## Developing Targeted Stimuli-Responsive Drug Delivery Systems for Cardiovascular Diseases

by

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I dedicate this thesis to the soul of my parents; **Refaat and Magda** who lost their lives fighting against cancer and thrombosis. I hope my research contributes towards the development of life-saving treatments for vulnerable patients suffering every day.

### Abstract

Stimuli-responsive release of therapeutic agents at the site of disease can prevent serious adverse effects. Near-infrared light (NIR) in the first window (650-900 nm) has shown success as an external stimulus for drug release, supported by its tissue penetration capability. In this context, we engineered a NIR light-responsive liposomal gold nanorodcontaining platform (Au-LRLs) for on-demand delivery of proteins. The developed platform was then evaluated for NIR-triggered release of urokinase plasminogen activator (uPA), a commonly used thrombolytic agent with potentially fatal bleeding adverse effects. Efficient low-dose, site-directed, and potentially bleeding-free thrombolysis can be achieved from our developed platform, augmented by the photothermal thrombolytic effect of incorporated gold nanorods (AuNRs). Liposomes were prepared and characterised for physicochemical properties, such as gold content, AuNRs localisation, photothermal transduction, biocompatibility, temperature-assisted and light-assisted release. Further, in vitro thrombolysis studies were performed to assess thrombolytic efficiency of uPA-Au-LRL. Localisation of ultrasmall AuNRs in/to liposomes was confirmed by TEM. Mild hyperthermia (~ 5 °C) was detected from the liposomes upon NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup>). In light-assisted release studies, ~ 55% of FITCovalbumin (model protein) was released in 15 min following NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup> for 5 min). In thrombolysis studies, uPA-Au-LRLs showed ~ 80 % lysis of an *in vitro* clot model in 30 min following NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup> for 5 min) compared to  $\sim 36$  % and  $\sim 15$  % clot lysis from equivalent free uPA and nonirradiated liposomes, respectively. In conclusion, the newly engineered, gold nanorodbased, NIR light-responsive liposome represents a promising drug delivery system for site-directed, photothermally-assisted thrombolysis.

Activated platelet targeting has attracted substantial attention as a tool for localised delivery of therapeutics and imaging agents to thrombi. In this context, we report on an activated platelet-targeted IR780-loaded liposome (Tar-IR-L) as a platform for sitedirected photothermal therapy of acute thrombosis. IR780 is a cationic, lipophilic, and photothermal dye capable of absorbing and emitting light in the NIR region. Here we prepared IR780-loaded liposomes and coated them with neutravidin before their conjugation to activated platelet-targeting scFv antibody using biotin-neutravidin chemistry. Photothermally-assisted thrombo-lysis was also investigated using targeted IR780 liposomes co-loaded with a single chain urokinase (scuPA) (Tar-scuPA-IR-L). The developed liposomes were characterised for physicochemical properties, photothermal transduction, biocompatibility, and photostability. Selective binding to activated platelets was assessed in vitro using both flow cytometry and confocal microscopy. In vitro photothermal thrombolysis was tested using a microfluidic thrombosis-on-a-chip model. Further, selective targeting and photothermal thrombolysis were tested in vivo using a FeCl3-induced thrombosis mouse model. Results showed successful incorporation of IR780 into liposomes with high cytocompatibility and high photostability in aqueous fluids, compared to the free dye. Selective binding of the targeted liposomes to activated platelets was confirmed using flow cytometry and confocal microscopy imaging, compared to non-targeted liposomes. In vitro NIR irradiation (785 nm, 1 W/cm<sup>2</sup>) of the developed liposomes generated mild hyperthermia (~ 5-10 °C). In the thrombosis-on-a-chip model, NIR irradiation of the targeted liposomeperfused channels resulted in a significant reduction in the mean fluorescence intensity (MFI) and area coverage (%) of the fluorescent microthrombi. Ex vivo fluorescence imaging of thrombosed arteries in mice revealed selective accumulation of targeted liposomes to the FeCl<sub>3</sub>-induced thrombi. Higher local temperature increments were achieved from targeted liposomes in vivo, when thrombosed left carotid arteries of anesthetised mice were NIR irradiated (785 nm, 1 W/cm<sup>2</sup>, 5 min). Clot areas showed  $\sim$ 52 % and  $\sim$  72 % decrease following NIR irradiation of mice treated with Tar-IR-L and Tar-scuPA-IR-L, respectively. In conclusion, activated platelet targeted IR780 liposomes represent a promising platform for targeted photothermal therapy of acute thrombosis.

Briefly, the thesis presents two promising liposomal platforms for NIR-responsive release and site-directed delivery of PAs to thrombosis based on photothermal-assisted therapy and antibody-mediated targeting. The developed systems showed great capabilities towards more efficient, low-dose, localised, and potentially bleeding-free thrombolysis which offers a safer alternative to current pharmacological approaches.

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### Declaration

This thesis 'contains no material that has been accepted for the award to the candidate of any other degree or diploma. To the best of the candidate's knowledge, this thesis contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome, and with permission received to republish the work in the thesis.' <sup>1</sup> This thesis was conducted under the supervision of Professor Simon E. Moulton, Professor Karlheinz Peter, Associate Professor Xiaowei Wang, and Doctor Blanca del Rosal.

Signature: Ahmed Refaat Abdelhamid Ahmed Ahmed

Date: 22 November 2022

<sup>1</sup>Declaration formatted according to the Swinburne Research Graduate Studies Research Training Statement of Practice Candidature Handbook Version 3.0 (July 2019).

# List of publications, conferences, talks, and workshops

### **Peer-reviewed Publications**

#### 1. Review articles:

**A. Refaat**, B. del Rosal, J. Palasubramaniam, G. Pietersz, X. Wang, K. Peter, S.E. Moulton, Smart delivery of plasminogen activators for efficient thrombolysis; recent trends and future perspectives, *Advanced Therapeutics*, 4 (2021) 2100047. doi: 10.1002/adtp.202100047

**A. Refaat, M. Yap, G. Pietersz**, A. Walsh, J. Zeller, B. del Rosal, X. Wang, K. Peter. *In vivo* Fluorescence Imaging: Success in Preclinical Imaging Paves the Way for Clinical Applications, *Journal of Nanobiotechnology*, 20 (2022) 450. doi: 10.1186/s12951-022-01648-7

#### 2. Research articles:

**A. Refaat**, B. del Rosal, J. Palasubramaniam, G. Pietersz, X. Wang, S.E. Moulton, K. Peter, Near-infrared light-responsive liposomes for protein delivery: Towards bleeding-free photothermally-assisted thrombolysis, *Journal of Controlled Release*, 337 (2021) 212-223. doi: 10.1016/j.jconrel.2021.07.024

**A. Refaat**, B. del Rosal, V. Bongcaron, A. Walsh, G. Pietersz, K. Peter, S. E. Moulton, X. Wang. Activated platelet targeted IR780 immunoliposomes for photothermal therapy of acute thrombosis, accepted in *Advanced Functional Materials* [October 2022].

### **Conference Abstract Presentations**

#### 1. Controlled Release Society Virtual 48<sup>th</sup> Annual Meeting (CRS2021)

**Abstract oral presentation** titled "Near-infrared light-responsive liposomes for protein delivery and its application in potentially bleeding-free photothermally-assisted thrombolysis" <u>https://2021.controlledreleasesociety.org/</u>

### 2. 6<sup>th</sup> Annual Scientific Symposium of the Australian Society of Molecular Imaging (ASMI 2021)

Abstract oral presentation titled "Activated Platelet-Targeted IR780 liposomes: NIR Light as a Tool for Image-Guided Photothermal Photodynamic Thrombolysis" <u>https://www.asmi2021.com/</u>

#### 3. The Aikenhead Centre for Medical Discovery Research Week (ACMD 2021)

Abstract poster presentation titled "Near-infrared light-responsive liposomes for protein delivery: Towards bleeding-free photothermally-assisted thrombolysis"

#### 4. Baker Department of Cardiometabolic Health Symposium (2021)

Abstract poster presentation titled "Near-infrared light-responsive liposomes for protein delivery: Towards bleeding-free photothermally-assisted thrombolysis"

#### Workshops and Talks

- Joint Australian Korean Workshop "Bioengineered Materials for Medicine" (2020). Oral presentation titled "Stimuli responsive nanodelivery systems for cardiovascular diseases"
- **2.** Soft Tissue Bioengineering Virtual Symposium (2020). Oral presentation titled "NIR light-responsive liposomes for treatment of thrombosis"
- **3.** Baker Institute Tuesday Talk (June 2021). Oral presentation titled "NIR lightresponsive liposomes for protein delivery: Towards bleeding-free, photothermallyassisted thrombolysis"

### Awards

**Outstanding Oral Presentation Prize,** 6<sup>th</sup> Annual Scientific Symposium of the Australian Society of Molecular Imaging (ASMI2021), Translational Research Institute (TRI), QLD, Australia

**Poster Presentation Prize (Runner up)**, Baker Department of Cardiometabolic Health Symposium (2021), University of Melbourne, VIC, Australia.

Further details on conference presentations can be found in Appendix I.

### Graduate Certificate of Research and Innovation Management

Unit	Status
ENT60010 - Creativity and Innovation in Research	Passed
INF60016 - Project Management for Research	Passed
COM80001 - Advanced Research Communication Skills in Sci, Eng, and Tech	Passed
MFP60004 - Research Engagement (STEM)	Passed

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### List of Acronyms

Abs	Antibodies
ACD	Acid citrated dextrose
ADP	Adenosine Diphosphate
AgNO <sub>3</sub>	Silver nitrate
AMF	Alternating magnetic field
Au-LRLs	Gold nanorods light-responsive liposomes
Au@MSNs	Gold-mesonorous silica nanonarticles
AuNRs	Gold nanorods
AuNRs-PFG	PEGylated gold nanorods
AuNS	Gold nanostars
	Gold hallostars
BBB	Blood-brain barrier
Brij58	Polyoxyethylene 20 cetyl ether
Brij78	Polyoxyethylene 20 stearyl ether
BSA	Bovine serum albumin
CaCh	Calcium chloride
	Computed tomography
CTAR	Cetyltrimethylammonium bromide
	Chalostaral
CHOL	Cholesterol
DCM	Dichloromethane
DHPE-RhB	Rhodamine-B-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine
DI	Deionised
DIO-C <sub>6</sub>	Dihexyloxacarbocyanine iodide
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle medium
<b>DOPE-biotin</b>	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- (cap biotinyl)
	(sodium salt)
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol
DVT	Deep vein thrombosis
DD	
EE	Entrapment efficiency
EGFR	Epidermal growth factor receptor
ELIPS	Echogenic liposomes
ELISA	Enzyme-linked immunosorbent assay
EPR	Enhanced permeability and retention
FBS	Foetal bovine serum
FeCl <sub>3</sub>	Ferric chloride
Fe <sub>3</sub> O <sub>4</sub>	Ferric oxide
FITC	Fluorescein isothiocyanate
FITC-Au-LRLs	FITC-ovalbumin & AuNRs-PEG co-loaded light-responsive liposomes
GPIID/IIIa	Glycoprotein IIb/IIIa

HAuCl <sub>4</sub>	Gold (III) chloride trihydrate
HEK	Human embryonic kidney cells
HSA	Human serum albumin
H&E	Haematoxylin and eosin
	<b>x</b> 1 ·
ICG	Indocyanine green
ICP-OES	Inductively coupled plasma atomic emission spectrometer
IR780	2-[2-[2-Chloro-3-[(1,3-dihydro-3,3-dimethyl-1-propyl-2 <i>H</i> -indol-2-
	ylidene) ethylidene]-1-cyclohexen-1-yl] ethenyl]-3,3-dimethyl-1-
	propylindolium iodide
Iso-FITC	FITC mouse IgG isotype control
<i>i.v</i> .	Intravenous
IVC	Inferior vena cava
IVIS	In vivo imaging system
LCST	Lower critical solubility temperature
LK	Lumbrokinase
LSPR	Longitudinal surface plasmon resonance
<b>A b</b>	Managlandihadu
MAD MD	Monocional antibody
	Microbubbles Magnetic nenematicles
IVIINP	Magnetic nanoparticles
mp	Meiting point
MPEG2000-SH	Methoxypolyethylene glycol thiol
	Magnetic resonance imaging
MPS	Mono-nuclear phagocytic system
	3(4,5-di metnyl thiazoi-2-yi)-2,5-dipnenyltetrazolium bromide dye
MWCO	Molecular weight cut off
NaBH <sub>4</sub>	Sodium borohydride
NIR	Near-infrared
NP	Nanoparticles
OCT	Optical coherence tomography
PAC-1	FITC mouse anti-human PAC-1 antibody
PAs	Plasminogen activators
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PDI	Poly dispersity index
PE	Pulmonary embolism
PE	R-Phycoerythrin
PEG	Polyethylene glycol
PEG-PCL	Poly (ethylene glycol)- poly(ε-caprolactone)
PEG-SH	Thiolated polyethylene glycol
PFA	Paraformaldehyde
PLA <sub>2</sub>	Phospholipase-A <sub>2</sub>
PLGA	Polylactic co-glycolic acid polymer
PMIN	Platelet microparticle-inspired nanovesicles

PNIPAM	Poly (N-isopropyl acrylamide) polymer
PRP	Platelet rich plasma
PS	Phosphatidylserine
RBCs	Red blood cells
RES	Reticulo-endothelial system (RES)
RGD	Arginine-glycine-aspartate peptide
rpm	Round per minute
scFv	Single chain variable fragment antibody
scuPA	Single chain variable fragment urokinase plasminogen activator
siRNA	small interfering RNA
SK	Streptokinase
SMANCS	Styrene-maleic acid conjugated neocarzinostatin
SPR	Surface plasmon resonance
STL	Sono-thrombolysis
t <sub>1/2</sub>	Half-life
T <sub>m</sub>	Gel to liquid crystalline transition temperature
TSLs	Thermo-sensitive liposomes
tPA	Tissue plasminogen activator
uPA	Urokinase-type plasminogen activator
US	Ultrasound

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### **Chapter 1** Introduction

### 1.1. Introduction

For decades, cardiovascular diseases have been one of the major causes of death globally. According to the world health organisation (WHO), the top global causes of deaths in 2019 were associated with three major disease categories: cardiovascular (ischaemic heart disease, stroke), respiratory (chronic obstructive pulmonary disease, lower respiratory infections) and neonatal conditions ("The top 10 causes of death,"). In Australia, coronary heart diseases came first in the leading cause of deaths in 2019, according to Australian Institute of Health and Welfare ("Deaths in Australia,"). New trends in treatment and diagnosis of cardiovascular diseases using nanotechnology have attracted attention as potential alternatives to conventional pharmacological approaches and surgeries, particularly after the surge of FDA approvals in the field of nanomedicine.

Acute thrombosis is the underlying cause for most life-threatening myocardial infarctions and strokes (Nigel Mackman, 2008). Timely reperfusion of occluded blood vessels using plasminogen activators (PAs), the gold standard pharmacological treatment in clinical settings, comes with the risk of systemic bleeding (Absar, Gupta, Nahar, & Ahsan, 2015). PAs are characterised by their short circulation half-lives (t<sub>1/2</sub>) and rapid clearance which necessitate administration of large doses in the form of *i.v.* infusions (Stepan Koudelka et al., 2016). Nano-sized drug delivery systems offered some solutions to circumvent PAs bleeding side effect, prolong their circulation time, and achieve efficient thrombolysis at subtherapeutic doses. Smart delivery of PAs to thrombosis, using stimuli-responsive systems and targeted-delivery, has proven success in preclinical models towards low dose, bleeding-free, and more efficient thrombolysis (D. Disharoon, D. Marr, & K. Neeves, 2019) (T. Huang, Li, & Gao, 2019) (Refaat, del Rosal, et al., 2021b).

Targeted thrombolysis can be achieved through direct conjugation of PAs-loaded nanocarriers to targeting moieties which specifically bind to the most abundant thrombus components namely, fibrin and activated platelets (Adzerikho et al., 2021; Xiaowei Wang et al., 2016; N. P. Zhang et al., 2018). Another successful approach involves engineering of recombinant fusion nanoconstructs of single chain PA and targeting antibody (Xiaowei Wang et al., 2014b).

Stimuli-responsive delivery systems have been investigated for on-demand release/activation of PAs, in response to internal or externally applied stimulus, at thrombosis site. This local release/activation of PAs can significantly reduce the therapeutic dose and minimise off-site bleeding adverse effects. Internal stimuli include local enzymes (such as thrombin) (Shahriar Absar, Young M. Kwon, & Fakhrul Ahsan, 2014), activated platelets (Yu Huang et al., 2019), and high shear stress (Netanel Korin et al., 2012). Externally-applied stimuli include ultrasound waves (Hua et al., 2014), magnetic fields (C.-H. Liu, H.-L. Hsu, J.-P. Chen, T. Wu, & Y.-H. Ma, 2019), and near-infrared (NIR) light (X. Wang et al., 2017b).

In this thesis, we hypothesise that a photothermal agent loaded-liposome would provide a promising platform for site-directed/on-demand delivery of medicines to thrombosis, besides augmenting thrombolysis through the photothermal effect.

### **1.2.** Statement of research questions

Through this research, the following questions will be addressed:

 Is it possible to achieve on-demand instantaneous release of macromolecular therapeutic proteins such as plasminogen activators (PAs) from liposomal nanocarriers relying on the plasmonic properties of AuNRs, when NIR illuminated within the safe exposure limits?

- Would the therapeutic protein maintain its therapeutic stability following photothermally-assisted release?
- Is it feasible to bioconjugate the developed delivery system to the single chain antibody (scFv) specifically targeting activated platelets without affecting the system integrity and functionality?
- Is it possible to achieve efficient photothermal thrombolysis using the targeted liposome *in vivo*?

### 1.3. Research aims

#### The research project aims to

- Develop a NIR light-responsive liposomal delivery system co-loaded with urokinase plasminogen activator (uPA) and ultrasmall gold nanorods (AuNRs).
- Test the developed system for stimuli-responsive release of the therapeutic protein under NIR irradiation.
- Assess the thrombolytic properties of the developed system in the absence and presence of NIR irradiation, using relevant *in vitro* and *ex vivo* clot models.
- Conjugate the NIR light-responsive liposome to scFv antibody targeting activated platelets and confirm specific binding to activated platelets both *in vitro and in vivo*.
- In vivo appraisal of the targeted liposomes towards efficient and low dose photothermal thrombolysis.

### 1.4. Research significance

#### Contribution to scientific knowledge and to the field of cardiovascular nanomedicine

Due to the multi-disciplinary nature of the research, it covers different technical aspects and discusses new methodologies for the development and evaluation of stimuliresponsive targeted liposomes and their application in thrombosis treatment.

Firstly, preparation of a biocompatible, ultrasmall AuNRs-loaded, thermosensitive liposome for protein delivery was described in detail. Limitations of loading metallic nanoparticles to liposomes relying solely on physical encapsulation was described and confirmed using TEM imaging. Mechanisms for both temperature and light-assisted release of therapeutic proteins from liposomes were discussed as well. In addition, retention of the enzymatic activity of the released protein was investigated using urokinase plasminogen activator (uPA) as a therapeutic protein model. These findings open the way to further research on the use of photothermal-responsive liposomes for protein delivery. It can be applied in the fields of cancer, skin disorders, brain disorders and ophthalmic diseases.

Secondly, photothermal therapy of acute thrombosis was investigated and proven efficient novel approach, where the generated local hyperthermia facilitated fibrin clot break and synergised with the co-administered or locally delivered low dose PAs. This comes in line with some recently published work from other groups working in the same area of research (T.-Y. Lu et al., 2021; X. Wang et al., 2017b; Jiasheng Xu et al., 2020; Z. Zhao et al., 2022).

Thirdly, an activated platelet-targeted, NIR fluorescent photothermal dye (IR780)loaded liposome was successfully prepared. Stabilisation of NIR fluorescent dyes (such as IR780) in liposomes provides another evidence to the need of FDA-approved liposomal dyes towards their clinical application in tumour guided-surgery, brain tumour fluorescence imaging, and others. Conjugation of activated platelet targeting-scFv antibody to IR780 liposomes represents a novel approach to achieve targeted photothermal therapy of thrombosis. Neutravidin-biotin conjugation chemistry, as a strong instantaneous non-covalent bonding, offers a quick approach to immediately conjugate biotinylated antibodies or proteins to neutravidin-coated nanocarriers before their administration to patient.

Fourthly, the work presents a detailed description of a thrombosis-on-a-chip model which utilises a commercially available collagen-coated microfluidic channel (ibidi®  $\mu$ -Slides), a hydrophobic fluorescent dye (DiO-C<sub>6</sub>), and freshly collected human blood to form homogenous sized and reproducible microthrombi *in situ*. This easy-to-setup model is a multi-purpose tool, where it can be used to screen thrombolytic nanoparticles, thrombosis targeting ligands, and molecular imaging probes under flow conditions. Further research is required to confirm the *in vivo/in vitro* correlation.

Fifthly, the use of FeCl<sub>3</sub>-induced injury thrombosis model to preclinically test thrombosis targeting and photothermal thrombolysis has revealed some important remarks which might help future research in the field. The FeCl<sub>3</sub>-induced injury model showed an ideal model to study thrombosis targeting. However, it was difficult to study the photothermal thrombolysis based on occlusion time measurements. Delay in occlusion time used to present a key parameter to judge efficiency of prophylactic anti-thrombotics and anti-platelets. Measurements of occlusion time delay is not practical in the case of externally-stimuli responsive delivery systems, where stimulus is applied after artery full occlusion. Here, histological examination of injured artery and photothermal transduction measurements presented a reliable approach to compare between the different test treatments.

#### **1.5.** Thesis outline

**Chapter 1** is an introductory chapter which provides a brief background of the research project and highlights the main research questions, aims, and significance. It further outlines the thesis structure.

**Chapter 2** is a literature review chapter. First, targeted and stimuli-responsive delivery systems are generally discussed with a focus on ligand-mediated targeting and NIR light as an external stimulus. Then, smart delivery of plasminogen activators, clot busters, is discussed in detail showing the latest advances in this research area and identifying research gaps. Lastly, the use of liposomes as a translational nanocarrier was discussed.

- Aspects of the work presented in chapter 2 were published in Advanced Therapeutics as: Smart delivery of plasminogen activators for efficient thrombolysis; recent trends and future perspectives, *Advanced Therapeutics*, 4 (2021) 2100047. doi: <u>10.1002/adtp.202100047</u>
- Copyright clearance was obtained to use the full article materials in thesis [License No: 5240771170945]- See Appendix II.

**Chapter 3** describes the development of a NIR light-responsive liposomal platform based on a hybrid formulation of thermosensitive lipids and ultrasmall gold nanorods. Synthesis method of ultrasmall gold nanorods, and their co-loading into thermosensitive liposomes with a fluorescently labelled model protein (FITC-ovalbumin, MW 45 kDa) is detailed in this chapter. Physicochemical properties of AuNRs and the prepared liposomes are then evaluated *in vitro* using TEM imaging, Uv-vis-NIR spectroscopy, photothermal transduction, ICP-OES, DLS measurements, and fluorescence spectroscopy. Cytocompatibility and haemolysis study was performed as well. Finally, NIR-assisted release of FITC-ovalbumin from the developed system was confirmed.

**Chapter 4** involves application of the NIR light-responsive liposomes, developed under chapter 3, in acute thrombosis treatment. The same liposomal platform was used to co-

load ultrasmall AuNRs with urokinase plasminogen activator (uPA), a commonly used fibrinolytic drug in clinical settings with fatal bleeding side effects. NIR-assisted release of uPA was tested using BCA and enzymatic activity assays. *In vitro* thrombolysis was evaluated using a halo-clot model.

Aspects of the work presented in chapter 3 and 4 were published in the Journal of Controlled Release as: Near-infrared light-responsive liposomes for protein delivery: Towards bleeding-free photothermally-assisted thrombolysis, *Journal of Controlled Release*, 337 (2021) 212-223. doi: <u>10.1016/j.jconrel.2021.07.024</u>
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**Chapter 5** describes the conjugation of an activated platelet-targeting scFv antibody to the surface of NIR light-responsive liposomes and their *in vitro* evaluation. Here, gold nanorods were replaced by a molecular NIR fluorescent and photothermal dye (IR780) to achieve more homogenous assembly into liposomes and to give potentials to NIR fluorescence imaging of acute thrombosis. A low-dose single chain urokinase (scuPA) was conjugated to the surface of the liposomes as well. Beside testing the specific binding of liposomes to activated platelets and microthrombi, photothermally-assisted thrombolysis was assessed using a microfluidic thrombosis-on-a-chip model.

**Chapter 6** extends assessments of the activated platelet-targeted IR780 liposomes to preclinical FeCl<sub>3</sub>-induced thrombosis mouse model. The *in vivo* experiments involve testing of clot targeting and photothermal thrombolysis.

**Chapter 7** presents conclusions of the research project. The main outcomes are highlighted and potentials/challenges to clinical translation is emphasised.

### **Chapter 2** Literature review

**Aspects of the work presented in this chapter were published as:** Smart delivery of plasminogen activators for efficient thrombolysis; recent trends and future perspectives, *Advanced Therapeutics*, 4 (2021) 2100047. doi: 10.1002/adtp.202100047

Advances in using nanoscale systems for drug delivery have recently led to the development of a tremendous number of nanotherapeutics and nanodiagnostics that can deliver drugs and imaging probes in spatiotemporal and dosage-controlled manners. These customised therapies aim to address the unmet needs for a myriad of diseases such as cancer, cardiovascular diseases, inflammatory diseases, and neurodegenerative disorders.

### 2.1. Targeted drug delivery systems

### 2.1.1. Levels of targeting

Targeted drug delivery systems have arisen to control the biodistribution of drugs within the body so that most of the dose is delivered selectively to the target tissues at cellular or sub cellular level. This would substantially reduce the total dose and side effects of highly toxic therapeutics, prolong duration of action from sustained release formulations and improve therapeutic outcome (Koning & Storm, 2003). Levels of targeting extends from organ to cellular and subcellular targeting. Brain targeting for Alzheimer's (Mourtas, Lazar, Markoutsa, Duyckaerts, & Antimisiaris, 2014) and lung targeting for cystic fibrosis (X. Li, Hayes, & Mansour, 2011) are examples for organ level targeting. Tissue level targeting for solid tumours (J. H. Kim et al., 2015) or inflammatory tissue can be likewise achieved to reduce non-specific interaction with other biological compartments. Additionally, cellular and subcellular levels targeting has been used for

gene delivery to nucleus (Thomas, Tajmir-Riahi, & Pillai, 2019) and mitochondrial delivery for cancer (Palacios, Crawford, Vaseva, & Moll, 2008) or Parkinson's treatment (Zanon, Hicks, Pramstaller, & Pichler, 2017).

# 2.1.2. Non-specific interaction with blood components and phagocytotic uptake

Efficient nanotherapeutic targeting following intravenous (*i.v.*) administration is challenged by several biological barriers. Clearance from blood stream by mono-nuclear phagocytic system (MPS) in dictated filtration organs (i.e. liver, spleen, kidney and lungs) is the most substantial barrier (Gustafson, Holt-Casper, Grainger, & Ghandehari, 2015). This natural mechanism of clearing colloidal medicines from the blood has been extensively studied to find out approaches to counter phagocytosis and prolong circulation.

The particle size, surface charge and surface hydrophilicity of the nanosystem are decisive parameters to control its pharmacokinetic profile and to escape phagocytosis for prolonged circulation. Particle size smaller than 100 nm showed high potential to escape uptake by MPS, while those larger than 1 µm demonstrated maximal phagocytic uptake. On the other hand, surface charge of delivery system is another important parameter, as cationic particles are taken up to a greater extent than their anionic or neutral counterparts besides imparting cytotoxicity (M. Zhu et al., 2012). Coating with hydrophilic polymers such as poly ethylene glycol (PEG) or dextran imparts surface hydrophilicity and steric stabilisation against opsonisation and subsequent phagocytic uptake which can greatly boost circulation course and biological half-life of the delivery system (Walkey & Chan, 2012).

### 2.1.3. Types of targeted drug delivery systems

Targeting can be classified into two major classes: passive and active targeting (Figure 2.1). The former is dictated by a natural mechanism or pathological condition which enhance the accumulation of the nanosystem at the target site, while the latter is driven by an affinity ligand interaction (biochemical targeting) or external force such as magnetic field (physical targeting) (Attia, Anton, Wallyn, Omran, & Vandamme, 2019).



**Figure 2.1.** Types of drug targeting; (a) Passive targeting and (b) Active targeting. Adapted with permission (Danhier, Feron, & Préat, 2010).

#### Passive targeting and the EPR effect

Passive targeting of therapeutic macromolecules to tumour tissue was the first studied form of drug targeting more than 30 years ago. In 1986, Matsumura and Maeda observed the preferential accumulation of their anti-cancer polymer conjugate styrene-maleic acid conjugated neocarzinostatin (SMANCS) to tumour tissue (Matsumura & Maeda, 1986) which lead them to the EPR pharmacokinetic principle (Enhanced permeability and retention) as an inherent pathological feature of solid tumours with leaky vasculature (Maeda, 2015). Since then, the EPR effect has been widely studied to reveal how heterogonous this phenomenon can be, and much research has been conducted to develop tumour targeted therapies based on the EPR effect. Doxil<sup>®</sup> (liposomal doxorubicin HCl) and Abraxane<sup>®</sup> (albumin bound paclitaxel) are of the earliest commercialised FDAapproved nanomedicines developed to passively accumulate in tumour with significant lower side effects compared to their prototype conventional medicines (Bobo, Robinson, Islam, Thurecht, & Corrie, 2016).

#### Ligand-mediated active targeting

Active targeting has evolved as a synergistic approach to capitalise on the full potential benefit of the nanotherapeutic through utilisation of an affinity ligand on the surface of nanosystem for specific recognition and binding to the targeted diseased cells (Attia et al., 2019). Ligand-mediated active targeting has been efficiently exploited for cancer (Qiu, Dong, & Kan, 2018; N. Zhang, J. N. Zhang, et al., 2018), Alzheimer's (Mourtas et al., 2014), thyroid gland (Martinez & Montero, 2012), cystic fibrosis (X. Li et al., 2011) and cardiovascular diseases (Kawata et al., 2012; X. Wang & Peter, 2016; N. P. Zhang et al., 2018) by bio-conjugating specific antibodies (N. Zhang, J. N. Zhang, et al., 2018),

peptides (Delehanty et al., 2010), aptamers (Dou et al., 2018) and even small molecules such as folic acid (Qiu et al., 2018) to the surface of the nanosystem (Table 2.1).

