Molecular engineering of biomolecule-based functional nanodots for biomedical applications

Xu Victor Hesheng

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**NANYANG
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SINGAPORE

**MOLECULAR ENGINEERING OF BIOMOLECULE-BASED
FUNCTIONAL NANODOTS FOR BIOMEDICAL
APPLICATIONS**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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FUNCTIONAL NANODOTS FOR BIOMEDICAL
APPLICATIONS**

XU HESHENG VICTOR

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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

2019

Statement of Originality

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This thesis contains material from 4 paper(s) published in the following peer-reviewed journal(s) in which I am listed as an author.

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The contributions of the co-authors are as follows:

- A/Prof Tan Yen Nee provided the overview for the review and edited the manuscript drafts.
- I prepared and organized the manuscript drafts for both articles. The manuscript was revised by Dr Zheng (1 and 2) and Dr. Yu (2).
- Ms Mok and Ms Salwa contributed ideas and plans for Functional Properties and Biomedical Applications of Bioinspired Nanostructures, and Nucleic acid–metal NCs respectively.

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The contributions of the co-authors are as follows:

- A/Prof Tan provided the initial project direction and edited the manuscript drafts.
- Prof Zhao provided guidance and suggestions to further improve the manuscript.
- I prepared and organized the manuscript drafts. The manuscript was revised by Dr Zheng.
- I co-designed the study with Dr Zheng and performed all the laboratory work at A*STAR's Institute of Materials Research and Engineering and NTU's School of Physical and Mathematical Sciences.
- All characterizations such as surface, optical and morphologies study, and sample preparations, were performed by me in the A*STAR's Institute of Materials Research and Engineering. Data analysis and comparison were also conducted by me.
- I performed cell culturing and *in vitro* studies including MTT test.
- Dr Zheng assisted in the collection of the Atomic Force Microscopy data and carried out Confocal Microscopy study for the samples.

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The contributions of the co-authors are as follows:

- A/Prof Tan suggested the materials area and edited the manuscript drafts.
- Prof Zhao provided guidance and suggestions to further improve the manuscript.
- I prepared and organized the drafts of the manuscript. The manuscript was revised together with Dr. Zheng and Dr. Wang.
- I performed all the materials synthesis, and characterizations which include Fourier-Transform Infrared Spectroscopy, X-ray Photoelectron Spectroscopy, ¹³C Nuclear Magnetic Resonance, Transmission Electron Microscopy, Scanning Electron Microscopy, UV-vis Spectroscopy, Zeta and Photoluminescent measurements. I also conducted the data evaluation.
- The microbial testing and staining were devised and conducted by me.
- Dr. Wang performed the Multi-drug Resistance microbial study and resistance development assay.
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- A/Prof Tan suggested the materials area and edited the manuscript drafts.
- Prof Zhao provided guidance and suggestions to further improve the project and the manuscript.
- I prepared and organized the drafts of the manuscript. The manuscript was revised together with Prof Zhao and A/Prof Tan.
- I conducted all the materials synthesis, and characterizations which include Fourier-Transform Infrared Spectroscopy, X-ray Photoelectron Spectroscopy, ^{13}C Nuclear Magnetic Resonance, Transmission Electron Microscopy, UV-vis Spectroscopy, Zeta and Photoluminescent measurements. I also performed the data analysis and evaluation.
- I developed the sensing assay for H_2O_2 and carried out the selectivity study.
- Glucose and cholesterol detection were devised by me.

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Abstract

In recent years, nanotechnology has gained considerable attention owing to its numerous attractive properties. Bestowed with quantum mechanical effects, nanomaterials exhibit unique properties including high surface-to-volume ratio, great strength and ductility, ease of functionalization and enhanced cargo loadings. This enabled great versatility in their application, especially in the field of biomedicine. Nonetheless, the preparation of these nanomaterials is often challenging, involving tedious multistep synthesis and expensive reagents. In addition, the fabrication usually utilises harsh and toxic reagents which are hazardous towards human and environmental health. Consequently, this restricts the effectiveness of their applications.

Unlike the conventional synthesis, bioinspired synthesis represents new facile and benign strategy for the fabrication of nanomaterials. This strategy offers a simple and green approach as it utilises mainly biomolecules ranging from macro biostructures such as protein, DNA, to poly saccharides, polypeptide and even the building units such as nucleotides and amino acid, and their intrinsic properties to facilitate the formation of nanomaterials. Moreover, the resulting bioinspired nanomaterials are often endowed with unique characteristics such as rich chemical functionalities, aqueous solubility, unusual optical features, and great biocompatibility. Besides, through careful selection of the biomolecule precursors, the resulting nanomaterials can be engineered with desired morphology and properties. Hence, this strategy creates a new programmable assembly of nanomaterials with multifunctional properties which enabled great versatility in their application, especially in the field of biomedicine.

In this thesis, bioinspired synthesis strategy was employed to prepare various unique biodots. The biodots were developed through critical combinations of mainly amino acid,

Serine, and carefully selected precursors such as PEI (polyethylenimine) and histamine. The resulting biodot (ie. Ser-dot, Ser-PEI biodot and Ser-Hist biodot) displayed enhanced properties which is suitable for applications including bioimaging, antibacterial therapy and biosensing respectively. These findings could provide essential insights towards interaction of the biomolecular precursors and the formation of the biodot, thus, potentially improving understanding towards more responsive and multifunctional nanostructure for complex applications such as theranostic, deep tissue diagnosis and nanorobots for surgery.

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Chapter 1

Introduction

1.1 Emergence of Nanotechnology

The emergence of nanotechnology has been made possible by the advancing science and technology over the past decades. The nanotechnology involves the defined manipulation of atoms and molecules for the synthesis of materials within nano-metric scale of at least one dimension. At this scale, the nanomaterials benefit from the quantum mechanical effects and are gifted with exceptional properties which cannot be achieved by their bulky counterparts. Thus, these nanomaterials present as a promising solution and create opportunities in various range of applications, particularly in field of biomedicine.

