

Engineering Catalytic Gold Nanoclusters and Nanovesicles for Detection of Disease

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Statement of Originality

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Abstract

Timely and accurate disease detection is a crucial first step in effective disease management, including personalised medicine treatments. Point-of-care (PoC) diagnostics offer numerous advantages, such as user-friendliness, speed, and cost-effectiveness, making them valuable in resource-limited settings. Nevertheless, their limited test offerings often result in lower sensitivity and specificity when compared to laboratory-based diagnostics. To address this limitation, this thesis focuses on leveraging ultra-small renal clearable gold nanoclusters (AuNCs) as the foundation for developing straightforward, sensitive and cost-effective biosensors for *in vivo* disease monitoring. These NCs possess several benefits, including biocompatibility and the ability to be readily eliminated from the body. In this thesis, the catalytic activity of NCs for signal amplification was optimised firstly while ensuring they retain their renal clearable size, which aimed to enhance the sensitivity of biosensors and guarantee renal clearance for *in vivo* sensing. The second aim focuses on creating an enzyme-responsive biosensor for the rapid and sensitive detection of malaria infection while malaria remains a substantial global health challenge, particularly in developing nations. The final project involves encapsulating nanoclusters within liposomes to create a biosensor tailored for the detection of bacterial infections. This innovative biosensor holds the potential to revolutionise the diagnosis of bacterial infections. In conclusion, this PhD research thesis represents an important step towards the development of biosensors for *in vivo* disease detection using ultra-small renal clearable gold nanoclusters. The successful development of these biosensors could have a significant impact on global health, enabling early detection and treatment of diseases, improving patient outcomes, and reducing healthcare costs.

Publications

Work in Chapter 2 and 4 is in the preparation for submission:

Kaili Chen, Adrian Najer, Molly M. Stevens. “Non-invasive *in vivo* sensing of bacterial implant infection using catalytically optimised gold nanocluster-loaded liposomes for urinary readout” (In preparation)

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List of abbreviations

ABN	Activity-based nanosensor
AuNCs	Gold nanoclusters
ACTs	Artemisinin combination therapies
ABR	Antibiotic resistance
BSM	Brain sphingomyelin
BPC	BSM:PC:CH
BI	Bacterial infection
CH	Cholesterol
CHCl ₃	Chloroform
COVID-2019	Coronavirus Disease 2019
DSPE-PEG2K	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
DPBS	Dulbecco's phosphate buffered saline
DLS	Dynamic light scattering
DMF	N,N-dimethyl formamide
DCM	Dichloromethane
DIEA	Diisopropylethylamine
DTT	Dithiothreitol
DMSO	2,2'-dipyridyl disulphide, dimethyl sulfoxide
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FBS	Fetal bovine serum
Fmoc	Fluorenylmethoxycarbonyl
FCS	Fluorescence correlation spectroscopy
GSH	Glutathione
HABA	4'-hydroxyazobenzene-2-carboxylic acid
HRP2	Histidine rich protein II
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate

HPLC	High-performance liquid chromatography
HC	Healthy control
HA	Hyaluronic acid
H ₂ PtCl ₆	Chloroplatinic acid hexahydrate
GSH	Glutathione
HAuCl ₄	Gold chloride trihydrate
HRP	Horseradish peroxidase
H ₂ O ₂	Hydrogen peroxide solution
HABA	4'-hydroxyazobenzene-2-carboxylic acid
HRP2	Histidine rich protein II
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HPLC	high-performance liquid chromatography
HC	Healthy control
HA	Hyaluronic acid
ICP-MS	Inductively coupled plasma mass spectrometry
MRI	Magnetic resonance imaging
MOFs	Metal-organic frameworks
MMP	Matrix metalloproteinase
MCM	Malaria culture medium
MS	Malaria sensors
MI	Malaria infection
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MeOH	Methanol
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega)
NPs	Nanoparticles
NIR	Near-infrared
NAv	NeutrAvidin
NaBH ₄	Sodium borohydride
NaOH	Sodium hydroxide
OPD	O-phenylenediamine dihydrochloride
OG488	Oregon Green 488 Carboxylic Acid, Succinimidyl Ester, 6-isomer

PEG	Polyethylene glycol
PEG-MAL	Polyethylene glycol maleimide
PoC	Point-of-care
PB	Prussian blue
<i>Pf</i> LDH	<i>Plasmodium falciparum</i> lactate dehydrogenase
PoC	Point-of-care
PB	Prussian blue
RES	Reticuloendothelial system
RBC	Red blood cell
RDT	Rapid diagnostic test
R	Ring stage
SPR	Surface plasmon resonance
SEC	Size exclusion chromatography
SPPS	Solid-phase peptide synthesis
S	Schizont stage
SSI	Surgical site infections
SPARTA	Single particle automated Raman trapping analysis
UV-vis	Ultraviolet-visible
<i>S. aureus</i>	Staphylococcus aureus
TEM	Transmission electron microscopy
Tris	Tris(hydroxymethyl)aminomethane
TIS	Triisopropylsilane
TSA	Tryptic soy agar
TSB	Tryptic soy broth

Chapter 1

Introduction

1 Introduction

1.1 Disease diagnosis

The first crucial step in effectively treating any disease through therapeutic intervention is the accurate and timely diagnosis and detection of the condition. Diagnosis holds immense significance in both patient care and research. Typically, this can be achieved by prioritising the identification of symptomatic patients. The process of diagnosis encompasses categorising symptoms and the examination of blood, urine, or nasal swab samples, which contain a wealth of information about a patient's health status. However, it is important to note that some diseases manifest as asymptomatic, devoid of any discernible signs or symptoms. Furthermore, certain symptoms, such as fever, can be indicative of multiple diseases, making it challenging to pinpoint a specific disease. Consequently, there is a growing demand for the early and specific detection of diseases, as it can significantly enhance diagnostic accuracy and increase the chances of successful treatment before the disease progresses further.

In this context, the effective early detection of diseases has a profound influence on the control and management of various conditions, including infectious (malaria, bacterial infections) and noncommunicable diseases (like cancer, chronic diseases and cardiovascular diseases). These diseases combined stand as the primary causes of mortality worldwide. For example, late diagnosis often leads to delayed access to life-saving antibacterial, antimalarial, and cancer treatment, especially in resource-constrained settings and among vulnerable populations. The consequence of delayed or unavailable treatment for these diseases can result in a reduction of survival rate.

Hence, achieving global accessibility to early detection and screening of diseases is of paramount importance to improve the treatment and outcomes^{1,2}. As highlighted by the World Health Organization (WHO), there exists an urgent demand for achieving precise and early diagnoses, as this can significantly reduce the number of cases diagnosed at advanced stages and ultimately enhance survival rates (Figure 1.1).

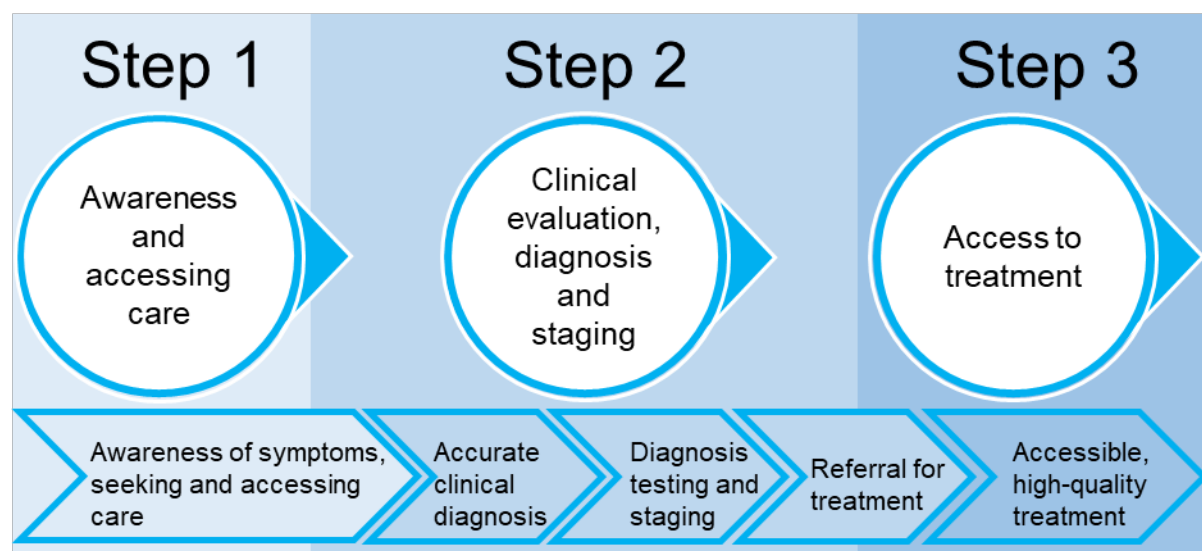


Figure 1.1 Three steps for early diagnosis of diseases to reduce cases diagnosed at a late stage and improve patient outcomes (reproduced from WHO³).

A comprehensive strategy must be implemented, commencing with an emphasis on raising awareness and enhancing self-diagnostic capabilities. It is imperative that individuals become knowledgeable about the telltale signs and symptoms of various diseases, such as cancer, inflammation and infectious diseases, as well as gaining access to efficient diagnostic methods. The second crucial aspect entails the enhancement of referral systems and the development of diagnostic infrastructure to mitigate the obstacles hindering timely disease detection. Lastly, there is a pressing need to improve the accessibility and affordability of treatment, particularly in low-income countries. It is crucial to create simple, low-cost and rapid diagnostic tools for a better outcome of treatment.

1.1.1 Laboratory-based diagnostics

Based on portability and complexity, diagnostic sensing systems can be categorised into two primary types: laboratory-based and point-of-care (PoC). Laboratory-based diagnostics are typically conducted within centralised laboratory settings. Samples are collected and preserved before being subjected to analysis. This method allows for in-depth analyses, including molecular analysis, genetic testing, and advanced immunoassays, providing detailed diagnostic reports. Laboratory-based diagnostics offer high sensitivity and precision, enhancing the accuracy of diagnoses and disease monitoring^{4–6}. However, there are notable drawbacks associated with laboratory-based diagnostics. Sample collection and transportation can introduce risks of contamination and are time-consuming. The extended turnaround time may be limiting for urgent and critical cases. Moreover, this approach necessitates access to large, complex, and specialised equipment or reagents, often requiring well-trained personnel. Consequently, costs can be prohibitively high, particularly in resource-constrained settings, and obtaining results may take hours to days. To address these limitations and meet the demand for cost-effective, rapid, and sensitive diagnostic methods, the development of PoC diagnostics is essential.

1.1.2 Point-of-Care diagnostics

Compared to laboratory-based diagnostics, PoC diagnostics offer the advantage of being commonly conducted at or near patients, whether in clinics or remote resource-limited settings. These systems are typically designed to be portable, compact, and user-friendly, requiring minimal training in techniques. This capability facilitates immediate clinical decision-making, especially in emergency situations. Furthermore,

PoC diagnostics often have the advantage of being cost-effective, which is particularly beneficial for frequent testing and in resource-limited environments^{7,8}. Nevertheless, PoC diagnostics come with their own set of challenges. They may not match the versatility of laboratory-based tests, offering a more limited menu of available tests. Additionally, they are often designed for initial screening assessments, which may result in lower sensitivity and specificity compared to laboratory-based assays. It is critical to develop further affordable, portable, and user-friendly PoC devices that combine both sensitivity and specificity to provide rapid and easily interpretable results. One crucial parameter in assessing sensitivity is the limit of detection (LoD), which measures the lowest concentration of the target analyte that can be reliably detected. A reduction in LoD can significantly enhance the sensitivity of the sensing platform. Specificity is another key factor for accurate disease diagnosis, and it can be improved by designing sensing systems responsive to multiple cues. Therefore, effective diagnostic devices should be designed with a focus on achieving enhanced accuracy and specificity. These improvements can lead to shortened diagnosis times, timely treatment, and ultimately better patient outcomes. The ASSURED criteria, introduced in 2006 for PoC diagnostics to control and prevent diseases and infections, encompass affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, delivered to end users. With advancements in digital technology, the ASSURED criteria have evolved into the REASSURED criteria, emphasising real-time connectivity and simplified specimen collection^{9,10}. Developing cost-effective and sensitive PoC diagnostic tools in accordance with these criteria is significant for early disease and infection detection, ensuring timely treatment and better patient outcomes.

1.1.3 Biosensors in diagnostics: *in vitro* and *in vivo* sensing

In vitro sensing refers to tests and analyses conducted on patient samples collected from the organism, including blood, tissues, fluids and so on. This is achieved by measuring the presence of biomarkers using external devices in a laboratory or PoC setting. *In vitro* sensing offers several advantages, such as the absence of ethical constraints since it does not involve living organisms. Additionally, it can be performed with greater control over variables, especially in laboratory settings, minimising potential influences on the results. However, replicating certain complex interactions and conditions, as seen in living organisms, can be challenging^{11,12}. When it comes to analysing samples, there are inherent risks associated with sampling, preparation, and storage. These processes can introduce artifacts or errors, potentially leading to erroneous diagnostic outcomes.

Unlike *in vitro* sensing, *in vivo* biosensing refers to diagnostic techniques performed within a living organism, which can include laboratory animals, human patients, or other organisms. In this approach, a biosensor is delivered or implanted into the body of a patient to respond to specific biomarkers. This can monitor target analytes and transmit health-related information. The resulting signal is then reported externally. *In vivo* biosensing holds significant potential for advancing healthcare¹³. One key advantage of *in vivo* biosensing is its real-time measurement of physiological processes, enabling immediate detection of abnormalities. Additionally, these biosensors offer dynamic insights by capturing real-time responses to external stimuli, facilitating the development of therapeutic interventions and personalised treatments based on up-to-date data. Furthermore, many *in vivo* biosensors are minimally

invasive, reducing the risk of contamination and patient discomfort^{14,15}. Despite these advantages, there are still several challenges to address, encompassing ethical, technical, and regulatory considerations^{12,16}. Developing rapid, cost-effective, and highly sensitive *in vivo* biosensors for early disease detection will create future tools to better tailor treatments to the need of each patient.

Recently, the Stevens research group developed ultrasmall renal-clearable catalytic gold nanoclusters (AuNCs) for *in vivo* colorectal cancer detection, employing a urinary colourimetric readout¹⁷. This innovative approach first capitalises on the size-selective properties of the glomerular filtration membrane in the kidneys¹⁸. The filtration capability of kidneys allows particles smaller than about 5.5 nm to pass into the urine while retaining larger substances within the bloodstream, facilitating diagnostic results through urine analysis^{19–23}. This *in vivo* biosensing platform has demonstrated its efficacy in achieving a simple, rapid, and highly sensitive detection of colorectal cancer in mice. When designing nanoscale sensors for *in vivo* biosensing to detect and monitor diseases, several critical considerations come into play. These sensors must prioritise biocompatibility to prevent triggering an immune response¹³. Moreover, they should be meticulously engineered for maximum sensitivity and specificity to ensure quick and accurate disease analysis, whilst they are preferably excreted from the body as rapidly as possible to avoid any potential long-term side effects.

1.2 Biomarkers and biomarker detection

1.2.1 Biomarkers

The analysis of biomarkers that play a pivotal role in disease progression and dissemination is crucial for achieving early disease detection. This step relies on the precise analysis and quantification of specific biomarkers, thereby discerning their presence or absence. Biomarkers employed in diagnostics encompass a wide range of substances and processes, including cells, antibodies, proteins, polysaccharides, enzymes, and nucleic acids. These biomarkers serve as measurable indicators of disease state and the health status of a patient^{24–26}. Information about the health status of a patient can be obtained from various biological samples, such as blood, urine, faeces, sweat, breath, and saliva. The analysis of biomarkers within these biological samples furnishes valuable insights into the disease state. For instance, foreign antigens or antibodies generated as part of an immune response to a specific antigen can serve as biomarkers, readily detectable in blood samples^{27,28}.

Additionally, the measurement of enzyme activity can serve as a disease indicator, with enhanced sensitivity often achieved through the assessment of its catalytic activity²⁹. Among these biomarkers, proteases stand out as enzymes capable of cleaving peptide bonds and playing crucial roles in both normal physiological functions and disease states. The measurement of proteolytic activity holds significant potential for amplifying disease detection capabilities, thereby advancing the field of diagnostics^{17,30,31}. The same applies for another class of enzyme, lipases, which are defined as enzymes involved in the digestion and metabolism of fats or lipids. They can break down triglycerides into smaller molecules like glycerol and fatty acids,

making these lipids more easily absorbed and utilised by the body. However, the lipase activity is closely associated to certain diseases and health conditions, including liver disease, obesity, pancreatitis and cardiovascular diseases and so on^{32–34}. Using enzyme activity as a biomarker for disease detection represents a valuable approach in medical diagnostics.

1.2.2 Proteases and lipases

Proteolysis is an important process in both normal and pathological physiology³⁵. Proteolytic enzymes, often referred to as proteases, can catalyse the degradation of proteins by cleaving peptide bonds between amino acids. These proteases can play a significant role in the pathogenesis, progression, or therapeutic response of the disease. Importantly, proteases typically exhibit specificity for particular protein substrates or peptides. Consequently, diseases can lead to alterations in protease activity, either an increase or decrease^{17,36}, or foreign proteases are introduced into the body, e.g. during an infection^{37,38}. By monitoring the presence of these foreign proteases or changes of host protease activity against these specific substrates, the disease state can be understood. Therefore, disease-associated protease activity has emerged as promising biomarkers for assessing the presence, severity, and therapeutic effect of disease, especially for early detection of cancers^{39,40} or infectious disease (such as bacteria, viruses, fungi, eukaryotes and prions)^{37,38,41,42}. This is owing to the functional and active roles that proteases play in cancer and infectious disease progression.

Lipases are a class of enzymes primarily responsible for catalysing the breakdown of triglycerides into glycerol and free fatty acids within living organisms, acting on the

ester bonds of lipid molecules. This process is crucial for facilitating lipid digestion and converting them into energy during digestion and metabolism⁴³. Various lipases are expressed and active in multiple tissues, including hepatic lipase in the liver, hormone-sensitive lipase in adipose tissue, lipoprotein lipase on the vascular endothelium, and pancreatic lipase in the small intestine, *etc.*^{44,45}. Dysfunction in lipase activity can contribute to the development of certain diseases. For instance, insufficient lipase activity can result in lipid metabolism disorders, which are associated with health issues like obesity, high cholesterol levels, and cardiovascular disease^{43,46,47}. Furthermore, certain rare inherited disorders related to lipase deficiencies, such as familial hypercholesterolemia, are also linked to lysosomal acid lipase deficiencies^{48,49}. In addition, certain pathogenic bacteria can secrete lipases during host infections, making them potential biomarkers for the detection of bacterial infections^{50,51}. Therefore, the study of lipase and its role in lipid metabolism is crucial for comprehending and treating diseases associated with lipid metabolism.

1.2.3 Disease-associated protease activity as a biomarker

Protease assessment can be achieved through affinity-based and activity-based assays. The affinity-based assay involves measuring protease concentration and quantifying target proteases using specific antibodies or ligands that bind to the enzyme of interest. These assays are generally highly specific, but sensitivity may vary depending on the affinity of the antibody or ligand used. Enzyme-linked immunosorbent assay (ELISA) is a well-known affinity-based assay commonly used for enzyme detection. ELISA is highly specific, but its sensitivity requires optimisation, and it may not always provide real-time or dynamic monitoring of enzyme activity. On

the other hand, the activity-based assay evaluates the enzymatic activity of the target enzyme, providing direct insights into the link between diseases and protease activities^{52–54}. These assays allow real-time monitoring of enzyme activity and are generally more sensitive to changes in enzyme levels than affinity-based assays. Activity-based assays are typically performed using fluorescent or chromogenic substrates, which detect enzyme activity by measuring the generation of a fluorescent or colorimetric signal. Although activity-based assays offer high sensitivity and real-time monitoring capabilities, there may still be limitations in specificity because they may detect the activity of associated enzymes or nonspecific reactions.

Both affinity-based and activity-based assays can achieve high levels of sensitivity and specificity with appropriate optimisation and validation. Affinity-based assays tend to provide higher specificity due to the use of specific antibodies or ligands, whereas activity-based assays provide higher sensitivity for detecting enzymatic activity. Advantages of affinity-based assays include high specificity, ease of use, and compatibility with high-throughput screening platforms. However, they may require the availability of specific antibodies or ligands and may not always provide real-time monitoring of enzymatic activity. Advantages of activity-based assays include real-time monitoring of enzyme activity, high sensitivity, and the potential for dynamic measurements. However, they may have limitations in specificity and may need to be optimised for different enzyme substrates and conditions. Affinity-based and activity-based assays can complement each other and provide comprehensive insights into enzyme detection and monitoring.

Dysregulation of protease activity is a common occurrence in pathological conditions, including cancer, inflammation, severe infections, thrombosis, and cardiovascular diseases⁵⁴. The activity-based approach for protease detection holds promise for disease detection. Proteases function by hydrolysing peptide bonds between amino acids or at amino and carboxyl terminal amino acids, leading to the generation of two or more peptide fragments. This hydrolytic function allows for the design and customisation of specific peptide substrates that can be selectively cleaved by a target protease using solid-phase peptide synthesis techniques⁵⁵. The cleaved fragments can then be analysed using mass spectrometry⁵⁶, which enables real-time monitoring of protease activities and serves as a valuable sensing tool for assessing protease activity via peptide bond hydrolysis. One notable advantage of utilising protease activities as biomarkers for diseases is their applicability in live cells and *in vivo* detection of conditions such as cancers, infections and other diseases.

Bacterial infections constitute a significant public health concern. Early and precise detection of infections is crucial for timely and effective treatment, preventing drug resistance and complicating treatment. Present diagnostic and monitoring technologies, including radiography and sputum cultures, often provide nonspecific or delayed results. They lack the specificity required to identify bacterial infections accurately and are time-consuming for determining the optimal use of antibiotics^{57,58}. Therefore, there is a crucial demand for infection-specific biomarkers to facilitate early and accurate diagnosis of bacterial infections. Proteases, a class of enzymes typically associated with diseases and bacterial infections, offer a potential avenue for biomarker development. The activity of proteases can serve as a biomarker, and the

process of proteases cleaving substrates inherently leads to signal amplification through catalysis.

An *in vivo* model of the detection of respiratory tract infections, conducted by Sangeeta N. Bhatia and colleagues, has demonstrated that an activity-based nanosensor (ABN) was developed for achieving a rapid and sensitive urinary readout for the successful detection of *P. aeruginosa* lung infections through the sensing of protease activity from both host and bacteria⁵⁹. In this study, the ABN consists of a specific peptide substrate conjugated to commercially available multivalent PEG-MAL, a multi-arm PEG derivative, with each of the eight arms terminated with maleimide, linked to a hexaglycerol core. The ABN was then administered intravenously into a mouse. The ABN underwent disassembly through *in situ* proteolysis, releasing the cleaved small report molecules that were subsequently filtered through kidneys into urine. Urine samples were collected, concentrated and subjected to sandwich ELISA, which revealed the protease activity as a signal detection and readout indicator for the detection of lung bacterial infection (Figure 1.2).

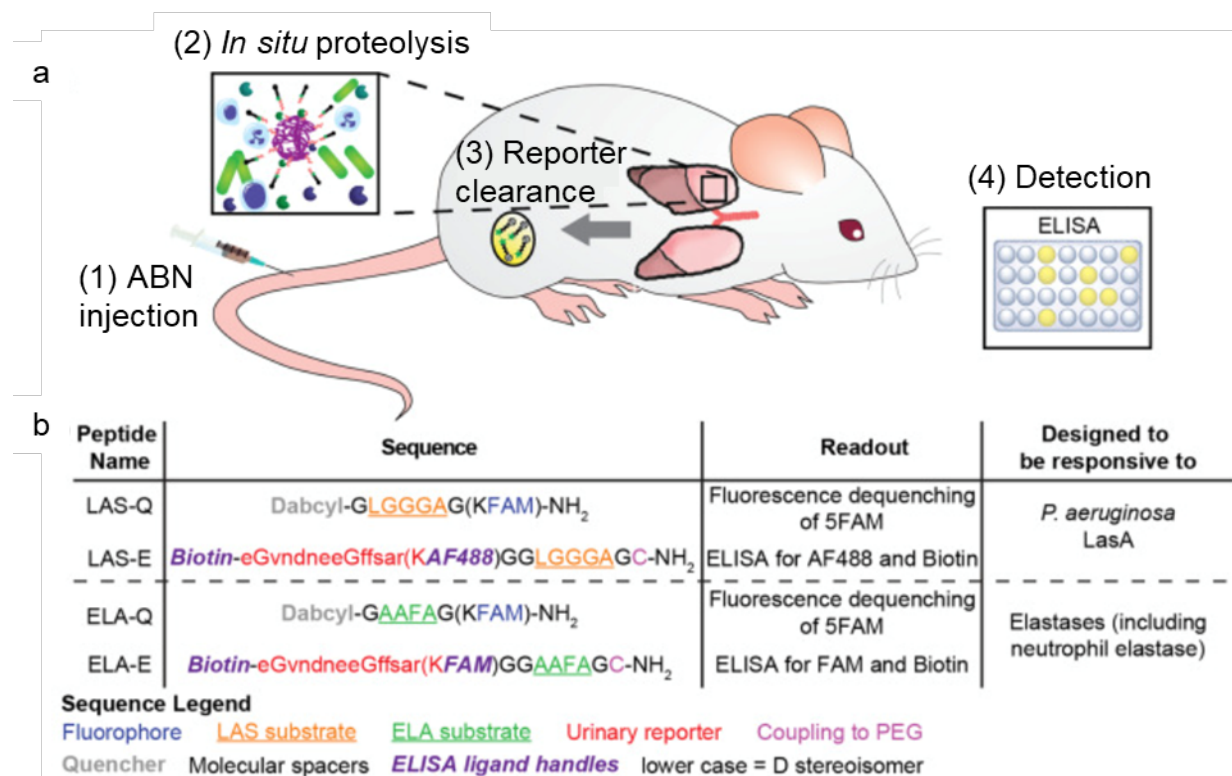


Figure 1.2 *In vivo* experiments demonstrating a protease sensor for the detection of bacterial infections. (a) Schematic of activity-based Nano sensor (ABN) system for detecting infection-related proteases. (1) Intravenous injection of ABNs into a mouse. (2) In situ proteolysis of nanosensor by host and bacterial proteases. (3) Kidney filtration of small reporters and urine collection. (4) Enzyme-linked immunosorbent assay (ELISA) on urine samples. (b) Development of substrates targeting proteases from both pathogens and host organisms (This figure was adapted from Ref. 59 with permission)⁵⁹.

Synthetic biomarkers are artificially created molecules, particles, or substances designed to serve as indicators of biological processes, disease states, or response to treatment in the body. The development of synthetic biomarkers was pioneered by Bhatia *et al.*, which is a type of activity-based probe and can be designed to detect disease-associated protease activity.^{60–64} Some synthetic biomarkers are comprised of nanoparticles adorned with tumour-targeting peptide linkers. These peptide linkers can be selectively cleaved by specific proteases present in the tumour environment⁶⁵.

In Kwon's work, upon administration of synthetic biomarkers with urine reporter MMP-9 substrate 'R' tethered to a nanoparticle *in vivo*, they accumulate at disease sites, where local enzymes cleave the peptide linker releasing urine reporter 'R'. The cleaved fragments are subsequently filtered through the kidneys into the urine, which is then collected for signal analysis and detection by measuring the peptide fragments (Figure 1.3).⁶⁴ The utilisation of synthetic biomarkers represents a promising approach for early cancer diagnosis, leveraging the activity of disease-associated proteases to provide a detectable signal through a cleverly designed catalytic process. This innovative method holds the potential to significantly improve the timely detection of tumours, thereby enhancing the prospects for successful treatment.

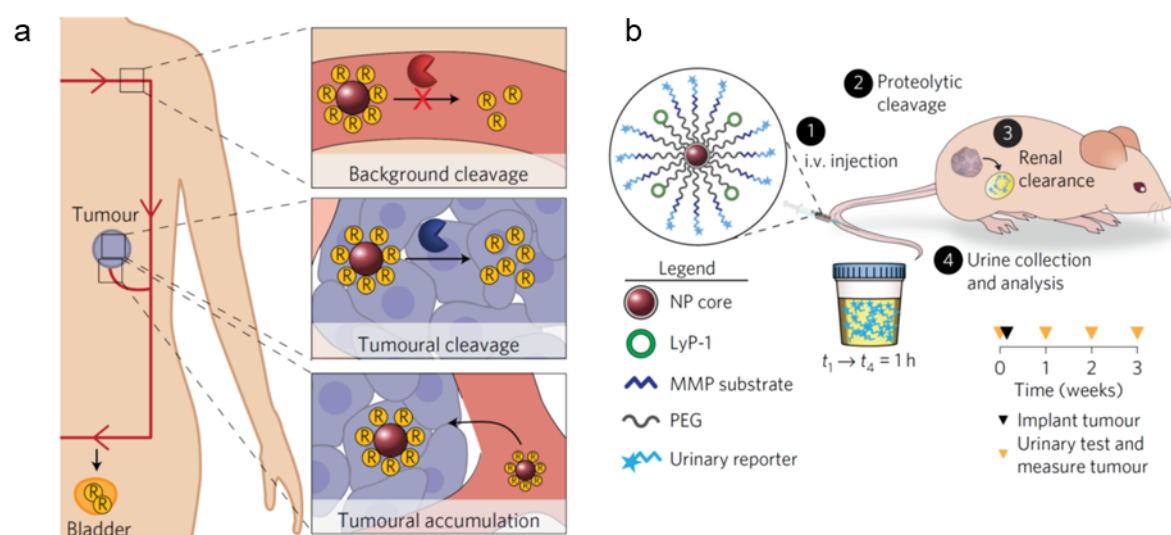


Figure 1.3 *In vivo* study demonstrating an activity-based nanosensor (ABN). (a) Schematic of *in vivo* nanosensor in a mathematical pharmacokinetic model. Encircled 'R's refer to urine reporters which are tethered to a nanoparticle core and filtered into bladder after release. (b) Schematic of urine testing in a mouse flank tumour model. i.v. refers to intravenous and t refers to time at given testing step (1 – 4) (This figure was adapted from Ref. 64 with permission)⁶⁴.

1.2.4 MMP activity and thrombin activity for disease detection

Matrix Metalloproteinases (MMPs), including MMP-7, MMP-8 and MMP-9, are significant proteases involved in various biological processes and hold a key role in the pathology of cancers^{66–69} and the progression of infection^{70–72}. The overexpression of MMP-9 often serves as a causal factor in the development of many malignant tumours^{67,73}. Consequently, there has been a growing focus on exploring and MMP-9 as potential biomarkers across a spectrum of cancer types. MMP-9 is intricately involved in a plethora of cancer-related processes, encompassing cancer cell migration, invasion, tumour metastasis, angiogenesis, inflammation, and proliferation^{74–76}. In numerous cases, MMP-9 plays a pivotal role in driving cancer development and progression, such as in colitis-associated colon cancer⁷⁷. Beyond cancer, the involvement of MMP-9 extends to the pathologies of other diseases, including cardiovascular diseases⁷⁸ and autoimmune diseases⁷⁹. This multifaceted role makes MMP-9 a promising biomarker for various cancer types. Nevertheless, harnessing MMP-9 as the sole biomarker for *in vivo* biosensing presents challenges due to its involvement in numerous diseases. Enhancing the specificity of nanosensors may necessitate a strategy that combines the detection of multiple MMPs within a single diagnostic platform. A sensitively optimised single sensor could be a viable approach for detecting MMP-9 to improve diagnostic accuracy. Another MMP, MMP-7, for instance, experiences upregulation in certain tumour cells, leading to an increase in the metastatic potential of these cells⁸⁰.

MMP-8, also known as collagenase-2, possesses the primary function to degrade collagens. It is often implicated with various diseases and infections, ranging from

tissue remodelling to inflammations. MMP-8 has been identified as the primary collagenase in human dentin and its disorder was found closely involved in periodontal disease⁸¹. It is produced in response to inflammation and bacterial infection in periodontal disease, which degrade extracellular matrix components to support periodontal tissues^{81,82}. Monitoring MMP-8 levels can be a valuable tool in tracking the treatment progress of periodontal disease⁸³. Dysfunction of MMP-8 can lead to a multitude of other diseases. For instance, MMP-8 deficiency has been associated with the development of skin cancer in a wound healing mouse model, underscoring its crucial role in resolving inflammation and facilitating tissue repair⁸⁴. Additionally, pathological overexpression of MMP-8 can contribute to cardiovascular diseases by promoting atherosclerotic plaque destabilisation and rupture⁸⁵. A diverse range of MMPs was observed in the lung tissue of patients during a COVID-19 infection study. Specifically, the study highlighted the expression of MMP-2 activity, along with the noteworthy finding of MMP-8 overexpression in severe COVID-19 cases. This overexpression of MMP-8 is associated with the extensive destruction of lung tissue (Figure 1.4)⁸⁶. These findings hold promise for the potential utilisation of MMP-2/MMP-8 activity as a biomarker for the detection of infectious diseases.

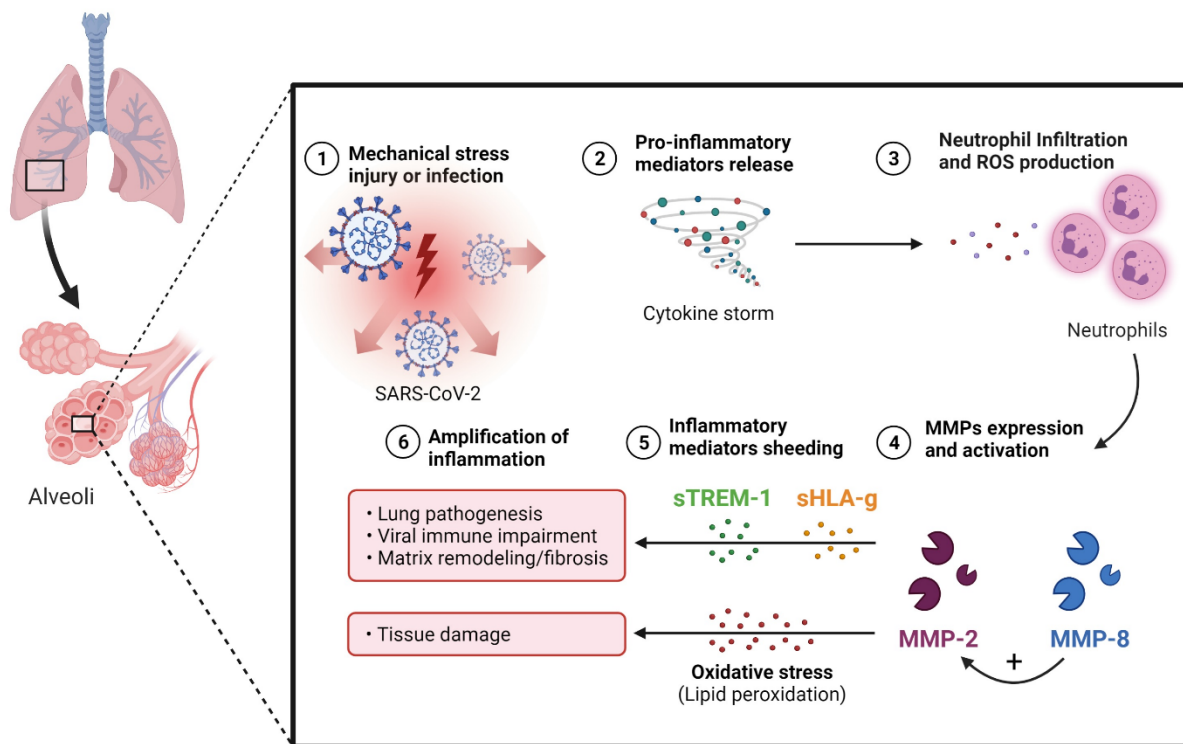


Figure 1.4 Role of matrix metalloproteinases (MMPs) in the pathogenesis of severe Coronavirus Disease 2019 (COVID-19) lung disease. Unregulated protease activity and overexpression of MMPs are associated with lung disease in severe cases of COVID-19. The overexpression of MMP-2 and MMP-8 intensifies the destruction of lung tissue in severe COVID-19 (This figure was adapted from Ref. 86 with permission)⁸⁶.

Thrombin, a serine protease, assumes an important role in a diverse array of both normal physiological processes and pathological conditions, including angiogenesis, cardiovascular diseases, and thrombosis. Among these, thrombosis, the formation of potentially life-threatening blood clots, stands out as a particularly concerning health issue for humans. The assessment of thrombin activity can be regarded as a valuable biomarker for the early detection of thrombotic events. Notably, Bhatia and her colleagues⁸⁷ have made significant strides in this area by pioneering the development of synthetic biomarkers, a category of protease-sensitive nanoparticles tailored to respond to dysregulated protease activity at disease sites, such as those associated

with thrombosis. These synthetic reporters are released into the circulatory system upon substrate cleavage and subsequently accumulate in the urine for analysis (Figure 1.5). Utilising thrombin activity as a biomarker provides a robust approach to sensing and diagnosing thrombosis, offering a promising avenue for early detection and intervention in this critical medical condition.

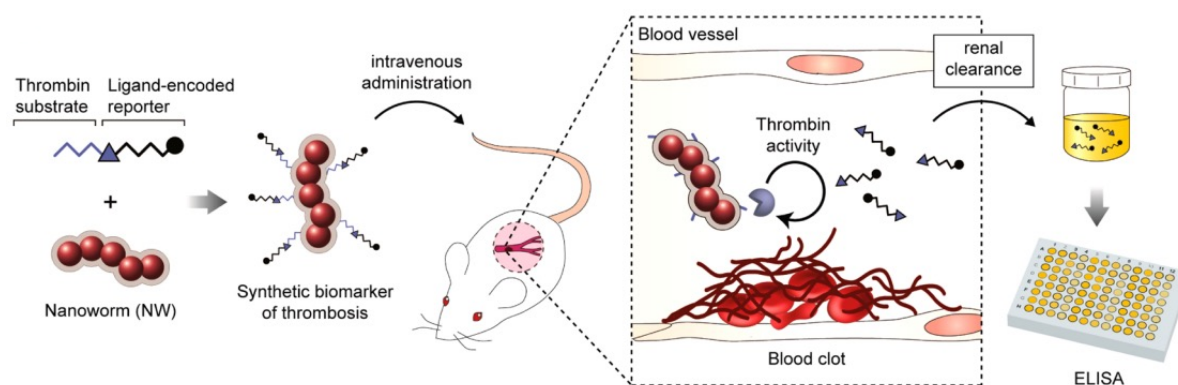


Figure 1.5 Schematic of synthetic biomarker sensing thrombin activity. The synthetic thrombosis biomarker comprised nanoworms conjugated to a thrombin-responsive substrate linked with a ligand-encoded reporter. This biomarker was subsequently administered intravenously to a mouse. Cleavage occurred in response to thrombin activity at the disease site, leading to the release of the reporter, which was subsequently filtered through the kidneys into the urine. Urine samples were analysed using ELISA for detection and analysis (This figure was adapted from Ref. 87 with permission)⁸⁷.

1.2.5 Lipase-activity for detection of bacterial infection

Lipases are enzymes of interest in bacterial infections, as they can be secreted by bacteria during host infections. Lipases have the capability to break down a variety of substrates, including phospholipids, sphingolipids, neutral lipids, ether lipids, and oxidised lipids, *etc*⁴³. Leveraging the enzymatic or membrane-damaging activities of virulence factors derived from bacteria, such as pore-forming toxins, enzymes (lipase activity), surface proteins (surfactant-like mechanisms), and adhesion proteins,

represents an emerging and promising alternative method for bacterial infection detection^{88,89}. Mechanistically, pore-forming toxins and lipases (e.g. beta toxin, a sphingomyelinase), notably implicated in *Staphylococcus aureus* (*S. aureus*) infections, are capable of creating pores and damaging host cell membranes. This toxin-driven and sphingomyelinase-like activity strategy was leveraged to create these liposomal systems responsive to bacterial growth, enabling the detection of bacterial infections^{90,91}. Since then, considering the substrate classification, sphingolipids have been utilised such as sphingomyelin, which can be broken down by sphingomyelinase-like toxin activity. This approach allows the detection of bacterial infections by monitoring the toxin activity.

The detection of toxin and lipase activity lays the foundation for designing various non-invasive diagnostic tools for the detection of *S. aureus*^{92–94}. One notable application of this approach is the utilisation of toxin-induced responses to engineer liposomal systems that respond to bacterial growth. Liposome systems are lipid-based nanoparticles composed of a phospholipid bilayer, similar to the structure of cell membranes. They are used as versatile drug delivery vehicles capable of encapsulating hydrophilic and hydrophobic compounds for targeted delivery to specific tissues or cells, providing controlled release and enhanced therapeutic efficacy in various biomedical and ingenious applications, including controlled antibiotic delivery^{95–97}, toxin sequestration to mitigate host toxicity⁹⁸, the on-demand activation of antibacterial nanoreactors⁹⁹, and bacterial infection detection^{90,91}. For instance, Thet *et al.* demonstrated the tunability of liposomes to elicit specific toxin responses by engineering membrane compositions¹⁰⁰. They recently developed a sensor swab incorporating self-quenched dye-encapsulated liposomes for infection detection in an

acute wound infection model, relying on the breakdown of liposomes, resulting in a noticeable colour change from yellow to green¹⁰¹.



Figure 1.6 A clinical study for assessing the degree of subjectivity in evaluating the observed colour of SPaCE indicator solution and converting it into a numerical value. (a) SPaCE pathogen-positive result (left: green) with evidence of burn wound infection (right). (b) SPaCE swab negative outcome (left: yellow) with no reports from patients of heat or pain (This figure was adapted from Ref. 101 with permission)¹⁰¹.

Liposomes, versatile vesicles composed of diverse lipid compositions, have gained prominence as carriers with a wide array of applications in drug delivery and biosensing over the past few decades^{102,103}. Thanks to their analogous components and structural similarity to cell membranes, liposomes serve as effective carriers for the encapsulation of both hydrophilic and hydrophobic cargos and can interact physiologically with cells¹⁰⁴. Liposomes have demonstrated their prowess in encapsulating various cargos, encompassing dyes, drugs, biomolecules, enzymes, and nanoparticles, thereby finding applications in diverse drug delivery scenarios and

serving as amplification strategies in biosensing^{105–110}. Liposomes formulated with a composition reminiscent of cell membranes, particularly by incorporating bacterial lipase-sensitive sphingomyelin lipids, have become a staple in specific drug delivery and sensing applications tailored for bacterial infections. This pioneering approach not only showcases the versatility of liposomes but also holds significant promise for the development of advanced tools for the detection and management of bacterial infections^{95,99,100,111}.

1.3 Renal clearable gold nanoparticles for *in vivo* biosensing

1.3.1 Metal nanoparticles for *in vivo* biosensing

The field of utilising metal nanoparticles in biosensing has gained substantial attention, thanks to their multifaceted properties. These properties encompass not only excellent stability for catalytic reactions but also a sufficiently large surface area for versatile functionalisation and modification¹¹². Their favourable biocompatibility and optical characteristics, such as surface plasmon resonance (SPR) exhibited by gold nanoparticles, further enhances their appeal for biosensing application^{113,114}. The SPR performance of nanoparticles refers to their characteristics in the surface plasmon resonance phenomenon and is affected by factors such as size, shape, surface modification, and material composition. This property plays a key role in applications such as sensor technology, optical imaging, photothermal therapy, and surface-enhanced Raman scattering, providing important means for detection and treatment with high sensitivity, high resolution, and high selectivity. Therefore, the SPR properties of nanoparticles have broad application prospects in the fields of

biomedicine and nanotechnology. Capitalising on these advantageous sensing properties, metal nanoparticles play an important role in the development of highly sensitive biosensors capable of detecting and quantifying various biomolecules and ions. They offer a powerful tool for signal amplification, elevating the sensitivity of biosensing techniques to new heights.

One of the most significant applications of metal nanoparticles is in the detection and diagnosis of various diseases, including cancer, infections, and cardiovascular conditions¹¹⁵. They can serve as probes for biomarkers associated with these diseases. By modifying their surfaces to interact with specific biomarkers, metal nanoparticles generate measurable signals, enabling the development of highly sensitive diagnostic assays. This breakthrough holds immense potential for early disease detection and accurate diagnosis, ultimately improving patient outcomes. Metal nanoparticles have also found widespread use in biomedical imaging. They serve as valuable contrast agents in techniques such as magnetic resonance imaging (MRI) and X-ray imaging, substantially enhancing image contrast and resolution¹¹⁶. This, in turn, facilitates the early detection and precise localisation of lesions and abnormalities within the body. Thus, metal nanoparticles are not only revolutionising biosensing but also contributing significantly to the advancement of biomedical imaging and disease management.

1.3.2 Size clearance consideration for *in vivo* biosensing

In the field of *in vivo* biosensing, noble metal nanoparticles are intentionally designed and introduced into the body. This interaction between the nanoparticles and the

biological environment occurs at various scales during *in vivo* transportation¹¹⁷. The clearance of nanoparticles from the body is typically accomplished through several primary pathways. The rate and manner in which these particles are cleared depend on the nature, size, surface properties of the particles, and the physiological conditions within the body. Initially, many nanoparticles are eliminated from the body through the liver¹¹⁸. The liver, being the principal metabolic organ capable of recognising and processing foreign invaders, plays an important role in this process. These nanoparticles may be sequestered by liver phagocytes, known as Kupffer cells, and subsequently broken down or transported into bile¹¹⁹. Eventually, these particles may be excreted into the small intestine and expelled from the body through faeces. In most cases, nanoparticles are ultimately cleared by the organs of the reticuloendothelial system (RES) or by the kidneys.

Smaller nanoparticles, typically measuring less than 5.5 nm and often referred to as nanoclusters, have the ability to pass through the glomerulus and enter the renal tubules^{19,120}. Within the renal tubules, they can either be reabsorbed back into the bloodstream or excreted in the urine, depending on factors such as their particle size, surface properties, and the presence of specific binding molecules, such as a polymer coating. Compared to the RES¹²¹, the renal clearance pathway offers a potential advantage in terms of reduced toxicity for small nanoparticles. As mentioned above, large nanoparticles tend to accumulate within the RES over the long term, whereas small nanoparticles can filter through the kidneys and be eliminated in the urine in as little as 1 h. This rapid filtration of small nanoparticles through the kidneys is facilitated by their small size, enabling them to pass through the filtration membrane of the glomerulus and enter the primary urine. The nanoparticles are also required to be

resistant to serum protein absorption. Renal clearable catalytic AuNCs were studied for early detection of cancer *in vivo*¹⁷, in which the catalytic ultrasmall nanoclusters around 1.5 nm in diameter could be filtered through the kidneys into the urine for subsequent analysis. Small nanoparticles also tend to exhibit good biocompatibility, making their use in the body relatively safe. This quality makes them a favourable choice for various applications, including drug delivery and biomedical imaging.

1.3.3 Enzyme-like activities of metal nanoparticles for biosensing

Catalysts play a crucial role in enhancing chemical reactions by reducing the activation energy, thereby increasing the reaction rate. In various biological immunoassay configurations, natural enzymes are employed to achieve sensitive analyte detection through amplification. However, natural enzymes come with several limitations, including high costs, low stability in harsh environments, and demanding storage requirements. Moreover, their utility at the PoC is hindered by their susceptibility to denaturation caused by proteases. To overcome these challenges, artificial enzymes and other enzyme-like nanomaterials have been developed. These materials mimic and replicate the fundamental properties and principles of natural enzymes while offering distinct advantages such as affordability, high stability, and scalability for mass production^{122,123}. One exciting category of signal amplification components in biosensing is enzyme-mimetic nanoparticles, often referred to as nanozymes. Additionally, even smaller-sized catalytically active nanoclusters have been explored for their potential in this context. This innovative approach represents a promising avenue for biosensing, combining the benefits of natural enzymatic activity with the enhanced properties of engineered nanomaterials^{124–126}.

Nanozymes have emerged as fascinating materials that can be purposefully engineered to replicate the catalytic functions of biological enzymes (such as peroxidase, oxidase, and hydrolase). Notably, these nanozymes possess substantially enhanced thermal and chemical stability when compared to their natural counterparts^{127,128}. Typically composed of noble or transition metals within the size range of 1 to 100 nanometers, nanozymes find diverse applications in biosensing and therapeutic fields. The catalytic activities of nanozymes can be precisely modulated by factors such as pH, hydrogen peroxide levels, glutathione (GSH) concentrations, and the oxygenation status of various microenvironments, including tumours. Nanozymes demonstrating superior catalytic performance are poised to revolutionize diagnostics and therapeutics. An early milestone in this realm was the discovery of ferromagnetic iron oxide nanoparticles in 2007, which exhibited peroxidase-like catalytic activity by converting colourless peroxidase substrates, such as 3,3',5,5'-Tetramethylbenzidine (TMB), 3,3' Diaminobenzidine (DAB) and o-phenylenediamine dihydrochloride (OPD), into their respective coloured products in the presence of H₂O₂ (Figure 1.7)¹²⁹. Fe₃O₄ MNP substitute for HRP as label for an immunoassay, and show a good stability in various temperatures, pH levels and ease of recycling.

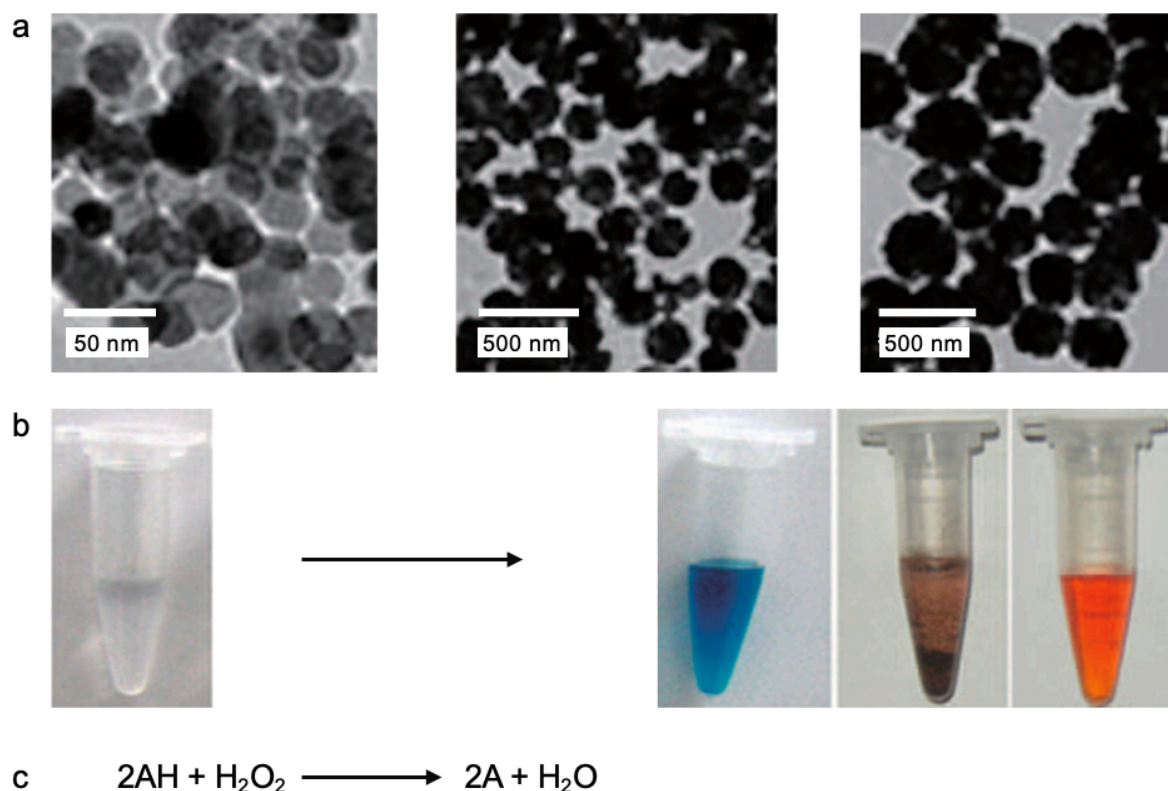


Figure 1.7 Fe₃O₄ MNPs exhibited peroxidase-like properties. (a) TEM images of Fe₃O₄ MNPs with different sizes. (b) The oxidation catalysis of various peroxidase substrates by Fe₃O₄ MNPs in the presence of H₂O₂ resulting in different colour changes. (c) The catalysis mechanism of by Fe₃O₄ MNPs. AH refers to the substrate as hydrogen donor (This figure was adapted from Ref. 129 with permission)¹²⁹.

Building upon this pioneering work, extensive research has focused on iron oxide-based peroxidase-mimicking nanozymes^{130–132}, as well as other materials like iron chalcogenides/phosphates and Prussian blue (PB) nanozymes. These nanozymes exhibit peroxidase-like properties, enabling them to facilitate the hydrolysis of hydrogen peroxide into water and catalyse the oxidation of colourimetric substrates. These remarkable properties have paved the way for numerous applications in magnetic resonance imaging, biosensing, and cancer therapy. In subsequent years, a wide array of peroxidase-like nanozymes were developed. These include vanadium-based mimics, as well as noble metal-based nanomaterials such as gold (Au), silver

(Ag), platinum (Pt), palladium (Pd), and multi-metallic nanoparticles, all of which have been extensively studied and employed in biosensors and therapeutic applications. Carbon-based nanomaterials entered the fray in 2010, with discoveries of their peroxidase-like capabilities¹³³. This development was followed by the exploration of other carbon-based materials, including graphene oxides, single-walled carbon nanotubes, carbon dots, and carbon nitride^{134,135}. Additionally, metal-organic frameworks (MOFs) have emerged as another class of nanozymes, leveraging their porous structures for biomedical applications¹³⁶.

Typically, metal nanoclusters are smaller nanoparticles, consisting of a metal core with a ligand shell, with the typical size of 2 nm in diameter (less than 5.5 nm) within kidney filtration threshold which is beneficial to the rapid *in vivo* biosensing application¹³⁷. Unlike the SPR absorption of large gold nanoparticles, gold nanoclusters exhibit good fluorescence, molecular-like properties and catalytic activity similar to natural enzymes because of their discrete electronic state owing to their ultrasmall size. Their atomically precise structure can provide a more detailed investigation on the catalytic performance at atomic level, revealing the relationship between the performance with their structures. Extensive research has demonstrated their potential in drug delivery^{138,139}, photothermal therapy¹⁴⁰, bioimaging^{141,142}, and biosensing^{143,144}, attributed to their photostability, specific recognition capabilities, small size, high surface-to-volume ratio, and strong absorbance in the near-infrared (NIR) region.

The use of gold nanoclusters as nanozymes has been investigated in biosensing applications to provide signal amplification to significantly improve diagnostic sensitivity¹⁷. Gold nanoclusters functionalised with biotinylated peptides, further

tethered to a protein carrier, were released and filtered into urine for a colourimetric readout after the cleavage of the peptide by an enzyme. The colourimetric readout was realised by the catalysis of the oxidation of TMB by hydrogen peroxide under the catalytic gold nanoclusters for signal amplification. Free hydroxyl radicals were generated through the decomposition of H_2O_2 , because H_2O_2 was not stable when exposed to gold nanoclusters with high catalytic activity. The surface of gold nanoclusters could facilitate to catalyse H_2O_2 for fastened decomposition. Generated free hydroxyl radicals on the surface of nanocluster can catalyse the oxidation of chromogenic substrate, like TMB. Colourless TMB can be oxidised with a blue colour change in the presence of H_2O_2 with catalytic nanomaterials, resulting a simple colourimetric readout (Figure 1.8). The colour change also represents the catalytic activity of nanocluster, which is advantageous for their applications for biosensing. Enhancing the intrinsic catalytic activity is attainable by optimising the synthesis conditions and keeping the size below 5 nm^{145,146}, which can significantly enhance the effectiveness of the gold nanocluster-based *in vivo* disease monitoring sensing system¹⁴⁷.

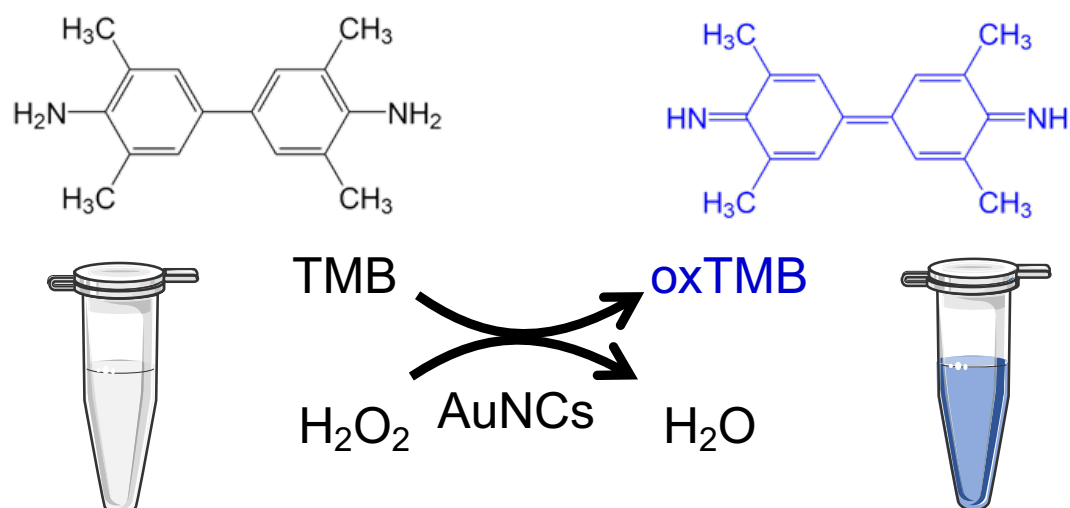


Figure 1.8 Schematic of oxidation of TMB. (AuNCs catalyse the decomposition of H_2O_2 , which can oxidise colourless TMB into blue oxTMB to form a readout signal.)

1.3.4 Peptide templated nanoclusters

Numerous methods are employed to functionalise nanoclusters for biosensing applications, each offering unique advantages. One particularly effective approach involves the templating of particle formation through the use of specific protease-cleavable peptides. In this technique, AuNCs, comprising clusters of precise atoms, are capped and templated by the peptides integrated during their synthesis. Jie Zheng and their dedicated team have made significant strides in advancing this field. They have not only showcased the remarkable biocompatibility of metal nanoclusters, protected by GSH ligands^{148,149}, but have also demonstrated their exceptional efficiency in renal clearance^{150,151}. These findings have paved the way for a wide spectrum of *in vivo* bioimaging applications, where the properties of these nanomaterials are harnessed to enhance the understanding of biological processes and facilitate early disease detection. Such innovative applications underscore the immense potential of peptide-templated nanoclusters in the realm of biosensing and medical diagnostics^{117,152–156}.

In 2019, the Stevens research group demonstrated a biosensor based on the utilisation of AuNCs for the early detection of cancer (Figure 1.9)¹⁷. This achievement marked a significant milestone in the field of rapid diagnostics. The synthesis process at the heart of this innovation relies on the reduction of gold chloride by GSH, a tripeptide featuring a thiol group. GSH plays a dual role as both a capping ligand and a reducing agent, orchestrating the transformation of Au^{3+} to Au^0 , leading to the formation of AuNCs. This chemical transformation is elegantly captured by the following equation:

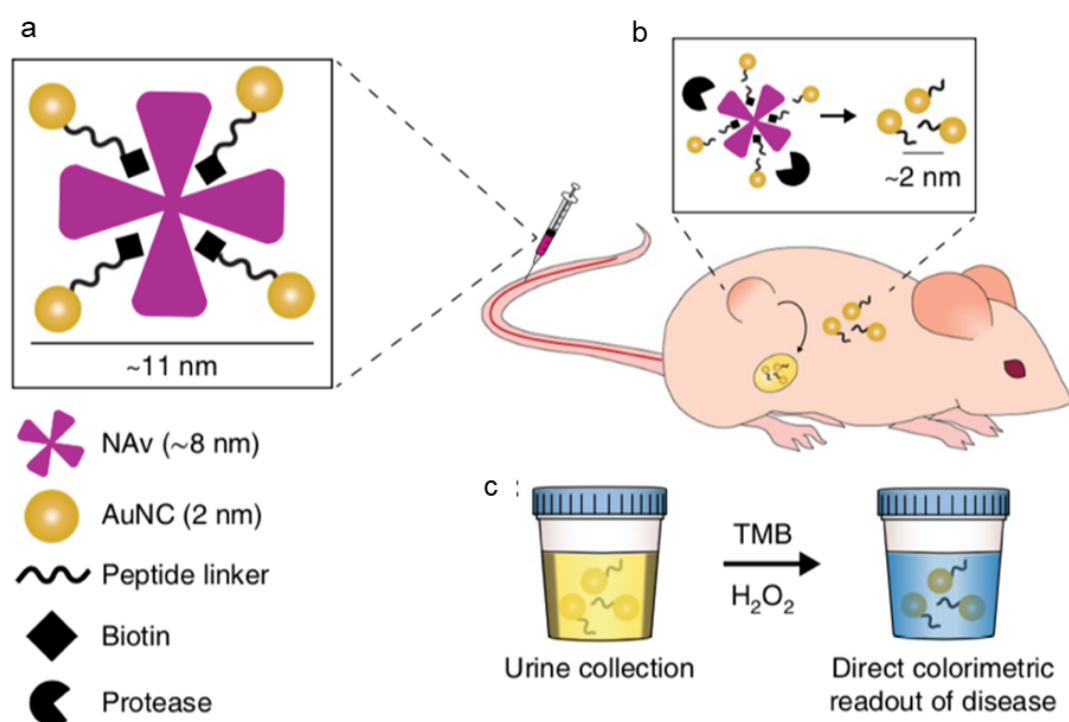
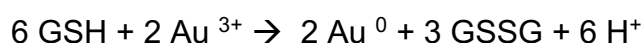


Figure 1.9 An overall schematic of a new design of an amplified sensing system by using nanocatalysts. (a) A nanosensor consists of catalytic gold nanoparticles tethered to a NeutrAvidin protein carrier. (b) Disassembly of nanosensors after arriving at the disease site. (c) A coloured signal readout by the detection of cleared AuNCs in collected urine by measuring their ability to oxidise TMB in the presence of hydrogen peroxide (This figure was adapted from Ref. 17 with the permission)¹⁷.

In this work, both GSH and protease-cleavable peptide linkers were used to template AuNCs, which were subsequently tethered to a protein carrier, NeutrAvidin (NAv) to construct a biosensor. The peptide substrate contains a cysteine with a thiol group and a biotin which can facilitate the connection between catalytic AuNCs and NAv. The choice of NAv as a protein carrier stems from its biocompatibility and its high affinity for biotin, coupled with low levels of non-specific binding properties. Prior to cleavage, this nanosensor exceeded the renal clearance threshold (approximately 11 nm). However, following cleavage by the target protease, small AuNCs were liberated and could traverse the kidney into the urine for subsequent analysis.

