Fabrication of stimuli responsive hyaluronic acid-based nanoparticles for cancer treatment

Phua, Fiona Soo Zeng

2019

Phua, F. (2019). Fabrication of stimuli responsive hyaluronic acid-based nanoparticles for cancer treatment. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/83246>

<https://doi.org/10.32657/10356/83246>

**NANYANG
TECHNOLOGICAL
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SINGAPORE

**Fabrication of Stimuli Responsive Hyaluronic Acid-Based
Nanoparticles for Cancer Treatment**

Phua Soo Zeng Fiona

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2019

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Phua Soo Zeng Fiona

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

A thesis submitted to the Nanyang Technological University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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19 Jun 2019

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Prof Zhao Yanli

Authorship Attribution Statement

This thesis contains material from 2 paper(s) published in the following peer-reviewed journal(s) where I was the first and/or corresponding author.

Chapter 2 is published as S. Z. F. Phua, C. Xue, W. Q. Lim, G. Yang, Y. Zhang, H. Chen, C. F. Wijaya, Z. Luo, Y. Zhao. Light-Responsive Prodrug-Based Supramolecular Nanosystems for Site-Specific Combination Therapy of Cancer. *Chem. Mater.* 31 (9), 3349-3358 (2019). DOI: 10.1021/acs.chemmater.9b00439

The contributions of the co-authors are as follows:

- Prof Zhao Yanli initiated the project and edited the manuscript drafts.
- Prof Luo Zhong provided advice on the *in vivo* studies.
- I did the synthesis, characterized the nanoparticles, carried out the *in vitro* studies and prepared the manuscript.
- The manuscript was revised by Dr Yang Guangbao and Lim Wei Qi.
- Xue Chencheng did the *in vivo* studies.
- The synthesis of the molecules was advised by Dr Chen Hongzhong and Dr Zhang Yuanyuan.
- Chintya Fransisca Wijaya assisted in the synthesis of the materials

Chapter 3 is published as S. Z. F. Phua, G. Yang, W. Q. Lim, A. Verma, H. Chen, T. Thanabalu, Y. Zhao. Catalase Integrated Hyaluronic Acid as Nanocarriers for Enhanced Photodynamic Therapy in Solid Tumour. *ACS Nano*, 13 (4), 4742–4751 (2019). DOI: 10.1021/acsnano.9b01087

The contributions of the co-authors are as follows:

- Prof Zhao Yanli provided the initial project idea, direction and edited the manuscript drafts
- I did the synthesis, characterisations of the nanoparticles, carried out the *in vitro* and *in vivo* studies and prepared the manuscript.

- Dr Yang Guangbao provided advice and helmed the *in vivo* studies
- Lim Wei Qi edited the manuscript
- Apoorva Verma and Prof Thirumaran Thanabalu provided advice on the H&E staining
- Dr Chen Hongzhong provided the materials for the synthesis

Note: If published materials are not inserted as thesis chapters, students must acknowledge co-worker contributions in the acknowledgement section of their thesis.

10 Jul 2019

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Date

Fiona Phua

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Phua Soo Zeng Fiona

Abstract

Non-selective cancer treatment causes low treatment efficacy and severe side effects in patients. Considering the current problem in oncology such as high systemic toxicity of chemodrug and poor efficiency of photodynamic therapy, the focus of this thesis is to design, fabricate and characterize hyaluronic acid based nanocarriers for stimuli-responsive targeted cancer therapy by supramolecular means. Various hyaluronic acid-based nano-sized systems have been designed and investigated for cancer treatment. Hyaluronic acid-based nanocarriers have numerous advantages such as its biodegradability and non-immunogenetic. Multiple functional groups on the hyaluronic acid also allows for facile modifications to allow for greater functionality. Formation of micelles, direct conjugation and formation of nanogels could be achieved. Hyaluronic acid could also target overly expressed receptors on cancer cells. Different hyaluronic acid-based nanocarriers will be explored in this thesis to prove its versatility as a polymer for delivery of cancer therapeutic agent. To further increase selectivity of treatment, stimuli-responsive groups will be incorporated. To better control the amount of therapeutic agents, supramolecular chemistry will be harnessed.

Chapter 1 describes the background of cancer, treatment modalities and current research using nanocarrier, and more specifically, using hyaluronic acid as the polymer for drug delivery of therapeutic agents. It also describes some current development in stimuli responsive drug delivery system and lastly introduced supramolecular chemistry as a mean for nanoparticle fabrication.

In Chapter 2, our primary objective was to design a hyaluronic acid-based nanocarrier based on self-assembly after formation of inclusion complex between the cyclodextrin-modified hyaluronic acid and adamantane-modified camptothecin drug and photosensitizers. The nanocarrier HA-aPS-aCPT was able to target the overly expressed CD44 receptors on

cancer cells. Furthermore, upon internalization, light irradiation was applied so that the photosensitizers produced reactive oxygen species. This was used for photodynamic therapy as well as the release of the caged prodrug via the reaction with the reactive oxygen species responsive linker. This resulted in cascaded release of active chemodrug. The system was applied on mice model and had shown to inhibit tumour growth.

Beside using hyaluronic acid polymer for encapsulation of drugs and photosensitizers, hyaluronic acid polymers could be used for direct conjugation with proteins or enzymes that can improve therapeutic outcome. Hence, in Chapter 3, we explored the possibility of direct conjugation of hyaluronic acid onto enzyme catalase. Direct conjugation of catalase increased its stability in the presence of proteinase K, which was essential for long term blood circulation in *in vivo* system. Photosensitizers was loaded into the nanocarrier by formation of inclusion complex of cyclodextrin modified on hyaluronic acid and the adamantane-modified chlorin e6 to form HA-CAT@aCe6 NPs. Modification with catalase in the nanocarrier could relieve hypoxia in cells. Because of the presence of hyaluronic acid, HA-CAT@aCe6 NPs was able to target CD44⁺ cells selectively. Upon irradiation of light, HA-CAT@aCe6 NPs inhibited the tumour growth in mice model as compared to hyaluronic acid with photosensitizers alone. This shows that modification of hyaluronic acid with enzymes were effective in preserving its activity and improve its biodistribution for application.

Beside using catalase to overcome the hypoxia in cells, another approach is to use prodrug that is responsive to hypoxia environment for release of drug. In Chapter 4, glucose oxidase, an enzyme that was able to convert glucose and oxygen to hydrogen peroxide was encapsulated in hyaluronic acid-based nanogels. Tirapazamine, a hypoxia-responsive drug was co-loaded into the HA nanogel to give HA@aCe6@GOD-TPZ. aCe6 in this case was the hydrophobic component for self-assembly of the HA nanogel and to perform imaging. Glucose oxidase can deplete the oxygen level in cells to release the prodrug. HA@aCe6@GOD-TPZ

was able to accumulate selectively in CD44⁺ cells. The NPs was shown to be able to exhibit greater cytotoxicity under hypoxic condition than HA@aCe6@GOD and TPZ alone.

Chapter 5 concludes the usage of hyaluronic acid as a polymeric nanocarrier in terms of formation of micelle, nanocarrier and nanogels. New insights for future research on nanocarrier for treatment of cancer was also provided.

Acknowledgements

In a blink of time, I have completed my PhD studies. In these four years, I have met many Good Samaritans who helped me in this tough journey. First, I would like to express my sincerest gratitude to Professor Zhao Yanli for the invaluable chance to do research in his laboratory. Towards the end of my undergraduate studies, he did not hesitate in accepting me as a Final Year Student and allowed me to work in his laboratory which gave me the first taste of what research is like. Under his supervision, I have developed technical and soft skills – research, writing, experimental as well as management and interpersonal skills. In addition, he gave me valuable suggestions and words of encouragement when I encounter problems in my research. His presence was always comforting and gave me a great sense of security because I know that I can rely on him for his advice whenever there is problem.

My heartfelt appreciations go to all the members in Prof Zhao's group. They are selfless in sharing their knowledge and extending their help. I thank Lim Wei Qi, my batchmate who has been through thick and thin together with me. She was the greatest source of encouragement and support in these four years. We had tough lessons and trainings together, discussion of project details and provision of suggestions and advice to each other. I would like to thank Dr Tham Huijun Phoebe for coaching me experiments such as cell studies, confocal microscopy, flow cytometry and also in bad times, pulling me through with words of encouragement and advice; Dr Wang Yang for his continual moral support; he was a friend, a mentor and someone who brought me out of my comfort zone; Dr Chen Hongzhong for his kind advice and encouragement, synthesis advice, exchange of ideas, he was selfless in providing ideas and advices; Dr Yang Chaolong for his support and encouragement; Dr Yang Guangbao for coaching me animal studies; Dr Nguyen Kim Truc for teaching me TEM; Dr Zheng Cunchuan for his insights on projects and life; Dr Xiang Huijing for her advice on graduation; Dr Qu

Qiuyu for teaching me confocal microscope sample preparation; Dr Zhang Yuanyuan for synthesis of hyaluronic acid polymer; Dr Shi Huifang who was my FYP mentor. I would also like to express my heartfelt gratitude to Dr Xing Pengyao, Dr Wang Dongdong, Dr Qian Cheng, Dr Liu Guofeng, Dr Zhou Weiqiang, Dr Zhao Lingzhi, Dr Sreejith Sivaramapanicker, Dr Quan Hongping, Dr Tan Si Yu, Dr Ang Chung Yen, Dr Feng Tao, Dr Li Menghuan, Ms Anivind Kuar Bindra, Mr Deblin Jana, Mr Ong Wee Kong, Mr Victor Xu Hesheng, Mr Eddy Wong Mun Fei and Mr Teo Wei Liang. I would also like to thank Dr Eric Shim and his mentor Prof Lee Soo Ying for his sharing of knowledge on gel electrophoresis. I would also like to thank Dr Shu Zhiyu and Prof Chen Gang for coaching me on gel electrophoresis preparation.

I would also like to thank my collaborator Mr Xue Chencheng and his mentor Prof Luo Zhong for the animal studies in my first project.

Last, I am grateful to my parents, sister and friends for their unconditional love and support as well as understanding from time to time.

I would like to thank School of Physical and Mathematical Sciences for providing me the research scholarship, facilities and services for pursuing my postgraduate studies. These eight years in Nanyang Technological University has made me feel like it's a second home to me.

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Chapter 1: Introduction

1.1 Drug delivery for Cancer treatment

Cancer resulted in high mortality rate despite advancement in medical technology. In 2012, 8.2 million death was due to cancer and 14 million new cases has been recorded.¹ Numbers continue to be on the rise. In Singapore, death due to cancer accounted for nearly 30% of the total deaths in 2015. Understanding of oncology is important in order to derive feasible remedies for cure. Cancer is defined as the rapid abnormal division of cells that could undergo continual proliferation, avoid growth suppressors, activate invasion and spreading of cancer cells, cause angiogenesis and resist apoptosis.² Cancer cells are rapidly proliferating cells that require nutrients and oxygen to grow.² As tumours grow, the oxygen levels are invariably lower than normal tissues, causing hypoxia in tumour.³ This leads to development of new blood vessels in the process called angiogenesis around the tumour cells.⁴ The growth of the blood vessels is often erratic, resulting in leaky vasculature in tumours with poor lymphatic drainage.⁵ Proliferating tumour cells also display increased glucose uptake known as the Warburg effect, whether is there excess or limiting amount of oxygen.⁶ As a consequence, concentration of lactic acid, the product of glycolysis, increases, resulting in an acidified cellular environment (~pH 5).⁷ Elevated amount of glutathione (GSH) levels are also measured in cancer cells, scavenging on the excess amount of reactive oxygen species (ROS) to resist cell death.⁸ Deregulations of receptors also elevate the cancer receptor proteins such as folate receptor,⁹ human epidermal factor growth receptor (HER2),¹⁰ CD (Cluster of Determinant) 44¹¹ and $\alpha_v\beta_3$ integrins.¹²

Several therapeutic methods have been explored for treatment of cancer including photodynamic therapy (PDT),¹³ radiation therapy,¹⁴ immunotherapy¹⁵ and

chemotherapy.¹⁶ Particularly, chemotherapy involves the administration of anti-cancer drugs into the body via different modes such as oral, intravenous injection and topical means. Some common chemotherapy drugs include doxorubicin,¹⁷ paclitaxel,¹⁸ gemcitabine¹⁹ and chlorambucil.⁹ For the reason that various anticancer drugs are non-selective and possess high cytotoxicity, cells are killed indiscriminately. This not only results in the apoptosis of cancer cells; normal cells are not spared. Side effects such as fatigue, pain, numbness, blood count disorders and hair loss are thus experienced by patients undergoing chemotherapy.

The emerging use of nanotechnology in cancer therapeutics is particularly attractive. Nanomedicine as a strategy to reduce side effects of chemotherapy has rendered better drug selectivity with reduced side effects at minimal dosage. Leaky vasculatures in tumours allow easy accumulation of the nanoparticles (NPs), but prevent the clearance of NPs from the interstitial space of tumours.²⁰ NPs could thus accumulate and retain for an extended duration in tumour site. This is known as the enhanced permeability and retention (EPR) effect.²⁰ Size of NPs could range from as small as 30 nm to 200 nm.²¹ NPs of diameter less than 30 nm would be cleared by the phagocytic uptake and hepatic filtration easily while NPs of diameter greater than 200 nm could be excreted by the spleen easily.²¹ One example of the earliest nanomedicine is the liposome.²² Among the NP drug delivery system developed for encapsulation of chemotherapy drugs (Figure 1), some organic nanomaterials include proteins,²³ liposomes²⁴ and polymers,²⁵ and just recently, inorganic nanomaterials such as silica,²⁶⁻²⁹ gold,³⁰ iron oxide,³¹ copper sulfide,³² graphene³³ and upconversion crystals NPs (UCNPs)³⁴ have been utilised. One major problem with most inorganic NPs is with its clearance from body after administrating in account of their poor biodegradability. Silica is biocompatible but non-biodegradable, resulting in accumulation in the body and could

not be excreted.³⁵ Biocompatibility of gold NPs is questionable to date.³⁶ On the other hand, UCNPs made from lanthanide elements remain toxic.³⁷ In recent times, a research by Peng et al. demonstrated that TiO₂, SiO₂, Au and Ag induce endothelial leakiness and unintentionally cause metastasis.³⁸ Due to the toxicity of inorganic nanomaterials, organic nanomaterial is preferred. Several NPs for anticancer treatment was approved by the U.S. Food and Drug Administration (FDA) including doxil (liposomal doxorubicin) and abraxane (albumin-paclitaxel).³⁹ Emulsifier Cremophor El was initially co-injected with the hydrophobic paclitaxel drugs for treating metastasized breast cancer, which has inherent toxicity to human. Using albumin as a carrier in abraxane eliminates such toxic emulsifier and thus increases the maximum tolerated dosage in patients.

Figure 1. Organic and inorganic NPs used for drug delivery.

Increasingly attractive strategy is the use of active targeting in nanomedicine to enable receptor-mediated endocytosis. Ligand–receptor interactions allow NPs to bind to the surface receptors of various cells, and then after which, allow endocytosis into the cells.⁴⁰ Different ligands are able to target different overly-expressed receptors on the cancer cells. Some common targeting ligands include folic acid, biotin, hyaluronic acid

(HA) and RGD peptide.⁴¹ As much as efforts were made, effectiveness of active targeting remains debatable, with researchers arguing that passive targeting of NPs still play the major role in accumulation.⁴²

Effort in increasing the precision of administrating and targeting of chemotherapy drugs to tumours does not discontinue with active targeting. Precise and controlled release of chemotherapeutics remains an issue. Nanomedicine has been constructed to be responsive to endogenous and exogenous stimuli. Endogenous stimuli are often associated with the microenvironment of the cancer cells. These include pH,⁴³ redox agents^{44, 45}, hypoxia environment⁴⁶ and enzymatic reactions while exogenous stimuli include irradiation of light,²⁴ ultrasound⁴⁷ and heat. Comparing these two types of stimuli in terms of selectivity, exogenous stimuli have an edge over their counterparts because of their higher selectivity and better controlled drug release profile to achieve non-invasive, on-demand treatment in desired targeted area in cells and in animal model.^{30, 48} Systematic control over the rate and time of release after administrating the drug is essential for targeted therapy. Most of the release mechanisms by endogenous stimuli are passive and very much rely on the local cellular environment which is essentially different for different cells.⁴⁹

1.2 Role of nanotechnology in Cancer treatment

Use of nanotechnology is a crucial milestone in cancer treatment over the past few decades. Some nanoparticulate drugs such as Doxil and Myocet were some of the therapeutic nanoparticles that were clinically approved. These drugs are liposomal based, which were found to improve pharmacokinetics and biodistribution.⁵⁰ Nanomedicine are developed for cancer treatment due to EPR effect of NPs in solid tumours as shown in Figure 2. Most drug delivery nanocarriers for tumour treatment are administered

systemically via intravenous means, after which they accumulate in the tumour through the EPR effect. Essentially, other than EPR effect, other factors could also be considered. NPs must evade elimination by the immune system (Macrophages and T cells) and avoid other macromolecules in the blood serum to selectively accumulate homogenously at heterogenous tumour site. Thus, by only relying on EPR effect on accumulation of NPs is overly simplified. Furthermore, in most studies, the assumption is that every system takes on the same effect. We should recognise that no one system is suitable for all and not all system is suitable for one. It is important to embrace the limitations of NPs and at the same time work on improving current system for cancer therapeutics.

Figure 2. EPR effect of NP on tumour sites.

1.3 Hyaluronic acid – a potential active targeting ligand

Passive targeting due to the EPR effect could effectively target tumour sites. However, to further increase specificity of the NP, active targeting could be harnessed. This translates to the functionalisation of the NPs with targeting ligands of the receptors found on tumour cells (Figure 2). One of the overly expressed receptors is the Cluster of Determinant (CD) 44. CD44 is a transmembrane glycoprotein in charge of numerous essential functions for example cell adhesion and migration.⁵¹ In addition, it can bind to HA. There are increasing evidences that suggest that CD44, especially the CD44v

isoforms could be a potential biomarker and therapeutic target for breast cancers and gastric cancers.⁵²⁻⁵⁴ CD44 also participates in many cellular functions such as tumour metastasis of cancers.⁵⁵⁻⁵⁷ Structure of CD44 proteins allows HA, an anionic, non-sulfated glycosaminoglycan to be a ligand and binds selectively to it.⁵⁸ HA was shown to target CD44 receptors. Many researches have been done to investigate on targeting capability of HA on CD44 receptors in oncology. One of the requirement for the binding of HA with a single CD44 receptor is that the HA polymer should possess at least six to ten monosaccharides conjugated consecutively.^{59, 60}

1.3.1 Current progress in using hyaluronic acid as a nanocarrier

In choosing the targeting ligand, HA is the choice because of several advantages such as its targeting ability to CD44 receptors,^{11, 61} hydrophilicity, biocompatible, possess carboxylic groups and hydroxy for easy conjugation and easily purified after conjugation.⁶² Internalisation of HA by cancer cells with overly expressed CD44 receptors enhances selectivity in drug delivery of siRNA, photosensitisers, proteins and other therapeutic agents. HA is a natural negatively charged polysaccharide composed of two moieties i.e. D-glucuronic acid and *N*-acetyl-D-glucosamine⁶³ that possesses inherent binding affinity with CD44 receptors.^{64, 65} Using HA can mediate endocytosis and assists in cellular uptake. HA was first used in drug delivery in 1994 by Yerushalmi and his group.⁶⁶ HA can be used to fabricate polymeric micelle,⁶⁷ conjugation on nanoparticle or formation of nanogels⁶⁸. It can also be chemically conjugated onto liposomes, inorganic NPs and polymeric NPs. Figure 3 shows the NPs involving HA that were used in drug delivery. Different types of HA NPs can be fabricated using different methods, which is discussed in the following subsections.

Figure 3. Different types of HA NPs that can be synthesized.

HA has several functional groups for easy modifications. Structure of HA is as shown in Figure 4 below. Functional groups such as carboxyl, hydroxyl and amine can be used for various modifications. Table 1 below summarized the modifications, conditions and reagents needed.

Figure 4. Structure of HA polymer and the functional groups identified.

Table 1. Summary of the HA target site, reaction type, activator, reagents and solvent needed for various modification of HA. (Reproduced with permission,⁶⁹ copyright 2011 Elsevier)

HA target site	Reaction type	Activator	Reagents	Solvent
-COOH	Amidation	Carbodiimides	EDC, NHS	Water or DMSO
		CMPI	CMPI, triethylamine	DMF or DMSO
		CDMT	CDMT, NMM	Water/ACN
		1,1'-carbonyl-diimidazole	1,1'-carbonyldiimidazole	DMSO
	Ugi Condensation		Formaldehyde, diamine, cyclohexyl isocyanide	Water (pH 3)
	Esterification	Diazomethane	Trimethylsilyl diazomethane, acetic acid	DMSO
		Alkyl halides	Alkyl iodides or bromides	DMSO
		Tetraethylene glycol tosylate	Tetraethylene glycol tosylate	DMSO
		Bisepoxides	Butanediol-diglycidyl ether	Water (acetic acid, pH 2-5)
	Oxidation	Sodium periodate	Sodium periodate	Water
-OH	Ether formation	Bisepoxides	1,2,3,4-diepoxybutane	Water (0.2 M NaOH, pH > 13)
			Butanediol-diglycidyl ether	Water (0.25 M NaOH, pH > 13)
			Ethyleneglycol diglycidyl ether and polyglycerol polyglycidylether	Water (1 M NaOH, pH 14)
			Epichlorohydrin or diepoxyoctane	Water (pH 10 then pH 4)
		Divinyl sulfone	Divinyl sulfone	Water (0.2 M NaOH, pH > 13)
		Ethylensulfide	Ethylensulfide, dithiothreitol (DTT)	Water (pH 8.5-10)
	Hemiacetal formation	Glutaraldehyde	Glutaraldehyde	Water (pH 2)
	Esterification	Alkyl succinic anhydrides	Octenyl succinic anhydride	Water (pH 9)
		Acyl-chloride activated carboxylate		DMSO
		Methacrylic anhydride		Water (pH 8-10)
	Carbamate formation	Cyanogen bromide (CNBr)		Water (pH 9-10)
-NHCOCH ₃	Deacetylation/amidation	Hydrazine sulfate		Water followed by DMSO

Purification of HA polymer can be done easily by dialysis to remove smaller precursors.

