosurvival rate was then defined as the number of live cells after thawing divided by the number of live cells before freezing as shown in following equation:

$$\text{Cryosurvival Rate (\%)} = \frac{\text{Cells}_{\text{after thawing}}}{\text{Cells}_{\text{Before freezing}}} \times 100$$

Equation 2 - 5

2.2.14.2 Cryopreservation of Jurkat cells

After trehalose loading, Jurkat cells were suspended in a cryo-solution containing trehalose at a specific cell density. A designated volume of the cell solution was then transferred to a 2 mL cryovial and placed in a 5×5 Nalgene™ cryogenic storage box, subsequently stored in a liquid

nitrogen tank. After a defined storage duration, the cell samples were removed from the liquid nitrogen tank and thawed in a water bath at a specified temperature for a particular period. Following thawing, 10 μL of the cell solution was extracted and diluted with PBS to achieve a cell density of approximately 10^6 mL^{-1} .

Next, 10 μL of the diluted cell solution was thoroughly mixed with 10 μL of trypan blue. A 10 μL portion of this mixture was then transferred to a haematocytometer for either manual cell counting under an optical microscope (Inverso TC100 Inverted Biological Microscope, Medline Scientific, UK) or automated cell counting using a Corning automatic cell counter (UK).

2.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.2.0 (283). Where appropriate, student's t-tests and one way analysis of variance (ANOVA) multiple comparison tests were performed. Statistical significance was determined as n.s. $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$. Statistical analysis was performed on technical repeats unless otherwise indicated. The confidence intervals were 95%

Chapter 3

Synthesis and characterisation of pH-responsive polymers

This chapter describes the synthesis and characterisation of a range of pH-responsive PLP-ADA polymers with varying degrees of grafting with hydrophobic side chains. The structures of the polymers and the modifications of the hydrophobic side chains were confirmed using FT-IR and ^1H -NMR. Their physicochemical properties in aqueous solutions at different pH and concentrations were characterized and the pH-responsive polymer conformational changes were demonstrated.

3.1 Introduction

Polymers employed for intracellular delivery are purposefully designed to overcome cellular barriers and enable the effective transport of therapeutic agents into the cytoplasm of cells. Polymer-based nanoparticles (NPs) have drawn much attention from researchers because of their inherent advantages such as superb biocompatibility, long-term stability, negligible toxicity, and easy conjugation with functional components (Li et al., 2023).

Over the last two decades, there has been significant interest in the development of pH-responsive polymers. pH-responsive polymers exhibit a binary response, transitioning abruptly between states (e.g., soluble and insoluble) at specific pH values. These polymers have found widespread applications in diverse fields, including drug delivery (Shi et al., 2017a, Xu et al., 2016, Nazila et al., 2016, Xu et al., 2017), micromechanical systems (Bashir et al., 2002, Andreeva and Shchukin, 2008), sensors (Bashir et al., 2002, Jinming and Shiyong, 2010, Cai et al., 2004), theranostics (Wang et al., 2017a, Caldorera-Moore et al., 2011, Yang et al., 2019),

biomimetics (Yu et al., 2014), surface functionalization (Barbey et al., 2009, Chen et al., 2010), catalysts (Gong et al., 2017, Jiang et al., 2017), and agriculture (Feng et al., 2015, Hill et al., 2015). In terms of drug delivery application, pH-responsive polymers can facilitate endosomal release of macromolecular drugs and other membrane-impermeable payloads from endosomes into the cytoplasm.

A variety of pH-responsive synthetic polymers have therefore been developed as non-viral vectors to mimic the behaviour of fusogenic viral peptides (El-Sayed et al., 2005, Chen and Chen, 2016, Wang et al., 2015). One significant example is the polyanions, such as polyacrylic acid, and poly(malic acid), which contain both hydrophobic and weakly ionizable carboxyl groups (Yessine and Leroux, 2004). They can exhibit more favourable biodistribution, relatively high transduction efficiencies, and lower cytotoxicity compared to polycations (Chen, 2013). When pH drops below their pKa, the polyanions may change their conformation from coiled hydrophilic structure to collapsed hydrophobic microdomains, which can cause destabilization of lipid membranes (Ho et al., 2011). To enhance membrane destabilization, the balance of hydrophobicity/hydrophilicity of the anionic polymers should be manipulated. This pH-responsive membrane-destabilizing activity has prompted their application in intracellular delivery (Chen et al., 2005). However, most current polyanions are not biodegradable, thus low polymer molecular weights are required. Increasing the molecular weight of polymers reduces renal filtration, leading to longer blood circulation times and greater tumor accumulation (Yang et al., 2015).

A pH-responsive, biodegradable pseudopeptidic polymer, poly(l-lysine iso-phthalamide) (PLP) was reported (Eccleston et al., 1999, Hoffman, 2013). However, its application for drug delivery is limited by its membrane destabilizing ability that is low and occurs in a low pH range from 4.5 to 5.0 (Chen et al., 2008). PLP has been modified by attaching functional groups as side chains, such as fatty (acid) groups, which act as hydrophobic anchors. They insert into the plasma membrane to facilitate membrane binding, resulting in pore formation (Eccleston et al., 1999, Chen et al., 2009a, Chen et al., 2009c, Chen et al., 2017). Incorporating different hydrophobic side chain or changing the length of hydrophobic side chains can lead to the shift of pKa (Tang et al., 2018, Chen et al., 2017). The polymer with the most hydrophobic pentyl group had the lowest pKa of 4.4, whereas the polymer with the least hydrophobic isopropyl

group as the amine substituent exhibited the highest pKa, nearly 6.6 (Tang et al., 2018, Li et al., 2016).

This chapter reports the synthesis and characterisation of a class of novel biodegradable pseudopeptides containing long hydrophobic fatty acids as side chains pendant to the PLP backbone for enhanced cytoplasmic delivery. Controlling the degree of side chain grafting was demonstrated to manipulate the pH-responsive phase separation and conformational changes, which are correlated with pH-responsive cell membrane destabilization reported in Chapter 4. The particle size, zeta potential and pKa were further investigated, and the effects of the degree of grafting were discussed.

3.2 Polymer synthesis and characterization

The pendant carboxylic acid groups along the PLP backbone were conjugated with ADA at a stoichiometric grafting percentage of 30, 45, 60, respectively, through DCC/DMAP mediated amide coupling chemistry. Their functional groups, molecular weight, structure, and thermal behavior were characterized by FT-IR, GPC, ¹H-NMR, and TGA.

3.2.1 FT-IR

After lyophilization, the white powder of PLP and PLP-ADA polymers in both acid and salt forms were obtained. The number ‘n’ in PLP-NDAn (i.e., PLP-ADA30, PLP-ADA45 and PLP-ADA60) denotes to a stoichiometrical degree of substitution of ADA pendant to the polymer backbone. The polymers in acid form were characterised by FT-IR. Figure 3-1 shows the characteristic peaks located at 3295 cm⁻¹ (N–H str and O–H str), 1723 cm⁻¹ (C=O acid str), 1625 cm⁻¹ (amide band I), 1533 cm⁻¹ (amide band II) and 1275 cm⁻¹ (C–O str). Notably, there was no characteristic absorption related to the C=O stretch of a carboxylic acid ester (1737 cm⁻¹). The results confirm the successful formation of amide bonds during polycondensation and the successful hydrolysis of ester groups into carboxylic acid. This is supported by the

observation that carboxylate ions, in their sodium form, exhibit a carbonyl stretching frequency at approximately 1600 cm^{-1} . However, this frequency could be masked or confused with the absorption of the amide band I in the same region.

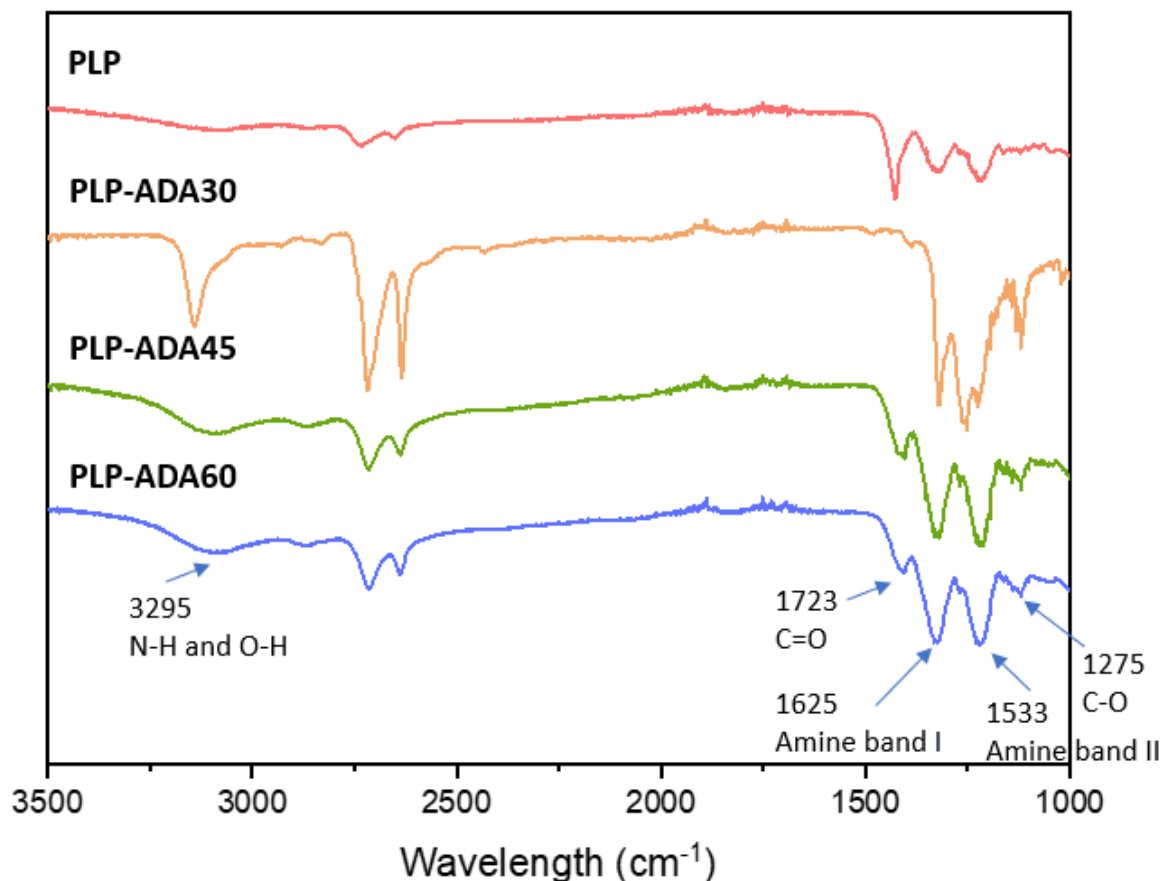


Figure 3-1. FT-IR spectra of PLP, PLP-ADA30, PLP-ADA45, PLP-ADA60 in acid form.

3.2.2 GPC

The polymers in acidic form were characterised using a DMF phase GPC system calibrated with poly(methyl methacrylate) standards. GPC profile of PLP-ADA30, PLP-ADA45, PLP-ADA60 in Figure 3-2 all showed one single peak. The retention time decreased with increasing degree of substitution, indicative of an increase in molecular weight. This is in agreement with the change of weight average from $10,765\text{ g mol}^{-1}$ for PLP-ADA30 to $15,642\text{ g mol}^{-1}$ for PLP-ADA45 and $32,582\text{ g mol}^{-1}$ for PLP-ADA60 (Table 3-1). However, the detected molecular

weights of the PLP-ADA polymers were even lower than that of the parent polymer, PLP (35.7k g mol^{-1} , $M_n = 17.9\text{k g mol}^{-1}$, $PDI = 1.99$) (Chen et al., 2009a). This suggests that the GPC profiles of PLP-ADA polymers failed to reflect their real molecular weights, which could be explained that the length and hydrophobicity of the fatty acid grafts might influence the elution time. Those profiles not reflecting a continuing rise in molecular weight of the grafted polymers was noted previously (Yue et al., 2005b, Yue et al., 2005a). This suggests that the side chain modifications had an overriding effect on the molecular structure of the polymers rather than simply increasing their molecular weight, thus making the conformation of PMMA standards unrepresentative of the resulting comb-like structure of PLP-ADA (Styring and Hamielec, 1989). It is also worth pointing out that the comb-like PLP-ADA polymers would have the increased interactions with the pore structure and bead materials in the GPC column in undesirable ways, leading to higher retention times and as a result lower apparent molecular weights relative to the parent polymer PLP. Therefore, the molecular weights of PLP-ADA polymers were calculated from the degrees of grafting and the M_n of the parent polymer (see Section 3.2.3), following the reported method for PP polymers (Chen et al., 2009a) and PLP-NDA polymers (Chen et al., 2017). The PDI (calculated as the ratio of the weight-average molecular weight to the number-average molecular weight) of PLP-ADA60 is 3.91, potentially attributable to polymer irregularities or experimental error. This variation could arise from inconsistencies in the degree of grafting, resulting in the presence of polymer molecules with differing degrees of grafting within the sample.

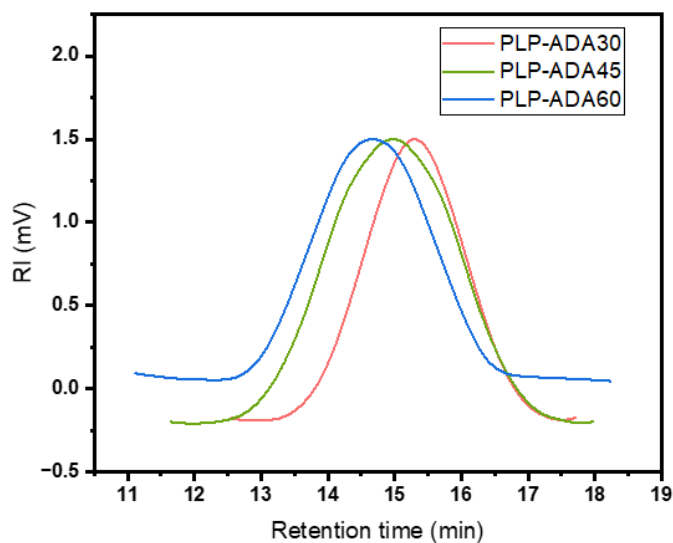


Figure 3-2. GPC traces for PLP-ADA30, PLP-ADA45, PLP-ADA60 prepared in DMF at 25 °C. *The sample preparation, figure, and analysis were conducted by me. The experimental work, data collection was conducted by Dr. Jirui Zhang (University of Warwick).*

Table 3-1. Apparent weight average molecular weights M_w and number average molecular weights M_n of PLP-ADA polymers. *The sample preparation, and analysis were conducted by me. The experimental work, data collection was conducted by Dr. Jirui Zhang (University of Warwick).*

	PLP-ADA30	PLP-ADA45	PLP-ADA60
M_n (g mol ⁻¹)	6,401	9,779	8,554
M_w (g mol ⁻¹)	10,765	15,642	32,582
Polydispersity index (PDI)	1.68	1.60	3.91

3.2.3 NMR

3.2.3.1 Structural characterization of PLP and PLP-ADA

In comparison to the ^1H -NMR spectrum of PLP (Figure 3-3), the comb-like PLP-ADA polymers that contain ADA side chains exhibited characteristic peaks at 2.10-2.29 ppm, attributed to the protons in the CH_2 group next to carboxyl group (Figure 3-4). The integral of each peak is proportional to the number of protons (Kemp and Kemp, 1991). Due to the chain dynamics, polymers can exhibit complex chain dynamics, such as segmental motions and entanglements, which can complicate NMR analysis (Kimmich and Fatkullin, 2004). These dynamics can cause broadening or overlapping of NMR signals, making it challenging to accurately assign and interpret the spectra (Kimmich and Fatkullin, 2004, Jelinski, 1985). To compared the calculated degree of grafting (DG), purple peak at 2.10-2.29 ppm (corresponding to protons next to carboxyl group on ADA) and red peaks at 7.38-8.42 ppm (corresponding to encompassing Ar aromatic protons on the PLP backbone) were separately choose to calculate the DG. The calculated DGs were summarized in Table 3-2.

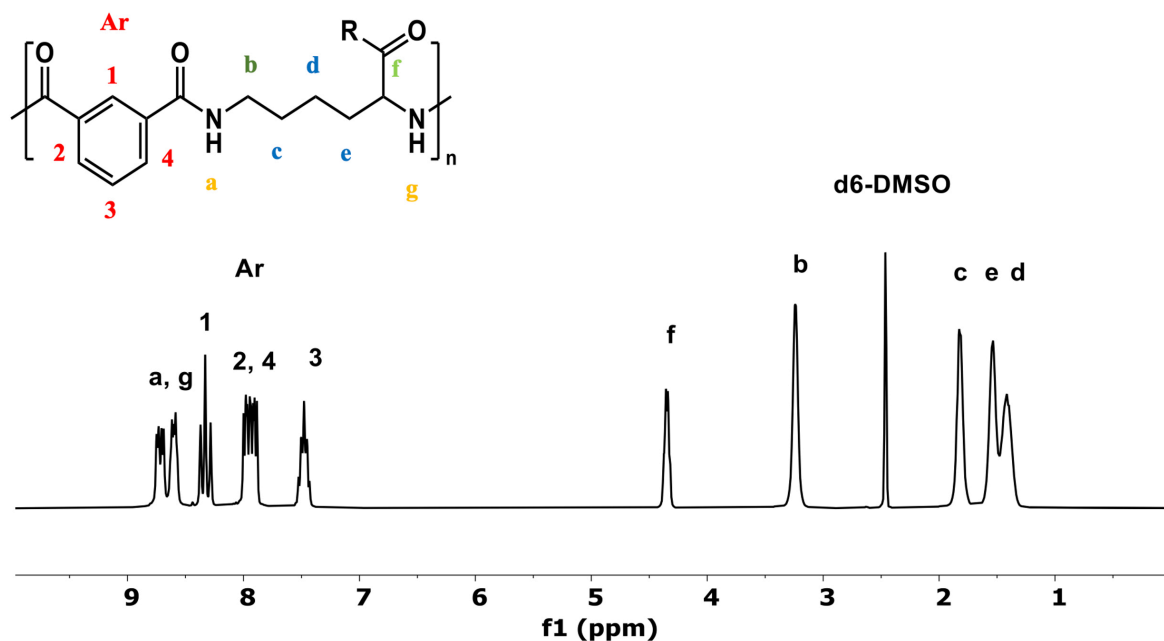


Figure 3-3. ^1H -NMR spectrum of PLP in acid forms in d_6 -DMSO at room temperature.

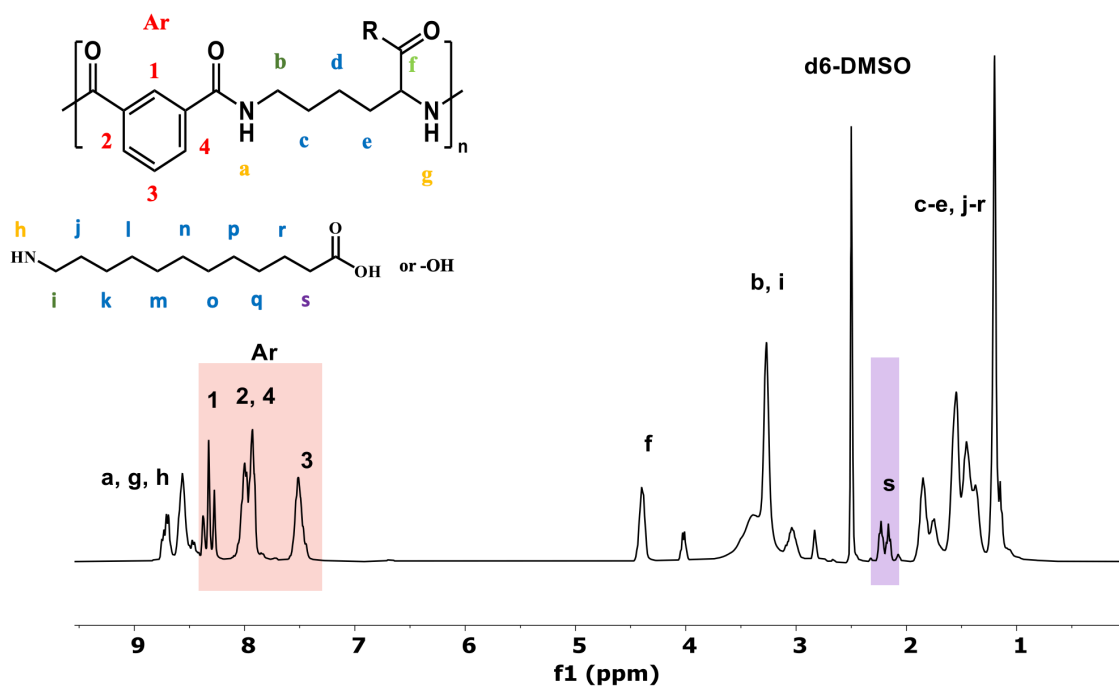


Figure 3-4. ^1H -NMR spectrum of PLP-ADA30 in acid form in d_6 -DMSO at room temperature.

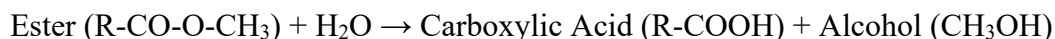
Table 3-2. Calculated degrees of grafting (DG) defined as the number of ADA grafts per 100 carboxylic acid groups pendant to the PLP backbone, in the ^1H -NMR spectrum in d_6 -DMSO at 25°C . Mean $\pm$ S.D. ($n=2$)

	PLP- ADA10	PLP- ADA20	PLP- ADA30	PLP- ADA40	PLP- ADA50	PLP- ADA60	PLP- ADA70	PLP- ADA80
DG (%)	10.0 $\pm$ 0.6	25.4 $\pm$ 0.0	36.4 $\pm$ 2.3	48.6 $\pm$ 0.0	61.3 $\pm$ 3.6	65.9 $\pm$ 0.4	62.6 $\pm$ 4.9	58.9 $\pm$ 4.7

3.2.3.2 Confirmation of complete ester hydrolysis

In DCC/DMAP-mediated coupling reaction, the ADA methyl ester was used with the ester bond acting as a protection group. Following the coupling reaction, the ester hydrolysis was

performed, transforming the methyl ester to a carboxylic acid. The equation for this ester hydrolysis can be described as follows:



During hydrolysis, the methyl group (CH₃) is replaced by a hydrogen group (H), culminating in the creation of a carboxylic acid. The progression of the alkaline hydrolysis of polymers with ester groups can be tracked through NMR spectroscopy. Specific peaks in the NMR spectra offer valuable clues about the degree of hydrolysis. The integral of peaks between 2.18-2.23 ppm (referred to as Peak 1) is attributed to the protons next to the methyl ester bond. The persistence of Peak 1 signifies that the ester hydrolysis is not yet complete. Conversely, the integral of peaks between 2.10-2.18 ppm (known as Peak 2) corresponds to the protons next to the carboxylic acid group. The emergence of this peak indicates the onset of the hydrolysis reaction. Ultimately, the vanishing of Peak 1 after a 12-hour hydrolysis period confirms the successful completion of the process, verifying that the methyl ester bond was effectively hydrolysed to yield the intended carboxylic acid.

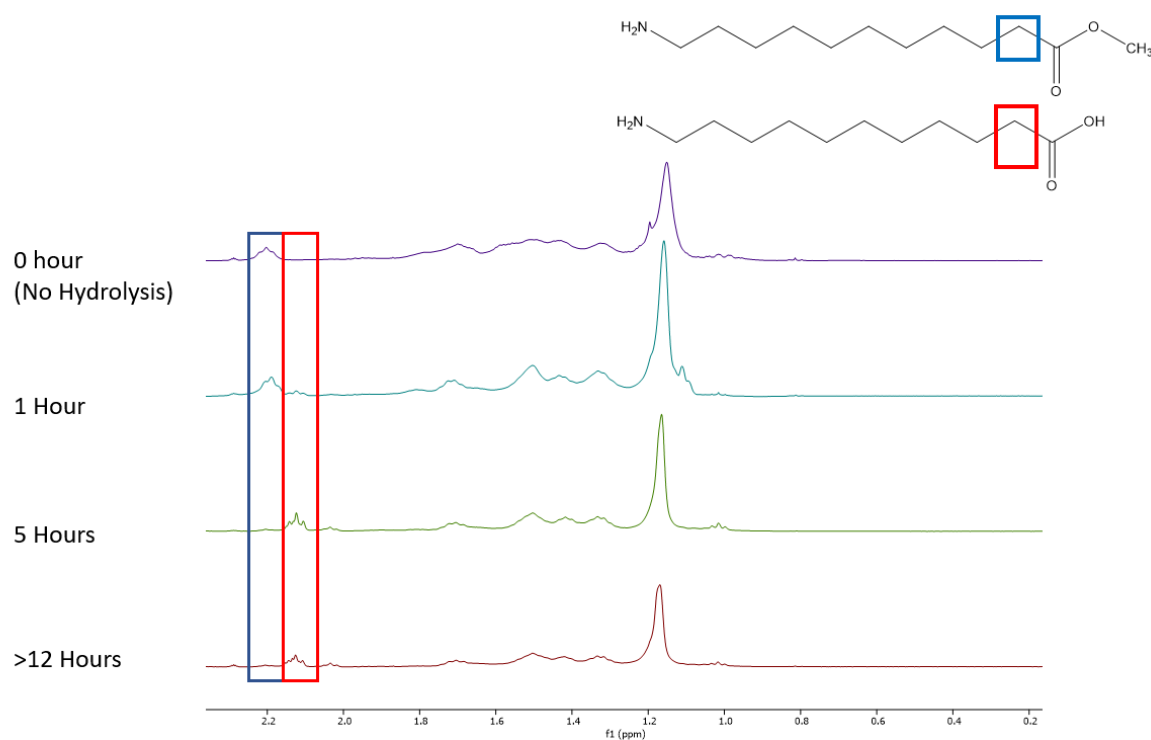


Figure 3-5. ¹H-NMR spectrum of PLP-ADA30 in their acid forms in d₆-DMSO at room temperature

3.2.3.3 Polymer stability during dialysis

Dialysis is a common method employed to purify polymer solutions, exchange solvents, or remove small molecules. This method involves adding the polymer solution to a dialysis membrane that permits small molecules to pass through while keeping the polymer chains confined. The stability of the process hinges on various elements such as the type of dialysis membrane used, the compatibility between the dialysis setup and the polymer and solvent, as well as the time span for which dialysis is conducted.

The polymer stability throughout the dialysis was evaluated by comparing NMR spectral data gathered after 7 and 10 days of the procedure. As listed in Table 3-3, there was little difference between the two datasets, implying that the dialysis operation had no adverse impact on the stability of the polymer's side chains.

Table 3-3. Compositions of PLP-ADA polymers after 7 and 10 days of dialysis.

	PLP- ADA 10	PLP- ADA 20	PLP- ADA 30	PLP- ADA 40	PLP- ADA 50	PLP- ADA 60	PLP- ADA 70	PLP- ADA 80
[NH ₂]/[COOH] ^I (%)	10	20	30	40	50	60	70	80
DG ^{II} (%) after 7 days dialysis	10.0	22.0	32.5	45.0	55.5	60.5	62.0	62.0
DG (%) after 10 days dialysis	8.5	21.0	30.0	43.0	51.0	60.0	59.6	57.0

^I Stoichiometric molar percentage of ADA relative to carboxylic acid pendant to the PLP backbone; ^{II} Degree of grafting, defined as the number of ADA to per 100 carboxylic acid groups as determined by ¹H-NMR in d₆-DMSO at 25 °C.

3.2.4 TGA

Thermogravimetric Analysis (TGA) is a method used to examine how the weight of a material varies with temperature, offering insights into its thermal decomposition and stability. In this study, the focus was on polymers with varying degrees of side chains to explore their impact on thermal stability. By subjecting these polymers to TGA, the temperature at which notable weight loss happened was identified. This critical temperature, often referred to as the onset or decomposition temperature, serves as an indicator of the polymer's thermal resilience. TGA can also shed light on the degradation patterns and the rate of weight reduction for polymers with different side chain configurations.

Specifically, TGA was carried out on several variations: PLP, PLP-ADAn (PLP-ADA30, PLP-ADA45, PLP-ADA60). As illustrated in Figure 3-6, PLP-ADAn undergoes a three-stage thermal decomposition process. The initial decomposition phase commences at 30°C and concludes at 243°C for PLP-ADA30, 260°C for PLP-ADA45, 270°C for PLP-ADA60, resulting in an approximate weight loss of 11.6%, 9.2%, 7.1% respectively. This might be due to the loss of water or loss of solvent. The decomposition temperature of PLP-ADAn remains consistent at a high threshold of 625°C. PLP starts degrading after its backbone decomposes at 185°C and decomposition at 342°C. TGA analysis revealed that the incorporation of side chains enhances the thermal stability, as evidenced by the TGA results.

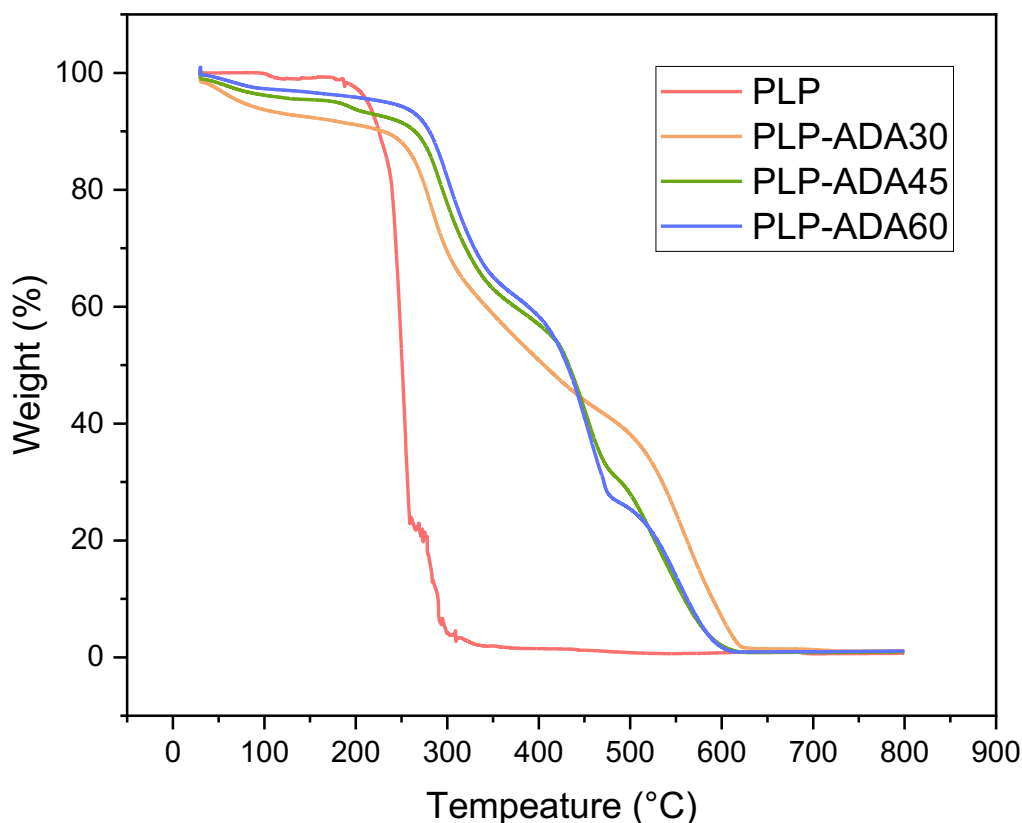


Figure 3-6. TGA plots of PLP, PLP-ADA30, PLP-ADA45, PLP-ADA60 in air at a heating rate of $5\text{ }^{\circ}\text{C min}^{-1}$. The figure and analysis were conducted by me. The experimental work, data collection was conducted by Mr. Yifan Ding (Imperial College London).