Targeting specificity and delivering capacity represent the most important evaluation parameters for any ligand-mediated targeted delivery system. Targeting specificity is usually determined by the pharmacokinetic behaviour of the ligand-conjugated nanosystem and the possible non-specific interaction with off-target cells; which is defined by the ligand and nanocarrier properties (N. Bertrand, J. Wu, X. Xu, N. Kamaly, & O. C. Farokhzad, 2014). The materials used and structure of the nanocarrier mostly control the delivering capacity and release properties at target sites (Kamaly, Xiao, Valencia, Radovic-Moreno, & Farokhzad, 2012).

**Antibodies (Abs)** are the first reported (Leserman, Barbet, Kourilsky, & Weinstein, 1980) and most widely investigated targeting moieties in drug delivery because of their specificity and high availability, though their efficient use is challenged by a number of factors which must be considered while formulating the targeted therapy. Firstly, antibodies usually have large molecular weights (Mwts) which would impede the surface conjugation process and add to the size of the nanosystem (Bartlett, Su, Hildebrandt, Weber, & Davis, 2007). Secondly, conjugation of Abs to the surface of the nanosystem sometimes accelerate the process of clearance from blood circulation following *i.v.* administration (Weinberg et al., 2005). The third limitation is the sensitivity of these proteins to temperature, ionic strength, organic solvents and other synthesis related-factors which may restrict their stability and necessitates careful design of the conjugation method with incorporation of stabilizers (N. Bertrand, J. Wu, X. Y. Xu, N. Kamaly, & O. C. Farokhzad, 2014). Despite these challenges, many antibody-linked delivery systems have found their way to the clinical stages where scientists used smaller fragments of Abs to circumvent problems associated with the high Mwt and to extend their circulation time.
Targeting ligand	Nanocarrier type	Application	Reference
Folic acid	PLGA (poly lactic co-glycolic) polymer	Targeted delivery of docetaxel to cancer cells	(Poltavets et al., 2019)
GE11 peptide, an anti-EGFR (epidermal growth factor receptor)	Cationic polymer	Intracellular delivery with endosomal escape of SiRNA for cancer treatment	(G. Chen, Wang, Xie, & Gong, 2017)
Anti-Transferrin antibody	Liposome	Amyloid targeting in brain for Alzheimer diagnosis & treatment	(Mourtas et al., 2014)
Activated platelets scFv antibody	Microbubbles	Ultrasound guided thrombolysis	(X. Wang & Peter, 2016)
Cyclic RGD peptide	Liposomes	Targeting urokinase to thrombus	(N. P. Zhang et al., 2018)
Aptamer	Albumin nanoparticles	Theranostic system for cancer imaging and treatment	(Baneshi et al., 2019)

**Table 2.1.** Examples of some ligand-mediated targeted delivery systems from literature.

## 2.2. Stimuli-responsive drug delivery systems

Owing to the stochastic nature of ligand-mediated interactions at target site and potential leakiness of drug from the targeted nanocarriers in blood circulation to non-specific cells, tissues or organs, the need for more efficient tailored drug delivery systems capable to achieve on-demand drug release at target site has arisen. This on-demand release of drug at the target site is becoming feasible through the design of stimuli-responsive delivery systems (Table 2.2) which can sense triggers in the tissue microenvironment (endogenous stimuli) such as temperature change, pH, redox gradients, or enzyme concentration and undergo conformational or structural changes to release the drug accordingly. Exogenous stimuli like variation in temperature, magnetic field, electric pulses, ultrasound waves and light have been also investigated (Mura, Nicolas, & Couvreur, 2013).

S	timuli type	Responsive nanocarrier	Drug	Application	References
Endogenous stimuli	Acidity (pH)	PLGA- polyhistidine- PEG triblock copolymer nanoparticles	Vancomycin antibiotic	Selective delivery of antibiotic to bacteria at low pH	(Radovic- Moreno et al., 2012)
	Redox gradient	Dextran nanoparticles with disulphide linker to photosensitiser	Chlorin e6 (Photosensitiser)	NIR imaging and photodynamic therapy of cancer	(P. Liu et al., 2014)
	Enzyme	Biotinylated gold-capped mesoporous silica nanoparticles through protease sensitive linker	Doxorubicin (Chemotherapeuti c agent)	Doxorubicin release in response to cancer proteases.	(Eskandari et al., 2019)
Exogenous stimuli	Temperatur e variation	Thermosensitiv e lysolipid containing liposomes (Thermodox <sup>®</sup> )	Doxorubicin (Chemotherapeuti c agent)	Selective delivery to cancer with radiofrequenc y ablation.	(Needham, Anyarambhatla, Kong, & Dewhirst, 2000)
	Magnetic field	Fe <sub>3</sub> O <sub>4</sub> nanoparticles- capped mesoporous silica	Proteins	Magnetic driven protein delivery platform	(Gan, Zhu, Yuan, Liu, & nanotechnology , 2016)
	Ultrasonic waves	Microbubbles	Chemotherapeutic s & Thrombolytics	Ultrasound image-guided delivery	(Osei & Al- Asady, 2019), (X. Wang & Peter, 2016)
	Light	Gold coated liposomes	Calcein	CT- imaging and photothermal therapy of breast cancer.	(Rengan, Jagtap, De, Banerjee, & Srivastava, 2014)

 Table 2.2. Stimuli-responsive drug delivery systems from literature.

The next section discusses the thermo-responsive delivery system as a promising approach to achieve on-demand drug release.

## 2.2.1. Thermo-responsive nanocarriers

Thermo-sensitive systems are the most investigated approach to achieve on-demand drug release where temperature variation is the only type of stimulus that can occur endogenously as in tumour microenvironment or be achieved exogenously using external heating or cooling sources (Davoodi et al., 2018). These smart systems are based on the concept of incorporating nanomaterials which exhibit a sharp change of its conformational properties with temperature change. An ideal thermosensitive nanocarrier retains its payload at physiological temperature (~ 37 °C) and promptly releases drug at the locally heated or cooled target site. Liposomes (Y. Dou, K. Hynynen, & C. J. J. o. c. r. Allen, 2017) and polymeric nanoparticles (Bordat, Boissenot, Nicolas, & Tsapis, 2019) are the most common nanocarriers for these platforms design.

Thermo-responsiveness of polymeric systems, usually (poly (N-isopropyl acrylamide), PNIPAM), arises from the change in polymer hydration state at threshold temperature called the lower critical solubility temperature (LCST) resulting in volume changes and subsequent drug release (Davoodi et al., 2018). Despite being the preferred building block polymer for thermosensitive polymeric nanocarriers, PNIPAM shows liquid to gel transitions at 32 °C which necessitates the incorporation of other co-polymers to elevate this transition temperature for spatial release at pathological or induced hyperthermia. Other polymeric nanomaterials like poly-ε-caprolactone have demonstrated higher transition temperatures allowing for drug release at mild hyperthermia (~ 40-42 °C) (Y. Cheng et al., 2012).

Thermo-sensitive liposomes (TSLs) can be formulated through the assembly of phospholipids with an average gel to liquid crystalline transition temperatures  $(T_m)$ 

slightly above the physiological temperature such as 1,2-dipalmitoyl-sn-glycero-3phosphocholine (DPPC,  $T_m \sim 41$  °C), where mild hyperthermia can increase the permeability of the phospholipid bilayer through phase transition from gel to liquidcrystalline state to release its payload (Yannan Dou et al., 2017). Liposomes as thermosensitive nanocarriers have found their way to clinical trials specially after Dewhirst and Needham breakthrough in 2000, when they succeeded in formulating a doxorubicin loaded thermosensitive liposomes with lysolipid which enabled the release of 80% of drug in 20 seconds at mild hyperthermia (Needham et al., 2000). Their formulation was afterward commercialised under the name Thermodox<sup>®</sup> by Celsion Corporation (Corporation, 2019) for cancer treatment.

# 2.2.2. Light and magnetic field as external stimuli for hyperthermia induction

Externally focussed hyperthermia provides a more reliable tool for local temperature control at target site which can be induced via exposure to light, magnetic field, and radiofrequency waves (Table 2.3).

Light as an exogenous release trigger is highly attractive as it can be applied with a high degree of spatiotemporal accuracy and controllable intensity (Mura et al., 2013). Near-infrared light in the first window (NIR-I) (700-1000 nm wavelength) has been studied for local hyperthermia induction (Ong et al., 2017) (A. Li et al., 2017) (L. An, Y. Wang, Q. Tian, & S. Yang, 2017) and biomedical imaging applications (Hong, Antaris, & Dai, 2017). NIR wavelengths are characterised by their high tissue penetration ability (**Figure 2.2**) which enable them to stimulate nanosystems in internal organs with minimal damaging effects to human tissues (Beaute, McClenaghan, & Lecommandoux, 2019).



**Figure 2.2. Tissue penetration of light stimulus.** a) Absorption spectra of oxyhaemoglobin (red) and deoxyhaemoglobin (blue) through a 1-mm-long path in human blood and b) Scattering coefficients of different biological tissues and of intralipid scattering tissue phantom as a function of wavelength in the 400–1,700 nm region. Adapted with permission (Hong et al., 2017).

Moreover, the capability of plasmonic materials such as gold (Au) nanostructures to convert the photon energy absorbed during NIR-I (700-1000 nm wavelength) irradiation into heat allowed the widespread use of NIR light for photothermal anticancer therapy (Nouri et al., 2019), photothermally controlled drug release (W. Z. Song et al., 2016) and neural stimulation (Eom et al., 2016). Due to their strong tuneable surface plasmon resonance (SPR) absorption, gold nanorods (AuNRs) have attracted attention as NIR-light mediated multifunctional platforms for many biomedical applications (L. An et al., 2017).

Magnetically-induced hyperthermia is another approach for local temperature increase through applying an external alternating magnetic field (AMF) to magnetic nanoparticles such as superparamagnetic iron oxide (Fe<sub>3</sub>O<sub>4</sub>) which can be simultaneously utilised for magnetic resonance imaging (MRI) (Gu, Wang, Toh, & Chow, 2018). DNA-capped Fe<sub>3</sub>O<sub>4</sub> mesoporous silica nanoparticles have been developed by Y. Zhu et al. for temperature controlled drug release and magnetic hyperthermia induction under alternating magnetic field (Y. Zhu & Tao, 2015). Another study reported the use of magnetic polymeric nanocarriers for targeted delivery of curcumin to tumours via magnetically-induced hyperthermia (Kuo, Liu, Hardiansyah, & Chiu, 2016).

N respo	NIR / Magnetic- onsive material	Delivery system design	Drug	Application	Reference
<b>NIR-responsive</b>	Gold (Au) nanoensembles	Au nanoensembles with oligonucleotide capped silica shell	siRNA	Gene delivery to cancer	(Chang et al., 2012)
	Indocyanine Green (ICG)	Folic acid labelled - ICG loaded block co-polymer nanocapsule	Doxorubicin HCl	Chemo- photothermal targeted therapy for cancer	(A. Li et al., 2017)
	Gold nanostars (AuNS)	AuNS coated thermosensitive liposomes	Calcein model drug	Photoacoustic imaging and drug release applications	(Sivasubrama nian et al., 2017)
	Gold Nanorods (Au-NRs)	AuNRs conjugated to thermosensitive liposomes	Tetrodotoxin and Dexmedeto midin	Ultrasensitive photo-triggered local anaesthesia	(C. Zhan et al., 2017)
<b>Magnetic-responsive</b>	Fe <sub>3</sub> O <sub>4</sub> nanoparticles	DNA-capped Fe <sub>3</sub> O <sub>4</sub> mesoporous silica nanoparticles	Doxorubicin	Controlled drug release to cancer	(Y. Zhu & Tao, 2015)
	Fe <sub>3</sub> O <sub>4</sub> -NH <sub>2</sub> Nanoparticles	Fe <sub>3</sub> O <sub>4</sub> -NH <sub>2</sub> grafted on the surface of tri- block co-polymer	Curcumin	hyperthermia	(Kuo et al., 2016)

Table 2.3. Summary for some of NIR/ magnetic-assisted thermal drug delivery.

# 2.2.3. Clinical trials of hyperthermia-assisted systems

Despite the tremendous preclinical studies in the area of stimuli-responsive systems, few nanodelivery systems reached different stages of clinical development. Thermosensitive and NIR light-responsive systems are well presented in those clinical trials. ThermoDox<sup>®</sup>, a doxorubicin-loaded thermosensitive liposome, was the first

thermosensitive liposomal formulation to reach phase III clinical trial in a study called HEAT. This study investigated the use of radiofrequency ablation (RFA) in combination with ThermoDox® to achieve site-directed release of doxorubicin to hepatocellular carcinoma (HCC). Unfortunately, ThermoDox<sup>®</sup> failed to show progression-free survival in comparison to RFA alone (Y. Dou, K. Hynynen, & C. Allen, 2017). Now, ThermoDox<sup>®</sup> is being re-evaluated in Phase III clinical trial called OPTIMA study. Here, ThermoDox<sup>®</sup> is used in combination with standardized RFA (45 min) for treating lesions three to seven centimetres, versus optimized RFA alone (OPTIMA, 2022). Another clinical trial investigated the use of photothermal ablation to treat atherosclerosis using NIR light-responsive plasmonic nanoparticles in a study called NANOM-FIM. The study demonstrated high safety with better rate of mortality through targeted lesion revascularisation in comparison to stent therapy (Kharlamov et al., 2017). A recent phase-I clinical trial explored the use of ultra-focal photothermal ablation of gold-silica nanoparticles to treat cancerous prostatic tumours. Nanoparticles-mediated focal laser ablation of prostate tumours presented a success rate of 94% (15/16) of patients with reduced risk of treatment-related side effects (Rastinehad et al., 2019).

Those clinical trials, among others, provide evidence to the potential of using thermosensitive and NIR-light responsive systems to treat serious diseases and address unmet needs in nanomedicine.

# 2.3. Smart delivery of plasminogen activators for efficient thrombolysis

Many nanotherapeutics (up to 51 products by 2016) have been approved by the FDA (Bobo et al., 2016) for commercialisation achieving minimal toxic side effects. A brief PUBMED search for targeted nanomedicines related studies, done in 2019 by Ana Cartaya et al., revealed a substantial discrepancy between studies conducted on cancer and cardiovascular diseases (CVD) in the past 5 years as shown in (Figure 2.3). Though experts expect that cardiovascular research will soon witness a shift towards these novel modalities for both treatment and diagnosis applications.



**Figure 2.3.** Number of publications in PUBMED through the past 5 years (2014–2018) comparing CVD to cancer targeted nanotherapies. Adapted with permission (Cartaya, Maiocchi, & Bahnson, 2019).

In the next section we will describe in detail the latest advances in the field of nanomedicine for thrombosis treatment using plasminogen activators as the gold standard clinical treatment of acute thrombo-embolic events.

## **2.3.1.** The need for nanodelivery systems for thrombosis

Haemostasis, mediated by platelet activation and blood coagulation, is the main physiological defence mechanism to stop bleeding after blood vessel injury. However, a pathological deviation of haemostasis in the form of intravascular clotting and subsequent occlusion results in thrombosis. Ischemic myocardial infarction and stroke can be fatal sequels of acute arterial thrombosis (N. Mackman, 2008; Palasubramaniam, Wang, & Peter, 2019). Likewise, pulmonary embolism, caused by a dislodged deep vein thrombus (DVT), is reported to be a major cause of mortality (Goldhaber & Bounameaux, 2012). In clinical practice, timely reperfusion of the occluded vessel can be achieved through intravenous (*i.v.*) administration of fibrinolytic drugs or with interventional percutaneous or surgical recanalisation. Plasminogen activators (PAs) such as streptokinase (SK), tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) are the primary fibrinolytics used for patients presented within the appropriate time window (D. Disharoon, D. W. M. Marr, & K. B. Neeves, 2019). Both uPA and tPA activate plasminogen directly, through proteolytic cleavage of Arg561-Val562 peptide bond, into active plasmin which subsequently initiates a fibrinolytic cascade. Meanwhile, SK lacks intrinsic enzymatic activity and activates plasminogen indirectly by complexation (S. Koudelka, R. Mikulik, J. Masek, et al., 2016).

PA-mediated fibrinolysis faces two major limitations: limited clot lysis efficiency and side effects, mainly bleeding complications, associated with substantial mortality and morbidity (J. D. McFadyen, Schaff, & Peter, 2018). The hydrophilic nature and low molecular weight (Mwt) of PAs contribute to their rapid renal clearance and short biological half-lives (t<sub>1/2</sub>) (Kuriakose & Xiao, 2020). For instance, the initial t<sub>1/2</sub> of alteplase (Activase<sup>®</sup>), an FDA-approved recombinant human tPA is less than 5 min ("RxList, Activase drug description,"). Further, plasminogen activator inhibitors (PAI-1

and PAI-2) are occasionally overexpressed and can lead to inactivation of the PAs. These two factors make large doses often necessary for strong thrombolytic effects. Larger administration doses can interfere with physiological haemostasis, resulting in a high risk of bleeding complications that imposes strict eligibility criteria for *i.v.* administration of PAs. In the case of myocardial infarction, percutaneous coronary intervention (PCI), using balloons and stents, has proven superior to intravenous thrombolytics (Palasubramaniam et al., 2019). Very recently, stroke interventional approaches based on clot aspiration have also been successful (Munich, Vakharia, & Levy, 2019). However, these interventional approaches are not readily available in sub-urban clinical settings or in countries with less developed healthcare systems.

Advances in nanoscale systems have led to new approaches to improve delivery of PAs. Encapsulation or immobilisation of PAs into nanoengineered polymeric or lipid-based delivery systems can effectively prolong their circulation time, mask immunogenicity, and enhance their stability in vivo (D. Disharoon et al., 2019; S. Koudelka, R. Mikulik, J. Mašek, et al., 2016; M. Zamanlu et al., 2018). Targeting of PAs to the thrombus microenvironment through conjugation to targeting moieties can also substantially decrease off-site bleeding side effects, achieving better therapeutic outcomes at lower doses (Pietersz, Wang, Yap, Lim, & Peter, 2017; N. Zhang, C. Li, et al., 2018). Ondemand PA release at thrombus site in response to external triggers such as magnetic fields and light, has also been described as a novel potentially bleeding-free therapy (C. H. Liu, H. L. Hsu, J. P. Chen, T. Wu, & Y. H. Ma, 2019; X. L. Wang et al., 2017). These external stimuli can also provide synergistic thrombolysis through mechanical or thermal clot ablation (A. Y. Prilepskii et al., 2018; N. Singh, A. Varma, A. Verma, B. N. Maurya, & D. Dash, 2016). Furthermore, theranostic platforms combining diagnostic and therapeutic agents have recently attracted attention as image-guided therapeutic tools to monitor and treat thrombo-embolic diseases (Pietersz et al., 2017).

Here, we first outline the different drug delivery systems that have emerged to improve the therapeutic outcomes from PAs. We then highlight the most abundant molecular targets identified in literature for the efficient delivery of thrombolytic drugs. Targeted, stimuli-mediated thrombolysis, as a state-of-the-art delivery approach, is discussed in detail, focusing on its advantages and translational limitations.

# 2.3.2. Types of plasminogen activator nanodelivery systems

Nanodelivery systems of PAs can be classified into three subclasses according to the type of nanocarrier used: 1) macromolecule conjugates/nanoconstructs, 2) particulate nanocarriers, and 3) biological/bio-inspired systems. These nanocarriers can be further decorated with targeting moieties and/or designed to respond to internal or external stimuli (Figure 2.4).



**Figure 2.4.** Illustrative diagram of PAs nanodelivery systems highlighting thrombus-targeting and different types of stimuli-assisted thrombolysis.

## Macromolecules-conjugates and nanoconstructs

One of the first trials towards improved delivery of PAs included their direct chemical conjugation to polyethylene glycol (PEG) and fibrin-targeting monoclonal antibodies (mAb) for prolonged circulation and bleeding-free enhanced fibrinolysis, respectively. A 1988 early study showed extended  $t_{1/2}$  for radiolabelled PEG-tPA in rat and mouse blood (H. J. Berger & S. Pizzo, 1988). Another early work by Runge et al. studied the thrombolytic efficiency of tPA coupled to an anti-fibrin antibody by disulphide bond in

neutral pH in a rabbit jugular vein model. The tPA-antibody conjugate was at least 2.8 times more potent than tPA alone (Runge, Bode, Matsueda, & Haber, 1987a). Albumin conjugates with PAs have also been shown to prolong circulation (Shahriar Absar et al., 2014; Breton et al., 1995).

Despite the promising results from PAs conjugates, non-specific coupling and reduced stability during coupling reactions are common drawbacks of chemical modification of PAs that affect therapeutic activity and antibody functionality. Enhanced immunogenicity, aggregation tendency and poor penetration are also observed from monoclonal antibody conjugates. As alternative, smaller fragments of antibodies and peptides allow diminished immunogenicity, easier production and higher stability (Victoria L. Stefanelli & Thomas H. Barker, 2015). Enzymatic bioconjugation can also overcome the limitations of chemical conjugation methods and provide robust and sitespecific coupling; such as staphylococcus aureus sortase-A enzyme-mediated conjugations (Ta et al., 2011). Nanoconstructs of recombinant PAs fused to targeting single-chain variable fragment antibody (scFv), produced by genetic engineering, have shown promising thromboprophylaxis and thrombolysis in pre-clinical models (Rudy E. Fuentes et al., 2016; Xiaowei Wang et al., 2014b). The recombinant single chain urokinase (scuPA)-scFv nanoconstructs engineered by Wang et al.(Xiaowei Wang et al., 2014b) prevented FeCl<sub>3</sub>-induced carotid artery thrombosis in C57BL/6 mice following prophylactic administration of small doses with no prolonging of tail bleeding time.

## **Particulate nanocarriers**

Particulate nanocarriers offer many advantages over direct conjugates for drug delivery including, but not limited to, drug stabilisation, higher drug loading capability, controlled payload release and potential for surface functionalisation without interference with

therapeutic activity of loaded drug. Physical encapsulation and surface immobilisation of PAs have been investigated using different nanocarriers such as polymeric nanoparticles (Juenet et al., 2018; Netanel Korin et al., 2012), liposomes (Yu Huang et al., 2019; J.-Y. Kim, J.-K. Kim, J.-S. Park, Y. Byun, & C.-K. Kim, 2009; C.-H. Liu et al., 2019; Stepan Koudelka & Andrew D. Miller, 2016; Tiukinhoy-Laing, Huang, Klegerman, Holland, & McPherson, 2007; N. P. Zhang et al., 2018), microbubbles (Ebben, Nederhoed, Lely, Wisselink, & Yeung, 2017; X. Wang & Peter, 2016), hydrogels, dendrimers (Mukhametova et al., 2017; Xiangtao Wang, Inapagolla, Kannan, Lieh-Lai, & Kannan, 2007) and others, to control their biodistribution and stabilize them against rapid clearance and possible inactivating enzymes. Surface modification (such as PEGylation) of nanocarriers prolongs circulation lifetimes, resulting in enhanced fibrinolytic activity, especially when conjugated to targeting antibody fragments.

Polymeric nanostructures offer a robust versatile platform for encapsulation and immobilisation of PAs, driven by the recent advances in material science and polymer synthesis techniques. For instance, tPA immobilised to poly (ethylene glycol)-poly(caprolactone) (PEG-PCL) polymeric nanoparticles (tPA-NP) revealed 10-fold dose reduction and 18 times extended half-life compared to free tPA (Jun Deng et al., 2018). Another polymeric system of polysaccharide-poly (isobutylcyanoacrylate) nanoparticles, loaded with tPA and functionalised with fucoidan-targeting moiety, was designed, and tested by Juenet et al. for enhanced targeted thrombolysis. The polymeric system showed that thrombus density decreases to 29.5% in a venous-thrombosis mouse model at 30 min after *i.v.* injection (Juenet et al., 2018). While synthetic polymeric nanoparticles have long shelf lives, they are limited by potential cytocompatibility and biodegradability issues that must be systematically assessed before their clinical use. Natural polymers such as

gelatin and chitosan have also been studied as potential biocompatible carriers for tPA (Uesugi, Kawata, Saito, & Tabata, 2012) (Chung, Wang, & Tsai, 2008).

Liposomes have been widely used for PAs delivery due to their biomimetic nature, low immunogenicity, ease of surface functionalisation, biodegradability and potential of coencapsulating other therapeutics and imaging probes. These include, for example, neuroprotective agents (Fukuta, Yanagida, Asai, & Oku, 2018), air bubbles in echogenic liposomes (Tiukinhoy-Laing et al., 2007), or inorganic nanoparticles (such as iron oxide) in magneto-liposomes (C. H. Liu et al., 2019) for image-guided stimuli-mediated thrombolysis. Conventional, PEGylated, and targeted liposomes have been studied for the delivery of PAs. Prolonged circulation and reduced uptake of liposomes by the reticulo-endothelial system are immediately achieved through the incorporation of 1,2distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol (DSPE-PEG) phospholipid in the lipid formulation at only 4-5 mol% (Woodle et al., 1992). An example is the PEGylated liposomal tPA reported by Kim et al., which showed a 21-fold increase in circulation half-life over free tPA in rats (J. Y. Kim, J. K. Kim, J. S. Park, Y. Byun, & C. K. Kim, 2009). Surface decoration of PEGylated liposomes with targeting moieties was further studied to ameliorate bleeding adverse effects of PAs and lower the administered doses. In a recent study by Zhang et al, significant reduction in tail-bleeding time was observed from uPA loaded, cyclic Arg-Gly-Asp peptide (cRGD)-labelled PEGylated liposomes in a mouse thrombus model. Compared to free uPA, equivalent thrombolysis was achieved with only 25% of the uPA dose (N. Zhang, C. Li, et al., 2018). However, low encapsulation efficiency and low storage stability of liposomal PAs remain major challenges for their clinical translation. Freeze thawing techniques and incorporation of cationic lipids have been reported to enhance PAs loading into liposomes (S. Koudelka, R. Mikulik, J. Mašek, et al., 2016; S. Liu, Feng, Jin, & Li, 2018; S. Naeem,

Viswanathan, & Bin Misran, 2018). Moreover, lyophilisation with cytoprotectants, such as trehalose, can improve the shelf life of PA-loaded liposomes (S. Koudelka, R. Mikulik, J. Mašek, et al., 2016; Ntimenou, Mourtas, Christodoulakis, Tsilimbaris, & Antimisiaris, 2006).

### **Biological and bio-inspired delivery systems**

Another approach towards delivery of PAs to the thrombus site is based on their coupling to blood components such as red blood cells (RBCs), the most abundant blood cell type, which is characterised by relatively long life-span and inherent homing properties (Villa, Anselmo, Mitragotri, & Muzykantov, 2016; Yoo, Irvine, Discher, & Mitragotri, 2011). Binding to or hitchhiking RBCs is reported to alter the functional profile of PAs and render them thrombo-prophylactic rather than thrombolytic (Kristina Danielyan et al., 2008; Murciano, Higazi, Cines, & Muzykantov, 2009; Zaitsev et al., 2010). This alteration can be of great clinical importance for preventing stroke and myocardial infarction, especially in high-risk patients. Danielyan et al. used the biotinstreptavidin conjugation method to couple tPA to RBCs ex vivo and then tested the tPA-RBCs conjugate in a mouse model of cerebral thromboembolism (Kristina Danielyan et al., 2008). Three advantages were observed following in vivo administration of this system. First, the relatively large size of RBCs (~7 µm) limits their mobility into preexisting haemostatic clots. Second, central nervous system toxicity and brain damage are mitigated by the restricted extravasation of tPA when coupled to RBCs. Finally, tPA carriage by RBCs significantly prolongs its circulation time and allows for in situ entrapment inside new intravascular clots to be lysed from within (Kristina Danielyan et al., 2008). Technical challenges, including cell stability, hemocompatibility issues and the need for autologous RBCs in most cases, complicate the clinical translation of this approach (Colasuonno et al., 2018).

Better understanding of the mechanisms underlying thrombosis together with the advance in bioengineering tools has encouraged researchers to develop bio-inspired systems towards more efficient PA delivery. As a recent example, the platelet microparticle-inspired nanovesicles (PMIN) designed by Pawlowski et al. (Figure 2.5a-b) use a multivalently ligand-decorated 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) liposomal platform to dual-target activated platelet microparticles towards thrombi. The streptokinase payload was released from the particles in response to the clot-abundant phospholipase-A<sub>2</sub> (PLA<sub>2</sub>) enzyme (Pawlowski et al., 2017). Similar thrombolytic effects were observed from SK-loaded PMIN and free SK in a FeCl<sub>3</sub>-induced carotid thrombosis mouse model. While tail bleeding time was dramatically prolonged in mice receiving free SK, targeted SK-loaded PMIN had no significant effect on haemostasis.

Erythrocyte-inspired PA delivery systems have been also investigated for thrombolytic applications. RBC mimetic systems allow for better penetration into the lungs and splenic parenchyma small capillaries due to their deformability under high shear stress. **Figure 2.5c-d** shows an example of tPA-loaded discoidal porous polymeric nanoconstructs (1  $\mu$ m) prepared by Colasuonno et al. via laser writing lithography (Colasuonno et al., 2018). The porous matrix was able to encapsulate tPA efficiently and preserve ~ 70% of its fibrinolytic activity after exposure to serum proteins for 3 h. Moreover, the shape and deformity of the nanoconstructs allowed for recanalisation of murine mesenteric venules at 1/10 of the clinical dose of tPA.



**Figure 2.5. Bio-inspired delivery systems of plasminogen activators.** a) Structure of platelet derived-microparticles (PMP) showing characteristic surface receptors. b) Design of the platelet microparticle inspired nanoliposomes (PMIN) showing dual- targeting ligands and PLA<sub>2</sub> degradable phospholipid membrane. Adapted with permission from (Pawlowski et al., 2017) Copyright 2017 Elsevier. c) Schematic presentation of erythrocyte-inspired polymeric nanoconstruct of tPA showing the crosslinked porous structure. d) TEM image (left side) and SEM image (right side) showing the discoid structure and dimensions of the nanoconstruct mimicking RBCs. Adapted with permission from (Colasuonno et al., 2018) Copyright (2018) American Chemical Society.