1.1.1 Properties and Applications of Nanomaterials

Up to date, various types of nanomaterials has been prepared, from organic nanomaterials such as polymer/dendrimer nanoparticles¹, to inorganic nanomaterials such as metallic nanoparticles² and ceramic nanoparticles³, to hybrid nanomaterials involving organic-inorganic composite⁴. Being at nano-metric scale, these nanomaterials exhibit unique physical properties such as high surface-to-volume ratio, great strength and ductility. This enables an ease of functionalisation on the surface of the nanomaterials and improves loading of cargoes including functional ligands, biomolecules and drug molecules. For instance, Li et al. reported the use of hollow mesoporous silica nanoparticles for controlled drug delivery.⁵ The hollow characteristics of the mesoporous silica nanoparticles enable the loading of drug molecule, doxorubicin (DOX), within the cavity. Upon entering intracellular environment, the drug can be released and delivered to the targeted site. Similarly, Nguyen et al. prepared an oral drug delivery system by developing a pH responsive bifunctional succinylated ϵ -polylysine-coated

mesoporous silica nanoparticles. The pH responsive ability of the nanoparticle serves as a nanogate which enabled controlled release of loaded drug molecule, prednisolone, in weakly acidic pH condition (pH 5.5-7.4). Thus, this allows selective delivery of the drug molecule to the disease site, the colon, whose pH condition falls within the responsive range of the nanoparticles (**Figure 1.11**).⁶

Figure 1.11 (a) Synthesis route of the drug delivery system from 3-aminopropyl-functionalized MCM-48 nanoparticles (MCM-NH₂), prednisolone, and SPL. (b) Schematic representative of pH-responsive release of drug/cargo under conditions simulating gastrointestinal transit. Reproduced with permission from Ref #6. Copyright 2017 American Chemical Society.

Furthermore, with the ability to fabricate and alter the structure of the nanomaterials, the nanomaterials can be prepared with unusual optical features including plasmonic effect, fluorescent and phosphorescent. As such, these nanomaterials are often utilised for biomedical applications such as bioimaging, therapy and sensing. For instance, Chetty et al. demonstrated

the bioimaging capability of the as-prepared $\text{CuInS}_2\text{-ZnS}$ alloyed nanocrystals in zebrafish-embryos (**Figure 1.12**).⁷ The cellular uptake and labelling can be achieved within 20 mins and the high stability of the nanocrystals suggested the potential for long-term in vivo tracking and intravital-fluorescence-bioimaging. On the other hand, Li et al. prepared an upconversion nanoparticles (UCNPs) capable of near-infrared triggered photodynamic therapy for deep tumors.⁸

Figure 1.12 Schematic illustration of preparing hydrophobic DDT-functionalized CIZS-NCs (Step I), and nano-xenotoxicity assessment and intravital fluorescence bioimaging application of nanocrystal in zebrafish (Step II). Reproduced with permission from Ref #7. Copyright 2016 Nature.

The UCNPs were grafted with pH-responsive polymeric ligands which aggregated and self-quenched under neutral pH condition. While at acidic pH condition, the nanoparticles displayed enhanced cellular uptake capability and dissembled into smaller nanoparticles, enabling efficient photodynamic effect. Thus, this greatly improved tumor-cell and effectively activated of the photosensitizers for the therapy. Conversely, Chen et al. reported an ultrasensitive

detection of thrombin with low detection limit of 1 pM, with high selectivity in presence of various interfering proteins. The detection assay was based on a colorimetric biosensor comprising of cationic aptamer and gold nanoparticles.⁹ The gold nanoparticles were used as sensing probes and thrombin-binding aptamer as recognition element. Upon the addition of thrombin, the aptamer would form a G-quadruplex structure, inducing an aggregation of the gold nanoparticles. This resulted in a colorimetric signal with visible color change from wine-red to blue-purple, which can be easily observed by naked eye.

1.1.2 Limitations of Current Nanomaterials

Despite the above-statement advantages of the current nanomaterials, the preparation of these nanomaterials is extremely challenging and difficult. Although the synthesis often has good controllability over the particle size, it is often in the expense of having a more tedious, and time-consuming process. For instance, the preparation of polymeric nanomaterials (e.g. dendrimer, polymer) usually comprises of tedious multi-step reactions including dimerization, cycloaddition, condensation and purification.¹⁰ As a result, the as-synthesized nanomaterials often suffer from low yields and poor biocompatibility which greatly limit the effectiveness of their applications. On the other hand, inorganic nanomaterials frequently require high manufacturing cost owing to the expensive precursors such as gold (e.g. gold nanoparticles) and in the case of heavy metals (e.g. semiconductor quantum dots), contributes to a greater environment footprint. In addition, the synthesis typically requires the use of strong base/reducing agent and surfactants.¹¹

This necessitates the search for a benign and facile method to synthesize the nanomaterials. Hence, in this thesis, bioinspired nanodot was formulated using a cheap and readily available biomolecule, serine, as a precursor. The preparation involved a simple one-step hydrothermal treatment without the use of any harsh and toxic reagents/solvents. The biodot formed was

found to have good size distribution ranging from 2 to 6 nm and possess unique properties suitable for application such as bio-imaging, antibacterial therapy and bio-sensing.

1.2 Bioinspired Nanomaterials

Nature, on the other hand, has given us many signs and hints on preparing unique materials. Typically, many living organisms utilize a unique process, biomineralization, to convert natural minerals into biominerals. For instance, biominerals such as carbonate and hydroxyapatite can be found in bones and shells of mammals¹², biogenic silica within radiolarians and marine sponges¹³⁻¹⁴, as well as magnetite in chiton teeth¹⁵, are natural materials which possess superior physiochemical properties including high flexibility, light-weight, exceptional mechanical strength and dynamic functions that support their daily essential physiological functions. The process is a highly intelligent biological mechanism which exploits the unique bio-recognition ability of the biomolecules. This enables the assembly of biological molecule and their respective substrates via various interaction, and eventually forming a unique complex material. Inspired by the distinctive characteristics of these biomolecules, many have shifted their focus towards understanding the formation mechanism of these exceptional biomaterials and mimicking the techniques by utilising biomolecules in designing and developing nanomaterials with superior properties. This has led to the emergence of “bioinspired” synthesis of nanomaterials.

1.2.1 Methodologies of Synthesizing Bioinspired Nanomaterials

In recent years, several biomimetic approaches have been developed to synthesize these bioinspired nanomaterials (**Figure 1.21**). These biomimetic approaches mimic the natural biochemical processes in hope to provide not only a green and facile preparation method, but also synthesizes a superior nanomaterial for desired application. For instance, the “bio-structure mimicking” approach is a technique whereby bioinspired nanomaterials were prepared by

imitating the nanoscaled architecture of natural materials.¹⁶⁻¹⁷ Particularly, Hensel et al. reported an omniphobic polymer coating mimicking the skin of a springtail. It involved a unique reverse imprint lithography technique which utilised a combination of overhanging cross-sections and arrangement into a self-supporting comb-like pattern to support a stable coating. The coating possesses excellent mechanical durability, long-term resistance against wetting and high pressure resistance which has potential for biofouling prevention.¹⁸ Likewise, by studying the structure of plant leaves, Wang et al. developed 2D/1D CoO_x heterostructure is composed of ultrathin CoO_x nanosheets which could be further assembled into a nanotube structure. In comparison to the enhanced surface reaction and efficient mass transport ability of the plant leaves, the bioinspired CoO_x heterostructure could conduct both effective oxygen evolution and interfacial electrochemical reaction on the surface while having improved transport of electrolyte and charge across the nanotube.¹⁹

Figure 1.21 Schematic representation of various bioinspired methodologies in preparing functional nanomaterials including bio-structure mimicking, bio-function anchoring, bio-

assembling and bio-templating approaches. Reproduced with permission from Ref #23. Copyright 2017 American Chemical Society.