The cleavable peptide linkers employed in this study include a thrombin peptide (P1) and a MMP-9 peptide (P2). These two peptide linkers confer specificity to the sensor, enabling it to target the respective proteases, thrombin or MMP-9, which are pivotal in cardiovascular diseases and cancer, respectively. Typically, encountering a disease site triggers the disassembly of the nanosensor through the cleavage of the specific peptide linkers. Subsequently, the small catalytic AuNCs are released and filtered through kidneys into the urine, where they catalyse the oxidation of TMB, resulting in a distinct blue-coloured oxidised TMB. A simple colour change in the urine serves as an indicator of the disparity between mice with tumours and healthy ones. Detecting catalytic AuNCs in the urine directly correlates with protease activity, thereby enabling disease detection. In this paper, they demonstrate the functionality of the MMP-9 responsive nanosensor both *in vitro* and *in vivo* using a mouse model of colorectal cancer. This approach enables the rapid detection of cancer with a straightforward colourimetric readout. This current nanosensor represents the cutting edge in point-of-care *in vivo* biosensing.

1.4 Scope of the thesis

Chapter 1: This chapter introduces the latest advances and technology applications in the field of disease diagnosis. Among them, laboratory-based and point-of-care diagnostic methods are introduced, as well as the importance of biosensors in diagnosis. Subsequently, the concept of biomarkers and their role in disease detection were focused on, especially the correlation between the activities of proteases and lipases and diseases. Moreover, the application of gold nanoparticles and nanocluster as *in vivo* biosensors is discussed, focusing on key factors such as their size, activity, and renal clearance. In summary, this chapter focuses on disease diagnosis and metal nanocluster-based biosensing technology, providing new ideas and directions for research in this field.

Chapter 2: The primary goal of this chapter is to improve the sensitivity of the biosensor based on our group previous work by optimising the signal amplification catalytic activity of nanoclusters while ensuring that they maintain the size of renal clearance, and to guarantee renal clearance when sensing *in vivo*. This goal aims to address a common problem in current biosensing technologies about how to ensure the safety and clearance of materials *in vivo* while increasing sensitivity.

Chapter 3: The goal in this chapter is to develop an enzyme-responsive biosensor for rapid and sensitive detection of malaria infection. Although the incidence of malaria is low in some developed countries, malaria remains a major global health challenge in developing countries. By introducing an enzyme response mechanism, this sensor is

expected to play an important role in the early diagnosis and surveillance of malaria, thereby helping to treat and control malaria outbreaks in a timely manner.

Chapter 4: This chapter involves encapsulating nanoclusters in liposomes to create toxin-responsive biosensors for detecting bacterial infections. This innovative biosensor has the potential to revolutionise the way bacterial infections are diagnosed and monitored. By combining the advantages of nanotechnology and liposomal carriers, this biosensor, consisting of catalytic AuNC in liposome, disassembles under the condition bacterial infection releasing AuNCs for detection. This liposomal sensing platform may have higher sensitivity, faster response time, and better adaptability, thus opening up new possibilities for early diagnosis and treatment of bacterial infections.

Chapter 5: This Chapter 5 firstly states the motivation and aims of this thesis. It subsequently summarizes the main findings for each main chapter, and finally concludes with an outlook and plans for future work.

Chapter 2

Optimisation of catalytic activity of nanoclusters with peroxidase-like activity

2 Optimisation of catalytic activity of nanoclusters with peroxidase-like activity

2.1 Aims

This chapter mainly builds on the previous research on gold nanoclusters (AuNCs) from our group. Due to the insufficient sensitivity of the nanozyme AuNCs studied previously, the goal here is to optimise the peroxidase-like catalytic activity of AuNCs by studying synthesis conditions to improve the sensitivity of biosensors designed based on AuNCs. Here another aim is to ensure that the size AuNC are controlled to be able renally clearable through multiple characterisation methods, while improving their catalytic performance to improve detection sensitivity. After optimising the catalytic performance of gold nanoclusters, a thrombin-responsive biosensor from previous can be used to verify whether the detection sensitivity of this sensing system is improved.

2.2 Introduction

Much attention has been attached to research on the application of metal nanoparticles/clusters in biosensing. Such applications take advantage of their fascinating properties, including high stability for reaction catalysis, a large enough surface for functionalisation and modification, good biocompatibility and optical properties. With these useful sensing properties, metal nanoparticles/clusters have become widely used in the design of biosensors with high sensitivity to detect and quantify different biomolecules and ions, whilst realising significant signal amplification.

2.2.1 Nanoparticles with enzyme-like activities

Both inorganic catalysts and biological enzymes facilitate chemical reactions by lowering the activation energy, thus increasing the rate of reaction, allowing it to proceed at reduced temperatures and pressures. Biological enzymes can be used in a variety of immunoassay configurations for amplification to enable sensitive analyte detection. Due to their catalytic activity and substrate specificity, biological enzymes have been extensively adopted in industrial, medical and biological fields, especially for sensing purposes. One of the most widely used enzymes for such applications is horseradish peroxidase (HRP), which catalyses the oxidation of specific substrates, such as 3,3',5,5'-Tetramethylbenzidine (TMB), when in the presence of H_2O_2 , resulting in the formation of oxidised blue TMB^{17,157,158}. However, there are some inherent disadvantages of biological enzymes, including high manufacturing cost, relatively low chemical and physical stability, and difficulty of recycling¹⁵⁹. Particularly, enzymes suffer from instability in harsh environments such as varying pH and high temperatures, and are susceptible to denaturation by proteases, complicating their application in medicine. To surmount these shortcomings, researchers have been exploring inorganic metallic nanomaterials as artificial enzyme mimics, for over a decade.

In 2007, Yan and coworkers¹²⁹ discovered that ferromagnetic nanoparticles (NPs) exhibited HRP-like activity. Nanomaterials functioning as biomimetic enzymes have aroused great interest and attention, with more and more research on artificial enzymes continuing to emerge over the past decade^{124,160,161}. These artificial enzymes are termed as 'nanozymes', which are based on nanomaterials with characteristics enzyme-like catalytic activity. Nanomaterials are engineered to effectively mimic the function, catalytic site or structures of biological enzymes, achieving e.g. peroxidase-,

oxidase-, hydrolase-, glucose oxidase-, and catalase-like activities¹²⁷. Alternatively, the nanomaterials can contain multivalent elements for reactions themselves, which can result in a nanozyme system that can replace traditional natural enzymes for catalysis. As the catalytic activity of the nanozymes are closely connected to their physiochemical properties, Yan *et al.*¹⁶² developed a standardised protocol to define nanozyme catalytic activity comparing different nanozyme formulations (Figure 2.1).

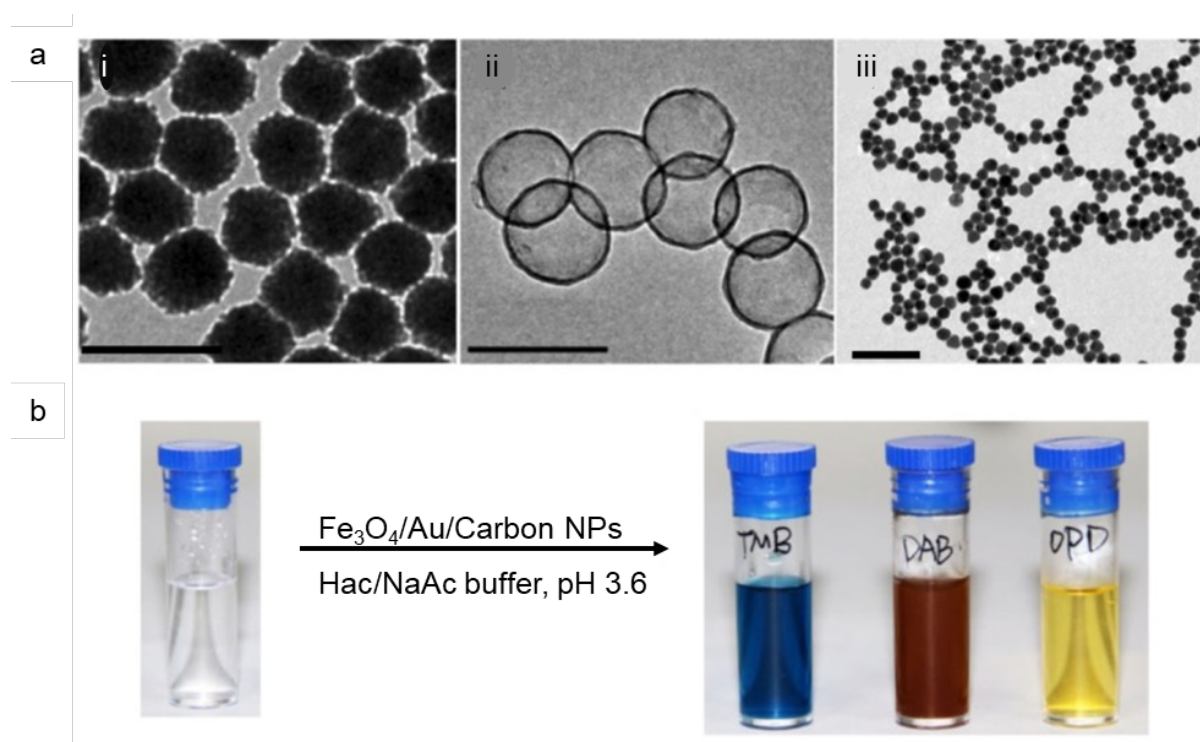


Figure 2.1 Nanozyme examples. (a) TEM images of three typical peroxidase-mimicking nanozymes: (i) Fe₃O₄, (ii) carbon, and (iii) AuNPs. Scale bars represent 200 nm, 200 nm and 100 nm, respectively. (b) Fe₃O₄, carbon and AuNPs show peroxidase-like activity and catalyse the oxidation of peroxidase substrates (TMB, diaminobenzidine (DAB) and o- phenylenediamine (OPD)) in the presence of H₂O₂ to produce colourimetric reactions (This figure was adapted from Ref. 162 with permission)¹⁶².

In consideration of both the significant role and the inherent shortcomings of biological enzymes, their substitutes, i.e. nanozymes, have been actively explored. Nanozymes can be mainly divided into two categories: (i) the oxidoreductase family which

catalyses the oxidation of substrates using hydrogen peroxide as an electron acceptor and (ii) the hydrolase family that catalyses hydrolysis reactions by using water to break a chemical bond. A wide range of enzyme-mimetic properties can be realised by designing nanomaterials with different elemental compositions (e.g. gold, platinum, palladium, iron oxide, *etc.*). Considering their diverse enzyme-mimetic activities, nanozymes have been used in a variety of applications, such as optical imaging¹⁶³, environmental treatment^{164,165}, drug delivery^{166,167}, antibacterial agents^{128,168–170}, cytoprotection^{171–173}, and biosensing^{174–180}. These applications include examples that require *in vivo* administration of nanoparticles. Since this thesis is focused on *in vivo* biosensing, nanozyme size is a key consideration.

2.2.2 Nanozyme size considerations for *in vivo* biosensing

As engineered nanoparticles are introduced into the body, the interaction of nanoparticles and their bioenvironment occurs at each step throughout their *in vivo* transport¹²⁰. Next, nanoparticles are often cleared out of the blood circulation by organs of the reticuloendothelial system (RES) or the kidneys. Blood filtration and waste elimination both rely on the kidneys. There is a lower potential toxicity of nanoparticles in renal clearance pathway compared with RES¹⁸¹. Nanomaterials can remain accumulated in the RES system for long times, increasing the potential for side effects, while it takes much less time (about 1 h) to filter through small nanoparticles into the urine via the kidneys. Transport and subsequent clearance of nanoparticles *in vivo*³² can take the advantage of the fact that the kidney is a good-size filter¹⁸². The kidneys can filter nanoparticles with diameters less than about 5 nm from blood into the urine^{17,19,20,183}. The design of nanoparticles, including their size, shape and surface

chemistry, has great impact on their excretion pathway. Nanoparticles can be designed to be renally clearable by reducing their size to be below the kidney filtration threshold and improving their stability in physiological conditions. The nanoparticles are also required to be resistant to serum protein absorption. This has been achieved by e.g. using GSH as zwitterionic, protein-repellent capping ligands on the nanoclusters^{17,139,184,185}. In the case of nanozymes, renal clearable catalytic AuNCs were studied for early detection of cancer *in vivo*¹⁷. In this study, catalytic ultrasmall nanoclusters of around 1.5 nm in diameter could efficiently be filtered through the kidneys into the urine for subsequent analysis. Hence, ensuring a small hydrodynamic size of below 5 nm, whilst minimising protein adsorption, are key to allow nanomaterial excretion through the kidneys.

2.2.3 Enzyme-like nanoclusters in biosensing

Typically, metal nanoclusters are smaller nanoparticles, composed of only ten to hundreds of atoms. These nanoclusters are typically less than 2 nm in diameter. Nanoclusters have unique physiochemical properties, distinct from larger nanoparticles and can be synthesised to be capped with various ligands, which enables them to be extraordinarily stable in harsh physiological environments. Moreover, nanoclusters can be produced in a wide range of precisely controlled sizes and shapes. The intrinsic catalytic activity of some of these nanoclusters can be advantageous for their application in biosensing. This catalytic activity can be further improved by optimising the synthesis conditions and ensuring that their size remains susceptible to renal clearance (Figure 2.5). The surfaces of nanoclusters can be modified and functionalised with different biomolecules, including peptide linkers, proteins, and nucleic acids, which can be applied to biosensing. For instance, a

peptide linker to a larger carrier above the kidney filtration size can be cleaved by a specific protease to release this type of nanomaterial for urinary biosensing¹⁷. Certain noble and transition metal nanomaterials have intrinsic peroxidase-like activity, which can oxidise both inorganic and organic compounds (such as TMB)¹⁸⁶ in the presence of hydrogen peroxide. AuNCs catalyse the decomposition of H₂O₂, which can oxidise colourless TMB into blue oxTMB to form a readout signal. Hydrogen peroxide is unstable and decomposes to generate hydroxyl radicals when exposed to catalytic metal nanoclusters. It is the surface of the nanoparticle that helps to catalyse the decomposition of hydrogen peroxide. Hydroxyl radicals produced on the surface of nanoparticles can facilitate the oxidation of a chromogenic substrate, like TMB^{187–189} (Figure 1.8). Therefore, it was hypothesised herein that AuNC catalytic activity could be fine-tuned by modifying the synthesis procedure.

2.3 Materials and methods

2.3.1 Materials

Gold chloride trihydrate (HAuCl_4), chloroplatinic acid hexahydrate (H_2PtCl_6), L-glutathione reduced (GSH), sodium borohydride (NaBH_4), sodium hydroxide (NaOH), Ethanol (EtOH) 30% (w:w) hydrogen peroxide solution (H_2O_2), Sephacryl® 200-HR, MW range 5-250 kDa (globular proteins), MW range 1-80 kDa (dextrans), Gold Standard for ICP, TraceCERT(R), 1000 mg/L Au in hydrochloric acid and Thrombin from human plasma were purchased from Sigma-Aldrich. Dulbecco's phosphate buffered saline (DPBS), NeutrAvidin Protein, Pierce™ Biotin Quantitation Kit, 3,3',5,5'-tetramethylbenzidine (1-Step™ Ultra TMB-ELISA Substrate Solution), and GlutaMAX (Gibco, 31966-021) were purchased from Thermo Fisher Scientific. Milli-Q water (18.2MΩ.cm) was used in all experiment.

2.3.2 Synthesis of ligand-capped nanoclusters in different conditions

The protocol for the synthesis and purification of nanoclusters was adapted from literature¹⁷. Many parameters for the reaction conditions were adjusted for the nanocluster synthesis to optimise the catalytic activity and retain the ultra-small size (Table 2.1). Various ratios of metal to ligand with different reducing agents were chosen to get ligand-capped nanoclusters (1:0.5, 1:0.8, 1:1, 1:1.5 and 1:2). Typically, solutions of HAuCl_4 or H_2PtCl_6 (20 mM in deionised water, 50 μL) were prepared in an Eppendorf tube, which was followed by quick addition of the reducing agent GSH (5 mM, 200 to 400 μL depending on the metal-to-GSH ratios) corresponding to the weak

reducing condition. Additional NaBH_4 (143mM, 20 μL) was added to yield the strong reducing condition. The whole reaction volume was topped up to 1 mL with H_2O . Subsequently, the weak reducing condition mixture was heated up to 70 °C, while the mixture of the strong condition was kept at room temperature (Figure 2.5). Here, solvent from aqueous to organic (ethanol) or inorganic (NaOH) was also used to investigate the influence on the final catalytic activity of nanoparticles. Here, another parameter was additionally employed for the initial reaction agents in the synthesis of AuNCs, as per the literature¹⁹⁰, to generate nanoclusters (denoted as ZP) at both 70 °C and 90 °C for comparative analysis. A thermomixer at gentle shaking speed of 500 r.p.m. was used and the reactions were allowed to continue for 24 h (weak reducing condition) and 2-3 h (strong reducing condition), respectively. Different reaction temperatures were evaluated for the synthesis in weak reducing condition, varying from 37 °C, 50 °C, 70 °C to 90 °C. The pH of the reaction was also varied from 2 to 10, by using NaOH and HCl for the adjustment. After the 24 h reduction reaction, nanoclusters were purified, and buffer exchanged with DPBS (pH 7.4) using Amicon Ultra-15 Centrifugal Filter Units (MWCO 10 kDa) at 5000 g and 15 min for three times¹⁷. Finally, the AuNCs in the concentrate were suspended in DPBS to yield 20 μM of nanoparticles (concentration given based on a previous study¹⁷) and subsequently transferred and filtered into a new Eppendorf tube using a sterile syringe filter (0.22 μm Millex-GV Filter) to remove any aggregates and sterilise the sample.

2.3.3 Analysis of nanocluster catalytic activity in physiological environments

For the study of catalytic activity of AuNCs in physiological environments, 50 μL of AuNCs (20 μM) was separately added into 50 μL of either DPBS, fetal bovine serum

(FBS) (Gibco), or synthetic urine (Surine Negative Urine Control) and then incubated at 37 °C for 1 h and 12 h. After incubation, the catalytic activity of AuNCs in each incubation solution was measured. For this, 10 µL of each sample was added into a 96-well plate (Corning), followed by an addition of 100 µL of 1-Step Ultra TMB ELISA Substrate Solution with hydrogen peroxide (final 2M H₂O₂) adjusted to a final concentration of 1 M. The absorbance of the reaction solution at 652 nm for each sample was recorded on a SpectraMax M5 plate reader immediately after mixing the sample with TMB and H₂O₂, and measurements were taken over 10 min to capture the kinetics of TMB oxidation by H₂O₂ with different catalytic activity of AuNCs. The catalytic activity retained before and after the incubation within different physiological environments were calculated and converted into the retained percentage. Each synthesis of different AuNCs and the catalytic activity measurements were repeated three times.

2.3.4 Optimisation of feed ratio GSH-to-peptide

To obtain functionalised AuNCs, a protease-cleavable peptide substrate was used in combination with GSH during the synthesis process. This allowed for subsequent connection to a protein carrier, NeutrAvidin (NAv), to form a biosensor. GSH served as a reducing agent to reduce HAuCl₄, and an appropriate peptide served as a linker to connect AuNCs and NAv. The feed ratio in the synthesis of AuNCs referred to the ratio of GSH to the peptide. In this study, a thrombin cleavable peptide was used to verify the cleavage efficiency¹⁷. The peptide sequence had biotin at one end and a cysteine containing a thiol at the other end, creating the sequence biotin-SGGfPR↓SGGSGGC (S1), where the arrow indicates the thrombin cleavage point. This peptide (S1) was sourced from the batch utilised in the previously published

study¹⁷. Biotin was used to conjugate AuNCs to NAv, and thiol-containing cysteine was utilised to couple the peptide to the gold surface^{191,192}. Different ratios of GSH to the thrombin peptide linker S1 were selected, namely 2:1, 5:1, 9:1, and 14:1. For typical 1 mL of reaction solution, 100 μ L of 20 mM HAuCl₄ was added into 750 μ L of H₂O, with a fast addition of 150 μ L (20 mM) GSH for non-peptide addition. According to the feed ratio of GSH-to-peptide S1, the volume of peptide (20 mM) was adjusted to meet the requirement, eg. 50 μ L (20 mM) peptide S1 and 100 μ L (20mM) GSH into the reaction solution. After producing and purifying AuNCs with these various feed ratios, a TMB oxidation assay was performed on each sample, measuring the absorbance at 652 nm (the oxidation of TMB) using a SpectraMax M5 plate reader. This measurement allowed for the determination of the catalytic activity of AuNCs in each sample.

2.3.5 Ligand exchange method for alternative AuNC synthesis

Instead of adding GSH and the sensor peptide at the same time, here, GSH AuNCs were formed first followed by ligand exchange by adding the peptide S1 in the next step. AuNCs were synthesised through the reduction of HAuCl₄ (20 mM, 100 μ L) with GSH (20 mM, 150 μ L) at the optimum pH 10 (using 1 M NaOH) and were then topped up with H₂O to a final reaction solution of 1 mL. After the AuNCs synthesis, a ligand exchange method was employed to cap peptides onto the AuNCs. This involved adding the desired amount of peptide to the crude AuNCs, following different feed ratios, and incubating the mixture at 70 °C for 24 h (Figure 2.17). After the reaction, nanoclusters were purified, and buffer exchanged with DPBS (pH 7.4) using the same

method as described earlier (Section 2.3.2) for the subsequent complex assembly (Section 2.3.7).

2.3.6 Biotin quantitation by using HABA/Avidin Premix

4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay was performed on each sample using Pierce™ Biotin Quantitation Kit, which was used for the quantification of the number of biotin ligands per AuNC (peptide per AuNC). The crude peptide templated AuNCs (500 µL) were loaded in Amicon Ultra 0.5 mL centrifugal filter (MWCO 10 kDa) and centrifuged at 14000g for 5 min, followed by filtrate collection containing the unbound peptides with biotins. After equilibrating the HABA/Avidin Premix at room temperature for 10 min, 100 µL of H₂O was added into a microtube of the reagent to get a homogeneous mixture. 160 µL of DPBS was added into a 96-well plate, followed by the addition of 20 µL of prepared HABA/Avidin Premix solution. Placed the plate on a plate reader, shaking for a few seconds. The absorbance of the solution was recorded at 500 nm as A₅₀₀-HABA/Avidin. 20 µL of the above obtained filtrate was added into the above pre-measured well plate. After shaking for a few seconds, the solution was measured again at 500 nm and values were recorded as A₅₀₀-Biotin. Finally, the data was processed based on the Beer Lambert Law equation.

$$A_{\lambda} = \varepsilon_{\lambda} b C$$

Where A is the wavelength of HABA at 500 nm, ε refers to the extinction coefficient, for HABA/Avidin at 500 nm at pH 7.0, b is the path length of the well plate (0.5 cm), and C represents the concentration of the measured sample (mmol/mL). Once the unbonded peptide-biotin in the filtrate was measured, the biotin concentration on the clusters was calculated by subtracting the unbound peptide-biotin from the initial

concentration of peptide-biotin used in the synthesis. AuNC-S1 with various feed ratios were further measured by TMB assay for the catalytic activity comparison.

2.3.7 Assembly of AuNC-NAv complex as a thrombin sensor

Briefly, 125 μ L of a protein carrier NeutraAvidin protein (NAv) was prepared in DPBS (8 mg/mL), which was followed by mixing it with 1 mL of the purified AuNC-S1 (20 μ M). The mixture was incubated at 37 °C, under a gentle stirring speed of 500 r.p.m. for 12 h. This was aimed to conjugate AuNC-S1 with NAv through the biotin binding to form a AuNC-NAv complex, namely thrombin sensors. After the incubation for 12 h, thrombin sensors were purified, and buffer exchanged with DPBS (pH 7.4) using Amicon Ultra-15 centrifugal filter units (MWCO 50 kDa) at 5000 g and 15 min at least for 5 times. This was used to remove the unbound AuNC-S1 filtered through membrane into the filtrate, while the thrombin sensors remained in the concentrate (Figure 2.5). The thrombin sensors were finally resuspended in DPBS and transferred to a new Eppendorf tube using sterile syringe filter (0.22 μ m Millex-GV Filter) to remove any aggregates.

2.3.8 Size exclusion chromatography (SEC)

Glass chromatography columns were packed with Sephacryl® 200-HR, which could be used for the separation of samples based on different sizes. The larger sensor complexes were eluted in the initial fractions, while the smaller free AuNCs were eluted more slowly in the later fractions. To perform SEC, the solution sample (500 μ L) was introduced into the column (ca. 30 cm long) and flowed through the resin bed.

Sufficient DPBS was added into the column for fraction collection. Each fraction was collected separately for a subsequent catalytic activity assay to determine the fractions containing the AuNC-NAv complexes and unbound AuNCs. After fraction collection (15 mL), 50 μ L of each diluted fraction was added in a 96-well plate, which was followed by the addition of a mixture of 200 μ L of mixture of TMB 1-Step and H₂O₂ (1:1) (the final concentration of H₂O₂ was 1 M). The absorbance at 652 nm of each sample fraction was measured to determine the catalytic activity of each fraction and the contents in the fractions. Dynamic light scattering (DLS) was operated to further determine which fractions contained the larger AuNC-NAv complexes and where the unbound free AuNCs eluted.

2.3.9 Characterisation of catalytic AuNCs and AuNC-NAv complex

SpectraMax M5 multimode microplate reader was used to measure the kinetics reaction (absorbance vs. time) of TMB oxidation by AuNCs/AuNCs-NAv, with the aid of SoftMax Pro software. The hydrodynamic diameters of nanoclusters and complexes were measured by using dynamic light scattering (DLS, Zeta Sizer Nanoseries, Malvern Instruments, Ltd). The optical properties and concentration of nanoclusters were obtained via Ultraviolet-visible (UV-vis) measurements on a SpectraMax M5 multimode microplate reader. Transmission electron microscopy (TEM) images were obtained on a JEOL 2100F. To prepare TEM samples, AuNCs solution needed to be desalted first with Zeba Spin Desalting Columns (7K MWCO) and 5 μ L of the sample was dropped on a carbon-coated copper grid and dried overnight.

2.3.10 *In vitro* thrombin cleavage assay

To study the sensitivity of AuNC-NAv nanosensors with respect to thrombin concentration *in vitro*, a range of thrombin concentrations (from 0 nM to 100 nM in PBS, Thrombin from human plasma was purchased from Sigma-Aldrich) were freshly prepared by the dilution of the enzyme stock. For each concentration, 50 μ L of purified AuNC-NAv nanosensor complex was mixed with 50 μ L of the freshly prepared thrombin aliquot, and the mixture was incubated at 37 °C for 4 h and 12 h. Following the incubation, 100 μ L of DPBS was introduced into the final solution, and subsequent centrifugal ultrafiltration was conducted using Amicon Ultra-0.5mL centrifugal filter units (MWCO 50 kDa) with all filtrates collected for the next assay. Finally, the TMB oxidation assay was carried out on all the filtrates. Each filtrate (50 μ L) was mixed with a solution composed of 1-Step TMB substrate (100 μ L) and H₂O₂ (2 M, 100 μ L). This mixture was employed to measure the absorbance over time at 652 nm and subsequently converted into the reaction velocity to compare the catalytic activity of released AuNCs, thus evaluating the efficiency of the thrombin cleavage. The limit of detection (LoD) was calculated by adding three times the standard deviation to the mean value of blank sample (0 nM thrombin).

2.4 Result and discussion

2.4.1 Optimisation of catalytic nanoclusters by screening reaction conditions

There are many parameters and factors that are needed to be considered for nanocluster synthesis. In pursuit of enhancing their catalytic properties, the synthesis process of nanoclusters was optimised to enhance their catalytic property. A range of conditions were explored, encompassing difference in core metals, core metal-to-thiol ratios, reducing agents, pH levels, and reaction temperatures. Noble metals, including gold, silver, platinum, and palladium, have been extensively researched and tailored for diverse applications such as bioimaging^{193,194}, drug delivery^{193,195} and biosensing^{193,196}. These applications leverage the distinctive properties of metal, encompassing catalytic activity. During this investigation, various metals were selected as potential nanocluster cores, ensuring their sizes remained within the range of the renal clearable threshold (5.5 nm).

Gold (Au) and platinum (Pt) were selected as the core metals of nanocluster synthesis, utilising different metal-to-ligand ratios following published protocols^{197,198} (Table 2.1). The peroxidase-like catalytic activity of nanoclusters was evaluated by monitoring the absorbance at 652 nm, arising from the oxidation of TMB by H₂O₂ catalysed by nanoclusters¹⁹⁹, which was employed for comparing catalytic activity. The assessment involved calculating the onset velocity, which was derived from the initial kinetics linear curve of the absorbance over time. Through comprehensive analysis, gold (Au) nanoclusters were found to exhibit superior catalytic activity compared to platinum (Pt) nanoclusters under various conditions. Notably, nanoclusters synthesised under weak

reducing conditions exhibited a more rapid increase in absorbance over time, revealing their superior catalytic activity (Figure 2.2). For either AuNCs or PtNCs, the kinetic reaction of the TMB assay after synthesising and purifying the nanoclusters under strong reducing conditions (Figure 2.2a) was markedly slower than that observed for synthesis in weak reducing conditions (Figure 2.2b). Meanwhile, a change in solvent from aqueous to organic (ethanol) or inorganic (NaOH) diminished catalytic activity. To facilitate a thorough comparison of catalytic activity, the onset velocities were calculated for each type of nanocluster. AuNCs synthesised under weak reducing conditions with a metal core-to-GSH ratio of 1:1, displayed significantly heightened catalytic activity in comparison to nanoclusters prepared in a previous study from the Stevens group¹⁷, where the metal core-to-GSH ratio of 1:1.5 in weak reducing condition was used for synthesis (Figure 2.2c). Furthermore, the sizes of these nanoclusters were confirmed to remain below the renal clearable threshold of 5.5 nm, which satisfies the size requirement for their potential in future *in vivo* studies (Figure 2.2d).

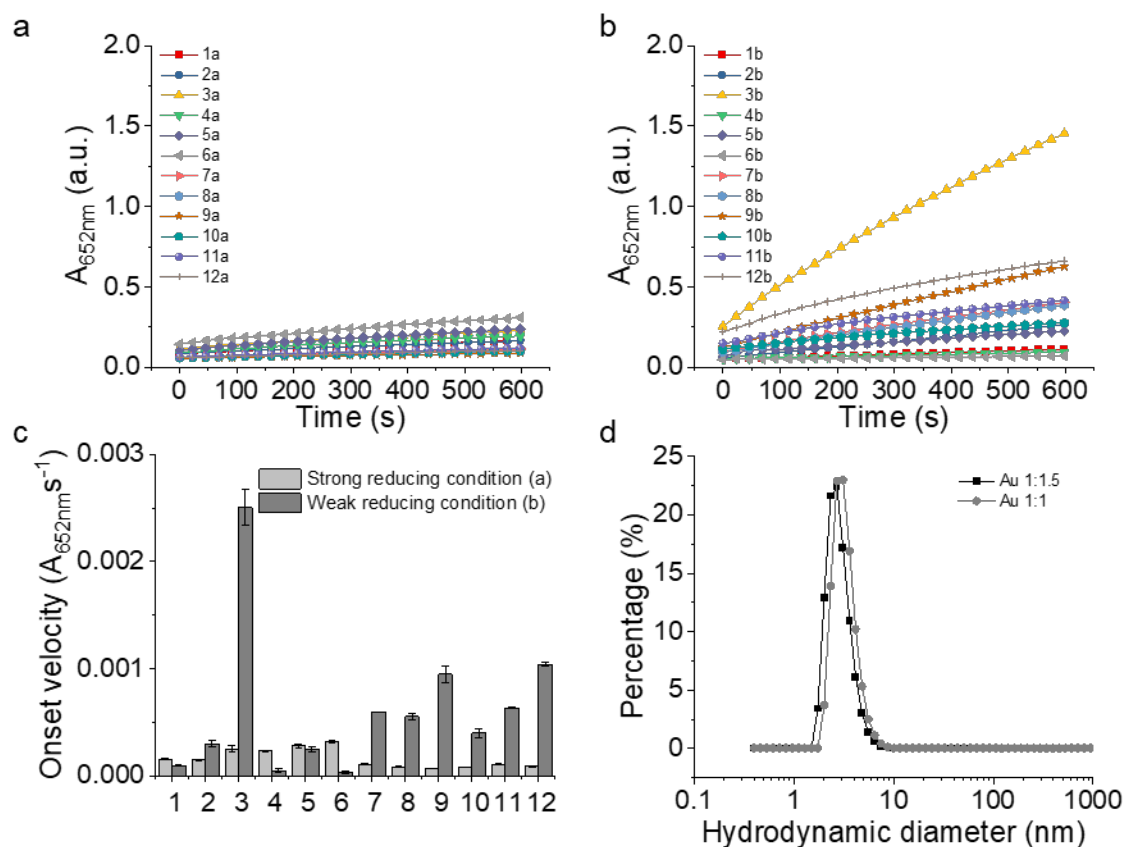


Figure 2.2 Nanocluster synthesis optimisation. (a, b) Absorbance over time measurement of catalytic activity assay (TMB/ H_2O_2 system) using gold and platinum nanoclusters synthesised in different reducing condition for (a) strong reducing condition and (b) weak reducing condition, with 1-12 corresponding to different metal-to-GSH ratios and different addition of ethanol or sodium hydroxide (sample numbers refer to Table 2.1). (c) Onset velocity calculated from (a) and (b) using linear fits ($N = 1$ independent experiment, $n = 2$ technical repeats). (d) Number distribution by dynamic light scattering of AuNCs at the gold-to-GSH ratio of 1:1 and 1:1.5 (two optimised nanocluster versions selected from the screening, curves are mean of $N = 1$ sample, $n = 3$ measurements).

Table 2.1 Synthesis conditions used for the optimisation of catalytic nanoclusters. Conditions varied from different metals, reducing conditions to various ratios of metal-to-ligands, etc.

	Strong reducing condition (a)		Weak reducing condition (b)	
	No.	Au or Pt : Ligand Ratio	No.	Au or Pt : Ligand Ratio
Au NCs (H ₂ O)	1a	1:2	1b	1:2
	2a	1:1.5	2b	1:1.5
	3a	1:1	3b	1:1
Au NCs (EtOH)	4a	1:2	4b	1:2
	5a	1:1.5	5b	1:1.5
	6a	1:1	6b	1:1
Pt NCs (H ₂ O)	7a	1:2	7b	1:2
	8a	1:1.5	8b	1:1.5
	9a	1:1	9b	1:1
Pt NCs (NaOH)	10a	1:2	10b	1:2
	11a	1:1.5	11b	1:1.5
	12a	1:1	12b	1:1

Further optimisation on improving the catalytic activity of nanoclusters was investigated by varying reaction temperatures, metal-to-GSH ratios and concentrations of reaction reagents. Alterations in synthesis temperature and metal-to-GSH ratio exerted an impact on both the size and catalytic activity of the nanoclusters (Figure 2.3). The evaluation of catalytic activity was based on the onset velocity of the kinetic reaction in the TMB oxidation assay. For AuNCs, the catalytic activity exhibited an upward trend with increasing reaction temperature, peaking at an optimal synthesis temperature of 70 °C (Figure 2.3a, c). To explore improved synthesis conditions for AuNCs, various initial reaction agents were employed in the process, following the procedure outlined in literature¹⁹⁰. Nanoclusters ZP synthesised at both 70 °C and 90 °C were included for comparative purposes. Notably, AuNCs featuring an intermediate metal-to-GSH ratio of 1:1 demonstrated relatively heightened catalytic activity. Moreover, achieving sizes below the renal clearance threshold of

approximately 5.5 nm was feasible only at metal-to-GSH ratios of 1:1 and 1:1.5 (Figure 2.3d). The observed relationship between the ratio of metal-to-GSH and size aligns with findings from previous literature, where it was shown that size decreases as the ratio increases²⁰⁰.

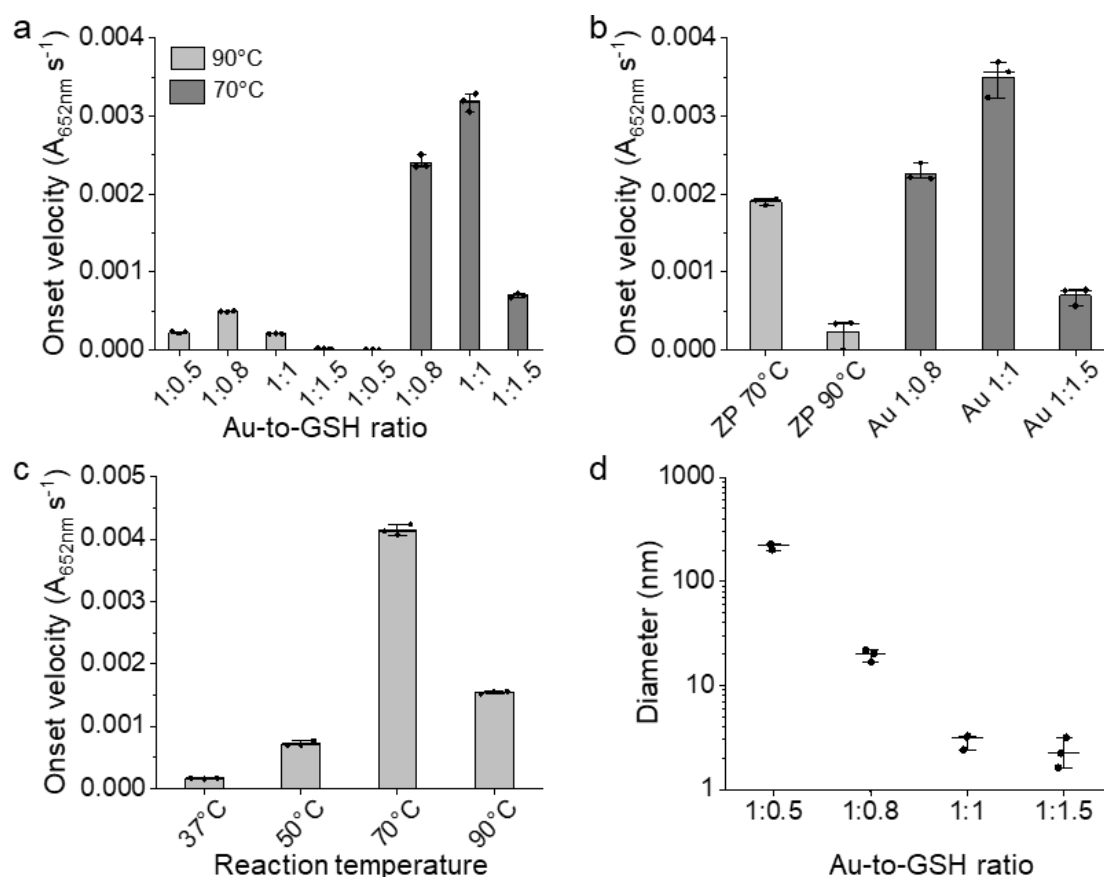


Figure 2.3 Further optimisation of nanocluster synthesis (different gold-to-GSH ratio and temperature). (a) Catalytic activity of different AuNCs synthesised with various Au-to-GSH ratios at 70 °C and 90 °C. AuNCs exhibited higher catalytic activity when synthesised at 70 °C (N = 1 independent experiment, n = 3 technical repeats). (b) Comparison of catalytic activity after using different initial reaction agents for synthesis of nanoclusters (nanoclusters ZP in 70 °C and 90 °C were adapted from literature¹⁹⁰ for comparison) (N = 1 independent experiment, n = 3 technical repeats). (c) The influence of different synthesis reaction temperature on the catalytic activity of AuNCs. 70 °C was the optimal temperature for the synthesis (N = 1 independent experiment, n = 3 technical repeats). (d) Hydrodynamic diameters of AuNCs prepared at different gold-to-GSH ratio to select AuNCs with sizes less than the kidney filtration threshold (5.5 nm) (mean of N = 1 sample, n = 3 measurements).

In the case of these optimised 1:1 and previously used 1:1.5¹⁷ samples, a comparison of their sizes and morphology was conducted using transmission electron microscopy (TEM) and derived histograms (Figure 2.4a-d). They showed an average diameter of 2.7 ± 0.6 nm and 2.2 ± 0.4 nm, respectively — both falling well below the 5.5 nm threshold based on the analysis of the size of 100 nanoclusters each. To investigate their catalytic functionality within different physiological environments, both AuNCs were subjected to incubation in fetal bovine serum (FBS), synthetic urine and DPBS as control, all at 50% v/v after incubation for 1 h and 12 h (Figure 2.4e-f). The catalytic activity was normalised in relation to the activity of AuNCs in DPBS. Notably, post-incubation in FBS and urine, only a marginal 10–20% decrease in catalytic activity was observed. This underscores the retention of over 80% of the catalytic activity, even following a 12-h incubation in physiologically relevant conditions. The assessment of retained catalytic activity unveiled that AuNCs sustained the most catalytic activity even after incubation in physiological environments, suggesting that urine measurements after *in vivo* administration and excretion should remain unaffected. These findings also align with those of a recently published study, demonstrating approximately 90% retention of catalytic activity for a similar AuNC synthesised at ratio 1:1.5²³.

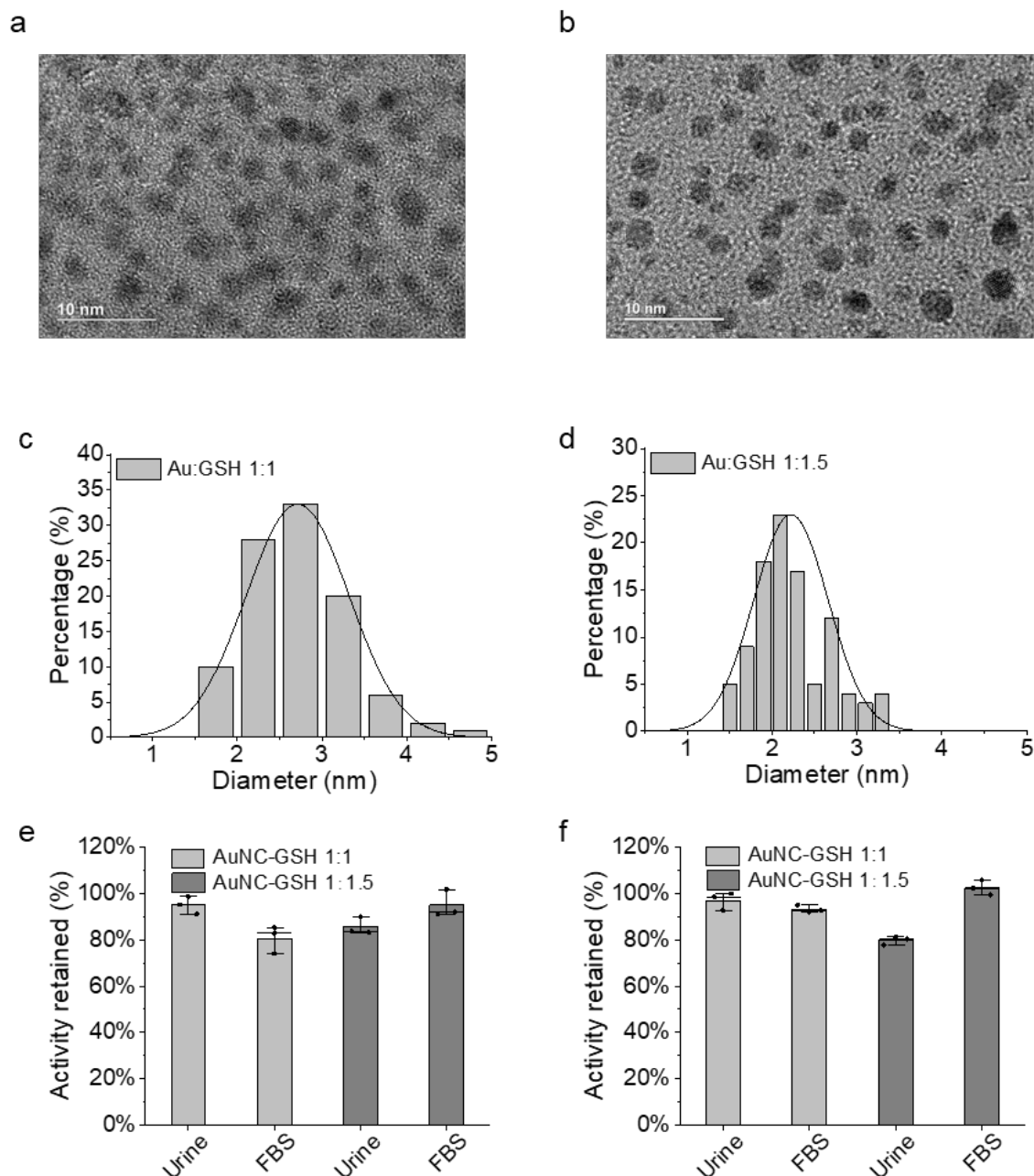


Figure 2.4 TEM and stability characterisation of AuNC 1:1 and 1:1.5. (a, c) TEM image and the derived histogram of AuNC-GSH 1:1 (mean diameter = 2.7 ± 0.6 nm (s.d.), $n = 100$). (b, d) TEM image and the derived histogram of AuNC-GSH 1:1.5 (mean diameter = 2.2 ± 0.4 nm (s.d.), $n = 100$). (TEM imaging was performed by Chalaisorn Thanapongpibul.) (e,f) Retention of catalytic activity of both AuNCs in physiology environment such as urine (50% v/v) and FBS (50% v/v) compared to PBS (100% v/v) after incubation for 1 h (e) and 12 h (f), respectively ($N = 1$, $n = 3$).

In order to investigate the augmentation of sensitivity when incorporating the above optimised AuNCs in an AuNC-based nanosensor complex, the sensing platform was utilised, involving the assembly of AuNCs onto a protein carrier NeutrAvidin (NAv) via a specific peptide linker derived from a previous study¹⁷. Peptide templated AuNC were synthesised through the reduction of Au (III) into Au (0) by GSH with peptide substrate in the synthesis reaction at 70 °C for 24 h (Figure 2.5). Those peptide templated AuNCs were mixed with the protein carrier NeutrAvidin (NAv) at room temperature and then incubated at 37 °C overnight. Subsequently, the resulting assembled nanosensors were purified using an Amicon filter device to remove unbound AuNCs, and the purified nanosensors were collected on the membrane (Figure 2.5).

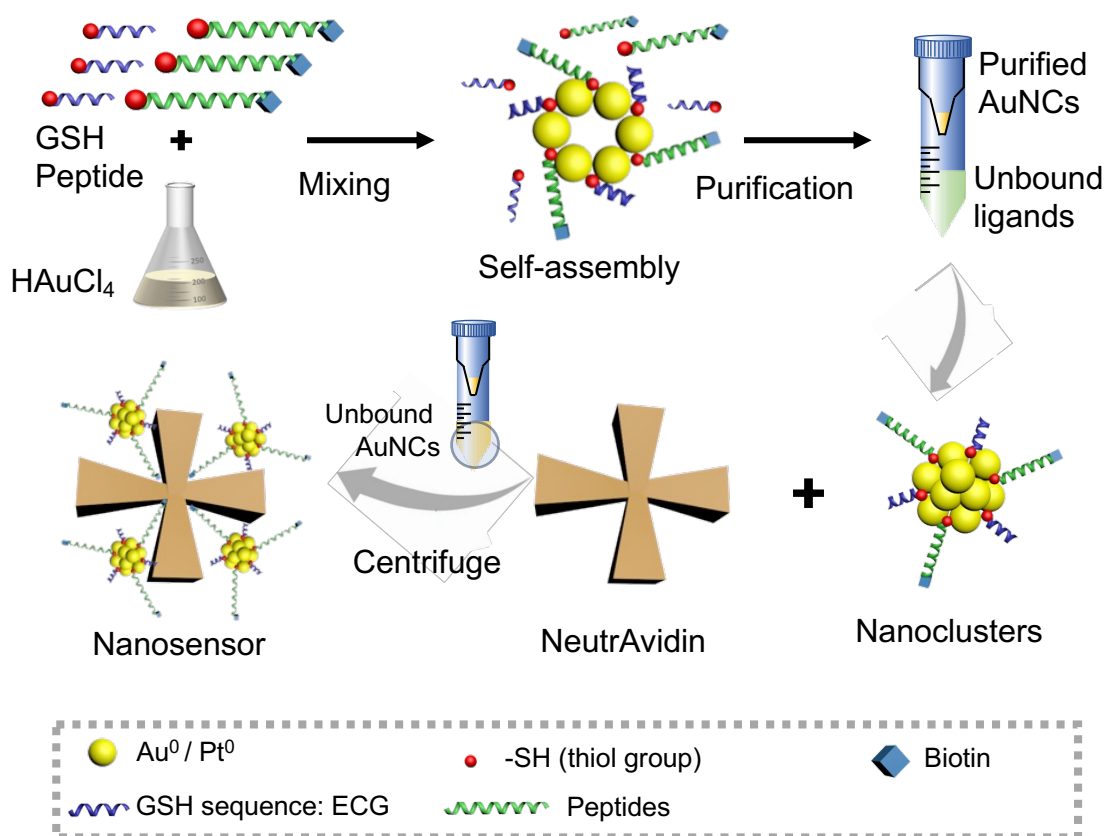


Figure 2.5 Schematic of AuNC synthesis and AuNC-NAV nanosensor complex assembly. The synthesis of nanoclusters. Metal iron Au is reduced by GSH and then self-assembled as nanocluster, which is followed by Amicon centrifugal filtration purification to obtain pure nanoclusters. The sequence of tripeptide GSH is γ -glu-cys-gly (ECG). Peptide is designed as a targeted substrate. The synthesis of AuNC-peptide-NAv complex nanosensor is reacted at 37 °C for 12 h and the purification is conducted using Amicon device.

Prior to assembling the nanosensor, the feed ratio in the peptide templated AuNC synthesis was optimised. The feed ratio pertained to the proportion of GSH ligands to the desired peptide (thrombin-cleavable peptide S1 was described in Section 2.3.4). GSH, a naturally occurring peptide, was utilised for the reduction of gold chloride during synthesis, leading to the conversion of gold (III) to gold (0) and subsequent self-assembly into GSH-stabilised AuNCs (Figure 2.5). Peptide linkers (S1) were employed to establish a connection between AuNCs and NAv through biotin binding.

The feed ratio plays an important role in determining the cleavage efficiency of peptide linker by a specific enzyme. This is because the peptide density, i.e. biotin density, has a significant impact on the ultimate performance of nanosensor complexes. This influence extends to both the catalytic activity of the final peptide-templated AuNCs and the accessibility of the enzyme to the peptide linker, facilitating efficient cleavage.

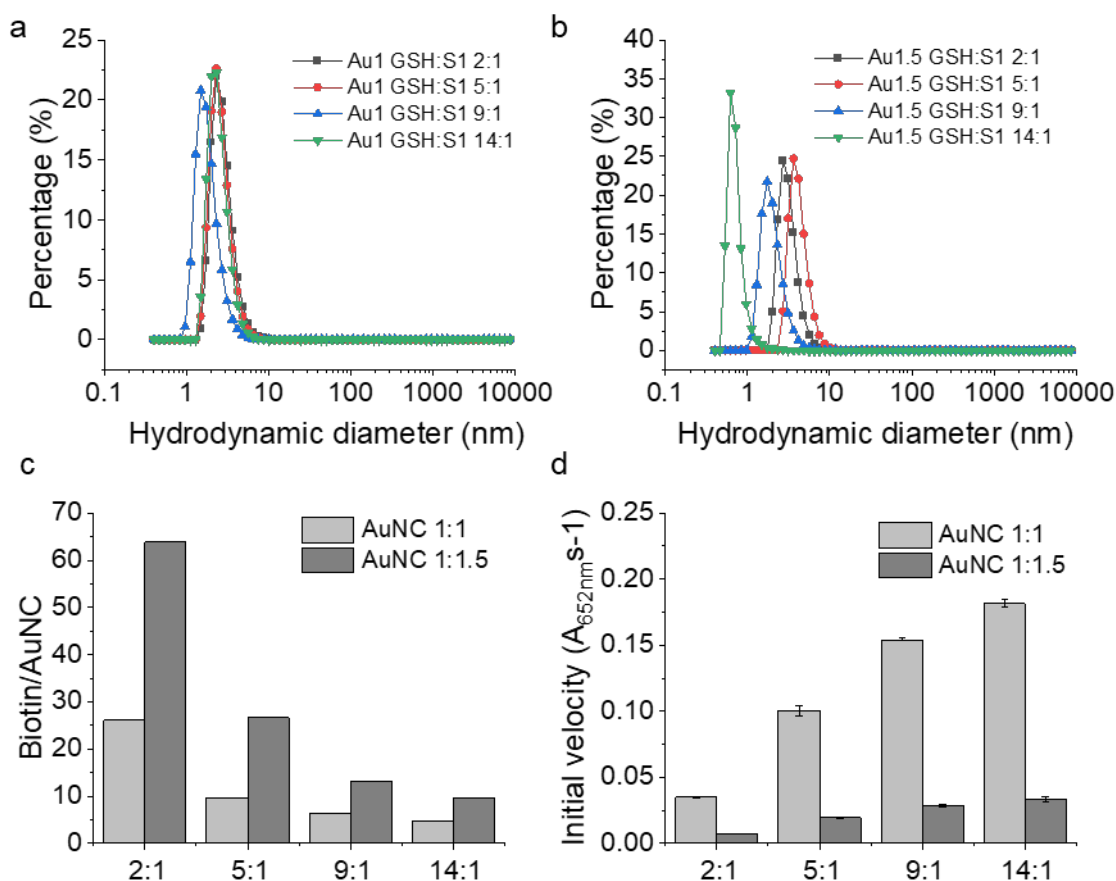


Figure 2.6 Characterisation of biotin density on optimised peptide templated AuNCs. (a,b) Hydrodynamic diameters of AuNCs of metal-to-GSH 1:1 and 1:1.5 at different feed ratio (GSH-peptide) from 14:1 to 2:1 by DLS (number distribution). (c) HABA assay for biotin quantification on AuNCs 1:1 and 1:1.5 synthesised using different feed ratios (GSH-to-S1) from 14:1 to 2:1 (N = 1, n = 1). (d) Catalytic activity assay of AuNCs 1:1 and AuNCs 1:1.5 with different feed ratio (GSH-to-S1) (initial velocity was calculated by averaging three independent samples, n = 3 measurements).

The quantity of peptide employed in AuNC synthesis plays a role in influencing the final size of peptide templated AuNCs. In the case of AuNC 1:1, the hydrodynamic diameters of all AuNCs with varying feed ratio (ranging from 2:1 to 14:1) exhibited a relatively consistent size of approximately 2 nm. However, in the AuNC 1:1.5 synthesis, the use of a reduced amount of peptide S1 led to slightly smaller sizes (Figure 2.6a-b). To determine biotin density, the HABA assay was employed, assessing the count of biotinylated ligands per AuNC. This assay is a colourimetric method for the quantification of the concentration of biotin in a sample, based on the binding of biotin to the molecule HABA, using a commercial Biotin Quantitation Kit. The binding of biotin to HABA causes an absorbance shift in the spectrum of HABA²⁰¹. As expected, it was found that biotin density decreased with decreasing peptide S1 usage in the AuNC synthesis. The AuNC 1:1.5 (gold-to-ligands, ligands include peptide S1 and GSH) configuration exhibited a higher biotin density on each cluster compared to the AuNC 1:1 configuration, across all feed ratios used in the synthesis. This divergence was due to the varying amounts of overall peptide employed in each (Figure 2.6c). An optimal balance between GSH ligand and biotin-containing S1 peptide is crucial for efficient assembly of nanosensors. An insufficient number of S1 peptides on the AuNC surface can limit the creation of nanosensors due to inadequate accessibility of linkers and biotins for forming effective AuNC-NAv connections. Conversely, excessive S1-biotin on AuNCs may hinder enzyme cleavage and result in cross-conjugation of multiple NAv carrier proteins. Ultimately, this could retard the release of AuNCs from the nanosensor complex, reducing sensitivity.

TMB oxidation assay conducted on all AuNCs suggested that both AuNC-S1 configurations (templated with thrombin cleavable peptide S1) exhibited superior

catalytic activity when more GSH and fewer peptide linkers S1 were utilised. This phenomenon might arise from the difference in GSH and S1 molecular weight. A higher amount of shorter GSH with fewer longer S1 peptide linkers could leave more AuNC surface exposed for the oxidation of the TMB substrate. In addition, a lower ligand density on the gold surface can enhance catalytic activity by allowing greater surface exposure to the substrate for reaction (Figure 2.6d). This also aligns with a previous publication where it was found that the utilisation of bulky ligands for protecting AuNCs resulted in a lower ligand density, and this gave higher oxygen reduction reaction and electrocatalytic activity²⁰². Consequently, the biotin density per cluster is important for the signal intensity, and a harmonious equilibrium between the catalytic activity and biotin density should be considered. With consideration for both catalytic activity and biotin concentration, the ideal final biotin density was determined to be around 1 to 2 biotins per AuNC. As a result, a feed ratio of GSH-to-S1 of 14:1 was chosen for AuNC synthesis, ensuring the desired biotin density.

2.4.2 Thrombin sensor designed with optimised peptide-templated AuNCs

The effect of using AuNCs with higher catalytic activity in the thrombin nanosensor design (Figure 2.5) was studied next. Nanosensor complexes were assembled by incubating both types of AuNCs-S1 (Au-to-GSH 1:1 and 1:1.5) at the feed ratio of 14:1 with NAv. Subsequently, the sensors were purified using an Amicon filter unit (MWCO 50 kDa). During the purification process, unbound AuNCs passed through the membrane into the filtrate waste, whereas the assembled nanosensor complexes were retained due to their size exceeding the membrane cut-off threshold. The

resulting complexes were concentrated to a desired concentration and revealed an approximate diameter of 11 nm (Figure 2.5).

To evaluate the enhancements achieved in the sensing system through the above optimisation, an *in vitro* thrombin cleavage assay was conducted by incubating the nanosensor complexes with various concentrations of thrombin enzyme for durations of 3.5 h and 24 h. During the incubation period, the complexes were disassembled through the cleavage of the specific peptide linkers S1 by thrombin, resulting in the release of AuNCs. These liberated were then separated from intact nanosensor complexes by using Amicon filter units (MWCO 50 kDa). During the filtration process, the intact complexes were retained within the membrane of the filter device, while the liberated AuNCs successfully passed through the membrane and were collected in the filtrate for a subsequent evaluation by TMB oxidation assay (Figure 2.7). The signal detected from the TMB assay was the measurement of the catalytic activity of the cleaved AuNCs, as a direct result of the enzyme cleavage of the peptide for the detection of thrombin.

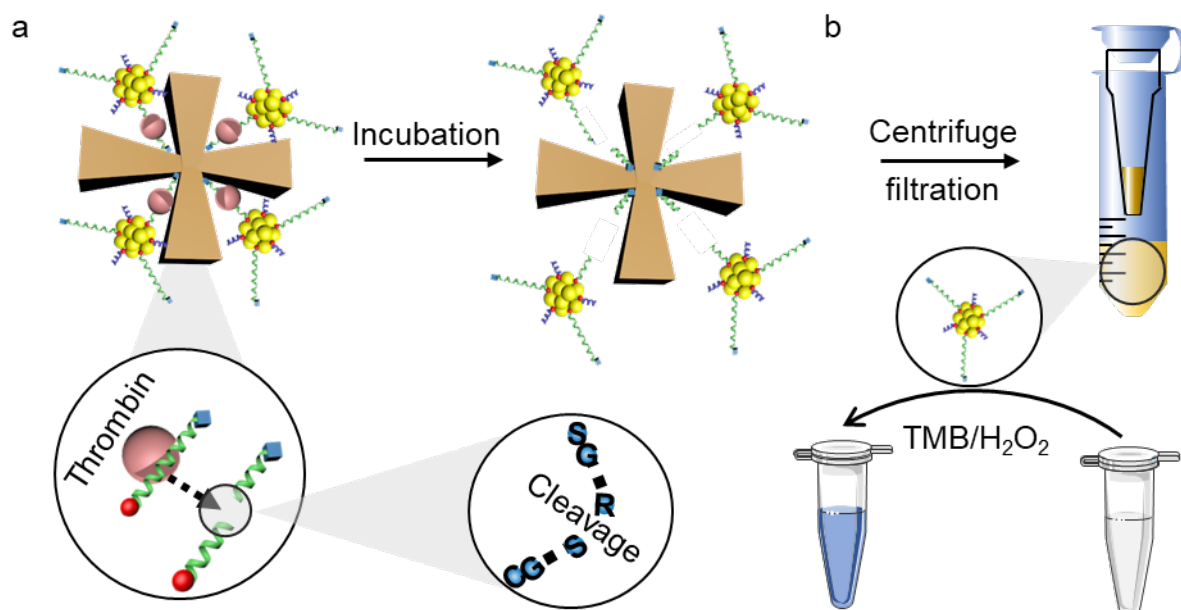


Figure 2.7 Schematic of the mechanism of enzyme cleavage of the nanosensor complex. (a) Enzyme cleavage of the peptide within the nanosensor complex, leading to the release of AuNCs, (b) TMB assay on the filtrate. The released AuNCs were subsequently filtered through the Amicon device. The filtrate was collected for the TMB oxidation assay, yielding a blue colour if thrombin was present in the sample.

Based on the catalytic activity assay conducted on the released AuNCs from the 3.5-h and 24-h incubation periods, it is evident that both AuNCs could be successfully incorporated in the thrombin sensor and delivered the desired sensing result (Figure 2.8). Both the optimised and previously studied¹⁷ AuNCs were effectively liberated through the cleavage of the peptide linkers by thrombin. Moreover, the results demonstrated that the optimised AuNC-functionalised nanosensors exhibited a substantially higher signal intensity subsequent to incubation with the thrombin enzyme. The limit of detection (LoD) was calculated by adding three times the standard deviation to the mean value of the background, resulting in values of 13 nM and 25 nM for sample ratios of 1:1 and 1:1.5 respectively after 3.5-h incubation period, with 1.6 nM and 5 nM respectively after 24-h incubation period. As a result, the

optimised AuNCs markedly enhanced the sensitivity of the sensor system by three-fold compared to the previously published work¹⁷.