## 2.3.3. Thrombosis targeting

The results discussed in the previous section provide evidence that thrombosis targeting is crucial to reduce the total dose of PAs and ameliorate any off-site bleeding. To date, multiple potential target epitopes such as fibrin and activated platelets have been identified for diagnostic and therapeutic targeting of thrombi. We will outline and discuss

the predominant molecular epitopes (Table 2.4) for thrombosis targeting below.

Site	Molecular target/epitope	Ligand	Ref.
Platelets	GP IIb/IIIa receptor (integrin αIIbβ3)	RGD peptide	(Y. Huang et al., 2019; Pawlowski et al., 2017; N. Zhang, C. Li, et al., 2018)
		scFv	(R. E. Fuentes et al., 2016; Pietersz et al., 2017; X. Wang & Peter, 2016; Zia et al., 2020)
	P-selectin	Fucoidan EWVDV peptide PSN peptide	(Juenet et al., 2018; B. Li et al., 2017) (Pawlowski et al., 2017)
	Phosphatidylserine	Annexin V	(X. Chen et al., 2018)
Fibrin	DD-Dimer domain, DD (E) complex, Fibronectin binding site, Knobs "B" and Knobs "A"	mAb, antibody fragments and Peptides	<ul> <li>(Pan et al., 2017; Sarda-Mantel et al., 2006)</li> <li>(Douketis et al., 2012; Kolodziej et al., 2012; Runge, Bode, Matsueda, &amp; Haber, 1987b; V. L. Stefanelli &amp; T. H. Barker, 2015)</li> </ul>

**Table 2.4.** Molecular targets and targeting ligands of activated platelets and fibrin covered in this review.

## Activated platelet targeting

Platelets play a key role in many pathophysiologic events, being involved in haemostasis, thrombosis, wound healing, inflammation, and cancer development (Estevez, Shen, & Du, 2015; Franco, Corken, & Ware, 2015; X. R. Xu, Yousef, & Ni, 2018). Under normal physiological conditions, resting platelets act as sentinels for vascular integrity. They are promptly activated in response to vascular injury, adhere to the injured vascular wall and ultimately seal the injury to maintain haemostasis. However, activation and aggregation of platelets can result in pathological events of thrombosis and

consequent ischemia (Yifei Lu, Quanyin Hu, Chen Jiang, & Zhen Gu, 2019; Nigel Mackman, 2008). Targeting activated platelets offers a logical approach for efficient delivery of antithrombotic and thrombolytic agents.

The most attractive targeting strategy of activated platelets explores the highly expressed glycoprotein GPIIb/IIIa ( $\alpha$ IIb $\beta$ 3) complex, which is the most abundant adhesion receptor on the surface of activated platelets (~ 60,000 to 80,000 receptors per platelet). Upon platelet activation, a calcium-dependent conformational change of GPIIb/GPIIIa occurs, exposing the high-affinity GPIIb/IIIa complex, which mediates binding to fibrinogen, fibrin, and other ligands (Armstrong et al., 2012; Saboor, Ayub, Ilyas, & Moinuddin, 2013). Several peptides and antibody fragments have been developed to bind to GPIIb/IIIa, including the fibrinogen mimetic RGD peptides and analogs (Cierniewski et al., 1999; Hu et al., 2012; G. Huang et al., 2008; Plow, D'Souza, & Ginsberg, 1992), the antibody fragment abciximab (Coller, 1999; Schwarz, Nordt, Bode, & Peter, 2002) and the scFv antibody generated by Schwarz et al. in 2006 (Meike Schwarz et al., 2006).

The RGD analogues and abciximab are not activation-specific and thus can bind to non-activated circulating platelets. They have also been shown to bind to several other integrins (Xiaowei Wang & Karlheinz Peter, 2017). Despite the non-specificity and cross-reactivity, successful thrombosis targeting has been reported using RGD analogs (Cierniewski et al., 1999; Hu et al., 2012; G. Huang et al., 2008; Plow et al., 1992) and Abciximab (Coller, 1999; Schwarz et al., 2002) for both molecular imaging and therapeutic purposes. This can be attributed to the accumulation of platelets in the thrombus that allows a relative targeting to the thrombus compared to circulating less concentrated platelets (Xiaowei Wang & Karlheinz Peter, 2017). On the other hand, the scFv antibody has shown specific binding to activated GPIIb/IIIa for specific targeting of

activated platelets. This scFv has been used highly successfully for molecular imaging (Ardipradja et al., 2014; Duerschmied et al., 2011; Elverfeldt et al., 2014; Heidt et al., 2011), targeted drug therapy, and theranostic approaches (Xiaowei Wang et al., 2016; Xiaowei Wang et al., 2014a) in several murine models of thrombosis and myocardial infarction.

Another receptor overexpressed on activated platelets (13,000 receptors per activated platelet) is the glycoprotein P-selectin (CD62P) which translocates from cytoplasmic  $\alpha$ -granules to the surface of the platelet membrane upon platelet activation (Z. Li & Smyth, 2019). Targeting of P-selectin has been investigated using fucoidan, a naturally occurring polysaccharide, which mimetics P-selectin glycoprotein ligand 1 (PSGL-1) on neutrophils (Chollet et al., 2016; Juenet et al., 2018). Further, several P-selectin-binding peptides have been engineered such as EWVDV and PSN sequences (Appeldoorn et al., 2003; X. Chen et al., 2018; Pawlowski et al., 2017). Early stages of platelet activation cause surface exposure of the intramembrane phosphatidylserine (PS), contributing to thrombus formation through interaction with blood coagulation factors (Whyte et al., 2015). Annexin V, a protein that specifically binds to PS, has been studied to target nanoparticles to thrombus sites for imaging applications and targeted thrombolysis (Pan et al., 2017; Sarda-Mantel et al., 2006)

## Fibrin as a thrombus-targeting epitope

Fibrin deposition is the hallmark for a variety of pathological conditions, including cancer, inflammatory conditions, neurodegenerative diseases, and notably atherothrombosis. Molecular targeting of fibrin has been studied to develop more reliable diagnostics and to enhance biotherapeutics delivery (Victoria L. Stefanelli & Thomas H. Barker, 2015). The success of different fibrin targeting approaches has been challenged

by the non-selective binding to its precursor fibrinogen, which circulates in blood at a concentration of 2-4 mg/mL and has a very similar structure to fibrin (98%) (Mosesson, 2005). Anti-fibrin mAb, antibody fragments, and small peptides engineered to specifically bind to fibrin-specific domains, including DD dimer, DD(E) complex, fibronectin binding sites, Knobs A and Knobs B, have overcome this limitation (Kolodziej et al., 2012; Marsh et al., 2011; Victoria L. Stefanelli & Thomas H. Barker, 2015). Despite being fibrin-specific structures, each of these molecular targets has its drawbacks. For instance, clot lysis can result in dispersion of the crosslinked DD-dimers of fibrin to the systemic circulation as free DD-dimers, which would interfere with the specific targeting. High levels of free DD-dimers, which have been detected in serum of pregnant or elderly patients, can reduce the specificity of the targeting (Kabrhel et al., 2010). Also, targeting fibrin knobs can be hindered by co-administration of anticoagulants such as heparin, which work through blocking fibrin polymerisation (Morris et al., 1997). That is the reason why, fibrin Knobs targeting may not be optimal for patients stabilised with anticoagulant therapies.

## 2.3.4. Stimuli-mediated thrombolysis

From a safety point of view, PAs enzymatic activity is better camouflaged/masked in systemic circulation and regenerated locally at the thrombus site through selective activation or site-specific release from nanocarriers. Several stimuli have been studied for the selective delivery of PAs and imaging probes to thrombosis including, both internal, such as enzymes, high shear stress, activated platelets or reactive oxygen species (Ziegler et al., 2019) and external such as ultra-sound waves, magnetic fields or near infrared (NIR) light.

#### Enzyme-triggered delivery system

Nanoconstructs of PAs with thrombin enzyme cleavable sites have been developed for both thrombo-prophylactic and therapeutic applications. Following preferential accumulation at the thrombus site driven by targeting moieties, PAs-prodrug activation or PAs release is achievable via cleavage of thrombin-sensitive sites, which is facilitated by the high abundance of active thrombin in thrombi (Stalker et al., 2014). A thrombinactivatable low-molecular weight prodrug of uPA, fused to an anti-GPIIb/IIIa scFvantibody was engineered and tested for thromboprophylaxis in murine models by Fuentes et al. (R. E. Fuentes et al., 2016). Studies on nascent thrombi versus pre-existing thrombi showed the prodrug could selectively prevent the development of FeCl<sub>3</sub>-induced nascent thrombi while sparing pre-existing clots induced by tail-clip injury (Rudy E. Fuentes et al., 2016). Absar et al. developed another thrombin-sensitive tPA delivery system for targeted thrombolysis by linking tPA to human serum albumin (HSA) via a thrombincleavable peptide (Figure 2.6). The nanoconstruct surface was further conjugated to a targeting peptide (CQQHHLGGAKQAGDV) derived from the C-terminal gamma-chain peptide sequence of fibrinogen that binds to GPIIb/IIIa on platelets. The enzymatic activity of tPA was camouflaged via a steric hindrance effect of albumin and reactivated in the presence of thrombin (S. Absar, Y. M. Kwon, & F. Ahsan, 2014).

PLA<sub>2</sub>, a phospholipid-metabolizing enzyme involved in cancer development (Sukocheva et al., 2019), atherogenesis, thrombosis and acute myocardial infarction (Olson et al., 2008; Takahashi et al., 2013), has been also utilised to release a drug payload from lipid carriers, specifically to target cancer and thrombosis (Arouri et al., 2015; Pawlowski et al., 2017; Sharipov, Tawfik, Gerelkhuu, Huy, & Lee, 2017; G. Zhu, Mock, Aljuffali, Cummings, & Arnold, 2011). Platelet microparticles-inspired nanovesicles (discussed under section 2.3) represent a PLA<sub>2</sub>-responsive liposomal delivery system of

SK (Pawlowski et al., 2017) where enzymatic cleavage of the sn-2 ester bonds in glycerophospholipids destabilizes the liposomes to release their SK payload. The cumulative release of SK from liposomes was four times higher in the presence of PLA<sub>2</sub>.



**Figure 2.6. Thrombin-sensitive camouflaged targeted tPA nanoconstruct.** a) Structure of the nanoconstruct showing albumins attached to tPA *via* a thrombin-cleavable peptide. b) Steric hindrance of tPA activity in systemic circulation followed by binding to activated platelets by homing peptides and thrombin-mediated release of tPA at thrombus site. c) Thrombin-mediated regeneration of camouflaged tPA activity was evaluated using chromogenic substrate S-225. d) *In vivo* thrombolysis study of free tPA and tPA nanoconstruct performed in inferior vena cava (IVC) rat thrombosis model. e) Fibrinogen levels in rat plasma measured by an ELISA sandwich assay following different treatments. Adapted with permission from (Shahriar Absar et al., 2014). Copyright 2014 Elsevier.

#### Shear-activated nanoconstruct

One of the pathophysiological features of atherosclerotic/thrombosed vessels is the high shear stress, which can exceed 1000 dyne/cm<sup>2</sup> in highly constricted arteries, compared to that below 70 dyne/cm<sup>2</sup> under normal flow conditions. Platelet activation in response to this high shear stress contributes to the atherosclerotic/thrombotic cascade (D. Kim, Bresette, Liu, & Ku, 2019). A promising strategy to target PAs to thrombosed vessels is through their release from shear-stress-sensitive nanoconstructs. This approach was investigated by Korin et al. (Netanel Korin et al., 2012) fabricating microaggregates of multiple smaller tPA-coated PLGA nanoparticles using a spray-drying technique and biotin-streptavidin coating chemistry. These microaggregates maintained their structure under physiological flow conditions in normal blood vessels but dispersed into individual nanoparticles under high local shear stress in partially occluded vessels. Fluorescent-labelled tPA showed preferential accumulation of the shear-dispersed nanoparticles at the stenosed vessel with a fast vessel reopening at a 100-fold lower dose of comparable active tPA. No bleeding side effects were observed and rapid clearance of microaggregates from systemic circulation was confirmed in mouse models.

### Activated platelet sensitive delivery

Targeting activated platelets can significantly improve delivery of PAs through selective distribution to thrombi, as discussed in **section 2.3.3**. Activated platelets can also destabilize liposomal nanocarriers for PAs selective release. This hypothesis was recently investigated by Huang et al. (Y. Huang et al., 2019) using cRGD-labelled liposomal tPA as shown in (Figure 2.7). Binding of cRGD-labelled liposomes to platelets was confirmed using flow cytometry. Calcein dequenching assay and fluorescence resonance energy transfer (FRET) assay confirmed destabilisation of liposomal membrane upon incubation with activated platelets through membranes fusion mechanism after selective binding to activated platelet surface (Y. Huang et al., 2019). 90% of tPA was released within 1 h in the presence of activated platelets, compared to only 10% release in the presence of resting platelets. Enhanced fibrinolysis was also reported from tPA liposomes in the presence of activated platelets using a fibrin clot model.



**Figure 2.7. Platelet-sensitive release of tPA for targeted thrombolysis.** Illustration of the designed liposomal delivery system (tPA-PEG-cRGD-lip), thrombus targeting and subsequent release of tPA. Adapted with permission (Yu Huang et al., 2019). Copyright 2019 Elsevier.

#### Magnetic-field driven thrombolysis

Externally applied magnetic fields can drive PA-loaded magnetic nanocarriers to the vicinity of thrombi, including those deeper in the tissue, thanks to the high tissue penetration ability of magnetic fields (Sharma & Guha, 1975). Superparamagnetic iron oxide nanoparticles and their derivatives are highly biocompatible and represent the gold standard for magnetic guided delivery of PAs (Bi, Zhang, Su, Tang, & Liu, 2009; J.-P. Chen et al., 2016; Hsu & Chen, 2017; C.-H. Liu et al., 2019; Artur Y. Prilepskii et al., 2018; Zhou et al., 2014). Different forms of magnetic-PA hybrid nanosystems have been reported; including direct conjugates (Bi et al., 2009), core-shell structures (Artur Y. Prilepskii et al., 2018), magnetoliposomes (Hsu & Chen, 2017; C.-H. Liu et al., 2019) and polymeric nanocarriers (J.-P. Chen et al., 2016; Zhou et al., 2014). These hybrid nanosystems have been investigated for site-specific delivery of PAs to the thrombus site through magnetic targeting and magnetic-assisted release of PAs payload. Chen et al. (J.-P. Chen et al., 2016) used ionic crosslinking of chitosan to co-encapsulate tPA and magnetite nanoparticles into a biocompatible hybrid nanosystem. This hybrid nanosystem showed magnet-sensitive release of tPA both in vitro and in vivo through reversible pellet formation under magnetic field. Blood flow was restored at 20% of the regular dose of tPA following magnetic guidance to the clot site in a rat embolic model. The same group recently (C.-H. Liu et al., 2019) reported targeted delivery of PA from thermosensitive PEGylated-magnetoliposmes co-encapsulating tPA and iron oxide nanoparticles. Therefore, magnetic guidance followed by the triggered release of tPA from thermosensitve liposomes under focal magnetic hyperthermia represents a promising approach towards targeted thrombolysis.

Prilepskii et al. (Artur Y. Prilepskii et al., 2018) utilised heparin, a common anticoagulant, to crosslink urokinase on the surface of magnetite nanoparticles (MNPs), as shown in **Figure 2.8**. The fabricated nanocomposites showed higher fibrinolytic efficacy compared to urokinase alone, with no side effects or hemorrhagic complications detected at therapeutic levels in different pre-clinical animal models (Figure 2.8f).

Furthermore, alternating magnetic fields (AMF) can improve PAs penetration into thrombi through the mechanical dragging force created by the magnetic nanoparticles (R. Cheng et al., 2014; Torno et al., 2008) and the induced localised hyperthermia (Voros et al., 2015a). Cheng et al. reported on accelerated thrombolysis in a rat embolic model at low doses of tPA when combined with magnetically powered, rotating nanorods (nanomotors) (R. Cheng et al., 2014). Magnetic hyperthermia-assisted thrombolysis was investigated by Voros et al. (Voros et al., 2015a) using tPA immobilised on iron oxide nanocubes. The authors reported enhanced clot dissolution rates (at least 10-fold) under AMF-induced mild hyperthermia.



Figure 2.8. Urokinase-magnetite nanocomposites for magneto-thrombolysis a) Schematic representation of the fabrication process of MNPs@uPA nanocomposite with  $\sim 100$  nm hydrodynamic diameter. b) SEM images of the synthesised MNPs. c) In vitro flow-chamber thrombolysis of the model clot treated with free uPA, MNPs@uPA and MNPs@uPA + magnet. d) In vivo thrombolysis study performed on FeCl<sub>3</sub> carotid artery injury model where 1) Animal carotid artery was first isolated and monitored by doppler ultrasonography for blood flow intensity. 2) Clot formation was inducted by application of a FeCl<sub>3</sub>-soaked cotton for 15 min. 3) MNPs@uPA were injected close to the clot. 4) Blood flow in the carotid artery was monitored under magnet. e) Time to blood reperfusion and rate of blood flow 24 h post injection. Adapted with permission.(Artur Y. Prilepskii et al., 2018). Copyright (2018) American Chemical Society.

#### Ultrasound mediated thrombolysis

The use of ultrasound (US) waves in thrombosis detection, monitoring and treatment represents a very attractive area for clinical research because of its advantages as a non-invasive, real-time, and broadly available technology. Besides being a common imaging modality, low-frequency US has been investigated for thrombus disruption in a process referred to as sonothrombolysis, where three mechanisms of thrombolysis enhancement have been identified as: acoustic streaming, inertial cavitation and local temperature rise (Bader, Bouchoux, & Holland, 2016; X. Chen, Leeman, Wang, Pacella, & Villanueva, 2014; Dmitry V. Sakharov, Rob T. Hekkenberg, & Dingeman C. Rijken, 2000).

Sonothrombolysis showed therapeutic advantages as an adjunct therapy to PAs specially in cases of fully occluded vessel with no blood flow in the clot microenvironment (D. V. Sakharov, R. T. Hekkenberg, & D. C. Rijken, 2000). Synergistic rapid and complete thrombolysis at lower doses of PAs has been achieved with the introduction of ultrasound contrast agents such as microbubbles (MB) and echogenic liposomes (Ebben et al., 2017; Marsh et al., 2011; Ren et al., 2015). Laing et al. (Susan T. Laing et al., 2011) reported on higher recanalisation rates from tPA-loaded echogenic liposomes in a rabbit aorta clot model when combined with 2 min pulsating doppler US. Another study by Flores et al. (Flores, Hennings, Lowery, Brown, & Culp, 2011) used MB-augmented sonothrombolysis in a rabbit model of acute ischemic stroke with and without intravenous tPA. The results showed diminished incidence of cerebral hemorrhage outside the infarct area with an infarct volume reduction comparable to tPA alone.

Targeting PA-loaded MB or liposomes to activated platelets via targeting ligands offers three advantages towards the treatment of thrombosis. First, rapid diagnosis and real-time monitoring of thrombolysis can be achieved using US imaging. Second, site-specific fibrinolysis can minimize off-site bleeding adverse effects. Third, the synergistic effect of US-contrast agents enhances PA biochemical effect at low clinical doses. An example is the targeted tPA-loaded MBs fabricated by Hua et al. (Hua et al., 2014) using an RGD targeting peptide. The system was tested for enhanced thrombolysis in a rabbit femoral artery thrombus model under US exposure to show higher recanalisation rates with lower doses of tPA. However, safety concerns of high-intensity low-frequency US application with microbubbles may be a major drawback to this approach. Another targeted PAloaded MB approach was innovated by Wang et al. (Xiaowei Wang et al., 2016) through dual-conjugation of both recombinant scuPA and an activated platelet targeting scFv to echogenic MBs. These targeted theranostic microbubbles (TT-MB) were tested in a FeCl<sub>3</sub>-induced thrombosis mouse model, and their efficacy was monitored using US imaging. TT-MB bind to thrombi in vivo and enable highly sensitive detection, as well as direct visualisation of TT-MB successfully breaking down the blood clot in real time. Overall, TT-MB allowed for a side effect-free theranostic strategy in a single approach, as shown in Figure 2.9.



**Figure 2.9.** Activated-platelet targeted theranostic microbubbles (TT-MB) (Xiaowei Wang et al., 2016). a) Monitoring of in vivo thrombolysis via molecular ultrasound imaging for 45 mins following injection of TT-MB and different treatments to FeCl<sub>3</sub>-induced thrombus model. (LIBS-MB are targeted microbubbles which bind to a ligand-induced binding site on activated GPIIb/IIIa complex) b) Thrombus size (%) after treatment with TT-MB and different controls. c) Molecular ultrasound images of thrombus at time of treatments injection and after 45 min showing the change in contrast intensity (%) after each treatment as per (d). e) Tail bleeding time measured by tail transection. f) Images for hematoxylin and eosin (H & E) stained thrombi in the carotid arteries of mice after different treatments: 1. after LIBS-MB and low dose commercial uPA, 2. after TT-MB administration with a partially lysed thrombus, 3. after LIBS-MB with saline as vehicle control and, 4. after high dose commercial uPA with a partially lysed thrombus.

## NIR-light mediated thrombolysis

Light as an exogenous trigger is highly attractive, especially as it can be applied with a high degree of spatiotemporal accuracy and controllable intensity (Mura et al., 2013). During the last decade, near-infrared light in the first window (NIR-I, 700-1000 nm wavelength) has been extensively used for anticancer photothermal therapy (L. An et al., 2017; A. Li et al., 2017; Ong et al., 2017) and real-time fluorescence imaging (Hong et al., 2017) encouraged by its high tissue penetration ability ( $\geq$  4 mm) and minimal damaging effects to human tissues (Beaute et al., 2019; Hudson, Hudson, Wininger, & Richardson, 2013). NIR-mediated photothermal thrombolysis has recently emerged as a new strategy for thrombus disintegration using gold nanostructures based on their surface plasmon resonance (SPR) properties which enable them to convert light into local hyperthermia (Dong et al., 2019; Shao, Abdelghani, Shen, Cao, Williams, & Hest, 2018; Shao, Abdelghani, Shen, Cao, Williams, & Loza, Williams, A. Verma, B. N. Maurya, & D. J. N. R. Dash, 2016; X. Wang et al., 2017a; Jiasheng Xu et al., 2020; Ting Yang et al., 2018). Dual-photothermal/photodynamic thrombolysis has also been reported using nanostructured porphyrin (F. Zhang, Liu, et al., 2019b).

In 2016, Singh et al. (Nitesh Singh et al., 2016) were the first to report on photothermal clot ablation using NIR irradiation (808 nm, 1.05 W/cm<sup>2</sup>) of gold nanorods (AuNRs). The generated local hyperthermia was tested for clot destabilisation using different fibrin clot lysis assays and a murine femoral vein thrombosis model. Up to ~ 16 % photothermal fibrinolysis was observed following 45 min NIR irradiation in fibrin clot models in the presence of AuNRs (18  $\mu$ g/mL), which resulted in a temperature increase to 50 °C. Confocal microscopy of fluorescence-labelled fibrin clots was also used to study molecular dynamics of fibrin monomers after 10 min NIR irradiation to confirm enhanced fibrin mobility and turnover kinetics within the thrombus. Addition of streptokinase at

sub-therapeutic doses to the photothermal therapy resulted in synergistic fibrinolytic effect (~ 40 % fibrinolysis) under arterial shear stress conditions in a parallel plate flow chamber. Restored blood flow in murine femoral vein-thrombosis models via *i.v.* injection of anti-fibrin conjugated AuNRs followed by local NIR irradiation of the clot site provided further evidence for the potential of photothermal thrombolysis. One of the major criticisms of NIR-induced photothermal thrombolysis is the risk of secondary embolism to small blood vessels by larger clot fragments (F. Zhang, Liu, et al., 2019b).

Other strategies have addressed this drawback combining photothermal ablation with thrombolytic agents or photodynamic therapy (Shao, Abdelghani, Shen, Cao, Williams, & Hest, 2018; Jiasheng Xu et al., 2020; F. Zhang, Liu, et al., 2019b). A NIR-driven biocompatible micromotor system was fabricated by Shao et al. through layer-by-layer assembly of chitosan and heparin followed by partial coating of the formed microcapsules with gold shells. Movement of the system was achieved through a self-thermophoresis effect upon pulsating NIR-irradiation for targeted photothermal ablation of clots. Efficient movement in biological fluids was augmented through coating with erythrocyte membranes. Photothermal thrombolysis was enhanced through site-specific release of heparin at the thrombus site (Shao, Abdelghani, Shen, Cao, Williams, & Hest, 2018). Besides, dual photothermal/photodynamic therapy has been recently explored by Zhang et al. using cRGD-labelled mesoporous carbon nanospheres containing porphyrin-like metal centres to combine hyperthermia and reactive oxygen species for efficient site-specific thrombolysis under NIR irradiation (F. Zhang, Liu, et al., 2019b).

Wang and co-workers developed another strategy for NIR-mediated thrombolysis combining gold nanoparticles and light-activated uPA release (X. Wang et al., 2017a; Jiasheng Xu et al., 2020; Ting Yang et al., 2018). Initially, uPA-loaded gold mesoporous silica core-shell nanospheres were developed and capped with thermosensitive

tetradecanol and tested for on-demand release of uPA upon NIR irradiation (808 nm, 5 W/cm<sup>2</sup>). Enhanced bleeding free-fibrinolysis was achieved in murine tail-thrombus model through synergistic photothermal-biochemical thrombolysis effect of gold and uPA, respectively (X. Wang et al., 2017a). Safety concerns of using high laser power density and reliability of the tail-thrombus model are the main criticism to this approach. Another study by the same group utilised platelet membrane-coated gold nanorods coloaded with uPA for NIR-assisted release of uPA in a murine pulmonary embolism (PE) model (T. Yang et al., 2018). In vitro blood clots showed maximal reduction in response to 30 min NIR irradiation (808 nm, 2 W/cm<sup>2</sup>) compared with treatments at 37 °C and 42 °C. Homing of platelet membrane coated gold nanorods to lungs in the murine PE model was confirmed using histological staining. However, NIR light's limited penetration ability into deep lung tissue and the unclear immune response to this system have restricted the use of the photothermal approach in vivo for pulmonary embolism treatment, and further studies are needed to address these limitations. Recently, the same group of researchers investigated bleeding-free NIR-triggered release of uPA from another gold mesoporous silica core-shell system capped with lauric acid and stearic acid eutectic mixture, as shown in Figure 2.10.



**Figure 2.10. NIR-mediated thrombolysis using UK-FA@Au@MSNs.** a) Scheme for the delivery system preparation and the proposed thrombolysis strategy. b) SEM image showing morphology of Au@MSNs. c) TEM image of the prepared Au@MSNs. d) Photothermal transduction of UK-FA@Au@MSNs under NIR irradiation (808 nm, 2 W/cm<sup>2</sup>). e) In vitro release study of UK from UK-FA@Au@MSNs at physiological temperature (37 °C) (black line) and under NIR irradiation (808 nm, 0.66 W/cm<sup>2</sup>) (red line). g) Ultrasound images of New Zealand white rabbit femoral vein thrombosis model under different

treatment (NIR power density was 2 W/cm<sup>2</sup> for *in vivo* thrombolysis study). f) H&E staining images of treated blood vessels. Reproduced from (Jiasheng Xu et al., 2020) with permission from The Royal Society of Chemistry (2020)

Advantages, limitations, and examples of the different systems discussed in this section are summarised in **Table 2.5** and **Table 2.6**.
**Table 2.5.** Summary for different plasminogen activators delivery systems highlighting their advantages and limitations with some illustrative examples from literature.

Delivery system	Advantage (s)	Limitation(s)	Examples from	literatur	e		
			Material description <sup>a)</sup>	PA <sup>a)</sup>	Targeting moiety/ site	Therapeutic ability/biological model	Ref.
1.Macromolecul e-conjugates							
PEG-conjugates	Prolonged circulation	Reduced activity, restricted stability during conjugation	PEG-5000	rtPA	N/A	Extended lifetime for radiolabelled PEG-tPA in CD-I female mice	(H. Berger, Jr. & S. V. Pizzo, 1988)
mAb- chemical conjugates	Thrombus targeting, lower doses, reduced off-site bleeding risk	Immunogenicity, loss of Ab functionality, restricted stability during conjugation	anti-fibrin mAb	sctPA	anti-fibrin mAb/ fibrin	2.8-9.6 more potent than free tPA in rabbit jugular vein model	(Runge et al., 1987b)
Recombinant scFv nanoconstruct 2. Particulate	Thrombus targeting, lower doses, bleeding free		scFv	scuPA	scFv antibody/ GPIIb/IIIa on activated platelets	FeCl <sub>3</sub> -induced carotid artery thrombosis prevention in C57BL/6 mice following prophylactic administration of small doses with no prolongation of tail bleeding time.	(X. Wang et al., 2014)
nanocarriers							
Polymeric nanocarriers	Increased stability, controlled release of encapsulated PAs, surface	Cytocompatibility, biodegradability issues for some synthetic polymers	PIBCA polymeric nanoparticles	tPA	Fucoidan/ P-selectin on activated platelets	Significant binding to activated platelets under venous flow conditions. Thrombus density reduction to 29.5 % at 30 min after injection into	(Juenet et al., 2018)

Delivery system	Advantage (s)	Limitation(s)	Material description <sup>a)</sup>	PA <sup>a)</sup>	Targeting moiety/ site	Therapeutic ability/biological model	Ref.
	functionalisation, high shelf- stability					venous thrombosis mouse model (C57BL/6J mice)	
	ŗ		PEG-PCL polymeric nanoparticles	rtPA	N/A	10-fold dose reduction and 18 times extended half-life compared to free tPA in in rat model of middle cerebral artery occlusion	(J. Deng et al., 2018)
			PCL- PDMAEMA- PHEMA Triblock polymeric micelle	LK	Annexin V/ PS on surface of activated platelets	Stronger thrombolysis abilities and reduced bleeding time compared to free LK in carotid artery thrombi mouse model	(Pan et al., 2017)
Liposomes	Biocompatibility, ease of surface modification, prolonged circulation via PEGylated phospholipid	Low encapsulation efficiency, limited storage stability	DPPC, CHOL, DSPE- PEG2000, DSPE- PEG2000- cRGD	uPA	cRGD peptide/ GPIIb/IIIa on activated platelets	Prolonged half-life in BALB/c male mice (t <sub>1/2</sub> of 2.5 hr compared to 15 min of free uPA). 75% dosage reduction of uPA and no significant prolongation in tail bleeding time in BALB/c male mice	(N. Zhang, C. Li, et al., 2018)
3. Biological and bio-inspired carriers							
RBCs	Prophylactic antithrombotic, inherent targeting with limited brain	Technical challenges with coupling techniques, need for autologous RBCs	Biotinylated RBCs bound to biotinylated tPA via	tPA	N/A	Rapid lysis of nascent cerebral thromboemboli following prophylactic injections of RBC- tPA to mouse model of cerebral thrombosis	(K. Danielyan et al., 2008)

Delivery system	Advantage (s)	Limitation(s)	Material description <sup>a)</sup>	PA <sup>a)</sup>	Targeting moiety/ site	Therapeutic ability/biological model	Ref.
	damage, long circulation time		streptavidin linker				
Platelet inspired systems	Biocompatibility, thrombus targeting		DSPC, DHPE- RhB, CHOL and DSPE- PEG-peptide conjugates)	SK	RGD peptide/ GPIIb/IIIa receptor + EWVDV peptide/ P- selectin	Similar thrombolytic effects to free SK in FeCl <sub>3</sub> -induced carotid thrombosis mouse model. No significant prolongation in tail bleeding time.	(Pawlowski et al., 2017)
RBCs inspired systems	Mimic RBCs deformability under high shear stress		Discoidal polymeric nanoconstructs (PLGA and PEG- diacrylate)	tPA	N/A	70 % recanalisation of murine mesentery venules at 1/10 of the clinical dose of tPA	(Colasuonno et al., 2018)

a) PEG: polyethylene glycol, mAb: monoclonal antibody, scFv: single chain variable fragment, PIBCA: polysaccharide-poly (isobutylcyanoacrylate), PEG-PCL: poly (ethylene glycol)- poly(e-caprolactone), PCL-PDMAEMA-PHEMA: polycaprolactone-*block*-poly(2-(dimethylamino) ethyl methacrylate)-*block*-poly (2-hydroxyethyl methacrylate), DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, CHOL: cholesterol, DSPC: 1,2-distearoyl-*sn*-glycero-3-phosphocholine, DSPE-PEG2000: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (polyethylene glycol)-2000, DHPE-RhB: Rhodamine-B-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine, PLGA: poly(lactic-co-glycolic) acid, rtPA: recombinant tissue plasminogen activator, scuPA: single chain urokinase plasminogen activator, LK: lumbrokinase, SK: streptokinase.

