Polymeric nanoparticles and organic fluorophores for cancer sensing and therapy

Ang, Chung Yen

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**Polymeric nanoparticles and organic fluorophores for cancer
sensing and therapy**

Ang Chung Yen

**School of Physical and Mathematical Sciences/Division of Chemistry and
Biological Chemistry**

2016

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Chemistry and Biological Chemistry

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Abstract

Cancer has been ranked as one of the top killer diseases around the world. Traditional therapeutic strategies often result in high side effect accompanied by the relatively low rate of survivability. More recently, various nano materials have been employed as delivery vehicle for the therapeutic agent, aiming to improve the therapeutic efficiency and at the same time, reducing the side effect experienced by the patient. Therefore, this research dissertation aims to provide a review on the various types of nanomaterials used in the biomedical field for cancer therapy application. With this, we have presented the synthesis and application of a polymer based nanoparticles for drug delivery application. We have extended this work to the in-vivo system and the nano formulation showed a positive effect on the tumor bearing nude mice.

Apart from tackling the disease by developing an efficient system for therapeutic purposes, diagnostic strategies developments are also a key aspect that the biomedical societies are focusing on. In various research, it was found that the intracellular compartment of cancer cells express high level of glutathione (GSH). Therefore, the detection in the rise of GSH level in the cellular compartment could serve as an early indication of cancer. One of the common ways in the qualitative detection of GSH is by means of using a reaction base fluorescence probe. Fluorescence probe allow us to detect the target analyte in a highly selective manner without the use of sophisticated instruments. In the second part of this dissertation, we have presented the development of two different fluorescence probes for the

detection of GSH and other biological thiol. In the first work, we have loaded the probe in a nano formulation that has shown positive response towards GSH in in-vitro environment. In the second work, we have designed a fluorescence probe that could detect biological thiol and hydrogen peroxide in an orthogonal fashion without compromising its selectivity. This could even be achieved with the use of a single excitation source and the generation of a different output depending on the analyte presence in the solution of interest. With this added advantage, the probe could also be used to detect H_2O_2 , which research has also shown that the H_2O_2 level also have certain correlation to other diseases.

With these three chapters, this dissertation is then concluded. Future work could then be geared towards the development of a single nanoparticles system that encompassed both therapy and diagnostic functionality. In this way, it was realized that the polymeric based nanoparticles are highly customizable whereby a total different functionality could be expressed by a simple change in the building component. Therefore, much work could be done to explore the various possible applications with this class of novel materials.

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Chapter 1: Introduction

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REVIEW ARTICLE

Yanli Zhao et al.

Recent advances in biocompatible nanocarriers for delivery of chemotherapeutic cargoes towards cancer therapy

A part of this chapter is adapted from an article published in Organic and Biomolecular Chemistry, Royal Society of Chemistry. I have contributed to the writing of the main part of the review and the article is adapted from

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1.1. Nanomaterial as promising tool for cancer treatment.

Cancer has been listed as the leading cause of death worldwide. Based on the information provided by the World Health Organization (WHO), it has accounted for a total death of 7.6 million (about 13% of all death) within the year of 2008 and 8.2 million within the year of 2012.¹ With this, there is an urgent need to address this issue and hoping to develop an efficient strategy to control this epidemic. Traditionally, cancers are being managed by the surgical removal of the tumor before the anticancer drugs are being administered, in hope to eradicate the remaining metastatic cancer cells. However, due to the lack of targeting properties of these anticancer drugs, the direct administration of anticancer drugs often brought about serious side effects to the patients (such as hair loss, loss of appetite, nausea etc...) and thus generated tremendous amount of suffering to the patient. Despite of these negative side effects that are being experienced by the patient, the chances of recovering and the success in the treatment are not always guaranteed. This is particularly for patients who are diagnosed with cancer at terminal stage when the cancer cells have migrated or metathesized to the various organs.^{2,3} In addition, many cancers do not show signs or symptoms in the early stages and this thus further increases the complication in combating this notorious diseases and reduce the chances of survival.

We have mentioned above that chemotherapy is usually being included during the course of cancer treatment and this mode of treatment usually brought upon various unpleasant side effect to the cancer patient. The use of

these anticancer drugs also allow the cancer cells to develop multidrug resistance (MDR) properties, thus making the drug ineffective in its treatment overtime.⁴ Furthermore, most of these anticancer drugs have limited solubility in biological system, which thus greatly reduces its pharmacokinetic properties. One of the approaches to this issue is the development of alternative treatment strategies. For instance, the advancement in photochemistry allows the utilization photodynamic and photothermal therapy to be applied for cancer treatment.⁵⁻⁷ This strategy could provide a localized and remote generation of phototoxic agent for cancer irradiation, thereby reducing the side effect of cancer therapy. Similar to the various anticancer drugs used, the organic nature of these photoactive agents reduces its solubility in biological environment, rendering their poor pharmacokinetic properties. One proposed solution to tackle this issue is the reliance of nanomaterials for target specific delivery and release of anticancer drug or photoactive agent into the tumor site or cancer cells.⁸⁻¹⁷ During this process, the delivery vehicle would increase the circulation lifetime of the cargo in the blood stream of patient and improve the biocompatibility of these agent. In addition, the target specific delivery of these nano vehicles into the tumor site or cells would improve the therapeutic efficiency accompanied by the reduction of the side effect brought about by the therapeutic process.

Figure 1. Applications of nanocarriers for the delivery of various cargoes for cancer therapy. Properties such as targeting and stimuli-responsive molecular switches can be integrated to customize the nanocarriers for applications.

The tremendous advancements in nanobiotechnology and material synthesis over the past decades have inspired the biomedical field to develop various novel designs of these delivery vehicles (Fig. 1). Other than performing their primary role as a delivery vehicle, these novel designs also possess “smart” properties such as the ability to release drug upon a remote trigger or conjugated with targeting ligand for the site specific release or even incorporated sensing moiety to fulfill the additional purpose of diagnostic applications. Hence, a careful engineered delivery design is an additional aspect to be considered in combating cancer apart from the mode of treatment to be utilized.

As of current stage, there are various successful examples of using these nanomedicines in cancer therapy. In fact, some of these drug-loaded nanovehicles have even pass through the third stage of the clinical trial and approved by the Food and Drug Administration (FDA) for commercial usage.^{9,18-21} One notable example is the Doxil[®], which has been available in the market since 1998. Doxil[®] is a liposome-encapsulated doxorubicin (DOX) and

was developed mainly by Gabizon and Barenholz.²² The primary aim for the development of Doxil[®] was to reduce the side effect, especially cardiotoxicity accompanied by the direct use of DOX during chemotherapy. Upon encapsulation of the drug in the liposome formulation, the DOX could escape the reticuloendothelial system (RES) and hence prolong the biodistribution of the drug. Even though Doxil[®] is not designed with a targeting ligand, the size of Doxil[®] (~100 nm) renders it suitable to be targeted to the tumor site by means of enhanced permeability and retention (EPR) effect. This effect allows retention and accumulation of the drug loaded liposome and the subsequent release of the anticancer drug in the tumor site.

Doxil[®] is by far one of the best anticancer drug formulations available in the market due to its strategic design that allowed the enhancement in its therapeutic effect accompanied by the reduction in side effect in the treatment process. As mentioned above, this can be achieved by the encapsulation of the therapeutic cargo within a delivery system. Thus, it is of utmost importance to understand the various types of delivery system that is currently being widely used in cancer therapy. This chapter would give a detailed introduction and discussion on the various types and properties of delivery systems used in combating cancer.

1.2. Classes of Nanomaterials

Cancer treatment that relies on nanocarrier usually includes the polymer-based nanoparticles, liposomal based nanopackages, and inorganic nanomaterials such as gold nanoparticles and mesoporous silica nanoparticles

(MSNPs). These materials are first loaded with anticancer agents, before delivering them to the tumor cells. This process improves the bioavailability of the agents and since they are design to deliver the payload to the cancer cells, the side effects from the usage of these agents can be reduced considerably. However, the type of the nanomaterials used differs from one to another. Hence, we also have to consider the advantages and drawbacks of a particular choice of nanomaterials in designing a delivery system. In this section, we will review the synthetic approaches used for some representative carriers and discuss how these carriers have been employed for delivery applications.

1.2.1. Polymer based nanocarriers

Polymer based nanocarriers have received tremendous amount of attention over the past decades owing to their versatility in its synthesis and functionalization.^{8,23-26} Furthermore with the development of some biodegradable polymer backbones, these classes of materials are well accepted by the medical community, particularly in response to the concerns of their fate after performing the delivery job. Thus, polymer based nanocarriers are often chosen as the delivery vehicle for various agents ranging from gene to drugs.²⁷⁻

Figure 2. Chemical structure and synthetic scheme for the formation of IT-101 nanocapsule in the delivery of camptothecin anticancer drug. Inset shows the electronic micrographs of the NPs. (Reproduce with permission from Ref. 36. Copyright 2009 Elsevier.)

Polymer based NPs are usually synthesized via the self-assembly technique, in which the nanoparticles are formed to achieve thermodynamic stability.³²⁻³⁵ One notable example is the IT-101 system developed by Davis and co-workers and this system is the first therapeutic design that has achieved the clinical trials for cancer therapy (Fig. 2).^{36,37} The polymeric nanoparticles system is synthesized by the self-assembly of a polymer containing an alternating segments of cyclodextrin and polyethylene glycol (PEG) together with functional carboxylate groups. Thereafter, the drug camptothecin (CPT) was conjugated onto the carboxylate group to form the polymer–drug conjugate. The polymer was then dispersed in water where the CPT formed an inclusion complex with the cyclodextrin ring, and hence the polymer self-assembled into polymer based NPs. The as-prepared NPs are spherical in shape and have a size range from 30 to 40 nm.

Figure 3. (a) Scheme showing the self-assembly process of PEI–adamantine conjugate for the delivery of drugs and genes. (b) SEM and TEM images of the polymeric nanocarriers. (Reproduce with permission from Ref. 28. Copyright 2011 Royal Society of Chemistry.)

Figure 4. Schematic illustration for the supramolecular self-assembly of polyamine based NPs. (Reproduce with permission from Ref. 42. Copyright 2009 Johnson Wiley and Sons.)

The drug delivery system as mentioned above relies on the formation of an inclusion complex between the drug and the cyclodextrin ring that is pre-conjugated onto the polymer chain. Due to the presence of a hydrophobic core, the cyclodextrin ring is capable of hosting a great variety of hydrophobic guest molecules such as azobenzene,³⁸ heterocyclic aromatics^{39,40} as well as bulky

alkanes⁴¹. In this way, the NPs can be prepared by the self-assembly of cyclodextrin-containing polymers with guest-containing polymers or drugs. In a recent work reported by Li et al., β -cyclodextrin was first conjugated onto a branched polyethylenimine (PEI), followed by the complexation of adamantane conjugated doxorubicin with the cyclodextrin on PEI (Fig. 3a).²⁸ Thereafter, the doxorubicin grafted PEI was combined with a DNA plasmid to achieve both drug and gene delivery both *in vitro* and *in vivo*. In this work, the authors have used transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to characterize the shape and size of the as-prepared NPs. From the electron microscope images, it was observed that the NPs have a polypetalled shape, having particle sizes from 200 nm to 500 nm with a relatively high polydispersity (Fig. 3b).

Another interesting concept of using the supramolecular self-assembly technique for the preparation of polymer based NPs was reported by Tseng et al. In this system, the NPs were prepared by the self-assembly of β -cyclodextrin conjugated PEI, adamantane conjugated PEG, and adamantane conjugated poly(amido amine) (PAMAM) (Fig. 4).⁴² Interestingly, this system has the advantage of controlling the particle size by changing the ratio of different components. The work showed the successful preparation of NPs with sizes of 30 nm and 100 nm in a good polydispersity. Furthermore, the as-prepared NPs served as good vectors for drugs and genes towards various interesting applications.⁴³⁻⁴⁵

Supramolecular interactions are interactions that are held by weak non-covalent interaction such as Van Der Waals forces, hydrogen bonding, dipole-dipole interaction or even metal ligand bonds. Due to these weak interactions,

supramolecular bonds are reversible dynamic bonds whereby the supramolecular assembly system could rearrange themselves into a new state with the lowest Gibbs free energy and hence a more thermodynamically stable state. As mentioned above that the guest/host complex involving cyclodextrin are commonly being employed in nanoparticles design as the hydrophobic guest could form a strong complex with cyclodextrin in aqueous environment. In aqueous environment, the complexation of the guest causes the water molecules to be expelled out of the cavity of the inner core of the cyclodextrin. The overall net result is the increase in entropy to the surrounding of the complex, thereby achieving a more thermodynamically favored state. This therefore concluded the mechanism explanation of the binding mechanism.

1.2.2. Dendrimers as drug and gene delivery vectors.

Figure 5. (a) Computer simulated conformation of the DOX-poly-L-lysine complex. (b) Conformation diagram of the DOX-poly-L-lysine complex with the interpolated charges. The blue surface represents the positive charges of poly-L-lysine. (Reproduce with permission from Ref. 60. Copyright 2013 American Chemical Society.)

Dendrimers belong to another class of organic material that has received significant amount of development in terms of its synthesis and applications.⁴⁶⁻⁵⁶ Recently, these classes of materials have emerged as promising candidates for biomedical applications such as for drug and gene delivery.^{53,55-57} As

compared with linear polymers, controlled multivalent dendrimers have the ability to host guest molecules (e.g., drugs, targeting ligands and solubilizing molecules) with different functionalities in a controlled fashion, thereby improving their delivery efficiency.^{48,58,59} In addition, the presence of a variety of functional groups on dendrimers impart conjugation flexibility, such that the guest entities could be functionalized onto dendrimers in many ways ranging from covalent to non-covalent methods.^{48,53}

In one study reported by Al-Jamal and co-workers, a poly-L-lysine dendrimer was used as a delivery vehicle for anticancer drug DOX.⁶⁰ The authors proposed that the DOX molecule complexes with the dendrimer through hydrogen bonding interaction (Fig. 5). In addition, the authors used molecular simulation and ¹H NMR spectroscopy to confirm the complexation between the drugs and the dendrimer. Thereafter, the drug delivery efficiency of the drug/dendrimer complex was investigated both *in vitro* and *in vivo*. The obtained results indicate that the complex could exhibit an enhancement in therapeutic efficiency as compared with that of DOX or dendrimer alone. It was further concluded that the dendrimer has good potential as a delivery vehicle for different types of therapeutic cargoes.

1.2.3. Liposome based nanocarriers

Liposomes are vascular NPs that are formed from the dispersion of amphiphiles in aqueous environment. Due to the entropy effect, the water molecules are expelled from the hydrophobic region to form a well-defined hydrophilic domain. Although the neat arrangement of the lipid bilayer does reduce the entropy of system, the overall expelled of the water molecules to the

surrounding increases the entropy of the surrounding which cause a net increment of the overall entropy. Similar to the discussion above on the cyclodextrin binding effect, the net increment of the entropy increases the thermodynamic stability, which therefore is a favorable process. In order to further minimize the interaction of the nanocarriers with the solvent molecules, the nanocarriers adopt a spherical shape having a maximum surface tension on the surface of the nanocarriers. Liposomal nanocarriers contain an aqueous core separated by a bilipid layer from the external aqueous solution, and the cargo is loaded within the aqueous core of the liposome. To date, there are several US FDA approved drugs that are actually in the form of liposomes, including the famous Doxil[®] as mentioned previously.^{18,19,61} The reason for this is due to the ease fabrication of the liposomes, and very often they are made of biodegradable materials, which render them suitability for *in vivo* applications.⁶²

Figure 6. Chemical structures of (a) poly(lactic-co-glycolic acid) (PLGA) and (b) polyethylene glycol (PEG).

One example of a liposome vesicle is poly(lactic glycolic acid) (PLGA) based nanomaterials. As a hydrophobic polymer, PEG modified PLGA could self-assemble into a liposome effectively. Being a polyester polymer, PLGA is biodegradable in nature, and hence becomes a popular polymer for designing a liposome delivery vehicle (Fig. 6a). On top of imparting hydrophilicity to the liposome, the PEG moiety (Fig. 6b) also provides the liposome with steric

hindrance to prevent the interaction of the liposome with blood proteins, thereby improving the bioavailability of the liposome.⁶³⁻⁶⁶

One useful concept was designed by Langer et al. in creating a liposome based nanocarrier.⁶⁷ In this work, lidocaine drug was loaded into the liposome, and then its bioavailability was investigated in mouse models. From these studies, they found that loading the drug into the liposome could greatly reduce the accumulation of the drug in the liver and dramatically increase the blood circulation time. In addition, a remarkably high loading capacity of the drug up to a value of 45% by weight was reported. In the same work, they also synthesized a series of liposomes by varying the PEG length. Interestingly, they observed that the blood circulation time of the liposomes increases when increasing the length of the PEG unit.

Figure 7. Schematic representation of HSPC, cholesterol and DOTMA based liposomes for inducing necrosis of cancer cells. Top left figure shows a TEM image of NH_4CO_3 loaded liposome. (Reproduce with permission from Ref. 68. Copyright 2012 Johnson Wiley and sons.)

Other than relying on PLGA and PEG based polymers for the preparation of the liposomes, other biocompatible materials could also be

employed. A recent work reported by Sung and co-workers used a mixture of hydrogenated soy phosphatidylcholine (HSPC), cholesterol and 1,2-*O*-dioctadecenyl-3-trimethylammonium propane (DOTMA) in phosphate buffered saline (PBS) for the preparation of liposomes (Fig. 7).⁶⁸ In this work, a simple inorganic reagent, NH_4HCO_3 , was loaded into the liposome, which was internalized into the intracellular domain of cancer cells. The as-prepared liposome was characterized accordingly and the results showed that the size of the liposome was around 295.0 nm with a surface potential of 41.1 ± 1.3 mV.

Another work reported by Szoka et al. utilized a disteryl modified phospholipid for the preparation of a liposome based nanocarrier.⁶⁹ In this work, they successfully loaded doxorubicin into the liposome, and demonstrated remarkable therapeutic effects of doxorubicin-loaded liposome, in which the efficiency was equivalent to Doxil®.

1.2.4. Inorganic nanocarriers

Over the years, inorganic nanocarriers have emerged as a potential and promising candidate for biomedical applications due to its relative ease of synthesis which possesses interesting properties. Many exciting results have been reported regarding their applications as drug delivery vectors. This section introduces some representative inorganic nanocarriers.

1.2.4.1. Mesoporous silica nanoparticles.

Figure 8. Schematic diagram illustrating the template assisted synthesis of mesoporous silica NPs. (Reproduce with permission from Ref. 70. Copyright 2014 Royal Society of Chemistry.)

Mesoporous silica NPs are a class of inorganic nanomaterials possessing porous structure and high surface area. This class of nanomaterials has been widely used as delivery vehicles for a number of therapeutic cargoes in cancer therapy.^{11,71-76} Mesoporous silica NPs are synthesized via the template assisted technique, where cetyltrimethylammonium bromide (CTAB) serving as the template for the pore formation of the NPs arranges itself into cylindrical arrays and directs the formation of a silica wall surrounding the arrays (Fig. 8). Thereafter, the micelle templates are removed and the resulted channels are used for drug loading towards delivery. The main advantage for the utilization of mesoporous silica NPs as delivery vectors is their remarkably high surface area ($>1000 \text{ m}^2\text{g}^{-1}$), which implies that a large amount of cargo can be loaded into mesoporous silica NPs for delivery applications. In addition to the large surface area, the silanol groups on the surface of the NPs allow for the grafting of various functional groups on the surface. Thus, various interesting ligands

and gatekeepers can be conjugated. The purpose of using the gatekeepers is to keep the loaded cargos within the pores of the NPs, and then release them upon a certain trigger or activation.

1.2.4.2. Gold nanoparticles.

Figure 9. (a) TEM images of Au NPs of various sizes: (i) 20 nm, (ii) 40 nm, (iii) 60 nm and (iv) 80 nm. (b) Photographs of Au NP solutions with different sizes. (c) UV-Vis absorption spectra of Au NPs with different sizes. (Reproduce with permission from Ref. 83. Copyright 2007 American Chemical Society.)

Gold NPs (Au NPs) are another class of materials that are widely used in various biological applications.⁷⁷⁻⁷⁹ On top of being non-toxic and non-immunogenic, Au NPs also exhibit interesting optical properties that allow the combination of several functions (e.g., drug delivery, sensing, and photothermal therapy) in a single system. In view of this capability, some Au NP-based systems have reached the clinical trial stage, demonstrating their incredible application potential.⁸⁰ Au NPs are typically synthesized by a simple reduction of an aqueous gold salt (such as tetrachloroaurate) in a controlled manner, yielding NPs with various sizes and shapes.^{81,82} Since Au NPs have the surface Plasmon resonance effect, the size of the NPs can directly influence their UV-

Vis absorption.⁸³ Hence, one can correlate the UV-Vis absorption or color of an Au NP sample to the size of the NPs (Fig. 9).

The as-synthesized Au NPs are usually stabilized by ligands added during the synthesis process. These ligands can either be used directly for further functionalization or be easily replaced by strong chelating ligands.⁸⁴⁻⁸⁶ In many cases, mercaptans are used as the ligands for the Au NP functionalization, since the thiophilic properties of Au could result in relatively strong bond formation between the thiol ligands and the Au NP surface. Thus, mercaptan ligands with various functionalities or properties could be easily introduced onto the Au NP surface by simple ligand exchange reactions. For instance, in a study reported by Brown et al., the anticancer drug oxaliplatin was anchored onto the Au NP surface through the use of lipoic acid ligand.⁸⁷ The authors have demonstrated that the Au NPs could successfully deliver oxaliplatin into the nucleus of cancer cells, indicating that the Au NPs have the capability to serve as the drug delivery vehicle.

1.2.4.3. Iron oxide nanoparticles.

Figure 10. TEM images of (a) γ -Fe₂O₃ NPs (Reproduced with permission from Ref. 102. Copyright 2009 John Wiley and Sons.) and (b) Fe₃O₄ NPs (Reproduced with permission from Ref. 107. Copyright 2004 American Chemical Society.). These two images clearly show that the size of γ -Fe₂O₃ NPs is significantly smaller than that of Fe₃O₄ NPs. (c) Magnetization curve of Fe₃O₄ NPs coated with a layer of mesoporous silica shell. The inset shows the photographs of Fe₃O₄ NPs captured by a 0.4 T magnet, which indicate that the dispersion and capturing processes of the NPs are reversible. (Reproduced with permission from Ref. 93. Copyright 2009 Royal Society of Chemistry.)

Another class of inorganic nanomaterials that has been employed as drug carriers is iron oxide NPs.⁸⁸⁻⁹⁰ Since the physical size of iron oxide NPs is much smaller than the ferromagnetism domain size of its bulk material, iron oxide NPs exhibit superparamagnetism properties.^{91,92} When an external magnetic field is applied to the NP samples, there would be an immediate saturation of magnetization, which is in alignment to the vector of the external field (Fig. 10).^{93,94} In addition, the primary elemental compositions of iron oxide NPs are none other than iron and oxygen. In view of this, iron oxide NPs are known to be biocompatible and even biodegradable.^{95,96} Thus, iron oxide NPs could serve as excellent contrast agents for magnetic resonance imaging (MRI) applications.⁹⁷⁻⁹⁹

Iron oxide NPs exist in two main forms, namely maghemite ($\gamma\text{-Fe}_2\text{O}_3$, Fig. 10a) and magnetite (Fe_3O_4 , Fig. 10b). These two types of NPs could be synthesized by three methods including the co-precipitation method, thermal decomposition of organoiron reagents, and the microemulsion technique.^{91,94,100-103} These methods produce iron oxide NPs in the size range from 2 to 17 nm, depending on the synthetic method and conditions used. In this section, we would not discuss the synthesis and properties of these materials in detail and these discussions can be found in several excellent review papers.^{90,100,101} In biological applications, Fe_3O_4 NPs are often preferred over $\gamma\text{-Fe}_2\text{O}_3$ NPs as Fe_3O_4 NPs have the ability to achieve higher magnetization saturation and magnetic susceptibility.¹⁰⁴ In other words, the magnetic saturation of Fe_3O_4 NPs could be achieved with the application of lower external magnetic field.

The Fe atoms on the surface of iron oxide NPs could act as an excellent Lewis acid for interacting with a variety of oxygen containing electron lone pair donors. For instance, when iron oxide NPs are dispersed in an aqueous environment, the water molecules act as ligands to chelate onto the NP surface. The consequence of such chelation changes the properties of the NPs. In addition, other types of functional groups such as carboxylate, phosphate, sulfate and phenoxylate can also bind with the Fe atoms effectively for further functionalizations of the NPs.¹⁰⁰ On top of using these molecular ligands for the functionalizations, macromolecules and inorganic materials such as silica are also often used to coat the iron oxide NPs, so as to impart higher stability and bio-availability on the NPs.¹⁰⁵⁻¹⁰⁸

To further elaborate the applicability of the iron oxide NPs for drug delivery and MRI imaging, a recent work reported by Yu et al. was selected as an example.¹⁰⁹ In this work, the authors prepared thermally cross-linked polymer coated Fe₃O₄ NPs followed by loading the anticancer drug DOX into the polymer network. The obtained nanocomposite was then injected intravenously into mice to perform T2-weighted MRI imaging. The imaging results showed that there was an accumulation of the nanocomposite in the tumor after 4.5 h from the time of the injection, indicating excellent uptake of the nanocomposite by the tumor as well as its high performance as an MRI contrast agent. Thereafter, the authors also studied the therapeutic efficiency of the nanocomposite and observed that the nanocomposite displayed remarkable tumor inhibition efficiency up to 63% relative to the control group. This work indicates that functional iron oxide NPs could perform as not only the MRI contrast agents, but also the drug delivery vehicle.

1.2.4.4. Quantum dots.

Figure 11. (a) Color photographs of UV-excited CdSe QD solutions. The QD solutions were excited by 365 nm UV light. (b) TEM images of CdSe QDs with maximum emission wavelengths ranging from 525 to 590 nm. (c) UV-Vis absorption spectra and (d) emission spectra of QDs synthesized under various conditions. The longer the growth time, the larger QD size produced. (Reproduced with permission from Ref. 111. Copyright 2013 IOP Publishing.)

Semiconductor nanocrystals or quantum dots (QDs) are a class of inorganic nanomaterials that have also been widely used in the biomedical field.¹¹⁰⁻¹¹³ This class of emissive nanomaterials exhibit interesting optical properties and are more stable from photobleaching than classical organic fluorophores.¹¹² In addition, QDs have narrower emission spectra than the organic fluorophores, allowing multicolor imaging without obvious overlapping in the emission signals when QDs with different colors are used simultaneously. Since the physical size of QDs is smaller than the Bohr radius of bulky materials, they exhibit the quantum confinement effect whereby their bandgap is strongly dependent on their size.¹¹³⁻¹¹⁵ Thus, the emission wavelength of QDs could be tuned by simply changing the size of the nanocrystals (Fig. 11).¹¹¹

Among many QDs developed, Cd-containing QDs, such as CdSe and CdTe, have received a great deal of attention, because the emission wavelengths of Cd based QDs fall in visible region.¹¹⁶⁻¹¹⁹ Various papers have reported the bioimaging applications of this type of nanomaterials.¹²⁰⁻¹²² Some studies have shown that growing a shell of higher bandgap materials over the QD cores (e.g. CdSe/ZnS) can further improve the luminescence efficiency and photostability.¹²³ Cd based QDs could be synthesized by either the high-temperature organometallic method or the aqueous synthesis method, producing Cd chalcogenide QDs in the size range from 3 to 4 nm.^{124,125} Similar to Au NPs, the as-synthesized QDs are stabilized by the ligands added during the synthesis process, allowing the QDs to grow in a controlled manner.^{126,127} These ligands can either be functionalized directly through chemical reactions or be replaced with other ligands for further modifications.¹²⁸⁻¹³¹ The surface of the QDs contains the Cd atoms, which act as good coordination sites for Lewis bases.

Typically, thiol groups are selected as suitable chelators for binding with the Cd atoms on the surface of the QDs.¹³² However, some studies have indicated that the ligand exchange process might cause a decrease in the luminescence efficiency of the QDs, especially for CdSe QDs.¹³³⁻¹³⁵ Hence, the compatibility of a ligand with the QDs is an important factor to be considered in functionalizing the QDs for applications.

In a work reported by Farokhzad et al., the authors have functionalized the QD surface with the A10 RNA aptamer followed by intercalating anticancer drug DOX within the aptamer network.¹³⁶ In this system (QD-Apt(Dox)), the energy from the QD emission was transferred to the DOX molecule through the Förster Resonance Energy Transfer (FRET) process, and the absorbed energy from the DOX molecule was then transferred to the RNA aptamer through a secondary FRET process. The net result of this bi-FRET system is total fluorescence quenching, indicating the successful loading of DOX within it. When QD-Apt(Dox) was delivered into cancer cells, the loaded DOX was gradually released from the aptamer network, leading to the fluorescence recovery of both DOX and the QDs. Thus, the QDs in the QD-Apt(Dox) system serves as both a fluorescence tracer and an indicator for the real time monitoring of the DOX release.

