Reactive oxygen species-mediated cancer therapy with silica-based nanoparticles

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2019

Ong, W. K. (2019). Reactive oxygen species-mediated cancer therapy with silica-based nanoparticles. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/137584>

<https://doi.org/10.32657/10356/137584>

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**Reactive Oxygen Species-Mediated Cancer Therapy with Silica-
Based Nanoparticles**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2019

Reactive Oxygen Species-Mediated Cancer Therapy with Silica-Based Nanoparticles

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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

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Abstract

Reactive oxygen species (ROS) is important for the well-being of cells since they served as messengers. However, too much ROS will kill them because of their high reactivity with DNA and surrounding organelles.

Nanoparticles made from silica and ROS-producing agents have been successfully synthesized. They can manipulate the ROS within cancer cells by raising it to apoptotic or necrosis levels.

The first project talks about the use of a nanoreactor that produces $\cdot\text{OH}$ radicals independently. A photosensitizer was loaded into it as well to generate even more radicals for enhanced therapy. This was subsequently subjected to both *in-vitro* and *in vivo* testing.

The second project explores the use of a new ligand to fabricate an organically modified silica nanocomposite capable of not only generating the ROS H_2O_2 , but reducing the antioxidant capabilities of cancer cells to enhance the effectiveness of the produced ROS.

The third project combined the use of the nanoreactor in the first project with a silane conjugated glucose oxidase for increased $\cdot\text{OH}$ radicals production.

In summary, this thesis showed that ROS can be effectively exploited to treat cancer.

Useful Abbreviations

MOF – Metal-organic frameworks

FDA – Food and Drug Administration

EPR – Enhanced permeability and retention

GSH – Glutathione

NIR – Near-Infrared

ISC – Intersystem crossing

ROS – Reactive oxygen species

$^1\text{O}_2$ – Singlet oxygen

$\cdot\text{OH}$ – Hydroxyl radical

H_2O_2 – Hydrogen peroxide

O_2^- – Superoxide anion

HOCl – Hypochlorous acid

ETC – Electron transport chain

MPTP – Mitochondrial permeability transition pore

XOD – Xanthine oxidase

EGF – Epidermal growth factor

$\text{TNF}\alpha$ – Tumor necrosis factor α

PDGF – Platelet derived growth factor

SODs – Superoxide dismutases

TRX – Thioredoxin

ASK1 – Kinase-1

DNA – Deoxyribonucleic acid

TEOS – Tetraethyl orthosilicate

PEG – Polyethylene glycol

CTAB – Cetyl trimethyl ammonium bromide

ORMOSIL – Organically Modified Silica

YSN – Yolk-shaped nanoreactor

HA – Hyaluronic acid

TEM – Transmission electron microscopy

XPS – X-ray photoelectric spectroscopy

BET – Brunauer–Emmett– Teller

BJH – Barret–Joyner–Halenda

PXRD – Powder X-ray diffraction

CLSM – Confocal laser microscopy

H&E – Haematoxylin and Eosin

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling

SEM – Scanning electron microscope

ADMA – 9,10-anthracenediyl-bis-(methylene)dimalonic acid

ZnPc – Zinc phthalocyanine

GRAS – “Generally Recognized As Safe”

QM – Quinone methide

GOx – Glucose oxidase

FITC – Fluorescein isothiocyanate

K_m – Michael-Menten constant

$V_{\max}$ – Maximum velocities

APTES-GOx – (3-Aminopropyl)triethoxysilane-conjugated glucose oxidase

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Professor Zhao Yanli for his support, guidance and suggestions. He trusted me to do good research even when I am struggling hard in my first three years to get results. Without him, I wouldn't have the drive to complete my manuscripts for submission together with this thesis.

I would like to express gratitude to my collaborators, Dr. Zhong Luo, Dr. Li Meng Huan and Xuemei Tao from Chongqing University for their valuable advices and *in-vivo* assistances.

I would also like to thank my other collaborators Dr Peizhou Li, Prof. Yang Chaolong and Dr. Liu Guofeng for their project partnership.

I would like to thank my friends and colleagues Eddy Lin, Fiona Phua, Deblin Jana, Anivind Kaur, Lim Wei Qi, Leong Wei Liang, Phoebe Lam and the many who I have meet for their support, energy and help.

I would also like to thank my family for their support in doing my PhD and long absence.

Lastly, I would also like to thank Nanyang Technological University for providing me the opportunity and funding through Nanyang President's Graduate Scholarship (NPGS) to fulfill my childhood dream to become a scientist.

Table of Contents

Abstract	1
Useful Abbreviation	2
Acknowledgements	5
Table of Contents	6
Chapter 1 Introduction	9
1.1 Nanoparticle in cancer therapy	9
1.1.1 Challenges and opportunity in cancer therapy	11
1.1.2 Passive accumulation of nanoparticles in cancer	12
1.1.3 Active accumulation of nanoparticles in cancer	13
1.1.4 Avoiding one of cancer's natural defenses	14
1.1.5 Exploiting high acidity in cancer	15
1.1.6 Exploiting glutathione (GSH) in cancer	16
1.1.7 Exploiting heat sensitivity in cancer	16
1.1.8 Exploiting singlet oxygen in cancer	17
1.1.9 Exploiting hypoxia in cancer	17
1.1.10 Exploiting high metabolism in cancer	18
1.2 ROS and its newfound role in cancer therapy	19
1.3 Properties of silica in ROS cancer therapy	25
1.4 Creating nanomaterials from silica for ROS cancer therapy	30
1.5 Research objective	32
References	33

Chapter 2 Investigation of Fe₃O₄ nanoparticle as a hyaluronic yolk-shaped nanoreactor for targeted ROS-mediated therapy *in vivo*

.....	40
2.1 Introduction	40
2.2 Experimental	44
2.3 Results & Discussion	52
2.4 Conclusion	82
References.....	83

Chapter 3 An anticancer glucose-depleting ORMOSIL nanocomposite for cascade reactive oxygen species therapy and glutathione scavenging

.....	87
2.1 Introduction	87
2.2 Experimental	91
2.3 Results & Discussion	97
2.4 Conclusion	116
References.....	117

Chapter 4 Yolk-shaped nanocomposite for simultaneous glucose starvation and enhanced ·OH production

.....	119
2.1 Introduction	119
2.2 Experimental	123
2.3 Results & Discussion	127
2.4 Conclusion	140
References.....	141

Conclusion and Outlook	144
-------------------------------------	------------

List of Publications	146
Manuscript In Preparation	146
Appendix.....	147

Chapter 1

Introduction

1.1 Nanoparticles in cancer therapy

Cancer is the second largest cause of death worldwide behind heart disease.¹ Unlike conventional diseases which have fixed characteristics, cancer manifests itself uniquely in each person.² This makes detection and curing cancer highly difficult. Cancer however does take time to become lethal and can be classified into stages depending on when it is discovered in the patient.³ Early stages are more localized and seen as more treatable while late stages mean that the cancer has spread. This late stage is typically the cause of death in diagnosed patients. Nevertheless, there are a variety of treatments available at each stage. Some common methods include surgery, radiotherapy and chemotherapy.⁴⁻⁷ While considered to be effective, these traditional methods suffered from drawbacks like being highly invasive and equally destructive to normal cells.^{8,9} Moreover, the low survival rate of patients and their high recurrence meant that more and better cure must be found.^{10,11} As such, it would be ideal to develop another way to kill cancer without any drawbacks and with all of the advantages. While it may seem like a tall order, the rise of nanomedicine has brought forth its realization closer than even before.¹²⁻¹⁴

The definition of nanomedicine varies across experts. However, according to the European Commission, nanomedicine refers to “a natural, incidental, or manufactured material comprising particles, either in an unbound state or as an aggregate wherein one or more external dimensions is in the size range of 1–100 nm for $\geq 50\%$ of the particles, according to the number size distribution”.¹⁵ The materials can be made using anything from organic polymers, small molecules, metal oxides, natural products, bacteria, viruses, cancer cell membranes to the recently popularized metal-organic frameworks (MOF).¹⁶ The one thing

that remains consistent regardless of the material used is that, they are usually nano-sized.

The question then would be why nano-sized? In order to answer this question, it is important to first delve into the characteristics of cancer and then learning how nano-sized particles can exploit these characteristics.

Figure 1. Different types of nanomaterials for cancer treatment (Reproduced with permission from ref 73. Copyright 2018 Springer Nature.)

1.1.1 Challenges and opportunity in cancer therapy

Most people will see cancer as a lump of tumor that has spiraled out of control. This is very well shown in popularized media and is very real indeed. Typically, surgeons would cut open the patient carefully to reveal a large tumor. The tumor would eventually be extracted and the site washed thoroughly before it is sewed up. However, cancer is much more complicated than depicted because it is a form of mutation. While the naked eye can help remove solid tumors, the real extent of cancer within the body is normally much larger and more difficult to detect. Not to mention that in later stages of cancer, the growth of tumor may be so extensive that surgery may not even be viable.¹⁷ Hence, an idea that the drug can be automatically delivered to tumor or activated upon tumor contact is highly attractive. In fact, a good number of nanoparticles are currently used to treat cancer patients. Those nanoparticles include Abraxane, Doxil, DaunoXome, Marqibo, Onivyde, Magnablate, Cornell Dots and AuroLase.¹⁸ Having said that, these nanoparticles are not complete cure all in cancer patients and regarded as having potential unknown side effects. Hence, they are subjected to constant monitoring by the Food and Drug Administration (FDA), even if they are approved for use in clinics and hospitals. Still, nanoparticles possess unique properties different from its macro-sized counterparts that have proven to be effective in killing cancer selectively.

For instance, nanoparticles can mainly (i) reduce the dosage required for therapeutic efficiency, thereby reducing the toxicity and unwanted accumulation in the body, (ii) provide specific targeting by exploiting a ligand to target overexpressed receptors on cancer cells, (iii) bypass biological barrier and accumulate selectively at tumor regions, and (iv) allow for multi-functionality on the same material.¹⁹

Presently, to successfully transition into clinical trial, nanoparticles still face a number of challenges. These challenges include (i) the difficulty in upscaling and reproducibility, (ii) unknown toxicological profile, (iii) overall cost of production, and (iv) hard-to-control changes to the physical and chemical properties from *in-vitro* to *in-vivo*.

In the following text, we will continue to discuss more on the unique advantages of nanoparticles, and on how we can easily solve some of these challenges by combining different materials.

1.1.2 Passive accumulation of nanoparticles in cancer

A well-known feature of tumor is that it grows rapidly. In order to achieve that, it requires a larger amount of nutrient and oxygen to be channeled its way.²⁰ The tumor achieves that by re-channeling the existing vessels to the tumor. This is a process known as neovascularization. While the process is able to supply more nutrients, it forms a network of disorganized vessels with leaky vasculature. This meant that nano-sized particles can be directed to tumor sites at greater volumes and flow through the leaks into the tumor. The lymphatic drainage system is also damaged, meaning that it is hard for anything to flow out of the system.²¹ This dynamic of easy accumulation but hard displacement meant that nanoparticles can preferentially build up in tumor sites over normal cells. In fact, one can be sure that whatever drugs or toxins that nanoparticles carry will result in a greater cancer cell death relative to normal cells. The whole phenomenon is termed Enhanced permeability and retention (EPR) effect and this is an explanation for most researchers when it comes to cellular uptake of nanoparticles.²² As a matter of fact, most commercially available nanoparticles made use of this phenomenon to accumulate at cancer sites for treatment.²³ This can be popularly termed as passive targeting

of cancer because no tools have been purposefully exploited to gravitate the nanoparticles to the cancer site except for its size.

1.1.3 Active accumulation of nanoparticles in cancer

With the previous concept in mind, active targeting will be discussed. Active targeting requires a specific understanding of the receptors that are present on the surface of cancer cells. These receptors should only be overexpressed in cancer cells over normal cells. In this way, the most suitable targeting ligand can be used. It is also important to know that each type of cancer cells have its own unique set of receptors. Hence, it must not be assumed that if HeLa cells have CD44 receptors, 4T1 cancer cells would also have them.²⁴ For instance, the folate receptors in most ovarian cancer cells are overexpressed.²⁵ To ensure that nanoparticles target these cancer cells, one can couple folic acid or antibodies to them since they have high affinity for folate receptors. However, if other ligands like a non-specific aptamer are used, there will be no enhancement of uptake in cancer. It is also noteworthy that if free drugs were directly conjugated to the targeting ligand, the uptake may not change and instead, the therapeutic properties of the drug may be compromised.²⁶ Hence, the use of nanoparticle as a separate vehicle for active targeting can help retain the killing efficiency of the drug while delivering more of them to tumor sites.²⁷

Figure 2. Illustration highlighting the movement in passive targeting and active targeting. (Reproduced with permission from ref 74. Copyright 2014 American Chemical Society.)

1.1.4 Avoiding one of cancer's natural defences

Cancer cells can adapt overtime to therapeutic drugs overtime and develop drug resistance.

While there are many ways to developing it, P-glycoprotein efflux pump is by far the most well investigated mechanism.²⁸ The transmembrane P-glycoprotein efflux pumps can actively remove any internalized free drugs out into the extracellular matrix before it can take effect.²⁹

This is a huge problem since free drugs can only be internalized via passive diffusion or membrane translocator. As a result, cancer cells cannot be properly treated. However, nanoparticles can avoid the pumps via endocytosis. The process begins when the nanoparticle is engulfed by the membrane and then contained within endosomes.³⁰ These endosomes transport the nanoparticles that are taken in away from the pumps and deeper into the cells for eventual release of drug cargo. In other words, the effect of the drugs can be prolonged, resulting in more effective cancer therapeutics.

1.1.5 Exploiting high acidity in cancer

Another feature of cancer is the tumor microenvironment that is created over time. Aerobic respiration cannot keep up with the high tumor metabolism. Hence, tumor resorts to anaerobic respiration as well to generate enough energy for proliferation and survival. The process is known as glycolysis where glucose is broken down by biological catalyst in the absence of oxygen to form pyruvic acid as the end-product.³¹ Now, it is also mentioned that things within tumor will find it hard to escape when it comes to the circulation at tumor sites. With this in mind, pyruvic acid or pyruvate will naturally accumulate increasing amounts at the site. This built-up of acid causes the pH to become significantly low relative to normal tissues.³² Hence, the design of the nanoparticle can also be tailored in a way that only trigger under acidic conditions. This would ensure that normal cells are not harmed during the treatment.

Figure 3. Illustration showing the movement of particles through the different mechanisms. (Reproduced with permission from ref 75. Copyright 2018 American Chemical Society.)

1.1.6 Exploiting glutathione (GSH) in cancer

It is discovered that cancer cells have a much greater amount of GSH compared to normal cells.³³ This is one form of adaptation that cancer cells take as an antioxidant system to balance the high amount of radicals generated within. This is a problem because some anticancer drugs destroy cancer cells by increasing the amount of radicals within the system, causing irreversible damage to DNA. The elevated GSH levels mean that the antioxidant will prevent the drug from working and confer multidrug resistance.³⁴ Hence, nanoparticles that can reduce the amount of intracellular GSH are potentially useful in improving drug treatment. Also, the design of the nanoparticle can be tuned in a way that releases drugs only under elevated GSH level. This would promote a much greater amount of drug selectivity in contrast to free drugs. In fact, a great number of polymers and hybrids have used GSH-cleavable linkages like –SS– thiol bonds to fabricate stimuli-responsive nanoparticles.³⁵

1.1.7 Exploiting heat sensitivity in cancer

Tumor seemed to undergo cell death when exposed to excess amount of heat. However, it is important to know that the temperature at the tumor site determines the type of change that occurs. Typically, temperature around 42 °C to 45 °C would only damage the cancer cells to an extent that desensitize them to further drug treatment.³⁶ However, much higher temperatures will result in apoptosis and burns. Some reports even showed that heating actually triggered an immune response in a separate tumor located on the same mice.³⁷ This is potentially groundbreaking in seeking a reliable method to treat metastatic cancer. In order to achieve these high temperatures, stable metallic oxides and even pure metals nanoparticles are typically required within the design.³⁸ It is also possible to turn to photothermal dyes like IR825 and IR820.³⁹ However, an external energy source is also needed to trigger heating from these nanoparticles and this commonly comes from a light source. Now, before going

further, it is crucial to know that all light does not simply pass through human although the tumor is located within the body. Only red light and near-infrared light are penetrating and safe enough to be used for triggering heat production.⁴⁰ In fact, a literature review by Nature Reviews indicated that “NIR light (700–900 nm) can travel millimetres—and up to centimetres—through blood and tissue”. Since light can be controlled to target a specific part of the body, it means that only the tumor region may be destroyed by the heat.