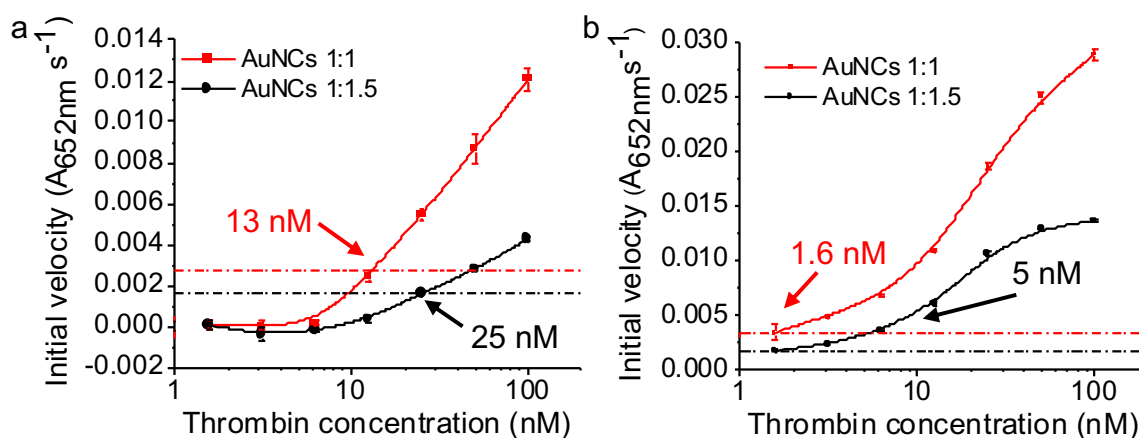


Figure 2.8 Sensitivity comparison of AuNC 1:1 and 1:1.5 thrombin sensors. (a, b) The catalytic activity comparison after 3.5-h and 24-h incubation ($N = 1$, $n = 3$). Thrombin concentrations ranging from 0 to 100 nM were employed for incubation with thrombin-responsive nanosensors, each for durations of 3.5 h and 24 h. Subsequently, these mixtures were subjected to filtration through an Amicon filter device to isolate the cleaved and released AuNCs. The filtrates were then subjected to a TMB oxidation assay, and the results were subsequently converted into velocity measurements for the purpose of comparing catalytic activity.

2.4.3 The influence of synthesis pH on the final AuNC catalytic activity

For the further improvement of the catalytic activity of AuNCs, the pH of the synthesis reaction condition was investigated in a next step. Notably, the obtained AuNCs synthesised at different pH exhibited different colours suggesting differences in size and/or other properties. Their catalytic activity was again evaluated through TMB oxidation assay (Figure 2.9a). The onset velocity served as the comparator of the catalytic activity, which revealed that AuNCs with a metal-to-GSH ratio of 1:1.5 at pH 10 exhibited the highest activity. The catalytic activity was approximately 33.5-fold

greater than that of ratio 1:1.5 synthesised at pH 2, which were the previously studied AuNCs¹⁷ as reported in the previous paper employing catalytic AuNCs as a cancer sensor with urinary readout¹⁷ (Figure 2.9b). This remarkable improvement in catalytic activity suggests that the sensitivity of the sensing platform could potentially be significantly increased when incorporating these AuNCs in the nanosensor. UV-Vis spectral analysis revealed a similar absorbance for the two similarly sized samples, confirming that variation in catalytic activity comes from improved catalytic activity not variation in concentration (Figure 2.9c, d). To investigate whether this substantial enhancement in catalytic activity was related to differences in particle concentration and/or size, DLS was used to measure corresponding sizes and derived count rate (Figure 2.9e, f). The sizes of samples synthesised at the same Au:ligand ratio of 1:1.5 but at different pH levels (2 and 10) were found to be similar and not significantly different. As their sizes were identical, their derived count rate, which is proportional to their concentration, could be compared. There was no significant difference between the derived count rate of these two samples, indicating they have almost the same concentration.

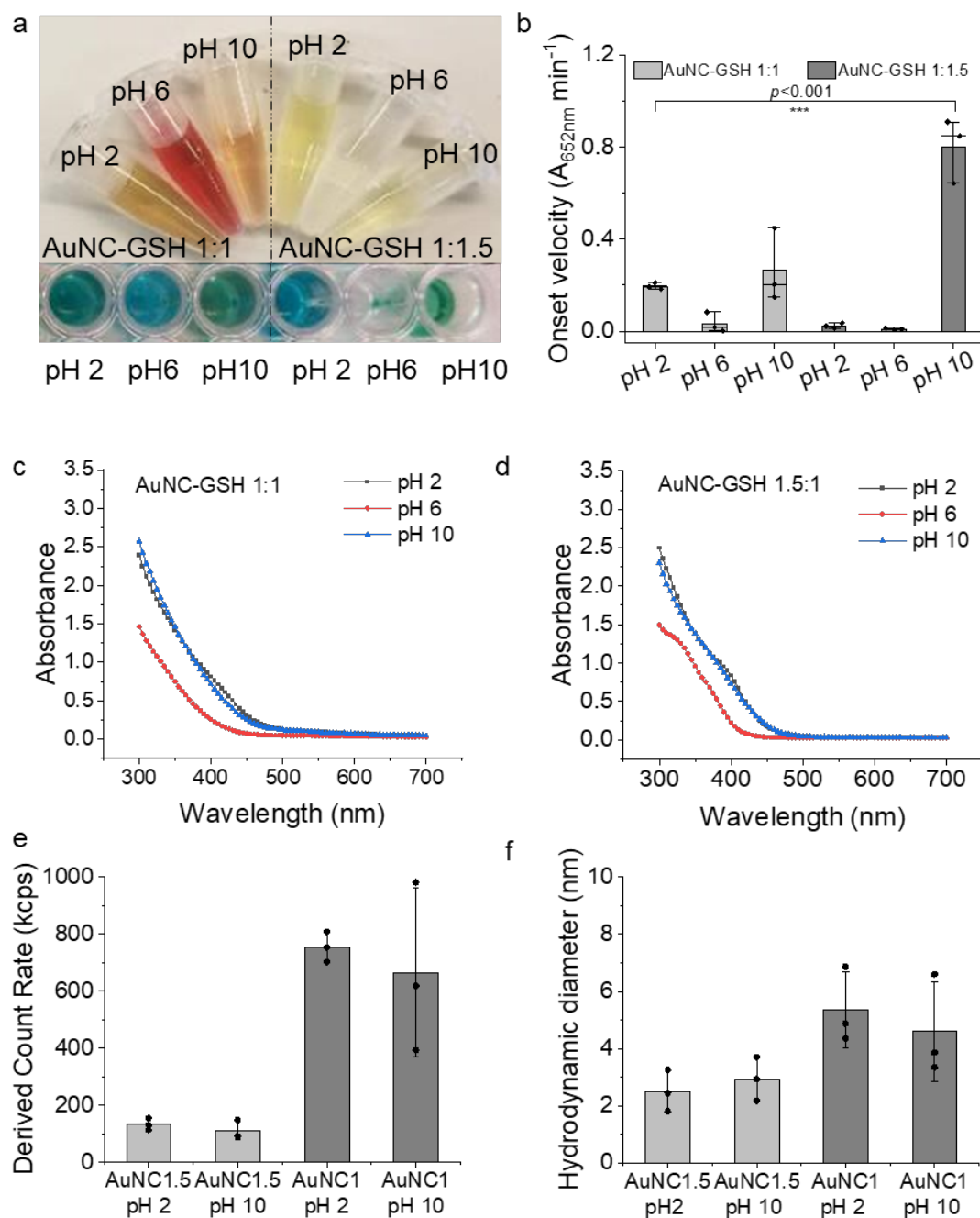


Figure 2.9 Characterisation of AuNC 1:1 and 1:1.5 synthesised at different pH levels. (a) Photographs of final products of all AuNCs synthesised at different pH conditions with different colours and the colourimetric readout results after TMB oxidation assay under of all AuNCs. This showed it could be read by eyes to identify how fast of the reaction was and indicated the catalytic activity of all AuNCs. This is corresponding to the catalytic activity measurement on the plate reader. (b) Catalytic activity of AuNCs in different pH reaction conditions. The catalytic activity was calculated from onset velocity ($A_{652nm} \text{ min}^{-1}$) when the TMB kinetics happened in a linear

trend (N = 3 independent samples, n = 3 technical repeats). (c, d) UV-vis measurement of both AuNC-GSH 1:1 and 1:1.5 at different pH reaction conditions. This showed AuNCs synthesised at pH 6 might have had a lower concentration, which could account for the lower catalytic activity. (e) Derived count rate measurement of AuNC 1:1 and 1:1.5 at pH 2 and 10 (N = 1, n = 3). (f) Hydrodynamic diameter measurement of AuNCs from DLS (N = 1, n = 3).

TEM images and the histogram derived from measurements indicate that the average size of the final optimised pH10 AuNCs at 1:1.5 Au-to-GSH ratio (1.7 ± 0.4 nm) falls below the renal clearable threshold imposed by the kidneys (5.5 nm), thereby theoretically enabling their efficient filtration through the urinary clearance system²⁰³ (Figure 2.10a,b). The limit of detection (LoD) when studying dilution series in synthetic urine for both types of AuNCs was determined to be 2.33 pmol and 0.028 pmol, respectively (Figure 2.10c, d). This indicates an 83-fold increase in sensitivity for the synthesis pH optimised AuNCs. Interestingly, the best sample after pH optimisation is the 1:1.5 ratio and not 1:1 as found in previous Section 2.4.2 . This could potentially be cause of the better stability of AuNCs without aggregations and reduced surface oxides.

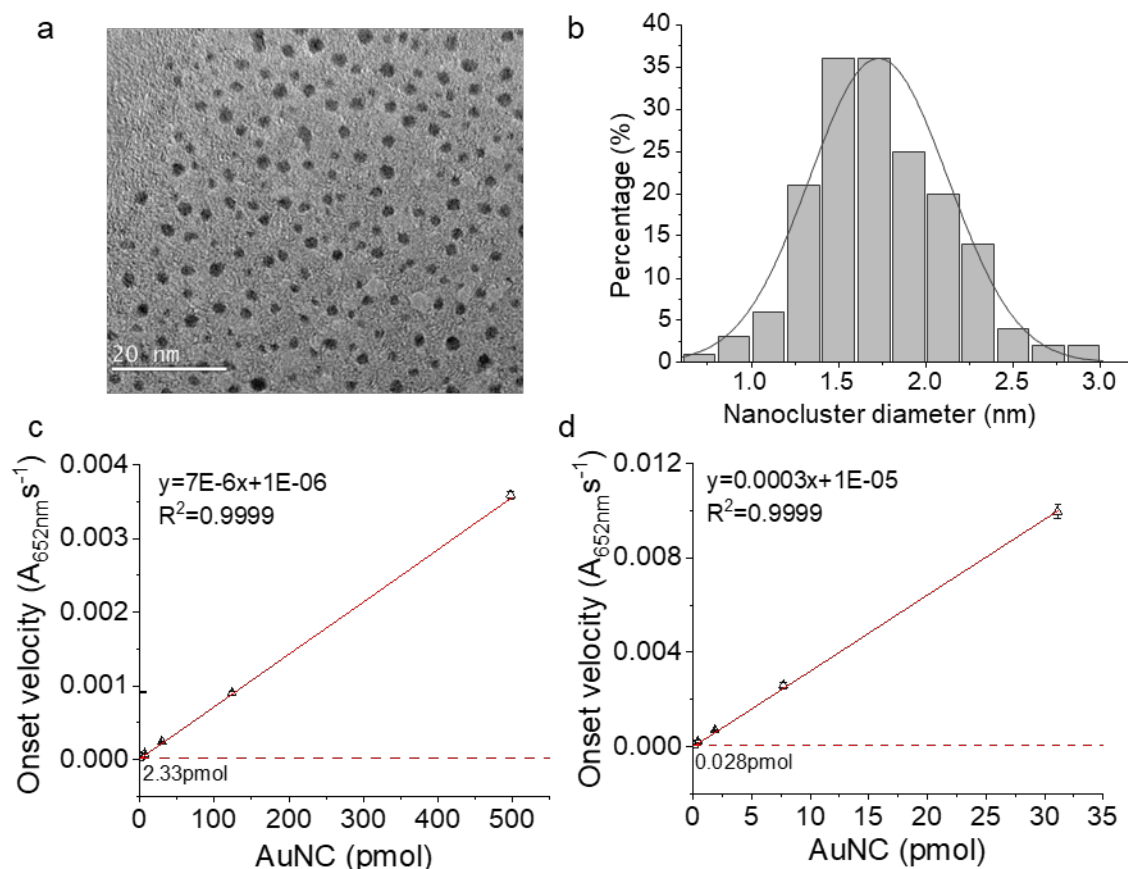


Figure 2.10 TEM image characterisation of the optimised AuNC pH 10 and LoD of both AuNC for sensitivity comparison. (a) TEM image of AuNCs (optimised synthesis at 1:1.5 Au-to-GSH ratio, pH 10). Scale bar 20 nm. (b) The size distribution histogram from TEM image of AuNCs. This shows the size analysis of AuNCs (mean diameter = 1.7 ± 0.4 nm (s.d.), $n = 170$ particles). (c, d) The limit of detection (LoD, calculated as 3 times of standard deviations plus the mean value of the background) of AuNCs 1:1.5 from pH 2 and pH 10 reaction conditions. The catalytic activity was measured with the AuNCs diluted in synthetic urine (50% v/v) and evaluated by the onset velocity analysis from the oxidation of TMB substrate ($A_{652nm} s^{-1}$). The LoD of AuNCs 1:1.5 from pH 2 and 10 synthesis condition was 2.33 and 0.028 pmol ($N = 1$, $n = 3$), respectively, corresponding to 83-fold enhancement of catalytic activity, with the higher number corresponding to the AuNCs of a related recent paper¹⁷.

UV-Vis spectral analysis revealed a similar absorbance for the two similarly sized samples, confirming that variation in catalytic activity comes from improved catalytic activity not variation in concentration (Figure 2.11). These results suggest that AuNCs,

particularly those with a specific metal-to-GSH ratio and alkaline pH synthesis conditions, exhibit significantly improved catalytic performance compared to previous findings¹⁷, which will allow creation of more sensitive diagnostic tests using these AuNCs in a variety of settings²⁰³. Adjusting the pH during synthesis has an important impact on the size and catalytic performance of nanoclusters. Changes in synthesis pH adjust the ion concentration, reaction rate, and species distribution in solution, which in turn affects the formation and growth of nanoclusters. Alkaline conditions can increase the reduction rate of gold salts, resulting in faster nucleation and growth of nanoclusters, which may lead to the formation of larger size. In addition, the alkaline environment will also change the morphology and adsorption properties of surfactants, affect the morphology and surface properties of nanoclusters, and thus affect their catalytic properties. The catalytic activity of gold nanocluster is related to their morphology, size, and surface activity, and the alkaline environment can regulate these factors. Therefore, a better understanding of the effects of alkaline environment on the synthesis and properties of gold nanoparticles is needed to help precisely regulate the preparation process of nanomaterials and improve their performance and efficiency in catalysis and other applications, which can be studied in the future work by characterising the difference in final AuNCs like ligand density on the surface.

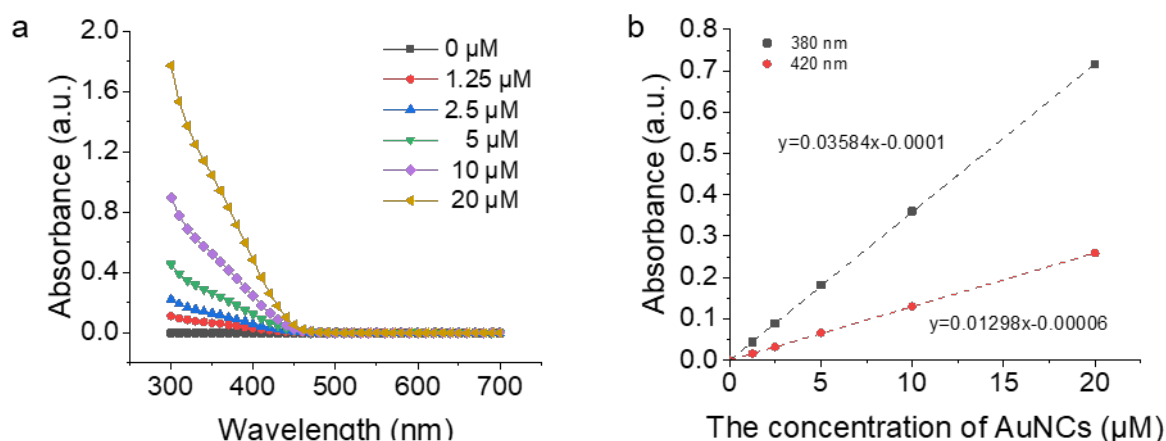


Figure 2.11 Characterisation of the optimised AuNCs (1:1.5, pH 10). (a) UV-vis measurement of the optimised AuNCs with serial dilution. Absorbance at 380/420 nm could be used for calibration of concentration for various batches. (b) The calibration curve for the absorbance of AuNCs at the wavelength of 380 nm and 420 nm from (a), allowed quantification of AuNC concentrations for batch comparisons ($N = 1$, $n = 1$).

Thrombin substrate peptide S1, with the sequence of Biotin- SGGfPRSGGSGGC, has an isoelectric point of 5.8. In the pH 10 synthesis condition, peptide S1 and GSH are negatively charged because of their isoelectric gold ions (Au^{3+}), on the other hand, are usually positively charged. The thiol group (-SH) of GSH and peptide can coordinate with gold ions to reduce Au^{3+} to gold atom form, promoting the formation and growth of AuNCs. In addition, alkaline conditions help increase the activity of reactants, speed up the reaction rate, and promote the formation and growth of AuNCs. Under alkaline conditions, the solubility of gold ions may increase, which is conducive to the binding and reduction of gold ions to ligands such as GSH. This may lead to the better reaction for AuNC formation and further improvement of the catalytic activity. The next optimisation step again involved the optimisation of S1 peptide to GSH ratio in order to produce more sensitive thrombin model sensors to evaluate sensitivity increase with the final optimised AuNCs.

2.4.4 Variation of peptide to GSH feed ratio in AuNC synthesis at pH 10

Since AuNCs exhibited the best catalytic activity when using an Au:GSH ratio of 1:1.5 for synthesis at pH 10, another factor, the feed ratio of S1 peptide to GSH, was investigated to determine its impact on the sensing efficiency of this nanosensor. The feed ratio refers to the ratio of S1 peptide to GSH in the AuNC synthesis reaction, which influences the peptide density on each AuNC and, consequently, affects the biotin binding between AuNCs and NAv to obtain the sensor (Figure 2.12). Having an excess of peptide on AuNCs could possibly result in more NAv binding to each AuNC, leading to a higher number of enzymes being required for the cleavage of peptide and the subsequent release of AuNCs (Figure 2.12a). This, in turn, could lead to a lower sensitivity for the sensing process. Therefore, it was crucial to optimise the feed ratio for achieving an optimal S1 peptide density on AuNCs for binding to NAv. This would allow efficient cleavage of the peptides even at low enzyme concentrations, thus enabling sensitive detection (Figure 2.12b).

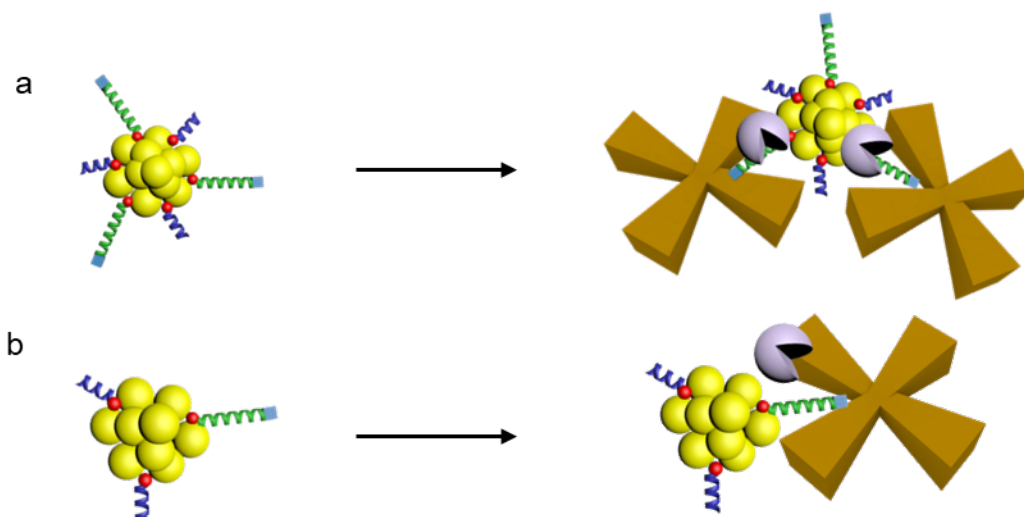


Figure 2.12 Schematic of different enzyme cleavage performances on nanosensors with various biotin densities. (a, b) Potential influence of higher (a) and lower (b) biotin density (on green S1 peptide, blue peptides represent GSH) on AuNCs within nanosensor on the enzyme cleavage efficiency.

Various feed ratios of peptide (S1) to GSH were used for AuNCs 1:1.5 (gold-to-ligands) synthesis at pH 10, from 1:5 to 1:100. This corresponded to S1 peptide percentage of 1% to 16.67% of the total ligands used in the synthesis, which resulted in biotin densities from 1 to 16 biotins per AuNC (Figure 2.13a). To assess the effect of feed ratio on catalytic activity of AuNC-S1, TMB oxidation assay was conducted for comparison, showing similar activities among most ratios. Only the 1:100 ratio displayed slightly higher activity, likely due to fewer long ligands on the AuNCs, resulting in more exposure to H_2O_2 for quicker TMB oxidation (Figure 2.13b).

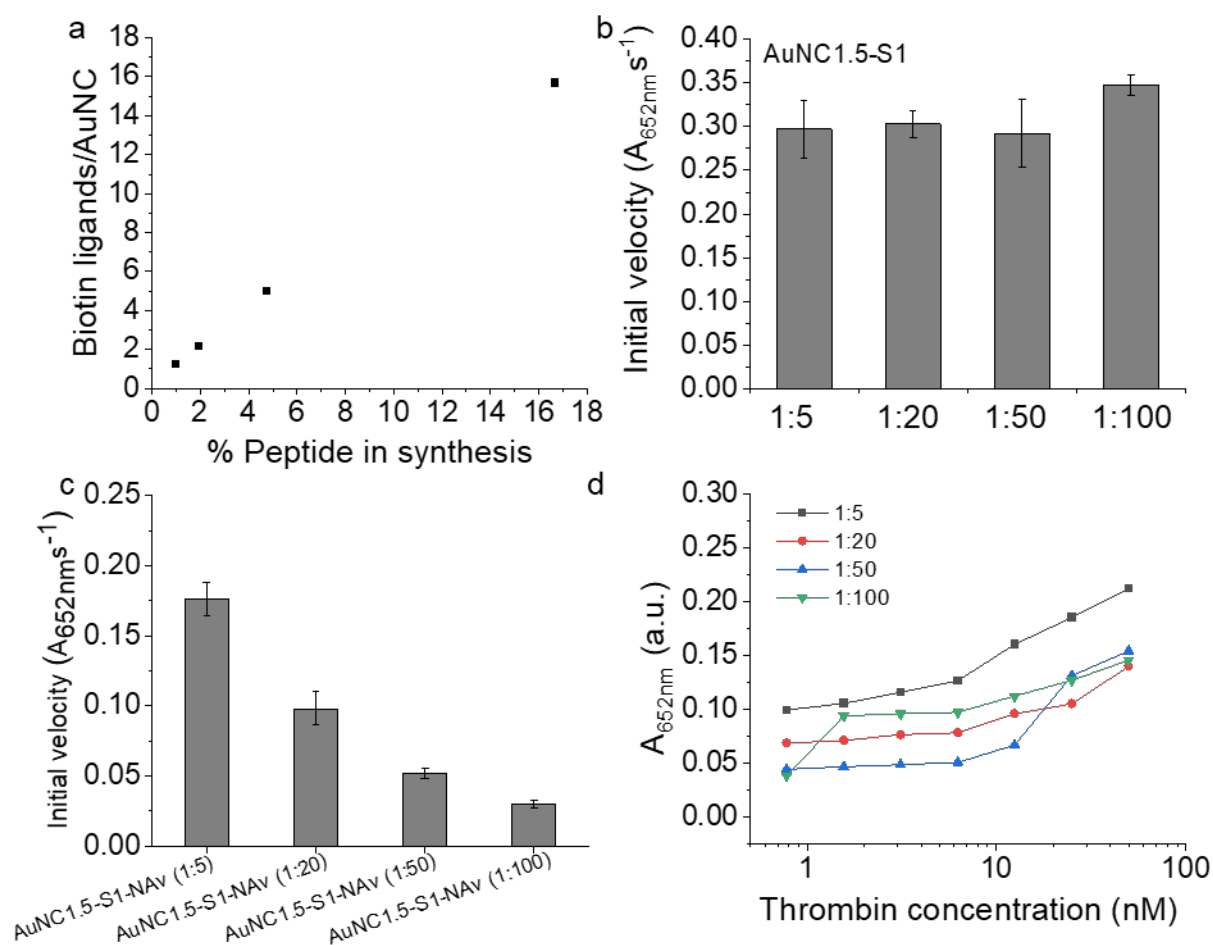


Figure 2.13 The influence of peptide density on the thrombin enzyme cleavage on the nanosensor platform. (a) HABA assay for biotin quantification of AuNCs 1.5 pH 10 synthesised using different feed ratios (S1-to-GSH) from 1:5 to 1:100 ($N = 1$, $n = 1$). (b, c) Catalytic activity measurements by TMB oxidation for all AuNC-S1 (b) and AuNC-S1-NAv nanosensor complex (c) ($N = 1$, $n = 3$). (d) The catalytic activity of the filtrates from the thrombin cleavage samples for the sensitivity assessment ($N = 1$, $n = 1$).

Subsequently, AuNCs with different biotin densities were used to assemble thrombin-responsive nanosensor complexes by incubating them with NAv. Similarly, TMB oxidation was employed to compare the catalytic activity of nanosensor, showing the complexes formed with AuNCs of higher biotin density exhibited enhanced activity (Figure 2.13c). A higher biotin density allowed more biotins to bind to NAv, which, in

turn, meant more AuNCs could be bound to NAv, even when using the same excess AuNCs for all samples. Following the assembly, the nanosensor complexes were incubated with varying concentrations of thrombin enzyme. Afterward, filtration using an Amicon filter unit (MWCO 50 kDa) and collection of the filtrate were carried out for the TMB oxidation assay. The nanosensor (1:5) displayed the best catalytic activity for all concentrations, consistent with its highest initial catalytic activity before thrombin cleavage. Notably, the nanosensor (1:100) exhibited relatively good cleavage efficiency, despite having the lowest catalytic activity before incubation with thrombin enzyme (Figure 2.13d). Additionally, the nanosensor (1:50) showed a similar trend with better cleavage efficiency compared to the nanosensor (1:20) even with higher initial catalytic activity, suggesting that the feed ratio significantly impacted the sensitivity of this sensing platform through the improvement of enzyme cleavage efficiency. For better comparison, it was crucial to maintain the same biotin density to ensure the same chances of nanosensor assembly and thus, fair comparisons of cleavage performance for all concentrations.

To facilitate a more precise comparison, a uniform biotin concentration was maintained across the AuNC-S1-NAv assembly using AuNC-S1 synthesised with various biotin densities. This was achieved by concentrating AuNC-S1 with lower biotin density more than samples where higher biotin density were used, before adding to NAv. This equalised the probability of biotin binding to a specific quantity of NAv. After purification, the nanosensor complexes exhibited more comparable catalytic activities. Interestingly, the complex formed with AuNC-S1 of lower biotin density displayed slightly higher catalytic activity, which could be attributed to the improved and more accessible binding between AuNC-S1 and NAv. The absorbance was

recorded over time, indicating that AuNC-S1 with lower biotin density but the same biotin concentration led to superior absorbance values as well as the catalytic activity for the resulting complexes (Figure 2.14a, b).

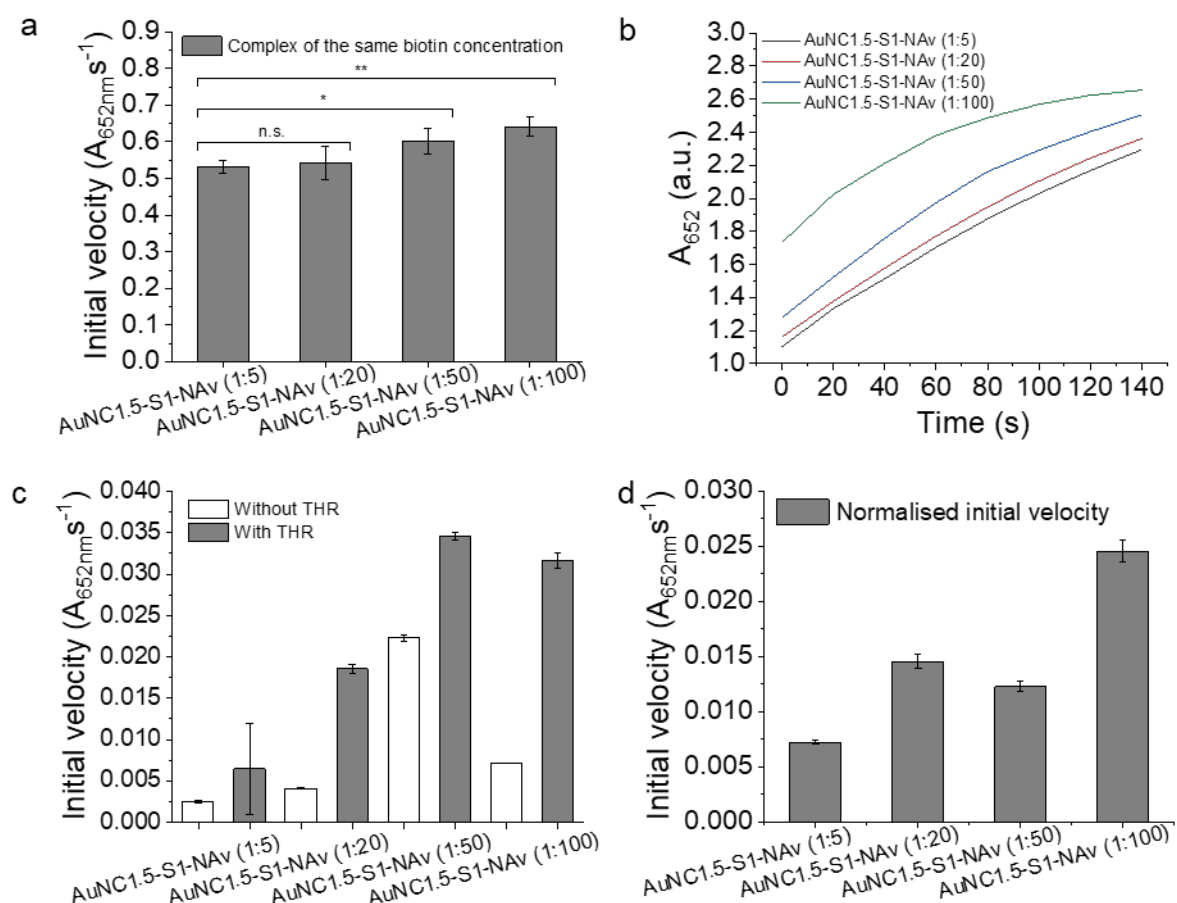


Figure 2.14 Cleavage performance of nanosensor complex with different feed ratios but same biotin concentration. (a) The catalytic activity of all nanosensor complexes using different feed ratios ($N = 1$, $n = 3$). (b) TMB oxidation absorbance measurement over time for all complexes ($N = 1$, $n = 1$). (c) TMB assays on all the filtrates of all complexes after thrombin enzyme incubation for 4 h ($N = 1$, $n = 3$). (d) The normalised catalytic activity of cleavage performance of all nanosensors from (c) after subtracting background values (0 nM thrombin) ($N = 1$, $n = 3$).

Subsequently, a thrombin enzyme cleavage assay was conducted on all nanosensor complexes by incubating them with thrombin (final concentration 50 nM) for 4 h. The liberated AuNCs were then separated by using Amicon filter unit (MWCO 50 kDa)

mimicking kidney filtration and collected for TMB oxidation assay. The nanosensor complexes formed with AuNC with lower biotin density displayed higher signal intensity for the detection, indicating enhanced accessibility for the enzyme to effectively cleave the peptide substrate. However, it is noteworthy that the nanosensor complexes with lower biotin density exhibited elevated background levels when no thrombin was present (Figure 2.14c), indicating certain instability issues with these sensors.

To compare the final detection sensitivity ignoring the background for now, the velocity of the filtrates of each nanosensor from TMB assay, obtained from the thrombin cleavage incubation, was normalised by subtracting their respective backgrounds. This revealed that the nanosensor (1:100) displayed notably higher signal intensity in the thrombin cleavage assay compared to those with higher biotin density. This observation underscores the fact that lower biotin density in the nanosensor complex assembly may lead to heightened cleavage efficiency, especially using same biotin concentration for the assembly of nanosensor complexes (Figure 2.14d). However, it is important to address the issue of high background levels in the optimised AuNC-NAv sensing platform, which could adversely impact the accuracy of disease detection and potentially result in false-positive outcomes. Therefore, ensuring the thorough purification of the nanocomplex is of paramount significance. This involves meticulous removal of excess unbound AuNC-S1 and weakly bound ligands from the nanosensor complex samples, effectively addressing potential issues related to background noise and false positives.

2.4.5 Purification of nanosensor complex using Amicon device and SEC

After the optimisation work on AuNCs and peptide templated AuNCs, the issue of elevated background from the final nanosensor complex due to potential impurities during purification emerged. Here, a combination of both Amicon filter devices (through a preliminary 5-time wash) and size elution chromatography (SEC) was evaluated to ensure comprehensive purification to hopefully better eliminating any residual unbound AuNCs. To gauge the efficiency of this purification process, two complexes with feed ratios of 1:5 and 1:100 using the optimised AuNCs (Au:GSH 1:1.5 at pH 10) were used.

In the chromatographic results, after the primary Amicon filtration purification, both complex samples exhibited comparable TMB oxidation absorbance within the first peak fractions corresponding to the sensor (AuNC-NAv, ca 6-9 mL fractions). However, the second shoulder (ca 10 mL fraction), containing unbound AuNCs, displayed notably lower signal intensity even after purification with Amicon devices, underscoring the need for further purification for both complex samples (Figure 2.15a). This also suggested Sephacryl® 200-HR was not good enough for the separation of complexes and AuNC, due to their small difference in size. Concentrating the complexes of the first peak fractions, followed by TMB oxidation assay, indicated equivalent catalytic activity post dual purification, using the same biotin concentration for sensor assembly (Figure 2.15b). In thrombin cleavage, both complexes displayed significantly higher catalytic activity than the background control group. Despite these positive results, it was noteworthy that the background issue from the sensing system remained relatively unaddressed and requires further investigation (Figure 2.15c, d). While dual

purification (Amicon and SEC) shows promise in reducing background noise, achieving higher sample purity is crucial to enhance system performance.

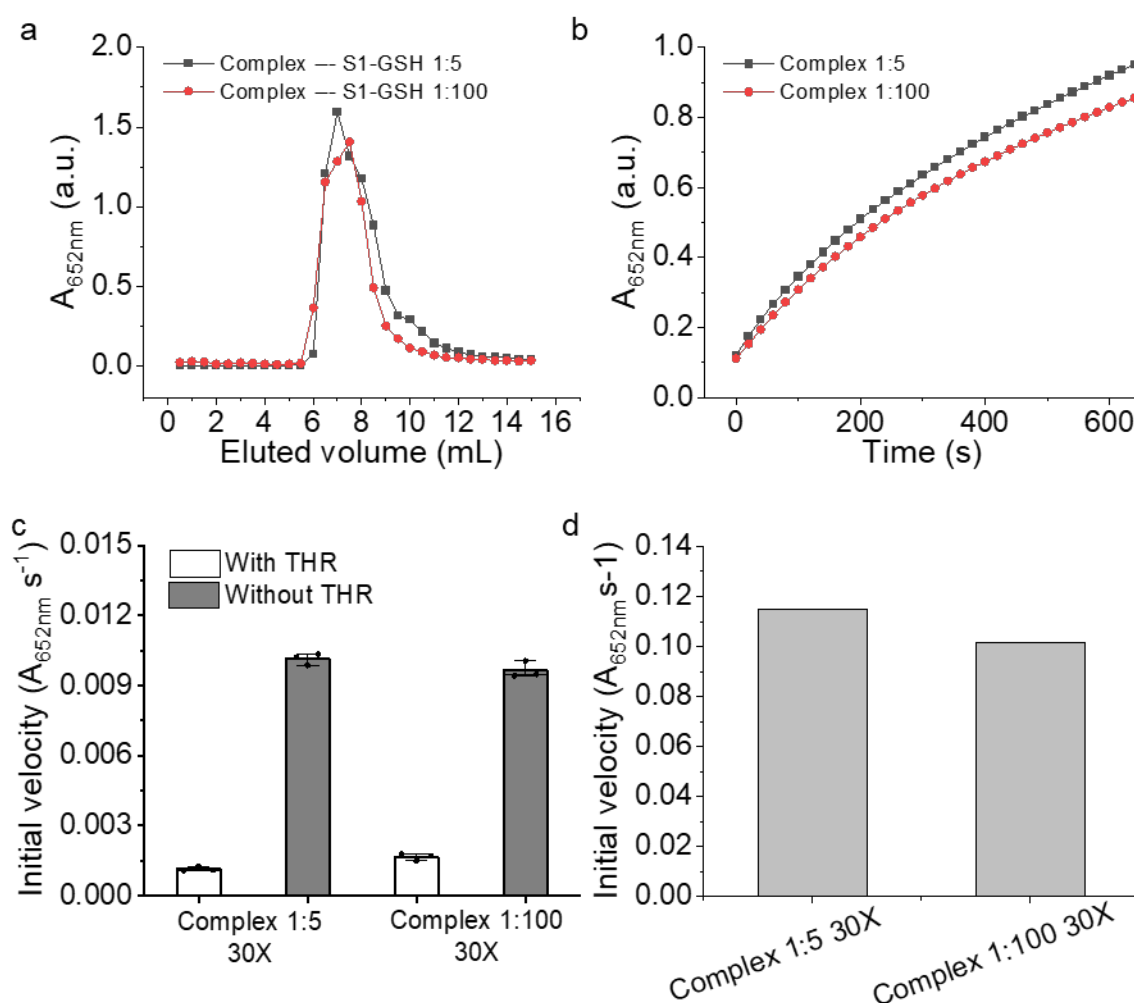


Figure 2.15 Characterisation of purified complexes through Amicon device and SEC. (a) TMB oxidation assay on the collected fractions of complexes of feed ratio of 1:5 and 1:100 from SEC after additional Amicon filtration. (b) Catalytic activity measurement of both complexed after two purification process by TMB assay. (c) Catalytic activity measurement on the filtrate after thrombin cleavage assay on both complexes (N = 1, n = 2). (d) The normalised result from (c) (background no thrombin removed) comparison of cleavage result (the mean value was used to get the normalised result, N = 1, n = 1).

Leveraging the separation process of SEC based on nanoparticle diameter variations, a heightened precision was utilised by extending the column length. This should

enhance the resolution for the separation and purification of nanosensor complexes. In this pursuit, a comparative evaluation unfolded between two sets of complexes, including sensors with the recently published AuNCs¹⁷ and with my optimised AuNCs synthesised at pH 10. The findings underscored that the optimised system (with pH 10 AuNCs) yielded significantly elevated absorbance signals within SEC purification fractions, including the complex peak as well as the second peak for unbound AuNCs (Figure 2.16a).

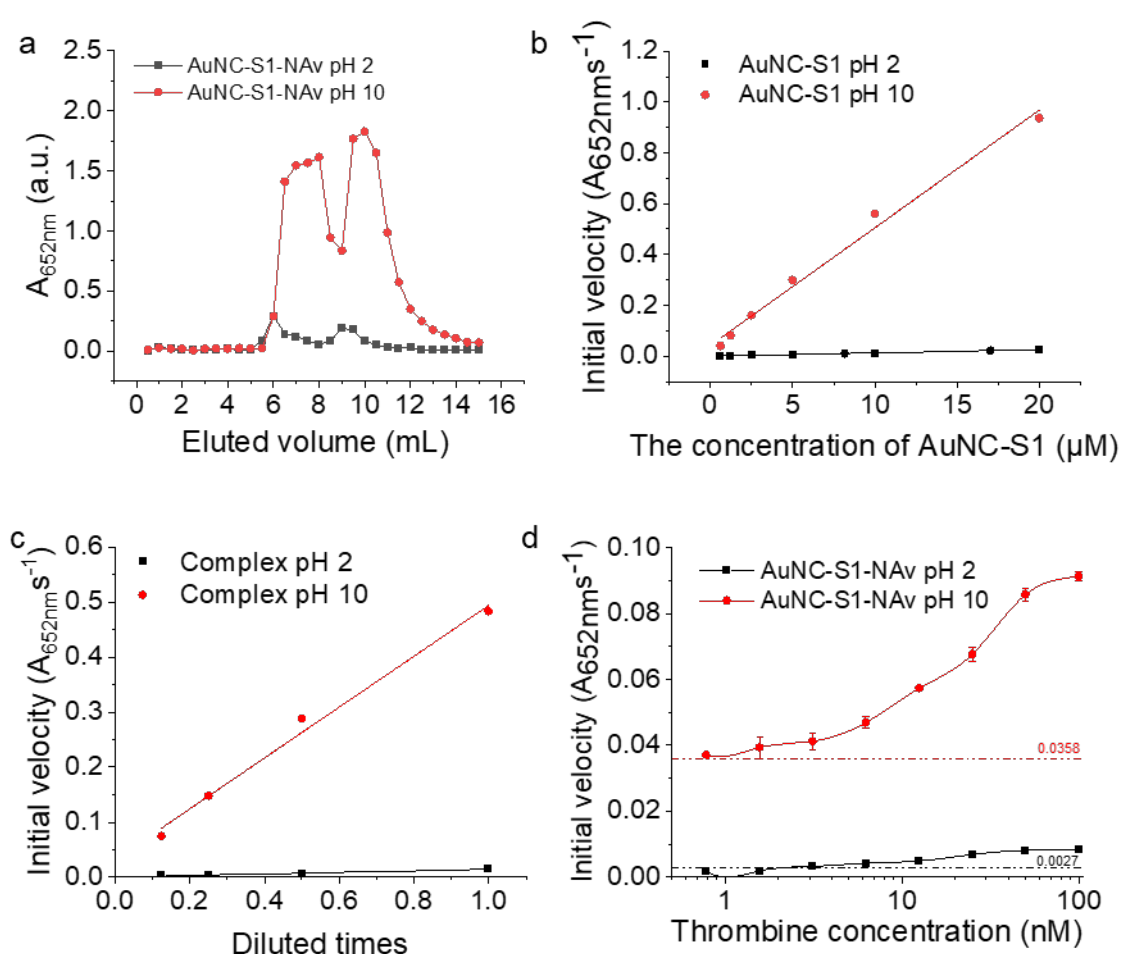


Figure 2.16 Characterisation of complex system of pH 2 and pH 10 after SEC for lowering the background of the sensing system. (a) TMB assay on all the collected fractions after SEC for both complexes ($N = 1$, $n = 1$). (b, c) Calibration curve of AuNCs pH 2 and 10 and their corresponding nanosensor complexes ($N = 1$, $n = 1$). (d) TMB assay on the filtrates after thrombin enzyme cleavage assay on both complex at different concentration ($N = 1$, $n = 3$).

To facilitate quantification calculations, calibration curves were constructed for both AuNCs-S1 and their corresponding nanosensor complexes using TMB assay (Figure 2.16b, c). Significantly, both AuNCs-S1 pH 10 and their complexes exhibited markedly higher catalytic activity compared to their unoptimised counterparts. Following characterisation, a thrombin cleavage assay was performed on the nanosensor complexes at varying thrombin concentrations. The optimised system consistently displayed superior signal detection after thrombin cleavage compared to the unoptimised system. However, the background from the optimised system was still elevated, posing a challenge for the accuracy of this sensing platform (Figure 2.16d). Addressing this high background requires more complex solutions beyond simple purification methods, possibly involving stabilisation of surface ligands. To tackle this, the AuNC synthesis was conducted by stepwise addition of the two ligands, GSH and S1 peptide, instead of the previous one-pot method.

2.4.6 Ligand swap on AuNC-GSH

Ligand exchange represents a post-synthesis chemical process harnessed for the further modification or functionalisation of metal complexes. In this transformation, the metal core retains its coordination with a specific ligand while permitting the replacement of one ligand with another. Numerous investigations have delved into the effects of ligand exchange on enhancing the physical and chemical properties of precisely synthesised thiolate-protected metal clusters^{185,204,205}. In this study, the process was initiated by employing GSH for the reduction of gold chloride hydrate, thereby yielding GSH-capped AuNCs under the above optimised pH 10 synthesis condition for 24 h. Subsequently, a carefully measured quantity of a different ligand

(peptide S1 in this instance) was introduced into the reaction mixture, followed by an additional incubation at 70 °C of up to 24 h to facilitate ligand exchange. The resultant mixture was then subjected to purification through an Amicon filter unit, effectively eliminating excess unbound ligands (Figure 2.17).

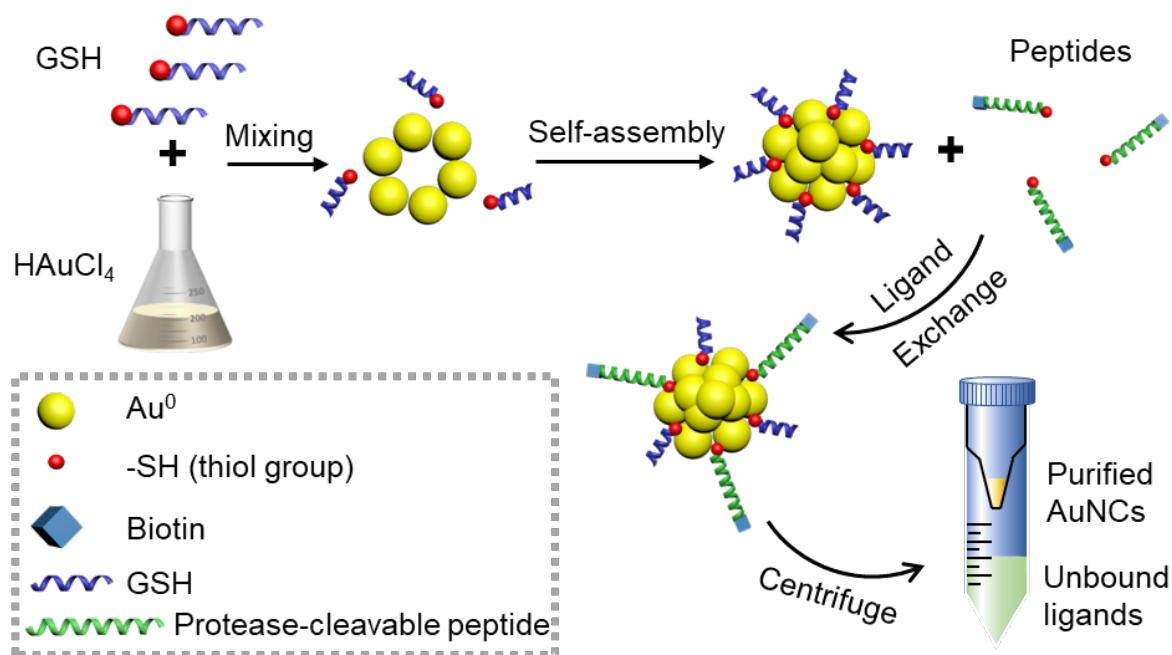


Figure 2.17 Ligand exchange method for peptide templated AuNC synthesis.

Gold chloride hydrate was reduced and capped with GSH at pH 10 over a 24-h period, resulting in GSH-AuNCs with significantly improved catalytic activity. Subsequently, peptide S1 was introduced into the reaction through a ligand exchange method for an additional 24 h. Finally, the resulting AuNC-S1 was purified using an Amicon filtration device to remove unbound ligands.

In this phase of this research, the effect of varying ligand exchange incubation times was investigated on the overall reaction, maintaining a constant temperature of 70 °C, ranging from 2 h to 24 h. Additionally, a control group subjected to a 2-h ligand exchange at room temperature was included. Employing a feed ratio of 1:5 for peptide S1 to GSH, as per a previously published study¹⁷, the biotin density was assessed through the HABA assay on the initial filtrate containing unbound peptide. By

subtracting the initial amount from the unbound peptide, the biotinylated peptide density was quantified. Surprisingly, regardless of the temperature and incubation time, biotin density remained consistent, seemingly unaffected by the conditions of the ligand exchange process (Figure 2.18a).

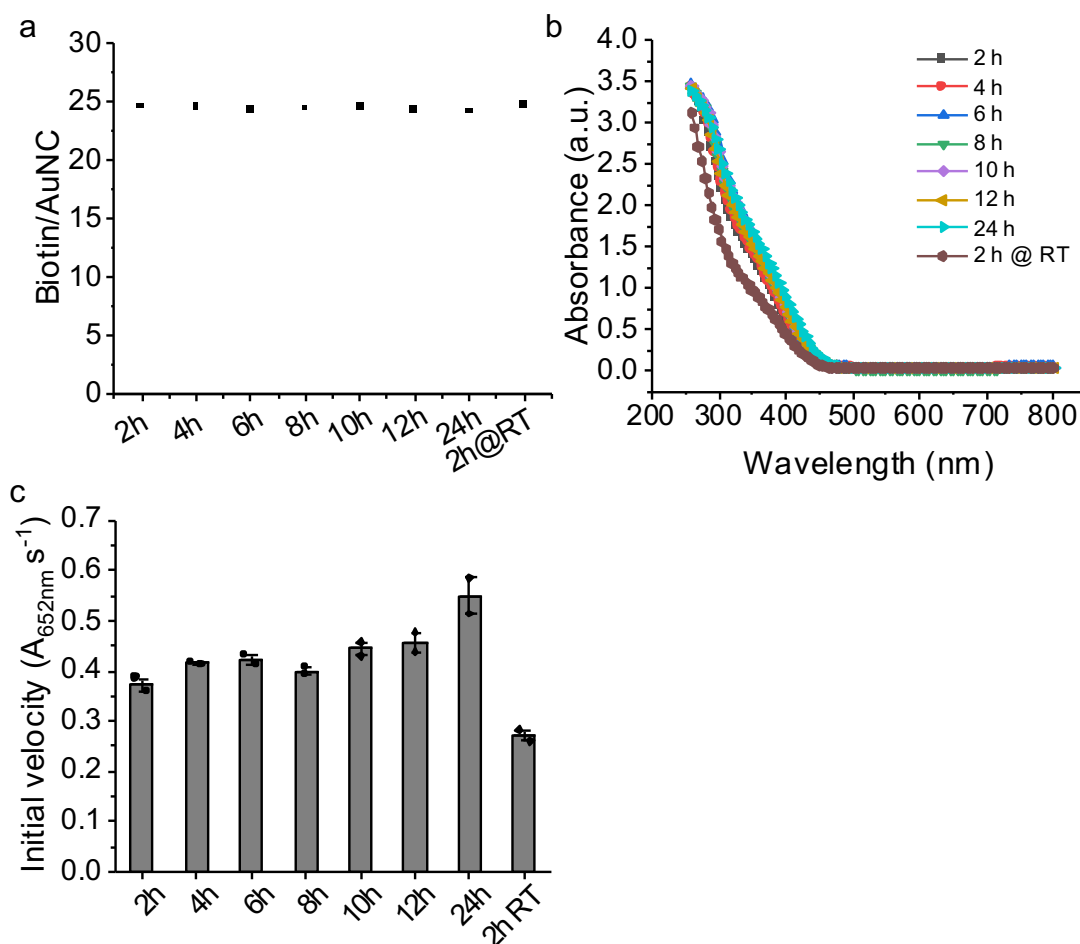


Figure 2.18 The influence of ligand exchange incubation time on the biotin density on the AuNC surface and their catalytic activity. (a) HABA assay for the biotin density quantification on AuNCs at different temperatures and for different incubation times ($N = 1$, $n = 1$). (b) UV-vis measurement of all peptide ligand exchanged AuNCs, indicating the concentration of AuNCs after the reaction ($N = 1$, $n = 1$). (c) Catalytic activity measurement of all AuNCs from different reaction conditions obtained by the TMB oxidation assay ($N = 1$, $n = 2$).

For a comparative assessment of AuNC concentration, UV-vis measurements were performed, indicating a notably weaker signal intensity for AuNCs synthesised at room temperature (Figure 2.18b). In contrast, AuNCs undergoing ligand exchange at 70 °C displayed analogous absorbance spectra, suggesting their concentration equivalence. Employing the TMB assay on these AuNCs, it was observed that catalytic activity from the low room temperature reaction markedly lagged behind that from the elevated temperature of 70 °C. Furthermore, the catalytic activity demonstrated a direct correlation with the duration of the ligand exchange process (Figure 2.18c). Consequently, the findings pinpointed the optimal conditions. Specifically, ligand exchange at 70 °C for 24 h was crucial for achieving highly efficient peptide-templated AuNCs. These conditions were subsequently employed to verify the enhanced sensitivity of the developed sensing platform.

2.4.7 *In vitro* thrombin cleavage assay with ligand exchanged AuNC-sensors

With the establishment of the optimal reaction conditions for ligand exchange, a thrombin cleavage assay was conducted aiming for a sensitivity comparison, utilising both unoptimised and optimised AuNC-GSH synthesised under pH 2 and pH 10. As seen before, the catalytic activity of the synthesised AuNC-GSH and the assembled thrombin-responsive nanosensor derived from pH 10 AuNC synthesis far surpassed that obtained from the pH 2 reaction condition (Figure 2.19a). Subsequently, both assembled thrombin sensors were subjected to incubation with varying concentrations of thrombin enzyme for a 4-h period. Following this, Amicon filtration (MWCO 50 kDa), simulating kidney filtration, was employed to segregate cleaved and liberated AuNCs for a subsequent TMB oxidation assay. In this context, the catalytic activity of filtrates

originating from the optimised AuNC-NAv thrombin sensor consistently outperformed those from the unoptimised sensor, while maintaining a comparably low background signal (Figure 2.19b). Regarding the sensitivity of thrombin detection, the optimised sensor achieved a limit of detection of 1.5 nM, marking a tenfold improvement over the unoptimised pH 2 counterpart. Collectively, the optimised AuNCs not only exhibited substantially enhanced catalytic activity but also facilitated the successful assembly of the sensor with sufficient stability. This enhancement translated into a promising augmentation of the sensing platform's sensitivity, further enhancing its potential for early disease detection and diagnosis.

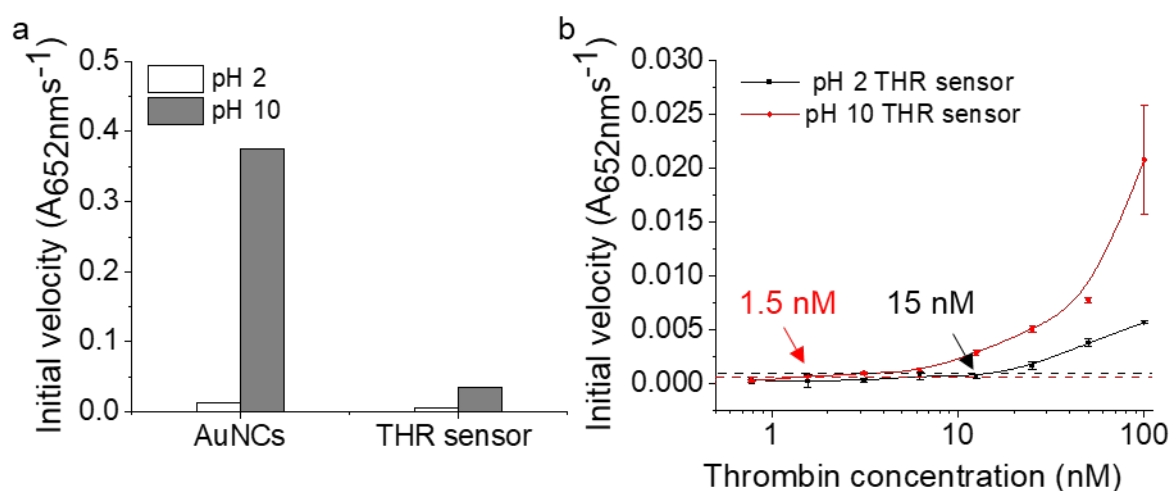


Figure 2.19 Thrombin cleavage assay on thrombin responsive sensors formed by peptide ligand exchange AuNCs. (a) Catalytic activity measurement of both unoptimised and optimised AuNCs and their corresponding thrombin responsive nanosensor ($N = 1$, $n = 1$). (b) Thrombin cleavage assay evaluated by TMB oxidation assay on the filtrate samples from nanosensors incubated with different concentrations of thrombin enzyme ($N = 1$, $n = 3$).

2.5 Conclusion

This chapter provides a comprehensive overview of the optimisation efforts undertaken in nanocluster synthesis aimed at achieving heightened catalytic activity. This enhancement has been assessed through TMB oxidation assays, while also ensuring that the size remained below the kidney filtration threshold. The exploration of various parameters in nanocluster synthesis aimed to develop a nuanced understanding of how different conditions impact both the catalytic activity and the size of the nanoclusters.

The findings of this study reveal that a synthesis condition involving a pH of 10 and a high temperature of 70 °C yielded a marked enhancement in catalytic activity (by 33.5-fold vs synthesis at pH 2), concurrently maintaining an ultra-small size of approximately 2 nm. Moreover, the study has established the favourable stability and retention of catalytic activity of AuNCs within physiological *in vitro* environments (in FBS or urine), thus indicating promising prospects for future *in vivo* applications. For a peptide templated AuNC-based nanosensor, of significance is the role played by the feed ratio, which refers to the peptide to GSH ligand ratio and the density of peptides on the nanocluster surface needed for conjugation to a protein carrier protein. This factor emerges as pivotal in the enzymatic cleavage of peptides and the efficient release of AuNCs from this nanosensor system. Notably, when the same amount of biotin is employed but with a lower biotin density, enzymes exhibit heightened accessibility to cleave peptide ligands, resulting in increased cleavage and release efficiency. This augments sensitivity levels substantially. Additionally, the technique of ligand exchange stands out as an effective approach for modifying AuNCs with

alternative ligands, all while maintaining structural stability ensuring a low background. In comparison to the one-pot synthesis method for AuNCs, sensors created using ligand-exchanged AuNCs demonstrate a lower background interference for the no thrombin control. It also achieved a remarkable tenfold improvement in sensitivity for enzyme cleavage for the optimised system when contrasted with the unoptimised system published previously¹⁷.

The findings in this chapter underscore the substantial enhancement of the catalytic properties of AuNCs attainable through meticulous screening of optimal nanocluster synthesis conditions. This achievement holds considerable significance for bolstering the sensitivity of sensing platforms. Further research endeavours hold promise for even greater performance enhancements (catalytic activity was 33.5 higher, but final sensor currently only 10x better) tailored to specific applications, while also ensuring a minimal background through the stabilisation of peptide templated nanoclusters.

Chapter 3

Towards a diagnostic test to predict severe malaria

3 Towards a diagnostic test to predict severe malaria

3.1 Aims

The goal of this chapter is to extend our previous research on gold nanocluster-based biosensors, enhancing their specificity, and developing an enzyme-responsive biosensor for swift and sensitive malaria infection detection. Despite low malaria incidence in some developed nations, it persists as a significant global health issue in developing regions. By incorporating an enzyme reaction mechanism involving malaria-related enzymes like SUB1 protease and MMP-8 for severe malaria, treatment can be employed for the control of the spread of malaria.

3.2 Introduction

Malaria is an infectious disease caused by parasites which can result in a significant and serious health burden. Numerous people are infected by malaria every year, and many lose their lives to this disease, with children and pregnant women being particularly vulnerable. Malaria is widespread, particularly in tropical and subtropical regions. The global popularity of travel has contributed to its spread around the world. Among the various types of malaria, *Falciparum* malaria, caused by *Plasmodium falciparum*, is the deadliest. Malaria infections can result in a wide range of adverse consequences, including life-threatening implications, social and economic burdens, on the affected areas. People infected with malaria require treatment, placing a significant financial burden on families and healthcare systems. Additionally, malaria pathogens are progressively developing resistance to antimalarial drugs, which further

complicates malaria treatment. Early detection and diagnosis of malaria infection are important tools for timely and effective treatment preventing and controlling malaria transmission. Children and pregnant women are at a higher risk of malaria and, consequently, require prompt testing and treatment. Enhancing the accuracy and accessibility of malaria detection is a crucial and essential step towards reducing morbidity and mortality rates.

3.2.1 The global burden of malaria infection

Malaria, an infectious disease caused by the parasite *Plasmodiums*, represents a significant public health burden globally, particularly in tropical and subtropical regions. The parasite is transmitted to humans through the bite of an infected female *Anopheles* mosquito. While the number of malaria cases had shown a steady decrease from 245 million in 2000 to 230 million in 2015, it regrettably experienced an upward trend since 2016. This situation was further compounded by disruptions caused by the COVID-19 pandemic, resulting in an annual increase of 13 million cases. Additionally, the elevated mortality rates observed among children under the age of 5 years heighten their susceptibility to severe malaria infections. In 2021, an estimated 247 million people were affected by malaria worldwide, marking an increase of 2 million cases compared to 2020²⁰⁶, and the number of deaths reached 619 thousand in 2021. The fight against malaria has been a challenging endeavour, with ongoing efforts to control mosquito vectors, improve access to prompt diagnosis, and provide effective treatment. The World Health Organization (WHO) and various stakeholders are actively engaged in strategies to mitigate the impact of malaria, and despite setbacks, progress has been made over the years. Sustained international collaboration and targeted interventions are essential in the battle to reduce malaria

incidence, prevent fatalities, and eventually eliminate this persistent global health threat.

3.2.2 Life cycle and development of severe malaria

The life cycle of the malaria parasites encompasses two distinct stages: an asexual phase within the human host and a sexual phase within mosquitoes^{207,208}. When mosquitoes carrying the infection bite humans, the parasite, in its sporozoite form, infiltrates the body and circulates through the bloodstream. Eventually, it reaches the liver, where it replicates asexually to form a stage known as merozoites. These merozoites enter the bloodstream and transform, subsequently infecting red blood cells (RBCs). This asexual cycle is responsible for all symptoms associated with the disease^{209,210}. Inside the RBCs, the parasite undergoes multiplication before rupturing and liberating additional parasites, to continue the cycle and infect more and more RBCs. In addition, merozoites in some infected RBCs develop into a sexual form called gametocytes, which subsequently enter the bloodstream. Gametocytes are taken up by mosquitoes when they bite an infected host, developing into mature sex cells called gametes (Figure 3.1).