Most of the QDs used for biomedical applications are Cd based QDs and Pb based QDs (NIR emissive QDs).¹¹⁶ The Zn based QDs are UV emissive and show little use for biomedical applications.^{137,138} On the other hand, the Cd based QDs have received a lot of concerns pertaining to their toxicity for practical biomedical applications.^{139,140} Hence, the QDs consisting of non-toxic materials need to be developed in future studies.¹⁴¹⁻¹⁴³

In this chapter, we have highlighted various type of nano carriers reported in literature for the development of an effective drug delivery system for controlled and targeted cancer therapy. Although a lot of efforts have been devoted to developing these novel therapeutic systems, only some of the systems have been demonstrated in clinical trials. Hence, the future research in this field will be (1) the further improvement of the existing technologies towards clinical applications, and (2) the development of new techniques for the next generation of therapeutics.

1.3. Fluorescence organic molecules for sensing and detection.

Fluorescence organic dyes are branch of organic molecules that possesses interesting optical properties. The high quantum yield and large stoke shift exhibited by these molecule allow them to be used as optical labels for bioactive molecules or macromolecules ranging from proteins to polysaccharides.^{112,144-146} In addition, they are also normally applied as cell labels that enable us to image the various components or even organelles of cells using a simple fluorescence microscope.¹⁴⁷⁻¹⁴⁹ Moreover, the various basic types of organic fluorophores exhibit different optical properties such that they possess different excitation and emission wavelength. This property can be harnessed such that multiple dyes can be used to stain the cells for the simultaneous multi wavelength imaging.

Figure 12. Jablonski diagram of chromophore. The diagram showed the possible transition pathways of electrons. The electrons could transit from the ground state (S_0) to the excited state of S_1 or S_2 . The excited electron in S_1 state could be further excited to S_2 by excited state absorption (ESA). The excited electrons could also relax by means of internal conversion (IC), fluorescence or transit to the triplet state in T_1 by means of intersystem crossing (ISC). The electron in T_1 state could relax back to S_0 state by the subsequent ISC or phosphorescence. Reproduce with permission from Ref. 150. Copyright 2003 Royal Society of Chemistry.)

In a typical photoexcitation of organic chromophore (usually $\pi \rightarrow \pi^*$ transition), the electrons from the ground state (S_0) would then be excited into the excited state (S_1) upon absorbing the photons, provided that the energy difference between the excited state and the ground state of the molecule matches the energy of the photon (Fig. 12).¹⁵⁰ Thereafter, the absorbed energy can be dissipated by the molecule by means of internal conversion ($S_1 \rightarrow S_0$, non-radiative), intersystem crossing ($S_1 \rightarrow T_1$) and the fluorescence ($S_1 \rightarrow S_0$, radiative) pathway. Fluorescence organic dyes exhibited unique molecular structure such that fluorescence is the dominating relaxation pathway. In the event that the fluorescence relaxation pathway is being disrupted (such as the missing of the excited electrons or the electrons are relaxed by the other two pathways as the preferred way of relaxation), the fluorescence quenching of the molecule would be quenched accordingly.

Figure 13. Molecular structures of various fluorophores.

1.3.1. Properties of Organic fluorophores

The presence of certain group on a fluorophore would alter the optical property of the fluorophore.¹⁵¹ Apart from the extended conjugated pi system on the fluorophore, these substituent groups have a heavy influence on the electronic properties on the pi system. We would discuss the effect of these substituents on the photo physical properties on the fluorophore or chromophore.

Figure 14. Molecular structure of fluorescein and Rose Bengal.

The presence of heavy atom (such as Br, I or heavy metal) on a chromophore usually resulting in the quenching of the fluorescence of the chromophore as compare to the same chromophore without the presence of heavy atom. This phenomenon is known as the heavy atom effect whereby the presence of heavy atom promotes the spin-orbit coupling of the chromophore in its excited state. The spin-orbit couple thus facilitates the quantum mechanically forbidden spin flip of the electrons and favors the intersystem crossing as the dominance relaxation pathway. For instance, the Rose Bengal dye is the halogenated analogue of the fluorophore fluorescein the two dyes are almost identical in terms of their chemical structure (Fig. 14). However, fluorescein exhibited a fluorescence quantum yield of 37%¹⁵² while Rose Bengal exhibited a low fluorescence quantum yield of 0.6%¹⁵³ when both dyes are under the same condition.

Figure 15. Molecular orbital diagram on the effect of electron donating group (EDG) on the conjugated system.

The presence of electron donating group (such as OR, NH₂, OH etc...) on the fluorophore would form a π bond with the π conjugation system. The

photo excitation of this system would cause a $\pi \rightarrow \pi^*$ transition whereby the electron density would be shifted from the substituent to the π conjugated system. This results in the intramolecular charge transfer (ICT) process particularly if there is a presence of an electron acceptor on the other end of the molecule. Usually, these fluorophores exhibit a solvent dependent fluorescence property especially the emission wavelength of these fluorophores would increase with the solvent polarity. Upon the photo excitation of the fluorophores, the photo induced ICT process causes a charge re-distribution around the fluorophores, resulting in an instantaneous change in dipole moment. The surrounding polar solvent will then undergo a re-organization process such as the solvent dipole will be in line with the dipole moment of the fluorophores. This reduces the energy level of the excited fluorophore prior to the radiative relaxation into the ground state whereby the dipole moment of the fluorophore would return to its original state. The rate of this relaxation occurs at a much faster rate than the rate of the solvent re-organization process. The mismatch in the dipole moment of the fluorophores and the dipole moment of the solvent result in the higher energy level of the fluorophore in its ground state. Therefore, the emission energy of the fluorophore would be much lower than the corresponding excitation energy. This thus explains the large Stoke shift experienced by this class of fluorophores particularly in polar solvent. For instance, Iñigo et. al. had shown that the Stoke shift of 7-aminocoumarin was pronounce in water than in the corresponding non-polar decanol.¹⁵⁴ Apart from looking at the effect of electron donating on the ICT processes, we will also analyzed the trend in the shifting of the fluorescence and absorption wavelength of a fluorophore with respect to the extent of electron donating capability of the

substituent. As mentioned above that the lone pair on the electron donating group has the ability to form the π conjugation with the fluorophore. When less electronegative group was being conjugated on the fluorophore, the corresponding p orbital of the substituent would be higher in energy. Upon forming the new molecular orbital with the π system, the resulting π orbital would be higher in energy and therefore the HOMO – LUMO gap would be smaller as compare to a more electronegative group being conjugated on the π system. This phenomenon can be observed when we compare the absorption and emission wavelength of Rhodamine B and fluorescein. We can observe that fluorescein has two oxygen atoms being conjugated on the xanthene group while Rhodamine B have two secondary amino groups being conjugated on the xanthene group. By considering that the two hydroxyl groups are more electronegative than the amino group, the fluorescein dye would exhibit a shorter absorption and emission wavelength of 484 nm and 513 nm as compare to Rhodamine B dye, which exhibits a longer absorption and emission wavelength of 540 nm and 565 nm. Lastly, the electrons on these electron donating groups could also interact with proton in the solvent particularly in aqueous environment. Therefore in many cases, these organic fluorophore would exhibit a pH dependent quantum yield and thus could be used as a tool to determine the pH of an aqueous sample or intracellular pH of cancer cells.¹⁵⁵⁻¹⁵⁷

Figure 16. Molecular orbital diagram on the effect of electron withdrawing group (EWG) on the conjugated system.

Electron withdrawing substituent usually contains a non-bonding molecular orbital that would not participate in any orbital interaction with the π conjugated system of the fluorophores. However, this non-bonding orbital usually assume the HOMO of the whole fluorophore. The direct transition from the HOMO to the LUMO ($n \rightarrow \pi^*$) is then forbidden due to the different symmetry between the two orbitals. Thus, the electrons in the excited state of the fluorophore are often being promoted by the $\pi \rightarrow \pi^*$ transition. At this junction, the promoted electrons would be relaxed predominantly via the internal conversion pathway over the fluorescence pathway due to the short decay life of internal conversion over the fluorescence lifetime. Unless in certain circumstances where there is a significant amount of hydrogen bonding form between the solvent molecules with the electrons in the non-bonding orbital, there would be significant lowering in the non-bonding orbital energy

and the HOMO of the chromophore would be lied on the π orbital. In this case, the chromophore would possess fluorescence properties and this can be observed in fluorophores such as fluorenone particularly in protic solvents.

1.4. Applications of organic fluorophores for sensing and detection.

More recently, these organic fluorophores are used as a biomedical tool for sensing and detection.¹⁵⁸⁻¹⁶¹ To further elaborate on this, chemical modification is usually performed on the basic fluorophores and these modifications are usually chemical responsive towards the analyte of interest. In this way, the intended chemical reaction would selectively changes the structure of the molecular probe thereby either resulting in the quenching of the initial fluorescence of the probe, enhancing the fluorescence of the probe or even changes in the emission wavelength. The changes in the emission properties of the probe were then utilized as a detection signal for a particular analyte of interest in a highly selective manner. However, the “turn-on” or fluorescence enhancement types of molecular probes are more favored than the other two types. This is due to its ease of design and synthesis of the “turn-on” type fluorophore and also the “turn-off” probe would have higher background fluorescence prior to its innate fluorescence property, resulting in the lower sensitivity than the “turn-on” type. In order to achieve the “turn-on” effect on some of these molecular probe, the initial structures of the fluorophores are designed such that the fluorescence are being quenched either by a quencher or the presence of certain groups that alters the electronic properties of the

conjugation thereby converting the dye into a non-fluorescence form. Upon reacting with the desired analyte, the molecular probe would be reverted back to its fluorescence structure thereby switching on the fluorescence properties of these molecular probes. We would provide some examples to further demonstrate the strategies used by various works in the design of these molecular probes.

Figure 17. (a) Chemical schematic illustration of the conversion of the non-fluorescence derivatives into its fluorescence form, mediated by Palladium species. Below: Photographs of Probe 1 solution in the absence (left) and presence of Palladium species (right). Image on the right shows the fluorescence images of the same set of solution. (b) Catalytic cycle for the generation of fluorescence Rhodamine species by active Pd(0) (Reproduced with permission from Ref. 160. Copyright 2010 American Chemical Society.)

In a work reported by Ahn et. al., the authors have developed at Rhodamine B based chromogenic and fluoregenic sensor for the detection of Palladium species (Fig. 17).¹⁶⁰ The spiro cyclic ester group on the original Rhodamine molecule was replaced with a spirolactam group, in which the spirolactam can be opened by a series of catalytic reaction mediated by palladium species, particularly the Palladium (0) species. This process would change the conjugation on the xanthenes group, thereby changing the electronic properties of the Rhodamine molecule. The outcome of this is the changes of the apparent color from colorless to pink with the turning on of the fluorescence property, exhibiting orange fluorescence. The advantage of this design is the involvement of the catalytic reaction between the palladium species with the molecular and therefore, the probe has an extremely high sensitivity. The authors reported that the probe could detect palladium species as low as 50 ppb in a given sample, displaying the remarkable property of this probe.

Figure 18. Schematic diagram illustrating the FRET mechanism and the various factors influencing the FRET efficiency. (A) Jablonski diagram of donor and acceptor and their relationship during the FRET process. (B) Absorption and fluorescence spectra of donor and acceptor. Region in yellow showed the spectra overlapped between the donor fluorescence spectrum and the acceptor absorption spectrum. (C) Figure illustration of the relationship between FRET efficiency and the distance between the donor and the acceptor. (D) Figure illustration of the orientation factor between the donor and the acceptor. Φ represent the angle between the donor and the acceptor. R depicts the transition dipole vector. (Reproduced with permission from Ref. 162. Copyright 2014 Royal Society of Chemistry.)

Another common type of fluorescence based sensor relies on the Förster Resonance Energy Transfer (FRET) mechanism in which the conditions must fulfill these criteria (Fig. 18)¹⁶²; there must be significant spectra overlapped between the absorption spectrum of the acceptor and the fluorescence spectrum of the donor; sufficient distance between the donor and the acceptor (1 – 10 nm); the direction of the transition dipole between the donor and acceptor must be approximately parallel; the fluorescence lifetime of the donor must be sufficiently long enough for the energy transfer to occur. To further elaborate on the FRET phenomenon, the FRET mechanism is a transfer of non-radiative energy during which the dipole from the donor interacts with the dipole in the acceptor in a Columbic fashion, as long as the distance between the donor and acceptor is shorter than the emission wavelength of the donor. This is especially when there is an absence of a spin flip phenomenon (singlet to triplet or triplet to singlet), which is a quantum forbidden phenomenon. In the Förster mechanism, the Columbic interaction can even occur in long range order (up to 10 nm), allowing the donor and acceptor to be separated by various bridges. Furthermore, the FRET efficiency (Φ_T) is also extremely sensitive to the distance, r , between the donor and the acceptor (as $\Phi_T = \frac{1}{(\frac{r}{R_0})^6 + 1}$ where R_0 is the critical distance between the donor and acceptor which give rise to 50% of the FRET efficiency). This allows us to utilize the FRET mechanism as a molecular ruler to gauge the distance between the donor and acceptor on a single entity (e.g. distance between one ends of protein to another end of protein).

Figure 19. Chemical schematic illustration of the naphthamide-Rhodamine dyad for the sensing of Cr^{3+} based on the FRET mechanism. (Reproduced with permission from Ref. 163. Copyright 2014 Royal Society of Chemistry.)

The FRET mechanism can also be incorporated into the design of fluorescence probe for sensing and detection. For instance in a work reported by Li et. al., the authors conjugated a naphthamide with a Rhodamine moiety, which the cyclic ester group was replaced by a spirolactam group (Fig. 19).¹⁶³ Similar to the previous example, the presence of a spirolactam group locks the spiro ring and therefore the resulting Rhodamine group would not possess any fluorescence property. Therefore in the initial state, the molecular probe would only exhibit a fluorescence signal at 536 nm upon the photo excitation with 405 nm. This fluorescence signal is being emitted by the naphthamide group especially when the Rhodamine group does not possess any absorption in this state. However, the careful design of the Rhodamine group allow $\text{Cr}(\text{III})$, Cr^{3+} , to be chelated and thereby regaining the fluorescence of the Rhodamine dye through the opening of the spirolactame ring. Thereafter, the energy from the photoexcitation of the naphthamide group would transfer its energy to the Rhodamine group and the Rhodamine would emit photons at 594 nm as a detection signal.

Figure 20. Reaction scheme (a) between GSH and the “Turn-On” probe for GSH fluorescence sensing and detection application as well as (b) between singlet oxygen and “Turn-On” fluorescence probe for singlet oxygen sensing and detection. Mechanistic diagram of electron transfer between (c) electron withdrawing quencher and fluorophores and (d) electron donating quencher and fluorophore.

The FRET mechanisms allow us to develop interesting novel design for the fluorogenic probe for sensing and detection. In addition, the reliance of the Photo Induced electron (PeT) transfer mechanism could also be utilized for the designing of various fluorogenic probes for detection and sensing application. We will first discuss the mechanism of the PeT process before listing an example to demonstrate this form of application. During the photoexcitation of a fluorophore, the electrons would receive the photo energy and undergo a vertical transition from the ground state to the excited state ($S_0 \rightarrow S_1$). This is assuming that the transition is an allowed transition such that there is a significant overlapped of the probability function of the ground state with the probability function of the excited state (Franck-Condon Principle). After the transition, there would be a hole left on the ground state energy level and an electron presence on the excited state energy level. Thereafter, the excited electron would be transported to an acceptor in the vicinity of the donor, through internal conversion. Finally, with the absence of this excited electron, there would not be any possible radiative relaxation thereby quenching the fluorescence of the fluorophore (Fig. 20a). Another possible mode of transfer is the movement of electrons from a donor which will then be filled into the hole left by the photo excited fluorophore (Fig. 20b). This would thus prevent the radiative relaxation of the excited electrons on the fluorophore and thereby quenching the fluorescence of the later. One example of the reliance of PeT mechanism in the design of a molecular probe was demonstrated by Xi et. Al (Fig. 20c).¹⁶⁴ for the detection of glutathione (GSH). In this work, a maleimide group was conjugated onto an umbelliferone group and the pi conjugate acts as an electron acceptor for the excited state of the umbelliferone, thereby

quenching the fluorescence of the umbelliferone. The sensor probe was designed based on the well-studied thiol maleimide reaction, which is a form of Michael addition reaction. Hence after the Michael addition reaction, the double bond on the maleimide was converted into a single bond and this result in the breaking of the π conjugated system. This process thus blocks off the electron transfer process and regain the fluorescence property of the umbelliferone. In another example, Nagano et. al. had reported the synthesis of a fluorescein based probe for the detection of singlet oxygen (Fig. 20d).¹⁶⁵ In this design, the spiro-phenyl group of a fluorescein was replaced with a spiro-anthracene moiety and the fluorescence of the xanthene group would be quenched off by the extended π conjugated system of the anthracene group by means of the PeT mechanism. Other than acting as a PeT quencher for the fluorescein moiety, the anthracene group has the ability to react with the high energy singlet oxygen through the 4 + 2 cycloaddition. This process would break the π conjugation system of the anthracene and inhibits the PeT processes. This results in the switching-on of the fluorescence of the fluorescein, thereby achieving the sensing application.

1.5. Research objectives and goals

This chapter has summarizes the current strategies that the biomedical community has adopted toward the combating of cancer, particularly relying on “smart” nanocarrier for delivery of therapeutic agent to the tumor site. We have discussed the various types of nanocarriers and the innate properties of these nanocarriers that can be utilized for the fabrication of a hybrid material that can

be used for delivery as well as imaging applications. This chapter has also discussed the principles of fluorescence and also the effect of various substituent on a chromophore and fluorophore. We have also discussed in detail the design strategy for the development of fluorophore for sensing and detection applications. Lastly, we have also described the various examples found in the literature that rely on fluorogenic probe for analyte specific detection. In our studies, we aimed to develop “smart” polymer based nanomaterials for drug delivery and sensing applications. We will also be discussing the development of two fluorogenic probes for the detection of thiol and also a molecular sensor for the dual responsive detection of thiol and hydrogen peroxide. Research have shown that the intracellular concentration of these two agent has a strong correlation with cancers or other diseases. With this, we envision that our research works will have a good implication towards the development of organic materials that encompassed both diagnostic and therapeutic properties.

In the first chapter of this dissertation, we will be presenting the development of a polymer based nanoparticles system for the target specific delivery of anticancer drug in both *in vitro* and *in vivo* systems. The nano vehicle used in this work can be prepared by the supramolecular self-assembly of three polymer chains where the doxorubicin was loaded during the preparation process. In addition, the fluorescence tracer and a targeting ligand, folic acid, can be incorporated into the nano-assembly to impart tumor target specificity to the drug carrier. The drug loaded nanoparticles showed excellent target specificity towards the cancerous MDA-MB231 breast cancer cell line and the B16-F10 mouse melanoma cell line whereas showed insignificant

toxicity towards the healthy HEK293 human embryonic kidney cell line. We have extended the work towards the *in vivo* drug delivery application in the MDA-MB231 tumor bearing nude mice. We have included controlled nanoparticles, which does not contain any targeting ligand, for the *in vivo* experiments. To our delight, our experiments showed that the nanoparticles functionalized with targeting ligand showed excellent therapeutic effect while the nanoparticles without the incorporation of targeting ligand showed a lower therapeutic effect. The post treatment analysis of the various organs of the mice showed that the organ does not exhibit significant damage from the treatment.

Apart from using the polymeric nanoparticles for drug delivery applications, our research has also demonstrated the application of this material for diagnostic application. In chapter 3, we have loaded a fluorescence probe into the polymeric nanoparticles for sensing application. The fluorescence probe that we have used is able to respond towards the reducing biological thiol, which is highly expressed in cancer cells. Our series of experiments showed that the probe loaded nanoparticles have the capability to respond to reducing thiol in a highly selectivity manner. Apart from this, the probe and the probe loaded nanoparticles could be prepared easily. We envision that this system could be combined with the co-loading of drugs and thereby achieving the theranostic application from this simple system.

As mentioned before that the fluorescence probe has been widely used as labels and detection tools. However, conventional fluorescence probe reported in the literature could only response to one analyte in a highly selective manner. In recent years, there have been emerging development of fluorescence probe that could generate different response to different analyte of a specific

class of analyte. These different responses could be either utilizing the different reaction rate from one type of analyte to another or in many cases, a different excitation source has to be used for the detection of the one analyte to another. In chapter 4, we have developed a novel type of fluorescence probe which is a constitution of two primary fluorophores. This probe could respond orthogonally to reducing biological thiols or hydrogen peroxide selectively through the use of a single excitation source. Thus, this allows the use of a single probe for achieving two different detection functions.

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Chapter 2: Supramolecular nanoparticle carriers self-assembled from cyclodextrin and adamantane functionalized polyacrylates for tumor-targeted drug delivery

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2.1 Introduction

Biocompatible nanoparticles (NPs) have received much attention over the past decades in terms their preparations¹⁻⁵, and applications for drug⁶⁻¹⁴ and gene^{1,15-19} delivery, as diagnostic²⁰⁻²³ purposes. Non-toxic nature of these NPs provides an excellent protection and delivery platform for transporting drugs or genes to target sites, with the aim of solving the issues of the side effects generated by the use of therapeutic agents. Among these biocompatible NPs, polymer-based NPs present promising potential in drug delivery applications, and some successful cases of employing polymer-based NPs as drug carriers have been approved by the US Food and Drug Administration (FDA) and have been applied in clinical cancer therapy.^{9,24-26}

Employing a supramolecular self-assembly strategy in the synthesis of polymer-based NPs offers several advantages.²⁷⁻²⁹ In addition to providing the versatility in terms of choice of design for various applications, a primary feature of using supramolecular self-assembly strategy is the ease of preparation of polymer-based NPs with high reproducibility. Several self-assembled polymer-based NPs have been used as carriers for drug delivery.^{18,19,30-32} For instance, Guo et al. relied on a supramolecular self-assembly approach driven by the complexation between β -cyclodextrin (β -CD) and adamantine (AD) using calcium carbonate as the template for the formation of hollow NPs.³³ The group demonstrated an excellent drug delivery capability of the hollow NPs in cancer cells. In another work reported by Aditya et al., they used a similar strategy to prepare a polymeric gene delivery system, which showed good transfection efficiency *in vitro*.³⁴ Although there are many promising reports in the literature demonstrating the drug and gene delivery

applications of supramolecular assemblies, the *in vivo* performance of such systems has been less investigated.

The use of NP-based drug carriers *in vivo* requires effective accumulation of the carriers in the tumor sites. By relying on the enhanced permeability and retention (EPR) effects, some rationally customized NPs have shown enhanced accumulation of released drugs at the target sites for therapy.^{14,31,35,36} Self-assembled polymer-based NPs should have suitable sizes (100 nm to 2 μ m) in order for effective EPR accumulation in the tumor sites, thus reducing the circulation time of the NPs in the body.^{21,37,38} However, the reliance on passive targeting (EPR in this instance) suffers from several limitations, such as the lack of tumor-targeting ability and low efficiency for certain tumors as the permeability of the vessels is not always the same for all tumor models.^{39,40} Furthermore, small NPs (<100 nm) are able to pass through the blood vessel wall, further improving the pharmacokinetic properties of delivered drugs.^{41,42} Although the use of small NPs has the above-mentioned advantages, the investigations on the utilization of small NPs to target tumor sites still remain a great challenge, particularly on account of their relatively poor EPR accumulation properties. Hence, the search for a delivery system with enhanced pharmacokinetic properties and effective tumor targeting capability is urgently required. In order to address this issue, we herein report the integration of targeting ligands onto polyacrylate based supramolecular NPs with a relatively small diameter of 35 nm, which were prepared by taking advantage of the supramolecular self-assembly approach. The size of polyacrylate based supramolecular NPs could be easily controlled during the synthesis. The designed drug delivery system could also be easily customized, since the

polyacrylate-based supramolecular NPs were synthesized from the “mix and match” of various components based on the properties of interest. In addition, the present delivery system exhibits an excellent therapeutic efficiency with a remarkably low drug dosage, which could significantly reduce the side effects caused by anticancer drugs.

Scheme 1. Schematic representation of the formation of ZG-01 (FA + VE) NPs through supramolecular self-assembly for target-specific drug delivery in cancer therapy.

Scheme 1 illustrates the overall approach employed in this work. The scheme begins with the preparation of doxorubicin (DOX)-loaded NPs (ZG-01 (FA + VE)) through supramolecular self-assembly. In this approach, the driving force for the formation of the NPs relies on the host–guest complexation between β -CD and AD. From this aspect, the NPs were formed through the self-assembly of β -CD modified polyacrylic acid (PAA-CD), AD modified polyacrylic acid (PAA-CD), and AD modified polyethylene glycol (PEG-AD) in the presence of DOX and AD conjugated fluorescein isothiocyanate (FITC-AD) for fluorescent tracing purposes. In order to address the specificity issue, a

targeting ligand, folic acid (FA), was also included as one of the key ingredients for the preparation of the ZG-01 (FA + VE) NPs to endow the folate-receptor mediated endocytosis of the NPs by cancerous cells.⁴³⁻⁴⁶ We envisioned that once the ZG-01 (FA + VE) NPs undergo a folate-receptor mediated endocytosis by cancerous cells, the loaded DOX is released into the intracellular compartment to achieve the target-specific anticancer therapy. Thereafter, the ZG-01 (FA + VE) NPs were utilized for both *in vitro* and *in vivo* drug delivery applications. We thus demonstrated the target-specific drug delivery of DOX loaded and folate-containing NPs to two cancerous cell lines, namely human breast cancer cells (MDA-MB231) and mouse melanoma cells (B16-F10), since the surfaces of the two cancerous cell lines are over-expressed by the folate receptors.⁴⁷⁻⁵⁰ For comparison purposes, a non-cancerous cell line, human embryonic kidney cell (HEK293), was employed to compare the delivery efficiency of the ZG-01 (FA + VE) NPs, demonstrating a high target-specific drug delivery to the cancer cells. Thereafter, *in vivo* experiments were conducted on tumor bearing nude mice, indicating the remarkable DOX delivery efficacy of the supramolecular NPs within living animal models for efficiently inhibiting the tumor growth.

2.2 Results and discussion

2.2.1 Preparation and characterization of ZG-01 (FA + VE) NPs

In this work, PAA with an average molecular weight of 1952 Da was synthesized through a two-step process based on a modified procedure.⁵¹ The synthesis involves the ATRP of *tert*-butylacrylate followed by the deprotection of the *tert*-butyl ester to yield the PAA polymer. The ATRP approach in the

PAA synthesis provides an advantage in controlling the polydispersity of the as-synthesized PAA polymer.⁵²⁻⁵⁴ GPC analysis shows that the as-synthesized PAA polymer has a low PDI value of 1.06, thereby confirming the excellent control over the size dispersity of the as-synthesized PAA. Subsequently, the AD and β -CD conjugations onto the as-synthesized PAA were performed to afford PAA-AD and PAA-CD with average molecular weights of 4924 Da and 5429 Da, respectively. Based on these molecular weight, we have calculated the degree of conjugation of PAA-AD and PAA-CD to be 89% and 12% respectively. The disparity in the degree of conjugation of these two polymers is due to the relatively molecular weight of the pendant group. The β -CD has a molecular weight of 1135 Da while the AD molecule has a molecular weight of only 135 Da. The larger molecular structure of β -CD impose significant amount of steric hindrance onto the next incoming β -CD during the conjugation onto the PAA polymer. On the other hand, the smaller adamantane molecule has imposed a comparable smaller amount of steric hindrance with each other during the conjugation process. Therefore, this explains the consequence of the great disparity in the degree of conjugation between PAA-CD and PAA-AD.