1.1.8 Exploiting singlet oxygen in cancer

It was also found that cancer cells were susceptible to attacks from singlet oxygen. Singlet oxygen generation can be briefly defined as $^3\text{O}_2$ (ground state) $\rightarrow$ $^1\text{O}_2$ (singlet oxygen).⁴¹ In the field of nanomedicine, this transition happens when a photosensitizer or a plasmonic nanoparticle absorbs photon energy. The photon energy then undergoes a process of intersystem crossing (ISC), causing $^3\text{O}_2$ to be excited to the highly reactive $^1\text{O}_2$ state. Some examples of photosensitizer include Photofrin sodium (Photofrin) or zinc phthalocyanine, which can be irradiated by laser for excitation.⁴² The energy absorbed from the laser can then be eventually transferred to excite the ground state electrons to the excited $^1\text{O}_2$ state. This species can interact with the surrounding organelles for cellular damage.

1.1.9 Exploiting hypoxia in cancer

Tumors grow rapidly to a point where there are much cells and activities that the initial oxygen supply can no longer match the demand for it.⁴³ This produces regions within tumors where the tumor tissues far from the vasculature lack oxygen. It is also generally accepted that tumor tissues have lower levels of oxygen compared to that of normal cell tissues.⁴⁴ The oxygen level in the deprived regions of tumor is on average between 1 to 2 % O_2 while the

level in normal tissues is between 9.5 % O₂ in the renal cortex to 4.6 % O₂ in the brain.⁴⁵ This means that a nanoparticle that responds well to hypoxia conditions can selectively activate in cancer cells. For instance, nitroimidazole moiety can be incorporated into a polymeric nanoparticle.⁴⁶ But within hypoxic cells, the hydrophobic -NO₂ group may be reduced to the hydrophilic -NH₂ group, causing the entire nanoparticle to destabilize and break apart to release the drug. In this way, selectivity towards cancer cells can again be achieved with a nanoparticle system.

1.1.10 Exploiting high metabolism in cancer

Cancer cells do require a large amount of nutrients for cellular growth and proliferation. As a result, it is logical to say that a disruption in the nutrient supply should inhibit cancer. For instance, if cancer cells are deprived of glucose, it will undergo cell death due to glucose starvation and inadequate nutrient for survival. Perhaps another but less utilized method is to form solid debris within tumor which blocks the flow of nutrient to tumor. This was demonstrated by the use of magnesium silicate nanocomposites in mice.⁴⁷

Figure 4. Illustration showing the different stimuli through which nanoparticles can be activated for cancer therapy. (Reproduced with permission from ref 76. Copyright 2018 Ivyspring International Publisher.)

1.2 ROS and its newfound role in cancer therapy

Reactive oxygen species (ROS) is a term used to classify a group of partially reduced oxygen species including singlet oxygen ($^1\text{O}_2$), hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hypochlorous acid (HOCl). ROS generally serves as messenger within the cell and is responsible for the proper biological functioning.⁴⁸ A well explored fact of cancer is that the cells have a much higher level of ROS compared to normal cells.⁴⁹ This phenomenon can be due to the increased metabolism from rapid growth, UV light exposure, heat and hypoxia which increases the oxidative stress on the cell. ROS can also be typically produced in the mitochondria because of oxidative phosphorylation.⁵⁰ This process occurred through the electron transport chain (ETC) which comprise of the protein complexes I to IV

and the enzyme ATP synthase located on the inner membrane of the mitochondrion. The complexes I and III produce superoxides (O^{2-}). Superoxide is leaked into the cytoplasm through the outer membrane of the mitochondrion via mitochondrial permeability transition pore (MPTP). The O^{2-} can then be dismutated to hydrogen peroxide (H_2O_2) either in the cytosol by Cu/ZnSOD or in the mitochondrial matrix by MnSOD. Beside the mitochondrion, peroxisomes are another main site of ROS production together with xanthine oxidase (XOD) in the peroxisomal matrix. In fact, the peroxisomal membrane itself is a producer of O^{2-} and H_2O_2 .⁵¹ Moreover, factors like epidermal growth factor (EGF), tumor necrosis factor α (TNF α), lysophosphatidic acid, platelet derived growth factor (PDGF) and angiotensin all stimulate the production of O^{2-} .⁵² The produced H_2O_2 is an important non-radical ROS because it can not only pass freely through cell membrane but can be further processed to create other kinds of ROS.⁵³ In this following chapters, we will discuss how we exploit ROS for cancer treatment. But before we move on, we like to address the conflicting reports on whether ROS can be used to treat cancer.⁵⁴ This is because while there are literature proving the effectiveness of ROS therapy, there's an equal number of them reporting on the cell proliferation nature of ROS.

Figure 5. Illustration showing the different ways ROS may be produced in cells and in nanoparticles for cancer therapy. (Reproduced with permission from ref 77. Copyright 2018 *IJMS*.)

In general, literature that investigated the side effects of ROS associated it with the initiation, progression and metastasis process within cancer.⁵⁵ When cells are under mild ROS stress, they can undergo adaptation to counteract the change. The adaptation involves the use of antioxidant enzymes and redox buffering systems.⁵⁶ The main antioxidant system would be the glutathione system together with the thioredoxin system for removal of ROS. Other antioxidant include catalase to convert H_2O_2 to the

harmless H_2O and O_2 as well as superoxide dismutases (SODs) to convert the destructive O_2^- to molecular oxygen and less harmful H_2O_2 .⁵⁷ Hence, cancer cells still survive at high level of ROS since this can be balanced with an equally high level of antioxidant. There are several implications since ROS serves as messengers in the signaling pathways. An increase in H_2O_2 can promote tumor progression and stimulate cell proliferation. For example, high level of ROS can oxidize thioredoxin (TRX), causing it to break away from the apoptosis signaling kinase-1 (ASK1), which is responsible for the metabolism of cancer cells and cell proliferation.⁵⁸ Another example would be the oxidation of p53 protein located in the cysteine residue. As a result, p53 protein is unable to bind properly to p53 promoter element in DNA and deregulate the expression of downstream genes.⁵⁹ Moreover, cancer cells may start to develop multi-drug resistance and become metastatic overtime.

However, there are also reports of ROS contributing to cancer cell death. ROS commonly cause DNA lesion and can induce gene mutation due to their high chemical reactivity.⁶⁰ Some consequences include DNA strand breakage, DNA-protein crosslinking, unintended oxidized bases and DNA intra-strand adduct. The O_2^- that is generated can be converted to H_2O_2 and pass through physical barriers into the nucleus compartment. The very same ROS that is generated at the mitochondrion is also capable of interacting with mitochondrial DNA. This ROS would disrupt the normal functioning of the mitochondrion, resulting in a cycle where ROS production would go out of control. Existing metal like iron can further react with H_2O_2 via fenton reaction to produce the even more highly toxic $\cdot\text{OH}$ radicals.⁶¹ Other parts of the cells like protein and lipid membranes can also be destroyed or altered in excess ROS. Oxidation of the amino acid side chain and peptide cleavages modified the protein and

render enzymes like cytochrome *c*, cytochrome *c* oxidase and catalases denatured.⁶² Since some of these enzymes are responsible for ROS removal and conversion, a positive feedback can happen where the ROS production increases uncontrollably. Again, the mitochondrion can be damaged at the membrane, which trigger the release of cytochrome *c* and activate cell apoptosis. As a matter of fact, necrosis may occur instead of apoptosis, highlighting the high reactivity of ROS and its potential in killing cancer.⁶³

While it seemed ROS is a dangerous double-edge sword, it was later discovered that it is possible to kill cancer with it under a certain condition. This discovery has reignited and spurred research in the proper use of ROS in treating cancer. Generally, at low level of ROS, cells are indeed in a state of homeostasis and at higher levels, cancer cells starts to become more aggressive and hard to treat overtime. However, it was later found that no matter how much cancer cells can counteract the high level of ROS, there is a threshold level. Once this threshold is exceeded, cancer cells will start to undergo apoptosis. In other words, it is possible to overload cancer cells with ROS so that they will die instead of proliferating or mutating. More importantly, ROS-mediated treatment is a relatively new form of therapy and is expected to improve or replace the current paradigm of using light, heat or synthetic drugs which may not be viable in deeper tissues or metastatic tumor and are costly to produce.

Figure 6. ROS increase helps in cancer survival until a threshold is reached where cell death starts to occur. (Reproduced with permission from ref 56. Copyright 2018 Springer Nature.)

1.3 Properties of silica in ROS cancer therapy

Since it is known that ROS can be used to kill cancer when overloaded, agents that can generate ROS become attractive candidates for exploration in nanomedicine. However, many popular anthracycline drugs like Doxorubicin, Epirubicin and Daunorubicin are already known to produce ROS to kill cancer.⁶⁴ Platinum-containing drug complexes, topoisomerase inhibitors, camptothecins, arsenic drugs and alkylating agents are also found earlier to do the same.⁶⁵

Figure 7. Illustration presenting the different advantages of silica-based nanomaterials for cancer therapy. (Reproduced with permission from ref 78. Copyright 2013 The Royal Society of Chemistry.)

The ROS generation during drug treatment is attributed to a couple of reasons. The two reasons are the elevated production of ROS in the mitochondrion and the damage sustained by the antioxidant system in cancer cells. For example, arsenic trioxide causes the loss of mitochondrial membrane potential and inhibition of the protein complexes I and II.⁶⁶ This process disrupted the ETC and triggers the increased production of ROS. The antioxidant system contributed to the increase in ROS because it is responsible for suppressing ROS. A failure in the system such as the scavenging of GSH or ascorbic acid prevents them from depleting the intracellular ROS. Some examples include the use of Imexon to bind thiols like GSH in cancer patients and Mangafodipir to inhibit the SOD enzymes for rising of H₂O₂ level.⁶⁷

Silica-based nanoparticle can help to deliver these agents to the right target safely. Silica nanoparticles are well known for their biocompatibility in cells.⁶⁸ Silica is also considered safe by FDA and hence holds a great potential as a reliable platform for fabrication as a delivery vehicle.⁶⁹ Free drugs do not have any differentiating ability towards cancer cells or healthy cells. This ambiguity meant that healthy cells will be subjected to an equal amount of cellular damage because free drugs will simply interact with anything they come in contact with.⁷⁰ Silica can come into the picture as a safe delivery vehicle because it can shield the healthy cells from the reactive drugs. This vehicle can be created via physical loading into the mesopores of silica or chemical conjugation with the silanol groups on the silica surface. When the silica nanoparticles reach the cancer cells, the drug cargo can then be released there, thereby avoiding unnecessary damage to healthy cells.⁷¹ As a result, patients would have less chance in contracting secondary diseases or experience any physical side effects from a failing immune system. However, it is important to know that depending on the

silica formation process, the availability and size of mesopores vary. For typical mesoporous silica nanoparticles, the pore size is generally around 2nm and allows for drugs to be loaded into the pores via diffusion. The drugs that are loaded can then be released via the same method. For non-porous silica nanoparticles, it is possible to covalently conjugate drugs via a stimuli-responsive linkage.⁷²

The sizes of silica nanoparticles are also tunable according to the reaction time, amount of tetraethyl orthosilicate (TEOS) added and any post-modifications that may be made to the surface.⁷³ The formation of silica nanoparticles can be done in air too. This air-insensitivity would not only mean less stringent conditions for fabrication, but allow for easy purification in air. Since the protocols for the size of silica nanoparticle are established, the correct size for the nanoparticles can be chosen so that the EPR effect can take place. Chemotherapeutic agents do not have targeting abilities to cancer cells and having the EPR effect can promote drug-containing silica nanoparticles to accumulate passively within them.⁷⁴ These would ensure that ROS therapy can take place in cancer cells selectively.

In addition, the surface properties of silica can be adjusted according to the user's desire. Silica nanoparticles have reactive silanol groups on the surface for any post-modifications to be done.⁷⁵ In many cases, immune proteins in the bloodstream tend to adsorb onto nanoparticles for subsequent clearance. However, if a layer of polyethylene glycol (PEG) can be coated onto the surface via reaction with the silica surface, it would not only allow for prolonged circulation time, but improved biocompatibility. It is also possible to coat the surface with active targeting ligands or organic layers like folic acid, aptamers, antibodies or hyaluronic acid for enhanced

targeting.⁷⁶ ROS therapy can then take place more selectively in tumor. A simple process of stirring them in water with the as-synthesized pure silica nanoparticles would suffice, making the use of silica nanoparticles for ROS therapy highly attractive.⁷⁷

Silica can be used to further fabricate multifunctional nanoparticles of unique architecture.⁷⁸ These nanoparticles usually take the form of core-shell, yolk-shaped or hollow spherical nanoparticles. This is because silica not only encapsulates smaller nanoparticles, but can also form empty usable pockets or cavities for containment of drugs. Fabrication can be done by using oleic acid stabilized nanoparticles or related hydrophobic long chain in the nanoparticles to be coated. Cetyl trimethyl ammonium bromide (CTAB) is then used to form micelle with them for stabilization in aqueous solution before the silica precursor is added. Typically, this is a process used to form simple core-shell nanoparticles. An additional shell can be chemically fabricated before etching using a base like ammonium hydroxide, or strong acid like hydrofluoric acid to remove the silica template, and reveal the yolk-shaped or hollow nanoparticle. This technique can create unique structures that can generate ROS while providing MRI, fluorescence and heating capabilities.

Silica nanoparticles can be synthesized in a large batch up to gram scale. This ability to up-scale gives a huge advantage over many existing nano-platforms that requires tedious and costly processes to synthesize.

Perhaps a less investigated area of silica is the integration of organic ligands into the silica matrix. This is known as the fabrication of Organically Modified Silica

(ORMOSIL).⁷⁹ Generally, instead of using only the common TEOS to form the siloxane network, a functional silane ligand can be added simultaneously to add a property into the nanoparticle depending on the ligand's function. For instance, if the ligand is acid-responsive or capable of producing ROS, adding that ligand during the fabrication process would produce a nanoparticle possessing that same capability. Since ROS therapy should be targeted towards cancer cells, silica can serve as the bridge to bring those two together for effective therapy.

Figure 7. Illustration showing the synthetic process of ORMOSIL nanoparticle.

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1.4 Creating nanomaterials from silica for ROS cancer therapy

Recent years have seen large increase in the use of ROS for cancer research. This is because researchers are starting to appreciate the ROS phenomenon of cancer and have the information about ROS threshold.⁸⁰ In fact, ROS has been applied in a good number of silica-based nanocomposites. However, not all ROS are equally exploited. Early research focused more on the use of $^1\text{O}_2$ since it is better understood for cancer therapy compared to other radicals like $\cdot\text{OH}$ and H_2O_2 .⁸¹⁻⁸³ Generally, whenever silica nanoparticles and $^1\text{O}_2$ are involved, it would usually include the use of photodynamic agents like organic dyes and organic polymers. Inorganic materials like carbon nitride, black phosphorus and gold nanoparticles can also produce $^1\text{O}_2$. Organic dyes include zinc phthalocyanine, methylene blue, Ce6, BODIPY, rose Bengal and ICG dyes.⁸³⁻⁸⁵ These dyes are loaded into the nanoparticles or chemically conjugated onto the nanoparticle for subsequent near-infrared light (NIR) irradiation. It is important that NIR light or red light is used because unlike UV light, they are able to penetrate skin-deep to reach cancer cells for therapy and are less harmful to human. Light also provide the external energy needed to excite the photodynamic dyes. H_2O_2 is another form of non-radical ROS. In the field of nanoparticles, H_2O_2 is typically produced through the use of catalysts. For instance, glucose oxidase can convert the existing glucose in cancer cells to H_2O_2 and gluconic acid. The functional group on the surface facilitates loading of glucose oxidase both physically and chemically as well. In the field of silica, glucose oxidase can be conjugated with silane group before it undergoes hydrolysis with the silanol surface of nanoparticles. Up to 80% of the enzymatic activities of the conjugated glucose oxidase retain according to a published study while a good number of publication have shown that glucose oxidase continued to act as a reliable source of H_2O_2 after the reaction.⁸⁶ Enzymes like catalase can also be

used to convert the already high level of H_2O_2 to O_2 to provide sufficient O_2 for photodynamic activities in hypoxic cells.⁸⁷ Another useful form of therapeutic ROS is $\cdot\text{OH}$. $\cdot\text{OH}$ is normally derived from naturally-occurring fenton reaction in cells or reaction of H_2O_2 with metal ions. This reaction is mostly exploited through transition metals like iron and manganese. For example, Fe_3O_4 nanoparticles can release Fe ions overtime which reacts with H_2O_2 via fenton reaction in acidic conditions to generate $\cdot\text{OH}$ radicals.⁸⁸ Since high levels of H_2O_2 and high levels of acidity coincide with the intracellular and tumor microenvironment of cancer, nanoparticles using fenton reaction naturally have huge selectivity towards cancer cells.⁸⁹

Figure 8. An example on the use of silica-based nanocomposite for ROS and NO gas production. (Reproduced with permission from ref 80. Copyright 2016 Angewandte Chemie International Edition.)

1.5 Research objective

ROS therapy is a new paradigm in the field of nanomedicine that involves the use of oxygen-containing species to eradicate tumor. Basically, when there are enough of these oxygen species, they will start to interact destructively in cancer cells, ultimately killing it.

In order to ensure that the therapy is done selectively in cancer, nanoparticles are used to deliver therapeutic cargos that generate ROS. It is also possible to fabricate nanoparticles that can generate ROS themselves.