1.3.2 Literature review on using hyaluronic acid for formation of micelle

NPs can be formed using HA polymer by introducing hydrophobic components onto the polymer so that self-assembly can occur. Some researchers have used other polymers such as the polyethylenimine conjugated to cyclodextrin (CD). Addition of hydrophobic components with adamantane lead to self-assemble of NPs suitable for drug delivery.^{70, 71}

In earlier attempts to modify HA, HA was simply conjugated to hydrophobic drug or photosensitizers to increase hydrophobicity. In a work by Lee and his group,⁷² paclitaxel was conjugated onto HA by different feed ratios to give polymer that could self-assemble into NPs. Using HA of 64 kDa, they synthesized the polymer with 10% degree of substitution of paclitaxel to give DLS size of 196 nm. The conjugation of hydrophobic paclitaxel allowed self-assembly of the polymer into its nanosize.

In a work by Choi et al., the HA (2.344×10^5 Da) was fabricated with different degree of substitution of hydrophobic bile acid (5 β -cholanolic acid).⁷³ The amphiphilic polymer was then fabricated into NPs through self-assembly. The particle sizes decreased when the degree of substitutions increased because a more hydrophobic core was formed. The NPs size obtained were in range of 237 – 424 nm. This research showed that the NPs can accumulate in tumour tissues by both passive and active targeting where the latter was through CD44 receptors mediated endocytosis. In another work by Yoon et al. (Figure 5), the HA was conjugated to cholanolic acid, black hole quencher 3 and PEG. Chlorin e6 (Ce6) was encapsulated within the core of this amphiphilic polymer as the photosensitizer.⁷⁴ PEG can increase the blood circulation of the NPs while the black hole quencher 3 was used to elicit fluorescent quenching of Ce6 so that the photoactivity of the Ce6 was reduced. This work shows that it is possible to encapsulate hydrophobic photosensitisers like Ce6 within the core of self-assembled

amphiphilic polymer. Conjugations of HA with hydrophobic components such as octadecyl group, poly (D,L-lactide-co-glycolide) (PLGA) polymer and catechin (-)-Epigallocatechin-3-O-gallate (EGCG) could form NPs with core shell morphology of suitable size upon self-assembly.⁷⁵⁻⁷⁷

Figure 5. Schematic diagram of how hydrophobic components conjugated to HA can help in its self-assembly. (Reproduced with permission,⁷⁴ copyright 2012 Elsevier)

In another work by our research team as shown in Figure 6, the HA was functionalised with β -CD.⁶⁷ A reduction sensitive prodrug with a fluorescence reporter was fabricated as the hydrophobic component. When the inclusion complex was formed between the β -CD and adamantane, it led to the formation of an amphiphilic polymer that could self-assemble to give a hydrophobic core. IR825, a photothermal dye was loaded within the hydrophobic core to carry out dual therapy of chemo and photothermal therapy. This strategy enables easy purification of the prodrug and adjustable addition of the adamantane moiety.

Figure 6. Schematic diagram of cyclodextrin conjugated HA polymer and hydrophobic prodrug used in drug delivery for photothermal and chemotherapy. (Reproduced with permission from reference,⁶⁷ copyright 2018 Elsevier)

1.3.3 Literature review on using hyaluronic acid for formation of nanoparticle

HA can be grafted or coated on to NPs so that the biocompatibility, stability and the dispersity of the NPs can be improved. These NPs can be inorganic NPs such as gold

NP,⁷⁸ CuS NP and silica NP and organic NPs like proteins. The method of conjugation is important in enhancing the NPs for uptake into cellular or animal model.

Thiolated HA can be used for NPs that has affinity with sulfide group. Modification of the HA polymer can be easily done by functionalisation with different groups. Lee et al. developed a thiolated HA grafted gold NPs for delivery of IFN α .⁷⁸ For NPs that contain amine group, simple amidation of using zero cross-linker EDC and NHS was used.⁷⁹ In one work by Ma et al., HA was conjugated on to mesoporous silica (MSN) via EDC/NHS method by amidation reaction of carboxyl group in HA and amino group on the mesoporous silica.⁸⁰ HA improved the dispersity of MSN and ensured better selectivity to tumour site through active targeting. In another work by Yang and his group as shown in Figure 7, a hybrid NPs of iron oxide core with Prussian blue, PEI and quantum dot were conjugated with HA in the similar method of amidation using EDC/NHS.⁸¹ On the other hand, for NPs that contain functional group such as epoxy, HA must be modified. One common modification is using adipic acid dihydrazide to give hydrazido group for conjugation to the functional groups on NPs. Song et al. modified HA with adipic acid dihydrazide (ADH) so as to provide pendant hydrazido group for the binding with graphene oxide (GO).⁸² Mild condition was used for binding of HA to GO which is by simple stirring at pH 7.4 in the dark for 24 h. Direct conjugation of HA by the means of chemical bond still enable targeting of HA to cells with over-expression of CD44 receptors in all of the above examples.

Figure 7. Schematic diagram showing fabrication of HA-coated NPs for bimodal imaging guided therapy in mice. (Reproduced from reference⁸¹)

HA can also be grafted on to NPs using electrostatic interaction through layer-by-layer assembly. In a work by Dreaden et al.,⁸³ a multilayer polyelectrolyte shell was grafted on to the NPs because of the opposite charges. Carboxy-modified polystyrene latex nanospheres were first coated with poly(L-lysine) which is positively charged. After which, HA which has net negative charge was added on to the NPs for coating. Such facile modification reduces the need for other chemicals precursor to be used for functionalisation.

1.3.4 Literature review on using hyaluronic acid for formation of nanogel

One type of nanoparticle is the nanogel. Nanogels are composed of hydrophilic or amphiphilic polymeric nanoparticles formed by crosslinking to keep the structure intact.⁸⁴ Such bonds can be chemical bonds such as covalent bonds or physical bonds such as non-covalent bonds like electrostatic interactions, hydrogen bonding and hydrophobic interactions. HA can be used to form nanogel due to its hydrophilicity.

Formation of such structure can be done by two approaches: “top-down” or “bottom up” approaches.^{84, 85} The former is the fabrication of NPs from larger particles or clusters while the latter is realized by designing smaller molecules or compounds and then building the NPs by cross-linking using chemical or physical bonds. The nanogels formation by crosslinking reactions can increase the nanocomplex’s stability in physiological condition, which enabled minimal leakage of the encapsulated drugs.

In one work by Bian et al., cysteine was conjugated on to HA to give thiolated HA.⁸⁶ HA-SH can form hydrogel by simple oxidation reaction in the air after addition of sodium hydroxide. The formed disulfide bond was responsive to huge concentration of glutathione in cancer cells and thus resulted in controlled drug release. However, one problem is the difficulty in controlling the rate of reaction and predictability of the reaction. In another work by Singh and his group, they encapsulated proteins within nanogel that was crosslinked by the enzymatic reaction of horse radish peroxidase (HRP).⁸⁷ Similar thiolated HA was used. However, in this case, HRP was introduced as the catalyst to crosslink such that nanosized particles can be obtained. HRP can enzymatically oxidise the cysteine terminated monomers to give disulfide without the addition of H₂O₂ to give nanogel. They were used to load large proteins β -galactosidase (β -Gal) of 464 kDa at encapsulation efficiency of 40-43% with retention of β -Gal of slightly more than 80%. What was remarkable is the easy and mild conditions for the formation of the nanogel by HRP.

Yang and his team⁸⁸ developed a HA-based nanogels for drug delivery. The methacrylate HA was formed into the nanogel by addition of diethylene glycol diacrylate under 70°C with the addition of H₂S₂O₈. Doxorubicin was then loaded by stirring overnight. Hyaluronidase in cancer cells could release the drugs in the nanogel for stimuli-responsive drug release. One disadvantage of this work is that elevated heat

is needed to form the nanogel. If the cargo is preloaded into the nanogel, heat sensitive cargoes will not be able to withstand the heat. This is often in the case of proteins. HA of 7 kDa was used and the size and zeta potential were appropriate for drug delivery into tumour site as shown by the *in vivo* results.

In a work by Mo and his group as shown in Figure 8,⁸⁹ a HA nanogel was synthesized to load doxorubicin for anticancer drug delivery. HA was modified with acrylamide and acrylate group. The crosslinking was achieved using UV irradiation. The system is responsive to ATP and in presence of ATP, doxorubicin can be released.

Figure 8. Schematic diagram of an ATP responsive HA nanocarrier. (Reproduced from reference,⁸⁹ copyright 2014 Springer Nature)

In a work by Zhu et al. as shown in Figure 9,⁶⁸ they fabricated a HA nanogel by conjugation of cholesterol (chol) on to HA using the “bottom up” approach. HA-chol can self-assemble to form NPs and encapsulate the proteins due to hydrophobic-hydrophobic interaction between chol and hydrophobic component of proteins. To prevent leakage of protein, radical polymerization of methacrylic moiety of the HA was

carried out and results showed that leakage was reduced. Furthermore, this method of crosslinking to form the nanogel preserve the protein activity due to the mild reaction conditions.

Figure 9. Schematic diagram of a HA nanogel that is fabricated from bottom up approach. (Reproduced from reference,⁶⁸ copyright 2018 Wiley)

1.4 Development of stimuli responsive nanoparticle system

Other than making use of passive and possible active targeting using HA based NPs, different components were incorporated into the NPs system to enable therapeutic effects in the presence of a stimuli to further increase selectivity.⁹⁰ Stimuli can be classified as endogenous or exogenous.⁹¹ Endogenous stimuli usually depend on the variances in the intrinsic properties of cancer cells and normal cells. Most cancer cells have lower cellular pH, greater concentration of glutathione (GSH) level and a higher level of certain enzymes such as matrix metalloproteinases (MMPs). In 1920s, Warburg observed that cancer cells were taking up enormous amount of glucose for aerobic

glycolysis to give lactate by-product which thus caused cancer cells to be more acidic than normal cells.^{6, 92, 93} Large amount of ROS has been detected in cancer. However, as a mechanism to protect itself, cancer cells also express higher concentrations of GSH.⁸ Many enzymes such as MMPs are also overly expressed.⁹⁴ Endogenous stimuli have been used in many NPs system for reduction mediated release of prodrug from its disulfide linkage, acidic cleavage of chemical bonds such as hydrazone bond and also cleavage of peptides sequence using MMPs. In a work from our group,⁶⁷ we synthesized a prodrug of camptothecin with adamantane via a redox-sensitive disulfide bond. When the prodrug enters the reducing environment of the cancer cell, active drug is released together with a recovery of the fluorescence emission. In another work by Rodrigues et al., chemodrug doxorubicin was conjugated to polyethylene glycol(PEG) polymer via acid-sensitive hydrazone bond and shown to be responsive to the tumour acidic pH. Zhu and his group synthesized a nanocarrier that is responsive to MMP2 in tumour cells by incorporation of MMP2-cleavable octapeptide.⁹⁵ Cleavage of this octapeptide revealed the cellular penetrating peptide for enhanced uptake of the NPs. Despite numerous nanosystems incorporating internal stimuli, they are less reliable because cells have varying amount of each stimuli and outcome of treatment is thus less predictable and beyond control. Exogenous stimuli, on the other hand, is much more controllable and can be used for spatiotemporal treatment. The therapeutic agent could first be administered before exogenous stimuli is applied for therapy. Examples of exogenous stimuli include ultrasound,^{96, 97} temperature, magnetic field and electromagnetic waves such as visible light, near-infrared light,⁹⁸⁻¹⁰¹ UV¹⁰² and X-ray.¹⁰³ In a work by You et al., ultrasound was used to generate ROS from the titanium dioxide NPs in sonodynamic treatment.⁹⁷ Ultrasound can penetrate deep into tissues and would not be absorbed by tissue to produce heat. In another work by our group as shown in Figure 10, adamantane

modified photosensitizer BODIPY together with adamantane modified paclitaxel (PTX) drug was incorporated into PEG polymer functionalized with CD to form NP.¹⁰¹ Upon intravenous injection of these NPs into the mice, the NP accumulated at the tumour site but did not elicit any toxicity. With irradiation of NIR light on tumour site, tumour growth was inhibited. In another work by Chen et al., X-ray was used to activate the photosensitizers to produce ROS for killing of cells.¹⁰³ All these examples have one common advantage which is the possibility of activation of treatment upon irradiation of the respective treatment modalities.

Figure 10. Schematic diagram of a light responsive NPs system that was fabricated from self-assembly due to formation of cyclodextrin and adamantane inclusion complex. (Reproduced with permission,¹⁰¹ copyright 2019 Wiley)

1.5 Current development of using light in drug delivery

Exogenous stimuli such as light has caught attention of the researchers on account of its orthogonality and reliability. Phototherapy is the use of light of various wavelengths in treatment of a physical condition. In the 1890s, ultraviolet (UV) was discovered to have strong anti-bacterial effects. In the 1903, treatment of lupus vulgaris using concentrated light irradiation in medical field received recognition through Nobel Prize award. Thereafter, light was recognised as essential treatment tool for many diseases such as jaundice in newborns, autoimmune diseases, dermatologic and oncologic diseases. Light-mediated controlled activation in various cancer therapy has emerged as a hopeful means for precise control and activation of therapeutic reagents.¹⁰² Advantages of such phototherapy include its minimum invasive nature, high spatiotemporal resolution and reliability as a stimuli. Thus, various nanotherapeutic system has been developed for various phototherapy including PDT and light-responsive drug delivery. It is important to relate these concepts to how cells are killed.

1.5.1 Photodynamic therapy

Currently in clinical oncology, PDT is used for skin cancer treatment. This involves the applying of the therapeutic agents which in this case the photosensitizers on to skin cancer lesions first. After which, light of appropriate wavelength is irradiated.^{104, 105} PDT is defined as a way of cancer treatment using light of some wavelength and photosensitizers. This therapy involves the administration of non-toxic photodynamic agents that localise or accumulate at the tumour site. Activation of the photosensitizers is done by the illumination of light of wavelength absorbed by the photosensitizers to generate ROS from dissolved O₂. With oxidative stress, cellular structures are damaged and thus lead to cell death. There are two types of photodynamic therapy. Upon absorption of light, the photosensitizers are excited to its triplet state from

ground state. The triplet can undergo either type I or II reaction pathways for achieve PDT. Type I reaction is when the photosensitizer reacts to form radicals which produce species such as H_2O_2 , radicals and superoxide. Type II reaction is when singlet oxygen is produced from triplet oxygen.¹⁰⁶⁻¹⁰⁸ Figure 11 shows the Jablonski diagram of the photosensitizers to produce singlet oxygen.

Figure 11. Jablonski diagram depicting the process of how a photosensitizer generates singlet oxygen. IC represents internal conversion. ISC represents intersystem crossing. VR represents vibrational relaxation.

In comparison to conventional cancer treatment method of cancer, PDT possesses several main advantages.¹⁰⁸⁻¹¹⁰ One of the main advantages is that the therapeutic effect of PDT agents is activated by light which can thus minimized systemic toxicity. Furthermore, PDT are usually allowed in multiple dosages as compared to chemotherapy or surgery. Treatment by PDT is also non-invasive in nature in contrast to surgical means. In addition, minimal or no resistance exists for PDT treatments. Versatility in PDT treatment also enables it to be coupled with other treatment methods.

Most of the PDT photosensitizers possess a heterocyclic ring structure like that of chlorophyll or heme in hemoglobin. Choice of photosensitizers affect the light needed to irradiate the tumour site. Photosensitizers can be categorized by their chemical structures and origins. In general, there are three different types of photosensitizers namely porphyrin-based photosensitizer,^{111, 112} chlorophyll-based photosensitizer¹¹³ and

newly synthesized dyes such as phthalocyanine¹¹⁰ and BODIPY.¹¹⁴ One of the earliest approved photosensitizers is photofrin which can be used for treatment of cervical, bladder and gastric cancers and brain tumours.¹¹⁵ Photosensitizers were traditionally applied on the skin for treatment of melanoma and other cancer that are on the surface of the skin. Incorporation of photosensitizers in NPs was done in recent years for treatment of tumours that were deeper in the body.

Photosensitizers are usually hydrophobic and could aggregate when dissolved in water or solutions with high ionic strength.¹¹² Attempts have made to incorporate photosensitizers into nanocarriers. Lee and his group has incorporated Ce6 by carbodiimide coupling with the acetylated HA polymer for release of doxorubicin encapsulated and photoactivation of Ce6 for dual therapy as shown in Figure 12.¹¹⁶ Modification of the polymer backbone with photosensitizers enable hydrophobic photosensitizers to be loaded and aided in the self-assembly of the NPs.

Figure 12. Schematic diagram showing the incorporation of Ce6 photosensitizers on to acetylated HA polymers. (Reproduced with permission,¹¹⁶ copyright 2014 American Chemical Society)

1.5.2 Light responsive drug delivery system (DDS)

Light responsive DDS was developed for targeted cancer drug delivery. In the infant stage of the development of such system, light responsive prodrugs were fabricated by relying on the concept of photocages and release of such prodrug to its

active drug form was achieved with irradiation of light. These prodrugs usually do not possess high toxicity unless activated. Photocages are the establishment of photolabile bond in the molecule conjugates or NP system, in which light is needed in order to release the “caged” prodrug, activating the therapeutic system.¹¹⁷ Release of parent drug is only possible when light is irradiated in a controlled timely manner. Conjugation of drug prevents premature leakage and better quantification of drugs. Examples of various photocages that have been established over the years in the field of biology and chemistry based on UV-light responsive aromatic ring including arylmethylcarbonyl group,¹¹⁸ o-nitrobenzyl¹¹⁹⁻¹²¹ and coumarinyl ester^{34, 122}, and established spatiotemporally release of therapeutic molecules.

In a study by Tan and his group, DNA strand was conjugated to camptothecin (CPT) through an o-nitrobenzyl photocage.¹²³ Upon UV light irradiation, the self-assembled NPs disassembled as the hydrophobic CPT was cleaved from the DNA, releasing the CPT drug. Another class of photoremovable group is the simple arylmethyl group.⁴⁸ In fact, the primary application of such photosensitive protecting moiety was back in 1962 where Schofield and his group successfully release glycine from its benzyloxycarbonyl derivative.¹²⁴ Arylmethyl are excellent protecting group for carboxylates, carbonates, phosphates and other good leaving group.

Despite tremendous effort in designing photocages for light activated system, disadvantages such as the lack of imaging capability of some photocages still renders its disability for a real-time monitoring of endocytosis of NPs. Short wavelength (254 nm) used for photocleavage also resulted in photocytotoxicity and thus could not be rendered for prolong usage. Production of toxic photolysis by-products also results in unsuitability of the photocages.

In recent work, photochemically activated drug delivery were designed and synthesized. These systems were light activated. However, instead of direct photolysis, the nanostructures disassembled or ruptured due to the singlet oxygen or radical species produced by photosensitizers when irradiated with light.

In a work by Rwei et al., photosensitizers were loaded into the liposomal carrier.¹²⁵ Reaction of $^1\text{O}_2$ with the unsaturated lipids resulted in peroxidation, disrupting the stability of the liposome and caused release of the hydrophilic drug loaded within the aqueous core. In a separate work by Li et al., light sensitive liposome was synthesized and photosensitizer ICG was loaded at the hydrophobic region of the liposome.¹²⁶ Upon light irradiation of the nanostructure, the photolabile bond could be cleaved to dissemble the liposomes and release the doxorubicin encapsulated within the core. These proved to be a feasible method of releasing of free drugs from the carrier. Attempts were also made to harness near infrared light by using two photon technique. The molecules that had large two photon cross-sections were conjugated on to the polymer. With two photons irradiation, the molecules absorbed light and were cleaved from the polymer. In a work by Kumar et al., coumarin was functionalised on to block polymer poly(ethyleneoxide) and poly(L-glutamic acid).¹²⁷ Irradiation of two photon light to cleave the coumarin caused the nanocarrier destabilisation and released the hydrophobic drug within the core. However, two photon irradiation has some disadvantages such as the small focal point and the complex equipment which can hinder its clinical applications. It is thus essential to develop system that can release drugs from near infrared light in controlled manner.

1.6 Current development of using ROS responsive drug delivery

1.6.1 Reactive oxygen species

ROS are reactive radicals, ions or compounds containing oxygen that possess singly unpaired electron in their outermost orbitals. Singlet oxygen,¹²⁸ hydrogen peroxide¹²⁹ and hydroxyl radicals are some example of the common ROS in oncology. ROS are commonly found in cells¹²⁹ and are deemed double-edged sword. The amount of ROS in cancer cells is higher because of oncogenic stimulation, greater metabolic activity and malfunctional mitochondria.² Low level of ROS in cells helps in the regulation of biological pathways, which facilitates cell survival. Excessive ROS can lead to non-specific damage of intracellular macromolecules i.e. cellular proteins, lipids and DNA, causing cell death. Damage in DNA strands imparts repair of cellular structures, leading to apoptosis. ROS can perform reaction with amino acids residues, altering functions of proteins. ROS is also able to react with the double bond in lipid structures in lipid peroxidation which lead to eventual cell death. Many of the chemotherapy drugs and radioactive therapy agents increase the amount of ROS in tumour in order to kill the cells. Some factors like dosage, time, type and site of ROS productions determine if the cells will survive or die.

1.6.2 Literature review on ROS responsive system

ROS responsive system has been developed because of the intrinsic and extrinsic ROS present in the tumour cells. Some functional groups that can react with ROS are as shown in Figure 13 below.¹³⁰

Figure 13. Summary of the functional groups that are responsive to ROS. (Reproduced with permission,¹³⁰ copyright 2017 Wiley)

Some functional groups include diselenide,^{131, 132} monoselenide,¹³³ telluride,¹³⁴ arylboronic ester,¹³⁵⁻¹³⁷ thioether,^{138, 139} thioketal,¹⁴⁰⁻¹⁴² vinylthioether,¹⁴³ aryloxalate¹⁴⁴ and ferrocene.¹⁴⁵ Reactions with endogenous or exogenous ROS can lead to stimuli responsive drug release of the nanocarrier. In a work by Chen and his group as shown in Figure 14, catalase was loaded into core of the NP together with methylene blue as the photosensitizer.¹⁴⁶ The photosensitizer was quenched by black hole quencher 3. When hydrogen peroxide diffused into the NP, catalase catalyzed the production of O_2 . It ruptured the polymeric shell and decreased the FRET to recover fluorescence of methylene blue and thus enhanced the singlet oxygen production when light was irradiated.