Stimulus type	Advantage (s)	tage (s) Limitation(s) Examples from literature						
			Material description <sup>a)</sup>	PA <sup>a)</sup>	Stimuli	Therapeutic ability/biological model	Ref.	
1.InternalStimuli(Enzymes, shearstressandactivatedplatelets)	Selective release, activation, or deposition of PA payload at thrombus site. Lower doses, decreased off-site	High interpatien variability. Shear stress responsive systems cannot be used fo fully occluded vessels.	t Pro-uPA with thrombin s cleavable site s fused to r αIIbβ3 scFv l antibody	uPA	Thrombin enzyme	Thromboprophylaxis in murine models while maintaining haemostasis	(Rudy E. Fuentes et al., 2016)	
	bleeding risk		tPA-Albumin nanoconstruct with thrombin cleavable linker peptide	tPA	Thrombin enzyme	Steric masking of tPA activity in systemic circulation with selective release of tPA at thrombus site confirmed in inferior vena cava (IVC) rat trombosis model	(S. Absar et al., 2014)	
			Microaggrega tes of multiple smaller tPA- coated PLGA nanoparticles	tPA	Shear stress	Preferential accumulation of the shear-dispersed nanoparticles at occluded site in FeCl <sub>3</sub> -induced arterial thrombus mouse. Clear vessel reopening in 5 minutes at a 100-fold lower doses of tPA	(N. Korin et al., 2012)	
			cRGD- liposomes (EPC, DSPE- PEG-cRGD and CHOL)	tPA	Activated Platelets	Enhanced tPA release (90% release in 1 hour) in presence of activated platelets through membranes fusion causing liposomes destabilisation.	(Y. Huang et al., 2019)	

 Table 2.6. Summary for some stimuli-enhanced delivery systems of plasminogen activators from literature.

Stimulus type	Advantage (s)	Limitation(s)	Material description <sup>a)</sup>	PA <sup>a)</sup>	Stimuli	Therapeutic ability/biological model	Ref.
2. External stimuli (Ultrasound waves magnetic field, and NIR light)	On-demand release of PA payload at thrombus site. Enhanced fibrinolysis through ultrasound cavitation and hyperthermia-	System complexity, bio-incompatibility of metallic nanoparticles used, need for external medical devices for stimulation, limited US, NIR deep tissue penetration	scuPA loaded microbubbles labelled with scFv antibody targeting αIIbβ3 receptor on activated platelets	scuPA	Ultrasound waves	Real time ultrasound imaging of carotid artery FeCl <sub>3</sub> - induced thrombus showing more than 50% reduction in clot size at 45 min of i.v. injection to mice models	(X. Wang et al., 2016)
	induced clot ablation. Lower doses and decreased off-site bleeding risk. Potential for		Echogenic liposomes (PC, PE, PG and CHOL)	tPA	Doppler ultrasound waves (5.7 MHz-2 min)	Enhanced thrombolysis with complete recanalisation of abdominal aorta in New Zealand White rabbits	(S. T. Laing et al., 2011)
	image-guided therapy monitoring		Magnetite- heparin-uPA nanocomposit e	uPA	Magnetic field	Blood flow restoration of the carotid artery in FeCl <sub>3</sub> -induced thrombus model within 0.5–10 min	(Artur Y. Prilepskii et al., 2018)
			Magneto- liposomes (Fe <sub>3</sub> O <sub>4</sub> , DPPC, DSPE- PEG2000 and CHOL)	rtPA	Magnetic field	Restored iliac blood flow in rat embolic model	(C. H. Liu et al., 2019)

Stimulus type	Advantage (s)	Limitation(s)	Material description <sup>a)</sup>	PA <sup>a)</sup>	Stimuli	Therapeutic ability/biological model	Ref.
			Platelets membrane coated Gold	uPA	NIR light (808 nm- 2 W/cm <sup>2</sup> )	Thrombus targeting in murine pulmonary embolism model	(Ting Yang et al., 2018)
			Gold- mesoporous silica core-shell nanospheres capped with lauric acid and stearic acid eutectic mixture	uPA	NIR light (808 nm- 2 W/cm <sup>2</sup> )	Enhanced thrombolysis in rabbit femoral vein thrombosis model	(J. Xu et al., 2020)

<sup>a)</sup> uPA: urokinase plasminogen activator, scFv: single chain variable fragment, tPA: tissue plasminogen activator, PLGA: poly(lactic-co-glycolic) acid, EPC: egg phosphatidylcholine, DSPE-PEG2000: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (polyethylene glycol)-2000, CHOL: cholesterol, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, Fe<sub>3</sub>O<sub>4</sub>: iron oxide, DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, scuPA: single chain urokinase plasminogen activator, rtPA: recombinant tissue plasminogen activator

Treatment of acute thromboembolic events with fibrinolytic drugs can be life-saving therapy. However, these drugs often fail to achieve clot lysis, while the bleeding adverse effects associated with the therapeutic doses of current fibrinolytic drugs result in high morbidity and mortality. These limitations impose narrow eligibility criteria for their administration, both regarding the therapeutic window and patient selection. Over the past few decades, research has focused on developing new fibrinolytic drugs and their most efficient use to treat thrombo-embolic events and their associated complications, such as stroke, myocardial infarction, and pulmonary embolism.

To increase efficiency and to reduce the bleeding risk, the loading of fibrinolytic drugs into various polymeric and liposomal systems have been developed and tested in preclinical animal models. Numerous challenges have been identified from such systems, which will potentially guide further translation to clinical use. The most important limitations are poor biodistribution and weak penetration into fully occluded blood vessels. Functionalisation of such nanocarriers with targeting moieties that specifically bind to activated platelets represent a highly promising strategy to increase the local concentration at the thrombus site and minimize any off-site bleeding. Among these targeting moieties, the scFv antibody, which binds to the activated GPIIb/IIIa on activated platelets, has shown the highest degree of specificity and binding affinity.

Targeted delivery systems responsive to external stimuli such as ultrasound waves, magnetic fields and NIR light can efficiently lyse clots through targeted release of fibrinolytic drugs and synergistic thrombolytic effects of the applied stimulus. Magnetic guidance, magnetic mechanical dragging (nanomotor) forces, sonothrombolysis, inertial cavitation, photothermal thrombolysis, and the actuated release of fibrinolytic drugs at the thrombus site are new features characteristic to these smart systems. These nextgeneration thrombolytic approaches are highly attractive. Furthermore, image-guided thrombolysis using ultrasound imaging, MRI, photoacoustic or NIR fluorescence imaging also provides a step towards real-time monitoring of the success or failure of thrombolysis. These promising approaches require further studies to optimize efficiency, dosing, storage stability and safety in pre-clinical models before they can ultimately be translated to clinical practice. Developing strategies to scale up the synthesis of these systems is also essential to allow their application in the clinics.

To improve the performance and clinical translation of stimuli-assisted thrombolysis using external stimuli, formulators should consider the following:

- The use of biocompatible materials such as phospholipids, lipid-based nanocarriers, and coated metal nanoparticles.
- Tuning the external stimuli to be within acceptable safety ranges while maintaining the functionality of site-directed release of PAs from the nanocarrier.
- Simple preparation methods that can be scaled-up such as film hydration method, high shear homogenisation of self-assembled systems, and recombinant technology.
- Long term stability of the final formulation through the incorporation of stabilizers, or lyophilisation with cryoprotectants.

# 2.4. Liposomes; Biocompatible nanocarriers for translational nanomedicine

Liposomes are among the most investigated nanocarriers for drug delivery applications due to their advantages which include, but not limited to, cytocompatibility, ease of preparation at large scale levels, ability to accommodate high drug payload, and possible physicochemical/biophysical modifications (Monteiro, Martins, Reis, & Neves, 2014) . For these reasons, they have reached the pharmaceutical market and been commercialised in several novel products for treatment of various life-threatening diseases such as systemic fungal infections (Ambisome<sup>®</sup>), respiratory distress syndrome (Curosurf<sup>®</sup>), Kaposi sarcoma (Doxil<sup>®</sup> and DaunoXome<sup>®</sup>), pancreatic cancer (Onivyde<sup>®</sup>), lymphomatous meningitis (Depocyt<sup>®</sup>) and acute lymphoblastic leukemia (Marqibo<sup>®</sup>) (Bobo et al., 2016). The Pfizer/BioNTech<sup>®</sup> and Moderna<sup>®</sup> novel Covid-19 vaccines are based on liposomal formulations of mRNA expressing spike protein of the virus.

Structurally, liposomes can be described as nanovesicles (Figure 2.11) consisting of one or more phospholipid bilayers enclosing an inner aqueous core which enable them to entrap both lipophilic drugs into the bilayer and hydrophilic ones in the aqueous core (Ulrich, 2002). Liposomes can be used to deliver a variety of small molecules such as chemotherapeutic agents, and macromolecules such as genetic materials and therapeutic proteins (Sumaira Naeem, Viswanathan, & Misran Misni, 2018). Furthermore, the surface properties of liposomes can be modified for prolonged systemic circulation through incorporation of low percentage of PEGylated phospholipids such as DSPE-PEG2000. Targeting ligand conjugation to liposome surface can be achieved using different conjugation chemistries. Imaging probes could be also loaded into the liposomes for theranostic applications (Sercombe et al., 2015).

Versatility of commercially available phospholipids offers a broad range of options to the formulator to control the physico-chemical and functional properties of liposomes including surface charge, surface hydrophilicity, liquid-glass transition temperature ( $T_m$ ), phospholipid membrane rigidity, and surface functionalisation with targeting ligands and imaging probes. Functional group tagged lipids are commercially available where different chemistries can be employed to achieve functionalisation to targeting ligands or imaging agents. Examples of those tagged phospholipids include biotinylated lipids for biotin-avidin conjugation, azide-tagged lipids for azide/DBCO click chemistry, aminetagged lipids for NHS/EDC chemistry, and NTA-Ni lipids for Histidine binding (Encapsula, 2022).



**Figure 2.11.** Schematic representation of liposome structure with possible surface modifications. Adapted with permission (Sercombe et al., 2015).

Surface charge could be also controlled for versatile applications. The use of cationic and ionisable phospholipids enables efficient transfection of genetic materials such as mRNA expressing spike protein of viruses. Those lipids are the cornerstone of the newly developed Pfizer/BioNTech<sup>®</sup> and Moderna<sup>®</sup> novel Covid-19 vaccines (X. Han et al., 2021). As well, metallic nanoparticles and quantum dots have been successfully loaded to liposomes for a wide variety of applications such as magnetic resonance imaging (Kostevšek et al., 2020), photothermal-photodynamic therapy (PTT) (Chauhan, Prasad, Devrukhkar, Selvaraj, & Srivastava, 2018), and NIR fluorescence imaging (Aizik et al., 2020).

Functionally, stimuli-responsive liposomes have been also investigated for on-demand delivery of therapeutics to diseased site. This approach can significantly limit off-site adverse effects and enhance local concentrations of non-selective therapies such as chemotherapeutic agents and thrombolytics. Examples of stimuli-responsive liposomes include thermosensitive liposomes, pH responsive- liposomes, enzyme-responsive liposomes, redox-sensitive liposomes, magneto-liposomes, echogenic liposomes, and light-responsive liposomes (Lee & Thompson, 2017; Zangabad et al., 2018). Those stimuli-responsive liposomes present great potentials towards improved and side effects free-treatment of wide range of diseases. They reached different stages of clinical trials such as the thermosensitive doxorubicin-loaded liposomes (ThermoDox<sup>®</sup>) for liver cancer treatment (OPTIMA, 2022).

Because of the properties of liposomes including biocompatibility, loading efficiency, stimuli-responsiveness, and ease of functionalisation to targeting ligands, they were selected as a potential nanocarrier in our work. NIR light responsive liposomes were developed using a hybrid formulation of photothermal agents and thermosensitive phospholipids. Further functionalisation of liposome surface to activated platelet-targeting scFv antibody was performed using a biotin-neutravidin chemistry, as will be described later.

# Chapter 3Near-Infrared (NIR)Light-Responsive Liposomes for Protein Delivery

Aspects of the work presented in this chapter were published as: Nearinfrared light-responsive liposomes for protein delivery: Towards bleedingfree photothermally-assisted thrombolysis, *Journal of Controlled Release*, 337 (2021) 212-223. doi: 10.1016/j.jconrel.2021.07.024

#### **3.1. Introduction**

Stimuli-responsive liposomes have received great attention for spatiotemporal delivery of therapeutics to disease sites. Thermo-sensitive liposomes (TSLs), which release their payload in response to mild hyperthermia (39-43 °C), were among the first studied of these smart systems. Drug release from such TSLs was entirely dependent on lipid bilayer permeation enhancement when heated above the average transition temperature (T<sub>m</sub>) of the lipid mixture (Yannan Dou et al., 2017; Xiaoyi Huang, Li, Bruni, Messa, & Cellesi, 2017). Incorporation of lysolipid in TSLs, first reported by Dewhirst and Needham, enabled the creation of stable nanopores in the lipid membrane in response to mild hyperthermia for more efficient release (Needham et al., 2000). Lysolipid-TSLs loaded with doxorubicin (ThermoDox®, Celsion Corporation) reached phase III trials for hepatocellular carcinoma treatment in combination with radiofrequency ablation(Mura et al., 2013). Many other non-invasive heating modalities have been reported for localised clinical hyperthermia, including focused ultrasound (Santos, Goertz, & Hynynen, 2017), magnetothermal (C. H. Liu et al., 2019) and photothermal (PT) approaches (Santos et al., 2017).

Near-infrared (NIR) light in the first window (NIR-I) (650-950 nm wavelength) is characterised by its relatively higher tissue penetration and minimal cytotoxicity, compared to visible and ultraviolet light (D. E. Hudson, 2013; Hong et al., 2017). Several studies have utilised NIR irradiation of photothermal agents such as gold nanomaterials and indocyanine green (ICG) to assist the release of small molecule drugs such as doxorubicin (Chauhan et al., 2018; Yan et al., 2016), calcein dye (Rengan et al., 2014; Viitala et al., 2016), tetradoxin (C. Zhan et al., 2017) and polyinosinic:polycytidylic acid immune-stimulant (L. Xu et al., 2019) from TSLs in response to hyperthermia. The NIR-assisted release/delivery from TSLs was also proven applicable for macromolecular agents such as siRNA (MW 14 kDa) (Kontturi, van den Dikkenberg, Urtti, Hennink, & Mastrobattista, 2019), FITC-dextran (MW 20 kDa) (Lajunen, Kontturi, et al., 2016), and DNA plasmids (Wiraja et al., 2016). To the best of our knowledge, the use of NIR-irradiation of incorporated gold nanorods to release macromolecular therapeutic proteins immobilised on and/or encapsulated in TSLs has not been reported yet.

In this study, we report on a NIR light-responsive liposomal platform (Figure 3.1) based on a hybrid formulation of ultrasmall gold nanorods (AuNRs), thermosensitive phospholipid (DPPC) and non-ionic surfactant (Brij58). Ultrasmall PEGylated AuNRs act as plasmonic agents for photothermal assisted release following NIR irradiation. Brij58, an amphiphilic non-ionic surfactant (melting point = 38-39 °C) works as channelling agent analogous to lysolipid to create stable nanopores upon heating. It is also reported to prolong liposome circulation time by imparting surface hydrophilicity (Tagami, Ernsting, & Li, 2011). The hybrid formulation was tested using FITC-ovalbumin (model fluorescent-labelled protein; MW 45 kDa) as a model payload.



**Figure 3.1.** Graphical presentation of the developed NIR light-responsive liposome for protein delivery and its potential application in photothermally-assisted thrombolysis.

#### **3.2.** Materials and methods

#### 3.2.1. Materials

Gold chloride trihydrate (HAuCl<sub>4</sub>. 3H<sub>2</sub>O), cetyltrimethylammonium bromide (CTAB), silver nitrate (AgNO<sub>3</sub>), hydrochloric acid (HCl), L-ascorbic acid, sodium borohydride (NaBH<sub>4</sub>), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay kit (tox-1), methanol (MeOH), dichloromethane (DCM), Triton X-100, and polyoxyethylene 20 cetyl ether (Brij58<sup>TM</sup>) were purchased from Sigma-Aldrich (Melbourne-Australia). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were obtained from Lipoid GmbH (Ludwigshafen-Germany). Dulbecco's modified Eagle medium (DMEM) culture medium, foetal bovine serum (FBS) and antibiotics were supplied by Gibco (Carlsbad, USA). Methoxypolyethylene glycol thiol (mPEG2000-SH) and ovalbumin-fluorescein isothiocyanate conjugate (FITC-ovalbumin, MW 45 kDa) were purchased from Nanocs<sup>®</sup> (New York, USA). All reagents were used as purchased.

#### **3.2.2.** Synthesis of gold nanorods (AuNRs)

The reported one-step seedless method (M. R. K. Ali, Snyder, & El-Sayed, 2012) was used with slight modifications to synthesize ultrasmall sized AuNRs. Briefly, 5 mL of 1 mM HAuCl<sub>4</sub> and 5 mL of 0.2 M CTAB were mixed in a glass vial and 260  $\mu$ L of 4 mM AgNO<sub>3</sub> was added with gentle stirring, then pH was adjusted to 1.15 using 8  $\mu$ L of HCl (37%) and subsequently 70  $\mu$ L of 79 mM ascorbic acid was added and gently shaken to change the solution colour from yellow to colourless. Immediately, 15  $\mu$ L of 0.01 M icecold NaBH<sub>4</sub> was injected, and the mixture was left to react for 6 h. The resulting AuNRs were purified via centrifugation at 20,000 g for 15 min and washed with deionised (DI) water twice to remove any residual CTAB. The synthesised AuNRs were further treated with mPEG2000-SH to replace the cytotoxic CTAB (Yasun et al., 2015) and impart stability in physiological fluids as follows; 10 mL of AuNRs dispersion was centrifuged at 20,000 g for 15 min, the supernatant was removed, and 1 mL of freshly prepared 4 mM mPEG2000-SH was added to the AuNRs pellet whilst vortexing for 30 seconds. The resulting mixture was stirred at room temperature overnight. Excess mPEG2000-SH was removed by two rounds of centrifugation and resuspended in 1 mL of DI water.

#### 3.2.3. Optimisation of liposome homogenisation method

Liposomes were prepared according to the film hydration method with Lipoid S-75 phospholipid (Tm = -20 °C) to study the different size optimisation/homogenisation techniques (probe sonication and microfluidisation) as follows; Lipoid S-75 (75 mg) was weighed in a 50 ml round bottom flask and dissolved in 3 ml DCM/MeOH mixture (2:1 v/v). Solvents were then evaporated using rotary evaporator (Buchi, Switzerland) at room temperature to produce a dry lipid film. The lipid film was then hydrated using 25 ml of (PBS, pH 7.4) with further mixing for 5 minutes to obtain multilamellar liposomes.

#### Microfluidisation

Liposomal dispersion samples (5 ml) were run through a microfluidizer (Microfluidics<sup>TM</sup>) under pressure range (2000- 20000 psi) for 1,3,5 and 7 runs for each pressure followed by particle size and polydispersity analysis using DLS (Zetasizer Nano ZS, Malvern Instruments).

#### **Probe sonication**

On an ice bath, liposomal dispersions were ultrasonicated using a probe sonicator (Misonix S-4000) for 5 minutes on continuous pulse mode using 20%, 50%, 75% and 100% power intensities. Pulsating sonication was also used (10 seconds on, 10 seconds off) for total 10 minutes process. Samples of liposomal dispersion were withdrawn at

different time intervals during sonication for particle size and polydispersity analysis using DLS (Zetasizer Nano ZS, Malvern Instruments).

### **3.2.4.Preparation of FITC-ovalbumin light responsive liposomes (FITC-Au-LRLs)**

Liposomes were prepared using the lipid film hydration method and then homogenised by probe sonication. Briefly, respective lipids **(Table 3.1)** were dissolved in DCM/MeOH mixture (2:1 v/v) in a round bottom flask, then solvents were rotary evaporated under vacuum to form lipid films (Büchi<sup>®</sup>, Switzerland). Dry lipid films were hydrated with premixed FITC-ovalbumin (0.5 mg/mL) and AuNRs-PEG (80 µg/mL gold content as per ICP-OES) dispersions in PBS buffer and agitated at either 45°C (for DPPC and Brij 58) or 60°C (for DSPC) for 30 min at a final lipid concentration of 3 mg/mL. Next, the multilamellar vesicles were probe sonicated for 10 min (10/10 s, on/off, 150 W) (Misonix<sup>®</sup> S-4000) at either 45°C (for DPPC and Brij 58<sup>TM</sup>) or 60°C (for DSPC). Unincorporated free protein and AuNRs were further separated using ultracentrifugal filters (MWCO 300 KDa) under 8000 g for 12 min at 4 °C for at least three washing cycles (Beckmann<sup>®</sup> ultra-centrifuge) and the purified liposomes were resuspended in PBS buffer and stored at 4 °C for later use. AuNRs-PEG loaded liposomes (Au-LRLs) were prepared using the same method. All liposomes were used within one month of preparation.

Liposome		Lipid composition (3 mg/mL)	Photothermal agent (80 μg/mL)	Model Protein (0.5 mg/mL)	
Au-LRLs	Au-NLR Au-LR1 Au-LR2	DSPC DPPC DPPC/Brij58 (96:4 molar ratio)	AuNRs-PEG AuNRs-PEG AuNRs-PEG	- -	
FITC-Au-LRLs	FITC-Au-NLR FITC-Au-LR1 FITC-Au-LR2	DSPC DPPC DPPC/Brij58 (96:4 molar ratio)	AuNRs-PEG AuNRs-PEG AuNRs-PEG	FITC-ovalbumin FITC-ovalbumin FITC-ovalbumin	

Table 3.1. Composition of the different prepared NIR-light responsive liposomes

## **3.2.5.** Characterisation of AuNRs and FITC-Au-LRLs

#### Absorption spectroscopy

Absorption spectra of the synthesised AuNRs, AuNRs-PEG and Au-LRLs were collected using a UV-vis-NIR spectrometer (Perkin Elmer-Lambda 1050) in standard quartz cuvettes with a path length of 1 cm. DI water and PBS were used as reference solutions accordingly.

#### Transmission electron microscopy (TEM)

The size, morphology, and localisation of AuNRs-PEG in/to the liposomes were investigated using JEOL 1010 TEM at an accelerating voltage of 100 kV. For AuNRs sample preparation, a drop of the dispersion was allowed to air-dry on carbon-coated grids at room temperature. FITC-Au-LRLs were incubated on carbon-coated grids for 30 minutes and then stained with 1% uranyl acetate.

#### Size, polydispersity, and zetapotential measurements

The hydrodynamic diameter, polydispersity, and zeta potential of FITC-Au-LRLs were analysed using DLS (Zetasizer Nano ZS, Malvern Instruments) at 25°C. Zeta potentials of AuNRs were also analysed before and after PEGylation to confirm CTAB replacement. Dispersions were diluted in filtered DI water at ratios of 1% and 10 % (v/v) for size and zeta potential measurement, respectively.

#### Determination of FITC-ovalbumin entrapment efficiency (EE %)

An aliquot of 100  $\mu$ L of FITC-Au-LRLs was treated with TritonX-100 (1% v/v) to lyse the liposomes. The amount of encapsulated FITC-ovalbumin was determined following the calibration curve shown in **Figure 3.5** using a fluorescence microplate reader (Fluostar<sup>®</sup>) at 485 nm as the excitation wavelength and 520 nm as the emission wavelength. Percentage entrapment efficiency was calculated as follows:

$$EE(\%) = \frac{We}{Wi} \times 100 \tag{Eq.3.1}$$

where  $W_e$  is the amount of FITC-ovalbumin encapsulated in the liposomes and Wi is the initial amount of FITC-ovalbumin used to hydrate the liposomes.

#### **ICP-OES** for gold content quantification

The gold content of AuNRs-PEG aliquots and FITC-Au-LRLs was measured using an inductively coupled plasma optical emission spectrometer (ICP-OES, PerkinElmer) following digestion in aqua regia (65% HNO<sub>3</sub>: 37% HCl, 1:4 v/v) for 24 h at 100 °C.

#### Photothermal transduction using NIR light

Prior to using NIR light for the photothermal assisted release from liposomes, the photothermal conversion efficiency of AuNRs-PEG and FITC-Au-LRLs dispersions was investigated under different laser powers. Briefly, 1 mL AuNRs-PEG dispersion (equivalent to 80  $\mu$ g/mL gold as per ICP-OES) was transferred into quartz cuvettes to be irradiated with a NIR laser (OptoTech, 785 nm). The bulk temperature was recorded using a thermal camera (FLIR ETS320 series) while the cuvette was illuminated using two

different power densities (0.64 W/cm<sup>2</sup> and 1.35 W/cm<sup>2</sup>) for 14 min. The return to baseline room temperature after switching the laser off was also recorded. The same experiment was performed on purified FITC-Au-LRLs.

#### In vitro release studies

#### **Temperature-assisted release**

The *in vitro* passive release of FITC-ovalbumin from different liposomes was evaluated at physiological temperature (37 °C) and mild hyperthermia (42 °C) using the dialysis bag diffusion method. Liposomes were transferred into dialysis bags (MWCO 300 KDa, Spectrum) immersed in PBS buffer at 37 °C and 42 °C under agitation (30 rpm). 100  $\mu$ L of the dialysate solution was withdrawn at (0.5, 1, 1.5, 2, 4 and 6 h) time intervals to measure the amount of FITC-ovalbumin released using a fluorescence microplate reader (Fluostar<sup>®)</sup> as previously described. Preheated PBS buffer aliquots (100  $\mu$ L each) were used to compensate the volume of the dialysis incubation medium after each sample withdrawal.

Cumulative FITC – ovalbumin released (%) =  $\frac{Wr \text{ at time point}}{We} \times 100$  (Eq.3.2) where Wr is the cumulative amount of FITC-ovalbumin released at the time (t) and  $W_e$  is the initial amount of FITC-ovalbumin encapsulated in liposomes at the time (0)

#### NIR-light assisted release

FITC-Au-LRLs dispersions were ultrafiltered and redispersed into PBS buffer (preheated to 37 °C, pH 7.4) and transferred into a 48-well microplate (Costar<sup>®</sup>). The samples were immediately irradiated with NIR laser light (OptoTech, 785 nm) at two different power densities (0.64 and 1.35 W/cm<sup>2</sup>) for 5 min. A 100  $\mu$ L liposomal aliquot was collected immediately after irradiation at different time points (0-60 min), replaced by fresh preheated PBS, and ultracentrifuged with cold PBS (20000 rpm, 30 min) at 4°C

for supernatant fluorescence analysis using a fluorescence microplate reader (Fluostar<sup>®</sup>) as

Cumulative FITC – ovalbumin released (%) = 
$$\frac{F_t - F_0}{F_{100\%} - F_0} \times 100$$
 (Eq.3.3)

where  $F_t$  is the fluorescence of the supernatant solution at time (t),  $F_{100\%}$  is the supernatant fluorescence of Triton-X100 treated liposomes, which represents 100% release of FITC-ovalbumin, and  $F_0$  is the fluorescence of the supernatant before NIR irradiation. The test was performed at 37 °C in a darkroom to control the light exposure conditions.

#### In vitro cytocompatibility studies

#### MTT assay

Cytocompatibility of AuNRs-PEG and Au-LRLs (Au-LR<sub>1</sub>) was tested using the MTT assay on human embryonic kidney (HEK) cell line cultured and maintained in Dulbecco's modified Eagle medium (DMEM, high glucose 4.5 g/L) supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) at 37 °C with 5% CO<sub>2</sub>. HEK cells were harvested and 100  $\mu$ L aliquots were added to a 96-well flat bottom plate (Sarstedt<sup>®</sup>, Inc) at a concentration of 10<sup>4</sup> cells per well and allowed 24 h for cell attachment. Serial dilutions of different test dispersions were added to cells as 10  $\mu$ L aliquots in triplicates. The medium was not changed during cells incubation for 48 h.