In this thesis, research has been successfully conducted to not only deliver ROS-producing cargo, but synthesize new nanoparticles capable of generating ROS.

These new nanoparticles are fabricated from silica due to its favorable yet poorly explored application in ROS therapy.

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Chapter 2

Investigation of Fe₃O₄ nanoparticle as a hyaluronic yolk-shaped nanoreactor for targeted ROS-mediated therapy *in vivo*

2.1 Introduction

Fe₃O₄ nanoparticle has been gaining popularity as an effective agent for ROS therapy.¹⁻⁷ The iron on Fe₃O₄ nanoparticle mostly exists in the heterogeneous form and high level of hydrogen peroxide can react with it via fenton reaction to produce a specific ROS known as ·OH radicals.^{8,9} Still, Fe₃O₄ nanoparticles alone are insufficient in killing cancer cells in small dose. They are normally combined with other materials to improve their performance. For instance, free Fe₃O₄ nanoparticles are commonly loaded into nano-vehicles as an iron source.¹⁰⁻¹⁴ Iron metal ions can be chemically doped into nanomaterials as well.^{15,16} However, Fe₃O₄ nanoparticles can also be used to further form yolk-shaped nanocomposites. Nevertheless, these nanocomposites were typically only used for MRI imaging or magnetic accumulation in the field of nanomedicine.^{17,18} While there were reports on fenton reaction by yolk-shaped nanocomposite, they were not explored in biological systems.¹⁹⁻²³ This gap meant that the therapeutic property that comes from fenton reaction in Fe₃O₄-based yolk-shaped nanocomposite may not be taken into account or not properly exploited. Hence, it is important to study the potential of Fe₃O₄ nanoparticle as a shell-bound nanocomposite for ROS-mediated therapy. Although there were a few close studies on iron use in cancer, there is a lack of animal studies which hinders further applications.²⁴

Scheme 1. Schematic representation of the components of YSN@ZnPc@HA for target-specific ROS therapy

Silica nanomaterials have good biocompatibility, scalability and can be tuned at the molecular level to fabricate new therapeutic vehicles.^{25,26} Well-developed protocols were available that uses silica to make stable core-shell, yolk-shaped, star-shaped, nano-rods and nano-spikes etc.^{27,28} The mesoporous nature of silica also allowed a variety of drugs to be loaded.²⁹ Moreover, silica surface can be modified to introduce targeting properties, further improved biocompatibility or better containment of drug cargo.³⁰ This meant that silica-based yolk-shaped nanocomposite is an ideal platform to study the fenton reaction of Fe_3O_4 nanoparticle for nanomedicine.

Herein, we report the fabrication of a $\text{Fe}_3\text{O}_4@\text{MgSiO}_3$ yolk-shaped nanoreactor (YSN) loaded with ZnPc and surface coated with hyaluronic acid. The individual components were selected to enhance the ROS therapeutic performance of the nanoreactor (Scheme 1). $\text{Fe}_3\text{O}_4@\text{MgSiO}_3$ contains a Fe_3O_4 core for fenton catalysis. This core is rigid, hence effectively preventing any potential leakage of nanoparticles. Furthermore, since fenton reaction can only occur in acidic medium and the Fe_3O_4 metal core only corrode rapidly under the same conditions, this nanoreactor will selectively kill cancer cells over healthy ones.³¹ Moreover, the empty cavity of the $\text{Fe}_3\text{O}_4@\text{MgSiO}_3$ nanocomposite can be exploited to reliably store greater amount of the hydrophobic ZnPc via its mesopores. ZnPc is a photodynamic agent capable of generating singlet oxygen upon red light irradiation.³² It can complement the $\cdot\text{OH}$ radicals as a secondary source of ROS in the form of singlet oxygen. The combination is expected to raise the overall ROS to a lethal level. Last but not least, the silanol surface of $\text{Fe}_3\text{O}_4@\text{MgSiO}_3$ nanocomposite can be coated with hyaluronic acid for active targeting towards the CD44 receptors of the chosen HeLa cell line.³³ The size of the nanoreactor also allows for passive targeting via EPR effect.³⁴ These properties mentioned above result in a nanoreactor that is not only highly activating, but selective towards cancer cells.

$\text{Fe}_3\text{O}_4@\text{MgSiO}_3$ yolk-shaped nanoreactor was fabricated precisely from a bottom-up approach to achieve the desired morphology and composition make-up. Crystalline Fe_3O_4 was first fabricated via decomposition of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in oleic acid at high temperature.³⁵ It was subsequently added into a sol-gel templated mixture of CTAB, mixed homogeneously and condensed with TEOS to form the spherical mesoporous core-shell structure of $\text{Fe}_3\text{O}_4@\text{SiO}_2$.³⁶ The nanoparticle was further reacted with $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to form an outer layer of magnesium silicate shell. The inner silica sacrificial template was simultaneously etched

with ammonium chloride in a hydrothermal process to further create a hollow cavity. The exterior was then post-modified with APTES to obtain a positively-charged amine functionalized silicate shell.³⁷ Hydrophobic ZnPc was subsequently loaded into the structure via stirring in DMF solvent overnight and washed extensively with water to remove any excess ZnPc and solvent. Finally, the carboxylic acid group of hyaluronic acid (HA) was chemically converted to succinimide by NHS coupling before reacting with the active amine group on the amine functionalized $\text{Fe}_3\text{O}_4@\text{MgSiO}_3$.³⁸ These gave rise to the formation of YSN@ZnPc@HA nanoreactor. In this chapter, we presented a proof-of-concept of an anticancer yolk-shaped nanoreactor for ROS-mediated therapy. It will selectively accumulate and activate its catalytic activity in cancerous environment to produce the toxic $\cdot\text{OH}$ radicals while having the ability to generate singlet oxygen upon on-demand light irradiation. This combination causes cancer cell death while leaving most healthy cells intact.

2.2 Experimental

Characterization of nanocomposites.

^1H NMR spectra were produced on a Bruker AV 300MHz NMR spectrometer and a Bruker AV 500MHz NMR spectrometer. CDCl_3 was used as the solvent. Transmission electron microscopy (TEM) images were obtained on JEM-1400 (JEOL) at an acceleration voltage of 100 kV. The UV–vis–NIR absorption and fluorescence emission spectra were performed on a Shimadzu UV-3600 and Shimadzu RF5301PC spectrophotometer, respectively.

Hydrodynamic diameters and zeta potential values were determined by a Malvern Zetasizer Nano-S dynamic light scattering (DLS) system at 25 °C. X-ray photoelectric spectroscopy (XPS) was carried out on a Phoibos 100 spectrometer. N_2 adsorption/desorption analyses were conducted using Quantachrome Instruments Autosorb-iQ (Boynton Beach, Florida USA) with extra-high pure gases. Specific surface areas were calculated from the adsorption data in low-pressure range using the Brunauer–Emmett–Teller (BET) model, and pore size was determined by following the Barret–Joyner–Halenda (BJH) method. Thermogravimetric analysis (TGA) was carried out for powder samples using a TGA Q500 recorded from 100–800 °C in an airflow at a heating rate of 10 °C min^{-1} . Powder X-ray diffraction (PXRD) patterns were collected using Shimadzu XRD-6000e quipped with Cu-K α radiation ($\lambda = 1.5418 \text{ \AA}$). Confocal laser microscopy (CLSM) images were acquired by a Leica TCS confocal microscope with a Nikon Eclipse TE2000-S objective (60 $\times$ oil). Flow cytometry experiments were conducted on BD LSRFortessa X-20 cell analyzer.

Synthesis of Fe_3O_4 Nanoparticles.

Fe_3O_4 nanoparticles are synthesized as according to literature by Park.³⁹ The synthesis was divided into two sections. Briefly, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (10.8 g, 40 mmol) and sodium oleate (36.5 g, 120 mmol) were added into a mixture of ethanol (80 ml), hexane (140 ml) and distilled water

(60 ml). The mixture was heated to 70 °C for 4 hours and cooled to room temperature. The organic layer was washed with distilled water before evaporating to dryness. A black wax was obtained as the iron-oleate complex. Next, the iron-oleate complex (36 g, 40 mmol) was dissolved in a mixture of oleic acid (5.7 g, 20 mmol) and 1-octadecene (200 g). The reaction vessel was purged with nitrogen gas for 15 minutes before heating to 320 °C and maintained for 1 hour. The mixture was then cooled to room temperature before ethanol (500 ml) was added to precipitate the nanoparticles. The precipitated nanoparticles can then be collected by centrifugation, washed multiple times with ethanol and finally vacuum dried until a viscous solid is formed.

Synthesis of Fe₃O₄@SiO₂ Core-shell Nanoparticles.

Fe₃O₄@SiO₂ core-shell nanoparticles are synthesized with slight modification as according to a literature by Pisani. Briefly, cetyltrimethylammonium bromide (CTAB) (125 mg) was dissolved in distilled water (60 g). A 2M NaOH solution (0.44 ml) was then added and left to stir for 1 h at 70°C with a stirring speed of 750 rpm under nitrogen. After which, a suspension of well-sonicated Fe₃O₄ nanoparticles (40 mg/ml, 0.5 ml) in chloroform was injected stepwise (0.1 ml × 5). Five minutes after each injection, a brief vacuum (3 s) was applied before switching back to nitrogen in order to remove the hot chloroform. Subsequently, the mixture was heated to 80 °C where a brief vacuum was applied once more to remove any residual chloroform and left to stabilize for 1h. Tetraethoxysilane (TEOS) (0.1 ml) was added to the mixture and left to stir for 30 minutes before the remaining TEOS (0.5 ml) was injected. The silica suspension was stirred for 1.5 hours before cooling to room temperature. The synthesized nanoparticles were collected by centrifugation, washed with distilled water (×5) and ethanol (×2) and finally kept in ethanol (20 ml) overnight at 4 °C before use.

Synthesis of Fe₃O₄@MgSiO₃ Yolk-shaped Nanocomposite (YSN).

Well-defined Fe₃O₄@MgSiO₃ was prepared according to a literature by Wang.⁴⁰ Briefly, MgCl₂·6H₂O (114 mg) and NH₄Cl (276.5 mg) were dissolved in a solution of distilled water, ethanol and NH₄OH solution (1 ml, 25 %) within a Teflon vessel. A well-sonicated solution of Fe₃O₄@SiO₂ was then added into the mixture and autoclaved at 160°C for 12 hours. After the autoclave cooled to room temperature, the nanocomposite was collected by centrifugation and washed thrice in ethanol before vacuum drying at 45°C overnight.

In order to synthesize the hollow nanocomposite (HSN) as the control, the previously-synthesized Fe₃O₄@MgSiO₃ was dispersed in methanol (200 ml), concentrated hydrochloric acid (37%, 16 ml) was then added to the suspension and refluxed at 80°C for 24 hours to remove the ferromagnetic Fe₃O₄ core. The final Fe concentration was determined by ICP-OES while the nanoparticle's structural integrity was verified by TEM, XRD and BET measurements.

Synthesis of YSN@NH₂ Nanocomposite.

YSN (56 mg) was dispersed in toluene (5 ml) and sonicated for 30 minutes. (3-Aminopropyl)triethoxysilane (APTES) (80 µl) was added and stirred at 80 °C for 24 hours with a stirring speed of 400 rpm. After cooling to room temperature, the APTES-modified nanocomposite was collected by centrifugation and subjected to several ethanol washing before vacuum drying at 45 °C overnight.

Loading of ZnPc into Nanocomposite.

The loading of ZnPc was done by first dispersing YSN@NH₂ (25 mg) in dimethylformamide (25 ml). The suspension was sonicated for 1 hour before ZnPc (2.5 mg) was added directly into the stirring suspension and left to stir for 24 hours. The ZnPc-loaded nanocomposite was

collected by centrifugation and washed with distilled water several times before lyophilization.

Determination of ZnPc Loading Capacity.

YSN@ZnPc (1 mg) was suspended in DMF solution (1 mL) containing ZnPc (0.1 mg mL^{-1}). After stirring for 24 h, ZnPc-loaded nanoparticles were collected by centrifugation at 13 000 rpm for 3 min. After washing with PBS twice, the nanoparticles were suspended in DMF, and the loaded ZnPc was extracted by thorough sonication for 30 min. Then, the suspension was centrifuged at 13 000 rpm for 3 min, and the supernatant was collected. The extraction process was performed repeatedly until a colourless supernatant was obtained. The ZnPc amount in all the collected supernatants was quantified by the UV–vis absorption intensity at 675 nm.

Synthesis of YSN@ZnPc@HA Nanocomposite.

Hyaluronic acid (1.04 mg, 10^6 MW.) was first completely dissolved in a solution of N-Hydroxysuccinimide (NHS) (3.4 mg), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (1.84 mg) and distilled water (1 ml). ZnPc-loaded nanocomposite (9.2 mg) was then added into the suspension with a small amount of triethylamine till pH 9.0. The mixture was stirred at 38°C for 12 hours. The fabricated YSN@ZnPc@HA nanocomposite was collected by centrifugation and washed with distilled water several times before lyophilization.

YSN@HA (30 mg) was etched in 4M NaOH solution (30 ml) at room temperature for 24 h to remove the inorganic framework. The suspension was dialyzed against deionized water prior to lyophilization and finally analyzed by NMR.

Iron Release Experiment.

YSN@HA (4 mg) was incubated in acetate (pH 5.5, 1.5 ml) and phosphate buffers (pH 7.4, 1.5 ml) at 37°C up to 72h. The samples were centrifuged for 10 min at 10000 rpm, and an aliquot (0.5 ml) of the supernatant was collected and isolated by filtration (0.45 μ m) before analysis. The solution is then topped up to 9 ml using 2% nitric acid. Iron concentration was determined by ICP-OES. Similarly, YSN@HA, YSN and HSN (2 mg each) were dispersed in 2% nitric acid (10 ml) before analysis.

Terephthalic Acid Probing Technique for $\cdot$ OH radical.

Solution of different concentration of YSN@HA and HSN@HA (0, 50, 100, 150, and 200 μ g/mL), H₂O₂ (1 mM), and terephthalic acid (2 mM) was shaken in acetate buffer (pH 5.5, 1.5 ml) and PBS buffer (pH 7.4, 1.5 ml) at 25°C. The photoluminescence spectra under the excitation wavelength of 312 nm were recorded after 24 hours using a spectrofluorophotometer.

Cellular Uptake.

Human cervical carcinoma HeLa cells, were cultured with Dulbecco's modified eagle medium (DMEM) mixed with 10% fetal bovine serum (FBS), penicillin (100 U mL⁻¹) and streptomycin (100 μ g mL⁻¹) under normoxic condition (5% CO₂ atmosphere) at 37 °C.

In the CLSM study, HeLa was seeded in a 6-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. Upon 24 hours incubation, the medium were replaced with fresh ones and an appropriate amount of nanoparticle suspension with the concentration of 10 μ g mL⁻¹ was added into the cell culture medium. After further incubation for 1 h and 4 h, the culture medium was removed and the cells were washed three times with phosphate

buffered saline (PBS). Finally, the cells on the slide were fixed with 4% formaldehyde at room temperature for 15 min and mounted for observation with CLSM.

In flow cytometry analysis, HeLa cells were seeded in a six-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. After incubation for 24 hours, the medium was replaced and nanoparticle suspension was added in the medium. After incubation for 1, 3, or 24 h, the cells were washed with PBS several times and trypsinized (0.5 mL) for 2 min. Then fresh medium (0.5 mL) was added into each culture well, and the cells were collected via centrifugation at 4000 rpm for 3 min. After one more round of washing with PBS through centrifugation, the cells were resuspended in PBS (1 mL) and subjected to the flow cytometry analysis.

***In vitro* Cell Viability Assay.**

Briefly, HeLa was seeded in 96-well plates containing 100 μ L medium each and incubated under normoxic condition for 24 hours. When the cell confluence reached around 60–70 %, the medium was replaced with fresh one (90 μ L), and depending on the desired concentration, nanoparticle suspensions of up to 10 μ L each were added. After 6 hours of incubation under normoxic condition or the hypoxic condition (1% O₂, 5% CO₂ balanced with N₂), the culture medium was replaced and the cells were irradiated with 630 nm laser at 50 mW cm⁻¹ for 3 minutes, while the cell groups without any treatment were used as controls. After further incubation for 24 hours, MTT solution (10 μ L, 5 mg L⁻¹) was added in the medium. The cells were incubated for another 4 hours before the medium was replaced by DMSO (100 μ L each) to dissolve the purple crystals. Finally, optical densities of the samples were measured using a microplate reader (infinite M200, TECAN) at 490 nm.

In-vitro observation of ROS and $\cdot\text{OH}$.