$^1\text{S}_0$, singlet ground state; S_1 , lowest singlet excited state; T_1 , lowest triplet excited state; ISC, intersystem crossing.

Figure 14. Schematic diagram showing a H_2O_2 responsive system. (Reproduced with permission,¹⁴⁶ copyright 2015 American Chemical Society)

In a separate work by Saravanakumar and his group, they fabricated a polymeric carrier with vinylthioether group in the polymeric backbone.¹⁴³ Both the photosensitizer Ce6 and doxorubicin were encapsulated in the core of the NPs. Light irradiation of Ce6 depleted the O_2 in the NPs and this generated $^1\text{O}_2$ which was used to

react with the vinylthioether functional group that was cleavable by $^1\text{O}_2$. The disassembly of the NPs released the encapsulated drug doxorubicin.

1.7 Current development of using Hypoxia responsive drug delivery

Prodrugs can also be stimulated by endogenous factors such as commonly hypoxia conditions in tumour cells. Some of the more common hypoxic activated drugs include Tirapazamine (TPZ), TH-302, TH-4000 and EO9.¹⁴⁷ These hypoxic activated drugs are activated when there is limiting amount of oxygen in the cells as this promotes reduction to give their toxic counterparts.

Hypoxic environment also encourages bio reduction due to the lack of oxygen. In a work by He and his group as shown in Figure 15, 2-nitroimidazole moiety was incorporated into polyethylenimine as the polymeric NP.¹⁴⁸ The hydrophobic 2-nitroimidazole moiety could be reduced to give hydrophilic 2-aminoimidazole, which enabled the disassembly of the NP. HA was conjugated with Ce6 and grafted on the PEI NPs. Doxorubicin was encapsulated within the NP. This gave a hypoxia responsive NP that released drugs after it was irradiated with light of 660 nm.

In a work by Kulkarni and his team, azobenzene was incorporated within the polymer chain of the polymeric nanocarrier.¹⁴⁹ Under hypoxia condition, azobenzene was cleaved. This disassembled the nanocarrier which enabled the release of the two drugs gemcitabine and erlotinib in the polymersomes. In another work by Wang et al. as shown in Figure 16, they developed a nanocarrier that target tumour under both hypoxic and normoxic condition.¹⁵⁰ Both photosensitizers ICG and hypoxia activated drug tirapazamine (TPZ) was incorporated into the system. When the NPs reached the normoxic portion of the tumour, light could be irradiated to increase ROS in the system. As the NP diffused into the hypoxic region, TPZ could be activated to give its toxic

radical species for therapeutic purpose. This enabled the killing of both normoxic and hypoxic cancer cells. However, this would also mean that upon light irradiation, TPZ could be activated before reaching the hypoxic cells.

Figure 15. Schematic diagram of a hypoxia responsive NPs system. (Reproduced with permission,¹⁴⁸ copyright 2018 Royal Society of Chemistry)

Figure 16. Schematic diagram of a drug delivery system that delivers TPZ, a hypoxia responsive drug. (Reproduced with permission,¹⁵⁰ copyright 2017 American Chemical Society)

1.8 Supramolecular Chemistry

With the goal of fabrication of nanocarrier using polymers in mind, various methods such as self-assembly, nanoprecipitation, emulsification and dialysis had been explored.^{151, 152} Therapeutic agents can be loaded on to the nanocarriers using physical encapsulation, hydrophobic-hydrophobic interaction, dipole-dipole interactions and covalent bonding, depending on the type, size and material of the nanocarrier. However, these methods lack specificity, tunability and flexibility in loading of the cargoes. One possible method in which these therapeutic agents could be loaded is by supramolecular means by formation of “host-guest” inclusion complex.¹⁵³ One class of molecules is the CD that is commonly used for nanocarrier fabrication. CDs are made of sugar molecules that are linked by α -1,4-glycosidic bonds, giving a hydrophilic exterior and hydrophobic interior as shown in Figure 17.^{25, 154, 155} There are different CDs (α , β , γ) that corresponds to 6, 7 or 8 glucopyranoside monomers. This gives CD a hydrophobic

interior in which hydrophobic moieties can be encapsulated within CD to increase its solubility in aqueous solution. CDs are “host” molecules. Hydrophobic components that can fit into the cavity of the CDs are known as “guest” molecule. Some common “guests” are adamantane, azobenzene, cholesterol and ferrocene (Figure 17).

Figure 17. Schematic diagram of a cyclodextrin (host, left) and its guests (right). (Reproduced with permission,¹⁵⁵ copyright 2014 American Chemical Society)

Namgung et al. designed a NP that contains poly-CD and poly-conjugated drug for anti-cancer treatment.¹⁵⁶ CD formed an inclusion complex with paclitaxel to self-assemble and gave NPs. In a work by our group, β -CD modified polyacrylic acid (PAA) was fabricated together with adamantane modified moieties.²⁵ The moieties included folic acid (targeting moiety), FITC (imaging moiety) and PEG (to increase biocompatibility). The versatility of incorporation of different adamantane entities for fabrication of NPs is an advantage. Furthermore, purification of individual adamantane modified molecules was easier. Modifications of the different molar ratios could also be done easily by mixing.

1.9 Objectives of this thesis

The aim of this dissertation is to fabricate HA based nanocarriers for stimuli responsive cancer treatment by supramolecular means. Advantages of HA such as its

good biocompatibility, biodegradability, multiple functional groups for conjugation and its active targeting properties render it a suitable candidate as the polymeric base for DDS. Furthermore, it can be fabricated to give micelle or NPs and can be grafted on to NPs easily give it an edge over other polymers or polysaccharides. This renders it suitable loading of different entities such as hydrophobic therapeutic agents, protein and hydrophilic drug. To further enhance the selectivity of our nanocarrier, stimuli responsive system can be fabricated. These stimuli can be in a form of either exogenous or endogenous. In the following chapter 2, camptothecin prodrug modified with adamantane was synthesized together with adamantane modified photosensitiser porphyrin. These two moieties were incorporated into a NPs by self-assembly with hydrophilic CD modified HA polymer. When the cells were incubated with this NP, light was shone which led to light activatable release of chemodrug camptothecin from the prodrug via the ROS responsive linker. This increased selectivity of the chemodrug and enabled dual therapy of both PDT and chemotherapy. In chapter 3, attempts were made to incorporate catalase, a type of enzyme, in which it can produce O_2 in the presence of H_2O_2 to alleviate tumour hypoxia. CD modified HA was grafted on catalase. Adamantane modified chlorin e6 was synthesized to be incorporated to form a catalase integrated HA base nanocarrier to treat solid tumour. Encouraged by the positive result of natural enzyme, in chapter 4, attempts were made to incorporate glucose oxidase, an enzyme, in the HA nanogel, together with hypoxia activated prodrug tirapazamine to give NPs that can induce apoptosis in MDA-MB-231 cells. Finally, conclusion on the three studies done as well as some further insights are given in Chapter 5.

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Chapter 2: Light-Responsive Prodrug-Based Supramolecular Nanosystems for Site-Specific Combination Therapy of Cancer

2.1 Introduction

Many of the anticancer drugs have high systemic toxicity when administrated into patients, which leads to the development of prodrugs that does not exhibit toxicity.¹ Such systemic toxicity could be alleviated by the design of on-demand release stimuli.² Some of the common endogenous stimuli³ include acidic cellular pH,⁵⁻⁷ highly reducing environment due to overly expressed amount of glutathione,⁸⁻¹⁰ various tumour microenvironment enzymes,^{11, 12} and lower oxygen partial pressure,^{13, 14} as well as stimuli introduced externally such as elevated heat,¹⁵ irradiated light,^{13, 16-20} and ultrasound^{21, 22} have been used for release of biologically active chemotherapeutic drugs from the inactive prodrugs. In particular, external stimuli have an edge over their endogenous stimuli because they do not depend on varying tumour microenvironment, neither does it exhibit non-invasiveness nor excellent spatiotemporal control.²³ One of the widely used exogenous stimulus for controlled release of photocaged prodrugs^{20, 24} including *o*-nitrophenyl^{25, 26} and coumarinyl ester²⁷ is light. However, the current design of prodrugs has to be released from low wavelength light which possessed inherent phototoxicity when administrated and low tissue penetration depth which rendered low treatment efficacy in solid tumours.^{25, 26, 28-30} As such, development of near infrared (NIR)-photoactivated prodrugs requires greater attention.³¹ Some recent progress has been made. Gorka and his group conjugated an anticancer drug with antibody to form heptamethine cyanine adducts that were cleaved selectively by NIR light.³²⁻³⁴ Another strategy involves the use of NIR light absorbing photosensitizers to harvest reactive

oxygen species (ROS) for cleavage of prodrugs for combinational therapy i.e. photodynamic therapy (PDT) and subsequent release of free drugs upon light irradiation. Some common examples of ROS-responsive linkers include thioether,^{35, 36} diselenide,³⁷ thioketal,¹⁷ arylboronic ester,³⁸ aminoacrylate³⁹ and peroxalate ester.⁴⁰ Liu et al. developed photosensitizer based prodrug where biological activities of the gemcitabine were inhibited by porphyrin via a singlet oxygen ($^1\text{O}_2$) responsive thioketal linker.¹⁷ However, such system has its disadvantage such as its difficult synthesis procedure, difficulty in purifications, inherently poor solubility for *in vivo* applications, as well as difficult storage because of its unexpected release of drugs when exposed to stray light. As such, nanoparticles (NPs) responsive to ROS were also fabricated to enhance tumour accumulation and better therapeutic outcome.^{18, 19, 41, 42} For instance, Yue et al. synthesized a NPs system that comprised of photosensitizer and camptothecin prodrug through a thioketal linker.¹⁸ Similarly, Pei et al. designed a red-blood cell membrane containing prodrug of paclitaxel and photosensitizers.⁴³ Although such systems showed enhanced solubility of prodrugs, the inability of adjustment of the ratio of photosensitizers and drugs hindered its applications greatly. There has been an increasing attention on precise control of the drugs to photosensitizers ratio for better therapeutic outcomes, as such systems allow autonomy in drug prescriptions for different patients.

Development of NIR activated prodrug has also lead to the development of inorganic nanomaterials³¹ such as upconversion crystals¹⁹ and gold NPs^{44, 45} that were used as a tool to facilitate release of prodrugs. However, various important pharmacokinetics of the drugs such as metabolism and biodistributions in the body still require much investigations.⁴⁶ Biodegradable polymer such as HA (HA) with high biocompatibility should be harnessed. It is type of natural occurring polysaccharide that

could also be found in various extracellular matrix of our body.⁴⁷⁻⁵⁰ Some of the advantages of HA include its high biocompatibility *in vivo* and multiple functional group for modifications and conjugations.⁵¹ Another excellent property of HA is that it is able to act as a targeting ligand to the overexpressed receptors on cancer cells i.e. the cluster determinant 44 (CD44) receptors which is often connected to tumour progression and metastasis.⁵²⁻⁵⁵ On account of over expression of the CD44 receptor on primary and metastatic tumour cells as compared to normal cells, NPs modified with HA would attain preferential uptake into cancer tissues *via* the receptor-mediated endocytosis.^{44, 48, 50, 54-56}

Having these considerations, we herein design a NP system through supramolecular means using a hydrophilic polymer encapsulated with hydrophobic prodrug and photosensitizer in a controllable ratio to realize PDT and controlled release of chemotherapeutic drugs (Scheme 1). In this system, HA is conjugated with β -cyclodextrin (CD) to give HA-CD, in which CD could form the inclusion complex with adamantane conjugated photosensitizer and prodrug for effective encapsulation and formation of NPs. When inclusion complex is formed by CD and adamantane, it gives the polymers amphiphilicity which then enables self-assembly into a NPs.^{10, 56-58} The two therapeutic components encapsulated include adamantane conjugated camptothecin (aCPT) as the prodrug *via* ROS-responsive thioketal bond and adamantane-conjugated 5,10,15,20-tetrakis(4-hydroxyphenyl)-21H,23H-porphine (aPS) as the photosensitizer. The supramolecular assembly of the three components (*i.e.*, HA-CD, aCPT and aPS) gives HA-aPS-aCPT NPs as shown in Scheme 1. To fulfil combinational therapy, the NPs synthesized should first be internalized via the EPR effect and CD44 mediated endocytosis.^{59, 60} Following which, the irradiation of NPs with light led to the generation of $^1\text{O}_2$ by aPS for PDT. The produced $^1\text{O}_2$ can also react with thioketal linker on the

prodrug of camptothecin (aCPT) to result in a cascaded release of free camptothecin (CPT). The advantages of this system are (1) passive targeting due to its nanoscale size and active targeting by HA *via* overly expressed CD44 receptor on cancer cells, (2) light activatable on-demand release of active drugs from prodrugs for spatiotemporal chemotherapy combined with photodynamic therapy, (3) easy purifications of individual molecules (aCPT and aPS) as opposed to some amphiphilic prodrugs, and (4) high tunability in controllable dosage and ratio of photosensitizer and prodrug for personalized cancer treatment. Thus, this superior NP system presented selective and higher killing efficacy of cancer cells *via* combined PDT and chemotherapy after systemic administration, reducing side effects of conventional chemotherapeutics.

Scheme 1. Schematic representation illustrating the treatment process using HA-aPS-aCPT NPs. The formation of HA-aPS-aCPT NPs was first achieved through self-assembly of HA-CD, aCPT and aPS (A). After which, the therapeutic efficacy was tested on mice model. Accumulation of HA-aPS-aCPT NPs was achieved after intravenous injection of NPs (B). Light of suitable wavelength i.e. 660 nm was then irradiated. The combinational therapy of photodynamic therapy (PDT) and chemotherapy is then achieved

after CD44 mediated endocytosis of HA-aPS-aCPT NPs coupled with light irradiation in five steps (C). Mechanism for the drug release was illustrated in A.

2.2 Materials and Methods

2.2.1 Materials

Sodium hyaluronate ($M_w = 8$ to 12 kDa), Camptothecin (CPT), 4-nitrophenylchloroformate and 1-adamantanecarbonyl chloride were purchased from Aaron Pharmatech Ltd (Shanghai, China). Dimethylaminopyridine (DMAP), anhydrous thioglycolic acid, triethylamine (TEA), 4-hydroxybenzaldehyde, 1-Hydroxybenzotriazole hydrate (HOBt), pyrrole and Amberlite® IR120 hydrogen form were purchased from Sigma Aldrich. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl), propionic acid and lithium aluminium hydride (LiAlH_4), β -CD, thiazolyl blue tetrazolium hydrate (MTT) were purchased from Tokyo Chemical Industry. Dry HCl gas was produced in situ by adding concentrated sulfuric acid to sodium chloride and then drying via concentrated sulfuric acid. Anhydrous THF and acetone were obtained by distilling over CaH_2 overnight. MDA-MB-231 and MCF-7 cells were obtained from American Type Culture Collection (ATCC). Dialysis membrane Spectra/Por 6 (MWCO:2 kDa) was purchased from Thermo Fisher Scientific Pte Ltd. All chemicals were of analytical grade and used without further purification if not indicated otherwise.

2.2.2 Instruments

^1H NMR spectra were probed on a Bruker BBFO400 spectrometer, and D_2O , DMSO CDCl_3 was used as the deuterated solvent. The UV-vis-NIR absorption and fluorescence emission spectra were measured using Shimadzu UV-3600 and Shimadzu RF5301PC spectrophotometer, respectively. Transmission electron microscopy (TEM)

images were taken at an acceleration voltage of 100 kV on JEOL JEM-1400. Hydrodynamic diameters and zeta potential values were measured at 25°C by a Malvern Zetasizer Nano-S system. HPLC analysis was performed with Shimadzu LC20AP with the Waters XSelect™ HSS C18 5 μm (4.6 x 250 mm) column at detection wavelength of 254 nm. ACN/water binary system containing 0.5% trifluoroacetic acid was used as the mobile phase with flow rate at 1 mL/min. Confocal laser scanning microscopy (CLSM) images were acquired by ZEISS LSM 800 Confocal Laser Scanning Microscope.

2.2.3 Synthesis of aCPT (adamantane modified prodrug)

Scheme 2. Synthesis procedure of prodrug Ada-TL-CPT (aCPT). Conditions: (a) DMAP, DCM, 0°C, yield: 88% (b) anhydrous acetone, HCl (g), yield: 56% (c) LiAlH₄ in dry THF, yield: (d) pyridine in anhydrous diethyl ether, yield: 34% (e) DMAP reflux in dry DCM, yield: 24%.

Synthesis of Compound 1. The synthesis was reported in previous literature.⁶¹

Briefly, camptothecin (CPT) (300 mg, 861 μmol) and 4-nitrophenylchloroformate (608 mg, 3.01 mmol) were dissolved in dry DCM (45 mL) at 0°C. Dimethylaminopyridine (DMAP, 631 mg, 5.17 mmol) was added. The solution was then stirred for 6 h at 0°C. The mixture was washed with water (60 ml). The organic layer was dried over sodium sulfate, filtered and excess solvents were removed in vacuo. Purification was done by

silica column chromatography, DCM (100 mL) was first used, follow by 50% DCM/EtOAc (300 mL) and then EtOAc (200 mL) to give product as a pale yellow solid (1, 265 mg, 88%). ^1H NMR (400 MHz, CDCl_3): δ 8.42 (1H, s), 8.22 (3H, m), 7.96 (1H, m), 7.86 (1H, m), 7.69 (1H, m), 7.38-7.41 (3H, m), 5.71 (1H, d, $J_{1,2}$ 17.3), 5.42 (1H, d, $J_{1,2}$ 17.3), 5.31 (2H, d), 2.21-2.41 (2H, m), 1.07 (3H, t, J 7.5 Hz).

Synthesis of Compound 2. The synthesis was reported in previous literature.¹⁷ Anhydrous thioglycolic acid (9.21g, 100 mmol) and anhydrous acetone (11.6g, 200 mmol) was purged with dry HCl gas with stirring at r.t.p. for 6 h. The mixture was then cooled in an ice bath. The mixture was filtered, washed with ice cold hexane and cold water to give white solid (2, 6.24 g, 56%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 12.6 (2H, s, br), 3.37 (4H, s), 1.54 (6H, s).

Synthesis of Compound 3. The synthesis was reported in previous literature with minor modification.¹⁷ 2 (1.5 g, 6.69 mmol) was dissolved in anhydrous THF (150 mL) and cooled at 0°C. LiAlH_4 (1.52 g, 40.1 mmol) was slowly added over 30 min, purging with N_2 to prevent built up of hydrogen gas. After addition was done, mixture was stirred at r.t.p. for 4 h. The reaction was quenched by addition of water (1 mL). After which, mixture was filtered. The filtrate was evaporated to dryness. DCM was added followed by Na_2SO_4 to remove water. The mixture was filtered and evaporate to obtain an orange liquid. Purification was done by silica column chromatography at 5% MeOH/DCM to give product as a yellow liquid (0.774 g, 59%). ^1H NMR (400 MHz, CDCl_3): δ 3.73 (4H, t, J 6.4 Hz), 3.33 (2H, s), 2.81 (4H, t, J 6 Hz), 1.574 (6H, s).

Synthesis of Compound 4 (TL). 1-Adamantanecarbonyl chloride (1.04 g, 5.24 mmol) and 3 (0.858 g, 4.37 mmol) were added to 100 mL of anhydrous diethyl ether and anhydrous pyridine (0.415 g, 5.24 mmol). The reaction was stirred at 23-25°C for 3 days. The organic layer was extracted with DCM and brine. Purification was done by

silica column chromatography using 10% EA/Hexane to give 4 as an orange liquid (0.530 g, 34%). ^1H NMR (400 MHz, CDCl_3): δ 4.21 (2H, t, 6.8 Hz), 3.79 (2H, t, 5.4 Hz), 2.86 (4H, m), 2.01 (3H, s), 1.89 (6H, s), 1.71 (6H, s), 1.62 (6H, s). HR-MS (ESI $^{+}$): m/z $\text{C}_{18}\text{H}_{30}\text{O}_3\text{S}_2\text{Na}$, $[\text{M}+\text{Na}]^{+}$ calcd. 381.1534, found 381.1541.

Synthesis of Compound 5 (aCPT). **1** (265 mg, 516 μmol) and **4** (278 mg, 775 μmol) were dissolved in dry DCM (60 mL). DMAP (95 mg, 775 μmol) was then added and the mixture was left to reflux overnight at 75 $^{\circ}\text{C}$. The mixture was then allowed to cool to r.t.p.. Purification was done by flash chromatography with DCM (100 mL), 5% EtOAc/DCM (100 mL), 10% EtOAc/DCM (100 mL), 15% EtOAc/DCM (300 mL) to give **5** which was abbreviated as Ada-TL-CPT as a pale yellow solid. ^1H NMR (400 MHz, CDCl_3): δ 8.39 (1H, s), 8.21 (1H, d, J 8.4 Hz), 7.93 (1H, d, J 8.4 Hz), 7.83 (1H, t, J 7.6), 7.67 (1H, t, J 7.6), 7.33 (1H, s), 5.71 (1H, d, J 17.2), 5.42 (1H, d, J 17.2), 5.29 (2H, s) 4.30-4.19 (2H, m), 4.15-4.08 (2H, m), HR-MS (ESI $^{+}$): m/z $\text{C}_{39}\text{H}_{45}\text{N}_2\text{O}_8\text{S}_2$, $[\text{M}+\text{H}]^{+}$ calcd. 733.2617, found 733.2631.

2.2.4 Synthesis of ada-THPP (aPS)

Scheme 3. Synthesis of aPS. Conditions: (a) TEA at 0 $^{\circ}\text{C}$ in THF (b) propionic acid, reflux 1 h.

Synthesis of 6. Triethylamine (1.53 mL) and 4-hydroxybenzaldehyde (1.24 g, 10.2 mmol) in THF (10 mL) was first cooled in ice bath before dropwise addition of 1-adamantanecarbonyl chloride (3.03 g, 15.2 mmol) in THF (10 mL) into the solution. The mixture was left to stir for 12 h at r.t.p. before THF was removed under vacuo. The

residue was dissolved in ether (50 mL), followed by washing with 1M Na₂CO₃ (3x 50 mL), followed by brine (50 mL). The resultant solution was dried over Na₂SO₄, filtered and excess solvents were removed in vacuo to yield **6** as a white solid (1.42 g, 50%). ¹H NMR (400 MHz, CDCl₃): δ 9.99 (1 H, s), 7.91 (2 H, m), 7.24 (2 H, m), 2.10 (3 H, s), 2.60 (6 H, s), 1.75 (6 H, m).