3.3 Physicochemical properties

3.3.1 Phase separation

The phase separation of PLP-ADA with different grafting percentages was investigated at 0.2 mg mL^{-1} . The transmittance value started to decrease at the onset of precipitation (pH_p). pH_p can be changed by varying the degree of grafting. As shown in the Figure 3-7, compared with the linear PLP, PLP-ADA showed a significant change in pH_p . With the increase DG of side

chains, the pH_p value increased from 4.5 for PLP to 5.0 for PLP-ADA30, 5.8 for PLP-ADA45 and 6.0 for PLP-ADA60. As for PLP-NDA18 (1.0 mg mL^{-1}), with a further increase in the hydrophobic alkyl side chain substitution, the pH_p value increased from 4.5 for PLP (1.0 mg mL^{-1}) to 5.0. This suggests that adding hydrophobic side chains can enhance the hydrophobicity. This observation aligns with reports showing that increasing the degree of grafting with hydrophobic amino acid pendant groups improves polymer hydrophobicity (Chen et al., 2009a, Chen et al., 2009c). At $pH > pH_p$, deprotonation of the side groups could lead to electrostatic repulsion causing the polymer to be soluble in water (Moon et al., 2009). In other words, the repulsive forces generated by the electrical charges help to disperse the polymer molecules in water, allowing them to dissolve and form a homogeneous solution. In addition, the polymer–water interactions increase. When the solution has a lower pH than the pH_p value ($pH < pH_p$), the hydrophobic interaction between polymers intensifies as the side chain grows, leading to phase separation at a specific pH (Moon et al., 2009, Gonçalves et al., 1997). As a result, it can be deduced that the hydrophobicity increases with decreasing pH. Consequently, it suggests that incorporating of the hydrophobic side chains can remarkably enhance the hydrophobicity of polymer (Chen et al., 2009c, Chen et al., 2009b).

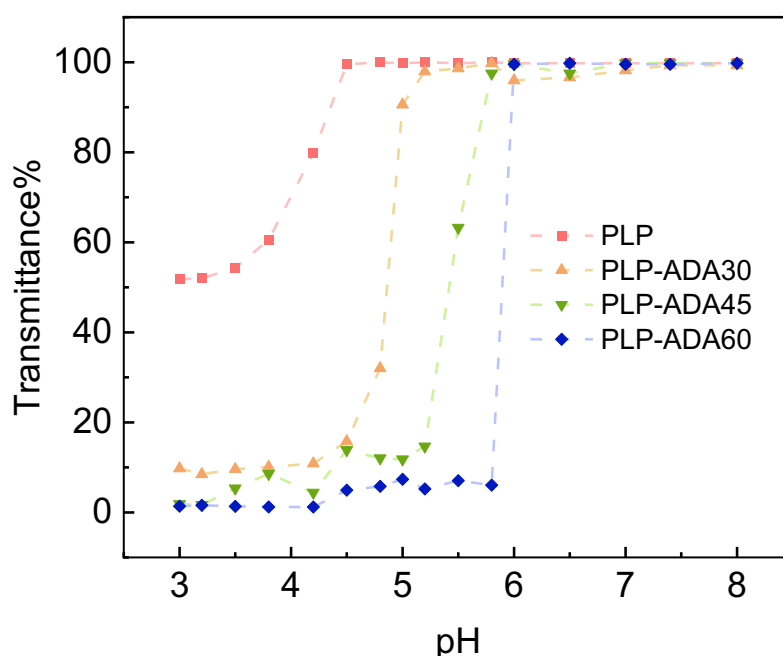


Figure 3-7. pH-Dependent transmittance of the aqueous solutions of PLP, PLP-ADA30, PLP-ADA45, PLP-ADA60 at 0.2 mg mL^{-1} in 100 mM phosphate or citrate buffer. Transmittance was measured at $\lambda = 480 \text{ nm}$. Mean $\pm$ S.D. ($n=3$).

3.3.2 Conformational change

Pyrene is a polarity-sensitive probe, which can be used to investigate hydrophobic association of polymers in aqueous solution (Chen et al., 2009b). The ratio of the fluorescence intensities at 338 and 333 nm (I_{338} , I_{333}) in the excitation spectrum of pyrene can be applied to express the hydrophobic association of PLP-ADA. The pH at onset of hydrophobic association named as pH_h , where I_{338}/I_{333} started to increase. As shown in Figure 3-8, the pH-mediated hydrophobic association was enhanced by incorporating the relatively long side chains onto the PLP backbone. Because of the hydrophobicity of the side chain, I_{338}/I_{333} increases from 5.0 for PLP to 8.5 for PLP-ADA60 and the pH range for the hydrophobic association was broadened. For the PLP-ADA polymers, the pH played a more significant role in determining the balance of hydrophilicity and hydrophobicity. When the pH exceeded pH_h , PLP-ADA underwent a coil-to-globule conformational change (Chen et al., 2009a, Chen et al., 2009b). Consequently, the hydrophobicity decreased at higher pH levels, causing the polymer to transition from a partially charged or uncharged collapsed state to a charged and extended coil structure (Schmaljohann, 2006). This process depends on the proportion of neutral particle (degree of ionisation) which increases with pH increase. This indicates that the high percentage of ADA enhances hydrophobic association. A further increase in the degree of substitution to 60 mol% led to the hydrophobicity even at high pH, suggesting the localization of pyrene in the hydrophobic core of compact aggregates that were formed throughout the pH range tested. (Chen et al., 2017, Kang et al., 2001) The enhanced hydrophobic association of the PLP-ADA polymers compared to their linear counterpart PLP can be ascribed to the enhanced hydrophobicity resulting from grafting with hydrophobic side chains. As summarized in Table 3-4, the pH_h value of the PLP-ADA increased with increasing the molar percentage of ADA grafts, which could be attributed to the enhanced polymer hydrophobicity.

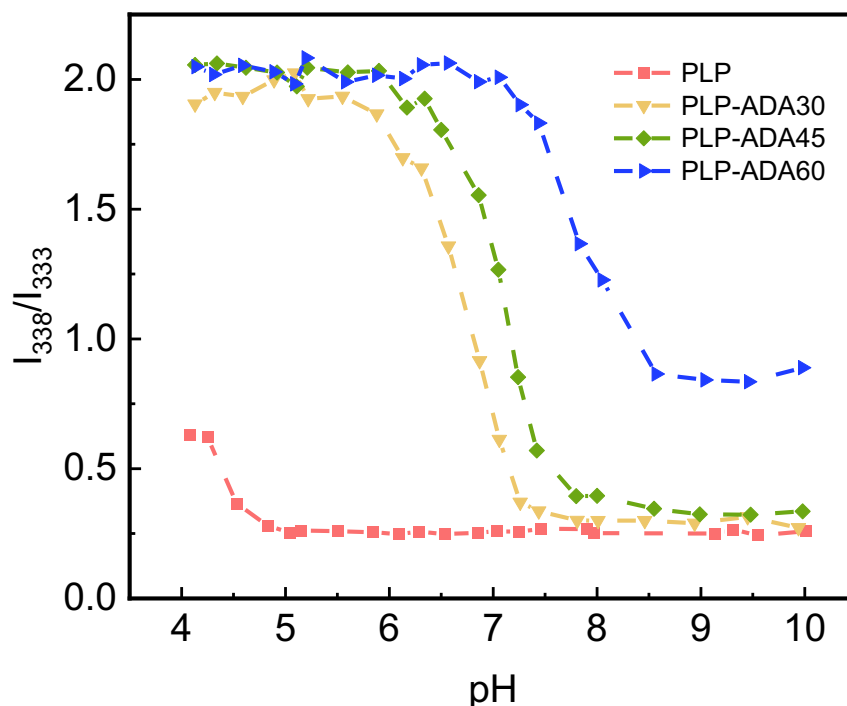


Figure 3-8. pH-Dependent I_{338}/I_{333} in the excitation spectra of pyrene (600 nM) dissolved in aqueous solutions of PLP, PLP-ADA30, PLP-ADA45 and PLP-ADA60 at 0.5 mg mL⁻¹. Mean $\pm$ S.D. (n=3).

In order to investigate the concentration-dependant hydrophobic association, PLP-ADA polymers were tested in the PB buffer at pH 7.4 with the concentration ranging from 0.001 to 5 mg mL⁻¹. The critical aggregation concentrations also known as CACs of the polymers were obtained from the concentration-dependent variations of I_{338}/I_{333} at pH 7.4, with the intersection of the line through the points on the rapidly varying part and the line through the points at the low-concentration extreme of the plot (Chen et al., 2017, Mao et al., 2016). For a concentration below the CAC, the ratio of fluorescence intensities increases upon its transfer into a less polar environment. When the concentration was above CAC, the polymer started to form micelles and tended to form multimolecular aggregates due to the predominant intermolecular interactions (Chen et al., 2009b). Herein, the changes in the ratio were utilized to assess the influence of the polymer concentration and the presence of relatively long side chains. Based on the depicted Figure 3-9, it can be deduced that as the degree of grafting decreases, the PLP-ADA exhibits an increased hydrophilicity. The grafting rate is adjustable, allowing for better control over the hydrophilic properties of the polymer. This observation can be attributed to

the incorporation of hydrophobic side chains, which leads to the strengthening of both intermolecular and intramolecular interactions (Deo et al., 2005, Kang et al., 2001).

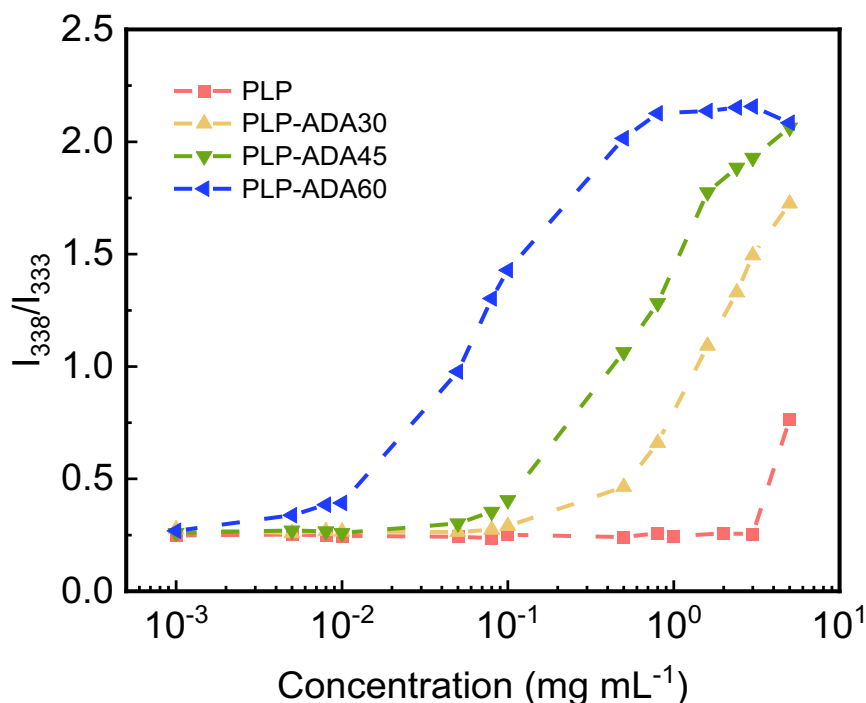


Figure 3-9. Concentration-dependent I_{338}/I_{333} in the excitation spectra of pyrene dissolved in PB buffer of PLP, PLP-ADA30, PLP-ADA45 and PLP-ADA60 at pH 7.4. Mean $\pm$ S.D. (n=3).

Table 3-4. The pH values at the onset of hydrophobic association (pH_h) and the pH ranges within which hydrophobic association occurs for PLP and PLP-ADA polymers at a concentration of 0.5 mg mL^{-1} , and the critical aggregation concentrations (CACs) of these polymers at pH 7.4. Mean $\pm$ S.D. (n=3).

	PLP	PLP-ADA30	PLP-ADA45	PLP-ADA60
pH_h	4.8 ± 0.2	7.2 ± 0.2	7.8 ± 0.2	8.5 ± 0.2
pH range	4.0-4.8	5.2-7.8	6.0-8.5	7.2-8.5
CAC (mg mL^{-1})	3.0	0.37	0.079	0.013

3.3.3 Particle size

The hydrodynamic size of the PLP-ADA polymers was determined using the dynamic light scattering (DLS) technique. Figure 3-10, Table 3-5 and Table 3-6 illustrate the results obtained at a concentration of 0.5 mg mL^{-1} , which was above the CACs of PLP-ADA30, PLP-ADA45 and PLP-ADA60 as shown in Table 3-4. At pH 7.4, PLP-ADA30 displayed a monodisperse population with a small particle size of $10.2 \pm 1.3 \text{ nm}$ due to its pH-induced coil-to-globule conformational change. In contrast, PLP-ADA45 and PLP-ADA60 displayed two size populations at pH 7.4 due to the formation of loose aggregates resulting from the enhanced hydrophobicity of the polymers with increasing the amount of hydrophobic side chains (Chen et al., 2008).

Upon reduction of pH to 5.5, PLP-ADA30 displayed the small primary and larger secondary aggregates with a mean size of small particle population of $19.54 \pm 1.725 \text{ nm}$ and a large particle population of $118.5 \pm 30.15 \text{ nm}$. This could be attributed to the formation of loose aggregates after protonation of carboxylic acid groups of the polymer. With increasing the degree grating percentage of hydrophobic ADA side chains, PLP-ADA45 and PLP-ADA60 displayed one single size population, with the mean hydrodynamic size decreasing from $188.0 \pm 14.21 \text{ nm}$ for PLP-ADA45 to $103.1 \pm 5.365 \text{ nm}$ for PLP-ADA60. This could be explained that the polymer with the increased amount of hydrophobic side chains exhibited the enhanced intermolecular and intramolecular interactions (Kang et al., 2002). At acidic pHs, the carboxyl ions became protonated, increasing the dominance of hydrophobic interactions and resulting in more compact aggregates (Chen et al., 2017), leading to a reduction in particle size. This observation aligns with previous research findings that PLP with PEG side chains exhibited a similar transition from a distribution with two size peaks to a single peak upon acidification (Chen et al., 2008).

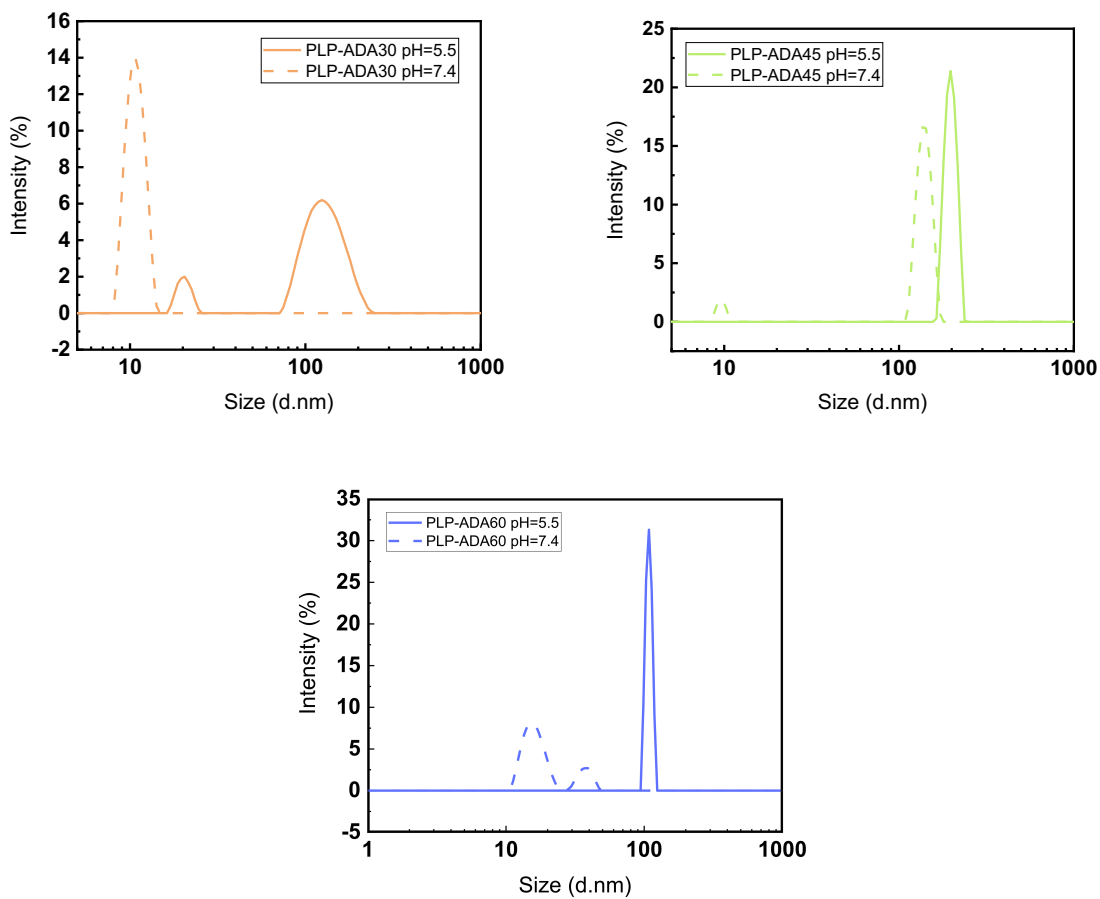


Figure 3-10. Particle size distributions of (A) PLP-ADA30, (B) PLP-ADA45 and (C) PLP-ADA60 at 0.5 mg mL^{-1} at pH 7.4 (dashed line) and 5.5 (solid line).

Table 3-5. The mean hydrodynamic diameters of PLP-ADA30, PLP-ADA45, and PLP-ADA60 at a concentration of 0.5 mg mL^{-1} at pH 7.4. Mean $\pm$ S.D. (n=6)

	PLP-ADA30	PLP-ADA45	PLP-ADA60
Population 1 mean size (nm)	10.2 ± 1.3	9.3 ± 0.2	14.8 ± 2.5
Population 2 mean size (nm)	N/A	129.9 ± 12.0	35.6 ± 4.0

Table 3-6. The mean hydrodynamic diameters of PLP-ADA30, PLP-ADA45, and PLP-ADA60 at a concentration of 0.5 mg mL⁻¹ at pH 5.5. Mean $\pm$ S.D. (n=6)

	PLP-ADA30	PLP-ADA45	PLP-ADA60
Population 1 mean size (nm)	19.5 $\pm$ 1.7	188.0 $\pm$ 14.2	103.1 $\pm$ 5.4
Population 2 mean size (nm)	118.5 $\pm$ 30.3	N/A	N/A

3.3.4 Zeta potential

Zeta potential is another key particle property, which could affect particle stability, interactions with living cells (Rasmussen et al., 2020), and particle toxicity (Valadi et al., 2007). The pH of a polymer solution was controlled using 100 mM phosphate or citrate buffer, leading to protonation or deprotonation of the carboxylic acid groups of PLP-ADA polymers. As the pH of the polymer solution changes, its electro-kinetic properties undergo variations, with the conformational change and the DG playing a crucial role.

Figure 3-11 shows the zeta potentials of PLP-ADA polymers at 0.5 mg mL⁻¹, above their CAC values as listed in Table 3-4. As expected, all those anionic polymers displayed negative zeta potential values at pH 7.4. At acidic pH ranging from 7.0 to 5.5, the zeta potentials of each PLP-ADA polymer solution were still negative, indicative of partial protonation. The zeta potentials of the polymer solutions at pH 5.5 were lower than those at higher pH values for PLP-ADA30 and PLP-ADA60, which might be due to the formation of aggregates resulting from protonation of the carboxyl groups. PLP-ADA45 and PLP-ADA60 exhibited lower zeta potentials than PLP-ADA30. This might be attributed to the increased amount of hydrophobic side chains and consequently enhanced intermolecular and intramolecular interactions (Kang et al., 2002) and formation of more compact aggregates (Chen et al., 2017) (Figure 3-10, Table 3-5 and Table 3-6).

The stabilizing effect of zeta potential arises from the electrostatic repulsion between particles carrying the same electric charge, which leads to the segregation of particles, preventing them from clustering together and ensuring the stable polymer solutions (Pochapski et al., 2021, Mohammadi-Jam et al., 2022). The negative charge of the PLP-ADA polymers is favorable for circulation in blood (due to electrostatic repulsion with negatively charged serum proteins) and contributes to reduced cytotoxicity (due to electrostatic repulsion with negatively charged cell membranes) (Tseu and Kamaruzaman, 2023, Chen et al., 2017).

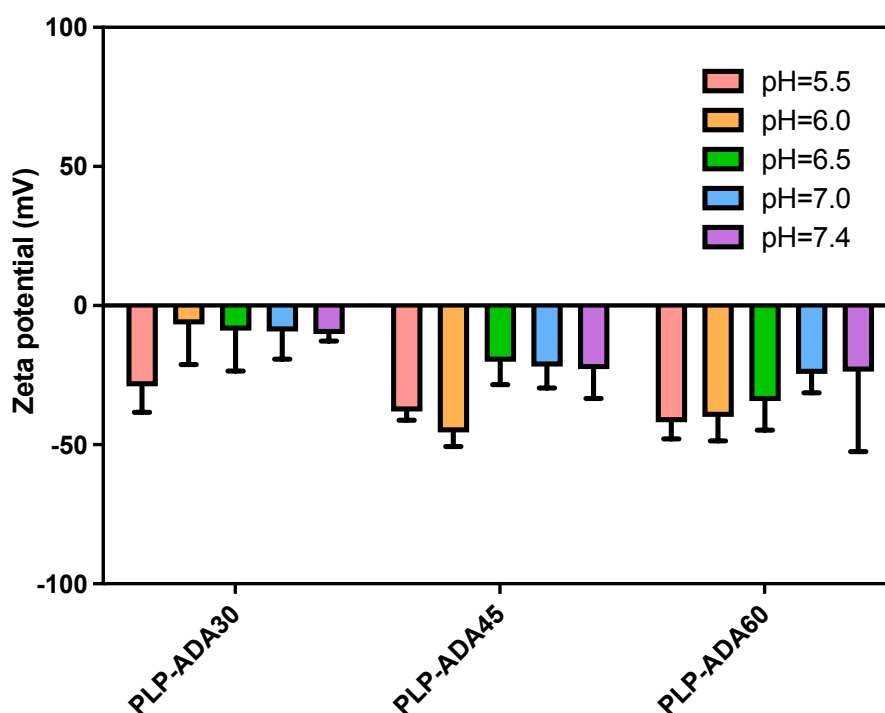


Figure 3-11. The averaged zeta potentials of PLP-ADA polymers. Each sample concentration was 0.5 mg mL^{-1} in 100 mM phosphate or citrate buffer with a different pH value at 25°C . Mean $\pm$ S.D. (n=6)

3.3.5 pKa

Variations in pH can be used to regulate enzyme activity or prevent protein aggregation (Ikebuchi et al., 1993, Ainis et al., 2019), modulate the release of anti-cancer medications (Ulbrich and Šubr, 2004), or manipulate protein sequestration within polyelectrolyte

complexes, brushes, or hydrogels (van Lente et al., 2019, Freudenberg et al., 2019, Schirmer et al., 2020, Del Castillo et al., 2020). When such systems incorporate both weak acid and weak base groups, alterations in pH can induce simultaneous changes in their ionization states. The apparent pK_a corresponds to the pH at which the system contains an equal number of ionized (protonated) and deionized groups and can be determined using titration. This indicating the point where half of the volume of base needed for reaching the equivalence point has been added. For PLP-ADA polymers, the titration involves a chemical process in which the pH of a solution containing carboxylic acid groups is gradually increased by the addition of 0.1M NaOH (titrant).

Figure 3-12 shows a typical titration curve of a PLP-ADA polymer. At the equivalence point, there is a significant shift in pH as the solution transitions from acidic to basic dominance. At the half equivalence point, the quantity of base added is sufficient to neutralize half of the moles of the acid in the solution. The halfway point in the titration, where the carboxylic acid has reacted with half of the titrant, corresponds to the half-equivalence point, which is indicative of the pK_a . The curve of the titration slope against the volume of titrant added is first-derivative plot ($\Delta pH/\Delta V$). This sharp transition resembling a spike, offering the most precise method for determining the equivalence point. There is additional pK_a values can be found halfway between two consecutive equivalence points (Kraft, 2003, Subirats et al., 2015, Lazzari et al., 2019, Kalka, 2021). The table lists two pK_a values, which result from the presence of two distinct ionizable groups: pK_{a1} represents the first protonation, while pK_{a2} corresponds to the second protonation.

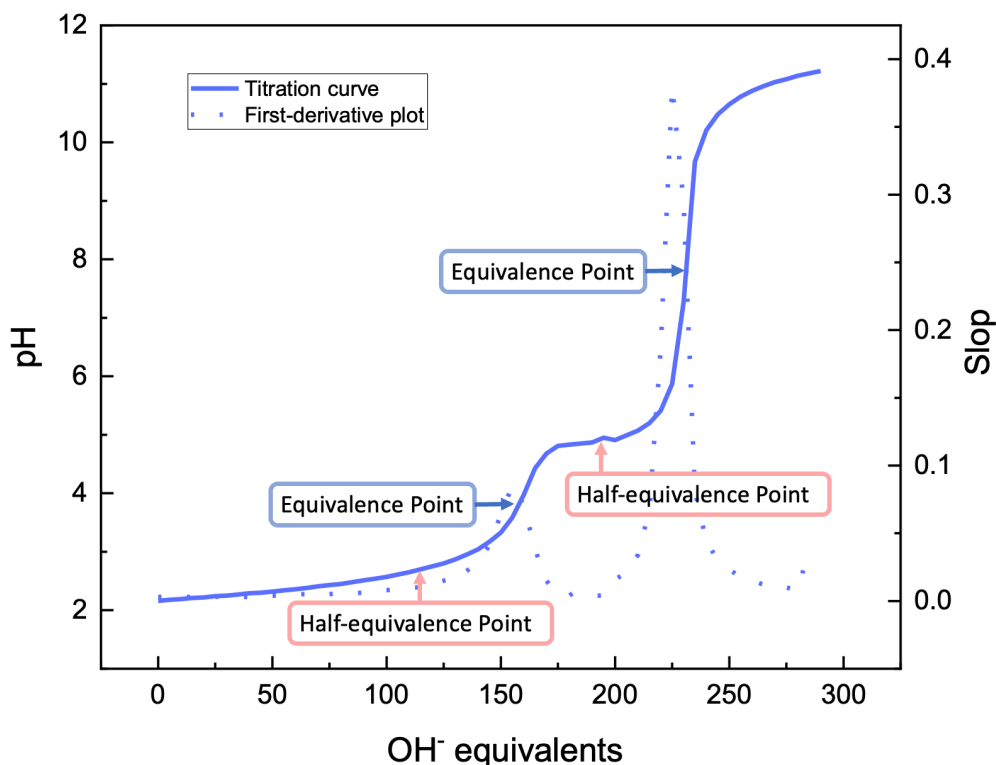


Figure 3-12. Typical titration curve and plot of slope, $\Delta\text{pH}/\Delta V$ on titration curve for 10 mL of PLP-ADA30 at 0.2 mg mL^{-1} .

Figure 3-13 shows the $\text{pK}_{\text{a}1}$ and $\text{pK}_{\text{a}2}$ value with different degree grafting. This $\text{pK}_{\text{a}1}$ due to the coil-to-globule conformational change of the PLP-ADA polymer. This ionization state leads to a coil conformation, which the carboxyl groups on the outer surface of the globule are protonated, where the uncharged side chain is aggregated and remains globule (Lunkad et al., 2021). After one equivalent of base was added, the pH of the solution increased steadily. Addition of a second equivalence of base led to the conversion to coil structure, which further exposed carboxyl groups for half-neutralization ($\text{pK}_{\text{a}2}$) (Aldabbagh, 2022). Additionally, there is a notable trend where PLP grafting with relatively long fatty acid side chains results in higher $\text{pK}_{\text{a}1}$ and $\text{pK}_{\text{a}2}$ values compared to the bare backbone. The increase in $\text{pK}_{\text{a}1}$ occurs due to increasing the degree of side chain would typically drive electrostatic attraction between oppositely charged groups transition from globule to coil conformation. This can lead to simultaneous changes in ionization states upon variations in pH in systems containing acid groups (Lunkad et al., 2021). The elevation in $\text{pK}_{\text{a}2}$ is attributed to the exposure of the additional carboxylic acid groups on the side chain deprotonated (Urry et al., 1992).

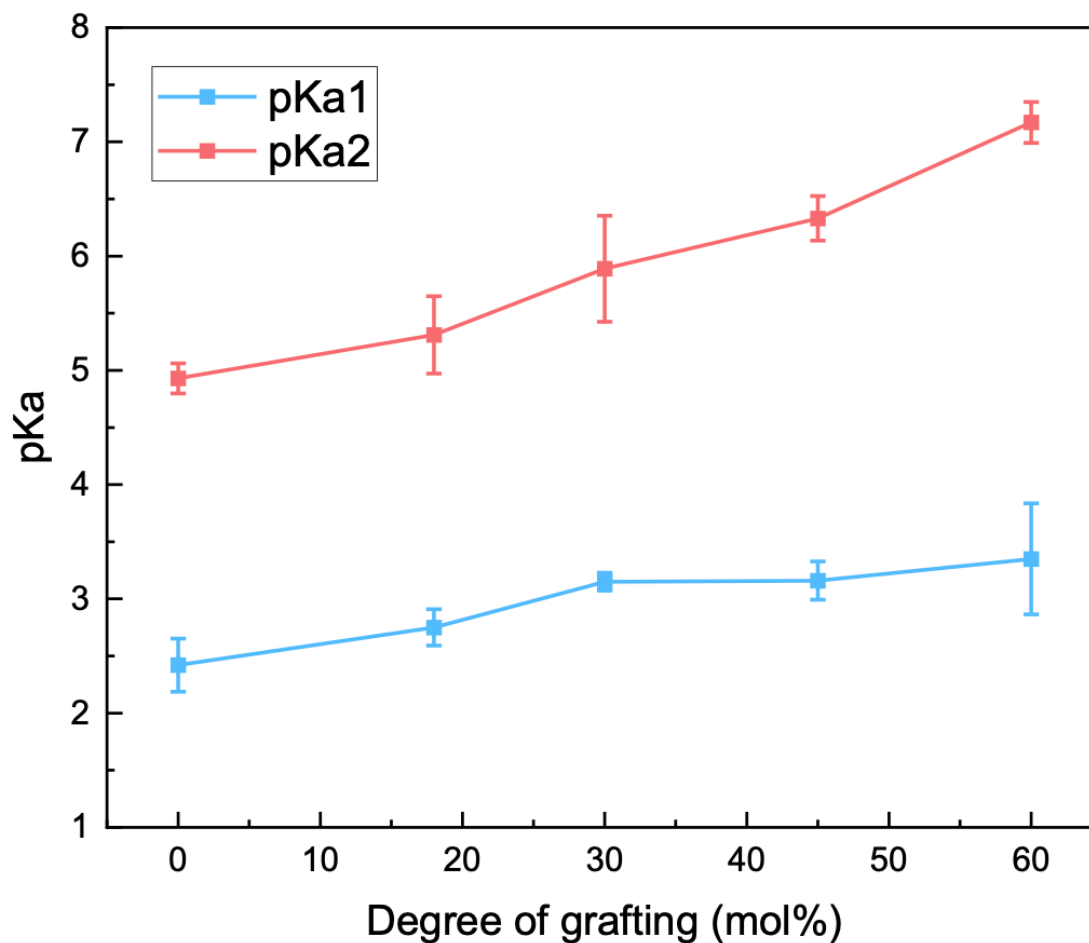


Figure 3-13. pKa values of PLP-ADA polymers with different degrees of grafting. Mean $\pm$ SD, (n = 3).