After incubation, cells were treated with filter sterilised (5 mg/mL) MTT solution (10  $\mu$ L/well) for a further 4 h. Afterwards, the medium was removed, and the insoluble formazan crystals were solubilised in the supplied solubilisation medium. Absorbance values were measured using a microplate reader (Fluostar<sup>®</sup>) at 570 nm and cell viability was calculated as a percentage to untreated control cells.

$$Cell \ viability \ (\%) = \frac{Absorbance \ of \ test}{Absorbance \ of \ control} \times 100$$
(Eq.3.4)

#### Haemolysis study

Hemocompatibility of AuNRs-PEG and Au-LRLs (Au-LR<sub>1</sub>) (80  $\mu$ g/mL gold content) was tested using haemolysis of red blood cells (RBCs). Briefly, human blood was freshly collected in citrated vacutainers. Then test samples (20  $\mu$ L) were incubated with the red blood cells (380  $\mu$ L blood) for 1 h and 24 h in a shaking incubator at physiological temperature (37 °C) under agitation (150 rpm). After incubation, blood was centrifuged at 2000 rpm for 10 minutes using ultra-centrifuge (Beckman<sup>®</sup>) for supernatant analysis.

Haemoglobin released from hemolyzed RBCs was quantified in the supernatant by measuring its absorbance using a microplate reader (Fluostar<sup>®</sup>) at 550 nm wavelength. The red blood cells treated with PBS and Triton X-100 were set as negative and positive control respectively.

$$Haemolysis(\%) = \frac{Absorbance of test - Absorbance of PBS treated}{Absorbance of Triton treated - Absorbance of PBS treated} \times 100$$

Sysmex<sup>®</sup> analysis of blood samples, treated with either PBS or Au-LRLs for 24 h, was also performed to compare the main haematological parameters including white blood cells count (WBCs), red blood cells count (RBCs), haemoglobin (HGB), haematocrit values (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelets count (PLT).

#### 3.2.5. Statistics

Data was expressed as mean ± standard deviations. Analysis of *in vitro* biocompatibility data was done with one-way repeated measures ANOVA comparing all groups with one

another using Tukey multiple-comparison post-test. GraphPad Prism 8 and OriginLab Pro were used to plot the figures and analyse the data.

#### 3.3. Results and discussion

#### **3.3.1.** Preliminary optimisation of liposome preparation

Liposomes in the size range of (100-150 nm) were suggested as a potential nanocarrier for co-loading of ultrasmall AuNRs-PEG and the model protein. These small sized liposomes are expected to escape opsonisation and further identification by the reticuloendothelial system and to show prolonged circulation following *i.v.* administration, as described previously under **section 2.1.2**. Microfluidisation and probe sonication were selected as suitable homogenisation methods. Membrane extrusion method was excluded because of the potential for membrane blockage with the adsorbed AuNRs-PEG. Blank S-75 ( $T_m = -20$  °C) liposomes were prepared and used to determine the optimal conditions for liposome homogenisation within the desired size range (**Figure 3.2 & Figure 3.3**).

Trials on using microfluidisation at different pressures (**Figure 3.2**) revealed the feasibility of liposomes preparation at sizes  $(116 \pm 15 \text{ nm and } 89 \pm 6 \text{ nm})$  and PDIs  $(0.331\pm 0.03 \text{ and } 0.282 \pm 0.03)$  after 7 runs under 5000 psi and 10,000 psi respectively. On the other hand, larger liposomes  $(189 \pm 12 \text{ nm})$  with higher PDIs  $(0.44 \pm 0.04)$  were produced after 7 runs under 2000 psi pressure. Small sized liposomes  $(46 \pm 6 \text{ nm})$  with PDIs  $(0.281 \pm 0.03)$  were produced at 20,000 psi pressure.

Probe sonication of liposomes was also tested using different amplitudes of 20%, 50% and 75%. The change of vesicles size and PDI with sonication time is demonstrated in **Figure 3.3**, where liposomes of sizes (133  $\pm$  12 nm) and PDIs (0.298  $\pm$  0.02) are successfully prepared using 50% power intensity. Larger liposomes (177  $\pm$  18 nm) with

higher PDIs  $(0.41 \pm 0.03)$  are obtained after 5 min treatment using 20% power intensity. Pulsating probe sonication (Figure 3.3d) was also tested as an alternative to continuous sonication, where the same energy is delivered over a prolonged time. This approach can help avoid intense heating from continuous sonication.



**Figure 3.2:** The Effect of microfluidisation pressure and number of samples runs on the **average hydrodynamic diameter and PDI of Lipoid S-75 liposomes** at (a) 2000 psi, (b) 5000 psi, (c) 10000 psi and (d) 20000 psi pressure (n=3).



**Figure 3.3: The Effect of probe sonication time on the average hydrodynamic Z-size and PDI of Lipoid S-75 liposomes using** a) 20 % power intensity-continuous mode, b) 75% power intensity-continuous mode, c) 50% power intensity-continuous mode and d) 50 % power intensity-pulsating mode (10 s on/ 10 s Off) (n=3).

Both microfluidisation (5000 psi, 7 runs) and probe sonication (50% power intensity, 5 min actual sonication time) proved efficient and suitable to prepare liposomes within the desired size range (100 -150 nm) with acceptable polydispersity (PDI < 0.3).

## **3.3.2.** Preparation and physical characterisation of AuNRs and FITC-Au-LRLs

Liposome-gold nanohybrids offer a unique delivery platform with versatile applications such as chemo-photothermal therapy of cancer (Chauhan et al., 2018; Wei Li et al., 2019; Nguyen et al., 2019; Rengan et al., 2014) and multifunctional imaging (Lozano et al., 2012; Rengan et al., 2014). Binding of gold nanomaterials to liposomes can be mediated via direct coating (Rengan et al., 2014), covalent bonding (C. Zhan et al., 2017), physical entrapment (Nguyen et al., 2019), or electrostatic interaction (Lozano et al., 2012). Among the different gold nanomaterials, AuNRs present a longitudinal surface plasmon resonance band (LSPR) whose spectral position can be tuned by adjusting the aspect ratio of the particles. AuNRs with aspect ratios around 4 present LSPR bands in the NIR, allowing the efficient conversion of NIR light into heat when optically excited at these wavelengths (Xiaohua Huang & El-Sayed, 2010). In this context, we report on the co-loading of ultrasmall AuNRs-PEG and macromolecular proteins to thermosensitive liposomes for NIR-triggered release.

The one-pot seedless method was used because it is reported to yield ultrasmall sized AuNRs (M. R. K. Ali et al., 2012; Nguyen et al., 2019; J. Song et al., 2015; F. Zhao et al., 2017) compared with the two-step seed-mediated method. Smaller sizes are preferred as they may incorporate more efficiently in/to nanoliposomes. TEM images (Figure 3.4a) of the prepared AuNRs confirmed the ultrasmall dimensions of  $23(\pm 6) \times 6.8(\pm 0.8)$  nm, with aspect ratio of ~ 3.4 (Figure 3.4c-d). As seen in the absorption spectra (Figure 3.4b), the longitudinal surface plasmon resonance (LSPR) peak of the synthesised AuNRs is centred at 785 nm, indicating their suitability for optical excitation in the NIR-I window. For biocompatibility, mPEG2000-SH was used to replace the cytotoxic surfactant CTAB via formation of strong Au-S covalent bonds (Lu An, Yuanyuan Wang, Qiwei Tian, & Shiping Yang, 2017). PEGylation was verified by measuring the zeta-potential of AuNRs, which significantly decreased from 39.50  $\pm$  0.6 mV to 3.11  $\pm$  0.3 mV after coating with mPEG2000-SH. A very slight blue shift of the LSPR to 780 nm was observed following AuNRs PEGylation, which may be attributed to slight changes

in the refractive index of the surrounding microenvironment (Chauhan et al., 2018; F. Zhao et al., 2017).



**Figure 3.4. Characterisation of AuNRs** a) Transmission electron microscopy (TEM) image of the prepared ultrasmall AuNRs (scale bar = 100 nm). b) Absorption spectra of AuNRs, AuNRs-PEG and Au-LR<sub>1</sub>. Size distributions of AuNRs; c) particle length and d) particle width. The small (< 20%) fraction of impurities (Au nanospheres and nanocubes) present in the samples were manually excluded from the size distribution calculations.

Lipids with different thermal sensitivities were selected to prepare the liposomes. DSPC, a saturated lipid with elevated  $T_m$  of 55 °C, was used to prepare non-light responsive liposomes (NLR) with no thermosensitive properties. DPPC, with  $T_m$  of ~ 41°C, was used to prepare both (LR<sub>1</sub> and LR<sub>2</sub>) liposomes (Table 3.1). DPPC is the main lipid component of thermosensitive liposomes, usually combined with lysolipid and PEGylated lipid (de Matos et al., 2018; Yannan Dou et al., 2017; Jose, Ninave, Karnam, & Venuganti, 2019; C. H. Liu et al., 2019). Brij58, a biocompatible non-ionic surfactant with a melting point of 38-39 °C was incorporated with DPPC (LR<sub>2</sub>) at small molar ratio (4 mol%). Brij58 can destabilize the liposome membrane for enhanced protein release by forming stable nanopores when heated up to mild hyperthermia. The poly-oxyethylene tail of Brij is also expected to increase the surface hydrophilicity of LR<sub>2</sub> liposomes for prolonged circulation in blood following *i.v.* administration. Tagami et al. (Tagami et al., 2011) previously reported on the efficient use of DPPC and Brij78 (polyoxyethylene 20 stearyl ether; mp = 40-45 °C) to prepare thermosensitive liposomal doxorubicin. It showed faster payload release, similar stability in serum and enhanced tumour regression, compared to lysolipid-TSLs. Brij58 (cetyl ether; mp = 38-39 °C) is proposed to assemble better within the DPPC lipid bilayer and show higher thermosensitivity than Brij78 to mild hyperthermia (42 °C). Cholesterol was not added to any of the formulations because it can broaden T<sub>m</sub> and interfere with ovalbumin binding to the lipid membrane (Xiaoyi Huang et al., 2017).

The average hydrodynamic diameters of the prepared FITC-Au-LRLs were below 200 nm, with PDI values close to 0.2 and negative zeta-potential values (**Table 3.2**), which suggests that the negatively charged FITC-ovalbumin is embedded into the liposome membranes as transmembrane protein (Xiaoyi Huang et al., 2017; Thakur, Das, & Chakraborty, 2014; X. Zhang, Luckham, Hughes, Thom, & Xu, 2011) besides being encapsulated inside the aqueous core. Fluorescence intensity measurement of Triton-X100 treated liposomes confirmed the entrapment of FITC-ovalbumin in different liposome formulation, with average loading level of ~ 34.22 µg/mg of FITC-ovalbumin/lipid (**Table 3.2**). Despite the steric nature of PEG on the surface of AuNRs, the slight positive charge of AuNRs-PEG is thought to encourage electrostatic binding to

negatively charged liposome surface and ovalbumin protein, which would subsequently detach following laser irradiation (Polo et al., 2019). The gold content of purified liposomes, measured using ICP-OES, revealed loading of AuNRs with good entrapment efficiencies ( $\geq$  18%) as shown in **Table 3.2**.

Liposome	d <sub>h</sub> (nm)	PDI	ζ-potential (mV)	FITC-ovalbumin EE (%)	AuNRs EE (%)
FITC-Au-NLR FITC-Au-LR1 FITC-Au-LR2	$150 \pm 11$ $138 \pm 09$ $146 \pm 15$	$\begin{array}{c} 0.258 \pm 0.04 \\ 0.246 \pm 0.04 \\ 0.260 \pm 0.06 \end{array}$	$-8.9 \pm 0.2$ $-8.4 \pm 0.2$ $-7.8 \pm 0.2$	$27.26 \pm 1.67$ $26.01 \pm 2.30$ $24.32 \pm 1.42$	$22.84 \pm 2.83 \\ 20.31 \pm 1.74 \\ 18.44 \pm 1.62$

Table 3.2. Size, PDI, Z-potential and entrapment efficiency (%) of FITC-Au-LRLs (n=3).

**Figure 3.5** presents a calibration curve of standard FITC-ovalbumin fluorescence measured using fluorescence microplate reader at  $\lambda ex = 485$  nm and  $\lambda em = 520$  nm. Here, serial dilutions of standard FITC-ovalbumin solutions (0-100 µg/mL) were split into a 96-well flat-bottomed microplates for fluorescence measurements (n=3). Fluorescence of test solutions of FITC-ovalbumin was measured accordingly to calculate the entrapment efficiency in liposomes and the subsequent temperature-assisted and NIR light-assisted release amounts using equations described under **section 3.2.5**. To quantify metallic gold content [Au<sup>0</sup>] of AuNRs dispersions and their entrapment efficiency in liposomes, a calibration curve was established using standard gold chloride solutions (0-10 µg/mL) as per ICP-OES (**Figure 3.6a**). Test solutions of AuNRs or liposomal AuNRs were digested in aqua regia and diluted before ICP-OES measurement. Absorbance of AuNRs dispersions at 400 nm showed a linear correlation (R<sup>2</sup>= 0.9425) with their metallic gold content [Au<sup>0</sup>] quantified by ICP-OES), as shown in **Figure 3.6b**. This correlation curve was further used as a quick tool to confirm gold content of AuNRs and liposomal AuNRs dispersions, relying on their absorbance at 400 nm with respective blanks.



Figure 3.5: Calibration curve of standard FITC-ovalbumin fluorescence measured using fluorescence microplate reader at  $\lambda_{ex} = 485$  nm and  $\lambda_{em} = 520$  nm). These results were used for entrapment efficiency calculations and release profiles establishment of FITC-ovalbumin from FITC-Au-LRLs (n= 3).



Figure 3.6: Gold content  $[Au^0]$  quantification using ICP-OES. a) Calibration curve of ICP-OES gold content  $[Au^0]$  measurements, b) Correlation curve between absorbance of AuNRs colloids at 400 nm and gold content  $[Au^0]$  obtained from ICP-OES analysis (n= 3).



**Figure 3.7. Characterisation of gold nanorods-loaded liposomes (FITC-Au-LRLs)** (a) TEM images of FITC-Au-LRLs showing localisation of AuNRs-PEG in/to the liposomes. (Scale bars are 100 nm) (b) Photothermal transduction of AuNRs-PEG and FITC-Au-LR<sub>1</sub> under NIR light irradiation (785 nm). The inset shows thermographic images at 5 seconds (left) and 4 min (right) of the NIR irradiation of AuNRs-PEG (1.35 W/cm<sup>2</sup>).

TEM images of the liposomes (Figure 3.7a) showed successful incorporation of AuNRs-PEG in/to the liposomes. However, we cannot exclude that some AuNRs are adsorbed onto the liposome surfaces. Both localisations would mediate photothermal sensitivity. The loading of AuNRs in/to the liposomes is showing a heterogenous distribution, where some liposomes have clusters of AuNRs while others encapsulate only a single or no AuNRs at all. The analysis of different TEM images showed that the proportion of liposomes having at least one AuNR was  $13.37 \pm 5.82$  %. Similar characteristics have been reported in other studies in regard to the loading of metallic nanoparticles to liposomes via physical entrapment and electrostatic interaction, although the exact proportion of metallic nanoparticle loaded liposomes was not reported (Chauhan et al., 2018; C. H. Liu et al., 2019; Lozano et al., 2012). This pattern of loading would not represent a concern for cancer therapy applications, where enhanced

permeability and retention (EPR) effects would likely result in a high local AuNRs concentration allowing for efficient bulk heating and subsequent release of payload from the thermosensitive liposomes (Agarwal, Mackey, El-Sayed, & Bellamkonda, 2011; Moustafa R. K. Ali et al., 2017). For thrombolysis application, future studies will be required to address dosing optimisation for maximal *in vivo* functionality and active targeting of thrombosis. Other photothermal agents, such as small molecule dyes, may be suitable alternatives as they may be more homogeneously entrapped within the liposomes.

The photothermal ability of AuNRs-PEG (equivalent to ~ 80 µg/mL gold) was tested through continuous irradiation with NIR light (785 nm) for 14 min at 0.64 and 1.35 W/cm<sup>2</sup> power densities. The bulk temperature of the solution started to increase immediately following irradiation and reached a plateau after 4-5 min, with temperature increments proportional to the laser power densities applied (Figure 3.7b). Upon NIR irradiation of FITC-Au-LR<sub>1</sub> dispersions, mild hyperthermia (~ 5° C average bulk temperature increase in response to 1.35 W/cm<sup>2</sup> laser) was also detected, which is consistent with the gold content of the liposomes. Comparable temperature increments were observed from FITC-Au-NLR and FITC-Au-LR<sub>2</sub>. This suggests that the loaded AuNRs can be used as photothermal agents to create local hyperthermia and trigger release of therapeutics from thermosensitive liposomes in response to NIR light.

The photothermal conversion efficiency of AuNRs-PEG, was calculated following the Roper method (Roper, Ahn, & Hoepfner, 2007) as follows;

$$\eta = \frac{hA(T_{max} - T_0) - Q_0}{I(1 - 10^{-0D})} \quad (Eq.3.6)$$

where *h* is the heat transfer coefficient and *A* is the surface area of the container where the dispersion is placed.  $T_{max}$  and  $T_0$  represent the maximum temperature reached by the dispersion containing the NPs and the temperature at the surroundings, respectively (44.3 °C and 22.8 °C in our case).  $Q_0$  corresponds to the power dissipated from the light absorbed by the solvent and container, I to the laser power (860 mW) and OD to the optical density of the sample (2.27). The product hA can be calculated from the properties of the solvent and the cooling curve once the laser is turned off as follows:

$$hA = \frac{m_D C_D}{\tau_s}$$
(Eq.3.7)

where  $m_D$  and  $C_D$  are the mass (1 g) and heat capacity (4.179 Jg<sup>-1</sup>s<sup>-1</sup>) of the solvent (water in this case), while  $\tau_s$  is the time constant of the system (~174 s) calculated from the fitting of the cooling curve to an exponential decay. The photothermal conversion efficiency ( $\eta$ ) of the AuNRs was found to be ~ 56%.

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#### **3.3.3. Release studies on FITC-ovalbumin model protein**

Prior to investigating the NIR-triggered release of FITC-ovalbumin from the prepared liposomes, time and temperature dependent release behaviour was studied using the dialysis method at 37 °C and 42 °C for 6 h. As shown in Figure 3.8a, all formulations displayed good stability at 37 °C (T < T<sub>m</sub>) with some passive protein leakage, which may be attributed to the detachment of some of the surface adsorbed FITC-ovalbumin to the release medium at physiological temperature. FITC-Au-NLR were not responsive to heating, showing comparable release behaviour at 37 °C and 42 °C. Temperaturedependent release was observed from both FITC-Au-LR1 and FITC-Au-LR2 (Figure **3.8b**) when the temperature was raised to 42  $^{\circ}$ C (T > T<sub>m</sub>) with an initial burst release over the first 30 min followed by a diffusion-controlled slower release. Defects created in the lipid packing at hyperthermia possibly results in immobilisation of the surface adsorbed and membrane-embedded FITC-ovalbumin from liposomes in the first instance. Encapsulated macromolecular ovalbumin is expected then to diffuse slowly through the fluid lipid bilayer (Al-Ahmady & Kostarelos, 2016; X. Zhang et al., 2011). Incorporation of Brij in FITC-Au-LR<sub>2</sub> triggered more initial release at a higher temperature compared to FITC-Au-LR<sub>1</sub> and the cumulative (%) release at 42 °C after 1 hour was 57.51 ( $\pm$  6.51) from LR<sub>2</sub> vs. 33.29 ( $\pm$  7.57) from LR<sub>1</sub>. Similar observations for initial burst release of albumin and lysozyme from lysolipid-TSLs at mild hyperthermia (42 °C) were reported by Huang et al. (Xiaoyi Huang et al., 2017) using gel filtration chromatography separation method.



**Figure 3.8. Temperature-assisted release profiles of FITC-ovalbumin from different liposomal formulations.** Release was measured at a) physiological temperature (37 °C) and b) mild hyperthermia (42 °C) in PBS, pH 7.4 using dialysis method.
Upon NIR irradiation (785 nm) of liposomes for 5 min, only low FITC-ovalbumin cumulative release percentages of 15.14 ( $\pm$  3.16) and 22.05 ( $\pm$  2.32) were detected from FITC-Au-NLR after 30 min at 0.64 and 1.35 W/cm<sup>2</sup> respectively (Figure 3.9a). These results suggest that the DSPC lipid membranes in FITC-Au-NLR retained their structural integrity under stimulation conditions where the average bulk temperatures did not exceed the T<sub>m</sub> of DSPC (55 °C). As the AuNRs act as nanoscale heat sources, disruption of the liposome membrane at points in very close proximity to AuNRs may have provoked this slight FITC-ovalbumin release. Similar release profiles (Figure 3.9b) were observed from FITC-Au-LR<sub>1</sub> at 0.64 W/cm<sup>2</sup>, where the bulk temperature increase to  $\sim$  39 °C was not sufficient to fully fluidize the DPPC membranes of the liposomes. On the contrary, efficient cumulative (%) release of 41.14 ( $\pm$ 5.37) and 55.64 ( $\pm$ 4.77) was observed after only 15 min from FITC-Au-LR1 and FITC-Au-LR2 respectively at 1.35 W/cm<sup>2</sup> as shown in **Figure 3.9b-c**. The average bulk temperature increment to  $\sim 42$  °C possibly resulted in fluidisation of DPPC lipid membranes and creation of stable water permeable nanopores after melting of the membrane embedded Brij at 38 °C. The initial burst release during the NIR irradiation phase (0-5 min) was higher from FITC-Au-LR<sub>2</sub> with steeper slope  $(I_2 > I_1)$ , where a combination of detachment of surface bound protein, diffusion of membrane-embedded and core-encapsulated protein through the formed nanopores is suggested to all contribute to release. The decline in the release rate ( $E_2 < E_1$ ) from FITC-Au-LR<sub>2</sub> during the early release stage (5-15 min) is proportionally related to the decrease in residual content of loaded FITC-ovalbumin. Later time points (15-60 min) show comparable release rates from both formulations ( $L_2 \approx L_1$ ), suggesting a ceased heating effect from laser irradiation and subsequent protein-release. The slow release of FITC-ovalbumin during late-stage release suggests minor leakage of protein at physiological temperature (below 5%). Overall, the release profile of FITC-Au-LR2 under NIR-irradiation seems more dependent on surface-bound and surface-embedded protein. The packing defects created by Brij58 under NIR-irradiation may be insufficient to allow free pore-diffusion of the macromolecular protein from the aqueous core of liposomes resulting in incomplete release.



**Figure 3.9. NIR (785 nm) light-assisted release of different liposomal formulations following 5 min irradiation a) FITC-Au-NLR, b) FITC-Au-LR<sub>1</sub> and c) FITC-Au-LR<sub>2</sub>. Liposomes were irradiated using 0.64 and 1.35 W/cm<sup>2</sup> power densities at 37 °C in the dark. Passive release at physiological temperature (37 °C) was used as a negative control.** 

# 3.3.4. In vitro cytocompatibility of Au-LRLs

MTT assays against HEK cells were performed to compare the cytotoxicity of AuNRs before and after PEGylation and to evaluate the cytocompatibility of AuNRs-loaded liposomes. As depicted in (Figure 3.10a), AuNRs were highly cytotoxic at different concentrations due to the remaining free CTAB on surface, a cationic stabilizing agent which imparts a highly positive charge to the surface of AuNRs (Z-potential, +39.5  $\pm$  0.6 mV). On the contrary, AuNRs-PEG showed dramatic decrease in cytotoxicity at high concentrations (~80 µg/mL) which is related to the replacement of cytotoxic CTAB by PEG, as indicated by the drop-in zeta potential (Figure 3.10c).

No significant cell death was observed from Au-LRLs liposomes at all gold concentrations studied, suggesting cytocompatibility of liposomes for *in vivo* applications. Furthermore, no significant RBCs haemolysis was observed (Figure 3.10b and Figure 3.11) in blood samples treated with Au-LRL after 24 h incubation, though AuNRs resulted in 13.89 ( $\pm$  5.2) % haemolysis after the same incubation time, providing more evidence of Au-liposomes biocompatibility for further thrombolysis applications.

Despite this promising data of *in vitro* biocompatibility, long-term toxicity of gold nanoparticles *in vivo* is still a controversial topic (Jia, Ma, Wei, & Qian, 2017). The clearance rate and pathway of metallic nanoparticles depends largely on their size, shape, and surface charge (Hoshyar, Gray, Han, & Bao, 2016; Jia et al., 2017; Sukhanova et al., 2018). In the case of AuNRs, smaller particles such as the ones synthesised in our work are cleared faster and more efficiently than larger AuNRs (Z. Li et al., 2016). However, more work is required to ascertain their *in vivo* biocompatibility, clearance pathways, and potential long-term accumulation.



Figure 3.10. *In vitro* cytocompatibility and hemocompatibility of AuNRs after PEGylation and loading to liposomes: a) MTT assay on HEK cells after 48 h treatment. b) Haemolysis test of human blood samples after 1 h and 24 h incubation. Analysis was done with One-way ANOVA using Tukey multiple comparisons (Mean  $\% \pm$  SD, <sup>ns</sup> p>0.05, \*\*\*p<0.001, \*\*\*\*p<0.0001, n=3). c) Zeta potentials of AuNRs, AuNRs-PEG2000 and FITC-Au-LRLs.



**Figure 3.11: (a) Haemolysis test of human blood samples** treated with 1) PBS negative control, 2) Triton X-100 positive control, 3) AuNRs, 4) AuNRs-PEG and 5) Au-LRLs (Au-LR<sub>1</sub>) for 24 hours at 37 °C. **(b) Haematological analysis of human blood samples** treated with PBS or Au-LRLs (Au-LR<sub>1</sub>) for 24 h using Sysmex<sup>®</sup> analyser.

## **3.4.** Conclusion

In conclusion, a biocompatible NIR-responsive protein delivery system was fabricated based on a hybrid liposomal formulation of ultrasmall PEG-coated gold nanorods, DPPC and non-ionic surfactant (Brij 58). Here, liposomes offered an optimal nanocarrier for coloading of ultrasmall AuNRs-PEG and FITC-ovalbumin model protein using a simple film hydration method. FITC-Au-LRLs showed acceptable entrapment efficiency of both FITC-ovalbumin and ultrasmall AuNRs-PEG as quantified using fluorescence measurement and ICP-OES, respectively. Coating of AuNRs with PEG and their further encapsulation into liposomes resulted in significant enhancement of their biocompatibility as per MTT assay and haemolysis testing. The prepared nanohybrid system showed significant thermosensitivity to the mild hyperthermia induced by NIR irradiation of the incorporated gold nanorods, which resulted in triggered and fast release of the loaded protein. Therefore, the system was selected for encapsulating and the ondemand release of the thrombolytic agent uPA, with the aim to avoid serious off-site bleeding adverse effect. The photothermal effect of incorporated AuNRs is expected to synergise with the biochemical effect of the release uPA to efficiently break clots at subtherapeutic dose of uPA. In addition, this NIR light-responsive liposomal platform offers a promising delivery system for on-demand release of therapeutic proteins to other disease candidates such as cancer, skin disorders, and ophthalmic disorders.

# Chapter 4 Urokinase, gold nanorods co-loaded liposomes towards photothermally-assisted thrombolysis

Aspects of the work presented in this chapter were published as: Nearinfrared light-responsive liposomes for protein delivery: Towards bleedingfree photothermally-assisted thrombolysis, *Journal of Controlled Release*, 337 (2021) 212-223. doi: 10.1016/j.jconrel.2021.07.024

# 4.1. Introduction

Acute thrombosis, caused by rupture of atherosclerotic plaques, is the underlying cause for most myocardial infarctions and many strokes, both associated with a high mortality and morbidity (Engelmann & Massberg, 2013; N. Mackman, 2008). In clinical practice, timely intravenous administration (*i.v.*) of plasminogen activators (PAs), such as tissue plasminogen activator (tPA) and urokinase (uPA), allows re-perfusion of occluded arteries. Their rapid clearance and short circulation half-lives (uPA  $t_{1/2} = 7-20$  min) impose injection of large doses for significant thrombolytic effects, which comes with the risk of haemorrhagic adverse effects (El-Gengaihy, Abdelhadi, Kirmani, & Qureshi, 2007; Refaat, del Rosal, et al., 2021a). Bleeding complications in general are being increasingly recognised as major limitations of the development of new antithrombotic drugs (James D. McFadyen & Peter, 2017). Liposomes, as a translational drug delivery platform, have been investigated to extend the circulation time of PAs and to target thrombus through surface conjugation to polyethylene glycol (PEG) and targeting moieties, respectively (S. Koudelka, R. Mikulik, J. Mašek, et al., 2016; Pawlowski et al., 2017; N. Zhang, C. Li, et al., 2018). Furthermore, smart liposomes responsive to external stimuli (such as ultrasound waves and magnetic fields) have been investigated to target PAs to a specific location and to release their payload on-demand to induce bleeding free thrombolysis (Kandadai, Meunier, Hart, Holland, & Shaw, 2015; C. H. Liu et al., 2019). It is also reported that hyperthermia, induced by alternating magnetic fields (C. H. Liu et al., 2019; Voros et al., 2015b) or photothermal agents (Dong et al., 2019; T.-Y. Lu et al., 2021; N. Singh et al., 2016; X. L. Wang et al., 2017) can accelerate clot lysis.

In this chapter, the NIR light-responsive liposomes (developed in chapter 3) are tested for encapsulation and on-demand release of urokinase plasminogen activator (uPA). We hypothesised that uPA-loaded NIR light-responsive liposomes would release their payload when irradiated with NIR light and show synergistic photothermal lysis of clot mediated by the incorporated AuNRs. This approach can achieve low-dose, site-directed, and potentially bleeding-free thrombolysis.

# 4.2. Materials and Methods

## 4.2.1. Materials

Genkinase<sup>®</sup> for injection (100,000 IU urokinase) was obtained from Alfred hospital pharmacy (Melbourne, Australia). Thromborel<sup>®</sup> S was purchased from Siemens Healthineers (Pennsylvania, USA). Calcium chloride (CaCl<sub>2</sub>) and adenosine diphosphate (ADP) were purchased from Sigma-Aldrich (Melbourne-Australia).