Synthesis of 7 (aPS). **6** (1.42 g, 5.00 mmol) and 4-hydroxybenzaldehyde (1.83 g, 15.0 mmol) first underwent reflux in propionic acid (150 mL). Upon the addition of pyrrole (1.34 g, 20.0 mmol), reaction mixture was refluxed for another 1 h. Reaction mixture was cooled to r.t.p. and then cooled further to 0°C for 15 mins. The cooled mixture was filtered, and purple solid residue was obtained. The solid was dissolved in DCM (250 mL) and filtered to remove impurities. The organic solution was washed with 1 M NaHCO₃ (50 mL), follow by brine (50 mL) and then dried over Na₂SO₄. Purification was done by flash chromatography with 2 % MeOH/DCM to gives Ada-THPP as a purple solid. ¹H NMR (400 MHz, CDCl₃): δ 8.86 (8 H, s), 8.20 (2 H, m), 8.05 (6 H, m), 7.45 (2 H, m), 7.19 (6 H, m), 2.38 (3 H, s), 2.24 (6 H, s), 2.18 (6 H, s). HR-MS (ESI⁺): m/z C₅₅H₄₅N₄O₅, [M+H]⁺ calcd. 841.3390, found 841.3378.

2.2.5 Synthesis of β-cyclodextrin modified hyaluronic acid (HA-CD)

Scheme 4. Synthesis procedure of HA-CD polymer. Condition: (a) NaOH, TsCl. (b) ethylenediamine, reflux in DMF under N₂ (c) EDC.HCl, HOBT for 2 days.

Sodium hyaluronate was pretreated with cationic exchange resin (Amberlite® IR120 hydrogen form) to give HA in the following procedure. Sodium hyaluronate (1

g) in deionized water (300 mL) was passed through the cationic exchange resin slowly and then lyophilized to give HA. EDC.HCl (0.232 g, 1.21 mmol) and HOBT (0.162 g, 1.05 mmol) were added into a solution of HA (0.1384 g, 14.6 μ mol) in DMSO (10 mL). The mixture was then stirred at 25°C for 30 min. Mono-6-deoxy-6-ethylenediamino- β -CD (0.8139 g, 0.69 mmol) in DMSO (10 mL) was added into the mixture and it was stirred for another 24 h at r.t.p.. The resulting mixture was dialyzed against DMSO (4 x 100 mL) and then with deionized water for 3 days (2 L x 10) and freeze-dried to give HA-CD as white solid. The degree of substitution of β -CD on to HA is defined as the no. of β -CD molecules per hundred sugar residues of HA was computed using ^1H NMR in D_2O . The degree of substitution (defined as the no. of β -CD per hundred sugar residues of HA polymer) value was around 6.56% for HA-CD. The concentration of CD in 1 mg/mL of HA-CD was calculated to be 135 μM .

2.2.6 Drug release using HPLC

A reverse phase HPLC was used. Buffer A was 0.5% trifluoroacetic acid (TFA) in H_2O while buffer B was 0.5% TFA in ACN. Analysis was carried out at 25°C with elution gradient. Briefly, the mixture of molecules was separated using condition as follows: 5% buffer B for 5 minutes, ramping of 5% buffer B to 95% buffer B in 30 minutes, followed by a decrease of 95% buffer B to 5% in 2 minutes. Retention times of CPT, aPS and aCPT were first determined to be 12.0, 15.3 and 16.1 min. 45 μM of aCPT with different concentrations of aPS (0, 45, 90, 135 μM) were prepared and illuminated with light of 660 nm (100 mW/cm²). The amount of free CPT released were then calculated from the standard calibration curve obtained from different CPT concentrations (0, 6.25, 12.5, 25, 50 and 100 μM) in 10% DMSO/ H_2O using the external standard method whereby the area under the curve of peak corresponding to CPT was directly proportionate to the concentrations of CPT prepared.

2.2.7 Stability constants as determined by Benesi-Hildebrand Plot

For determination of the stability constants of aPS, the Benesi-Hildebrand method was employed. UV vis spectrum of aPS (2×10^{-5} M) were measured in the dark. After which, β -CD (20.0 mg mL⁻¹, 5 μ L) in water was added to the aPS solution. After which, the absorbance spectrum of the resulting solution was obtained. The process was repeated systematically by addition of β -CD (20.0 mg/mL) in water into the same solution. The absorbance at 435 nm was noted down. Benesi-Hildebrand plot was then illustrated taking $\frac{1}{\Delta A}$ against $\frac{1}{[H]_0}$ by following equation (1):

$$\frac{1}{\Delta A} = \frac{1}{b\Delta\epsilon[G]_0[H]_0K_a} + \frac{1}{\Delta\epsilon[G]_0} \quad (1)$$

Where ΔA is the change in absorbance of the aCPT at the respective concentration of β -CD, b is the path length, $\Delta\epsilon$ is the change in molar absorptivity of the guest (in this case aPS), $[G]_0$ is the concentration of the guest (in this case aPS), $[H]_0$ is the concentration of the host (in this case β -CD) and K_a is the association or stability constant.

The experiment was repeated for aCPT (4×10^{-5} M) and 10 μ L of 20.0 mg mL⁻¹ of β -CD was added instead. The absorbance were taken at 370 nm.

The association or stability constant can be calculated by the following equation (2):

$$K_a = \frac{y \text{ intercept}}{\text{slope}} \quad (2)$$

2.2.8 Determination of critical aggregation concentration (CAC) by pyrene probe

Pyrene (1 mg) was first dissolved in acetone (4 mL) to yield a concentration of 1.24 mM. 1.6 μ L of this pyrene solution was added to 4 mL glass vial and it was left to evaporate overnight in dark. Subsequently, the solution of HA-aPs-aCPT of different

concentrations (0.02 mg/mL to 1 mg/mL) were then prepared and added to the vials to stir overnight to give final concentration of 1 μ M of pyrene. The solutions were left to stir overnight in dark. Fluorescences of pyrene were then measured from 350 to 450 nm by excitation at 335 nm. The pyrene emission wavelengths at 373 nm and 383 nm were recorded for different concentrations. Pyrene 1:3 ratios were calculated to determine the critical micelle concentration.

2.2.9 Preparation of HA-aPS-aCPT NPs

HA-CD (1 mg) was first dissolved in DI water (0.948 mL). A separate aliquot of aPS (0.0755 mg, 90 μ M) and aCPT (0.0324 mg, 45 μ M) in 52.6 μ L of DMSO (5 %) was prepared and mixed well. Under sonication, the aliquot of aPS and aCPT in DMSO was added dropwise into the HA-CD suspension. The mixture was then dialyzed against 500 mL of phosphate buffer saline (PBS) to give HA-aPS-aCPT NPs. The loading capacity was computed by using the following:

$$\text{Loading capacity of aCPT(\%)} = \frac{\text{Mass of aCPT}}{\text{Mass of nanoparticles}} \times 100\%$$

$$\text{Loading capacity of aPS(\%)} = \frac{\text{Mass of aPS}}{\text{Mass of nanoparticles}} \times 100\%$$

2.2.10 Singlet oxygen detection using singlet oxygen sensor green (SOSG)

Singlet oxygen detection was determined by following previously reported protocol with slight modification.⁶² Briefly, SOSG dissolved in methanol was incubated with different samples ([SOSG] = 2.5 μ M, [aPS] = 4.5, 9 and 13.5 μ M) to measure $^1\text{O}_2$ generation after irradiation of 660 nm light (65 mW/cm²). The solution was diluted 5 times with DI water and then 150 μ L of the diluted solution was pipetted into 96 well

plate and then measurement was taken. The generated $^1\text{O}_2$ was determined by measuring SOSG fluorescence maximum at 530 nm under 494 nm excitation.

2.2.11 Cell culture

MDA-MB-231 (CD44^+) and MCF-7 (CD44^-) human breast carcinoma cells were cultured in cell incubator with high glucose DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin under 5% CO_2 at 37°C .

2.2.12 *In vitro* ROS detection

The generation of $^1\text{O}_2$ from aPS was qualitatively determined using 2',7'-dichlorofluorescein diacetate (carboxy- H_2DCFDA). HA-aPS-aCPT NPs (50 $\mu\text{g/mL}$) in deionized water were first incubated with MDA-MB-231 cells for 4 h. Following which, the sample was irradiated with light of 660 nm (65 mW/cm^2) for 30 mins. The cells were then dyed with carboxy- H_2DCFDA (25 μM) in PBS for 30 mins at 37°C , protected from light. At the 25th minutes, Hoechst 33342 dye was added to stain the nucleus. The dyes were then removed, and excess dyes were washed away with phosphate buffer saline (PBS) and cells were visualized under the confocal microscope immediately. The excitation/emission of carboxy- H_2DCFDA was taken as 495/592 nm. The excitation/emission of Hoechst 33342 was taken as 350/461 nm.

2.2.13 Cellular uptake and *in vitro* targeting ability

MDA-MB-231 cells were seeded on to 6-well plate with coverslips at the bottom at the density of 2×10^5 cells/ well and left to adhere for 12 h. To determine cellular uptake by confocal laser scanning microscope (CLSM), HA-aPS-aCPT NPs (100 $\mu\text{g/mL}$) in deionized water was incubated at different duration of 2 h and 4 h. The cells were then washed thrice with PBS and fixed with 4% formaldehyde for visualization under the CLSM. To validate targeting ability of the HA-aPS-aCPT NPs, MDA-MB-231 (CD44^+) and MCF-7 (CD44^-) cells were cultured and seeded on to 6-well plates at

a density of 5×10^5 cells/ well and left to adhere for 12 hours. After which, equivalent concentrations of HA-aPS-aCPT NPs (100 $\mu\text{g/mL}$) in deionized water were added to both MDA-MB-231 and MCF-7 cells for two different durations of 2 h and 4 h. The cells were then washed thrice with PBS and trypsinized for flow cytometry analysis using em/ex 633/730 nm. Both flow cytometric analysis and CLSM imaging were done for HA competitive assays to test the targeting ability of the HA-aPS-aCPT NPs to CD44 receptors in MDA-MB-231 cells. In both experiments, MDA-MB-231 cells of 2×10^5 /well were pretreated with 5 mg/mL of free HA for 2 h. HA-aPS-aCPT NPs (100 $\mu\text{g/mL}$) were then incubated for 2 h. The treated cells were then washed thrice with PBS. To do flow cytometry analysis, the cells were trypsinized and collected. For CLSM imaging, cells were fixed with 4% formaldehyde, mounted and visualized under excitation/emission 640/730 nm.

2.2.14 *In vitro* cytotoxicity tests of HA-PS-CPT NPs and apoptosis study

The cytotoxicity of the various materials was investigated with MDA-MB-231 cells by MTT assay. MDA-MB-231 cells (1×10^4 cells per well) were seeded onto 96-well plates and incubated for 12 h. After cell confluency reached 60 - 70%, culture medium was replaced with 100 μL of medium containing CPT drug, aCPT prodrug, HA-aPS-TL NPs (6.25, 12.5, 25, 50, 100 and 200 $\mu\text{g/mL}$) and HA-aPS-aCPT NPs (6.25, 12.5, 25, 50, 100 and 200 $\mu\text{g/mL}$) followed by incubation for 48 h at 37°C in the dark. The cell viability was evaluated by MTT assay. The MTT dye dissolved in DMEM high glucose (5 mg/mL) was incubated for four hours to form the formazan crystals. DMSO (100 μL) was then added. Measurements of the absorbance in each well were then taken at 570 nm with reference to 630 nm. The percentage cell viabilities of each sample at different concentrations were calculated using the following formula:

$$\frac{Abs_{570\text{ nm}}(\text{sample}) - Abs_{630\text{ nm}}(\text{sample})}{Abs_{570\text{ nm}}(\text{control}) - Abs_{630\text{ nm}}(\text{control})} \times 100$$

To test for the cytotoxicity of HA-aPS-aCPT NPs under light irradiation, the above procedure was repeated with the same concentrations of NPs added. However, instead of incubation of 48 h in the dark, light of 660 nm at 70 mW/cm² were irradiated on the cells for 30 mins. After incubation for another 44 h in the dark, the cell viability was measured in like previous procedure.

To visualize the phototoxicity using CLSM, MDA-MB-231 cells were seeded on to petric dish (2 x 10⁵) and incubated for 48 h. The medium was replaced with fresh medium containing HA-aPS-aCPT (50 µg/mL) and incubated in dark for 6 h. After which, light of 660 nm (110 mW/cm²) is used. After 18 h of incubation in dark, the cells were washed with PBS and stained with calcein-AM (3 µM, ex/em: 488/515 nm) and propidium iodide (PI) (8 µM, ex/em: 488/610 nm) for 2 h before visualizing under the microscope.

2.2.15 Animal model

Female BALB/c nude mice (6 weeks old) were obtained from the Animal laboratory of Xinqiao Hospital (Chongqing). To develop the MDA-MB-231 tumour xenograft model, 5 × 10⁵ MDA-MB-231 cells suspended in 100 µL of 3:2 Matrigel:phosphate buffered saline (PBS) were subcutaneously injected on to the mice. The tumours were left to grow to about 60 to 80 mm³. The tumour volumes were computed following this formula: (width² × length)/ 2

2.2.16 *In vivo* optical imaging

Female BALB/c nude mice were intravenously injected with 200 µL of mixture of free aPS and aCPT as well as HA-aPS-aCPT NPs ([aPS] = 4.0 mg/kg, [aCPT] = 2.0 mg/kg) *via* their tail vein. At 2, 6 and 24 h interval, the mice were anesthetized and

visualized using *in vivo* imaging system (IVIS) under the excitation wavelength of 670 nm and emission wavelength of 740 nm. To visualize the bioaccumulation, after 24 h, the mice were sacrificed and major organs such as heart, liver, spleen, lung, kidney, brain as well as tumour were collected and imaged using the same excitation/emission wavelength.

2.2.17 *In vivo* combinational therapy

BALB/c nude mice bearing subcutaneous MDA-MB-231 tumours (~80 mm³) were randomly divided into five groups (n=6 per group). The respective groups were: (I): Saline (II): HA-aPS-aCPT (III): CPT (IV): THPP (V): HA-aPS-aCPT + Light irradiation. Intravenously injections were used on all mice with volume of 100 µL administrated each time. Mice in group I were treated with saline, group II were treated with HA-aPS-aCPT NPs in the dark, group III were treated with camptothecin drugs, group IV were treated with THPP and group V were treated with HA-aPS-aCPT NPs and irradiated by the 660-nm light (100 mW/cm² for 30 minutes) 24 h after administration. Concentrations of different moieties administrated each time are as following: [HA-CD] = 0.5 mg/kg, [aPS]=[THPP]=0.375 mg/kg and [CPT]=[aCPT]=0.16 mg/kg of mice. The mice were administrated with its respective treatment once every three days for a total of six times in 21 days period. The tumour sizes were recorded every 2-3 days for 21 days, with their widths as well as lengths measured by a digital vernier caliper. The weight of the mice was also recorded using a digital weighing balance every 2-3 days for 21 days. The tumour volumes were computed according to the following formula: (width² × length)/ 2. On the 21st day, all the mice were euthanized and major organs such as heart, liver, spleen, lung, kidney were harvested and fixed by 10% formalin for two days at 4°C. Subsequently, these fixed tissues were embedded with paraffin and then sectioned for histological studies.

2.2.18 TUNEL and histological assay

To investigate the tumour apoptosis in BALB/c nude mice, tumour biopsies were performed using a TUNEL apoptosis detection kit (Beyotime biotechnology, China). The samples were stained using Hoechst 33258 to mark the nuclei for CLSM observation. Standard protocol was followed for hematoxylin and eosin (H&E) staining and observed under an optical microscope.

2.3 Results and Discussions

2.3.1 Synthesis

The synthetic procedures of HA-CD, aCPT and aPS are illustrated in Schemes 2 to 4. The successful synthesis of aCPT and aPS was confirmed by ^1H NMR spectra and ESI-MS. HA-CD polymer was characterized via ^1H NMR spectrum. The conjugation degree of CD was determined to be 6.56%, which is equivalent to 15.2 HA monomer per 1 CD. This observation indicates that HA-CD is ideal for targeting purpose, as some reports showed that at least 6 consecutive HA sugar monomers are required for targeting CD44 receptor.^{63, 64}

2.3.2 Cleavage of thioketal linker from aCPT.

The cleavage of the thioketal linker on aCPT was investigated using ^1H NMR spectra and high performance liquid chromatography (HPLC). When $^1\text{O}_2$ reacts with the thioketal linker, the intermediate product could undergo nucleophilic attacking and release acetone as one of the by-products (Scheme 1). Upon irradiation of the mixture of aPS and aCPT with light, the formation of acetone was indicated by the presence of the resonance at 2.17 ppm in the NMR spectrum as shown in Figure 1a. Under longer irradiation time, the integral of this resonance increased. Concurrently, the integral of the resonance at 1.55 ppm assigned to the methyl group on the thioketal unit decreased,

indicating that the degradation of the thioketal linker by the generated $^1\text{O}_2$ occurred. To further confirm that free CPT could be released under light irradiation, the HPLC study of the reaction was performed (Figure 1b). The amount of CPT was quantified by external standard calibration method as presented in Figure 1c, showing linear dependence of the area with respect to the concentration of CPT. Under longer irradiation time, the released amount of CPT increased as indicated by greater area at the retention time of 12.0 min. This result shows that the amount of free CPT released was dependent on the irradiation time as shown in Figure 1d. When the photosensitizer with higher concentrations was co-doped in the mixture, the amount of free CPT released under the same irradiation time and light power density was higher, revealing that the release of free CPT was also dependent on the amount of photosensitizer. The generated CPT was further proven using high-resolution mass spectrometry (HRMS) (calculated $[\text{M}+\text{H}^+] = 349.1188 \text{ m/z}$, found = 349.1189). These results indicated that the synthesized prodrug was indeed capable of releasing free CPT in ROS-responsive release manner.

2.3.3 Photophysical properties of aPS and ROS detection using singlet oxygen sensor green.

The photophysical properties of aPS were investigated through UV-vis-NIR spectroscopy as shown in Figure 1f. Porphyrins are known to contain a Soret band and four Q bands in their absorbance.⁶⁵ In the case of aPS, the absorbance maximum obtained was at 431 nm assigned as the Soret band, and four Q bands at 530, 572, 603 and 657 nm were also observed. This indicated that the photophysical properties of porphyrins were generally well preserved despite functionalization of adamantane done. To investigate the ROS generation ability of aPS, SOSG was utilized for capturing the $^1\text{O}_2$ generated upon irradiation of light on aPS, where the amount of $^1\text{O}_2$ produced was

correlated to the increase in SOSG fluorescence. HA-aPS samples with three concentrations ($[aPS] = 4.5, 9.0, \text{ and } 13.5 \mu\text{M}$) were examined for the singlet oxygen generation. HA-aPS with $[aPS] = 9.0 \mu\text{M}$ showed the highest increase in fluorescence as compared to the other two samples (Figure 1h). This could be because, at lower concentration of aPS ($4.5 \mu\text{M}$), the singlet oxygen generated was limited, while at higher concentration ($13.5 \mu\text{M}$), the quenching of the photosensitizer led to a decrease in the $^1\text{O}_2$ generation. Thus, the amount of aPS chosen in the final NPs was $9.0 \mu\text{M}$. After which, $^1\text{O}_2$ generated by free aPS, HA-aPS-TL, and HA-aPS-aCPT ($[aPS] = 9.0 \mu\text{M}$) was investigated in aqueous solution. TL is a control linker with the thioketal bond but has no CPT drug attached. The amount of $^1\text{O}_2$ generated follows this sequence: free aPS > HA-aPS > HA-aPS-TL $\approx$ HA-aPS-aCPT. HA-aPS had lower $^1\text{O}_2$ generation yield than free aPS, because aPS was encapsulated within the HA-aPS NPs having possible aggregation effect.⁶⁶ Comparing HA-aPS, HA-aPS-TL and HA-aPS-aCPT, HA-aPS-TL and HA-aPS-aCPT had lower $^1\text{O}_2$ yield than HA-aPS, because some of the $^1\text{O}_2$ generated was utilized for the cleavage of thioketal linker. Since the same molar ratio of TL linker and aCPT ($[TL] = [aCPT]$) was added in HA-aPS-TL and HA-aPS-aCPT, they gave similar $^1\text{O}_2$ yield. Despite some of the $^1\text{O}_2$ was used for the cleavage of thioketal bond, the generated $^1\text{O}_2$ was still high enough for possible PDT of cancer, fulfilling the dual therapeutic treatment.

Figure 1. (a) ^1H NMR analysis for the cleavage of thioketal linker after irradiation for 30 min and 1 h. (b) HPLC analysis for CPT release from aCPT (retention time of CPT, aPS and aCPT were 12.0, 15.3 and 16.1 min respectively). (c) Calibration curve of CPT obtained from corresponding HPLC curve. (d) Drug release profile determined by HPLC when 45 μM aCPT was co-incubated with aPS with concentrations of 45, 90 and 135 μM .

2.3.4 Self-assembly characterisations

Employment of CD and adamantane was due to the high association constant of approximately 1×10^5 mol/L in water.^{67, 68} We hypothesized that the adamantane of aPS and aCPT would form an inclusion complex with CD of the HA-CD to give an amphiphilic polymer. NPs were then formed by self assembly of this amphiphilic polymer to give a HA-aPS-aCPT NPs with hydrophobic core (aPS and aCPT) and hydrophilic shell (HA). The association constant of the adamantane moieties synthesized were calculated from Hildebrand-Benesi plot⁶⁹ with β -CD as the reference

host. aPS and aCPT were found to have association constants of 3.58×10^3 L/mol and 2.55×10^3 L/mol respectively (Figure 2), which shows their capability to bind to β -CD. After which, the critical aggregation concentration (CAC) of HA-aPS-aCPT was determined by pyrene probe.^{10, 70} Table S1 revealed that the CAC was lower as the amount of hydrophobic components increased. The CAC was found to be lower with increased hydrophobic component indicating stronger hydrophobic interactions within the hydrophobic core (Figure 3).^{56, 58, 71} Furthermore, as the amount of hydrophobic adamantane moieties increased, the size measured by dynamic light scattering (DLS) decreased because of the more compact hydrophobic core⁵⁶ which also in turns give better distribution as shown by the lower polydispersity index (PDI).