3.4 Conclusions

This chapter explored the synthesis and characterization of a range of pH-sensitive PLP-ADA polymers with hydrophobic, relatively long fatty acid chains of varying degrees of grafting to the backbone of a linear pseudopeptide, PLP. This series of PLP-ADA polymers exhibited a remarkable ability to undergo conformational changes in response to pH. PLP-ADA were characterized using analytical techniques including NMR, GPC, particle size analysis, pyrene probe, zeta potential, and determination of pKa values. NMR and GPC analysis provides insights into the molecular structure and composition, molecular weight and polydispersity, confirming the successful grafting of hydrophobic alkyl acid chains onto the PLP backbone.

Particle size analysis reveals the hydrodynamic dimensions of the polymer. It is crucial for understanding its behavior in solution and potential applications in drug delivery. Zeta potential measurements provide insights into the surface charge of the polymer particles, influencing their stability and interactions with biological membranes. Determination of pKa and CAC values offers critical information about the pH-responsive behavior, and conformational change, guiding its design for specific environmental conditions. The presence of hydrophobic alkyl acid chains influences the pKa values, modulating the response to changes in pH.

In conclusion, these analyses underscore the versatility and potential of PLP-ADA as a pH-responsive polymer for targeted drug delivery applications. By harnessing its unique properties, including pH sensitivity, membrane-disrupting ability, and tunable surface charge, PLP-ADA holds promise for the development of advanced therapeutic delivery systems tailored to specific physiological conditions.

Chapter 4

Intracellular delivery by pH-responsive polymers and cancer treatment

This chapter describes PLP-ADA-mediated intracellular delivery. A systematic investigation was conducted to examine the effects of pH, concentration, incubation time, and the degree of grafting on the cell membrane activity by haemolysis assay. The mechanism through which these polymers destabilize cell membranes was explored. Delivery of different sized payloads into different types of mammalian cells and multicellular spheroids was demonstrated by laser scanning confocal microscopy and flow cytometry. The intracellular delivery pathways and the mechanism controlling were elucidated. In-vitro cytotoxicity of the PLP-ADA polymers at different concentrations was examined. Furthermore, intracellular delivery of a ribosome-inactivating protein, saporin, was demonstrated for cancer treatment.

4.1 Introduction

Endosomal release is a crucial process in cellular biology that involves the escape of cargo molecules from endosomes into the cytoplasm. This process is vital for many biological functions, including viral infection, drug delivery, and intracellular signaling. However, it faces challenges in practical applications due to the potential damage of endocytosed contents by lysosomal enzymes after cellular uptake (Hoffman, 2013, Canton and Battaglia, 2012). Techniques like using recombinant viruses and fusogenic viral peptides for gene transfection are limited due to safety concerns and problems in large-scale production (Han et al., 2001). Effective intracellular delivery systems are needed that can release drugs into the cytoplasm under mildly acidic conditions by destabilizing endosomal membranes. Consequently, research has focused on endosomolytic non-viral vectors.

Synthetic polymers have been engineered as non-viral vectors to mimic fusogenic viral peptides (El-Sayed et al., 2005, Chen and Chen, 2016, Wang et al., 2015). One notable example involves the utilization of polyanions which possess hydrophobic and weakly ionizable carboxyl groups (Yessine and Leroux, 2004). These polymers exhibit favourable biodistribution, relatively modest transduction efficiencies, and reduced cytotoxicity (Chen, 2013). Under certain conditions, such as a decrease in pH below their pKa, polyanions may undergo a conformational alteration, leading to the destabilization of lipid membranes (Ho et al., 2011). The balance between hydrophobicity and hydrophilicity of these anionic polymers can be adjusted to modulate their membrane acidity. This pH-responsive membrane-destabilizing activity has motivated their utilization in intracellular delivery (Chen et al., 2005). However, the majority of existing polyanions lack biodegradability, necessitating the use of low molecular weights to ensure renal clearance.

To address this concern, researchers have developed a biodegradable pseudopeptidic polymer known as poly(L-lysine iso-phthalamide) (PLP) (Eccleston et al., 1999). However, the utilization of this polyanion for drug delivery is constrained by its relatively low membrane destabilizing activity, which is restricted to a narrow pH range. Various modifications to the side chains of PLP have been explored, including the attachment of hydrophilic polyethylene glycol (such as PA polymers), hydrophobic amino acids (such as PP, PL, PV, and PY polymers) (Chen et al., 2009a, Chen et al., 2009b), and lengthy aliphatic chains (PLP-NDA polymers) (Chen et al., 2017, Chen et al., 2020). These modifications enable the polymers to embed into the plasma membrane, facilitating membrane binding. This can lead to pH-mediated pore formation, direct membrane penetration, or membrane solubilization. A potential side effect is that altering cell membrane permeability can lead to the loss of electrolytes, specifically small water-soluble ions and molecules, from the cell (Benos et al., 1999). However, further research is needed to enhance membrane interaction and solubilization properties, thus improving drug delivery efficiency. To overcome this, explored modification to PLP side chains, a novel polymer incorporating extended aliphatic side chains and carboxylic groups leading to the development of a novel polymer.

This chapter reports a series of pH-responsive PLP-ADA polymers which could cause the fast, controllably cell membrane-destabilizing activity by varying the degree of grafting with ADA.

The mechanism of their membrane activity was explored. Endosomal escape was realized by PLP-ADA using membrane-impermeable Calcein and FITC-labelled Dextran with different molecular weights as different sized model drugs. The results indicate that the polymers were effective at destabilising endosomal membranes and releasing endocytosed materials into the cytoplasm of different types of mammalian cells. It was also found that multiple pathways are involved in endosomal escape, and the dominant pathway was identified by observing the decrease in fluorescence intensity in the presence of various inhibitors. Multicellular cancer spheroids were then utilized to better mimic the tumour microenvironment (Abdolahinia et al., 2022, Popescu et al., 2023) and the PLP-ADA mediated successfully three-dimensional (3D) delivery was demonstrated. The PLP-ADA polymers displayed negligible in-vitro cytotoxicity against different mammalian cell lines. By contrast, PLP-ADA mediated intracellular delivery of a ribosome-inactivating protein, saporin, into cancer cells led to efficient cell killing activity.

4.2 Cell membrane activity

Sheep erythrocytes (RBCs) were used as a model to study the pH-dependent endosomolytic capabilities of PLP-ADA polymers. The integrity of the RBC membrane was assessed through a haemolysis assay, which measured the retention or leakage of haemoglobin. The ability of polymers to induce haemolysis is believed to be affected by various factors such as the type and concentration of hydrophobic components, molecular weight, and structure. Previous research has shown that the membrane-disrupting capabilities of vinyl-based polymers, pseudopeptides, polycarbonate copolymers, comb-like polymer can be adjusted by fine-tuning the hydrophobicity-hydrophobicity balance, achieved by altering the attached groups and the degree of grafting (Chen et al., 2009a, Chen et al., 2017, Murthy et al., 1999, Murthy et al., 2001, Yessine and Leroux, 2004, Chen et al., 2020, Qiao et al., 2012, Chen et al., 2009b). Polymers of higher molecular weight have been found to exhibit pH-responsive membrane activities at higher, near-physiological pH levels, attributed to an increase in pKa values (Bulmus et al., 2003). Additionally, polymers with more branched and flexible structures have been reported to be more effective in compromising cell membranes compared to those that are linear, globular, hyperbranched, or rigid (Fischer et al., 2003, Chen et al., 2020, Wang and Chen, 2017, Chen et al., 2009a, Chen et al., 2009c). As such, it may be feasible to engineer

polymers to disrupt membranes selectively either during the early or late phases of endosomal acidification (Chen et al., 2020, Chen et al., 2009c, Chen et al., 2008).

4.2.1 pH dependant haemolysis

The haemolysis model was employed to evaluate the membrane-destabilizing activity of pseudopeptide polymers, PLP-ADA with varying degrees of grafting. The chosen pH range of 4.5 to 7.4 mimics the acidification process through early endosomes, late endosomes and lysosomes that the polymers encounter after endocytosis. As shown in Figure 4-1A, at 0.2 mg mL⁻¹ and pH levels ranging from 4.5 to 7.4, the linear PLP exhibited little to no haemolysis due to limited interactions with the cell membrane despite undergoing a coil-to-globule conformational change below pH 4.39 (Eccleston et al., 2000). To address this limitation, fatty acids with relatively long hydrophobic chains were introduced as side chains to the PLP backbone in this thesis work. It has been reported in Section 3.3.2 above that upon acidification the PLP-ADA polymers exhibited hydrophobic association, resulting in formation of more hydrophobic compact microdomains than the parent PLP polymer. The resulting microdomains significantly enhanced hydrophobic interactions with the cell membrane by outweighing the electrostatic repulsion. The pH corresponding to the maximum haemolysis increased from pH 6.0 (lower end of the early endosomal pH range) for PLP-ADA30 to pH 6.5 (typical early endosomal pH) for PLP-ADA45 and pH 7.4 (physiological pH) for PLP-ADA60. Also interestingly, increasing the degree of grafting with ADA led to the onset of haemolytic activity approaching higher pH value (Figure 4-1A and B). This change consequently enabled the PLP-ADA to enhance haemolytic activity within a higher pH range even at physiological pH. This shift occurred because of the increased membrane interactions due to the increased polymer hydrophobicity, thus more readily overcoming the electrostatic repulsion from the negatively charged cell membrane. The polymer exhibits lower haemolysis upon acidification, there is a sharp decrease in haemolysis due to the conformational change of the polymer and subsequent precipitation of the polymer, as depicted in Figure 3-7 above. These findings align closely with previous reports on PLP grafted polymers, comb-like polymers and hyperbranched polymer behaviours (Chen et al., 2009c, Chen et al., 2017, Wang and Chen, 2017).

Figure 4-1B shows that at a low concentration of 0.01 mg mL^{-1} , PLP-ADA60 still displayed excellent haemolytic activity at physiological pH. By contrast, PLP-ADA30 and PLP-ADA45 were non-haemolytic at pH 7.4. The onset of haemolytic activity of PLP-ADA45 was pH 7.0 and then peaked at pH 6.5-6.0. As comparison, PLP-ADA30 started to lyse erythrocytes at pH 6.5 and the maximum haemolysis reached at pH 6.0. The results indicate that the haemolytic effects of the PLP-ADA polymers are concentration-dependent. This is because at low polymer concentrations and high pHs, the dominant force would become the electrostatic repulsion between carboxylate ions and the negatively charged cell membrane, leading to reduced membrane interactions. However, sufficiently high polymer concentrations could cause the cell membrane destabilization at physiological pH, which could be due to the enhanced driving force for the polymer binding with the cell membrane regardless of the negative polymer charge (Chen et al., 2009c, Thomas et al., 1994).

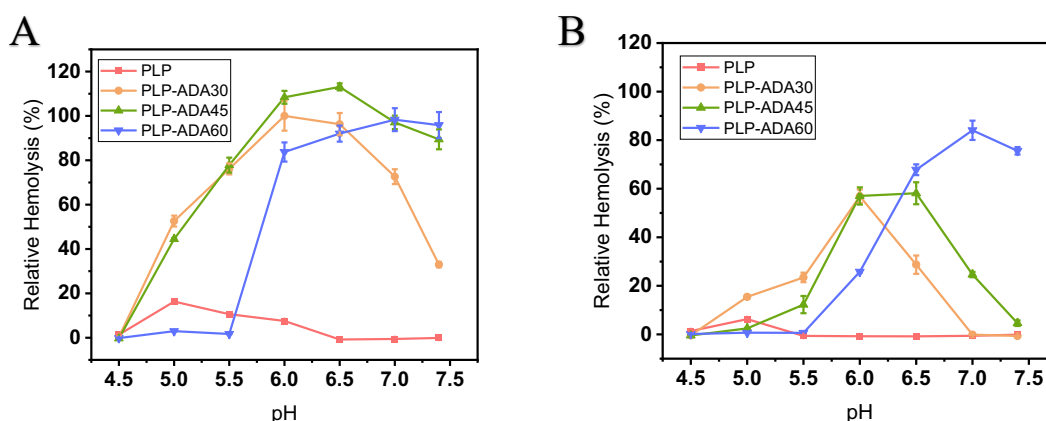


Figure 4-1. pH-dependent relative haemolysis of sheep RBCs incubated for 1 h with PLP, PLP-ADA30, PLP-ADA45 and PLP-ADA60, respectively, at (A) 0.2 mg mL^{-1} or (B) at 0.01 mg mL^{-1} . Mean $\pm$ S.D. (n=3).

4.2.2 Concentration dependant haemolysis

Figure 4-2 shows the concentration-dependant relative haemolysis of PLP and PLP-ADA polymers. The parent PLP polymer displayed limited haemolysis at both pH 6.5 and pH 7.4 within the concentration range tested. By contrast, at pH 6.5, with increasing the polymer concentration, PLP-ADA30 showed the increased haemolytic activity from $23 \pm 0.1\%$ at 0.005 mg mL^{-1} to $95 \pm 0.2\%$ at 2 mg mL^{-1} . However, at the same early endosomal pH, PLP-ADA60

displayed the higher haemolysis ($53 \pm 5.7\%$) than PLP-ADA45 ($34 \pm 5.4\%$) at a dilute concentration of 0.005 mg mL^{-1} , while at the rest higher concentrations both polymers displayed the similar variation of haemolysis, which peaked ($96 \pm 1.0\%$) at 0.2 mg mL^{-1} and then decreased afterwards due to the polymer precipitation as shown in Figure 3-7 (A). At pH 7.4, PLP-ADA30 and PLP-ADA45 were non-haemolytic at dilute concentrations and their haemolytic activities increased significantly with concentration increase. For comparison, PLP-ADA60 showed a high level of haemolysis ($64 \pm 2.6\%$) at the physiological pH even at the dilute concentration of 0.005 mg mL^{-1} and its haemolytic activity further increased gradually with increasing concentrations, achieving the complete haemolysis at the same concentration (0.2 mg mL^{-1}) with PLP-ADA45. PLP-NDA18 demonstrated minimal hemolysis at pH 7.4 across the tested concentration range ($0.005\text{--}10 \text{ mg/mL}$) (Chen et al., 2017). However, at pH 5.5, it displayed outstanding membrane-disruptive activity that was independent of dilution, effectively working over a broader concentration range ($0.5\text{--}10 \text{ mg/mL}$)(Chen et al., 2017).

The results showed that the haemolytic activity of the PLP-ADA polymers was strongly influenced by concentration. This could be attributed to the increased concentration gradients acting as the driving force for the migration of polymer molecules to the membrane surface. The ranking of haemolytic activity amongst those polymers tested was $\text{PLP} < \text{PLP-ADA30} < \text{PLP-ADA45} < \text{PLP-ADA60}$. This is because with increasing the degree of ADA grafting, the polymer hydrophobicity was enhanced, leading to the enhanced hydrophobic interactions and membrane destabilizing activity. At a sufficiently high degree of ADA grafting and/or at a sufficiently high polymer concentration, the haemolytic activity of PLP-ADA could be high at pH 7.4. PLP-ADA60 was the most membrane-active amongst the PLP-ADA polymers. Upon a 400-fold dilution from 2 to 0.005 mg mL^{-1} , PLP-ADA60 retained a high level of haemolysis at pH 7.4. It needs to be pointed out that the membranes of different cell types may have different membrane properties and sheep RBC membranes are rather fragile.

This desired profile of membrane activity for intracellular drug delivery applications (this Chapter) is that the polymers should be non-membrane-active at physiological pH but display strong membrane activity at endosomal pH. However, for other applications such as

intracellular trehalose loading for cell cryopreservation (Chapter 5), the membrane activity at physiological pH could be favorable.

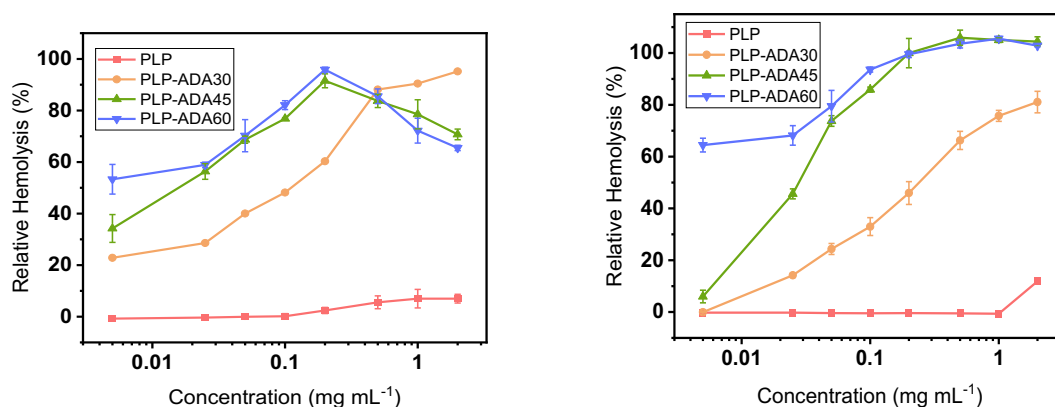


Figure 4-2. Concentration-dependent relative haemolysis of sheep RBCs incubated for 1 h with PLP, PLP-ADA30, PLP-ADA45 and PLP-ADA60, respectively, at (A) pH 6.5 or (B) pH 7.4. Mean $\pm$ S.D. (n=3).

4.2.3 Time dependant haemolysis

The kinetics of haemolysis was investigated by treating sheep RBCs with PLP-ADA polymers at 0.2 mg mL⁻¹ at pH 7.0 for different time durations. As shown in Figure 4-3, all the PLP-ADA polymers showed the strong membrane activity with a high kinetics, in the order of PLP-ADA30 < PLP-ADA45 < PLP-ADA60. After only 5 minutes of incubation, almost 100% of RBCs were haemolyzed by PLP-ADA60. By contrast, no haemolysis was detected for the parent PLP polymer within the time period tested. This indicates that increasing the degree grafting could enable the comb-like PLP-ADA polymers to display the considerably stronger hydrophobic interactions with cell membranes and the increased number of interaction sites, leading to rapid and efficient membrane destabilization at early endosomal pH. This would enable the effective endosomal escape during the endocytic pathway which is favorable for intracellular drug delivery.

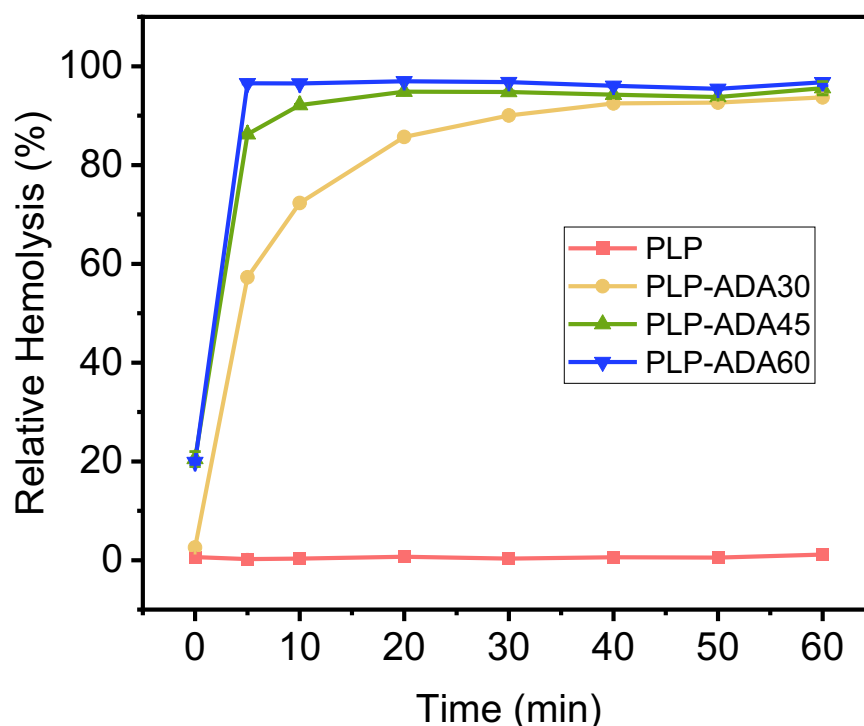


Figure 4-3. Time-dependant relative haemolysis of sheep RBCs in the presence of PLP, PLP-ADA30, PLP-ADA45, PLP-ADA60, respectively, at 0.2 mg mL^{-1} at pH 7.0. Mean $\pm$ S.D. ($n=3$).

4.3 Mechanism of cell membrane disruption

4.3.1 Inhibition of haemolysis

The leakage of lipid membranes can occur through different mechanisms, such as membrane fusion, pore formation, or membrane solubilization (Linhardt and Tirrell, 2000, Matsuzaki et al., 1997, Thomas et al., 1994). To obtain a deeper understanding of how cell membranes are destabilized, the haemolytic effects of PLP-ADA60 when combined with PEGs of different molecular weights (ranging from 200 Da to 20 kDa) were investigated. PEGs were shown to offer osmoprotection to RBCs against polymer-induced pore formation (Chen et al., 2009a). Polymers or peptides that disrupt membranes were observed to create an osmotic imbalance in cells by forming channels or pores in the cell membrane. These openings allowed substances

with low molecular weights to pass through, while obstructing the leakage of haemoglobin (64.5 kDa) (Hajizadeh et al., 2018). This led to swelling of the cells due to osmotic pressure, eventually causing them to rupture and release their contents (Makadia and Siegel, 2011).

Figure 4-4 illustrates that PLP-ADA60 caused the complete haemolysis at 0.2 mg mL^{-1} , while the co-treatment with PEG 200 and PEG 400 led to limited inhibition of haemolysis. It is interesting to note that as the PEG molecular weight increased, the inhibition of haemolysis was significantly enhanced. In the presence of 10 mM PEG 8K, the PLP-ADA60 induced haemolysis decreased to $57 \pm 4.1\%$, while the presence of PEG 20 kDa almost completely inhibited haemolysis. This result indicates that larger PEG molecules reduced the osmolality difference between the cell cytoplasm and the buffer solution, thereby protecting RBCs against haemolysis. This finding supports the hypothesis that the haemolysis occurred due to polymer-induced pore formation on the cell membrane.

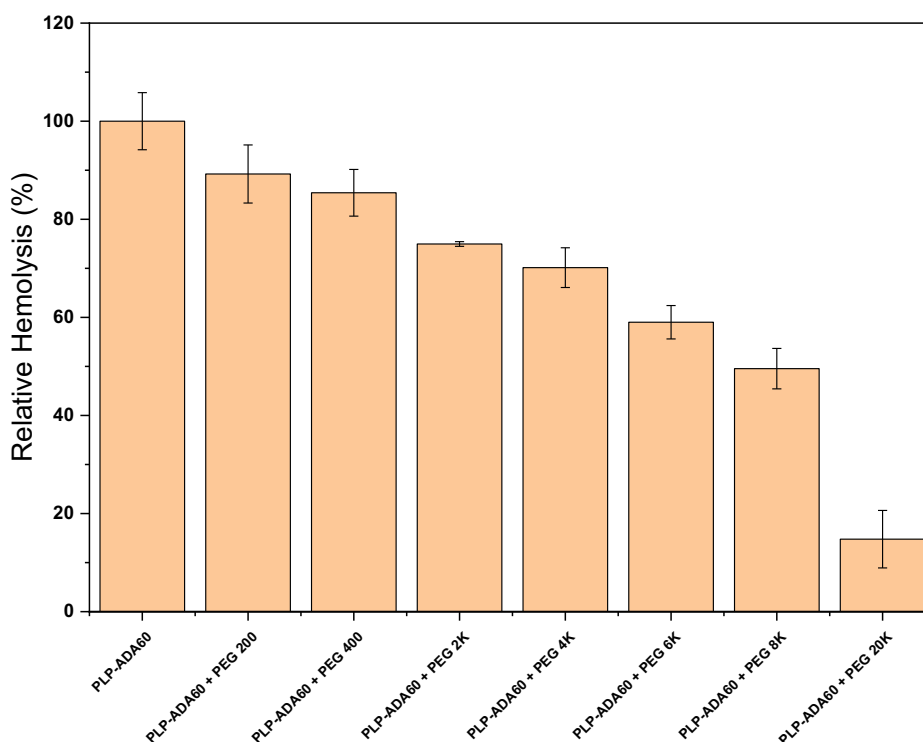


Figure 4-4. Relative haemolysis of sheep RBCs incubated with 0.2 mg mL^{-1} PLP-ADA60 at pH 7.0 for 1 h, with or without the presence of 10 mM PEG with different molecular weights. Mean $\pm$ S.D. (n=3).

4.3.2 Erythrocyte ghost formation

To further explore the mechanism of cell membrane disruption induced by the PLP-ADA polymers, laser scanning confocal microscopy was employed to investigate the integrity of RBC membranes treated with the polymers. A set of images were captured, featuring both fluorescence and differential interference contrast (DIC) visuals of RBCs. These RBCs were incubated with 10 μM of FITC-Dextran ($M_w = 4 \text{ kDa}$) and 1 μM of Texas Red hydrazide, in the presence of PBS buffer, PLP or PLP-ADA60. The green and red channels are represented by FITC-Dextran 4k and Texas Red, respectively.

As shown in Figure 4-5, neither FITC-dextran 4k nor Texas Red hydrazide could pass through the membrane of RBCs treated with PBS only or 0.2 mg mL^{-1} PLP at pH 7.4. PLP has been reported to exhibit a coil-to-globule change of conformation at acidic pH (Eccleston et al., 2000). However, the electrostatic repulsion between the negatively charged cell membrane and anionic parent PLP polymer could play a dominant role in preventing effective membrane binding and insertion of the linear polyanion, which led to no or low haemolysis due to the limited polymer-membrane interactions (see Section 4.2 above).

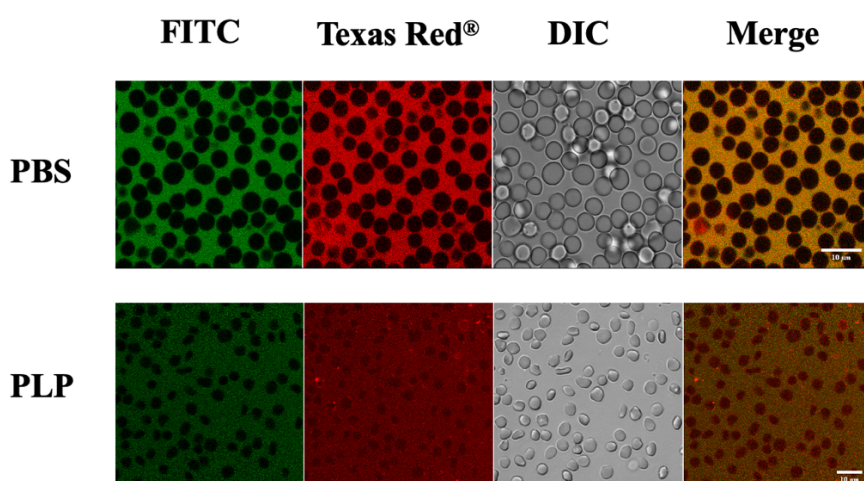


Figure 4-5. Laser scanning confocal microscopy fluorescence and differential interference contrast (DIC) images of sheep RBCs incubated with 10 μM FITC-Dextran ($M_w = 4 \text{ kDa}$) and 1 μM Texas Red hydrazide in the absence of PBS buffer or 0.2 mg mL^{-1} PLP at pH 7.4 in a shaking bath for 10 minutes. Scale bar 10 μm .

In the case of comb-like PLP-ADA polymers, the considerably increased hydrophobicity could outweigh the electrostatic repulsion, and additionally, the hydrophobic side chains functioned as anchors to the membrane, which in combination could enhance the polymer-membrane interaction and aid in the creation of pores within the cell membrane. As shown in Figure 4-6, this process led to the formation of ghost cells (empty RBCs) in the presence of 0.2 mg mL^{-1} PLP-ADA60 at both pH 7.4 and 6.5. These ghost cells displayed a vivid red fluorescence due to the cell membrane staining with Texas Red hydrazide, which can interact with peroxidised lipids during ghost cell formation, resulting in a bright red fluorescence (Matteo et al., 1998). FITC-Dextran 4k was observed inside the RBC ghosts, and there was no difference in the green fluorescence intensity between the inside and the outside. This indicates that FITC-Dextran effectively passed through the RBC membrane treated with PLP-ADA60 under different pH conditions. This result is consistent with the pH-dependant haemolysis profile of PLP-ADA60 shown in Figure 4-1.

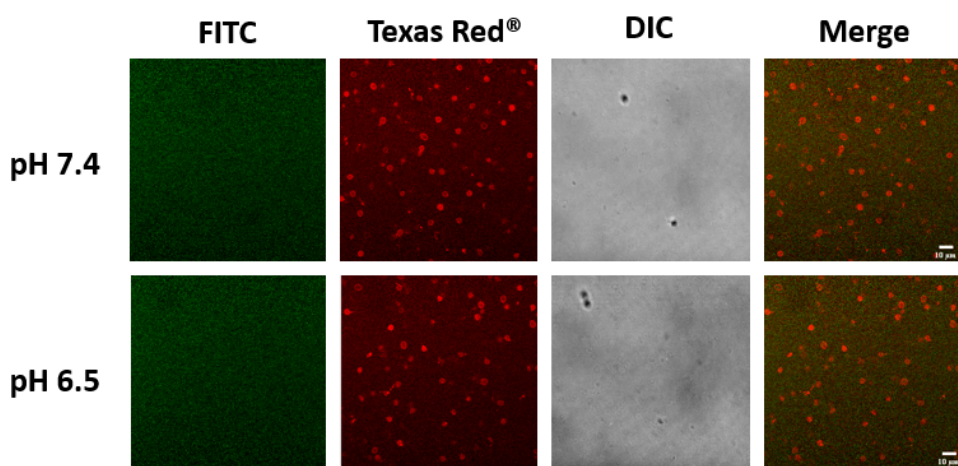


Figure 4-6. Laser scanning confocal microscopy fluorescence and differential interference contrast (DIC) images of sheep RBCs incubated with $10 \text{ }\mu\text{M}$ FITC-Dextran ($M_w = 4 \text{ kDa}$) and $1 \text{ }\mu\text{M}$ Texas Red hydrazide in the presence of 0.2 mg mL^{-1} PLP-ADA60 at different pHs in a shaking bath for 10 minutes. Scale bar $10 \text{ }\mu\text{m}$.

Figure 4-7 illustrates the sheep erythrocytes treated with FITC-Dextran ($10 \text{ }\mu\text{M}$, $M_w = 4 \text{ kDa}$) in the presence of different concentration of PLP-ADA60 at pH of 7.4 for 10 minutes in a shaking water bath maintained at 37°C . As shown in Figure 4-7, neither FITC-Dextran 4k nor Texas Red hydrazide could penetrate the RBC membrane in the presence of 0.02 mg mL^{-1} PLP-

ADA60 . In contrast, exposure of the RBCs to 0.05 mg mL^{-1} PLP-ADA60 at pH 7.4 resulted in the transformation of over 60% of these cells into erythrocyte ghosts. As the concentration of PLP-ADA60 was elevated to 0.2 mg mL^{-1} , approximately 90% of RBCs were turned into ghost cells. FITC-Dextran 4k effectively penetrated the membrane, implying that the polymer compromised the cell membrane at physiological pH by creating pores. This process occurred without causing membrane fusion or rupture, which is favorable for cross-membrane drug delivery. It is in agreement with previous reports that the PEGylated pseudopeptide, i.e., PLP bearing 1.3 mol% long methoxy PEG ($M_n = 4.4 \text{ kDa}$) as side chains could release haemoglobin (65 kDa) (Hajizadeh et al., 2018) into the extracellular medium without lysing RBC membranes (Chen et al., 2008).