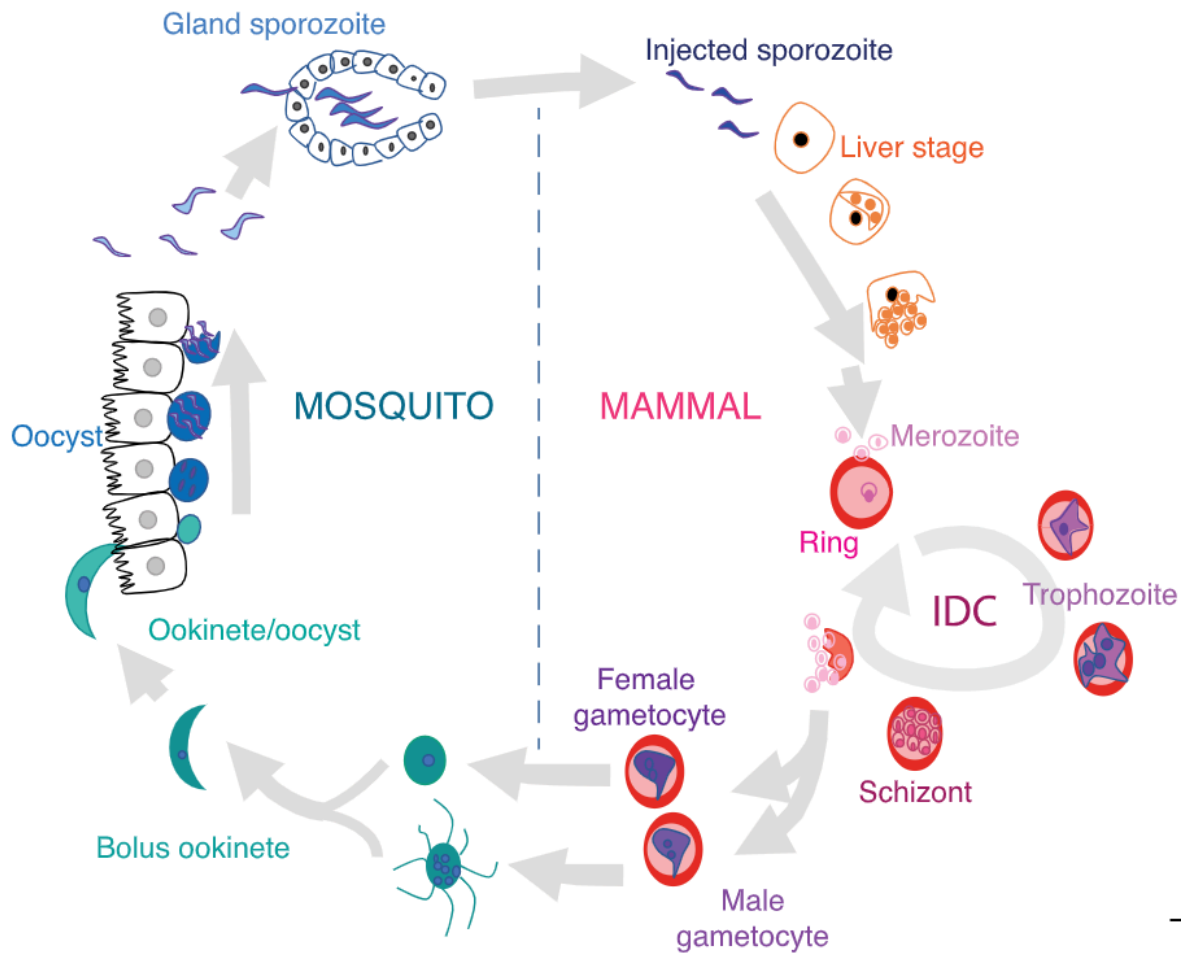


Figure 3.1 Malaria parasite life cycle. The malaria parasite life cycle begins when an infected female *Anopheles* mosquito bites and injects sporozoites into the host. These sporozoites enter the bloodstream, travel to the liver, and multiply asexually there. Subsequently, they transform into the merozoite stage and infect RBCs, where they replicate and eventually burst, re-invading RBCs cyclically. Additionally, some merozoites develop into a sexual form known as gametocytes, which can be taken up by a mosquito when it bites an infected host (This figure was adapted from Ref. 210 with permission)²¹⁰.

Severe malaria is defined as infections caused by *Plasmodium falciparum* with detection of vital organ dysfunction²¹¹. This involves sequestration of infected RBCs to endothelial walls, obstructing blood flow in various organs, and extensive damage to the host glycocalyx^{212,213}. Damage of this protective sugar layer is one characteristic part of severe malaria infection^{214,215}, and it was found to be mediated by host

neutrophils and includes various upregulated host enzymes such as olfactomedin 4, defensin A3, neutrophil elastase, and MMP8^{216,217}.

3.2.3 The diagnosis and therapy of malaria

To control the spread of malaria effectively and also achieve timely treatment of malaria infection in an early stage, it is essential to use early and accurate diagnostic tools. Traditional diagnostic methods include microscopy of blood smears, but this technique is limited due to the requirement of considerable expertise, technology, and equipment^{218,219}. Rapid Diagnostic Tests (RDTs) for malaria detection were developed already in early 1990s. RDT serve as a convenient and reliable method to diagnose malaria, especially in resource-limited settings, determining the presence of the malaria parasite infection by determining *Plasmodium*-specific antigens in blood specimen without the use of a microscope, which are now very widely employed in the field²²⁰. In recent years, molecular biology techniques were developed to obtain rapid and highly sensitive diagnostic methods, such as polymerase chain reaction (PCR). It provides more accurate identification of different species of *Plasmodium* parasites with a significant higher sensitivity²²¹. These methods and other highly sensitive methods, such as spectroscopy-based techniques²²², are commonly valuable and used in outbreak investigation and scientific research.

The described methods are applied differently in various contexts, from simple and rapid microscopy techniques and RDTs to highly accurate molecular biology methods, offering a variety of options for early diagnosis of malaria. In the prevention and control of the spread of malaria, testing methods have to be selected to be suitable for local

conditions, which can assist the achievement of detection of cases in an early stage and to initiate effective treatment and control measures immediately.

When it comes to malaria therapy, the selection of appropriate antimalarial drugs is pivotal, contingent upon factors such as the *Plasmodium* species involved, the severity of infection, and prevailing drug resistance patterns. A significant stride has been taken with the introduction of artemisinin combination therapies (ACTs) for treating uncomplicated malaria, along with the development of artesunate for severe cases. These advancements have shown potential in curbing mortality resulting from malaria infection^{223–225}. However, the emergence of drug resistance poses a substantial challenge to effective antimalarial treatment. Hence, adopting a multifaceted therapeutic approach becomes imperative for addressing malaria infection^{226,227}. Furthermore, the accessibility to prompt and efficacious treatment remains a notable concern in certain remote and resource-limited regions, where obtaining timely healthcare facilities and antimalarial drugs can be difficult. Focusing on the innovation of antimalarial drugs, vaccines, and combination therapies is paramount to addressing this disparity.

With respect to severe malaria, the current treatment scheme is still insufficient. Malaria infection is typically associated with low mortality, but when reaching the severe stage, mortality can be up to 15-20%, even when reaching a state-of-the-art hospital and applying modern drugs such as intravenous artesunate²²⁸. This drug acts by killing intracellular parasites, which does not impact sequestration nor glycocalyx degradation, two of the hallmarks of severe malaria. Hence, current research focuses on finding specific new treatments for severe malaria, including small molecules²²⁹,

heparin-like molecules²³⁰, and nanomedicine-based solutions^{231,232}. Inhibition of certain host enzymes responsible for glycocalyx might be another option for severe malaria mitigation²¹⁵. However, there is no accurate tool yet available to predict whether a malaria-infected patient develops severe malaria, often, it is only clinically detected once the patients are already in a very severe stage. Hence, the development of an affordable, rapid, and sensitive diagnostic tool for early severe malaria detection holds great significance, as it could be facilitating timely treatment with novel more specific drugs e.g. by reversing sequestration or inhibiting host enzyme, which can ultimately yield improved patient outcomes.

3.2.4 SUB1 and MMP-8 as biomarkers for diagnosis of severe malaria

Given the profound impact of malaria infection on public health, especially the impact of severe malaria, the development of an affordable, swift, and highly sensitive diagnostic tool for detecting severe disease is of paramount importance. As discussed in Section 3.2.3, traditional methods for detecting malaria parasites have limitations that underscore the need for innovative approaches, especially for severe cases that are associated with high mortality. Previous biosensor systems have already played a pivotal role in addressing the need of cost-effective, user-friendly, rapid, and targeted approach to malaria diagnosis. These tests are mostly based on the detection of parasite-derived proteins, peptides, and nucleic acids combined with a suitable detection system to provide a measurable signal^{233–235}.

Numerous biomarkers have been used for malaria diagnostic purposes. These encompass histidine rich protein II (HRP2), *Plasmodium falciparum* lactate

dehydrogenase (*Pf*LDH), both detected with RDT devices, and *Plasmodium* DNA or RNA (detectable through PCR) among others^{236–239}. Herein, the focus was shifted to a biomarker with enzymatic activity, as the system developed herein is enzyme responsive. Hence, SUB1, subtilisin-like protease 1, was identified as an interesting biomarker candidate. SUB1 has been identified as an essential enzyme of the parasite that is involved in various stages of the life cycle, mainly in egress/invasion processes^{240–243}. For example, SUB1 plays a pivotal role in the replication of the parasites, as it is an essential part of the host RBC rupture at the end of the cycle to liberate parasites. Consequently, the hypothesis herein is that the activity of SUB1 protease might be harnessed as a biomarker indicative of the presence of malaria parasites. Since severe malaria is associated with high parasite numbers, developing a SUB1 sensor that only detects high concentrations of this enzyme could function as part of a tool for a severe malaria sensor that makes it malaria-specific.

For a second biomarker of interest, the focus of the research aim here was to host enzymes that are upregulated in severe malaria. This search was performed with the idea in mind that a future SUB1 and host enzyme dual-responsive sensor could be an ideal setup where the test would confirm malaria infection (SUB1) and provide information on the severity (host enzyme). Many reports have discussed and underscored the strong association between MMP-8 and severe malaria, particularly in cases of fatal cerebral malaria^{217,244,245}. Importantly it was found that enzymatic activity seems to be localised *in vivo*, hence a simple blood test might not be sufficient for such a test^{216,217}. Drawing inspiration from this literature, the concept of developing an enzyme-responsive biosensor for rapid and efficient detection was demonstrated in this Chapter. This entails the creation of a biosensor utilising protease substrate

peptides specific to SUB1 and MMP-8, incorporated onto ultra-small AuNCs. These AuNCs are then tethered to a protein carrier NeutrAvidin (NAv), culminating in the formation of a biosensor platform. Upon exposure to SUB1 or MMP-8 protease, the AuNCs are released, facilitating a colourimetric response of blue colour change under the oxidation of TMB. This response is observed post-separation through an Amicon filter device, simulating renal filtration processes (Figure 3.5). In essence, the envisioned biosensor has the potential to enable rapid and highly sensitive detection of severe malaria infection via a kidney-filtration mechanism, all within the timeframe of 1 h, which was investigated in this Chapter. The ultimate goal, not yet explored herein, would be the combination of both enzyme-responsive systems. This could in the future be achieved, by e.g. formulating the SUB1 sensor as normal and then adding a PEGylated version (>20kDa) of the MMP-8-responsive peptide additionally to the AuNCs of the SUB1-sensor. If only SUB1 is present, the liberated AuNCs would still be too big for renal clearance due to the large PEG connected via the MMP-8 links, hence, only if also cleaved by MMP-8, a signal would be detected in the urine through TMB assay. Due to time constraints, in this thesis, pioneering work on SUB1 and MMP-8 responsive sensors was performed separately, leaving the combination of the two as future work.

3.3 Materials and methods

3.3.1 Materials

Calcium chloride, zinc chloride, sodium chloride, Brij-35, tris(hydroxymethyl)aminomethane (tris), piperidine, N,N-dimethyl formamide (DMF), Dichloromethane (DCM), diisopropylethylamine (DIEA), triisopropylsilane, dithiothreitol (DTT), trifluoroacetic acid (TFA), methanol, 2,2'-dipyridyl disulphide, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and dimethyl sulfoxide (DMSO), formic acid, Fmoc-protected amino acids were purchased from Sigma-Aldrich. -Chlorotriyl chloride resin was purchased from Iris Biotech.

3.3.2 Solid phase peptide synthesis (SUB1 and MMP8)

Peptides were synthesised using the general solid-phase peptide synthesis (SPPS) method, employing fluorenylmethoxycarbonyl (Fmoc) chemistry. The peptide sequences are summarised in Table 3.1. The end group design followed a previous paper⁴², yielding a cysteine at the C-terminal end and a biotin at the N-terminal end for subsequent sensor formation. The middle parts of the sequences were chosen from references^{243,246} for SUB1 and references^{216,236,237,247} for MMP8, as they contain the specific peptide for enzymatic cleavage by the respective enzyme.

A 2-Chlorotriyl chloride resin served as an insoluble support for assembling the peptide chain stepwise, with each amino acid added sequentially. The initial step involved loading the first C-terminal carboxylic acid (Fmoc-Cys-OH) onto the resin,

which was left overnight. Following this, the resin was subjected to five washes with dichloromethane (DCM). To cap any unreacted sites, a capping solution composed of DCM:MeOH:DIEA (9:1:0.5) was introduced to the resin and agitated for 20 min. Subsequently, the capping solution was expelled, and the resin was thoroughly washed with DCM and N,N-Dimethylformamide (DMF), each cycle repeated five times, in preparation for the upcoming Fmoc-deprotections and amino acid coupling steps. For Fmoc protecting group removal, a mixture of piperidine:DMF (20:80) was applied to the resin and shaken for 1 min. The resin was then washed five times with DMF. This process was repeated for another 15 min to ensure complete deprotection, followed by another five washes with DMF. To facilitate amino acid coupling, a coupling solution comprising Fmoc amino acid (3 molar equivalents), HATU (2.95 molar equivalents), and DIEA (6 molar equivalents) was prepared and agitated on a shaker for 15 min. The coupling solution was then introduced to the resin, where it was shaken for 30 min before undergoing five DMF washing cycles. The precise peptide sequences were achieved through repeated Fmoc-deprotections and coupling steps using specific amino acids. For biotin coupling, the compound was dissolved in dimethyl sulfoxide (DMSO) and combined with HATU and DIEA for an overnight coupling reaction. Subsequently, the peptides were cleaved using a solution of trifluoroacetic acid (TFA), triisopropylsilane (TIS), dithiothreitol (DTT), and water (87.5:2.5:7.5:2.5) for 3 h. The resulting cleaved peptides were collected in a 50-mL Falcon tube, followed by drying under nitrogen gas and centrifuge precipitation using cold ether three times at 500 rpm for 5 min (Figure 3.2). The resulting peptide pellets were then dried under nitrogen gas and desiccated under vacuum to prepare for subsequent high-performance liquid chromatography (HPLC) purification. Peptides were synthesised as designed sequences in Table 3.1.

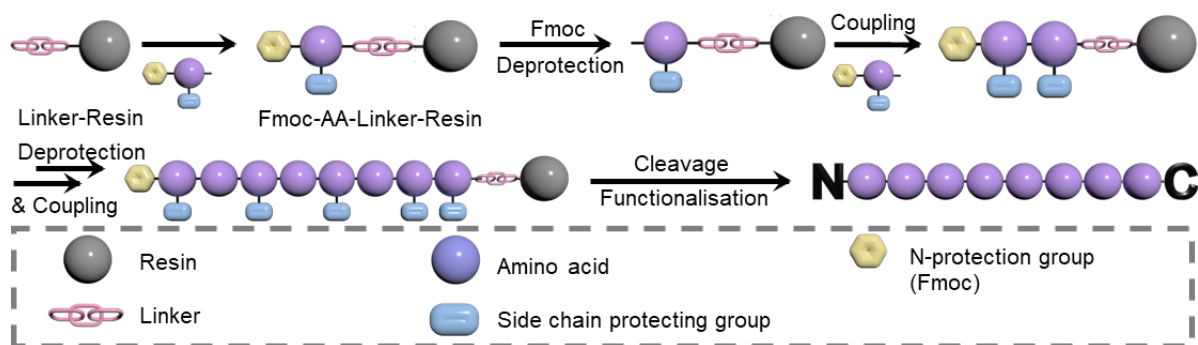


Figure 3.2 Solid phase peptide synthesis. It included the first amino acid loading on the resin, Fmoc-deprotection, amino acid coupling and cleavage of peptide chain.

3.3.3 Purification and characterisation of peptides (HPLC and LC-MS)

The purification of crude peptide products was meticulously conducted through reversed-phase high-performance liquid chromatography (HPLC), utilising a Phenomenex C12 column (Jupiter 4 μm Proteo 90 Å, LC Column 250 x 10 mm). In preparation for HPLC, the crude peptides were dissolved in a mixture of acetonitrile and water (5% v/v), supplemented with 0.1% v/v trifluoroacetic acid (TFA). To eliminate insoluble aggregates, this solution was carefully filtered through a 0.45 μm syringe filter before being introduced into the HPLC apparatus. An acid condition method was employed for the pre-HPLC process, optimising the separation of the peptide components. During this phase, all discernible peak fractions were collected. These fractions were then subjected to further analysis to determine molecular weight, a crucial step in confirming the composition of the peptide library. This analysis was conducted through liquid chromatography-mass spectrometry (LC-MS), allowing for a comprehensive evaluation of the synthesised peptides.

3.3.4 Synthesis and purification of peptide-templated gold nanoclusters

The process of peptide templated AuNC synthesis was elucidated in Sections 2.3.2 and 2.3.9 , which was repeated here using the final optimised protocol. In brief, the optimised AuNCs were synthesised through the reduction of HAuCl_4 (20 mM, 100 μL) using GSH (20 mM, 150 μL) at the precisely determined pH of 10, which was achieved by utilising 1 M NaOH. The reaction mixture was then adjusted to a final volume of 1 mL with the addition of H_2O . After the successful synthesis of the AuNCs, a carefully devised ligand exchange technique was implemented to firmly cap the synthesised peptides onto the AuNCs. This process encompassed the gradual addition of the specified quantity of peptides to the crude AuNCs with different peptide-to-GSH feed ratio described in Section 3.3.5 , followed by an incubation period at 70 °C that extended for 24 h for ligand exchange. Following the completion of this reaction, the resultant nanoclusters underwent a purification procedure, coupled with buffer exchange to ensure optimal characteristics for subsequent experiments. This involved use of DPBS (pH 7.4) as the buffering solution with Amicon filters, applying the same method detailed earlier in Section 2.3.2 .

3.3.5 Feed ratio variation in SUB1-29 templated AuNC synthesis

The investigation of feed ratio of peptide to GSH in nanosensor assembly was previously addressed in Section 2.3.4 . In order to ascertain the impact of feed ratio on peptide-templated AuNCs using this distinct peptide sequence SUB1-29, this aspect was revisited to assess its effect on the cleavage efficiency within the context

of the SUB1-29 malaria sensor. The feed ratio, which governs the ratio of peptide to GSH in the AuNC-GSH synthesis (Au:GSH 1:1.5), was systematically explored at varying proportions: 1:12.5, 1:25, 1:50, and 1:100 (12, 6, 3, 1.5 μ L of 20 mM SUB1-29 peptide for each ratio). This exploration was carried out through ligand exchange as described above, where a predetermined quantity of peptide was introduced into the synthesis subsequent to the formation of the crude GSH-AuNCs at pH 10. Following the reaction, the peptide templated AuNCs were subjected to purification with DPBS, utilising an Amicon centrifugal filter device (MWCO 10 kDa) at 5000 g for 5 min in three sequential rounds. This procedure ensured the removal of any unbind ligands. The final step involved resuspending the purified AuNCs in DPBS and passing them through sterile filters (0.2 μ m) using a syringe filtration approach. This preparation rendered the AuNCs ready for characterisation and subsequent assembly into sensors.

3.3.6 Assembly of AuNC-NAv complexes as severe malaria sensors

This process was elaborated upon in Section 2.3.7 . Briefly, a protein carrier NAv solution (125 μ L, 8 mg/mL) was prepared in DPBS. Subsequently, this NAv solution was combined with 1 mL of the purified AuNC-SUB1/MMP-8 (20 μ M) solution. The incubation of these components was carried out at a temperature of 37 °C, employing a gentle stirring speed of 500 revolutions per minute (r.p.m) over a period of 3 h. The incubation was to facilitate the conjugation of AuNC-SUB1/MMP-8 with NAv through the specific interaction of biotin binding. This interaction culminated in the formation of a complex named SUB1/MMP-8 sensors, wherein the AuNCs served as a probe for detection and peptide SUB1/MMP-8 served as linker for connection, and NAv acted as a protein carrier. Following the 3-h incubation, the malaria sensors underwent a

purification process to eliminate any unbound AuNC-SUB1/MMP-8. This was achieved through the use of Amicon Ultra-15 centrifugal filter units (MWCO 50 kDa), with the samples subjected to centrifugation at 5000 g for a minimum of 15 min. This process was iterated at least five times, or until no signal of TMB oxidation was detected in the filtrate. The goal here was to retain the malaria sensors in the concentrate while effectively removing any unbound components that had permeated through the membrane, ensuring the purity of the final product. The sensors were finally resuspended in DPBS and transferred using sterile syringe filter (0.22 μ m Millex-GV Filter) to remove any aggregates and sterilise the sample.

3.3.7 Characterisation of templated gold nanoclusters and malaria sensors

SpectraMax M5 multimode microplate reader was used to measure the kinetics reaction (absorbance vs. time) of TMB oxidation by AuNC-SUB1/MMP-8 and malaria sensors, with the aid of SoftMax Pro software as described in Section 2.3.9 . The hydrodynamic diameters of nanoclusters and complexes were measured by using dynamic light scattering (DLS, Zeta Sizer Nanoseries, Malvern Instruments, Ltd). The optical properties and concentration of nanoclusters were obtained via Ultraviolet-visible (UV-vis) measurement on SpectraMax M5 multimode microplate reader. Transmission electron microscopy (TEM) images were obtained on a JEOL 2100F. To prepare TEM samples, AuNCs solution needed to be desalted first with Zeba Spin Desalting Columns (7K MWCO) and 5 μ L of the sample was dropped on a carbon-coated copper grid and let to dry overnight.

3.3.8 *In vitro* malaria parasite detection with SUB1 sensors

In order to investigate the detection capability of the SUB1 malaria sensor, the study involved the cultivation of *Plasmodium falciparum* parasites due to the inaccessibility of recombinant SUB1. *Plasmodium falciparum* strain 3D7 was maintained in culture in human O+ RBCs as described elsewhere²⁴⁸ using RPMI-HEPES (Sigma-Aldrich, R5886) medium with 5 g/L Albumax II (Gibco)²⁴⁹, 0.292 g/L L-glutamine, 0.05 g/L hypoxanthine, and 0.025 g/L gentamicin. The full medium is henceforward referred to as malaria culture medium (MCM). 5% (w/v) sorbitol was employed for synchronisation²⁵⁰. Compound C1 at 2 μ M for 4-8 h in MCM was employed to tightly synchronise schizonts, which were washed in MCM just prior to performing the assays²⁵¹. In the specific context of the experiment, malaria sensors (60 μ L, 1 mg/mL) were subjected to incubation with either medium only (540 μ L MCM), RBCs only in MCM (540 μ L MCM, at 5% haematocrit), a diluted (540 μ L at 5% haematocrit, ca 1% schizonts) or undiluted parasite culture (540 μ L at 5% haematocrit, ca 3-4% schizonts) for durations of 1 h or 3 h, at a temperature of 37 °C, under shaking at 500 RPM. During the incubation time, schizonts burst open and parasites reinfected RBCs, yielding ca 3-4% or 10-15% ring stage infected RBCs at the end, for the diluted and undiluted samples, respectively. Following the incubation, the solution underwent centrifugation at 800 g for 5 min, followed by sterile filtration of the supernatant with 0.22 μ m syringe filters to remove any residual parasites. This filtered solution was then transferred to Amicon centrifugal filtration units (MWCO 30 kDa), and a further centrifugation step was performed at 14000g for 15 min, which yielded the liberated AuNCs in the filtrate. The resultant filtrate was collected and employed in the subsequent TMB oxidation assay. To carry out the TMB oxidation assay, the collected

filtrate (50 μ L) was mixed with 1-Step TMB (100 μ L) and H_2O_2 (100 μ L, 2 M). The absorption of light at 652 nm was measured using a plate reader, providing data for our analysis (Figure 3.5). The process of cultivating malaria parasites and conducting the incubation of the malaria sensor with parasites was performed by Dr Adrian Najer in Prof. Jake Baum's laboratory (Imperial College London, DoLS).

3.3.9 *In vitro* MMP-8 enzyme cleavage assay and limit of detection (LoD)

To explore the detection of MMP-8 enzyme activity, commercially acquired MMP-8 (brand and catalogue number) enzyme fragment was employed for incubation. MMP-8 sensors (50 μ L, 1 mg/mL of NAv) were incubated with MMP-8 enzyme (50 μ L, 100 nM) for 3 h at 37 °C and 500 r.p.m. Subsequently, the incubation solution was transferred to Amicon centrifugal filtration units (MWCO 30 kDa) and centrifuged at 14000 g for 15 min, with the collected filtrates designated for the TMB oxidation assay. For the TMB oxidation assay, the collected filtrate (50 μ L) was combined with 1-Step TMB (90 μ L) and H_2O_2 (90 μ L, 2 M). The absorption of light at 652 nm was measured using a plate reader.

In the context of the limit of detection (LoD), varying concentrations of MMP-8 enzyme (0-100 nM) were employed for incubation with MMP-8 sensors under the same conditions at 37 °C for 3 h. The resulting incubation solution was filtered through Amicon centrifugal filtration units (MWCO 30 kDa), with the filtrate collected for the TMB oxidation assay. This assay involved the combination of the collected filtrate (50 μ L) with 1-Step TMB (90 μ L) and H_2O_2 (90 μ L, 2 M). The subsequent measurement of light absorption at 652 nm using a plate reader provided the final data.

3.3.10 *In vitro* cytotoxicity studies of gold nanoclusters and sensor

RAW 264.7 cells were cultured in DMEM 1X medium supplemented with 5% FBS and 0.5% penicillin-streptomycin. For cell seeding, the desired RAW 264.7 cell concentration was suspended in culture media and introduced into sterile 96-well plates (20,000 cells/well). This was followed by a 24-h incubation to promote adhesion. After discarding the culture media from the wells with adherent RAW 264.7 cells, different concentrations of peptide-templated AuNCs and malaria sensors (20 μ L each in PBS) were added to the wells yielding the final mixture comprised of 180 μ L of medium (9 parts) and PBS +/- particles (1 part). The plates were then incubated at 37 °C with 5% CO₂ for an additional 24 h. To assess cell viability, the MTS assay (MTS) was employed. Cell supernatants were replaced with 120 μ L of MTS stock solution, consisting of DMEM medium without phenol red (31.5 mL), MTS (2 mg/mL, 6 mL), and PMS (0.92 mg/mL, 0.3 mL). After 1 h of incubation, the absorbance was measured at 490 nm to quantify the results.

3.3.11 *In vivo* pilot test

All animal experiment procedures were approved by the Imperial College Animal Welfare and Ethical Review Board and conducted under a Home Office Project License (PP7225449), following LASA good practice guidelines. A severe malaria mouse model was established by intraperitoneally administering a lethal *Plasmodium* strain, *Plasmodium yoelii* 17XL, with an inoculum of 8×10^6 for the infection, into a mouse (wild-type C57BL/6 mice purchased from Charles River, female, 8 weeks old).

For the administration of the MMP-8 sensor, the assembled and purified MMP-8 sensor (2 mg/mL of NAv, 100 μ L) was administered to both a healthy control mouse and a severe malaria-infected mouse. After a 1-h period following injection, urine samples were collected and stored in Eppendorf tubes for further colourimetric analysis. Each collected urine sample (20 μ L) was mixed with 60 μ L of TMB: H₂O₂ solution (final concentration of 5 M) in a 384-well plate, and the absorbance was subsequently measured at 652 nm. The severe malaria mouse model was established and the administration of the MMP-8 sensor into mice were performed by Dr. Athina Georgiadou in Prof. Aubrey Cunningham's laboratory (Imperial College London).

3.4 Result and discussion

3.4.1 Peptide synthesis

A multitude of studies have explored proteases associated with malaria infection, including parasite-derived SUB1^{243,246} and host MMP-8^{216,247}. In alignment with this, a protease substrate library was designed and synthesised, featuring specific peptide sequences primed for cleavage by corresponding enzymes (SUB1 and MMP-8). This was accomplished through solid-phase peptide synthesis (SPPS), as detailed in (Table 3.1)^{216,243,246,247}.

Table 3.1 Malaria responsive peptide sequences designed using literature information^{216,243,246,247}.

Pathological state	Enzyme specificity	Substrate Sequence	Substrate	MW (g/mol)
Malaria	SUB1	Biotin-GGGG ITAQSDEE GGGGC	SUB1-17	1677
Malaria	SUB1	Biotin-GGGGGGGGGGG ITAQSDEE GGGGGGGGGGC	SUB1-29	2361
Severe malaria	MMP-8	Biotin-SGGSGG TMNAPDFLRG SGGSGG C	MMP-8	2254

The peptide design criteria with biotin on one and cysteine on the other side adhered to the guidelines outlined in a recent paper¹⁷. These peptides exhibit a three-domain structure, encompassing a protease recognition motif, a C-terminal cysteine with a thiol group to facilitate attachment to AuNCs, and an N-terminal region incorporating biotin for subsequent conjugation to the protein carrier NAv. This approach offers the dual benefit of functionalising AuNCs with the desired peptide template and designing the peptide sequence to be specifically cleaved by the appropriate enzyme. The influence of peptide length and additional spacers on accessibility to the scissile bond was previously investigated¹⁷. Extending this exploration, the impact of an elongated SUB1 peptide was examined on cleavage performance within this sensing platform.

The specific SUB1 sequence was chosen from reference that showed various target peptide sequences have already been evaluated and found to be cleaved by SUB1²⁴⁶. The structure of the enzyme has been solved and a consensus recognition sequence identified that was cleaved equally efficiently by both human and rodent-infecting *Plasmodium* species (in single-letter notation, ITAQ↓SDEE)²⁴⁶.

After synthesis via SPPS, crude peptides underwent purification through prep-HPLC, with peak fractions collected for subsequent analysis via LC-MS. These mass spectrometry results provided insight into the final product's molecular weight post HPLC purification (Figure 3.3) and confirmed successful synthesis of the desired SUB1-responsive peptides (SUB1-17 and SUB1-29).

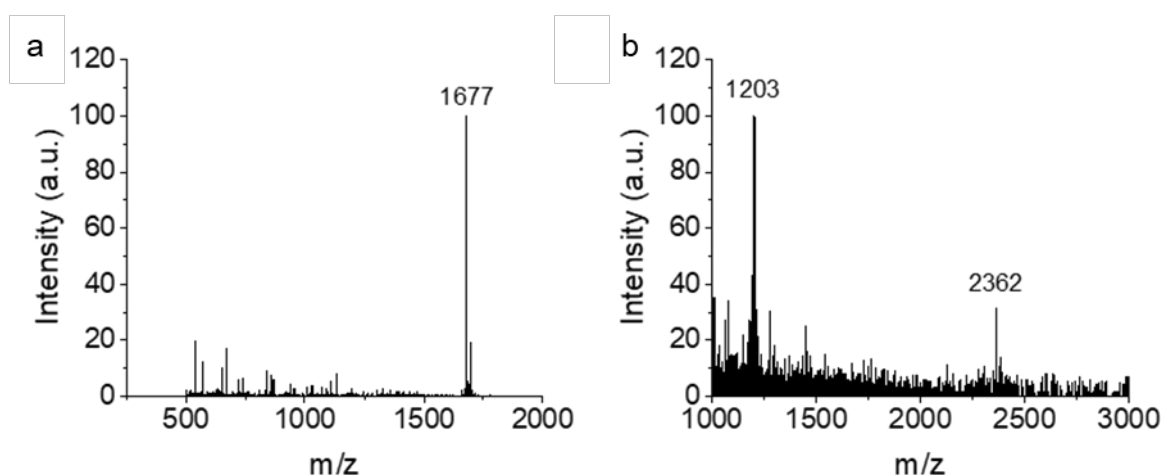


Figure 3.3 LC-MS chromatograms of peptides of (a) SUB1-17 and (b) SUB1-29 products after HPLC purification.

Investigations into the substrate specificity of the MMP family within the literature have highlighted the significance of incorporating aspartic acid at the P' position. This modification enhances substrate selectivity, enabling discrimination against closely related MMP-1 and other MMPs²⁴⁷. Drawing on this insight, the core sequence of the substrate was chosen as TMNAPDFLRG. Further refinements included introducing

additional spacer elements in the form of SGG and functionalising the C-terminal with cysteine to facilitate interactions with gold, while the N-terminal was coupled with biotin for binding with the protein carrier NAv. This design culminated in the sequence structure Biotin-SGGSGGSGGT**TMNAPDL**RGSGGSGGSGGC (Figure 3.4a), with the SGG spacers strategically incorporated to extend the peptide length, rendering it more accessible for MMP-8 cleavage. Incorporation of S in SGG rather than just using G was previously shown to yield thrombin-cleavable peptide¹⁷. Subsequent to the synthesis of the MMP-8 substrate peptide, purification was carried out using prep-HPLC. The resultant crude peptide underwent analysis utilising LC-MS to determine the final product and assess its purity (Figure 3.4b).

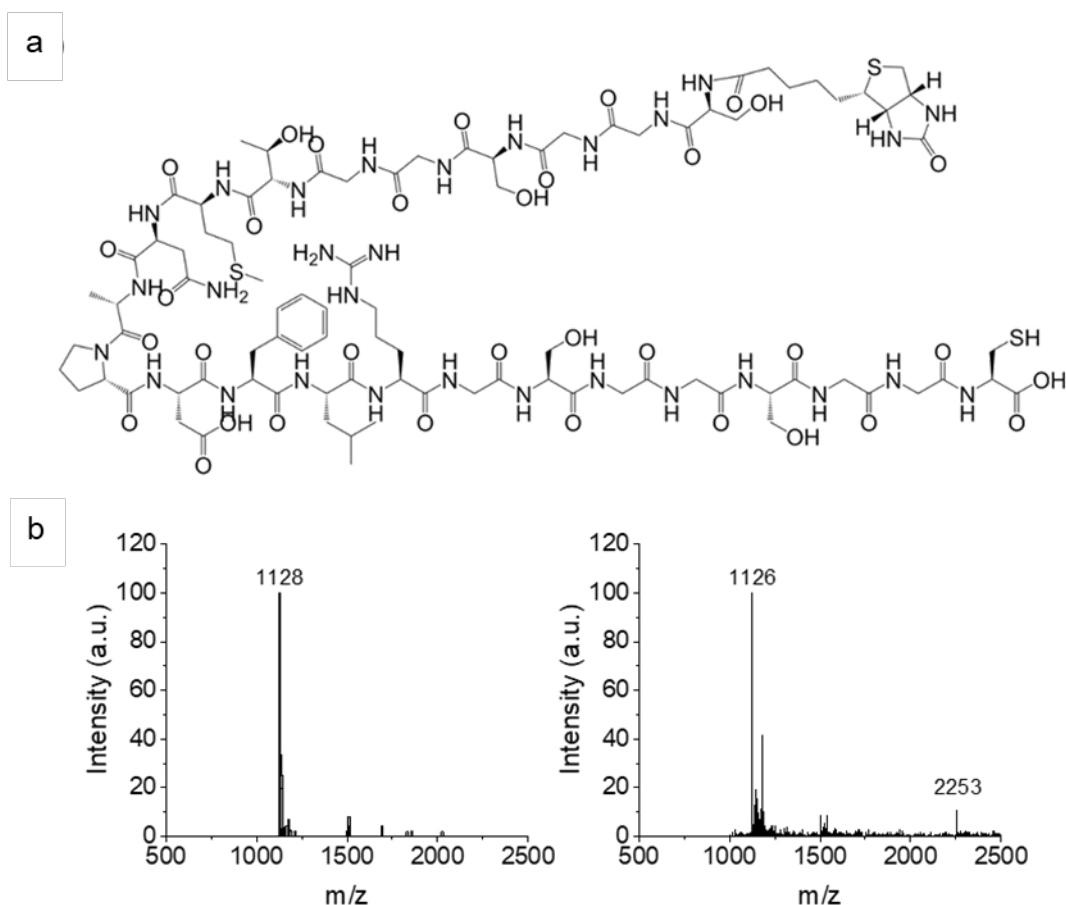


Figure 3.4 The design and synthesis of MMP-8 substrate. (a) Chemical structure of the sequences of MMP-8 substrate. (b) LC-MS analysis of MMP-8 substrate.

After successful synthesis of the desired peptides, the sensor setup of Chapter 2 was used again to build nanosensors for the potential detection of malaria and disease severity.

3.4.2 Synthesis and characterisation of peptide templated AuNCs using the original and the optimised AuNCs for sensor assembly (SUB1-17)

In Chapter 2, the optimisation of AuNCs was explored, resulting in a significant enhancement of catalytic activity while maintaining a size of approximately 2 nm below the renal clearance threshold. Building on this foundation, the optimised (synthesis pH 10) AuNCs was employed for the assembly of a malaria sensor designed to leverage SUB1 protease activity (Figure 3.5).

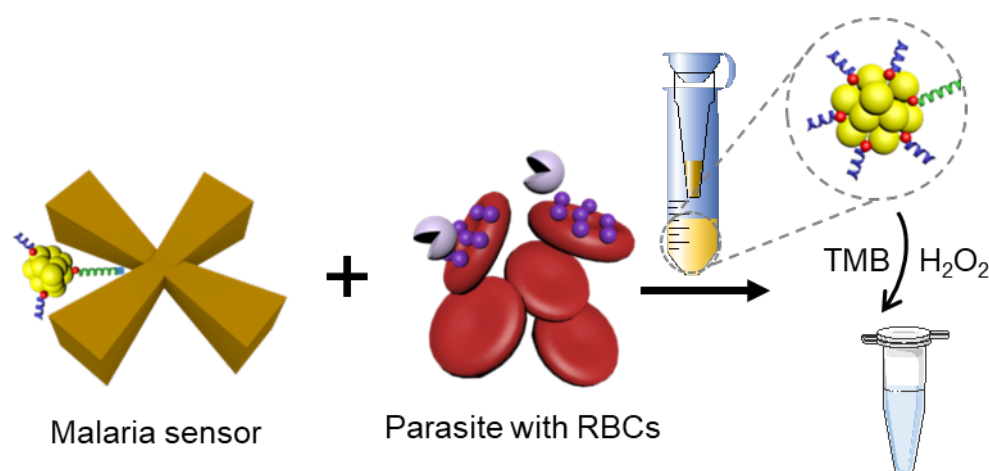


Figure 3.5 Overview of the design of malaria sensor and the cleavage mechanism by SUB1 associated with malaria parasites. Amicon filtration after parasite incubation was again used to separate intact sensor from liberated AuNCs to mimic the kidney filtration process. TMB assay on the filtrate corresponds to the final signal detected.

For assembling a first malaria sensor, the SUB1 peptide with 17 amino acids (SUB1-17) was utilised with optimised AuNCs from Chapter 2 . Notably, a one-pot not the ligand exchange AuNC synthesis approach was adopted here first. Following the assembly, a crucial step involved the purification of the sensors using size exclusion chromatography (SEC). This separation strategy was essential to purify the sensors from any unbound AuNCs present. Subsequently, the eluted fractions obtained from the SEC purification were subjected to analysis using the TMB oxidation assay, with absorbance measurements taken at 652 nm. The intensity of fraction signals originating from the sensor assembled with optimised AuNCs exhibited a considerable enhancement in comparison to those associated with unoptimised AuNCs as found in the previous Chapter 2 . Two peaks were found in the SEC fractions and to ensure a minimal amount of the free cluster peak being collected with the sensor, only fractions 8 to 10 from the sensor peak were used. Future optimisation could involve use of longer SEC columns or different column material to improve separation.

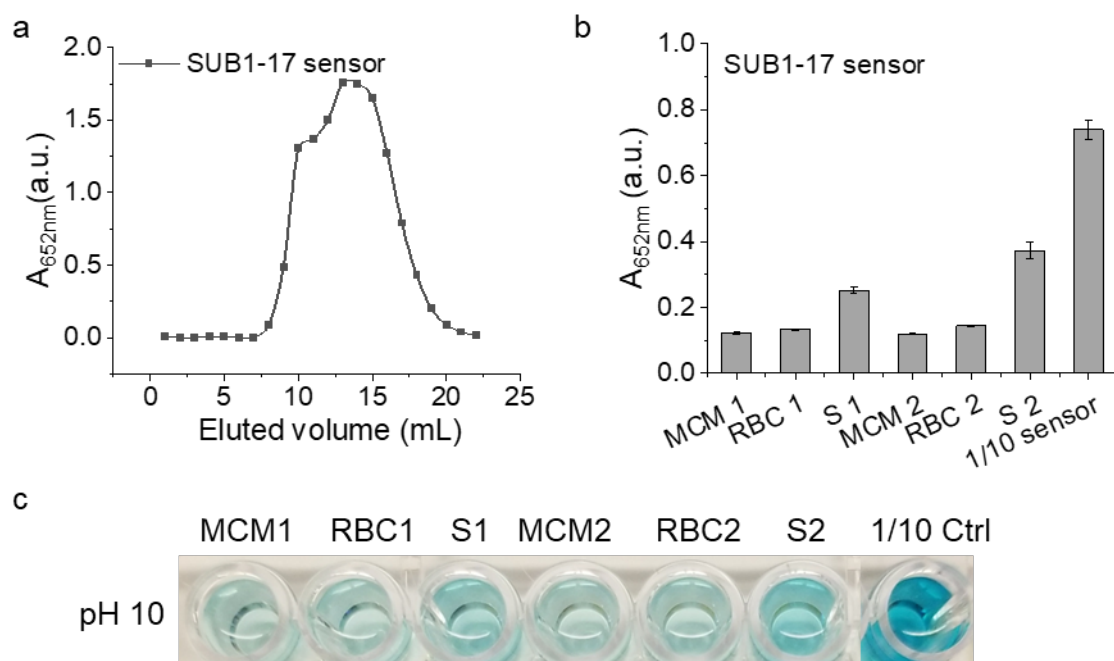


Figure 3.6 The detection of malaria parasites by SUB1-17 malaria sensors using the optimised AuNCs. (a) TMB oxidation assay analysis on the SEC fractions from SUB1-17 sensors formed with the optimised AuNCs one-pot synthesised at pH 10. (b) *In vitro* assay of parasite incubation with SUB1-17 sensors and running TMB on the Amicon filtrates, showing AuNC liberation (MCM and RBC refer to incubation with medium control and RBC control, S represents schizont stage used at the beginning of the assay, which burst and formed new rings during incubation, 1/10 sensor is the 100% signal control) (N = 1, n = 3). (c) Photograph of the final TMB assay results analysed in (b).

Following purification of the SUB1 malaria sensors, they underwent an incubation process with cultured malaria parasites. This experiment encompassed the culture medium only (MCM), RBCs (5% haematocrit in MCM), and schizont stage *P. falciparum* infected RBCs (5% haematocrit in MCM at ca 3-4% parasitaemia). During the subsequent 3 h incubation time, the schizonts burst open, which involves SUB1 action, and parasites invaded neighbouring RBCs to form ring stage parasites. This 3-h incubation was followed by Amicon separation to isolate the released AuNCs into

the filtrate. Subsequent TMB assays were conducted within the filtrate to confirm presence or absence of AuNCs. It was indeed found that schizont containing samples liberated higher amounts of AuNCs from the sensor. However, background signals after sensor incubation in MCM and RBC control groups alone were elevated. This elevation in background signals potentially poses a challenge by introducing the possibility of false positive signals (Figure 3.6b, c). The elevated background signal was similar for these two control groups, meaning incubation in the culture medium alone seems to be problematic. Addressing this issue necessitates the exploration of alternative methods aimed at enhancing the stability of peptide-template AuNC-based nanosensors within the physiological environment. The background signals from the control group might come from the unbound AuNCs or the breakage of the unstable bond between Au and peptide.

Aiming for a more comprehensive purification of malaria sensors, ensuring better separation from unbound AuNCs, Amicon centrifugal filter devices was first employed followed by SEC. The fractions obtained after SEC were subjected to measurement using the TMB assay, enabling the assessment of signal intensity within each fraction. However, only a small particle elution fraction was observed, possibly indicating the presence of only a small residual AuNCs that were not entirely removed during the prior Amicon filtration step (Figure 3.7a). To ensure high purity of the sensor, only fractions 5.5 mL to 6 mL, instead of the whole peak, were selectively collected for the subsequent *in vitro* assay. Before proceeding, the sensor and free AuNC hydrodynamic diameters were determined through dynamic light scattering (DLS), confirming approximate sizes of 2 nm and 10 nm respectively, aligning well with the experimental design requirements (Figure 3.7b).

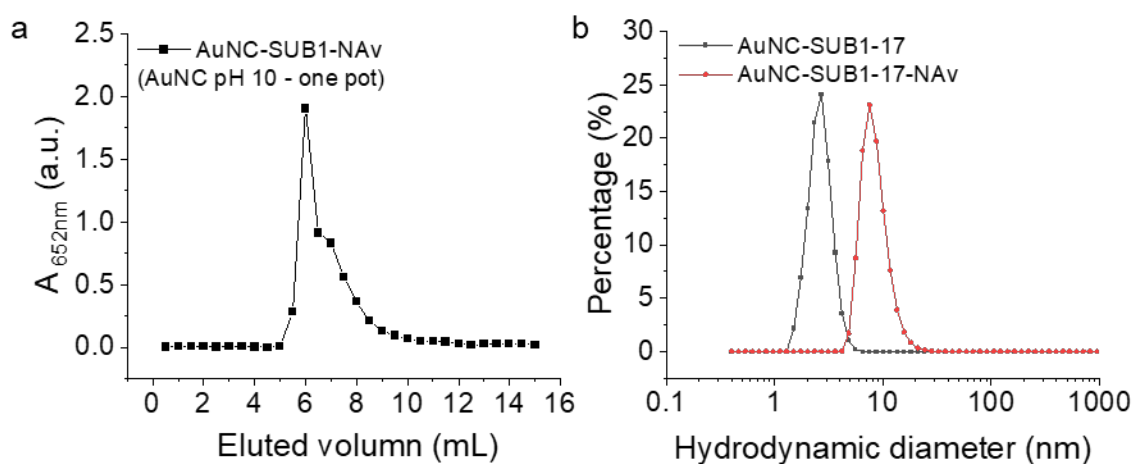


Figure 3.7 SEC fraction analysis and DLS of AuNC-SUB1-17 and SUB1 malaria sensor. (a) TMB assay on the SEC fractions of SUB1 malaria sensor after prior purification by Amicon filtration (N = 1, n = 1). (b) DLS measurement of the hydrodynamic diameters of AuNC-SUB1-17 and SUB1-17 malaria sensor (N = 1, n = 3).

Having undergone dual purification via Amicon and SEC, the SUB1 malaria sensor was subjected to another *in vitro* assay. The sensor was again incubated with parasite-infected RBCs (dilution series starting from 3-4% parasitaemia) alongside blank controls consisting of MCM and RBC. Additionally, another positive malaria parasite control group encompassing ring stage (R) was included (5% haematocrit at ca 3-4% parasitaemia), where no schizonts were present, hence release of SUB1 will not happen during the incubation time. The 3-h incubation was again succeeded by Amicon centrifugal filtration, enabling the isolation of released AuNCs. Notably, the schizont stage group (S) displayed a heightened signal compared to the other control groups. However, still the elevated background observed in the medium control (MCM) and RBC control hindered the establishment of a definitive negative reference for detection. Ring stage parasites did not yield increased signal, confirming that the

elevated signal after incubation with schizont cultures (egress during incubation period) is likely due to liberated SUB1 involved in that process (Figure 3.8).

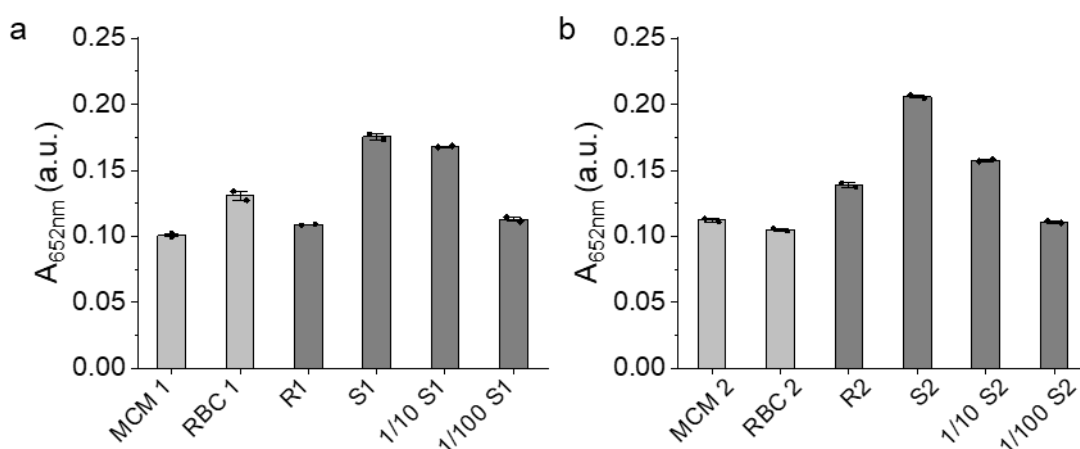


Figure 3.8 TMB analysis on the filtration from parasite incubation with SUB1-17 malaria sensor. (a, b) TMB analysis on filtrates from the independent repeats of incubation (N = 2, n = 2) after 3-h incubation with various samples and Amicon filtration.

Given that the additional purification approach did not yield improved results compared to above, the exploration of a longer SUB1 peptide was pursued with 29 sequences, including a longer spacer sequence with the hypothesis that better enzyme access could yield more cleavage, hence, larger difference to the background signal. Moreover, a ligand exchange method was implemented in order to achieve a more stable structure.

3.4.3 The influence of longer peptides on the cleavage of the sensing system (SUB1-29)

Extended peptides attached to the surface of AuNCs, then coupled to the protein carrier NAv, can influence the accessibility of enzymes to the scissile bond, subsequently impacting cleavage efficiency. In light of this, a SUB1 peptide consisting of 29 amino acids (SUB1-29) was synthesised, utilising additional glycine residues to

extend the peptide length. As previously detailed in Section 2.3.9 , ligand exchange was investigated as an additional means to enhance the stability of peptide-templated AuNCs and their corresponding sensors. The process involved the synthesis of GSH-AuNCs at pH 10, followed by ligand exchange with a peptide-to-GSH feed ratio of 1:5, employing a 48-h reaction duration in this case. The subsequent sensor incubation extended for 3 h and was prepared freshly for the ensuing parasite incubation.

Following the purification of the SUB1-29 malaria sensor, they underwent a 1-h incubation with the schizont stage (S) of the malaria parasite, alongside MCM and RBC controls. This was followed by separation using Amicon filtration. The resulting filtrates were subjected to analysis utilising the TMB oxidation assay (Figure 3.9). The blank signals (no sensor added) stemming only from RBCs (Ctrl RBC) or schizont stage culture (Ctrl S) yielded no response, confirming that any signal detected in these types of assays comes from AuNC catalysis only. In the case of the background control involving the incubation of sensors with MCM and RBC, only a slightly elevated signal in TMB oxidation was measured in one experiment.

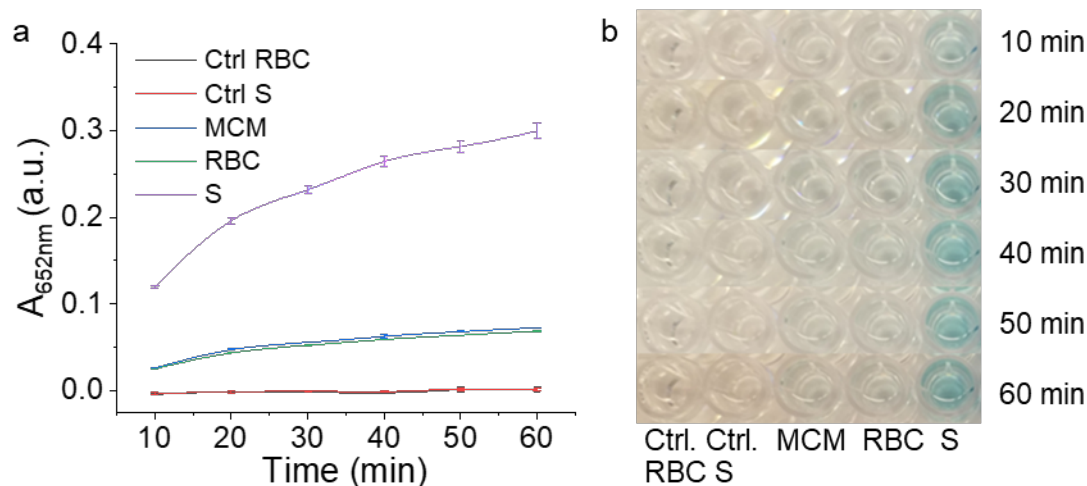


Figure 3.9 *In vitro* parasite incubation with sensor using longer peptide SUB1-29. (a) TMB assay result from the filtrate after parasite incubation with SUB1-29 malaria sensor and the corresponding photograph (N = 1, n = 2). (b) The photograph of the final TMB result from measured data in (a).

However, in repetition experiments the background problem came up again (Figure 3.10). Nevertheless, incubation with the parasite sample (S) always gave rise to elevated signals even when background signal stemming from sensor instability in the medium is present. This problem could be mitigated by plate reader measurements and quantitative comparison for diagnostics, however, further optimisation to remove the background completely would be beneficial as it would allow simple and rapid readout by the naked eye.

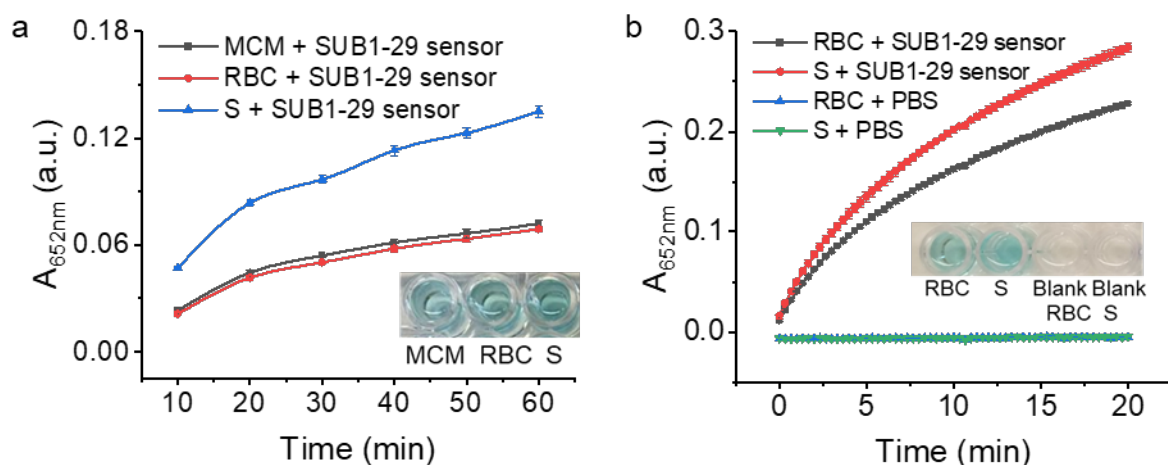


Figure 3.10 TMB analysis on the filtration from *in vitro* parasite incubation with SUB1-29 sensor. (a, b) TMB analysis on filtrates from the independent repeats of parasite incubation with the sensor using longer peptide SUB1-29 after Amicon filtration (N = 2, n = 2).

3.4.4 Cleavage performance of SUB1-29 sensor using AuNCs with different feed ratios (peptide-to-GSH)

Besides changing the peptide length, there remain further opportunities for optimisation to amplify the sensitivity of this sensing platform. The pivotal role of the peptide-to-GSH feed ratio in enzyme-mediated peptide cleavage warrants attention as found in Section 2.3.4. An excessive number of peptides on the AuNCs can result in their binding to more than one protein carrier, NAv. This scenario would also demand a higher enzyme concentration or longer incubation time to cleave all peptides for the release of AuNCs. Conversely, if only one peptide is present on the AuNC surface, it has the potential to bind solely to one NAv. Consequently, this implies a reduced enzyme requirement for the cleavage of the peptide, facilitating the release of AuNCs and enabling their detection (Section 2.3.4).

A variety of peptide-to-GSH feed ratios were explored, ranging from 1:12.5, 1:25, 1:50, to 1:100, resulting in distinct biotin densities: 6, 3, 2, and 1 peptide per AuNC, respectively, as found by the quantification of biotin via the HABA assay (Figure 3.11a). Across all AuNCs with differing feed ratios, a consistent catalytic activity was observed in the TMB assay. This indicated that despite the utilisation of varying peptide amounts, a uniform quantity of AuNCs was produced (Figure 3.11b). Their hydrodynamic diameters, as well as the derived count rate, were measured using DLS, revealing an approximate size of 2 nm. Notably, AuNCs with lower peptide content displayed even smaller sizes. Given the non-identical sizes, comparing their concentration based on the derived count rate was not feasible (Figure 3.11c). To confirm their concentration, UV-vis measurements were conducted, validating a similar concentration. This outcome was consistent with the findings from catalytic activity measurements (Figure 3.11d).

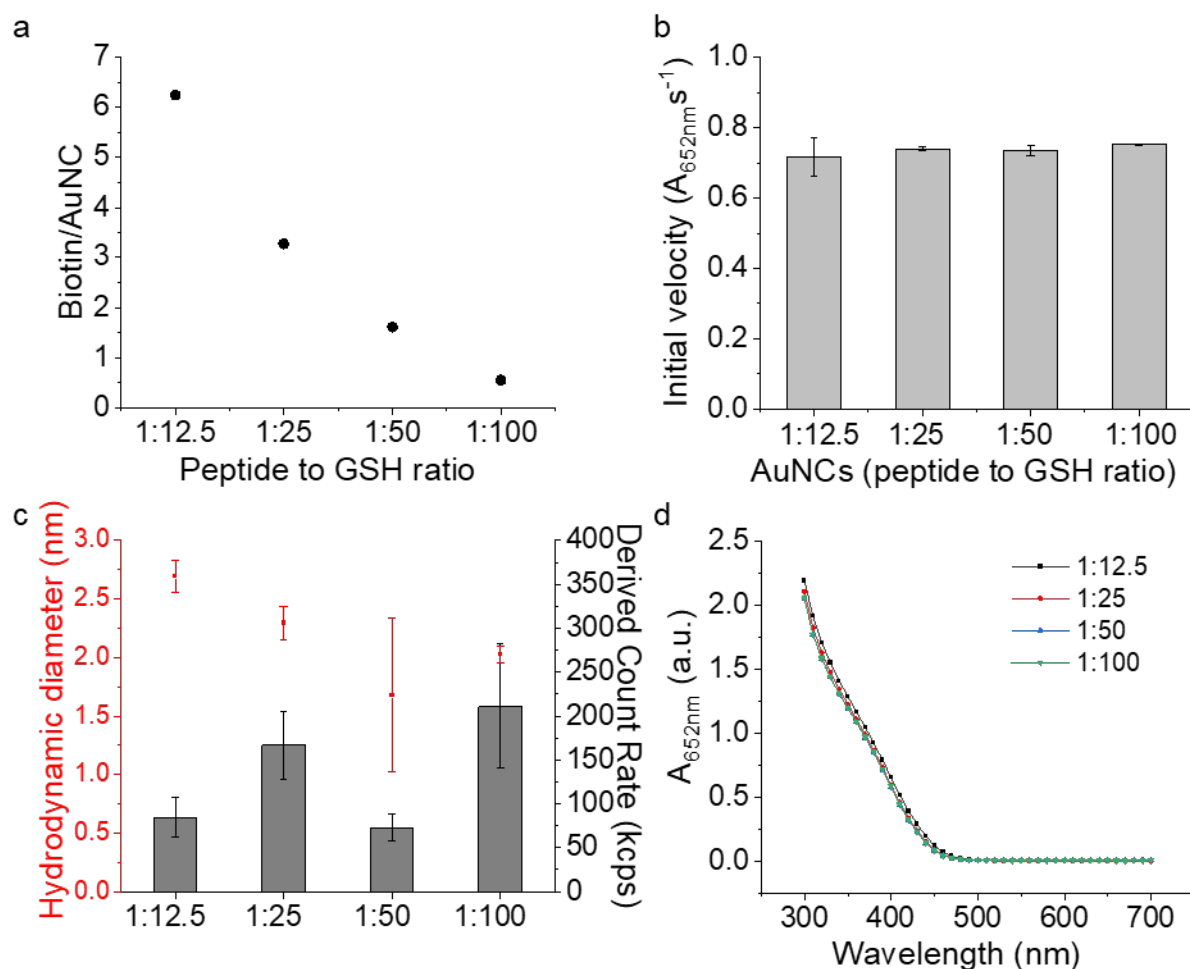


Figure 3.11 Characterisations of AuNCs-SUB1-29 after ligand exchange using different feed ratios. (a) Biotin or peptide density on AuNCs. HABA assay was used for the biotin quantification which also referred to the peptide density ($N = 1$, $n = 1$). (b) TMB assay on AuNCs synthesised with different feed ratios. The absorbance at 652 nm was measured over time, from which the velocity could be converted for the comparison of the catalytic activity. (c) Hydrodynamic diameter and derived count rate measurements using DLS. (d) UV-vis measurements for concentration comparison.

To ensure equitable opportunities for protein carrier NAv to bind to biotinylated-peptide templated AuNCs, it was imperative to unify the biotin concentration across all AuNCs with distinct biotin densities, rather than maintaining a uniform concentration of AuNCs. In this context, employing an excess of AuNCs during the conjugation with NAv facilitated an equal likelihood of biotin binding to the available binding sites on NAv.

This resulted in a consistent sensor concentration in terms of NAv content. Following the concentration step, the size of AuNCs remained stable, hovering around 2 nm below the 5.5 nm threshold. As expected, after concentrating the samples differently to yield the same biotin concentration, all data suggested successful adjustment of AuNC concentration (Figure 3.12).