Figure 2. Hildebrand-Benesi plot for (a) aPS and (b) aCPT.

Figure 3. Critical aggregation concentration (CAC) determination using pyrene probe by taking the ratio of fluorescence at 373 nm and 383 nm at different concentration of hydrophobic components and keeping HA-CD polymer constant.

Table 1. Polymer concentration, hydrophobic component and its respective CAC as determined by pyrene probe from Figure S12 and hydrodynamic diameter and PDI value.

HA-CD/ mg	[Hydrophobic component] (μM)	CAC (mg/mL)	Hydrodynamic diameter (nm)	Polydispersity index (PDI)
1	3.375	0.49	135.1	0.342
1	6.75	0.16	102.7	0.360
1	13.5	0.047	76.3	0.06

2.3.5 Characterizations of NPs

The NPs were formed by the supramolecular assembly of hydrophilic HA-CD and hydrophobic aPS and aCPT to give a HA-aPS-aCPT NPs with hydrophobic core (aPS and aCPT) and hydrophilic shell (HA). One of the advantages of such supramolecular system is that the amount of photosensitizers and drugs could be facilely adjusted. Six NPs with different photosensitizer to prodrug ratios (1:0, 3:1, 2:1, 1:2, 1:3 and 0:1) were mainly fabricated. The morphologies of the NPs were found to be spherical as visualized by transmission electron microscopy (TEM). The measurement of their hydrodynamic diameters using dynamic light scattering (DLS) gave 76.3 to 302.4 nm with good monodispersity (Table 2 and Figures 4 and 5). The absorption spectra of the six NPs indicated successful encapsulation of different amounts of aCPT and aPS with almost 100% loading efficiency. Hypsochromic shifts were observed for the NPs with increasing amounts of photosensitizers due to the aggregation effect of the photosensitizers within the NPs (Figure 6).

Table 2. Compilation of the NP labelled, ratio of photosensitizer and camptothecin prodrug, and corresponding hydrodynamic size and polydispersity index.

Name	Ratio of aPS : aCPT	Hydrodynamic size (nm)	Polydispersity index (PDI)
NP1	1:0	81.82	0.074
NP2	3:1	78.09	0.083
NP3	2:1	76.32	0.056
NP4	1:2	100.9	0.088
NP5	1:3	95.13	0.062
NP6	0:1	302.4	0.567

Figure 4. TEM images of (a) NP1, (b) NP2, (c) NP3, (d) NP4, (e) NP5, and (f) NP6. Scale bar: 200 nm.

Figure 5. Hydrodynamic diameters of (a) NP1, (b) NP2, (c) NP3, (d) NP4, (e) NP5, and (f) NP6 measured using DLS.

Figure 6. Absorption curves of NP1 (black), NP2 (red), NP3 (green), NP4 (blue), NP5 (cyan), and NP6 (pink).

Finally, HA-aPS-aCPT NPs with photosensitizer to prodrug ratio of 2:1 ($[HA] = 1 \text{ mg/mL}$, $[aPS] = 9.0 \text{ }\mu\text{M}$, and $[aCPT] = 4.5 \text{ }\mu\text{M}$) were chosen for further cell studies and *in vivo* applications. A concentration of $9.0 \text{ }\mu\text{M}$ was determined to present the highest $^1\text{O}_2$ production from the SOSG experiment (Figure 7a). With greater proportion

of photosensitizers, a portion of the photosensitizers can be used to fulfill on-demand release of chemotherapeutic drugs by the cleavage of ROS-responsive bond, and the other portion can be employed for the PDT purpose. In this specific case, the drug loading capacity for aCPT and aPS was determined to be 3.24% and 7.55%, respectively. The morphology of HA-aPS-aCPT NPs visualized by TEM (Figure 7b) showed monodispersed spherical NPs. DLS measurements gave hydrodynamic size of 76.3 nm and zeta potential of -26.7 ± 8.2 mV as shown in Figure 7c. The nanosize of the NPs could thus indicate that it is suitable for accumulation for EPR effect. The negatively charged zeta potential also possibly indicated that HA was on the surface of the NP, which was typical of many HA system.^{50, 72, 73} The CAC was found to be 0.047 mg/mL of the polymer which showed that the micelle is stable even at low concentrations of polymer. The chosen HA-aPS-aCPT NPs were found to have characteristic absorbance peaks of aCPT at 361 nm and aPS at 427, 524, 560 and 598 nm (Figure 7d), indicating successful encapsulation of both of these components.

Figure 7. (a) SOSG results obtained for different samples (HA-aPS at different concentrations of 4.5, 9.0 and 13.5 μM ; aPS, HA-aPS-TL and HA-aPS-aCPT NPs with an equivalent aPS concentration of 9.0 μM) after irradiation of light (65 mW/cm^2) at different time intervals measured at 530 nm when excited at 494 nm. (b) TEM image of HA-aPS-aCPT NPs. Scale bar: 100 nm. (c) Hydrodynamic diameter of HA-aPS-aCPT NPs using DLS. (d) Absorbance of aPS, aCPT and HA-aPS-aCPT NPs.

2.3.6 Cellular uptake and *in vitro* targeting ability

MDA-MB-231 were chosen as the cell line because their overly expressed CD44 could be targeted by HA.⁴⁷ The accumulation of HA-aPS-aCPT NPs in cells was visualized using confocal laser scanning microscope (CLSM) and measured using flow cytometry. Confocal images showed time-dependent uptake, *i.e.*, greater fluorescence with longer incubation time (Figure 8a). To determine the capability of the HA-aPS-aCPT NPs for targeting CD44 receptors, MDA-MB-231 (CD44-rich: CD44⁺) and MCF-7 (CD44-poor: CD44⁻) cells were selected for cellular comparative studies. Using flow cytometry analysis (Figure 8b,c) after the incubation of HA-aPS-aCPT NPs at 2 h and 4 h, the mean fluorescence intensity (MFI) of MDA-MB-231 cells was 1.93-folds and 2.05-folds higher than that of MCF-7 cells respectively, indicating higher uptake of HA-aPS-aCPT NPs by MDA-MB-231 cells possibly due to the rich presence of CD44 receptors for efficient receptor-mediated cellular uptake. In addition, competitive inhibition study was executed to confirm the cellular uptake of HA-aPS-aCPT NPs *via* CD44-mediated endocytosis using confocal imaging (Figure 8d) and flow cytometric analysis. The MDA-MB-231 cells pretreated with excess serum-free HA polymer showed 1.90-folds lower uptake of HA-aPS-aCPT NPs than untreated ones (Figure 8e,f). This observation suggested that both free HA and HA-aPS-aCPT NPs could competitively bind to CD44 receptors, confirming that the internalization pathway of HA-aPS-aCPT NPs was through CD44 receptor-mediated endocytosis.

Figure 8. (a) Fluorescence images of MDA-MB-231 (CD44⁺ cells) when incubated with HA-aPS-aCPT NPs (100 $\mu\text{g/mL}$) for 2 h and 4 h. (b) Representative plots of cell populations determined by flow cytometric analysis for MDA-MB-231 (CD44⁺) and MCF-7 (CD44⁻) cells internalized by HA-aPS-aCPT NPs at different incubation time (2 h and 4 h). (c) MFI of HA-aPS-aCPT NPs in different cells at different incubation times. (d) Fluorescence images of MDA-MB-231 (CD44⁺ cells) incubated with HA-aPS-aCPT NPs (100 $\mu\text{g/mL}$) for 2 h when pretreated without and with free HA (5 mg/mL). (e) Representative plots and (f) MFI of cell populations determined by flow cytometric analysis for cellular uptake of HA-aPS-aCPT NPs pretreated without and with free HA. Scale bar: 50 μm .

2.3.7 *In vitro* ROS detection

After confirming the internalization pathway of the NPs, we evaluated whether ROS could be produced *in vitro* when HA-aPS-aCPT NPs were illuminated with light. The intracellular ROS production induced by HA-aPS-aCPT NPs was determined in MDA-MB-231 cells using a general oxidative stress indicator, carboxy-DCFDA (Figure 9a). The fluorescence intensity of cells incubated with NPs and irradiated with light was

the highest amongst other control groups, indicating the successful production of ROS by HA-aPS-aCPT NPs *in vitro*. The produced ROS from HA-aPS-aCPT NPs is important for subsequent cleavage of the thioketal linker for the drug release as previously discussed. Cleavage of the prodrug into its active form allows chemotherapy to then take place and lead to combinational therapy.

2.3.8 Cell viability and apoptosis study

The cytotoxicity of the NPs was evaluated using MTT assays (Figure 9c). At the same equivalent molar concentrations, aCPT was found to be less toxic than free CPT due to the functionalization of the hydroxy group on the lactone ring that is important in the topoisomerase inhibition to result in the apoptosis in cells.^{74, 75} At high concentrations of NPs ([NPs] = 200 µg/mL), HA-aPS-aCPT displayed low cellular toxicity in the dark, showing high cell viability of 74.4%. Comparing HA-aPS-TL and HA-aPS-aCPT NPs under light irradiation, at low concentrations of 0 to 100 µg/mL, HA-aPS-aCPT NPs displayed higher cytotoxicity because the contained aCPT could be released as free CPT upon light irradiation, leading to synergistic PDT and chemotherapy. At the concentration of 200 µg/mL, however, HA-aPS-aCPT and HA-aPS-TL NPs showed similar high cytotoxicity possibly because PDT predominated. Flow cytometric analysis was used to quantify the amount of apoptotic MDA-MB-231 cells when treated with HA-aPS-aCPT NPs. When cells were incubated with HA-aPS-TL and HA-aPS-aCPT NPs in the dark, the total amounts of apoptotic cells were low at 3.0% and 4.9%, respectively (Figure 9d). The numbers of apoptotic cells for both HA-aPS-TL and HA-aPS-aCPT NPs under light irradiation were significantly more than that in the dark. These results showed that the NPs displayed minimal dark cytotoxicity and light irradiation was essential to induce their cellular toxicity.

According to the cellular apoptosis results, there was higher percentage of apoptotic cells incubated with HA-aPS-aCPT (72.8%) as compared to that of HA-aPS-TL NPs (27.8%), validating that CPT was released upon light irradiation to result in higher cellular toxicity. Live/dead confocal images of the cells irradiated with HA-aPS-aCPT NPs under light irradiation also showed higher amount of red cells (propidium iodide (PI) stained cells) indicative of higher cellular toxicity (Figure 9b).

Figure 9. (a) Detection of ROS using carboxy-DCFDA (green). Nuclei were stained with DAPI (blue). Scale bar: 50 μ m. (b) Live/dead assay on MDA-MB-231 (CD44+ cells) with and without NPs as well as with and without light irradiation (IRR), where + means related treatment was done, and - means no such treatment was done. Scale bar: 100 μ m. (c) Cell viability of MDA-MB-231 cells when incubated with

free CPT, aCPT, HA-aPS-TL with and without light irradiation, and HA-aPS-aCPT with and without light irradiation at different concentrations. (d) Quantitative numbers of different cells obtained from apoptotic study using Annexin V FITC and PI.

2.3.9 *In vivo* biodistribution

Encouraged by the *in vitro* therapeutic results, the biodistribution and treatment efficiency of mixture of free aPS and aCPT as well as HA-aPS-aCPT NPs were assessed *in vivo* using xenografted nude mouse models with MDA-MB-231. After HA-aPS-aCPT NPs were intravenously injected into the nude mice bearing tumours, it was visualized (Figure 10a) using the *in vivo* imaging system (IVIS) at the wavelength corresponding to aPS under different time intervals. Accumulation was observed in both treatments initially. A progressive accumulation of the HA-aPS-aCPT NPs in tumours was observed over time. In contrast, free aPS was not accumulated in tumour at the end of the 24 h. After 24 h, the mice were euthanized, and major organs and tumours were removed and visualized. The NPs were accumulated in tumours, liver and spleen but free aPS does not accumulate in tumours (Figure 10b) indicating accumulation of HA-aPS-aCPT in tumours via EPR effect. Free aPS accumulated in liver and spleen for excretion because its high hydrophobicity causes large aggregates to be formed when dissolved in PBS.⁷⁶ HA-aPS-aCPT NPs, despite having the ability to target tumour, also could not escape the fate of being excreted and thus also shown accumulation in organs such as liver and spleen. (Figure 10c)

Figure 10. *In vivo* studies on anti-tumour therapeutic effect. (a) Biodistribution of free aPS and aCPT and HA-aPS-aCPT NPs at different time intervals after intravenous injection into mice. The tumours were circled. (b) *Ex vivo* fluorescence image of major organs and tumour excised from mice injected with free aPS and aCPT and HA-aPS-aCPT NPs. H, L, S, Lu, K, B and T stands for heart, liver, spleen, lung, kidney, brain and tumour, respectively. (c) Biodistribution of HA-aPS-aCPT NPs in various organs determined from (b).

2.3.10 *In vivo* antitumour efficacy

The antitumour efficacy of the NPs was examined by using similar nude mice model. Five groups of mice were monitored over a period of 21 days after treated with different conditions (I: saline; II: HA-aPS-aCPT NPs; III: CPT; IV: tris(3-hydroxypropyl)phosphine (THPP); V: HA-aPS-aCPT + Light irradiation). THPP is the free photosensitizer that was not modified by adamantane. As shown in Figure 11a,b, HA-aPS-aCPT NPs with light irradiation could largely suppress tumour growth as compared to the saline as well as THPP groups. The mean tumour volume ($253.09 \pm 50.27 \text{ mm}^3$) resulted from HA-aPS-aCPT NPs with light irradiation was significantly smaller than the saline ($902.35 \pm 178.48 \text{ mm}^3$) and THPP ($939.98 \pm 131.02 \text{ mm}^3$) groups. Although the administration of free CPT also delayed the growth of tumour ($561.08 \pm$

67.43 mm³), it exhibited obvious systemic toxicity as indicated by the lowest average body weight (Figure 5c). HA-aPS-aCPT NPs in the dark also exhibited certain toxicity as shown by smaller mean tumour size (429.68 ± 54.01 mm³). This observation could be explained by its better passive accumulation in tumour and high amount ROS already in cancer cells for releasing free CPT from aCPT.

In addition to the significant inhibition of tumour growth, HA-aPS-aCPT NPs accumulated mostly at tumour sites did not cause as much systemic toxicity as free CPT, indicated by the increased body weight of mice and Hematoxylin and Eosin (H&E) staining from various mouse organs treated with HA-aPS-aCPT NPs (Figures 11c and 10). H&E staining of tumours was conducted on all five groups of mice. Some minor damages in the tumour tissues when treated with HA-aPS-aCPT NPs and free CPT were observed, while serious damage was observed when mice were treated with HA-aPS-aCPT NPs under light irradiation (Figure 11d). This difference indicated that there was an enhancement of PDT with cascaded chemotherapy as compared to free CPT. Similar conclusion was drawn from TUNEL assay that detected apoptotic cells. After various treatments, the amount of TUNEL-positive cells was found to be in this order: V > II > III > IV > I. More red fluorescence from Cyanine 3 observed for tumour tissues of mice treated with HA-aPS-aCPT NPs under light irradiation indicated the greatest number of apoptotic cells in these groups. After treatment with HA-aPS-aCPT NPs, the H&E staining on major organs in different mice showed no noticeable systematic toxicity (Figure 12). All these results demonstrated the feasibility of HA-aPS-aCPT NPs for combinational PDT and chemotherapy by inducing the tumour cell death, showing high potency of HA-aPS-aCPT NPs as a therapeutic protocol for tumour treatment.

I : Saline II: HA-aPS-aCPT III: CPT IV: THPP V: HA-aPS-aCPT + Light irradiation

Figure 11. (a) Time-dependent tumour growth on mice with different treatments over a period of 21 days. (b) Three excised tumours from each group at the end of day 21 from euthanized mice. (c) Body weights of mice with different treatments monitored over a period of 21 days. (d) H&E staining and TUNEL assay of tumour tissues from euthanized mice with different treatments (I: saline; II: HA-aPS-aCPT; III: CPT; IV: THPP; V: HA-aPS-aCPT + Light irradiation).

Figure 12. H&E staining on major organs for different groups of mice (Group I: Saline, II: HA-aPS-aCPT NPs, III: CPT, IV: THPP, V: HA-aPS-aCPT NPs + Light irradiation).

2.4 Conclusion

In conclusion, HA-aPS-aCPT NPs have been fabricated using three components (HA-CD, aPS and aCPT) *via* simple supramolecular means. The obtained NPs were monodispersed with almost 100% loading efficiency. The thioketal linker conjugated between adamantane and CPT in aCPT could be cleaved in a controlled manner when singlet oxygen is produced from aPS photosensitizer. The as-synthesized aPS photosensitizer was able to produce $^1\text{O}_2$ upon the irradiation of light. Targeting capability of HA was clearly demonstrated at cellular level using competitive HA assays. High cellular toxicity was demonstrated when cancer cells were incubated with HA-aPS-aCPT NPs under light irradiation. Excellent tumour accumulation of HA-aPS-aCPT NPs in xenograft mouse models bearing MDA-MB-231 cancer was clearly due to passive targeting and CD44 receptor-mediated endocytosis. HA-aPS-aCPT NPs did slow down the tumour growth as compared with other control groups. Importantly, the present system capable of controlled release of chemotherapeutic drugs and PDT displayed no obvious systemic toxicity, enabling its useful applications for practical cancer treatment. Future investigations should also address pharmacokinetics and pharmacodynamics of our NPs system for possible clinical applications.

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Chapter 3: Catalase Integrated Hyaluronic Acid as Nanocarriers for Enhanced Photodynamic Therapy in Solid Tumour

3.1 Introduction

Photodynamic therapy (PDT) involves light illumination of appropriate wavelength on photosensitizers at the tumour site, converting endogenous molecular oxygen to singlet oxygen ($^1\text{O}_2$) *in vivo* for inhibition of tumour growth.¹⁻³ It has been investigated in different cancer treatment such as skin tumours,^{4, 5} head and neck cancer,⁶ prostate,² non-small cell lung and other cancers.⁷ Advantages of PDT in cancer treatment includes its minimal invasiveness in comparison to chemotherapy and radiotherapy, possible repeatable dose, and low systematic toxicity to the body.^{1, 8-10} Despite its extensive use in cancer treatment, the efficacy of PDT is reduced due to issue such as the low penetration depth of lower wavelength light in deep-situated tumour, non-specific accumulation of photosensitizers and the lack of endogenous oxygen in tumours.¹¹ Hypoxia, a term used to describe the lack of oxygen,¹² arises in many tumours, especially in areas that are at a distant from the blood vessels in tumours.¹³ Many research has been done on imaging¹⁴ as well as therapy in hypoxic tumour.¹¹ Hypoxic tumours are often resistant to drug which thus limit chemotherapy efficacy.¹⁵ PDT as a treatment modality has limitations due to the lack of oxygen which thus impeded PDT efficacy.

To date, different methods have been used for overcoming the limited supply of oxygen in tumours such as direct delivery of oxygen,^{16, 17} *in situ* generation of oxygen¹⁸⁻²² and improving blood flow.^{11, 23} Specifically, *in situ* generation of oxygen using

catalase as an enzyme is attractive because it is self-responsive to hydrogen peroxide (H_2O_2) and does not require pre-treatment with oxygen ($^3\text{O}_2$).^{18, 19, 24} It has been shown that excessive reactive oxygen species (ROS) such as H_2O_2 are produced in cancer cells.²⁵ Catalase could catalyze H_2O_2 to $^3\text{O}_2$ for relieving hypoxia in tumours.²⁶ Production of oxygen could ameliorate tumour hypoxia, leading to better PDT efficacy. Delivery of proteins such as catalase raises a few concerns such as its instability *in vivo* due to the presence of numerous physiological proteases, unpredictable protein immunogenicity, and poor *in vivo* half-life.^{27, 28} Thus, design of suitable nanocarriers that can overcome these problems is highly needed.

Many researchers have tried to improve the protein stability by encapsulation them within inorganic NPs (NPs).²⁹⁻³¹ These methods often involved complicated synthesis procedures and the inorganic materials used often have issues being metabolized. In contrast, using organic materials as nanocarriers lead to good biodistribution, easy metabolism and better biodegradability.³²⁻³⁵ PEGylation is widely used in such modification for the delivery of proteins/peptides into the human body.³⁶ Numerous advantages of such polymer modification include the improved stability, solubility and pharmacokinetics.^{37, 38} An example of the US FDA approved PEG-protein conjugates is Oncaspar (mPEG-L-asparaginase).³⁹ However, high molecular weight PEGylation has its disadvantage of poor biodegradability.³⁷ In light of this, polysaccharides such as dextran and hyaluronic acid (HA) as biodegradable polymers^{37, 40} have been explored for modifications on proteins. HA, a natural polysaccharide composed of alternate units of two sugar monomers *i.e.*, glucuronic acid and N-acetylglucosamine,⁴¹ is biodegradable and biocompatible with excellent biosafety. HA is known to target the overly expressed CD44 receptors in several tumours including MDA-MB-231 human breast cancer⁴² and A549 lung cancer.^{43, 44} In addition, HA is

highly amenable to functionalization due to the availability of reactive chemical groups such as acetamide, carboxyl, hydroxyl and aldehyde groups.³⁸ Many studies have been reported on using HA as a therapeutic carrier with covalently attached photosensitizers for targeted therapy.⁴⁵⁻⁴⁷ On the other hand, such direct conjugation onto the polymer results in the difficulties in controlling the amount of photosensitizers delivered and prolonging the storage of light sensitive photosensitizers. Therefore, an alternative strategy as opposed to direct conjugation of photosensitizers should be devised.