The in-vitro oxidative stress was analyzed using the Image-IT LIVE Green reactive oxygen species detection kit from Thermo Fisher according to the manufacturer's instructions. HeLa cells were seeded in a 8-well plate. Nanoparticle suspension was added at a final concentration of 10 $\mu\text{g/mL}$. After incubation for 1 and 4 hours, the cells were labeled with carboxy- H_2DCFDA for 30 minutes. After the treatment, the plate was used directly for live-imaging by CLSM. Carboxy H_2DCFDA $\lambda_{\text{ex/em}}$: 488/529 nm. Hydroxyl radical was visualized with the same setup as shown above except with the addition of coumarin to generate the fluorescent 7-hydroxycoumarin (100 $\mu\text{g/mL}$). 7-hydroxycoumarin $\lambda_{\text{ex/em}}$: 400/500 nm

***In Vivo* Anticancer efficiency**

Normal nude mice were purchased from Xinqiao Hospital for establishing a tumor model. Briefly, HeLa cells (1×10^6 cells) were injected into the tail vein of nude mice. When the tumor volume reached around 100 mm^3 , the nude mice were randomly divided into 6 groups (5 mice per group). Nude mice were injected with various nanoparticles (saline, HSN@ZnPc@HA (-L), HSN@ZnPc@HA (+L), YSN@ZnPc@HA (-L), YSN@ZnPc@HA (+L) of 2 mg ZnPc equivalent per kg mice every 3 days. After 1 hour, the tumor site is irradiated with 630 nm laser (500 mW cm^{-2}) for 5 minutes. All mice were treated twice a week and the treatment period lasted for 21 days. Meanwhile, the mice had their weight and tumor volume measured every day. The tumor sizes were calculated using the following equation: $V_{\text{tumor}} = LW^2/2$ (L: tumor length, W: tumor width). After 21 days of treatment, the mice were sacrificed for further analysis.

***In Vivo* Pharmacokinetic Evaluation.**

The tumors and main organs (heart, liver, lung, spleen, and kidney) were taken out at the 21st day and then embedded with paraffin and sliced for staining with H&E. To evaluate the apoptosis in tumor tissue, tumor tissue was stained with a TUNEL apoptosis detection kit (Beyontime, China). The nuclei of cells within tumor tissue were stained with Hoechst 33258.

***In Vivo* Biodistribution Study.**

One hour after the injection of nanoparticle, mice were sacrificed at 24 h post i.v. injection. Heart, liver, spleen, lung, kidney and tumor from each mouse were collected and manually ground before transferring into test tubes.

2.3 Results & Discussion

Figure 1. TEM images of (a) Fe₃O₄ nanoparticles, (b) Fe₃O₄@SiO₂ nanoparticles, (c) MgSiO₃ nanocomposite, (d) YSN@HA nanocomposite and (d) HSN@HA nanocomposite. The scale bars are 100 nm.

Each successive fabrication steps up to YSN@ZnPc@HA nanoreactor were well-characterized by TEM, XRD, BET, Zeta, DLS, TGA, SEM, UV-Vis, Fluorescence, ICP-OES and MRI. A control without the Fe₃O₄ nanoparticle as the core, termed Hollow Silica Nanoparticle (HSN) was synthesized by etching YSN@NH₂ in 1M HCl at 80 °C overnight and characterized separately to check the structural integrity. Fe₃O₄ nanoparticles were synthesized via high temperature decomposition to form monodispersed crystalline nanoparticles stabilized by the hydrophobic oleic acid (Figure 1). To start off, TEM depicted Fe₃O₄ as having a size of approximately 10 ± 2 nm while DLS calculations revealed a size of 14.3 ± 0.9 nm. Moving on, Fe₃O₄@SiO₂ was synthesized from this oleic stabilized Fe₃O₄ to

form core-shell nanoparticles of around $85.5 \pm 3.6\text{nm}$ by TEM and $80.7 \pm 0.3\text{nm}$ by DLS.

Figure 2. DLS size of nanoparticles at each successive fabrication step.

Subsequently, YSN nanocomposite was formed by base etching of the inner silica content in $\text{Fe}_3\text{O}_4@\text{SiO}_2$ and addition of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to constitute the outer shell. The YSN nanocomposite has a TEM size of around $101.7 \pm 2.1\text{nm}$ and $98.8 \pm 11.0\text{nm}$ by DLS (Figure 2). The shell thickness was determined to be around $13.5 \pm 1.2\text{nm}$ and a single Fe_3O_4 nanoparticle could be observed in every yolk-shaped YSN nanocomposite. YSN was then post-modified with APTES to functionalize it with amine group and further surface coated with HA. The final YSN@HA had a TEM size of $117.8 \pm 5.0\text{nm}$ and DLS size of $119.6 \pm 2.9\text{nm}$, indicating the ability to accumulate not only actively, but passively by EPR effect.

Figure 3. SEM image of YSN@HA nanocomposite. The scale bars are 100 nm.

SEM of YSN@HA also showed that the nanocomposite is spherical in morphology and had a size of around $106.7 \pm 11.9\text{nm}$ (Figure 3). Interestingly, we compared the ZnPc-loaded version of YSN@HA and found the DLS size to have slightly increased to $129.2 \pm 5.5\text{nm}$. Again, all sizes were found to be within the EPR range, meaning that an increased accumulation in cancer cells could be expected.

Figure 4. Zeta potential of nanoparticles at each successive fabrication step.

Zeta potential measurements were performed alongside DLS for all samples (Figure 4). The zeta potentials of Fe_3O_4 , $\text{Fe}_3\text{O}_4@\text{SiO}_2$, YSN, $\text{YSN}@\text{NH}_2$, $\text{YSN}@\text{HA}$, $\text{YSN}@\text{ZnPc}$, $\text{YSN}@\text{ZnPc}@\text{HA}$ were $-23.5 \pm 1.2\text{mV}$, $-19.3 \pm 0.6\text{mV}$, $-23.5 \pm 0.2\text{mV}$, $+38.0 \pm 0.4\text{mV}$, $-35.7 \pm 2.0\text{mV}$, $-9.1 \pm 0.1\text{mV}$ and $-13.3 \pm 1.3\text{mV}$ respectively. This is as expected of Fe_3O_4 because of the negatively-charged oxide layer as well as for $\text{Fe}_3\text{O}_4@\text{SiO}_2$ and YSN due to their silanol surface groups. The zeta potential quickly changed to positive in $\text{YSN}@\text{NH}_2$ because of the positively charged amine groups but a final coating of the carboxylic group-abundant HA swing the zeta potential back to negative. The negative charges were expected to reduce the amount of protein adsorption by the immune system, thereby increasing the chance of $\text{YSN}@\text{ZnPc}@\text{HA}$ to reach cancer cells.

Figure 5. XRD of nanoparticles at each successive fabrication step.

XRD was used to characterize Fe_3O_4 , $\text{Fe}_3\text{O}_4@\text{SiO}_2$ and YSN as well (Figure 5). The sharp peaks at (111), (311), (400) and (440) matched that of Fe_3O_4 , but were overshadowed by a silica core-shell layer in $\text{Fe}_3\text{O}_4@\text{SiO}_2$ as shown by the distinct broad peak between 15 to 35°. Two additional peaks were observed upon etching and yolk-shell formation in YSN, indicating the successful incorporation of magnesium. Nitrogen adsorption-desorption isotherms of both $\text{Fe}_3\text{O}_4@\text{SiO}_2$ and YSN exhibited a typical IV-type isotherm pattern, revealing the presence of mesopores (Figure 6). Deeper BET analysis saw the surface area of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ increased from 199 m^2/g to 324 m^2/g in YSN. The pore volume rose from 0.543 cm^3/g to 0.754 cm^3/g . Similarly, the pore size increased from 1.4 nm to 2.0 nm when $\text{Fe}_3\text{O}_4@\text{SiO}_2$ was etched to form YSN.

Figure 6. BET of Fe₃O₄@SiO₂, Fe₃O₄@MgSiO₃ and HSN.

This finding is encouraging since more ZnPc can be loaded into the nanocomposite for photodynamic therapy and we know that the inner Fe₃O₄ core is more exposed to cancer environment changes. Direct colour visualization of HSN@HA, YSN@HA, YSN@ZnPc and YSN@ZnPc@HA showed the colour change that came with successful removal of the inner Fe₃O₄ core, dye loading and HA coating (Figure 7). The original Fe₃O₄@MgSiO₃ is orange, which changes to white after etching, revealing the distinctive white of silica. On the other hand, the use of ZnPc transforms the colour to blue. A further coat of HA induced another colour change to green.

Figure 7. Visualization of HSN, Fe₃O₄@MgSiO₃, YSN@ZnPc and YSN@ZnPc@HA.

Figure 8. TGA comparison of YSN@NH₂ and YSN@HA.

TGA analysis determined the mass of HA that was covalently coated onto YSN@NH₂ to be 3 wt.% (Figure 8). FITC plot showed that there was a new peak at 1412 cm⁻¹ corresponding to the carboxylate symmetric stretching of HA (Figure 9).⁴¹ All in all, the fabrication of an YSN@ZnPc@HA nanoreactor was proved to be successful.

Figure 9. FTIR comparison of YSN@NH₂ and YSN@HA.

The fenton capability was assessed in the YSN@HA nanoreactor without ZnPc to exclude any photodynamic capability (Figure 10). Inductively coupled plasma optical emission spectroscopy (ICP-OES) determined the Fe content of YSN@HA to be 0.3895 wt.% but HSN@HA to be negligible at 0 wt.%. The respective nanoreactor and control were incubated in distilled water and acetate buffer and aliquots were taken at different time points for further ICP-OES quantification. The total amount of Fe content released by YSN@HA at pH 5.5 after 72h was calculated to be 3.3 % while the Fe content released at pH 7.4 was 0.7 %. This translated to a 4.7 times selectivity in the acidic lysosome of cancer cells compared to the neutral environment of healthy cells. Nonetheless, the release of Fe content should be accompanied with a fenton reaction with H₂O₂. Hence, the ability of YSN@HA to perform fenton reaction was tested at different pH as well.

Figure 10. Fe release comparison of YSN@HA under different pH.

The bleaching of methylene blue (MB) was a common indicator of $\cdot\text{OH}$ radicals and the concentration can be quantified using UV-Vis (Figure 11).⁴² Results shown that the control, YSN@HA incubated in pH 7.4 and 5.5 aqueous solution at the 72h mark showed a 22.6 %, 67.6 % and 91.6 % degradation in MB respectively. This meant that YSN@HA can generate $\cdot\text{OH}$ radicals preferentially in acidic pH. The large degradation seen in pH 7.4 was due to the ability of YSN@HA to potentially trap MB in its porous cavity and ability of silica to generate small amounts of radicals. As such, an additional fluorescence test using terephthalic acid (TA) was performed (Figure 12). TA produces terephthalic acid hydroxide (TAOH), which fluorescence at 425 nm, in the presence of $\cdot\text{OH}$ radicals.⁴³ A greater number of controls were used to assess the $\cdot\text{OH}$ radical generating ability and to confirm the selectivity of YSN@HA for fenton therapy in cancer cell environment.

Figure 11. Methylene blue degradation comparison of YSN@HA under different pH.

The experiments demonstrated that only in the presence of H_2O_2 and acidic pH that TAOH was increasingly generated in a dose-dependent manner up to the tested 200 ppm. The other fluorescence graphs proved that if any one of the factors was missing, be it the Fe_3O_4 core, H_2O_2 or acidic medium, there will not be any production of $\cdot OH$ radicals. This trend could be interfered from the unchanged fluorescence intensity of TAOH in all three controls. The observable drop in the fluorescence was likely due to the degradation of H_2O_2 to form water. From the studies mentioned above, the fenton therapy from YSN@HA nanoreactor was found to be very selective towards cancer cell environment.

Figure 12. TaOH concentration change with/without H_2O_2 in pH 5.5/7.4.

To design a nanocomposite can effectively inhibit and kill cancer, ZnPc was simultaneously loaded into the mesopores as the secondary source of ROS. The hydrophobicity of ZnPc and availability of cylindrical mesopores meant that ZnPc photodynamic dye can be reliably loaded and stored into YSN. The loading wt.% is 0.54 %, which is low but typical of ZnPc loading.⁴⁴ The leakage of ZnPc over a period of 48h in water was tested and determined to be 7.3 %. It was noteworthy that it stopped leaking after the 2 h mark (Figure 14), implying that the release may have originated from the loosely surface-bound ZnPc. The quantum yield (ψ_F) of the YSN@ZnPc@HA nanocomposite was 0.12 (Figure 13).

Figure 13. Best-fit line for YSN@ZnPc@HA and ZnPc in DMF respectively.

This value of 0.12 is characteristic of ZnPc-loaded nanomaterials used for cancer treatment. It also showed that ZnPc can continue to generate ROS effectively in YSN@ZnPc. A direct change in colour from orange to green was also observed, confirming the successful loading of ZnPc into YSN.

Figure 14. ZnPc release YSN@ZnPc@HA from in water overtime.

Furthermore, UV-Vis analysis was conducted to study the nature of ZnPc in the YSN@ZnPc@HA nanocomposite (Figure 15). A two times bathochromic red-shift of absorption maximum from 670 nm in free ZnPc to 675 nm in YSN@ZnPc and finally to 678 nm in YSN@ZnPc@HA demonstrated the characteristic pattern of monomeric ZnPc in the mesopores.

Figure 15. UV-Vis of ZnPc in respective forms.

The photoinduced singlet oxygen ($^1\text{O}_2$) production capability was assessed via the use of 9,10-anthracenediyl-bis-(methylene)dimalonic acid (ADMA) as the $^1\text{O}_2$ trap (Figure 16). The nanocomposite was dissolved in an aqueous solution of ADMA and irradiated at different time intervals. The resulting UV-Vis absorption spectrum has two main area of interest. The first being the region between 350 to 415 nm since it represents the absorption of ADMA and the second being 650 to 690 nm since it corresponds to the absorption of ZnPc. Across the samples, the absorbance of the nanocomposite remained relatively constant. However, the absorbance of ADMA decreased substantially as the irradiation time increased. This observation meant that more $^1\text{O}_2$ was successfully generated overtime if subjected longer to irradiation. This also proved that the nanocomposite was able to preserve the monomeric configuration of the ZnPc for photodynamic property and protected the loaded dye from

aggregation.

Figure 16. ADMA absorbance change when incubated with YSN@ZnPc@HA overtime. Increasing degradation of ADMA reflected the increasing production of singlet oxygen.

YSN@ZnPc@HA could be used as a T_2 contrast agent for MRI. Results showed that the darkening from YSN@ZnPc@HA is concentration-dependent. Calculations revealed a high transverse relaxivity (r_2) of $109.2 \text{ mM}^{-1} \text{ s}^{-1}$ (Figure 17)

Figure 17. T₂ weighted MR images of YSN@ZnPc@HA nanocomposites at different concentration of Fe; Plot of R_2 over Fe concentration of YSN@ZnPc@HA nanocomposites.

In-vitro studies were conducted to assess the cancer killing efficiency of YSN@ZnPc@HA and the individual components. The test was planned to first assess the fenton therapy capabilities, followed by the photodynamic efficiency. HSN@HA and YSN@HA were tested against each other in different cell lines. Two types of cell lines, HeLa cancer cells and Hek293 healthy cells, were used to evaluate the selectivity of the nanocomposite. MTT assay

test in HeLa cell line revealed that the cell viability of YSN@HA was at 33 % at 200 ppm while the viability of HSN@HA was only at 88 % at the same concentration. A similar pattern was seen across all concentrations between 50 to 200 ppm. This pattern meant that the Fe_3O_4 core was fundamental in killing cancer cells. Fe_3O_4 core can release iron ions or react with H_2O_2 at the surface to produce $\cdot\text{OH}$ radicals in the acidic pH and high H_2O_2 environment of cancer.

Figure 18. MTT assay in Hek293 normal cells and HeLa cancer cells.

A similar experiment was done in Hek293 healthy cell line to ascertain any toxic effects (Figure 18). As expected, a high biocompatibility of 93 % and 85 % cell viability using HSN@HA and YSN@HA respectively was found. This finding suggested that the therapeutic effects of the Fe_3O_4 core was non-existence in healthy cells but was manifested itself in cancer cells. The phenomenon was in line with the nature of fenton reaction, where toxic radicals were produced only under acidic conditions and promoted by high level of hydrogen peroxide, both of which are characteristic of cancer cells. It also showed that the fenton capabilities of Fe_3O_4 were retained in the yolk-shaped structure. Furthermore, flow cytometry apoptotic study showed that 98.6 %, 83.4 % and 28.9 % of HeLa cells survived the treatment when incubated with the control, HSN@HA and YSN@HA respectively (Figure 19). In Hek293 cells, the survival rate was found to be 99.9 %, 98.3 % and 88.1 %

respectively. These matched well with the MTT findings and highlighted the high fenton selectivity of the nanoreactor towards cancer cell lines.

Figure 19. Flow cytometry results of blank control, HSN@HA and YSN@HA in Hek293 normal cells(top row) and HeLa cancer cells(bottom row).