The self-assembly of 8 equiv. of AD from PEG-AD, 8 equiv. of AD from PAA-AD, 5 equiv. of β -CD from PAA-CD, 0.8 equiv. of FA-AD, and 0.8 equiv. of FITC-AD formed supramolecular NPs that can encapsulate DOX/HCl. A mixture of PAA-AD and PAA-CD in the absence of PEG-AD resulted in significant precipitation of large polymeric aggregates, which was observed under TEM (Fig. S3.1A in the appendix). This observation indicates that PEG-AD is required for the preparation of stable and size-controllable polymer-based NPs. Here, PEG-AD has the ability to prevent continuous propagation of

the cross-linking network formed from PAA-CD and PAA-AD. In the NP preparation, FA-AD and FITC-AD were also included in the self-assembly in order to provide the target specificity and fluorescent tracer, respectively. Rational integration of the six components led to the formation of the desired ZG-01 (FA + VE) NPs, which were then used for target-specific drug delivery to the cancer cell lines. It can be proposed that hydrophobic AD moieties could form a core for subsequent complexation by PAA-CD. The hydrophobic core enables the DOX molecule to be encapsulated within it. We presume that such a type of encapsulation relies on the formation of electrostatic interaction between the amino group of DOX and the free carboxylate groups on the PAA backbone within the NPs.⁵⁵ In addition, the ketone unit on DOX may also form a dynamic iminium bond with the amino group on PAA-CD, which reinforces the integration of the drug within the polymer-based NPs.^{18,56} Lastly, the post conjugation of AD groups on the polymer chains facilitates the hydrophobic interactions between the AD moiety with the aromatic groups of the DOX. Together, these combinations enable the efficient loading of DOX molecules within the polymeric network. Thus, the synthetic strategy involves the mixing of all the AD-containing components followed by the addition of PAA-CD into the mixture to induce the self-assembly in water. The obtained aggregates were then centrifuged at a high-speed rate to precipitate the NPs to the bottom of the sample tube. The DOX concentration left in the supernatant was measured via UV-Vis spectroscopy and compared to the amount used during the preparation. It was found that there was a reduction in the DOX concentration. From this comparison, we calculated that the loading capacity of DOX in the NPs was 0.35 wt%.

Table 1. Characterization results of ZG-01 (FA+VE) and ZG-02 NPs.

Entry	NPs	Zeta-potential (mV) ^a	NP diameter (nm) ^b
1	ZG-01 (FA+VE)	-0.376±3.61	35.14±7.15
2	ZG-02	-0.363±3.04	30.83±5.60

^aAverage zeta-potential values from 40 times of zeta measurements.

^bAverage NP diameter from 30 times of measurements in the TEM images, using the software Nanomeasure[®] as the measurement tool.

Figure 1. TEM images of (A) ZG-01 (FA + VE) and (B) ZG-02 NPs. The scale bars are 100 nm. SEM images of (C) ZG-01 (FA + VE) NPs and (D) ZG-02 NPs. The scale bars are 100 nm. (E) DOX release profile of ZG-01 (FA + VE) NPs upon the addition of ABS (pH changes from 7.4 to 5.5). The released DOX is presented as mg of DOX per mg of NPs on the y-axis.

To investigate the morphology and surface charge of the ZG-01 (FA + VE) NPs, a series of characterization techniques was employed. For comparison, we also prepared ZG-02 NPs without the DOX loading, which were characterized with the same set of characterization techniques. TEM and SEM images (Fig. 1) of the NPs show that both NPs are uniformly spherical in shape. The aggregation of polymeric materials was not observed for both samples, demonstrating that the self-assembly for the formation of the NPs occurs in a controlled fashion. The TEM images indicate that the diameter (about 35 nm) of the ZG-01 (FA + VE) NPs is bigger than that (about 31 nm) of the ZG-02 NPs (Table 1). The larger NP size of ZG-01 (FA + VE) could be a result of the integration of DOX within the NPs. As mentioned above, DOX/HCl was integrated into the ZG-01 (FA + VE) NPs through the dynamic iminium bond formation and the non-covalent electrostatic interaction. We expect that both interactions could be weakened under the acidic intracellular compartment of cancerous cells, eventually leading to the release of DOX.^{18,56} Various groups have reported that the incorporation of DOX within a self-assembly ensemble would because the quenching of the DOX fluorescence.^{30,57,58} Since a similar DOX fluorescence quenching phenomenon was observed in this work, the fluorescence changes of DOX can be utilized to study the DOX release behavior of the ZG-01 (FA + VE) NPs under different pH conditions. Fig. 1E shows the release profile of DOX from the ZG-01 (FA + VE) NPs. The NPs were first dispersed in 0.1X PBS (pH 7.4) over a period of 70 min, when there was no significant increment in the fluorescence intensity of DOX. The observation clearly indicates that DOX was still intact and packed within the NPs. Upon the addition of 10% v/v of 10X ABS (pH 5.5), which was

used to simulate the acidic environment within the endosome of cancerous cells, the fluorescence of DOX was increased significantly, and reached a release half-life in about 37 min and the maximum release at 200 min from the time of addition. The obtained results demonstrate that DOX was released from the ZG-01 (FA + VE) NPs under acidic conditions. In order to further study the effect of acidity on the ZG-01 (FA + VE) NPs, the suspension of the ZG-01 (FA + VE) NPs was treated with 1X ABS, and the mixture was left for a day prior to the TEM studies. The TEM image shows that the NPs remained intact with their original morphology (Fig. S3.1D in the appendix), however, with a decrease in the NP size to 30.99 nm. Thus, the observations indicate that the weak iminium bond was hydrolyzed and the electrostatic interaction could be disturbed in acidic medium, resulting in the release of DOX from the NPs.

Zeta-potential measurements of the two NP samples were then conducted. As seen from Table 1, the two types of NPs have very similar surface potentials with slightly negative values below the neutral point. We observed no aggregation of the NPs despite the low zeta potential values. This phenomenon may be a result of the presence of the hydrophilic PEG units on the surface of the NPs, which greatly enhance the stability of the NPs in water. In addition, the negatively charged surface of the NPs could be attributed to the free carboxylic acid groups on the PAA components. This property favors the subsequent drug delivery application, as the negative surface charge of drug loaded NPs could prevent non-specific interactions with the negatively charged cell surface.^{6,59,60} Hence, the interaction between the NPs and the cell surface would rely largely on endocytosis, mediated by the incorporated FA units as the targeting ligands.

2.2.2 *In vitro* drug delivery applications

Figure 2. Cytotoxicity assay of ZG-01 (FA + VE), ZG-02, and free DOX/HCl on (A) MDA-MB231 cells, (B) B16-F10 cells, and (C) HEK293 cells. (D) A summary of the cytotoxicity data for the ZG-01 (FA + VE) NPs on the three cell lines. Error bars for each condition represent the standard deviation of 8 replicates.

To demonstrate the potential of ZG-01 (FA + VE) NPs for target specific *in vitro* drug delivery towards cancer therapy, we conducted a series of biological experiments. The toxicity of the ZG-02 NPs without the DOX loading on the three cell lines was first measured by the MTT assay. We found that the ZG-02 NPs showed no toxicity towards the three cell lines under the concentrations used, indicating that the self-assembled NPs are biocompatible. We then assessed the toxicity of the ZG-01 (FA + VE) NPs containing DOX on the three cell lines in order to evaluate the specificity of the drug-loaded NPs. The comparison studies show that ZG-01 (FA + VE) NPs were toxic to the MDA-MB231 and B16-F10 cells, whilst being biologically benign to HEK293 cells (Fig. 2A–D). These results suggest that the FA units in the ZG-01 (FA + VE) NPs could assist the targeting of the DOX-loaded NPs towards cancerous cell lines, while preventing them from non-specific internalization by a healthy

cell line. In addition, we also evaluated the toxicity of DOX and compared it to the ZG-01 (FA + VE) NPs on all three cell lines. The cytotoxicity profiles of DOX show that DOX could cause systemic toxicity to the three cell lines. Thus, free DOX has the ability to be internalized by the cells and exhibits non-specific toxicity effects on all three cell lines. Comparatively, the ZG-01 (FA + VE) NPs prevent the non-specific internalization of DOX from healthy cell lines.

Figure 3. Fluorescence microscope images of (A) MDA-MB231, (B) HEK293 and (C) B16-F10 cells, visualizing (from left to right) the bright field images of the cells followed by DOX channel (excitation filter: 540/25 nm; emission filter: 605/55 nm), FITC channel (excitation filter: 480/40 nm; emission filter: 535/50 nm), DAPI channel (excitation filter: 350/50 nm; emission filter: 460/50 nm), and merged images within the cellular compartments after 4 h incubation with the ZG-01 (FA + VE) NPs (0.6 mg mL^{-1}). Three sets of imaging experiments were performed under the same microscope settings. The scale bar in each is 20 μm .

These observations provide an important basis for performing the target specific drug delivery using the present NP system. Fluorescence microscope studies were conducted to investigate the uptake of the DOX-loaded NPs on the three types of cell line. In a typical imaging experiment, we incubated the cells with the samples for 4 h before the culture medium was replaced with a fresh medium. The cells were then incubated for another 24 h before fixing the cells followed by staining the cell nucleus with DAPI. Thereafter, the fluorescence microscope images of these cells were recorded. Fig. 3 shows the fluorescence

microscope images of the three cell lines after treatment with the ZG-01 (FA + VE) NPs. The images clearly exhibit that the MDA-MB231 and B16-F10 cells have multiple green and red fluorescence spots within the cell endosome, which are originated from the FITC-labeled NPs and the released DOX, respectively. In contrast, similar fluorescence spots cannot be observed in the fluorescence images of the HEK293 cell, confirming that the ZG-01 (FA + VE) NPs are specific towards the cancerous cells. We also performed the fluorescence imaging experiments on the three cell lines treated with free DOX under the same experimental conditions. The fluorescence images show the presence of free DOX in all three cell lines (Fig. S4.1–S4.3 in the appendix), indicating that free DOX has no specificity towards these cell lines. Thus, the comparison of various fluorescence images confirms that the ZG-01 (FA + VE) NPs could deliver the anticancer drug DOX specifically into the cancerous cells but not to the healthy cells.

2.2.3 *In vivo* drug delivery applications

Encouraged by the results from the *in vitro* experiments, we then proceeded to study the drug delivery efficiency of the NPs *in vivo* using nude mice with xenograft tumors. Fourteen mice were subcutaneously injected with MDA-MB231 cancer cells. After seven days from the injection, the mice were segregated into four groups (3, 3, 4, 4) for the *in vivo* studies, where the four groups of mice were intravenously (tail vein) injected with various samples (100 μ L) every other day over a period of twenty-one days. The first group of three mice received the injection of saline solution, which was used as a negative control group. In the *in vivo* studies, we carried out a set of control

experiments to confirm the targeting role of the FA unit on the ZG-01 (FA+VE) NPs. The comparison study was performed by using the ZG-01 (FA+VE) NPs with the FA targeting ligand and the ZG-01 (FA-VE) NPs without FA. The second group of three mice was treated regularly with the ZG-01 (FA-VE) NPs (4 mg mL^{-1}). The last two groups of four mice were each treated with DOX solution (14 mg mL^{-1}) and the ZG-01 (FA + VE) NPs (4 mg mL^{-1}), respectively. The dosage of 70 mg DOX per kg of mouse is 10 times to 50 times lower than that used for other NP systems for achieving therapeutic effects.^{50,56,57,61} The utilization of the low dosage may further reduce the side effects brought about by chemotherapy and also decreases the chance of imparting multiple drug resistance to the tumor cells.

Figure 4. Effects of ZG-01 (FA + VE), ZG-01 (FA-VE), DOX\$HCl\$, and saline on the tumor growth. (A) The observed size of tumors upon treatment with these samples over a period of 25 days (* $p < 0.05$). (B) Weights and (C) photographs of tumors obtained from mice that were treated with these samples (* $p < 0.05$). (D) Histology (H&E staining) images of the tumors isolated from mice that were treated with these samples. H&E staining shows that the tumors treated with the ZG-01 (FA + VE) NPs had less cell density than those treated with the other samples.

During the course of treatment, the sizes of the tumors on the mice were measured from time to time, as plotted in Fig. 4A. After twenty-one days of treatment, the mice were sacrificed and the tumors were isolated for a series of analysis. Fig. 4B shows the weight summary of the tumors isolated from the various groups of mice, and Fig. 4C exhibits the photographs of the tumors after the isolation. Together with the growth profile of the tumors, the results indicate that the group of mice treated with the ZG-01 (FA + VE) NPs has the slowest growth rate in the tumor size, and the resulting tumors have the lowest weight. Statistical comparison (Student t-test) was then conducted on the tumor weights between the mice treated with the ZG-01 (FA + VE) NPs and the mice treated with free DOX, yielding a *p* value less than 5.0%. The weight of the tumors from the group of mice treated with the ZG-01 (FA + VE) NPs was also compared with that from the group of mice treated with the ZG-01 (FA-VE) NPs. The same statistical comparison indicates that the tumors from the group of mice treated with the ZG-01 (FA + VE) NPs were significantly smaller than those from the group of mice administered with the ZG-01 (FA-VE) NPs. The photographs of the tumors in Fig. 4C shows the order of tumor sizes after treatment with the samples.

In addition to studying the tumor size in determining the effect of various samples on the tumor growth, the histology studies of these tumors were also performed. Fig. 4D shows the H&E staining of the tumors isolated from the various mouse groups. The staining images indicate that the tumors isolated from the mice treated with the ZG-01 (FA + VE) NPs have significant tissue damage, as compared with those treated from free DOX/HCl and the ZG-01 (FA-VE) NPs. It should be mentioned that the H&E staining images of the

tumors from the mice treated with the ZG-01 (FA-VE) NPs show a similar morphology to the tumors from the mice treated with saline solution. These observations demonstrate that the therapeutic effect of DOX was enhanced by loading it into FA-functionalized NPs, i.e., ZG-01 (FA + VE) NPs. However, the absence of FA on the DOX-loaded NPs, i.e., ZG-01 (FA-VE) NPs, lowers the therapeutic effect of DOX significantly, confirming that the ZG-01 (FA + VE) NPs provide the targeting delivery of DOX into tumors with enhanced therapeutic efficiency.

Major concerns over the use of chemotherapeutic drugs include the toxicity of the drug package and their specificity towards the tumor. In order to address these concerns, various organs were isolated from the mice for histological studies. Interestingly, no necrosis was observed in these organs of the mice treated with the NPs (Fig. S5 in the appendix). This could be attributed to the low dosage of DOX used in these *in vivo* experiments, which led to significantly low levels of damage to the organs. Hence, it can be concluded that the ZG-01 (FA + VE) NPs only cause significant damage to the tumor tissues, but not to the healthy organ tissues of the mice.

2.3 Conclusion

In summary, we have prepared polyacrylate-based supramolecular nanoparticles via a facile self-assembly approach. The supramolecular nanoparticles have been integrated with a folic acid unit as the targeting ligand for target specific drug delivery in cancer therapy. The morphology of the supramolecular nanoparticles has been well characterized by several techniques, showing an average nanoparticle size of 35 nm in diameter, which is small

enough for excellent blood circulation. The *in vitro* tumor-targeted drug delivery of doxorubicin-loaded nanoparticles has been carried out by evaluating their cytotoxicity on two types of cancerous cells as compared to that on the HEK293 normal cell. The fluorescence microscopy experiments have been performed to further confirm the target specificity of the supramolecular nanoparticles towards cancerous cell lines. The *in vivo* drug delivery of the doxorubicin-loaded supramolecular nanoparticles was then carried out on mouse models with xenograft tumors, showing much better tumor-targeted specificity for more efficiently inhibiting the tumor growth than the nanoparticles without the folic acid targeting ligand. Furthermore, this work has highlighted the use of low doxorubicin dosage for achieving therapeutic effects. Thus, the present research paves the way for developing the next generation of nanotherapeutics towards improved cancer treatment.

2.4 Experimental

General

The reagents and solvents were used directly as received unless otherwise stated. The water used (Ultrapure, 18.2 U cm resistivity at 25°C and <10 ppb Total Organic Carbon) was dispensed from Sartorius Ultrapure Water. Copper(I) bromide was treated with glacial acetic acid for 24 h followed by washing with absolute ethanol three times prior to drying under vacuum. Cyclohexanone, methyl 2-bromopropionate (MBrP) and *tert*butylacrylate (stabilized with 15 ppm 4-methoxyphenol) were distilled from CaH₂ and kept in a Schlenk flask prior to use. Dialysis tubing, i.e., cellulose membrane (MWCO 10,425), was pre-treated based on the procedure provided by the supplier.

Briefly, the dialysis tubing was treated with hot water (80 °C) for 5 min followed by treatment with 0.2% H₂SO₄ and rinsing with hot water again prior to storage in the ultrapure water. 10X acetate buffer saline (ABS, pH 5.5) was prepared in a solution (500 mL) containing acetic acid (16.8 mM), sodium acetate (101.2 mM), sodium chloride (1.37 mM) and potassium chloride (27 mM). 10X phosphate buffer saline (PBS) was used as purchased. Both 1X ABS and 1X PBS were prepared from 10 times diluted buffer with water, and 0.1X ABS was prepared from 100 times diluted 10X buffer with water. Flash column chromatography was performed with Merck Geduran® Si 60 silica gel with particle sizes ranging from 40 to 63 μ m. The column was packed by loading the slurry and equilibrated with eluting solvent.

¹H NMR spectra were recorded on a Bruker AMX 300 spectrophotometer at ambient temperature. All of the ¹H NMR spectra are reported as chemical shift δ in units of parts per million (ppm) downfield from tetramethylsilane (TMS, δ = 0.0 ppm) or relative to deuterated solvents (7.26 ppm for CDCl₃, 2.50 ppm for DMSO-*d*₆, 4.80 ppm for D₂O, as well as 3.58 ppm and 4.78 ppm for MeOD-*d*₄). Multiplicities are provided as: s (singlet), d (doublet), t (triplet), and m (multiplet). The coupling constants *J* values are expressed in Hz. The number of protons (*n*) for a given resonance are reported as *n*H. The ¹³C spectra were recorded on a Bruker AMX 300 spectrophotometer at ambient temperature. The spectra were reported as chemical shift (δ) in ppm with reference to the residual solvent signal (77.0 ppm for CDCl₃, 39.81 ppm for DMSO-*d*₆, and 49.3 ppm for MeOD-*d*₄).

The melting points (MP) of the compounds were determined with an OptiMelt Automated Melting point system. High-resolution mass spectra

(HRMS) were recorded on a Waters Q-tof Premier MS. High-resolution matrix-assisted laser desorption/ionization (HR-MALDI) mass spectra were measured on a MALDI TOF/TOF ABI4800. Gel permeation chromatography (GPC) was recorded with an Alliance Waters 2690 separation module using water as the mobile phase and the refractive index (RI) detector. FT-IR measurements were conducted on a SHIMADZU IR Prestige-21 spectrophotometer. UV-Vis spectroscopy studies were carried out using a SHIMADZU UV-3600 UV-VIS-NIR spectrophotometer. The fluorescence kinetic experiment was performed on a Varian Cary Eclipse fluorescence spectrophotometer, equipped with a water circulated temperature controller. Transmission electron microscope (TEM) images were recorded on a JEOL JEM 1400 thermionic emission electron microscope operating at an acceleration voltage of 100 kV. Scanning electron microscope (SEM) images were recorded on a JOEL JSM-6340 Field emission electron microscope operating at an acceleration voltage of 30 kV. Zeta potential measurements were performed on a Zetasizer, Malvern Instrument Ltd., Worcestershire, UK. NPs were dispersed in 1X PBS at a concentration of 1.0 mg mL^{-1} prior to the measurements. All measurement settings were set at 40 times per measurement.

Synthesis of mono-6-deoxy-6-(p-toluenesulfonyl)- β -CD (1)

β -CD (74 g, 0.0652 mol) was suspended in water (550 mL), and then NaOH (7.5 g, 0.188 mol) in water (22.5 mL) was added dropwise over a period of 10 min. The slightly yellow solution was added dropwise along with p-toluenesulfonyl chloride (11.25 g, 0.059 mol) in acetonitrile (37.5 mL) over the course of 1 h, when the solution turned cloudy again. The mixture was allowed

to stir for 3 h before the precipitate was removed by filtration. When the pH of the filtrate was adjusted to 7 – 8 with HCl, flocculation occurred. The mixture was left undisturbed overnight before the precipitate was isolated by suction filtration. The precipitate was then re-crystallized from water twice before drying in vacuo overnight to afford compound (1) as a white solid (7.67 g, 10.0%). MP: 171.6–171.8°C (decomposition temperature). IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3387.0, 2926.0, 1654.9, 1363.7, 1157.3, 1022.3. ^1H NMR (300 MHz, DMSO- d_6): d 7.74 (d, $J = 8.10$ Hz, 2H), 7.42 (d, $J = 8.10$ Hz, 2H), 5.63–5.79 (m, 14H), 4.77–4.84 (m, 10H), 4.19–4.34 (m, 7H), 3.16–3.65 (m, 58H) (HDO overlapped), 2.42 (s, 3H). ^{13}C NMR (75.4 MHz, DMSO- d_6): δ 162.5, 145.3, 133.1, 130.3, 128.0, 102.4, 82.0, 72.5–73.5, 60.4, 21.7. HRMS (H_2O –MeOH): m/z : calcd: 1289.3864 $[\text{M}]^+$; found: 1289.3845.

Synthesis of 6-mono-(N-aminoethyl)amino- β -CD (2)

Mono-6-deoxy-6-(p-toluenesulfonyl)- β -CD (1) (2.0 g, 1.55 mmol) was charged into a 2-neck round bottom flask (50 mL), and then the reaction flask was evacuated to vacuo. The flask was back filled with N_2 gas before evacuating to vacuo again. This process was repeated twice, and ended with back filling with N_2 gas. To the reaction flask was added dimethylformamide (6 mL), and the mixture was stirred until the solid had completely dissolved. After which, ethylene diamine (5 mL, 74.8 mmol) was swiftly injected into the reaction mixture, and the mixture was heated to 65°C. The reaction temperature was maintained for another 3 h before cooling down to room temperature. The excess ethylene diamine in the reaction mixture was removed under reduced pressure followed by pouring the mixture into acetone (200 mL) to induce

precipitation. The slurry was heated up until the acetone solution began boiling when the mixture was then cooled down to room temperature. The isolated solid was then dissolved in the minimum amount of water followed by pouring into acetone (200 mL). This process was repeated twice. The slurry was filtered and dried in vacuo overnight to afford compound (**2**) as a white solid (1.52 g, 83%). IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3373.5, 2926.0, 1647.2, 1363.7, 1157.3, 1030.0. ^1H NMR (300 MHz, DMSO- d_6): δ 5.74 (s, 1H), 4.83 (s, 7H), 4.43 (s, 4H), 3.56–3.67 (m, 24H), 3.32–3.35 (m, 24H). ^{13}C NMR (75.4 MHz, DMSO- d_6): δ 102.4, 84.0, 72.5–73.5, 60.4, 55.3, 31.1. HRMS (H_2O –MeOH): m/z : calcd: 1177.4358 $[\text{M}]^+$; found: 1177.4314.

Synthesis of poly(*tert*-butyl acrylate) (**3**)

Atom transfer radical polymerization (ATRP) for the synthesis of poly(*tert*-butyl acrylate) (**3**) was adopted from the literature report.⁵¹ A 2-neck round bottom flask (50 mL) was charged with CuBr (39.1 mg, 0.273 mmol). The flask was then evacuated and purged with N_2 gas for three cycles before the addition of *tert*-butyl acrylate (4 mL, 27.3 mmol) and cyclohexanone (2 mL). Subsequently, pentamethyldiethylenetriamine (57.0 μL , 0.273 mmol) was added into the mixture via a syringe, and the cloudy mixture turned into a clear light green solution. The mixture was allowed to stir for 20 min before the addition of MBrP (122 μL , 1.09 mmol). Immediately, the flask was placed in a preheated oil bath (65°C) and the mixture was allowed to react overnight, resulting in a dark green viscous solution. The solution was diluted with acetone (10 mL) and flushed through a column packed with neutral alumina to remove the copper catalyst. The mixture was concentrated by removing acetone

with a rotary evaporator and the polymer was precipitated out from 1:2 methanol–water mixture solution (400 mL). The solvent was drained off before the polymer was re-dissolved in toluene. The solvent was evaporated by rotary evaporation. The polymer was then re-dissolved in acetone and precipitated out from 1:2 methanol–water mixture (400 mL) again. The solvent was drained off and the obtained polymer (**3**) was dried in vacuo overnight as a gummy gel (2.98 g, 85%). IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 2980.0, 1724.4, 1458.2, 1367.5, 1145.7. ^1H NMR (300 MHz, CDCl_3): δ 3.56 (s, 3H), 2.07–2.26 (m, 24H), 1.35 (s, 207H). ^{13}C NMR (75.4 Hz, CDCl_3): δ 176.1, 80.1, 51.2, 41.7, 27.8.

Synthesis of polyacrylic acid (**3'**)

To a solution of poly(*tert*-butylacrylate) (**3**) (0.5 g, 0.148 mmol) in dichloromethane (3 mL) was added trifluoroacetic acid (3 mL, 39.17 mmol). The resulting solution was stirred for 4 h before the addition of water (0.5 mL), and the cloudy suspension was further stirred for 3 h. The solvent and trifluoroacetic acid were removed under reduced pressure and the resulting mixture was dissolved in water (10 mL) to obtain a clear brownish solution. The brownish solution was dialyzed with Slide-A-Lyzer dialysis cassette (MWCO 2000) against dilute aqueous NaHCO_3 solution for one day before dialyzing against water for 2 days to afford a colorless solution. Finally, the solution was lyophilized to obtain the polymer (**30**) as a white solid (0.217 g, 74%). IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3095.8, 2966.5, 1718.6, 1247.9, 1170.8. ^1H NMR (300 MHz, D_2O): δ 3.62 (s, 3H), 2.36 (s, 21H), 1.59–1.89 (m, 40H), 1.38 (s, 6H), 1.08 (d, $J = 6.6$ Hz, 4H). ^{13}C NMR (75.4 MHz, D_2O): δ 183.6, 162.3, 44.4–45.2,

36.6–37.4. MS (HR-MALDI): $m/z = 1952.1105$. GPC: polydispersity index (PDI) = 1.06.

Synthesis of poly-N-adamantylacrylamide (PAA-AD)

A solution containing polymer (30) (32 mg, 16.1×10^{-3} mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (76 mg, 0.397 mmol) and N-hydroxysuccinimide (46 mg, 0.400 mmol) in dimethylformamide (4 mL) was stirred for 2 h. After which, 1-adamantanamine hydrochloride (75 mg, 0.398 mmol) and triethylamine (55.6 mL, 0.400 mmol) were added to the stirring solution when the solution turned slightly turbid. The reaction was allowed to stir for 2 days before adding into aqueous acetic acid (200 mL, 0.1 M) to induce the precipitate. The slurry was centrifuged and the supernatant was discarded. The precipitate was re-suspended in aqueous acetic acid (0.1 M) and subjected to centrifugation again. The process was repeated twice with aqueous acetic acid (0.1 M) followed by repeating trice with water. Finally, the precipitate was suspended in a minimal amount of water and the slurry was lyophilized to obtain the PAA-AD polymer as a white solid (42 mg). MS (HR-MALDI): $m/z = 4924.3286$.

Synthesis of poly-N-[6-mono-(N-aminoethyl) amino- β -CD]-acrylamide (PAA-CD)

A solution containing polymer (30) (16 mg, 8.05×10^{-3} mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (38 mg, 0.198 mmol) and N-hydroxysuccinimide (23 mg, 0.200) in dimethylformamide (4 mL) was stirred for 2 h. After which, 6-mono- (N-aminoethyl)amino- β -CD (2) (235 mg, 0.199

mmol) was added and the reaction was further stirred for 2 days. Then, the clear solution was diluted with water (10 mL) before loading into dialysis tubing (MWCO 10,425), which was dialyzed against water for 2 days. Finally, the resulting solution was lyophilized to afford PAA-CD as a fluffy white solid (65.6 mg). The molecular weight was determined by UV-Vis: 5429.

Synthesis of N-adamantyl-2-poly(oxyethylene)acetamide (PEG-AD)

PEG-AD was synthesized based on a literature procedure. 1-Adamantanamine hydrochloride (100 mg, 0.534 mmol) was dissolved in dichloromethane (5 mL) together with trimethylamine (90 mL, 0.647 mmol). The solution turned turbid after a while and the stirring was continued for 3 h. After which, methoxy PEG succinimidyl carboxymethylester (200 mg, 0.04 mmol) was slowly added and the reaction was stirred overnight. Subsequently, the solvent was removed by rotary evaporation and the residue was suspended in water (5 mL). The resulting turbid solution was filtered to remove the insoluble solid before the filtrate was loaded into the Slide-A-Lyzer dialysis cassette (MWCO 2000). The solution was dialyzed against water overnight and finally lyophilized to obtain PEG-AD as a white powder (202 mg, 98%). IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3483.4, 2889.4, 1668.4, 1467.8, 1342.5, 1112.9. ^1H NMR (300 MHz, CDCl_3): δ 3.39–3.91 (m, 504H), 1.67–2.06 (m, 15H). ^{13}C NMR (75.4 MHz, CDCl_3): δ 168.6, 72.6, 71.9, 70.5, 61.6, 59.0, 41.5, 36.3, 29.4.