Herein, we report a therapeutic system (HA-CAT@aCe6) consisting of (1) CD44-targeting HA, (2) catalase (CAT) as an enzyme to catalyze the production of H₂O and O₂ from endogenous H₂O₂, and (3) adamantane modified Chlorin e6 (aCe6) photosensitizer to produce ¹O₂ from the ground state ³O₂ (Scheme 1). In this system, β-CD was first functionalized onto HA, followed by conjugation with catalase to form HA-CAT NPs. It was hypothesized that the integration of HA with CAT improves physiological stability of the system in presence of body proteases, and at the same time, enables active targeting to tumour. aCe6 photosensitizer is loaded into HA-CAT by supramolecular means between β-CD and adamantane to give HA-CAT@aCe6 that can target tumours possessing overly expressed CD44 receptors. Such supramolecular loading of drugs enable separation storage of different components and easy adjustment of photosensitizers is to catalase ratio, is highly tunable and predictable.⁴⁸ Upon light irradiation, aCe6 in HA-CAT@aCe6 produces ¹O₂ for PDT, even in hypoxic tumour. Some advantages of this system are (1) active targeting capability, (2) suitable nanosize for accumulation in tumour, (3) presence of catalase to produce oxygen for overcoming hypoxia in solid tumour, and (4) facile and controlled loading of photosensitizers using supramolecular means, all of which affording the enhanced PDT efficacy in mice model.

Scheme 1. Schematic illustration of the processes after administration of HA-CAT@Ce6 NPs into tumour mice model. Process A: HA-CAT@Ce6 NPs were prepared by the conjugation of cyclodextrin modified HA with catalase, followed by supramolecular encapsulation of Ce6. Process B: HA-CAT@Ce6 NPs can accumulate in hypoxic tumour and catalyze intracellular H_2O_2 to ground state oxygen $^3\text{O}_2$. $^3\text{O}_2$ is then converted to singlet oxygen $^1\text{O}_2$ upon 660 nm light irradiation for PDT of cancer.

3.2 Materials and Methods

3.2.1 Materials

1-Adamantanecarbonyl chloride, chlorin e6, and sodium hyaluronate (MW = 8 to 12 kDa) were purchased from Aaron Pharmatech Ltd (Shanghai, China). Amberlite® IR120 hydrogen form, catalase, hydroxybenzotriazole hydrate, and methyl iodide were purchased from Sigma Aldrich. β -CD, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl), thiazolyl blue tetrazolium hydrate (MTT), triethylamine, and trifluoroacetic acid were purchased from Tokyo Chemical Industry. MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC). Dialysis membrane Spectra/Por 6 (molecular weight cut off (MWCO): 2 kDa) was purchased from Thermo Fisher Scientific Pte Ltd. ROS-ID® hypoxia/oxidative stress detection kit was obtained from Enzo Life Sciences Inc. Pimonidazole, CD31 and

HIF-1 α antibodies were from Hypoxypore, Inc. In situ BrdU-Red DNA Fragmentation (TUNEL) Assay Kit were obtained from Abcam. All chemicals were of analytical grade and used without further purification if not indicated otherwise.

3.2.2 Instruments

^1H NMR spectra were probed on a Bruker BBFO400 spectrometer, and D_2O , DMSO-d_6 , and CDCl_3 were used as the deuterated solvents. The UV-vis-NIR absorption and fluorescence emission spectra were measured using Shimadzu UV-3600 and Shimadzu RF5301PC spectrophotometer, respectively. Transmission electron microscopy (TEM) images were taken at an acceleration voltage of 100 kV on JEOL JEM-1400. Hydrodynamic diameters and zeta potential were measured at 25 °C by a Malvern Zetasizer Nano-S system. Confocal laser scanning microscopy (CLSM) images were acquired by ZEISS LSM 800 Confocal Laser Scanning Microscope. Flow cytometry were taken on BD LSRFortessa™ X-20 cell analyzer. H&E staining images were taken by Leica DM5500B upright widefield fluorescence and BF microscope.

3.2.3 Synthesis of aCe6

Scheme 2. Synthesis procedure of ada-Ce6. Conditions: (a) TEA in anhydrous DCM, 0°C (b) TFA in anhydrous DCM, 3 h at r.t.p. (c) EDC, TEA, 2 h follow by CH_3I and K_2CO_3 , 2 h.

Synthesis of 1. 0.735 mL of anhydrous triethylamine (TEA) (0.533 g, 5.30 mmol) was added to N-Boc-ethylenediamine (0.850 g, 3.31 mmol) and to 20 mL of anhydrous dichloromethane (DCM) at 0°C. Subsequently, 1-adamantanecarbonyl chloride (0.630 g, 3.17 mmol) in 10 mL of anhydrous DCM was added dropwise for 30 mins. The

reaction mixture was stirred overnight at r.t.p. Extraction was carried out with dilute HCl, brine and water and the organic layer was collected. Purification was done using column chromatography with 50% ethyl acetate (EA)/hexane to obtain a white solid as product (0.834 g, 82 %) ^1H NMR (CDCl_3 , 400 MHz): δ 1.45 (s, 9H), 1.72 (m, 6H), 1.85 (s, 6H), 2.03 (s, 3H), 3.32 (m, 4H), 4.88 (s, 1H), 6.35 (s, 1H).

Synthesis of 2. Trifluoroacetic acid (TFA) (1.061 g, 9.30 mmol) in 10 mL of anhydrous DCM was added to 1 (0.30 g, 0.90 mmol) and stirred at r.t.p. for 3 h. Subsequently, the reaction mixture was evaporated to dryness to obtain the product as white solid. The product was used without further purification. ^1H NMR (DMSO-d_6 , 400 MHz): δ 1.66 (m, 6H), 1.77 (m, 6H), 1.97 (s, 3H), 2.83 (m, 2H), 3.26 (m, 2H), 7.54 (t, 1H, $J=5.6$ Hz), 7.69 (s, 2H).

Synthesis of aCe6. The reaction was carried out in the dark and under N_2 atmosphere. EDC.HCl (0.039 g, 0.201 mmol, 1.2 equivalent) was added to Chlorin e6 (0.100 g, 0.168 mmol, 1 equivalent) dissolved in 5 mL DMF. Reaction mixture was then stirred for 2 h at 25°C . 2 (0.045 g, 0.201 mmol) and 28 μl TEA in 1 mL of DMF was then added and stirred for 30 mins. Then, anhydrous potassium carbonate (K_2CO_3) (0.070 g, 0.504 mmol, 3 equivalent) and CH_3I (1.189 g, 8.38 mmol, 50 equivalent) was added and stirred for another 2 h at 25°C . The reaction mixture was then diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The residue was then purified using the silica column chromatography, eluting at 10% MeOH/DCM to give the product aCe6 as a green solid (0.035 mg, 25%). ^1H NMR (CDCl_3 , 300 MHz): δ 1.10 (d, 3H, $J=14.4$ Hz), 1.22 (d, 6H, $J=6.12$ Hz), 1.31 (s, 2H), 1.35 (s, 1H), 1.50 (s, 3H), 1.59 (s, 9H), 1.71 (t, 6H, $J=6.3$ Hz), 2.14 (m, 2H), 2.46 (m, 1H), 3.01 (s, 1H), 3.28 (t, 7H, $J=6.6$ Hz), 3.48 (s, 4H), 3.56 (s, 3H), 3.78 (q, 2H, $J=7.7$ Hz), 4.33 (s, 3H), 4.43 (m, 2H), 5.22 (t, 2H, $J=21$ Hz), 5.93 (t,

J=6.3 Hz, 1H), 6.17 (d, 1H, J=13.5Hz), 6.36 (d, , 1H, J=19.5 Hz), 6.57 (s, 2H), 4.33 (s, 1H), 8.06 (dd, 1H J=5.7 Hz, 30.3 Hz,), 8.77 (s, 1H), 9.59 (s, 1H), 9.69 (s, 1H)

3.2.4 Synthesis of β -cyclodextrin modified hyaluronic acid (HA-CD)

Scheme 3. Synthesis procedure of HA-CD polymer. Condition: (a) NaOH, TsCl. (b) ethylenediamine, reflux in DMF under N₂ (c) EDC.HCl, HOBT for 2 days.

Sodium hyaluronate was pretreated with cationic exchange resin (Amberlite® IR120 hydrogen form) to give HA in the following procedure. Sodium hyaluronate (1 g) was first dissolved in deionized water (300 mL) and then passed through the cationic exchange resin slowly and then lyophilized to give HA. EDC.HCl (0.232 g, 1.21 mmol) and HOBT (0.162 g, 1.05 mmol) were added into a solution of HA (0.1384 g, 14.6 μ mol) in DMSO (10 mL). The mixture was then stirred at 25°C for 30 min. Mono-6-deoxy-6-ethylenediamino- β -CD (0.8139 g, 0.69 mmol) in DMSO (10 mL) was added into the mixture and it was stirred for another 24 h at r.t.p.. The resulting mixture was dialyzed against DMSO (4 x 100 mL) and then deionized water for 3 days (2 L x 10) and freeze-dried to give HA-CD as white solid. The degree of substitution of β -CD on to HA was around 6.56% for HA-CD conjugates. The concentration of CD in 1 mg/mL of HA-CD was calculated to be 135 μ M.

3.2.5 Synthesis of HA-CAT NPs

Various ratio of HA-CD polymers to catalase were synthesized. Briefly, HA-CD polymers (20 mg, 40 mg, 80 mg) were added with EDC.HCl (2 molar equivalent of the

HA) for 30 minutes in deionised water. Catalase (12 mg) were then added with HOBt (2 molar equivalent of the HA) and was stirred for 18 h at r.t.p. Thereafter, the mixtures were then centrifuge at 5000 rpm using molecular weight cut off (MWCO) ultracentrifuge tubes of 100,000 Da for 1 h and washed with deionised water thrice. The final concentrations of the catalase were measured using BCA assay using various concentrations of free catalase as the reference. The mass of HA conjugated were obtained from the mass difference between the freeze-dried HA-CAT NPs and CAT mass obtained from BCA assay.

3.2.6 Synthesis of HA-CAT@aCe6 NPs

HA-CAT NPs with concentration of catalase 400 µg/mL were added with aCe6 dissolved in DMSO to obtain 12 µM. After which, the mixture was then dialysed against PBS of pH 7.4. The loading efficiency were calculated using the following formula:

$$\text{Loading efficiency (\%)} = \frac{\text{Photosensitizer loaded}}{\text{Photosensitizer added}} \times 100\%$$

The amount of aCe6 were calculated by measuring the absorbance of the aCe6 at wavelength of 404 nm using calibration curve of aCe6 in 10% DMSO/H₂O. The loading capacity were computed using the following formula:

$$\text{Loading capacity (\%)} = \frac{\text{Mass of photosensitizers}}{\text{Mass of nanoparticles}} \times 100\%$$

3.2.7 Generation of O₂ from H₂O₂ and catalase activity assay

To compare the relative activity of the free CAT, HA-CAT and HA-CAT@aCe6 NPs and HA@aCe6 NPs where [CAT] = 400 µg/ mL, [aCe6] = 90 µM, a solution of H₂O₂ (0.5 mM) was prepared. The concentration of H₂O₂ was chosen such that the generated O₂ is detectable by the oxygen probe (Lovibond SD 400 OXIL). Endogenous H₂O₂ in human was 100 µM.⁴⁹ The amount of HA-CAT@aCe6 added such that it is proportionate to the [CAT] used in cellular studies (40 µg/mL) and endogenous H₂O₂

(approximately 100 μM). The increased in the O_2 concentration with respect to the initial O_2 concentration was calculated.

To measure and compare the stability of free catalase and HA-CAT, the Góth method was used.²⁴ Both free catalase and HA-CAT with the final concentration of 300 $\mu\text{g/mL}$ were incubated with Proteinase K (60 $\mu\text{g/mL}$) at 37 °C. At 0, 0.5, 1, 2, 3, 4 and 5 h, aliquots of sample were used for immediate catalase activity assay. To determine the catalase activity, H_2O_2 (50 mM, 1 mL) was added with free catalase or HA-CAT (300 $\mu\text{g/mL}$, 0.2 mL) at 37 °C for 60 s, and then ammonium molybdate (32.4 mM, 1 mL) was added to terminate the reaction. The relative catalase activity could be determined by the absorbance of the solution at 405 nm.

3.2.8 Detection of $^1\text{O}_2$

$^1\text{O}_2$ was determined by the following protocol.²⁴ In brief, SOSG dissolved in methanol was incubated with different samples ($[\text{SOSG}] = 2.5 \mu\text{M}$, $[\text{aCe6}] = 4.9 \mu\text{M}$, $[\text{H}_2\text{O}_2] = 0.25 \text{ mM}$) to measure singlet oxygen generation after light irradiation (630 nm, 15 mW/cm^2) purged under nitrogen. The amount of H_2O_2 was chosen such that the generated $^1\text{O}_2$ can be detected by SOSG and that the fluorescence of SOSG falls in the detection limit of our spectrometer. The generated $^1\text{O}_2$ was determined by measuring SOSG fluorescence signal at 528 nm under 494 nm excitation.

3.2.9 Immunofluorescence for detection of HIF-1 α in cells

The MDA-MB-231 cells were seeded onto 6 well plates with cover slip and incubated at normoxic (21% O_2) and hypoxic conditions (1% O_2) for 24 h. HA-CAT@aCe6 NPs ($[\text{CAT}] = 40 \mu\text{g/mL}$, $[\text{aCe6}] = 10 \mu\text{M}$) were first incubated with the cells for 4 h. They were then quickly fixed with 4% paraformaldehyde for 10 mins at rtp. It is then washed with PBS thrice. After which, the cells were permeabilised using 0.1% Triton X-100 in PBS for 10 mins and washed thrice with 0.1% Tween-20 in PBS.

After which blocking buffer was added and incubated for 1 h at r.t.p.. Primary anti-HIF-1 α antibody was diluted in blocking buffer (2:1000) to yield a 2 μ g/mL and incubated for 1.5 h at r.t.p. After which the cells were washed thrice with 0.1% Tween-20 in PBS. It is then incubated with the secondary Alexa Fluor®488-conjugated Goat Anti-Rabbit IgG H&L antibody diluted in blocking buffer (5:1000) with final concentration of 5 μ g/mL for 1 h at r.t.p. in the dark. It is then washed thrice with 0.1% Tween-20 in PBS. Finally, DAPI with working concentration of 300 mM was used to stain the nucleus for three min. The cells were washed again and then visualised using the confocal microscopy with excitation wavelength of 488 nm and emission max at 525 nm.

3.2.10 Immunofluorescence for detection of CD44 receptors in different cells

The MDA-MB-231 and MCF-7 cells were seeded onto six well plates with cover slip for 24 h at cell density of 200000 cells/well separately. The cells were then fixed with 4% paraformaldehyde for ten mins at r.t.p.. It is then washed with PBS thrice. After which, the cells were permeabilized using 0.1% Triton X-100 in PBS for 10 mins and washed thrice with 0.1% Tween-20 in PBS. After which blocking buffer was added and incubated for 1 h at r.t.p.. The primary anti-CD44 antibody was diluted in blocking buffer (1:1000) to yield a final concentration of 1 μ g/mL and incubated for 1 h at rtp. After which the cells were washed thrice with 0.1% Tween-20 in PBS. It is then incubated with the secondary Alexa Fluor®488-conjugated Goat Anti-Rabbit IgG H&L antibody diluted in blocking buffer (1:1000) with final concentration of 2 μ g/mL for 1 h at r.t.p. in the dark. It is then washed thrice with 0.1% Tween-20 in PBS. Finally, DAPI with working concentration of 300 mM was used to stain the nucleus for 3 min. The cells were washed again and then visualised using the confocal microscopy with excitation wavelength of 488 nm and emission max at 525 nm.

3.2.11 Cellular experiments

MDA-MB-231 cells were cultured in high glucose DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO₂. To study the dark cytotoxicity of HA@aCe6 and HA-CAT@aCe6 NPs with different concentrations at both normoxic (74% N₂, 5% CO₂, 21% O₂) and hypoxic (94% N₂, 5% CO₂, 1% O₂) conditions, the cells were seeded into 96-well cell culture plates at cell density of 10,000/well until adherent and incubated with respective normoxic and hypoxic conditions for 24 h. The cells were then incubated with various concentrations of HA@aCe6 and HA-CAT@aCe6 NPs for 24 h before cell viability in dark conditions was determined. To study the photodynamic efficacy, the cells were seeded until adherent and incubated at various conditions for 24 h. The cells were then incubated with different concentrations of HA@aCe6 and HA-CAT@aCe6 NPs for 4 h before irradiated with light (630 nm, 3.1 mW/cm²) for 5 min. The standard MTT assay was used to determine the cell viability relative to untreated control cells.

3.2.12 Animal model

Female BALB/c nude mice were purchased from InVivos Pte Ltd and used under protocol approved by Institutional Animal Care and Use Committee (IACUC). To develop the tumour model, 2×10^6 MDA-MB-231 cells suspended in 3:2 matrigel:PBS (60 µL) were subcutaneously injected onto the back of each mouse. Experiments on the mice commenced when tumour volumes reached about 50 – 60 mm³.

3.2.13 *In vivo* imaging

For animal imaging, HA@aCe6 and HA-CAT@aCe6 NPs (200 µL) with equivalent concentration of aCe6 (0.4 mg/mL) were intravenously injected into the mice (aCe6 dose: 4 mg/kg). *In vivo* fluorescence imaging (IVIS) was conducted at wavelength of 675 nm and emission of 735 nm using an *in vivo* optical imaging system. The mice

were sacrificed at 24 h after intravenous injection, with their major organs including tumour, liver, heart, lung, spleen, and kidney collected for imaging. The fluorescence intensity was analyzed by ImageJ. To evaluate the tumour hypoxia post treatment, BALB/c nude mice bearing MDA-MB-231 tumour were intravenously injected with HA-CAT@aCe6 NPs. After 24 h, each mouse was injected with pimonidazole hydrochloride (30 mg kg⁻¹, hypoxyprobe-1 plus kit, Hypoxyprobe Inc). The mice were sacrificed after 90 mins for the collection of tumour slices for immunofluorescence staining. Finally, the slices were observed using CLSM.

3.2.14 *In vivo* photodynamic therapy

For *in vivo* therapy, 25 tumour-bearing nude mice were divided into five groups (five mice per group): I) control group with saline injection only, II) free Ce6 with light irradiation, III) HA@aCe6 NPs with light irradiation, IV) HA-CAT@aCe6 NPs in the dark, and V) HA-CAT@aCe6 NPs with light irradiation. Then, the mice were injected with the materials, and after 24 h, group II, III and V received light irradiation (50 mW/cm²) on the tumour for 30 min. The doses of aCe6, catalase and Ce6 were 4 mg/kg, 18.4 mg/kg and 2.9 mg/kg, respectively. The lengths and widths of the tumour were measured using a digital caliper for fourteen days. Volumes of the tumour were computed using the standard formula where volume = $\frac{1}{2} \times \text{width}^2 \times \text{height}$. The tumour growth curve was processed by comparing tumour volumes from each day to the initial tumour volumes at day 0. Evaluation of the treatment efficacies for each group was done by sacrificing mouse at day 4 for the collection of the tumours for H&E staining.

3.3 Results and Discussions

3.3.1 Synthesis and characterizations

CD-modified HA was synthesized by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxybenzotriazole (HOBt) coupling. Low molecular weight HA (8 – 15 kDa) was conjugated to β -CD to produce HA-CD with a conjugation degree of 6.56%. The photosensitizer Ce6 was modified with adamantane for easy loading with HA-CD *via* supramolecular means. The compounds synthesized were all characterized by ^1H NMR spectra. Following which, HA-CD was reacted with catalase *via* the EDC and HOBt coupling to produce HA-CAT in different mass ratios (1:1, 3:1 and 6:1) of HA-CD:catalase. Catalase, an enzyme that catalyzes the production of O_2 from H_2O_2 , was introduced for promoting the PDT efficiency in hypoxic tumour. HA-CD:catalase mass ratio of 3:1 was eventually chosen for further studies, since the obtained HA-CAT has a reasonable hydrodynamic diameter suitable for the accumulation in tumour⁵⁰ and better retained activity of catalase (Figure 1 and Table 1). The concentrations of catalase in all catalase-containing NPs were determined by the bicinchoninic acid protein assay. The mass amount of HA-CD to catalase was approximately 1.4:1 as determined by the subtraction of the CAT mass (measured by the BCA protein assay) from the known dried mass of HA-CAT. For the same concentration of catalase in HA-CAT and free catalase, the activity of HA-CAT was estimated to be 15.3% of free catalase, determined using the Amplex Red Catalase Kit. For controlled loading of photosensitizer, ada-Ce6 was loaded into HA-CAT *via* the formation of inclusion complex between adamantane on aCe6 and β -CD modified on HA to give HA-CAT@aCe6.

Figure 1. (a) Hydrodynamic diameters, (b) zeta potential and (c) relative activity of HA-CAT synthesized using different mass ratios of HA-CD : catalase. The measurements were taken in H₂O as the solvent at room temperature.

Table 1. Results obtained from Figure 1.

Ratio of HA-CD : catalase	Final ratio of HA-CD: Catalase	Hydrodynamic diameter (nm)	Zeta potential (mV)	Activity of catalase (U/mg)	Relative activity of catalase
0 : 1	-	9.4	-3.4	7464	100*
1.5 : 1	1.27 : 1	504.5	-10.3	3128	41.9
3 : 1	1.32 : 1	224.0	-20.3	1144	15.3
6 : 1	3.19 : 1	216.7	-21.7	304	4.1

HA-CAT and HA-CAT@aCe6 NPs were fully characterized. Transmission electron microscopy (TEM) images show a spherical morphology for both HA-CAT and HA-CAT@aCe6 NPs (Figure 2a,b). HA-CAT NPs in water have a hydrodynamic diameter of 224.0 nm as measured by the dynamic light scattering (DLS) with zeta potential of -20.3 mV, while free catalase has a diameter of approximately 9.4 nm with zeta potential of -3.4 mV. The increase in size and reduction in zeta potential from highly negatively charged HA-CAT demonstrated the successful conjugation of HA-CD with catalase (Figure 2c,d). HA-CAT@aCe6 NPs are slightly larger with a hydrodynamic

diameter of 233.6 nm and zeta potential of -13.6 mV (Figure 2c,d). Vertical gel electrophoresis was used to verify the successful synthesis of HA-CAT (Figure 2e). Catalase is made up of 4 monomeric proteins and gives a single 66 kDa band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by taking the reference with the known mass of protein in lane 1 where lane 1 is the protein ladder. After the conjugation of HA-CD onto catalase to form HA-CAT, a trailing band was observed in the SDS-PAGE lane (lane 3), which was a result of the increased molecular weight of HA-CAT. After the photosensitizer loading, the presence of characteristic absorbance bands of aCe6 at around 400 nm and 660 nm and the aggregation induced blue shift of aCe6 confirmed the successful entrapment of aCe6 in HA-CAT@aCe6 NPs (Figure 2f). The entrapment efficiency of aCe6 by HA-CAT NPs ([CAT] = 40 $\mu\text{g/mL}$) was found to be 80.1 %. The final concentration of aCe6 was determined to be 9.7 μM (Figure 3b) by referencing to the standard calibration curve of aCe6 (Figure 3c). The photosensitizer loading efficiency was found to be 2.0 % (w/w), with reference to the mass of HA-CAT.