Although the ROS producing ability of YSN@HA were confirmed, it is important to assess the effects in cancer cells. A ROS fluorescence probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used to observe the amount of ROS before and after treatment (Figure 20). DCFH-DA test revealed that when YSN@HA was incubated, a 3.7 times increase in green fluorescence was generated compared to the blank. However, only a 1.2 times insignificant increase occurred in HSN@HA, which does not produce sufficient amount of ROS. This test proved that the YSN@HA nanoreactor alone can cause the overall ROS level in cancer cells to significantly increase.

Figure 20. ROS fluorescence of blank control, HSN@HA and YSN@HA in HeLa cancer cells. The scale bars are 20 μm .

Coumarin was used to further analyse the nature of our ROS (Figure 21).⁴⁵ The hydrogen peroxide and acidic lysosome within cancer cells triggered the fenton production of $\cdot\text{OH}$ radicals. This radical can react immediately with the colourless coumarin to produce the blue-coloured 4-hydroxylcoumarin. Again, an obvious blue fluorescence could be seen when YSN@HA nanocomposite was incubated with HeLa cells over 12 h. Surprisingly, a 51 times

increase in blue fluorescence was generated compared to the blank (Figure 22). However, only a 1.8 times insignificant increase occurred in HSN@HA, which does not promote fenton reaction. This increase in fluorescence was a direct evidence of the $\cdot\text{OH}$ radicals generating ability of YSN@HA.

Figure 21. Coumarin fluorescence of blank control, HSN@HA and YSN@HA in HeLa cancer cells. The scale bars are 20 μm .

Figure 22. Quantitative analysis of ROS and coumarin fluorescence of blank control, HSN@HA and YSN@HA in HeLa cancer cells.

A more comprehensive MTT analysis was conducted using HSN@ZnPc@HA and YSN@ZnPc@HA in the absence and presence of 630 nm red light at the 48 h mark (Figure 23). It was seen in the absence of light that the cell viabilities of HSN@ZnPc@HA and YSN@ZnPc@HA were 87 % and 41 % at 200 ppm.

Figure 23. MTT comparison of the therapeutic components in YSN@ZnPc@HA in HeLa cancer cells.

This is reminiscent of the in-vitro results done using YSN@HA. The presence of the Fe_3O_4 core served as a source of metal ions for fenton reaction to occur in-vitro. In the presence of light, the cell viabilities were improved to 36 % and 14 %, showcasing the effectiveness of the combinational treatment. The combined ROS from the generation of both $\cdot\text{OH}$ radicals and singlet oxygen gave the best therapeutic efficiency.

Figure 24. Flow cytometry results (from top left to right) of blank control (-L), blank control (+L), HSN@ZnPc@HA (-L), (from bottom left to right) HSN@ZnPc@HA (+L) YSN@ZnPc@HA (-L) and YSN@ZnPc@HA (+L) in HeLa cancer cells.

Flow apoptosis analysis at the 40 h mark further revealed that YSN@ZnPc@HA was the most effective combination in killing cancer cells (Figure 24). Moreover, the Calcein/PI test at the 6 h mark emphasized that the Fe_3O_4 and ZnPc were crucial in eradicating the most number of cancer cells in the shortest amount of time (Figure 25).

Figure 25. Calcein/PI results in HeLa cells. The scale bars are 20 μm .

Since hyaluronic acid have a strong affinity for CD44 receptors in HeLa cells and the size of the nanocomposites can accumulate via EPR effect, these properties were evaluated.⁴⁶ Two derivatives, one with and one without hyaluronic acid were compared in HeLa cells. At the 6h incubation mark, there was an obvious preferential uptake CLSM observation in YSN@ZnPc@HA and interestingly, the trend remained unchanged after 24 h (Figure 26). Flow cytometry was also used to analyse the fluorescence of YSN@ZnPc@HA in cancer cells (Figure 27). Again, the uptake of YSN@ZnPc@HA was more substantial.

Figure 26. CSLM images of nanocomposite uptake in HeLa cells. The scale bars are 20 μm .

In vivo studies were conducted with BALB/C nude mice bearing subcutaneous HeLa tumor and they were randomly divided into six groups. HeLa cells were used to ensure that the previous analytical and in-vitro studies were properly represented and validated. The tumor size of each mouse was measured at different incubation time points over 21 days to monitor the therapeutic progress of YSN@ZnPc@HA and its control groups (Figure 28). After which, the mice were euthanized and their tumors were extracted for in-depth analysis. The first group was intravenously injected with PBS saline as the control, and the subsequent groups were injected with YSN@HA, HSN@ZnPc@HA and YSN@ZnPc@HA in the absence of 630 nm irradiation.

Figure 27. Flow cytometry analysis of nanocomposite uptake in HeLa cells.

The final two groups were injected with HSN@ZnPc@HA and YSN@ZnPc@HA as well but were irradiated by 630 nm red light for 5 minutes after administration of the drug. As expected, the tumor grew rapidly throughout the 21 days treatment with only saline injection up to a size of 1038 mm³. The tumor growth in mice injected with saline, YSN@HA, HSN@ZnPc@HA and YSN@ZnPc@HA without irradiation followed a similar growth profile throughout the 21 days. This observation meant that amount of ROS generated by YSN@HA as well as YSN@ZnPc@HA without irradiation was insufficient to inhibit tumor growth. A final tumor size of 484 mm³ was reached in HSN@ZnPc@HA in the presence of light irradiation. The photo-generation of ¹O₂ was able to produce enough ROS to cause the tumor to undergo apoptosis. While the tumor size still grew linearly like the previous four groups discussed, the rate of growth was significantly inhibited to more than half. Nevertheless, it is not sufficiently potent to stop the tumor growth. The tumor size in YSN@ZnPc@HA with irradiation was 190 mm³. Considering the initial size of tumor was 112 mm³ and irradiation time was only 5 minutes, this is a 1.7 times growth compared to 8.7 times in the control. Photographs taken at Day 0, 7, 14 and 21 revealed that the tumor grew aggressively during the 21 days in all controls. However, the tumor size remained relatively constant across the days.

Figure 28. In vivo therapeutic study of (from left to right) tumor growth curve and body weight change in mice.

Direct visualization and measurement of the final tumor sizes again showed a similar order where the YSN@ZnPc@HA treatment group was the most successful example with the smallest tumors (Figure 29). The weight of the controls and nanoreactors were taken alongside during the treatment. Interestingly, the weight of the mice increased at a relatively similar rate during the 21 days treatment. A 1.2 times increase in weight was seen across all mice while drugs were administrated every three days. This increase in weight highlighted the high biocompatibility of the hyaluronated nanoreactors. The hyaluronic acid coating and the nature of the magnesium silicate nanoreactor meant that its toxicity towards the mice was negligible and indeed, it seemed that no side effects were observed. From the in-vivo therapy conducted, results showed that the utilization of photodynamic therapy or Fe_3O_4 fenton reaction alone was insufficient. However, the combinational treatment with both was able to successfully enhance the overall ROS treatment.

PBS

YSN@HA

HSN@ZnPc@HA(-L)

YSN@ZnPc@HA (-L)

HSN@ZnPc@HA (+L)

YSN@ZnPc@HA (+L)

Figure 29. Photographs of the solid tumors removed from six different treatment groups after 21 days therapy. Experiment was repeated five times for each treatment group.

Histological examinations with hematoxylin–eosin (H&E) and terminal deoxynucleotidyltransferase(TdT)-mediated dUTP nick end labeling (TUNEL) suggest that the tumor growth inhibitory effect was the highest in YSN@ZnPc@HA (Figure 30). TUNEL analysis across all samples indicated that the blue fluorescence of the tumor cells' nuclei fully dominated within tumor tissues after the 21 days treatment in most control groups. However, a pink fluorescence took over instead in the YSN@ZnPc@HA combinational treatment group. This meant that the greatest amount of apoptosis happened within this group. Although a good amount of apoptosis took place in HSN@ZnPc@HA, the extent was not as predominant as the one in YSN@ZnPc@HA. H&E revealed that most tumor tissues had been

destroyed in YSN@ZnPc@HA group. The purple colour corresponding to the DNA and RNA in tumor had been greatly reduced with respect to all other control groups.

Figure 30. Micrographs of H&E stained and TUNEL stained organ slices from after treatment.

The major organs and tumor tissues were extracted after 21 days of incubation and stained with H&E for histological analysis (Figure 31). No significant changes were observed in the nuclei and cell morphology for all groups. In comparison, the tissue samples showed no apparent sign of histological damage, suggesting the excellent biocompatibility.

Figure 31. Histological evaluations of major organs (heart, liver, spleen, lung and kidney) of mice with H&E staining. Scale bar: 100 μ m.

NIR fluorophore dye Cy5.5 was used to label the hyaluronic acid attached to YSN@ZnPc@HA (Figure 32). As seen from the image, the accumulation increased from the 6 hour mark until it became the most prominent at the 24 hour mark. This increased accumulation highlighted the successful targeted accumulation in mice overtime with YSN@ZnPc@HA. MRI imaging with YSN@ZnPc@HA alone also showed a similar effect

with a time-dependent accumulation of nanocomposite at the tumor site.

Figure 32. (Top) *In vivo* T₂ MR images of tumor-bearing mice with intravenous injected YSN@ZnPc@HA. (Bottom Left) *In vivo* fluorescence imaging after intravenous injection of YSN@ZnPc@HA nanocomposites. (Bottom right) *Ex vivo* imaging of tumor and organs extracted from HeLa tumor-bearing mice.

2.4 Conclusion

In summary, a nanoreactor exploiting Fe_3O_4 nanoparticle for fenton reaction and the active targeting of hyaluronic acid was fabricated. The nanoreactor can successfully convert intracellular H_2O_2 into $\cdot\text{OH}$ radicals upon preferential internalization into cancer cells. The loaded ZnPc can also be activated by red light to generate $^1\text{O}_2$ on demand in cancer cells. The combinational ROS treatment is able to raise the ROS level within tumor to apoptotic level, thereby inhibiting tumor growth.

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Chapter 3

An anticancer glucose-depleting ORMOSIL nanocomposite for cascade reactive oxygen species therapy and glutathione scavenging

3.1 Introduction

The use of ROS for therapy is controversial because it has been reported to promote cancer growth.^{1,2} However, more comprehensive studies have proved that ROS can kill cancer by elevating oxidative stress to a toxic level.^{3,4} Therapeutic effects can be achieved by increasing ROS and reducing antioxidant.⁵ To date, this strategy revolves around the use of polymers and prodrugs.⁶⁻⁸ Nevertheless, challenges such as synthetic complexities, impractical scalability and insufficient targeting hindered their progression towards clinical translation.⁹ Moreover, most strategies require external energy sources or drug cargo, which limit their effectiveness and result in leakages.¹⁰⁻

12

Silica nanomaterials are endowed with superior stability, biocompatibility, scalability, simple synthesis and are accepted as “Generally Recognized As Safe” (GRAS) by the FDA.^{13,14} The abundance of silanol groups on the surface also facilitates post-modifications for additional functions.¹⁵ Moreover, protocols based on Stöber method enable precise control of nanoparticle size.¹⁶ This control allows for EPR effect for passive targeting.¹⁷ These properties would compensate for the challenges as mentioned previously. However, there has been no report on the use of organically-modified silica (ORMOSIL) nanomaterial for simultaneous GSH depletion and ROS generation.¹⁸

Scheme 1. Schematic illustration of (a) fabrication of ORMOSIL and ORMOSIL@GOx and (b) therapeutic mechanism via ROS generation, QM ROS-triggered release and glucose depletion. (c) Schematic illustration for

catalytic and therapeutic mechanism. Cell image taken from Medira (2012) on Wikimedia Commons.

Herein, we report the fabrication of an organically modified silica nanocomposite (ORMOSIL@GOx) in two steps.¹⁹ First, a boronate ester-protected quinone methide silane ligand was co-condensed with tetraethyl orthosilicate (TEOS) in a templated sol–gel methodology, followed by a post-modification with APTES-GOx (Scheme 1.). Each component added is critical in treating cancer. The boronate ester-protected quinone methide silane is the first silica precursor. It is cleaved to release quinone methide (QM) in response to hydrogen peroxide.²⁰ The resulting QM will in turn react with GSH to form a GSH-QM adduct. This process will not only deplete the level of GSH but recover the amine group of APTES. While it is well-known that cancer cells have a higher level of hydrogen peroxide, it is insufficient to release QM rapidly.²¹ As such, it is essential to include a component that can generate hydrogen peroxide. Glucose oxidase (GOx) is an enzyme which converts intracellular glucose in cancer cells into hydrogen peroxide.^{22,23} The carboxylic groups on the enzymes can react with APTES in the presence of N-Hydroxysuccinimide to form the second silica precursor, APTES-GOx.²⁴ After incorporation onto the nanocomposite's surface, APTES-GOx will not only produce additional hydrogen peroxide, but starve cancer cells of their glucose supply.²⁵ Equally importantly, hydrogen peroxide from APTES-GOx serves as a source of ROS. This increase in ROS and decrease in GSH raises oxidative stress levels in cancer cells above apoptosis threshold.²⁶ One may argue that it is sufficient to simply further increase ROS to kill cancer cells. Nevertheless, cancer cells can adapt by generating GSH to counteract ROS.²⁷ Hence, the released QM is important in weakening the antioxidant ability of cancer cells.²⁸ In this chapter, we show a proof-

of-concept of an anticancer silica nanocomposite. It will trigger the simultaneous starving of glucose supply, increase in intracellular oxidative stress and suppression of antioxidant.

3.2 Experimental

Synthesis of CDI-PE

4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl 1*H*-imidazole-1-carboxylate
Chemical Formula: C₁₇H₂₁BN₂O₄

CDI-PE was synthesized according to a reported procedure.²⁵ 4-

(Hydroxymethyl)phenylboronic acid (1 g, 4.27 mmol) was dissolved in anhydrous dichloromethane (10 ml). Carbonyldiimidazole (CDI) was subsequently added and the mixture was stirred for 2h. The mixture was then evaporated and re-dissolved in ethyl acetate (15 ml), followed by washing with deionized water (3 × 25 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered, and concentrated to afford CDI-PE as a white solid (1.22 g, yield: 87%). ¹H NMR (300 MHz, δ, ppm, DMSO-*d*₆): 8.30 (s, 1H), 7.71 (d, 2H), 7.62 (d, 1H), 7.51 (d, 2H), 7.09 (d, 1H), 5.47 (s, 2H), 3.31 (s, 2H), 1.45 (m, 2H), 1.30 (s, 12H). ¹³C NMR (The used frequency in ¹³C NMR instrument is 400 MHz, δ, ppm, DMSO-*d*₆): 148.7, 138.4, 137.8, 135.1, 130.9, 127.9, 118.0, 84.23, 79.5, 69.4, 25.1.

Synthesis of Si-PE

4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl (3-(triethoxysilyl)propyl)carbamate
Chemical Formula: C₂₃H₄₀BN₂O₇Si

CDI-PE (0.610 g, 1.86 mmol) was dissolved in anhydrous dichloromethane (10 ml).

Dimethylaminopyridine (2.2 mg, 0.01 eq) and (3-Aminopropyl)triethoxysilane (513

mg, 1.3 eq) was subsequently added and the mixture was stirred for 24h at room temperature. The mixture was then evaporated and purified by column chromatography (silica gel, hexane/ethyl acetate (10 : 1, v/v)) to afford Si-PE as a yellow oil (0.45 g, yield: 50%). ¹H NMR (300 MHz, δ, ppm, DMSO-*d*₆): 7.65 (d, 2H), 7.33 (d, 2H), 7.28 (t, 1H), 5.03 (s, 2H), 3.72 (q, 6H), 3.31 (s, 9H), 2.96 (q, 2H), 1.45 (m, 2H), 1.29 (s, 12H), 1.13 (t, 10H), 0.51 (m, 2H). ¹³C NMR (The used frequency in ¹³C NMR instrument is 400 MHz, δ, ppm, DMSO-*d*₆): 156.47, 141.26, 134.92, 127.22, 127.18, 84.12, 58.14, 56.49, 25.42, 25.13, 19.02, 18.66. HRMS (EI): *m/z* [M]⁺ calcd for C₂₃H₄₀BNO₇Si: 481.27, found: 482.2751.

Synthesis of monodispersed Organically-Modified Silica Nanoparticle (ORMOSIL)

The synthesis of ORMOSIL followed a modified Stöber process. Typically, 0.125 g of CTAB was placed in a 250 mL round bottom flask containing ultrapure water (60 g) and a solution of 2M NaOH (0.44 ml). This solution was heated to 70 °C under vigorous stirring (750 rpm). After 1 hour of stabilization, an initial amount of TEOS (0.1 ml) was added to the mixture. Finally, after 30 min of the reaction, an additional TEOS (0.5 ml) and Si-PE (130 mg) was added to complete the reaction. The condensation process was conducted for 1 h 30 min. After the reaction, the mixture was slowly cooled down at room temperature while stirring, and then collected in Nalgene™ tubes for centrifugation at 9000 rpm for 10 min. To extract the surfactant from the nanoparticles, the solid was redispersed in a 100 mL round bottom flask containing concentrated 37% HCl (8 ml) in methanol (50 ml) and was refluxed at 80 °C for 24 h. The nanoparticles were then collected by centrifugation and washed with water and ethanol twice respectively before vacuum dried at 50°C overnight.