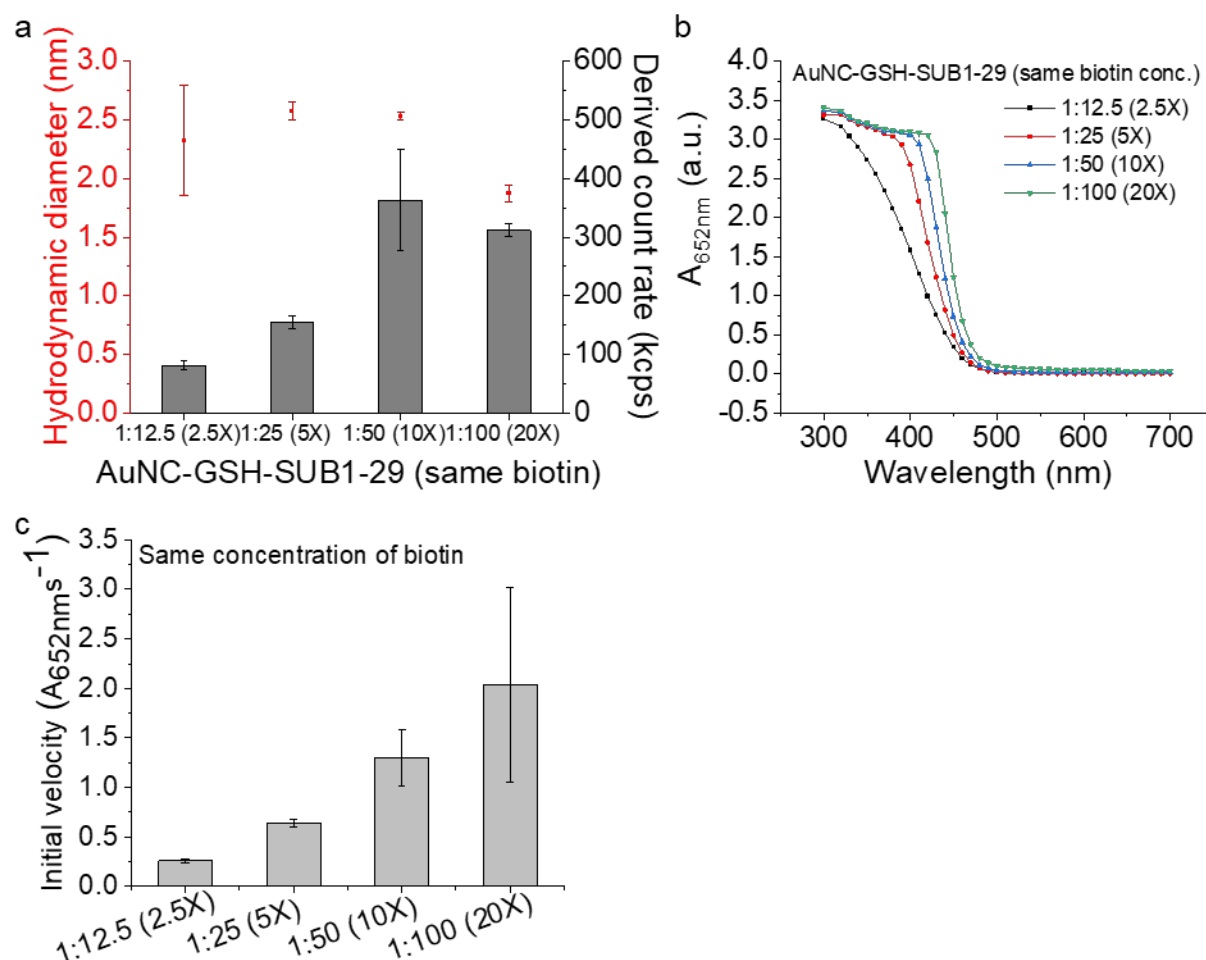


Figure 3.12 Re-characterisations of AuNC-SUB1-29 after concentrating to the same biotin concentration. (a) Hydrodynamic diameter and derived count rate measurements of all concentrated AuNCs using DLS. (b) UV-vis measurements for the concentration comparison. (c) TMB assay on concentrated AuNCs with different feed ratios. The absorbance at 652 nm was measured over time, from which the velocity could be converted for the comparison of the catalytic activity.

After obtaining AuNCs using different feed ratios while maintaining a constant biotin concentration, the SUB1-29 malaria sensors were assembled for subsequent incubation with malaria parasites for the sensing evaluation. This endeavour aimed to determine the optimal feed ratio within the malaria sensors, ensuring enhanced cleavage efficiency in response to malaria parasite-derived enzyme. As the biotin concentration and the carrier protein NAv concentration remained consistent across all malaria sensors (MS), the sensor concentrations were equated. The variation in feed ratios during AuNC synthesis could influence the binding opportunities and accessibility to protein carrier NAv, potentially leading to distinct quantities of AuNCs bound to the protein. The difference in UV-vis and DLS measurements for all malaria sensors reveal that the sensor utilising AuNCs with a peptide-to-GSH ratio of 1:12.5 was lost during the Amicon purification process (Figure 3.13a, b). Consequently, it is advisable to exclude this particular sample from further incubation in the malaria parasite incubation experiment.

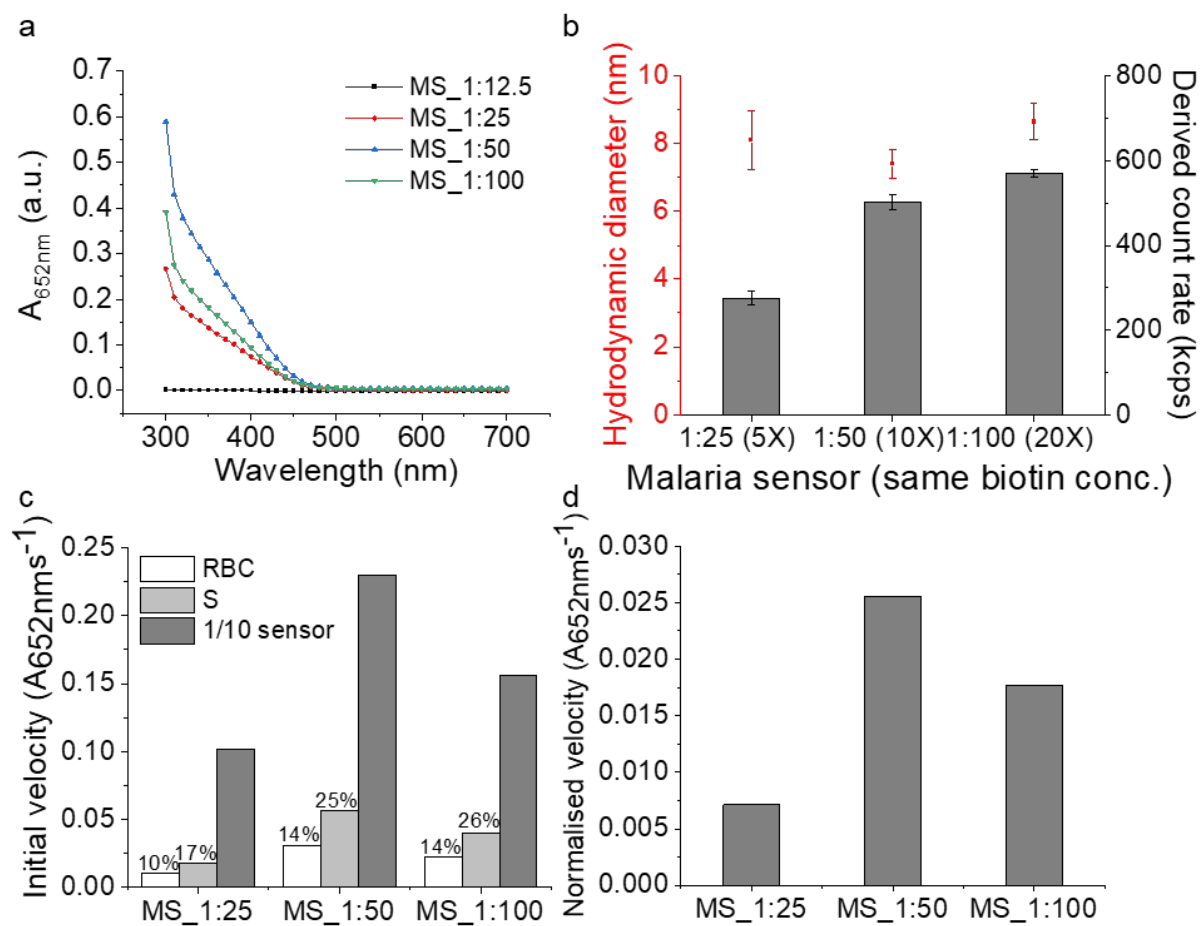


Figure 3.13 Variation in feed ratio for formation of malaria sensor SUB-1. (a) UV-vis measurements of SUB1-29 malaria sensors after Amicon purification and using AuNCs prepared with different feed ratios of SUB1-29-to-GSH from 1:12.5 to 1:100. (b) Hydrodynamic diameter and derived count rate measurements by DLS ($N = 1$, $n = 3$). (c) TMB assay measurements on the filtrates after parasite incubation ($N = 1$, $n = 1$). (d) Normalised TMB assay from (c) after subtracting the velocity from RBC background measurements.

To assess and compare the sensitivity of the malaria sensors with different feed ratios, each of them was subjected to incubation with an identical batch of malaria parasite culture. Following the incubation, the released AuNCs were isolated through centrifugation using an Amicon filter device, effectively segregating them into the filtrate. These AuNCs were subsequently collected for a subsequent TMB assay, characterised by a distinct blue colour shift. Malaria sensors configured with lower feed

ratios exhibited slightly higher signal intensities following their incubation with the parasites, achieving release efficiency of 25% and 26% for ratio of 1:50 and 1:100 when juxtaposed with the output from sensors with higher feed ratio (1:25), which yielded a signal of 17%. However, the background after incubation in medium only also exhibited a slight elevation in the case of malaria sensors with lower feed ratios (Figure 3.13c). To mitigate the influence of this background signal, a process of normalisation was employed by subtracting the background values. Consequently, the malaria sensor crafted with a feed ratio of 1:50 emerged as the frontrunner in generating the most pronounced signal for malaria parasite detection (Figure 3.13d). The key future optimisation step will be the reduction of this background signal, as outlined in the conclusion and outlook parts.

3.4.5 Specificity of SUB-1 sensor

To investigate the specific cleavage of the SUB-1 sensor by malaria parasite enzymes, a SUB1 peptide-conjugated malaria sensor was utilised along with a randomly selected thrombin sensor, as outlined in Chapter 2 . The optimal feed ratio for the malaria sensor (SUB1-29) was established at 1:50 above, while for the thrombin sensor assembly, the established feed ratio of 1:5 was adopted, as previously reported in a recent study⁴². Once both sensors were assembled, they were incubated with the same batch of malaria parasite culture for a duration of 1 h. Following the sensor-parasite incubation, the potentially released AuNCs were separated using an Amicon filter device, and the resulting filtrate was collected for subsequent TMB assays. After incubation of the malaria sensor with S parasites, the signal detected in the filtrate exhibited a clear increase over time when compared to the negative control involving the incubation of the malaria sensor with RBCs only. Notably, a distinct blue colour

change was observed in the positive sample derived from the schizont stage incubation, whereas the control sample involving incubation of the sensor with RBCs only showed no visible colour change (Figure 3.14a).

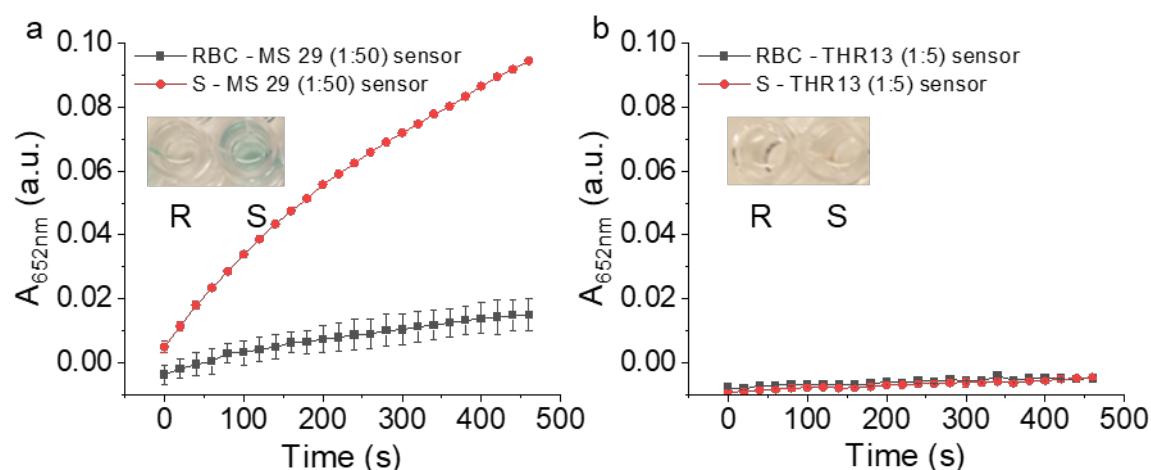


Figure 3.14 Cleavage assay incubating malaria sensors (SUB1-29) and thrombin sensors with malaria parasites. (a, b) TMB assay on the filtrates from the incubation of malaria parasites with malaria sensor using SUB1 and thrombin sensors using SUB1-29 (a) and THR-13 (b) (S1 used in Chapter 2) thrombin cleavable peptides for sensor formation, respectively.

Importantly, for the incubation of the thrombin sensor with parasites, both the signals from the RBC control and the schizont stage parasite samples remained consistently low (Figure 3.14b). This observation strongly implies that the SUB1 sequence was selectively cleaved by the SUB1 protease released exclusively from schizont stage parasite-infected samples, demonstrating its specificity. This cleavage event did not occur with other peptides possessing an unrelated sequence (thrombin-responsive). After evaluating this parasite enzyme-sensitive sensor, it was important to shift towards the host response and develop and study an MMP-8-responsive sensor instead.

3.4.6 *In vitro* cytocompatibility of AuNCs and corresponding sensors

Ensuring the biocompatibility of AuNCs and corresponding sensors holds paramount importance, particularly when considering their potential for future *in vivo* studies. The assessment of biocompatibility for both AuNCs and malaria sensors (SUB1-29 sensor) was executed through their incubation with RAW 264.7 cells at varying concentrations. Subsequent employment of the MTS assay enabled the evaluation of cell viability, yielding definitive results that underscored the high biocompatibility of both the AuNCs and the malaria sensors (Figure 3.15). These findings resonate harmoniously with existing literature. GSH-AuNCs have been extensively investigated for their biocompatibility in diverse *in vitro* and *in vivo* applications^{17,183,252}. NAv, a protein derivatised of avidin renowned for its robust affinity to biotin, has been extensively studied in the context of biotechnological applications. It has been demonstrated that NAv displays excellent stability in binding to biotin and exhibits commendable biocompatibility at reasonable concentration^{253,254}.

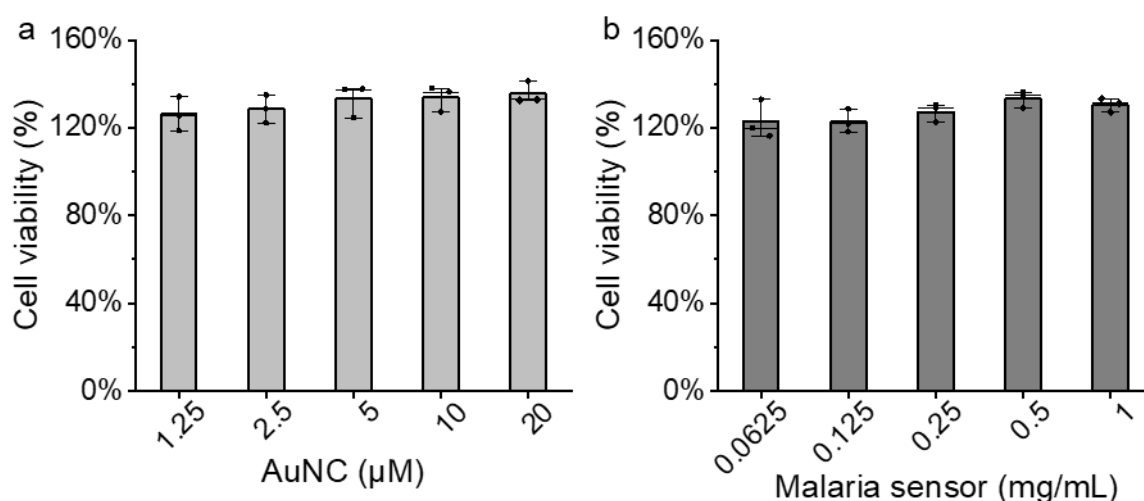


Figure 3.15 Cell viability of AuNCs and malaria sensors. (a, b) *In vitro* cytocompatibility MTS cell viability assay after incubation of AuNCs (a) and malaria sensors (b) with RAW 264.7 cells (N = 3, n = 3).

3.4.7 MMP-8-responsive nanosensor

Recent focus has turned towards unravelling the molecular mechanisms underpinning severe malaria, with particular emphasis on the role of MMP-8, which is notably associated with and abundantly expressed in severe malaria, particularly in cases of cerebral malaria^{244,245,255–257}. In light of this, an MMP-8 sensor was assembled utilising an MMP-8 peptide substrate. This design aimed to yield a sensor capable of responding to MMP-8, thereby enabling the detection of severe malaria.

After obtaining the purified MMP-8 substrate peptide (see Section 3.4.2), it was utilised in ligand exchange during the synthesis of peptide-templated AuNCs, followed by the assembly of the MMP-8 sensor by using protein carrier NAv. The investigation into optimal feed ratios encompassed two prominent ratios from prior studies: 1:5 and 1:50. Subsequently, the two MMP-8 sensors, formed with these distinct feed ratios, were subjected to *in vitro* cleavage testing by recombinant MMP-8. This involved incubation with PBS as a control and with MMP-8 at a final concentration of 50 nM for 3 h to induce sensor cleavage. The AuNCs released due to MMP-8 substrate cleavage were isolated using an Amicon filter device, and the collected filtrates underwent TMB assay to gauge cleavage performance. In both MMP-8 sensor configurations, the signals from the incubation with MMP-8 enzyme far surpassed those of the PBS negative control (Figure 3.16a, b).

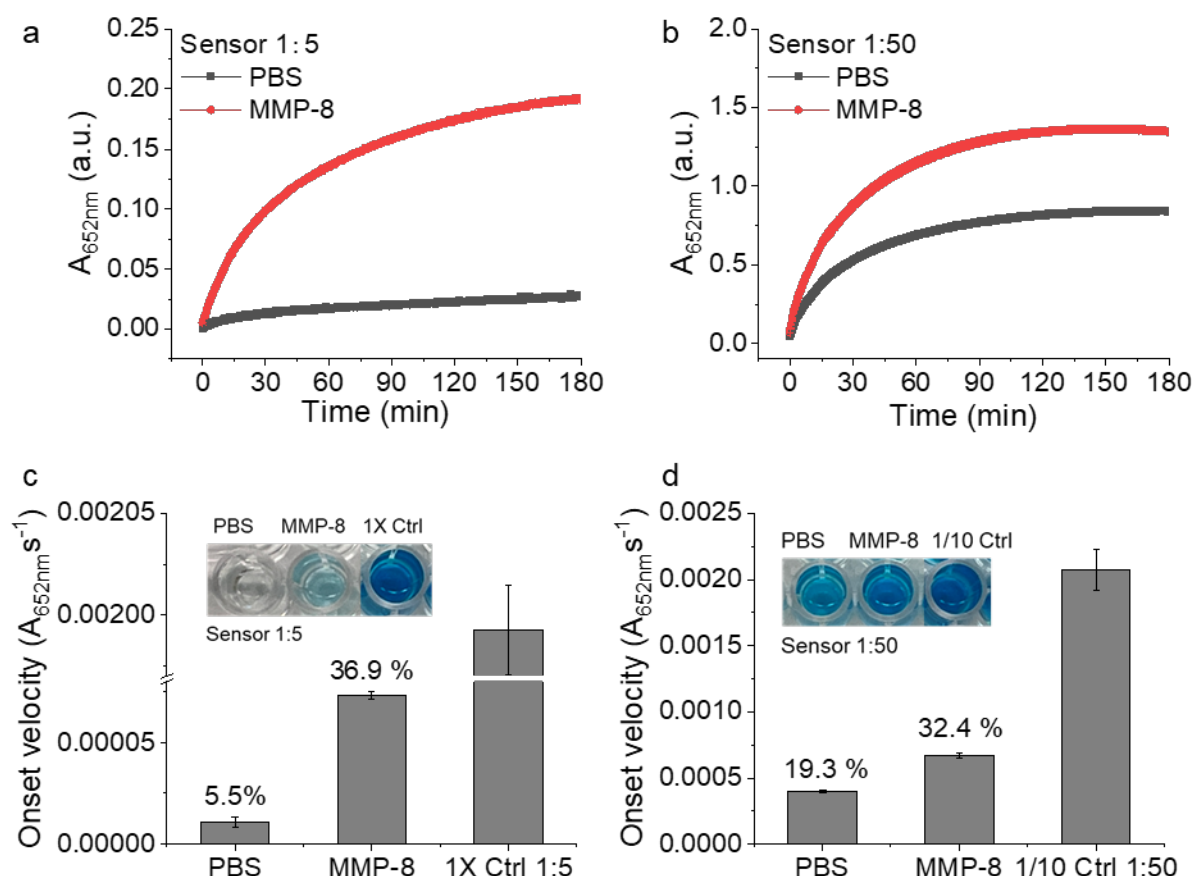


Figure 3.16 *In vitro* cleavage testing of MMP-8 sensor by MMP-8 using feed ratio of 1:5 and 1:50 during sensor synthesis. (a, b) Absorbance measurements at 652 nm on the filtrates after incubation of PBS or MMP-8 enzyme with MMP-8 sensor using feed ratio of 1:5 and 1:50, respectively. (c, d) The onset velocity for the catalytic activity comparison converted from (a, b) ($N = 1$, $n = 3$). Note the y-axis break in (c), as this experiment included a measurement with an undiluted control sample (1X Ctrl 1:5), while the PBS/MMP-8 samples always included a 1/10 dilution. All the efficiency comparisons were based on a 1/10 dilution of the original sensor.

This To corroborate the stability and sensitivity achieved with the 1:5 feed ratio in this sensing platform, two additional repetitions were performed (Figure 3.17).

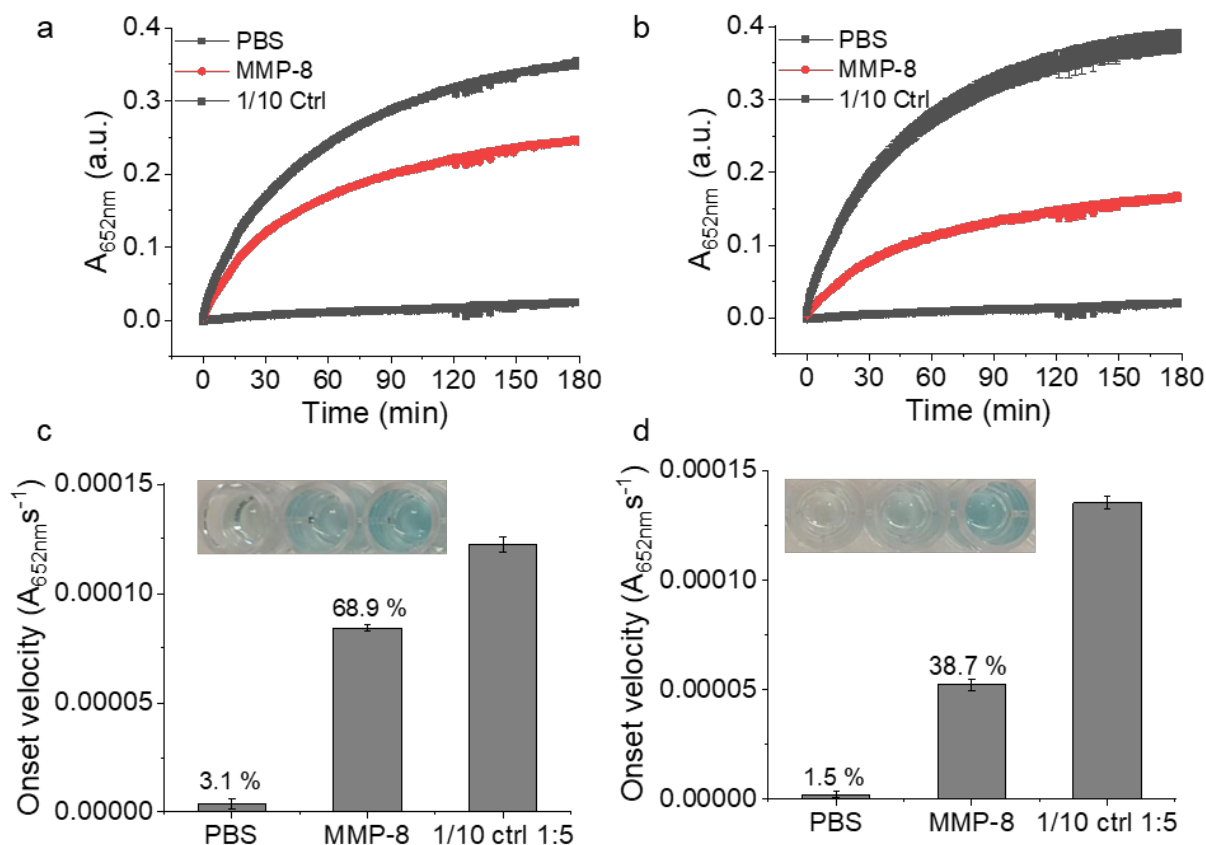


Figure 3.17 Repeats of *in vitro* cleavage of MMP-8 sensor (1:5 feed ratio) by MMP-8 enzyme. (a, b) Absorbance measurements at 652 nm on the filtrates after incubation of PBS or MMP-8 enzyme with MMP-8 sensor and the sensor 10X dilution control for the calculation of cleavage efficiency. (c, d) The onset velocity for the catalytic activity comparison conversed from (a, b) and cleavage efficiency comparison (N = 2, n = 3).

The background signal from the PBS incubation sample remained consistently low with no accompanying blue colour change. In contrast, the MMP-8 incubation triggered a distinct blue colour change, accompanied by heightened signals of 68.9% and 38.7% for cleavage efficiency. This gives an average of 48.2 +/- 17.9% across the three independent repeats. These three distinct and independent experiments collectively underscored the stability and successful detection of MMP-8 with this

sensing platform. Based on previous research, the current MMP-8 sensing platform holds promise for detecting MMP-8, which is linked to severe malaria.

3.4.8 *In vivo* colourimetric urinary readout for severe malaria detection

Having validated the response of the MMP-8 sensor to the MMP-8 enzyme *in vitro*, a pilot experiment was performed by employing a severe malaria infection model in mice. Notably, MMP-8 has been extensively investigated in association with various severe malaria infections, particularly cerebral malaria^{244,245,255,256}. Further, it was found that *Plasmodium yoelii* 17XL infection in mice best represents *P. falciparum* severe infection in humans²⁵⁸. The MMP-8 sensor, comprising MMP-8 substrate peptide templated AuNCs tethered to the protein carrier NAv, was administered intravenously (i.v.) to the model after establishment of severe infection, which was previously found to give increased MMP-8 in this model²⁵⁸. This administration was carried out in two distinct groups, each consisting of only a single mouse in this pilot experiment: a healthy control mouse (HC) and a *Plasmodium yoelii* 17XL-infected mouse with a severe malaria infection (MI). The administration was performed via the i.v. route, and urine samples were collected 1-h post-administration (Figure 3.18a). Before administering the MMP-8 sensor, a control experiment was conducted by administering 100 μ L of PBS instead of MMP-8 sensor into both HC and MI mice, followed by the collection of urine samples from each mouse. The TMB oxidation assay was subsequently conducted mixing 20 μ L of each urine with 60 μ L of 1-Step TMB and 5 M H₂O₂. The absorbance at 652 nm was measured and remained constant for both urine samples from both HC and MI mice, indicating that there were no substances in the urine capable of catalysing the oxidation of TMB (Figure 3.18b).

After 1-h period post injection of MMP-8 sensor, urine was collected from both HC and MI mice, followed by a TMB oxidation assay conducted on 20 μ L of the urine to quantify the catalytic activity of the released AuNCs. The oxidation of TMB was promptly triggered upon the mixture of urine and reagents. The absorbance at 652 nm of endpoint at the initial assessment of the assay indicated a substantial elevation in signal from the mouse with severe malaria infection (MI) compared to the healthy control (HC) mouse (Figure 3.18c).

Furthermore, visual observation revealed a distinct blue colouration in the MI group and a yellow hue (reflecting the natural colour of urine) in the HC group (Figure 3.18c). This underscores the successful detection of severe infection with the MMP-8 sensor. This sensing platform has the potential to rapidly detect severe malaria through a colourimetric readout within a remarkably short timeframe of 1 h. However, experimental repeats are necessary to corroborate the finding of this pilot experiment, as only one mouse each was used, which is not sufficient for drawing definitive conclusions. In addition, another control group has to be included, mice with uncomplicated malaria, to demonstrate detection of only severe cases with this method. Nevertheless, the observation of very clearly distinguishable signals for the samples from the control vs severe infected mouse are highly promising for these future steps.

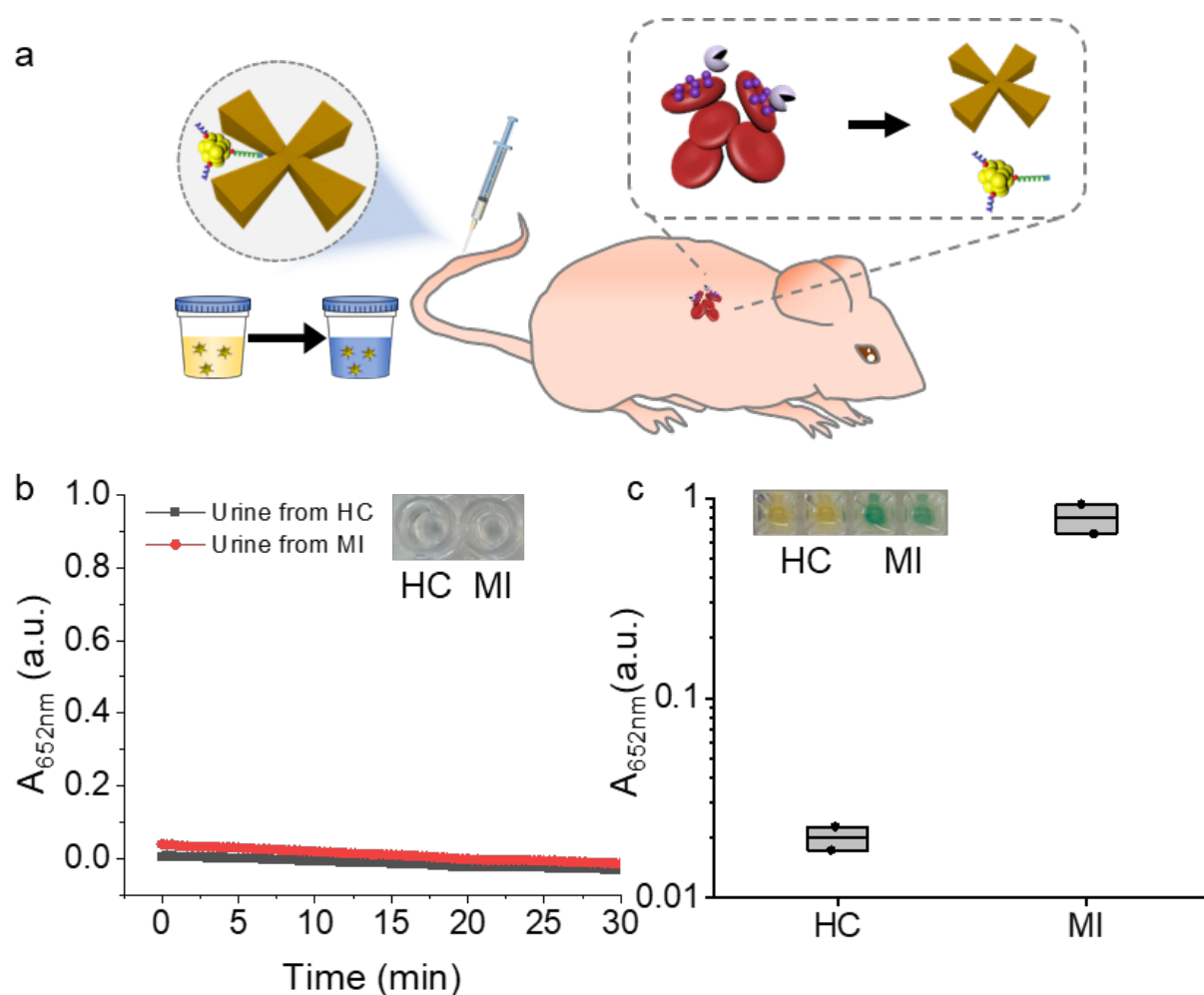


Figure 3.18 MMP-8 sensing platform enabling a colourimetric urinary readout for detecting severe malaria. (a) Schematic of mouse i.v. administration with MMP-8 sensor and AuNC catalysis detection in urine. (b) TMB absorbance assay on the urine collected from both healthy control and malaria infected mice without the administration of MMP-8 sensors (N = 1 mouse each, n = 1 technical repeat of the TMB assay). (c) TMB reaction endpoint measurements on the undiluted urine collected from administration of MMP-8 sensors in healthy control mouse and severe malaria infected mouse (N = 1 mouse each, n = 2 technical repeats of the TMB assay). The mouse experiment was conducted by Dr Athina Georgiadou.

3.5 Conclusion

In this Chapter, it demonstrated that a biosensor system was successfully engineered and tailored for the rapid detection of enzymes associated with malaria infections, with particular emphasis on severe cases, achieved through a non-invasive colourimetric readout mechanism within a short 1-h time window. This research endeavours focused on several pivotal aspects: the synthesis of substrate peptides specifically responsive to SUB1 and MMP-8, *in vitro* enzyme detection, and the fine-tuning of this sensing platform. The synthesis of the relevant substrate peptides was achieved using SPPS and followed by meticulous purification and thorough analysis employing HPLC and LC-MS techniques. With these robust methodologies, the production of the desired substrates was achieved successfully. Subsequent investigations involved varying the feed ratio to scrutinise its impact on the ultimate cleavage of AuNCs from the sensor unit, thereby optimising the sensitivity of the biosensor platform. Those results conclusively demonstrated that an optimal feed ratio not only heightened positive signal in response to SUB1 or MMP-8 in the biosensor platform but also simultaneously maintained a desirable low background measurement in case of the MMP-8 sensor, whilst further optimisation is necessary for the SUB1 sensor.

Regarding SUB1 detection, the specificity of the sensor for malaria was demonstrated as a thrombin-responsive biosensor equivalent exhibited no response to the same parasite sample under identical incubation conditions. Similarly, for MMP-8 detection, the capability of the MMP-8 sensor was successfully showcased to yield a substantial signal following incubation with MMP-8 *in vitro*. Finally, the capacity of the MMP-8 biosensor for *in vivo* application was indicated by demonstration in a pilot experiment,

although repeats will be necessary to corroborate this exciting finding. A positive blue signal was detected after administration of the MMP-8 sensor in a mouse with a severe malaria infection, whilst no signal was detected from the urine of a control mouse. This achievement further highlights the proficiency of the biosensor platform for rapid and sensitive diagnostic applications in real-world scenarios.

Chapter 4

Non-invasive *in vivo* sensing of bacterial implant infection using catalytically optimised gold nanocluster-loaded liposomes for urinary readout

4 Non-invasive *in vivo* sensing of bacterial implant infection using catalytically optimised gold nanocluster-loaded liposomes for urinary readout

4.1 Aims

The primary objective of this chapter is to develop a toxin-responsive biosensor for detecting bacterial infections. Building upon the optimisation of gold nanocluster outlined in Chapter 2, the optimised nanoclusters need to be encapsulated in liposomes forming a biosensor. The liposome formulation requires investigation to enhance responsiveness to toxins secreted by bacteria, which can improve the overall biosensor sensitivity. This innovative biosensor leverages the benefits of nanotechnology and liposome carriers, featuring catalytic AuNCs within liposomes. Upon bacterial infection, these liposomes break down releasing the encapsulated AuNCs which can be subsequently detected. Developing this liposomal sensing platform can facilitate early diagnosis and treatment of bacterial infections.

4.2 Introduction

4.2.1 The global burden of bacterial infections

Staphylococcus aureus is responsible for a raft of superficial and serious infections of humans and the leading cause of surgical implant-associated infection^{259–261}. Surgical site infections (SSI) are a major complication of procedures involving indwelling medical devices, accounting for 24.3% of health care-associated infections in Egypt

(2017). Among these cases, 21.21% were attributed to *S. aureus* as the isolated bacteria²⁶². Infection of implanted devices typically results in hard-to-treat biofilm formation and necessitates removal and replacement, significantly increasing morbidity, mortality and costs^{263,264}. Antimicrobial resistance (AMR), which can occur in a wide range of microorganisms, represents another factor that significantly increases the global health burden of bacterial infections. AMR is associated with high morbidity and mortality, as it threatens the effectiveness of treatment for infectious diseases and increases the risk of disease recurrence, complications and deaths (1.27 million in 2019 in Figure 4.1)²⁶⁰. Antibiotic resistance (ABR) is a specific subset of AMR, summarising the reduction in the efficacy of antibiotics, which increases the challenge of successfully treating Gram-positive and Gram-negative bacterial infection^{265,266}. As an example, ABR in *S. aureus* has increased the frequency of complications, like prolonged and severe infection and cross resistance, and costs for antibacterial treatment, with methicillin-resistant *Staphylococcus aureus* (MRSA) representing a leading cause of mortality due to bacterial infection. Therefore, earlier diagnosis of bacterial infection is key to the clinical management of this potentially life-threatening condition to enable accurate and rapid treatment. This will reduce the chance of complications, save patient lives, prevent the spread of bacterial infection within hospitals and the population, and also eliminate unnecessary antibiotic use to avoid the development of further antibiotic resistance.

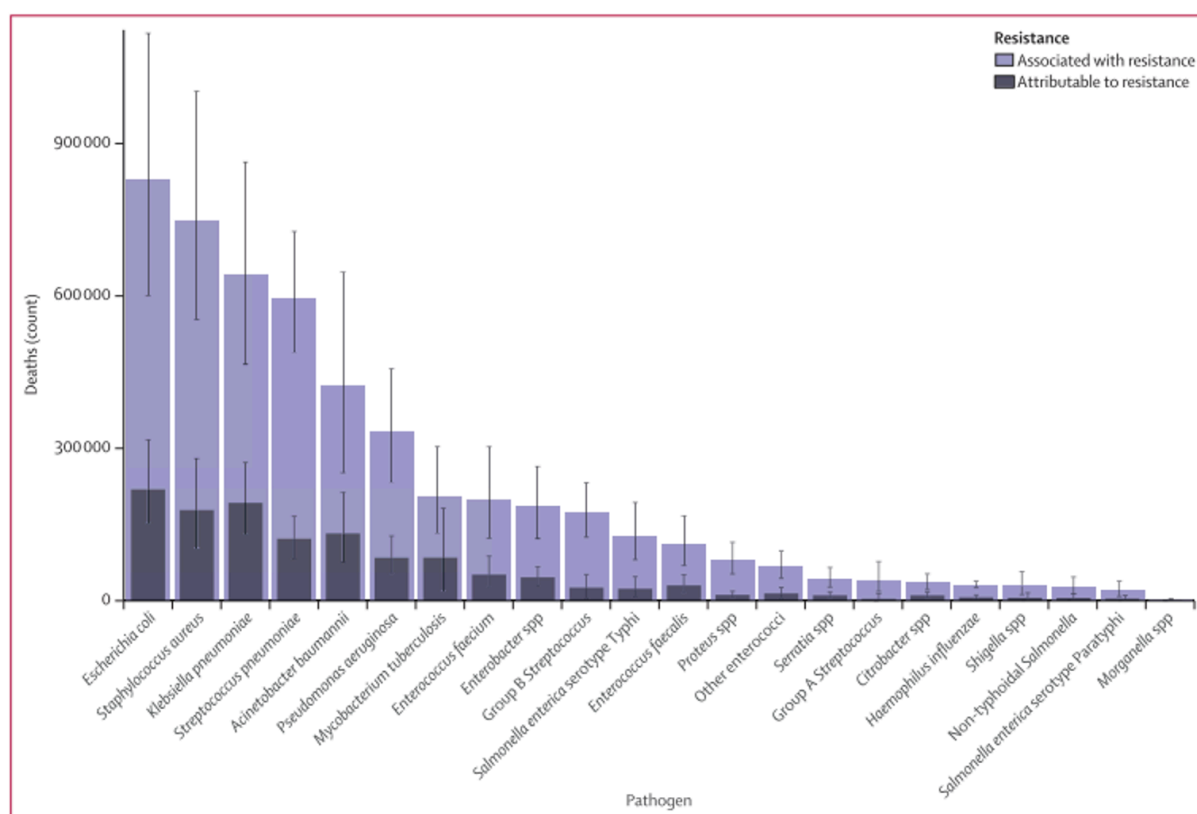


Figure 4.1 The global count of deaths associated with bacterial antimicrobial resistance by various pathogens, 2019. (This figure was adapted from Ref. 260 with permission²⁶⁰.)

4.2.2 Diagnostic tools for bacterial infections

To identify infection at the site of an implant, it is necessary to collect samples from patients using swabs, aspirations, and even tissue biopsy or removal of the implant^{267–269}, which is invasive, challenging and carries the potential risk of causing additional infections. Traditional bacterial culture is the gold standard method for the detection of bacterial infection. Bacterial species can be identified by inoculating clinical samples in bacteria culture setups and observing the growth characteristics²⁶⁸. However, it requires long incubation times, often several days, which is a shortcoming for early diagnosis in emergency situations²⁷⁰. Molecular biology methods, including polymerase chain reaction (PCR)^{271,272} and real-time fluorescent PCR^{273,274} can

amplify and detect specific bacterial nucleic acid fragments to identify and quantify the bacteria in a short period of time. Similarly, immunological methods, like enzyme-linked immunosorbent assay (ELISA)^{275,276}, immunofluorescent staining²⁷⁷, and mass spectrometry (MS)^{278,279}, can also serve as highly sensitive, specific and accurate methods for bacterial identification by analysing bacterial components. However, these advanced assays require highly specialised equipment and lab-based technical support, resulting in high costs and limiting their application in resource-limited laboratories and medical institutions. In addition, these methods may not be able to detect low concentrations of bacterial infection and some bacteria may have mutated, leading to a reduction in the specificity of the assay. Using enzymatic or membrane-damaging activities of bacteria-derived components is an upcoming alternative method to detect bacterial infection^{88,89}. These virulence factors include toxins (pore-formation, lipase activity), enzymes, surface proteins (surfactant-like mechanisms) and adhesions for which different non-invasive diagnostic tools can be designed to realise the detection of *S. aureus*^{92–94,280–282}. As such, there is a great demand to develop simple, low-cost, rapid and sensitive diagnostic tool for the detection of these components.

4.2.3 Liposomes as biosensors for detection of bacterial toxins

Liposomes, vesicles composed of varied compositions of lipids, have emerged as carriers with a wide range of applications in drug delivery and biosensing over the past few decades^{102,103}. Due to their similar components and structures to cell membranes, liposomes are effective carriers of both hydrophilic and hydrophobic cargos and are capable of interacting physiologically with cells¹⁰⁴. Liposomes have been shown to

encapsulate a variety of cargos, including dyes, drugs, biomolecules, enzymes, nanoparticles for various drug delivery applications and as amplification strategies in biosensing^{105–110}. In particular, toxin-action has been leveraged for creating bacteria growth responsive liposomal systems for controlled antibiotic delivery^{95–97}, capturing toxins to reduce host toxicity⁹⁸, switch on an antibacterial nanoreactor on-demand⁹⁹, and to detect bacterial infection^{91,283}. Thet *et al.* demonstrated liposomes could be tuned for specific toxin response by engineering the membrane compositions¹⁰⁰ and recently developed a sensor swab consisting of self-quenched dye-encapsulated liposomes for infection detection through the breakdown of liposomes and a colour change from yellow to green²⁸³ (Figure 4.2).

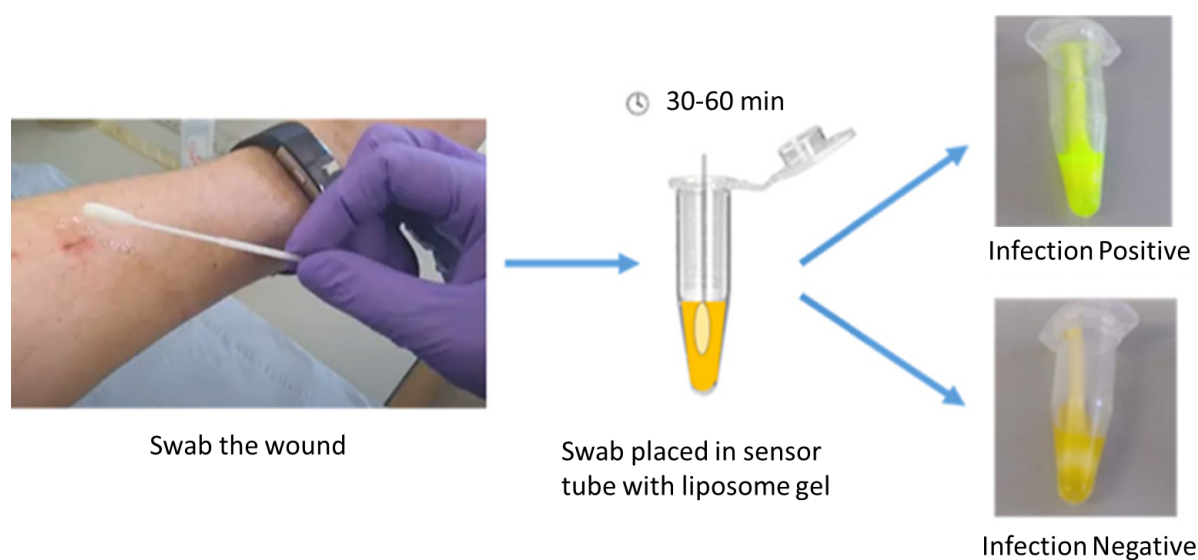


Figure 4.2 Rapid and simple detection of wound infection using liposome sensor encapsulated self-quenched fluorescent dye. (This figure was adapted from Ref. 283 with permission²⁸³.)

4.2.4 Catalytic gold nanoclusters for *in vivo* biosensing

Biosensors with simple urinary readout are an upcoming and promising strategy to designing non-invasive advanced biomedical diagnostics approaches^{17,59,284–287}.

Rapid kidney filtration can effectively filter nanoparticles smaller than 5.5 nm within hours, facilitating the rapid detection of probes in urine^{17,19}. A new class of signal amplification components for biosensing are enzyme-mimetic nanoparticles (nanozymes) or even smaller sized catalytically active nanoclusters^{124–126}. Catalytic gold nanoclusters (AuNCs) consist of dozens to hundreds of gold atoms with an ultra-small size of about 1 to 5 nm (normally around 2 nm)¹³⁷. In contrast to gold nanoparticles, AuNCs exhibit a unique discrete electronic structure and molecular-like surface properties. Extensive research has demonstrated their potential in drug delivery^{138,139}, photothermal therapy¹⁴⁰, bioimaging^{141,142}, and biosensing^{143,144}, attributed to their photostability, specific recognition capabilities, small size, high surface-to-volume ratio, and strong absorbance in the near-infrared (NIR) region. The catalytic activity of AuNCs was recently used for an *in vivo* sensing application for cancer¹⁷. In this work, AuNCs were covalently linked to a protein carrier *via* enzyme-responsive peptides. Upon enzyme cleavage *in vivo*, the clusters were liberated from the complex and cleared into the urine of tumour-bearing mice, which then turned blue due to hydrogen peroxide (H₂O₂)-mediated oxidation of a chromogenic substrate catalysed by AuNCs (Figure 1.9).

4.2.5 Chapter overview

In this chapter, an AuNC-based sensing system was designed which assembles a large number of AuNCs (>100) per sensor unit and responds to pathogenic bacterial growth in the vicinity for detecting bacterial implant infection. First, the catalytic activity of AuNCs was optimised and improved over the previous report¹⁷ by varying the conditions of the synthesis in Chapter 2 . These ultra-small catalytic AuNCs (1-2 nm to ensure they are renally clearable) was encapsulated in 100-nm liposomes serving as a nanocarrier, which could be disassembled by exposure to toxins secreted by pathogenic bacteria. The supernatant of *S. aureus* culture was utilised to optimise the compositions of liposomes by quantifying the percentage of AuNCs released from the various biosensors. The optimised sensor was then embedded in hyaluronic acid (HA) hydrogels, which are clinically used as dermal fillers. This chapter demonstrated successful response of the developed biosensor to *S. aureus* growth in contaminated HA hydrogel implants both *in vitro* and *in vivo*. The mouse model confirmed the usability of the sensor for the non-invasive detection of *S. aureus* implant infection *in vivo* with a simple urinary readout. This sensing platform took advantage of the toxin activity to trigger the disassembly of liposomes in combination with the catalytic activity and renal clearance ability of inorganic AuNCs. This sensor system yielded a colourimetric readout in urine, achieving a versatile biosensing platform for bacterial infection detection.

4.3 Materials and methods

4.3.1 Materials

Triton X-100 and Sepharose CL-2B was purchased from Sigma-Aldrich. Brain sphingomyelin (BSM), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (16:0-18:1 PC), cholesterol (CH), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2K) were purchased from Avanti Polar Lipids. Methanol (MeOH) and chloroform (CHCl_3) were used as purchased. DMEM without phenol red (1X, high glucose, GlutaMAX, Gibco, 31966-021) and Oregon Green 488 Carboxylic Acid, Succinimidyl Ester, 6-isomer (OG488) were purchased from Thermo Fisher Scientific. RAW 264.7 cells were obtained from ATCC. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega) was purchased from Abcam. Tryptic soy agar (TSA) and tryptic soy broth (TSB) were purchased from BD biosciences. HA hydrogel (Belotero intense) was purchased from Fillerworld.

4.3.2 Synthesis of glutathione capped gold nanoclusters (AuNCs)

AuNCs were catalytically optimised in Chapter 2. HAuCl_4 (20 mM, 100 μL) was added into 700 μL of H_2O , followed by the fast addition of GSH (20 mM, 150 μL) and pH adjustment to 10 by using NaOH (1 M) and finally topped up with H_2O to 1 mL for final reaction solution. The reaction mixture was heated up to 70 $^\circ\text{C}$ for 24 h reaction. AuNCs were scaled up from 1 mL to 300 mL. After the reaction, 300 mL of crude

nanoclusters were purified, and buffer exchanged with DPBS (pH 7.4) using Amicon Ultra-15 Centrifugal Filter Units (MWCO 10 kDa) at 5000 g and 15 min for washing three times. Finally, AuNCs were concentrated suspended in DPBS to yield 1 mL as 2 mM for the concentration, which subsequently transferred and filtered into a new Eppendorf tube using a sterile syringe filter (0.22 μ m Millex-GV Filter) to remove any aggregates and sterilise the sample for encapsulation use.

4.3.3 Liposome preparation and optimisation of encapsulation efficiency using different concentrations of AuNCs

Liposomes were encapsulated with the catalytically optimised AuNCs by the methods of hydration and extrusion following the recently published protocol⁹⁹. The lipid film formulation was composed of BSM (25 mg/mL, 100 μ L) and CH (25 mg/mL, 100 μ L) with 1 mol% DSPE-PEG2K to BSM (25 mg/mL, 2.63 μ L) as stabilizer preventing aggregations. The above lipid films were mixed in CHCl_3 :MeOH (9:1 used to the lipid stock preparation) in 1.5-mL glass vials, followed by drying the solvent with N_2 gas and for desiccation overnight. The optimised AuNCs synthesised at pH 10 were prepared at different concentrations, ranging from 12.5x (0.25 mM) to 200x (4 mM), for hydration in liposomes. The lipid films were hydrated with AuNCs cargo solutions (500 μ L) at various concentrations for at least 1 h. Subsequently, liposomes were vortexed and conducted five freeze-thaw cycles by heating up to 50 $^\circ\text{C}$ and freezing at -80 $^\circ\text{C}$. The lipid suspensions were then extruded by using 200 nm polycarbonate membranes (6X) and 100 nm membranes (21X). Finally, the extruded liposome suspension was purified by using size exclusion chromatography (SEC), using a 30-cm column packed with Sepharose CL-2B, which was equilibrated in PBS. Eluted

fractions (30 mL) were collected for analysis of the liposome fractions and free unloaded AuNC fractions, respectively, through TMB oxidation assay. To increase liposome concentrations, Amicon Ultra-15 Centrifugal Filter Units (MWCO 100 kDa) were used to concentrate the eluted liposome fraction. The samples were finally sterilised by filtration through a sterile syringe filter (0.45 μ m Millex-GV Filter) to remove any aggregates and sterilise the sample.

4.3.4 Size exclusion chromatography (SEC)

Identical glass chromatography columns were packed with Sepharose CL-2B resin, which could be used for the separation of samples based on different sizes. The larger liposomes were eluted in the initial fractions, while the smaller free AuNCs were eluted more slowly in the later fractions. To perform SEC, the solution sample (500 μ L) was introduced into the column and flowed through the resin bed. Sufficient phosphate-buffered saline (PBS) was added into the column for fraction collection. Each fraction was collected separately for a subsequent catalytic activity assay to determine the fractions containing the AuNC-loaded Liposomes and unloaded AuNCs. After fraction collection (30 mL), the first 10 fractions (containing AuNC-loaded liposomes) were diluted 20 fold and the last fractions (10 – 30 mL) (unloaded AuNCs) were diluted 100 fold due to the optimised activity. Subsequently, 100 μ L of each diluted fraction was added in a 96-well plate, which was followed by the addition of a mixture of 100 μ L of mixture of TMB and H₂O₂ (1:1) (the final concentration of H₂O₂ was 1 M). The absorbance at 652 nm of each sample fraction was measured to determine the catalytic activity of each fraction and the contents in the fractions. Dynamic light scattering (DLS) was operated to further determine which fractions contained the larger AuNC-loaded liposomes and where the unloaded free AuNCs eluted.

4.3.5 Inductively coupled plasma mass spectrometry (ICP-MS)

Aqua regia was freshly prepared with a molar ratio of 3:1 of HCl (35%, 8 mL) to HNO₃ (65%, 2 mL). Liposomes loaded with varying concentrations of AuNCs, ranging from 12.5x (0.25 mM) to 200x (4 mM), or initial AuNC batches for loading (200 µL), were digested in 500 µL of aqua regia overnight and then diluted with H₂O up to 5 mL. A gold standard for ICP was prepared to calibrate the machine. The gold content in the digested and diluted samples was measured using ICP-MS (8900 Triple Quadrupole ICP-MS), and the data were recorded on iTEVA iCAP Software (Version 2.2.0.51).

4.3.6 Characterisation of AuNCs and AuNCs-loaded liposomes

The hydrodynamic diameter of nanoclusters and liposomes was measured by using dynamic light scattering (DLS, Zeta Sizer Nanoseries, Malvern Instruments, Ltd). SpectraMax M5 multimodal microplate reader was used to measure the kinetics reaction of TMB oxidation by AuNC catalysts as well as AuNC-loaded liposomes in the presence of H₂O₂. Transmission electron microscopy (TEM) images of AuNCs were obtained on a JEOL 2100F. To prepare TEM of AuNC samples, AuNCs solution needed to be desalted first with Zeba Spin Desalting Columns (7K MWCO) and 5 µL of the sample was dropped on a carbon-coated copper grid and let to dry overnight for imaging. Nanoparticle Tracking Analysis (NTA) was used to obtain the hydrodynamic diameters, size distribution and count-based concentration of liposomes. For Cryo-Transmission electron microscopy (cryo-TEM), 5 µL of 5 mg/mL and 20 mg/mL

liposome solution was dropped on a holey carbon-coated copper grid and subsequently blotted with Whatman 1 filter paper, followed by vitrification into liquid ethane at -180 °C. Frozen grids were placed on a Gatan Multi-Specimen cryo-holder Model 910 and transferred into a Talons L120C Electron microscope. Micrographs were recorded using 4K x 4K Ceta CMOS camera, at an accelerating voltage of 120 kV at -175 °C using a low-dose system (40 e-/Å²).

4.3.7 Optimisation on liposome formulations

To optimise the responsiveness of the liposome system, different lipid compositions were used, including BSM, PC, CH and DOTAP. 1 mol % DSPE-PEG2K was added in all the formulations for prevention of liposome aggregation. The optimised liposome composition was 33.3% BSM, 33.3% PC and 33.3 %CH (mass percentage) with 1 mol% of DSPE-PEG2K (all the other compositions tested are summarised in **Error! Reference source not found.**). The lipid films (typically 5 mg total) were prepared in 1.5-mL glass vials through evaporation of CHCl₃:MeOH (9:1 used to prepare the lipid stocks) by drying with N₂ gas, then storing with desiccant overnight. The lipid films were hydrated with AuNCs cargo solutions in PBS (500 µL, 2 mM as the optimal concentration for encapsulation) for at least 1 h. The process of hydration, extrusion, SEC and TMB assay was conducting following Section 4.3.3 .

4.3.8 *In vitro* toxin-triggered release of AuNC from liposome

Toxin was collected from an overnight culture supernatant of *S. aureus* SH1000 cultured in TSB, centrifuged away the bacteria, and sterile filtered the supernatant

before use. Liposomes (5 mg/mL) with all formulation (250 μ L) were mixed with the toxin supernatant (250 μ L) and incubated at 37 °C for 2 h at a shaking speed of 500 r.p.m. As a control group without toxin, and a positive full release control, liposomes were separately incubated with the same volume of TSB and Triton X-100 in PBS (10% v/v), respectively. After incubation, the reaction solutions were centrifuged and filtered through Amicon Ultra-0.5 Centrifugal Filter Units (MWCO 100 kDa) to separate the released AuNCs from the intact liposomes. The collected filtrates were collected for TMB assay for detection of released AuNCs. To perform the TMB assay, all the collected filtrates (100 μ L) were added into a 96-well plate, followed by the addition of 1-Step™ Ultra TMB-ELISA Substrate Solution (50 μ L) and H₂O₂ (2 M, 50 μ L). The kinetics of the reactions were measured by monitoring the absorbance at a wavelength of 652 nm using a SpectraMax M5 plate reader.

4.3.9 *In vitro* AuNC release from bacterial incubation with liposome

S. aureus SH1000 was cultured overnight to obtain 1X stocks which were subsequently diluted at a 1:100 ratio. BPC (BSM:PC:CH in % w/w 33:33:33) liposomes were prepared at a concentration of 20 mg/mL in a total volume of 2 mL. The prepared liposomes (1 mL) were incubated with 5 mL of the diluted *S. aureus* SH1000 in TSB (1:100) or 5 mL TSB only was used as the control. The incubation period lasted for 8 h. At various time points (0 to 8 h) with 1-h intervals, 500 μ L of the reaction mixture solution was sampled. Each sample was then centrifuged and filtered using Amicon Ultra-0.5 Centrifugal Filter Units (MWCO 100 k Da). All the collected filtrates (100 μ L), which contained the released AuNCs, were used for the TMB assay (60 μ L 1-Step

TMB and 40 μL H_2O_2). This assay allows for the quantification of the released AuNCs by measuring oxidised TMB absorbance.

4.3.10 Fluorescence correlation spectroscopy (FCS)

FCS using fluorescently labelled AuNCs was performed similarly to the previous report from the Stevens group¹⁷. Briefly, AuNCs (1 mM, 50 μL) were labelled by adding a 20-molar excess of NHS-activated Oregon Green 488 (OG488) through reactive amine (GSH on AuNCs) coupling. This labelling process lasted for 3-h incubation at room temperature. Subsequent PDminitrapp and PDmiditrap columns equilibrated in PBS were then utilised to remove any unreacted OG488 dye to obtain purified AuNCs-OG488. Subsequently, 5 mol% labelled AuNCs-OG488 were mixed with 95 mol% unlabelled AuNCs (overall final conc. 2 mM) and encapsulated in BPC liposomes, which were again purified by SEC using a Sepharose CL-2B column. The purified AuNC-liposomes were then incubated 1:1 with either TSB or *S. aureus* SH1000 overnight culture supernatant containing the secreted toxins in TSB and incubated for 2 h, at 37 °C. FCS was then performed on a commercial LSM 880 (Carl Zeiss, Jena, Germany) using 5 μL droplets on glass-bottom ibidi 8-well plates (80827, ibidi, Germany), equilibrated at 37 °C. The setup included an Ar^+ laser (488 nm excitation), 40X C-Apochromat water immersion objective (NA 1.2), and a LP 505 filter. All measurements were performed 200 μm above the ibidi bottom plate. A serial dilution of OG488 diluted in PBS or TSB were used for calibration ($D = 5.49 \times 10^{-6} \text{ cm}^2/\text{s}$ at 37 °C)²⁸⁸ and obtaining the x-y dimension of the confocal volume (ω_{xy}^2) to enable calculation of diffusion coefficients (D) and use Stokes-Einstein equation to convert to hydrodynamic diameter (D_h). Intensity traces (25 x 5s) were recorded and analysed for each sample. The autocorrelation curves shown in the figures represent the

average curve for the whole 125 s measurement. All curves were fitted and analysed using PyCorrfit program 1.1.6²⁸⁹. Free AuNCs-OG 488 were first fitted with a one component fit to obtain their characteristic diffusion time and the liposome samples were then analysed with a two-component fit, fixing one component to free cluster diffusion. The ratio of height to width of the confocal volume was fixed to 5 and a triplet fraction with triplet time of 1-10 μ s was included in all fits. The number of clusters loaded per liposome were obtained by using the counts per particle (cpp in kHz) signal of free AuNCs-OG488 and loaded liposomes, using a correction due to the disproportional effect of bright slow diffusing species on average autocorrelation curves²⁹⁰, and multiplying by 20 because only 5 mol% of loaded AuNCs was used in the encapsulation procedure.

4.3.11 Single particle automated Raman trapping analysis (SPARTA™)

SPARTA™, as previously reported²⁹¹, is a label-free method that enables high-throughput analysis of single nanoparticles with Raman spectroscopy to provide population level information with single particle detail about nanoparticle size and chemical composition. For sample preparation, the optimal BPC liposomes were prepared and loaded with catalytically optimised AuNCs through lipid film hydration and extrusion. To collect fresh toxin supernatant, *S. aureus* SH1000 strain was cultured in TSB overnight for bacterial growth and toxin secretion. For sample incubation, BPC liposomes (10 mg/mL, 200 μ L) were incubated with DPBS (200 μ L) as control or 4-fold diluted fresh toxin supernatant in DPBS (200 μ L) for 2 h. SPARTA measurements were conducted using the SPARTA Alpha prototype. The system consisted of a spectrograph (HoloSpec-F/1.8-NIR, Andor, UK) coupled with an iDus

416ALDC-DD (Andor, UK) thermoelectrically cooled ($-60\text{ }^{\circ}\text{C}$) back-illuminated CCD camera on a Cera platform (Thorlabs, UK). A 63 $\times$ /1.0 NA WI objective (W Plan-Aprochromat, Zeiss, Oberkochen, Germany) was used for immersing the sample solution, and a 785 nm laser (200 mW, Cheetah, Sacher Laser Technik, Germany) was employed for the optically trapping of particles while simultaneously providing Raman excitation. A 22 mm diameter coverglass with thickness 0 (VWR) was affixed to a microscope slide. Onto the coverglass was placed 200 μL of either particle suspension or DPBS for background measurement. During measurements, liposomes in DPBS or with toxin were trapped and analysed with a 10 s acquisition time. The laser was disabled for 1 s to release the trapped particle and allow a new particle to diffuse into the confocal volume. 20 spectra of blank DPBS at 10 s acquisition time was measured for background subtraction. Raman spectra were analysed using custom MATLAB scripts for cosmic spike removal and spectral response correction (785 nm reference standard National Institute of Standards and Technology, US). Aggregates or empty traps were then manually removed by minimum and maximum thresholding, before a background subtraction of 95% blank DPBS was performed. A Whittaker baseline correction was then applied, before each spectrum was then smoothed using a Savitzky-Golay smoothing filter with order 1 and frame size 7. Each particle spectrum was normalised to the area under the whole spectrum to remove effects such as laser power fluctuation between samples. Mean spectra and standard deviation were calculated across each sample in Matlab R2020a.