Figure 2. Characterisations of HA-CAT@aCe6 NPs. TEM images of (a) HA-CAT NPs and (b) HA-CAT@aCe6 NPs. The scale bar is 50 nm. (c) The hydrodynamic diameter measured from dynamic light scattering of free catalase, HA-CAT NPs and HA-CAT@aCe6. (d) Zeta potential of various materials. (e) Gel obtained from running HA-CAT NPs. Lane 1 is the protein ladder which contains protein of known mass. (f) The absorbance of HA-CAT NPs, aCe6 and the HA-CAT@aCe6 NPs.

Figure 3. (a) Absorbance curve of Ce6 and aCe6 (b) Absorbance of HA-CAT@aCe6 NPs before (black) and after (red) dialysis. (c) Calibration curve of aCe6. Amount of aCe6 was calculated based on the Beer-Lambert law.

3.3.2 Evaluation of catalase activity

Following the preparation of HA-CD and HA-CAT@aCe6 NPs, their abilities to generate O_2 when incubated with H_2O_2 were investigated. As shown in Figure 4, H_2O_2 and HA@aCe6 NPs (control group without CAT loaded) did not have a significant effect on the generation of O_2 in the solution. The slight increase of the O_2 concentration in the H_2O_2 sample was probably caused by inherent decomposition of H_2O_2 . On the other hand, HA-CAT and HA-CAT@aCe6 NPs produced significantly higher amounts of O_2 when incubated with H_2O_2 . Comparatively, free catalases produced O_2 at the fastest rate, reaching the maximum concentration of O_2 at 90 s. HA-CAT and HA-CAT@aCe6 showed a steady increase in the O_2 concentration upon time. They did not show obvious differences in the production of O_2 from H_2O_2 , suggesting that the photosensitizer loading did not affect the activity of catalase. The activities of HA-CAT and HA-CAT@aCe6 NPs were lower than that of free catalase, since the conjugation of HA-CD onto catalase made it less available for the catalytic reaction. Nonetheless, the capacity of HA-CAT and HA-CAT@aCe6 NPs is high enough for catalytic production of O_2 from H_2O_2 .

Figure 4. Change in oxygen concentration when different samples (H_2O_2 only, free catalase, HA-CAT, HA@aCe6 and HA-CAT@aCe6 NPs) were incubated with H_2O_2 .

3.3.3 Verification of production of singlet oxygen using singlet oxygen sensor green.

After the verification that O_2 could be produced from HA-CAT and HA-CAT@aCe6 NPs upon the addition of H_2O_2 , we tested their efficiency in the generation of 1O_2 using SOSG. SOSG has weak fluorescence, but when it reacts with 1O_2 , the fluorescence increases at 530 nm under the excitation at 494 nm. Modification of Ce6 with adamantane to give aCe6 and verification is needed to ensure that aCe6 is also able to produce 1O_2 . When only H_2O_2 was present in the solution, no fluorescence was observed. Obvious fluorescence increase was measured for HA-CAT@aCe6 or aCe6 with H_2O_2 when irradiated under light, indicating their comparable 1O_2 production capability. Considering effective generation of oxygen *via* the catalysis of loaded catalase on H_2O_2 , the production of 1O_2 by HA-CAT@aCe6 with H_2O_2 presented the highest fluorescence change at 530 nm at all irradiation time (Figure 5). This observation indicates that the production of 1O_2 was enhanced in the presence of both catalase and H_2O_2 , which could possibly promote the PDT efficacy of HA-CAT@aCe6 NPs in cellular and animal studies.

Figure 5. Change in fluorescence of SOSG over irradiation time for different samples such as H_2O_2 only, free Ce6 with H_2O_2 , HA-CAT@aCe6 only and HA-CAT@aCe6 with H_2O_2 .

3.3.4 Stability examination of conjugated catalase

Catalase might rapidly lose its stability and activity *in vivo* during the blood circulation owing to the degradation by other enzymes especially the proteases. The conjugation of enzymes to polymers was known to improve the stability of the enzymes due to the polymer protection from the proteases.³⁸ To verify that the present modification could lead to better protein stability, free catalase or HA-CAT was incubated with proteinase K for a specific duration and their corresponding ability to decompose H₂O₂ was verified using the Gøth method.⁵¹ As shown in Figure 6a, the activity of free catalase quickly decreased to near zero within 2 h after the incubation with proteinase K. In contrast, HA-CAT was still able to retain more than 90% of its activity after 2 h, and the activity remained high even after 4 h. Therefore, the conjugation of catalase with HA-CD polymer did improve its stability in the presence of proteinase K, which is important for tumour targeted delivery *in vivo*. The minimal change in size of the HA-CAT@aCe6 in different solution (PBS, cell culture medium and water) over three days also indicated good physiological stabilities (Figure 6b).

Figure 6. (a) The relative enzymatic activity of free catalase and HA-CAT@aCe6 NPs after proteinase K digestion over 5 h. (b) Hydrodynamic size of HA-CAT@aCe6 NPs monitored over three days in three different solutions.

3.3.5 Cellular uptake and cytotoxicity of HA-CAT@Ce6 NPs

After verifying the sufficient capability of HA-CAT@aCe6 NPs for the $^1\text{O}_2$ production in the presence of H_2O_2 , its cellular uptake in MDA-MB-231 cells under both 21% O_2 and 1% O_2 was studied by the confocal microscopy and flow cytometry (Figure 7). Under both incubation conditions, the confocal images of MDA-MB-231 cells showed brighter fluorescence resulted from the loaded photosensitizer at longer incubation period, showing time-dependent uptake of HA-CAT@aCe6. Flow cytometric results also exhibited (Figure 7b and c) greater uptake of HA-CAT@aCe6 with longer incubation time by having higher mean fluorescence intensity (MFI).

Figure 7. (a) Fluorescence images of the time dependent uptake of HA-CAT@aCe6 NPs in cells at different time period (2 h, 4 h and 8 h) [aCe6] = 4.7 μM , [CAT] = 20 $\mu\text{g/mL}$, DAPI channel: Ex:405 nm, Em: 400 to 450 nm. HA-CAT@aCe6 NPs channel Ex: 488 nm, Em: 600 – 700 nm, Scale bar: 50 μm .

Uptake of NPs at different incubation time point (2 h, 4 h and 8 h) in **(b)** normoxic and **(c)** hypoxic condition. [aCe6] = 2.5 μ M, [CAT] = 10 μ g/mL and ex/ em: 488/710 nm.

As HA was known to target CD44 receptors over expressed on some cancer cells, the targeting ability of HA-CAT@Ce6 was verified by using CD44⁺ MDA-MB-231 and CD44⁻ MCF-7 cell lines. The levels of CD44 receptors on these cell lines were first evaluated, and CD44⁺ MDA-MB-231 cells expressed greater amount of CD44 receptors as compared to CD44⁻ MCF-7 cells (Figure 8a). Following the incubation of HA-CAT@aCe6 NPs with CD44⁺ MDA-MB-231 and CD44⁻ MCF-7 cells respectively (Figure 8b), the fluorescence from MDA-MB-231 cells was brighter as compared to that of MCF-7 cells at incubation times of both 2 h and 4 h. This result indicates that the uptake of HA-CAT@aCe6 by MDA-MB-231 cells was greater, probably through CD44 receptor-mediated endocytosis. Furthermore, competitive assay of HA was used to further support the targeting capability of HA-CAT@aCe6 (Figure 8c). Free HA with different concentrations (0, 5, 15, and 30 mg/mL) was incubated with MDA-MB-231 cells for 4 h before the addition of HA-CAT@aCe6. This process allowed the CD44 receptors on the cells to be occupied by free HA, and thus they were less available to facilitate the endocytosis of HA-CAT@aCe6. Indeed, with an increasing amount of free HA preincubated, the uptake of HA-CAT@aCe6 by both normoxic and hypoxic cells decreased, confirming that the HA component in HA-CAT@aCe6 provides targeting property, enabling specific accumulation to CD44 receptor-overexpressed cancer cells (Figure 8d and e).

Figure 8. (a) Fluorescence images of MDA-MB-231 cells and MCF-7 cells stained by immunostaining with primary Anti-CD44 antibody, followed by secondary Alexa Fluor®488-conjugated Goat Anti-Rabbit IgG H&L antibody. Scale bar: 20 μ m. (b) Fluorescence images of the time-dependent uptake of HA-CAT@aCe6 NPs in respective cell lines at different time periods (2 h and 4 h) [ada-Ce6] = 4.7 μ M, [CAT] = 20 μ g/mL, DAPI channel: Ex:405 nm, Em: 400 to 450 nm. HA-CAT@aCe6 NPs channel Ex: 488 nm, Em: 600 – 700 nm, Scale bar: 50 μ m. (c) Uptake of NPs at 2 h in the absence and presence of preincubation of HA under normoxic and hypoxic conditions. Uptake of HA-CAT@aCe6 NPs at (d) normoxic and (e) hypoxic conditions. [ada-Ce6] = 2.5 μ M, [CAT] = 10 μ g/mL and ex/em: 488/710 nm.

Then, immunostaining was used to measure the expression level of hypoxia-induced factor 1 α (HIF-1 α) in MDA-MB-231 cells. HA-CAT@Ce6 NPs were incubated with the cells under hypoxic condition of 1% O₂. High green fluorescence intensity was

observed from the hypoxic cells, indicating the presence of HIF-1 α proteins in the cells. (Figure 9) Conversely, cells incubated with HA-CAT@Ce6 NPs showed less green fluorescence signal, indicating that the expression in HIF-1 α was reduced and HA-CAT@Ce6 NPs were able to alleviate the hypoxia in the cells.

Figure 9. Fluorescence images of the hypoxic markers HIF-1 α detected in cells, with and without HA-CAT@Ce6 NPs. Scale bar: 50 μ m.

HA@aCe6 as a negative control was employed to investigate the catalytic effect of catalase in relieving hypoxia. TEM and DLS results of HA@aCe6 NPs showed that they were also suitable for cellular studies (Figure 10).

Figure 10. (a) TEM image of HA@aCe6 NPs and (b) zoomed version of (a). (c) Hydrodynamic diameter of HA@aCe6 NPs measured by DLS.

Hypoxia/ROS detection assay was done, and the cells were visualized using confocal under both normoxic and hypoxic condition (Figure 11,12). Red fluorescence could be induced when the nitro group of the hypoxia detection probe reduced to

hydroxylamine and amino groups while green fluorescence was induced from DCFH-DA when ROS was present to produce DCF. Results show that hypoxia could be relieved using HA-CAT and HA-CAT@aCe6 NPs and upon irradiation of light under both normoxic and hypoxic condition, the HA-CAT@aCe6 NPs were able to induce the greatest amount of ROS due to the highest intensity of green fluorescence from DCF.

Figure 11. Fluorescence images of cells with different treatments as monitored by the ROS-Hypoxia Kit under normoxic condition. Red fluorescence indicates that the cells were hypoxic, while green fluorescence indicates the presence of ROS. Scale bar: 100 μ m.

Figure 12. Fluorescence images of MDA-MB-231 cells with different treatments as monitored by the ROS-Hypoxia Kit under hypoxic condition. Red fluorescence indicates that the cells were hypoxic, while green fluorescence indicates the presence of ROS. Scale bar: 100 μm .

Cellular cytotoxicity was studied to test the therapeutic efficiency of different systems under normoxic and hypoxic conditions (Figure 13). When incubated with aCe6, HA@aCe6 and HA-CAT@aCe6 NPs at different concentrations (concentration of aCe6 was as high as 4.3 μM) in the dark, a little cytotoxicity was observed, showing high cell viability of over 90% in normoxic cells. The half maximal inhibitory concentration (IC_{50}) was computed as 2.2 μM and 1.4 μM for HA@aCe6 and HA-CAT@aCe6 NPs under normoxic condition, respectively. The same study was conducted in hypoxic condition where the concentration of oxygen was set at 1%. The IC_{50} of HA@aCe6 increased to 4.8 μM , while the IC_{50} of HA-CAT@aCe6 drastically

reduced to 0.72 μM under hypoxic condition. Comparing HA@aCe6 with the same concentration at normoxic and hypoxic conditions, its cytotoxicity greatly decreased at low concentration of oxygen under hypoxic condition. This is because the oxygen required for the generation of $^1\text{O}_2$ to kill cancer cells was lower under hypoxic condition, thus impeding the therapeutic efficacy of the photosensitizer in HA@aCe6. On the contrary, HA-CAT@aCe6 at the same concentration under hypoxic condition still displayed high cell killing efficacy. At the same concentration of the photosensitizer in both HA@aCe6 and HA-CAT@aCe6 NPs with light irradiation, the cytotoxicity of HA-CAT@aCe6 NPs was greater than that of HA@aCe6. This outcome could be due to that the intrinsic H_2O_2 in cancer cells is catalyzed by catalase in HA-CAT@aCe6 to produce more O_2 for improving the PDT efficacy. HA-mediated cellular accumulation coupled with catalase-catalyzed oxygen production²⁵ from endogenous H_2O_2 increased the amount of singlet oxygen generated, promoting the cell killing efficacy of HA-CAT@aCe6 even under hypoxic condition. Therefore, HA-CAT@aCe6 was proven to be an efficient system for tumour-specific PDT.

Figure 13. Cell viability after incubation with HA-CAT@aCe6 NPs or HA@aCe6 NPs with or without 630 nm light irradiation (3.1 mW/cm², 5 min) under (a) Normoxic and (b) Hypoxic conditions.

Apoptotic studies done using PI and Annexin IV FITC for labelling of cells also showed similar results. When MDA-MB-231 cells were incubated with HA-

CAT@aCe6 NPs, with light irradiation, the apoptotic cells detected were the highest (Figure 14).

Figure 14. Apoptosis assay by flow cytometry under normoxic (first row) and hypoxic (second row) conditions: HA@aCe6 NPs and HA-CAT@aCe6 NPs in the dark and with light irradiation with concentrations of [CAT] = 1.25 μ g/mL, [aCe6] = 0.33 μ M and [CAT] = 2.5 μ g/mL, [aCe6] = 0.65 μ M.

3.3.6 *In vivo* biodistribution of HA-CAT@aCe6 NPs.

Before the application of HA-CAT@aCe6 NPs for *in vivo* PDT, its biodistribution was first investigated. aCe6, HA@aCe6 and HA-CAT@aCe6 NPs were individually injected into MBA-MD-231 tumour-bearing nude mice intravenously. The fluorescence signal at the tumour sites was monitored using IVIS (Figure 15a). At 2 h, the tumours of all treated mice showed similar fluorescence intensity. Upon time, the fluorescence intensity from aCe6 group decreased, while HA@aCe6 and HA-CAT@aCe6 groups showed an enhancement in fluorescence at 24 h time point (Figure 15a, b). After 24 h, the mice treated with HA-CAT@aCe6 NPs were sacrificed for *ex vivo* imaging of their major organs (Figure 15c). The tumours were found to have the highest average radiant efficiency, translating to highest accumulation of HA-CAT@aCe6 NPs in tumours (Figure 15d). However, the liver showed weaker fluorescence, and other major organs such as spleen, kidney, heart and lungs exhibited minimal fluorescence signal. Free Ce6 were not able to accumulate in the tumour site since it is a small molecule and could diffuse in and out of the tumour freely, resulting in being metabolized.⁵² On the other hand, both HA@aCe6 and HA-CAT@aCe6 NPs could accumulate in tumour due to the EPR effect.⁵³ In addition, the ability of HA-CAT@aCe6 NPs to overcome hypoxia in tumours was also evaluated using fluorescent tagged antibody that binds to endogenous HIF-1 α (green) and CD31 (red) on blood vessels.⁵⁴ Typically, downregulation of both HIF-1 α and CD31 indicates the relief of hypoxia in solid tumours. MDA-MB-231 tumours were sectioned and stained by fluorescent tagged antibody and visualized under the microscope. The tumours treated with HA-CAT@aCe6 NPs showed remarkably alleviated hypoxia as compared to those without the treatment, as indicated by less intense fluorescence by HIF-1 α and CD31.

These immunofluorescence staining results indicate that HA-CAT@aCe6 NPs are indeed capable of overcoming hypoxia associated tumours for the PDT treatment.

Figure 15. (a) Biodistribution of HA-CAT@aCe6 NPs in mice at time interval of 2 h, 8 h and 24 h after IV injection (b) Relative mean fluorescence intensity obtained from the tumours of the mice using ImageJ (c) Ex vivo fluorescence image of various important organs as well as the tumour dissected from mice 24 h after an intravenous injection of HA-CAT@aCe6s. T, Li, S, H, K and Lu stand for tumour, liver, spleen, heart, kidney and lung, respectively. (d) Average radiant efficiency of the organs compiled from (c). (e) Immunofluorescence staining of tumour sections excised from mice with treatment with PBS and HA-

CAT@aCe6 NPs. Nuclei was stained blue with Hoechst 33342, blood vessels were stained red with anti-CD31 antibody and hypoxic tumour areas stained green with HIF-1 α antibody. Scale bar: 100 μ m.

3.3.7 *In vivo* photodynamic therapy

Because of the remarkable accumulation of HA-CAT@aCe6 in tumour sites, its efficacy in the eradication of tumours was evaluated *in vivo* using nude mice bearing MDA-MB-231 tumours. Five distinct therapeutic groups comprising of five mice per group were monitored for 14 days. The five groups include I: control in which only PBS was injected, II: Free Ce6 with light irradiation, III: HA-CAT@aCe6 NPs in the dark, IV: HA@aCe6 with light irradiation, and V: HA-CAT@aCe6 NPs with light irradiation. The relative tumour growth curve was obtained (Figure 16a). The growth of tumour from group V was the most inhibited one among all groups, indicating the highest anti-tumour efficacy of HA-CAT@aCe6. On the other hand, HA@aCe6 NPs showed moderate inhibition of tumour growth because the accumulated NPs at the tumour site were not able to exhibit enough toxicity to ablate the whole tumour. This led to the continual growth of the tumour. On account of the non-accumulation of free Ce6 at tumour site, the irradiation of light did not result in the inhibition of tumour growth. HA-CAT@aCe6 NPs in the dark also showed little therapeutic efficacy. In general, all the systems administrated did not show any major systemic toxicity as the weights of the mice all maintained at a healthy level (Figure 16b). The tumours from all treatment groups were excised and weighed at the end of day 14. Group V had the lowest tumour mass, followed by groups IV, III, II and I (Figure 16c). The tumours in group V were the smallest among all the tumours (Figure 16d).

To further evaluate the anti-tumour efficacy of PDT using HA-CAT@aCe6 NPs, H&E stain and TUNEL were utilized to stain the tumours from groups I to V for histological analysis. Based on obtained results (Figure 16e), the most severe

morphological change and necrosis for tumour slices were observed in group V, while moderate damages were observed from group IV and minimal damages from groups I-III. For studying the systemic toxicity of HA-CAT@aCe6, healthy nude mice were injected with HA-CAT@aCe6 NPs and the major organs were harvested for similar histological analysis. No major organ damage was observed (Figure 17), indicating good biocompatibility of HA-CAT@aCe6. All in all, HA-CAT@aCe6 NPs displayed excellent ability to target the tumour and reduce hypoxia in solid tumour to ensure effective anti-tumour PDT.

Figure 16. Relative tumour volume of MDA-MB-231 tumour-bearing mice for the five groups in 14 days. (b) Average weight of the different group (I-V) of mice (c) Mean tumour weight of mice excised on the 14th day. (d) Excised tumours from the five mice 14 days after treatment (e) Histological section of tumours stained with hematoxylin and eosin and TUNEL of tumours sectioned from different groups of mice where DAPI (blue) and TUNEL (red) are stained. Scale bar: 100 μ m Light of 660 nm was irradiated for 20 mins at 50 mW cm⁻². Data are presented as mean $\pm$ standard error mean (SEM). *, $p \leq 0.05$; **, $p \leq 0.01$, ***, $p \leq 0.001$ as calculated by Student's t test.

Figure 17. Histological section of various organs stained with H&E after different treatments. Scale bar: 100 μm .

3.4 Conclusion

In all, we have successfully fabricated HA-CAT@aCe6 NPs capable of targeting CD44 overexpressed cancer cells and tumours as well as reducing the hypoxia to ensure high efficacy of PDT both in cells and animal model. This method of catalase conjugation is feasible in protecting the catalase from the action of proteinase K. The protein stability was greatly enhanced through the conjugation with HA polymer. Thus, the loaded catalase was able to efficiently catalyze H_2O_2 to generate additional O_2 toward the production of ROS for PDT. Systemically administered HA-CAT@aCe6 NPs could accumulate in the tumour and attenuate the tumour hypoxia for improved PDT. Therefore, HA-CAT@aCe6 NPs displayed a potential for tumour-targeted treatment. The present approach could be extended to other protein-based therapeutics or enzymes.