Synthesis of FITC-labelled glucose oxidase (FITC-GOx)

FITC-GOx was prepared according to the reported method. Briefly, FITC in DMSO (25 μ L, 10 mg/mL) was added into GOx aqueous solutions (1 mL, 10 mg/mL). After incubation at 4 °C for 12 h, the reaction mixture was dialyzed against deionized water overnight in the dark, followed by lyophilization. FITC numbers conjugated to GOx were determined by the extinction coefficients of 81,000 M⁻¹ cm⁻¹ at 495 nm (FITC). The number of conjugated FITC per GOx was estimated to be 4.2.

Synthesis of GOx-conjugated ORMOSIL (ORMOSIL@GOx)

GOx (4 mg) was first dissolved in deionized water (4 mL). An aliquot (1 mL) of this GOx stock solution was then added into an aqueous solution consisting of deionized water (2 mL), EDC (20 mg), NHS (30 mg) and APTES (23 μ L) and then stirred for 8h at room temperature. A well-sonicated suspension of ORMOSIL (10 mg) was subsequently added into the amino-functionalized GOx and stirred for 24 h at room temperature to form ORMOSIL@GOx. ORMOSIL@GOx was washed thrice with deionized water to remove the unreacted GOx.

Cell culture

MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, 0.03% *L*- glutamine and 1% penicillin-streptomycin under 5% CO₂ atmosphere at 37 °C. DMEM was replaced every two days.

In vitro cytotoxicity assay

The anticancer efficiency of SiNP, GOx, ORMOSIL and ORMOSIL@GOx were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on the metastasis MDA-MB-231 breast cancer cells. Briefly, cancer cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM medium (100 μ L) containing 10% FBS and 1% penicillin at 37 °C in 5% CO₂ humidified atmosphere. After 24 h incubation, the medium was replaced with fresh one (85 μ L) and doped with 20 mg/mL glucose (5 μ L), followed by addition of free GOx, ORMOSIL or ORMOSIL @GOx at various concentrations of GOD (1-10 mU/mL). After 24h incubation, the medium was again replaced with fresh one (90 μ L), followed by addition of MTT solution (10 μ L, 5 mg/mL in PBS buffer). The plate was incubated further for 4 h, before the medium was removed and replaced with DMSO (100 μ L) to dissolve the purple formazan crystals. Finally, the optical density of the sample in each well was analyzed at 490 nm by a microplate reader (infinite M200, TECAN). The cell viability (%) was calculated based on the following equation, $(A_{\text{sample}}/A_{\text{control}}) \times 100\%$, where A_{sample} and A_{control} represent the absorbance of the sample and control groups respectively.

Intracellular ROS and GSH quantification

MDA-MB-231 breast cancer cells were seeded at a density of 2×10^5 cells per well into a 8-well glass plate and incubated for 24h in 200 μ L DMEM high glucose medium with 10% FBS and 1% penicillin at 37 °C with 5% CO₂ humidified atmosphere. The medium was replaced with fresh DMEM containing 1 mg/ml glucose, followed by addition of free GOx, ORMOSIL, or ORMOSIL@GOx at GOx concentration of 7.5 mU/mL. After 24 h, ROS probe (DCFH-DA) was added at the final concentration of

10 μ M and incubated for 20 min. Then, the cells were directly imaged with CLSM. DCF decomposed from DCFH-DA was excited at 488 nm, and fluorescence was detected from 500 to 550 nm.

For quantification of intracellular GSH level, MDA-MB-231 breast cancer cells were seeded at a density of 2×10^5 cells per well into a 6-well glass plate and incubated for 24h in 2mL DMEM high glucose medium with 10% FBS and 1% penicillin at 37 °C with 5% CO₂ humidified atmosphere. After treatment, the cells were incubated with ThiolTracker™ Violet dye working solution (20 μ M) for 30 min and washed three times with PBS for flow cytometry measurements. ThiolTracker™ Violet was excited at 405 nm, and fluorescence was detected from 500 to 600 nm. Data were analyzed with Flowjo software.

Flow cytometric apoptosis and flow uptake studies

For the flow cytometric apoptosis study, cancer cells were seeded in 6-well plates at a density of 2×10^5 cells per well in DMEM medium (2 mL) containing 10% FBS and 1% penicillin at 37 °C in 5% CO₂ humidified atmosphere. After 24 h incubation, the medium was replaced with fresh one (1.7 mL) and doped with 20 mg/mL glucose (0.1mL), followed by addition of free GOx, ORMOSIL or ORMOSIL@GOx. After 24h incubation, cells in each well was collected in a 2mL eppendorf tube and washed twice with PBS buffer solution. The cells were washed twice with PBS and treated with trypsin (0.5 mL) for 2 min. Subsequently, the cells were re-suspended in fresh medium (0.5 mL) and collected by centrifugation at 3500 rpm for 3 min. After one more round of washing with PBS (0.5 mL) and centrifugation, the cells were re-suspended in $1 \times$ Annexin V binding buffer (100 μ L), followed by addition of FITC annexin V (5 μ L) and 100 μ g/mL PI working solution (1 μ L). After incubation for 15

minutes at room temperature, $1\times$ Annexin V binding buffer (400 μL) was added and the samples were subjected to the flow cytometry analysis.

Cellular uptake study

MDA-MB-231 breast cancer cells were seeded on glass cover slip in 6-well plates and allowed to grow for 24 h. The cells were then incubated with cell culture medium containing 250 mU mL^{-1} FITC-labelled GOx and ORMOSIL@GOx for 2h. After which, the cells were washed with PBS three times, and LysoTracker™ Deep Red, followed Hoechst 33342 was used to stain the nucleus. After the treatment, cells were imaged by CLSM (ZEISS LSM 800). Fluorescence was examined under excitation at 405 nm for Hoechst 33342, 488 nm for FITC and 630 nm for LysoTracker™ Deep Red.

3.3 Results & Discussion

The boronate ester-protected quinone methide silane ligand was prepared in a two-step synthetic route by first reacting 4-(hydroxymethyl)phenylboronic acid with carbonyldiimidazole, followed by a conjugation with (3-aminopropyl)triethoxysilane with dimethylaminopyridine. The chemical structures of successive compounds were well characterized by ^1H and ^{13}C NMR.

Figure 1. Characterizations of the catalytic performances of ORMOSIL@GOx in QM release.

Before utilizing the ligand for the templated sol-gel synthesis, we investigated whether the ligand can be specifically cleaved by hydrogen peroxide to release QM. ^1H NMR analysis was employed to study the release of QM in deionized water and 1mM H_2O_2 aqueous solution. Five new NMR peaks at corresponding to 4-hydroxybenzyl alcohol were identified only in the H_2O_2 aqueous solution. The phenolic proton at 9.2 ppm,

two aromatic doublets at 6.8 ppm and 7 ppm, hydroxyl peak at 5.9 ppm and aliphatic singlet at 4.3 ppm correspond to the protons of 4-hydroxybenzyl alcohol (Figure 1). 4-hydroxybenzyl alcohol is the hydrolysed product of QM which meant that the incorporation of the silane ligand was successful. The silane ligand is dormant in deionized water but will react with the hydrogen peroxide provided to generate QM.

Figure 2. (left to right) XRD and BET of nanocomposites at each successive fabrication step.

Encouraged by the above results, the silane ligand was added jointly with TEOS to form the primary organically modified silica nanoparticle (ORMOSIL).²⁹ ORMOSIL was later layered with APTES-GOx to form ORMOSIL@GOx. Pure silica nanoparticle (SiNP) was also fabricated as the unmodified control to ORMOSIL. The fabrication of the respective nanocomposites was characterized by XRD, BET, SEM, TEM, XPS, DLS and Zeta instruments. XRD analysis revealed a broad peak between 15 to 35° for all nanocomposites (Figure 2). This matched well with the reported peaks of amorphous silica.³⁰ BET isotherm pattern further confirmed the non-porous property of the as-synthesized ORMOSIL and ORMOSIL@GOx nanocomposites (Figure 2)

Figure 3. SEM and TEM characterizations of ORMOSIL@GOx. The scale bars are 100 nm.

TEM images show ORMOSIL and ORMOSIL@GOx nanocomposites as having spherical structures of 45 ± 3 and 75 ± 2 nm in diameter, respectively (Figure. 3 And Figure 4). Indeed, SEM of ORMOSIL@GOx showed most nanocomposites were around 100nm in actual size and had a spherical morphology. Zoomed-in images validated the interpreted data that ORMOSIL and ORMOSIL@GOx nanocomposites were non-porous in nature (Figure 4). These observations confirmed that the nanocomposites synthesized can passively accumulate in cancer cells.

Figure 4. TEM characterization and TEM closed-up image of ORMOSIL. The scale bars are 50 nm and 20 nm respectively

Figure 5. DLS of nanocomposites at each successive fabrication step.

DLS results nevertheless presented a larger hydrodynamic size of $155 \pm 25\text{nm}$, $108 \pm 10\text{nm}$ and $150 \pm 10\text{nm}$ for SiNP, ORMOSIL and ORMOSIL@GOx respectively (Figure 5). Another conclusion is the lack of mesopores hindered the access of hydrogen peroxide to the silane ligand and could encourage sustained release of QM.

Figure 6. Zeta potential of nanocomposites at each successive fabrication step.

Zeta potential studies showed the successive treatment of the nanocomposites.

Obvious zeta fluctuation after each modification showed the integration of the silane ligand from -21.7 mV in SiNP to +26 mV in ORMOSIL, the effective removal of the CTAB template in ORMOSIL from +26 mV to +7.79 mV and the incorporation of APTES-GOx from +7.79 mV to 18.2 mV (Figure 6).

Figure 7. XPS Characterizations of SiNP and ORMOSIL.

XPS comparison between SiNP and ORMOSIL revealed an additional boron 1s peak in the latter, further confirming the successful incorporation of the silane ligand (Figure 7). Again, a new UV-Vis absorbance peak corresponding to 4-(hydroxymethyl)phenylboronic acid pinacol ester (4-HPBA) appeared upon incorporation of the silane ligand into ORMOSIL (Figure 8). 4-HPBA has an intense absorbance peak at 225 nm because of its aromaticity. However, the peak at 225 nm drastically dropped after covalent conjugation with APTES-GOx. This observation could be attributed to the effective surface loading of APTES-GOx on the surface of ORMOSIL.

Figure 8. UV-Vis absorbance showing the absence and presence of 4-HPBA in SiNP, ORMOSIL and ORMOSIL@GOx.

To confirm the attachment of APTES-GOx on the nanocomposite's surface, fluorescence measurement of fluorescein isothiocyanate (FITC) and thermogravimetric analysis (TGA) were used. In the former study, GOx was initially labelled with FITC before conjugation with APTES.²⁵ It was calculated via a given formula that the number of labelled FITC per GOx was 4.2 units. The FITC-labelled GOx was further reacted with APTES then ORMOSIL to form FITC-labelled ORMOSIL@GOx. Lastly, by matching the fluorescence of the nanocomposite to the calibration curve of FITC-labelled GOx, the GOx loading was determined to be 9.3 wt.%. Michaelis-Menten steady-state kinetics was used to determine the catalytic performance of ORMOSIL@GOx towards glucose conversion to hydrogen peroxide

(Figure 9). The Michael-Menten constant (K_m) and maximum velocities (V_{max}) of ORMOSIL@GOx were calculated to be 44.7 mM and $12.8 \times 10^{-8} \text{ M s}^{-1}$ respectively. The K_m value is comparable to the commercially available GOx of between 33 to 110 mM. This finding meant that in 44.7 mM of glucose, half of the maximum catalytic activity will be achieved.

Figure 9. Michaelis-Menten kinetics and lineweaver-Burk plotting of ORMOSIL@GOx.

It is also important to characterize the QM content of ORMOSIL@GOx. TGA comparison between SiNP and ORMOSIL showed a weight difference of 11%, which correspond to the additional weight from the silane ligand (Figure 10).

Figure 10. TGA comparison of SiNP and ORMOSIL@GOx in QM loading.

From this result, the loading efficiency of the silane ligand in ORMOSIL is calculated to be 59%. Since each silane ligand holds a unit of QM, the total QM content can similarly be calculated to be 0.229 mmol/g of ORMOSIL. The cumulative QM release profile showed that the release of QM started after the 7 h mark (Figure 11). This delay in release is attributed to the non-porous nature of ORMOSIL@GOx, which restricted access to the silane ligands. The release occurred rapidly until it plateau after 50 h to give an approximately 60% total release.

Figure 11. Release of QM from ORMOSIL@GOx (10 mg mL^{-1}) in 5 mg mL^{-1} glucose. Mean $\pm$ s.d., $n = 3$

QM released from the immolation of the boronate ester-protected QM silane ligand is very reactive. It will eventually be hydrolyzed to produce 4-hydroxybenzyl alcohol within aqueous medium. Within GSH-rich medium such as the lysosome of cancer cells, 4-hydroxybenzyl alcohol can produce GSH-adduct via thiol substitution at the allylic alcohol (Scheme. 2).

Figure 12. LCMS analysis of ORMOSIL@GOx incubated in 1mM glucose and 1mM GSH over 24h.

LCMS analysis of ORMOSIL@GOx incubated in 1 mM glucose and 1 mM GSH over 24 h revealed a distinct peak at 412 m/z attributing to the GSH-adduct (Figure 12). This new peak is a direct evidence of its GSH scavenging ability.

Previous results have shown that the silane ligand in ORMOSIL can break down when subjected to high levels of hydrogen peroxide over time. Hydrogen peroxide from the catalysis of glucose allowed a cascade reaction to cleave the C-O bond of pinacol ester.

Figure 13. TEM of ORMOSIL nanocomposite in H_2O and H_2O_2 over 48 h.

Since the content of silane ligand in ORMOSIL is significant, the morphological change was investigated. ORMOSIL was incubated in both deionized water and 1mM H_2O_2 over three days and monitored by TEM and DLS. It was observed that the spherical shape was intact over 48 h in the aqueous sample. However, in the H_2O_2 sample, the nanocomposite aggregated after 24 h and eventually merged into a lump after 48 h (Figure 13). This aggregation is due to the attack by H_2O_2 on the framework of boronate ester, disrupting the surface charges that separate the nanoparticles. DLS comparison between the two again confirmed that the H_2O_2 sample was significantly more aggregated at each time point (Figure 14). In fact, a greater difference was found

after 1 week of study.

Figure 14. DLS comparison of ORMOSIL nanocomposite in H₂O and H₂O₂ over 1 week.

In-vitro studies were conducted to investigate the biocompatibility and effectiveness of the nanocomposites. The IC₅₀ of SiNP is 2140 ppm while the IC₅₀ of ORMOSIL is 753 ppm (Figure 15). This drop is attributed to the incorporation of the silane ligand which increases the hydrophobicity of the nanocomposite. At the same time, the QM released can disrupt the ROS homeostasis in cancer cells. Nonetheless, a high concentration of 200 ppm only yielded a 29% killing efficiency, indicating that QM alone is inadequate for effective cancer therapy. Still, the results reflected the

exceptionally high biocompatibility of ORMOSIL relative to other types of nanocomposites.

Figure 15. (left to right) The IC_{50} of SiNP is calculated to be 2140 ppm while the IC_{50} of ORMOSIL is 753 ppm.

GOx served as the main source of hydrogen peroxide and was subsequently conjugated to the surface of ORMOSIL. Before that, it is important to determine the toxicity of GOx so that a healthy amount of GOx equivalent is used for biocompatibility studies. GOx's cytotoxicity in the presence of 1mg/mL glucose was concentration dependent. The IC_{50} of the free GOx in MDA-MB-231 breast cancer cells was determined to be 41 mU/mL (Figure 16). MTT assay was carried out up to a GOx concentration of 10 mU/mL for all components in the ORMOSIL@GOx nanocomposite. At this range of GOx, it was found that both GOx and ORMOSIL were highly biocompatible in cancer cells with cell viability of more than 75% at 10 mU/mL. However, the therapeutic efficiency greatly increased when both GOx and ORMOSIL combined as ORMOSIL@GOx. The IC_{50} of ORMOSIL@GOx in MDA-MB-231 breast cancer cells was determined to be 0.576 ppm or 5.4 mU/mL GOx equivalent. This is a 7.6 times improvement in anticancer therapy (Figure 16).

Figure 16. (Top left to right, Bottom right to left) The IC₅₀ of the free GOx in MDA-MB-231 breast cancer cells was determined to be 41 mU/mL. In-vitro cytotoxic profile of GOx, ORMOSIL and ORMOSIL@GOx at varying GOx concentration. IC₅₀ comparison between SiNP and ORMOSIL. IC₅₀ comparison between free GOx and ORMOSIL@GOx.