4.3.12 *In vitro* tests with HA hydrogels

BPC liposomes were prepared at a concentration of 20 mg/mL. *S. aureus* SH1000 was cultured in TSB overnight and subsequently purified by pelleting and

resuspension in DPBS. The purified SH1000 was then diluted to a concentration range of 10^9 to 10^8 colony-forming units per millilitre (CFU/mL). 100 μ L of BPC liposomes were added to 100 μ L of HA hydrogel (Belotero intense). Subsequently, either TSB or the diluted SH1000 (10^8 CFU/mL) was added to the mixture (1000 μ L). After thorough mixing, the resulting mixtures were pipetted and injected into 700 μ L of TSB for incubation over a period of 0 to 3 h. To analyse the release of AuNCs, 200 μ L of supernatant was collected from each incubation solution and filtered using a 0.45 μ m syringe filter into an Amicon Ultra-0.5 mL Filter Unit (100 kDa). This filtration step allowed for the separation of the released AuNCs into the filtrate, which was then used for the subsequent TMB assay. For the TMB assay, 20 μ L of the filtrate was mixed with 180 μ L of TMB:H₂O₂ solution (final concentration 1 M H₂O₂) for the generation of a colourimetric signal. The absorbance of the reaction mixture was measured over time on the plate reader.

4.3.13 *In vitro* cytotoxicity studies

RAW 264.7 cells were cultured in culture media consisting of DMEM 1X medium with 5% of FBS and 0.5% of penicillin-streptomycin. To seed the cells, the desired concentration of RAW 264.7 cells were prepared in culture media and then transferred into sterile 96-well plates (20,000 cells/well), which is followed by incubation for 24 h for adhesion. The culture media from the wells with the adherent RAW 264.7 cells was removed and different concentration of AuNC-loaded liposomes and AuNCs (20 μ L both in PBS) were added in the wells with a final mixture of 180 μ L of medium:PBS (9:1). The plates were incubated at 37 °C with 5% CO₂ for another 24 h. The cell viability assay was evaluated by using reagents MTS assay (MTS) by removing the cell supernatants and replacing with 120 μ L of MTS stock solution containing DMEM

medium without phenol red (31.5 mL), MTS (2 mg/mL, 6mL), PMS (0.92 mg/mL, 0.3 mL), incubation for 1 h and measurement of absorbance on 490 nm.

4.3.14 *In vivo* injection and urine analysis

All animal experiments were conducted according to the Animals (Scientific Procedures) Act 1986, following ethical review by Imperial College London Animal Welfare and Ethical Review Body (PF93C158E), and with approval from the UK Home Office authority. For AuNC clearance study, catalytically optimised AuNCs (10 μ M, 200 μ L) or DPBS (200 μ L) were injected i.p. into each mouse. Urine samples were collected in Eppendorf tubes after 1-h and 3-h period post injection for TMB assay and then diluted to get enough volume for the assay. The diluted urine sample (10 μ L) was mixed with 40 μ L of TMB:H₂O₂ solution (final concentration of 2.5 M) in 384-well plate for the absorbance measurement at 652 nm. The results were normalised by dilution factors for comparison. A mixture of liposomes (60 mg/mL, 100 μ L), hyaluronic acid (HA) (100 μ L), and either DPBS or *S. aureus* SH1000 (3x10⁸ CFU/mL, 100 μ L) was prepared at room temperature. Subsequently, 200 μ L of this mixture with PBS (healthy control, HC) or SH1000 (bacterial infection, BI) was injected i.p. into each mouse (wild-type C57BL/6 mice purchased from Charles River, female, 6-14 weeks old). Adult mice were housed no more than five per cage with Aspen chip 2 bedding with a 12-h light and 12-h dark cycle at 20–22 °C. Mice were randomly assigned to experimental groups, water was provided freely, and mice were fed RM1 (Special Diet Services). Groups consisted of 6 animals each. After a 4-h period after injection, urine samples were collected in Eppendorf tubes for further analysis. The collected urine (20 μ L) was subjected to the TMB assay in 384-well plate, where it was mixed with 60 μ L of TMB:H₂O₂ solution (final concentration of 5 M).

4.4 Result and discussion

4.4.1 Design of *in vivo* bacteria sensor

Catalytic AuNCs (see optimisation in Chapter 2) were chosen as the signal generating component of this liposomal biosensor. These AuNCs were subsequently encapsulated within liposomes to finalise the sensing system (Figure 4.3a). Detection of catalytic activity provided by the released AuNCs, due to the action of bacterial toxins on the lipid membrane, directly serves as an indicator of bacterial infection state. This detection leverages the use of sphingomyelin lipids in the liposome formulation, which can be hydrolysed by the toxins secreted by *S. aureus* (Figure 4.3c). AuNCs capped with GSH were selected as probes due to their low cytotoxicity, high stability against harsh environments, high signal amplification with a high surface-to-volume ratio, stable catalytic activity in complex physiological environments, and small size to be rapidly and efficiently cleared by the kidneys^{17,292}. The AuNC-loaded liposomes together with *S. aureus*-contaminated or bacteria-free HA hydrogels were intraperitoneally (i.p.) administrated to serve as an *in vivo* model to detect implant infection^{19,112} (Figure 4.3b). Once liberated from the liposomal sensor unit by exposure to toxins produced by nearby pathogenic bacteria, AuNCs rapidly reached the kidneys and filtered through into the urine since they are smaller than the threshold of 5.5 nm of renal filtration^{17,19}. A colourimetric assay utilising 3,3',5,5'-tetramethylbenzidine (TMB) oxidation through AuNC catalysis was performed in the collected urine, creating

a blue colour change to verify the presence of AuNCs for the indication of bacterial infection state of the implant (Figure 4.3d).

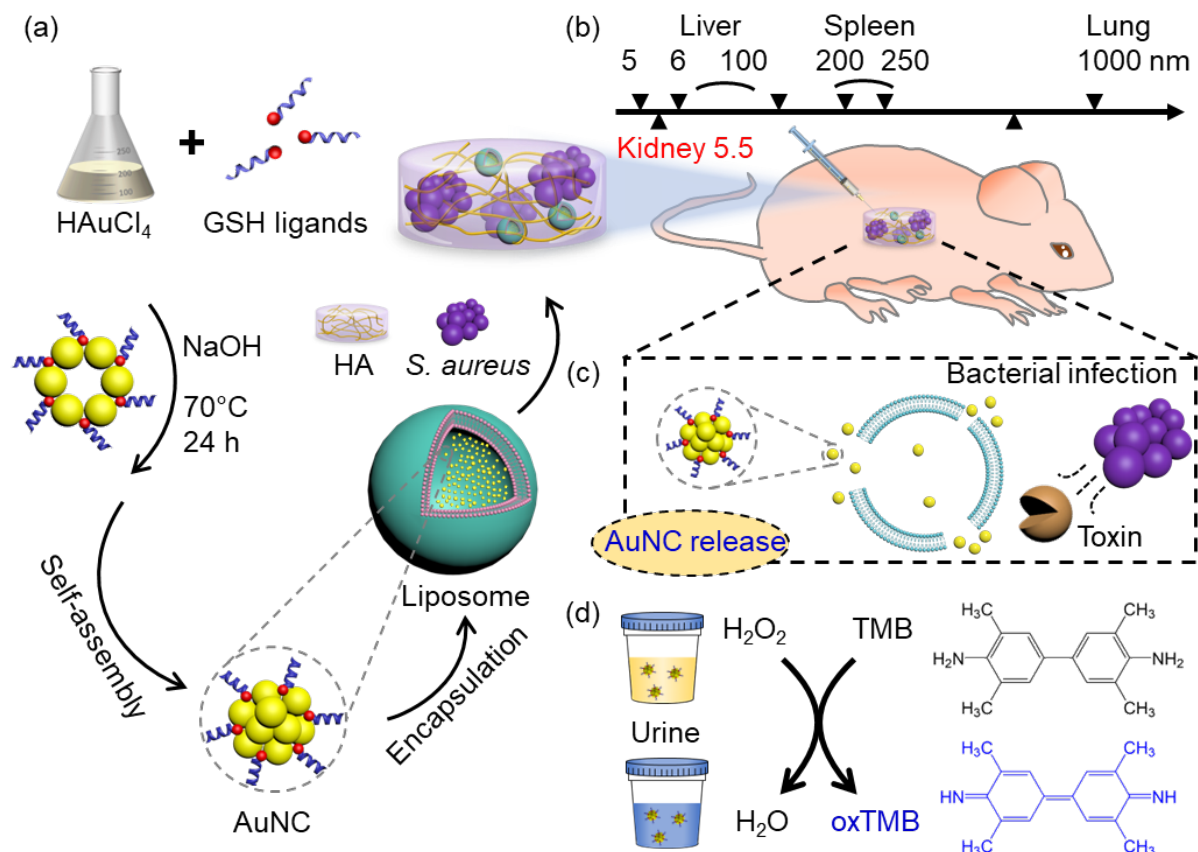


Figure 4.3 Design of AuNC-loaded liposome biosensors and mechanism for detecting bacterial implant infection. (a) AuNC synthesis. Gold (III) was reduced to gold (0) to self-assemble as GSH-stabilised AuNCs, which were further encapsulated in liposomes as a sensing system. HA hydrogels were used as model implants, embedded with AuNC-loaded liposome with or without bacterial contamination. (b) Size threshold of particle clearance from different organs^{17,112}. The ultra-small AuNCs were below the kidney filtration threshold (5.5 nm) and were able to be rapidly cleared through the urinary system. (c) Response mechanism of AuNC-loaded liposomes sensing system in presence of pathogenic bacteria growth concomitant with the secretion of membrane-damaging toxins. (d) Colourimetric TMB oxidation assay in collected urine. The colour change of TMB substrate from colourless to blue demonstrated the presence of AuNCs which is the indicator of bacterial infection state of the implant. Schematic was created using Autodesk 3ds Max and Power Point.

4.4.2 Comparison of liposomal sensor sensing platform loaded with unoptimised and optimised AuNCs pH 2 and 10

Liposomes (lipid vesicles) were selected as nanocarriers for the signal-generating AuNCs as they are composed of biocompatible lipids with the properties of low toxicity, potential to encapsulate hydrophilic cargos (GSH-AuNCs), and the ability to rupture in presence of bacterial toxins for cargo release. To create the sensing unit, liposomes were self-assembled in the presence of aqueous solutions of AuNCs. The ratios of lipid components were varied and included brain sphingomyelin (BSM), 1-palmitoyl-2-oleoyl-glycerol-3-phosphocholine (16:0-18:1 PC), cholesterol (CH), 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2k), and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Figure 4.4, **Error! Reference source not found.**).

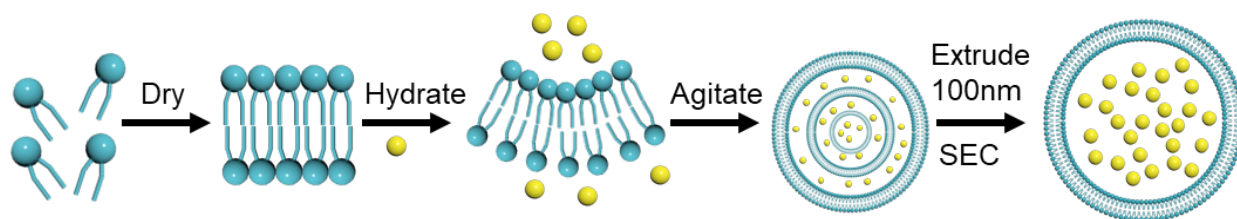


Figure 4.4 Schematic of the self-assembly formation of liposome with the encapsulation of AuNCs. Schematic was created using Autodesk 3ds Max.

BSM was specifically chosen as a crucial component in liposomes due to the secretion of beta-toxin by *S. aureus*, which is a sphingomyelinase C^{293,294}. This toxin turns sphingomyelin into ceramide, which induces negative membrane curvature, eventually causing liposome rupture^{295,296}. To validate the effect of enhanced catalytic activity of AuNCs (Figure 4.5) for this sensing platform, both optimised (1:1.5, pH 10, see

Chapter 2) and unoptimised AuNCs (1:1.5, pH 2, from previous study¹⁷) were encapsulated in liposomes and purified by size exclusion chromatography (SEC). The AuNC-GSH synthesised at pH 2 and pH 10 displayed similar size, measuring about 2 nm, and their corresponding liposomes exhibited an identical size of about 120 nm (Figure 4.5a, b). To assess the maximum signal achievable after complete release of AuNCs from the liposomes, Triton X-100 was added for the incubation before running the TMB assay on the SEC fractions. The liposome sensing system utilising the optimised AuNCs demonstrated significantly higher catalytic activity following the lysis of the relevant liposome fractions (Figure 4.5c). Furthermore, the calibration curve of liposomal sensors using optimised AuNCs also exhibited notably higher signal intensity for each concentration of samples compared to that generated by unoptimised AuNCs. (Figure 4.5d).

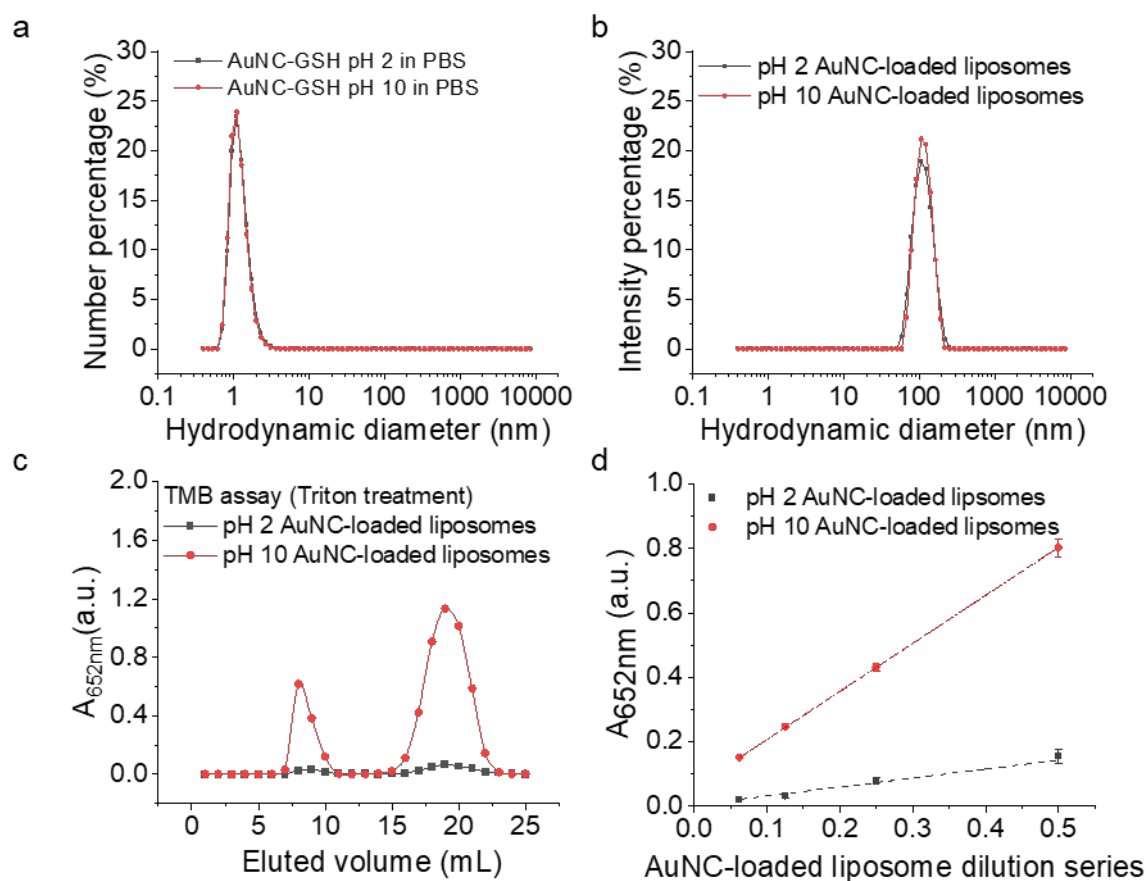


Figure 4.5 Characterisation of AuNC pH 2 and pH 10 loading into liposomes. (a) Number distribution of AuNC pH 2 and pH 10 by DLS. It showed the size of both AuNCs were similar at ~2 nm (curves are mean of N = 1 sample, n = 3 measurements). (b) Intensity distribution of liposomes loaded with AuNC pH 2 and pH 10 by DLS. It showed the size of both liposomes were similar at ~120 nm (curves are mean of N = 1 sample, n = 3 measurements). (c) TMB oxidation assay on the eluted fractions of both liposomes through SEC with subsequent Triton X-100 treatment to lyse the samples. This showed that both the liposome fractions and unloaded AuNCs fractions exhibited significantly higher catalytic activity for the samples with the optimised AuNCs pH 10, with a similar trend observed when the assay was performed with Triton X-100 treatment (N = 1, n = 1). (d) Calibration curve of both liposomes loaded with AuNCs pH 2 and 10. This could be used for the quantification of released AuNCs (N = 1, n = 2).

To verify and compare the sensitivity of the liposomal sensor formed by both AuNCs, comprehensive characterisations of both AuNCs and toxin response of liposomal

sensors were conducted. To assess the sensitivity of both liposomal sensors to bacterial toxins, the liposomes in PBS were incubated after mixing one to one with either bacteria medium (TSB) only, or freshly prepared bacterial culture supernatant from overnight cultures (*S. aureus* SH1000 cultured in TSB) containing the secreted toxins, henceforward this is abbreviated by just saying “toxins” if not specified otherwise. The incubation solution was subsequently filtered using Amicon filter units (100 kDa, to mimic kidney filtration), which will contain only the released AuNCs, whilst the intact liposomes stay in the filter. Filtrates were then measured through the oxidation of TMB catalysed by the released AuNCs in the presence of hydrogen peroxide (Figure 4.6a). Before the toxin cleavage assay, the calibration curves of both AuNCs were measured in DPBS + 5% v/v Triton X-100 as controls. Triton X-100 decreased the catalytic activity of AuNCs compared to that in DPBS, but it was necessary for the full release control of the liposomal sensor for calculation of the release efficiency. The catalytic activity of AuNCs synthesised at pH 10 in both DPBS +/- Triton X-100 was significantly higher than that of unoptimised AuNCs pH 2 (Figure 4.6b, c). Following incubation of the liposomal sensors with toxins secreted by bacteria, filtrates were collected for TMB oxidation for the evaluation of the signal intensity as well as release efficiency of AuNCs for sensitivity comparison. The oxidised TMB absorbance signal from the liposomal sensor made with the optimised AuNC was significantly higher than that of unoptimised AuNCs, demonstrating better sensitivity for toxin detection (Figure 4.6d). Complete release of AuNCs from liposomal sensors was achieved by incubating them (50:50 % v/v) with 5% v/v Triton, which enabled efficiency comparison. The release efficiency of liposomal sensor made with unoptimised AuNC pH 2 was 62%, which was higher than that of optimised AuNC pH 10, which was calculated as 49% (Figure 4.6e). However, when comparing the signal

intensity, the liposomal sensor of AuNC pH 10 exhibited a much higher signal, suggesting superior overall sensitivity in the detection of toxin despite the slightly lower efficiency of release. This outcome was expected due to passive loading of hydrophilic components (GSH-AuNCs), which ended up in the liposome core, generally reaching similar encapsulation efficiencies of a few percent limited only by the internal volume available. Hence, the difference in catalytic activity between AuNCs pH 2 and 10 directly translated to the final liposomal sensor sensitivities.

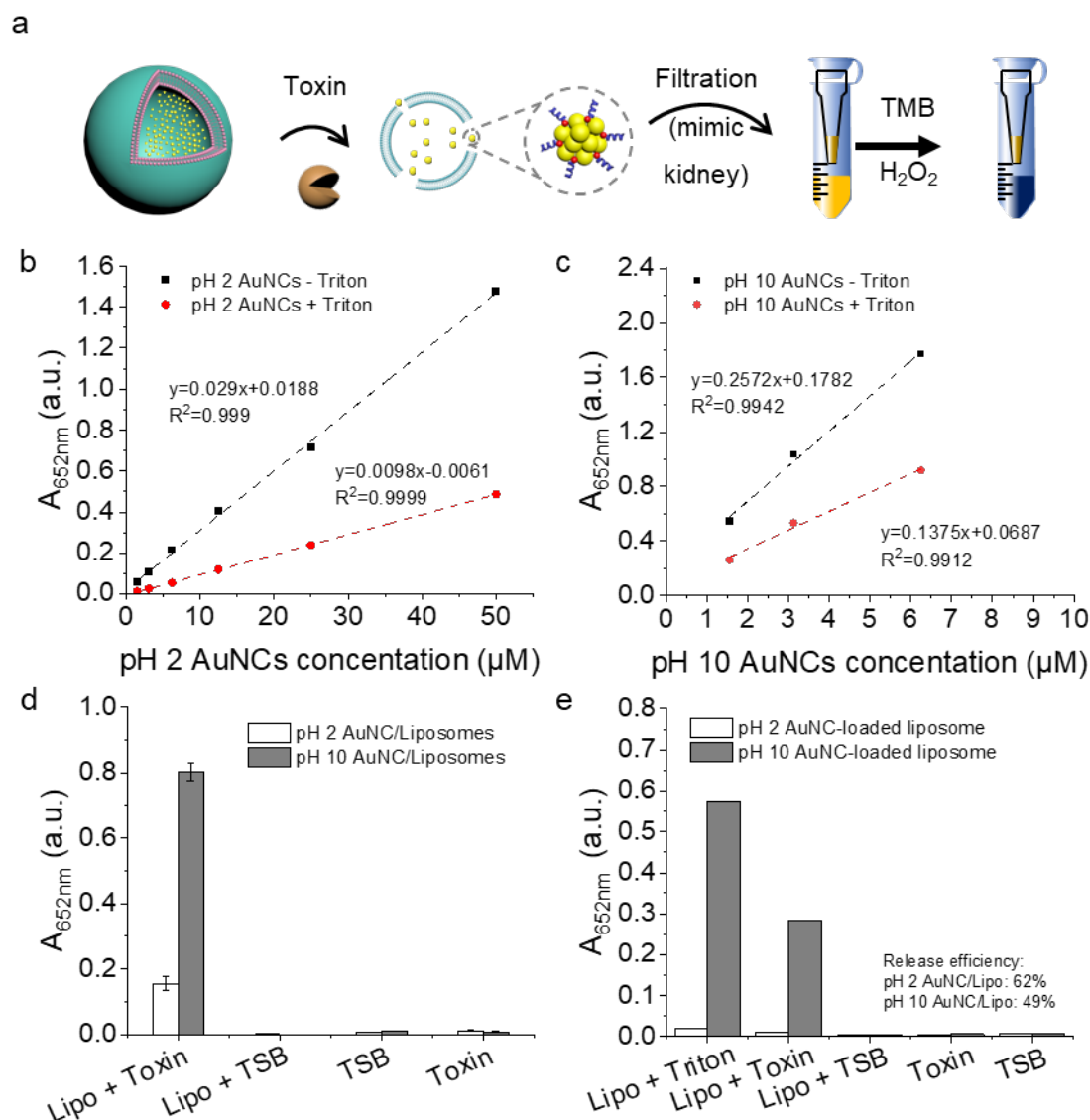


Figure 4.6 The mechanism of the toxin incubation assay with liposomes and calibration curves for AuNCs. (a) Schematics of toxin incubation with AuNC-loaded liposome and separation of released AuNCs for TMB assay. (b, c) Calibration curves for TMB assay endpoint measurements using AuNCs pH 2 and 10 in PBS or 10% v/vTriton X-100. TMB assay endpoint for AuNCs pH 2 was plotted at 100s and for AuNCs pH 10 at 80s. This provided a reference for the accurate calculation of the amount of both AuNCs when measured in an unknown sample. (d, e) TMB assay endpoint measurements after incubation of liposomes with toxin or TSB as controls or with 10% v/v Triton X-100 representing full release control. All data is after Amicon filtration and TMB assay performed on filtrate (N = 1, n = 2 for (d) and N = 1, n = 1 for (e)).

4.4.3 Optimisation of loading efficiency of AuNCs in liposomes

The optimal concentration of AuNCs was further investigated for the encapsulation in liposomes. Various characterisation methods were employed to quantify the amount of AuNCs loaded into liposomes. TMB oxidation assay was conducted on eluted fractions of all liposomes after purification using SEC. This analysis revealed that the liposomal sensor had higher catalytic activity for liposome peak fractions when loaded with higher concentration of AuNCs (Figure 4.7a). However, liposomes loaded with 150X and 200X concentration of AuNCs showed a much higher signal for the unloaded AuNC peak, indicating a significant amount of waste, unloaded AuNCs. Concerning the catalytic activity of the concentrated liposomal sensor from the first peak, the one loaded with 100X concentration of AuNC exhibited the highest signal for the pooled liposome fractions, even though the concentration was not the highest for loading (Figure 4.7b).

Zeta potential measurements were performed on all samples, revealing that the concentrated AuNCs exhibited a negative charge of less than -30 mV, whereas the bare liposomes possessed a slight positive charge at about 10 mV. Importantly, the final AuNC-loaded liposomes exhibited a slightly negative charge, which is favourable for conducting *in vivo* administration studies (Figure 4.7c). The hydrodynamic diameters of AuNCs and their corresponding liposomal sensors were measured by DLS, showing consistent size of 2 nm for AuNC even at a high concentration, and the AuNC-loaded liposomal sensors also maintained a consistent size of 120 nm also after loading AuNCs and SEC purification (Figure 4.7d).

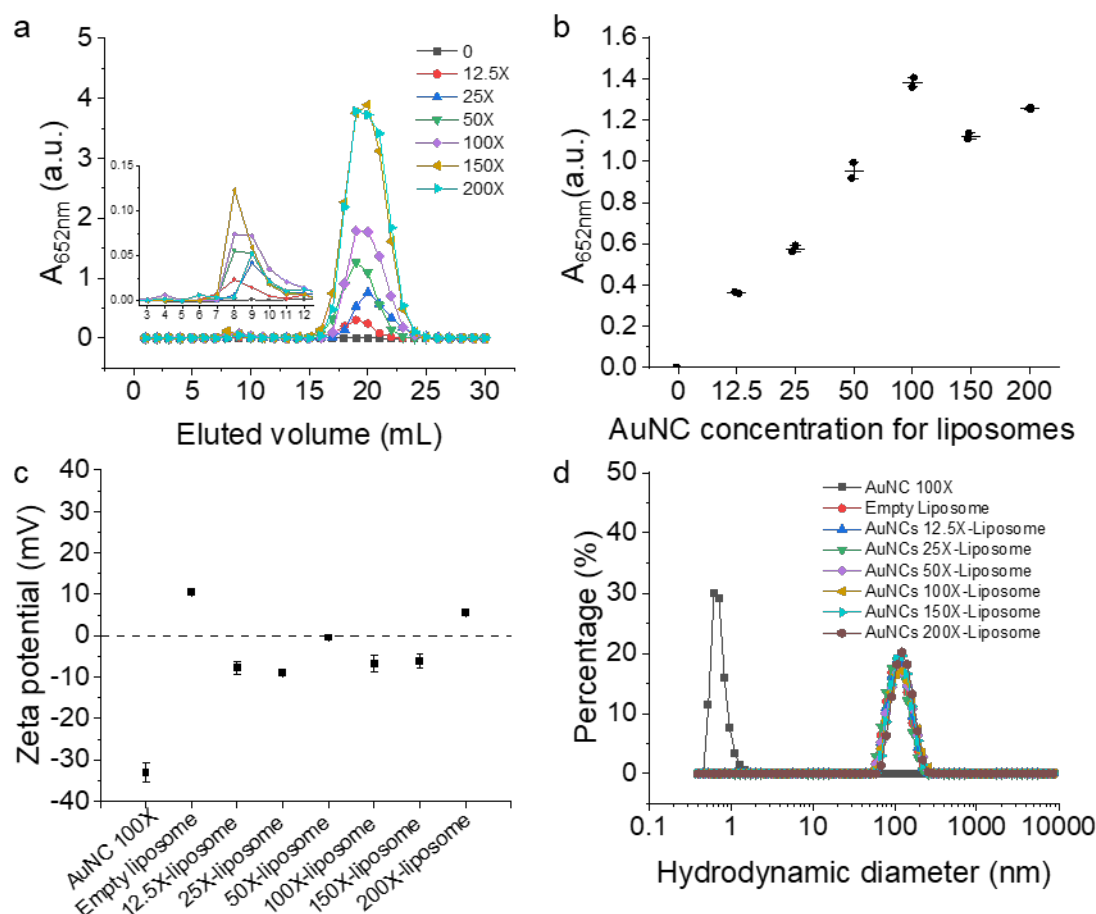


Figure 4.7 Optimisation of AuNC concentration for encapsulation in liposomes.

(a) TMB oxidation assays on the eluted fractions in PBS after SEC of AuNC-loaded liposomes with different concentrations of AuNCs (12.5X corresponds to 0.25 mM during loading up to 200X, which was 4 mM). Larger liposomes were eluted faster (first collection peak, zoom insert) than AuNCs (second, much larger peak). All fractions were diluted 20X for TMB measurement ($N = 1$, $n = 1$). (b) Catalytic activity of all pooled and concentrated liposome fractions from (a). Liposomes loaded 100X of AuNCs (2 mM) performed with the optimal catalytic activity according to the endpoint at 60s ($N = 1$, $n = 2$). (c) Zeta potential measurements of AuNCs, bare BSM:CH (50:50 % w/w) liposomes and the liposomes loaded with different concentrations of AuNCs. (d) DLS size distributions of AuNCs (number distribution), bare liposomes and AuNC-loaded liposomes (intensity distributions) (curves are mean of $N = 1$ sample, $n = 3$ measurements).

To further investigate and precisely compare the encapsulation efficiency of AuNCs within liposomes, inductively coupled plasma mass spectrometry (ICP-MS) were employed to accurately measure the gold contents in liposomes after SEC purification. This method allowed us to quantitatively determine the encapsulation efficiency of AuNCs within the liposomes. For this analysis, all concentrations of AuNCs and the corresponding formed liposome were digested in aqua regia overnight and further diluted for the ICP-MS measurement. Gold standard calibration was conducted each time before gold content measurements using ICP-MS. Through these measurements, it is discovered that the liposome sensing platform loaded with an initial concentration of 2 mM AuNCs (100X sample) exhibited the best catalytic activity, along with an encapsulation efficiency of 2.5% (Table 4.1). Hence, this formulation was taken forward for the subsequent experiments.

Table 4.1 ICP-MS measurement of gold contents in AuNC-loaded liposomes for encapsulation efficiency calculation. 12.5X corresponds to 0.25 mM, going up to 200X, which corresponds to 4 mM AuNCs used during loading.

AuNC	ppm 100x dil.	STDEV	AuNC-liposome	ppm	STDEV	Encapsulation efficiency (%)
AuNC 0 (PBS)	0.0341	0.0112	Empty liposomes	0.0434	0.0311	0%
AuNC 12.5x	2.12	0.006	AuNC 12.5x-loaded liposomes	6.992	0.04	3.30%
AuNC 25x	3.645	0.028	AuNC 25x-loaded liposomes	11.12	0.03	3.05%
AuNC 50x	9.415	0.069	AuNC 50x-loaded liposomes	20.31	0.11	2.16%
AuNC 100x	14.36	0.12	AuNC 100x-loaded liposomes	36.3	0.25	2.53%
AuNC 150x	25.57	0.09	AuNC 150x-loaded liposomes	25.2	0.28	0.99%
AuNC 200x	31.04	0.07	AuNC 200x-loaded liposomes	36.92	0.17	1.19%

4.4.4 Variation of lipid composition for design of liposomal sensor

To investigate the influence of different lipid formulations of liposomes on the toxin response sensitivity of this sensing platform, seven formulations were designed for the assembly of liposome by utilising the following lipids: BSM, PC, CH, DOTAP and DSPE-PEG2k (**Error! Reference source not found.**). In all the formulations, 1 mol% DSPE-PEG2k was always incorporated as a stabiliser for liposome formation, a strategy previously studied and reported^{99,297}. The formulations without DOTAP were studied firstly. Liposomes were assembled using BSM:CH (% w/w 50:50), PC:CH (% w/w 50:50), and BSM:PC:CH/BPC (% w/w 33:33:33), all including the 1 mol% DSPE-

PEG2k, and involving a series of steps, including hydration, extrusion, and SEC purification.

Table 4.2 Liposome composition (in wt%) using brain sphingomyelin (BSM), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (16:0-18:1 PC), cholesterol (CH), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), all including with 1 mol% of DSPEPEG-2K.

Lipid formulation	BSM:PC	PC:CH	BSM:PC:CH /BPC	BPC+1% DOTAP	BPC+5% DOTAP	BPC+10 %DOTAP	BPC+25 %DOTAP
BSM	50%	N/A	33.3%	33%	31.7%	30%	25%
PC	N/A	50%	33.3%	33%	31.7%	30%	25%
CH	50%	50%	33.3%	33%	31.7%	30%	25%
DSPE-PEG2k (mol% to lipids)	1%	1%	1%	1%	1%	1%	1%
DOTAP	N/A	N/A	N/A	1%	5%	10%	25%

The TMB signals on all eluted fractions of three formulations of liposomes, after SEC purification, exhibited similar trends for two eluted peaks of both the liposome and the unloaded AuNC, indicating that they had the same encapsulation efficiency in the loading process (Figure 4.8a). Their hydrodynamic diameters were measured using DLS, along with their derived count rates. All three formulations showed an identical size of 120 nm, with a slight difference in the derived count rate (a rough concentration estimate for comparison of same sized particles with similar compositions), ranked from high to low in the order of BC, BPC, and PC (Figure 4.8b). To further confirm the difference in their concentration, nanoparticle tracking analysis (NTA) was utilised to

measure the particle concentration as well as molar concentration after conversion. The results suggested that BPC had the lowest concentration, and PC had a slightly larger diameter than the other two liposomes, which could account for the higher intensity of derived count rate (Table 4.3). TMB oxidation assay was characterised on the purified and concentrated liposomes by measuring the absorbance over time. PC liposomes exhibited a slightly quicker reaction in TMB assay compared to the other two liposomes (Figure 4.8c). The onset velocity of their initial reaction was determined by analysing the increase in absorbance over time within the initial linear reaction range, which was consistent with the findings in the reaction process (Figure 4.8d).

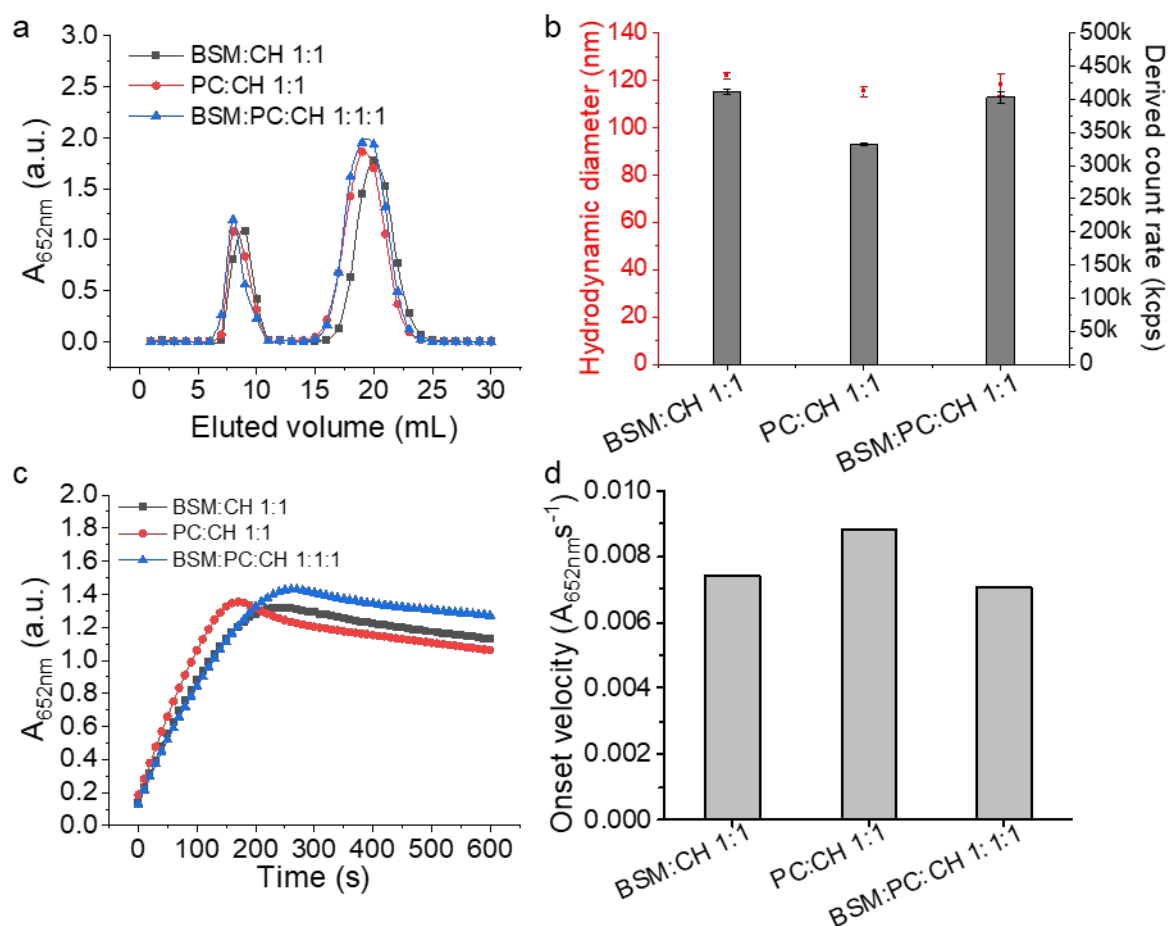


Figure 4.8 The characterisation and toxin release performance of liposomes with different components including BSM, PC and CH. (a) TMB oxidation on SEC eluted fractions for three different formulations of liposomes. The absorbance of all samples had similar performance of catalytic activity. (b) Hydrodynamic diameter and dederived count rate measurements of all three liposomes by DLS. It showed they had similar size and roughly same concentration by comparison of derived count rate. (c, d) Characterisation of the catalytic activity on the three liposomes. The increase of absorbance over time for three liposomes were measured on a plate reader and the onset velocity was derived from the absorbance curve.

Table 4.3 NTA characterisation of all three liposome formulations for the comparison of their concentration

NTA measurement	Mean size	SD	Concentration (particles/mL)	c (mol/mL)
BSM:CH	118.5 nm	27.3 nm	2.23e+013 +/- 5.02e+07	3.703e-11
PC:CH	123.1 nm	27.3nm	2.05e+013 +/- 1.14e+08	3.404e-11
BSM:PC:CH	115.6 nm	23.8nm	1.71e+013 +/- 2.12e+08	2.8395e-11

To compare responsiveness of these different liposome formulations to bacterial toxins, they were incubated with a dilution series of toxin for 2 h, followed by Amicon centrifugal filtration for the separation of the released AuNCs from toxin-lysed liposomes. Subsequently, the TMB assay was conducted on the filtrates to measure the catalytic activity of the released AuNCs and evaluate the toxin release efficiency of the liposomal system.

Among the three liposome types, BSM:CH (% w/w 50:50) liposomes showed the lowest response to toxin, compared to the other two liposomes. The incubation and toxin mediated release assays were repeated three times, and the same trend was consistently observed for all liposomes. Notably, BPC exhibited the highest signal value from the released AuNCs after toxin incubation, followed by PC:CH (% w/w 50:50) liposomes (Figure 4.9). The signals obtained from the released filtrates were converted into release efficiency, which was calculated as the signal after toxin-mediated release divided by the full release signal obtained by measuring the catalytic activity using Triton X-100 for complete disruption of all liposomes (Figure 4.9c). This confirmed that BPC is the optimal formulation among the three, whilst further fine-tuning of the liposome formation was attempted in the next section.

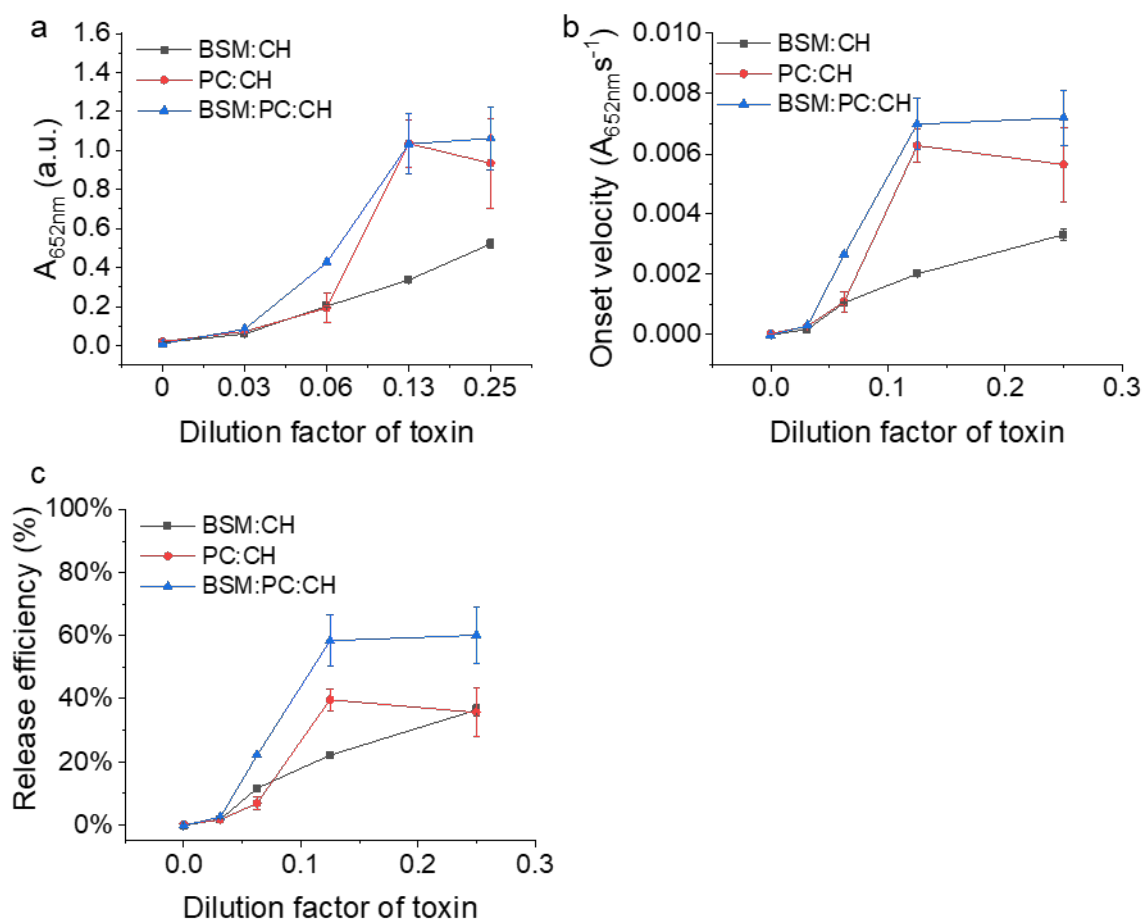


Figure 4.9 LoD comparison for the three liposome formulations after incubation with a toxin dilution series. The end point absorbance after TMB assay (a), the derived onset velocity (b) and the release efficiency (c) of filtrates analysed after toxin incubation and Amicon filtration (N = 3, n = 3).

4.4.5 Incorporation of DOTAP in the liposomal sensor

DOTAP is a cationic lipid with an ammonium headgroup, resulting in a positive charge, which is popular for drug and gene delivery²⁹⁸. It is commonly used in liposome formulations for various applications, as it influences the size, zeta potential and stability of liposomes. Numerous studies have shown that the positive charge of DOTAP can facilitate cellular uptake, enhance transfection efficiency²⁹⁹, improve the stability of liposomes³⁰⁰.

In this studies, different % w/w of DOTAP was incorporated in the before optimised BPC formulation, ranging from 1% to 25%. Due to its positive charge, DOTAP was hypothesised to potentially interact with negatively charged AuNCs and may lead to higher encapsulation efficiency and responsiveness to toxins. However, yellow aggregations were observed when using over 15% w/w DOTAP in the liposome formulation, and complete precipitation occurred with 25% w/w DOTAP, indicating an intense electrostatic reaction with AuNCs (Figure 4.10a). Comparing TMB oxidation assay results on the eluted fractions of all liposomes after SEC, similar peak intensities for liposomes and unloaded AuNCs fractions was observed. However, using over 10% w/w DOTAP in the formulation resulted in less liposome production, which aligned with the aggregations observed (Figure 4.10b). Despite these observations, all liposomes showed similar hydrodynamic diameters, except for the formulation with 25% w/w DOTAP, which had a larger size. Comparing their derived count rate related to their concentration, the one with 25% w/w DOTAP exhibited a significantly lower value even with a larger size, most-likely due to aggregation (Figure 4.10b, c). Additionally, it was noticed that when more than 5% w/w of DOTAP was added in the liposome formulation, even in the presence of 1 mol% DSPE-PEG2k, the zeta potential of liposomes became positive, which is not favourable for conducting *in vivo* administration studies in the future (Figure 4.10d).

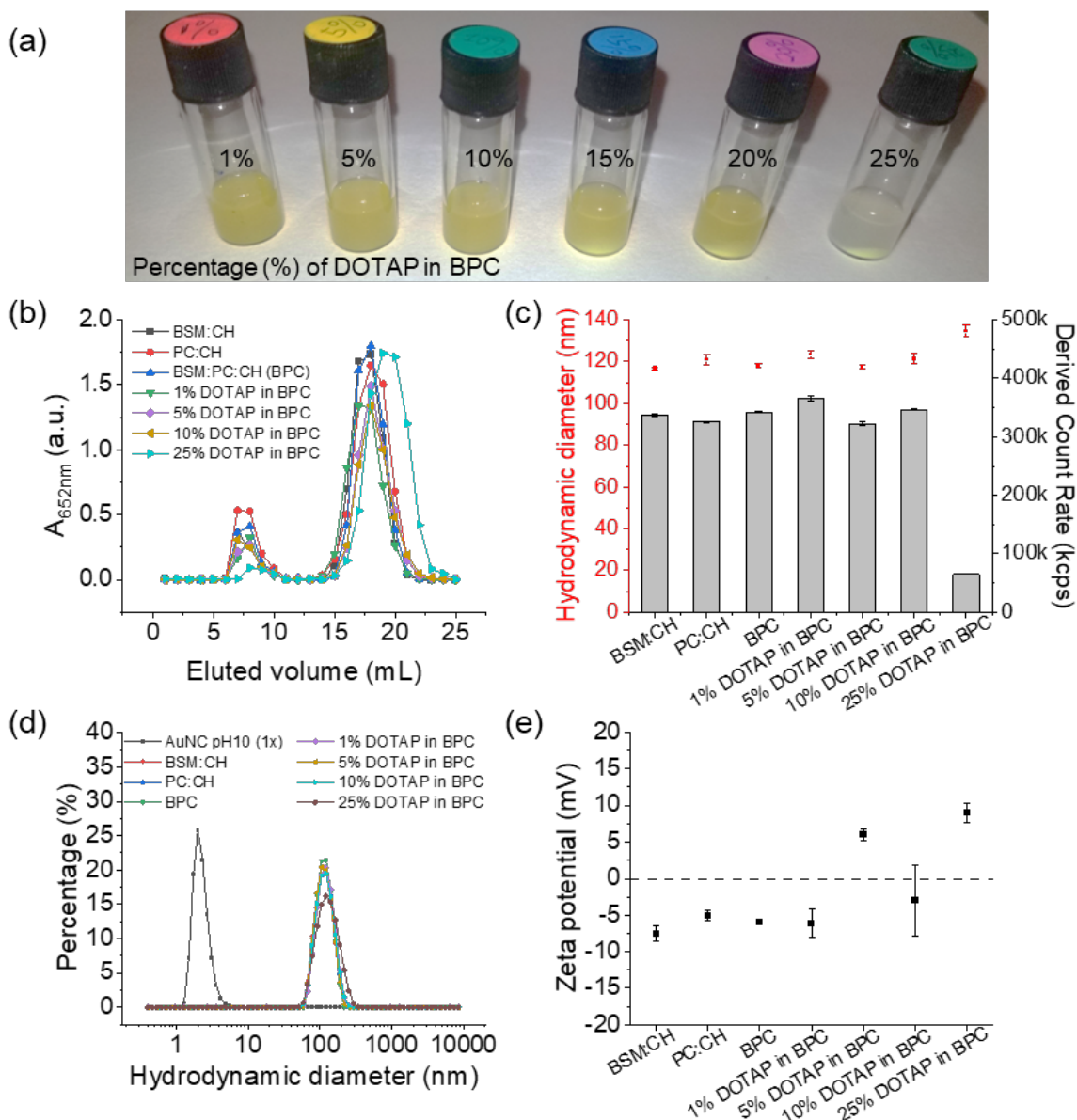


Figure 4.10 Further optimisation and characterisation of liposome compositions incorporating DOTAP. (a) Photograph of all liposomes containing different percentages of DOTAP. It shows some aggregation occurred when adding more than 15% of DOTAP in the compositions. (b) TMB oxidation assay of SEC elution fractions for all liposomes. Notably, liposomes with 25% w/w DOTAP exhibited a much lower absorbance signal in the liposome fractions, which corresponded to the observed aggregations. (c,d) Characterisation of the size of AuNCs and AuNC-loaded liposomes by DLS and derived count rate. (e) Zeta potential measurement of all liposomes. The addition of cationic DOTAP lipids led to a positive charge in the AuNC-loaded liposomes at higher DOTAP contents.

To compare their responsiveness to toxins, all liposomes were incubated after mixing one to one with either toxin or 5% v/v Triton X-100 for 2 h, followed by filtration to collect the released AuNCs for TMB oxidation assay. Importantly, all the liposomal sensors remained intact when incubated with bacteria medium only (TSB), as observed by negligible signal from the AuNC-catalysed reaction using the Amicon filtrate (Figure 4.6 d,e and Figure 4.9). In contrast, in presence of toxin and among all the different formulations, the triple-lipid liposome (BPC) exhibited the highest signal and release efficiency compared to the other two liposomes composed of two lipids (BC and PC) without DOTAP, same as above (Figure 4.9). Liposomes containing 10% and 25% of DOTAP exhibited an optimal toxin release efficiency. However, the intensity of the final TMB assay for liposomes with DOTAP, used as an indicator of toxin detection, was lower than the previous best liposome (BPC) without any DOTAP. Considering both the toxin release efficiency and the intensity of the final assay as well as a more favourable slightly negative surface charge, BPC liposomes stood out as the best formulation and were therefore used in all the next sections (Figure 4.11).

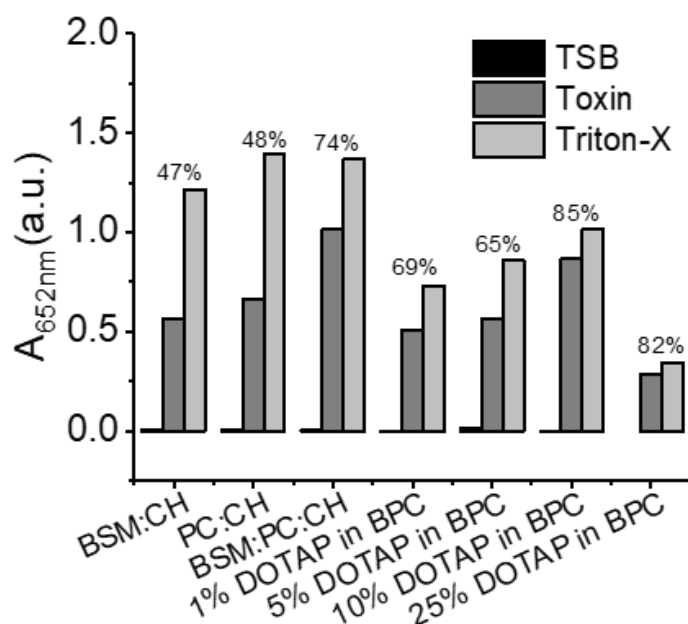


Figure 4.11 Toxin release efficiency comparison for all liposomes. Liposomes with 10% and 25% DOTAP had an optimal toxin release efficiency followed by liposomes (BSM:PC:CH) without DOTAP. Considering the surface charge and aggregation, as well as the overall catalytic activity of the final filtrates, liposome (BSM:PC:CH) without DOTAP was the best formulation candidate for this toxin sensing platform (N = 1, n = 1).

4.4.6 Detailed characterisation of the BPC liposomal sensor

Cryo-TEM was employed to obtain a picture of the liposomal sensor. It was interesting to see where the AuNCs are located and the integrity of the membranes. Both empty BPC liposomes and AuNC-loaded BPC liposomes displayed well-defined membranes, albeit with limited instances of multilinearity (Figure 4.12). Their sizes are around 100 nm which aligns with DLS measurement in Figure 4.10d. In the case of AuNC-loaded BPC liposomes, it was evident that the AuNCs were encapsulated within the liposomes, confirming the successful encapsulation of AuNCs, which were discernible as dark dots (Figure 4.12b, d, f).

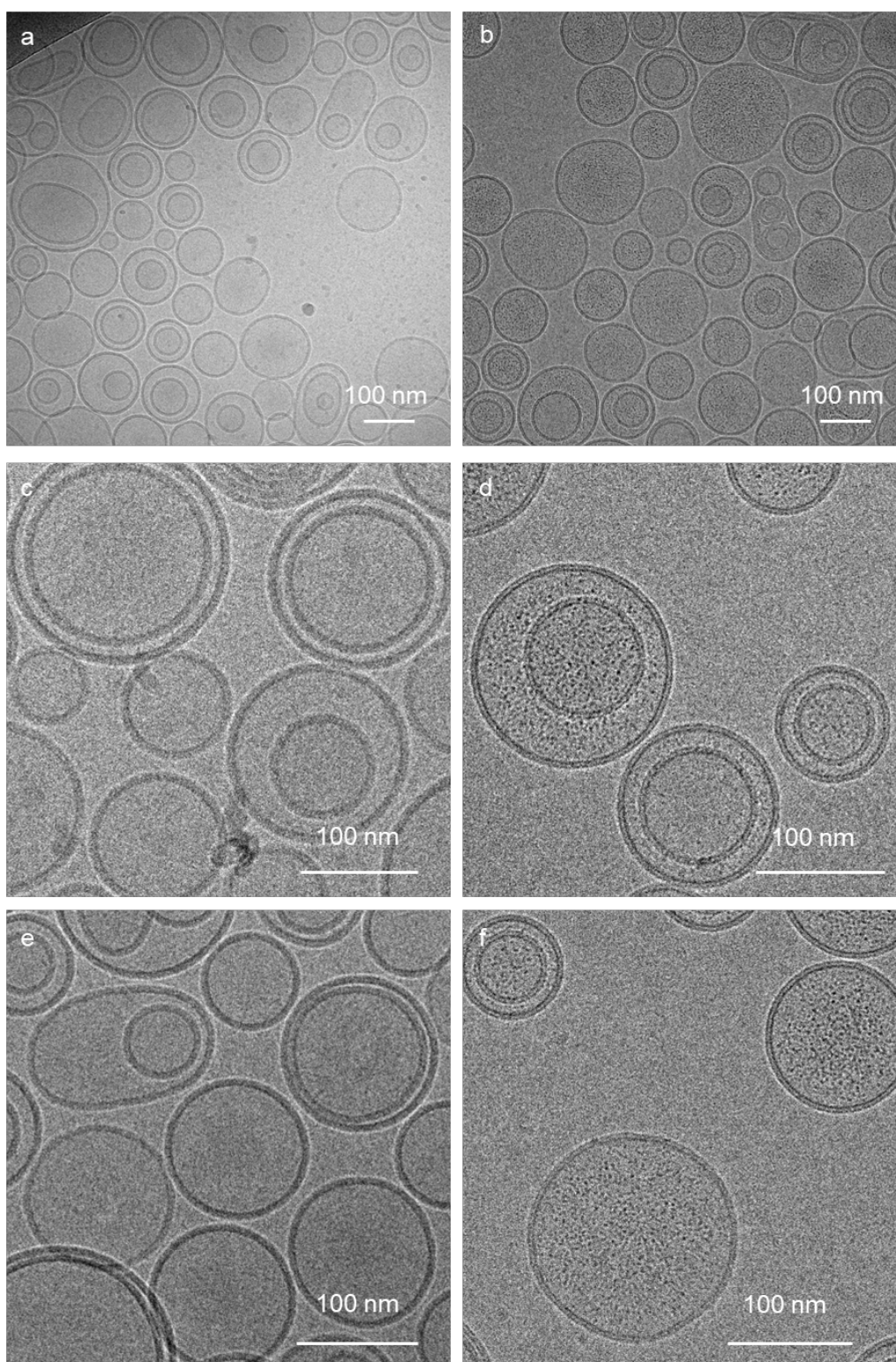


Figure 4.12 Cryo-TEM images of empty liposome controls and AuNC-loaded liposomes. Scale bars = 100 nm. (a, c, e) Empty liposome control (BSM:PC:CH). They have a clear core and a membrane. (b, d, f) AuNC-loaded liposomes (BSM:PC:CH), showing the successful loading of AuNCs (dark dots) inside liposome (Cryo-TEM imaging was performed by Dr Mohamed Chami).

To investigate the toxin-mediated release from the liposomes, fluorescence correlation spectroscopy (FCS) was employed. FCS allows characterisation of the diffusion properties (hence the hydrodynamic size) of a fluorescent sample and the amount of fluorescent cargo per liposome can be calculated using the molecular brightness (counts per particle, cpp, in kHz) information. After incubation with the toxin, the diffusivity of liposomes completely shifted to that of freely diffusing OG488-labelled AuNCs and release analysis indicated 99% release AuNCs-OG488 upon toxin incubation (Figure 4.13a). This release number is higher than found above *via* catalytic activity assessment (Figure 4.11), which can be attributed to the fact that FCS was performed without purification after toxin-incubation. The centrifugal filters used above have likely adsorbed a fraction of the released AuNCs. The hydrodynamic diameters of both the AuNCs-OG488 and AuNC-OG488-loaded liposomes calculated from the FCS autocorrelation analysis (Figure 4.13b) were consistent with the sizes obtained from DLS. Brightness analysis (cpp) also revealed that in average >100 AuNCs were loaded per liposome, in agreement with the cryo-TEM images (Figure 4.12). DLS measurements of different dilutions of toxin-containing bacterial culture supernatants revealed the impact on size and relative concentration (derived count rate as a rough estimate) of intact liposomes, which dropped with increasing amount of toxin added (Figure 4.13c).

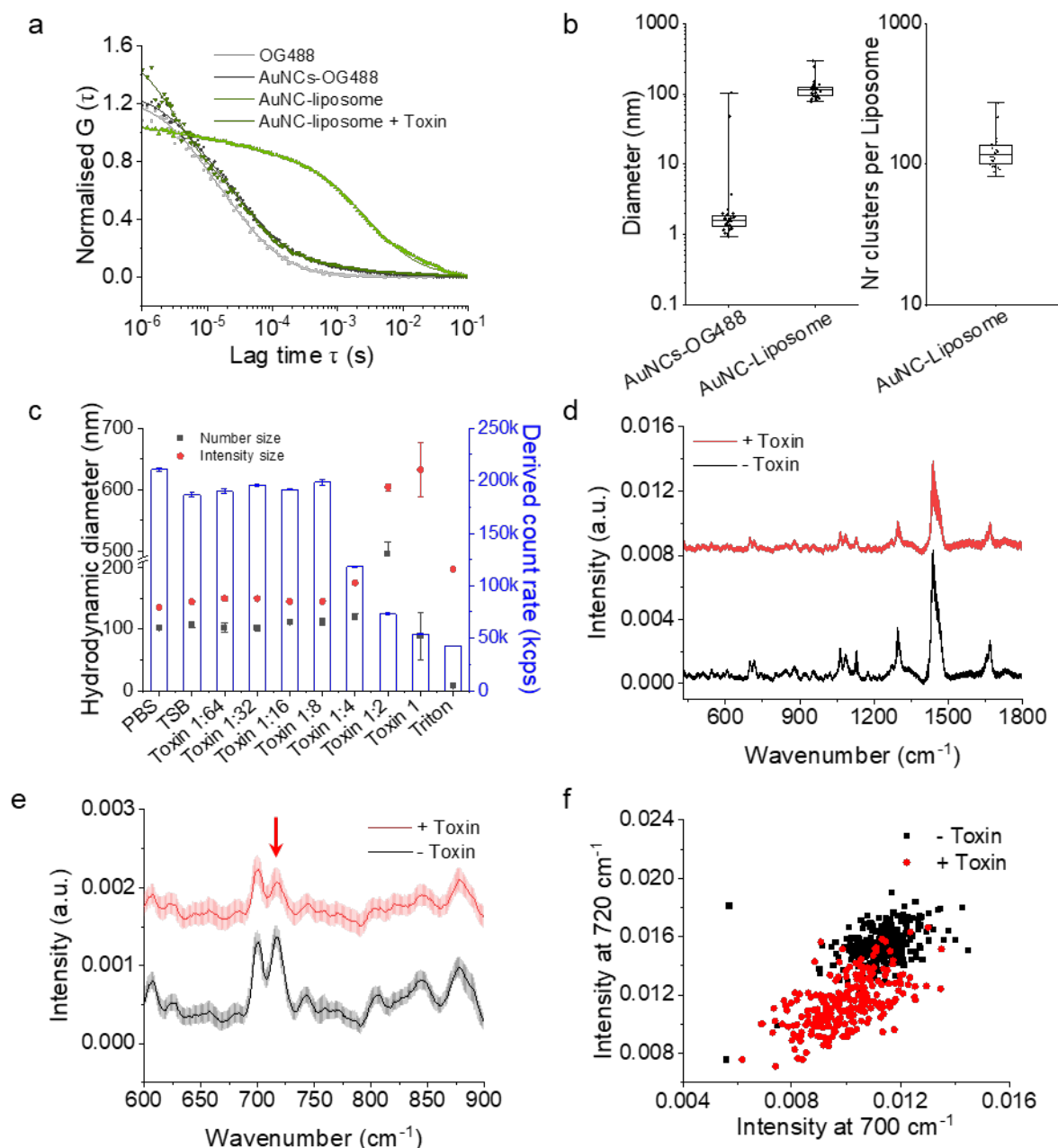


Figure 4.13 Characterisation of BPC. (a) Normalised average autocorrelation curves from FCS measurements of free dye (OG488), dye-labelled AuNCs (AuNCs-OG488), liposomes loaded with AuNCs-OG488, and the toxin-treated liposomes ($n = 25$ individual measurements per sample). (b) Hydrodynamic diameters of AuNCs-OG488 and AuNC-OG488-loaded liposomes and the number of AuNCs per liposomes calculated from FCS molecular brightness analysis of data from (a). (c) DLS measurements of BPC liposomes incubated with a dilution series of toxin, including hydrodynamic diameter (intensity and number distribution) and the derived count rates as an approximate guide of concentration ($n = 3$). (d, e) Single particle automated

Raman trapping analysis (SPARTA) spectrum measurements of AuNC-liposome before (red line) and after toxin treatment (black line) ($n > 3$) of the full spectra and range from 600 to 900 cm^{-1} . (f) Scatter plots of peak signal intensity from (e) of both samples at 700 cm^{-1} and 720 cm^{-1} . SPARTA measurements were performed by Catherine Saunders.