On the other hand, “bio-function anchoring” approach is a technique where active functioning biomolecules are chemically conjugated onto as-synthesized nanomaterials. This bestows the resulting nanomaterials with desired properties such as enzymatic activity, solubility and biocompatibility from the conjugated biomolecule.²⁰⁻²¹ In a typical example, Yin et al. demonstrated a rapid conjugation of RGD peptides onto gold nanocluster. The specific binding affinity of RGD peptides permitted the nanocluster to selectively target the Melanoma A375 cells, enhancing the intracellular uptake activity of the gold nanocluster. In addition to the bright fluorescence capability, the nanocluster exhibited good biocompatibility and stability, which is suitable to be used as a contrast agent for fluorescence imaging of the Melanoma A375 cells (**Figure 1.22**).²² Similarly, Reuter et al. has reported the use of transferrin, a cancer targeting ligand, to coat of silica nanoparticles. By retaining the functional properties of transferrin, the nanoparticles exhibited enhanced cellular uptake ability in human cancer cells which could potentially be used for drug delivery application.²³

Figure 1.22 (a) Schematic representation of the synthesis route to prepare RGD peptide conjugation gold nanocluster. (b) Confocal fluorescence images of Melanoma A357 cells (left) and MCF-7 cells (right) after treatment with RGD peptide conjugation gold nanocluster. Reproduced with permission from Ref #22. Copyright 2015 American Chemical Society.

In contrary, “bio-assembling” approach is a method which utilises the unique molecular recognition ability of the biomolecule to invoke self-assembling of the biomolecule into a functional nanomaterial.²⁴ For instance, Wei et al. exploited the cognate Watson-Crick base-pairing and assembled several strands of nucleic acids into a robust framework. The framework consists of nucleic acids with concatenated sticky ends and that binds to four local neighbours. This enabled the self-assembly of the nucleic acids, thus, forming a complex two-dimensional nanostructure.²⁵ Given the availability and vast amount of existing naturally occurring biomolecules, this indicates a new strategy to prepare highly biocompatible nanomaterials which can be further functionalised for any desired applications.

Nonetheless, these techniques face some drawbacks which may limit their applications. For instance, the “bio-structure mimicking” is often tedious due to the multi-step synthesis and requires sophisticated equipment such as lithography to complete the construction of the complex structure. Furthermore, the “bio-function anchoring” method may alter the structure of the biomolecule during the chemical conjugation, and thus, causing the biomolecule to lose its intended biofunction. Lastly, the “bio-assembling” approach is currently limited to the use of nuclei acids and it involves a complicated polymerase chain reaction technique to fabricate a various long chain nucleic acid with different sticky ends in order to assemble the desired nanostructure. Hence, these approaches need to be refined so that they can be further used for desired applications.

1.2.2 Bio-templating Synthesis of Nanomaterials

Among the various methodologies of bio-inspired synthesis, “bio-templating” technique represents the most promising technique to prepare nanomaterial for biomedical application. This technique offers a facile and efficient preparation route and involves the use of simple biomolecules to fabricate the nanomaterials. In the synthetic process, the biomolecules can either serve as preserved templates to be integrated into the final nanohybrids or will be sacrificed at the end of synthesis leaving the nanomaterial product with well-defined size, shape, and structure. Although the bio-function of the biomolecule maybe lost in the process, it introduces other unique characteristics such as rich chemical functionality, good biocompatibility, high aqueous solubility, unusual optical features, and tunable physiochemical properties to the resulting nanomaterial. In addition, this approach does not require the use of toxic reagents and harsh condition in the synthesis, and thus, represents a green, energy-efficient and eco-friendly way for nanomaterial synthesis.²⁶⁻²⁷

1.2.2.1 Design of Preserved Bio-template for Metallic Nanostructures Synthesis

Nucleic acids as bio-templates

Nucleic acids such as DNAs are natural biopolymers formed by long chain of nucleotides consisting of nucleobases such as Cytosine (C), Guanine (G), Adenine (A) and Thymine (T). The nucleobases are able to interact with their corresponding pair via Watson-Crick base pairing. Upon annealing with the complementary strand, the two ssDNAs would hybridise into a double helix structure with the negatively charged phosphate groups forming the backbone to minimise repulsion. As the phosphate backbones are highly negatively charged, they are able to bind cationic species such as metal ions via electrostatic interactions.²⁸ Such interactions are able to stabilise the unstable intermediate cationic complexes. Likewise, the nucleobases such as C, G, A and T that contains the electron-rich nitrogenous group could potentially bind

to and stabilise the cations and their intermediate complexes. Furthermore, they could also reduce electron-poor complexes through electron transfer mechanism.²⁹

In a typical synthesis of metal NPs, metal precursors (e.g. Ag^+) are first reduced to form nuclei and then grow into different nanostructures which are stabilized by capping agents. Since the formation of metal NPs is driven by the capping and reducing capabilities of the ligands, these corresponding properties of nucleic acids are critical in the bio-template design for NP synthesis. For instance, the negatively charged phosphate groups are able to bind to and stabilise the positively charged metal ions such as Ag^+ and Au^+ via electrostatic interactions.³⁰ External stimuli such as UV light could be irradiated to induce a reducing environment allowing metal NP nucleation.³¹ Since double-stranded DNAs (dsDNAs) are rigid molecules with linear structures, the metal nuclei could subsequently grow along the dsDNA nanostructure and gradually forming a nanowire.³² It is worth noting that the stabilisation of Ag/Au intermediate complexes by the phosphate backbone is sufficient to prevent aggregation of the resultant NPs. Besides dsDNA, ssDNA has also demonstrated high efficiency in seed-mediated growth of metal NPs. Unlike dsDNA, ssDNA has exposed nucleobases which are strong electron donors. Thus, they are not only capable of binding to the electron-poor metal ions, but also able to reduce the metal ions for nucleation and growth. For example, it is found that the nucleobases in ssDNA could bind to Ag^+ with different affinities (i.e. $\text{C} > \text{G} > \text{A} > \text{T}$) and different Ag nanostructures such as nanocubes, nano-octahedron and nanoflowers have been obtained by varying the sequences.³³

Figure 1.23 (A) Schematic representation of using DNA-templated AgNPs and intercalating dye Genefinder (GF) for dopamine (DA) detection. (B) Fluorescence emission spectra of GF/dsDNA–silver nanohybrids in the presence of increasing DA concentrations (0-0.5 μM). (C) Fluorescence intensity at 525nm as a function of the DA concentration (0-0.5 μM). Reproduced with permission from Ref# 34 Copyright 2011 John Wiley and Sons Ltd.