Synthesis of N-adamantyl folate amide (FA-AD)

To a solution of folic acid (500 mg, 1.13 mmol) in DMF (10 mL) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (146.1

mg, 0.762 mg) and N-hydroxysuccinimide (1.133 mmol, 130.4 mg), and the resulting slurry was stirred for a day. In a separate vial, a mixture of adamantamine hydrochloride (212.6 mg, 1.133 mmol) and triethylamine (157.9 mL, 1.133 mmol) in DMF (1 mL) was stirred for 4 h before adding to the above solution, and the resulting mixture was stirred for 2 days. Thereafter, the reaction mixture was added into 30% acetone in ether to induce precipitation. The precipitate was re-dissolved in DMF and re-precipitated in 30% acetone in ether. The final solid was washed with 1X PBS three times and purified via column chromatography with silica gel (DCM/MeOH = 90 : 10 then H₂O–MeOH = 80 : 20) to obtain FA-AD as a brown solid (170mg, 39%). IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3304.1, 2910.6, 1697.4, 1606.7, 1508.3, 1452.4, 1190.1, 839.0, 767.7. ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.66 (s, 1H), 7.54 (m, 3H), 7.34 (m, 3H), 6.94 (s, 1H), 6.67 (d, *J* = 7.20 Hz, 2H), 4.50 (s, 2H), 1.91–2.23 (m, 18H), 1.60–1.64 (m, 6H). ¹³C NMR (75.4 MHz, DMSO-*d*₆): 174.7, 174.6, 171.6, 166.2, 161.6, 156.8, 154.4, 151.2, 149.0, 129.4, 128.4, 121.9, 111.7. HRMS (H₂O–MeOH): *m/z*: calcd: 575.2730 [M + H]⁺; found: 575.2725.

Synthesis of fluorescein adamantylthiourea (FITC-AD)

A solution of adamantamine hydrochloride (40 mg, 0.213 mmol) in anhydrous THF (10 mL) was added to trimethylamine (160 mL, 1.148 mmol), and the resulting mixture was stirred for 3 h. Thereafter, FITC (40 mg, 0.103 mmol) was added and the orange-yellow suspension was further stirred for a day. Subsequently, water (500 mL) was added to the stirring mixture and the reaction was continued for 3 h. THF was then removed under reduced pressure and the obtained mixture was dissolved in ethyl acetate (50 mL). The yellow

solution was washed with 0.5 N HCl and brine three times before drying over Na₂SO₄. The ethyl acetate was evaporated and the product was purified by column chromatography with silica gel (DCM/MeOH = 99 : 1 – 90 : 10) to afford FITC-AD as an orange-yellow solid (36 mg, 64%). IR (KBr) $\bar{\nu}$ /cm⁻¹: 3294.4, 3063.0, 2906.7, 2848.9, 1714.7, 1635.64, 1599.0, 1456.3, 1305.8, 1207.4, 1118.7, 852.5. ¹H NMR (300 MHz, MeOD-*d*₄): δ 8.18 (d, *J* = 1.50 Hz, 1H), 7.76 (dd, *J* = 8.25 Hz, 1.95 Hz, 3H), 7.13 (d, *J* = 8.1 Hz), 6.68 (dd, *J* = 5.40 Hz, 3.00 Hz, 4H), 6.55 (dd, *J* = 8.70 Hz, 2.40 Hz, 2H), 2.36 (s, 6H), 2.13 (s, 3H), 1.77 (s, 6H). ¹³C NMR (75.4 MHz, MeOD-*d*₄): 171.0, 159.2, 134.0, 132.8, 131.0, 130.6, 128.8, 126.6, 125.5, 119.7, 117.1, 116.7, 102.0, 54.23, 40.9, 36.2, 29.8. HRMS (H₂O–MeOH): *m/z*: calcd: 541.1797 [M + H]⁺; found: 541.1802.

Preparation of NPs

To a solution of PAA-AD (12 mL, 26 mg mL⁻¹) in DMSO was added DOX/HCl (36 mL, 1 mg mL⁻¹) in water, FA-AD (12 mL, 6.7 mg mL⁻¹) in DMSO and FITC-AD (12 mL, 6.3mgmL⁻¹) in DMSO. The resulting mixture was mixed thoroughly using a vortex before the addition of PEG-AD (264 mL, 8.4 mg mL⁻¹) in water. Subsequently, PAA-CD (294 mL, 4.3 mg mL⁻¹) in water and 10X PBS (60 mL, pH 7.4) were added into the mixture followed by sonication for 60 min to ensure complete equilibration in the formation of the NPs. The solution was centrifuged at 14,800 rpm into a pellet and the supernatant was discarded. The NP pellet was redispersed in PBS (1mL) and the mixture was centrifuged again to wash off any free DOX. The process was repeated until the supernatant became colorless. The NP pellet was re-

suspended back into solution form with 1X PBS (690 mL) to achieve a final concentration of 12 mg mL^{-1} as ZG-01 (FA + VE). ZG-02 and ZG-01 (FA-VE) NPs were prepared by following the same procedure, without the addition of DOX and FA-AD, respectively.

DOX release measurement

The DOX release profile of the NPs was measured by dispersing the as-prepared NP solution in 0.1X PBS (0.9 mL) in a fluorescence cuvette and then recording time-dependent fluorescence intensity changes at 593 nm every one min over a period of 70 min. Thereafter, the solution was added to 10X ABS (100 mL) and the time-dependent fluorescence intensity changes were measured for another 280 min.

Materials for biological experiments

Mouse melanoma cells (B16-F10), human breast cancer cells (MDA-MB231) and Human Embryonic Kidney 293 cells (HEK293) were obtained from ATCC. The three types of cell line were maintained in 37°C and 5% CO₂ environment and cultured in Dulbecco Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). The FBS and PS supplemented media were used for all of the biological experiments. Both DMEM with sodium pyruvate and L-glutamine, as well as FBS, were purchased from PAA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar. The MTT solution was prepared by dissolving the MTT crystal in 1X PBS (5 mg mL^{-1}) and stored in the dark prior to use. The cytotoxicity measurements

were carried out with a Tecan Infinite 200 PRO microplate reader. The fluorescence images for the cell studies were acquired using a Nikon Eclipse TE2000-E Confocal fluorescence microscope with a Nikon TE2-PS100W high-pressure mercury lamp. For the *in vivo* animal studies, Balb/c Nude female mice 6–7 weeks of age were purchased from the Jackson laboratory (Bar Harbor, Maine, USA). The mice were housed in a specific pathogen-free animal facility in Nanyang Technology University (NTU). All experiments were carried out in accordance with the NTU guidelines under the protocols approved by the institutional animal care and use committee.

Cell viability studies

In a typical cell viability study, the respective cell lines were seeded on a 96 well plate at a density of 10,000 cells per well and maintained in growth media (100 mL) for 24 h. Thereafter, respective sample solutions (10 mL) were added into each well while water was used as a negative control. The plates were then returned to the incubator for 4 h, and subsequently the culture medium was replaced with fresh medium (100 mL). After 24 h, the medium was replaced once again and MTT solution (10 mL) was added into each well followed by the incubation for another 4 h. Then, the medium was removed and DMSO (100 mL) was added to dissolve the purplish crystals. Finally, absorbance readings of the wells were carried out at 560 nm with the microplate reader. The results of cell viability were expressed by taking the percentage of readings to the readings of the negative control. The measurements were repeated for 8 replicates for each sample, and the results were expressed as

mean values while the error bars represent the standard deviation of these readings.

Confocal fluorescence microscope studies

In a typical confocal fluorescence microscope study, the cells of the respective cell lines were seeded at a density of 2×10^6 cells per well on a 6 well plate, which was preloaded with a 22 mm X 22 mm microscope glass cover slip. The cells were maintained in the growth medium for 24 h. Subsequently, DOX solution (200 mL, 21 mg mL^{-1} in water) or respective NP solution (200 mL, 6 mg mL^{-1}) was added into each well and the plate was returned to the incubator for a period of 4 h. Then, the medium was removed, and the cells were washed once with 1X PBS (1 mL), which was added to a fresh medium (2 mL). The cells were incubated for another 24 h. The medium was then removed and the cells were washed trice with 1X PBS. The cells were then fixed with 10% formaldehyde in 1X PBS, and then the cells were left in the incubator for 20 min. Thereafter, the formaldehyde solution was removed and the cells were washed trice with 1X PBS again before staining with 4',6-diamidino-2-phenylindole (DAPI) solution in 1X PBS (1 mL, 1 mg mL^{-1}). After incubating the fixed cells in DAPI solution for another 20 min, the cells were washed trice with 1X PBS. The cells were ready for fluorescence microscope imaging. The imaging experiment was performed by removing the glass cover slip from the cell culture well, and then the cells were sandwiched between the cover slip and a piece of microscope glass slide.

***In vivo* studies of therapeutic efficacy**

To prepare the nude mice for the *in vivo* experiments, the MDA-MB231 cells were cultured in a T-75 tissue culture flask supplemented with complete DMEM medium. Thereafter, the adherent tumor cells were washed with PBS and harvested from the flask by 3 min exposure to 0.25 percent trypsin in an incubator. Trypsinization was quenched with the culture medium before centrifugation at 100 g under 40°C for 4 min, and the floating cells in the supernatant were discarded. The cell pellet was re-suspended and diluted to a cell count of 2.5×10^6 per 100 mL in the cell growth medium containing 30% matrigel (BD Biosciences, Bedford, MA, USA). The prepared cell solution was subcutaneously injected into the right flank area of each mouse. The mice were then reared for a period of seven days before randomly segregating them into various treatment groups, where each mouse has a tumor with a size of approximately 7.5 mm^3 . The mice were treated with (i) saline (100 mL), (ii) free DOX (0.21 mg kg^{-1} per week), (iii) ZG-01 (FA–VE) NPs (60 mg kg^{-1} per week), and (iv) ZG-01 (FA + VE) NPs (60 mg kg^{-1} per week). The tumor dimensions were measured using a linear caliper and the tumor volumes were calculated using the following equation.

$$V (\text{mm}^3) = a \times \frac{b^2}{2}$$

where V represents the tumor volume, a represents the largest diameter of tumor, and b represents the smallest diameter of tumor. All of the mice were sacrificed after twenty-one days of post inoculation by exposure to carbon dioxide. The dissected organs and tumor tissues of the sacrificed mice were fixed in 4% paraformaldehyde and processed routinely into paraffin, sectioned

at 5 mm, and stained with hematoxylin & eosin (H&E). The examined tissues include the liver, lung, heart, kidney and spleen.

2.5 Appendix

2.5.1 Synthesis scheme of various compounds

Scheme S1. Schematic representation for the synthesis of PAA-AD, PAA-CD, PEG-AD, FA-AD and FITC-AD.

2.5.2 Analysis of PAA-AD and PAA-CD

2.5.2.1 UV-Vis studies for determination of the conjugation degree of β -CD on PAA-CD

The conjugation degree of β -CD onto PAA was determined by the UV-Vis spectroscopy technique. As the β -CD molecule does not possess any UV-Vis absorption, conventional UV-Vis standard calibration plot for β -CD could not be prepared. Thus, we relied on an indirect UV-Vis spectroscopy method for the determination of the conjugation degree of β -CD on PAA-CD. The hydrophobic cavity of β -CD enables it to host a variety of guest molecules including azobenzene compound (Fig. S1.1) with a certain binding constant. In a recent work,⁶² the binding constants (k) of β -CD with azobenzene compounds were determined via the reciprocal plot of Hidebrand-Benesi equation (Fig. S1.2). Upon complexation with β -CD, there is a significant change in the UV-Vis spectrum of azobenzene. The change of the absorbance can be used for the preparation of the reciprocal plot from the Hindebrand-Benesi equation as shown below.

Figure S1.1. Schematic illustration for the complexation of β -CD with azobenzene.

$$\text{a) } k = \frac{[\text{Complex}]}{[\beta\text{-CD}][\text{AZB}]}$$

$$\text{b) } \frac{I}{|\Delta A|} = \frac{I}{\Delta \varepsilon k [\text{AZB}][\beta]} - \frac{I}{\Delta \varepsilon [\text{AZB}]}$$

$|\Delta A|$ is the modulus change in the abs of AZB upon the complexation with β -CD, $\Delta \varepsilon$ is the change in the molar extinction coefficient of AZB at the π - π^* wavelength, k is the binding constant between AZB and β -CD, $[\text{AZB}]$ is the concentration of AZB, and $[\beta]$ is the concentration of β -CD.

Figure S1.2. Formation equilibrium of the β -CD/azobenzene complex. a) The expression of the reaction equilibrium. b) The reciprocal plot of the Hindebrand-Benesi equation.

The binding constant reveals the crucial information regarding the stability of the resultant complex, which is one of the determining factors for its self-assembly application. In this work, we relied on the reciprocal plot of the Hindebrand-Benesi equation as the standard calibration plot for the determination of the β -CD concentration in the given solution. Thereafter, the degree of conjugation on PAA can be calculated by taking the ratio of the absolute mole of β -CD to the absolute mole of PAA polymer. Owing to its excellent aqueous solubility, sodium 4-((4-hydroxyphenyl)diazenyl)benzenesulfonate (HBS) (Fig. S1.3) was chosen as the azobenzene model molecule for the preparation of the reciprocal plot.

Figure S1.3. Molecular structure of sodium (*E*)-4-((4-hydroxyphenyl)diazenyl)benzenesulfonate (HBS).

Preparation of Hindebrand-Benesi plot

In the preparation of the reciprocal plot from the Hindebrand-Benesi equation, the UV-Vis spectrum of aqueous HBS (8.00×10^{-5} M, 3.0 mL) was measured in the absence of light. Thereafter, aqueous β -CD (20.0 mg mL⁻¹, 15 μ L) was added to the same HBS solution and the UV-Vis spectrum of the resulting

solution was measured again. This process was repeated by systematically adding aqueous β -CD (20.0 mg/mL) into the same solution, and the absorbance at 353 nm (π - π^*) was recorded accordingly (Fig. S1.4 and Table S1.1). Lastly, the reciprocal plot of the Hindebrand-Benesi equation was prepared by plotting the values from the $\frac{1}{[\beta]}$ column versus the values from the $\frac{1}{|\Delta A|}$ column (Fig. S1.5).

Figure S1.4. UV-Vis absorption spectra of HBS solution (8.00×10^{-5} M) containing various amount of β -CD.

Entry (n)	Volume of addition ^[a] (μ L)	$[\beta]$ ^[b] (mg mL ⁻¹)	$[\beta]$ (M)	$\frac{1}{[\beta]}$ (M ⁻¹)	Abs ₃₅₃ (A_n)	$ \Delta A = A_n - A_0 $	$\frac{1}{ \Delta A }$
0	0	0	0	-	1.549	0	-
1	15	0.1	8.810×10^{-5}	11350	1.534	0.015	66.7
2	30	0.2	1.762×10^{-4}	5675	1.523	0.026	38.5
3	45	0.3	2.643×10^{-4}	3783	1.512	0.037	27.0
4	60	0.4	3.524×10^{-4}	2837	1.499	0.05	20.0
5	75	0.5	4.405×10^{-4}	2270	1.491	0.058	17.2
6	105	0.7	6.167×10^{-4}	1621	1.472	0.077	13.0
7	150	1	8.811×10^{-4}	1135	1.445	0.104	9.62
8	225	1.5	1.322×10^{-4}	756.7	1.408	0.141	7.09

[a] Aqueous β -CD with a concentration of 20.0 mg mL⁻¹, [b] final concentration of β -CD in the solution prior to UV-Vis spectroscopy measurement

Table S1.1. Conditions and results of the UV-Vis spectroscopy measurements for the preparation of the reciprocal plot from the Hindebrand-Benesi equation.

Figure S1.5. The reciprocal plot of the Hindebrand-Benesi equation for the complexation between HBS and β -CD.

Determination of the conjugation degree of β -CD on PAA-CD

The conjugation degree of β -CD onto PAA was determined indirectly through the use of UV-Vis spectroscopy technique. The determination was done by first measuring the absorbance of the HBS solution ($8.00 \times 10^{-5} \text{ M}$, 3.00 mL) in the absence of light. After which, aqueous PAA-CD (44 mg mL^{-1} , 50 μL) was added into the same HBS solution and the absorbance was measured once again under the same conditions (Fig. S1.6). Thereafter, the difference in the absorbance at the wavelength of 353 nm was determined and applied onto the reciprocal plot of the Hindebrand-Benesi equation to obtain the concentration of β -CD in the polymer solution. Subsequently, the mass of the PAA polymer was calculated and the molar ratio of β -CD to the PAA polymer was determined, and this can be re-expressed as the conjugation degree of β -CD on PAA. The results of various measurements and calculations are summarized in Table S1.2.

Figure S1.6. UV-Vis absorption spectra of HBS solution ($8.00 \times 10^{-5}\text{M}$) with and without PAA-CD.

Abs ₃₅₃ of HBS solution in the absence of PAA-CD (A_0)	1.471
Abs ₃₅₃ of HBS solution in the presence of PAA-CD (A)	1.417
$ \Delta A = A - A_0 $	0.054
$1/ \Delta A $	18.51
$1/[\beta]$	2567 M^{-1}
Concentration of β -CD in HBS solution (3 mL), $[\beta]$	$3.895 \times 10^{-4} \text{ M}$

Table S1.2. Results obtained from the UV-Vis spectroscopy measurement of the HBS solution containing the PAA-CD polymer.

After obtaining the concentration of β -CD from the HBS solution upon the titration of PAA-CD, the value was used to calculate the ratio between the moles of the β -CD molecule to the moles of PAA polymer. Based on the results obtained from this experiment, it was found that the ratio of β -CD to PAA polymer is approximately 3:1. The ratio obtained was then re-expressed as the conjugation degree of β -CD onto PAA and the value was calculated to be 12%.

Calculations

Concentration of β -CD in 3 mL of HBS solution, $[\beta] = 3.895 \times 10^{-4} \text{ M}$

Concentration of β -CD in 50 μL of PAA-CD solution = $3.895 \times 10^{-4} \text{ M}$

$$\times \frac{3000\mu\text{L} \times}{50\mu\text{L}}$$

$$= 0.02337 \text{ M}$$

Mass of β -CD in 50 μL of PAA-CD solution = $0.050 \text{ mL} \times 0.02337 \text{ M} \times 1177$

$$\frac{\text{mg}}{\text{mmol}}$$

$$= 1.375 \text{ mg}$$

Mass of PAA-CD in 50 μL of PAA-CD solution = $44 \frac{\text{mg}}{\text{mL}} \times 0.050 \text{ mL}$

$$= 2.2 \text{ mg}$$

Of 2.2 mg of PAA-CD, mass contributed from PAA backbone = $2.2 \text{ mg} - 1.375 \text{ mg}$

$$= 0.83 \text{ mg}$$

Mole of PAA in 0.83 mg = $\frac{0.83 \text{ mg}}{1983 \frac{\text{mg}}{\text{mmol}}} = 0.419 \mu\text{mol}$

Mole of β -CD in the same 50 μL of PAA-CD solution = $0.050 \text{ mL} \times 0.02337 \text{ M} = 1.168 \mu\text{mol}$

Mole ratio of β -CD to PAA = $\frac{1.168\mu\text{mol}}{0.419\mu\text{mol}} = 2.79 : 1 \approx 3 : 1$

Number of conjugation sites per PAA = 25

Conjugation density = $\frac{3}{25} \times 100\% = 12\%$

2.5.2.2 Conjugation degree of AD on PAA evaluated through MALDI-TOF MS

The conjugation degree of AD on PAA was calculated based on the result obtained from MALDI-TOF MS. The m/z value obtained from MALDI-TOF MS of PAA-AD was 4924. The m/z value of PAA was 1952 and thus there are 25 repeat units on the synthesized PAA.

Define the number of conjugation site of AD onto PAA to be n :

Molecular weight of AD-NH₂ = 151

Molecular weight of the MBrP initiator = 183

Molecular weight of every repeat unit of acrylic acid = 72

Molecular weight of PAA-AD will be:

$$183 + 72 \times 25 - n \times 18 + n \times 151 = 4924$$

Solving for n gives $n = 22$

Thus, percent conjugation = $\frac{22}{25} \times 100\% = 89\%$

2.5.3 Electron microscopy studies

2.5.3.1 Transmission electron microscope (TEM) studies

The morphology and size of the self-assembled nanoparticles were studied by TEM. The TEM samples were prepared by dropping the sample solution (10 μ L) onto pieces of hydrophilic copper grids. Excess amount of liquid was absorbed and removed by a piece of filter paper. The sample grids were then allowed to dry overnight and directly taken for TEM studies. No staining was involved for all TEM studies.

Figure S2.1: TEM images of A) polymeric aggregates prepared from the mixture of PAA-CD and PAA-AD without the addition of PEG-AD, B) ZG-01 NPs, C) ZG-02 NPs, and D) ZG-01 NPs after the treatment with 1M acetate buffer (pH 5.5).

2.5.3.2 Scanning electron microscope (SEM) studies

The morphology and size of the self-assembled nanoparticles were also studied by SEM. The SEM samples were prepared by dropping the sample solution (10 μ L) onto pieces of double coated conductive carbon tapes. Excess amount of liquid was drained off and the tapes were dried with compressed air. The process was repeated for 3 times before a layer of gold was sputtered on top of the samples for SEM studies.

2.5.4 Confocal fluorescence microscopy images

Figure S3.1. Confocal fluorescence microscope images of MDA-MB231 cells upon treatment with A) ZG-01 NPs (0.6 mg mL^{-1}), B) ZG-02 NPs (0.6 mg mL^{-1}) and C) Free DOX ($30 \text{ } \mu\text{g mL}^{-1}$) after 4 hours incubation, visualizing (from left to right) the bright field image of the cells followed by DOX channel (excitation filter: 540/25 nm; emission filter: 605/55 nm), FITC channel (excitation filter: 480/40 nm; emission filter: 535/50 nm), DAPI channel (excitation filter: 350/50 nm; emission filter: 460/50 nm), and merged images of all fluorescence images within the cellular compartments.

Confocal fluorescence images of MDA-MB231 cells under various conditions were presented in Fig. S3.1. The multiple red and green spots on Fig. S3.1A indicate that there was an efficient uptake of the ZG-01 NPs by the cells. Fig.

S3.1B confirmed that folic acid can mediate the endocytosis of the ZG-02 NPs, in which DOX was absence in the NP system. Lastly, Fig. S3.1C shows that there was a positive uptake of free DOX by MDA-MB231 cells.

Figure S3.2. Confocal fluorescence microscope images of B16-F10 cells upon treatment with A) ZG-01 NPs (0.6 mg mL^{-1}), B) ZG-02 NPs (0.6 mg mL^{-1}) and C) free DOX ($30 \text{ } \mu\text{g mL}^{-1}$) after 4 hours incubation, visualizing (from left to right) the bright field image of the cells followed by DOX channel (excitation filter: $540/25 \text{ nm}$; emission filter: $605/55 \text{ nm}$), FITC channel (excitation filter: $480/40 \text{ nm}$; emission filter: $535/50 \text{ nm}$), DAPI channel (excitation filter: $350/50 \text{ nm}$; emission filter: $460/50 \text{ nm}$), and merged images of all fluorescence images within the cellular compartments.

Confocal fluorescence images of B16-F10 cells under various conditions were presented in Fig. S3.2. Similar to the fluorescence images of MDA-MB231 cells, the multiple red and green spots on Fig. S3.2A indicate that there was an efficient uptake of the ZG-01 NPs by the cells. Fig. S5.2B confirmed that folic acid can mediate the endocytosis of the ZG-02 NPs, in which DOX was absence in the NP system. Lastly, Fig. S3.2C shows that there was a positive uptake of free DOX by B16-F10 cells.

Figure S3.3. Confocal fluorescence microscope images of HEK293 cells upon treatment with A) ZG-01 NPs (0.6 mg mL^{-1}), B) ZG-02 NPs (0.6 mg mL^{-1}) and C) free DOX ($30 \text{ } \mu\text{g mL}^{-1}$) after 4 hours incubation, visualizing (from left to right) the bright field image of the cells followed by DOX channel (excitation filter: $540/25 \text{ nm}$; emission filter: $605/55 \text{ nm}$), FITC channel (excitation filter: $480/40 \text{ nm}$; emission filter: $535/50 \text{ nm}$), DAPI channel (excitation filter: $350/50 \text{ nm}$; emission filter: $460/50 \text{ nm}$), and merged images of all fluorescence images within the cellular compartments.

Confocal fluorescence images of HEK293 cells under various conditions were presented in Fig. S3.3. In this set of imaging experiments, only the cell image from the treatment of free DOX shows positive signal of DOX within the cellular endoplasm, while the remaining two sets of images do not show signals of DOX or NPs within the cells. The experiments conclude that the absence of folate receptor on the HEK293 cell surface prevents the non-specific delivery of DOX into the healthy cell line. Together with imaging experiments from the two cancerous cell lines, it was confirmed that the NP system has the ability to perform target-specific drug delivery towards cancerous MDA-MB231 and B16-F10 cells, while preventing the non-specific uptake of the anticancer drug by healthy cells.

2.5.5 Histology studies of various organs of mice

Figure S4. H&E staining images of major organs from mice treated with various samples. No obvious differences are observed from these images.

2.6 References

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Chapter 3: “Turn-on” fluorescence probe integrated polymer nanoparticles for sensing biological thiol molecules

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3.1 Introduction

Thiols in biological systems play a significant role in the maintenance of life. Owing to their oxidative properties, biological thiols serve as antioxidants,¹⁻³ aid in the inhibition of cellular apoptosis,⁴⁻⁷ and participate as scaffolds for the formation of complex three-dimensional protein structures. All of these activities occur through the formation or cleavage of disulfide bond. Among biological thiols, a lot of attention has been given to glutathione (GSH), cysteine (Cys) and homocysteine (Hcy). Exceptionally high concentration of any of these thiol compounds could act as a signal for the abnormality of cells including cancer, cardiovascular disease, AIDS and angiogenesis.⁸⁻¹³ Therefore, the detection of these thiol-containing compounds is crucial as a form of diagnostics. In view of this fact, various strategies, such as chromatography techniques, nanoparticle (NP) based reporters, and fluorescence assays, have been developed for the detection and sensing of intracellular thiol compounds.¹⁴⁻²⁰ Of these techniques, the fluorescence assays are often highly favored over other techniques owing to their high selectivity, low cost and simplicity, which can be applied without the use of sophisticated equipment.²¹ However, lipophilic nature of these fluorescence probes has greatly reduced their pharmacokinetic properties, and hence their uses for *in vivo* detection and sensing are still far from reach.²² Furthermore, these probes normally do not possess cancer cell-targeting property, significantly limiting their specificity for *in vivo* applications.²³⁻²⁴ In this work, we developed a “proof of concept” solution to address these issues by designing a molecular probe and then integrating it into biocompatible polymer based NPs for sensing intracellular thiols. The utilization of the polymer NP carriers could protect potential

biodegradation of the molecular probe during the circulation before reaching the target cells. In addition, a folic acid targeting ligand was introduced into the NP system for targeted thiol sensing.

Figure 1 (A) Supramolecular formation of PySSCCou-containing ZG-20 NPs. The chemical structures of the components for the formation of PySSCCou-containing ZG-20 NPs are shown in Figure S1. The ZG-20 NPs experience a fluorescence enhancement upon reaction with biological thiol compounds. (B) Schematic illustration of the working principle for the thiol-responsive PySSCCou probe.

The use of nanocarriers for the delivery of cargoes has shown improved bioavailability of the later significantly in biological systems.²⁵⁻²⁸ Recently, we reported the preparation of polyacrylate based NPs (ZG-20 NPs) as a carrier for *in vivo* drug delivery.²⁹ The ZG-20 NPs were fabricated by a supramolecular self-assembly of β -cyclodextrin (β -CD) conjugated polyacrylic acid (PAA-CD),

adamantane (AD) conjugated polyacrylic acid (PAA-AD), and AD conjugated PEG (PEG-AD). Anticancer drug doxorubicin was loaded into the NP system, showing enhanced chemotherapeutic effect as compared with free doxorubicin. In the continuation of this work, we herein integrated a “turn-on” thiol-responsive fluorescence probe (PySSCou) into the same NP system for sensing biological thiol compounds (Fig. 1A). The PySSCou molecule was designed as a responsive probe towards thiols. Its main fluorescence signal originates from the coumarin group in the probe. Initially, the fluorescence is mainly suppressed by the pyridine group, which is connected to the coumarin group by a disulfide bond-containing linkage. It was proposed that the pyridine group might quench off the fluorescence of the coumarin group by photoinduced electron transfer (PET) process (Fig. 1B).³⁰⁻³¹ Together with the coumarin group, the pyridine group could also form a host-guest complex with β -CD in PAA-CD, thereby facilitating the integration of the probe into the NPs.³²⁻³³ With this design, the biological thiols can enhance the fluorescence of the ZG-20 NPs by catalyzing the hydrolysis of the carbonate group on the 7th position of the coumarin unit and also the reduction of the disulfide bond in PySSCou to remove the pyridine group from the molecule. The removal of the pyridine group inhibits the PET process on the fluorescence probe, hence recovering the fluorescence from the coumarin unit. Although there are several thiol-responsive probes reported in literature,³⁴⁻⁴² the integration of a fluorescence probe into a nanocarrier to realize fluorescent thiol sensing has not been investigated so far.