To investigate the lipid transformations in liposomes exposed to toxin at a signal-particle level, the single-particle automated Raman trapping analysis (SPARTA) technique was employed²⁹¹. SPARTA was recently reported as a label-free method to provide high-throughput information about the precise chemistry compositions among a population of the nanocarriers and their cargo within a population³⁰¹ and to measure time-resolved enzymatic conversion of lipid headgroups³⁰². Here, SPARTA measurements were performed on toxin-treated and untreated liposomal sensors. The mean Raman spectra across all liposomes exhibited similarities between the samples, with characteristic lipid peaks at 1298 cm^{-1} and 1438 cm^{-1} (CH_2 deformation), and 1670 cm^{-1} (overlapping cholesterol, ceramide backbone, $\text{C}=\text{C}$). However, the signal intensity of toxin-treated liposomes was slightly lower than of the non-treated ones, likely due to less integrity after exposure to toxin (Figure 4.13d, e). Notably, there was a reduction relative to the rest of the spectrum in intensity of the 717 cm^{-1} peak after incubation with toxin, which can be attributed to the C-N symmetric stretching of the choline group³⁰³. This suggests that liposomes were lysed due to the hydrolysis of sphingomyelin lipids by sphingomyelinase-like beta-toxin, secreted by *S. aureus*³⁰⁴, into ceramide and soluble phosphorylcholine. After cleavage the phosphorylcholine was likely released into solution, so was no longer spatially associated with the nanoparticle, leading to the loss of choline headgroup signal in SPARTA data after toxin incubation. This observation is consistent with previous literature on the transformation of sphingomyelin to ceramide by sphingomyelinase^{303,305,306}.

Additionally, the peak signal at 700 cm^{-1} is associated with cholesterol^{303,307}, which is a component present in the liposome formulation. The consistent presence of the stable signal at 700 cm^{-1} was attributed to the cholesterol lipids in the liposome formulation, contributing to enhanced stability³⁰⁸. Scatter plots comparing the peak signal at 700 cm^{-1} against the choline headgroup peak at 717 cm^{-1} can be used to show the single particle-level variation of choline signal with cholesterol signal for both samples (Figure 4.13f). Untreated liposomes formed a homogenous population with cholesterol and choline peak areas centred around 0.011 and 0.016 respectively. After toxin treatment, choline signal in the whole population notably decreased relative to the cholesterol signal which demonstrates relatively homogenous cleavage of the choline headgroup. Overall, the optimised liposome composition, BPC, was demonstrated to be responsive to toxin secreted from *S. aureus in vitro* and achieve an almost complete release of AuNCs, whilst the sensor remained intact when incubated without the toxins.

4.4.7 AuNC-loaded liposomes respond to *S. aureus in vitro*

To assess the responsiveness of AuNC-loaded liposomes to bacteria growth in their vicinity – a more clinically relevant scenario –incubation experiments with *S. aureus* cultures instead of toxin supernatant were conducted as above. This approach aimed to mimic the sensing process during bacterial infection. Following incubation of the liposomal sensor with *S. aureus* cultures or with TSB only, the supernatant was obtained by centrifugation after different time points. The supernatant of each incubation was then filtered through a centrifugal filter (100k Da) to collect the filtrate, which then only contains AuNCs released from the liposomes. The collected filtrates

were subjected to TMB assays, where a colour change to blue indicated the release of AuNCs from the liposomes in the presence of bacterial growth (Figure 4.14a).

To ensure consistent *S. aureus* SH1000 growth behaviour, an overnight bacterial culture was diluted at ratios of 1:100 and 1:1000, followed by subsequent serial dilutions. The diluted bacteria were streaked on tryptic soy agar (TSA) plates and allowed to grow overnight to obtain colony-forming units (CFU) for determining the bacterial concentration. The 1:100 diluted bacteria exhibited rapid growth in the initial 5 h and reached a maximum concentration of 10^9 CFU/mL (Figure 4.14b). The 1:100 dilution was taken forward for testing with the liposome sensor. The filtrates obtained from liposomes incubated with bacteria or TSB were mixed with the TMB substrate, and the absorbance at 652 nm was monitored to assess the kinetics of the reaction. The absorbance endpoint reaching the highest intensity from TMB assay indicated the catalytic activity of the released AuNCs, reflecting the extent of their release. After a 3-h incubation, a blue colour change was observed in wells where liposomes were incubated with bacteria. This indicated the onset of liposome lysis by the bacterial toxins, which correlated with the endpoint measurements of the TMB assay. The catalytic activity reached its highest value after 5 h, as indicated from their onset velocity (Figure 4.14g). In contrast, the filtrates from liposomes incubated with TSB culture medium showed no colour change, indicating the stability of liposomes in the absence of bacteria in medium (Figure 4.14c). The kinetics reaction curve also demonstrated a similar trend, with the fastest reaction observed at 5 h of incubation. The onset velocity of the reaction was consistent with the endpoint measurements, and the reproducibility of the assay was confirmed (Figure 4.17). The reason for reaching a plateau after 5 h was attributed the blockage of Amicon filter units caused

by bacterial growth and the accumulation of liposome debris following disassembly. To estimate the minimum amount of sensor that needs to be disassembled to achieve a detectable signal, the calibration curve for AuNC-loaded liposomes (BSM:PC:CH) was conducted and determined to be 19 $\mu\text{g/mL}$ of lipid in the presence of 86 pmol of AuNC by calculation (Figure 4.16a).

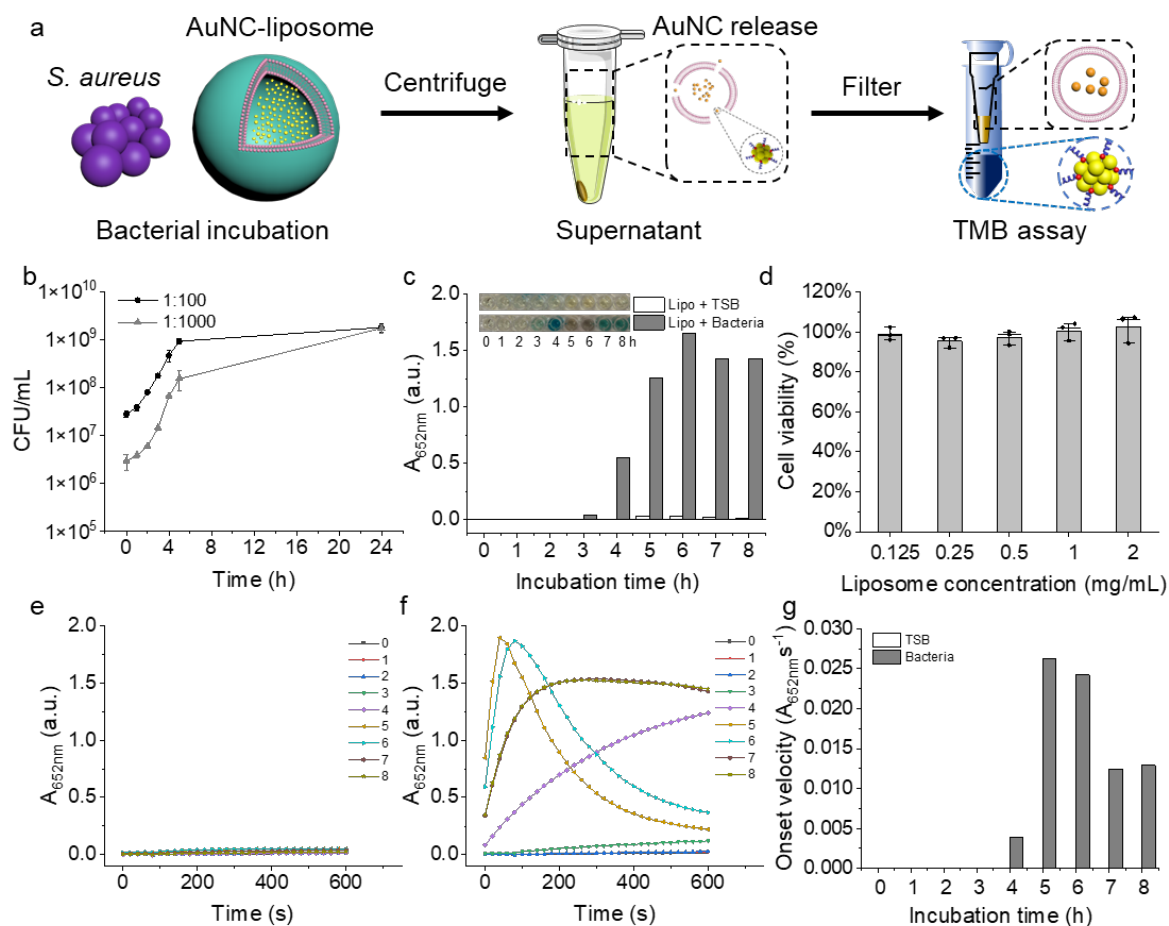


Figure 4.14 *In vitro* incubation of bacteria with AuNC-loaded liposomes and characterisation of bacterial growth. (a) Schematic of *in vitro* incubation of AuNC-loaded liposomes with bacteria directly, and subsequent separation of released AuNCs from intact liposomes. Schematic was created using Autodesk 3ds Max and Power Point. (b) Bacterial growth curve of *S. aureus* SH1000 by CFU counting method (N = 1, n = 2). (c) Filtrate analysis by TMB oxidation assay (140s endpoint) for different incubation time points (from 0h to 8h, n = 1, repeats in Figure 4.17). (d) *In vitro* cytocompatibility MTS assay after incubation of AuNC-loaded liposomes with RAW 264.7 cells (N = 3 individual experiments, n = 3 measurements). (e, f) The measurement of absorbance over time for TMB assay on the filtrates collected after various time periods after incubation of AuNC-loaded liposome with TSB and TSB + bacteria. (g) Onset velocities calculated from (e, f).

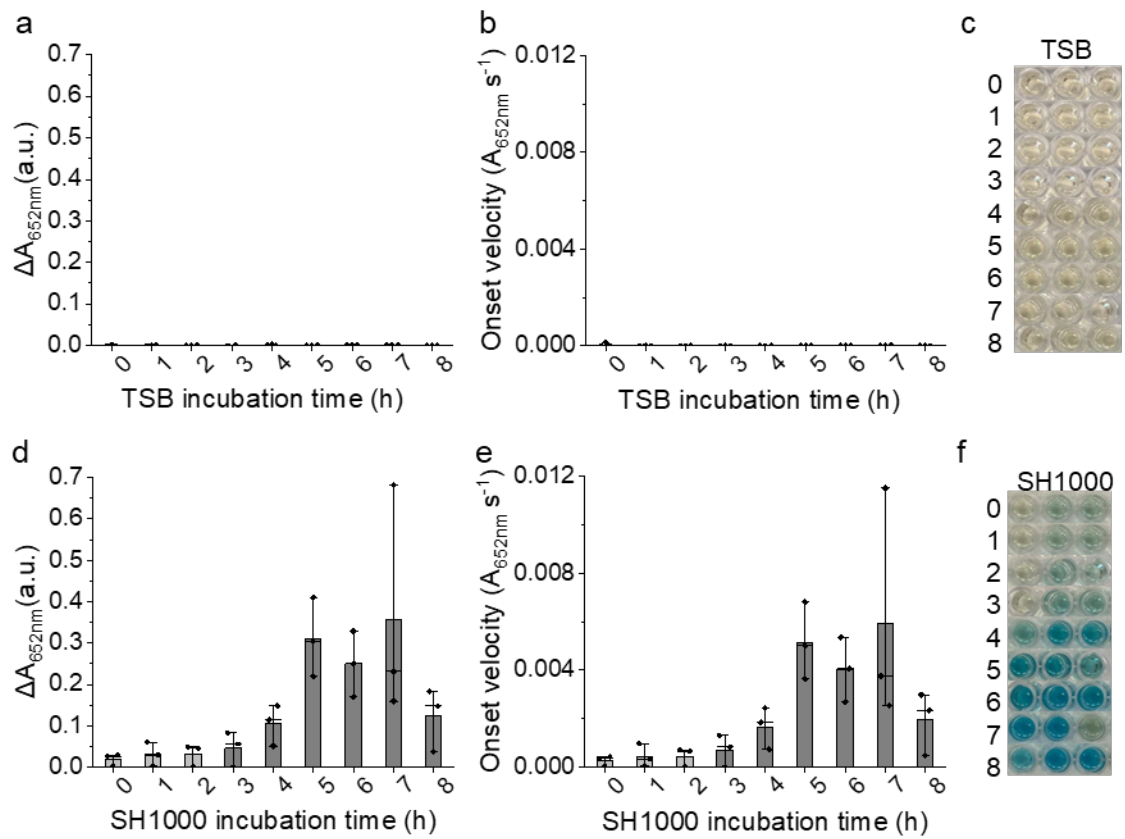


Figure 4.15 Response performance of AuNC-loaded liposomes to toxin by co-incubation with *S. aureus* SH1000. Three more repeats of incubation of AuNC-loaded liposomes with TSB (a-c) or TSB + bacteria (d-f) (N = 3, n = 1).

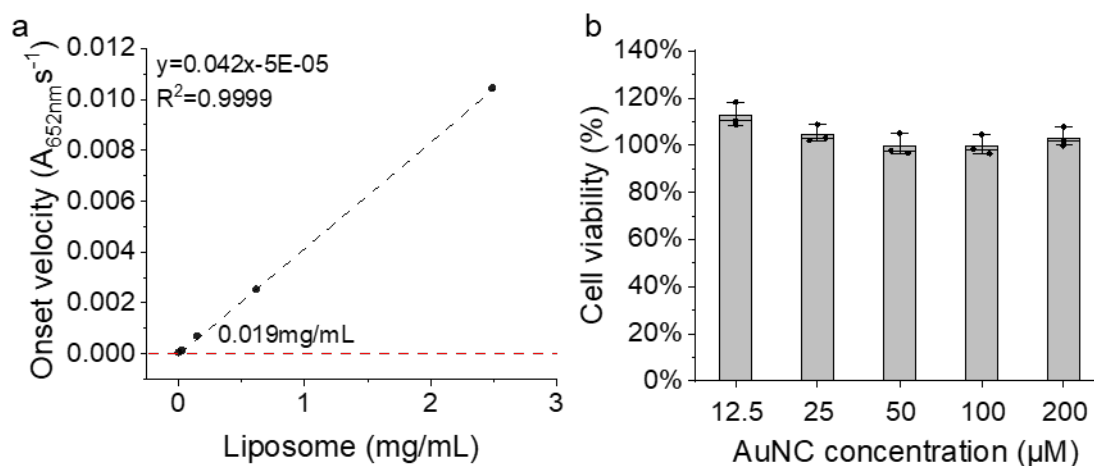


Figure 4.16 The calibration curve and LoD of the BSM:PC:CH liposomal sensor and cytocompatibility of AuNCs. LoD measurement of the AuNC-loaded liposomes. The liposomal sensor was diluted in urine (50/50 % v/v) to get different concentration (mg/mL). 25 μL of diluted liposomal sensor was mixture with 100 μL of 1-step TMB substrate and 100 μL H_2O_2 (2 M). Absorbance at 652 nm of the mixture was measured on the plate reader. The onset velocity was calculated by the increased absorbance signal over time, representing the catalytic activity determined. (b) *In vitro* cytocompatibility MTS cell viability assay after incubation of AuNCs with RAW 264.7 cells (N = 3, n = 3).

An important consideration for the use of any biosensor is their biocompatibility. To assess the cytocompatibility of AuNC-loaded liposomes, RAW 264.7 cells were exposed to serial dilutions of the liposomal sensor (Figure 4.14d) or AuNCs alone (Figure 4.16b), and cell viability was evaluated using MTS assays. The results indicated that the sensor and the AuNCs exhibited excellent biocompatibility and the cells remained viable. This is in agreement with previous literature, liposomes of this composition are known for their biocompatibility^{309,310} and GSH-AuNCs have also previously been tested and shown to be biocompatible *in vitro* and *in vivo*^{17,142,252}. In conclusion, a biocompatible sensor was synthesised that responds to *S. aureus* growth *in vitro*.

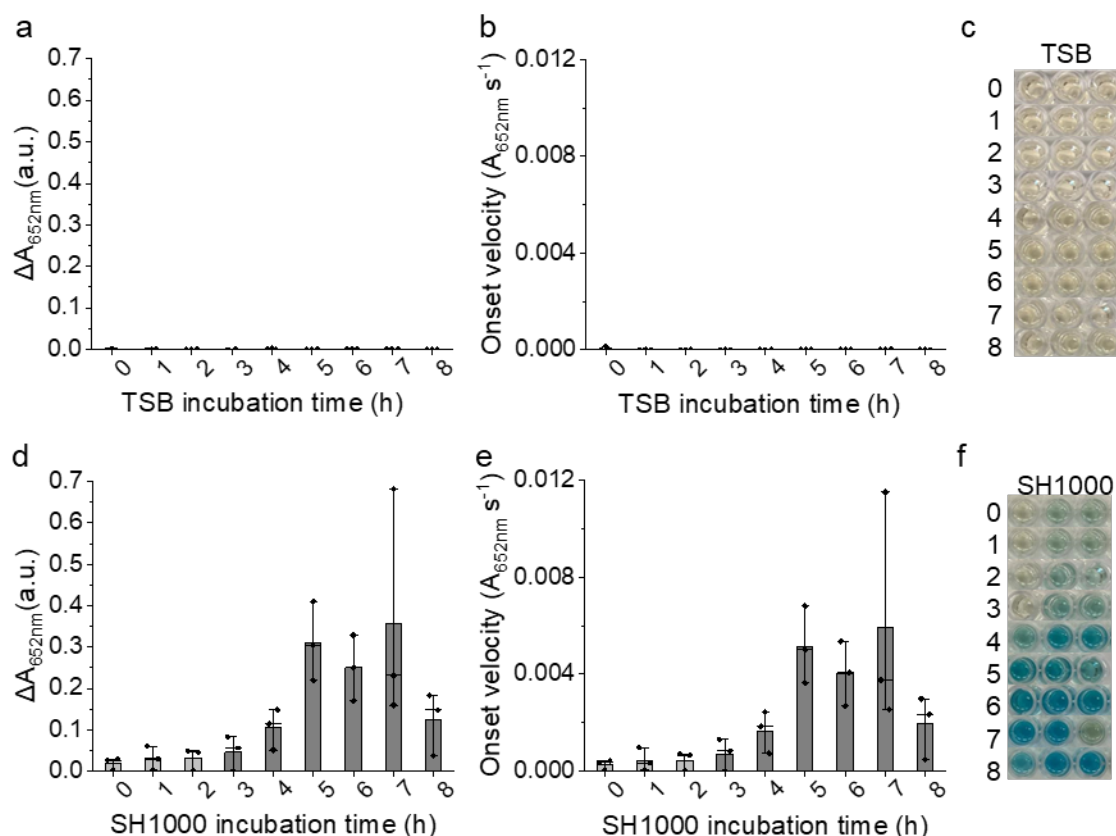


Figure 4.17 Response performance of AuNC-loaded liposomes to toxin by co-incubation with *S. aureus* SH1000. Three more repeats of incubation of AuNC-loaded liposomes with TSB (a-c) or TSB + bacteria (d-f) (N = 3, n = 1).

4.4.8 HA hydrogels as an implant model for the liposomal bacteria sensor

Various hyaluronic acid (HA) hydrogels have been approved for human use as injectable dermal fillers^{311,312}. HA has the advantage of high content of water, tissue-like properties and ease of implantability^{313,314}. Here, a commercial HA hydrogel (Belotero intense) was used as an implant model, which allows for simple physical loading of the liposomal sensor. To test the suitability of this implant system, free AuNCs or AuNC-liposomes were mixed with HA hydrogels and injected into PBS or TSB culture medium, incubated over time, followed by supernatant collection and TMB

assay. These tests were performed to investigate the timescales of retention, diffusion, and release of free AuNCs and AuNC-liposomes out of the HA hydrogels. AuNCs readily diffuse out of the HA hydrogel within the first hour, demonstrating that liberated AuNCs from the liposomal sensor will be able to pass through the gel efficiently. In contrast, AuNC-liposomes were mostly remained within the hydrogel up to 6 h of incubation and the centrifugal filter test confirmed that liposomes largely remained intact and the AuNCs remained encapsulated (Figure 4.18).

The catalytic activity measurements of the supernatants from HA and AuNCs incubation, along with and the reaction photograph, were conducted. The onset velocity was measured to indicate the quantity of AuNCs diffused from the incubation. Remarkably, even after a 1-h incubation, a noticeable blue colour change was observed in the photograph, demonstrating the ease of AuNCs dispersing out of HA. Similarly, the catalytic activity measurements of the supernatants from HA and AuNC-loaded liposomes incubation, along with the reaction photograph, were performed. The onset velocity was measured to indicate the diffusion behaviour of AuNC-loaded liposomes over the incubation time. Interestingly, in the photograph, the blue colour change was observed only after several hours of incubation, highlighting the stability of AuNC-loaded liposomes and successfully controlling their diffusion. Overall, the HA hydrogel system was deemed suitable for the subsequent proof-of-concept tests in a mouse model of acute implant infection, as the liposomes largely remained with the hydrogel for the timescale of the below *in vivo* experiments and the mixture remained readily injectable. A covalent coupling strategy to anchor the sensor to the implant has not yet incorporated, but this will be investigated as future work.

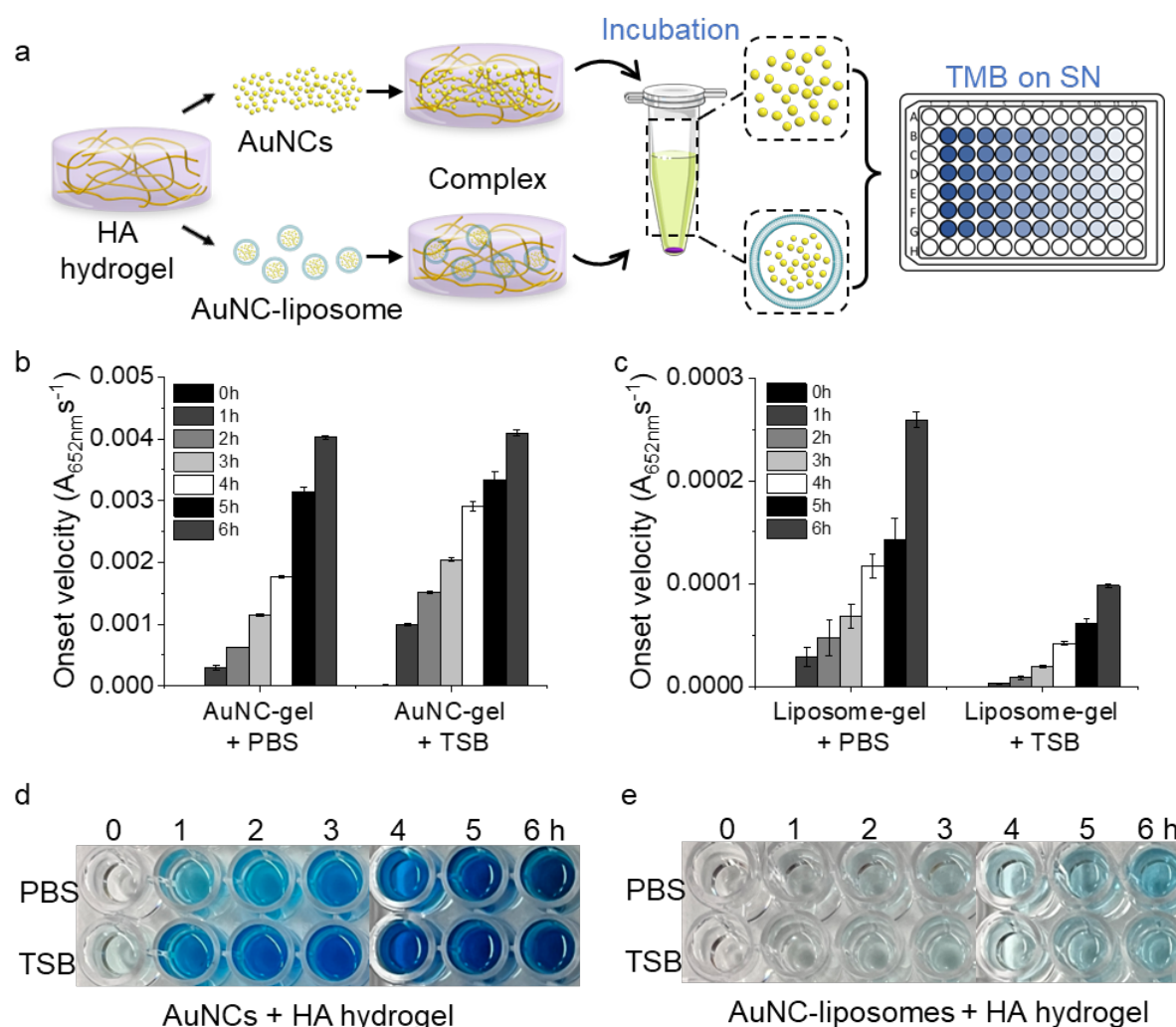


Figure 4.18 The utilisation of HA hydrogel to study the diffusion of AuNCs and AuNC-loaded liposomes during incubation. (a) Schematic of incubation of HA hydrogels with AuNCs and AuNC-loaded liposomes and evaluating release out of the gels. The supernatants, containing the released AuNCs or AuNC-liposomes, were collected after incubation of these mixtures, followed by TMB assay. (b-e) Onset velocity calculated from catalytic activity measurements and photographs of the wells after collecting supernatants from AuNC- or AuNC-liposome-loaded HA hydrogels incubation samples.

Using the experimental setup described above, bacterial contamination was then incorporated into the system. AuNC-liposomes mixed with HA hydrogel were either kept bacteria-free or they were intentionally contaminated with *S. aureus*. The mixture

was again injected into TSB for incubation, supernatant collection, and centrifugal filtration. The filtrates were collected for TMB assay to detect any released AuNCs from the liposomal sensor (Figure 4.19a). Again, the sensor remained intact when incubated in bacteria-free HA hydrogels, whilst AuNCs were released from the sensor and detected by TMB assay for the bacteria-contaminated gels, as early as 1 h after start of the incubation (Figure 4.19b and c, repeats in Figure 4.21). Those repeats confirmed that this sensing system could generate obvious signal after 1-h contamination with controlled low background. Before *in vivo* administration, it was confirmed that bacteria growth was also efficient in the presence of HA hydrogels. Bacteria incubated in TSB with or without HA hydrogel had the same growth curve (Figure 4.19d) and CFU counts were identical over time (Figure 4.20). The growth of SH1000 strain was confirmed by streaking the plate for colony forming unit (CFU) counting from serial dilutions of samples. The SH1000 strain exhibited normal growth in tryptic soy broth (TSB). The CFU measurement of the samples from the incubation of SH1000 with and without HA showed that SH1000 batches in both TSB and HA incubation followed the same growth trend, which was also corresponding to the absorbance measurements. Hence, the HA hydrogel implant model was found to be a suitable system to verify this sensor *in vivo*, as the implant is prone to bacterial infection and growth.

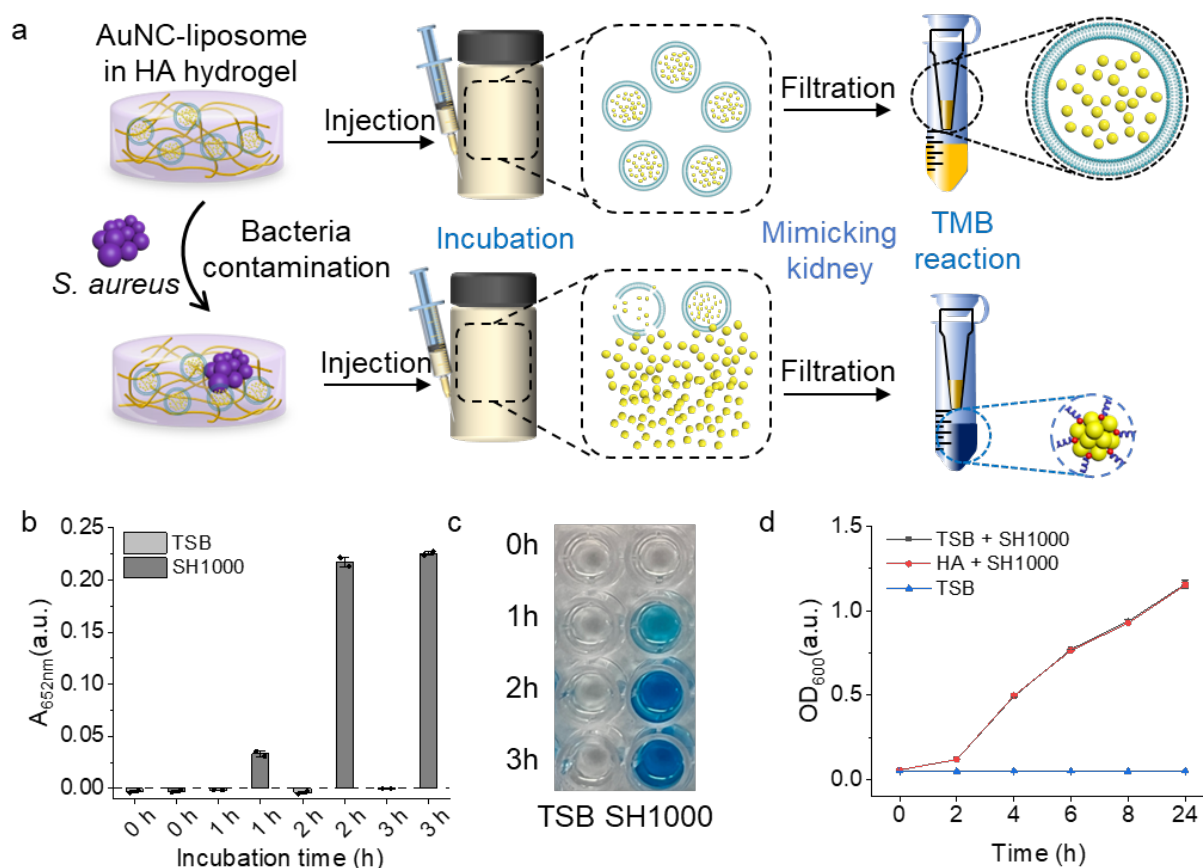


Figure 4.19 AuNC-liposome sensing system enabling a colourimetric readout of the bacterial infection state. (a) Schematic of HA utilisation for the diffusion control of AuNC-loaded liposomes for implant infection and *in vitro* release of AuNCs detection. (b) TMB catalytic activity on the filtrates from HA-liposome mixture incubation with bacteria. The release AuNCs can be filtered through the Amicon device to verify the efficiency of the responsiveness of liposome to bacteria within HA. (c) Photograph of the filtrates reaction with TMB substrate. The blue colour change demonstrated the presence of AuNCs, which indicated the success of AuNC release from liposomes by toxin activation. (d) Absorbance measurement at 600 nm of incubation solution of TSB only, SH1000 in TSB and SH1000 with HA in TSB. The same growth curve for SH1000 incubated with and without HA showed that HA would not inhibit the growth of bacteria.

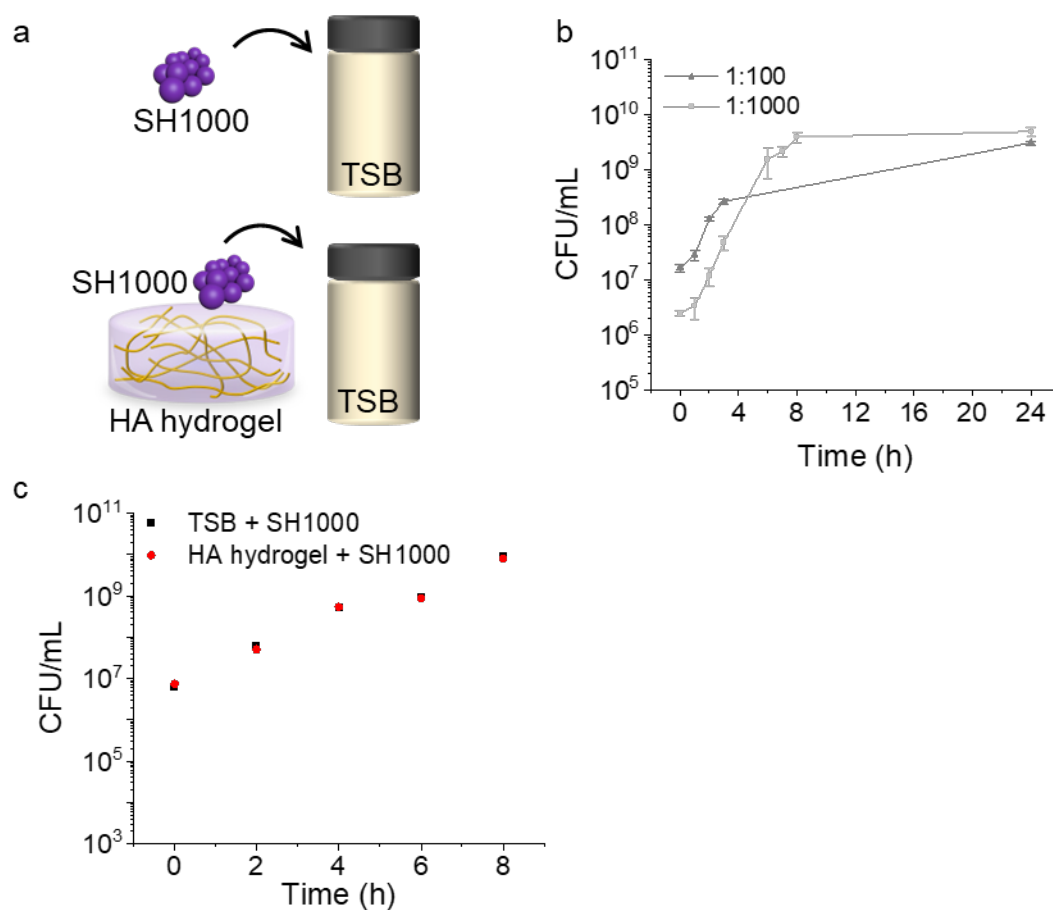


Figure 4.20 Anti-bacterial property measurement of HA. (a) Schematic of incubation of *S. aureus* SH1000 strain with and without HA hydrogel. (b) CFU measurements of the samples from SH1000 incubation with and without HA hydrogel demonstrating the gel had no effect on bacteria growth, corresponding to the absorbance measurement (Figure 4.19d).

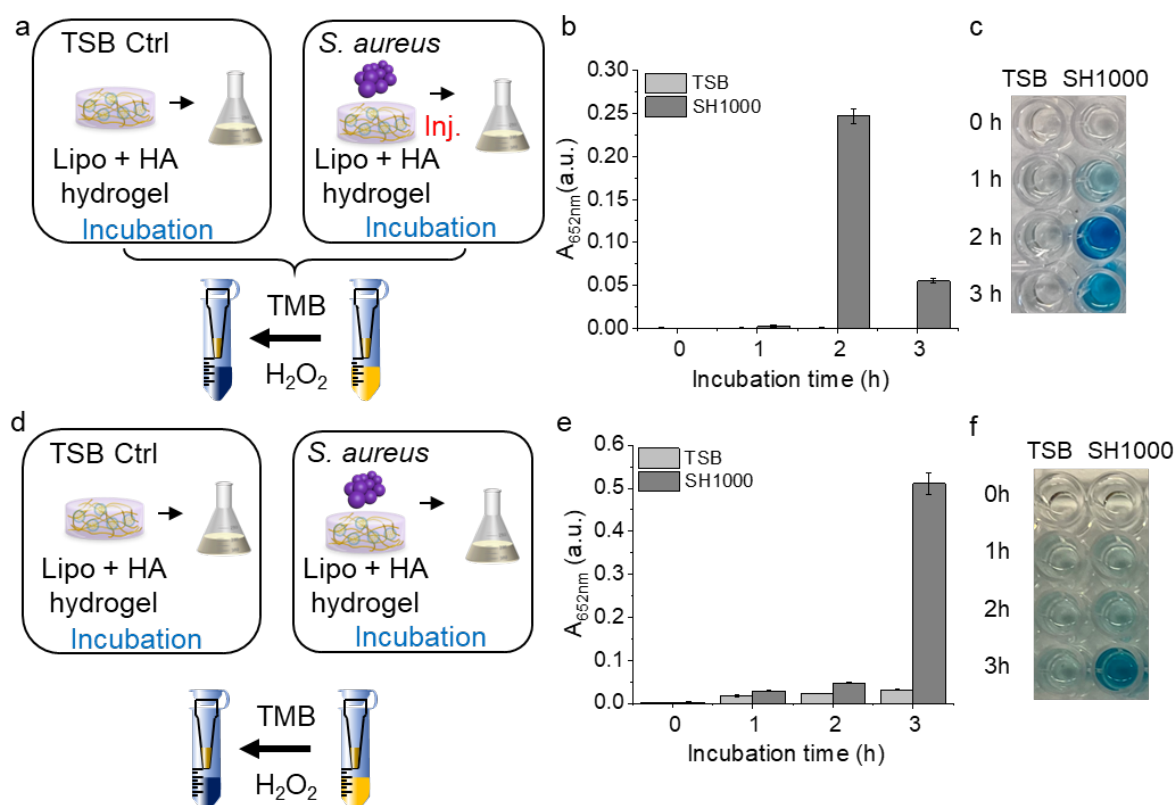


Figure 4.21 The bacteria-triggered release performance of HA mixture complex loaded with AuNC-liposomes. (a) The schematic of the incubation of AuNC-liposome HA complex with TSB and bacteria by injection mimicking. (b) and (c) TMB oxidation catalytic activity assay on the filtration of the collected supernatant and the reaction photograph. There was barely AuNCs detection in samples collected TSB incubation, while the catalytic activity was measured from bacterial incubation even after only 1 h. The photograph showed clear blue colour for the samples from bacterial incubation samples but no change in TSB incubation collections with a good stability. (d) The schematic of the incubation of AuNC-liposome HA complex with TSB and bacteria by mild topping up with TSB. (e) and (f) TMB oxidation assay on the filtration of the collected supernatant and the reaction photograph. The repeat was similar to the injection mimicking. TMB signal was lower in TSB control with not obvious blue colour change in the wells, while bacterial incubation performance demonstrated a high A_{652nm} measurement and reached a high level after 3-h incubation with dark blue colour change observation.

4.4.9 AuNC-liposome biosensor responds to bacteria growth *in vivo* and provides simple urinary readout

GSH-AuNCs at a size of around 2 nm have been extensively studied with regard to biocompatibility, biodistribution, clearance through the kidneys into the urine, and catalytic activity retention in urine¹⁷. This previous work demonstrated rapid elimination of this type of nanoclusters into the urine after i.v. administration using TMB assay to verify their presence. To confirm renal clearance of the bare AuNCs also after i.p. administration, urine samples were collected from mice injected i.p. with AuNCs. The catalytic activity of cleared AuNCs was monitored by TMB oxidation reaction. PBS control injections gave no signal, whilst the urine samples after AuNCs i.p. injection and TMB reaction turned blue indicating successful clearance of AuNCs into the urine (Figure 4.22). The urine sample from 3 h post injection exhibited higher catalytic activity than that from 1 h, after normalisation for different dilution factors (less urine was available for 3 h sample, hence it was tested at a higher dilution than the 1 h sample). A calibration curve of onset velocity of AuNCs in urine was measured for the clearance efficiency of the AuNC clearance at 1 h and 3 h as 6.3% and 2.5%. This data provides insights into the renal clearance dynamics of bare AuNCs in mice^{315–317}. In previous work, the cleared AuNCs in urine were measured with up to 73% clearance efficiency compared to the injected dose after 1 h postinjection by intravenous (i.v.) administration¹⁷. This suggests that AuNCs could be cleared through kidneys after i.p. administration but the clearance after i.p. administration was slower than i.v. administration.

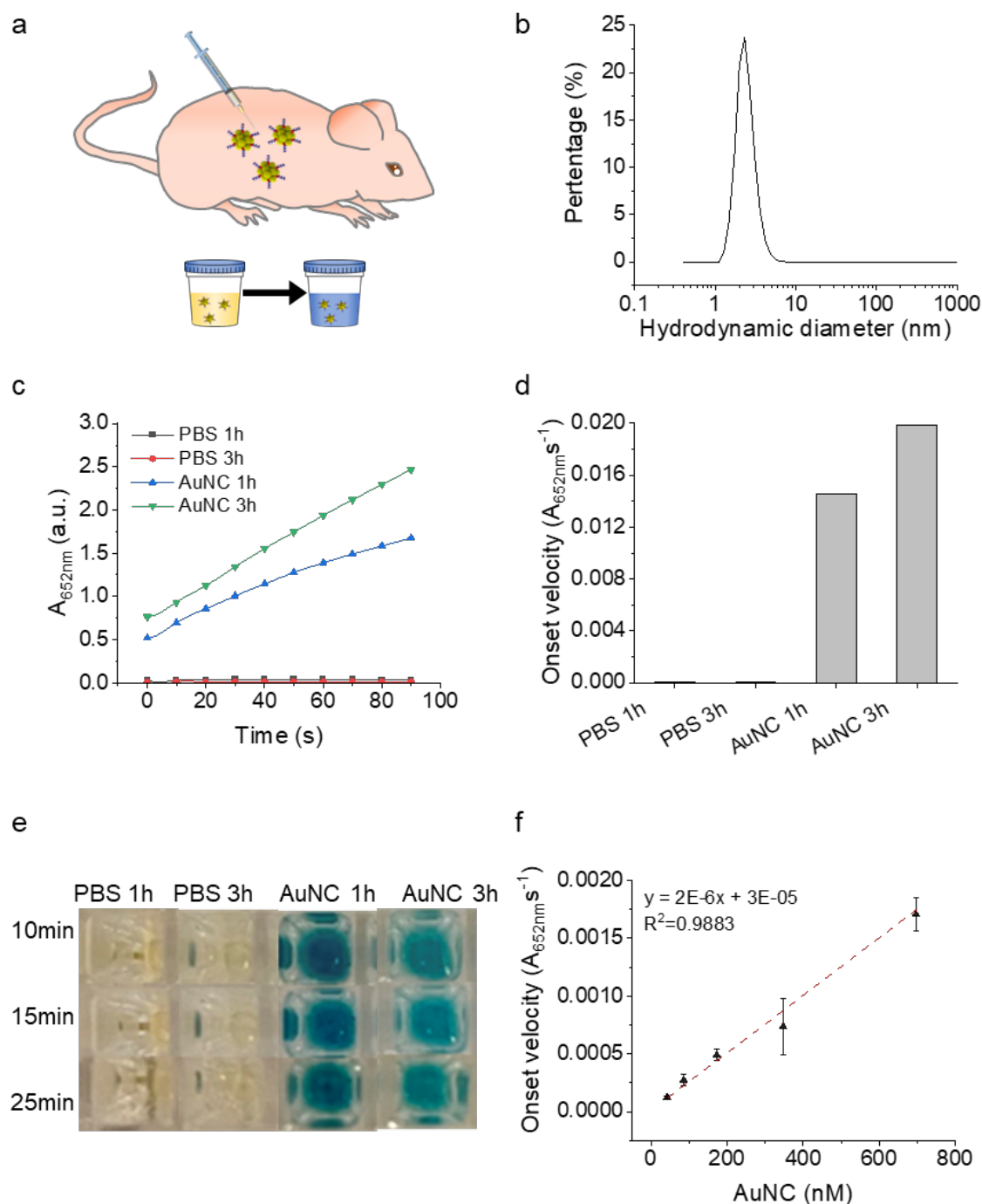


Figure 4.22 The renal clearance of AuNCs *in vivo*. (a) The Schematic of intraperitoneal (i.p.) administration of AuNCs in mice. (b) The number PSD measurement of AuNCs by DLS. This confirmed the hydrodynamic diameter of AuNCs to be ~ 2 nm less than the kidney threshold. (c) The measurement of absorbance over time in the collected urine from PBS control and AuNC-administrated mice by TMB oxidation assay. (d) The normalised onset velocity comparison derived from the measurement of absorbance over time. (e) The photograph of TMB oxidation with collected urine. The blue colour from 3 h post i.p. administration was less obvious than

that from 1 h, which was due to less volume of urine sample and a higher dilution for enough volume. (f) The calibration curve of AuNCs in synthetic urine for clearance calculation.

After confirming above the *in vitro* response of the liposomal biosensor to bacterial infection in HA hydrogels and the successful clearance of AuNC through the kidneys, an acute infection model was employed in mice. *S. aureus* SH1000 strain has previously been used for the study of surgical bacterial implant infection^{318–320}. The implant with biosensor (AuNC-liposome-loaded HA hydrogels) was administered to the model, either with or without bacterial contamination (*S. aureus* SH1000 strain), *via* i.p. route using two groups of six mice each: the healthy control group (HC) and the bacterial infection (BI) group. Urine collection was performed 4 h after the injection (Figure 4.23a). The TMB catalytic activity kinetics assay was measured using 20 μ L of the collected urine. The raw absorbance curves for the TMB reaction increased over time in the bacterial infection (BI) group (pink lines), whereas the healthy control (HC) group did not increase (grey, Figure 4.23b). This confirmed the *in vitro* data, showing that only in the presence of bacterial contamination significant amounts of AuNCs were released from this sensor and cleared into the urine. Comparing the calculated onset velocities showed significantly higher signals from BI versus HC group (Figure 4.23c). In the current setting, only the highest signals were clearly observable as a blue colour by the naked eye. Further optimisation of AuNC catalytic activity, loading amount of AuNCs per liposomes, liposome composition, or timescale for urine collection are all future means to optimise the signal. These results demonstrate that the biosensor, combining catalytic AuNCs and toxin-responsive liposomes, can realise rapid detection of implant infection with a colourimetric readout using a non-invasive method.

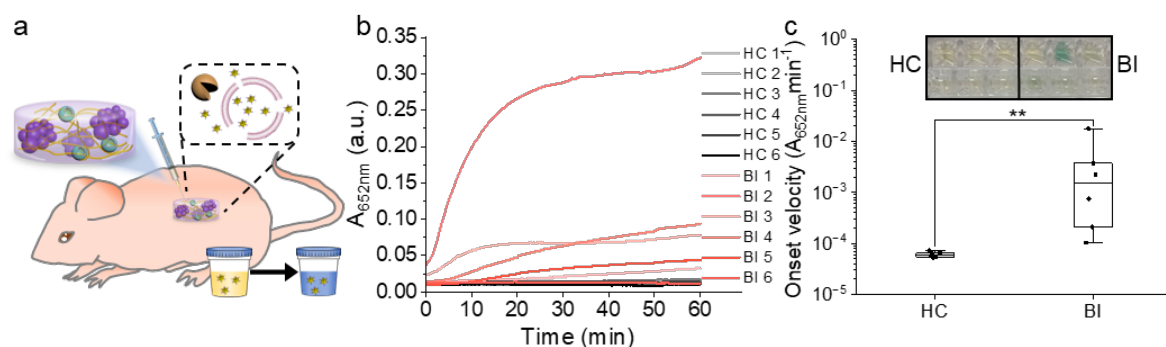


Figure 4.23 AuNC-liposome sensing system enabling a colourimetric readout of the bacterial infection state. (a) Schematic of mouse i.p. administration by implant infection through HA-liposome with bacteria. (b) TMB kinetics reaction measurements on the urine collected from mouse administration of PBS and bacteria included HA-liposomes. HC meant the healthy control mice group and BI meant the bacterial infected mice group (N = 6, n = 1). (c) TMB catalytic activity assay on urine samples collected from healthy control and bacterial infected mice (N = 6, n = 1).

4.5 Conclusion

In this chapter, a biosensor platform was developed as a rapid and sensitive colourimetric method for detecting bacterial infections within a 4-h timeframe. This research focused on the synthesis and optimisation of ultra-small AuNCs with significantly enhanced catalytic activity (33.5-fold higher than AuNCs in previous work¹⁷, with 83-fold lower LoD). The composition of liposomes was varied to develop a biosensing platform with high sensitivity and responsiveness. It demonstrated that the biosensors could respond to toxins secreted by *S. aureus in vitro*. Finally, the biosensor platform was validated in solution, culture, and in hydrogels and this successfully demonstrated the detection of released AuNCs in the urine from mice with a model implant-associated bacterial infection. Overall, this work emphasised the significance of enhancing the catalytic activity of AuNCs and tailoring the liposome compositions to engineer a sensitive biosensor platform for the detection of bacterial infection *in vivo*, in which the catalytic activity of AuNCs can be used as a readout for disease monitoring. This development holds great promise for early detection of implant infection for timely treatment to limit the chance of developing severe complications. The findings in this chapter highlight the potential of this sensing system to be developed into a rapid and effective diagnostic tool for detecting implant infection and potentially also for other scenarios of bacterial infection. For example, the liposomal sensor could be injected locally in any area of suspected bacterial infection and urine could be collected at a defined later timepoint for sensing *via* the catalytic reaction. Further research and refinement of this biosensor platform could lead to its practical application in the clinics for early detection and timely treatment of bacterial infections, especially in resource limited settings.

Chapter 5

Conclusions and future work

5 Conclusions and future work

5.1 Motivation

Timely and accurate diagnosis during the early stages of disease development not only facilitates the application of effective treatments but also significantly enhances the prospects of successful recovery. Many diseases become increasingly challenging to cure as they advance to more severe stages. Early diagnosis is important in mitigating the need for prolonged medical interventions and hospitalisation, resulting in substantial savings on healthcare expenses. Furthermore, it serves as a proactive measure to avoid the exorbitant costs associated with managing diseases at later, more critical stages. Early disease diagnosis serves as a bulwark against the worsening of health conditions, thereby enhancing the overall quality of life for patients. This holds particular significance for chronic illnesses such as cancer, diabetes, heart diseases and infectious diseases. In the case of contagious diseases, early diagnosis is instrumental in promptly isolating affected individuals and curbing the further spread of the illness. Recognising the importance of diagnosing diseases at their early stages is paramount, as it enables the timely and judicious administration of treatments, ultimately leading to better outcomes for patients. Numerous diseases present a formidable challenge in their early stages due to the absence of conspicuous symptoms, rendering early diagnosis a daunting task. This predicament extends to specific cancers, neurological disorders, and infectious diseases. The existing diagnostic arsenal, although valuable, is not without its limitations when it comes to early detection and often demands significant financial investments or intricate technology. For instance, medical imaging techniques may necessitate substantial

exposure to radiation, while the identification of biomarkers calls for highly sensitive detection methodologies. Even for easily transmissible diseases, the pursuit of an economical and sensitive detection method, one that is minimally invasive, remains an arduous undertaking. It is significant to develop a sensitive and low-cost diagnostic method to realise diagnosis of disease at an early stage for a timely and rational treatment for the disease.

This thesis is dedicated to exploring the utilisation of gold nanocluster-based biosensors for disease detection. Through the fine-tuning of AuNCs catalytic performance, coupled with the incorporation of peptide modification, protein conjugation and liposome encapsulation technology, this thesis has successfully demonstrated a range of biosensors tailored for the swift detection of a wide spectrum of diseases, spanning from malaria infections to bacterial infections. The research efforts have yielded highly favourable and promising results.

5.2 Conclusion

5.2.1 Catalytic nanoclusters for biosensing

The sensing platforms developed in this thesis are focused on the catalytic activity of nanoclusters, based on academic literature exploring nanoclusters^{17,142,252}. Chapter 2 explored how the catalytic performance of nanoclusters could be substantially improved by a thorough examination of synthesis reaction conditions. Various synthesis variables were explored, including different reducing agents, diverse metal cores, metal-to-ligand ratios, reaction temperatures, and pH levels, all in the pursuit of identifying the optimal parameters that would result in significantly enhanced catalytic

activity, while still maintaining nanocluster sizes below the kidney filtration threshold for rapid *in vivo* detection. Characterisation methods were employed to assess their properties, such as the ultra-small size and catalytic activity, ultimately paving the way for the identification of the ideal conditions for producing highly catalytic AuNCs. One notable approach involved using a colourimetric method through the TMB oxidation assay to evaluate nanocluster catalytic activity while simultaneously gauging their size using DLS.

Notably, the investigations in Chapter 2 unveiled a fascinating revelation: gold outperforms platinum and their hybrid counterparts in catalytic nanocluster formation. Moreover, the strength of the reducing agents was found to be a crucial determinant of final catalytic performance. Specifically, employing weaker reducing conditions for an extended duration yielded favourable results with significantly heightened catalytic activity. In addition, the ratio of gold to ligands, such as GSH, emerged as a critical factor in controlling nanocluster size and influencing the exposure of the gold surface for catalytic processes. In theory, fewer ligands result in larger sizes but more exposed surface area for heightened catalytic activity, which also aligns with findings in other literature²⁰⁰. A delicate balance that must be struck according to the specific application requirements. Furthermore, the pH level during the synthesis reaction was identified as a significant factor impacting the ultimate outcome. Basic pH synthesis condition could result in a great enhancement of the catalytic property of AuNC. The discoveries made in Chapter 2 establish a robust foundation for the further application of gold nanoclusters and provide essential support for enhancing the sensitivity and specificity of biosensors based on the catalytic AuNCs. This work not only contributes

to the academic understanding of nanocluster catalysis but also has practical implications for the development of advanced biosensing technologies.

5.2.2 Protease-responsive biosensors for malaria infection detection

Malaria infection constitutes a substantial health burden, particularly in developing countries like those in Africa, where limited resources further exacerbate the gravity of infectious disease-related fatalities²⁰⁶. Traditional methods of malaria detection cannot meet the requirement of easy and quick diagnosis, especially because their time-consuming nature and lack of suitability for point-of-care applications. Chapter 3 focused on the development of peptide-templated AuNCs-based biosensors designed to detect severe malaria infections, both *in vitro* and *in vivo*. To achieve this, a comprehensive screening of biomarkers with protease activity was undertaken, with a keen eye on SUB1 and MMP-8 protease as potential candidates intricately involved in malaria infections based on research of those relevant proteases involved in malaria infection^{217,243,246}. Subsequently, the respective substrate sequences for these biomarkers were determined, and the corresponding substrate peptides were successfully synthesised using the solid-phase peptide synthesis technique.

Expanding upon the groundwork laid out in Chapter 2, where investigated the performance of peptide-templated nanoclusters and optimised enzyme-responsive biosensors, here a protease-responsive biosensor was created by assembling catalytically optimised gold nanoclusters templated with targeted peptides onto biocompatible protein carriers. The presence of SUB1 in malaria parasite infections was subsequently validated *in vitro* through an experiment involving the incubation of

the SUB1-responsive biosensor with parasite culture, with the colourimetric TMB oxidation assay serving as the readout on the filtrate after Amicon filtration. The filtrate contained the potentially cleaved and release AuNCs from the biosensor upon the exposure to SUB1 during parasite culture. In addition, the detection of the MMP-8 enzyme, both *in vitro* and *in vivo*, was achieved through the incubation of the MMP-8 responsive biosensor with MMP-8 itself *in vitro*, and the administration of the MMP-8 sensor into a severe malaria-infected mouse model. This breakthrough represents a significant step toward realising effective detection of severe malaria infection. However, it is crucial to note that the *in vivo* experiment conducted here serves as a pilot test for severe malaria infection detection. Further *in vivo* tests will be imperative to validate the effectiveness and reliability of this groundbreaking sensing platform.

5.2.3 AuNC-loaded liposomal sensors for implant bacterial infection detection

Bacterial infection remains a significant global health concern, exacerbated by antibiotic resistance, which poses a formidable challenge in treating infections effectively and promptly, often resulting in fatalities²⁶⁰. In the context of implant-related infections, the complexities arise from the difficulty and high risk associated with obtaining samples for detection. Moreover, the traditional bacterial culture method is time-consuming, and the adoption of advanced techniques is constrained in many developing countries with the requirement of specialised equipment.

Chapter 4 in this thesis focused on the development of an innovative approach involving the application of liposomes encapsulating gold nanoclusters with catalytic properties as a biosensor which could respond to bacterial infection-associated

diseases. This novel method enables both *in vitro* and non-invasive *in vivo* detection of implant-associated bacterial infections. The optimised AuNCs were utilised for the encapsulation within liposomes, forming a liposomal sensor. The optimal concentration of AuNCs for the encapsulation and the composition of the liposomes were further explored to achieve efficient release of AuNCs upon exposure to the toxin supernatant secreted by bacteria. *In vitro* assays were conducted through the incubation of the liposomal sensor with bacterial toxin supernatant. Subsequently, potential gold nanoclusters were separated using Amicon filtration, and a colourimetric reaction were employed to verify the successful release of gold nanoclusters, thereby confirming the efficacy of bacterial infection detection. During these experiments, the adjustment of the lipid composition ratio in liposomes was fine-tuned to maximise the responsiveness to toxins from bacteria and enhance liposome stability. This step is important to ensure that these sensing systems are highly sensitive and selective in detecting bacterial toxins, facilitating efficient *in vitro* bacterial detection.

Furthermore, the incubation of the liposomal sensor with bacterial cultures directly, instead of just the secreted toxin, was performed to mimic *in vivo* conditions for further study. This approach yielded similar results to the incubation with the toxin alone, expanding the scope of research to simulate implant infections *in vitro*. This was achieved through the incubation of the liposomal sensor with HA hydrogel as implants within bacteria, effectively replicating implant conditions using HA hydrogels. The AuNC-loaded liposome sensing system was further assessed in an *in vivo* context by administering this liposomal sensor into a mouse. The detection of bacterial infection was confirmed through a colourimetric method in urine analysis that generated a blue signal, serving as a clear indicator of the disease state. This illustrates a novel, non-

invasive, quick, and straightforward biosensor was successfully developed for detecting implant-associated infections.

This research opens up a promising avenue for biosensing applications of AuNC-loaded liposomes, offering potential benefits in the realms of non-invasive bacterial detection and graft monitoring. It holds the potential to significantly impact the field of biosensing and improve healthcare outcomes.

5.3 Future work

Biosensors based on gold nanoclusters (AuNCs) in this thesis utilise the renal clearance and catalytic activity of gold nanoclusters to achieve a colourimetric readout for rapid and sensitive disease detection. Firstly, further research into AuNCs of different shapes, sizes, and surface modifications is essential to enhance their catalytic performance and biocompatibility. This, in turn, will improve the sensitivity of biosensors based on AuNCs. Optimising sensor construction and refining detection methods are also crucial to attain higher specificity and sensitivity, meeting the requirements for early disease detection.

Secondly, additional *in vivo* experiments need to be conducted to confirm the feasibility of gold nanoparticle-based biosensors in clinical diagnosis and potential commercial applications. For the detection of malaria, further investigation is needed to confirm the identification of severe malaria infections. Given the promising results from the pilot experiment in Chapter 3, more repetitions of *in vivo* malaria infection mouse models should be carried out for system validation. Additionally, there is value in

developing a sensing platform that simultaneously utilises parasite-derived enzymes, such as SUB1, and host MMP-8 for enhanced detection for severe malaria infection detection. This approach can expedite testing and confirmation of severe malaria, ensuring timely, specific, and accurate treatment.

Furthermore, liposomal sensors can be locally administered to suspected areas of bacterial infection, and urine can be collected at a later time point post injection for sensing via a catalytic reaction. Collaboration with researchers in biomedicine, chemistry, materials science, and clinical medicine is essential to explore multiple applications of AuNC-based biosensors, including early diagnosis of cancer, infectious disease and drug delivery. Considerations of sustainable production and safety of AuNCs are crucial to ensure their widespread and sustainable use in the medical field.

In future research, AuNC-based biosensors will continue to drive innovation in the field of disease diagnosis and treatment, contributing positively to global health improvement. Through those ongoing efforts, more work needs to be done to develop more precise, rapid, and reliable diagnostic tools that enhance human health and quality of life.

6 References

1. Forouzanfar, M. H. *et al.* Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. *The Lancet* **386**, (2015).
2. Selmouni, F. *et al.* Tackling cancer burden in low-income and middle-income countries: Morocco as an exemplar. *Lancet Oncol* **19**, e93–e101 (2018).
3. World Health Organization. *Guide to Early Cancer Diagnosis*.
4. Kuwana, M. *et al.* Preliminary laboratory based diagnostic criteria for immune thrombocytopenic purpura: evaluation by multi-center prospective study. *Journal of Thrombosis and Haemostasis* **4**, 1936–1943 (2006).
5. Andryukov, B. G., Besednova, N. N., Romashko, R. V., Zaporozhets, T. S. & Efimov, T. A. Label-Free Biosensors for Laboratory-Based Diagnostics of Infections: Current Achievements and New Trends. *Biosensors 2020, Vol. 10, Page 11* **10**, 11 (2020).
6. Siddig, E. E., Van De Sande, W. W. J. & Fahal, A. H. Actinomycetoma laboratory-based diagnosis: a mini-review. *Trans R Soc Trop Med Hyg* **115**, 355–363 (2021).
7. Busin, V., Wells, B., Kersaudy-Kerhoas, M., Shu, W. & Burgess, S. T. G. Opportunities and challenges for the application of microfluidic technologies in point-of-care veterinary diagnostics. *Mol Cell Probes* **30**, 331–341 (2016).
8. Xiao, M. *et al.* Virus Detection: From State-of-the-Art Laboratories to Smartphone-Based Point-of-Care Testing. *Advanced Science* **9**, 2105904 (2022).

9. Otoo, J. A. & Schlappi, T. S. REASSURED Multiplex Diagnostics: A Critical Review and Forecast. *Biosensors (Basel)* **12**, (2022).
10. Land, K. J., Boeras, D. I., Chen, X. S., Ramsay, A. R. & Peeling, R. W. REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes. *Nature Microbiology* **2018 4:1** **4**, 46–54 (2018).
11. Kubota, R. & Hamachi, I. Protein recognition using synthetic small-molecular binders toward optical protein sensing in vitro and in live cells. *Chem Soc Rev* **44**, 4454–4471 (2015).
12. Jiang, C. *et al.* Antifouling Strategies for Selective in Vitro and in Vivo Sensing. *Chem Rev* **120**, 3852–3889 (2020).
13. Soto, R. J., Hall, J. R., Brown, M. D., Taylor, J. B. & Schoenfish, M. H. In Vivo Chemical Sensors: Role of Biocompatibility on Performance and Utility. *Anal Chem* **89**, 276–299 (2017).
14. Wilson, G. S. & Hu, Y. Enzyme-based biosensors for in vivo measurements. *Chem Rev* **100**, 2693–2704 (2000).
15. Eckert, M. A. *et al.* Novel Molecular and Nanosensors for In Vivo Sensing. *Theranostics* **3**, 583 (2013).
16. Downs, A. M. & Plaxco, K. W. Real-Time, in Vivo Molecular Monitoring Using Electrochemical Aptamer Based Sensors: Opportunities and Challenges. *ACS Sens* **7**, 2823–2832 (2022).
17. Loynachan, C. N. *et al.* Renal clearable catalytic gold nanoclusters for in vivo disease monitoring. *Nat Nanotechnol* **14**, 883–890 (2019).
18. Soo Choi, H. *et al.* Renal clearance of quantum dots. *Nature Biotechnology* **2007 25:10** **25**, 1165–1170 (2007).