As the molecular structures of DNA templates are preserved throughout the synthesis, some of these DNA-templated NPs retain the molecular recognition functions of the DNAs, endowing them with intrinsic sensing capabilities for diagnostic applications. This is especially true with plasmonic NPs such as AuNPs or AgNPs as they possess unique interparticle-distance dependent localized surface Plasmon resonance properties.³⁵ For instance, their high extinction coefficient are sensitive enough to induce noticeable colour change and detection by naked eyes.³⁶ With the target-specific binding of DNA preserved, these DNA-AuNPs or DNA-AgNPs could form aggregation or dispersion depending on their biomolecular interactions with target analytes, providing a basis for colorimetric sensing.³⁷ Particularly, Liu et al. have demonstrated the use of DNAzyme-AuNP as a highly sensitive and selective colorimetric assay for Pb(II) detection.³⁸ Upon hybridisation, the AuNP aggregates, resulting in a blue-coloured solution. In the presence of Pb²⁺, hydrolytic cleavage occurs, preventing the aggregation, producing a red-

coloured solution of well-dispersed AuNPs. In another report, Lin et al. have described the use of a DNA-AgNP as a platform for a simple fluorescence turn-on detection of dopamine (DA) (**Figure 1.23**).³⁴ Since DAs are able to form strong Ag-catechol bonds, DA addition released AgNPs from the DNA and a fluorescent signal was produced in the presence of intercalating DNA dyes. Using a similar strategy, detection of thiol-containing biomolecules was also reported.³⁹

Figure 1.24 (A) Schematic Illustration of an aptamer based AgNC assay for the label-free and fluorescent turn-on detection of cancer cell. The fluorescence responses of TD05 involved in recognition probe to assay target Ramos cancer cells by (B) flow cytometry and (C) confocal microscopy images. Reproduced with permission from Ref# 40 Copyright 2013 American Chemical Society.

More recently, nucleic acids were also exploited as templates to mediate the synthesis of metal nanoclusters (NCs), a new class of luminescent nanomaterial. These metal NCs exhibit ultrasmall size of < 2 nm, which lead to strong quantum confinement effect, thus bestowing them with bright photoluminescence.⁴¹ In particular, AgNCs are commonly synthesized using nucleic acids due to the strong binding affinity between Ag^+ and nucleobase C, which could stabilise the intermediate complex and serve as a nucleation site for AgNCs.⁴² Compared to typical NP synthesis, these ultra-small fluorescent NCs require a more meticulous and stringent preparation. For instance, it was found that slight variation in the overall DNA length and/or sequences would alter the fluorescence properties of the AgNCs such as the emission wavelength and the photostability.⁴³ As such, a careful design of DNA sequences is critical. Notably, Guo et al. reported a C6-loop with C-C mismatch to first entrap the Ag^+ and then gradually reducing them to form AgNCs.⁴⁴ By manipulation of the base pair mismatches and a basic sites to produce Ag binding sites, other DNA supramolecular structures such as the i-motif,⁴⁵ DNA duplex⁴⁶ and the G-quadruplex⁴⁷ have also been explored to synthesize bio-templated AgNCs.

Similar to DNA-templated metal NPs, these DNA-AgNCs could also function as an excellent sensing platform. Instead of colorimetric assay, they could be developed as fluorometric assays due to their intrinsic fluorescence characteristics. For example, Yeh et al. have observed that the fluorescence of DNA-AgNCs could be enhanced when comes in close proximity with G-rich DNA sequences.⁴⁸ Based on this observation, they have further developed a NanoCluster Beacon (NCB) to detect an influenza sequence. Applying a similar strategy, Yin et al. prepared a system of DNA-AgNCs for cancer cell detection (**Figure 1.24**).⁴⁰ The system consists of two tailored DNA probes, one containing sequence for AgNCs template synthesis while the other comprises of G-rich DNA sequence at 5'-end and a cancer targeting

aptamer sequence at 3'-end. Upon detecting CCRF-CEM cancer cell, the recognition probe would undergo conformation change, giving out a fluorescent signal.

Further on, photoinduced electron transfer of DNA-AgNCs was also explored. Wang's group prepared a functional DNA-AgNCs that is capable of detecting hemin biomolecule using a parallel G-quadruplex and a hemin specific sensing sequence.⁴⁹ Upon detection of complementary sequence, the G-quadruplex would be released and subsequently captures the hemin biomolecule, forming a stable G-quadruplex/hemin complex. This formation promotes electron transfer from DNA-AgNCs to the hemin complex, thus reducing the fluorescent intensity of the former. Other DNA templated NCs such as DNA-CuNCs have also shown potential to be used as a biosensor. For instance, Zhou et al. developed a label-free aptamer sensor for adenosine triphosphate (ATP) by controlling the formation of DNA-CuNCs.⁵⁰ Since CuNCs would only form in the presence of dsDNA but not ssDNA, the existence of ATP would bind strongly to one of the strands, inhibiting the growth of CuNCs. This simple detection for ATP has a preferable linear range (0.05-500 μ M) and a high sensitivity with detection limit of 28 nM. Likewise, Wang's group exploited this strategy to test for single nucleotide polymorphism.⁵¹ Compared to the perfectly matched DNA, it was found that the mismatch base pair site would provide an appropriate environment for CuNCs, altering their fluorescent intensity. The differences in fluorescence intensity were highly dependent on the mismatch sequence which allows the detection of more than one mismatches in a specific DNA sequence. Besides sensing of biomolecules, detections of other ions such as Pb^{2+} and S^- have also been demonstrated.⁵²

In general, nucleic acids are good stabilising and reducing agent which serves as an excellent designable bio-template to direct the synthesis of metal NPs. Such methodology promotes the green synthesis of metal NPs and renders inherent biocompatibility to the nanohybrid.

Furthermore, as the bio-templates are preserved after the synthesis, it endows them with the unique biorecognition capability for direct biosensing and diagnostic applications without post functionalization.