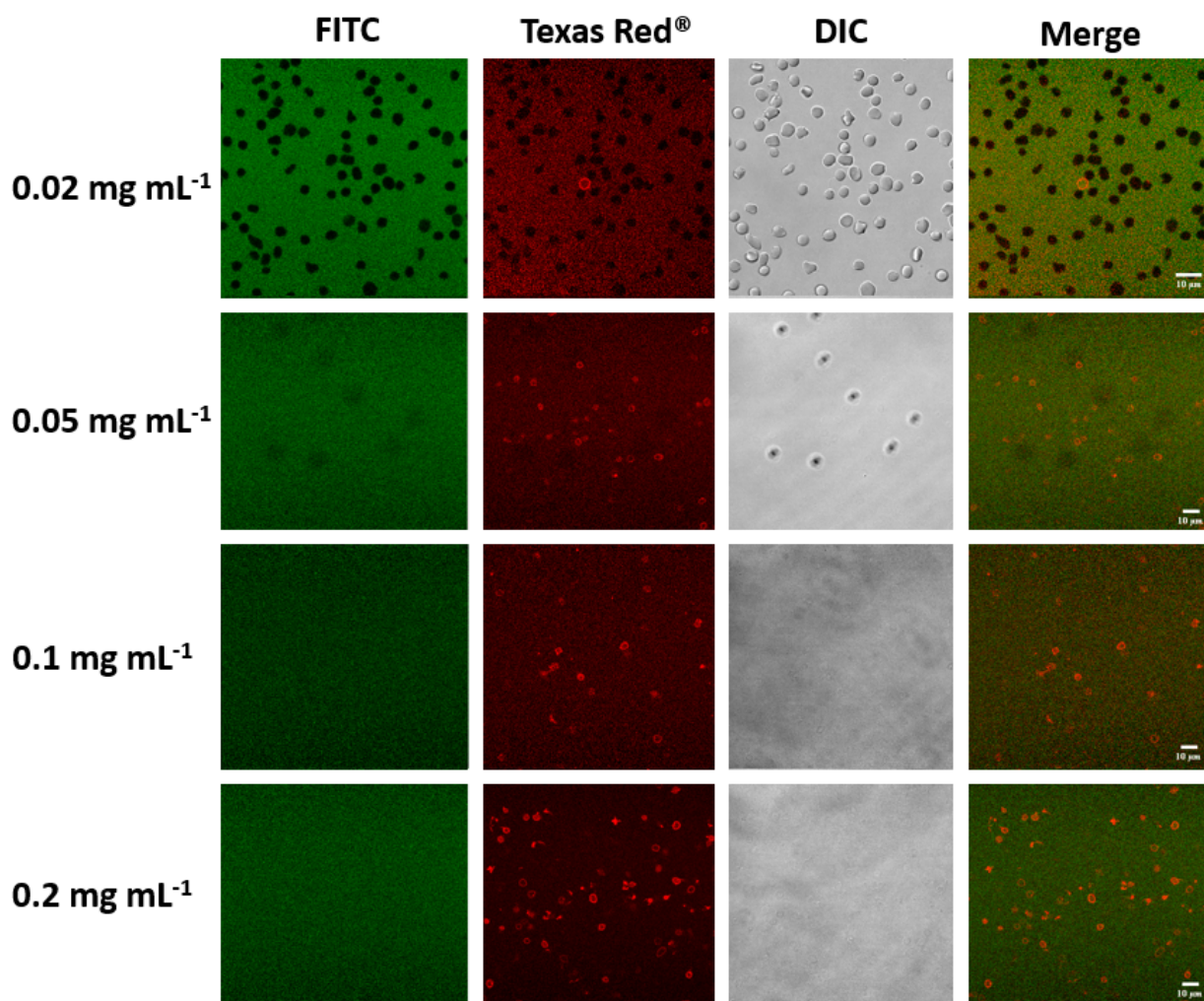


Figure 4-7. Laser scanning confocal microscopy fluorescence and differential interference contrast (DIC) images of sheep RBCs incubated with $10 \mu\text{M}$ FITC-Dextran ($M_w = 4 \text{ kDa}$) and $1 \mu\text{M}$ Texas Red hydrazide in the presence of PLP-ADA60 at different polymer concentrations and pH 7.4 in a shaking bath for 10 minutes. Scale bar $10 \mu\text{m}$.

4.4 Intracellular delivery

4.4.1 Release of endocytosed materials into the cytoplasm

The PLP-ADA mediated efficient membrane destabilization, as described above, has prompted the investigation into intracellular delivery to different nucleated mammalian cells, including HeLa cells from human cervical carcinoma, A549 cells from human lung carcinoma, and non-cancerous CHO cells. Calcein is a membrane-impermeable dye that can be used as a model drug to trace the endosomolytic activity of the polymers in cells. In the absence of PLP-ADA60, all three cell lines treated with 2.0 mg mL^{-1} Calcein displayed green punctate spots, which co-localized with red lysotracker labelling endosomes/lysosomes (Figure 4-8). This was caused by the Calcein entrapment within the intracellular vesicles following endocytosis (Chen et al., 2009c).

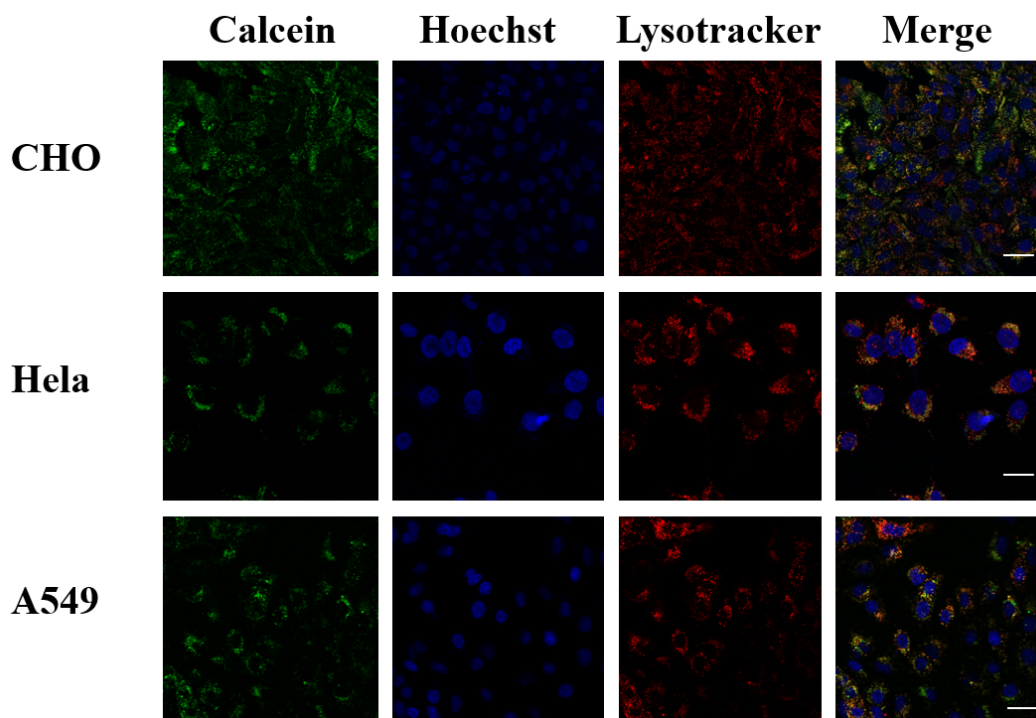


Figure 4-8. Laser scanning confocal microscopy images of CHO, HeLa, A549 cells showing the subcellular distribution of Calcein fluorescence. The cells were treated with 2.0 mg mL^{-1} Calcein only. Images were acquired after 2 h of uptake and 2 h of further incubation in fresh DMEM. Scale bar: $10 \text{ }\mu\text{m}$.

As shown in Figure 4-9, after co-incubation with 2.0 mg mL^{-1} Calcein and 0.2 mg mL^{-1} PLP-ADA60 for 2 hours, bright green diffuse fluorescence was observed throughout all three cell lines, indicative of efficient endosomal escape of Calcein into the cytosol after endocytosis. It shows the applicability of the pseudopeptidic polymer for cytoplasmic delivery to various cancerous and non-cancerous cells. Intriguingly, this green model payload was consistently observed to co-localize with blue Hoechst staining nuclei. Additionally, the green fluorescence in the nuclear region appeared stronger in cancerous cell lines HeLa and A549 as compared to that in the cytosol, although the underlying reason was not readily apparent.

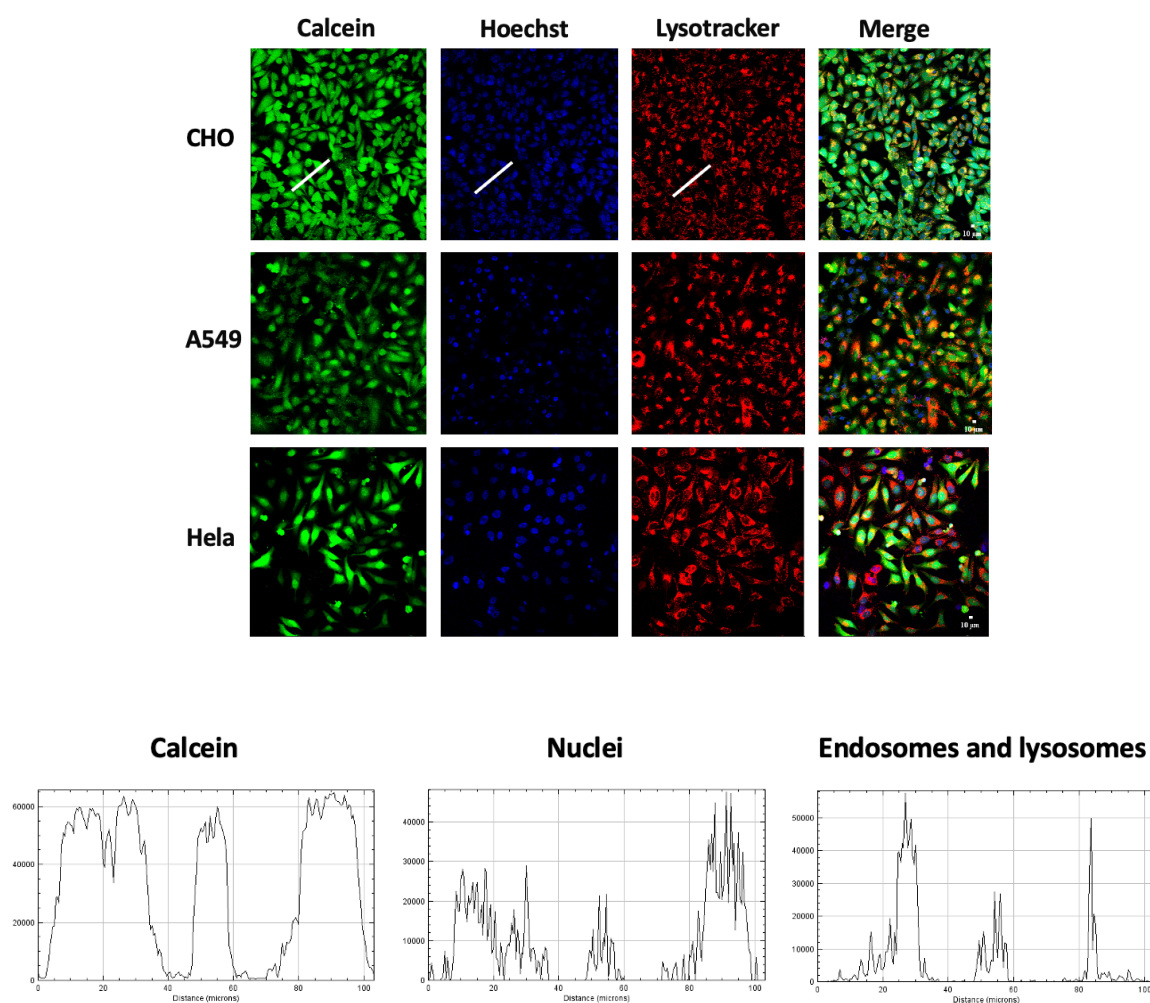


Figure 4-9. Laser scanning confocal microscopy images of CHO, A549, HeLa cells (top) showing the subcellular distribution of Calcein fluorescence. Cells were cotreated with 2.0 mg mL^{-1} Calcein and 0.2 mg mL^{-1} PLP-ADA60. Images were acquired after 2 h of uptake and 4 h of further incubation in fresh DMEM. The white line represents the cross-sectional area utilized for generating the intensity profiles (bottom), achieved through the application of ImageJ. Scale bar $10 \mu\text{m}$.

4.4.2 PLP-ADA60 mediated cytoplasmic delivery of macromolecular payloads

4.4.2.1 Delivery of different sized macromolecular payloads

It remains a significant hurdle to achieving effective intracellular delivery of macromolecules (Canton and Battaglia, 2012, Sahay et al., 2010). Typically, macromolecules enter cells through an endocytic pathway, therefore endosomal escape is essential to avoid their degradation in lysosomes (Iversen et al., 2011, Varkouhi et al., 2011, Yessine and Leroux, 2004). Herein, FITC-Dextran molecules were selected as model macromolecular payloads due to (i) their weak negative charge, which minimizes nonspecific binding to negatively charged cell surface, and (ii) easy accessibility to a wide range of molecular weights. To evaluate the applicability of this polymeric delivery system for intracellular delivery of macromolecules, A549 cells were co-incubated with PLP-ADA60 and FITC-Dextran of different molecular weights, specifically 10, 40, 70, 150 and 2000 kDa, at pH 7.4. These Dextran variants cover a broad spectrum of sizes, corresponding to the typical sizes of peptides, proteins, antibodies, RNAs, and plasmids (Watkins and Chen, 2015).

As shown in Figure 4-10A and B, the diffuse green fluorescence was noticeable throughout the A549 cells treated with FITC-Dextran with a wide range of molecular weights from 10 kDa to 2000 kDa in the presence of 0.1 mg mL⁻¹ PLP-ADA60 at pH 7.4 for just 30 minutes. This suggests that the pH-responsive polymer could facilitate the efficient cytoplasmic delivery of a diverse array of therapeutic agents. It is consistent with the results in Figures 4-6 and 4-7 showing the PLP-ADA60-mediated ghost red blood cell formation as a result of release of haemoglobin with a molecular weight of 65 kDa without causing cell membrane lysis and the cross-membrane delivery of 4 kDa FITC-Dextran. It is noted that the fluorescence intensity in the cells varied and those co-incubated with the polymer and the lower molecular weight FITC-Dextrans (below 40 kDa) was appeared to be lower. This might be due to the pH-sensitivity and photobleaching in FITC imaging (Le Guern et al., 2020, Benson et al., 1985).

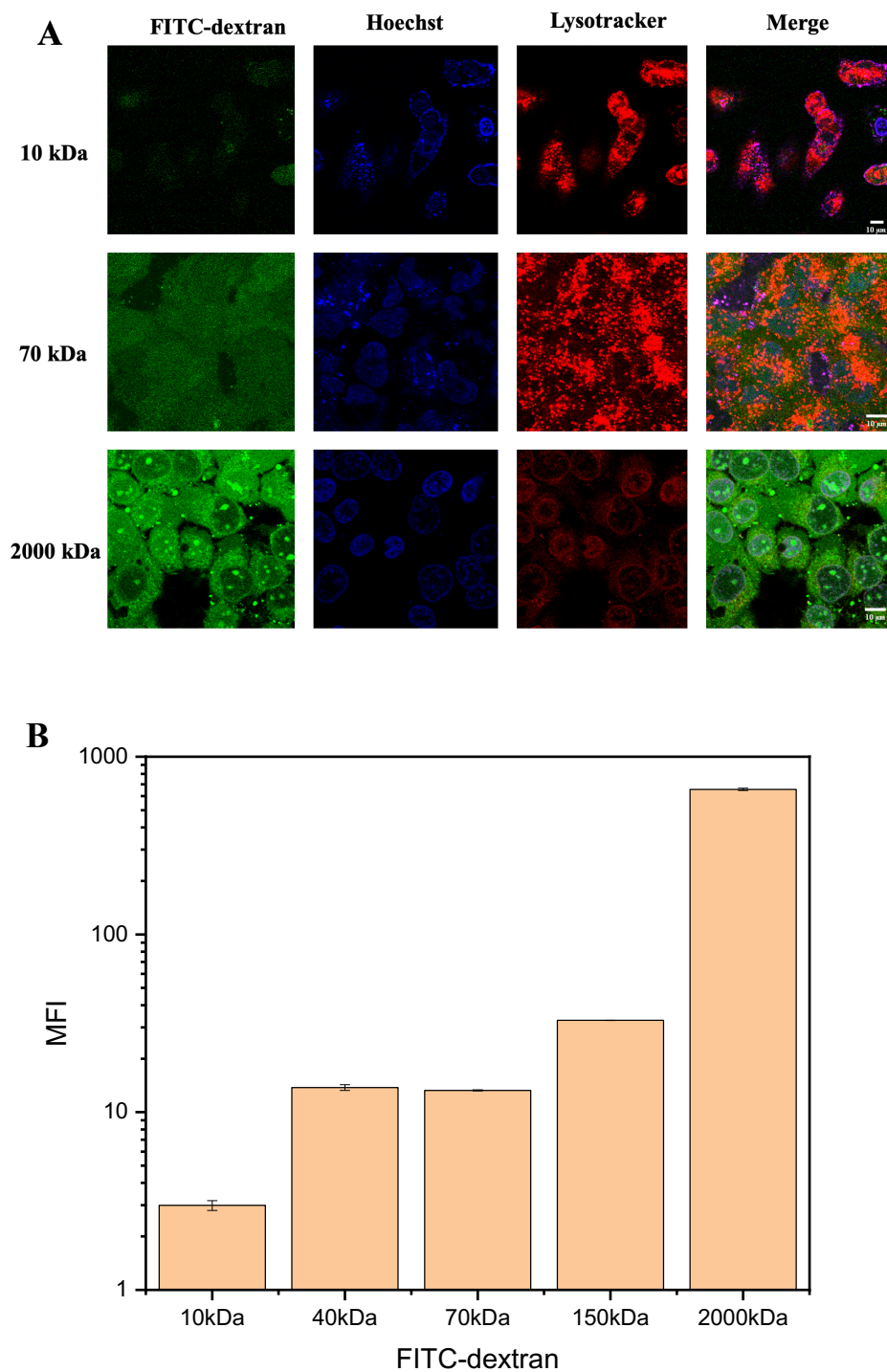


Figure 4-10. Flow cytometry analysis of mean fluorescence intensity (MFI) in macromolecular delivery method characterization. (A) Laser scanning confocal microscopy images and (B) mean fluorescence intensity (MFI) in A549 cells after a treatment with a mixture of PLP-ADA60 (0.1 mg mL^{-1}) and different sized FITC-Dextran ($10 \text{ }\mu\text{M}$) at pH 7.4 for 30 minutes. Mean $\pm$ S.D. ($n=3$).

4.4.2.2 Effect of extracellular acidity

A majority of solid tumors have an acidic microenvironment with a dysregulated pH ranging from 5.3–7.2 (Wike-Hooley et al., 1984, Gillies et al., 2002, Van Sluis et al., 1999). The acidification mainly results from factors including the high glycolytic rate of tumor cells, production and accumulation of lactic acid, increased secretion of protons, and chaotic tumor vasculature (Stubbs et al., 1994, Schornack and Gillies, 2003, Gillies et al., 1990). The pH-sensitive membrane-destabilizing capabilities of PLP-ADA60 has prompted a further investigation into how extracellular acidity impacts polymer-mediated intracellular delivery of macromolecules by co-incubation. A 150 kDa FITC-Dextran was employed as a model of large protein payload, as its molecular weight is similar to that of IgG antibodies (Chandran Suja et al., 2023).

Figure 4-11 shows the quantitative flow cytometry analysis of mean fluorescence intensity (MFI) in A549 cells co-treated with PLP-ADA60 and 150 kDa FITC-Dextran at different extracellular pH from 5.5 to 7.4. As expected, for the untreated cells and the cells treated with 150 kDa FITC-Dextran only, there was negligible intracellular fluorescence. As comparison, after co-incubation with PLP-ADA60 and 150 kDa FITC-Dextran for 30 minutes, a significant increase in intracellular fluorescence was observed at all those extracellular pHs, indicative of the efficient PLP-ADA60 mediated intracellular delivery. Notably, co-incubation at pH 6.5 led to the highest MFI among all those pHs tested, showing approximately a 13-time higher MFI the 150 kDa FITC-Dextran only control group. The significantly higher intracellular delivery at an extracellular pH 6.5 compared to treatments at physiological and lower acidic pHs is favorable for cancer treatment.

It is well-known that cell-penetrating peptides (CPPs) can deliver cargos into cells via direct translocation or endocytosis (Desale et al., 2021) and they can switch between different entry mechanisms depending on exogenous factors (Ruseska and Zimmer, 2020). Similarly, the thesis results suggest that there are also two distinct pathways for PLP-ADA60 to penetrate the cells. As shown in Figures 4-6 and 4-7 above, PLP facilitated leakage of haemoglobin with a molecular weight of 65 kDa and enabled the cross-membrane delivery of 4 kDa FITC-Dextran

into the resulting ghost of red blood cells without endocytic pathways (Van Wijk and Van Solinge, 2005), indicative of direct membrane penetration. In addition, Figure 4-9 above shows that PLP-ADA could efficiently release endocytosed materials into the cytosol through endosomal escape. The endocytic mechanisms will be further consolidated in Section 4.4.3 below. Therefore, it is likely that the membrane-destabilizing activity of the polymer can be activated before endocytosis occurs, thus speeding up and enhancing the delivery of cargos into the cytoplasm. This is particularly favorable for treatment of tumors with an acidic microenvironment.

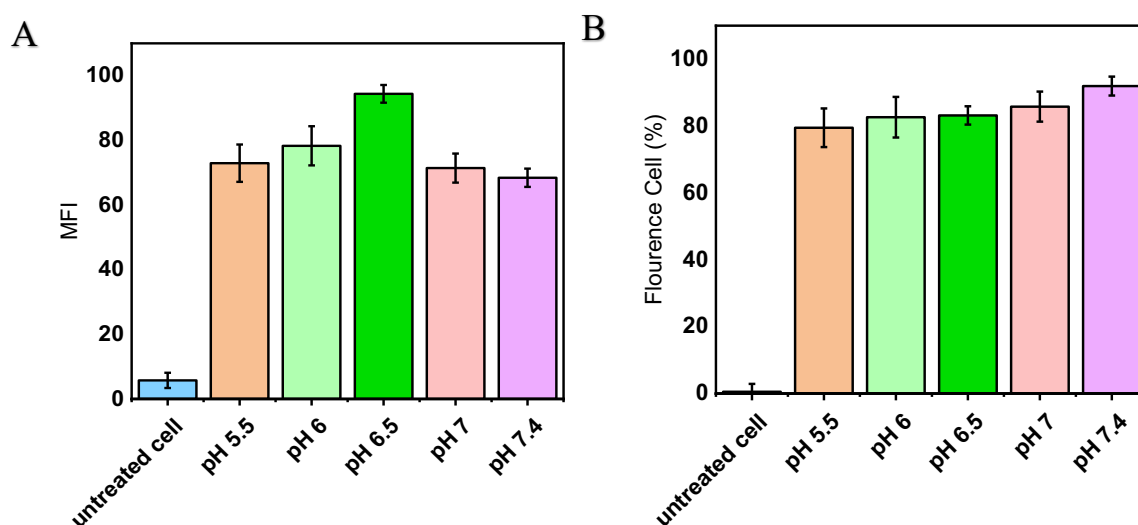


Figure 4-11. Flow cytometry analysis of A549 cells illustrating (A) MFI and (B) Fluorescence cells percentage of after co-incubated with PLP-ADA60 (0.1 mg mL^{-1}) and 150 kDa FITC-Dextran ($10 \text{ }\mu\text{M}$) at different pHs for 30 minutes. Results were based on a minimum of 10,000 events analysed. Mean $\pm$ SD, (n = 3).

4.4.2.3 Effect of polymer structure

Furthermore, A549 cells were co-incubated with 150 kDa FITC-Dextran and different PLP-ADA polymers at the same concentration of 0.1 mg mL^{-1} at pH 6.5. After 30 minutes of treatment, flow cytometry analysis was performed to assess the effect of polymer structure on intracellular delivery efficiency. As shown in Figure 4-12A, the fluorescence intensity in A549 cells after co-incubation of PLP-ADA and 150 kDa FITC-Dextran was substantially stronger compared to the control group in the absence of polymer. This finding was further consolidated

by the percentage of FITC-positive cells shown in Figure 4-12B, with over 80% of A549 cells showing successful intracellular delivery of 150 kDa FITC-Dextran at pH 6.5 in the presence of PLP-ADA30 and PLP-ADA45. Despite both PLP-ADA polymers led to a nearly identical percentage of fluorescent cells, the MFI was higher for the PLP-ADA45 group as compared to the PLP-ADA30 group. This increased intracellular delivery efficiency of PLP-ADA45 was due to its stronger pH-induced hydrophobic association (Figure 3-8) and enhanced membrane-destabilizing activity (Figure 4-1) as compared to PLP-ADA30. It is noteworthy that there was a slight reduction in both MFI and percentage of FITC-positive cells for the PLP-ADA60 group at pH 6.5 as compared to PLP-ADA45. This decrease is attributed to a conformational change for the polymer, leading it to create a globular structure that is less conducive to membrane insertion at slightly lower pH values, as shown in Figure 3-7 above.

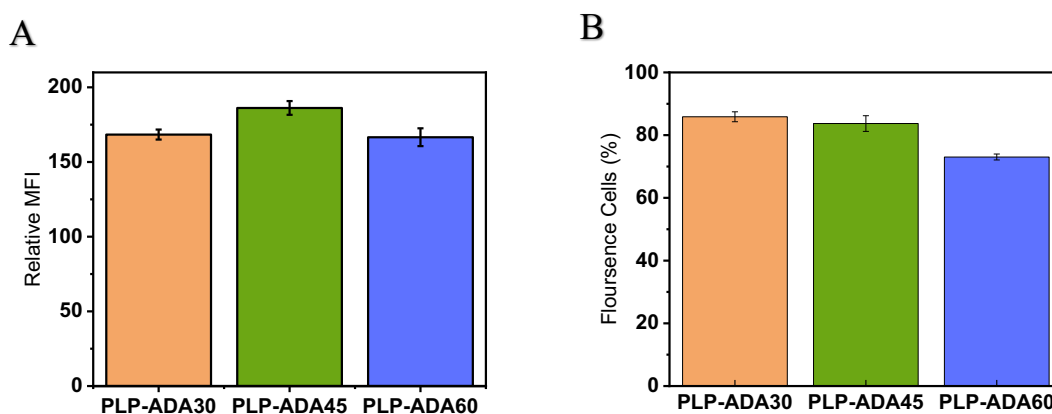


Figure 4-12. Flow cytometry analysis of A549 cells illustrating (A) MFI and (B) percentage of fluorescent cells after co-incubation of 150kD FITC-Dextran (10 μ M) by co-incubation with PLP-ADA30, PLP-ADA45, PLP-ADA60 (0.1 mg mL⁻¹) at pH 6.5 for 30 minutes. Results were based on a minimum of 10,000 events analysed. Mean $\pm$ SD, (n = 3). Relative MFI is measured by comparing it to control groups, which consist of cells treated only with FITC-dextran in the absence of polymer.

4.4.2.4 Effect of polymer concentration

The impact of polymer concentration on intracellular delivery efficiency of PLP-ADA60 was then quantitatively assessed using flow cytometry. Previous studies have shown that a higher

concentration of polymers, like poly(acrylic acid), PP pseudopeptides (such as PP75 and PP50), and PLP-NDA pseudopeptides (such as PLP-NDA18) can enhance membrane binding and permeability (Chen et al., 2009c, Murthy et al., 1999, Lee and Li, 2010, Chen et al., 2020). Herein, to investigate the polymer concentration-dependent delivery efficiency, A549 cells co-incubated with 150 kDa FITC-Dextran and PLP-ADA60 across a polymer concentration range of 0.01 - 0.2 mg mL⁻¹ at pH 7.0 and 7.4 (Figure 4-13). Notably, a considerable MFI relative to the FITC-Dextran only group was observed even at the lowest concentration tested (0.01 mg mL⁻¹). As anticipated, the MFI in the cells was dependent on the polymer concentration. Figure 4-13A shows that at pH 7.0, there was no notable difference in MFI seen in cells with increasing the PLP-ADA60 concentration from 0.01 to 0.025 mg mL⁻¹. This suggests that a PLP-ADA60 concentration of 0.01 mg mL⁻¹ was adequate for promoting intracellular delivery at this pH, implying that a saturation point existed for the polymer, beyond which the additional polymer would not enhance delivery. Interestingly, a further increase in polymer concentration to 0.2 mg mL⁻¹ led to a gradual reduction in MFI, possibly due to the polymer aggregation at higher concentrations at pH 7.0. As shown in Figure 4-13B, at pH 7.4, the maximum MFI reached at a PLP-ADA60 concentration of 0.1 mg mL⁻¹. This was because at the physiological pH there was no aggregation observed in the concentration range tested (Figure 3-7), and the marked enhancement in MFI at higher polymer concentrations resulted from the increased membrane destabilizing activity, driven by the stronger driving force for polymer molecules to migrate and binding to the lipid membrane (Chen et al., 2009c). Similar concentration-dependent effects have been reported by other researchers on cell-penetrating poly(disulfide)s (Wan et al., 2023), transactivator of transcription (TAT) (De Martini et al., 2023), and poly(acrylic acid) derivatives (Arkaban et al., 2022). This finding is aligned with the concentration-dependent haemolysis of PLP-ADA60 (Figure 4-2B), where its membrane destabilizing activity at physiological pH increased with increasing polymer concentration until a point of maximum haemolysis.

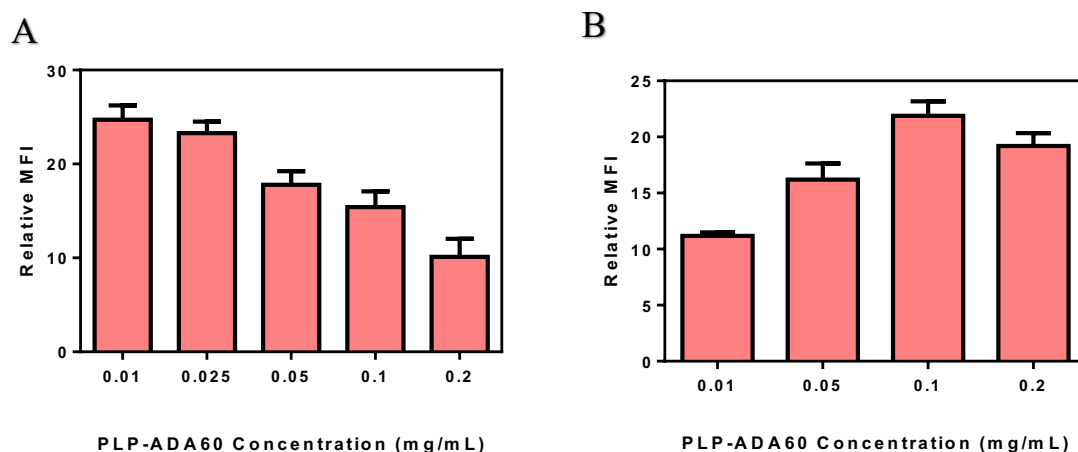


Figure 4-13. MFI in A549 cells after treatment with a mixture of PLP-ADA60 and 150 kDa FITC-Dextran (10 μ M) at (A) pH 7.0 and (B) pH 7.4 for 30 minutes. Mean $\pm$ SD, (n = 3). Relative MFI is measured by comparing it to control groups, which consist of cells treated only with FITC-dextran in the absence of polymer.