# 4.2.2. Preparation of light-responsive urokinase loaded liposomes (uPA-Au-LR<sub>2</sub>)

The film hydration method described previously (under section 3.2.4) was used to load urokinase to LR<sub>2</sub> formulation, where (DPPC/ Brij58 = 96:4 molar ratio) lipid films were hydrated with urokinase (30.000 IU/mL) and AuNRs-PEG (80  $\mu$ g/mL gold content) dispersions in PBS buffer at 45 °C and agitated for 30 min at a final lipid concentration of 3 mg/mL, then homogenised and purified as aforementioned.

# 4.2.3. Characterisation of uPA-Au-LR<sub>2</sub>

### Size, PDI, zetapotential, and storage stability of uPA-Au-LR<sub>2</sub>

The hydrodynamic diameter, PDI and zetapotential of  $uPA-LR_2$  were measured using DLS for 28 days post preparation to assess their stability under storage conditions (PBS buffer, pH 7.4, 4 °C). At the predetermined time intervals, aliquots of stored liposomes were diluted in filtered DI water at ratios of 1% and 10% (v/v) for size and zeta potential measurement, respectively.

### Urokinase encapsulation efficiency (%)

The entrapment efficiency of uPA in the prepared liposomes was measured using Pierce<sup>™</sup> BCA protein assay kit (Thermo Fisher, Australia), using the supplier protocol with slight modifications. Briefly, serial dilutions (0-2000 µg/mL) of standard bovine serum albumin (BSA) solution were prepared. Then, aliquots (50  $\mu$ L each) of BSA standards or uPA test solutions were transferred into a 96-well flat-bottomed plate (Costar<sup>®</sup>). The working reagent was freshly prepared (50:1, BCA Reagent A: B) and added to each well (200  $\mu$ L). The plate was covered and incubated at 37°C for 30 min, then absorbance was measured at 562 nm. Calibration curve was established through plotting absorbance at 562 nm against concentration of BSA standards. Entrapment efficiency of uPA was calculated through quantification of the total amount of free protein in the supernatant after liposomes ultrafiltration as follows:

$$EE(\%) = \frac{Wi - Wf}{Wi} \times 100 \tag{Eq.4.1}$$

where  $W_f$  is the free amount of uPA in the supernatant after liposomes ultrafiltration and Wi is the initial amount of uPA used to hydrate liposomes.

### Enzymatic activity of urokinase

To confirm the NIR-assisted release of uPA and to assess the therapeutic stability of the released uPA following NIR irradiation, samples (1 mL) of uPA-LR<sub>2</sub> were irradiated with NIR light (785 nm, 1.35 W/cm<sup>2</sup>) for 5 min and then ultracentrifuged at 20,000 rpm for 30 min at 4 °C to separate free released uPA in the supernatant. The total amount (in  $\mu$ g) of released uPA (active + denatured) was quantified using BCA protein assay as previously. Enzymatic activity (in IU) of released uPA in the supernatant was determined with a chromogenic substrate assay. Samples of uPA test aliquots (80  $\mu$ L) were transferred to a 96-well flat-bottom plate (Costar<sup>®</sup>). Then, 20  $\mu$ L of assay buffer and 100  $\mu$ L of 1mM Biophen CS-61(44) chromogenic substrate were added. Absorbance was measured at a wavelength of 405 nm over a period of 60 min. Standard uPA solutions (0–1000 IU/mL) were used as positive controls to establish a calibration curve. Specific enzymatic activity of the release uPA was expressed in IU/ $\mu$ g.

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Furthermore, the specific enzymatic activity of non-formulated free uPA was investigated under different conditions including incubation at mild hyperthermia (42 °C) for 1 h and NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup>) for 5 min in the absence/presence of equivalent amount of free AuNRs-PEG.

Enzymatic activity (%) = 
$$\frac{Specific enzymatic activity after treatment (IU/µg)}{Specific enzymatic activity of standard uPA (IU/µg)} \times 100$$

(Eq.4.2)

### In vitro static thrombolysis assay

Freshly collected human blood from healthy volunteers (1 mL) was mixed with 25  $\mu$ L of 2.68 M calcium chloride, 20  $\mu$ L of thromborel<sup>®</sup> S, and 20  $\mu$ L of 10 mM ADP, leading to platelet activation. Aliquots of the mixture (25  $\mu$ L each) were immediately transferred to a flat bottomed 96-well plate (Costar<sup>®</sup>) and deposited on the inner walls of wells to create halo-shaped clots then incubated at 37 °C for 2 h. The halo-shaped clots were washed twice with PBS, before being treated with equal volumes (75  $\mu$ L) of different treatments with and without NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup>- 5 min). Whole blood (25  $\mu$ L) was treated with 75  $\mu$ L of PBS and used as a positive control for complete lysis. Samples were measured using the Clariostar<sup>®</sup> microplate reader at a wavelength of 510 nm over a period of 60 min at 37 °C. The effect of mild hyperthermia (42 °C) on clot lysis was also investigated using different treatments.

Clot lysis (%) at time (t) = 
$$\frac{Abs(t) - Abs0}{Abs Total - Abs0} \times 100$$
 (Eq.4.3)

where  $Abs_{(t)}$  is the sample absorbance at time *t*,  $Abs_0$  is the absorbance of PBS-treated clots (negative control) at zero time and  $Abs_{Total}$  is the absorbance of the whole blood samples (positive control) that represent 100 % clot lysis.

Blood collection from healthy volunteers was approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (**627/17**).

# 4.2.4. Statistics

Data was expressed as mean  $\pm$  standard deviations. Analysis of *in vitro* enzymatic activity and thrombolysis data was done with either one or two-way repeated measures ANOVA comparing all groups with one another using Tukey multiple-comparison posttest. GraphPad Prism 8 was used to plot the figures and analyse the data.

## 4.3. Results and discussion

Analogous to FITC-ovalbumin, the optimised light-responsive formulation (LR<sub>2</sub>) was used to prepare hybrid liposomes of urokinase and gold nanorods (uPA-Au-LR<sub>2</sub>) to be tested for NIR-triggered release. In this case, the photothermal effect is expected to synergise with the thrombolytic effect of the released uPA.

As shown in Figure 4.1a, the average hydrodynamic diameter of the prepared liposomes was less than 200 nm with PDI values of  $0.296 \pm 0.04$  and zeta potential of - $4.26 \pm 0.2$  mV, which suggests membrane attachment of uPA analogous to that of FITCovalbumin, besides entrapment into the aqueous core of the liposomes. Under storage conditions (PBS buffer, pH 7.4, 4 °C), the size, PDI and zeta potential of the prepared uPA-Au-LR<sub>2</sub> were stable for at least 28 days post-preparation (Figure 4.1a). The entrapment efficiency of uPA was quantified using Pierce<sup>™</sup> BCA protein assay and was found to be  $27.18 \pm 3.6$  (%) with average loading level of ~ 2718 iu/mg of uPA/lipid. Upon NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup>, 5 min) of uPA-Au-LR<sub>2</sub> samples, the release of uPA was confirmed (46.04  $\pm$  1.53 % released in 15 min) through analysis of the ultracentrifuge supernatant for the total amount of free released uPA using BCA protein assay. Enzymatic activity of the free, released uPA was further tested using the Biophen CS-61(44) chromogenic substrate assay and compared to standard uPA solutions. The results ascertain a compromised enzymatic activity after NIR-assisted release, which may be caused by the intense heating from AuNRs to the surrounding microenvironment at the nanoscale (Figure 4.1b). Similar observations were made when free non-formulated uPA was NIR-irradiated in the presence of equivalent amount of free AuNRs-PEG. Neither mild hyperthermia (42 °C) nor NIR-irradiation (in absence of AuNRs-PEG) resulted in a significant change in the enzymatic activity of non-formulated uPA.



Figure 4.1. Characterisation of uPA-Au-LR<sub>2</sub>. a) Storage stability of uPA-Au-LR<sub>2</sub> in PBS buffer (pH 7.4) at 4 °C for 28 days post-preparation. b) Enzymatic activity (%) of uPA assessed using Biophen CS-61(44) chromogenic substrate assay under different conditions. Bulk temperature increase under NIR irradiation was controlled at ~ 5 °C through using equivalent amounts of AuNRs-PEG. Analysis was done with One-way ANOVA using Tukey multiple comparisons (Mean %  $\pm$  SD, <sup>ns</sup> p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n≥3).



Figure 4.2. Calibration curves of a) Pierce<sup>™</sup> BCA protein assay kit and b) Biophen CS-61(44) chromogenic substrate assay kit. These results were used for entrapment efficiency, cumulative release (%) and enzymatic activity calculations of uPA from uPA-Au-LR<sub>2</sub>.

A calibration curve of bovine serum albumin (BSA) serial dilutions (0-2000 µg/mL) was established (Figure 4.2a) using a Pierce<sup>™</sup> BCA protein assay kit, as described under section 4.2.3. Protein content of test solutions of uPA was further quantified and used to calculate uPA entrapment efficiency in liposomes using equation 4.1. (See section 4.2.3). To calculate enzymatic activity of uPA under different treatments, test samples were quantified using Biophen CS-61(44) chromogenic substrate assay, as described by manufacturer (See section 4.2.3.). Here, a calibration curve of standard solutions of commercial uPA (0-1000 iu/mL) was established and used accordingly (Figure 4.2b).

In vitro thrombolytic effect of uPA-Au-LR<sub>2</sub> was then assessed using 25 µL halo-clots (Figure 4.3) at physiological temperature (37 °C), mild hyperthermia (42 °C), and under NIR-irradiation. As shown in Figure 4.3a-b, under mild hyperthermia, uPA-Au-LR<sub>2</sub> showed relatively high clot lysis of 52.84 % ( $\pm$  7.44) after 60 min, compared to 32.99 %  $(\pm 5.5)$  clot lysis at 37 °C. These results further confirm the liposome thermosensitivity and suggest better clot lysis effect from uPA at mild hyperthermia. Enhanced clot lysis was observed from equivalent strength of free uPA at mild hyperthermia as well (Figure 4.3a-b). To judge the photothermal thrombolytic effect of AuNRs in the prepared liposomes, NIR irradiation (785 nm-1.35 W/cm<sup>2</sup> for 5 min) of Au-LR<sub>2</sub> (without uPA) was tested on similar clots to show 29.97 % ( $\pm$  2.86), and 61.13 % ( $\pm$  9.14) clot lysis after 5 min and 60 min respectively (Figure 4.3c-d). Our uPA-Au-LR<sub>2</sub> were further tested for clot lysis under laser irradiation (785 nm-1.35 W/cm<sup>2</sup> for 5 min), where 55.65 % ( $\pm$  5.04) clot lysis was detected immediately following NIR irradiation compared to only 5.21 %  $(\pm 2.37)$  clot lysis from non-irradiated liposomes. After 60 min (Figure 4.3f), NIRirradiated uPA-Au-LR<sub>2</sub> showed almost complete clot lysis compared to  $32.99 \% (\pm 5.5)$ lysis from non-irradiated liposomes.



Figure 4.3. In vitro thrombolysis assay in a 96-well plate using 25 µL Halo-clots. a) Clot remaining (%) following exposure to different treatments of PBS, upA-Au-LR<sub>2</sub>, and equivalent amount of free uPA at 37 °C and 42 °C. b) Clot lysis (%) measured after 60 min of treatment in a). c) Clot remaining (%) following exposure to PBS or Au-LR<sub>2</sub> with and without NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup>, 5 min). d) Clot lysis (%) measured after 60 min of treatment in c). e) Clot remaining (%) following exposure to PBS or uPA-Au-LR<sub>2</sub> with and without NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup>, 5 min). d) Clot lysis (%) measured after 60 min of treatment in c). e) Clot remaining (%) following exposure to PBS or uPA-Au-LR<sub>2</sub> with and without NIR irradiation (785 nm, 1.35 W/cm<sup>2</sup>, 5 min). f) Clot lysis (%) measured after 60 min of treatment in e). Bulk temperature increase under NIR irradiation was controlled at ~ 5 °C through using liposomes loaded with equivalent amounts of AuNRs-PEG. Analysis was done with Two-way ANOVA for clot remaining (%) curves and One-way ANOVA for clot lysis (%) using Tukey multiple comparisons. (Mean % ± SD, <sup>ns</sup> p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, n≥3).

Three observations can be concluded from these results. First, the AuNRs themselves possess a strong photothermal thrombolytic effect on halo-clots under NIR stimulation. Second, uPA on-demand release from NIR-irradiated liposomes is achievable after 5-min irradiation. Third, enhanced thrombolysis is observed from NIR-irradiated liposomes through combined photothermal effects of AuNRs and biochemical effects of immobilised/released uPA.

As a novel approach towards photothermal thrombolysis, our liposomal uPA-AuNRs hybrid system offers several potential advantages over some of the previously reported work. Secondary embolism of large clot fragments to small blood vessels, reported with photothermal clot lysis using AuNRs solely (N. Singh et al., 2016; F. Zhang, Liu, et al., 2019a), can potentially be avoided through the combined effect of uPA released in response to NIR light. Besides, AuNRs allow more efficient heating upon NIR irradiation, as the LSPR band can be tuned to match the laser wavelength. Other gold nanostructures require high irradiation power densities; for example, Wang and co-workers (X. L. Wang et al., 2017) used high laser power (808 nm, 5 W/cm<sup>2</sup>) to create mild hyperthermia and to release uPA from tetradecanol capped gold-mesoporous silica core-shell nanospheres. Furthermore, liposomes are biocompatible nanocarriers and their surface can be easily modified for extended circulation in blood and ligand-mediated targeting to thrombus sites (Y. Huang et al., 2019; X. Wang et al., 2016; X. Wang et al., 2014). Ligand-mediated targeting will facilitate the delivery of high local concentrations of the liposomes to thrombi, allowing more efficient bulk heating and faster and larger amounts of uPArelease. Most importantly, this will be a release of uPA that is localised at the site of the thrombus, with the clinically highly desirable avoidance of systemic bleeding side effects. The same liposome-gold nanorod platform may also be applied for light-triggered delivery of cytotoxic macromolecular proteins, such as mistletoe lectin I, Trichosanthin and gelonin, in the case of chemo-photothermal therapy to cancer (Asrorov, Gu, Min, Shin, & Huang, 2020; de Matos et al., 2018).

More recently, photothermal therapy and NIR light-triggered nanodelivery systems attracted major interest as efficient non-invasive delivery platforms, as it had been successfully applied in preclinical animal models. However, its clinical translation is still hampered by limitations such as the limited light penetration ability into deep tissue and the need for local or targeted systemic delivery (Deng, Shao, & Zhao, 2021). Several studies have addressed ex vivo and in vivo penetration of NIR light into tissues such as skin, skeletal muscles, skull, and brain and demonstrated penetrations between 2 mm to few cm (Bashkatov, Genina, Kochubey, & Tuchin, 2005; D. E. Hudson, 2013; Weissleder, 2001). Examples of NIR light stimulation being directly translatable are skin cancer chemo-photothermal therapy (W. Chen, Goldys, & Deng, 2020), ocular drug delivery (Lajunen, Nurmi, et al., 2016), ocular histoplasmosis photodynamic therapy (Visudyne<sup>®</sup>) (W. Chen et al., 2020) and brain surface irradiation (Henderson & Morries, 2015; Zivin et al., 2009). For deep tissue irradiation, guided light delivery using magnetic resonance imaging (MRI), ultrasound (US), or computed tomography (CT), will be needed. Examples include the FDA-approved MRI-guided laser ablation (Visualase Thermal Therapy<sup>®</sup>) and (NeuroBlate<sup>®</sup>) for high-grade glioma therapy. The US-guided AuroLase<sup>®</sup> therapy utilizes NIR irradiation of PEG gold-silica core-shell nanoparticles to treat prostatic cancer in patients (H. S. Han & Choi, 2021). In this context, our designed light-responsive liposomal platform can be potentially used for transcranial laser therapy of venous thrombosis and ischaemic strokes (Zivin et al., 2009). Alternatively, a guide wire with optic fibres or an optical coherence tomography (OCT) catheter system could be used to deliver NIR light to deep tissue clots such as in coronary thrombosis, deep vein thrombosis, or pulmonary embolism. Overall, there is clearly the need for further development and improvement of photothermal thrombolysis to ultimately allow clinical translation.

# 4.4. Conclusion

A NIR light-responsive liposomal platform was successfully developed for on-demand release of uPA, a commonly used thrombolytic drug in clinical settings with fatal bleeding adverse effects. The uPA-Au-LR<sub>2</sub> presented a small size range (less than 200 nm) and small PDI values (<0.3), which were retained for at least 28 days postpreparation under storage conditions (PBS buffer, pH 7.4, 4 °C). Enhanced thrombolysis was achieved in vitro using a halo-clot model immediately after 5 min NIR irradiation of the prepared uPA-Au-LR<sub>2</sub>, making use of the combined photothermal/biochemical effects of AuNRs and released uPA. Here, the photothermal effect of the incorporated AuNRs is suggested to stimulate release of uPA from the thermosensitive liposome and assist penetration of the released uPA into clot. Importantly, photothermal irradiation resulted in only a slight decrease in the therapeutic efficacy of the released uPA, as confirmed by the enzymatic activity assay. These observations open the way to the development of NIR light-responsive liposomes for therapeutic protein delivery to other diseases such as cancer, skin disorders, brain disorders, and ophthalmic diseases. Ondemand site-directed release of low doses of uPA at the thrombus site using this approach is expected to achieve bleeding-free thrombolysis *in vivo*. Furthermore, liposomes can be easily modified for extended circulation in blood and ligand-mediated targeting to thrombus sites. Ligand-mediated targeting will facilitate the delivery of high local concentrations of the liposomes to thrombi, allowing more efficient bulk heating and faster and larger amounts of uPA-release. These highly promising data indicates the potential of our newly engineered NIR light-responsive liposomes as a drug delivery tool for site-directed, photothermally-stimulated therapeutic protein release.

# Chapter 5 Activated Platelet-Targeted IR780 Immunoliposomes for Photothermal Therapy of Acute Thrombosis

Aspects of the work presented in this chapter are submitted to Advanced Functional Materials for publication as: Activated Platelet-Targeted IR780 Immunoliposomes for Bleeding-Free Photothermal Therapy of Acute Thrombosis

# **5.1. Introduction**

Treatment of acute thrombosis using plasminogen activators (PAs), the gold standard clot-busters in clinical settings, comes with some limitations such as poor thrombus penetration, the need for long infusions, and subsequent risk of fatal systemic bleeding (S. Liu et al., 2018; Nigel Mackman, 2008). Targeted therapy to thrombosis offers a promising tool towards bleeding-free and more efficient treatment (Absar et al., 2015; Masumeh Zamanlu et al., 2018). Activated platelets, a predominant component of acute thrombi, represent a very attractive target for localised delivery of PAs and diagnostic probes to acute thrombosis (Y. Lu, Q. Hu, C. Jiang, & Z. Gu, 2019; Palasubramaniam et al., 2019). Both peptides (such as cRGD) (Yu Huang et al., 2019; Pawlowski et al., 2017; Nengpan Zhang et al., 2018) and single chain variable fragment antibodies (scFv) (M. Schwarz et al., 2006; Xiaowei Wang et al., 2016; Xiaowei Wang et al., 2014b) have been used to target the overexpressed glycoprotein complex (GPIIb/IIIa) on the surface of activated platelets. Antibodies showed higher binding specificity to activated platelets compared to RGD peptides (X. Wang & K. Peter, 2017). Targeted delivery of PAs to

thrombosis results in more efficient thrombolysis at low doses with reduced bleeding side effects (Xiaowei Wang et al., 2014b; Nengpan Zhang et al., 2018).

Photothermal therapy has recently attracted attention as a new technology for treatment of thrombosis (Nitesh Singh et al., 2016; X. Wang et al., 2017b; F. Zhang, Liu, et al., 2019b; Z. Zhao et al.). Local hyperthermia mediated by photothermal agents, such as gold nanomaterials illuminated with NIR light, can break up clots and enhance the effect of co-administered low-dose PAs. Localised release of PAs from thermoresponsive nanocarriers, under NIR irradiation, has been also investigated for low-dose thrombolysis (Refaat, del Rosal, Palasubramaniam, Pietersz, Wang, Moulton, et al., 2021; X. Wang et al., 2017b; Jiasheng Xu et al., 2020; Z. Zhao et al.). Because of the potential long-term accumulation and subsequent toxicity of metallic photothermal nanoparticles (Jia et al., 2017), a shift towards the use of organic molecular dyes (such as ICG and IR780) represents a rational alternative. Interestingly, these molecular dyes can be simultaneously used for NIR fluorescence imaging of thrombosis.

IR780 is a cationic lipophilic NIR fluorescent photothermal dye which has been explored for cancer photothermal therapy and imaging applications (Palao-Suay et al., 2017; Tran et al., 2017; Yue et al., 2017; Y. Zhan et al., 2017). Because of its rigid cyclohexenyl ring with a central chlorine atom, IR780 is brighter and more photostable than FDA-approved NIR dye indocyanine green (ICG) (K. Wang et al., 2016; Yue et al., 2013; C. Zhang et al., 2010). IR780, like other organic dyes, has limited solubility in biological fluids, low targeting ability, toxicity at large doses, and rapid clearance from the body, all of which limit its clinical potential (Alves, Lima-Sousa, de Melo-Diogo, Louro, & Correia, 2018). These limitations have been addressed through encapsulation of IR780 molecules in different nanostructures such as lipid nanoparticles, micelles, albumin conjugates and liposomes (Yan et al., 2016) (Alves et al., 2018) (Figure 5.1).



**Figure 5.1.** Potential applications of IR-780 nanostructures in cancer therapy and diagnosis. Adapted with permission from (Alves et al., 2018). Copyright 2018 Elsevier.

We hypothesised that targeted delivery of liposomal IR780 to activated platelets (Tar-IR-L) will offer efficient photothermal thrombolysis and give potential for NIR fluorescence imaging of acute thrombosis. Photothermally-assisted thrombolysis in the presence of low-dose, co-loaded single chain urokinase (scuPA) was also be investigated using targeted IR780/scuPA liposomes (Tar-scuPA-IR-L). In this context, liposomes represent an optimal delivery system for loading of the hydrophobic IR780 dye within its phospholipid membranes, while Tar-scFv and scuPA molecules can be functionalised to liposome surfaces. In this chapter, we report on an activated platelets-targeting IR780 immunoliposomes for site-directed photothermal thrombolysis (**Figure 5.2**). Conjugation of liposomes to Tar-scFv/scuPA was achieved using biotin-neutravidin chemistry. Selective binding of liposomes to activated platelets was confirmed using both flow cytometry and confocal microscopy. Photothermal thrombolysis was then investigated *in vitro* using a microfluidic thrombosis-on-a-chip model.



Figure 5.2. Schematic diagram showing formulation of neutravidin-coated IR780 liposomes and their conjugation to scFv antibody targeting activated platelets

(Tar-scFv), in absence or presence of single chain urokinase (scuPA).

# 5.2. Materials and methods

# 5.2.1. Materials

2-[2-[2-Chloro-3-[(1,3-dihydro-3,3-dimethyl-1-propyl-2*H*-indol-2-ylidene) ethylidene ]-1-cyclohexen-1-yl]ethenyl]-3,3-dimethyl-1-propylindolium iodide (IR-780 iodide), 3,3'-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>), absolute ethanol (EtOH), chloroform (CHCl<sub>3</sub>), Triton X-100, polyoxyethylene 20 cetyl ether (Brij58<sup>™</sup>), bovine serum albumin (BSA), paraformaldehyde (PFA), apyrase, acid citrated dextrose (ACD), and adenosine diphosphate (ADP) were purchased from Sigma-Aldrich. 1,2-dipalmitoyl-*sn*-glycero-3phosphocholine (DPPC) was obtained from Lipoid GmbH (Ludwigshafen-Germany). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- (cap biotinyl) (sodium salt) (DOPEbiotin) was purchased from Avanti Polar Lipids. Urokinase 100.000 IU (Actosolv<sup>®</sup>) was supplied by Alfred hospital pharmacy (Melbourne-Australia). NeutrAvidin and R-Phycoerythrin (PE)-labelled streptavidin were purchased from ThermoFisher. FITC mouse anti-human CD41, FITC mouse anti-human PAC-1 antibody (PAC-1) and FITC mouse IgG isotype control (Iso-FITC) were obtained from BD Biosciences. Human collagen was obtained from Takeda<sup>®</sup>.

# 5.2.2. Biotinylated activated platelet-targeting single chain antibody (Tar-ScFv) and biotinylated single chain urokinase plasminogen activator (scuPA) production

The activated platelet-targeting scFv and its mutated form were generated, expressed, and purified as previously described (Xiaowei Wang et al., 2016; X. Wang et al., 2012). The recombinant scuPA was generated, expressed, and purified as previously described (Xiaowei Wang et al., 2016). Vector-maps, generation and purification are shown in **Figure 5.3**.



**Figure 5.3. Vector-map, generation, and purification of biotinylated scuPA and scFv. a)** Gene-map of scuPA in pSectag2A vector for mammalian expression. **b)** Electrophoresis with 0.8% agarose gel: pSectag2A plasmid (5137bp) after double cut restriction digest, and scuPA (924bp) after polymerase chain reaction amplification. **c)** Western blot analysis of scuPA after protein purification. **d)** Gene-map of scFv<sub>anti-LIBS</sub> in pAC6 vector for biotinylation. **e)** Electrophoresis with 0.8% agarose gel: pAC6 plasmid (4186bp) after double cut restriction digest, and scuPA (925bp) after polymerase chain reaction amplification. **f)** Western blot analysis of scFv after protein purification. Adapted with permission from (Xiaowei Wang et al., 2016). Copyright 2012 Ivyspring.

# 5.2.3. Preparation of neutravidin-coated IR780 liposomes (IR-L-Neut)

Biotinylated IR780 liposomes (IR-L) were prepared using the reported film hydration method and size optimisation was done using a membrane extruder (Avanti<sup>®</sup>, 0.2  $\mu$ m). Aliquots of DPPC, Brij58 and DOPE-biotin (**Table 5.1**) in chloroform were mixed with IR780 in ethanol at 2:1 volume ratio. Organic solvent mixture was evaporated at 50 °C under vacuum using rotary evaporator. The formed lipid/IR780 films were then hydrated at 50 °C with PBS and rotated for 30 min. Liposomal dispersion was then passed through preheated thermostated membrane extruder (0.2  $\mu$ m) for 20 times at 50 °C and left to equilibrate at room temperature for 30 min. Neutravidin was then added to biotinylated liposomes (IR-L), while vortexing, at 10 molar excess of neutravidin and incubated for 1 hr at room temperature. Excess unbound neutravidin and free IR780 was removed through dialysis for 24 h using membranes of MWCO 300 kDa in PBS under stirring (100 rpm) at 4 °C.

Table 5.1.	Composition	of different IR7	80 liposomes.
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Formula symbol	Composition	Molar ratio (%)
Blank L IR-L IR-L-Neut	DPPC/Brij58 DPPC/Brij58/IR780/DOPE-biotin DPPC/Brij58/IR780/DOPE-biotin/Neutravidin	96/4 93.9-39.8/4/2/0.1-0.2 93.9-39.8/4/2/0.1-0.2/0.1-0.2
Tar-IR-L Mut-IR-L Tar-scuPA-IR-L Mut-scuPA-IR-L	IR-L-Neut (0.1) /Tar-scFv (0.3) IR-L-Neut (0.1) /Mut-scFv (0.3) IR-L-Neut (0.2) /scuPA(0.3)/Tar-scFv (0.3) IR-L-Neut (0.2) /scuPA(0.3)/Mut-scFv(0.3)	3 molar excess of scFv to neutravidin Equimolar scuPA & scFv antibodies

# 5.2.4. Conjugation of activated platelet-targeting scFv antibodies and scuPA to liposomes

Neutravidin-coated liposomes (IR-L-Neut) were conjugated to biotinylated scFv antibodies using biotin-neutravidin conjugation method. Biotinylated scFv antibodies

(Tar-scFv and its mutated form) were added at 3:1 molar ratio of scFv to neutravidin for conjugation and incubated for 30 min at room temperature, immediately before use. Tar-scFv-labelled IR780 liposomes are referred as Tar-IR-L and Mutated scFv-labelled IR780 liposomes are referred as Tar-IR-L and Mutated scFv-labelled IR780 liposomes are referred as Mut-IR-L (Table 5.1).

For co-loading of scFv and scuPA to liposomes, equimolar amounts of biotinylated scFv antibodies and biotinylated scuPA were added to the neutravidin-coated IR-liposomes (0.2% neutravidin) while vortexing and incubated for 30 min at room temperature, immediately before use.

# 5.2.5. Characterisation of the prepared liposomes

### Size, PDI, and zeta potential measurement

Size distribution, PDI, and zeta potential of the prepared liposomes were measured using DLS (Zetasizer Nano ZS, Malvern Instruments). Dispersions were diluted in filtered DI water at ratios of 1% and 10 % (v/v) for size and zeta potential measurement, respectively.

### **IR780** content

For IR780 content quantification, liposomes were dissolved in absolute ethanol, then absorbance was measured at 780 nm wavelength to quantify the amount of IR780, using Enspire Perkin Elmer 2300 microplate reader.

### **UV-vis-NIR spectrophotometry**

Absorbance spectra of freshly dispersed free IR780 and liposomal IR780 (IR-L) in PBS (equivalent to ~ 12.5  $\mu$ g/mL IR780) were collected using (Cary50Bio) spectrometer in standard (1 cm pathlength) quartz cuvettes. PBS was used as reference solution accordingly.

Day-light photostability was also assessed over the period of 48 h for both the free and liposomal dye in PBS at 25°C, through monitoring of the changes in absorbance spectra using (Cary50Bio) spectrometer.

### NIR- photothermal transduction using 785 nm laser

IR780 liposomal dispersions (0.5 mL) of different IR780 content were transferred to a 48-well plate (Costar<sup>®</sup>) and irradiated with a NIR laser (OptoTech, 785 nm) at 1 W/cm<sup>2</sup> power density continuously for 5 mins. A thermal camera (FLIR ETS320) was used to record the bulk temperature change in well during irradiation and after switching the laser off. The same experiment was performed on freshly dispersed IR780 in PBS.

### Fluorescence storage stability

Fluorescence of free IR780 dye and IR-L in PBS buffer (pH 7.4) was measured using Enspire Perkin Elmer 2300 microplate reader at 780 nm as the excitation wavelength and 810 nm as the emission wavelength. Stability of NIR fluorescence was assessed for 28 days post preparation under storage in dark conditions at 4 °C. Normalised fluorescence intensity ( $I_t/I_0$ ) was used for comparison.