Proteins as bio-templates

Proteins are a class of biomacromolecules with complex three-dimensional (3D) architecture. The protein structures which constitute of amino acids as basic building blocks, have diverse functional groups such as -NH_2 , $\text{-CO}_2\text{H}$, -OH , -SH in their side chains for chemical synthesis and modifications. Some of the functional groups of amino acids residues such as tyrosine (Tyr)⁵³, aspartic acids (Asp)⁵⁴, tryptophan (Trp)⁵⁵, lysine (Lys)⁵⁶ and cysteine (Cys)⁵⁷ have been shown to be good reducing agents and/or stabilising agents, which could provide specific binding and nucleation sites for metal ions. For example, Tyr residues in the BSA protein template are found to be responsible for the reduction process of Au^+ , leading to the formation of spherical AuNPs.⁵⁸ Conversely, Casein protein which forms micelle structure in aqueous environment, uses its Asp residues to bind to and reduce Au^+ , producing anisotropic Au nanoplates.⁵⁹ As mentioned previously, the formation of NCs involves a more rigorous requirement in the bio-template design. In particular, the bulky proteins could introduce steric hindrance, providing sufficient stabilizing effect to promote the formation of NCs. For instance, bulky proteins such as transferrin,⁶⁰ lactoferrin,⁶¹ lysozyme type VI,⁶² horseradish peroxidase,⁶³ trypsin⁶⁴ and insulin⁶⁵ which contain Cys residues could provide sufficient steric hindrance as well as forming Au^+ -thiolate intermediates to stabilise the growth of protein-templated AuNCs effectively.⁶⁶

Similar to DNA-templated metallic nanostructures, the protein templates also retain their initial bio-recognition functions after the bio-templating synthesis, enabling them to act as diagnostic sensors. For instance, lysozyme-AuNCs were found to be able to retain their

bioactivity, allowing them to label bacteria such as *E. coli* and inhibit their growth.⁶⁷ In another example, Wang et al. prepared a transferrin-AuNCs coupled with sheets of graphene oxide (GO) for cancer cells diagnosis and imaging.⁶⁸ The nanocomposite has a “turn-on” fluorescent feature which could display near infrared (NIR) fluorescence upon identification of the transferrin receptor on the surface of the cancerous cell both in vitro and in vivo. In addition, Ranjita et al. utilised protein seeding of AuNPs via glycosylated haemoglobin to study the mechanism of glycosylation and sense the glycosylated end products by solution color changes due to the changes in AuNPs size.⁶⁹ The size increase of the AuNPs could be observed through transmission electron microscopy (TEM) while the structural alterations of the proteins were investigated using infrared spectroscopy and circular dichroism. Besides the bio-recognition ability, BSA-AgNCs synthesized by Yu et al. displayed strong singlet oxygen generation capacity with a quantum yield of ~1.26 and exhibited excellent cellular uptake and biocompatibility which demonstrated a high anticancer efficacy via photodynamic therapy.⁷⁰

Using similar strategies of reduction and stabilisation, protein cages such as apoferritin, viral capsid, heat shock protein and lumazine synthase have also been demonstrated as efficient templates for the preparation of nanomaterials.⁷¹ In particular, apoferritin has been used to synthesize magnetic NPs such as Fe,⁷² while other metal NPs like Ni,⁷³ Cr,⁷⁴ Cu,⁷⁵ Au,^{12b} Ag,⁷⁶ and semiconductor NPs such as CdS⁷⁷ and CdSe⁷⁸ have also been reported. Ferritin (Fn) has a nearly spherical structure with a negatively charged channels containing amino acids such as Asp and Glu, could bind to and transport positively charged metal ions to its hollow cavity.⁷⁹ The encapsulated metal ions could be reduced using UV light or heating and the resulting NPs would be stabilised by the neighbouring carboxylate groups in the cavity.⁸⁰ The as-synthesized Fn-NP could be used for diagnostic or therapeutic applications due to the distinctive properties of the cage-enclosed metal NPs. For example, Li et al. prepared a Fn-Fe₃O₄ nanostructures for tumor sensing and imaging.⁸¹ The surface of Fn was conjugated with cancer targeting RGD

peptide and a green fluorescent protein, combined with the magnetic resonance imaging capability of Fe₃O₄ NPs, the nanoparticle could specifically target and track cellular uptake by tumor cells. On the other hand, Wang et al. prepared a CuS-Fn nanocage which achieved superior cancer therapeutic efficiency using photothermal therapy (**Figure 1.25**).⁸² Moreover, the nanocages are also excellent positron emission tomography (PET) and photoacoustic imaging (PAI) agent which provide real-time monitoring and guidance of the nanocage in vivo. The theranostic potential of Fn-NP has also been illustrated recently by Ceci's group.⁸³ By decorating the surface of Fn with melanoma targeting peptide and poly(ethylene) glycol (PEG), and doping the Fe₃O₄ core with Co²⁺, the resulting magnetoferritin exhibit excellent targeting properties, outstanding in vivo stability, enhanced magnetic anisotropy and hyperthermic effect toward melanoma cancer cells.

Figure 1.25 (A) The preparation procedure of CuS–Fn NCs; TEM images of (B) iron free Fn and (C) CuS–Fn NCs stained with 1% uranyl acetate; (D) Temperature recording of U87 MG tumour mice upon 5 min laser exposure of different powers; (E) The variation of temperature in tumour area upon laser irradiation; (F) Images of U87MG tumour mice at various days after treatment. Reproduced with permission from REF# 82 Copyright 2016 American Chemical Society.

Peptides as bio-templates

In contrary to proteins, peptides compose of shorter chain of amino acids, which provide a more versatile platform for bio-templating synthesis due to the absence of complex secondary

and tertiary structures. Through careful selection of amino acid residues, the peptide could be programmatically designed into an efficient template to direct the synthesis of metal NPs. For instance, Tan et al. conducted a systematic study to uncover the design rules for peptide synthesis of AuNPs (**Figure 1.26**).⁸⁴ They demonstrated that through combining amino acids such as Tyr and Trp with the shape-directing sequences respectively, the resulting peptides (i.e. SEKLWWGASL and SEKLYYGASL) were able to synthesize Au nanoplates from AuCl₄⁻ precursors in one pot solution. Moreover, other peptides such as Ac-TLHVSSY-CONH₂ and SSFPQPN were also designed to facilitate the formation of platinum nanocrystals.⁸⁵