4.4.2.5 Effect of incubation time

The rate of intracellular delivery facilitated by the comb-like PLP-ADA60 polymer was further explored by flow cytometry. As depicted in Figure 4-14, the MFI for A549 cells co-incubated 150 kDa FITC-Dextran (10 μ M) and PLP-ADA60 (0.1 mg mL⁻¹) at pH 7.4 for 5 minutes was comparable to that of the cells treated with 150 kDa FITC-Dextran only. However, when the incubation period was extended to 15 minutes, evidence of polymer-mediated intracellular delivery began obvious. The MFI in cells surged significantly with further increasing the treatment time. Specifically, the MFI in cells co-incubated with FITC-Dextran and the polymer at pH 7.4 for 120 minutes was approximately 23 times higher than that for cells treated with FITC-Dextran only under the same conditions. This rapid intracellular delivery is consistent with the time-dependent haemolysis profile of PLP-ADA (Figure 4-3), which could be due to rapid direct membrane penetration and swift escape from endosomal compartments into the cytosol.

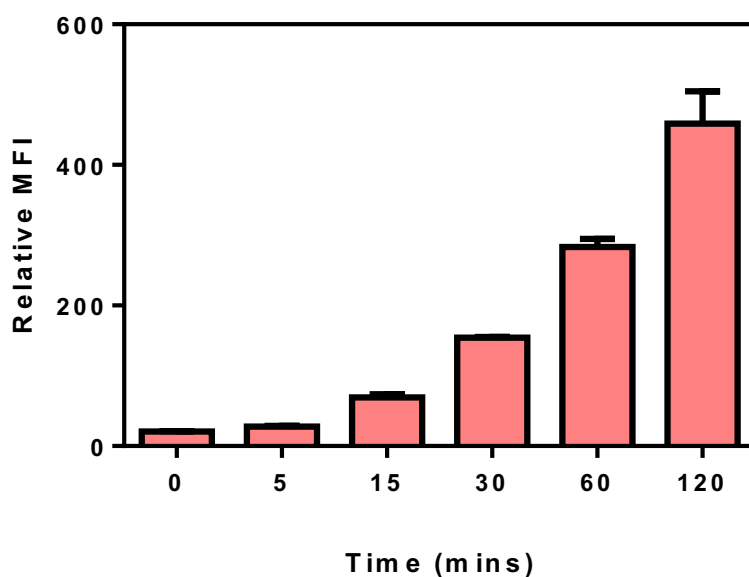


Figure 4-14. Time-dependent MFI in A549 cells after treatment with a mixture of PLP-ADA60 (0.1 mg mL^{-1}) and 150 kDa FITC-Dextran ($10 \text{ }\mu\text{M}$) at pH 7.4. Mean $\pm$ SD, ($n = 3$). Relative MFI is quantified in comparison to control groups, which consist solely of cells without any experimental treatment or modification.

4.4.3 Endocytosis and endosomal escape mechanisms

It has been reported that temperatures below the normal physiological level of 37°C can impede transport mechanisms within cells that require energy, including the endocytic route (Hohn et al., 2009). To confirm the cell internalization by endocytosis, HeLa cells were co-incubated with Calcein and PLP-ADA60 at both 4°C , to simulate a low-energy environment, and at 37°C , to allow for normal cellular activity. Figure 4-13 shows the bright diffuse green fluorescence throughout the cell at 37°C , which is consistent with the results shown in Figure 4-9. By contrast, there was negligible green fluorescence within the cytoplasm at 4°C . This observation suggests that the PLP-ADA60 mediated delivery of Calcein into cells is an endocytic process requiring cellular energy, which could be significantly reduced at lower temperatures, thereby hindering the transport process.

To further investigate the endocytic pathways, different inhibitors were used to block specific pathways. HeLa cells were treated with nystatin ($10\ \mu\text{g mL}^{-1}$) to inhibit caveolae-mediated endocytosis (Khormaei et al., 2013), or M β CD ($1.5\ \text{mg mL}^{-1}$) to inhibit lipid raft-mediated endocytosis (Greenlee et al., 2021) or chlorpromazine ($3\ \mu\text{g mL}^{-1}$) to inhibit clathrin-mediated endocytosis (Chang et al., 2014). HeLa cells were treated with Calcein ($0.2\ \text{mg mL}^{-1}$) only as negative control or co-incubation with Calcein ($2\ \text{mg mL}^{-1}$) and PLP-ADA60 ($0.2\ \text{mg mL}^{-1}$) in the absence of inhibitors as positive control. As shown in Figures 4-16 and 4-17, as expected, the negative control group showed a punctate staining pattern within cells, indicative of endosomal entrapment, while the positive control group showed bright diffuse fluorescence throughout the cytoplasm, indicative of efficient endosomal escape. The results with the use of endocytosis inhibitors suggest that PLP-ADA60 mediated intracellular Calcein delivery could involve a combination of one or more endocytic pathways, among which, the lipid raft-mediated endocytosis was dominant.

Ammonium chloride was then used to inhibit endosomal acidification (Khormaei et al., 2013). It was interesting to find that there was only a small decrease in the intracellular fluorescence intensity as compared to positive control. This could be due to the strong membrane activity of PLP-ADA60 in a high pH range of 7.0-7.4, making the endosomal escape and direct membrane processes not necessarily rely on acidification to trigger its membrane activity.

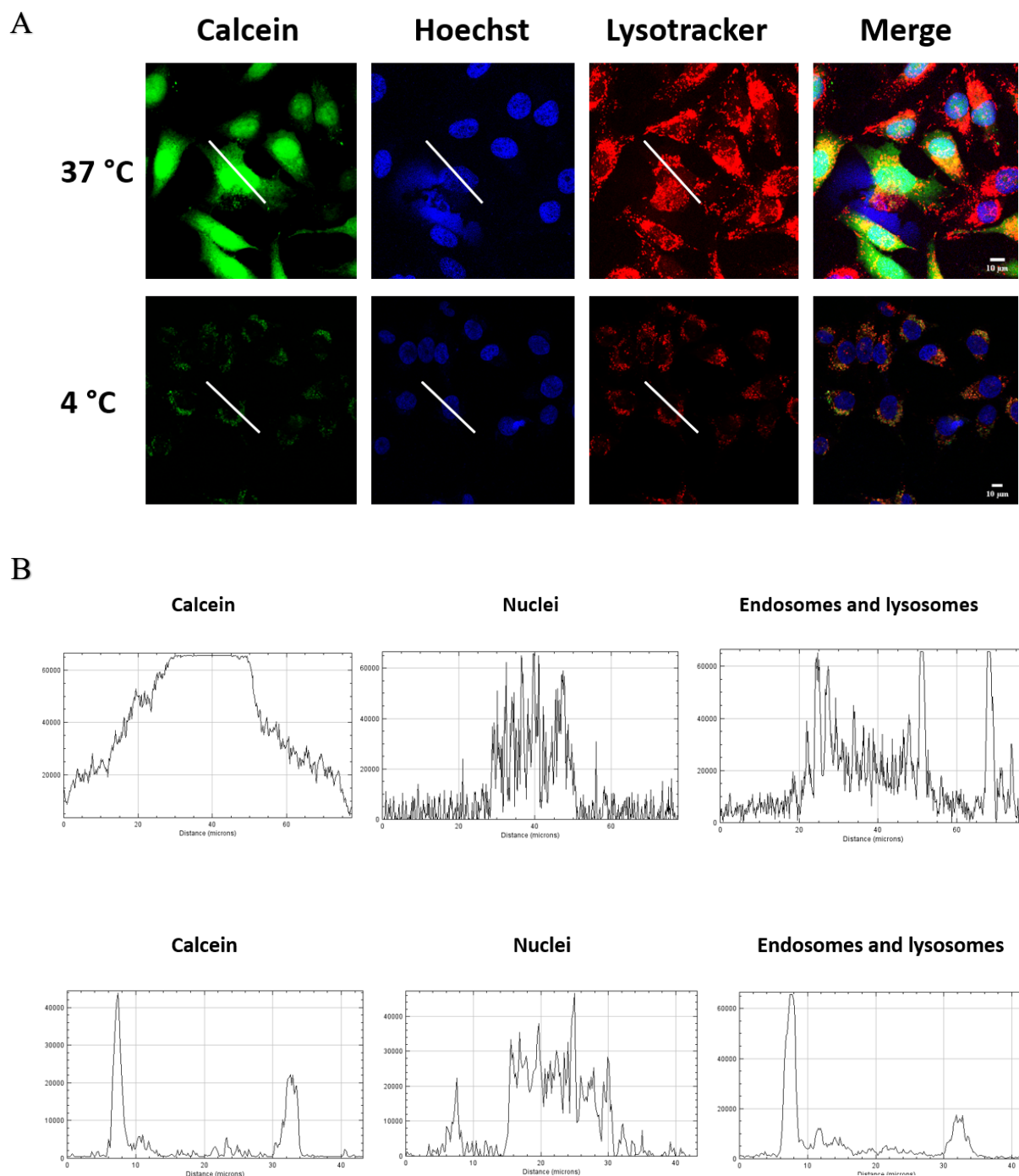


Figure 4-15. Delivery of Calcein (2 mg mL^{-1}) to HeLa cells by co-incubation with PLP-ADA60 (0.5 mg mL^{-1}) for 30 minutes at 37°C and 4°C , respectively, as visualised by (A) laser scanning confocal microscopy, Scale bar $10 \mu\text{m}$. White line indicates the cross section used to produce the intensity profiles shown in (B) with the use of ImageJ.

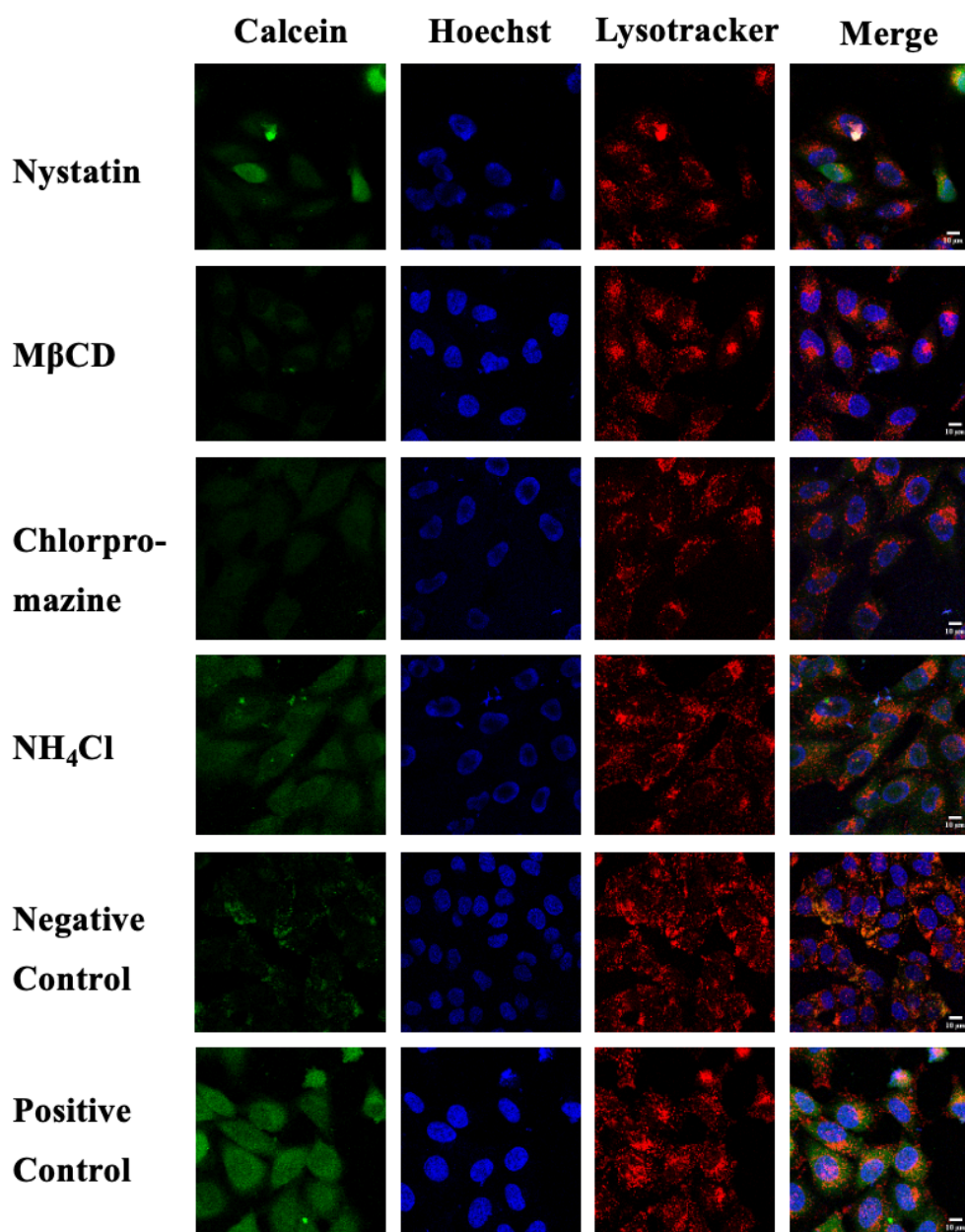


Figure 4-16. Laser scanning confocal microscopy of effects of endocytosis inhibitors on the intracellular uptake and distribution of Calcein in HeLa cells. Cells were pre-treated with nystatin ($10 \mu\text{g mL}^{-1}$), M β CD (1.5 mg mL^{-1}), chlorpromazine ($3 \mu\text{g mL}^{-1}$), or NH_4Cl ($30 \mu\text{M}$) for 1 hour followed by treatment of Calcein (2 mg mL^{-1}) and PLP-ADA60 (0.2 mg mL^{-1}) in the presence of inhibitors for 1 hour. Negative control cells were treated with Calcein (2 mg mL^{-1}) only. Positive control cells were treated with Calcein (2 mg mL^{-1}) and PLP-ADA60 (0.2 mg mL^{-1}) in the absence of inhibitors.

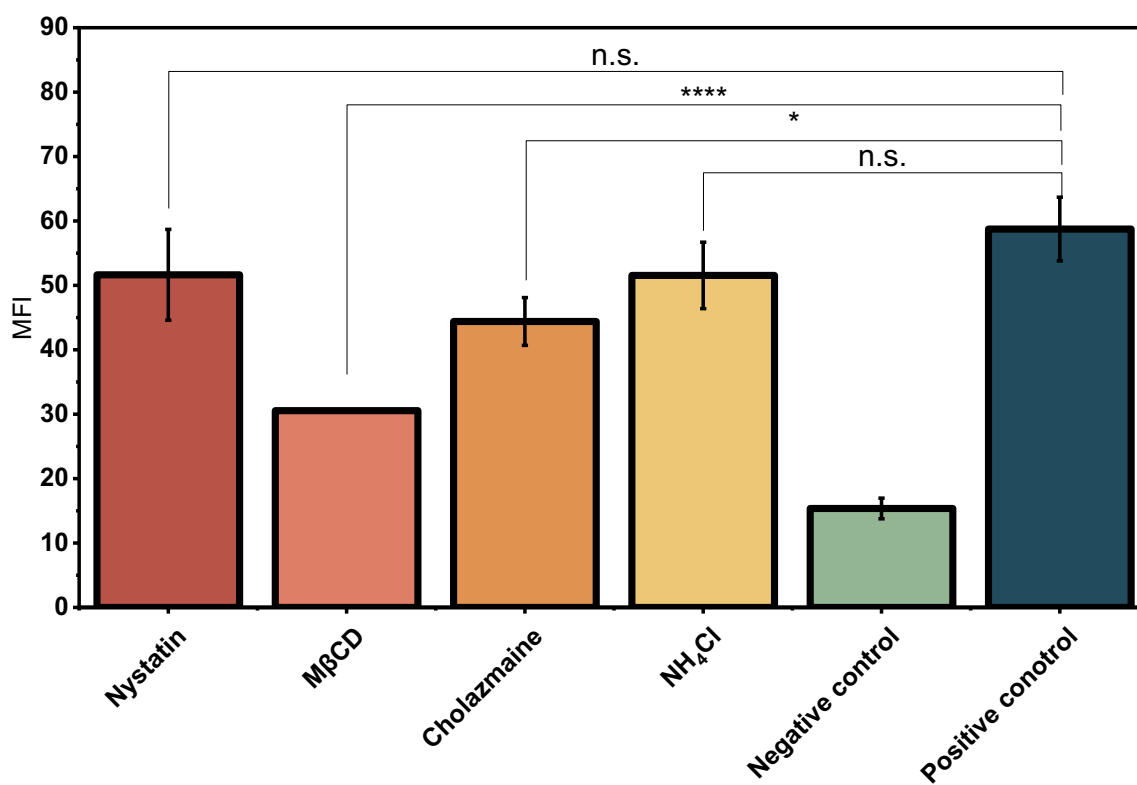


Figure 4-17. Flow cytometry analysis of effects of endocytosis inhibitors on the intracellular uptake and distribution of Calcein in HeLa cells. Cells were pre-treated with nystatin (10 $\mu\text{g mL}^{-1}$), M β CD (1.5 mg mL^{-1}), chlorpromazine (3 $\mu\text{g mL}^{-1}$), or NH₄Cl (30 μM) for 1 hour followed by treatment of Calcein (2 mg mL^{-1}) and PLP-ADA60 (0.2 mg mL^{-1}) in the presence of inhibitors for 1 hour. Negative Control cells were treated with Calcein (2 mg mL^{-1}) only. Positive control cells were treated with Calcein (2 mg mL^{-1}) and PLP-ADA60 (0.2 mg mL^{-1}) in the absence of inhibitors. Mean $\pm$ S.D. (n=3).

4.5 Delivery to multicellular spheroids

Three-dimensional (3D) multicellular spheroids serve as valuable models that mimic the complex architecture and cellular dynamics of tumors and tissues (Abdolahinia et al., 2022,

Popescu et al., 2023). The efficient transport of large molecules into such spheroids holds significant promise for advancing research in basic cellular processes and tumor biology. In an experimental setup to evaluate the effectiveness of PLP-ADA60 in mediating macromolecular delivery, 3-day-old A549 spheroids were prepared and treated with a combination of Calcein (2 mg mL^{-1}) and PLP-ADA60 (0.2 mg mL^{-1}). The subsequent analysis through laser scanning confocal microscopy, as depicted in Figure 4-18, revealed the successful 3D transport of Calcein, as evidenced by strong green fluorescence within the cancer spheroids. This suggests that PLP-ADA60 can enable the transport across extracellular barriers in the spheroids and the subsequent cytoplasmic delivery of Calcein in cells. By contrast, A549 spheroids in the absence of polymer displayed only marginal fluorescence, indicative of the limited 3D transport and endosomal/lysosomal entrapment of the payload only (Kopytynski et al., 2020).

In conclusion, the evidence from confocal microscopy underscores the potential of PLP-ADA60 as a powerful delivery vehicle for transporting therapeutic agents into spheroids. Its capability to facilitate the cytoplasmic localization of payloads like Calcein, which typically do not transverse cell membranes independently, is particularly noteworthy. This characteristic could be exploited in targeted cancer therapies, where the delivery of drugs to the cytoplasm of cancer cells within a 3D tumor environment is crucial. Such targeted delivery could enhance the efficacy of therapeutic agents and minimize side effects, marking a significant step forward in the treatment of complex tumor environments.

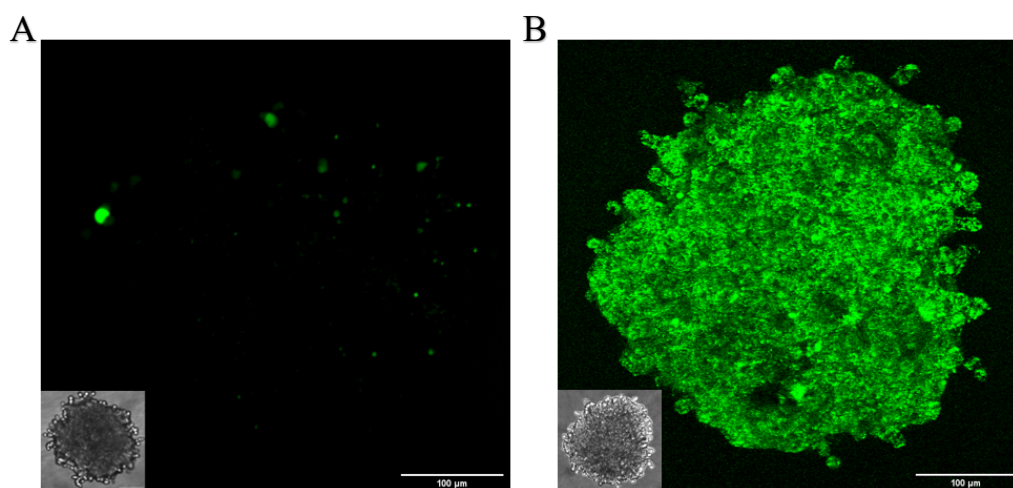


Figure 4-18. Laser scanning confocal microscopy images of A549 human lung cancer cell spheroids co-incubated with Calcein (2 mg mL^{-1}) and without (A) and with (B) PLP-ADA60 (0.2 mg mL^{-1}) for a period of 2 h. The 3D projections were shown from the top. Each inset showed the bright field images of the corresponding spheroid.

4.6 In-vitro cytotoxicity

The Alamar Blue assay, a widely used method for assessing cell viability (Rampersad, 2012), was employed to determine the cytotoxicity of PLP-ADA30, PLP-ADA45, and PLP-ADA60 toward CHO, HeLa, and A549 cells. As shown in Figure 4-19, after 12 hours of incubation at the polymer concentration ranging from 0.02 mg mL⁻¹ to 2 mg mL⁻¹, all the three PLP-ADA polymers exhibited no or marginal cytotoxic effects toward the two cancerous and one non-cancerous cell lines. The results imply that incorporating hydrophobic and comparatively lengthy ADA side chains onto the PLP backbone does not significantly increase cytotoxicity. This aspect of the polymer design is crucial because it suggests that enhancing the hydrophobicity of the polymer—often necessary for efficient cross-membrane drug delivery—does not compromise cell viability. It also opens the possibility that these polymers could be used to deliver a range of drug molecules without eliciting adverse effects from the delivery vehicle itself.

In conclusion, the data gathered from the Alamar Blue assay substantiates the potential of PLP-ADA polymers as safe drug delivery vehicles. The absence of significant cytotoxicity across a range of concentrations and cell types highlights the biocompatibility of these polymers. Given their non-toxic nature at effective delivery concentrations, PLP-ADA polymers can be considered promising candidates for further development in various biomedical applications, where cell viability is paramount. This could pave the way for their use in therapeutic treatments requiring the safe and efficient transport of drugs to target cells.

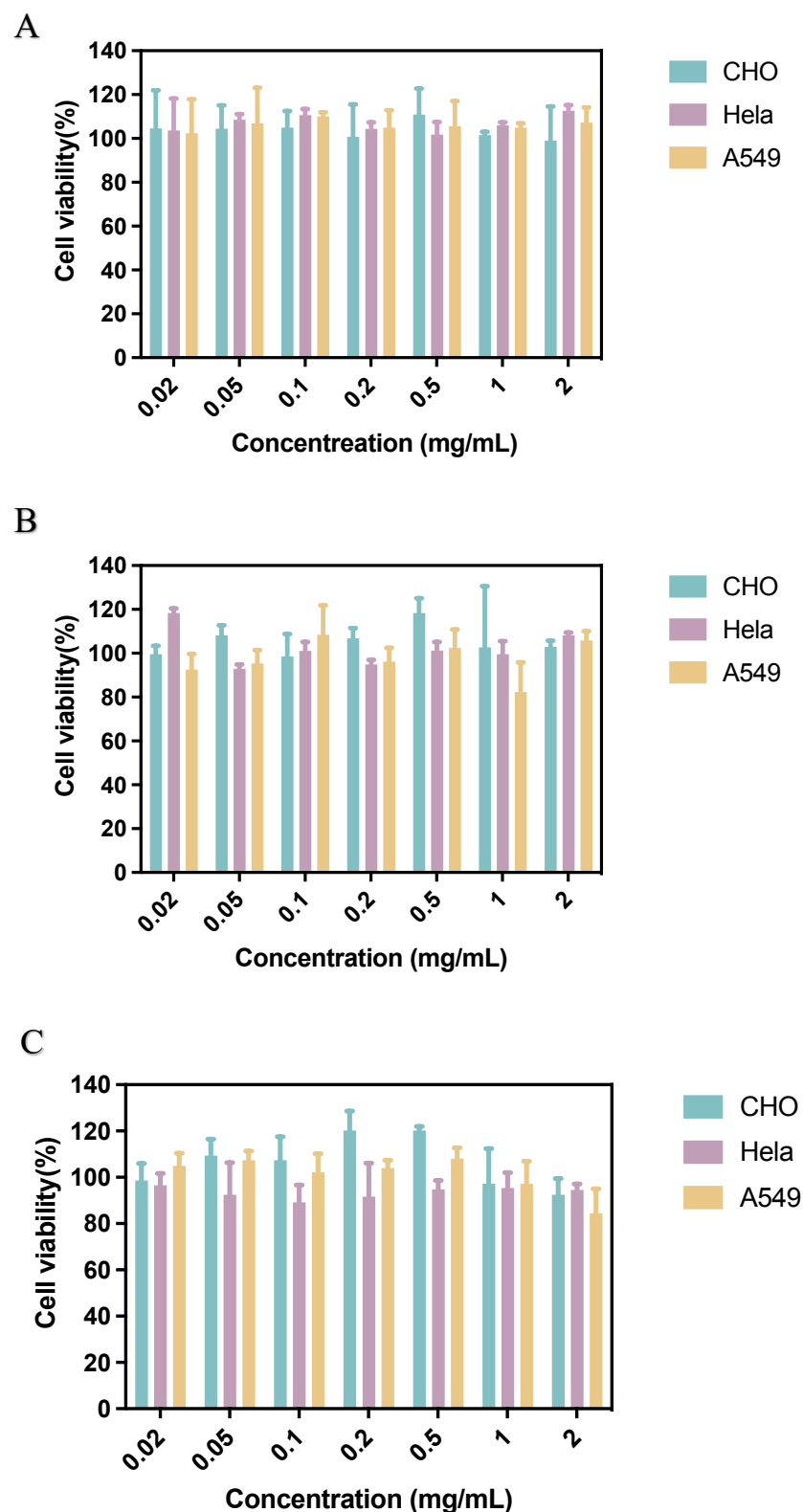


Figure 4-19. Relative viabilities of CHO, HeLa, A549 cells, as measured by Alamar Blue assay, after incubation with (A) PLP-ADA30, (B) PLP-ADA45, and (C) PLP-ADA60 at different concentrations for 12 hours. Mean $\pm$ S.D. (n=6).

4.7 Intracellular delivery of a therapeutic protein to enhance cytotoxicity for cancer therapy

The ability of PLP-ADA60 to deliver therapeutic macromolecules into the cell cytoplasm for enhanced cancer therapy was investigated in A549 cells using a ribosome-inactivating protein as a macromolecular drug. Saporin, a 30 kDa protein isolated from the seeds of *Saponaria officinalis* and possessing rRNA N-glycosidase activity, is commonly studied as an anticancer agent due to its potent ribosome-inactivating ability. It has been reported to irreversibly inhibit protein synthesis and cell death via apoptosis. However, the cytoplasmic localization is a major rate-limiting factor for Saporin to exert its therapeutic potential (Narayanan et al., 2005). Therefore, functional delivery of Saporin using PLP-ADA60 was explored in the thesis work.

Successful functional delivery was measured by determining the cell viability of A549 cells, as Saporin has been reported to induce cell death after entering the cell interior. Cells were co-incubated with 1 nM Saporin and PLP-ADA60 at four polymer concentrations: 0.025 mg mL⁻¹ (PLP-ADA60-0.025/Saporin), 0.05 mg mL⁻¹ (PLP-ADA60-0.05/Saporin), 0.1 mg mL⁻¹ (PLP-ADA60-0.1/Saporin), or 0.2 mg mL⁻¹ (PLP-ADA60-0.2/Saporin) for 1 hour, followed by a further incubation for 72 hours. As a control, the dose-dependent cytotoxicity of Saporin in its free form (free Saporin) was compared. Cell viability was measured using the CellTitre-Glo® 2.0 Cell Viability Assay.

Figure 4-20A shows that the half maximal effective concentration (EC₅₀), here defined as the concentration of Saporin that reduces the cell viability by half, of free Saporin remained above 1000 nM (Alexander et al., 1999). In the presence of PLP-ADA60 at the lowest concentration tested, the EC₅₀ of PLP-ADA60-0.025 was reduced to 73 nM. It is interesting to note that the increased PLP-ADA60 concentration in PLP-ADA60-0.05/Saporin and PLP-ADA60-0.1/Saporin resulted in the substantially higher cell death corresponding to the decreased EC₅₀ values to 0.0037 nM and 0.0034 nM, respectively. The further increased polymer concentration in PLP-ADA60-0.2/Saporin did not further reduce the EC₅₀, suggesting that the saturation concentration was reached. As shown in Figure 4-20B, PLP-ADA60 alone at the same tested

concentrations did not display any toxicity to cells, consistent with the cytotoxicity results shown in Figure 4-19. The results confirm that the enhanced cytotoxicity of Saporin was due to the polymer-induced intracellular delivery of Saporin, not the PLP-ADA60 delivery platform itself. For targeted deliver, Saporin can be selectively delivered to cancer cells by conjugating it with monoclonal antibodies that target tumor-specific or overexpressed receptors, such as the TCbIR receptor, which is upregulated in certain cancer cells (Quadros et al., 2010). This targeted approach ensures that saporin is primarily internalized by cancer cells, minimizing the impact on normal cells and reducing systemic toxicity.

The promising enzymatic activity of membrane-impermeable ribosome-inactivating proteins, such as Saporin, after they cross the plasma membrane, provides incredible opportunities for cancer therapy (Polito et al., 2013). This work presents PLP-ADA polymers as an excellent delivery platform for intracellular delivery of Saporin and other therapeutic macromolecules for potential application in cancer therapy fields.