The QM released brought the level of antioxidant GSH to a sufficiently low concentration that enhanced the killing effects of ROS. Moreover, the simultaneous depletion of glucose nutrient and continued generation of hydrogen peroxide exerted immense stress on cancer cells. The results of the therapy were again confirmed by flow cytometry analysis at a GOx concentration of 10 mU/mL. 20.6 % of the incubated cancer cells reached late apoptotic stages in ORMOSIL@GOx compared to the 1.61 % and 2.53 % in GOx and ORMOSIL respectively (Figure 17). These

experimental findings showed the good biocompatibility of both ORMOSIL and GOx. It also highlighted the synergy between ORMOSIL and GOx towards inhibiting cancer cell growth.

Figure 17. Live/Dead cells apoptotic assay of control, GOx, ORMOSIL, ORMOSIL@GOx after 24 h incubation with 10 mU/mL GOx equivalent.

Taking reference to Scheme 1, ORMOSIL@GOx initially catalyse β -D-glucose into β -D-glucono-1,5-lactone and hydrogen peroxide via GOx. The hydrogen peroxide produced will raise the level of ROS in cancer cells. The subsequent QM released reduced the amount of ROS scavenged by GSH. These meant that the overall oxidative stress and ROS will be substantially higher than normal. In order to confirm that a ROS-mediated therapy has happened, a ROS fluorescence probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used. MDA-MB-231 cells have the highest level of green fluorescence when after incubation with 7.5 mU ml^{-1} GOx equivalent of ORMOSIL@GOx over 12 h (Figure 18). As expected, GOx and ORMOSIL exhibited fluorescence exceeding that of the control but still beneath ORMOSIL@GOx. The observations suggested that GOx produced a good amount of ROS while ORMOSIL promoted a smaller amount of additional ROS. It further

proved that both components can work together to kill cancer cells by raising ROS beyond apoptotic levels.

Figure 18. CLSM images of MDA-MB-231 cells after co-incubation with PBS, GO_x, ORMOSIL and ORMOSIL@GO_x in 1mg mL⁻¹ glucose DMEM high glucose solution for 4 h and subsequently stained with ROS fluorescence probe DCFH-DA.

FITC-labelled ORMOSIL@GO_x and GO_x were simultaneously incubated with MDA-MB-231 cells over 2 hours to evaluate their uptake (Fig. 3b). Fluorescence analysis from flow cytometry indicated that GO_x cannot be endocytosed into cancer cells. However, additional fluorescence detected meant that ORMOSIL@GO_x nanocomposite can be accumulated in the cell effectively over time. This is because nanoparticle-based material can be absorbed through the endocytosis pathway.³¹ This finding also validated that the therapeutic effects of free GO_x is limited to the extracellular environment and does not directly treat cancer cells on its own. The result matched with that of confocal spectroscopy observation (Figure 19).

ORMOSIL@GOx nanocomposite was seen within the lysosome regions of cancer cells whereas no fluorescence appeared for GOx.

Figure 19. (left to right) CLSM images of MDA-MB-231 cells incubated with FITC-labelled GOx and ORMOSIL@GOx after 2h. Cell nuclei were stained by H33342. Scale bar: 20 μ m. Flow cytometry profiles of MDA-MB-231 cells incubated with FITC-labelled GOx and ORMOSIL@GOx after 2h. Cellular GSH level in MDA-MB-231 cells after incubation with PBS, GOx, ORMOSIL and ORMOSIL@GOx at GOx concentration of 10 mU/mL in the presence of 1 mg/mL glucose for 24 h. Mean $\pm$ s.d., $n = 3$. ** $p < 0.05$, * $p < 0.005$ (t-test).**

QM generated by ORMOSIL@GOx can reduce the level of GSH antioxidant in cancer cells. The intracellular GSH was investigated at a concentration of 5 mU/mL by flow cytometry where higher fluorescence detected corresponds to higher GSH level

(Figure 19). The fluorescence reduced to 90 %, 82 % and 64 % within 24h when incubated with GOx, ORMOSIL and ORMOSIL@GOx respectively. This trend reflected the better scavenging ability of ORMOSIL@GOx in cancer cells. The QM released was able to be released in larger amounts compared to ORMOSIL alone where the latter relied solely on the intracellular hydrogen peroxide for triggered release.

3.4 Conclusion

In summary, a glucose-depleting ORMOSIL nanocomposite was successfully fabricated via a modified Stöber process and post-modification. The nanocomposite can accumulate passively via EPR effect and convert intracellular glucose supply to the hydrogen peroxide ROS, which in turn trigger the release of QM to scavenge GSH for synergistic anticancer treatment. Furthermore, it does not rely on any external input or drugs which offer an easy method for cancer therapy. More interestingly, in-vitro experiments had proved that the strategy of exploiting ROS level for therapy can be used in ORMOSIL silica material. This work provides an alternative platform for ROS therapy and is expected to pave the way for new strategies in the field of cancer treatment.

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Chapter 4

Yolk-shaped nanocomposite for simultaneous glucose starvation and enhanced $\cdot\text{OH}$ production

4.1 Introduction

Fenton therapy involves the reaction of transition metal ions with hydrogen peroxide to generate $\cdot\text{OH}$ radical at the specific cancer site.¹⁻³ These transition elements with multiple redox states can decompose H_2O_2 into $\cdot\text{OH}$ radical via conventional Fenton-like pathways.⁴⁻⁷ $\cdot\text{OH}$ radical is the most toxic known ROS within a biological system.⁸ As such, therapeutic vehicles that can release these ions and promote $\cdot\text{OH}$ radical formation are highly effective in killing cancer cells. However, there are a few main problems. While the level of H_2O_2 within most cancer cells is higher than healthy cells, it is typically insufficient to produce enough $\cdot\text{OH}$.^{9,10} This problem can be overcome with solutions like co-delivering hydrogen peroxide or enzymes and inducing intracellular hydrogen peroxide production with drugs.¹¹ Nevertheless, premature toxic ion release can happen since they are highly soluble in aqueous environment and most vehicles have free metal ions loaded in them.^{12,13} This problem is worsened by the fact that metallic-based vehicles typically suffer from inherent toxicity.¹⁴⁻¹⁶ So, it is ideal to not only design a therapeutic vehicle that can increase hydrogen peroxide, but also selectively release metal ion in cancer cells and kills them.

Fe_3O_4 nanoparticles are used for MRI imaging in human because of its high biocompatibility and low toxicity.¹⁷ In the last two decades, it has been investigated as a promising source of $\cdot\text{OH}$ radical.^{18,19} It was found to have catalase-like activities in neutral medium but undergoes Fenton reaction under acidic conditions.²⁰ Since cancer environment is acidic

compared to the neutral pH in healthy cells, this meant that Fe_3O_4 nanoparticles can do fenton therapy selectively.^{21,22} Another important advantage is the size of Fe_3O_4 nanoparticles is in the range for EPR effect.^{23,24} EPR effect allows the nanoparticle to preferentially accumulate in cancer cells. Being heterogeneous nanoparticles also meant that the iron ions are not free. However, once the nanoparticle reaches the cancer cells, the acidic pH trigger the release of iron for fenton reaction. This eliminates unwanted release of metal ions and ensures that metal ions are still available for therapy. Furthermore, Fe_3O_4 nanoparticles do not require external energy sources. This meant potential to expand their application *in vivo* and beyond. Lastly, Fe_3O_4 nanoparticles can be further used to fabricate multifunctional materials.²⁵⁻²⁸ Hydrophobic oleic acid is bounded to the nanoparticle's surface after formation.²⁹ This layer of oleic acid allowed for further coating with silica. In fact, porous yolk-shaped nanocomposite has traditionally been synthesized with Fe_3O_4 nanoparticles.

We have previously proved that yolk-shaped nanocomposite with Fe_3O_4 as the core can do fenton therapy to kill cancer cells. However, there has been no report on the use of both Fe_3O_4 in a yolk-shaped nanocomposite and catalytically-produced hydrogen peroxide for enhanced fenton therapy.

Scheme 1. Schematic illustration of the therapeutic mechanism via enhanced ROS generation in YSN@GOx.

Herein, we report the fabrication of a catalytic yolk-shaped silica nanocomposite (YSN@GOx). There are two main components. Briefly, a yolk-shaped silica nanocomposite (YSN) formed the base for covalent conjugation with glucose oxidase (GOx). YSN consists of a 13nm Fe_3O_4 nanoparticle core bounded within a porous magnesium/silica yolk shell. This porosity meant that aqueous solution can enter in and out of the shell to interact with the core. This previously-synthesized YSN was added into (3-Aminopropyl)triethoxysilane-conjugated glucose oxidase (APTES-GOx) and stirred for a day in aqueous solution. The two components can undergo further silica surface modifications via hydrolysis between the $\text{Si}(\text{OEt})_3$ groups of APTES-GOx and the dense but porous silica network of YSN to finally form YSN@GOx. The bounded GOx acts as a biological enzyme to produce hydrogen peroxide from the existing glucose supply in cancer cells when internalized. The increased hydrogen peroxide supply can then react with the free iron or Fe_3O_4 core under the acidic pH of cancer to selectively produce more $\cdot\text{OH}$ radicals. In other words, the ability of YSN to

generate $\cdot\text{OH}$ radicals for therapy is greatly improved because of increased hydrogen peroxide supply for the fenton catalysis. Arguably, hydrogen peroxide on its own can also induce cell death in high amounts, rendering fenton catalysis from YSN redundant. However, it is critical to note that $\cdot\text{OH}$ is preferred radical since it is more reactive and toxic to cancer cells. Moreover, free GOx on its own may not be able to be internalized into cancer cells and that YSN can provide a reliable vehicle for intracellular catalysis. It is also important to note that the cancer cells will be starved of intracellular glucose, resulting in eventual cell death.³⁰ Unlike most literature reports where external sources of energy like light, heat or ultrasound are needed, YSN@GOx is a fully independent form of nanomedicine.³¹ This is expected to not only simplify its fabrication process and administration, but improve the reliability of its therapeutic effects *in-vitro* and *in-vivo*. The yolk-shaped nanocomposite as mentioned is a proof-of-concept for simultaneous glucose starvation and enhanced $\cdot\text{OH}$ production in a single self-sustaining vehicle.

4.2 Experimental

Characterization of nanocomposites.

Transmission electron microscopy (TEM) images were obtained on JEM-1400 (JEOL) at an acceleration voltage of 100 kV. The UV–vis–NIR absorption and fluorescence emission spectra were performed on a Shimadzu UV-3600 and Shimadzu RF5301PC spectrophotometer, respectively. Hydrodynamic diameters and zeta potential values were determined by a Malvern Zetasizer Nano-S dynamic light scattering (DLS) system at 25 °C. X-ray photoelectric spectroscopy (XPS) was carried out on a Phoibos 100 spectrometer. N₂ adsorption/desorption analyses were conducted using Quantachrome Instruments Autosorb-iQ (Boynton Beach, Florida USA) with extra-high pure gases. Specific surface areas were calculated from the adsorption data in low-pressure range using the Brunauer–Emmett–Teller (BET) model, and pore size was determined by following the Barret–Joyner–Halenda (BJH) method. Thermogravimetric analysis (TGA) was carried out for powder samples using a TGA Q500 recorded from 100–800 °C in an airflow at a heating rate of 10 °C min⁻¹. Powder X-ray diffraction (PXRD) patterns were collected using Shimadzu XRD-6000 equipped with Cu-K α radiation ($\lambda = 1.5418 \text{ \AA}$). Confocal laser microscopy (CLSM) images were acquired by a Leica TCS confocal microscope with a Nikon Eclipse TE2000-S objective (60 $\times$ oil). Flow cytometry experiments were conducted on BD LSRFortessa X-20 cell analyzer.

Synthesis of GOx-conjugated YSN (YSN@GOx)

GOx (4 mg) was first dissolved in deionized water (4 mL). An aliquot (1 mL) of this GOx stock solution was then added into an aqueous solution consisting of deionized water (2 mL), EDC (20 mg), NHS (30 mg) and APTES (23 μ L) and then stirred for 8h at room temperature. A well-sonicated suspension of YSN (10 mg) was subsequently added into the amino-

functionalized GOx and stirred for 24h at room temperature to form YSN@GOx.

YSN@GOx was washed thrice with deionized water to remove the unreacted GOx.

Cell culture

MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, 0.03% *L*- glutamine and 1% penicillin-streptomycin under 5% CO₂ atmosphere at 37 °C.

DMEM was replaced every two days.

In vitro cytotoxicity assay

The anticancer efficiency of GOx, YSN and YSN@GOx were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on the metastasis MDA-MB-231 breast cancer cells. Briefly, cancer cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM medium (100 μ L) containing 10% FBS and 1% penicillin at 37 °C in 5% CO₂ humidified atmosphere. After 24 h incubation, the medium was replaced with fresh one (85 μ L) and doped with 20 mg/mL glucose (5 μ L), followed by addition of free GOx, YSN and YSN@GOx at various concentrations of GOD (1-10 mU/mL). After 24h incubation, the medium was again replaced with fresh one (90 μ L), followed by addition of MTT solution (10 μ L, 5 mg/mL in PBS buffer). The plate was incubated further for 4 h, before the medium was removed and replaced with DMSO (100 μ L) to dissolve the purple formazan crystals. Finally, the optical density of the sample in each well was analyzed at 490 nm by a microplate reader (infinite M200, TECAN). The cell viability (%) was calculated based on the following equation, $(A_{\text{sample}}/A_{\text{control}}) \times 100\%$, where A_{sample} and A_{control} represent the absorbance of the sample and control groups respectively.

Flow cytometric apoptosis and flow uptake studies

For the flow cytometric apoptosis study, cancer cells were seeded in 6-well plates at a density of 2×10^5 cells per well in DMEM medium (2 mL) containing 10% FBS and 1% penicillin at 37 °C in 5% CO₂ humidified atmosphere. After 24 h incubation, the medium was replaced with fresh one (1.7 mL) and doped with 20 mg/mL glucose (0.1 mL), followed by addition of free GOx, ORMOSIL or YSN@GOx. After 24 h incubation, cells in each well were collected in a 2 mL eppendorf tube and washed twice with PBS buffer solution. The cells were washed twice with PBS and treated with trypsin (0.5 mL) for 2 min. Subsequently, the cells were re-suspended in fresh medium (0.5 mL) and collected by centrifugation at 3500 rpm for 3 min. After one more round of washing with PBS (0.5 mL) and centrifugation, the cells were re-suspended in 1× Annexin V binding buffer (100 µL), followed by addition of FITC annexin V (5 µL) and 100 µg/mL PI working solution (1 µL). After incubation for 15 minutes at room temperature, 1× Annexin V binding buffer (400 µL) was added and the samples were subjected to the flow cytometry analysis.

Cellular uptake study

MDA-MB-231 breast cancer cells were seeded on glass cover slip in 6-well plates and allowed to grow for 24 h. The cells were then incubated with cell culture medium containing 100 mU mL⁻¹ FITC-labelled GOx and YSN@GOx for 2 h. After which, the cells were washed with PBS three times, and LysoTracker™ Deep Red, followed by Hoechst 33342 was used to stain the nucleus. After the treatment, cells were imaged by CLSM (ZEISS LSM 800). Fluorescence was examined under excitation at 405 nm for Hoechst 33342, 488 nm for FITC and 630 nm for LysoTracker™ Deep Red.

In-vitro observation of ROS and $\cdot\text{OH}$.

The in-vitro oxidative stress was analyzed using the Image-IT LIVE Green reactive oxygen species detection kit from Thermo Fisher according to the manufacturer's instructions. MDA-MB-231 breast cancer cells were seeded in a 8-well plate. Nanoparticle suspension was added at a final concentration of 10 mU mL^{-1} and 7.5 mU mL^{-1} respectively for ROS and coumarin experiments. After incubation for 4 hours, the cells were labeled with carboxy- H_2DCFDA for 30 minutes. The plate was subsequently used directly for live-imaging by CLSM.

Carboxy H_2DCFDA , $\lambda_{\text{ex/em}}$: 488/529 nm. Hydroxyl radical was visualized with the same setup after 12 h as shown above except with the addition of coumarin to generate the fluorescent 7-hydroxycoumarin ($100\text{ }\mu\text{g/mL}$). 7-hydroxycoumarin, $\lambda_{\text{ex/em}}$: 400/500 nm

4.3 Results & Discussion

The yolk-shaped nanocomposite YSN@GOx was fabricated from two components. The first component being the magnesium silicate yolk-shell YSN while the second being APTES-GOx. The morphology and composition of YSN and YSN@GOx were well characterized by TEM, XRD, BET, Zeta and DLS. TEM images showed the 2D cross-sectional view of YSN as having a circular yolk-shell structure. A singular dark spot within the shell correspond to the 13 nm Fe_3O_4 nanoparticle. The hollow interior of YSN confirmed the successful etching of the inner silica with NH_4Cl and the exterior shell lining confirmed the successful binding of magnesium ions to form the non-etchable MgSiO_3 shell.

Figure 1. TEM of YSN and YSN@GOx. The scale bars are 100 nm.