3.2 Results and discussion

3.2.1 Preparation and characterization of probe-integrated ZG-20 NPs.

a)

b)

Scheme 1. A) Synthesis scheme of PySSCou. B) Self-assembly scheme of ZG-20 NPs.

Figure 2 (A) TEM image and (B) Zoomed-in TEM image of ZG-20 NPs. (C) Histogram showing the size distribution of ZG-20 NPs measured from 200 NPs from TEM image.

The PySSCou molecule was synthesized from commercially available starting materials (Scheme 1). The molecule was dissolved in dimethyl sulfoxide (DMSO) to yield a stock solution, which was then used for the preparation of ZG-20 NPs. In a typical preparation of ZG-20 NPs, 0.1 equiv. of PySSCou was mixed with PAA-AD containing 0.25 equiv. of the AD unit. To this mixture was added with 0.2 equiv. of PEG-AD followed by the addition of 0.1 equiv. of PAA-CD. The mixture was then washed thoroughly with phosphate buffered saline (PBS) so as to ensure that all unbound precursors were removed. Finally, the NPs were re-suspended in PBS for subsequent characterizations and applications. The size and morphology of the as-prepared ZG-20 NPs were characterized by transmission electron microscope (TEM). The TEM image (Fig. 2) shows that the NPs are spherical in shape and have the mean diameter of 18.7 ± 3.8 nm measured from 200 particles. The TEM image also exhibits the absence of large polymeric aggregates, confirming that the NPs were formed under a controlled manner. The surface charge of the NPs was measured using the Zeta potential technique, which was found to be 24.56 mV. The slight negative surface charge may be attributed to the free carboxylate groups on the PAA conjugates. The negatively charged surface of NPs is in favor of minimizing non-specific interactions between the NPs and

cells with negatively charged surface. Furthermore, as mentioned in chapter 2, the narrow distribution in the particles size is attributed to the presence of PEG-AD as a mono functional modulator. Having only one adamantane group on the terminal of PEG, the mono functional modulator prevents the uncontrollable crosslinking between the multifunctional PAA-AD and PAA-CD, which would result in the formation of large polymer mass (See Fig S2.1A of Chapter 2).

3.2.2 Photophysical properties of probe-integrated ZG-20 NPs.

The UV-Vis spectrum of the ZG-20 NPs shows that the NPs exhibit the maximum absorption at the wavelength of 330 nm (Fig. S5 in the Appendix). Based on this observation, the fluorescence spectrum of the ZG-20 NPs was measured. The NPs exhibit a weak fluorescence signal at 417 nm. In addition, UV-Vis spectroscopic studies reveal that the ZG-20 NPs have a loading capacity of 2.75 w/w% for the PySSCou probe molecule (see Section 6 in the appendix). The XPS (X-ray photoelectron spectroscopy) measurement also indicates that the NPs contain 2.65 mol% of S relative to N and C elements (Fig. S9 in the SI). These measurements confirmed that the PySSCou molecule was successfully integrated into the polymeric NP system, as the NPs without the PySSCou loading did not exhibit these properties. Thus, the NP system could serve as a host for effective loading of the PySSCou molecule.

3.2.3 Detection selectivity.

Figure 3 (A) Fluorescence intensity (l 5 477 nm) of ZG-20 NPs after overnight co-incubation with various amino acids and thiol compounds (5.0 mM) in PBS (pH 5.7.4) at 37°C (lex 5 330 nm). Error bar: n = 3. Inset: Fluorescence images of the resulted NP solutions under UV lamp. From bottom left to top right: ZG-20 NPs treated with DTT, Cys, Hcy, GSH, Methionine (Met), Alanine (Ala), Glycine (Gly), Leucine (Leu), Histidine (His), Isoleucine (Ile), Tryptophan (Trp), Tyrosine (Tyr), Aspartic acid (Asp), Arginine (Arg), Asparagine (Asn), Lysine (Lys), Phenylalanine (Phe), Threonine (Thr), Proline (Pro), Serine (Ser), Valine (Val), Glutamine (Gln) and Glutamic acid (Glu). (B) Fluorescence spectra of the NP solution after overnight co-incubation with various concentrations of Cys in PBS (pH 5.7.4) at 37°C.

The detection selectivity of probe-integrated ZG-20 NPs was evaluated by conducting the co-incubation of the NPs (0.66 mg mL^{-1}) with various amino

acids and thiol compounds (5.0 mM) in PBS buffer over 24 h. In this study, we used dithiolthretol (DTT) to investigate the response of the NP system. Although DTT is not considered as a biological thiol compound, it is a well-known thiolated reducing agent and could serve as a positive control for comparison. After the overnight incubation, we measured the fluorescence intensity of the NP solution at 477 nm under the excitation wavelength of 330 nm. The measurement results show that the ZG-20 NPs possess an excellent selectivity, i.e., the NPs only respond to various thiol compounds but not to the nonthiolated amino acids (Fig. 3A). Similarly, the selectivity experiments of the naked PySSCou molecule with these amino acids and thiol compounds also show the same trend, thereby confirming that the integration of PySSCou into the polymeric NPs did not compromise its selectivity (Fig. S4 in the appendix).

3.2.4 Detection limits determination.

In order to study the sensitivity of the NP system, its response upon changing the concentration of Cys was recorded. It was observed that the response decreased with the decrease in the Cys concentration (Fig. 3B). By using the same set of experiment data, the plot of the intensity at the maximum wavelength (477 nm) versus the Cys concentration gave a linear relationship between the two variables (Fig. S6 in the appendix). The detection limit (5.75 μM) of the NP system was obtained based on the linear plot.

3.2.5 Quantum yield calculation.

In the design of the fluorescence probe, we proposed that the reaction of the integrated probe with thiol compounds could release highly fluorescent coumarin molecule (CouSH) into the testing buffer. In order to confirm this mechanism, we measured the quantum yields of PySSCou as well as its coumarin product CouSH after reaction with DTT (see Section 5 in the appendix). The measurement results reveal that the corresponding quantum yields increased from 1.0% to 12.0%, indicating that the reaction released highly fluorescent CouSH into solution in order to serve as a signal for the sensing application. We also performed the GC EI-MS (gas chromatography electron ionization–mass spectrometry) analysis of DTT-treated NP solution (Fig. S10 in the appendix). The GC EI-MS analysis shows the presence of an m/z value at 281, corresponding to the $[M+H]^+$ peak of the released CouSH molecule.

3.2.6 Kinetic studies.

Figure 4. (A) Kinetic measurements of the ZG-20 NPs with and without (Blank) various thiol compounds. λ_{ex} 5 330 nm, λ_{em} 5 477 nm. (B) Fluorescence spectra of ZG-20 NPs (λ_{ex} 330 nm) after incubation with and without (Blank) various thiol compounds (5 mM). Prescan curve refers to the fluorescence spectrum of the NPs before the incubation, and Blank curve means that the fluorescence spectrum of the NPs was recorded after the same incubation time without the addition of thiol compounds.

In order to further probe the reaction kinetics of NPs with various thiol compounds, we carried out the time-dependent fluorescence measurements of the NP solution with the incubation of various thiols at pH 7.4 and 37°C over a period of 3 h (Fig. 4A). The results of the kinetic experiments show that the treatment of the NPs with various thiol compounds induces the enhancement of the fluorescence at 477 nm under the excitation of 330 nm. DTT led to the highest rate of response, followed by Cys, Hcy and GSH. In addition, a blank run where no additive was added into the NP solution during the incubation period was performed as a control. In the control case, there was no increase in the fluorescence intensity at 477 nm over 3 h, while a decrease in the fluorescence intensity was observed. We speculated that the decrease in

the fluorescence intensity was a result of the hydrolysis of the carbonate group on the 7th position of the coumarin molecule. In the initial state, electron-withdrawing carbonate group exhibits an inductive effect on the PySSCou molecule, contributing to the slight inhibition of the PET process to the pyridine group. The unstable carbonate group is prone to hydrolyze, converting into electron-donating phenoxide anion. The resulted phenoxide anion further promotes the PET process, which leads to the decrease of the fluorescence for the blank experiment. The presence of the carbonate group in the PySSCou molecule has two reasons. One is for synthesis reason that the incorporation of the carbonate group prevents the nucleophilic attack of the phenoxide group onto the 4-nitrophenyl chloroformate unit. The second reason is to employ it as a normalization signal during all the kinetic studies. It was observed from the kinetic studies that all the kinetic curves begin at the same starting point, having absolute fluorescence intensity at about 40 a.u. This observation indicates that all the kinetic experiments were conducted with the same initial NP concentration. At the end of the kinetic experiments, the fluorescence spectra of the NPs were recorded under the excitation wavelength of 330 nm (Fig. 4B). Initially quenched fluorescence of probe-integrated NPs showed a fluorescence enhancement upon the reactions with various thiol compounds. In addition, kinetic studies were also performed using the naked PySSCou molecule and a same trend of reactivity was observed (Fig. S3 in the appendix).

3.2.7 Electrochemical and Gibbs free energy measurements.

We then carried out the cyclic voltammetry (CV) measurements of PySSCou and thiol compounds in order to work out their reduction potentials and subsequently to estimate the Gibbs free energy of the reactions (Table 1). The Gibbs free energy estimation indicates that all the thiol compounds could react with PySSCou spontaneously. It was observed that the trend in the Gibbs free energy agrees with the trend obtained from the kinetic experiments.

Table 1. Summary of the results from the electrochemical analysis. All potentials reported were measured against the Ag/AgCl electrode.

	PySSCou	Cys	DTT	GSH	Hcy
E_{ox} [V]	-0.590	-0.869	0.360	-0.565	-0.589
E_{red} [V]	-1.010	-1.080	-3.690	-1.050	-1.030
$E_{1/2}^{\circ}$ [V]	-0.805	-0.975	-1.665	-0.808	-0.810
$\Delta E_{\text{Cell}}^{\circ}$ [a] [V]	0	0.170	0.860	0.003	0.005
ΔG° [b] [J/mol]	0	-2900	-15000	-51	-87

a) Difference between the standard reduction potentials ($E_{1/2}^{\circ}$) of PySSCou and respective thiol compounds. b) Estimated Gibbs free energy based on $\Delta G_o = -nFE_{\text{ocell}}$, where n is the number of electrons involved (2 electrons for disulfide exchange reaction) and F is Faraday's constant.

3.2.8 Targeted intracellular imaging of biological thiols.

Figure 6 Fluorescence microscopy images of B16-F10 cells after co-incubation with FA+VE ZG-20 NPs, FA-VE ZG-20 NPs, and PySSCou molecule. Upper three: Fluorescence images were taken in the DAPI (49,6-diamidino-2-phenylindole) channel. Excitation filter: 350/50 nm and emission filter: 460/50 nm. Middle three: Bright field images of the respective cells. Bottom three: Merged images of the respective bright field and fluorescence cell images. Scale bar is 20 μ m.

In order to assess the bio-applicability of the probe-integrated ZG-20 NPs for intracellular sensing of thiol compounds, two types of ZG-20 NPs were prepared. One of them (FA+VE ZG-20 NPs) was integrated with the AD conjugated folic acid (FA-AD), while the other one (FA- VE ZG-20 NPs) did not contain FA-AD. In our previous work, we have demonstrated that the integration of FA-AD into the NPs could induce the folate mediated endocytosis of the PAA NPs by cancer cells.²⁹ Hence, we would like to prove that the sensing of intracellular thiols could also be highly selective through the introduction of a targeting ligand into the NP system. The two types of NPs were incubated with a pre-cultured B16-F10 mouse melanoma cells followed by taking the fluorescence microscopy images of the cells. From the

fluorescence microscopy images (Fig. 6), it was observed that the cells treated with the FA+VE ZG-20 NPs show a positive fluorescence signal (blue color), particularly within the cytoplasm of the cells. However, the same experiment conducted using the FA-VE ZG-20 NPs exhibit the absence of the fluorescence signal. These experiment results indicate that the selectivity of the probe can be altered by the conjugation of folate group on the NPs.

It is known that cancer cells exhibit higher intracellular thiol concentration, and non-cancerous cells express a low amount of thiol compounds in the intracellular environment. Thus, non-specific targeting of NPs may provide false positive results during the cancer imaging. In addition, the fluorescence images of the cells upon the incubation with the naked PySSCou molecule were recorded. In this control experiment, no fluorescence from the treated cells was observed. This observation agrees with the literature report that free dye molecules have certain difficulties in the trans-membrane trafficking process.⁴⁷ The control experiment confirms that the folate-containing NPs have the ability to carry the PySSCou probe into the intracellular environment. Furthermore, we conducted the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay for the cells treated with the probe-integrated NPs and the naked PySSCou molecule. The experiments showed that the NPs exhibit insignificant toxicity to the cells under the concentrations measured (Fig. S16 in the appendix), demonstrating their suitability for biological applications. Thus, the application of the NPs for intracellular sensing of thiol compounds was successfully demonstrated.

3.3 Conclusion

By utilizing the intramolecular PET mechanism, we rationally designed and synthesized a coumarin based “turn-on” fluorescence probe that consists of a disulfide bond connected to a quencher. The pyridine moiety in the PySSCou probe not only serves as an electron reservoir for the quenching of the coumarin fluorescence, but also allows easy integration of the probe into polymeric NPs for *in vitro* sensing of biological thiol molecules. To confirm the presence of the PySSCou probe in the polymeric NPs, various characterization techniques such as XPS and UV-Vis spectroscopy were employed. The characterization results proved that the PySSCou probe was indeed incorporated into the polymeric NPs. Together with our previous work on the loading and delivery of doxorubicin using this NP system, the research demonstrated the versatility of the NP system for the integration of different cargos capable of multiple uses.

In terms of the selectivity towards the thiol compounds, the disulfide bond on PySSCou enables the probe to react with thiols in a highly selective manner, which was proven by a series of control experiments against the non-thiolated amino acids. The electrochemical studies confirmed that the reaction between biological thiols and PySSCou was a spontaneous process with a negative Gibbs free energy. In addition, various *in vitro* thiol detection studies showed that the probe, either as a free molecule or being integrated into the NPs, could present excellent responses to biological thiol compounds, concluding that the integration process does not influence the selectivity and reactivity of the probe. On the other hand, this NP system allows for the incorporation of cancer cell-

targeting ligand for enhancing the sensing selectivity to the intracellular thiol compounds within cancerous cells.

In conclusion, we have successfully developed a novel “turn-on” fluorescence probe that has been integrated into polymeric NPs for sensing applications. The as-prepared NPs have the capability to respond to various biological thiol compounds against the non thiolated amino acids. Intracellular sensing of biological thiols in B16-F10 mouse melanoma cells using the probe-integrated NPs has been carried out, indicating that the sensitivity and selectivity of the NPs could be altered by the integration of the targeting ligand. Since the NP system has the capability to selectively deliver the cargoes into the tumor sites in tumor-bearing nude mouse models²⁹, the probe-integrated NPs show a promising potential to be utilized in the sensing of biological thiol compounds *in vivo*.

3.4 Experimental

General procedure

Acetic anhydride (98%, Sigma Aldrich), *N*-acetylglycine (ReagentPlus[®], Sigma Aldrich), 1-adamantamine hydrochloride (99%, Alfa Aesar), 4-aminopyridine (98%, Alfa Aesar), *N,N'*-dicyclohexylcarbodiimide (99%, Merck), 2,4-dihydroxybenzaldehyde (98%, Sigma Aldrich), 2,2'-dipyridyl disulfide (98%, Alfa Aesar), di-*tert*-butyl dicarbonate ($\geq 97\%$, Alfa Aesar), *L*-glutathione reduced ($\geq 98\%$, Sigma Aldrich), *DL*-homocysteine ($\geq 95\%$, Sigma Aldrich), *N*-hydroxysuccinimide (98%+, Alfa Aesar), 2-mercaptoethylamine hydrochloride (98%+, Alfa Aesar), 3-mercaptopropionic acid ($\geq 99\%$, Sigma

Aldrich), 4-nitrophenyl chloroformate (97%, Alfa Aesar), sodium acetate (anhydrous, Sigma Aldrich), trifluoroacetic acid (Alfa Aesar), triisopropylsilane (99%, Sigma Aldrich), trityl chloride (Sigma Aldrich), and a series of amino acids (Amino acids kit, Sigma Aldrich) were used as received. PAA-CD, PAA-AD and PEG-AD were synthesized based on our previously reported procedure.²⁹ All solvents and inorganic bases or acids were of analytical grade and used as per received. Water (Ultrapure, 18.2 $\Omega \cdot \text{cm}$ resistivity at 25 °C and < 10 ppb Total Organic Carbon) used in this work was dispensed from Sartorius Ultrapure Water dispenser system.

All reactions performed in this work were under ambient conditions (approximately 23-25°C, atmospheric pressure: 1 atm), unless otherwise stated.

Purification of compounds by flash column chromatography was performed with Merck Geduran[®] Si 60 silica gel with particles size of 40 to 63 μm . Column was stacked with the slurry mixture of the silica gel and the eluting solvent.

¹H and ¹³C NMR spectra were recorded on a Bruker AMX 400 MHz NMR spectrophotometer at ambient temperature. All chemical shifts δ from ¹H NMR spectra were reported in unit of parts per million (ppm) downfield from TMS (δ 0.00 ppm) or relative to the deuterated solvents (either δ 7.26 ppm for CDCl₃ or 2.54 ppm for DMSO-*d*₆) used. Multiplicities for ¹H NMR spectra were reported as: s (singlet), d (doublet), t (triplet), dd (doublet of doublet), dt (doublet of triplet) and m (multiplet). Coupling constants, *J* values, were reported in units of Hz and the number of equivalent protons for a given resonance was reported as *n*H. Chemical shifts δ from ¹³C NMR spectra were

reported in units of parts per million (ppm) relative to the residual signals from the deuterated solvents (either δ 77.0 ppm for CDCl₃ or 39.81 ppm for DMSO-*d*₆) used.

Melting point of solid compounds was measured on OptiMelt Automated Melting point instrument. High resolution mass spectrometry (HRMS) was recorded on Waters Q-tof Premier MS and electron ionized mass spectrometry (EI-MS) was measured on ThermoFinnigan PolarisQ MS coupled with Thermo Trace gas chromatography (GS) setup. FTIR measurements were performed on a SHIMADZU IR Prestige-21 spectrophotometer. UV-Vis spectroscopy studies were carried out using a SHIMADZU UV-3600 UV-VIS-NIR Spectrophotometer using a 10-mm-by-10-mm 3.5 mL quartz cuvette. Fluorescence emission spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer, equipped with water circulated temperature controller. Transmission electron microscope (TEM) images were recorded on JEOL: JEM 1400 thermionic emission electron microscope operating at an acceleration voltage at 100 kV. Zeta potential measurements were performed on the Malvern Zetasizer Nano ZSP. X-Ray Photoelectric Spectroscopy measurements were carried out on a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany) for the wide-range and high resolution S 2p scans at 12.53 kV.

For *in vitro* biological experiments, mouse melanoma cells B16-F10 cells were obtained from ATCC and cultured with DMEM (High glucose with sodium pyruvate and *L*-Glutamine), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. The cells were grown in 5% humidified CO₂ incubator. Fluorescence images of the cells were obtained by a Nikon

Eclipse TE2000-E Confocal fluorescence microscope with Nikon TE2-PS100W high-pressure mercury lamp.

Synthesis of compounds

3-amino-7-hydroxy-2H-chromen-2-one (1)

3-Amino-7-hydroxycoumarin was synthesized in accordance to reported work.^{31, 43} Briefly, to a mixture of 2,4-dihydroxybenzaldehyde (1.104 g, 8.0 mmol), *N*-acetylglycine (936 mg, 8.0 mmol) and anhydrous sodium acetate (1.968 g, 24.0 mmol) in acetic anhydride (40 mL) were added. The mixture was brought to reflux under stirring condition over a period of 4 h. Thereafter, the mixture was cooled down to room temperature and poured into ice water to yield a yellow slurry. The water was decanted off carefully and the slurry was brought to reflux in a solution of concentrated HCl in ethanol (2:1, 30 mL) over a period of 1 h. The mixture was cooled and poured into ice water (approximately 50 to 100 mL) before the solution was carefully neutralized to a pH of 5.0 with 30% NaOH in water. The solid from the resultant slurry was isolated by centrifugation and washed twice with water to yield **1** as a brown solid (260 mg, 18%). M.P.: 242.9-246.0°C. FTIR $\bar{\nu}$ /cm⁻¹: 3441.01, 3350.35, 3226.91 (broad), 3057.17, 1681.93, 1614.42, 1463.97, 1288.45, 1128.36, 881.47, 844.82, 802.39, 767.67. ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.23 (d, *J* = 8.37 Hz, 1H), 6.69 (d, *J* = 10.89 Hz, 3H), 5.63-5.79 (m, 14H), 5.22 (s, 2H), 5.23 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 190.0, 159.5, 156.7, 149.8, 130.7, 126.2, 113.9, 113.5, 110.4, 102.3. HRMS (H₂O/MeOH): *m/z*: calcd: 178.0504 [M + H]⁺; found: 178.0502.

3-amino-2-oxo-2H-chromen-7-yl *tert*-butyl carbonate (2)

To a stirring solution of **1** (488 mg, 2.76 mmol) in THF (10 mL) was added dropwise with di-*tert*-butyl dicarbonate (654 mg, 3.0 mmol) and triethylamine (384 μ L, 2.76 mmol) in THF (20 mL). The resulting solution was stirred for 24 h before the solvent was removed by rotary evaporation. The mixture obtained was purified by flash chromatography with ethyl acetate and hexane (1:1) as eluent to afford a yellow solid **2** (601 mg, 79%). M.P.: 96.4–99.8°C. FTIR $\bar{\nu}$ /cm⁻¹: 3425.58, 3365.78, 2983.88, 2935.66, 1751.36, 1707.00, 1635.64, 1600.92, 1435.04, 1371.39, 1242.16, 1145.72, 975.98, 887.26, 825.53, 771.53. ¹H NMR (400 MHz, CDCl₃): δ 7.29 (d, J = 8.34 Hz, 2H, overlapped with CDCl₃), 7.16 (s, 1H), 7.07 (dd, J = 8.53 Hz and 1.51 Hz, 1H), 6.70 (s, 1H), 4.26 (s, 2H), 1.56 (s, 29H, overlapped with H₂O). ¹³C NMR (100 MHz, CDCl₃): δ 159.1, 149.0, 146.7, 131.5, 125.4, 118.9, 118.1, 110.3, 109.5, 85.2, 27.4. HRMS (H₂O/MeOH): m/z : calcd: 278.1028 [M+H]⁺; found: 278.1013.

4-nitrophenyl**7-(*tert*-butoxycarbonyloxy)-2-oxo-2H-chromen-3-ylcarbamate (3)**

A stirring solution of **2** (601 mg, 2.16 mmol) and 4-nitrophenyl chloroformate (503.8 mg, 2.50 mmol) in THF (40 mL) was added with triethylamine (306 μ L, 2.20 mmol). Thereafter, white precipitate of triethylamine hydrochloride was formed and the solution was further stirred for 24 h. The white precipitate was then removed by filtration

and the solvent of the filtrate was removed by rotary evaporation. The resulting solid was dissolved in DCM (about 2 mL) and the mixture was added into 5% ethyl acetate in hexane to induce precipitation. The precipitate was isolated by filtration and re-dissolved in DCM (about 2 mL). The mixture was further purified by re-precipitation in 15% ethyl acetate in hexane and the resulting white solid **3** (168.4 mg, 18%) was isolated by filtration. M.P.: 151.0-152.2°C (Decomposition temperature). FTIR $\bar{\nu}$ /cm⁻¹: 3317.56, 3086.11, 2985.81, 1749.44, 1716.65, 1629.85, 1616.35, 1550.77, 1527.62, 1492.90, 1373.32, 1348.24, 1217.08, 1145.72, 1002.98, 860.25. ¹H NMR (400 MHz, CDCl₃): δ 8.34 (dd, J = 6.91 and 2.13 Hz, 3H), 7.93 (s, 1H), 7.53 (d, J = 8.54 Hz, 1H), 7.44 (d, J = 9.09 Hz, 2H), 7.21 (dd, J = 8.55 and 2.19 Hz, 1H), 1.60 (s, 9H). ¹³C NMR (100 MHz, CDCl₃): δ 158.0, 154.8, 152.1, 151.0, 150.2, 150.0, 128.3, 125.4, 125.3, 125.1, 122.9, 122.1, 118.9, 117.0, 109.8, 84.6, 53.4, 27.7. HRMS (H₂O/MeOH): m/z : calcd: 443.1091 [M+H]⁺; found: 443.1092.

3-(tritylthio)propanoic acid (**4**)

Compound **4** was synthesized in accordance to the reported procedure.⁴⁴ 3-Mercaptopropionic acid (6.0 g, 56.6 mmol) in DCM (50 mL) was added dropwise with trityl chloride (17.3 g, 62.3 mmol) in DCM (30 mL). The resulting white slurry was stirred for 16 h. The precipitate formed was isolated by filtration and washed with DCM repeatedly to yield **4** as white solid (16.4 g, 83%). M.P.: 210.1 – 210.8°C. FTIR $\bar{\nu}$ /cm⁻¹: 3400 – 2400 (broad), 3061.03, 3032.10, 2883.58, 2883.59, 1703.14, 1591.27, 1487.12, 1448.54, 1431.18, 1408.04, 1253.73, 1253.73, 958.62,

746.45, 702.09. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 7.33 (t, $J = 4.35$, 12H), 7.25 (dt, $J = 8.47$ and 4.27 Hz, 3H), 2.31 (t, $J = 6.99$ Hz, 2H), 2.18 (t, $J = 6.94$ Hz, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): 173.2, 144.8, 129.6, 128.5, 127.2, 66.7, 33.4, 27.2. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 349.1262 $[\text{M}+\text{H}]^+$: found: 349.1255.

***N*-(pyridin-4-yl)-3-(tritylthio)propanamide (5)**

A mixture of **4** (4.0 g, 11.48 mmol), *N,N'*-dicyclohexylcarbodiimide (2.85 g), *N*-hydroxysuccinimide (1.59 g, 13.81 mmol) was stirred in THF (60 mL) for 4 h. Thereafter, 4-aminopyridine (1.30 g, 13.80 mmol) and triethylamine (1.92 mL, 13.80 mmol) were added into the solution and the mixture was brought to reflux for 24 h. The mixture was cooled down to room temperature and the white precipitate of *N,N'*-dicyclohexylurea was removed by filtration. The solvent of the filtrate was removed by rotary evaporation and the resulting syrup was purified by flash chromatography with ethyl acetate and hexane (8:2) as eluent to afford a white solid **5** (2.18 g, 45%). M.P.: 134.6 – 136.2°C. FTIR $\bar{\nu}$ / cm^{-1} : 3250.05, 3159.40, 3082.25, 3057.17, 3030.17, 3018.60, 2997.38, 2981.95, 2929.87, 2910.58, 2854.65, 2818.00, 1705.07, 1593.20, 1519.91, 1444.68, 1417.68, 1294.24, 1001.06, 827.46, 742.59, 700.16. ^1H NMR (400 MHz, CDCl_3): δ 8.49 (dd, $J = 4.83$ and 1.55 Hz, 2H), 7.48-7.46 (m, 6H), 7.41 (dd, $J = 4.86$ and 1.52 Hz, 2H), 7.34-7.25 (m, 11H), 2.63 (d, $J = 7.09$ Hz, 2H), 2.20 (d, $J = 7.11$ Hz, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 150.7, 144.5, 129.6, 128.0, 126.9, 113.4, 52.3, 36.9, 27.3. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 425.1688 $[\text{M}+\text{H}]^+$: found: 425.1676.

3-mercapto-*N*-(pyridin-4-yl)propanamide (6)

Compound **5** (1.85 g, 5.66 mmol) was charged into a round bottom flask where trifluoroacetic acid (4 mL) was added. The resulting brown colored mixture was stirred vigorously for 3 h, and then triisopropylsilane (2.5 mL) was added. The mixture immediately turned colorless with the formation of white precipitates. Stirring was then continued overnight before the reaction was quenched off with the addition of DCM. The solvent of the mixture was then removed under *vacuo* and the resulting syrup was purified by flash chromatography with 1% MeOH in DCM to afford a white solid **6** with pungent odor (460 mg, 45%). M.P.: 158.5 – 160.1°C. FTIR $\bar{\nu}$ /cm⁻¹: 3246.20, 3130.47, 3099.61, 3045.60, 2567.25, 2129.41, 1724.36, 1678.07, 1597.09, 1512.19, 1435.04, 1321.24, 1205.51, 842.89, 721.38. ¹H NMR (400 MHz, MeOD): δ 8.61 (d, *J* = 7.20 Hz, 2H), 8.14 (d, *J* = 6.80 Hz, 2H), 3.37-3.32 (m, 4H, overlapped with MeOD). ¹³C NMR (100 MHz, MeOD): δ 172.3, 153.4, 114.4, 40.8, 18.5. HRMS (H₂O/MeOH); *m/z*: calcd: 183.0592 [M+H]⁺; found: 183.0595.