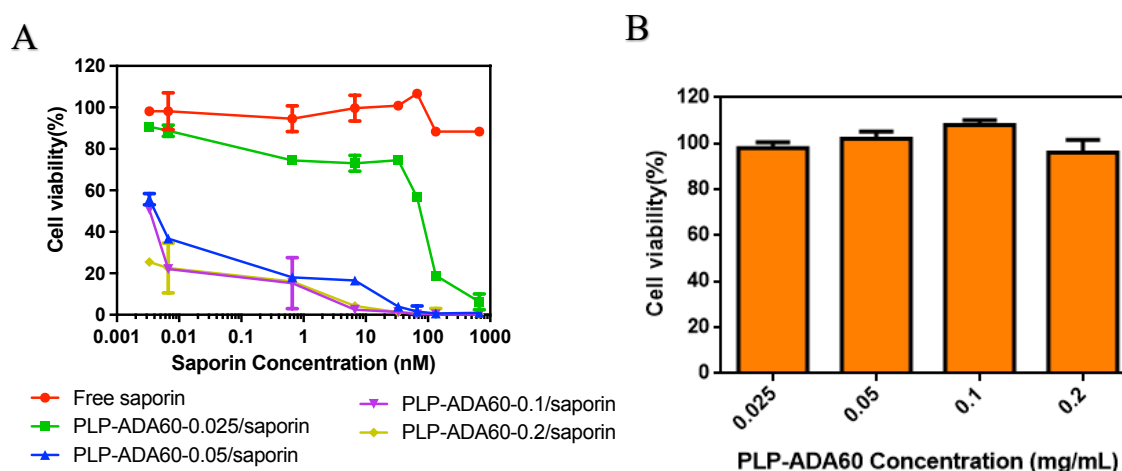


Figure 4-20. (A) Viabilities of A549 cells after 1 hour of treatment at pH 7.4 with PLP-ADA60/Saporin at varying PLP-ADA60 concentrations or Saporin alone plus 72 hours of further incubation, as determined by the CellTitre-Glo® 2.0 cell viability assay. Mean results $\pm$ SD, (n=6). (B) Viabilities of A549 cells after 2 hours of treatment at pH 7.4 with PLP-ADA60 alone plus 72 hours of further incubation, as determined by the CellTitre-Glo® 2.0 cell viability assay. Mean results $\pm$ SD, (n=6).

4.8 Conclusions

The utilization of pH-responsive polymers for intercellular delivery enhancing the efficiency delivery in diverse applications. This chapter presents a detailed investigation into PLP-ADA-mediated intracellular delivery, encompassing various factors such as pH, concentration, incubation time, and degree of grafting, evaluated through haemolysis assays to assess cell membrane activity. Mechanistic insights into the destabilization of cell membranes by PLP-ADA were elucidated. The efficacy of PLP-ADA in delivering payloads of different sizes into diverse mammalian cells and multicellular spheroids was demonstrated through techniques including laser scanning confocal microscopy and flow cytometry. Endosomal escape was tracked by using Calcein and FITC-dextran with different molecular weight as model drugs. PLP-ADA was found to be highly effective in delivering Calcein and FITC-Dextran of varying molecular weights. The results indicate that the polymers are effective at disrupting cell membranes and releasing endocytosed materials into the cytoplasm of mammalian cells, such as cancerous and non-cancerous cells. Furthermore, the intracellular delivery pathways and associated mechanisms were thoroughly explored. The results showed that multiple pathways are involved in endosomal escape, rather than a single pathway. To quantify the delivery pathway, flow cytometer used to measure the decrease of the mean fluorescence intensity with inhibitors. HeLa cells were treated with various inhibitors to block specific endocytic pathways, revealing that PLP-ADA60 primarily utilizes lipid raft-mediated endocytosis for intracellular delivery of Calcein. Further experiments with ammonium chloride showed only a slight decrease in intracellular fluorescence, suggesting that PLP-ADA60's strong membrane activity at higher pH levels enables endosomal escape without relying heavily on acidification. Notably, the chapter shows the successful intracellular delivery of Saporin, underscoring the potential of PLP-ADA as a platform for cancer treatment modalities. This study also describes the use of spheroids, as models to mimic the *in vivo* tissue environment of tumour cells. Furthermore, Successful uptake of Saporin was determined by increased cytotoxicity, measured by a decrease in cell viability, compared to free Saporin when Saporin was co-incubated PLP-ADA. Importantly, polymers alone were found to be non-cytotoxic to A549 cells. Importantly, the polymers showed minimal cytotoxicity. These benefits indicate the significant potential of this comb-like polymer for use in intracellular drug delivery applications. However, the pH-responsive polymers have faced challenges, this approach lacks tumor selectivity, such as the

minimal pH difference between healthy and tumor tissues and the uneven pH distribution within the tumor interstitium. Additionally, there's a conflict between the characteristics that promote long-term circulation of drug delivery systems and the properties that enhance their efficient internalization by cancer cells, further limiting the effectiveness of pH-responsive polymers.

This chapter provides an overview of PLP-ADA-mediated intracellular delivery, mechanistic investigations, payload delivery, cytotoxicity assessments, and therapeutic applications, thereby highlighting the considerable promise of these polymers in efficacy transporting various macromolecules into cells. In conclusion, intercellular delivery using pH-responsive polymers holds great promise for improving the efficiency.

Chapter 5

Intracellular trehalose delivery by co-incubation with PLP-ADA polymers for cell cryopreservation

This chapter describes the intracellular delivery of membrane-impermeable bioprotectant trehalose, facilitated by the comb-like PLP-ADA pseudopeptidic polymers, and the application for cell cryopreservation. Trehalose delivery into different mammalian cells, such as unnucleated red blood cells and nucleated Jurkat cells, was optimized by adjusting various parameters, including osmolality of washing buffer, polymer structure, polymer concentration, pH, and incubation time. Cryopreservation of trehalose-loaded red blood cells and Jurkat cells was demonstrated and the effects of intracellular trehalose, extracellular trehalose and storage conditions were investigated.

5.1 Introduction

The long-term preservation of cells is critically important for various medical fields, including cell transplantation and therapeutic applications. Cryopreservation stands as the most employed technique for the long-term preservation of cellular and tissue vitality and functionality. Nonetheless, the primary concern during the freezing and thawing process is the potential harm posed by the formation of ice crystals inside and outside cells and tissues. To mitigate ice crystal formation, cryoprotective agents (CPAs) are essential additives to cells and tissues to confer cryoprotection. However, CPAs currently in use, mainly dimethyl sulfoxide or glycerol, have toxic effects, limiting their suitability for use in cell therapies. Consequently, a labor-intensive purification process is often required to eliminate toxic CPAs for subsequent applications in translational medicine, which may result in the loss or damage of valuable cells. Sugars offer a non-toxic alternative with cryoprotective capabilities. Despite these advantages,

sugars are seldom used in cryopreservation. This is primarily because they are not membrane-permeable under physiological conditions, leading to the perception that they cannot offer intracellular protection during the freezing process. It's worth noting, however, that membrane permeability characteristics can undergo significant alterations in the presence of ice. This affects not only the permeability to water (Akhoondi et al., 2011) but also to molecules that are generally considered membrane-impermeable (Stoll et al., 2012, Glärfke et al., 2012, Zhang et al., 2016, Oldenhof et al., 2013), thus leading to cell damage. Presently, trehalose is a nontoxic disaccharide consisting of two glucose molecules and a promising cryoprotection agent for medically applicable cells (Dovgan et al., 2021) due to its cryoprotective efficacy and stability throughout cryopreservation. Nevertheless, trehalose is a non-membrane-permeable CPA, and the primary challenge lies in the absence of a method for efficient intracellular trehalose delivery without causing cell damage, which hampers its adoption in cryopreservation (Hu et al., 2023).

In this chapter, the use of PLP-ADA was explored to facilitate the delivery of trehalose into the cytoplasm of mammalian cells by interacting with the cell membrane and temporarily enhancing its permeability. Importantly, it was demonstrated in Chapter 4 PLP-ADA polymers exhibited non-cytotoxic effects. Additionally, the enhancement of membrane permeability achieved by this type of pseudopeptides could be reversed to the intact form of the membrane through a simple wash with a buffer solution (Lynch et al., 2010, Chen et al., 2020, Lynch et al., 2011). These polymers could be transformed into a membrane-active state in response to pH reduction, typically by undergoing conformational changes in response to acidic extracellular and/or intracellular environments. The pH-responsive nature of the polymer-based delivery system facilitates efficient cytoplasmic delivery of trehalose into mammalian cells either through direct membrane penetration (e.g., unnucleated red blood cells without endocytic pathways) or through a combination of endosomal escape and direct membrane penetration (e.g., nucleated cells). The safeguarding of cells using pH-responsive polymer-facilitated trehalose delivery holds substantial promise in diverse biomedical contexts.

In summary, as per studies on the trehalose concentration, it has been determined that an extracellular trehalose concentration of 360 mM resulted in intracellular trehalose levels ranging from 80 mM to 250mM, which proved sufficient for the successful cryopreservation

of cells. The utilization of pH-responsive PLP-ADA polymers for delivering trehalose holds great promise as an approach to enhance cell protection and bolster cellular viability under challenging conditions. The use of biopolymer-mediated trehalose uptake enables the transport of high-concentration trehalose into cells at relatively low osmotic pressures. This approach can be harnessed to enhance cell preservation during cryopreservation and other medications within the same delivery platform, showing the potential for synergistic protective effects and therapeutic advantages to be realized.

5.2 Characterization of PLP-ADA mediated intracellular trehalose loading

5.2.1 Intracellular delivery of trehalose to red blood cells

Trehalose, a glucose disaccharide, is highly concentrated in various organisms that withstand diverse environmental stresses. It is naturally produced by yeasts, plants, and some animals like tardigrades and nematodes, helping to maintain structural integrity of cells under extreme conditions like severe dehydration and cold (Chapman, 1994, Crowe and Crowe, 2000, Drennan et al., 1993, de-Araujo, 1996). In biotechnology, trehalose is widely used as an effective stabilizer for frozen or dry macromolecules (like antibodies and enzymes) (Nielsen, 1995, March and Clark, 2000), as well as for both artificial and natural membranes (Crowe et al., 1985, Crowe and Crowe, 1988, Hianik et al., 1996, Newman et al., 1993, Panek, 1995), and yeast cells (Diniz-Mendes et al., 1999). Its medical uses extend to the lyopreservation and cryopreservation of blood, sperm, tissues, and organs, aiding in their storage, transport, and use in surgical transplants (Aisen et al., 2000, Pellerin-Mendes et al., 1997, de Castro and Tunnacliffe, 2000, Guo et al., 2000, Storey et al., 1998, Woelders et al., 1997, Beattie et al., 1997). Herein, PLP-ADA mediated trehalose delivery into red blood cells was optimized by adjusting various parameters, including osmolality of washing buffer, polymer structure, polymer concentration, pH, and incubation time.

5.2.1.1 Effect of osmolality of washing buffer

Osmotic fragility indicates the likelihood of cell rupture based on the osmotic strength of the surrounding solution. Previous research has shown that intracellular trehalose can enhance the osmotic resilience of human erythrocytes in low osmolarity environments (Satpathy et al., 2004, de Castro and Tunnacliffe, 2000). However, most previous studies on osmotic fragility have mainly focused on the effects of moderate intracellular trehalose concentrations, ranging from 10-60 mM for red blood cells and approximately 80 mM for nucleated mammalian cells (Satpathy et al., 2004, de Castro and Tunnacliffe, 2000). In the context of washing buffers, trehalose may be included to protect cells during the washing process, which is often a necessary step in various laboratory protocols such as cell sorting, staining, or cryopreservation.

In order to deeper understand the impact of buffer osmolality on the protective effects of red blood cells, trehalose loading concentrations were fixed to establish a consistent osmolality inside of the cells between the groups (William et al., 2023). In the washing step following the loading of intracellular trehalose, cells were subjected to centrifugation and then resuspended in PBS. This was done to eliminate any residual free trehalose and polymer, while also restoring the pH to its normal physiological level. Since the cells had been loaded with intracellular trehalose during the incubation process, resulting in a higher intracellular osmolality, it was crucial to carefully consider the osmolality of the PBS used for resuspending the cell pellet. This was necessary to maintain the osmotic balance.

To evaluate the osmotic resilience of erythrocytes after trehalose was delivered by PLP-ADA60, cells were exposed to PBS with osmolarities of 306 mOsm, 660 mOsm, and 800 mOsm, as determined by osmometers. As shows in Figure 5-1, during the co-incubation with 360 mM trehalose and 0.5 mg mL^{-1} PLP-ADA60, the osmolality of the intracellular solution of RBCs could be adjusted to an isotonic level (approximately 660 mOsm) due to the uptake of trehalose and possible cell shrinkage. Consequently, any extracellular osmolality below 600 mOsm in the subsequent washing step would be considered hypotonic for the RBCs, potentially causing cell swelling and bursting. As the osmolality of the PBS increased to 660 mOsm, there was a significant rise in intracellular trehalose concentration, reaching $187 \pm 27 \text{ mM}$. This

increase was likely due to the fact that, at an osmolarity below 660 mOsm, the osmolality of the PBS was lower than that of the intracellular environment, resulting in cell bursting and subsequent trehalose leakage. However, when the osmolality of the PBS was further increased to 800 mOsm, there was a decrease in intracellular trehalose concentration compared to the 660 mOsm group. The reduction in intracellular trehalose levels could be attributed to the high concentration of the washing buffer, which induced cell shrinkage and facilitated trehalose leakage from the cell due to the kinetic energy of concentration gradient. This indicated that using PBS with an osmolality of 660 mOsm was recommended as the optimal choice for the washing step to prevent trehalose leakage.

Resulting from the use of biopolymers to facilitate trehalose loading, erythrocytes were shown to achieve intracellular trehalose concentrations of up to around 200 mM. When the cell volume is isotonic, the addition of 360 mM trehalose increases the intracellular osmolarity from the standard 306 mOsm to approximately 506 mOsm, nearly doubling the normal physiological levels. Similar to how cells contract to counteract osmotic imbalances in hypertonic external conditions during trehalose loading and freezing (Lynch et al., 2011, Meryman, 1968), cells expand to balance out the osmotic difference when trehalose-enriched cells are reintroduced into a buffer with physiological osmolarity.

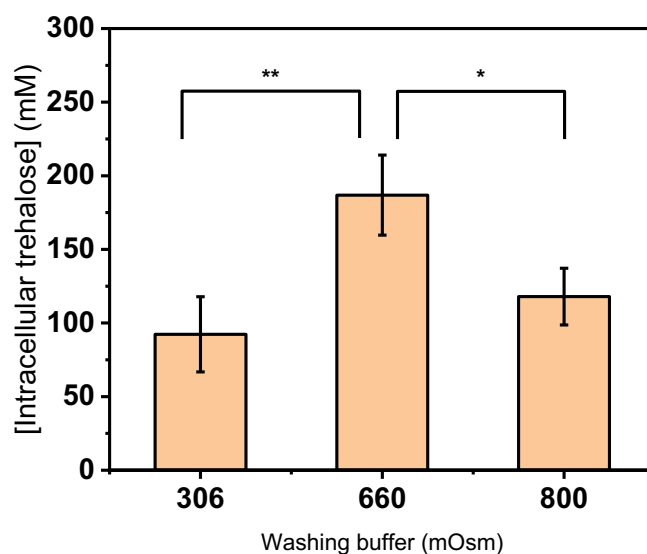


Figure 5-1. Effect of washing buffer osmolarity on intercellular trehalose concentration in sheep erythrocytes, as measured by the anthrone method. Cells were loaded with trehalose via co-incubation for 30 minutes with 360 mM extracellular trehalose and 0.5 mg mL⁻¹ PLP-ADA60 at 37°C and pH 7.0. Error bars represent standard deviations (n = 3). Data were analysed for statistical significance, *p<0.05, and **p<0.01, respectively.

5.2.1.2 Effect of pH using different PLP-ADA polymers

Modulating the pH of the surrounding environment allows for manipulation of the interaction between the cell membrane and pH-responsive polymer, which results in alterations in intracellular trehalose concentration. The initial intracellular trehalose content in RBCs, before exposure to polymer and trehalose solutions, was approximately 0.05 - 0.2 mM (Zhang et al., 2021). Subsequent treatment with PLP-ADA notably enhanced the loading of trehalose into RBCs. Figure 5-2 illustrates the extent of intracellular trehalose loading induced by the three different grafted polymers. PLP-ADA exhibited a pronounced effect in promoting intracellular trehalose. The intracellular trehalose loading content was significantly higher compared to cases without polymers (around 5 mM). In comparison between the different PLP-ADA polymers, it was demonstrated in Chapter 4 that the content of hydrophobic groups conferred membrane-disruptive properties, which is in agreement with the findings reported by other researchers (Liu et al., 2019, Chen et al., 2009c, Khormaei et al., 2010). Specifically, the PLP-

ADA30-induced loading of intracellular trehalose decreased as the extracellular pH increased from 6.0 to 7.4, showing intracellular trehalose concentrations of 189 ± 24 mM, 164 ± 18 mM, and 127 ± 12 mM at pH levels of 6.5, 7.0, and 7.4, respectively. As the pH increased, the PLP-ADA30 became less effective due to its low degree of grafting with hydrophobic ADA side chains, resulting in weaker interactions of the polymer with the RBC membrane (Figure 4-1). This is because as the pH drops, the carboxyl groups on the pseudopeptide gets protonated, leading to stronger hydrophobic association, which causes the polymer conformation to change from expanded coils to more condensed globules (Figure 3-8), enhancing membrane interaction significantly.

Furthermore, this observation was not consistent with PLP-ADA45 and PLP-ADA60. PLP-ADA45 displayed a more pronounced intracellular trehalose concentration, reaching 140 ± 29 mM, 193 ± 11 mM, and 209 ± 29 mM at pH levels of 6.5, 7.0, and 7.4, respectively. Attaining an elevated intracellular trehalose concentration within a physiological pH range is a desirable objective. Notably, PLP-ADA45 and PLP-ADA60 exhibit the highest intracellular trehalose concentration at pH 7.4, with a slight reduction observed for these two polymers at pH 7.0. The amphipathic polymers containing hydrophobic fatty acid side chains exhibit notable membrane-disrupting and pore-forming effects on cell membranes (Liu et al., 2022). Hydrophobic side chains disrupted the arrangement of the lipid bilayer, increasing the permeability of RBC membrane, and creating a pathway for the loading of trehalose into the cells. The higher degree of grafting indicated their possession of stronger membrane-disruptive activity, which could potentially enhance the loading of trehalose into RBCs (Moreau et al., 2002, Fischer, 2003, Li et al., 2009). As the grafting degree of fatty acid side chains further increases, this effect becomes less pronounced. This might be due to that the interplay between the polymer and the cell membrane influences the trehalose loading process during incubation, potentially resulting in membrane rupture and subsequent trehalose leakage as Figure A-4 in Appendix D shows the haemolysis data. As a result, elevating the degree of ADA grating from 45 mol% to 60 mol% led to the comparable intracellular trehalose concentrations. However, under optimized conditions, the delivery of trehalose using PLP-NDA18 in an acidic environment successfully loaded 177.9 ± 8.6 mM (Chen et al., 2020). Additionally, acidic conditions may affect the stability and activity of cells (Zhang et al., 2011b). However, PLP-ADA, which can deliver trehalose into cells at physiological pH, offers a significant advantage, as it allows for intracellular delivery without the need for acidic conditions. This approach

effectively tackled the challenge of intracellular loading of trehalose, which cannot permeate the membrane into RBCs that lack endocytic pathways, without causing significant cellular damage.

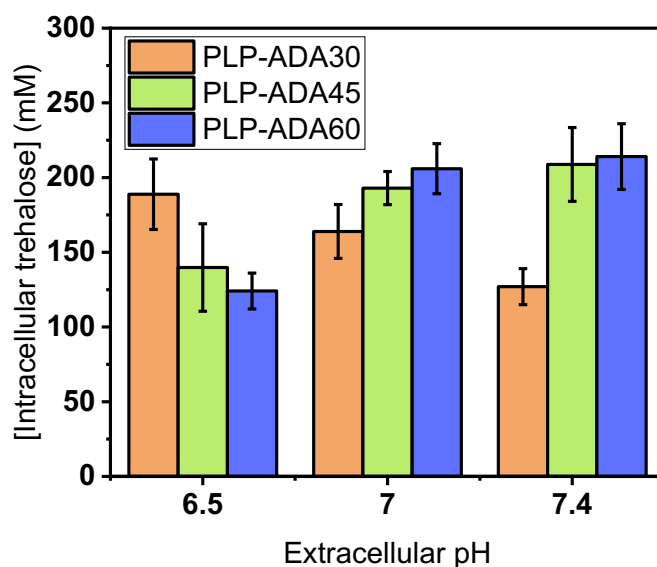


Figure 5-2. Effect of extracellular pH on intracellular trehalose concentration in sheep erythrocytes, as measured by the anthrone method. Cells were loaded with trehalose via co-incubation for 30 minutes with 360 mM extracellular trehalose and 0.2 mg mL⁻¹ indicated PLP-ADA polymer at 37°C and different pHs. Error bars represent standard deviations (n = 3).

5.2.1.3 Effect of polymer concentration and structure

As depicted in Figure 5-3, there is a clear correlation between the polymer concentration and the intracellular trehalose loading for all the three PLP-ADA polymers tested. The intracellular trehalose concentration demonstrated an upward trend as the concentration of PLP-ADA polymers increased within the range of 0.2 – 0.8 mg mL⁻¹ at pH 7.0, as depicted in Figure 5-3. At pH 7.0, after a 30-minute incubation at 0.2 mg mL⁻¹, a desirable intracellular trehalose concentration reached, with 168 ± 5 mM, 212 ± 18 mM, and 231 ± 18 mM observed for PLP-ADA30, PLP-ADA45, and PLP-ADA60, respectively. However, when the concentration was raised to 0.5 mg mL⁻¹, The intracellular trehalose concentration rose by approximately 1.2-fold

for both PLP-ADA30 and PLP-ADA45, and by about 1.3-fold for PLP-ADA60. This substantial increase could be attributed to the enhanced sensitivity of erythrocyte membrane lipids to polymer concentration variations. Specifically, at the pH 7.0, the cell membrane becomes more fluid and PLP-ADA polymers at a higher concentration exhibit the stronger driving force for migration to the membrane. These likely increased driving force for migration to the membrane, thus enhance the interaction between the polymer and the lipids and, leading to improved intracellular delivery (Chawla and Amiji, 2003, William et al., 2023, Wong et al., 2009) (William et al., 2023, Wong et al., 2009, Chawla and Amiji, 2003) Moreover, the faster molecular diffusion at a high polymer concentration further boosts intracellular loading. Nevertheless, a further concentration increments from 0.5 mg mL⁻¹ to 0.8 mg mL⁻¹ reach a plateau leading to no significant difference ($P > 0.05$) in intracellular trehalose concentration. It is worth noting that the maximum permeability to trehalose was achieved with PLP-ADA60 at pH 7.0, as well as pH 7.4 (Figure A-5 in Appendix E). This polymer-concentration-dependent trehalose loading profile aligns with the findings of polymer-concentration-development cell membrane activity displayed in Figure 4-2 above, indicating that increasing polymer concentration can increase the driving force for membrane binding, leading to the improved membrane permeabilization (Chen et al., 2009c, Chen et al., 2009b, Thomas et al., 1994).

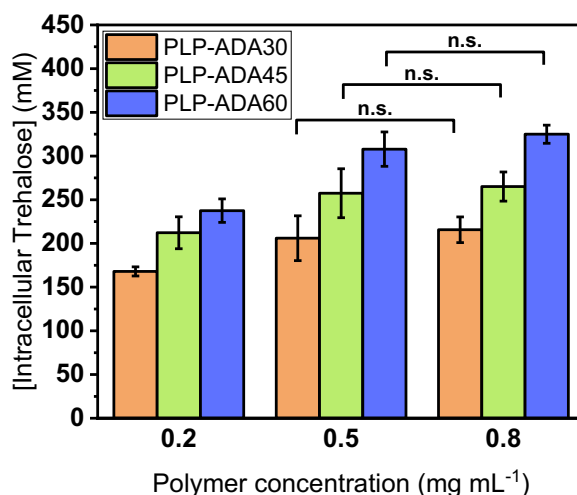


Figure 5-3. Effect of polymer concentration on intercellular trehalose concentration in sheep erythrocytes, as measured by the anthrone method. Cells were loaded with trehalose via co-incubation for 30 minutes with 360 mM extracellular trehalose and different concentrations of the indicated PLP-ADA polymer at 37°C and pH 7.0. Error bars represent standard deviations ($n = 3$). Data were analysed for statistical significance, n.s.(not significant) : $p > 0.05$.

5.2.1.4 Effect of incubation time

Figure 5-4 and Table 5-1 illustrate the swift and effective intracellular loading of membrane-impermeable trehalose, facilitated by using 0.5 mg mL^{-1} PLP-ADA at a temperature of 37°C and pH 7.4. Incubation of erythrocytes with 360 mM trehalose alone for 60 minutes resulted in a minimal delivery efficiency (defined as a percentage of intracellular trehalose concentration relative to the total trehalose loading concentration) of only $0.3 \pm 0.0\%$. In comparison, a higher level of hydrophobic ADA grafts led to the more rapid and effective trehalose loading due to the increased membrane activity of the polymer. The use of PLP-ADA60 enabled the most rapid loading of $214 \pm 36 \text{ mM}$ trehalose (equivalent to a delivery efficiency of $59.4 \pm 0.1\%$) into the erythrocytes within just 15 minutes. As the incubation duration increased, the intracellular trehalose loading showed an uptrend. By extending the incubation from 30 to 60 minutes, PLP-ADA60 facilitated an increase in intracellular trehalose concentration from $69.7 \pm 0.1\%$ to $88.3 \pm 0.0\%$. Notably, this latter concentration was 294 times higher than that of the trehalose only group in the absence of PLP-ADA60.

In previous studies, PP50 (i.e., PLP grafted with 50 mol% L-phenylalanine) was used to improve intracellular trehalose loading for effective cell cryopreservation (Lynch et al., 2010, Lynch et al., 2011, Lynch and Slater, 2011). But this PP50-based method was significantly less efficient and slower, achieving an intracellular trehalose concentration of approximately 10 mM (equivalent to a delivery efficiency of about 2.8%) after a 1.5-hour incubation and reaching intracellular trehalose threshold concentrations (100–200 mM) required for bioprotection of mammalian cells in approximately 9 hours (Lynch et al., 2010). As for the PLP-NDA18-based approach, which achieved the intracellular trehalose concentrations at $178 \pm 9 \text{ mM}$ (delivery efficiency, $49.4 \pm 2.4\%$) and $300 \pm 23 \text{ mM}$ (delivery efficiency, $83.4 \pm 6.4\%$) after 15 minutes and 1 hour of incubation, respectively. However, the loading conditions of pH 6.1 and 0.8 mg mL^{-1} PLP-NDA18 are unfavourable for the cells. By contrast, as depicted in Figure 5-4 and Table 5-1, Under the milder loading conditions that are more favorable for cells, PLP-ADA60 can achieve the similar intracellular trehalose loading kinetics and efficiency with PLP-NDA18. Co-incubation of erythrocytes with 0.5 mg mL^{-1} PLP-ADA60 and 360 mM trehalose at pH 7.4 for only 15 minutes led to the intracellular trehalose concentration of $214 \pm 36 \text{ mM}$ (delivery efficiency, $59.4 \pm 0.1\%$), reaching the required level of intracellular trehalose concentrations

for bioprotection of mammalian cells during cell cryopreservation. This observation underscores the preference for an extracellular pH that is more favourable for the cells.

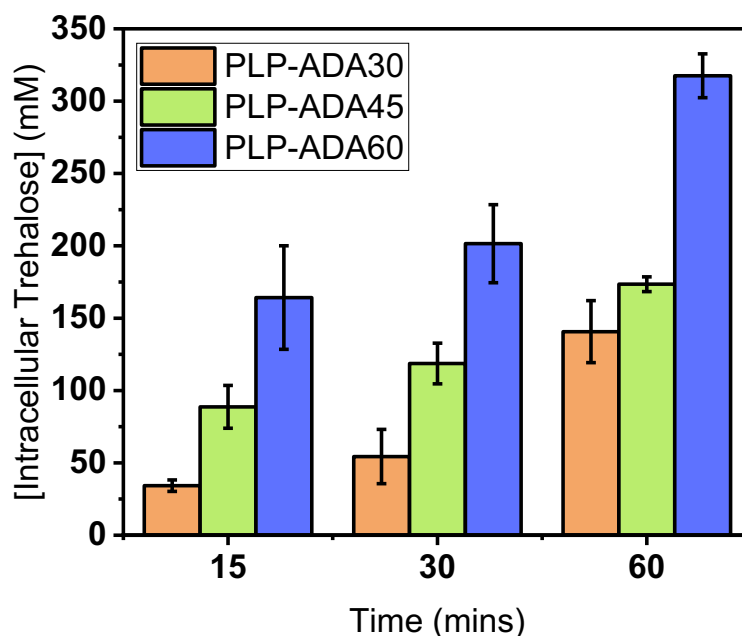


Figure 5-4. Time-dependent intracellular trehalose concentrations mediated by PLP-ADA, as measured by the anthrone method. Erythrocytes were treated with the PBS containing 360 mM trehalose presence of 0.5 mg mL^{-1} PLP-ADA for specific time periods at 37°C and pH 7.4. Error bars represent standard deviations ($n = 3$).

Table 5-1. Comparison of delivery efficiencies. Erythrocytes were treated with the PBS containing 360 mM trehalose in the absence or presence of 0.5 mg mL⁻¹ PLP-ADA for specific time periods at 37 °C and pH 7.4. Mean ± S.D. (n = 3).

Formulation	Time (min)	Delivery efficiency (%)
Trehalose only	60	0.3 ± 0.0
Trehalose + PLP-ADA30	15	37.2 ± 0.0
	30	42.8 ± 0.1
	60	66.9 ± 5.6
Trehalose + PLP-ADA45	15	52.5 ± 0.0
	30	60.8 ± 0.0
	60	75.8 ± 0.0
Trehalose + PLP-ADA60	15	59.4 ± 0.1
	30	69.7 ± 0.1
	60	88.3 ± 0.0

5.2.2 Intracellular trehalose delivery to Jurkat cells

5.2.2.1 Intracellular delivery of model payload

After effectively delivering trehalose into red blood cells under the desired conditions, mammalian cells were used to validate the applicability of this delivery system across various cell lines. In this investigation, Jurkat cells, an immortalized line of human T lymphocyte cells that was originally obtained from the peripheral blood of a boy with T cell leukemia, were used as a model system for researching cryopreservation for cell-based therapies (Meneghel et al., 2019). The work in Chapter 4 has indicated that PLP-ADA 60 was not toxic toward mammalian cell lines with the concentration range tested. First, 150 kDa FITC-Dextran was used as a model payload in this study to quantify the PLP-ADA60 mediated delivery efficiency into Jurkat cells. Intracellular delivery to immune cells to enable DMSO-free cryopreservation (Section 5.3.2) is crucial for immune cell therapies, yet it often poses challenges in terms of efficiency.

As shown in Figures 5-5 and 5-6, the MFI in Jurkat cells was considerably enhanced after co-treatment with PLP-ADA60 and 150 kDa FITC-Dextran, as compared to those treated with 150 kDa FITC-Dextran alone. Contrasting a mildly acidic pH with a physiological pH, an acidic extracellular environment resulted in elevated intracellular FITC-Dextran concentrations. The polymer interacted with cell membranes in a pH-dependent manner, which could lead to the controllable cytoplasmic delivery of the payload through the direct membrane penetration and/or endosomal escape (Seki and Tirrell, 1984, Yang and Hinner, 2015). These results are in good agreement with the PLP-ADA mediated pH-responsive cell membrane activity using haemolysis as a model (Section 4.2) and intracellular delivery to different lines of nucleated mammalian cells (Section 4.4).