Other than synthetic peptides, naturally occurring peptides such as glutathione (GSH) were utilised in the synthesis of ultrasmall AuNCs. Being a short chain peptide, the GSH peptides could facilitate the reduction of Au⁺ to Au⁰ while stabilising the Au⁺ intermediate by binding through carboxylate and thiol functional groups.⁸⁶ The as-synthesized protein- and peptide-based metallic nanostructures could also be used as promising biosensing probes owing to the conserved biorecognition functions of the template. This includes the detection of enzymes,⁸⁷ small molecules⁸⁸ and metal ions.⁸⁹ Remarkably, peptide-NCs are able to produce near-infrared emission wavelength upon excitation, allowing them to activate any photosensitizer to yield reactive oxygen species (ROS) such as singlet oxygen for potential photodynamic therapy (PDT). For instance, Zhang et al. fabricated a GSH-AuNCs functionalized with folic acids and PEG on the surface, to enable the entrapment of photosensitizer chlorin e6.⁹⁰ The in vitro and in vivo studies show the enhanced cellular uptake and satisfactory PDT effectiveness toward cancerous MGC-803 cells. More recently, Vankayala et al. reported a TAT (cell penetrating peptide)-AuNCs that could perform simultaneous in vitro and in vivo fluorescence imaging, gene delivery, and NIR activated photodynamic therapy for effective anticancer therapy.⁹¹ The positively charged nanoprobe could load the negatively charged DNAs via electrostatic interactions and transport them into the nucleus for successive transfection. Furthermore, the

nanoprobe could also generate ROS to induce cell apoptosis without the use of any organic photosensitizer.

Proteins/peptides offer an excellent platform for the bioinspired design and engineering of metallic nanostructures of different size, shape and properties. Specifically, the biomolecular shell of the metallic nanostructures could endow them with good biocompatibility and a diverse functional group such as NH_2 , COOH and SH for further conjugation of therapeutic and/or diagnostics agents for biomedical applications.

Figure 1.26 (A) Schematic illustration for the peptide mediated synthesis of AuNPs in aqueous solution. The peptides and chloroaurate ions were first interacted to form peptide- AuCl_4 complexes, facilitating the reduction of Au ions to $\text{Au}(0)$ in forming nuclei. It is followed by the growth of nuclei into crystalline particles induced by the addition of more Au atoms from the solutions or by fusion with other nuclei. (B) The molecular interactions between peptide and Au ion, as well as peptide and gold atoms which determined the reactivity of functional peptide template for metal NPs synthesis can be designed by the selection of amino acids and their sequences. TEM images of AuNPs synthesized from using multifunctional

peptides (C) SEKLWWGASL and (D) SEKLYYGASL. Reproduced with permission from Ref# 84 Copyright 2010 American Chemical Society.

1.2.2.2 Design of Sacrificial Bio-templates for Carbon Nanomaterials Synthesis

Besides functioning as a bio-template that can be preserved throughout the bioinspired synthesis process, biomolecules such as nucleic acids, proteins, peptides, amino acids and carbohydrates could also be used as sacrificial templates in the synthesis of carbon nanomaterials. Particularly, “bio-dots”, the biomolecule-derived fluorescent nanodot, represent a new class of fluorescent nanomaterial synthesized using biomolecules as the sacrificial templates.⁹² Abundant with elements such as oxygen, nitrogen, phosphorous and sulphur, biomolecules are good doping agents in the preparation of the bio-dots. By introducing heteroatom doping, it provides various trapping sites of different series of energy levels in bio-dots.⁹³ This enables electronic transitions such as $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, allowing emission of photons with varying excitation energy. Therefore, the use of biomolecules could endow these bio-dots with interesting optical properties that are desirable for biomedical applications.⁹⁴

In one example, Du et al. synthesized nitrogen doped nanodots using glucose and serine as precursor.⁹⁵ The surface of the nanodots were passivated with functional groups such as C-O, C=O and O=C-OH which provided surface defects with different energy levels, granting the nanodots with excitation-dependent properties. Upon incubation with A549 cells, the nanodots could be readily uptaken by the cells, displaying multiple fluorescence emission wavelengths (i.e. blue, green and red). Correspondingly, Sun’s group prepared Asp derived bio-dots to diagnose brain cancer (**Figure 1.27**).⁹⁶ The Asp-dots (CD-Asp) not only exhibit tunable full-colour emission with a quantum yield of 7.5%, but also possess intrinsic selectivity and targeting affinity towards cancerous C6 glioma cells. Both in vitro and in vivo studies showed

high biodistribution of Asp-dots located to the brain tumor indicating their potential application as an excellent bio-imaging and diagnostic agent.

In addition to surface passivation, the biomolecules also supply the bio-dots with rich chemical functionalities for linking targeting moieties and/or drug molecules. For instance, Sharon and co-workers prepared a fluorescent dot from sorbitol via microwave-assisted heating.⁹⁷ The sorbitol bio-dots were first attached onto BSA surface via electrostatic attractions and then further functionalized with cancer targeting ligand, folic acids and anticancer agent, doxorubicin (dox). The resulting complex could be applied as anticancer theranostics. On the other hand, Shen's group utilised DNA from *E. coli* to synthesize fluorescent bio-dots.⁸⁸ The surface of DNA dots was grafted with functional groups such as C–OH, N–O, and N–P resulting in a negative zeta potential, enabling electrostatic dox loading. Due to the inherent fluorescence from DNA dots, the nanocomplex was able to induce cell apoptosis and at the same time allowed real-monitoring of the drug release process.

Figure 1.27 (A) TEM, (B) High-resolution TEM images of CD-Asp; (C) In vivo imaging of glioma-bearing mice at different time points after injection with CD-Asp, CD-G, and CD-A. Reproduced with permission from Ref# 96 Copyright 2015 American Chemical Society.

On the whole, biomolecules could serve as an efficient precursor in the synthesis of carbon nanomaterials such as bio-dots. Although the overall secondary or tertiary structure of the biomolecule may be altered or completely destroyed in the process, their inherent properties such as aqueous solubility, rich chemical functional groups and excellent biocompatibility are still imparted to the bio-dots. This makes them potentially useful in various biomedical applications such as imaging, diagnostic and therapeutic delivery.

1.2.3 Advantages of Bioinspired Nanomaterials

In comparison, the bioinspired synthesis of nanomaterials presents many advantages over the conventional synthetic route. For instance, the cheap and abundance nature of biomolecules reduces the manufacturing cost of the nanomaterials, while exploiting the unique recognition ability of the biomolecule ensures a facile and simple preparation method for nanomaterials. Moreover, bioinspired synthesis of nanomaterials reduces the environmental footprint as such approach does not require the use of toxic and harsh reagent in the synthesis.⁹⁹ Furthermore, the resulting nanomaterials often display unique characteristics such as rich chemical functionality, good biocompatibility, highly aqueous solubility, unusual optical features, and tunable physiochemical properties.¹⁰⁰⁻¹⁰¹ In addition, with careful selection of the biomolecule precursors, the resulting nanomaterials can be engineered with desired morphology and properties. Hence, this strategy creates a new programmable assembly of nanomaterials with multifunctional properties for specific applications.