2-(pyridin-2-yl)disulfanyethanaminium chloride (7)

In a typical synthesis, 2,2'-dipyridyl disulfide (4.41 g, 20mmol) and AcOH (0.8 mL) were dissolved in MeOH (20 mL), and then 2-mercaptoethylamine hydrochloride (1.14 g, 10 mmol) in MeOH (10 mL) was added dropwise. The resulting yellow solution was further stirred for 1 day and MeOH was removed via rotary evaporation. The resulting syrup was then added into diethyl ether (100 mL) to induce precipitation. The solid was

isolated by filtration and the yellow filtrate was discarded accordingly. The solid was then re-dissolved in MeOH (approximately 5 mL) and the solution was added into diethyl ether to induce precipitation again. The process was repeated for another 4 times to obtain **7** as a white solid (1.16 g, 52%). FTIR $\bar{\nu}$ /cm⁻¹: 3124.68, 2949.16, 2912.51, 1608.63, 1573.91, 1558.48, 1465.90, 1448.54, 1112.93, 869.90, 765.74. ¹H NMR (400 MHz, MeOD): δ 8.56 (d, J = 4.62 Hz, 1H), 7.84-7.80 (m, 1H), 7.68 (d, J = 8.04 Hz, 1H), 7.33 (dd, J = 7.25 and 5.05 Hz, 1H), 3.31 (t, J = 6.01 Hz, 4H, overlapped with MeOD), 3.16 (t, J = 6.15 Hz, 2H). ¹³C NMR (100 MHz, MeOD): δ 158.1, 149.5, 137.7, 121.9, 121.5, 37.4, 35.5. HRMS (H₂O/MeOH): m/z : calcd: 187.0364 [M-Cl]⁺: found: 187.0370.

2-((3-oxo-3-(pyridin-4-ylamino)propyl)disulfanyl)ethanaminium chloride (8)

To a stirring solution of **7** (488 mg, 2.20 mmol) in MeOH (20 mL) was added dropwise with **6** (400 mg, 2.197 mmol) in MeOH (10 mL). The resulting yellow solution was further stirred for 1 day and MeOH was removed via rotary evaporation. The resulting syrup was then added into diethyl ether (100 mL) to induce precipitation. The solid was isolated by centrifugation and the yellow supernatant was discarded accordingly. The solid was then re-dissolved in MeOH (approximately 5 mL) and the solution was added into diethyl ether to induce precipitation again. The process was repeated for another 4 times to obtain **8** as a white solid (520 mg, 81%). FTIR $\bar{\nu}$ /cm⁻¹: 3188.33-2530.61 (broad), 1703.14, 1637.56, 1571.99, 1508.33, 1481.33, 1417.68, 1157.29, 1062.78, 819.75, 765.74. ¹H NMR (400 MHz, MeOD): δ 8.58 (d, J = 6.00 Hz, 2H), 8.10 (d, J = 5.20 Hz, 2H), 3.34-3.28 (m,

4H), 3.15-2.98 (m, 7H, overlapped with MeOD). ^{13}C NMR (100 MHz, MeOD): δ 171.5, 149.4, 146.6, 113.7, 38.4, 36.1, 35.6, 32.7. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 258.0735 $[\text{M}-\text{Cl}]^+$; found: 258.0737.

***tert*-butyl**

2-oxo-3-(3-(2-((3-oxo-3-(pyridin-4-

ylamino)propyl)disulfanyl)ethyl)ureido)-2H-chromen-7-yl carbonate (9) or

PySSCou

PySSCou or **9** was synthesized as follows. A mixture of **8** (64.5 mg, 0.250 mmol) and **3** (80 mg, 0.180 mmol) was charged into a round bottom flask where DCM (25 mL) was

added to the mixture under stirring condition. Thereafter, triethylamine (84 μL , 0.60 mmol) was slowly added to the stirring solution, and the color of the solution turned yellow gradually. The solution was further stirred for 24 h, and then DCM was removed by rotary evaporation. The resulting residue was purified by flash chromatography with ethyl acetate and hexane (1:1) as eluent to yield **9** as a white solid (92 mg, 92%). M.P.: 150.8–152.5°C (decomposition temperature). FTIR $\bar{\nu}/\text{cm}^{-1}$: 3356.14, 2980.02, 1761.01, 1716.65, 1699.29, 1670.35, 1614.42, 1548.84, 1431.18, 1369.84, 1278.81, 1251.80, 1141.86, 896.90, 823.60. ^1H NMR (400 MHz, CDCl_3): δ 8.55 (s, 2H), 8.153 (m, 3H), 8.145-8.176 (m, 3H), 7.46 (d, J = 8.40 Hz, 2H), 7.111 (d, 8.80 Hz, 2H), 6.787 (d, J = 6.0 Hz, 1H), 3.66-3.70 (m, 4H), 2.92-2.95 (m, 4H), 1.585 (s, 19H, Overlapped with H_2O). ^{13}C NMR (100 MHz, CHCl_3): δ 159.4, 155.4, 151.0,

149.3, 128.0, 124.8, 120.6, 118.7, 109.4, 84.3, 60.4, 39.0, 38.1, 27.7, 21.1, 14.2.

HRMS (H₂O/MeOH): *m/z*: calcd: 561.1478 [M+H]⁺: found: 561.1500.

Preparation of NPs

Prior to the preparation of NPs, all components were prepared as stock solutions in their respective concentrations. PAA-CD: 8.6 mg/mL in water, PAA-AD: 26.0 mg/mL in DMSO, PEG-AD: 16.8 mg/mL in water, PySSCou: 11.7 mM in DMSO, and FA-AD: 11.7 mM in DMSO.

Preparation of ZG-20 NPs or FA-VE ZG-20 NPs

PAA-AD solution (4 µL) was added with PEG-AD solution (66 µL) and PySSCou solution (12 µL). The mixture was stirred thoroughly before the addition of PAA-CD solution (66 µL) and PBS (1 mL). The mixture was sonicated for 15 min and the NPs formed were isolated by high speed centrifugation. The supernatant was discarded and the NPs were re-dispersed in PBS (1 mL). The NPs were again isolated by high speed centrifugation and the process was repeated for two addition cycles to ensure the thorough washing of the NPs. Finally, the NPs were re-dispersed either in PBS (3 mL) to achieve a concentration of 0.66 mg/mL for fluorescence and kinetic measurements or in PBS (0.3 mL) to achieve a concentration of 6.6 mg/mL for bioimaging experiments.

Preparation of FA+VE ZG-20 NPs

The procedure for the preparation of FA+VE ZG-20 NPs was similar to the preparation of FA-VE ZG-20 NPs, while adding the FA-AD solution (12 µL) to the mixture prior to the addition of PAA-CD solution. The washed NPs were re-dispersed in PBS (0.3 mL) for bioimaging experiments.

3.5 Appendix

3.5.1 Characterization of FA+VE ZG-20 NPs

Figure S1. (A) TEM image and (B) Zoomed-in TEM image of FA+VE ZG-20 NPs.

Figure S2. Histogram showing the size distribution of FA+VE ZG-20 NPs measured from 200 NPs from Figure S2A.

The particle size and the surface charge of FA+VE ZG-20 NPs were characterized using TEM, DLS and Zeta potential measurements. TEM images showed that these NPs are spherical in shape with the diameter size of 21.3 ± 4.36 nm. Zeta potential measurement indicated that the NPs have a surface charge of -21.1 mV.

3.5.2 Response and selectivity of PySSCOu

Figure S3. (A) Kinetic measurements of PySSCOu with and without (Blank) thiol compounds. $\lambda_{\text{ex}} = 330 \text{ nm}$, $\lambda_{\text{em}} = 477 \text{ nm}$. (B) Fluorescence spectra of PySSCOu ($\lambda_{\text{ex}} = 330 \text{ nm}$) after incubation with and without (Blank) the thiol compounds (5 mM).

Figure S4. Fluorescence intensity ($\lambda = 477$ nm) of PySSCou after overnight co-incubation with various amino acids and thiol compounds (5.0 mM) in PBS (pH = 7.4) at 37°C ($\lambda_{\text{ex}} = 330$ nm). Error bar: $n = 3$. Inset: Fluorescence images of the resulted PySSCou solutions under UV lamp. From bottom left to top right: PySSCou treated with DTT, Cys, Hcy, GSH, Met, Ala, Gly, Leu, His, Ile, Trp, Tyr, Asp, Arg, Asn, Lys, Phe, Thr, Pro, Ser, Val, Gln and Glu.

3.5.3 Additional spectroscopic data (UV-Vis and detection limit calculation)

The absorption spectrum and the linear plot of the Cys concentration versus the fluorescence intensity ($\lambda_{\text{ex}} = 330$ nm, $\lambda_{\text{em}} = 477$ nm) are shown below.

Figure S5. UV-Vis absorption spectrum of ZG-20 NP solution.

Figure S6. Linear plot of cysteine concentration versus fluorescence intensity ($\lambda_{\text{ex}} = 330 \text{ nm}$, $\lambda_{\text{em}} = 477 \text{ nm}$) for cysteine treated ZG-20 NPs.

Detection limit calculation:

From linear plot, fluorescence intensity = 359,225 [Cysteine] + 2.0655

Hence, solving for absolute value of [Cysteine] when fluorescence intensity = 0 gives

$$\left| \frac{-2.0655}{359,335} \right| = 5.749 \times 10^{-6} M = 5.749 \mu M$$

3.5.4 Quantum yield computation

Quantum yield computation was conducted for PySSCou and PySSCou (50 mM) after treatment with 5 mM DTT in 1 X PBS. In this study, we chose 7-hydroxycoumarin (QY. 15%)⁴⁵ as the standard for quantum yield computation using the formula shown below.

$$\Phi_{\text{Sample}} = \Phi_{\text{Std}} \frac{1 - 10^{Abs_{\text{Std}}}}{1 - 10^{Abs_{\text{Sample}}}} \frac{\int Em_{\text{Sample}} d\lambda}{\int Em_{\text{Std}} d\lambda}$$

Where,

Φ is the quantum yield of sample or standard (Std)

Abs is the absorbance at λ_{max} (330 nm) of sample or standard

$\int Em d\lambda$ is the integrated area under the emission spectrum (Ex. 330 nm) with respect to the wavelength of sample or standard.

Figure S7. Absorption and Emission spectra (Ex. 330 nm) of 7-hydroxycoumarin in 1 X PBS.

3.5.5 Loading capacity determination of PySSCou in ZG-20 NPs

Loading capacity of PySSCou in ZG-20 NPs was determined by measuring the absorbance of the supernatant of the first centrifuged ZG-20 NPs during the NP preparation. Thereafter, the absorbance was compared to the absorbance of PySSCou without the addition of the polymeric precursors. The difference in the absorbance was then used to compare with a standard curve to obtain the loading capacity of PySSCou in ZG-20 NPs.

Figure S8. (A) Absorption spectrum of PySSCou solution before and after loading into the polymeric NPs. (B) Standard curve of PySSCou.

3.5.6 X-ray photoelectric spectroscopy (XPS) study

The integration of PySSCou into the NPs was confirmed via XPS analysis of the ZG-20 NPs. The NP solution (6.6 mg/mL) was prepared as per above and the solution was drop-casted onto a copper tape mounted onto an aluminum XPS holder. Drop-cast was performed by adding the NP solution (10 μ L) onto the copper tape and the solution was allowed to dry before the addition of another 10 μ L of the NP solution. The process was repeated for another three times before placing the sample in the vacuum desiccator overnight. The sample was then analyzed accordingly.

Figure S9. XPS spectrum of ZG-20 NPs with the relative percentage of respective elements. Inset: the zoomed-in scan of the S 2p.

3.5.7 Mass spectrometry study

To confirm the release of CouSH from the ZG-20 NPs upon treatment with reducing thiols, we performed the GC EI-MS of the NP solution after incubation with DTT.

Figure S10. EI-MS spectrum of ZG-20 NP solution after treatment with DTT.

The m/z value of 281.0 corresponds to the $[M+H]^+$ of CouSH, confirming that the treatment of the ZG-20 NP solution with DTT produces the CouSH fragment.

3.5.8 Electrochemical studies

All cyclic voltammetry experiments were performed using tetramethyl ammonium hexafluorophosphate in DMF as the electrolyte solution. All measurements were carried out by dissolving the respective analyte (5 mg) in electrolyte solution (about 30 mL).

Figure S11. Cyclic voltammogram of PySSCou.

Figure S12. Cyclic voltammogram of DTT.

Figure S13. Cyclic voltammogram of GSH.

Figure S14. Cyclic voltammogram of HCY.

Figure S15. Cyclic voltammogram of Cys.

3.5.9 Intracellular sensing of biological thiol compounds and cytotoxicity evaluation.

Intracellular sensing of biological thiol compounds was conducted on mouse melanoma B16-F10 cells. In a typical imaging experiment, 8.0×10^4 cells were seeded into a SPL 200350 coverglass-bottom dish and the cells were incubated overnight. Thereafter, FA-VE ZG-20 NP solution or FA+VE ZG-20 NP solution (200 μ L, 1.0 mg/mL) was added, and the cells were then incubated overnight. The medium was removed. The cells were washed 3 times with PBS and then added with fresh medium prior to imaging.

In the MTT cytotoxicity assay, the cells were first seeded into a 96 well plates at a seeding density of 1.0×10^4 cells per well prior to overnight incubation. Thereafter, 10 μ L per well of FA+VE ZG-20 NPs or PySSCou in PBS with various concentrations were added and followed by a further overnight incubation. The medium was removed and replaced by 100 μ L per well of fresh medium containing 0.5 mg mL⁻¹ of MTT. The cells were returned to the incubator for 4 h before removing the medium again. The residual purple crystal was dissolved in DMSO (100 μ L per well) and the absorbance of the purple solution was measured at 562 nm using a plate reader (Infinite® 200 PRO plate reader). The viability of each well was determined as a relative percentage to the control well where only water was added. Each data was reported as a mean of eight replicates and the error bars were the results of the standard deviations of these replicates.

Figure S16. Cytotoxicity profiles of (a) FA+VE ZG-20 NPs and (b) PySSCou.

3.6 References

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Chapter 4: A dual responsive “turn-on” fluorophore for orthogonal selective sensing of biological thiols and hydrogen peroxide

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4.1 Introduction

Stimuli-responsive fluorescent probes have received a great deal of attention on account of their special applications for sensing and imaging.¹⁻⁸ Fluorescence-based sensing is a cheaper technique and preferred over other methods, since the preparation of fluorescent probes is relatively easy and the sensing applications can be realized without utilization of sophisticated instrument.⁹⁻¹⁴ Fluorescent probes exhibit interesting properties, which react with the species of interest in a chemoselective manner. Hence, these probes are carefully integrated with designed functional groups that only interact with the substrate to induce an intended reaction for the generation of highly fluorescent adducts. Recently, Guo et al. have reported a coumarin based fluorescent probe that has the ability to discriminate glutathione (GSH), cysteine (Cys) and homocysteine (Hcy) in a simultaneous fashion.¹⁵ In this work, the fluorescent probe reacts with these three biological thiol compounds differently and produces fluorescent adducts with different excitation and emission. Other than this work, there are various reported examples for the highly selective detection of thiols by means of the fluorescence technique.¹⁶⁻²⁸ However, dual-responsive fluorescent probes that can respond to two different types of analytes with different signals have not been commonly reported.

It is well known that the presence of a high intracellular concentration of thiols has a great correlation with certain types of diseases.²⁹⁻³⁶ In particular, it was reported that the intracellular environment of most cancer cell lines expresses a high concentration of GSH.³⁷⁻⁴¹ In view of this fact, various drug delivery systems were designed for GSH-triggered release of anticancer drugs within cancer cells.^{31,}

⁴²⁻⁵⁰ Additionally, H_2O_2 was also reported as one of the major reactive oxygen species (ROS) that are capable of inducing oxidative stress to the physiological environment.⁵¹⁻⁵⁴ A high concentration of physiological H_2O_2 is known to be associated with various issues such as aging, cancer, Alzheimer's disease and cardiovascular disorder.⁵⁵⁻⁶¹

Herein, we have introduced the concept of orthogonality into our fluorescence sensing probe in which a single molecule is designed with a multi-responsive capability to two different analyte. In this work, we designed a “turn-on” fluorescent probe that is not only responsive to biological thiol compounds and H_2O_2 , but also capable of discriminating these two types of analytes in a highly selective manner by generating different responses. Furthermore, it is note-worthy our probe could discriminate H_2O_2 and thiol compounds, which are either oxidative or reductive in nature. This is an added remarkable property of our probe that the probe could detect analytes which exhibits exactly two different properties. Thus the concept of orthogonality fits extremely well to our probes.

Scheme 1. Schematic illustration of the working mechanisms of the dual responsive “turn-on” fluorescent probe FLB2SSCou when interacting with biological thiols and H_2O_2 .

The design of this “turn-on” probe was based upon a coumarin containing group that was conjugated to a diboron xanthene spiro isobenzofuran bridged by a disulfide bond (FLB₂SSCou in Scheme 1). In the initial form, the probe would be in its dark state whereby the fluorescence of the coumarin is quenched by non fluorescing diboron xanthene spiro isobenzofuran through the photo-induced electron transfer (PET) mechanism. The PET mechanism was supported by the absence of spectral overlap between the absorption peak of diboron xanthene spiro isobenzofuran and the emission peak of coumarin. We thus ruled out the possibility of Förster resonance energy transfer (FRET) between the two moieties in the dark state. Upon reaction with a biological reducing thiol, the disulfide bond would be cleaved and the coumarin unit would be separated from diboron xanthene spiro isobenzofuran. This process terminates the electron transfer from coumarin to diboron xanthene spiro isobenzofuran, restoring the fluorescence from the coumarin moiety. When the probe reacts with H₂O₂, the diboron functional group on xanthene would be oxidized into the corresponding fluorescein entity.⁶² This process converts the PET mechanism from coumarin into the FRET mechanism, thereby turning on green fluorescence under the same excitation of the coumarin moiety. This phenomenon is understandable, since coumarin/fluorescein is a common FRET pair reported in the literature.⁶³⁻⁶⁶ Hence, the diboron xanthenes spiro isobenzofuran group serves as not only a quencher for the coumarin group, but also a secondary probe.

With this design in mind, we conducted this work in a systematic manner, beginning with the synthesis of the probe, followed by the studies of its various

optical properties, and finally proving the PET mechanism by utilizing density functional theory (DFT). Thereafter, we proceeded to test the applicability of the probe for the simultaneous imaging of intracellular thiol compounds and H_2O_2 in live HeLa cancer cells.

4.2 Results and discussion

4.2.1 Synthesis of FLB₂SSCou

Scheme 2 Synthetic scheme for coupling a disulfide bond with a coumarin unit.

Scheme 3. Synthesis of the H_2O_2 responsive diboron xanthene spiro isobenzofuran precursor and FLB₂SSCou probe.

The synthesis of the FLB₂SSCou probe was accomplished in a total of 8 synthetic steps (Schemes 2 and 3). The synthetic strategy begins with the preparation of 7-diethylamino-3-aminocoumarin (2) from 4-diethylamino-salicylaldehyde through a two-step reaction. Firstly, 4-diethylamino-salicylaldehyde reacted with methyl-2-nitroacetate to afford a coumarin compound (1) by a Knoevenager condensation using piperidine as the amine catalyst in the presence of acetic acid. Thereafter, the nitro group on compound 1 was reduced to the corresponding amino group on compound 2 in a good yield. The amino group of the resulting compound 2 was functionalized with an activated thiol by reaction with 3-(pyridin-2-ylidisulfanyl)-propanoic acid (3) through dicyclohexylcarbodiimide (DCC)-N-hydroxysuccinimide (NHS) coupling, affording compound 4. With the availability of a pyridin-2-ylidisulfanyl group on compound 4, it can easily react with the thiol group of 2-mercaptoethanamine hydrochloride to afford compound 5. Thus, compound 5 contains a coumarin moiety and an amino group bridged by a disulfide bond.

In order to obtain the H₂O₂ responsive diboron xanthenes spiro isobenzofuran unit of the probe, we adopted a two-step synthetic procedure (Scheme 3).⁶⁷ It was reported that the diboron group in molecular probes has the ability to react with H₂O₂ in a highly selective manner through oxidative deprotection of the boronic ester group.^{64, 68-77} Thus, it was expected that the introduction of the diboron unit could endow the highly selective detection of H₂O₂ on the dual responsive probe.⁶⁸ In the synthesis, 2 equivalents of benzene-1,2,4-tricarboxylic acid condensed with one equivalent of 3-bromophenol to form the dibromo xanthene spiro

isobenzofuran derivative 6. Due to unsymmetrical nature of benzene-1,2,4-tricarboxylic acid, the crude product consisted of a mixture of two isomers in which the carboxylic acid unit was on either the 5- or 6-position of the isobenzofuran site. Based on the reported procedure, the isomer with carboxylic acid on the 6-position was isolated by salting out as a pyridine salt in an acetic anhydride–pyridine mixture. Thereafter, the pyridine salt was neutralized back into the carboxylic acid group. The two bromine groups on the xanthene moiety in compound 6 were then converted into the pinacolato boronic esters by a palladium mediated coupling reaction with bis(pinacolato)diboron, affording compound 7. Finally, the carboxylic acid group on compound 7 was used as the coupling site with compound 5 to achieve the target probe 8 (or FLB₂SSCou).

4.2.2 Spectroscopic properties and optical responses of FLB₂SSCou toward H₂O₂ and biological thiols

Figure 1. (A) UV-Vis and (B) fluorescence spectra of FLB₂SSCou (50 mM) in the absence and presence of H₂O₂ (1.0 mM) or DTT (5.0 mM) after overnight incubation at 37°C. Fluorescence spectra were measured under the excitation wavelength of 408 nm. The inset of B shows photoluminescence images of the three samples upon irradiation at 365 nm by a handheld UV lamp. Images from left to right are FLB₂SSCou, FLB₂SSCou after treatment with H₂O₂, and FLB₂SSCou after treatment with DTT.

After obtaining FLB₂SSCou, we performed a series of optical studies of this molecule to investigate its response toward biological thiols and H₂O₂. We first measured the absorption spectra of FLB₂SSCou before and after the introduction of 1.0 mM H₂O₂ and 5.0 mM dithiothreitol (DTT), respectively (Fig. 1A). It was observed that all the absorption spectra exhibited a common maximum peak at the wavelength of 408 nm. However, the absorption spectrum of FLB₂SSCou after the reaction with H₂O₂ showed a new signal at 497 nm. This new absorption peak coincides with the typical absorption signal of a fluorescein molecule, indicating that the oxidation of the two boronic acid units on diboron xanthene spiro isobenzofuran indeed produced the corresponding fluorescein moiety.

We next performed fluorescence measurements of FLB₂SSCou before and after the DTT and H₂O₂ treatments (Fig. 1B). The probe without any treatment exhibited a weak fluorescence peak at 497 nm ($\Phi = 0.001$). Upon treatment with DTT, the fluorescence intensity at the same wavelength of 497 nm was enhanced ($\Phi = 0.168$). On the other hand, when the probe was treated with H₂O₂, it showed a fluorescence enhancement at 525 nm and a fluorescence decrease at 497 nm ($\Phi = 0.101$). As mentioned above, treatment of the probe with H₂O₂ led to the emergence of a new absorption peak at 497 nm. Thus, a complete spectral overlap occurs between the emission of the coumarin unit at 497 nm and the absorbance of the fluorescein moiety at 497 nm. This observation indicates that there is FRET in the probe upon the treatment with H₂O₂. The photographs in the inset of Fig. 1B are the luminescence images of the probe upon excitation at 365 nm by a UV lamp before and after the DTT or H₂O₂ treatment. A distinctive difference in the

fluorescence color between the three samples was observed, thus confirming that the probe indeed possesses the capability to show different responses upon treatment with thiols and H_2O_2 . However, if we place the probe in the co-existence of H_2O_2 and thiol, we would expect that the H_2O_2 would result in the stoichiometric oxidation of the thiol into sulfoxide, disulfide or sulfonate, thereby cancelling the activity of the thiol and H_2O_2 towards the probe⁷⁸. With this, we speculate that there would have no observable response from the probe should the H_2O_2 and thiol are existed in stoichiometric ratio or otherwise we would observe the response from reagent are which are presence in stoichiometric excess.

We also studied the responsivity of the probe toward thiol (DTT) and H_2O_2 in acidic conditions by measuring the time dependent fluorescence response (Fig. S1 in appendix). When comparing the fluorescence responsivity of FLB₂SSCou to DTT at pH 7.4 and 5.5, it was observed that the probe exhibits a faster response to DTT at pH 7.4 than that at pH 5.5, which may be due to the presence of a higher percentage of protonated thiol groups on DTT at pH 5.5 than at pH 7.4. The protonation of the thiol group on DTT in acidic conditions could decrease its nucleophilicity, and thus have a negative effect in reducing the disulfide bond of the probe. Interestingly, a different trend was observed when comparing the responsivity of the probe toward H_2O_2 in both pH conditions. Within the first 10 min, there was a higher responsivity of the probe to H_2O_2 at pH 7.4. Then, the rate decreased as compared to the case at pH 5.5. Similar to the thiol-responsive mechanism, higher pH could induce the deprotonation of H_2O_2 and thus increase the initial response rate. On the other hand, lower pH could increase the hydrolysis

rate of the borate group into the corresponding hydroxyl group after the oxidation occurs. This explained the unexpected increment in the response rate of the probe to H_2O_2 at pH 5.5.

4.2.3 Selectivity and sensitivity studies.

The primary advantage of the fluorescent probe is its dual responsive property and its capability of discriminating between reducing thiols and oxidizing H_2O_2 . It is hence important to evaluate the selectivity and sensitivity of this probe toward biological thiols and H_2O_2 . Fig. 2 shows the fluorescence responses of FLB₂SSCou upon treatment with various biological agents. As expected, FLB₂SSCou showed positive responses to DTT, GSH, Hcy, Cys and H_2O_2 only, whereas the rest of the amino acids did not induce any significant responses. Moreover, H_2O_2 exhibited a different response as compared with DTT, GSH, Hcy and Cys. This set of experiments confirmed that only thiol-containing biological agents have the ability to react with FLB₂SSCou by inducing a reductive cleavage of the disulfide bond to enhance the coumarin fluorescence. On the other hand, H_2O_2 can trigger a different response upon reaction with FLB₂SSCou by oxidative deprotection of the boronic ester group into a highly fluorescent fluorescein derivative. It could be concluded that the probe has the capability to sense biological thiols and H_2O_2 without compromising its selectivity.

Figure 2 Fluorescence responses of FLB₂SSCou (50 mM) toward various amino acids (5.0 mM), biological thiols (5.0 mM), and H₂O₂ (1.0 mM). All emission spectra were measured under excitation of 408 nm, and the value of the emission intensity was obtained at the wavelength of 497 nm (except for H₂O₂). The emission intensity for H₂O₂ was obtained at the wavelength of 525 nm. Inset: fluorescence images of the FLB₂SSCou solution after the addition of various additives upon excitation at 365 nm by a handheld UV lamp. Images from bottom left to top right are for: DTT, GSH, Hcy, Cys, H₂O₂, Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

We then studied the sensitivity of the probe toward H₂O₂ and biological thiols.

A study of the time-dependent optical responses of the probe to these agents was performed first. The experiments were conducted by the co-incubation of FLB₂SSCou (50 mM) and an individual biological agent before measuring the emission spectra on a time-dependent basis under the excitation wavelength of 408 nm over a period of 120 min (Fig. S2 in appendix). As a control, we also performed a similar time-dependent fluorescence measurement of FLB₂SSCou in the absence of any biological agent.

Figure 3. Time dependent fluorescence response of FLB₂SSCou (50 mM) (A) in the absence (Blank) and presence of various biological thiol compounds (5.0 mM) as well as (B) by co-incubation with H₂O₂ (1.0 mM). Emission intensity in (A) was obtained at the wavelength of 497 nm under the excitation wavelength of 408 nm. Emission intensity in (B) was obtained at the wavelengths of 497 and 525 nm under the excitation wavelength of 408 nm.

The real time fluorescence measurements of FLB₂SSCou (50 mM) in the presence of various biological agents (Fig. 3A) showed that DTT could induce the fastest and highest response rate as compared to other biological agents, reaching a fluorescence intensity approximately 6 times higher than the starting fluorescence. Cys and Hcy were second, with similar response rates. GSH exhibited the slowest

rate as compared to DTT, Cys, and Hcy. This is probably due to the larger molecular weight of GSH, and hence, GSH would tend to induce a higher activation energy during the reaction with the disulfide bond of the fluorescent probe as compared to smaller biological thiols.