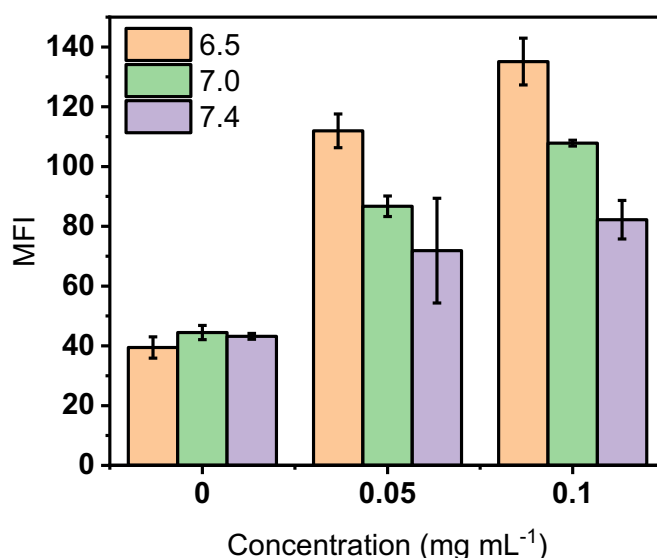


Figure 5-5. Flow cytometry analysis of Jurkat cells treated with 150 kDa FITC dextran (10 μ M) in the absence or presence of PLP-ADA60 for 30 minutes at different pHs. Error bars represent standard deviations ($n = 3$).

Specifically, at a PLP-ADA60 concentration of 0.1 mg mL⁻¹, the intracellular delivery of FITC-Dextran was greater at an extracellular pH of 6.5 as compared to an extracellular pH of 7.4. This is because at a lower pH PLP-ADA displays the stronger hydrophobic association (Figure 3-8), leading to more pronounced interactions with the membrane (Figure 4-1) (Chen et al., 2009c, Thomas et al., 1994). At a lower PLP-ADA60 concentration of 0.05 mg mL⁻¹, a slightly lower MFI was detected in Jurkat cells. The results suggest that parameters like extracellular

pH and polymer concentration could be controlled to increase the intracellular loading of polysaccharides to Jurkat cells.

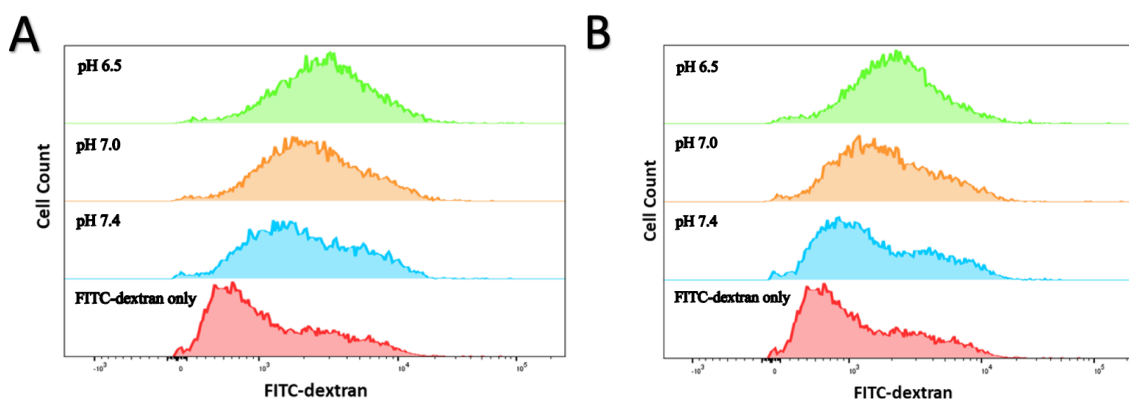


Figure 5-6. Flow cytometry analysis of Jurkat cells treated with 150 kDa FITC dextran (10 μ M) in the absence or presence of PLP-ADA60 at (A) 0.1 mg mL⁻¹ and (B) 0.05 mg mL⁻¹ for 30 minutes at different pHs.

5.2.2.2 Intracellular delivery of trehalose

Trehalose appears to protect mammalian cells against dehydration or freezing by stabilizing cellular proteins and membranes (de-Araujo, 1996). Additionally, it can prevent ice formation within cells by creating stable glass-like structures (Oliver et al., 1998). While extracellular trehalose can reduce cryodamage and lyodamage, optimal protection is achieved when the disaccharide is present on both sides of the cell membrane (Diniz-Mendes et al., 1999, Pellerin-Mendes et al., 1997, Eleutheria et al., 1993, Paiva and Panek, 1996). However, no mammalian species have been found to synthesize or actively gather trehalose, nor do they display the anhydrobiosis or cryotolerance observed in other species that produce this disaccharide (Guo et al., 2000). It has been reported that introducing 0.1-0.2 M of trehalose inside mammalian cells significantly enhances their survival during cryopreservation (Eroglu et al., 2000) compared to traditional protocols that use 1.0-2.0 M of penetrating cryoprotective agents (CPAs) such as DMSO, glycerol, and ethylene glycol (Mazur, 1984).

5.2.2.2.1 Effect of polymer concentration and structure

Cell entry involve different pathways such as endocytosis and direct membrane penetration (Sahay et al., 2010, Ray et al., 2024, Chen et al., 2022). A significant limitation of the trehalose loading method, which simply relies on the endocytosis-based cell entry, is that the endosomal escape often leads to the more time-consuming loading process. Consequently, alternative entry pathways that do not rely on endocytosis, such as polymer-mediated direct membrane penetration, have been explored to not only enhance the intracellular delivery efficiency but also shorten the time required for this process (Kaljot et al., 1988, Goda et al., 2010). In subsequent experiments, the trehalose assay kit was used to assess the intracellular trehalose concentrations. As shown in Figure 5-7, the intracellular trehalose concentration in Jurkat cells was influenced by the concentration and structure of PLP-ADA polymers. Treatment with a low polymer concentration of 0.3 mg mL^{-1} resulted in comparable, low intracellular trehalose concentrations of below 60 mM induced by PLP-ADA30, PLP-ADA45 and PLP-ADA60. There was a significant increase in the intracellular trehalose concentration for all the three polymer groups when the polymer concentration increased from 0.3 mg mL^{-1} to 0.5 mg mL^{-1} . Typically, a high polymer/cell ratio could result in the increased driving force for migration of polymer molecules to the cell membrane and the enhanced membrane interaction, which leads to the sufficient intracellular delivery (Chen et al., 2017). PLP-ADA60 enabled the intracellular trehalose concentration at $128 \pm 3 \text{ mM}$ reaching the threshold concentrations (100–200 mM) required for bioprotection of mammalian cells (Lynch et al., 2010). This level of intracellular trehalose concentration was comparable with that facilitated by PLP-ADA45, but significantly higher than the PLP-ADA30 group. This further confirm that the PLP-ADA polymers with the higher content of hydrophobic ADA grafts can exhibit the increased membrane interaction and intercellular delivery efficiency. For PLP-NDA18, a concentration of 0.8 mg mL^{-1} is needed to achieve the desired trehalose concentration, but increasing the polymer concentration also leads to greater hemolysis. In contrast, PLP-ADA achieves comparable trehalose loading with just 0.5 mg mL^{-1} , avoiding the adverse effects associated with higher concentrations.

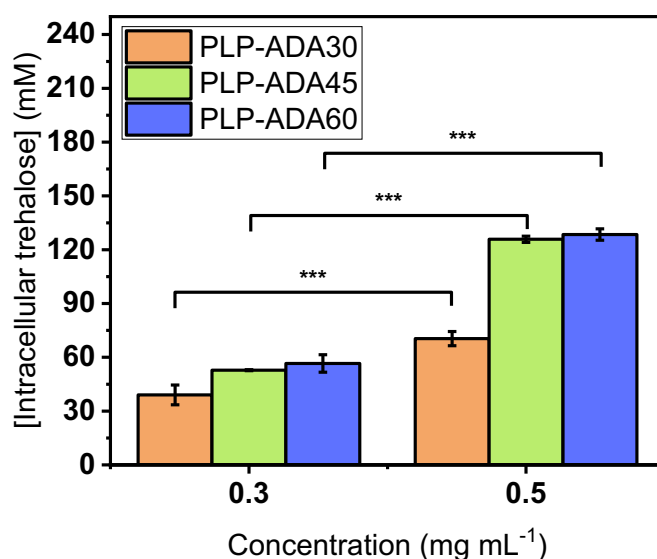


Figure 5-7. Effect of pH on intercellular trehalose concentration in Jurkat cells, as measured by the trehalose assay kit. Cells were loaded with trehalose via co-incubation for 30 minutes with 360 mM extracellular trehalose and different concentrations of the indicated PLP-ADA polymers at 37°C and pH 7.0. Error bars represent standard deviations (n=3). Data were analysed for statistical significance. ***P< 0.001.

5.2.2.2.2 Effect of pH using different PLP-ADA polymers

The pH-dependent trehalose loading into Jurkat cells, facilitated by PLP-ADA30, PLP-ADA45, and PLP-ADA60 at pH ranging from 6.5 to 7.4, was investigated to further understand the polymer-mediated membrane permeability to trehalose. As shown in Figure 5-8, For the PLP-ADA30 group, the intracellular trehalose concentration gradually increased from 30 ± 6 mM at pH 7.4 to 82 ± 10 mM at pH 6.5. The is because the anionic amphiphilic polymer displays the expanded hydrophilic coil structure at pH 7.4, leading to the reduced membrane-permeabilizing activity due to the electrostatic repulsion between the negatively charged polymer and the negatively charged cell membrane. Upon acidification to pH 6.5, the protonation of carboxylate groups resulted in a conformational change to the compact hydrophobic globular structure, leading to enhanced membrane interaction. With increasing the degree of grafting with the ADA side chains, PLP-ADA45 displayed stronger membrane interactions at higher pHs due to its enhanced hydrophobic association (Chen et al., 2009c),

thus loading substantially more trehalose into Jurkat cells at pH 7.0 and pH 7.4, with the maximum intracellular trehalose concentration of 130 ± 4 at pH 7.0. For PLP-ADA60 with a further increased degree of ADA grafting, the maximum intracellular trehalose concentration of 134 ± 1 mM, at a similar level with PLP-ADA45 but observed at a higher pH of 7.4, was within the intracellular trehalose concentration range required for bioprotection during cryopreservation. A significant reduction in the PLP-ADA60 induced intracellular trehalose concentration at pH 6.5 was attributed to the aggregation of the polymer with the most hydrophobic ADA grafts upon protonation of carboxylate groups. These results are in good agreement with the pH-responsive haemolysis profile of the PLP-ADA polymers as shown in Figure 4-1.

Previous studies have indicated that neutral/physiological pH environments are advantageous for preserving Jurkat ATP concentrations *ex vivo*. Maintaining a neutral/physiological pH level can sustain an intracellular ATP level adequate for apoptotic mechanisms, whereas an acidic extracellular pH can induce ATP depletion, directly leading to necrosis (Lan et al., 2007, Bohloli et al., 2016, Souza et al., 2023, Kruse et al., 2017, Maurer, 2005). The findings provide a crucial insight for optimizing the intracellular trehalose delivery in the context of cell cryopreservation and underline the significance of extracellular pH environment for preserving cell viability.

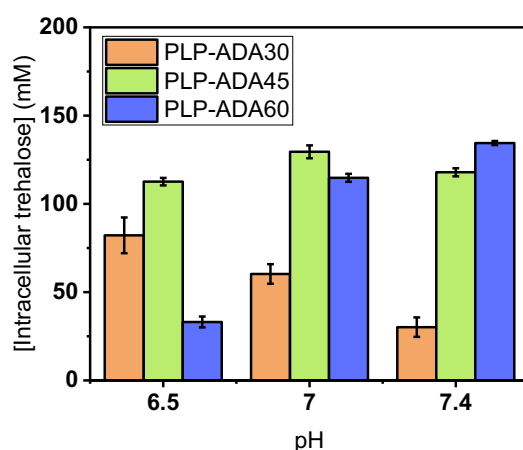


Figure 5-8. Effect of pH on intercellular trehalose concentration in Jurkat cells, as measured by the trehalose assay kit. Cells were loaded with trehalose via co-incubation for 30 minutes with 360 mM extracellular trehalose and 0.5 mg mL^{-1} of the indicated PLP-ADA polymer at 37°C and different pHs. Error bars represent standard deviations ($n=3$).

5.2.2.2.3 Effect of incubation time

Figures 5-9 shows how incubation time impacts PLP-ADA60 mediated trehalose loading into Jurkat cells at different pHs. At each pH tested (6.5, 7.0 and 7.4), there was no significant difference in intracellular trehalose concentration when the incubation period extended from 30 to 60 minutes. This might be attributed to the Jurkat cell membrane lipid phase behavior in the presence of polymer interaction being not time-dependant after 30 minutes (Meneghel et al., 2019, Bochicchio et al., 2017). In this context, there could be dual effects on the time dependence of the Jurkat cell membrane permeability. Intracellular trehalose loading occurs not only due to an osmotic driving force that enhances diffusion rates as the membrane becomes progressively more fluid over time but also because of the increased membrane permeability facilitated by the interactions between biopolymers and the lipid bilayer (Dougherty et al., 2019). The amplification of these interactions occurs as the membrane fluidity rises, particularly as it approaches physiological pH. Acidification could result in aggregation of PLP-AD60 due to protonation of its carboxylate groups, compromising this amplification effect. Additionally, the polymer-induced membrane destabilization might result in the loss of structural integrity of the cell during extended incubation, leading to the leakage of trehalose (Choi et al., 2015). Therefore, a 30-minute period proved sufficient to attain the desired intracellular trehalose concentration required for bioprotection during cell cryopreservation.

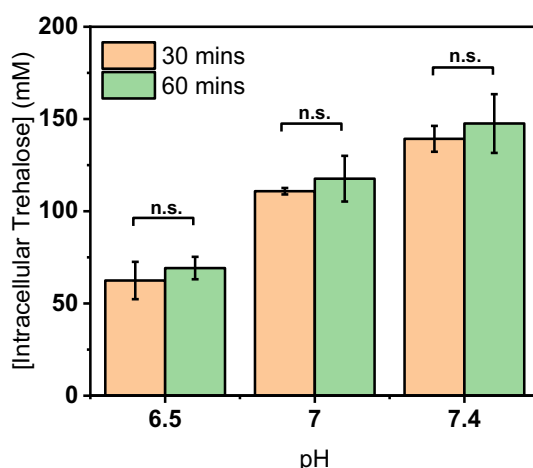


Figure 5-9. Effect of incubation time on intercellular trehalose concentration in Jurkat cells, as measured by the trehalose assay kit. Cells were loaded with trehalose via co-incubation for 30 or 60 minutes with 360 mM extracellular trehalose and 0.5 mg mL⁻¹ PLP-ADA60 at 37°C and different pHs. Error bars represent standard deviations (n=3).

5.3 Cell cryopreservation

5.3.1 Cryopreservation of trehalose-loaded erythrocytes

5.3.1.1 Effect of extracellular trehalose concentration

Most of the reported studies on trehalose for cell cryopreservation have focused on the use of extracellular trehalose only (Pellerin-Mendes et al., 1997, Erdag et al., 2002, Eroglu et al., 2000). In this thesis, the effect of extracellular trehalose concentration in the presence of the pH-responsive PLP-ADA60 polymer on cell cryosurvival rates was investigated by comparing the total live cell counts in each group before and after cryopreservation following the freezing and thawing process. As shown in Figure 5-10, the cryosurvival rate was found to be notably influenced by the concentration of extracellular trehalose. Without trehalose, only a minimal fraction of RBCs ($5.48 \pm 0.26\%$) survived the freeze-thaw cycle. As reported in Figure 5-4 above, the 30 minutes of co-incubation of sheep RBCs with 0.5 mg mL^{-1} PLP-ADA60 and 360 mM extracellular trehalose at pH 7.4 resulted in an intracellular trehalose concentration at $251 \pm 27 \text{ mM}$. The maximal survival rate was observed when the extracellular trehalose concentration ranging from 300 to 400 mM was used in combination with 0.5 mg mL^{-1} PLP-ADA60, suggesting that the co-existence trehalose both inside and outside the cell significantly enhanced the cell recovery after cryopreservation due to its vitrification effects. Specifically, the 400 mM extracellular trehalose group led to the cryosurvival rate of $84.3 \pm 7.6\%$. The decrease in the extracellular trehalose concentration to 200 mM caused a significantly reduced cryosurvival rate to $71.2 \pm 8.0\%$. With increasing the extracellular trehalose concentrations above 400 mM, the cell cryosurvival rate was gradually reduced, reaching $84.3 \pm 7.6\%$ for the 600 mM extracellular trehalose group, which might stem from solution-based factors such as osmotic imbalances and cellular dehydration (Schliess and Häussinger, 2002, Lang, 2011).

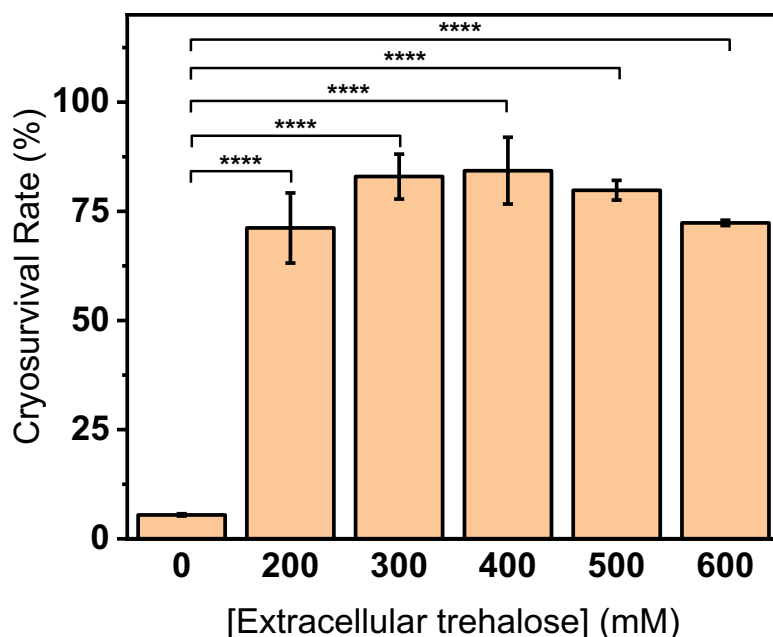


Figure 5-10. Effect of extracellular trehalose concentration on RBC cryosurvival rate. Cells were loaded with trehalose via co-incubation for 30 minutes with different concentrations of extracellular trehalose and 0.5 mg mL^{-1} PLP-ADA60 at 37°C and pH 7.4. These samples were stored in a liquid nitrogen tank for 2 hours before thawing in a 37°C water bath for 5 minutes. Error bars represent standard deviations ($n=3$). Data were analysed for statistical significance by ordinary one-way ANOVA. **** $P < 0.0001$.

5.3.1.2 Effect of intracellular trehalose

RBCs were then co-incubated with 360 mM trehalose and different concentrations of PLP-ADA60 at different pHs to achieve different intracellular trehalose concentrations. Figure 5-11 shows the effects of intracellular concentration on cryosurvival rates. For negative control with no incubation step for intracellular trehalose loading, i.e. 360 mM extracellular trehalose only for cell cryopreservation, the RBC cryosurvival rate was $48.5 \pm 2.3\%$. By comparison, in the presence of the same concentration of extracellular trehalose, as the intracellular trehalose concentration increased to $252.5 \pm 10.4 \text{ mM}$ (loaded into RBCs using 0.5 mg mL^{-1} PLP-ADA60 at pH 7.4, the cryosurvival rate of RBCs exhibited a marked improvement to $82.6 \pm 1.3\%$. However, further elevating the intracellular trehalose concentration to $307.9 \pm 19.7 \text{ mM}$

(loaded into RBCs using 0.5 mg mL^{-1} PLP-ADA60 at pH 7.0) did not yield any additional significant enhancement in the cryosurvival rate.

The findings underscore the importance of maintaining appropriate intracellular trehalose concentrations. While adequate intracellular trehalose is crucial for optimal cryopreservation, excessive amounts do not contribute further to improving cryosurvival rates. This is favorable for avoiding potential adverse effects associated with overly high intracellular trehalose concentrations. Furthermore, the results in Figures 5-10 and 5-11 highlight the importance of having both extracellular and intracellular trehalose for favorable bioprotection, including the inhibition ice crystal formation both inside and outside the cell. This dual emphasis on both intracellular and extracellular trehalose concentrations provides a valuable insight into refining cryopreservation strategies and offer a better understanding of the factors influencing RBC cryosurvival rates.

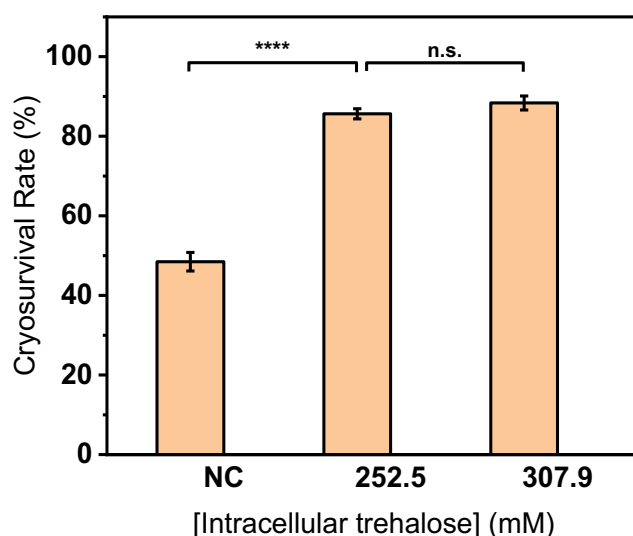


Figure 5-11. Effect of intracellular trehalose concentration on RBC cryosurvival rate. Cells were loaded with trehalose via co-incubation at 37°C for 30 minutes with 360 mM extracellular trehalose, together with 0.5 mg mL⁻¹ PLP-ADA60 at pH 7.4 or 0.5 mg mL⁻¹ PLP-ADA60 at pH 7.0. Following incubation, RBCs underwent washing with 660 mOsm PBS at pH 7.4 and were then resuspended in 360 mM extracellular trehalose for cryopreservation. For negative control (NC), cells were added in 360 mM extracellular trehalose alone without incubation, followed by immediate washing with 660 mOsm PBS at pH 7.4 and resuspension in 360 mM extracellular trehalose. The cell density during freezing was 3×10⁹ mL⁻¹. These samples were stored in a liquid nitrogen tank for 2 hours before thawing in a 37 °C water bath for 5 minutes. Error bars represent standard deviations (n=3). Data were analysed for statistical significance by ordinary one-way ANOVA. ****P < 0.0001, n.s.: P > 0.05.

5.3.1.3 Effect of storage duration

To further investigate the impact of cryostorage time on cell cryosurvival rates, the erythrocytes, which were pre-loaded with 252.5 ± 10.4 mM intracellular trehalose and resuspended in 360 mM extracellular trehalose, were frozen in liquid nitrogen followed by thawing in a 37 °C water bath for 5 minutes. The results in Figure 5-12 revealed a robust cryostorage protocol. After 12 hour of cryostorage the cryosurvival rate was measured to be 86.8 ± 3.4%, and extending the cryostorage time to 120 hours caused no significant change. This result signifies the efficacy

of trehalose as an effective cryoprotective agent. The stable cryosurvival rates achieved, particularly with the rapid PLP-ADA60-mediated intracellular trehalose delivery, suggest the potential for producing reliable and effective cell therapy products (Meneghel et al., 2020). These findings hold promise for the development of a robust cryopreservation method for cell therapy applications.

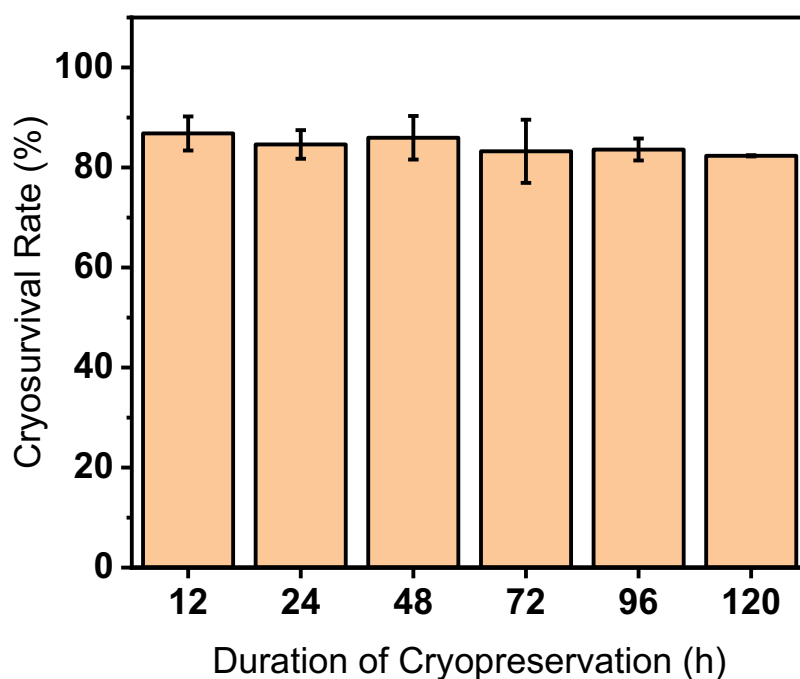


Figure 5-12. Time-dependent cryosurvival rates of RBCs. RBCs were pre-loaded with 252.5 ± 10.4 mM intracellular trehalose and re-suspended in 360 mM extracellular trehalose in PBS pH 7.4. The samples were kept in the liquid nitrogen tank for one day before undergoing thawing in a 37°C water bath for a duration of 5 minutes. Error bars represent standard deviations (n=3).

5.3.2 Cryopreservation of trehalose-loaded Jurkat Cells

5.3.2.1 Effect of intracellular trehalose

Figure 5-13 illustrates the impact of intracellular trehalose on the cryosurvival rate of Jurkat cells. For negative control with no incubation step for intracellular trehalose loading, i.e. 360 mM extracellular trehalose only for cell cryopreservation, the cryosurvival rate of Jurkat cells was $55.5 \pm 6.5\%$. With the extracellular trehalose concentration fixed at 360 mM, the co-existence of 114.8 ± 2.3 mM intracellular trehalose elevated the cryosurvival rate to $81.5 \pm 9.7\%$. This further confirms the crucial role of intracellular trehalose in enhancing the survival of cells during cryopreservation. However, there was no significant change in the cryosurvival rate upon a further increase in intracellular trehalose concentration to 134.5 ± 1.2 mM. This was in good agreement with the RBC cryosurvival results shown in Figures 5-10 and 5-11, further consolidating that the presence of intracellular trehalose is essential for enhancing the cryosurvival rate of cells during cryopreservation, while an excess of intracellular trehalose is deemed unnecessary (Eroglu et al., 2000). Therefore, maintaining an optimal concentration of intracellular trehalose is critical for achieving favorable cell cryopreservation outcomes, reinforcing the importance of a balanced approach in the trehalose loading process.

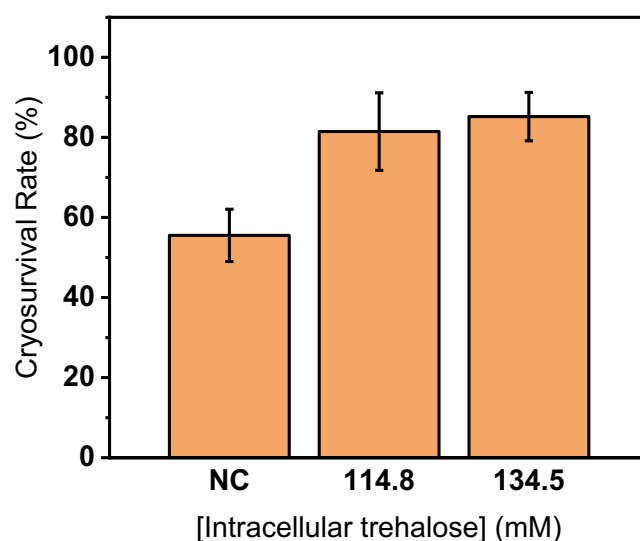


Figure 5-13. Effect of intracellular trehalose concentration on cryosurvival rate of RBCs. RBCs were pre-loaded with intracellular trehalose using 0.5 mg mL^{-1} PLP-ADA60 at 37°C and then re-suspended in 360 mM extracellular trehalose for cryopreservation. For negative control (NC), cells were added in the same concentration of extracellular trehalose alone without incubation, followed by immediate washing and resuspension in 360 mM extracellular trehalose. The cell density during freezing was $3 \times 10^9 \text{ mL}^{-1}$. Samples were stored in the liquid nitrogen tank for one day before being thawed in a 37°C water bath for 5 min. Error bars represent standard deviations ($n=3$).

5.3.2.2 Effect of recovery time

Several studies have provided insights into the potential benefits of the post-thaw resting process in aiding the recovery of functions impacted during cryopreservation (Weiner et al., 2015, Cottle et al., 2022). Figure 5-14 illustrates the cryosurvival rates of Jurkat cells under varying post-thaw recovery periods in a humidified incubator with $5\% \text{ CO}_2$ at 37°C for up to 1 hour. The extension of the recovery time from 0 to 60 minutes resulted in an increase in cryosurvival rate of Jurkat cells from $82.9 \pm 2.4\%$ to $91.2 \pm 2.7\%$. This indicates that the post-thaw resting step has a positive impact on cell recovery, contributing to enhanced viability. This notable improvement might be attributed to the uniform distribution of temperature in the incubator, where samples are surrounded by 37°C air, creating an environment with the

appropriate humidity and CO₂ levels favorable to cell survival (Talebipour et al., 2024, Wang et al., 2017b).

The finding suggests that the post-thaw resting step plays a role in promoting the recovery of cells following freeze-thaw processes. This knowledge contributes to a valuable insight into the development of effective protocols in cell cryopreservation, ensuring the preservation of cellular functions and viability, ultimately enhancing the success of cell-based therapies.