### Fluorescence stability during continuous NIR irradiation

Fluorescence of freshly dispersed free IR780 dye and IR-L in PBS buffer (pH 7.4) was monitored, under continuous laser irradiation using a Lumics laser diode (808 nm, 15 mW/cm<sup>2</sup>) and a Princeton Instruments NIRvana InGaAs CCD cooled to -70 °C. The laser background was filtered out using a Semrock notch filter. Normalised fluorescence intensity ( $I_t/I_0$ ) was used for comparison.

#### In vitro cytocompatability of IR-L using MTT assay

Cytocompatibility of free IR780 dye and IR-L was tested using a Chinese Hamster Ovary (CHO) cell line and MTT assay kit. Cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM, high glucose 4.5 g/L) supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) at 37°C with 5% CO<sub>2</sub>. Cells were split as 100  $\mu$ L aliquots into a 96-well flat-bottomed plate (Costar<sup>®</sup>) at a density of 10<sup>4</sup> cells/well and incubated, at 37°C with 5% CO<sub>2</sub>, for 24 h to allow cells attachment. Aliquots (10  $\mu$ L) containing different concentrations of free IR780, or IR-L were then added to cells and incubated for another 24 h or 48 h at 37°C with 5% CO<sub>2</sub>.

After that, MTT assay kit was used to detect cell viability. Each well was treated with 10  $\mu$ L of filter sterilised MTT solution (5 mg/mL) for 4 hrs. Then, the medium was carefully tipped off and the insoluble formazan crystals were solubilised in the solubilisation medium. Absorbance was measured at 570 nm using a microplate reader (PerkinElmer 2300 EnSpire) microplate reader and cell viability was calculated as follows:

$$Cell \ viability \ (\%) = \frac{Absorbance \ of \ test \ cells}{Absorbance \ of \ control \ untreated \ cells} \times 100$$
(Eq.5.1)

### Haemolysis study

Haemocompatibility of free IR780 dye and liposomal dye (IR-L) in PBS (~ 50 µg/mL dye content) was tested using haemolysis of red blood cells (RBCs). Human blood was freshly collected in citrated vacutainers, and then 380 µL of blood was incubated with 20 µL of test samples for 24 h at 37 °C under agitation (150 rpm). Blood was centrifuged at 2000 rpm for 10 minutes using ultra-centrifuge, and supernatant was 10-fold diluted with PBS and quantified for haemoglobin release through absorbance measurement using a

(PerkinElmer 2300 EnSpire) microplate reader at 550 nm. The red blood cells treated with PBS and Triton X-100 were set as negative and positive control respectively.

$$Haemolysis(\%) = \frac{Absorbance of test - Absorbance of PBS treated}{Absorbance of Triton treated - Absorbance of PBS treated} x100 \quad (Eq.5.2)$$

Sysmex<sup>®</sup> analysis of blood samples, treated with either free IR780 or IR-L for 24 h, was also performed to compare the main haematological parameters including white blood cells count (WBCs), red blood cells count (RBCs), haematocrit values (HCT), mean corpuscular volume (MCV), and platelets count (PLT).

### Neutravidin coating of liposomes

Successful neutravidin coating of liposomes was tested by flow cytometry (BD LSRFortessa<sup>TM</sup>) using R-PE streptavidin at 1:1000 strength. Data for 10<sup>4</sup> gated events were collected and analysed accordingly. Blank L were used as negative control.

### ScFv antibody and scuPA content

The scFv antibody and scuPA content was quantified using Pierce<sup>™</sup> BCA protein assay kit following supplier protocol, as described previously under **Section 4.2.3**.

For scuPA enzymatic activity calculation, Biophen® CS-41(03) chromogenic substrate assay was used following the supplier protocol with some modifications. Serial dilutions of commercial uPA (0 – 2000 iu/mL) and our scuPA (0-10  $\mu$ g/mL) were prepared in assay buffer (38 mM NaCl, 5 mM Tris-HCl, 0.1% BSA, pH 8.8). Aliquots of 50  $\mu$ L were split into a 96-well flat-bottomed plate (Costar ®) and incubated with 25  $\mu$ L of plasmin (0.005 iu/mL) for 2 h at 37 °C. Then, 50  $\mu$ L of plasminogen (0.02 iu/mL) was added to each well. After 5 min, 100  $\mu$ L of the chromogenic substrate (1.25 mg/mL) was added to each well using a multichannel pipette. Absorbance was measured at 405 nm every 5 min for 20 min at 37 °C. Specific enzymatic activity was then expressed as iu/ $\mu$ g.

### Binding affinity to activated platelets

### a) Flow cytometry

Platelet-rich plasma (PRP) was separated from freshly collected citrated human blood by centrifugation at room temperature (1000 rpm) for 10 min (Acc: 4, Decc: 0) using Eppendorf<sup>®</sup> ultracentrifuge (5810). PRP was then 20-fold diluted with PBS containing  $Ca^{+2}$  and  $Mg^{+2}$ . For platelets activation, ADP was then added at 20 µM concentration and incubated for 5 min at room temperature.

Aliquots (50  $\mu$ L) of resting platelets and ADP-activated platelets were incubated with different treatments (5  $\mu$ L) of IsoFITC, PAC1, IR-L, Mut-IR-L, Tar-IR-L, Mut-scuPA-IR-L, and Tar-scuPA-IR-L (at final lipid concentration ~0.125 mg/mL) for 30 min before being fixed with PFA (2%) and tested on flow cytometer (BD LSRFortessa<sup>TM</sup>). Data for 10<sup>4</sup> gated events were collected and analysed accordingly.

Selective binding of Tar-scFv to activated platelets was confirmed following similar protocol, where platelets were incubated with either Tar-scFv or its mutated form at final concentration of 8  $\mu$ g/mL for 30 min. Then Streptavidin PE (1:1000) was added immediately before fixing using PFA (2%) and testing on flow cytometer (BD LSRFortessa<sup>TM</sup>). Data for 10<sup>4</sup> gated events were collected and analysed accordingly.

### b) Confocal laser scanning microscopy

**Platelet washing:** 1 mL acid citrated dextrose (ACD) and 0.2 U apyrase were added to 10 mL of freshly collected human blood (healthy volunteer) before centrifugation at 180 g for 15 min (Acc: 4, Decc: 0). The supernatant (PRP) was collected, supplemented with apyrase (0.02 U/mL), and centrifuged again at 1500 g for 7 min (Acc: 7, Decc: 4) to separate a platelet pellet. The platelets pellet was resuspended in a platelet-washing buffer containing apyrase (0.02 U/mL), then further centrifuged at 1200 g for 7 min (Acc: 7, Decc: 7).

Decc: 4). The platelet pellet was resuspended in Tyrode buffer containing apyrase (0.01 U/mL) to 2 x  $10^7$  platelets/mL as per Sysmex count. Washed platelets were allowed to rest at 37 °C for 30 min.

**Platelet spreading and staining:** 100  $\mu$ L of washed platelets was added to each well of 8-well chambered glass coverslip at a density of 2 x 10<sup>6</sup> cells/well and allowed to spread on glass for 30 min. For platelet activation, 10  $\mu$ L of ADP (20  $\mu$ M final conc.) was added to the desired wells and incubated for another 15 min. Any suspended /non-adhered platelets were tipped off and 100  $\mu$ L PBS was added. 10  $\mu$ L of different liposome treatments (at final lipid concentration ~0.125 mg/mL) were added for 30 min. Then, platelets were rinsed three times with PBS and blocked with 2% BSA for 30 min. Platelets were further rinsed three times with PBS and fixed using PFA (2%).

Selective binding of scFv antibody to activated platelets was tested following a similar protocol, where platelets were spread on glass, blocked with 2% BSA, and then incubated with either Tar-scFv or its mutated form (8 µg/mL final concentration) for 30 min. After rinsing with PBS for 3 times, platelets were incubated with Streptavidin PE (1:1000) and CD41-FITC (1:100) for 30 min, rinsed three times with PBS and fixed using PFA (2%). Confocal imaging of platelets was performed using a confocal laser scanning microscope (Nikon A1R Plus si NIR Modified, Japan) at excitation wavelengths of 488, 561, and 785 for CD41-FITC, Streptavidin PE, and IR780 respectively. Semrock<sup>®</sup> long pass filters 450/50, 525/50, and 796/41 were used to filter out background laser accordingly.

### In vitro photothermal thrombolysis studies using microfluidic thrombosis-on-a-chip

Fluorescent microthrombi were formed *in situ* using microfluidic channels (ibidi<sup>®</sup>  $\mu$ -Slide VI 0.4). The channels were coated with human collagen (100  $\mu$ g/mL, Takeda<sup>®</sup>-Australia) overnight at 4 °C and then blocked with BSA (1%) for 1 h at room temperature. Whole blood from healthy volunteers was freshly collected in citrated vacutainers, then preincubated with DiOC<sub>6</sub> dye (5  $\mu$ g/mL) for 30 min at 37 °C. Citrated blood was then recalcified with CaCl<sub>2</sub> at 67 mM and perfused immediately through the channels for 5 min at shear rate of 200 s<sup>-1</sup>, using a syringe pump (PhD 2000, Harvard Apparatus, USA) (**Figure 5.4**). The formed fluorescent microthrombi were further perfused with washing buffer (PBS with Ca<sup>+2</sup> and Mg<sup>+2</sup>) for 5 min. Different liposome treatments (equivalent to ~ 1.25 mg total lipid content, ~ 22.5  $\mu$ g IR780 dye) were injected into the washing buffer and allowed to flow through the channels for 5 min. To study the thrombolytic effect of liposomes under NIR irradiation, the channels were further NIR irradiated (785 nm, 1 W/cm<sup>2</sup>) for 5 min and then perfused with washing buffer for 5 min.

Formation of fluorescent microthrombi, binding of different liposomal treatments, and the subsequent thrombolytic effect in absence or presence of NIR irradiation were analysed using confocal laser scanning microscopy (Nikon A1R Plus si NIR Modified, Japan) at excitation wavelengths of 488 and 785 for DiOC<sub>6</sub> and IR780 respectively. Semrock<sup>®</sup> long pass filters 450/50, and 796/41 were used to filter out background laser accordingly.



Figure 5.4. Microfluidic thrombosis-on-a-chip model setting to form fluorescent microthrombi *in-situ*.

# 5.2.6. Statistics

Data was expressed as mean ± standard deviations. Analysis was done with either one or two-way repeated measures ANOVA comparing all groups with one another using multiple-comparison post-test. Multiple student t-test was used as well. GraphPad Prism 9 was used to plot the figures and analyse the data.

# 5.3. Results and Discussion

# 5.3.1. Physicochemical characterisation of IR-L

Biotinylated IR780 liposomes (IR-L) of hydrodynamic diameter ~ 100 nm and PDI of ~ 0.1 were prepared using film hydration-membrane extrusion method. IR-L in PBS showed a strong and slightly shifted absorbance peak at 790 nm (Figure 5.5a). Similar shift in absorbance peak of liposomal IR780 was reported in literature (S. Li, Johnson, Peck, & Xie, 2017) (Y. J. Lu, A. T. S, C. C. Chuang, & J. P. Chen, 2021) which might be attributed to slight changes in the refractive index of the surrounding microenvironment . The absorbance decreased significantly when free IR780 was freshly dispersed in PBS because of the instantaneous aggregation of lipophilic dye molecules in aqueous media. In addition, the free dye photobleached rapidly – within first 6 hours – under room lights illumination, while IR780 liposomes were stable under the same conditions for at least 48 h (Figure 5.5a), consistent with previously published data (Y.-J. Lu, A. T. S, C.-C. Chuang, & J.-P. Chen, 2021). Figure 5.5b presents digital photograph of free IR780 dye (freshly dispersed in PBS), and freshly prepared liposomal dye (IR-L) in glass vials.

To study the photothermal effect of the prepared system, aliquots of IR-L with different content of IR780 were irradiated in a 48-well plate using NIR light (785 nm, 1 W/cm<sup>2</sup>), while recording the average bulk temperature increase using a thermographic camera. As shown in **figure 5.5c**, NIR irradiation of IR-L (equivalent to  $50\mu$ g/mL IR780) produced an average temperature increase of ~ 10 °C, compared to the negligible heating (< 2 °C) observed under the same conditions for an equivalent concentration of IR780 dispersed in PBS. These results suggest IR780 incorporates into liposomes mostly in monomeric forms, which can absorb light efficiently and partly convert it into heat, as previously reported (Kuang et al., 2017; K. Wang et al., 2016). On the other hand, free IR780 is

reported to exhibit high photothermal heating efficiency in ethanolic solutions and low efficiency in aqueous dispersions, probably due to IR780 aggregation and molecular stacking of aromatic ring structure in aqueous solutions (S. Li et al., 2017; K. Wang et al., 2016).

**Figure 5.5d** shows thermographic images of IR-L (equivalent to  $50\mu$ g/mL IR780) captured during the first 60 s of NIR irradiation (785 nm- 1 W/cm<sup>2</sup>), using a thermographic camera installed directly over the samples. To measure the average bulk temperature increase of the test dispersion, a region of interest was set on borders of the well. The laser spot (~ 8 mm in diameter) was directed to the centre of the well using a collimator. Before each experiment, laser power was calibrated using a thermal power meter placed at the same distance from the laser exit.




Fluorescence storage stability of free IR780 dye and IR-L in PBS buffer (pH 7.4) at 4 °C for 4 weeks. f) Fluorescence stability under continuous NIR irradiation (15 mW/cm<sup>2</sup>) of free IR780 dye and IR-L in PBS buffer. Analysis was done with Two-way ANOVA for fluorescence storage stability, and unpaired student t-test for stability under NIR irradiation (Mean  $\pm$  SD, ns p>0.05, \*p<0.05, \*\*\*\*p<0.0001, n=3).

Incorporating IR780 into liposomes enhances the photostability of the dye as shown in **figure 5.5e**, which represents the time evolution of the NIR fluorescence of IR-L and free IR 780 stored at 4 °C and protected from exposure to light. The fluorescence intensity of IR-L remained stable for at least 28 days after preparation, while the free dye in PBS lost over 50 % of its fluorescence intensity in one week. IR-L also showed a higher photostability compared to free IR780 under continuous laser irradiation (808 nm, 15 mW/cm<sup>2</sup>). The fluorescence of free dye decreased exponentially with time, falling to  $\sim$  32 % of its initial value after 6 min (**Figure 5.5f**), whereas IR-L photobleached at a much slower rate, requiring over 10 minutes of continuous irradiation for their NIR fluorescence to decrease by 50 %. This photostability of IR-L would potentially allow more efficient photothermal therapy *in vivo*, compared to the free dye.

# 5.3.2. In vitro cytocompatibility study

IR-L also showed higher cytocompatibility than IR780 dispersed in PBS, as indicated by MTT cytotoxicity and haemolysis assays. While both IR-L and IR780 showed low cytotoxicity at concentrations between 0.1 and 20 µg/mL for incubation times up to 48 hours, higher concentration (50-100 µg/mL) resulted in a much higher cytotoxicity for the free dye (see figure 5.6a-b). This can be attributed to the cationic nature of the dye, which is associated to cytotoxicity (Alves et al., 2018; Jiang et al., 2015). Incorporating IR780 into liposomes masks its cationic nature, as evidenced by a slightly negative  $\zeta$ potential (- 2.76 ± 0.2 mV). Compared to IR-780, IR-L also causes significantly lower haemolysis (Figure 5.6c). Further, Sysmex<sup>®</sup> analysis was used to compare the main haematological parameters of human blood samples treated with either free IR780 or IR-L for 24 h. The following haematological parameters were quantified: white blood cells count (WBCs), red blood cells count (RBCs), haematocrit values (HCT), mean corpuscular volume (MCV), and platelets count (PLT). While triton-X (the positive control) showed significant drop in RBCs count and haematocrit due to cell lysis, both free IR780 and IR-L did not show significant differences, compared to PBS negative control (Figure 5.6d). This enhanced biocompatibility of IR-L would potentially allow safer administration of higher equivalent doses of IR780 *in vivo* for more efficient photothermal applications.



Figure 5.6. *In vitro* cytocompatibility of free IR780 and IR-L in PBS. a) Cell viability of CHO cells after 24 h treatment, and b) 48 h treatment. c) Haemolysis test after incubation of free IR780 and IR-L ( $50\mu g/mL$ ) with human blood samples for 24 h. d) Haematological analysis of human blood samples measured using Sysmex<sup>®</sup> analyser after 24 h incubation. Analysis was done using multiple unpaired student t-test (Mean % ± SD, <sup>ns</sup> p>0.05, \*p<0.05, \*p<0.01, n=3).

# **5.3.3.** Neutravidin coating of IR-L and their conjugation to biotinylated scFv antibodies and scuPA

The avidin-biotin bonding is one of the strongest biological non-covalent interactions between a protein and a ligand, with versatile applications in the field of biotechnology and drug delivery (Akshay Jain & Cheng, 2017). The affinity constant of avidin to biotin is extremely high ( $K_d = 10^{-15}$  M) which results in a very rapid and strong bond formation (Livnah, Bayer, Wilchek, & Sussman, 1993). Avidin, streptavidin, and neutravidin are functional analogues with different sources and physicochemical properties. Avidin is derived from the eggs of aves, while streptavidin is purified from *Streptomyces avidinii* bacteria. Neutravidin (MW = 60 kDa) is a chemically de-glycosylated form of avidin. It shows the lowest non-specific binding properties to cells and other proteins because of its near-neutral isoelectric point (pI = 6.3) (A. Jain, Barve, Zhao, Jin, & Cheng, 2017). That is the reason why neutravidin was selected to be used as a bridging agent between our biotinylated-liposomes (IR-L) and biotinylated scFv /scuPA.

As shown in **Table 5.2.**, neutravidin binding to liposomes surface did not result in significant change in hydrodynamic size and PDI, where crosslinking of liposomes was avoided through adding neutravidin at 10 molar excess during the coating process (Hermanson, 2013), as described under **Section 5.2.3**. Success of liposome coating with neutravidin was further confirmed using flow cytometry. Here, liposomes gate was first set using a typical dot plot of side scatter (SSC) vs forward scatter (FSC) (Figure 5.7a).

Exposure of biotin on the surface of IR-L was detected using the fluorescently labelled streptavidin-PE. On the other hand, neutravidin-coating shielded biotin groups on the surface of coated-liposomes (IR-L-Neut) and prevented further binding of the fluorescently labelled-streptavidin. Non-biotinylated blank liposomes of similar size and lipid composition were used as a negative control.

Strong binding of streptavidin-PE to biotinylated IR-L surface was observed with strong fluorescence intensity (29449  $\pm$  5662), as compared to low signals (744  $\pm$  273) from non-biotinylated blank liposomes of similar size and lipid composition (**Figure 5.7b-c**). Binding of streptavidin-PE was sterically hindered after coating of IR-L with neutravidin (IR-L-Neut) showing significantly low fluorescence intensity (247  $\pm$  179) which suggests complete occupation of biotin molecules on the surface of IR-L-Neut.

Conjugation of neutravidin-coated liposomes (IR-L-Neut) to the targeting antibody Tar-scFv, in the absence or presence of equimolar scuPA, resulted in significant increase in the average hydrodynamic diameter of liposomes and polydispersity (**Table 5.2. & Figure 5.7d**). Surface charge, as indicated by  $\zeta$ -Potential values, showed a significant drop after conjugation of liposomes (**Figure 5.7e**). These changes in liposomes size and surface charge following conjugation suggest the formation of a protein corona on the surface of liposomes comprised of either scFv alone in the case of Tar-IR-L, or scFv with scuPA in the case of Tar-scuPA-IR-L (Hama, Sakai, Itakura, Majima, & Kogure, 2021). All liposomal formulations showed high entrapment efficiency of IR780 (91.39 ± 5.38%) with average loading level of ~ 18 µg/mg of IR780/lipid.

Liposome	d (nm)	PDI	ζ-potential (mV)	IR780 EE (%)
IR-L IR-L-Neut Tar-IR-L Tar-scuPA-IR-L	$85 \pm 06$ $96 \pm 10$ $141 \pm 16$ $195 \pm 07$	$\begin{array}{c} 0.084 \pm 0.02 \\ 0.179 \pm 0.04 \\ 0.304 \pm 0.06 \\ 0.302 \pm 0.14 \end{array}$	$-2.76 \pm 0.2$ $-4.80 \pm 0.8$ $-6.80 \pm 1.6$ $-14.27 \pm 0.6$	EE (%) = 91.39% (± 5.38)

**Table 5.2.** Hydrodynamic size, PDI,  $\zeta$ -potential and entrapment efficiency (EE %) of IR780 liposomes ( $n \ge 3$ ).



**Figure 5.7.** Neutravidin coating of IR-L and their conjugation to Tar-scFv alone or with scuPA. a) Flow cytometry gating of liposomes to test neutravidin coating. b) Flow cytometry histograms of blank L (non-biotinylated), IR-L (biotinylated), and IR-L-Neut after treatment with Streptavidin-PE at 1:1000. c) Mean fluorescence intensity (MFI) of the different liposomes after treatment with Streptavidin-PE. d) Hydrodynamic size distribution and e) Zeta potential measurements of IR-L, IR-L-Neut, Tar-IR-L, and Tar-scuPA-IR-L. Analysis was done

with One-way ANOVA using Tukey multiple comparisons. (Mean  $\pm$  SD, <sup>ns</sup> p>0.05, \*\*\*\*p<0.0001, n=3).

As described previously under Section 5.2.3, IR780 content of liposomes was quantified by dissolving liposomes in absolute ethanol and then measuring absorbance at 780 nm. A calibration curve of standard ethanolic solutions of IR780 was plotted (Figure 5.8a) and used to calculate IR780 content of liposomes. Before conjugating scFv and scuPA to the surface of IR-L-Neut, their protein content was measured using a Pierce<sup>TM</sup> BCA protein assay kit as described previously under Section 4.2.3 and using the calibration curve in Figure 5.8b.

The enzymatic activity of scuPA was also measured using a Biophen CS41(03) chromogenic substrate assay as described under **Section 5.2.3**. Here, the increase in absorbance at 405 nm was measured over the period of 20 min at 37 °C. Enzymatic activity of scuPA was then identified based on a serial dilution of standard commercial urokinase solutions (0-2000 iu/mL) as shown in **Figure 5.8c**. Specific enzymatic activity was calculated by dividing enzymatic activity of scuPA (iu/mL) by the concentration of scuPA ( $\mu$ g/mL) and expressed as iu/ $\mu$ g. For example, this batch of scuPA (**Figure 5.8c**) showed an enzymatic activity of ~ 250 iu/mL and protein content of ~ 6  $\mu$ g/mL (1:100 diluted stock of 600  $\mu$ g/mL), which can be expressed as ~ 42 iu/ $\mu$ g.



Figure 5.8. Calibration curves for quantification of IR780, scFv antibody, and scuPA content. a) Calibration curve of absorbance of standard IR780 ethanolic solutions measured using a microplate reader at  $\lambda = 780$  nm. b) Pierce<sup>TM</sup> BCA protein assay used to measure protein content of scFv and scuPA prior to conjugation (n=3). c) Biophen CS41(03) chromogenic substrate assay used to determine scuPA enzymatic activity.

# 5.3.4. Selective binding to activated platelets studies

#### Flow cytometry & confocal microscopy

To test the binding of liposomes to activated platelets, we used both flow cytometry and confocal microscopy. Activated platelets show overexpression of the GPIIb/IIIa surface receptor which can be confirmed using PAC-1, a FITC-labelled mouse antihuman IgG specifically binding the GPIIb/IIIa receptor. Here, platelet gates were first set using a typical dot plot of side scatter (SSC) vs forward scatter (FSC), where activated platelets showed slightly higher degree of forward- and side-scattering, compared to resting platelets (**Figure 5.9a**). These changes in scattering behaviour might be related to the morphological changes of platelets following activation and possible clustering of activated platelets (Downing & Klement, 2012). When treated with PAC-1, activated platelets showed higher fluorescence intensity (1454.2  $\pm$  280.69) compared to resting platelets (38.2  $\pm$  18.06) (**Figure 5.9b**). This result confirms over-expression of GPIIb/IIIa receptors on the surface of ADP-activated platelets. Selective binding of our Tar-scFv to activated platelets was further confirmed using streptavidin-PE as a 2<sup>nd</sup> stain, where activated platelets showed higher fluorescence intensity (1106  $\pm$  285.24) compared to (105  $\pm$  64.51) from resting platelets (**Figure 5.9c**).

Further, the selective binding of Tar-IR-L and Tar-scuPA-IR-L to activated platelets was confirmed using flow cytometry (**Figure 5.10a-b**). Here, the NIR fluorescence of the incorporated IR780 was used to quantify binding to platelets. Higher fluorescence intensity was observed from activated platelets compared to resting platelets following incubation with Tar-IR-L and Tar-scuPA-IR-L, respectively. Some degree of unspecific binding of non-targeted liposomes (IR-L, Mut-IR-L, and Mut-scuPA-IR-L) to both resting and activated platelets was observed, which resulted in relatively high background signals. This unspecific binding was mitigated through washing of glass-spread platelets with PBS multiple times after their incubation with different liposomal treatments and before confocal imaging.



Figure 5.9. Flow cytometry showing platelets activation using ADP and selective binding of Tar-scFv to activated platelets. a) Flow cytometry gating of platelets. b) Platelets activation was confirmed using PAC-1, after incubation with 20  $\mu$ M ADP for 5 min. c) Selective binding of free Tar-scFv to activated platelets was confirmed using streptavidin-PE 2<sup>nd</sup> labelling. Analysis was done using multiple student t-test, one per treatment, (Mean  $\pm$  SD, <sup>ns</sup> p>0.05, \*\*p<0.01, \*\*\*\*p<0.0001, n≥3).



Figure 5.10. Flow cytometry demonstrating selective binding of a) Tar-IR-L and b) TarscuPA-IR-L to activated platelets. NIR-fluorescence signals from the incorporated IR780 dye were used to detect binding of liposomes to platelets. Analysis was done using multiple student t-test, one per treatment, (Mean  $\pm$  SD, <sup>ns</sup> p>0.05, \*p<0.05, \*\*p<0.01, n=5).

Analogous to flow cytometry data, confocal images showed a similar pattern of selective binding of Tar-scFv, Tar-IR-L, and Tar-scuPA-IR-L to activated platelets (Figure 5.11). Here, platelets spreading on glass allowerd further washing of platelets with PBS after incubation with different liposomal treatments. This washing step resulted in detachement of passively bound liposomes from the surface of platelets before their confocal imaging. Most of platelets presented a high degree of spreading on glass after 30 min incubation, as shown using CD41-FITC staining (Figure 5.11). CD41, a platelet specific glycoprotein also known as gpIIb, is expressed on the surface of both resting and activated platelets and commonly used as conventional marker for platelet identification (Bagamery, Kvell, Landau, & Graham, 2005). Streptavidin-PE was used to confirm selective binding of Tar-scFv to activated platelets as shown in Figure 5.11a, where the Tar-scFv tended to bind predominantely to the activated platelet granulomere, the central area of platelet (Firkin, 1984). This pattern of binding is attributed to receptor migration and clustering to granulomere, upon platelet activation (Lewis et al., 1990). Figure 5.11b shows significant increase in mean fluorescecene intensity of activated platelets treated with Tar-scFv and streptavidin-PE, compared to resting platelets. On the other hand, MutscFv failed to show specific binding to platelets, regardless the activation status. Both Tar-IR-L and Tar-scuPA-IR-L showed higher mean fluorescence intensities from activated platelets, compared to all other non-targeted liposomes (Figure 5.11c-d). Here, the NIR fluorescence of the incorporated IR780 was used to quantify binding to platelets. Stronger signals were observed from activated platelet granulomere, which is consistent with the Tar-scFv binding pattern observed in **figure 5.11b**. These results further confirm the targeting efficiency of our targeted liposomes to activated platelets in vitro.



Figure 5.11. Confocal microscopy imaging confirming selective binding of Tar-scFv, Tar-IR-L, and Tar-scuPA-IR-L to activated platelets. Representative images of spread platelets treated with a) Tar-scFv or Mut-scFv and streptavidin PE, c) different liposomal treatments. Platelets are counterstained with FITC-labelled CD41antibody (scale bar = 10  $\mu$ m). Mean fluorescence intensity (MFI) of spread platelets following 30 min incubation with b) Tar-scFv or Mut-scFv and streptavidin PE, d) different liposomal treatments (n=3). Analysis was done with Two-way ANOVA (Mean ± SD, ns p>0.05, \*\*\*p<0.001, \*\*\*\*p<0.0001, n=3).

# 5.3.5. *In vitro* photothermal thrombolysis study using microfluidic thrombosis-on-a-chip model

To investigate the photothermal thrombolysis effect of our targeted liposomes, collagen-coated microfluidic channels (ibidi® µ-slides VI 0.4) were used to form DiOC<sub>6</sub>stained microthombi in situ (average size~ 20 µm), as seen in Figure 5.12. Collagen coating of microfluidic channels was used to induce platelet activation and adhesion, as the most potent sub-endothelial thrombogenic component (Roberts, McNicol, & Bose, 2004). DiOC<sub>6</sub>, a lipophilic fluorescent dye, was used to label platelets to study thrombi formation in the microfluidic setting (Berry et al., 2021; de Witt et al., 2014; Swieringa et al., 2016). Platelet-rich fluorescent microthrombi were formed, when recalcified citrated human blood was perfused in the collagen-coated channels for 5 min at shear rate of 200 s<sup>-1</sup> (Figure 5.12). Smaller and less dense microthrombi were obtained when TarscuPA-IR-L was perfused through the channels. Selective binding of targeted liposomes (Tar-IR-L and Tar-scuPA-IR-L) to microthrombi was further investigated relying on the NIR fluorescence of the incorporated IR780 dye. While non-targeted liposomes (IR-L, Mut-IR-L, and Mut-scuPA-IR-L) presented weak binding and low fluorescence signals in our preliminary data, strong fluorescence signals were observed in the IR780 channel from targeted liposomes (Tar-IR-L and Tar-scuPA-IR-L). Following 5 min-NIR irradiation (785 nm, 1 W/cm<sup>2</sup>) of channels treated with either Tar-IR-L or Tar-scuPA-IR-L, both mean fluorescence intensity (MFI) and percentage area covered by fluorescent microthrombi were significantly decreased (Figure 5.13a). Here, the photothermal effect of the IR780 dye present in bound Tar-IR-L is expected to exert a fibrinolytic action on the surface of microthrombi before liposome detachment from the surface. This effect is suggested by the decreased MFI and areas of DiOC<sub>6</sub>-stained microthrombi. In scuPAcoloaded targeted liposomes (Tar-scuPA-IR-L) perfused channels, the photothermal

effect synergised with the fibrinolytic effect of low-dose scuPA to efficiently break the microthrombi. The localised heating effect at nanoscale level can potentially increase the penetration of liposomes into clots, leading to a more efficient fibrinolysis by the co-loaded scuPA (F. Zhang, Han, et al., 2019; F. Zhang, Liu, et al., 2019b; Z. Zhao et al.). **Figure 5.13b** shows 3D construction of the fluorescent microthrombi under different treatments (Tar-IR-L and Tar-scuPA-L) before and after NIR irradiation (785 nm, 1 W/cm<sup>2</sup> - 5 min). The 3D construction was performed using Imaris<sup>®</sup> software analysis for z-stacks of microthrombi (150 µm x160 µm) at 20 µm height. Decrease in both MFI and microthrombi areas can be observed following NIR irradiation (785 nm, 1 W/cm<sup>2</sup> – 5 min) of the channels (**Figure 5.13b**). These observations confirm the efficiency of photothermal effect to loosen and break clots and give potential for further testing the targeted liposomes *in vivo*.