1.2.3 Biomedical Applications of Bioinspired Nanomaterials

Biomolecule-derived nanodot, “biodot”, are carbon-based nanomaterials similar to carbon quantum dots. The biodots are a new form of zero-dimensional nanoparticles with sizes below 10 nm. They are derived mainly from biomolecules ranging from macro-biomolecules such as proteins, nucleic acids and carbohydrates to simple biomolecules building blocks including amino acids, saccharides and nucleotides, where they could serve sacrificial precursor in the synthesis, directing the formulation of the nanomaterials. The biodot displayed unique optical properties such as bright photoluminescent and photo-stability, which have made them highly attractive in the field of luminescent materials. Such feature is due to the rich chemical functionality of the biomolecule which endows the heteroatom doping within the biodot with different elements such as oxygen, nitrogen, sulphur and/or phosphorus. Consequently, this grants the biodot with unusual optical properties by creating various trapping sites with different energy levels within the biodot.¹⁰²⁻¹⁰³ Thus, this enables electronic transitions among bonding, antibonding and nonbonding orbitals (ie. $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions), allowing emission of photons with varying excitation energy, displaying bright luminescent.

Furthermore, the biodot presents highly advantageous over the conventional semiconductor quantum dots including cadmium and palladium based QDs, owing to the benign, abundant and readily available nature of the biomolecule precursor. Additionally, the biodot also exhibits unique characteristics such as facile surface modification, good aqueous solubility, excellent biocompatibility and high resistance towards photobleaching which are highly desirable for biomedical applications such as bioimaging, therapy and sensing.¹⁰⁴⁻¹⁰⁵

Figure 1.28 (a) Schematic illustration of DNA-CD synthesis. (b) LSCM images revealing the localisation of DNA-CD and drug release within the *S. cerevisiae* cells. Reproduced with permission from Ref #106. Copyright 2015 American Chemical Society.

For instance, Ding et al. demonstrated a fluorescent nanodot, DNA-CDs, prepared from hydrothermal treatment of DNA obtained from *Escherichia coli* (**Figure 1.28**). The resulting DNA-CDs were found to be fluorescence with emission wavelength 445 nm when excited by wavelength at 366 nm. Furthermore, the DNA-CDs was capable of loading anticancer drug molecule, DOX, via electrostatic interaction and used for drug delivery and bioimaging.¹⁰⁶ Similarly, Liu et al. reported fluorescent carbon dot derived from D-glucosamine hydrochloride and sodium pyrophosphate. The amino-functionalised carbon dot was observed to be positively charge and emits green fluorescence with excellent stability against different pH conditions. Coupled with negatively charged hyaluronate stabilised gold nanoparticles, the carbon dot/gold nanoparticle system which could be used to detect for hyaluronidase. The assay involved utilisation of hyaluronidase to break down the hyaluronate stabilised gold nanoparticles, restricting surface energy transfer between the carbon dot and the gold nanoparticles and thus, producing both fluorometric and colorimetric signals.¹⁰⁷

1.3 Research Objectives

As mentioned previously, bioinspired synthesis of nanomaterials has gained much attraction in recent years. They are highly advantageous in comparison to the conventional synthesis of nanomaterials. Specifically, the bioinspired approach enables a green and more environmentally synthesis as it utilises cheap and readily available biomolecular reagent while reduces the usage of harsh and toxic reagent. Furthermore, they often consist of simple and facile preparation route in fabricating nanomaterials. Most importantly, the biomolecular precursors such as amino acids could serve as building block to guide the synthesis and ultimately enabling the ability to fine-tune the properties of the nanomaterial. In addition, the bio-derived nanomaterials are often bestowed with unique properties inherited their biomolecular precursor. These properties include rich chemical functionality, good aqueous solubility and excellent biocompatibility which are extremely difficult to instill into the conventional nanomaterials. Hence, this brands the bioinspired synthesis highly versatile and programmable which cannot be easily achieved in the traditional synthetic route.

Particularly, biodot, a new class of zero-dimensional nanoparticle which are derived from biomolecule precursor, have been a rising star in the luminescent field. Besides the inherited properties, these biodots possess unusual optical features such as bright photoluminescent with high quantum yield, high photostability and long photoluminescent lifetime. Hence, these makes them which are highly suitable in the biomedical applications such as imaging, therapy and sensing. Despite the attractive benefits of these biodots, there has been a lack of extensive studies in these materials. Explicitly, the programmability of the biomolecular precursor has not been fully explored as most of the precursors were chosen randomly and arbitrary under different experimental conditions. As such, most of the reported biodots has highly restricted

application as they could not attain their desired properties such as bright photoluminescent, excellent photostability and tunable surface properties.

In this thesis, we will be exploring the potential programmability of the biomolecular precursor such as amino acids in the synthesis of biodot and further apply them for suitable biomedical application. By Nature, there are 20 natural occurring amino acids which plays an essential role as building blocks for proteins. Their structure comprises of amine and carboxyl functional group, along of a unique side chain group which are specific to each amino acid. Particularly, the unique side chain groups offer great programmability owing to the highly versatile combinations. Couple with the inexpensive and abundance nature, amino acids were designated as the biomolecular precursor for the synthesis of biodot. Through careful selection of the amino acids precursor, biodot with desired properties could be engineered and thereafter, the biodot will be applied to specific application such as imaging, therapy and sensing.

In Chapter 2, we have conducted a systematic study to unravel the material design rule of biodot synthesis from 20 naturally occurring amino acids. A structure-property relationship between the amino acids precursor and the corresponding biodot was established via comprehensive characterisation of the biodots. It was found that the amino acids with reactive side chain group, forms unique chemical bonds within the biodot which stabilising surface defects. This translate into brighter photoluminescent biodot. On the other hand, the length of the side chain group affects the final morphology of the biodot which could influence their photoluminescence properties. Among the amino acids derived biodot, Ser and Thr biodots exhibit the brightest photoluminescent with high quantum yield and excellent photostability. Interestingly, by combining Ser or Thr with another amino acid precursor, the resulting mixed biodot could inherit unique characteristics such as improved photostability and red shifting in their emission wavelength. Equipped with these exceptional optical features, the bioimaging

capability of the biodot were also investigated. It was observed that the biodots demonstrated superb biocompatibility and excellent intracellular uptake activity which are highly suitable for *in vivo* bioimaging application.