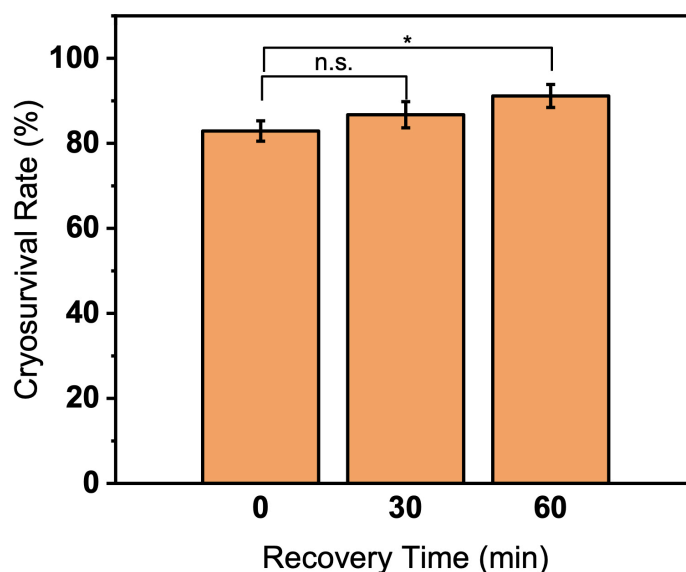


Figure 5-14. Effect of recovery time in a 37 °C water bath on cryosurvival rate of Jurkat cells. The intracellular and extracellular trehalose concentrations were 134.5 ± 1.2 mM and 360 mM, respectively. The cell samples were frozen in a -80 °C freezer for 2 h and then immediately immersed in the liquid nitrogen tank for one-day storage. Cell samples were then thawed in a 37 °C water bath for 3 min. After thawing, without dilution, the cell samples were left in a humidified incubator with 5% CO₂ at 37 °C for recovery. After that, cells were stained with trypan blue, and the viability was then measured by the automatic cell counter (Corning, UK). Error bars represent standard deviations (n=3). Data were analysed for statistical significance by ordinary one-way ANOVA. *P < 0.05, n.s.: P > 0.05

5.4 Conclusions

This chapter provides an exploration of the intracellular delivery of the membrane-impermeable bioprotectant trehalose, facilitated by the comb-like PLP-ADA pseudopeptidic polymers, and its application for cell cryopreservation. The study optimized trehalose delivery into different cells, such as unnucleated RBCs and nucleated Jurkat cells, by adjusting various parameters including the osmolality of the washing buffer, polymer structure, polymer concentration, pH, and incubation time. Notably, variations in pH played an important role in determining the optimal conditions for intracellular trehalose concentration, with different degree of grafting impacting hydrophobic associations. RBCs were loaded with internal trehalose concentrations using the biopolymer PLP-ADA while maintaining an external trehalose concentration of 360 mM. Across all tested freezing and thawing rates, this method consistently enhanced cell cryosurvival compared to cells solely surrounded by external trehalose. Under ideal conditions, the infused cells achieved a cryosurvival rate of $82.6 \pm 1.3\%$, marking a $34.2 \pm 3.6\%$ improvement over non-extracellular trehalose RBCs. This significant enhancement supports the argument that relying solely on external trehalose may not be optimal for cryoprotection. The incubation time also proved to be influential in trehalose loading, with a 30-minute duration is sufficient for achieving desired concentrations. The study emphasizes the importance of balanced intracellular trehalose concentrations for favorable cryopreservation outcomes, with post-thaw resting further enhancing cell recovery.

In summary, this study is the demonstration of PLP-ADA polymers as a powerful tool for improving cryopreservation outcomes. These findings offer insights into refining cryopreservation protocols, potentially advancing cell-based therapies and ensuring the preservation of cellular integrity and viability. Future research could focus on scaling up this approach, exploring its applicability to a broader range of cell types, and integrating it with other bioprotectants to further enhance cryosurvival rates. Continued development and optimization of intracellular delivery systems hold promise for significantly advancing cell preservation techniques and broadening their practical applications in medicine and biotechnology.

Chapter 6

Conclusions and future work

6.1 Conclusions

6.1.1 Synthesis and characterisation of the pH-responsive pseudopeptide polymers

The synthesis and characterization of bio-inspired polymers for pH-mediated intracellular delivery and therapeutic applications highlights the development of a range of pH-sensitive polymers. The structural and modifications of polymers were confirmed using FT-IR and ^1H -NMR spectroscopy. The physicochemical properties of these polymers in aqueous solutions were thoroughly investigated across different pH levels and concentrations, revealing critical insights into their behaviour and responsiveness. The study successfully demonstrated the pH-responsive conformational changes of the PLP-ADA polymers. In summary, the exploration of pH-sensitive PLP-ADA polymers with varying degrees of grafting of hydrophobic fatty acid chains onto the PLP backbone has revealed their remarkable ability to undergo conformational changes in response to pH variations.

6.1.2 pH-responsive polymers enable membrane activities and mechanisms using RBCs as a model

RBCs were utilized as a model to investigate the pH-dependent endosomolytic properties of PLP-ADA polymers. The RBCs membrane was evaluated through a haemolysis assay, which measured the leakage of haemoglobin. The ability of these polymers to induce haemolysis is

influenced by various factors such as the pH and concentration. The selected pH range from 4.5 to 7.4 simulates the acidification to the progression through early endosomes, late endosomes, and lysosomes, which the polymers undergo endocytosis. Polymers with higher degree grafting tend to exhibit pH-responsive membrane activity at higher, near-physiological pH levels due to increased pKa values. Consequently, it is possible to engineer polymers to selectively disrupt membranes either during the early or late phases of endosomal acidification. An investigation was undertaken to evaluate the effects of pH, polymer concentration, incubation time, and the degree of grafting on cell membrane activity using haemolysis assays. These studies revealed the mechanisms by which PLP-ADA polymers destabilize cell membranes, facilitating efficient intracellular delivery. Advanced imaging techniques, such as laser scanning confocal microscopy and flow cytometry, demonstrated the successful delivery of payloads of varying sizes into different RBCs. Exploring the membrane activity of pH-dependent PLP-ADA polymers uncovered their potential for facilitating efficient intracellular delivery systems.

6.1.3 The co-incubation of cells with PLP-ADA enables the intracellular delivery of 2D monolayer cell lines and 3D spheroids facilitating an effective approach for delivery of therapeutic protein for cancer therapy

The polymers have demonstrated effectiveness in transporting a wide range of macromolecules. This includes the delivery of molecules such as Calcein, FITC-Dextran of varying molecular weights into a variety of cell types, including cancerous and non-cancerous cells. Furthermore, PLP-ADA60 has been particularly effective in delivering substances into 3D spheroids, a method advantageous for studying cells and tumor. Importantly, the polymers were shown minimal cytotoxicity across all tested cell types to be non-cytotoxic to a range of cell types. It underscores the significant potential of pH-responsive polymers for intracellular drug delivery applications. Successful intracellular delivery of Saporin, a toxic protein, was evidenced by increased cytotoxicity upon co-incubation with PLP-ADA, resulting in decreased cell viability compared to free Saporin. This highlights the effectiveness of PLP-ADA in facilitating the uptake of therapeutic payloads into cells. This bio-inspired polymers for pH-

mediated intracellular delivery and therapeutic applications emphasizes the promising nature of pH-responsive polymers in the field of drug delivery and biomedical applications.

6.1.4 Enhanced cell cryopreservation through biopolymer-mediated trehalose delivery

A practical, glycerol-free, and DMSO-free cryopreservation protocol is established, relying solely on trehalose as the cryoprotective agent (CPA). The study presents an investigation into intracellular trehalose delivery using PLP-ADA for cell cryopreservation. Optimization of trehalose delivery into various mammalian cells, including unnucleated red blood cells and nucleated Jurkat cells, was achieved through adjustments in parameters such as the osmolality of washing buffer, polymer structure, concentration, pH, and incubation duration. It study the importance of intracellular trehalose for enhancing cryosurvival rates and optimizes various parameters, such as osmolality and duration of recovery. By utilizing biopolymer-mediated trehalose uptake, high-concentration trehalose can be transported into cells, offering a potential strategy to improve cell preservation during cryopreservation. This results in intracellular trehalose levels ranging from 80 mM to 250 mM, which has proven effective for successful cell cryopreservation.

6.2 Future plan

6.2.1 Subcellular fate and biodegradability

The confocal microscopy results indicate that PLP-ADA facilitate the release of endocytosed materials into the cytoplasm. However, further confirmation of the subcellular fate of these polymers is essential. Utilizing fluorescent labeling of the polymers would enable the visualization of membrane binding, internalization, and endosomal escape through confocal microscopy, with quantification possible through flow cytometry. Common intracellular organelle markers, such as EEA1 for early endosomes, ESCRTs for late endosomes, and

LAMP-1 for lysosomes, will be employed to assess the co-localization of the polymers with these biomarkers (Shearer and Petersen, 2019). This approach will offer valuable insights into the stage at which polymer-mediated endosomal escape occurs (Jiang et al., 2020). Furthermore, evaluating the biodegradability of the polymers through enzymatic degradation is crucial to ensuring their excretion from the body after degradation, preventing undesirable accumulation (Mallakpour and Dinari, 2011). The information gleaned from these studies will enhance comprehension of polymer-membrane interactions and membrane disruption, contributing to the exploration of their potential applications in drug delivery.

The delivery of proteins, exemplified by the 150 kDa IgG antibody, has proven challenging, potentially attributed to steric hindrance interactions between the macromolecular payload and the polymer. These interactions may impede successful coil-to-globule transition and/or binding onto the membrane, thereby hindering permeabilization. A potential solution involves conjugating polymers to proteins to enhance their inherent properties, increase activity, and improve stability in environments where they are usually unstable (Kaupbayeva et al., 2021). Alternatively, employing a longer crosslinker between PLP-ADA and the cargo could mitigate unwanted interactions. To address this limitation comprehensively, a broader range of proteins, varying in size, surface charge, and shape, including different antibodies, transcription factors, and enzymes, should be investigated. This exploration aims to identify critical parameters impeding successful delivery while further elucidating and expanding the PLP-ADA platform's potential for protein delivery, acknowledging its limitations.

6.2.2 Conjugation with alternative membrane anchors

Enhancing the membrane binding and disruption activity of the comb-like polymer was achieved by conjugating relatively long alkyl side chains as membrane anchors onto the PLP backbone. Drawing inspiration from naturally occurring membrane proteins, future research can explore the conjugation of alternative membrane anchors, such as cholesterol, phospholipid, and glycosyl phosphatidylinositol (GPI) (Khandelia et al., 2008), onto the PLP backbone to create innovative membrane-anchoring polymers. The investigation will encompass the influence of the degree of grafting, hydrophobic-hydrophilic balance, and types of side chains on polymer properties in aqueous solutions, membrane disruption, and cytotoxicity. This

systematic exploration aims to establish a foundation for designing more efficient and intelligent membrane-disruptive polymers. However, it is important to adjust their concentration carefully to achieve optimal responsiveness and accurate pH sensing.

6.2.3 Intracellular delivery of therapeutic genes

The potential of gene therapy to impart inhibitory activity in specific cells offers an appealing avenue for cancer treatment. Nevertheless, the effective delivery of genetic therapeutic agents poses a significant challenge owing to their limited *in vivo* stability, low cellular uptake, and vulnerability to lysosomal degradation. Consequently, the biomimetic nanoscale delivery systems developed in this project will undergo further optimization. The objective is to enhance their capability to efficiently deliver and release genes into the cell cytoplasm while concurrently bolstering stability and prolonging blood circulation time.

6.2.4 Utilization of saccharide mixtures as cryoprotective agent formulation

Various attempts have been made to enhance cryosurvival rates by employing a combination of different CPAs, including permeating and non-permeating CPAs, as demonstrated in prior studies (Yang et al., 2020, Joules et al., 2023). However, limited research has explored the impact of a saccharide mixture, with only one study incorporating trehalose and dextran as additives in the DMSO cell cryopreservation method. Considering the diverse mechanisms of saccharides as CPAs—such as hydrogen bonding for small saccharides and viscosity enhancement for large saccharides—a mixture of different saccharides could yield multiple protective effects during cell cryopreservation. Further exploration is encouraged, including the investigation of diverse formulations and excipients in the cryo-solution. This could involve the use of saccharide mixtures as CPAs or the addition of different excipients like FBS for mammalian cell cryopreservation.

6.3 Closing remarks

In this project, innovative biomimetic nanoscale systems were successfully engineered to enable pH-triggered drug delivery into the cytoplasm. Particularly noteworthy is the development of pH-responsive polymer PLP-ADA, which holds considerable promise for advancing the applications of PLP-ADA in drug delivery. Additionally, the introduction of bio-inspired comb-like pseudopeptidic polymers, featuring hydrophobic long fatty acid side chains as membrane anchors, introduces a novel class of potential candidates for efficient intracellular delivery. The optimal comb-like polymer exhibits significantly improved intracellular delivery across diverse cell lines, accommodating a broad range of payload sizes through a straightforward co-incubation process under acidic extracellular pH conditions. Cell cryopreservation and cell therapy treatment have undergone initial testing with promising results. While pH-responsive polymers have shown considerable promise in various applications, their lack of targeting specificity to individual cells remains a significant limitation. Despite this challenge, these polymers offer substantial advantages with ongoing research poised to improve targeting and broaden their biomedical applications.

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Appendix A

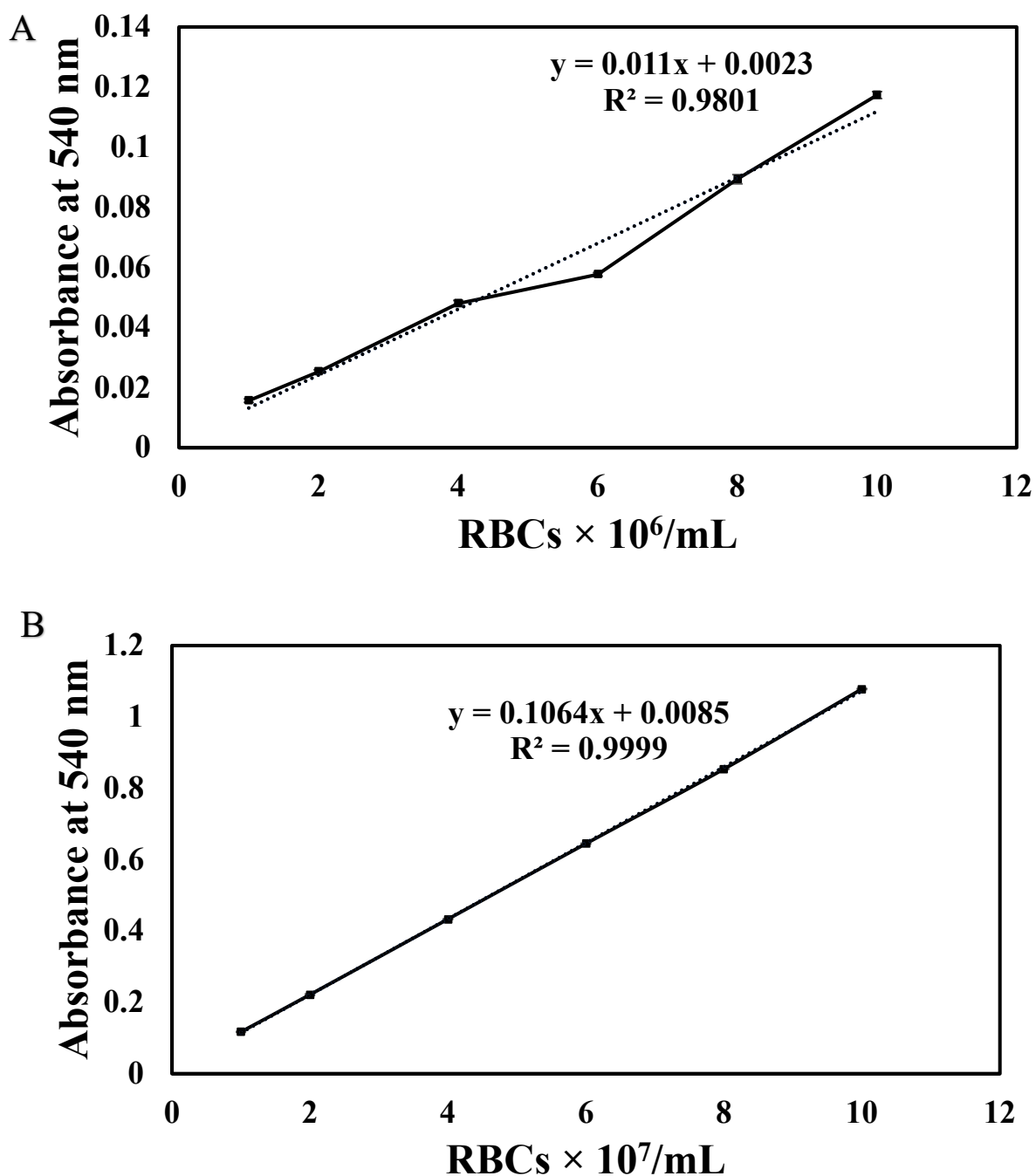


Figure A-1. Haemoglobin standard curve at different cell densities. (A) 10^6 RBCs mL^{-1} (B) 10^7 RBCs mL^{-1} . Mean $\pm$ S.D. (n =3).

Appendix B

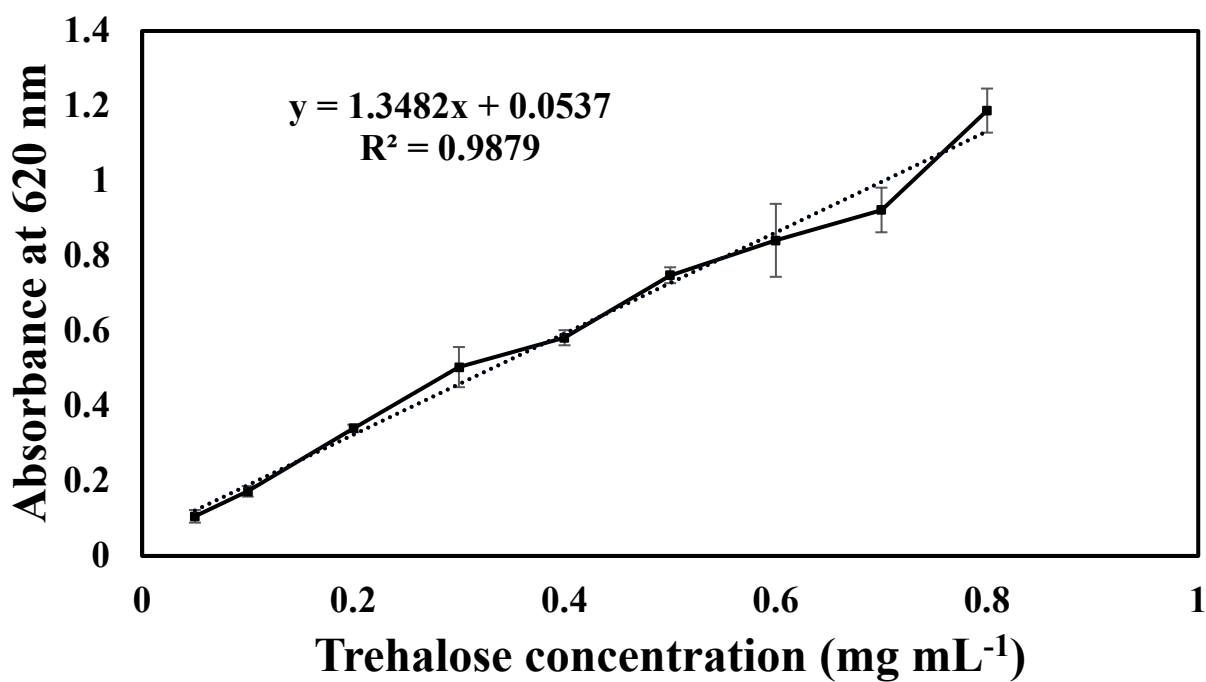


Figure A-2. Trehalose standard curves as measured by the anthrone method. Mean $\pm$ S.D. (n = 3).

Appendix C

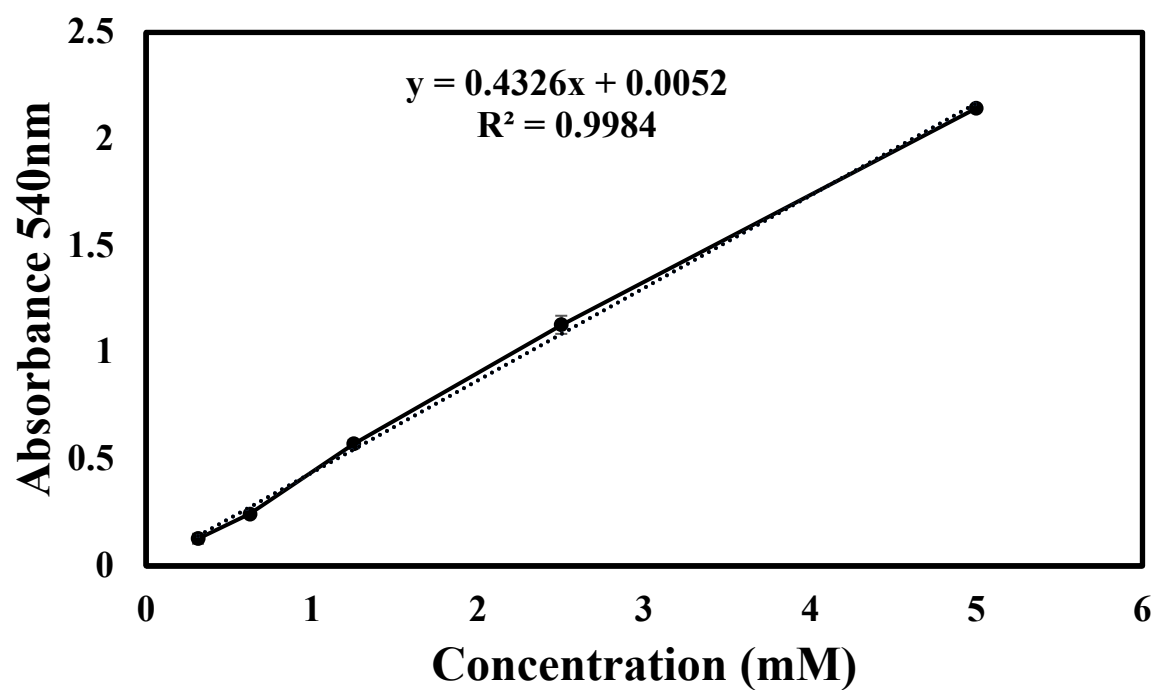


Figure A-3. Trehalose standard curves as measured by the trehalose assay kit. Mean $\pm$ S.D. (n = 3).

Appendix D

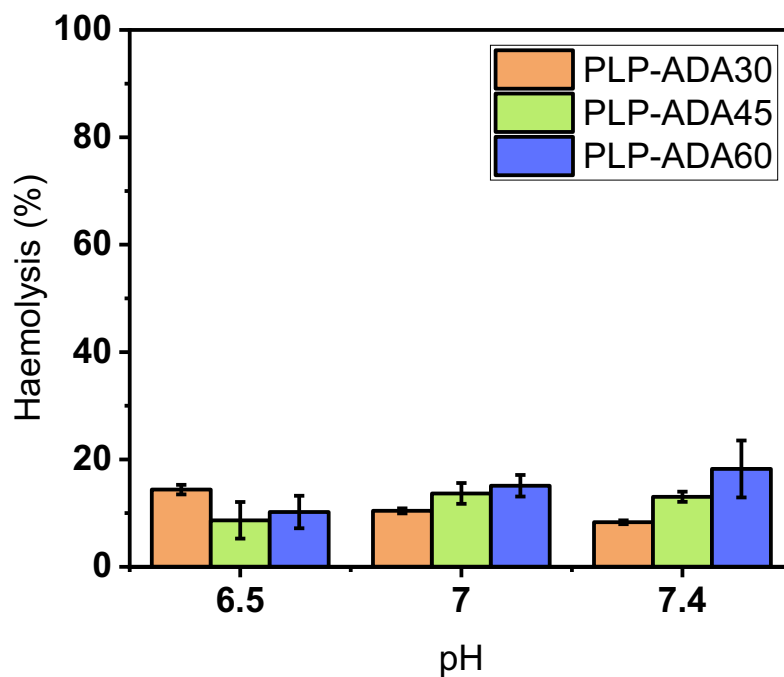


Figure A-4. Influence of extracellular pH on haemolysis of sheep erythrocytes during trehalose loading. Cells were loaded with trehalose via co-incubation for 30 minutes with 360 mM extracellular trehalose and 0.2 mg mL⁻¹ PLP-NDA polymer at 37°C and different pHs. Error bars represent standard deviations (n = 3).

Appendix E

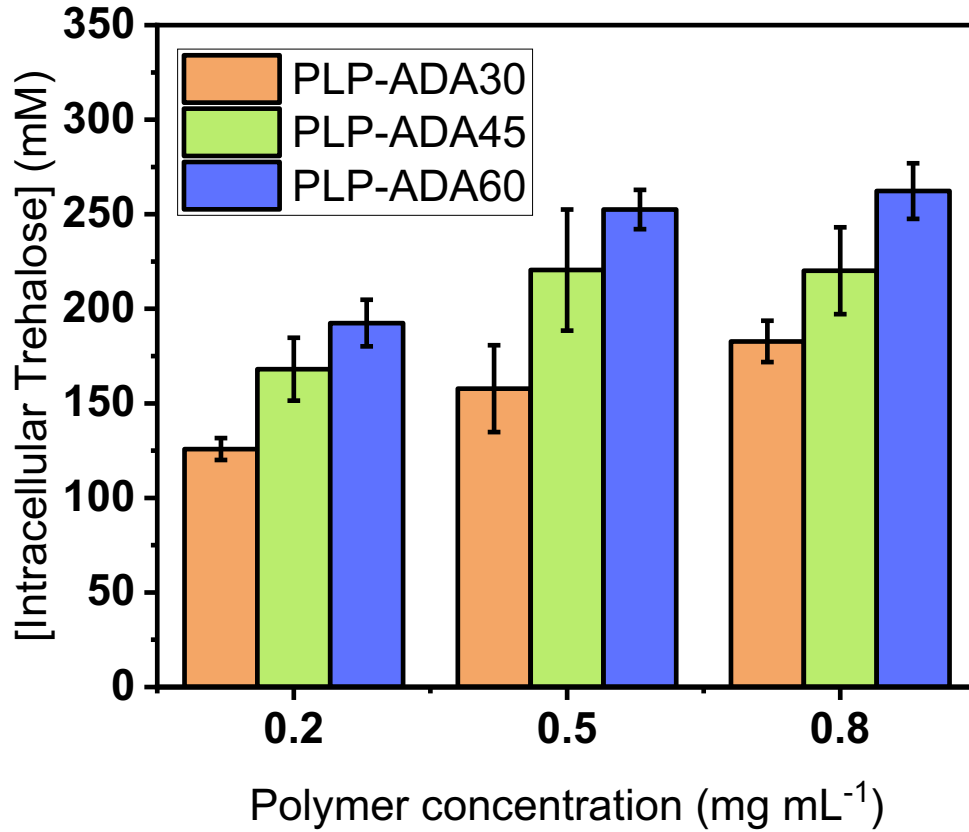


Figure A-5. Influence of polymer concentration on intercellular trehalose concentrations in sheep erythrocytes. Cells were loaded with trehalose via co-incubation for 30 minutes with 360 mM extracellular trehalose and different concentrations of indicated PLP-ADA polymer at 37°C and pH 7.4. Error bars represent standard deviations (n = 3).

Appendix F

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Figure 1-1. Graphical figure of viral and non-viral vector for drug delivery	Technological advances in the use of viral and non-viral vectors for delivering genetic and non-genetic cargos for cancer therapy. Drug Delivery and Translational Research, 13(11), pp.2719-2738.	©2023 Springer Nature	N/A	Yes	This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license
Figure 1-2. Basic depiction of lipid nanoparticle and its constituent elements, the central theme of this review.	The role of lipid components in lipid nanoparticles for vaccines and gene	© 2022 Elsevier	07/04/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1470357-1

	therapy. Advanced drug delivery reviews, 188, p.114416.				
Figure 1-3. Internalization and intracellular trafficking of therapeutics and nanoparticles involve diverse mechanisms	Delivery of cancer therapeutics to extracellular and intracellular targets: Determinants, barriers, challenges and opportunities. Advanced drug delivery reviews, 97, pp.280-301.	© 2015 Elsevier	04/04/2024	Yes	Rightslink® by Copyright Clearance Centre License Number : 5761740243565
Figure 1-4. Polymeric chain states of (A) polyacids and (B) polybases depending on the ionization degree	pH-sensitive polymers: Classification and some fine potential applications. Polymers for Advanced Technologies, 32(4), pp.1455-1484.	© 2021 John Wiley & Sons Ltd	22/02/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1453238-1

Figure 1-5. pH-responsive polymers of different architectures: (a) unimer-micelle, (b) micelle-reverse micelle, (c) nanogels or microgels, (d) hollow-reverse hollow, (e) dendrimer, (f) hyper-branched, (g) micelle morphology changes (from worm-like to hollow), and (h) polymer brushes	pH-Responsive polymers. Polymer Chemistry, 8(1), pp.144-176.	© Royal Society of Chemistry	23/02/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1453287-1
Figure 1-6. The 'proton sponge' hypothesis proposed by Behr and colleagues	The proton sponge hypothesis: Fable or fact? European Journal of Pharmaceutics and Biopharmaceutics, 129, pp.184-190.	© 2018 Elsevier	07/04/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1470368-1
Figure 1-7. pH-Responsive protonation/deprotonation of Poly (N,N'-dimethyl aminoethyl methacrylate) (PDMAEMA).	Revisiting structure-property relationship of pH-responsive polymers for drug delivery applications. Journal of	© 2017 Elsevier	07/04/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1470492-1

	Controlled Release, 253, pp.46-63.				
Figure 1-8. Summary of the polyanionic barriers encountered by polycationic drug delivery systems following dermal (polyanionic skin), mucosal (polyanionic mucus gel layer), or parenteral administration (polyanionic extracellular matrix, polyanionic cellular membranes, polyanionic red blood cells, polyanionic serum proteins) route to their target cells.	Strategies to overcome the polycation dilemma in drug delivery. Advanced drug delivery reviews, 136, pp.62-72.	© 2018 Elsevier	07/04/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1470377-1
Figure 1-9. (A) Illustration pH-responsive membrane permeabilizing polymers and their role in facilitating endosomal escape. (B) General design principles of carboxylated polyanions for endosomal escape applications. (C) Timeline highlighting the chronological	pH-Responsive amphiphilic carboxylate polymers: Design and potential for endosomal escape. Frontiers in Chemistry, 9, p.645297.	© Frontiers Media	N/A	Yes	This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license

development of carboxylated polymers for endosomal escape applications. The time plotted signifies the initial application for pH-dependent membrane permeabilization rather than the first reported synthesis of the polymer.					
Figure 1-13. Potential mechanisms of cellular damage during cryopreservation	Chemical approaches to cryopreservation. Nature Reviews Chemistry, 6(8), pp.579-593.	© 2022 Springer Nature	21/02/2024	Yes	Rightslink® by Copyright Clearance Centre License Number: 5733491073712
Figure 1-14. Overview of key approaches for substituting DMSO in the cryopreservation of therapeutic cells	Dimethyl sulfoxide-free cryopreservation for cell therapy: A review. Cryobiology, 94, pp.9-17.	© 2020 Elsevier	21/02/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1452388-1
Figure 1-15. Cell cryopreservation employing poly(vinyl alcohol) (PVA). (A) RBCs could be	Polymer mimics of biomacromolecular antifreezes. Nature	© 2017 Springer Nature	N/A	Yes	This article is an open access article distributed under the terms and

cryopreserved without a solvent by adding 1 mg mL ⁻¹ PVA, with micrographs revealing the preservation of intact RBCs. (B) recovery of somatic cells post-thaw was successful in mixtures of PVA and dimethyl sulfoxide (DMSO)	Communications, 8(1), p.1546.				conditions of the Creative Commons Attribution (CC BY) license
Figure 1-16. Trehalose loading of erythrocytes in 360 mM trehalose solution (□) and with the addition of 50 ug mL ⁻¹ of polymer PP-50 (▲), 100 ug mL ⁻¹ PP-50 (●), 150 ug mL ⁻¹ PP-50 (■), and 300 ug mL ⁻¹ . Temperature at 37 °C; pH at 7.05.	Biopolymer mediated trehalose uptake for enhanced erythrocyte cryosurvival. Biomaterials, 31(23), pp.6096-6103.	© 2010 Elsevier	21/02/2024	Yes	Rightslink® by Copyright Clearance Centre Order License ID: 1452394-1
Figure1-17. Schematic of poly(L-lysine iso-phthalamide)s grafted with NDA. Copyright 2017, with permission from American Chemical Society.	Membrane-anchoring, comb-like pseudopeptides for efficient, pH-mediated membrane destabilization and intracellular delivery.	© 2017 American Chemical Society	N/A	Yes	This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license

	ACS applied materials & interfaces, 9(9), pp.8021-8029.				
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