#### **Before NIR irradiation**

### After NIR irradiation

Figure 5.12: Confocal imaging of microthrombi in microfluidic channels after exposure to different treatments (PBS, Tar-IR-L, and Tar-scuPA-IR-L) before and after NIR irradiation (785 nm- 1 W/cm<sup>2</sup>, 5 min), showing *in vitro* photothermally-assisted thrombolysis effect (scale bar =  $20 \mu$ m).



Figure 5.13. Analysis of confocal images of the stained microthrombi in microfluidic channels. a) Mean fluorescence intensity of microthrombi and (%) area covered following different treatments. b) 3D construction of microthrombi under Tar-IR-L and Tar-scuPA-IR-L treatments, collected before and after NIR irradiation using z-stack imaging and Imaris software analysis. Analysis was done with Two-way ANOVA using multiple comparisons (Mean  $\pm$  SD, ns p>0.05, \*p<0.05, \*p<0.01, \*\*\*\*p<0.0001, n=9).

#### **5.4.** Conclusions

Biotinylated IR780 liposomes were successfully prepared using film hydrationmembrane extrusion method and compared to free dye for photostability, photothermal effect, and cytocompatibility. Liposomal IR780 showed higher degree of photostability and cytocompatibility, compared to free dye. Incorporating IR780 dye molecules within the liposome phospholipid bilayers resulted in significant increase in the dye fluorescence stability under both NIR irradiation and storage condition. Temperature increase (~ 10 °C) was produced when liposomal dye (equivalent to 50 µg/mL) was NIR irradiated (785 nm, 1 W/cm<sup>2</sup>), compared to the negligible heating (< 2  $^{\circ}$ C) observed under the same conditions for an equivalent concentration of IR780 dispersed in PBS. Then, activate platelet-targeted IR780 immunoliposomes (Tar-IR-L) were successfully prepared through conjugation of IR780-loaded liposomes to a scFv antibody which binds specifically to the glycoprotein (GP IIb/IIIA) on the surface of activated platelets. The biotin-neutravidin chemistry was used as an instantaneous, robust, and irreversible noncovalent bonding. Tar-IR-L showed selective binding to activated platelets in vitro, as demonstrated by flow cytometry and confocal microscopy imaging. In a thrombosis-ona-chip model, the photothermal effect of Tar-IR-L under NIR irradiation resulted in significant decrease in both fluorescence intensity and (%) microthrombi area. Coloading of a single chain urokinase (scuPA) to the surface of the targeted liposomes (TarscuPA-IR-L) resulted in enhanced thrombolysis of the fluorescent microthrombi. In conclusion, activated platelets-targeted IR780 immunoliposomes represent a promising delivery system for low-dose, potentially bleeding-free, photothermal thrombolysis.

# Chapter 6 *In vivo* studies of activated platelettargeted IR780 immunoliposomes

Aspects of the work presented in this chapter are submitted to Advanced Functional Materials for publication as: Activated Platelet-Targeted IR780 Immunoliposomes for Bleeding-Free Photothermal Therapy of Acute Thrombosis

# **6.1. Introduction**

In this chapter, the clot targeting ability and thrombolytic properties of the prepared Tar-IR-L and Tar-scuPA-IR-L were tested *in vivo* using a ferric chloride (FeCl<sub>3</sub>)-induced injury mouse model, as one of the most widely used and well-established acute thrombosis preclinical animal models.

FeCl<sub>3</sub>-induced injury is widely used to provoke arterial occlusive thrombosis in mice to study thrombogenesis, antiplatelet drugs, and antithrombotic agents preclinically (Grover & Mackman, 2020). The mechanism of thrombosis induction is still not fully understood, and several underlying pathways are suggested. Oxidative stress-induced vascular injury (ECKLY et al., 2011) and red blood cells (RBCs)-mediated platelet recruitment (Barr, Chauhan, Schaeffer, Hansen, & Motto, 2013) are the main suggested underlying causes of thrombosis induction. RBCs exposure to FeCl<sub>3</sub> results in lipid peroxidation and haemoglobin oxidation which was proven a contributor to vascular injury and platelets recruitment (Kevin, Sharelle, Jaye, Hatem, & Shaun, 2009). The model is characterised by simplicity of surgical procedure, reproducibility, sensitivity, and ease of application to several arteries of different diameters ranging from large carotid artery to small mesenteric arterioles (Bonnard & Hagemeyer, 2015) (W. Li, Nieman, & Sen Gupta, 2016).

The procedure simply involves surgical isolation of the artery of interest, followed by exposure to a small piece (1 mm x 2 mm) of filter paper impregnated in FeCl<sub>3</sub> solution (2.5-20 %) for 2-5 min. Assessment of thrombogenesis is usually performed using intravital imaging of fluorescent-labelled thrombus over time in small mesenteric arterioles (Bonnard & Hagemeyer, 2015) (W. Li et al., 2016). Alternatively, occlusion time of large carotid artery can be measured to confirm clot formation. Occlusion time is defined as the time between the vessel exposure to FeCl<sub>3</sub> to the time of complete blood flow cessation (Grover & Mackman, 2020; Shuai et al., 2021). The latter is monitored using an ultrasonic flow probe. Here, delay in occlusion time represents a key parameter to assess efficiency of antiplatelet drugs and prophylactic antithrombotic agents (Surin, Prakash, Barthwal, & Dikshit, 2010) (Shuai et al., 2021). Moreover, histological examination has been frequently used to assess the therapeutic efficiency of thrombolytic agents (Seo et al., 2018; Y. Wang, Xu, Zhao, & Yin, 2021), and external stimuli-responsive nanoparticles (Guan et al., 2020; T.-Y. Lu et al., 2021; Z. Zhao et al., 2022).

Due to the technical constraints of intravital imaging of thrombus size under NIR irradiation using externally applied class-I laser, we selected to use histological analysis, besides photothermal measurements, to investigate the thrombolytic efficiency of our liposomes *in vivo*.

### 6.2. Materials and methods

## 6.2.1. Materials

Ketamine was obtained from Parnell Laboratories, NSW, Australia. Xylazine was obtained from Troy Laboratories, NSW, Australia. Ferric chloride, neutral buffered Formalin solution (10%), Xylene, absolute ethanol, sodium bicarbonate, Haematoxylin stain, eosin stains, and D.P.X. neutral mounting medium were purchased from Sigma Aldrich, Australia. Enoxaparin sodium (Clexane<sup>®</sup>) was ordered from Alfred hospital pharmacy, VIC, Australia. Tissue-Tek O.C.T compound was obtained from Sakura Finetek, USA.

# 6.2.2. Ferric chloride-induced injury

Male C57BL/6 mice of 6 weeks age (20 to 25g) were used. Animals were anesthetised through intraperitoneal (*i.p.*) injection of ketamine/xylazine mixture at dose of 100 mg/kg and 10 mg/kg, respectively. FeCl<sub>3</sub>-induced injury was performed to induce thrombosis in the left carotid artery through introduction of a small filter paper (2mm x 1mm) saturated with 5% ferric chloride to the left carotid artery for 2 min.

### 6.2.3. *Ex-vivo* thrombosis fluorescence imaging

FeCl<sub>3</sub>-induced injury was performed as mentioned above. Then, different animal groups were immediately (*i.v.*) injected, using tail injection, with the different liposomal formulations including PBS, IR-L, Mut-IR-L, Tar-IR-L, Mut-scuPA-IR-L, and Tar-scuPA-IR-L (equivalent to 0.05  $\mu$ g IR780/g body weight) and PBS was used as the vehicle control. Mice were humanely killed after 1 h through (*i.p.*) injection of another dose of ketamine/xylazine mixture at dose of 300 mg/kg and 30 mg/kg, respectively. After perfusing heart with PBS, mice were dissected, and different organs (carotid arteries, liver, spleen, kidney, lung, and skeletal muscle) were collected for fluorescence imaging using IVIS at 780/845 nm excitation/emission wavelength filter. Biodistribution

and imaging experiments were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (**P1967**).

# 6.2.4. In vivo photothermally-assisted thrombolysis

After *i.p.* anaesthesia of mice, the left carotid artery was isolated and FeCl<sub>3</sub>-induced injury was performed as previous. Different treatments including PBS, IR-L, Mut-IR-L, Tar-IR-L, Mut-scuPA-IR-L, and Tar-scuPA-IR-L (equivalent to ~ 0.3 µg IR780, ~ 45 IU scuPA /g body weight) were immediately injected to mice (n=5), using tail vein injection. When full occlusion of artery was achieved (within 10 min) as confirmed by blood flow measurement using doppler flow probe, local area of thrombosis was exposed to NIR irradiation (785 nm, 1 W/cm<sup>2</sup> – spot diameter  $\sim$  4 mm) for 5 min. The real-time increase in local temperature was recorded using a thermographic camera. Here, a region of interest was set at the site of NIR irradiation and average increase in temperature was recorded. The experiment was performed in an enclosed laser setting (Class- I), as shown in Figure 6.1. The enclosed laser setting was exclusively designed by Optotech Pty LTd with dimensions of 60 x 60 cm (Figure 6.1a). The system is equipped with a FLIR thermogenic camera, a 785 nm laser source, optic fiber, collimator, laser safety interlock and an iDS camera (Figure 6.1b). For NIR irradiation, anesthetised mouse on a heating mat is transferred into the enclosed system, where the mouse and its body temperature changes can be monitored using iDS camera and FLIR thermal camera, respectively (Figure 6.1c). After 30 min from injection, mice were humanely killed as described in 6.2.3 and left injured carotid arteries were excised and embedded into Tissue-Tek® O.C.T. compound and snap-frozen without previous fixation. Cryostat (Leica CM1950) was used to section the frozen arteries, where 8 transversal histology sections were cut across the thrombosed artery and stained using Hematoxylin and Eosin (H & E) staining. Bright field microscopy (Olympus BX43) and ImageJ software were used to accurately measure

average clot area  $(\mu m^2)$  following different treatments. Regions of interest were set around the borders of each clot as discussed later in **section 6.3**.

Photothermal thrombolysis experiments were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (**P8196**).



**Figure 6.1.** Photos of the enclosed laser setting (class I) for *in vivo* laser experiments a) The enclosed setting has dimensions of 60x60x60 cm. b) The system is equipped with a fiber-coupled 785 nm laser source, collimator, iDS camera, FLIR thermal camera, and safety laser interlock. c) Anesthetised mouse on a heating mat is transferred into the enclosed system for laser irradiation. The mouse and its body temperature changes are monitored during laser irradiation using iDS camera and FLIR thermal camera.

# 6.2.5. Statistics

Data was expressed as mean  $\pm$  standard deviations. Analysis was done with either one or two-way repeated measures ANOVA comparing all groups with one another using multiple-comparison post-test. GraphPad Prism 9 was used to plot the figures and analyse the data.

#### 6.3. Results and discussion

In vivo targeting efficiency and photothermal thrombolysis of liposomal formulations were tested using FeCl<sub>3</sub>-induced thrombosis mouse model. As shown in Figure 6.2a, the left carotid artery was first exposed and treated with a small filter paper (2mm x 1mm) saturated with 5% ferric chloride for 2 min. Acute thrombosis started to form and the artery was fully occluded within  $\sim$  7-9 min, as confirmed by a doppler flow probe. Both thrombosed left carotid arteries and healthy right carotid arteries were collected after 1 h of administration of liposomal treatments. Ex vivo NIR fluorescence imaging of both thrombosed left carotid arteries and right healthy carotid arteries was performed using IVIS fluorescence imaging. Both Tar-IR-L and Tar-scuPA-IR-L treated-mice (n=5) showed significant signals comparted to Mut-IR-L and Mut-scuPA-IR-L treated-mice, respectively (Figure 6.2b). On the other hand, non-conjugated liposomes (IR-L) failed to show significant change compared to PBS treatment (Figure 6.2b). These observations confirm the efficiency of scFv-conjugation and subsequent activated platelets targeting in acute thrombosis and give potential for targeted thrombolysis and NIR-fluorescence imaging of acute thrombosis in vivo. Furthermore, different organs were ex vivo imaged using IVIS fluorescence imaging following different liposomal formulations. Liposomes tended to accumulate mainly in liver as presented by the strong NIR fluorescence signals in figure 6.3a. All the liposomal formulations presented similar distribution behaviour after 1 h of *i.v.* administration where the short time interval (1 h) was not enough to show if conjugation to scFv antibodies would influence liposomes distribution to different organs (Figure 6.3b). A longer timeframe (24 h) might be needed to evaluate the influence of liposome conjugation on their biodistribution and pharmacokinetics in vivo.



**Figure 6.2.** *In-vivo* **clot-targeting study.** a) Representative images of the left carotid artery before and after the FeCl<sub>3</sub>-induced injury. b) IVIS images and average radiant efficiency for the excised thrombosed left carotid arteries (left column) and healthy right carotid artery (right column), after 1 h from *i.v.* administration of liposomal treatments. Analysis was done with Two-way ANOVA using multiple comparisons. (Mean  $\pm$  SD, ns p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n=3 mice for PBS and n=5 for all other treatments).



Figure 6.3. *Ex vivo* biodistribution of different liposomes after 1 h of *i.v.* administration to mice. a) IVIS images of different organs. b) Average radiant efficiency of different organs showing comparable biodistribution behaviour from all liposomes. Liposomes tend to accumulate in mainly liver within 1 h of *i.v.* administration, regardless of their conjugation status. Analysis was done with Two-way ANOVA using Tukey multiple comparisons (Mean  $\pm$  SD, ns p>0.05, n=5 mice).

To study the photothermal effect, NIR irradiation (785 nm, 1 W/cm<sup>2</sup>- 5 min) of thrombosed carotid arteries was then performed and real-time temperature increase was measured using a thermographic camera. Higher average temperature increments (12.65  $\pm 2.95$  °C and  $11.15 \pm 0.85$  °C) were recorded from mice treated with targeted liposomes (Tar-IR-L and Tar-scuPA-IR-L), respectively (Figure 6.4a). Lower average temperature increments  $(5.94 \pm 2.15 \text{ °C}, 6.48 \pm 1.31 \text{ °C}, \text{ and } 4.29 \pm 1.22 \text{ °C})$  were recorded from other non-conjugated liposomes (IR-L), Mut-IR-L, and Mut-scuPA-IR-L, respectively. PBStreated mice showed around 3 °C background local temperature increment (Figure 6.4a). Real-time temperature increases during NIR irradiation (785 nm, 1 W/cm<sup>2</sup>) of thrombosed arteries of mice treated with PBS, IR-L, and Tar-scuPA-IR-L, was plotted against time to show the difference in photothermal behaviour of conjugated and nonconjugated liposomes (Figure 6.4b). Thermographic images captured at zero time and after 5 min-NIR irradiations show the changes in mice body temperature and the localised temperature increase at thrombosis site following different liposomal treatments (Figure **6.4c)**. These observations come in line with the clot-targeting study and further confirm the efficiency of targeted liposomes to elicit photothermal effect towards photothermal thrombolysis, where the localised temperature increase is expected to fibrinolytic action on acute thrombi and synergise with the delivered low dose of co-loaded scuPA to break clots in vivo.



Figure 6.4. *In vivo* photothermal transduction under NIR-irradiation (785 nm, 1 W/cm<sup>2</sup>). a) The average temperature increment of the thrombosed-left carotid artery as measured by FLIR thermal camera after 5 min-NIR irradiations of different treatments (n=5). b) Real time average temperature increment measurements of the thrombosed-left carotid artery under continuous laser irradiation, after *i.v.* injection of PBS, non-conjugated IR-L, and Tar-scuPA-IR-L. c) Representative thermographic images of an anaesthetised mouse at zero time (before laser irradiation), and at 5 min of continuous laser irradiation following i.v. injection of PBS, non-conjugated IR-L, and Tar-scuPA-IR-L. Analysis was done with One-way ANOVA using multiple comparisons (Mean  $\pm$  SD, ns p>0.05, \*\*\*p<0.001, \*\*\*\*p<0.0001, n=5 mice).

Histological examination of the excised thrombosed carotid arteries was then performed to study the photothermal effect on clot size. Here, average clot areas were accurately measured through averaging clot areas from sliced thrombosed vessel (8 slices) as shown in Figure 6.5a-b. A region of interest was set using ImageJ software around the borders of each clot as shown in figure 6.5b. Clot area measurements showed  $\sim$  52 % decrease in clot size from mice treated with Tar-IR-L (equivalent to  $\sim$  0.3  $\mu g$ IR780/g body weight), compared to PBS-treated mice (Figure 6.5c). Co-loading of lowdose scuPA (~ 45 IU scuPA/g body weight) to targeted liposomes (Tar-scuPA-IR-L) resulted in a larger (~ 72 %) but not significantly different reduction in clot areas, compared to Tar-IR-L (Figure 6.5c). The synergistic fibrinolytic effect of low-dose scuPA was not sufficient to eradicate the FeCl3-induced platelet aggregation and continuous recruitment of platelets to site of injury. Representative images of H&Estained sections of thrombosed left carotid arteries following exposure to different liposomal treatments under NIR irradiation (785 nm, 1 W/cm<sup>2</sup>, 5 min) are demonstrated in Figure 6.6. Here, a series of slices (8 slices - 6 µm each) across the thrombosed artery was obtained at 200 µm intervals to allow more representative analysis of clot area change.



Figure 6.5. Histological analysis of thrombosed left carotid arteries following exposure to different liposomal treatments under NIR irradiation (785 nm, 1 W/cm<sup>2</sup>, 5 min). a) Schematic indicating the approach for cryostat sectioning of the excised artery into 8 sections at 200  $\mu$ m intervals. b) Representative H&E images of a fully occluded and a partially occluded section with clot area shaded using ImageJ software to calculate the region of interest area. c) Average clot area ( $\mu$ m<sup>2</sup>) in left carotid arteries following exposure to different liposomal treatments under NIR irradiation. Analysis was done with One-way ANOVA using multiple comparisons (Mean ± SD, ns p>0.05, \*p<0.05, \*p<0.01, n=4 mice for PBS and n=5 mice for all other treatments).



Figure 6.6. Representative images of H&E stained-sections of healthy right carotid arteries and thrombosed left carotid arteries following exposure to different liposomal treatments under NIR irradiation (785 nm, 1 W/cm<sup>2</sup>- 5 min). Sections of healthy right carotid artery collapsed due to the absence of luminal thrombosis (Scale bars are 200  $\mu$ m).

Recently, the use of NIR light-induced photothermal effect has attracted attention to break clots and enhance clot penetration of PA-loaded-nanoparticles to acute clots, encouraged by the high tissue penetration depth of NIR light into tissues (F. Zhang, Liu, et al., 2019b; Z. Zhao et al.). However, clinical translation of this technology is still limited by the inferior localised accumulation of non-targeted photothermal agents at the thrombus site. Targeted antibody-nanoconjugates can increase localised accumulation of photothermal agents together with therapeutic agents and imaging probes to clot site. The later would allow successful photothermal therapy at lower laser powers within the accepted exposure limits. To circumvent the storage stability issues associated with antibody-nanoconjugates, neutravidin-biotin conjugation chemistry offers a rational and robust approach to conjugate neutravidin-coated nanoparticles to biotinylated antibodies immediately before their administration to patients (Xiaowei Wang et al., 2016; X. Wang et al., 2012). In the context of thrombosis treatment, targeted NIR light-assisted photothermal therapy offers opportunities for both PA-free thrombolysis and low-dose photothermally-assisted thrombolysis using low doses of co-loaded PA, which would potentially avoid the bleeding side effect associated with the current pharmacological treatment. PA-free photothermal therapy of thrombosis can be attributed to three main mechanisms: 1) structural fission of the fibrin skeleton, 2) protein dehydration and alteration of membrane fluidity of red blood cells, and 3) mechanical energy from water vapor bubble generation at the nanoscale (Maheshwari, van der Hoef, Prosperetti, & Lohse, 2018; Rehman et al., 2021; Nitesh Singh et al., 2016; Walski et al., 2015; F. Zhang, Liu, et al., 2019b).

### 6.4. Conclusion

Activated platelets-targeting in the context of acute thrombosis was confirmed in vivo using the FeCl<sub>3</sub>-induced thrombosis mouse model. NIR fluorescence of the incorporated IR780 dye was used to assess the targeting ability of the prepared liposomes. In vivo photothermal transduction of the thrombosed vessels further confirmed efficiency of targeted delivery of liposomes, where targeted liposomes (Tar-IR-L and Tar-scuPA-IR-L) showed higher local temperature increments compared to the non-targeted formulations. The developed targeted liposome (Tar-IR-L) showed ~ 2-fold decrease in clot size, following 5 min-NIR irradiation of clot area. Co-loading of a low-dose scuPA (~ 45 IU/g) resulted in lower but not statistically significant clot areas in vivo, which might be attributed to the continuous activated platelets recruitment to the FeCl3-induced injury site. Future studies will be required to assess the efficiency of targeted photothermal therapy in different thrombosis animal models. Incorporation of the NIR fluorescent dye within liposome bilayers gives potential for NIR fluorescence guidedthrombolysis as demonstrated by the ex vivo fluorescence imaging of the excised thrombosed arteries. In conclusion, activated platelets-targeted liposomes present a promising delivery system for safer, single-dose, and potentially bleeding-free therapy of acute thrombosis.
# **Chapter 7** Conclusions and future perspectives

Acute thrombosis treatment using currently available fibrinolytics (PAs) is limited by their short biological half-lives, the need for large doses using long infusions, and their fatal bleeding adverse effects. Nanotechnology offers novel approaches towards more efficient and safer thrombolysis through targeted and stimuli-responsive delivery of fibrinolytics. Liposomes, as biocompatible versatile nanocarriers, represent a promising delivery system for targeted and stimuli-responsive delivery of fibrinolytics and imaging probes to thrombosis. The surge in FDA-approvals of liposomal products during the last decade encourages further research to develop an optimal targeted liposomal PA. Prolonged circulation, less-frequent administration, and low-dose thrombolysis was achieved from liposomal systems in preclinical animal models (Stepan Koudelka et al., 2016; C.-H. Liu et al., 2019; N. P. Zhang et al., 2018).

Photothermally-assisted thrombolysis has proven an efficient novel technique to break clots at low-doses of co-administered or locally released PA. Liposomes loaded with photothermal agents such as gold nanorods or NIR molecular dyes can be efficiently stimulated using externally applied NIR light. The photothermal effect synergises with the biochemical effect of the co-administered or locally released PAs. Clinical translation of this approach still needs further development for enhanced deep tissue stimulation in the NIR window. Advances in nanoparticle designs and their conjugation to targeting ligands are expected to revolutionise photothermal therapy in the next few years. This technique with its high spatial and temporal resolution might be of great interest in treatment of strokes through transcranial irradiation; an area of research which needs further investigation. Acute thrombosis targeting, through selective binding of the highly expressed glycoprotein (GP IIb/IIIa) on the surface of activated platelets, represents a very attractive and reliable tool for site-directed treatment and diagnosis of thrombosis. A single chain antibody (scFv) showed the highest specificity and less cross-reactivity for binding activated platelets, compared to RGD peptides and Abciximab. Liposomes can be efficiently conjugated to the scFv using biotin-neutravidin chemistry for targeted delivery of payloads such as PAs, photothermal agents, and fluorescent dyes. Clinical translation of the liposome-scFv antibody conjugate is challenged by the limited stability of liposomes and antibodies upon long-term storage at ambient or refrigerator temperature. This stability issue can be mitigated through preparation of freeze-dried neutravidin-coated liposomes and biotinylated antibodies, to be reconstituted and conjugated immediately before administration to the patient. Here, neutravidin-biotin bonding represents a suitable conjugation technique as an instantaneous, robust, and stable non-covalent bonding. Further studies will be required in this area to confirm long-term stabilisation.

*In vivo* NIR fluorescence imaging has attracted great attention recently as a noninvasive, radiation-free, and highly sensitive diagnostic imaging modality. Its clinical application is currently limited to image-guided surgery of cancer using the FDA approved ICG dye and pafolacianine. Advances in fluorophores and scanner technologies is expected to open the way for further FDA-approved applications such as acute thrombosis monitoring. Here, targeted-liposomal dye denotes a promising delivery system for diagnostic and therapeutic applications. In addition, liposomes tend to stabilise the incorporated NIR-fluorescent dye for more efficient and prolonged imaging. In conclusion, the research work involved in this thesis presented two promising liposomal platforms for NIR-responsive release and targeted delivery of therapeutics to thrombosis based on photothermal therapy and antibody conjugation. The research opens further opportunities to the use of external stimuli and antibody delivery to efficiently treat acute thrombosis using nanotechnology. The promising results presented in this thesis come with some shortcomings which need to be addressed in the future work before clinical translation of this technology as follows:

- Long term storage stability of the developed systems needs further investigation employing freeze-drying as a well-established method to prolong storage stability.
- Different conjugation methods of activated platelets-targeting antibody (scFv) to liposomes surface should be tested in comparison to the biotin-avidin method. Click chemistry using Azide/DBCO might be another good option for one-step conjugation.
- Future studies will be required to assess the efficiency of targeted photothermal therapy in different thrombosis animal models and using different dosing regimens.
- Sex-sensitive approaches should be included using female mice to ultimately support the translational relevance of the research findings.

# **Ethics and authorisations**

The cell culture work, hemocompatibility studies, *In vitro* thrombus and *In vivo* animal studies were carried out within Baker Institute for Heart and Diabetes (Level 4) in both

- 1. Atherothrombosis and Vascular Biology Laboratory, and
- 2. Molecular Imaging and Theranostic Laboratory.

Relevant training and access authorisations have been done accordingly.

### **Ethics approvals for animal work:**

- Biodistribution and IVIS-imaging experiments were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (P1967).
- 2. In vivo photothermal experiments were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (P8196).

Alfred Research Alliance

# **Animal Ethics Committee**

### MEMORANDUM

#### TO: Xiaowei Wang

FROM: Long Bui, Centre For Thrombosis

SUBJECT: Chair Review APPLICATION P1967 - APPROVAL

#### Dear Karlheinz Peter, Xiaowei Wang

The Chair Review has considered and approved your application for a amendment of your project P1967 - E/1967/2019/B Molecular imaging for diagnostic and/or therapeutic approaches in models of thrombosis, atherosclerosis and inflammation V3.4.

Study Title: E/1967/2019/B Molecular imaging for diagnostic and/or therapeutic approaches in models of thrombosis, atherosclerosis and inflammation

Project Number: P1967 V3.4

Approval Date: 05/07/2021

Expiry Date: 08/11/2022

Name of Responsible Person/ Principal Investigator: Xiaowei Wang

Name of Committee that Approved the Application: Chair Review

Official Comments:

Dear Xiaowei,

Your application is now approved.

Kind regards,

AEC Secretary

Alfred Research Alliance

# **Animal Ethics Committee**

### MEMORANDUM

TO: Mitchell Moon

FROM: Judy Nash, Centre For Thrombosis

SUBJECT: Chair Review APPLICATION P8196 - APPROVAL

Dear Karlheinz Peter, Mitchell Moon

The Chair Review has considered and approved your application for a **amendment** of your project **P8196 - Developing new drugs to** treat blood clots. V1.2.

Study Title: Developing new drugs to treat blood clots.

Project Number: P8196 V1.2

Approval Date: 08/11/2021

Expiry Date: 07/11/2024

Name of Responsible Person/ Principal Investigator: Mitchell Moon

Name of Committee that Approved the Application: Chair Review

Official Comments:

Dear Mitchell,

Your application has been reviewed by the AEC Chair and is now approved.

Kind regards,

AEC Secretary

# **List of References**

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# **Appendix I: Conference presentations**

# 1. Outstanding Oral Presentation Prize (ASMI2021)



# The Australian Society of Molecular Imaging (ASMI) presents the

Outstanding Oral Presentation Prize

to

Ahmed Refaat

In recognition of excellence for their contribution, entitled

Activated Platelet-Targeted IR-780 Liposomes for Photothermal/photodynamic Thrombolysis

Presented this 23rd day of June, 2021 at the 6th Annual Scientific Symposium of the Australian Society of Molecular Imaging (ASMI2021), Translational Research Institute (TRI), Woolloongabba, QLD, Australia

A/Prof Xiaowei Wang ASMI Board President & ASMI2021 Steering Committee



A/Prof Hang Ta ASMI Board Secretary & ASMI2021 Committee Chair

### 2. CRS2021 Oral Presentation Certificate



The Controlled Release Society (CRS) proudly presents this

**Certificate of Presentation to:** 

# Ahmed Refaat

Who presented the abstract Near-infrared light-responsive liposomes for protein delivery and its application in potentially bleeding-free photothermally-assisted thrombolysis at

the Controlled Release Society 2021 Virtual Annual Meeting

July 25 - July 29, 2021



Mark Prausnitz, PhD 2021 CRS AMPC Chair

# **3. Student presentation prize (Runner up)- Baker Department of Cardiometabolic Health Symposium- Melbourne University**



# **Appendix II: Copyright Clearance**

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<u>A. Refaat</u>, B. del Rosal, J. Palasubramaniam, G. Pietersz, X. Wang, K. Peter, S.E. Moulton, Smart delivery of plasminogen activators for efficient thrombolysis; recent trends and future perspectives, Advanced Therapeutics, 4 (2021) 2100047. doi: 10.1002/adtp.202100047

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