19. Soo Choi, H. *et al.* Renal clearance of quantum dots. *Nature Biotechnology* 2007 25:10 **25**, 1165–1170 (2007).
20. Liu, J., Yu, M., Zhou, C. & Zheng, J. Renal clearable inorganic nanoparticles: a new frontier of bionanotechnology. *Materials Today* **16**, 477–486 (2013).
21. Tang, S. *et al.* Tailoring Renal Clearance and Tumor Targeting of Ultrasmall Metal Nanoparticles with Particle Density. *Angewandte Chemie - International Edition* **55**, 16039–16043 (2016).
22. Xu, J. *et al.* In Vivo X-ray Imaging of Transport of Renal Clearable Gold Nanoparticles in the Kidneys. *Angewandte Chemie - International Edition* **56**, 13356–13360 (2017).
23. Liu, S. J. *et al.* Point-of-care non-invasive enzyme-cleavable nanosensors for acute transplant rejection detection. *Biosens Bioelectron* **215**, 114568 (2022).
24. Coghlin, C. & Murray, G. I. Biomarkers of colorectal cancer: Recent advances and future challenges. *Proteomics Clin Appl* **9**, 64–71 (2015).
25. Falanga, A. & Marchetti, M. Hemostatic biomarkers in cancer progression. *Thromb Res* **164**, S54–S61 (2018).
26. Hansson, O. Biomarkers for neurodegenerative diseases. *Nature Medicine* 2021 27:6 **27**, 954–963 (2021).
27. Tissot, A., Danger, R., Claustre, J., Magnan, A. & Brouard, S. Early identification of chronic lung allograft dysfunction: The need of biomarkers. *Front Immunol* **10**, 462150 (2019).
28. Yadav, S. *et al.* Autoantibodies as diagnostic and prognostic cancer biomarker: Detection techniques and approaches. *Biosens Bioelectron* **139**, 111315 (2019).

29. Soleimany, A. P. & Bhatia, S. N. Activity-Based Diagnostics: An Emerging Paradigm for Disease Detection and Monitoring. *Trends Mol Med* **26**, 450–468 (2020).
30. İNCİR, İ., KAPLAN, Ö., BİLGİN, S. & GÖKÇE, İ. Development of a Fluorescent Protein Based FRET Biosensor for Determination of Protease Activity. *Sakarya University Journal of Science* **25**, 1235–1244 (2021).
31. Caminero, A. *et al.* Duodenal bacterial proteolytic activity determines sensitivity to dietary antigen through protease-activated receptor-2. *Nature Communications* 2019 10:1 **10**, 1–14 (2019).
32. Cridge, H., Sullivant, A. M., Wills, R. W. & Lee, A. M. Association between abdominal ultrasound findings, the specific canine pancreatic lipase assay, clinical severity indices, and clinical diagnosis in dogs with pancreatitis. *J Vet Intern Med* **34**, 636–643 (2020).
33. Tardelli, M., Bruschi, F. V. & Trauner, M. The Role of Metabolic Lipases in the Pathogenesis and Management of Liver Disease. *Hepatology* **72**, 1117–1126 (2020).
34. Silbernagel, G. *et al.* LDL triglycerides, hepatic lipase activity, and coronary artery disease: An epidemiologic and Mendelian randomization study. *Atherosclerosis* **282**, 37–44 (2019).
35. Dudani, J. S., Warren, A. D. & Bhatia, S. N. Harnessing Protease Activity to Improve Cancer Care. *Annu Rev Cancer Biol* **2**, 353–376 (2018).
36. Mayerle, J. *et al.* Genetics, Cell Biology, and Pathophysiology of Pancreatitis. *Gastroenterology* **156**, 1951-1968.e1 (2019).

37. Agbowuro, A. A., Huston, W. M., Gamble, A. B. & Tyndall, J. D. A. Proteases and protease inhibitors in infectious diseases. *Medicinal Research Reviews* vol. 38 1295–1331 Preprint at (2018).
38. Cheng, Z. *et al.* Pathogen-secreted proteases activate a novel plant immune pathway. *Nature* 2015 521:7551 **521**, 213–216 (2015).
39. Nomura, D. K., Dix, M. M. & Cravatt, B. F. Activity-based protein profiling for biochemical pathway discovery in cancer. *Nat Rev Cancer* **10**, (2010).
40. Mac, Q. D. *et al.* Urinary detection of early responses to checkpoint blockade and of resistance to it via protease-cleaved antibody-conjugated sensors. *Nature Biomedical Engineering* 2022 6:3 **6**, 310–324 (2022).
41. Beltrame, M. H. *et al.* MBL-associated serine proteases (MASPs) and infectious diseases. *Mol Immunol* **67**, 85–100 (2015).
42. Roy, K. K. Targeting the active sites of malarial proteases for antimalarial drug discovery: approaches, progress and challenges. *Int J Antimicrob Agents* **50**, 287–302 (2017).
43. Liu, T. T., Liu, X. T., Chen, Q. X. & Shi, Y. Lipase Inhibitors for Obesity: A Review. *Biomedicine & Pharmacotherapy* **128**, 110314 (2020).
44. Pirahanchi, Y. & Sharma, S. Biochemistry, Lipase. *StatPearls* (2019).
45. Holmes, R. S., Cox, L. A. & Vandeberg, J. L. Comparative studies of mammalian acid lipases: Evidence for a new gene family in mouse and rat (Lipo). *Comp Biochem Physiol Part D Genomics Proteomics* **5**, 217–226 (2010).
46. Stadler, J. T. & Marsche, G. Obesity-Related Changes in High-Density Lipoprotein Metabolism and Function. *International Journal of Molecular Sciences* 2020, Vol. 21, Page 8985 **21**, 8985 (2020).

47. Ministrini, S. *et al.* Lysosomal Acid Lipase as a Molecular Target of the Very Low Carbohydrate Ketogenic Diet in Morbidly Obese Patients: The Potential Effects on Liver Steatosis and Cardiovascular Risk Factors. *Journal of Clinical Medicine* 2019, Vol. 8, Page 621 **8**, 621 (2019).
48. Benito-Vicente, A. *et al.* Familial hypercholesterolemia: The most frequent cholesterol metabolism disorder caused disease. *International Journal of Molecular Sciences* vol. 19 Preprint at (2018).
49. Chora, J. R. *et al.* Lysosomal acid lipase deficiency: A hidden disease among cohorts of familial hypercholesterolemia? *J Clin Lipidol* **11**, 477-484.e2 (2017).
50. Ding, M., Zhao, W., Song, L. J. & Luan, S. F. Stimuli-responsive nanocarriers for bacterial biofilm treatment. *Rare Metals* **41**, 482–498 (2022).
51. Singh, H. *et al.* Lipase-Responsive Electrospun Theranostic Wound Dressing for Simultaneous Recognition and Treatment of Wound Infection. *ACS Appl Bio Mater* **2**, 2028–2036 (2019).
52. Song, Y. *et al.* Quantitative Detection of Cathepsin B Activity in Neutral pH Buffers Using Gold Microelectrode Arrays: Toward Direct Multiplex Analyses of Extracellular Proteases in Human Serum. *ACS Sens* **6**, 3621–3631 (2021).
53. Ong, I. L. H. & Yang, K. L. Recent developments in protease activity assays and sensors. *Analyst* **142**, 1867–1881 (2017).
54. Turk, B. Targeting proteases: Successes, failures and future prospects. *Nat Rev Drug Discov* **5**, 785–799 (2006).
55. Merrifield, R. B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J Am Chem Soc* **85**, 2149–2154 (1963).

56. Pan, B., Ricci, M. S. & Trout, B. L. Molecular mechanism of acid-catalyzed hydrolysis of peptide bonds using a model compound. *Journal of Physical Chemistry B* **114**, 4389–4399 (2010).
57. Ruiz, M., Arosio, C., Salman, P., Bauer, T. T. & Torres, A. Diagnosis of pneumonia and monitoring of infection eradication. *Drugs* **60**, 1289–1302 (2000).
58. Huang, J. H., Kao, P. N., Adi, V. & Ruoss, S. J. Mycobacterium avium-intracellulare Pulmonary Infection in HIV-Negative Patients Without Preexisting Lung Disease: Diagnostic and Management Limitations. *Chest* **115**, 1033–1040 (1999).
59. Buss, C. G., Dudani, J. S., Akana, R. T. K., Fleming, H. E. & Bhatia, S. N. Protease activity sensors noninvasively classify bacterial infections and antibiotic responses. *EBioMedicine* **38**, 248–256 (2018).
60. Kwong, G. A. *et al.* Mass-encoded synthetic biomarkers for multiplexed urinary monitoring of disease. *Nat Biotechnol* **31**, 63–70 (2013).
61. Kwong, G. A. *et al.* Mathematical framework for activity-based cancer biomarkers. *Proc Natl Acad Sci U S A* (2015) doi:10.1073/pnas.1506925112.
62. Warren, A. D., Kwong, G. A., Wood, D. K., Lin, K. Y. & Bhatia, S. N. Point-of-care diagnostics for noncommunicable diseases using synthetic urinary biomarkers and paper microfluidics. *Proc Natl Acad Sci U S A* (2014) doi:10.1073/pnas.1314651111.
63. Schuerle, S., Dudani, J. S., Christiansen, M. G., Anikeeva, P. & Bhatia, S. N. Magnetically Actuated Protease Sensors for in Vivo Tumor Profiling. *Nano Lett* (2016) doi:10.1021/acs.nanolett.6b02670.
64. Kwon, E. J., Dudani, J. S. & Bhatia, S. N. Ultrasensitive tumour-penetrating nanosensors of protease activity. *Nat Biomed Eng* **1**, (2017).

65. Kwong, G. A. *et al.* Synthetic biomarkers: a twenty-first century path to early cancer detection. *Nat Rev Cancer* **21**, 655–668 (2021).
66. Juurikka, K., Butler, G. S., Salo, T., Nyberg, P. & Åström, P. The Role of MMP8 in Cancer: A Systematic Review. *International Journal of Molecular Sciences* 2019, Vol. 20, Page 4506 **20**, 4506 (2019).
67. Mondal, S., Adhikari, N., Banerjee, S., Amin, S. A. & Jha, T. Matrix metalloproteinase-9 (MMP-9) and its inhibitors in cancer: A minireview. *Eur J Med Chem* **194**, 112260 (2020).
68. Liao, H. Y., Da, C. M., Liao, B. & Zhang, H. H. Roles of matrix metalloproteinase-7 (MMP-7) in cancer. *Clin Biochem* **92**, 9–18 (2021).
69. Quintero-Fabián, S. *et al.* Role of Matrix Metalloproteinases in Angiogenesis and Cancer. *Front Oncol* **9**, 480558 (2019).
70. da Silva-Neto, P. V. *et al.* Matrix Metalloproteinases on Severe COVID-19 Lung Disease Pathogenesis: Cooperative Actions of MMP-8/MMP-2 Axis on Immune Response through HLA-G Shedding and Oxidative Stress. *Biomolecules* **12**, 604 (2022).
71. Keskin, M. *et al.* Active matrix metalloproteinase-8 and interleukin-6 detect periodontal degeneration caused by radiotherapy of head and neck cancer: a pilot study. *Expert Rev Proteomics* **17**, 777–784 (2020).
72. Palomar, Q., Xu, X. X., Selegård, R., Aili, D. & Zhang, Z. Peptide decorated gold nanoparticle/carbon nanotube electrochemical sensor for ultrasensitive detection of matrix metalloproteinase-7. *Sens Actuators B Chem* **325**, 128789 (2020).

73. Dong, H. *et al.* Overexpression of matrix metalloproteinase-9 in breast cancer cell lines remarkably increases the cell malignancy largely via activation of transforming growth factor beta/SMAD signalling. *Cell Prolif* **52**, e12633 (2019).
74. Mehner, C. *et al.* Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. *Oncotarget* (2014) doi:10.18632/oncotarget.1932.
75. Kessenbrock, K., Plaks, V. & Werb, Z. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell* Preprint at <https://doi.org/10.1016/j.cell.2010.03.015> (2010).
76. Farina, A. R. & Mackay, A. R. Gelatinase B/MMP-9 in tumour pathogenesis and progression. *Cancers (Basel)* **6**, 240–296 (2014).
77. Garg, P. *et al.* Matrix metalloproteinase-9 functions as a tumor suppressor in colitis-associated cancer. *Cancer Res* (2010) doi:10.1158/0008-5472.CAN-09-3166.
78. Rahimi, Z. *et al.* Functional Promoter Polymorphisms of MMP-2 C-735T and MMP-9 C-1562T and Their Synergism with MMP-7 A-181G in Multiple Sclerosis. *Immunol Invest* (2016) doi:10.1080/08820139.2016.1180303.
79. Yabluchanskiy, A., Ma, Y., Iyer, R. P., Hall, M. E. & Lindsey, M. L. Matrix metalloproteinase-9: Many shades of function in cardiovascular disease. *Physiology* Preprint at <https://doi.org/10.1152/physiol.00029.2013> (2013).
80. Zeng, Z. S., Shu, W. P., Cohen, A. M. & Guillem, J. G. Matrix metalloproteinase-7 expression in colorectal cancer liver metastases: Evidence for involvement of MMP-7 activation in human cancer metastases. *Clinical Cancer Research* (2002).

81. Sulkala, M. *et al.* Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Arch Oral Biol* **52**, 121–127 (2007).
82. Kiili, M. *et al.* Collagenase-2 (MMP-8) and collagenase-3 (MMP-13) in adult periodontitis: Molecular forms and levels in gingival crevicular fluid and immunolocalisation in gingival tissue. *J Clin Periodontol* **29**, 224–232 (2002).
83. Mäntylä, P. *et al.* Gingival crevicular fluid collagenase-2 (MMP-8) test stick for chair-side monitoring of periodontitis. *J Periodontal Res* **38**, 436–439 (2003).
84. Gutiérrez-Fernández, A. *et al.* Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *The FASEB Journal* **21**, 2580–2591 (2007).
85. Sorsa, T. *et al.* Collagenase-2 (MMP-8) as a point-of-care biomarker in periodontitis and cardiovascular diseases. Therapeutic response to non-antimicrobial properties of tetracyclines. *Pharmacol Res* **63**, 108–113 (2011).
86. Huang, H. Matrix metalloproteinase-9 (MMP-9) as a cancer biomarker and MMP-9 biosensors: Recent advances. *Sensors (Switzerland)* Preprint at <https://doi.org/10.3390/s18103249> (2018).
87. Lin, K. Y., Kwong, G. A., Warren, A. D., Wood, D. K. & Bhatia, S. N. Nanoparticles that sense thrombin activity as synthetic urinary biomarkers of thrombosis. *ACS Nano* (2013) doi:10.1021/nn403550c.
88. Marie Makabenta, J. V *et al.* Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. *Nature Reviews Microbiology* **19**, 23–36 (2021).
89. Valiūnienė, A. & Valinčius, G. Tethered Bilayer Membranes as Impedimetric Biosensors for Detection of Pore-forming Biological Agents. *Curr Opin Electrochem* 101344 (2023).

90. Thet, N. T. *et al.* SPaCE Swab: Point-of-Care Sensor for Simple and Rapid Detection of Acute Wound Infection. *ACS Sens* **5**, 2652–2657 (2020).
91. Li, Y., Liu, G., Wang, X., Hu, J. & Liu, S. Enzyme-Responsive Polymeric Vesicles for Bacterial-Strain-Selective Delivery of Antimicrobial Agents. *Angewandte Chemie International Edition* **55**, 1760–1764 (2016).
92. Kong, C., Neoh, H. M. & Nathan, S. Targeting Staphylococcus aureus toxins: A potential form of anti-virulence therapy. *Toxins (Basel)* **8**, 72 (2016).
93. Tam, K. & Torres, V. J. Staphylococcus aureus Secreted Toxins and Extracellular Enzymes. *Microbiol Spectr* **7**, (2019).
94. Kwon, E. J. *et al.* Porous Silicon Nanoparticle Delivery of Tandem Peptide Anti-Infectives for the Treatment of Pseudomonas aeruginosa Lung Infections Silicon Nanoparticles. *Adv. Mater* **29**, 1701527 (2017).
95. Tonkin, R. L. *et al.* Bacterial Toxin-Triggered Release of Antibiotics from Capsosomes Protects a Fly Model from Lethal Methicillin-Resistant Staphylococcus aureus (MRSA) Infection. *Adv Healthc Mater* **11**, (2022).
96. Gonzalez Gomez, A. & Hosseinidoust, Z. Liposomes for Antibiotic Encapsulation and Delivery. *ACS Infect Dis* **6**, 896–908 (2020).
97. Ferreira, M. *et al.* Liposomes as Antibiotic Delivery Systems: A Promising Nanotechnological Strategy against Antimicrobial Resistance. *Molecules* **26**, 2047 (2021).
98. Zhou, J., Krishnan, N., Jiang, Y., Fang, R. H. & Zhang, L. Nanotechnology for virus treatment. *Nano Today* **36**, 101031 (2021).
99. Potter, M. *et al.* Controlled Dendrimersome Nanoreactor System for Localized Hypochlorite-Induced Killing of Bacteria. *ACS Nano* **14**, 17333–17353 (2020).

100. Thet, N. T. *et al.* Visible, colorimetric dissemination between pathogenic strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* using fluorescent dye containing lipid vesicles. *Biosensors and Bioelectronics* **41**, 538–543 (2013).
101. Tun Thet, N. *et al.* SPaCE Swab: Point-of-Care Sensor for Simple and Rapid Detection of Acute Wound Infection. *ACS Sens* **5**, 2652–2657 (2020).
102. Edwards, K. A. & Baeumner, A. J. Liposomes in analyses. *Talanta* **68**, 1421–1431 (2006).
103. Liu, Q. & Boyd, B. J. Liposomes in biosensors. *Analyst* **138**, 391–409 (2013).
104. Duzgunes, N., Duzgunes, D. & Nir, S. Mechanisms and kinetics of liposome-cell interactions. *Adv Drug Deliv Rev* **40**, 3–18 (1999).
105. Park, J. W. Liposome-based drug delivery in breast cancer treatment. *Breast Cancer Research* **4**, 1–5 (2002).
106. McNamara, K. P. & Rosenzweig, Z. Dye-encapsulating liposomes as fluorescence-based oxygen nanosensors. *Anal Chem* **70**, 4853–4859 (1998).
107. Chaize, B., Colletier, J. P., Winterhalter, M. & Fournier, D. Encapsulation of Enzymes in Liposomes: High Encapsulation Efficiency and Control of Substrate Permeability. *Artif Cells Blood Substit Immobil Biotechnol* **32**, 67–75 (2004).
108. Hwang, S. Y. *et al.* Effects of operating parameters on the efficiency of liposomal encapsulation of enzymes. *Colloids Surf B Biointerfaces* **94**, 296–303 (2012).
109. Kunisawa, J. *et al.* Fusogenic liposome delivers encapsulated nanoparticles for cytosolic controlled gene release. *Journal of Controlled Release* **105**, 344–353 (2005).
110. Couvreur, P., Elias Fattal & Antoine Andremont. Liposome and Nanoparticles in the Treatment of Intracellular Bacterial Infections. *Pharm Res* **8**, 1079–1086 (1991).

111. Camussone, C. M. *et al.* Efficacy of immunization with a recombinant *S. aureus* vaccine formulated with liposomes and ODN-CpG against natural *S. aureus* intramammary infections in heifers and cows. *Res Vet Sci* **145**, 177–187 (2022).
112. Yang, G. *et al.* Degradability and Clearance of Inorganic Nanoparticles for Biomedical Applications. *Advanced Materials* **31**, 1805730 (2019).
113. Amendola, V., Pilot, R., Frasconi, M., Maragò, O. M. & Iatì, M. A. Surface plasmon resonance in gold nanoparticles: a review. *Journal of Physics: Condensed Matter* **29**, 203002 (2017).
114. Bai, X. *et al.* The Basic Properties of Gold Nanoparticles and their Applications in Tumor Diagnosis and Treatment. *International Journal of Molecular Sciences* 2020, Vol. 21, Page 2480 **21**, 2480 (2020).
115. Hlaváček, A. *et al.* Bioconjugates of photon-upconversion nanoparticles for cancer biomarker detection and imaging. *Nature Protocols* 2022 17:4 **17**, 1028–1072 (2022).
116. Jeon, M., Halbert, M. V., Stephen, Z. R. & Zhang, M. Iron Oxide Nanoparticles as T1 Contrast Agents for Magnetic Resonance Imaging: Fundamentals, Challenges, Applications, and Prospectives. *Advanced Materials* **33**, 1906539 (2021).
117. Du, B., Yu, M. & Zheng, J. Transport and interactions of nanoparticles in the kidneys. *Nat Rev Mater* **3**, 358–374 (2018).
118. Poon, W. *et al.* Elimination Pathways of Nanoparticles. *ACS Nano* **13**, 5785–5798 (2019).
119. Ni, D. *et al.* Ceria Nanoparticles Meet Hepatic Ischemia-Reperfusion Injury: The Perfect Imperfection. *Advanced Materials* **31**, 1902956 (2019).

120. Du, B., Yu, M. & Zheng, J. Transport and interactions of nanoparticles in the kidneys. *Nat Rev Mater* **3**, 358–374 (2018).
121. Yu, M. & Zheng, J. Clearance Pathways and Tumor Targeting of Imaging Nanoparticles. *ACS Nano* **9**, 6655–6674 (2015).
122. Wei, H. & Wang, E. Nanomaterials with enzyme-like characteristics (nanozymes): next-generation artificial enzymes. *Chem Soc Rev* **42**, 6060–6093 (2013).
123. Wei, H. *et al.* Chem Soc Rev Chemical Society Reviews Nanomaterials with enzyme-like characteristics (nanozymes): next-generation artificial enzymes (II). *Chem. Soc. Rev* **48**, 965–1230 (2019).
124. Shamsabadi, A., Haghighi, T., Carvalho, S., Frenette, L. C. & Stevens, M. M. The Nanozyme Revolution: Enhancing the Performance of Medical Biosensing Platforms. *Advanced Materials* 2300184 (2023).
125. Das, B. *et al.* Nanozymes in Point-of-Care Diagnosis: An Emerging Futuristic Approach for Biosensing. *Nanomicro Lett* **13**, 1–51 (2021).
126. Zhang, Y., Jin, Y., Cui, H., Yan, X. & Fan, K. Nanozyme-based catalytic theranostics. *RSC Adv* **10**, 10–20 (2020).
127. Wei, H. & Wang, E. Nanomaterials with enzyme-like characteristics (nanozymes): Next-generation artificial enzymes. *Chem Soc Rev* **42**, 6060–6093 (2013).
128. Wu, J. *et al.* Nanomaterials with enzyme-like characteristics (nanozymes): Next-generation artificial enzymes (II). *Chem Soc Rev* **48**, 1004–1076 (2019).
129. Gao, L. *et al.* Intrinsic peroxidase-like activity of ferromagnetic nanoparticles. *Nat Nanotechnol* **2**, 577–583 (2007).

130. Zhang, T. *et al.* Enhanced peroxidase activity and tumour tissue visualization by cobalt-doped magnetoferritin nanoparticles. *Nanotechnology* **28**, 045704 (2016).
131. Xiao, P. *et al.* Highly selective colorimetric determination of catechol based on the aggregation-induced oxidase-mimic activity decrease of δ -MnO₂. *RSC Adv* **10**, 6801–6806 (2020).
132. Wu, Y. H. *et al.* The screening of metal ion inhibitors for glucose oxidase based on the peroxidase-like activity of nano-Fe₃O₄. *RSC Adv* **7**, 47309–47315 (2017).
133. Chen, Q. *et al.* Hemin-functionalized WS₂ nanosheets as highly active peroxidase mimetic for label-free colorimetric detections of H₂O₂ and glucose was reported. Hemin-functionalized WS₂ nanosheets as highly active peroxidase mimetic for label-free colorimetric detections of H₂O₂ and glucose. *J. Name* **00**, 1–3 (2013).
134. Sun, H., Gao, N., Dong, K., Ren, J. & Qu, X. Graphene quantum dots-band-aids used for wound disinfection. *ACS Nano* **8**, 6202–6210 (2014).
135. Yang, W. *et al.* High peroxidase-like activity of iron and nitrogen co-doped carbon dots and its application in immunosorbent assay. *Talanta* **164**, 1–6 (2017).
136. Chen, W. H., Vázquez-González, M., Kozell, A., Cecconello, A. & Willner, I. Cu²⁺-Modified Metal–Organic Framework Nanoparticles: A Peroxidase-Mimicking Nanoenzyme. *Small* **14**, 1703149 (2018).
137. Casteleiro, B. *et al.* Encapsulation of gold nanoclusters by photo-initiated miniemulsion polymerization. *Colloids Surf A Physicochem Eng Asp* **648**, 129410 (2022).

138. Han, L. *et al.* Protein-Stabilized Gadolinium Oxide-Gold Nanoclusters Hybrid for Multimodal Imaging and Drug Delivery. *ACS Appl Mater Interfaces* **9**, 6941–6949 (2017).
139. Fu, C., Ding, C., Sun, X. & Fu, A. Curcumin nanocapsules stabilized by bovine serum albumin-capped gold nanoclusters (BSA-AuNCs) for drug delivery and theranosis. *Materials Science and Engineering: C* **87**, 149–154 (2018).
140. Hong, E. J., Kim, Y. S., Choi, D. G. & Shim, M. S. Cancer-targeted photothermal therapy using aptamer-conjugated gold nanoparticles. *Journal of Industrial and Engineering Chemistry* **67**, 429–436 (2018).
141. Wang, Y., Chen, J. T. & Yan, X. P. Fabrication of transferrin functionalized gold nanoclusters/graphene oxide nanocomposite for turn-on near-infrared fluorescent bioimaging of cancer cells and small animals. *Anal Chem* **85**, 2529–2535 (2013).
142. Bai, Y., Shu, T., Su, L. & Zhang, X. Fluorescent Gold Nanoclusters for Biosensor and Bioimaging Application. *Crystals (Basel)* **10**, 357 (2020).
143. Zheng, Y., Lai, L., Liu, W., Jiang, H. & Wang, X. Recent advances in biomedical applications of fluorescent gold nanoclusters. *Adv Colloid Interface Sci* **242**, 1–16 (2017).
144. Liu, J. M., Chen, J. T. & Yan, X. P. Near infrared fluorescent trypsin stabilized gold nanoclusters as surface plasmon enhanced energy transfer biosensor and in vivo cancer imaging bioprobe. *Anal Chem* **85**, 3238–3245 (2013).
145. Mousavi, H. *et al.* Factors Influencing Catalytic Activity of Size-Specific Triphenylphosphine-Ligated Gold Nanoclusters in the Electrocatalytic Hydrogen Evolution Reaction. *Journal of Physical Chemistry C* **126**, 246–260 (2022).

146. Zhang, A. *et al.* Novel approach to immobilize Au nanoclusters on micro/nanostructured carbonized natural lotus leaf as green catalyst with highly efficient catalytic activity. *Chemical Engineering Journal* **371**, 876–884 (2019).
147. Sun, F., Liang, Y., Jin, L., Shi, J. & Shang, L. Weak Interaction-Tailored Catalytic Interface of Ultrasmall Gold Nanoclusters as Enzyme Mimics for Enhanced Colorimetric Biosensing. *ACS Appl Mater Interfaces* **13**, 58209–58219 (2021).
148. Xu, J. *et al.* Dose Dependencies and Biocompatibility of Renal Clearable Gold Nanoparticles: From Mice to Non-human Primates. *Angewandte Chemie* **130**, 272–277 (2018).
149. Yu, M., Xu, J. & Zheng, J. Renal Clearable Luminescent Gold Nanoparticles: From the Bench to the Clinic. *Angewandte Chemie International Edition* **58**, 4112–4128 (2019).
150. Yu, M. & Zheng, J. Clearance Pathways and Tumor Targeting of Imaging Nanoparticles. *ACS Nano* **9**, 6655–6674 (2015).
151. Xu, J., Peng, C., Yu, M. & Zheng, J. Renal clearable noble metal nanoparticles: photoluminescence, elimination, and biomedical applications. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **9**, e1453 (2017).
152. Zhou, C., Long, M., Qin, Y., Sun, X. & Zheng, J. Luminescent gold nanoparticles with efficient renal clearance. *Angewandte Chemie - International Edition* (2011) doi:10.1002/anie.201007321.
153. Xu, J. *et al.* In Vivo X-ray Imaging of Transport of Renal Clearable Gold Nanoparticles in the Kidneys. *Angewandte Chemie - International Edition* (2017) doi:10.1002/anie.201707819.
154. Ning, X. *et al.* Physiological stability and renal clearance of ultrasmall zwitterionic gold nanoparticles: Ligand length matters. *APL Mater* **5**, (2017).

155. Xu, J. *et al.* Dose Dependencies and Biocompatibility of Renal Clearable Gold Nanoparticles: From Mice to Non-human Primates. *Angewandte Chemie - International Edition* **57**, 266–271 (2018).
156. Luo, Z., Zheng, K. & Xie, J. Engineering ultrasmall water-soluble gold and silver nanoclusters for biomedical applications. *Chemical Communications* (2014) doi:10.1039/c3cc47512c.
157. Mesulam, M. M. Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: A non-carcinogenic blue reaction-product with superior sensitivity for visualizing neural afferents and efferents. *Journal of Histochemistry and Cytochemistry* **26**, 106–117 (1978).
158. Li, M. *et al.* Development and Application of an Efficient Medium for Chromogenic Catalysis of Tetramethylbenzidine with Horseradish Peroxidase. *ACS Omega* **4**, 5459–5470 (2019).
159. Jiang, D. *et al.* Nanozyme: New horizons for responsive biomedical applications. *Chem Soc Rev* **48**, 3683–3704 (2019).
160. Wen, M. *et al.* Artificial Enzyme Catalyzed Cascade Reactions: Antitumor Immunotherapy Reinforced by NIR-II Light. *Angewandte Chemie International Edition* **58**, 17425–17432 (2019).
161. Yao, J. *et al.* A novel biomimetic nanoenzyme based on ferrocene derivative polymer NPs coated with polydopamine. *Talanta* **195**, 265–271 (2019).
162. Jiang, B. *et al.* Standardized assays for determining the catalytic activity and kinetics of peroxidase-like nanozymes. *Nat Protoc* **13**, 1506–1520 (2018).
163. McAteer, M. A. *et al.* In vivo magnetic resonance imaging of acute brain inflammation using microparticles of iron oxide. *Nat Med* **13**, 1253–1258 (2007).

164. Huang, Y., Ran, X., Lin, Y., Ren, J. & Qu, X. Self-assembly of an organic-inorganic hybrid nanoflower as an efficient biomimetic catalyst for self-activated tandem reactions. *Chemical Communications* **51**, 4386–4389 (2015).
165. Gao, L. & Yan, X. Nanozymes: An emerging field bridging nanotechnology and biology. *Sci China Life Sci* **59**, 400–402 (2016).
166. Wijaya, A., Schaffer, S. B., Pallares, I. G. & Hamad-Schifferli, K. Selective release of multiple DNA oligonucleotides from gold nanorods. *ACS Nano* **3**, 80–86 (2009).
167. Emerich, D. F. & Thanos, C. G. The pinpoint promise of nanoparticle-based drug delivery and molecular diagnosis. *Biomol Eng* **23**, 171–184 (2006).
168. Wu, J., Li, S. & Wei, H. Integrated nanozymes: Facile preparation and biomedical applications. *Chemical Communications* **54**, 6520–6530 (2018).
169. Niu, J. *et al.* Photomodulated Nanozyme Used for a Gram-Selective Antimicrobial. *Chemistry of Materials* **30**, 7027–7033 (2018).
170. Chen, Z., Wang, Z., Ren, J. & Qu, X. Enzyme Mimicry for Combating Bacteria and Biofilms. *Acc Chem Res* **51**, 789–799 (2018).
171. Li, W. *et al.* Manganese Dioxide Nanozymes as Responsive Cytoprotective Shells for Individual Living Cell Encapsulation. *Angewandte Chemie - International Edition* **56**, 13661–13665 (2017).
172. Batrakova, E. V. *et al.* A macrophage-nanozyme delivery system for Parkinson's disease. *Bioconjug Chem* **18**, 1498–1506 (2007).
173. Popov, A. L. *et al.* Intracellular Delivery of Antioxidant CeO₂ Nanoparticles via Polyelectrolyte Microcapsules. *ACS Biomater Sci Eng* **4**, 2453–2462 (2018).

174. De La Rica, R. & Stevens, M. M. Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. *Nat Nanotechnol* **7**, 821–824 (2012).
175. Gormley, A. J., Chapman, R. & Stevens, M. M. Polymerization amplified detection for nanoparticle-based biosensing. *Nano Lett* **14**, 6368–6373 (2014).
176. Kumar Sharma, T. *et al.* Aptamer-mediated “turn-off/turn-on” nanozyme activity of gold nanoparticles for kanamycin detection. *Chemical Communications* **50**, 15856–15859 (2014).
177. Qiu, H. *et al.* Nanozyme as Artificial Receptor with Multiple Readouts for Pattern Recognition. *Anal Chem* **90**, 11775–11779 (2018).
178. Tian, L. *et al.* Planar intercalated copper (II) complex molecule as small molecule enzyme mimic combined with Fe₃O₄ nanozyme for bienzyme synergistic catalysis applied to the microRNA biosensor. *Biosens Bioelectron* **110**, 110–117 (2018).
179. Brangel, P. *et al.* A Serological Point-of-Care Test for the Detection of IgG Antibodies against Ebola Virus in Human Survivors. *ACS Nano* **12**, 63–73 (2018).
180. D. Howes, P., Rana, S. & M. Stevens, M. Plasmonic nanomaterials for biodiagnostics. *Chem Soc Rev* **43**, 3835–3853 (2014).
181. Yu, M. & Zheng, J. Clearance Pathways and Tumor Targeting of Imaging Nanoparticles. *ACS Nano* **9**, 6655–6674 (2015).
182. Du, B., Yu, M. & Zheng, J. Transport and interactions of nanoparticles in the kidneys. *Nat Rev Mater* **3**, 358–374 (2018).
183. Zhang, X. D. *et al.* In vivo renal clearance, biodistribution, toxicity of gold nanoclusters. *Biomaterials* **33**, 4628–4638 (2012).

184. Ning, X. *et al.* Physiological stability and renal clearance of ultrasmall zwitterionic gold nanoparticles: Ligand length matters. *APL Mater* **5**, 53406 (2017).
185. Niihori, Y. *et al.* Perspective: Exchange reactions in thiolate-protected metal clusters. *APL Mater* **5**, (2017).
186. Cao, G. J., Jiang, X., Zhang, H., Croley, T. R. & Yin, J. J. Mimicking horseradish peroxidase and oxidase using ruthenium nanomaterials. *RSC Adv* **7**, 52210–52217 (2017).
187. Chen, Q., Gao, R. & Jia, L. Enhancement of the peroxidase-like activity of aptamers modified gold nanoclusters by bacteria for colorimetric detection of *Salmonella typhimurium*. *Talanta* **221**, 121476 (2021).
188. Fan, L. *et al.* Green synthesis of stable platinum nanoclusters with enhanced peroxidase-like activity for sensitive detection of glucose and glutathione. *Microchemical Journal* **166**, 106202 (2021).
189. He, W. *et al.* Intrinsic catalytic activity of Au nanoparticles with respect to hydrogen peroxide decomposition and superoxide scavenging. *Biomaterials* **34**, 765–773 (2013).
190. Peng, C. *et al.* Targeting orthotopic gliomas with renal-clearable luminescent gold nanoparticles. *Nano Res* **10**, 1366–1376 (2017).
191. Santhana, A., Kumar, K. & Tseng, W.-L. Perspective on recent developments of near infrared-emitting gold nanoclusters: applications in sensing and bio-imaging. **12**, 1809 (2020).
192. Zheng, X. *et al.* Improved sensitivity of gold nanoclusters toward trypsin under synergistic adsorption of CdTe quantum dots. *Microchemical Journal* **187**, 108457 (2023).

193. Zhang, J. *et al.* Ultrasmall Au and Ag Nanoclusters for Biomedical Applications: A Review. **8**, 1019 (2020).
194. Kumar Kailasa, S., Borse, S., Reddy Koduru, J. & Murthy, Z. V. P. Biomolecules as promising ligands in the synthesis of metal nanoclusters: Sensing, bioimaging and catalytic applications. *Trends in Environmental Analytical Chemistry* **32**, 140 (2021).
195. Matus, M. F. & Häkkinen, H. Understanding ligand-protected noble metal nanoclusters at work. *Nature Reviews Materials* 2023 8:6 **8**, 372–389 (2023).
196. Zhang, Y. *et al.* Ultrasmall Au nanoclusters for biomedical and biosensing applications: A mini-review. *Talanta* **200**, 432–442 (2019).
197. Cook, A. W. & Hayton, T. W. Case Studies in Nanocluster Synthesis and Characterization: Challenges and Opportunities. *Acc Chem Res* **51**, 2456–2464 (2018).
198. Yuan, X. *et al.* Synthesis of highly fluorescent metal (Ag, Au, Pt, and Cu) nanoclusters by electrostatically induced reversible phase transfer. *ACS Nano* **5**, 8800–8808 (2011).
199. Abarghoei, S., Fakhri, N., Borghei, Y. S., Hosseini, M. & Ganjali, M. R. A colorimetric paper sensor for citrate as biomarker for early stage detection of prostate cancer based on peroxidase-like activity of cysteine-capped gold nanoclusters. *Spectrochim Acta A Mol Biomol Spectrosc* **210**, 251–259 (2019).
200. Zhao, R.-X. *et al.* Glutathione stabilized green-emission gold nanoclusters for selective detection of cobalt ion. *Spectrochim Acta A Mol Biomol Spectrosc* **154**, 119628 (2021).

201. Zhao, G., Tong, L., Cao, P., Nitz, M. & Winnik, M. A. Functional PEG-PAMAM-tetraphosphonate capped NaLnF₄ nanoparticles and their colloidal stability in phosphate buffer. *Langmuir* **30**, 6980–6989 (2014).
202. Mousavi, H., Sharma, S. K., Golovko, V., Shearer, C. J. & Metha, G. F. Methanol Tolerant Oxygen Reduction Reaction Electrocatalysis using Size-Specific Triphenylphosphine-Ligated Gold Nanoclusters. *ChemNanoMat* **8**, e202200122 (2022).
203. Zhang, X. D. *et al.* In vivo renal clearance, biodistribution, toxicity of gold nanoclusters. *Biomaterials* **33**, 4628–4638 (2012).
204. Kang, X. & Zhu, M. Transformation of Atomically Precise Nanoclusters by Ligand-Exchange. *Chemistry of Materials* **31**, 9939–9969 (2019).
205. Wang, Y. & Bürgi, T. Ligand exchange reactions on thiolate-protected gold nanoclusters. *Nanoscale Adv* **3**, 2710–2727 (2021).
206. Health Organization. WHO. *World Malaria Report 2022*. (2022).
207. Soulard, V. *et al.* Plasmodium falciparum full life cycle and Plasmodium ovale liver stages in humanized mice. *Nature Communications* **2015 6:1 6**, 1–9 (2015).
208. Talapko, J., Škrlec, I., Alebić, T., Jukić, M. & Včev, A. Malaria: The Past and the Present. *Microorganisms* **2019, Vol. 7, Page 179 7**, 179 (2019).
209. Josling, G. A. & Llinás, M. Sexual development in Plasmodium parasites: knowing when it's time to commit. *Nature Reviews Microbiology* **2015 13:9 13**, 573–587 (2015).
210. Howick, V. M. *et al.* The malaria cell atlas: Single parasite transcriptomes across the complete Plasmodium life cycle. *Science (1979)* **365**, (2019).
211. Health Organization, W. Management of severe malaria – A practical handbook. Third edition. (2012).

212. Knackstedt, S. L. *et al.* Neutrophil extracellular traps drive inflammatory pathogenesis in malaria. *Sci Immunol* **4**, 336 (2019).
213. Katsoulis, O., Georgiadou, A. & Cunningham, A. J. Immunopathology of Acute Kidney Injury in Severe Malaria. *Front Immunol* **12**, 651739 (2021).
214. Yeo, T. W. *et al.* Glycocalyx Breakdown Is Associated With Severe Disease and Fatal Outcome in Plasmodium falciparum Malaria. *Clin Infect Dis* **69**, 1712 (2019).
215. Georgiadou, A. & Cunningham, A. J. Shedding of the Vascular Endothelial Glycocalyx: A Common Pathway to Severe Malaria? *Clinical Infectious Diseases* **69**, 1721–1723 (2019).
216. Lee, H. J. *et al.* Integrated pathogen load and dual transcriptome analysis of systemic host-pathogen interactions in severe malaria. *Sci Transl Med* **10**, 3619 (2018).
217. Georgiadou, A. *et al.* Localised release of matrix metalloproteinase 8 in fatal cerebral malaria. *Clin Transl Immunology* **10**, e1263 (2021).
218. Organization, W. H. Basic malaria microscopy: Part I. Learner's guide. *Basic malaria microscopy: Part I. Learner's guide.* (2010).
219. Kettelhut, M. M., Chiodini, P. L., Edwards, H. & Moody, A. External quality assessment schemes raise standards: evidence from the UKNEQAS parasitology subschemes. *J Clin Pathol* **56**, 927–932 (2003).
220. Cunningham, J. *et al.* A review of the WHO malaria rapid diagnostic test product testing programme (2008-2018): Performance, procurement and policy. *Malar J* **18**, 1–15 (2019).

221. Leski, T. A. *et al.* Use of real-time multiplex PCR, malaria rapid diagnostic test and microscopy to investigate the prevalence of Plasmodium species among febrile hospital patients in Sierra Leone. *Malar J* **19**, 1–8 (2020).
222. Goh, B. *et al.* The application of spectroscopy techniques for diagnosis of malaria parasites and arboviruses and surveillance of mosquito vectors: A systematic review and critical appraisal of evidence. *PLoS Negl Trop Dis* **15**, e0009218 (2021).
223. Kenangalem, E. *et al.* Malaria morbidity and mortality following introduction of a universal policy of artemisinin-based treatment for malaria in Papua, Indonesia: A longitudinal surveillance study. *PLoS Med* **16**, e1002815 (2019).
224. Nureye, D. & Assefa, S. Old and Recent Advances in Life Cycle, Pathogenesis, Diagnosis, Prevention, and Treatment of Malaria including Perspectives in Ethiopia. *Scientific World Journal* **2020**, (2020).
225. Hanboonkunupakarn, B. & White, N. J. Advances and roadblocks in the treatment of malaria. *Br J Clin Pharmacol* **88**, 374–382 (2022).
226. Alven, S. & Aderibigbe, B. Combination Therapy Strategies for the Treatment of Malaria. *Molecules* 2019, Vol. 24, Page 3601 **24**, 3601 (2019).
227. Cock, I. E., Selesho, M. I. & van Vuuren, S. F. A review of the traditional use of southern African medicinal plants for the treatment of malaria. *J Ethnopharmacol* **245**, 112176 (2019).
228. Miller, L. H., Ackerman, H. C., Su, X. Z. & Wellems, T. E. Malaria biology and disease pathogenesis: insights for new treatments. *Nat Med* **19**, 156 (2013).
229. Gullingsrud, J. *et al.* High-Throughput Screening Platform Identifies Small Molecules That Prevent Sequestration of Plasmodium falciparum–Infected Erythrocytes. *J Infect Dis* **211**, 1134 (2015).

230. Leitgeb, A. M. *et al.* Inhibition of merozoite invasion and transient de-sequestration by sevuparin in humans with *Plasmodium falciparum* malaria. *PLoS One* **12**, e0188754 (2017).
231. Patra, S. *et al.* Polymeric Nanoparticle Based Diagnosis and Nanomedicine for Treatment and Development of Vaccines for Cerebral Malaria: A Review on Recent Advancement. *ACS Appl Bio Mater* **4**, 7342–7365 (2021).
232. Waknine-Grinberg, J. H. *et al.* Glucocorticosteroids in Nano-Sterically Stabilized Liposomes Are Efficacious for Elimination of the Acute Symptoms of Experimental Cerebral Malaria. *PLoS One* **8**, e72722 (2013).
233. Nate, Z., Gill, A. A. S., Chauhan, R. & Karpoormath, R. Recent progress in electrochemical sensors for detection and quantification of malaria. *Anal Biochem* **643**, 114592 (2022).
234. Dutta, G. Electrochemical biosensors for rapid detection of malaria. *Mater Sci Energy Technol* **3**, 150–158 (2020).
235. Krampa, F. D., Aniweh, Y., Kanyong, P. & Awandare, G. A. Recent Advances in the Development of Biosensors for Malaria Diagnosis. *Sensors 2020, Vol. 20, Page 799* **20**, 799 (2020).
236. Foko, L. P. K. *et al.* The spectrum of clinical biomarkers in severe malaria and new avenues for exploration. *Virulence* **13**, 634–653 (2022).
237. Minopoli, A. *et al.* Ultrasensitive antibody-aptamer plasmonic biosensor for malaria biomarker detection in whole blood. *Nature Communications* **2020 11:1** **11**, 1–10 (2020).
238. Jain, P., Chakma, B., Patra, S. & Goswami, P. Potential biomarkers and their applications for rapid and reliable detection of malaria. *Biomed Res Int* **2014**, (2014).

239. Sahu, P. K. *et al.* Pathogenesis of cerebral malaria: New diagnostic tools, biomarkers, and therapeutic approaches. *Front Cell Infect Microbiol* **5**, 165630 (2015).
240. De Monerri, N. C. S. *et al.* Global identification of multiple substrates for *Plasmodium falciparum* SUB1, an essential malarial processing protease. *Infect Immun* **79**, 1086–1097 (2011).
241. Tawk, L. *et al.* A key role for *Plasmodium* subtilisin-like SUB1 protease in egress of malaria parasites from host hepatocytes. *Journal of Biological Chemistry* **288**, 33336–33346 (2013).
242. Thomas, J. A. *et al.* A protease cascade regulates release of the human malaria parasite *Plasmodium falciparum* from host red blood cells. *Nature Microbiology* **2018 3:4 3**, 447–455 (2018).
243. Withers-Martinez, C. *et al.* The malaria parasite egress protease SUB1 is a calcium-dependent redox switch subtilisin. *Nat Commun* **5**, (2014).
244. Dietmann, A. *et al.* Matrix Metalloproteinases and Their Tissue Inhibitors (TIMPs) in *Plasmodium falciparum* Malaria: Serum Levels of TIMP-1 Are Associated with Disease Severity. *J Infect Dis* **197**, 1614–1620 (2008).
245. Bruschi, F. & Pinto, B. The Significance of Matrix Metalloproteinases in Parasitic Infections Involving the Central Nervous System. *Pathogens* **2013, Vol. 2, Pages 105-129 2**, 105–129 (2013).
246. Withers-Martinez, C. *et al.* *Plasmodium* subtilisin-like protease 1 (SUB1): Insights into the active-site structure, specificity and function of a pan-malaria drug target. *Int J Parasitol* **42**, 597–612 (2012).

247. Kukreja, M. *et al.* High-Throughput Multiplexed Peptide-Centric Profiling Illustrates Both Substrate Cleavage Redundancy and Specificity in the MMP Family. *Chem Biol* **22**, 1122–1133 (2015).
248. Moll, K., Kaneko, A., Scherf, A. & Wahlgren, M. *METHODS IN MALARIA RESEARCH Sixth Edition.* (2013).
249. Dorn, A., Stoffel, R., Matile, H., Bubendorf, A. & Ridley, R. G. Malarial haemozoin/ β -haematin supports haem polymerization in the absence of protein. *Nature* 1995 374:6519 **374**, 269–271 (1995).
250. Lambros, C. & Vanderberg, J. P. *Synchronization of Plasmodium Falciparum Erythrocytic Stages in Culture.* Source: *The Journal of Parasitology* vol. 65 <https://www.jstor.org/stable/3280287?seq=1&cid=pdf-> (1979).
251. Taylor, H. M. *et al.* The malaria parasite cyclic GMP-dependent protein kinase plays a central role in blood-stage schizogony. *Eukaryot Cell* **9**, 37–45 (2010).
252. Van De Looij, S. M. *et al.* Gold Nanoclusters: Imaging, Therapy, and Theranostic Roles in Biomedical Applications. *Bioconjug Chem* **33**, 4–23 (2021).
253. Kim, Y. & Jo, K. Neutravidin coated surfaces for single DNA molecule analysis. *Chemical Communications* **47**, 6248–6250 (2011).
254. Jain, A., Barve, A., Zhao, Z., Jin, W. & Cheng, K. Comparison of Avidin, Neutravidin, and Streptavidin as Nanocarriers for Efficient siRNA Delivery. *Mol Pharm* **14**, 1517–1527 (2017).
255. Polimeni, M. & Prato, M. Host matrix metalloproteinases in cerebral malaria: new kids on the block against blood–brain barrier integrity? *Fluids and Barriers of the CNS* 2014 11:1 **11**, 1–24 (2014).
256. Georgiadou, A. *et al.* Localised release of matrix metalloproteinase 8 in fatal cerebral malaria. *Clin Transl Immunology* **10**, (2021).

257. Lee, H. J. *et al.* Integrated pathogen load and dual transcriptome analysis of systemic host-pathogen interactions in severe malaria. *Sci Transl Med* **10**, 3619 (2018).
258. Georgiadou, A. *et al.* Comparative transcriptomic analysis reveals translationally relevant processes in mouse models of malaria. *Elife* **11**, e70763 (2022).
259. Howden, B. P. *et al.* Staphylococcus aureus host interactions and adaptation. *Nature Reviews Microbiology* 2023 21:6 **21**, 380–395 (2023).
260. Murray, C. J. L. *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet* **399**, 629–655 (2022).
261. Wang, J. *et al.* Magneto-Based Synergetic Therapy for Implant-Associated Infections via Biofilm Disruption and Innate Immunity Regulation. *Advanced Science* **8**, 2004010 (2021).
262. Hassan, R., El-Gilany, A. H., Abd elaal, A. M., El-Mashad, N. & Azim, D. A. An overview of healthcare-associated infections in a tertiary care hospital in Egypt. *Infection Prevention in Practice* **2**, 100059 (2020).
263. Li, Y. *et al.* Near-Infrared Light Triggered Phototherapy and Immunotherapy for Elimination of Methicillin-Resistant Staphylococcus aureus Biofilm Infection on Bone Implant. *ACS Nano* **14**, 8157–8170 (2020).
264. Yang, C. *et al.* Inorganic nanosheets facilitate humoral immunity against medical implant infections by modulating immune co-stimulatory pathways. *Nature Communications* 2022 13:1 **13**, 1–16 (2022).
265. Frieri, M., Kumar, K. & Boutin, A. Antibiotic resistance. *J Infect Public Health* **10**, 369–378 (2017).
266. Macgowan, A. & Macnaughton, E. *Antibiotic Resistance*.

267. Holm Jakobsen, T. *et al.* Sampling challenges in diagnosis of chronic bacterial infections. *J Med Microbiol* **70**, 1302 (2021).
268. Zhang, J. *et al.* High-throughput cultivation and identification of bacteria from the plant root microbiota. *Nature Protocols* 2021 16:2 **16**, 988–1012 (2021).
269. Nagy, E., Boyanova, L. & Justesen, U. S. How to isolate, identify and determine antimicrobial susceptibility of anaerobic bacteria in routine laboratories. *Clin Microbiol Infect* **24**, 1139 (2018).
270. Ghorbanpoor M, Seyfiabad shapouri M, Moatamedi H, Jamshidian M & Gooraninejad S. Comparison of PCR and bacterial culture methods for diagnosis of dairy cattle's subclinical mastitis caused by *Staphylococcus aureus*. *J Vet Res* **62**, 87–91 (2007).
271. Nagaraj, S., Ramlal, S., Sripathy, M. H. & Batra, H. V. Development and evaluation of a novel combinatorial selective enrichment and multiplex PCR technique for molecular detection of major virulence-associated genes of enterotoxigenic *Staphylococcus aureus* in food samples. *J Appl Microbiol* **116**, 435–446 (2014).
272. Jeyasekaran, G. *et al.* Simultaneous detection of *Staphylococcus aureus* enterotoxin C-producing strains from clinical and environmental samples by multiplex PCR assay. *Ann Microbiol* **61**, 585–590 (2011).
273. Warren, D. K., Liao, R. S., Merz, L. R., Eveland, M. & Dunne, W. M. Detection of methicillin-resistant *Staphylococcus aureus* directly from nasal swab specimens by a real-time PCR assay. *J Clin Microbiol* **42**, 5578–5581 (2004).
274. Hein, I. *et al.* Comparison of Different Approaches to Quantify *Staphylococcus aureus* Cells by Real-Time Quantitative PCR and Application of This Technique for Examination of Cheese. *Appl Environ Microbiol* **67**, 3122–3126 (2001).

275. Poli, M. A., Rivera, V. R. & Neal, D. Sensitive and specific colorimetric ELISAs for *Staphylococcus aureus* enterotoxins A and B in urine and buffer. *Toxicon* **40**, 1723–1726 (2022).
276. Kuang, H. *et al.* Monoclonal antibody-based sandwich ELISA for the detection of staphylococcal enterotoxin A. *Int J Environ Res Public Health* **10**, 1598–1608 (2013).
277. Schellenberg, J., Smoragiewicz, W. & Karska-Wysocki, B. A rapid method combining immunofluorescence and flow cytometry for improved understanding of competitive interactions between lactic acid bacteria (LAB) and methicillin-resistant *S. aureus* (MRSA) in mixed culture. *J Microbiol Methods* **65**, 1–9 (2006).
278. Sospedra, I., Soler, C., Mañes, J. & Soriano, J. M. Rapid whole protein quantitation of staphylococcal enterotoxins A and B by liquid chromatography/mass spectrometry. *J Chromatogr A* **1238**, 54–59 (2012).
279. Harris L. G. *et al.* Rapid identification of staphylococci from prosthetic joint infections using MALDI-TOF mass-spectrometry. *Int J Artif Organs* **33**, 568–574 (2010).
280. Foster, T. J., Geoghegan, J. A., Ganesh, V. K. & Höök, M. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat Rev Microbiol* **12**, 49–62 (2014).
281. Cheung, G. Y. C., Bae, J. S. & Otto, M. Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence* **12**, 547–569 (2021).
282. Chan, L. W. *et al.* Selective Permeabilization of Gram-Negative Bacterial Membranes Using Multivalent Peptide Constructs for Antibiotic Sensitization. *ACS Infect Dis* **7**, 721–732 (2021).

283. Tun Thet, N. *et al.* SPaCE Swab: Point-of-Care Sensor for Simple and Rapid Detection of Acute Wound Infection. *ACS Sens* **5**, 2652–2657 (2020).
284. Mac, Q. D. *et al.* Non-invasive early detection of acute transplant rejection via nanosensors of granzyme-B activity HHS Public Access Author manuscript. *Nat Biomed Eng* **3**, 281–291 (2019).
285. Hao, L. *et al.* nature nanotechnology CRISPR-Cas-amplified urinary biomarkers for multiplexed and portable cancer diagnostics. *Nature Nanotechnology* | **18**, 798–807 (2023).
286. Chan, K. M., Gleadle, J. M., O'callaghan, M., Vasilev, K. & Macgregor, M. Prostate cancer detection: a systematic review of urinary biosensors. *Prostate Cancer Prostatic Dis* **25**, 39–46 (2021).
287. Buss, C. G., Dudani, J. S., Akana, R. T. K., Fleming, H. E. & Bhatia, S. N. Protease activity sensors noninvasively classify bacterial infections and antibiotic responses. *EBioMedicine* **38**, 248–256 (2018).
288. Müller, C. B. *et al.* Precise measurement of diffusion by multi-color dual-focus fluorescence correlation spectroscopy. *EPL* **83**, (2008).
289. Müller, P., Schwille, P. & Weidemann, T. PyCorrFit-generic data evaluation for fluorescence correlation spectroscopy. *Bioinformatics* **30**, 2532–2533 (2014).
290. Massi, L. *et al.* Tuneable peptide cross-linked nanogels forenzyme-triggered protein delivery. *J Mater Chem B* **8**, 8894–8907 (2020).
291. Penders, J. *et al.* Single Particle Automated Raman Trapping Analysis. *Nat Commun* **9**, 1–11 (2018).
292. Wang, X., Zhong, X., Li, J., Liu, Z. & Cheng, L. Inorganic nanomaterials with rapid clearance for biomedical applications. *Chem Soc Rev* **50**, 8669–8742 (2021).

293. Gaskin, D. K., Bohach, G. A., Schlievert, P. M. & Hovde, C. J. Purification of Staphylococcus aureus β -Toxin: Comparison of Three Isoelectric Focusing Methods. *Protein Expr Purif* **9**, 76–82 (1997).
294. Walev, I., Weller, U., Strauch, S., Foster, T. & Bhakdi, S. Selective Killing of Human Monocytes and Cytokine Release Provoked by Sphingomyelinase (Beta-Toxin) of Staphylococcus aureus. *Infect Immun* **64**, 2974–2979 (1996).
295. Tam, K. & Torres, V. J. Staphylococcus aureus Secreted Toxins and Extracellular Enzymes. *Microbiol Spectr* **7**, 7–2 (2019).
296. Divyakolu, S. *et al.* Hemolysins of Staphylococcus aureus—An Update on Their Biology, Role in Pathogenesis and as Targets for Anti-Virulence Therapy. *Adv Infect Dis* **9**, 80–104 (2019).
297. Shohda, K., Takahashi, K. & Suyama, A. A method of gentle hydration to prepare oil-free giant unilamellar vesicles that can confine enzymatic reactions. *Biochem Biophys Rep* **3**, 76–82 (2015).
298. Bose, R. J. C., Lee, S. H. & Park, H. Lipid-based surface engineering of PLGA nanoparticles for drug and gene delivery applications. *Biomater Res* **20**, 1–9 (2016).
299. Soema, P. C., Willems, G. J., Jiskoot, W., Amorij, J. P. & Kersten, G. F. Predicting the influence of liposomal lipid composition on liposome size, zeta potential and liposome-induced dendritic cell maturation using a design of experiments approach. *European Journal of Pharmaceutics and Biopharmaceutics* **94**, 427–435 (2015).
300. Holsæter, A. M. *et al.* How docetaxel entrapment, vesicle size, zeta potential and stability change with liposome composition—A formulation screening study. *European Journal of Pharmaceutical Sciences* **177**, 106267 (2022).

301. Saunders, C. *et al.* Revealing Population Heterogeneity in Vesicle-Based Nanomedicines Using Automated, Single Particle Raman Analysis. (2023).
302. Barriga, H. M. G. *et al.* Coupling Lipid Nanoparticle Structure and Automated Single-Particle Composition Analysis to Design Phospholipase-Responsive Nanocarriers. *Advanced Materials* **34**, (2022).
303. Czamara, K. *et al.* Raman spectroscopy of lipids: A review. *Journal of Raman Spectroscopy* **46**, 4–20 (2015).
304. Astley, R., Miller, F. C., Mursalin, H., Coburn, P. S. & Callegan, M. C. An Eye on Staphylococcus aureus Toxins: Roles in Ocular Damage and Inflammation. *Toxins (Basel)* **11**, (2019).
305. Mir, I. H. & Thirunavukkarasu, C. The relevance of acid sphingomyelinase as a potential target for therapeutic intervention in hepatic disorders: current scenario and anticipated trends. *Arch Toxicol* **97**, 2069–2087 (2023).
306. Krafft, C., Neudert, L., Simat, T. & Salzer, R. Near infrared Raman spectra of human brain lipids. *Spectrochim Acta A Mol Biomol Spectrosc* **61**, 1529–1535 (2005).
307. Movasaghi, Z., Rehman, S. & Rehman, I. U. Raman spectroscopy of biological tissues. *Appl Spectrosc Rev* **42**, 493–541 (2007).
308. Nakhaei, P. *et al.* Liposomes: Structure, Biomedical Applications, and Stability Parameters With Emphasis on Cholesterol. *Front Bioeng Biotechnol* **9**, (2021).
309. Dymek, M. & Sikora, Z. Liposomes as biocompatible and smart delivery systems-the current state. *Nat Commun* **9**, 2839 (2022).
310. Inglut, C. T. *et al.* nanomaterials Immunological and Toxicological Considerations for the Design of Liposomes. *Nanomaterials* **10**, 190 (2020).

311. Kaya, G. & Oytun, F. Rheological Properties of Injectable Hyaluronic Acid Hydrogels for Soft Tissue Engineering Applications. **11**, 8424–8430 (2021).
312. Zerbinati, N. *et al.* Toward Physicochemical and Rheological Characterization of Different Injectable Hyaluronic Acid Dermal Fillers Cross-Linked with Polyethylene Glycol Diglycidyl Ether. *Polymers (Basel)* **13**, 948 (2021).
313. Buckley, C., Murphy, E. J., Montgomery, T. R. & Major, I. Hyaluronic Acid: A Review of the Drug Delivery Capabilities of This Naturally Occurring Polysaccharide. *Polymers (Basel)* **14**, (2022).
314. Hou, X. *et al.* Recent advances in hyaluronic acid-based nanomedicines: Preparation and application in cancer therapy. *Carbohydr Polym* **292**, 119662 (2022).
315. Ning, X. *et al.* Physiological stability and renal clearance of ultrasmall zwitterionic gold nanoparticles: Ligand length matters. *APL Mater* **5**, (2017).
316. Yu, M. *et al.* Noninvasive Staging of Kidney Dysfunction Enabled by Renal-Clearable Luminescent Gold Nanoparticles. *Angewandte Chemie - International Edition* **55**, 2787–2791 (2016).
317. Liu, J. *et al.* Passive tumor targeting of renal-clearable luminescent gold nanoparticles: Long tumor retention and fast normal tissue clearance. *J Am Chem Soc* **135**, 4978–4981 (2013).
318. Mandell, J. B. *et al.* Large variations in clinical antibiotic activity against *Staphylococcus aureus* biofilms of periprosthetic joint infection isolates. *Journal of Orthopaedic Research* **37**, 1604–1609 (2019).
319. Bispo, M. *et al.* Fighting *Staphylococcus aureus* infections with light and photoimmunoconjugates. *JCI Insight* **5**, (2020).

320. El-Mahdy, T. S. *et al.* Interaction of implant infection-related commensal bacteria with mesenchymal stem cells: a comparison between *Cutibacterium acnes* and *Staphylococcus aureus*. *FEMS Microbiol Lett* **368**, 14 (2021).