In the presence of H_2O_2 (Fig. 3B), FLB₂SSCou showed fluorescence signals at 497 nm and 525 nm under the excitation wavelength of 408 nm. It was found that the emission intensity at 497 nm decreased continuously, whereas the emission intensity at 525 nm increased correspondingly. This observation is in line with the result obtained from the fluorescence spectra of FLB₂SSCou upon treatment with H_2O_2 (Fig. 1B). From these observations, it could be further confirmed that the FRET process is indeed present between the coumarin moiety and the fluorescein group on the probe after the reaction with H_2O_2 . The time dependent decreases in the emission signal at 497 nm correspond to the time dependent increases in the emission signal at 525 nm, due to the energy transfer upon the “turn-on” effect. This hence explained the similarity in the rate changes of the two emission intensities.

Fig. 4 Changes in the emission spectra of FLB₂SSCou (50 mM) upon treatment with different amounts of (A) GSH and (B) H₂O₂ under the excitation wavelength of 408 nm.

After performing these measurements, the response of the probe at different concentrations of GSH and H₂O₂ was investigated (Fig. 4). The probe showed a good linear response toward GSH and H₂O₂ in the concentration ranges from 0.1 to 100 mM and 0.1 to 500 mM, respectively. Based on the linearity within these ranges, we computed the detection limits of the probe for GSH and H₂O₂ to be 4.98×10^{-6} M and 1.37×10^{-5} M, respectively. The relatively high detection limit of

H₂O₂ could be attributed to the loss in efficiency during the FRET process from the coumarin group to the fluorescein moiety. Note that the excitation wavelength used for the detection of H₂O₂ remained the same as in the coumarin case at 408 nm instead of the direct excitation at 497 nm.

4.2.4 Computational studies.

Based on experimental observations, it was speculated that the fluorescence of the initial dark state of FLB₂SSCou was quenched by the PET mechanism from the coumarin moiety to the diboron xanthene spiro isobenzofuran group. To confirm the existence of the PET phenomenon within the FLB₂SSCou probe, computational calculations on the molecule orbital locations were performed for the ground state and excited state. The ground state density functional theory (DFT) optimization of FLB₂SSCou in an aqueous environment was first carried out with the B3LYP/6-31+G(d,p) basis set using the Gaussian 09 package.⁷⁹ Prior to the reaction with thiol compounds or H₂O₂, it was observed that the diboron xanthene group adopted a spiro conformation with respect to the isobenzofuran group having the lactone ring intact in its closed confirmation. The same optimization calculations on the probe upon the oxidative reaction with H₂O₂ were then performed. Since the reaction was conducted in neutral conditions, it was expected that the fluorescein group existed in the dianionic state during the calculation process. After the optimization process, we observed that the fluorescein group remained in its spiro conformation, while the lactone ring was broken into the benzoic derivative. It is well documented that open ring form of fluorescein would exhibit high

photoluminescence efficiency, whereas the lactone ring form shows no fluorescence or weak fluorescence.⁸⁰⁻⁸²

Thereafter, we performed time dependent-DFT (TD-DFT) calculations with the same basis set on the excited state in order to have a better understanding of the electronic transition pathway during excitation. The same DFT optimization and TD-DFT calculations were also performed on the molecule after treatment with H₂O₂ and thiol compounds. The TD-DFT calculation results showed a lot of useful information, including the calculated excited wavelength and oscillator strength (f) together with the corresponding contributing orbitals (Table 1). Based on Kasha's law, the fluorescence process is a result of direct relaxation from the 1st excited state (S_1) to the ground state (S_0).⁸³⁻⁸⁴ Hence, we paid attention to the first excited state of FLB₂SSCou before and after reaction with thiols and H₂O₂ from the TD-DFT calculations. It was observed that the electronic transition to the first excited state was mainly contributed from the respective highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) transition.

Table 1. TD-DFT//B3LYP/6-31+G(d,p) calculation results of FLB₂SSCou, FLB₂SSCou + thiol and FLB₂SSCou + H₂O₂.

Molecule	Electronic transition ^a	Excitation wavelength (nm)	f^b	Contributed orbitals ^c	CI coefficient
FLB ₂ SSCou	$S_0 \rightarrow S_1$	394.02	0.0000	H $\rightarrow$ L	0.70704
	$S_0 \rightarrow S_2$	377.44 (408)	0.6912	H $\rightarrow$ L+1	0.70002
FLB ₂ SSCou + Thiol	$S_0 \rightarrow S_1$	388.58 (408)	0.8414	H $\rightarrow$ L	0.70089
FLB ₂ SSCou + H ₂ O ₂	$S_0 \rightarrow S_1$	447.77 (497)	0.7204	H $\rightarrow$ L	0.69911

^aDisplay of selected transition. ^bOscillator strength. ^cH represents HOMO, L represents LUMO and L+1 represents LUMO + 1. Value in parenthesis represent the observed excitation wavelength.

Figure 5. Calculated frontier molecular orbitals of (A) FLB₂SSCou, (B) FLB₂SSCou after reacting with thiols, resulting in the reductive cleavage of the disulfide bond to give a coumarin derivative, and (C) FLB₂SSCou after reacting with H₂O₂, resulting in the oxidation of the boronic group into the phenoxyl group.

Based on this information, we extracted the HOMO and LUMO orbital diagrams of these systems (Fig. 5). After a careful examination of the transition, it was found that the S_0 to S_1 transition for FLB₂SSCou was accompanied by the oscillator strength of $f = 0$. This means that the direct excitation from its ground state to its first excited state is forbidden. The extracted HOMO and LUMO orbital diagrams of this molecule reveal that there is a re-distribution in its electronic density from the coumarin group to the isobenzofuran group during this transition (Fig. 5A). We observed that the lowest allowed transition lies on S_0 to the 2nd

excited state (S_2) transition with an oscillator strength of $f = 0.6912$. For the cases of FLB₂SSCou after reaction with thiols and H₂O₂, the S_0 to S_1 transition being accompanied by a non-zero f value provides a clear indication that the transition is an allowed process. The TD-DFT calculation results demonstrate that these transitions are contributed by the HOMO to LUMO transition, and a detailed observation of the orbital diagram shows no re-distribution of the electronic density during this HOMO to LUMO transition (Fig. 5B and C). Based on these observations, we sketched out the orbital energy levels to explain the detailed mechanism of the “turn-on” effect (Fig. 6).

Figure 6. Energy level diagrams of (A) FLB₂SSCou, (B) FLB₂SSCou treated with thiol, and (C) FLB₂SSCou treated with H₂O₂, showing the electronic excitation as well as radiative and non-radiative relaxation pathways.

From the TD-DFT calculations (Fig. 6A), we can conclude that the photo-excitation of FLB₂SSCou induces an allowed electron excitation from the HOMO (-5.66 eV) to LUMO+1 (-2.05 eV) orbitals ($S_0 - S_2$, $f = 0.6912$), where there is no re-distribution of the electronic density. Thereafter, the excited electron is transferred to the LUMO (-2.34 eV) of the molecule with a net loss of 0.29 eV worth of energy. The electron transfer process is evident from the re-distribution of the electron density from the coumarin moiety to the isobenzofuran group on the orbital diagrams. The electron is then re-transferred back to the coumarin group by means of internal conversion, which is non-radiative in nature. This complete PET cycle explains the initial dark state of the molecule. By applying the same method of analysis to the molecule after the reductive cleavage of the disulfide bond and the oxidation of the diboron group into the phenoxide group, the chemically induced “turn-on” effect of FLB₂SSCou could be well explained. As seen from Fig. 6B, the direct transition from HOMO to LUMO leads to no re-distribution in the electron density. Based on Kasha’s law, the absence of any intermediate orbital between the S_1 and S_0 states confirms that the LUMO to HOMO relaxation is emissive. Similarly, we also did not observe any sign of PET from Fig. 6C, thus concluding the conversion of the emissive FLB₂SSCou into two emissive adducts by terminating the PET process.

4.2.5 *In vitro* intracellular imaging of thiols and H₂O₂.

Figure 7. *In vitro* bioimaging of HeLa cells. Confocal scanning microscope images of cells treated with 10 mM FLB₂SSCou and 20 mM β-CD under the (A) 4',6-diamidino-2-phenylindole (DAPI) channel and (B) FITC channel. (C) Merged image of A and B. Confocal scanning microscope images of cells treated with 10 mM FLB₂SSCou and 20 mM β-CD followed by post-treatment with 100 mM H₂O₂ under the (D) DAPI channel and (E) FITC channel. (F) Merged image of D and E. Flow cytometry dot-plot diagram of HeLa cells (G) without any additives (blank control), (H) after treatment with 10 mM FLB₂SSCou and 20 mM β-CD, and (I) after treatment with 10 mM FLB₂SSCou and 20 mM β-CD followed by post-treatment with 150 mM H₂O₂.

In order to evaluate the applicability of the FLB₂SSCou probe for biological applications, we conducted imaging of thiols and H₂O₂ in an intracellular environment. Since it is well documented that most cancer cells possess a high concentration of intracellular GSH, HeLa cells as the cancer cell model were selected in the studies. The biocompatibility of FLB₂SSCou was first evaluated

using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cell viability results showed that FLB₂SSCou exhibited insignificant toxicity to the cells (Fig. S3 in appendix), making it suitable for subsequent biological applications. Intracellular imaging using FLB₂SSCou was then carried out. In this case, the pre-seeded cells were treated with FLB₂SSCou (10 mM) and β -cyclodextrin (β -CD, 20 mM) for 24 h prior to the imaging experiments. Based on the confocal scanning microscopy images (Fig. 7A–C), strong blue fluorescence from the cells was observed accompanied by some negligible green fluorescence signals.

The applicability of FLB₂SSCou for the imaging of intracellular H₂O₂ was then evaluated. Similarly, the pre-seeded HeLa cells were treated with FLB₂SSCou (10 mM) and β -CD (20 mM) for 24 h before the post-treatment of H₂O₂ for 45 min. As compared to previous images, a great increment in the green fluorescence of the cells under the fluorescein isothiocyanate (FITC) channel (Fig. 7D–F) was observed. In addition, similar blue fluorescence intensity from the cells with and without the treatment with H₂O₂ was shown. From the fluorescence studies, it was concluded that the treatment of FLB₂SSCou with H₂O₂ resulted in the emergence of a fluorescence signal at 525 nm (green emission) accompanied by the quenching of the blue emission due to the FRET mechanism. The spectroscopic observations seem different to the *in vitro* imaging results in that the blue fluorescence remained the same for the cells with and without the post-treatment of H₂O₂. As observed from the UV-Vis studies, the treatment of FLB₂SSCou with H₂O₂ led to the emergence of a new absorption signal at 497 nm, which overlapped with the

emission of the coumarin unit at 497 nm. The presence of the disulfide bridge within the molecule allows the FRET process to occur especially when the distance between the FRET donor (coumarin) and the FRET acceptor (fluorescein) is not longer than 10 nm. Since both GSH and H_2O_2 exist in the intracellular environment, the thiol-induced reductive cleavage of the disulfide bond terminates the FRET process. This explains the presence of blue emission from the cells even after the H_2O_2 treatment. Although it is mentioned earlier that the H_2O_2 could oxidize the thiol compounds into the oxidized form thereby cancelling the stimulus effect of H_2O_2 and thiol, the post-treatment of H_2O_2 to the cell samples would cause the oxidative deprotection of the diboron group on the cleaved FLB₂SSCou, which was induced by the intracellular thiol compounds. Therefore in this case, we could observe the fluorescence signal from both the coumarin and the fluorescein group.

Flow cytometry experiments were carried out to further confirm the applicability of FLB₂SSCou for the intracellular imaging of thiols and H_2O_2 . We performed the same process of treatment on the cells before harvesting them for flow cytometry analysis. The analysis was performed with the brilliant violet 421 (BV421) channel and FITC channel, and the intensity of these measurements was then compared with blank cells, i.e. cells without treatment with any agent (Fig. 7G). Similar to the fluorescence images, a large percentage of the cells treated with FLB₂SSCou (10 mM) and β -CD (20 mM) showed emission signals from the BV421 channel accompanied by weak emission signals from the FITC channel (Fig. 7H). It was observed that 19.75% of the cell population occupies the first

quadrant of the dot plot, which is much higher than the cell population (0.01%) on the first quadrant in the control group (Fig. 7G).

The same flow cytometry experiment was also performed on FLB₂SSCou-treated cells with the post-treatment with H₂O₂. From the dot plot (Fig. 7I), it was observed that the cell population in the first quadrant (2.88%) greatly reduced, while having a significant population increase in the second quadrant (93.98%). This set of cells had the largest population in the second quadrant as compared to the other two cell groups, indicating that the post-treatment of H₂O₂ indeed turns on the green emission of internalized FLB₂SSCou measured by the FITC channel. When comparing the FITC signals from the one dimensional histogram plots (Fig. S4 in appendix), this group of cells show the highest FITC signal. Similar to the fluorescence microscopy images, we did not observe any significant decrease in the BV421 signal as compared to that of the cells treated with FLB₂SSCou and β -CD, once again confirming that the FRET process was terminated by the intracellular thiols.

4.3 Conclusion

In summary, we have developed a novel “turn-on” dual responsive fluorescent probe, FLB₂SSCou, for the detection of intracellular thiols and H₂O₂. In contrast to conventional “turn-on” fluorescent probes, FLB₂SSCou is capable of generating different emission responses when treated with thiols and H₂O₂. In the probe, a non-fluorescent diboron xanthene spiro isobenzofuran and a coumarin unit are

bridged by a disulfide bond. The initial dark state of the probe is due to the PET process that occurs from the coumarin group to the isobenzofuran group. Upon treatment with thiols, reductive cleavage of the disulfide bond terminates this intramolecular PET process, thus initiating the emission from the coumarin unit. On the other hand, upon treatment with H_2O_2 , the diboron xanthene spiro isobenzofuran group undergoes oxidation into a highly fluorescent fluorescein adduct, whereby the intramolecular PET process is converted into the intramolecular FRET process. A series of detection experiments have shown that the probe has the ability to sense thiols and H_2O_2 in a highly selective manner against over 20 amino acids.

In order to prove the intramolecular PET mechanism of FLB_2SSCou , the TD-DFT calculations have been carried out. A different distribution of the electron density between the ground state (S_0) and the first excited state (S_1) has been observed, since the emission process is a result of the electronic relaxation from S_1 to S_0 . Moreover, the transition to the first excited state is a forbidden transition process ($f = 0.0000$), and instead, the first allowed transition is to the second excited state ($S_0 - S_2, f = 0.6912$), where there is no re-distribution of the electron density. Thus, it has been confirmed that the PET process occurs during a process where the electron first gains its energy to excite into the S_2 state followed by transferring into the S_1 state, since the direct excitation to the S_1 state is forbidden and there is inadequate energy for the spontaneous transfer.

Lastly, the *in vitro* intracellular imaging of thiols and H_2O_2 using FLB_2SSCou has been conducted. The fluorescence microscopy images show the presence of

blue fluorescence within the intracellular environment of HeLa cells after treatment with FLB₂SSCou, due to the interaction of FLB₂SSCou with intracellular thiols. On the other hand, the post-treatment with H₂O₂ on FLB₂SSCou-treated cells exhibits an enhancement in green fluorescence as compared to the cells without this post treatment, indicating that exogenous H₂O₂ could induce a “turn-on” effect on the probe even in the intracellular environment. These results have been further supported by flow cytometry experiments, confirming the reproducibility of the probe for the detection and imaging of intracellular thiols and H₂O₂. All in all, this work has successfully demonstrated the synthesis and applications of a dual responsive fluorescence probe for the simultaneous detection of thiols and H₂O₂. The present research might inspire further development of “turn-on” fluorescent probes having multiple responsive properties.

4.4 Experimental

General procedure

All chemicals, reagents and solvents were used as obtained from commercial sources without further purification. Unless otherwise stated, the purification of compounds by flash chromatography was performed using Merck Gedurans Si 60 silica gel with particle sizes from 40 to 63 μ m. The column was stacked with the slurry mixture of the silica gel and the eluting solvent. All ¹H NMR and ¹³C NMR measurements were performed on a Bruker AMX AV400 MHz spectrophotometer at ambient temperature. The ¹H two-dimensional correlation spectroscopy (COSY) spectrum of FLB₂SSCou was measured with a Bruker AVIII 400 MHz NMR

BBFO2 probe spectrometer at ambient temperature. Signals from the ^1H NMR spectra were reported as chemical shifts in parts per million (ppm) calibrated using either deuterated solvent (7.26 ppm for CDCl_3 and 2.50 ppm for $\text{DMSO-}d_6$) or downfield from tetramethylsilane (0.00 ppm). Numbers of equivalent resonance protons are reported as nH and multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), ddd (doublet of doublet of doublet) and m (multiplet). Corresponding coupling constants (J values) for these multiplicities are reported in Hz. Signals from the ^{13}C NMR spectra were reported as chemical shifts in parts per million (ppm) calibrated using deuterated solvent (77.23 ppm for CDCl_3 and 39.51 ppm for $\text{DMSO-}d_6$). Fourier transform infrared spectroscopy (FTIR) measurements were performed on a SHIMADZU IR Prestige-21 spectrophotometer. Melting points of solid compounds were recorded on an OptiMelt Automated Melting point instrument. High-resolution mass spectrometry (HRMS) was performed on a Waters Q-tof Premier MS. The UV-Vis spectroscopy studies for all optically active compounds were performed using a SHIMADZU UV-3600 UV-Vis-NIR spectrophotometer with a 10 mm-by-10 mm 1.4 mL quartz cuvette. Fluorescence emission spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a water-circulated temperature controller. Except for time-dependent fluorescence spectroscopy analysis, all fluorescence studies were carried out at 37°C using a 10 mm-by-10 mm 1.4 mL fluorescence cuvette. Time-dependent fluorescence spectroscopy analysis was performed with a 10 mm-by-10 mm 3.5 mL fluorescence cuvette at 37°C. Unless otherwise stated, all UV-Vis and fluorescence spectroscopy

experiments were performed in 0.1X phosphate buffered saline (PBS). Spectroscopic studies in acidic conditions were conducted by dispersing the fluorophores in 0.1 M acetate buffer solution (pH 5.5).

HeLa cells used in this work were obtained from ATCC. The cells were maintained in a humidified CO₂ (5%) incubator and proliferated in Dulbecco's Modified Eagle Medium (DMEM) supplement with 10% fetal bovine serum and 1% penicillin streptomycin. Confocal microscopy experiments were conducted on a confocal microscope (Leica TCS SP5, 40× oil objective). Absorbance for the MTT cytotoxicity assay was measured with an Infinite 200 PRO multimode reader. Flow cytometry experiments were performed on a BD LSRFortessa X-20 cell analyzer.

Synthesis of compounds

7-(Diethylamino)-3-nitro-2H-chromen-2-one (1)

The synthesis of compound 1 was performed based on a reported procedure.⁸⁵ Briefly, to a 50 mL flask containing n-butanol (20 mL) was added 4-diethylaminosalicylaldehyde (0.97 g, 5.0 mmol), methyl 2-nitroacetate (502 mL, 5.15 mmol), piperidine (100 mL) and glacial acetic acid (2.5 mL). The mixture was heated to 110°C under stirring condition for 18 h. The resulting dark brown solution was cooled down in an ice bath when orange crystals were formed in the

reaction mixture. The crystals were isolated by filtration and washed three times with ice cold n-butanol before drying under *vacuo* to afford **1** as a red-orange crystal (0.37 g, 28%). M.P.: 198.6–199.3°C. FTIR $\bar{\nu}/\text{cm}^{-1}$: 2980.02, 2935.66, 1743.65, 1631.78, 1591.27, 1544.98, 1477.47, 1427.32, 1327.03, 1188.15, 823.60, 767.67. ^1H NMR (400 MHz, CDCl_3): δ 8.72 (s, 1H), 7.44 (d, J = 9.08 Hz, 1H), 6.70 (dd, J = 9.09 & 2.37 Hz, 1H), 6.48 (d, J = 2.22 Hz, 1H), 3.50 (q, J = 7.16 Hz, 4H), 1.27 (t, J = 7.16 Hz, 6H). ^{13}C NMR (100 MHz, CHCl_3): δ 158.9, 154.7, 153.6, 143.5, 132.7, 127.0, 111.3, 106.4, 97.0, 45.7, 12.6. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 263.1032, found: 263.1038 $[\text{M} + \text{H}]^+$.

3-Amino-7-(diethylamino)-2H-chromen-2-one (**2**)

Compound **2** was synthesized based on a reported procedure.⁸⁶ To 25 mL flask containing 37% HCl (5 mL) was added stannous chloride (1.35 g, 7.12 mmol) under stirring condition. Thereafter, compound **1** (0.25 g, 0.95 mmol) was added slowly into the stirring solution and the resulting mixture was stirred for 4 h before pouring it into crushed ice (~20 g) with aqueous NaOH (5 M) in an ice bath. The resulting suspension was saturated with NaCl before extracting three times with diethyl ether. The organic layer was then washed with brine and dried over Na_2SO_4 before the solvent was removed under pressure to afford **2** as an orange solid (0.17 g, 77%). M.P.: 89.6–90.3°C. FTIR $\bar{\nu}/\text{cm}^{-1}$: 3400.50, 3338.78, 2970.38, 1695.43,

1624.06, 1591.27, 1523.76, 1134.14, 813.96, 798.53. ^1H NMR (400 MHz, CDCl_3): δ 7.11 (d, J = 8.68 Hz, 1H), 6.70 (s, 1H), 6.57 (dd, J = 8.69 & 2.51 Hz, 1H), 6.53 (d, J = 2.44 Hz, 1H), 3.37 (q, J = 7.08 Hz, 4H), 1.18 (t, J = 7.07 Hz, 6H). ^{13}C NMR (100 MHz, CHCl_3): δ 160.3, 151.6, 147.5, 127.5, 126.0, 114.5, 109.7, 109.3, 98.0, 44.6, 12.5. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 233.1290, found: 233.1296 $[\text{M} + \text{H}]^+$.

3-(Pyridin-2-yl)disulfanylpropanoic acid (3)

Compound **3** was synthesized based on a reported procedure.⁸⁷ To a stirring solution of 2,2'-dipyridyl disulfide (3.75 g, 17 mmol) in ethanol (10 mL) was added acetic acid (0.4 mL). Thereafter, 3-mercaptopropionic acid (0.90 g, 8.47 mmol) in ethanol (5 mL) was added dropwise into the above solution, and the solution turned yellow gradually. The mixture was stirred for 20 h before ethanol was removed by rotary evaporation. The resulting yellow syrup was dissolved in CH_2Cl_2 /ethanol (3:2, 10 mL) and loaded into a neutral alumina column, equilibrated with CH_2Cl_2 /ethanol (3:2). The column was flushed with CH_2Cl_2 /ethanol (3:2) until the yellow fraction was completely eluted out of the column. Thereafter, the column was flushed with CH_2Cl_2 /ethanol (3:2) containing 4% acetic acid to elute the target product out of the column. The solvent was removed by rotary evaporation, and the remaining liquid was re-dissolved in CH_2Cl_2 and washed three times with water to remove the acetic acid completely. The organic layer was washed with brine and dried over Na_2SO_4 before the solvent

was removed by rotary evaporation. Finally, the residue was dried under *vacuo* to afford **3** as a white solid (1.42 g, 78%). M.P.: 61.2–62.6°C. FTIR $\bar{\nu}/\text{cm}^{-1}$: 3300–2400 (broad), 2912.51, 1728.22, 1583.56, 1446.61, 1423.47, 1188.15, 1002.98, 765.74, 715.59. ^1H NMR (400 MHz, CDCl_3): δ 10.21 (broad s, 1H), 8.47 (m, 1H), 7.66 (m, 2H), 7.13 (ddd, $J = 6.58$ & 4.85 & 1.85 Hz, 1H), 3.04 (t, $J = 6.95$ Hz, 2H), 2.79 (t, $J = 6.94$, 2H). ^{13}C NMR (100 MHz, CHCl_3): δ 176.2, 159.5, 149.4, 137.7, 121.3, 120.5, 34.0, 33.7. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 216.0157, found: 216.0153 $[\text{M} + \text{H}]^+$.

***N*-(7-(Diethylamino)-2-oxo-2H-chromen-3-yl)-3-(pyridin-2-yl)disulfanylpropanamide (4)**

To a stirring solution of compound **3** (0.24 g, 1.10 mmol) in THF (5 mL) were added dicyclohexyl carbodiimide (0.23 g, 1.10 mmol) and *N*-hydroxysuccinimide (0.12 g, 1.07 mmol). The reaction was stirred for 2 h with the formation of a white precipitate. Compound **2** (0.23 g, 1.00 mmol) was then added, and the resulting solution was refluxed for 24 h. The solid was removed by filtration, and the filtrate was concentrated by rotary evaporation. The obtained residue was purified by column chromatography eluted with 1:1 hexane/ethyl acetate to afford **4** as a yellow solid (0.16 g, 37%). M.P.: 130.8–131.9°C. FTIR $\bar{\nu}/\text{cm}^{-1}$: 3338.78, 2972.31, 2929.87, 1699.29, 1668.43, 1627.92, 1539.20, 1444.68,

1261.45, 1192.01, 1132.21, 763.81. ^1H NMR (400 MHz, CDCl_3): δ 8.59 (s, 1H), 8.54 (d, J = 4.83 Hz, 1H), 8.15 (s, 1H), 7.63 (m, 2H), 7.11 (m, 1H), 6.62 (dd, J = 8.81 & 2.46 Hz, 1H), 6.50 (d, J = 2.39 Hz, 1H), 3.40 (q, J = 7.10 Hz, 4H), 3.17 (t, J = 6.71 Hz, 2H), 2.85 (t, J = 6.75 Hz, 2H), 1.20 (t, J = 7.07 Hz, 6H). ^{13}C NMR (100 MHz, CHCl_3): δ 169.7, 159.6, 152.8, 150.1, 149.6, 137.1, 128.8, 126.2, 122.6, 121.1, 120.3, 118.8, 109.8, 108.3, 105.8, 97.5, 44.6, 36.9, 34.6, 12.6. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 430.1259, found: 430.1249 $[\text{M} + \text{H}]^+$.

3-((2-Aminoethyl)disulfanyl)-N-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)propanamide HCl salt (5)

A 25 mL round bottom flask was loaded with compound **4** (0.22 g, 0.50 mmol) and acetic acid (30 μL) in methanol (5 mL). To the stirring solution was then added mercaptoethanamine hydrochloride (0.05 g, 0.48 mmol). The reaction was allowed to react for 24 h and then the solvent was removed by rotary evaporation. The residue was re-dissolved in a small amount of methanol, which was poured into diethyl ether (100 mL) to induce the precipitation. The solid was isolated by filtration and then re-dissolved in a small amount of methanol before re-precipitating in diethyl ether (100 mL). The process was repeated two more times until the supernatant turned colorless after the precipitation. The resulting yellow solid was dried under vacuo to afford compound **5** (0.15 g, 67%). FTIR

$\bar{\nu}/\text{cm}^{-1}$: 3327.21, 2970.38, 1734.01, 1707.00, 1670.35, 1602.85, 1535.34, 1411.89, 1261.45, 1130.29, 823.60, 763.81. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 9.64 (s, 1H), 8.45 (s, 1H), 8.30 (broad s, 1H), 7.46 (d, $J = 8.80$ Hz, 1H), 6.73 (dd, $J = 9.0$ & 2.20 Hz, 1H), 6.56 (d, $J = 8.81$ & 2.0 Hz, 1H), 3.44 (q, $J = 6.80$ Hz, 7.5H, overlapped with HDO), 3.13 (t, $J = 6.40$ Hz, 2H), 3.04 (t, $J = 6.00$ Hz, 4H), 2.90 (t, $J = 7.20$ Hz, 2H), 1.15 (t, $J = 6.80$ Hz, 6H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 170.7, 158.7, 153.2, 149.7, 129.3, 128.2, 119.0, 109.9, 107.9, 97.1, 44.5, 38.2, 36.1, 34.4, 34.0, 12.8. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 408.1416, found: 408.1432 $[\text{M} - \text{Cl}]^+$.