The successive modification and chemical make-up of YSN was further analysed with XRD. The XRD patterns mentioned above match the reported peaks found in literature. XRD peaks at (111), (311), (400) and (440) represent the characteristic peaks of Fe_3O_4 nanoparticles.³² The subsequent formation of silica layer over the nanoparticle resulted in a silica broad peak between 15 to 35° and overshadowed the

Fe_3O_4 nanoparticle's peaks. The final chemical modification with $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to form the MgSiO_3 shell leads to the appearance of new peaks at 35° and 60° .

Figure 2. BET of YSN nanocomposite.

BET showed a Type IV adsorption/desorption isotherm pattern corresponding to the presence of cylindrical channel-like mesopores.³³ The pore volume and surface area of YSN was determined to be $1.389 \text{ cm}^3/\text{g}$ and $377 \text{ m}^2/\text{g}$ respectively. The average pore size was analyzed by Quantachrome software to be 1.9 nm. This small pore size meant that GOx loading on YSN is covalent in nature rather than being physically loaded into the pores. This is because the dimensions of GOx are $6.0 \times 5.2 \times 7.7 \text{ nm}^3$, which is too large to fit through the pores.³⁴

Figure 3. (Left to right) XRD and DLS & Zeta of nanocomposites at each successive fabrication step.

The coupling of APTES-GOx on YSN was characterized by TEM, Zeta, DLS, UV-Vis and fluorescence techniques. TEM image of YSN@GOx depicts the nanocomposite as having the same yolk-shaped structure as YSN but having an additional translucent layer enveloping the structure. This layer was made up of the organic GOx enzyme and further DLS hydrodynamic size change from 132 nm to 202 nm substantiate the claim. Although minor, the zeta potential slightly increased from -16.4 mV in YSN to -15.5 mV in YSN@GOx because of the coupling of the positively charged APTES-GOx.

Figure 4. (Left to right) UV-Vis absorbance showing the absence and presence of GOx in YSN and YSN@GOx. TGA comparison of YSN and YSN@GOx in GOx loading.

UV-Vis study highlighted the appearance of new peaks after the successful coupling of GOx. Fluorescence reading taken at 518 nm showed that the loading of FITC-labelled GOx is 4.16 wt.% with a loading efficiency of 41.6%. Direct visualization of the nanocomposites was done to study the change in the nanocomposite's solubility (Figure 5). It was found that when YSN was sonicated in aqueous solution, sedimentation starts to form at the 15 minutes mark. However, after modification with GOx, the YSN@GOx nanocomposite was able to remain

dispersed even after one hour. These studies highlighted that GOx had been successfully conjugated onto the silica surface of YSN.

Figure 5. Comparison of the aqueous solubility of YSN nanocomposite and YSN@GOx nanocomposite over a period of (from left to right) 0 min, 15 min, 60 min.

Nevertheless, the properties of the conjugated GOx should be analysed to ensure that hydrogen peroxide continued to be produced from glucose. The enzymatic activities were monitored by the $\cdot\text{OH}$ degradation of methylene blue, hydrogen peroxide assay, dissolved oxygen probe and pH probe techniques (Figure 6).

Figure 6. Characterizations of the catalytic performances of YSN@GOx. (Left to right) Production of H_2O_2 by YSN, YSN@GOx and free GOx in glucose solution (1 mg/mL). Consumption of O_2 by YSN, YSN@GOx and free GOx in glucose solution (1 mg/mL). Change in pH by YSN, YSN@GOx and free GOx in glucose solution (1 mg/mL). Change in methylene blue absorbance by YSN, YSN@GOx and free GOx in glucose solution (1 mg/mL).

Hydrogen peroxide (H_2O_2) analysis revealed that naked YSN does not produce any significant H_2O_2 overtime. This shows that YSN does not have any strong enzymatic property on its own. This observation is in stark contrast to YSN@GOx, where results showed that the relative amount of H_2O_2 produced by YSN@GOx nanocomposite was almost similar to that of free GOx overtime. Since the graph pattern matches that of free GOx, the enzyme GOx had been successfully grafted onto the surface of YSN. To further confirm the hypothesis,

YSN@GOx and its control were subjected to O₂ concentration probing study and pH analysis. This is because the enzymatic reaction of GOx uses up O₂ during the process and converts the neutral D-glucose to the acidic D-gluconic acid.³⁵ As expected, the concentration of O₂ overtime ranked in the following ascending order: The control, YSN, YSN@GOx and GOx. It was clear that O₂ was consumed by GOx enzyme to produce H₂O₂, as depicted by a sharp drop from 8.9 ppm to 8.6 ppm in YSN@GOx in 4 h. Similarly, there is a drop from 8.9 ppm to 8.4 ppm within 4h in free GOx. The trend was observed over 24h and was found to have remained unchanged. The pH of both YSN@GOx and GOx dropped drastically from between 6.5 – 6.7 to around 4.8 within 24 h. In contrast, the pH of YSN and D-glucose blank control remained constant at 6.4. Indeed, the D-glucose solution was consumed and converted to D-gluconic acid in YSN@GOx. In fact, the differentiation in pH across the samples started to happen quickly at the 4h mark, emphasizing the occurrence of the enzymatic process. Knowing that the first enzymatic process was successful, the presence of fenton reaction as the secondary enzymatic reaction was tested. Methylene blue is a common test for ·OH radicals. Methylene blue dissolves in water as a light blue solution but can be bleached in the presence of ·OH radicals.³⁶ The level of methylene blue absorbance remained constant for both the blank aqueous solution and solution incubated with GOx in the 75 minutes period that was tested. This meant that GOx and the H₂O₂ it produced did not generate any ·OH radicals. However, when GOx and YSN combined to form YSN@GOx, the degradation became a lot more significant. YSN@GOx showed a greater bleaching compared to YSN over a short period of time. Seemingly, the H₂O₂ produced by GOx did not cause any discolouration but the ·OH radicals did in both YSN and YSN@GOx. The most amount of degradation and corresponding amount of ·OH radicals produced indicated that YSN@GOx relied upon the additional H₂O₂ to produce more ·OH radicals in a cascade reaction.

Figure 7. In-vitro cytotoxic profile of GOx, YSN and YSN@GOx at varying GOx concentration.

The in-vitro studies were carried out to assess the effectiveness of fenton therapy by the individual components of YSN@GOx (Figure 7). According to the MTT assay results, the biocompatibility of YSN is high at 93 % even at the highest tested concentration of 10 mU mL⁻¹ GOx equivalence. The cell viability of free GOx was slightly lower at 78 % at the same concentration after 24h incubation. This finding showed that both components did not effectively kill cancer cells on their own. However, a drastic therapeutic effect was observed in the case of YSN@GOx, where cell survival dropped to 26 %. The same trend happened across all concentrations tested, highlighting the synergistic actions of both components in killing cancer cells. Comparison of IC₅₀ between GOx and YSN@GOx presented a great improvement in

therapy from 41 mU mL⁻¹ to 3.3 mU mL⁻¹.

Figure 8. Live/Dead cells apoptotic assay of control, GOx, YSN, YSN@GOx after 24h incubation with 10mU/mL GOx equivalent.

Flow apoptosis analysis of the therapeutic efficiency also supported the synergistic effect of the two components (Figure 8). A direct comparison across GOx, YSN and YSN@GOx highlighted the effect again with late apoptosis values of 3.50 %, 3.65 % and 97 % respectively. The early apoptosis value highlighted a less obvious but similar trend of YSN@GOx achieving the best therapy at 2.70 % in contrast to 1.13 % and 1.64 % in GOx and YSN. Although YSN can produce $\cdot\text{OH}$ radicals to increase the overall ROS level, it was not enough to induce apoptosis. It seemed to be the same for GOx, where the production of H_2O_2 did not impact the cells enough to cause any critical cancer cell death. However, a combinational treatment with both GOx and YSN in YSN@GOx can raise the ROS concentrations to a lethal level. From these two in-vitro studies, it can be concluded that YSN and GOx are biocompatible up to 10 mU mL⁻¹ but can serve as effective therapeutics when combined.

Figure 9. CLSM images of MDA-MB-231 cells incubated with FITC-labelled GOx and YSN@GOx after 2 h. Cell nuclei were stained by H33342. Scale bar: 20 μm .

Nevertheless, the good killing efficiency should be accompanied with good accumulation in cancer cells. The EPR effect of YSN@GOx was tested out in the same MDA-MB-231 breast cancer cell line. The 2 h accumulation confocal laser scanning microscopy (CLSM) observation showed that FITC-labelled free GOx does not accumulate within cancer cells while the FITC-labelled YSN@GOx can be endocytosed in good amount into the cytoplasm (Figure 9). This observation emphasized the importance of YSN as not only a source of iron to react with H_2O_2 , but as a vehicle for GOx to be internalized into cells for intracellular glucose starvation and H_2O_2 generation.

Figure 10. Flow cytometry profiles of MDA-MB-231 cells incubated with FITC-labelled GOx and ORMOSIL@GOx after 2 h.

Flow cytometry analysis further validate the point as seen in the diagram where more cells with higher intensity of FITC was measured compared to the blank control and free GOx. This analysis highlighted the effective internalization of YSN@GOx into cancer cell line (Figure 10).

While the successful accumulation and therapeutic efficiency were achieved, it is also important to determine the mechanism of therapy involved. The GOx coupled to YSN starts off by catalysing the β -D-glucose into β -D-glucono-1,5-lactone and H_2O_2 . The H_2O_2 generated will not only raise the overall level of ROS in MDA-MB-231 cancer cells but also provide an additional source of H_2O_2 for the iron to do fenton reaction. The fenton reaction will convert the non-radical H_2O_2 into a more potent form of ROS

in the form of $\cdot\text{OH}$ radicals.

Figure 11. CLSM images of MDA-MB-231 cells after co-incubation with PBS, GOx, YSN and YSN@GOx in 1 mg mL^{-1} glucose DMEM high glucose solution for 4 h and subsequently stained with ROS fluorescence probe DCFH-DA.

To confirm that the as-fabricated YSN@GOx is a far better ROS producer, a ROS fluorescence probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was first used (Figure 11). It can be interpreted that the higher the green fluorescence, the higher the level of intracellular ROS. As seen in the CLSM images, the blank control have a small amount of natural ROS within cells and were relatively low comparable to the fluorescence generated in YSN. The fluorescence in YSN is 1.7 times brighter than that in the blank control. This increase in fluorescence meant that YSN alone did produce ROS from the conversion of small amounts of H_2O_2 , but did not produce a substantial amount in MDA-MB-231 cells.

However, an improvement in overall ROS level could be observed in free GOx likely due to the ability for GOx to generate H_2O_2 in the extracellular matrix where high glucose DMEM medium was used. The fluorescence in free GOx is 2.5 times brighter than that in the blank control. The externally produced H_2O_2 can then diffuse into cancer cells since it is membrane-permeable. Looking at YSN@GOx, the highest level of green fluorescence was generated. The combination of GOx enzymatic actions to produce H_2O_2 with the fenton reaction from the Fe_3O_4 core was able to enhance the overall ROS level to a toxic stage. This was evident from the 16.6 times increase in green fluorescence compared to the blank control. As expected, the combination of YSN as an effective delivery vehicle for GOx and the ability of GOx to enhance the level of ROS contributed to the result.

An in-depth probe into the type of ROS was conducted. Coumarin exists as a colourless compound but can react with $\cdot OH$ radicals to produce a blue coloured compound known as 7-hydroxycoumarin.³⁷ Coumarin at 100ppm was doped into each control and YSN@GOx within the confocal plates for 12h. The fluorescence of the blank control, GOx and YSN were negligible under CLSM observation. However, a distinct blue fluorescence could be seen in the cells incubated with YSN@GOx. This distinction highlighted the substantial amount of $\cdot OH$ radicals that can be produced if more hydrogen peroxide could be supplied for fenton reaction. Interestingly, the morphology of the cells incubated with YSN@GOx was more clumped. This was attributed to greater level of cell apoptosis happening from increased amount of toxic $\cdot OH$ radicals. Using ImageJ software, analysis showed that the fluorescence was 0.8 times in GOx, 1.2 times in YSN and 30 times in YSN@GOx. The lack of blue fluorescence in YSN could be explained by the low concentration used in cancer cells. This could be greatly improved by the added supply of H_2O_2 which could in turn be converted

to $\cdot\text{OH}$ radicals.

Figure 12. CLSM images of MDA-MB-231 cells after co-incubation with PBS, GOx, YSN and YSN@GOx in 1 mg mL^{-1} glucose DMEM high glucose solution for 12 h and subsequently stained with $\cdot\text{OH}$ fluorescence probe coumarin (100 ppm).

4.4 Conclusion

In summary, a glucose-depleting YSN@GOx nanocomposite was successfully fabricated. The nanocomposite can accumulate passively via EPR effect and convert intracellular glucose supply to the hydrogen peroxide ROS, which in turn undergo a cascade fenton reaction to eventually form $\cdot\text{OH}$ radicals for synergistic anticancer treatment. Furthermore, it does not rely on any external input or drugs which offer an easy method for cancer therapy. More interestingly, in-vitro experiments had proved that the strategy of exploiting fenton reaction for therapy can be used in yolk-shaped silica material. This work provides an alternative platform for fenton therapy and is expected to pave the way for new strategies in the field of cancer treatment.

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CONCLUSION AND OUTLOOK

Silica-based nanocomposite has great potential in not only accumulating in cancer cells but producing ROS for effective therapy. Silica's tunable properties and favorable biocompatibility in cells open up a world of novelty when it comes to fabrication of ROS-mediated therapeutic nanocomposites. The scalability and reproducibility also meant that if it made it into clinical applications, the money and time cost can be drastically reduced.

Nonetheless, silica-based nanomaterials have been poorly explored when it comes to ROS therapy because the concept of ROS overloading has been new.

The wish is that, this dissertation can contribute in laying a good foundation for ROS therapy through a proof-of-concept via new ligand additions and integration with transition metal nanoparticles. This is demonstrated by the three projects found in the 2nd, 3rd and 4th chapter in this thesis.

The first project highlighted the effectiveness of combinational ROS production through the use of a ROS-producing nanoreactor and a photosensitizer. In-vitro as well as *in-vivo* studies showed that the combination of the nanoreactor and photosensitizer is most effective in treating cancer. As seen in Chapter 2, Figure 29, tumor sizes were substantially reduced in mice that were subjected to the combinational treatment.

The second project proved that silica can be modified via the addition of novel ligands to create new nanocomposites. The nanocomposite can lower ROS threshold while increasing ROS to kill cancer. As seen in Chapter 3, Figure 16, there is a 7.6 times increase in cancer

killing efficiency as compared to free GOx. This implied that the strategy of lowering ROS threshold can be highly effective in treating cancer.

The final project showed that biological catalyst like GOx can be used easily on the tunable silica surface to indirectly enhance ROS production and lethality. Briefly, we found that the H_2O_2 generated by GOx can be converted by the silica-based nanoreactor into the more toxic $\cdot\text{OH}$ radicals. In fact, the IC_{50} dropped from 41 mU mL^{-1} in GOx to 3.3 mU mL^{-1} in the final YSN@GOx nanocomposite.

Still, future work should seek to improve the antioxidant suppressing ability and solubility of the as-synthesized nanocomposites. ROS-mediated therapy using silica-based nanocomposites is still in its infancy and holds huge potential in solving many of the issues still plaguing the field of nanomedicine.

List of Publications

1. Y. Su, S. Z. F. Phua, Y. Li, X. Zhou, D. Jana, G. Liu, W. Q. Lim, W. K. Ong, C. Yang and Y. Zhao, *Science Advances*, 2018, **4**, eaas9732.
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3. W. K. Ong, M. F. Wong, C. Y. Ang and Y. Zhao, *Journal of Controlled Release*, 2017, **259**, e132.
4. W. K. Ong, D. Jana and Y. Zhao, *Chemical Communications*, 2019, 55, 13374-13377.

Manuscript In Preparation

1. Investigation of Fe₃O₄ nanoparticle as a hyaluronic yolk-shaped nanoreactor for targeted ROS-mediated therapy *in vivo*.

APPENDIX

Chapter 2.

Scheme S1. Schematic illustration of the successive fabrication of the YSN@ZnPc@HA nanocomposite.

Chapter 3.

Scheme S1. Schematic illustration of the conjugation mechanism between glucose oxidase and silica nanoparticles. Briefly, glucose oxidase have $-\text{COOH}$ groups for conjugation with the $-\text{NH}_2$ group of Si-PE. The resulting modified glucose oxidase can then use the $-\text{Si}(\text{OEt})_3$ groups for conjugation with the silica nanoparticles via silica hydrolysis.

Figure S1. ^1H NMR (top) and ^{13}C NMR (bottom) spectra of Si-PE.

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

17 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 23-23 H: 41-41 B: 0-1 N: 0-1 O: 7-7 127I: 0-2 Si: 0-1

C23H40BNO7Si

ZYH-9 6 (0.129)

1: TOF MS ES+
3.46e+002

Figure S2. HRMS of Si-PE.

Chapter 4.

Figure S1. TEM of monodispersed Fe_3O_4 nanoparticles (left) and $\text{Fe}_3\text{O}_4@\text{SiO}_2$ (right), before solvothermal treatment to form YSN.