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Chapter 4: Hyaluronic acid based nanogels for encapsulation of glucose oxidase and hypoxia responsive prodrug

4.1 Introduction

Tumour hypoxia is a phenomenon linked to solid tumour, as it describes an inadequate supply of O₂ in neoplasm.¹⁻⁷ When tumour grew, blood vessels were further from the center of the tumour, causing a limiting amount of O₂ in the tumour. Tumour hypoxia can affect tumourigenesis as well as treatment efficacy. Tumour hypoxia is related to increased expression of hypoxia-induced factors (HIF), a transcription factor that mediates angiogenesis, metabolism, cell proliferation and differentiation.⁸⁻¹⁶ Many hypoxic tumours are resistant to chemotherapy drugs due to several reasons like drug efflux,^{17, 18} apoptosis inhibition^{19, 20} and autophagy induction.²¹ Furthermore, some therapeutic methods such as photodynamic therapy and radiotherapy that require oxygen to generate reactive oxygen species (ROS) led to consumption of O₂ in cancer cells, which further exacerbates tumour hypoxia.^{2, 22-24} This further translates to low efficacy of such therapeutic methods. It is thus crucial to develop methods to either overcome hypoxia or to increase therapeutic performance in hypoxic tumour models. Attempts have been made to overcome hypoxia by reoxygenation of the tumour using materials such as perfluorocarbon^{25, 26} and artificial red blood cell²⁷ or introduce materials that can generate oxygen endogenously such as catalase²⁸⁻³³ and MnO₂.³⁴⁻³⁸ Some problem faced include its dependence on the endogenous environment, unknown toxicity or immunogenic effect from inorganic material as well as the need for stimuli or precursors for reaction to proceed. Another approach is to harness the hypoxic environment and design a hypoxia activated drug delivery system for cancer treatment. Hypoxia activated prodrugs (HAP) has been designed to undergo oxygen-inhibited enzymatic reduction.³⁹

Upon bio reduction of the HAP, it would generate toxic radicals.⁴⁰⁻⁴³ Such HAP is hydrophilic and selective to hypoxic cells. Many researches have combined oxygen consuming photodynamic therapy (PDT) with hypoxia activated prodrug for fabrication of such system. In a work by Liu et al.,⁴⁴ a nanosystem that contains upconversion nanoparticle (UCNP), photosensitizer and tirapazamine (TPZ) was developed. The UCNP was able to convert near infrared (NIR) light to uv-visible light to activate the photosensitizer which resulted in a depletion of oxygen. TPZ could then undergo a bio reduction to give its toxic radical. However, the efficiency of UCNP in light conversion and the depth of penetration of light remain a problem. Furthermore, UCNP being made of rare earth metal, could induce unknown toxicity and thus must be reconsidered when used.

In recent years, protein therapeutics is preferred over small therapeutic cargoes because of its numerous advantages such as high selectivity and less side effects due to low immunogenicity.⁴⁵ Proteins can be in a form of therapeutics, diagnostics or vaccine type. Because of the Warburg effect in cancer, there is a large uptake of glucose in cancer cells.⁴⁶⁻⁴⁸ Glucose oxidase (GOD) enzyme, a natural enzyme found in fungi and insects is able to catalyze the D-glucose into gluconic acid and H_2O_2 in the presence of O_2 as an electron acceptor.⁴⁹ GOD as a therapeutic enzyme can lead to glucose consumption which in turn produce reactive oxygen species in this case H_2O_2 .⁵⁰⁻⁵³ By reducing the glucose consumption of cancer cells, tumour growth can be impeded. Furthermore, it depletes the oxygen in the cells. However, such treatment needs to be enhanced by combination with another form of therapy. The reduction in oxygen due to the application of GOD can lead to a tumour hypoxia. In a work by Zhang et al.,⁵² liposome loaded GOD was applied to deplete the glucose and oxygen in tumour. After which, a hypoxia drug AQ4N was loaded into the liposome and the drug can be activated

due to the depleted oxygen level. However, there is no single system that incorporates both GOD and HAP.

Herein, we report a NPs system HA@aCe6@GOD-TPZ in scheme 1 that can 1) target CD44 overly expressed receptors, 2) responsive to glucose level in the cells to induce toxicity, 3) release radical species from tirapazamine (TPZ) prodrug under hypoxic condition. aCe6 was added with HA-CD to give an amphiphilic polymer that can self-assemble into nanoparticle before the structure of the NPs was stabilized by crosslinking using APS and TEMED. This allows GOD to be encapsulated within the HA nanocarrier without leakage. Because of the hydrophilic nature of TPZ, HA nanogel is suitable for encapsulation of these drugs. HA@aCe6@GOD-TPZ could undergo a CD44 mediated endocytosis into cancer cells. After which, glucose and oxygen can diffuse into the NP, GOD can catalyze the production of H_2O_2 , a ROS to kill the cancer cells. Concurrently, the O_2 consumption activates TPZ to undergo bio reduction to give its TPZ toxic radical. This led to dual therapy of cancer cells.

Scheme 1. Assembly of the HA@aCe6@GOD-TPZ NPs how it can lead to cell death.

4.2 Materials and Methods

4.2.1 Materials

Sodium hyaluronate ($M_w = 8$ to 12 kDa), 1-adamantanecarbonyl chloride and chlorin e6 were purchased from Aaron Pharmatech Ltd (Shanghai, China). Methyl iodide, Amberlite® IR120 hydrogen form, Hydroxybenzotriazole hydrate, Methacrylic acid and Glucose oxidase (GOD) were purchased from Sigma Aldrich. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl), Triethylamine, trifluoroacetic acid β -cyclodextrin (β -CD), N,N,N',N'-tetramethylethane-1,2-diamine (TEMED), ammonium persulfate (APS) and thiazolyl blue tetrazolium hydrate (MTT) were purchased from Tokyo Chemical Industry. MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC). Dialysis membrane Spectra/Por 6 (MWCO:1 kDa) was purchased from Thermo Fisher Scientific Pte Ltd. All chemicals were of analytical grade and used without further purification if not indicated otherwise.

4.2.2 Instruments

^1H NMR spectra were probed on a Bruker BBFO400 spectrometer, and D_2O , d-DMSO, CDCl_3 were used as the deuterated solvent. The UV-vis-NIR absorption and fluorescence emission spectra were measured using Shimadzu UV-3600 and Shimadzu RF5301PC spectrophotometer, respectively. Transmission electron microscopy (TEM) images were taken at an acceleration voltage of 100 kV on JEOL JEM-1400. Hydrodynamic diameters and zeta potential values were measured at 25°C by a Malvern Zetasizer Nano-S system. Confocal laser scanning microscopy (CLSM) images were acquired by ZEISS LSM 800 Confocal Laser Scanning Microscope. Flow cytometry were taken on BD LSRFortessa™ X-20 cell analyzer.

4.2.3 Synthesis of HA-Me-CD

Scheme 2. Synthesis procedure of HA-Me-CD polymer.

Methacrylated HA was prepared by following previous protocol. HA (8 to 15 kDa, 2.5 g) was dissolved in 100 mL of DI water and stirred in ice bath. The pH of the solution was adjusted to 8.5 using 5.0 NaOH. Methacrylic acid (MA, 5.6 mL was added) and the pH was adjusted repeated to 8.0 to 8.5 using a syringe pump for at least 4 h. The reaction was then stirred at 4°C overnight. After which, acetone was added to precipitate to give HA-Me, washed with ethanol, and dissolve in water. The solution was dialyzed against DI water for 3 days and lyophilized to give HA-Me. After which HA-Me (100 mg) was dissolved in DMSO (10 mL) and EDC.HCl (0.232 g) was added in and stirred for 2 h. After which, HOBt (0.162 g) and CD-NH₂ (0.8139 g) was added. Reaction was left to stir for 24 h before it was dialyzed against DI water using MWCO 2 kDa to yield HA-Me-CD. The polymer was characterized by NMR.

4.2.4 Synthesis of ada-Ce6

Scheme 3. Synthesis procedure of aCe6. Conditions: (a) TEA in anhydrous DCM, 0°C (b) TFA in anhydrous DCM, 3 h at r.t.p. (c) 1.EDC, TEA, 2 h follow by CH₃I and K₂CO₃, 2 h.

Synthesis procedure of this molecule can be found in Chapter 3, Section 3.2.3.

4.2.5 Synthesis of HA@aCe6@GOD-TPZ

HA-Me-CD (1 mg/ mL, 5 mL) in PBS was gently ultrasonicated for ten min under an ice bath by an ultrasonicator then filtered through a 0.22 µm microfilter and store at 4°C until further use. In order to fabricate HA@aCe6@GOD-TPZ, HA-Me-CD (800 µL) dissolved in PBS was added to GOD (10 U/ mL in DI water, 100 µL) and TPZ (1 mg/mL in DMSO, 32 µL) and then stirred in ice bath for 1 h. After which, aCe6 (1000 µM, 16 µL) was added and stirred for 1 h in the ice bath. Then 10% APS and TEMED (10 µL and 1 µL) was added to react for another 1 h at r.t.p.. After which, mixture was dialyzed against PBS for 4 h to give HA@aCe6@GOD-TPZ.

The loading efficiency of HA@aCe6@GOD-TPZ can be calculated using the following equation:

$$\text{Loading efficiency of TPZ (\%)} = \frac{\text{TPZ loaded}}{\text{TPZ added}} \times 100$$

The amount of TPZ was calculated by taking the absorbance at 460 nm and substituting it into the linear regression line from the standard calibration curve of TPZ.

The loading capacity of TPZ was calculated using the following equation:

$$\text{Loading capacity (\%)} = \frac{\text{Mass of TPZ}}{\text{Mass of Nanoparticles}} \times 100$$

HA@aCe6@GOD was synthesized using same procedure as HA@aCe6@GOD-TPZ but DMSO (32 µL) was added in replace of TPZ.

4.2.6 Evaluation of GOD activity in NPs

The change in concentration of oxygen was measured by incubating different sample GOD, HA@aCe6@GOD and HA@aCe6@GOD-TPZ where ([GOD]=100 mU/mL) with glucose (4 mg/mL) and the oxygen was recorded every thirty seconds.

The change in H_2O_2 was measured using hydrogen peroxide kit. Fix concentration of GOD was incubated with different concentrations of glucose. The H_2O_2 was measured 5 minutes after incubation.

4.2.7 Cellular experiments

Breast cancer MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC) and cultured in DMEM High glucose containing 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin at 37 °C under 5% CO_2 . To study the cytotoxicity of cells incubated with cell killing efficacy of those different concentrations HA@aCe6@GOD-TPZ, HA@aCe6@GOD NPs and free TPZ at both normoxic (74% N_2 , 5% CO_2 , 21% O_2) and hypoxic (94% N_2 , 5% CO_2 , 1% O_2) conditions, MDA-MB-231 cells were seeded into 96-well cell culture plates at cell density of 10000/ well until adherent and incubated with the respective normoxic and hypoxic conditions for 24 h. The cells were then incubated with various concentrations of HA@Ce6@GOD-TPZ, HA@Ce6@GOD NPs and free TPZ for 24 h before cell viabilities are determined. The standard thiazolyl tetrazolium (MTT, Sigma Aldrich) assay was carried out in both experiments to determine the cell viabilities relative to control untreated cells.

4.3 Results and discussions

4.3.1 Synthesis of HA-Me-CD

The synthesis of HA-Me-CD was successful as characterized by ^1H NMR in Figure 1. The degree of conjugation was calculated by taking integration of the anomeric proton of β -CD ($\delta = \sim 5.10$ ppm [7H, -CH-]) and the ethylene proton of Me ($\delta = \sim 5.77$ ppm [1H, =CH]) with the characteristic peak of the N-acetyl group in HA ($\delta = \sim 2.03$ ppm [3H, -COCH₃-]) to give 12.4% and 46% of conjugation respectively.

Figure 1. ^1H NMR of HA-Me-CD.

4.3.2 Characterisations of HA@aCe6@GOD-TPZ

After HA@aCe6@GOD-TPZ was fabricated, the successful synthesis must be verified. HA@aCe6@GOD was also synthesized as a control. The HA@aCe6@GOD and HA@aCe6@GOD-TPZ NP were shown to have a spherical morphology when visualized under the TEM (Figure 2a, b). The size distribution of the NPs was determined by dynamic light scattering (DLS). Glucose oxidase (GOD) was found to have a size of 8.5 nm while HA@aCe6@GOD and HA@aCe6@GOD-TPZ NPs were found to have a hydrodynamic diameter of 155.9 nm and 171.8 nm respectively (Figure 2c). Since the NPs are between the size of 1-200 nm, they are suitable for drug delivery purposes by EPR effect to accumulate in tumour sites. Absorption spectrum of the NPs were also taken to verify that the drugs TPZ and aCe6 was encapsulated in the NP and standard calibration curve of TPZ was plotted by taking absorbance maxima at 460 nm of varying concentrations. It was found that there are absorbance maxima of TPZ at 460

nm and aCe6 at 400 nm (Figure 2e). This shows that both TPZ and aCe6 were present in the NP. The loading efficiency of TPZ was found to be 63.3% and the loading capacity of TPZ was 2.0%. After the successful fabrication of the NPs were confirmed, the abilities to produce H_2O_2 as the ROS species must be verified. Glucose and oxygen in the cells are catalyzed by GOD to produce ROS. This process thus depletes the amount of O_2 in the cells. Fixed amount of glucose was incubated with the same [GOD] and the amount of dissolved O_2 was measured. It was found that the [O_2] decreases with increasing incubation concentration. After the encapsulation of GOD in HA@aCe6@GOD and HA@aCe6@GOD-TPZ NP, there is no obvious decrease in GOD activity as shown by the similar decreasing O_2 concentration trend. The depletion of O_2 is important as it means that the drug TPZ can be activated in presence of low oxygen level. Similarly, the concentration of H_2O_2 produced was measured using Pierce™ Quantitative Peroxide Assay Kit. The free GOD, HA@aCe6@GOD and HA@aCe6@GOD-TPZ was incubated with glucose at varying concentration for five minutes. The amount of H_2O_2 were calculated from the standard calibration curve obtained from known concentration of H_2O_2 (Figure 2g). It was found that there was an increasing trend in the H_2O_2 produced with no significant difference in the two different samples (Figure 2h). This shows that with glucose and oxygen, depletion of glucose and oxygen by GOD does produce H_2O_2 and that it is dependent on glucose concentrations. Encapsulation of GOD in NP does not affect the capability of GOD for catalysis.

Figure 2. Characterisations of HA@aCe6@GOD and HA@aCe6@GOD-TPZ. TEM images of (a) HA@aCe6@GOD and (b) HA@aCe6@GOD-TPZ. (c) Dynamic Light Scattering (DLS) of GOD and NPs. (d) Standard calibration curve of TPZ (e) Absorbance curves of TPZ and the NPs. (f) The change in oxygen when glucose (4 mg/mL) is incubated with free GOD and the NPs over 5 minutes. (g) Standard calibration curve of H₂O₂. (h) The change in H₂O₂ concentrations with GOD and the NPs are incubated with increasing glucose concentration (0, 0.156, 0.0313, 0.0625, 0.125, 0.25 mg/mL).

4.3.3 Cellular uptake and targeting effect on CD44 receptors

The HA@aCe6@GOD-TPZ NPs were then incubated with MDA-MB-231 cells under both normoxic as well as hypoxic conditions for different time intervals and visualized using the confocal microscope at the excitation wavelength of aCe6. It was

found to have a time dependent cellular uptake (Figure 3). Comparing the cells at 2 h and 4 h interval, the fluorescence intensities of the cells at 4 h incubation is greater than that of 2 h. HA competitive assay was also used to verify the targeting effect of HA@aCe6@GOD-TPZ in CD44⁺ MDA-MB-231 cells. Free HA (10 mg/mL) was first incubated for 2 h and then the NP was added. The fluorescence of cells preincubated with HA was much less than as compared to cells incubated with NP for the same incubation time. This shows that CD44 mediated endocytosis has increased the cellular uptake of NPs.

Figure 3. Fluorescence images of cellular uptake of HA@aCe6@GOD-TPZ at different time interval of 2 h and 4 h. A separate set of cells were pre incubated with free HA and then incubated with NP for 2 h for targeting effect. Scale bar: 100 μ m.

4.3.4 ROS detection and *In vitro* cytotoxicity

When incubating the cells with HA@aCe6@GOD and HA@aCe6@GOD-TPZ, they should be able to produce H₂O₂ which is a type of ROS. ROS can be detected by general ROS indicator carboxy-H₂DCFDA, where the nonfluorescent molecule is converted to a green-fluorescent form in presence of ROS. The results showed that ROS was produced when the cells were incubated with HA@aCe6@GOD and HA@aCe6@GOD-TPZ at both normoxic and hypoxic conditions (Figure 4a). Live/dead assay was also carried out. Live cells were stained using calcein-AM to give

green fluorescence while dead cells were stained using propidium iodide to give red fluorescence. The results indicated that the greatest cellular apoptosis was detected when incubated with the NPs at normoxic conditions (Figure 4b). When the cells were in hypoxic condition, HA@aCe6@GOD-TPZ NPs possessed more cytotoxicity as compared to HA@aCe6@GOD as shown in Figure 4c. This is because the TPZ is also able to convert into radical under hypoxic condition. This causes the amount of ROS in the cells to exceed the threshold in which the cells can take and thus leading to apoptosis.

Figure 4. (a) Detection of ROS using carboxy-DCFDA (green). Scale bar: 100 μm. (b) Live/dead assay of MDA-MB-231 cells with different treatments (i.e. PBS, HA@aCe6@GOD and HA@aCe6@GOD-TPZ). Scale bar: 200 μm.

The NPs were then incubated with MDA-MB-231 cells to test its cytotoxicity as shown in Figure 5. In general, TPZ gave low cytotoxicity even at high concentration of 9 μM under normoxic conditions. This is because the reduction of the drug can reverse to its non-toxic form when there is O₂ in the cells and thus TPZ is unable to convert into its toxic radical form to induce much apoptosis.

Comparing both HA@aCe6@GOD and HA@aCe6@GOD-TPZ NPs under normoxic condition (Figure 5a), low concentration of GOD (6.25 mU/mL) was needed to induce high cytotoxicity, giving low cell viability of less than 20%. This is because both glucose and oxygen are in excess amount, which drives the catalytic reaction

between glucose and oxygen in the medium as well as the GOD in these NPs to produce sufficient H_2O_2 , which killed the MDA-MB-231 cells. In contrast, under hypoxic condition, even high concentration of GOD (25 mU/mL) were not enough to induce high cytotoxicity (Figure 5b). Cell viability remained high above 80%. When the system is coupled with TPZ (2.3 μM), the cytotoxicity was increased as evident by the drastic decrease in cell viability to be less than 20%. Free TPZ at this concentration gave a high cell viability of more than 80%. This is because even though the cells are hypoxic (1% O_2), it is not low enough to induce toxicity in the cells with solely TPZ. This shows that GOD is needed to deplete the amount of oxygen in cells in order for TPZ to be reduced to its toxic radical, killing the cancer cells.

Figure 5. Cell viabilities of MDA-MB-231 when treated with different NPs at various concentrations for 24 h under (a) normoxic and (b) hypoxic conditions.

Apoptotic studies done using PI and Annexin IV FITC for labelling of cells also showed similar results as shown in Figure 6 below. When cells were incubated with HA@aCe6@GOD-TPZ NPs, the apoptotic cells detected were the highest for both normoxic and hypoxic conditions. At normoxic condition, HA@aCe6@GOD and HA@aCe6@GOD-TPZ NPs were able to cause similar amount of apoptotic cells. At hypoxic condition, the HA@aCe6@GOD-TPZ performed better than HA@aCe6@GOD which shows that TPZ was effective in causing apoptosis in cells.

Figure 6. Apoptosis assay by flow cytometry under normoxic (first row) and hypoxic (second row) conditions. [GOD] = 6.25 mU/mL.

4.4 Conclusion

In conclusion, HA@aCe6@GOD-TPZ NPs was fabricated successfully and shown to produce H_2O_2 from glucose and oxygen. This depleted the oxygen within the cells. The nanocarrier is also able to encapsulate hydrophilic TPZ for hypoxia activated treatment. Activation of TPZ drug to give TPZ radical was toxic to the cells and selectively when O_2 was depleted in the cells by GOD. This resulted in high cytotoxicity in MDA-MB-231 cells.

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Chapter 5 Conclusion

5.1 Conclusion

This dissertation describes the progresses and results of the work that were done on usage of HA for various modality of cancer treatment which included the chemotherapy and photodynamic therapy. In all three works, the usage of passive targeting was harnessed by fabrication of polymeric NPs of suitable sizes for accumulation at tumour site by enhanced permeation and retention effect. Furthermore, active targeting of CD44 receptors was done by the utilization of HA. These systems rendered efficient accumulation of NPs in MDA-MB-231 cells and tumour model in BALB/c nude mice. Furthermore, in all three chapters, reactive oxygen species (ROS) was harnessed. In the first work, HA-aPS-aCPT was fabricated for dual therapy in tumours. HA was proven to be CD44 targeting. The singlet oxygen produced from the adamantane modified porphyrin was used for both photodynamic therapy and the cascaded release of chemotherapy drugs from the prodrug synthesized from camptothecin. This system enabled multimodal therapy which led to ablation of tumour. Subsequently, in the second work, HA-CAT@Ce6 NP was harnessed for overcoming hypoxia in solid tumours. With the help of catalase for generation of in situ oxygen for photodynamic therapy, hypoxia in tumours was alleviated which led to better therapeutic efficacy. In the third project, HA@aCe6@GOD-TPZ NP was synthesized. It was able to also target MDA-MB-231 cells. It was responsive to endogenous glucose to cause hypoxia in the cells. When there was excessive glucose in the cells, H_2O_2 was produced to cause an elevated amount of ROS. In the process, O_2 was consumed leading to hypoxia in the tumour. This activated the TPZ drug which was hypoxia responsive.

Production of TPZ toxic radical further increased the cytotoxicity of the NP. This led to dual modal killing of the cells.

5.2 Future Outlook

Despite much work being done for investigation of HA as a polymer in oncology, it is still far from clinical application. The biosafety and biodistribution of NPs still need to be evaluated in clinical settings. Furthermore, the long-term side effects of such system must be evaluated. The effect of HA should be further evaluated. Studies on HA found that with HA polymers of a size of higher molecular weight (e.g. 107 kDa) generally have anti-angiogenic and immunosuppressive properties while the lower molecular weight (e.g. 20 kDa) counterparts are angiogenic, immune-stimulatory and inflammatory material.¹ Since different molecular weights of HA polymers have different effects on its bioactivity *in vivo*, we suspect that other factors such as shape and size should also affect their bioactivity and safety.

In all study, HA nanocarriers were able to selectively accumulate at the tumour site due to the overly expressed CD44 receptors on tumour. The accumulation of HA thus may be a double edge sword. While the utilisation of HA allowed therapeutic agents to be successfully delivered into the tumour site, it may induce other unknown or unexplored immune response. Thus, the effect of HA-aPS-aCPT NPs, HA-CAT@aCe6 NPs and HA@aCe6@GOD-TPZ NPs on metastasis as well as its effect on angiogenesis and immune response of the biological system must be done systematically. This should be an area for future exploration in development of HA-based NPs.

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