3',6'-Dibromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid

(6)

3-Bromophenol (27.64 g, 159.80 mmol) and benzene-1,2,4- tricarboxylic acid (16.80 g, 79.90 mmol) were charged into a 3-neck flask equipped with a reflux condenser. Gaseous exchange was then performed, cycling between N_2 and vacuo at least three times. Thereafter, methanesulfonic acid (60 mL) was injected into the flask and the reaction was heated at 140°C for 72 h. The reaction was cooled down to ambient temperature before pouring it into ice water (400 mL). The precipitate was then collected by filtration and dried in vacuo. The resulting brown solid was dissolved in an acetic anhydride/pyridine mixture (3:1, 160 mL) to induce the

precipitation. The white precipitate was isolated and the red filtrate was discarded. The white precipitate was washed with an acetic anhydride/pyridine mixture (2:1, 60 mL) six times to ensure the complete removal of any pink stain. The resulting white solid was then washed with aqueous HCl (1 M, 100mL) three times followed by washing with water three times. The solid was dried under vacuo to afford compound **6** as a brownish white solid (9.14 g, 23%). M.P.: 89.6–90.3°C (decomposition temperature). FTIR $\bar{\nu}/\text{cm}^{-1}$: 3300–2400 (broad), 2974.23, 2766.80, 1720.50, 1593.20, 1479.40, 1230.58, 1074.35, 921.97, 817.82, 754.17. ^1H NMR (400 MHz, DMSO- d_6): δ 8.61 (s, 1H), 8.30 (dd, J = 8.01, 1.26, 1H), 8.21 (d, J = 7.97 Hz, 1H), 7.89 (s, 1H), 7.73 (d, J = 1.90 Hz, 2H), 7.36 (dd, J = 8.51 & 1.92Hz, 2H), 6.91 (d, J = 8.52 Hz, 2H). ^{13}C NMR (100MHz, DMSO- d_6): δ 168.0, 166.4, 152.7, 151.2, 149.8, 138.3, 137.0, 132.0, 130.5, 129.0, 128.1, 126.2, 125.2, 124.5, 81.3. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 500.8973 found: 500.8986 $[\text{M} + \text{H}]^+$.

3-Oxo-30,60-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,90-xanthene]-6-carboxylic acid (7)

An oven-dried 3-neck flask was charged with compound **6** (2.00 g, 4.00 mmol), [1,10-bis(diphenylphosphino)ferrocene]dichloro palladium(II) dichloromethane adduct (0.98 g, 1.20 mmol), potassium acetate (3.94 g, 40.10

mmol) and bis(pinacolato)-diboron (4.00 g, 15.80 mmol). The flask was evacuated before back filling with N₂ gas. The process was repeated twice before anhydrous DMF (50 mL) was injected into the flask. The mixture was stirred at room temperature for 5 min, and then stirred at 80°C for 12 h. Thereafter, DMF was distilled off under reduced pressure and the resulting black mass was suspended in ethyl acetate (~30 mL). The mixture was washed three times with aqueous HCl (0.1 M) and once with water. The organic layer was subjected to filtration to remove any insoluble solid, and the filtrate was washed with brine and dried over Na₂SO₄ to remove any trace of water. The solvent was then removed by rotary evaporation. The obtained crude product was purified by flash column chromatography equilibrated with hexane, eluting with hexane/ethyl acetate mixture (gradient from 50 : 50 to 10 : 90). The solvent was removed under pressure to obtain a brown slurry, and Et₂O was added to the slurry. The solid was then obtained by vacuum filtration and washed with Et₂O to afford compound **7** as a white solid (0.52 g, 22%). M.P.: 265.0–265.8°C (decomposition temperature). FTIR $\bar{\nu}/\text{cm}^{-1}$: 3600–2400 (broad), 3145.90, 2980.02, 1737.86, 1406.11, 1359.82, 1143.79, 1087.85, 974.05, 854.45, 825.53, 688.59. ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.28 (dd, *J* = 8.04 & 1.05 Hz, 1H), 8.22 (d, *J* = 8.02 Hz, 1H), 7.71 (s, 1H), 7.65 (s, 2H), 7.44 (d, *J* = 7.84 Hz, 2H), 6.98 (d, *J* = 7.85 Hz, 2H), 1.35 (s, 24H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 168.3, 166.3, 153.6, 150.3, 138.2, 131.8, 130.0, 128.6, 128.0, 126.2, 124.8, 123.3, 121.2, 84.7, 81.6, 25.1. HRMS (H₂O/MeOH): *m/z*: calcd: 596.2504, found: 596.2534 [M + H]⁺.

N-(2-((3-((7-(Diethylamino)-2-oxo-2H-chromen-3-yl)amino)-3-oxopropyl)disulfanyl)ethyl)-3-oxo-30,60-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,90-xanthene]-6-carboxamide (8) or FLB₂SSCou

To a stirring solution of compound 7 (0.14 g, 0.23 mmol) in THF (5 mL) were added *N*-hydroxysuccinimide (0.03 g, 0.25 mmol) and dicyclohexylcarbodiimide (0.05 g, 0.25 mmol). The solution was stirred for 3 h when a white precipitate of dicyclohexylurea was formed. Thereafter, compound 5 (0.10 g, 0.23 mmol) and Et₃N (70 mL, 0.50 mmol) were added to the above solution for refluxing overnight. The solvent was removed by rotary evaporation and the residue was purified by flash chromatography, eluting with an ethyl acetate/hexane mixture (1 : 1) to afford compound 8 as a yellow solid (30 mg, 13%). M.P.: 140.2–141.7°C. FTIR $\bar{\nu}/\text{cm}^{-1}$: 3313.71, 2976.16, 2926.01, 2908.65, 1772.58, 1676.14, 1654.92, 1608.63, 1560.41, 1533.41, 1508.33, 1406.11, 1357.89, 1141.86, 1087.85, 974.05, 854.47, 825.53, 692.44. ¹H NMR (400 MHz, CDCl₃): δ 8.38 (s, 1H), 8.09 (s, 2H), 8.02 (s, 1H), 7.72 (s, 2H), 7.54 (s, 1H), 7.40 (d, J = 7.84

Hz, 2H). 7.14 (d, $J = 8.63$ Hz, 1H), 6.82 (d, $J = 7.75$ Hz, 2H), 6.62–6.59 (m, 1H), 6.49 (s, 1H), 3.71 (q, $J = 5.91$ Hz, 2H), 3.45–3.38 (m, 4H), 3.00 (t, $J = 6.56$ Hz, 2H), 2.86 (t, $J = 6.05$ Hz, 2H), 2.76 (t, $J = 6.55$ Hz, 2H), 1.33 (s, 24H), 1.23 (t, $J = 10.85$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ 169.7, 168.8, 165.9, 159.6, 154.5, 152.8, 150.4, 149.7, 141.0, 129.5, 129.0, 128.7, 127.6, 126.8, 126.3, 125.6, 123.7, 122.7, 120.7, 118.4, 109.8, 108.0, 97.4, 84.3, 82.2, 44.8, 39.0, 37.9, 37.3, 33.9, 33.5, 24.8. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 972.3542, found: 972.3682 $[\text{M} + \text{H}]^+$.

Quantum yield measurements

The relative quantum yields of various compounds were calculated using the following formula using fluorescein as the standard reference ($\Phi_{\text{std}} = 0.85$ in 1 N NaOH).⁸⁸

$$\Phi_{\text{sample}} = \Phi_{\text{std}} \frac{\int Fl_{\text{sample}} d\lambda}{\int Fl_{\text{std}} d\lambda} \frac{1 - 10^{-Ab_{\text{std}}}}{1 - 10^{-Ab_{\text{sample}}}}$$

Where Φ represents the quantum yield, $\int Fl d\lambda$ represents the integrated area in fluorescence spectra, and Abs represents the absorbance at the λ_{max} of sample (sample) and standard (std).

Detection limit measurements

Detection limit measurements of FLB₂SSCou with respect to GSH and H_2O_2 were performed based on a reported procedure.²² The measurements were

carried out by incubating the analyte (various concentrations) with the probe in PBS at 37 °C overnight followed by measuring the emission spectra. The maximum emission intensities were plotted against the concentrations to obtain a linear plot, and the gradient (k) of the plot was then obtained. The maximum emission intensity of FLB₂SSCou was measured five times, and the standard deviation (σ) of these measurements was obtained. Lastly, the detection limit was calculated using the following formula:

$$Detection\ Limit = \frac{3\sigma}{k}$$

Computational studies

Theoretical calculations on various adducts were performed using the Gaussian 09 package. DFT optimizations of the adducts were performed using the basis set of B3LYP/6-31+G(d,p). Water as the solvent was simulated with the polarizable continuum model (PCM) using the integral equation formalism variant (IEFPCM) during the calculation process. The optimized structures were used to proceed with the TD-DFT calculation using the same basis set and solvent simulation.

Confocal scanning microscopy experiments

HeLa cells were first seeded on a SPL 200350 coverglass-bottom dish at a seeding density of 2 × 10⁵ cells (2 mL) per well. The cells were incubated overnight prior to the addition of FLB₂SSCou (10 mM) and β-CD (20 mM) solution. The

cells were then incubated overnight with the samples followed by the removal of the medium. The cells were washed three times with PBS and finally fresh medium (2 mL) was added. For the post-treatment with H_2O_2 , H_2O_2 was added to the cells right after the medium replacement to achieve a concentration of 150 mM. All the cells were incubated at 37 °C for another 20 min prior to washing the cells 3 times with PBS and then fixing with 4% formaldehyde. The cells were washed once with PBS again before imaging.

Flow cytometry experiments

HeLa cells were first seeded on a 6-well plate at a seeding density of 2×10^5 cells (2 mL) per well. The cells were incubated overnight prior to the addition of FLB₂SSCou (10 mM) and β -CD (20 mM) solution. The cells were then incubated with the samples overnight before the removal of the medium. The cells were washed three times with PBS and finally trypsin (500 mL) was added to induce cell detachment. Thereafter, fresh medium (500 mL) was added to quench off trypsin before harvesting the cells. The cells were isolated by low speed centrifugation (2000 rpm, 3min), and the obtained cell pellets were washed two times with PBS and finally re-suspended in PBS (1 mL). For the post-treatment with H_2O_2 , H_2O_2 was added to the cells right after the PBS suspension so as to achieve a concentration of 150 mM. All the cells were incubated at 37 °C for another 45 min prior to flow cytometry experiments. Flow cytometry experiments were performed at the gated population of 10 000 events.

4.5 Appendix

4.5.1 Time-dependent fluorescence spectra measurements

Figure S1. Time-dependent fluorescence response of FLB₂SSCou with (a) DTT ($\lambda_{\text{Ex}} = 408$ nm, $\lambda_{\text{Em}} = 497$ nm) and (b) H₂O₂ in pH 5.5 and pH 7.4.

The solution containing FLB₂SSCou (50 μ M) in 0.1 X PBS was treated with the various thiol reagents (DTT, GSH, Cys and Hcy. Conc. 5.0 mM) and the emission spectra (λ_{Ex} . 408 nm) was measured at a constant time interval. The same set of experiment was also performed with the addition of H₂O₂ (1.0 mM).

Figure S2. Time-dependent fluorescence measurement ($\lambda_{\text{Ex}} = 408 \text{ nm}$) of FLB₂SSC_{0u} upon the addition of (a) Cys (5.0 mM), (b) DTT (5.0 mM), (c) GSH (5.0 mM), (d) Hcy (5.0 mM) and (e) H₂O₂ (1.0 mM).

4.5.2 Computation details

Theoretical calculations of the various molecules were performed with the Gaussian 09 package.⁸⁹ DFT optimizations and TD-DFT calculations were performed using the basis set of B3LYP/6-31+G (d,p). The polarization continuum model using the integration formalism variant (IEFPCM) with water as solvent was included in the optimization and TD-DFT calculations process.

6	7.110539	-0.684044	1.017588	1	8.815012	-1.528697	0.032079
6	6.109253	0.303573	1.014177	6	5.127024	0.434428	2.157395
6	6.086053	1.205031	-0.055615	6	4.020423	1.415601	1.837927
6	7.028749	1.136170	-1.087110	6	4.109352	2.256464	0.723290
6	8.027419	0.157678	-1.075725	6	3.124991	3.212942	0.451717
6	8.051900	-0.757660	-0.000893	1	3.243399	3.839925	-0.426448
1	7.149788	-1.397500	1.834866	6	2.017050	3.354057	1.292817
1	6.963447	1.858768	-1.894507	6	1.919670	2.503551	2.415712

6	2.901031	1.556407	2.677681	1	8.702003	-1.051487	-4.932654
1	2.806061	0.914155	3.547727	6	10.866197	1.665032	-4.829017
8	5.147727	2.199372	-0.171683	1	11.638462	1.316692	-5.522210
1	1.067960	2.588634	3.082966	1	10.176309	2.305684	-5.386320
8	5.884295	0.985806	3.334613	1	11.341024	2.267870	-4.052599
6	4.633741	-0.894767	2.705207	7	1.806232	-3.806534	1.299723
6	5.070126	-1.055597	4.014862	1	1.601187	-2.827468	1.154943
6	5.854606	0.136357	4.397551	6	0.957376	-4.784206	0.625625
8	6.409252	0.397647	5.448063	1	0.706368	-4.385460	-0.360273
6	4.768417	-2.199449	4.756778	1	1.533229	-5.701849	0.492022
6	4.006721	-3.187727	4.139413	6	-0.322343	-5.075839	1.418542
1	3.754081	-4.098591	4.670301	1	-0.081655	-5.544126	2.378559
6	3.545359	-3.027274	2.817138	16	-1.426811	-6.298230	0.593650
6	3.864532	-1.873578	2.084153	16	-2.171171	-5.281894	-1.070017
1	3.547064	-1.755493	1.053026	6	-3.652709	-4.402367	-0.397211
1	5.118202	-2.312242	5.777522	1	-3.333294	-3.705564	0.378717
6	2.737973	-4.153631	2.222012	1	-4.324945	-5.144123	0.040440
8	2.937012	-5.327102	2.565656	6	-4.331859	-3.650209	-1.543245
5	0.919003	4.423163	0.988758	1	-4.662740	-4.344994	-2.321861
5	9.081597	0.084251	-2.226557	1	-3.622608	-2.956397	-2.013642
8	9.143441	0.992914	-3.253290	6	-5.504334	-2.808867	-1.046297
8	10.039294	-0.895151	-2.308971	8	-5.508223	-2.265714	0.060627
8	0.925674	5.215049	-0.132104	7	-6.538758	-2.686513	-1.928602
8	-0.147412	4.663852	1.818065	1	-6.466694	-3.154338	-2.823710
6	10.101862	0.481686	-4.241871	6	-7.646423	-1.821430	-1.713179
6	10.972416	-0.515071	-3.377784	6	-7.420815	-0.390158	-1.842301
6	-1.067441	5.563774	1.110022	6	-8.893389	-2.306772	-1.434108
6	-0.119572	6.233150	0.036575	6	-10.002276	-1.434472	-1.256417
6	-1.675727	6.526495	2.126275	1	-9.038945	-3.380228	-1.347891
1	-2.319017	7.257223	1.625838	6	-9.778014	-0.045463	-1.352873
1	-2.290743	5.966514	2.837033	8	-6.366364	0.160833	-2.123492
1	-0.908649	7.063741	2.687202	8	-8.517204	0.429073	-1.628288
6	-0.762685	6.491916	-1.323190	6	-10.778760	0.895938	-1.179895
1	-1.591861	7.200111	-1.227662	1	-10.509489	1.939608	-1.260347
1	-0.022339	6.928861	-1.999919	6	-12.106800	0.477487	-0.903143
1	-1.139490	5.572960	-1.776204	6	-12.340815	-0.932538	-0.788555
6	0.582432	7.499595	0.541150	6	-11.323197	-1.845363	-0.964973
1	1.348037	7.793002	-0.182890	1	-11.535115	-2.906742	-0.873190
1	-0.123543	8.328288	0.646441	1	-13.328546	-1.304793	-0.555209
1	1.070747	7.334058	1.505859	7	-13.126364	1.382769	-0.764267
6	-2.159896	4.678051	0.498952	6	-12.876583	2.827878	-0.860635
1	-2.646965	4.112980	1.298891	1	-13.825530	3.297914	-1.129976
1	-2.920939	5.279880	-0.005932	1	-12.197006	3.016189	-1.698274
1	-1.747089	3.964618	-0.220124	6	-12.331796	3.472725	0.421207
6	11.415951	-1.782001	-4.104016	1	-12.148835	4.538474	0.246565
1	12.061208	-1.531598	-4.952073	1	-13.047265	3.381067	1.243366
1	11.988624	-2.414637	-3.419343	1	-11.389783	3.014283	0.736418
1	10.565236	-2.358844	-4.471723	6	-14.500623	0.950490	-0.470517
6	12.171660	0.151266	-2.692843	1	-15.164333	1.744194	-0.822239
1	12.607316	-0.552102	-1.977341	1	-14.735640	0.070156	-1.076611
1	12.943179	0.420785	-3.419715	6	-14.782872	0.667395	1.011056
1	11.876611	1.052934	-2.148374	1	-14.647285	1.567183	1.617992
6	9.276226	-0.210831	-5.332821	1	-15.818321	0.330158	1.129578
1	8.573115	0.511380	-5.757671	1	-14.124649	-0.112295	1.405976
1	9.916236	-0.579652	-6.139448				

Table S1. Coordinates of the optimized structure of FLB₂SSCou. From left to right: Atomic number, X coordinates, Y coordinates and Z coordinates.

6	-2.623582	0.733790	0.188499	1	-7.291049	-2.220987	-0.529663
6	-1.306570	0.368188	-0.033957	1	-7.018186	-0.620329	-1.239380
6	-0.893444	-0.972608	-0.178191	1	-5.756114	-1.859978	-1.338435
6	-1.900643	-1.960541	-0.086708	6	0.488016	-1.228401	-0.396904
6	-3.219727	-1.628161	0.135186	6	1.394508	-0.208649	-0.478613
6	-3.628832	-0.263083	0.294481	6	0.965868	1.175326	-0.351371
1	-2.847447	1.787876	0.272350	8	-0.384242	1.384155	-0.120064
1	-1.624153	-3.005025	-0.198422	8	1.671991	2.170101	-0.432403
1	-3.948084	-2.425496	0.184285	1	0.832088	-2.254088	-0.499659
7	-4.934733	0.063940	0.550955	6	5.156318	-0.643365	-0.206137
6	-5.978512	-0.967098	0.647913	1	5.226603	-0.620156	-1.297998
1	-6.786416	-0.544120	1.249985	1	5.399852	-1.664782	0.113166
1	-5.590194	-1.816531	1.218206	6	6.142502	0.346323	0.418184
6	-5.352192	1.465885	0.693135	1	6.067834	0.315578	1.505528
1	-4.610165	1.998228	1.297062	1	5.935491	1.362721	0.075593
1	-6.274593	1.465892	1.278948	16	7.861415	-0.113791	-0.084493
6	-5.587924	2.203565	-0.632045	1	8.494954	0.868558	0.585388
1	-6.398495	1.740487	-1.201979	7	2.775052	-0.452732	-0.719658
1	-5.865695	3.243445	-0.428881	6	3.728552	-0.365417	0.252739
1	-4.690738	2.208478	-1.258135	8	3.454703	-0.114346	1.429392
6	-6.539607	-1.442172	-0.699080	1	3.071628	-0.618352	-1.673323

Table S2. Coordinates of the optimized structure of FLB₂SSCou after treatment with thiol. From left to right: Atomic number, X coordinates, Y coordinates and Z coordinates.

4.5.3 MTT cytotoxicity assay of FLB₂SSCou

HeLa cells were first seeded in a 96 well plate at a seeding density of 1×10^4 cells/well prior to overnight incubation. Thereafter, FLB₂SSCou and 2 equivalent of β -CD were added into the cells at different concentrations. The cells were then incubated overnight again before the medium were replaced with medium containing 0.5 mg/mL of MTT. The cells were allowed to incubate for another 4 h

before removing the medium. The residual purple crystals were then dissolved in DMSO (100 μL /well). The cell viability was then calculated by taking the absorbance at 562 nm relative to the control well, where the cells were treated with water only. The viability data were then expressed in percentage. The MTT cytotoxicity results showed that the FLB₂SSCou exhibits insignificant toxicity to the HeLa cells, confirming its biocompatibility for biological applications.

Figure S3. MTT cell viability profile of FLB₂SSCou on HeLa cells.

4.5.4 Flow cytometry assay (Histogram)

Figure S4. (A) BV421 channel and (B) FITC channel for 1D histogram plots of flow cytometry analysis with HeLa cells treated with blank control (black line), FLB₂SSCou (10 μM) with β -CD (20 μM) (red line), and FLB₂SSCou (10 μM) with β -CD (20 μM) followed by post-treatment with H₂O₂ (150 μM) (purple line). Note the shift in the BV421 signal upon treatment with the FLB₂SSCou probe (Figure 3A) and the shift in the FITC signal upon treatment with H₂O₂ (Figure 3B).

4.5.5 Spectroscopic data of FLB₂SSCou

Figure S5. ¹H NMR spectrum of N-(2-((3-((7-(diethylamino)-2-oxo-2H-chromen-3-yl)amino)-3-oxopropyl)disulfanyl)ethyl)-3-oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxamide (8).

Figure S6. ¹H NMR 2-dimension correlation spectroscopy (COSY) spectrum of N-(2-((3-((7-(diethylamino)-2-oxo-2H-chromen-3-yl)amino)-3-oxopropyl)disulfanyl)ethyl)-3-oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxamide (8).

Figure S7. ¹³C NMR spectrum of N-(2-((3-((7-(diethylamino)-2-oxo-2H-chromen-3-yl)amino)-3-oxopropyl)disulfanyl)ethyl)-3-oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxamide (8).

Figure S8. IR spectrum of N-(2-((3-((7-(diethylamino)-2-oxo-2H-chromen-3-yl)amino)-3-oxopropyl)disulfanyl)ethyl)-3-oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxamide (8).

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Conclusion and outlook

The development of therapeutic and diagnostic reagents have shown a great deal of potential for future clinical applications particular towards the treatment of cancer in human. In view of this, this dissertation mainly focus on development of a polymeric based nanoparticles as drug carrier and the delivery of fluorescence sensor.

Cancer treatment often relies on the direct administration of anticancer drug into the patient, causing the patient to suffer from various side effects arise from the anticancer drug. The side effect from the anticancer drug is caused by it low biocompatibility and it non-specificity delivery into the various healthy tissue. A promising solution involves in development of nanomaterial to prevent the premature released of the anticancer drug. Due to its high biocompatibility, the polymer based material was used in this dissertation as the delivery material. Furthermore, the polymeric nanomaterial used in this work was prepared by supramolecular approached and thus, a folate ligand could be used to specifically delivery the nanocarrier to the tumor side effectively. Our *in vivo* experiments show that nanomaterials functionalized with folate ligand possesses excellent antitumor effect over its non-functionalized counterpart.

As mentioned that the supramolecular preparation approached could allow us to integrate various useful functionality into the nanoparticles system depending on the desired outcome. In this dissertation, the loading of a thiol responsive fluorescence probe into the same nanoparticles system could be realized for the

detection of biological thiol in the cancer cells. The probe consist of a fluorescence dye conjugated to a quencher by a disulfide bond. Upon the reductive cleavage of vthe disulfide bond, the fluorescence could be turn on as an indication of thiol reduction. It was found that the detection functionality was not compromised even when the probe was loaded into the nanoparticle system.

Encouraged by the desirable outcome from the design of the thiol responsive fluorescence probe, a second molecular design of a detection probe was therein generated. In this case, a secondary fluorescence probe was conjugated to the primary probe by means of a disulfide bond. The secondary probe contains a boronic ester group whereby the reaction with hydrogen peroxide could switch on the fluorescence properties of the second probe. Apart from this, the secondary fluorescence moiety serve as a quencher to the primary probe by the photoinduced electron transfer mechanism. Therefore, this molecular system is capable of detecting the reducing biological reducing thiol and hydrogen peroxide in an orthogonal and highly selective manner. This means that our single molecular probe possesses multi responsive property, which could generate a different output signal upon reacting with different analyte with the use of a single excitation source. This outcome is highly desirable especially when the elevation in the concentration of biological thiol and hydrogen peroxide could be an early sign for certain class of diseases.

In conclusion, this dissertation highlighted the development of a polymeric nanoparticles as a nanocarrier of anticancer drug and thiol responsive probe. This clearly demonstrate the versatility of this novel class of nanomaterial for biological

applications. In addition, a special class of dual responsive fluorescence probe for the detection of reducing thiols and hydrogen was designed and synthesized. In this way, it was demonstrated that a multi responsive properties could be incorporated into on single molecular system and the response outcome could be realized in an orthogonal fashion. All in all, it was found that the polymeric based nanoparticles are highly customizable whereby a total different functionality could be expressed by a simple changing in the building component. Future work could be focus on the optimization of current NPs system such as the direct utilization of tumor targeting aptamer as delivery platform so as to achieve the synergistic effect of targeting and delivery in one single system for tumor therapy. In addition, future smart nano delivery system could also incorporate a fluorescence probe together with anticancer drug so as to achieve the combination therapy and diagnostic or theranostic effect.

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1. S. Y. Tan, **C. Y. Ang**, A. Mahmood, Q. Qu, P. Li, R. Zou, Y. Zhao. "Doxorubicin-loaded metal-organic gels for pH and glutathione dual-responsive release" *ChemNanoMat* **2016**, Accepted. DOI: 10.1002/cnma.201600078. **(Co-first author)**
2. L. Bai, Y. Xia, A. Jana, **C. Y. Ang**, L. Zhao, Z. Fan, Y. Zhao. "Perylenetetracarboxylic-metal assemblies and anisotropic charge transport in a Cu II assembly" *Nanoscale* **2016**, 8, 9134-9140.
3. Y. Zhang, **C. Y. Ang**, M. Li, S. Y. Tan, Q. Qu, Y. Zhao. "Polymeric Prodrug Grafted Hollow Mesoporous Silica Nanoparticles Encapsulating Near-Infrared Absorbing Dye for Potent Combined Photothermal-Chemotherapy" *ACS Appl. Mater. Interfaces* **2016**, 8, 6869-6879.
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6. **C. Y. Ang**, S. Y. Tan, S. Wu, Q. Qu, M. F. E. Wong, Z. Luo, P. Z. Li, S. T. Selvan, Y. Zhao. "A dual responsive "turn-on" fluorophore for orthogonal selective sensing of biological thiols and hydrogen peroxide" *J. Mater. Chem. C* **2016**, 4, 2761-2774. **(Cover art)**
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27. **C. Y. Ang**, L. Giam, Z. M. Chan, A. W. H. Lin, H. Gu, E. Devlin, G. C. Papaefthymiou, S. T. Selvan, J. Y. Ying. "Facile Synthesis of Fe₂O₃ Nanocrystals without Fe(CO)₅ Precursor and One-Pot Synthesis of Highly Fluorescent Fe₂O₃-CdSe Nanocomposites" *Adv. Mater.* **2009**, 21, 869-873.
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Book Chapter contribution

1. Si Yu Tan, Chung Yen Ang, Yanli Zhao,* Smart therapeutics achieved via host-guest assemblies, in Comprehensive Supramolecular Chemistry II, Elsevier, 2016, accepted.

Conference attended and poster/oral presented

1. The International Chemical Congress of Pacific Basin Societies 2015 (Pacifichem 2015), Honolulu, Hawaii, USA (December 2015).

Oral presentation: **C. Y. Ang**, S. Y. Tan, Y. Zhao. "Supramolecular nanoparticle carriers self-assembled from cyclodextrin and adamantane-functionalized polyacrylates for tumor-targeted drug delivery in both in-vitro and in-vivo model."

2. 3rd Symposium on Innovative Polymers for Controlled Delivery, Suzhou, China (September 2014).

Poster presented: **C. Y. Ang**, S. Y. Tan, X. Wang, N. S. Tan, S. T. Selvan, Y. Zhao. "Preparation of Polyacrylate-based Nanoparticles through Supramolecular Selfassembly for Target Specific Drug Delivery"

3. SOKENDAI Asian Winter School "Innovations and Challenges in Molecular Science: From Basics to Cutting-edge Researches", Okazaki, Japan (December 2013).

Poster presented: **C. Y. Ang**, S. Y. Tan, L. Bai, P. Li, S. T. Selvan, Y. Zhao. "The supramolecular self-assembly of coumarin integrated polymer based nanoparticles for the "Turn-on" sensing of biological thiols compounds."

4. 8th International Symposium on Macrocyclic and Supramolecular Chemistry, Arlington, Virginia, USA (July 2013).

Poster presented: **C. Y. Ang**, S. Y. Tan, X. Wang, N. S. Tan, S. T. Selvan, Y. Zhao. "Preparation of Polyacrylate-based Nanoparticles through Supramolecular Selfassembly for Target Specific Drug Delivery"

5. 7th International Conference on Materials for Advanced Technologies, Singapore (June 2013).

Poster presented 1: **C. Y. Ang**, S. Y. Tan, X. Wang, N. S. Tan, S. T. Selvan, Y. Zhao. "Preparation of Polyacrylate-based Nanoparticles through Supramolecular Selfassembly for Target Specific Drug Delivery"

Poster presented 2: **C. Y. Ang**, S. Y. Tan, S. T. Selvan, Y. Zhao. "Synthesis and Application of Polyacrylic Acid -based Nanoparticles for Photodynamic Therapy"

6. The International Congress for Innovation in Chemistry (PERCH-CIC Congress VIII), Pattaya, Thailand (May 2013).

Poster presented: **C. Y. Ang**, S. Y. Tan, X. Wang, N. S. Tan, S. T. Selvan, Y. Zhao.
“Preparation of Polyacrylate-based Nanoparticles through Supramolecular Selfassembly for Target Specific Drug Delivery” **Best poster award.**