Upon understanding the material design rule of biodot synthesis using amino acids, the biodot was carefully designed and constructed, using natural amino acids, serine, and a cationic polymer, polyethylenimine to engineer a biodot with unique surface properties. Hence, in Chapter 3, we reported this unique biodot for antimicrobial application. The resulting biodot has ultrasmall size with charged neutral surface which reduces non-specific interaction with the mammalian cell membrane, promoting good biocompatibility. Moreover, the biodots were found to exhibit amphiphilic and zwitterionic-like properties owing to the unique chemical groups present on the surface of the biodot. This resulted in an effective bacteria membrane permeabilisation which prohibited resistance development within the bacteria. Additionally, the biodot could exert the rapid bactericidal effect and significant biofilm removal, improving the therapeutic efficacy and indicating its potential as antimicrobial agent.

Similarly, based on the learning from previous studies, serine was combined with biomolecular precursor, histamine to form a biodot with bright luminescent and distinctive surface properties capable of anchoring silver ions (Ag^+) and facilitating the growth of silver nanoparticles (AgNP) on the surface of the biodot. Thus, in Chapter 4, we developed a novel nanohybrid system (Bio@AgNPs) for bio-sensing application. The nanohybrid displayed distinct SPR AgNP characteristic absorption band while significantly quenching the luminescent of biodot. As such, this enabled the Bio@AgNPs hybrid to act as a sensing probe, detecting glucose and cholesterol via a dual mode detection including fluorometric and colorimetric sensing. The sensing of glucose and cholesterol could achieve low detection limits

with high sensitivity and selectively, while the practicability of the detection was also evaluated using artificial urine and human plasma sample.

In summary, we have explored the potential programmability of amino acids for the synthesis of biodot, the biodot can be carefully engineered to achieve unique properties such as bright photoluminescent, high photostability, and tunable surface properties to include amphiphilic and zwitterionic-like characteristics or even serve as a template for the synthesis of hybrid material. These features have demonstrated to be highly suitable for application such as imaging, therapy and sensing. Future development could include detailed study of the interaction of the biomolecular precursor and the formation of the biodot. These would enhance understanding towards more responsive and multifunctional nanostructure for complex applications such as theranostic, deep tissue diagnosis and nanorobots for surgery.

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Chapter 2

Uncovering the Design Principle of Amino acid-Derived Photoluminescent Bio-dots with Tailored-made Structure-Properties and Application for Cellular Bioimaging

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Abstract

Natural amino acids possess side chains with different functional groups (R groups), which make them excellent precursors for programmable synthesis of biomolecule-derived nanodots (bio-dots) with desired properties. Herein, we reported the first systematic study to uncover the material design rules of bio-dots synthesis from 20 natural α -amino acids via a green hydrothermal approach. The as-synthesized amino acids bio-dots (AA-dots) are comprehensively characterised to establish a structure-properties relationship between the amino acid precursors and the corresponding photoluminescent properties of AA-dots. It was found that the amino acids with reactive R groups, including amine, hydroxyl and carboxyl functional groups form unique C-O-C/C-OH and N-H bonds in the AA-dots which stabilise the surface defects, giving rise to brightly luminescent AA-dots. Furthermore, the AA-dots were found to be amorphous and the length of the R group was observed to affect the final morphology (e.g., disc-like nanostructure, nanowire or nanomesh) of the AA-dots which in turn influence their photoluminescent properties. It is noteworthy to highlight that the hydroxyl-containing amino acids, i.e., Ser and Thr, form the brightest AA-dots with quantum yield of 30.44%, and possess high photostability with negligible photobleaching upon continuous UV exposure for 3 h. Intriguingly, by selective mixing of Ser or Thr with another amino acid precursor, the resulting mixed AA-dots could inherit unique properties such as improved photostability and significant red-shift in their emission wavelength, producing enhanced green and red fluorescent intensity. Moreover, our cellular studies demonstrate that the as-synthesized AA-dots display outstanding biocompatibility and excellent intracellular uptake, which are highly desirable for imaging applications. We envision that the material design rules discovered in this study will be broadly applicable for the rational selection of amino acids precursors in the tailored synthesis of bio-dots.

2.1 Introduction

Fluorescent imaging has emerged as an important technology for real-time monitoring of biological processes in living cells. The current imaging probes such as organic fluorophore and semiconductor quantum dots, display bright photoluminescence suitable for *in vitro* imaging.¹⁻² Nonetheless, the poor photostability of organic fluorophores hinders their use in long term or real-time tracking and the inherent high toxicity of semiconductor quantum dots severely limit their biomedical applications.³⁻⁴ This necessitates the development of a new environmental-friendly synthetic approach to synthesize imaging probes with bright photoluminescence, excellent photostability and biocompatibility.

Biomolecules, which possess diverse molecular structures and chemical functionalities, are essential components that govern sophisticated biological processes in living organisms. Their inherent bio-recognition capabilities enable selective binding towards target molecules and also direct the formation of hierarchical biomaterials with superior qualities. Exploiting their unique properties, various biomolecules have been applied to guide the programmable synthesis or assembly of nanomaterials, leading to the emergence of “bioinspired synthesis”.⁵⁻⁶ Bioinspired approaches enable green synthesis of a variety of nanostructured materials from different designer building blocks such as amino acids or nucleic acids to allow the fine tuning of their extraordinary properties that are not achievable *via* conventional synthetic routes.

Bio-dots represent a new class of zero-dimensional carbon nanomaterials derive from biomolecular precursors with photoluminescent properties, whose size commonly ranges between 1 nm to 10 nm.^{5, 7} This novel fluorophore possesses unique properties such as bright photoluminescence, superb aqueous solubility, excellent chemical stability and inertness.⁸⁻¹⁰ Furthermore, the utilisation of naturally occurring biomolecules ensures the biocompatibility of the resulting bio-dots. These outstanding features brand the bio-dots as a promising

nanoprobe for biomedical applications ranging from diagnostics to therapeutic deliveries.¹¹⁻¹⁴ Biomolecules like nucleic acids and proteins provide a natural doping of elements such as nitrogen, oxygen, phosphorous and sulphur, endowing bio-dots with unusual optical properties. For instance, bio-dots derived from bovine serum albumin (BSA) exhibited excellent biocompatibility and bright blue fluorescence, which were applied for imaging, sensing and drug delivery.¹⁵⁻¹⁷ In contrast to the macrobiomolecules such as proteins, their basic building blocks, amino acids are expected to offer much greater programmability in synthesis due to their highly versatile combinations. Previously, Zeng *et al.* reported the use of serine and cysteine to synthesize a N, S co-doped bio-dots with orange photoluminescence.¹⁸ Histidine was also used to prepare bio-dots with enhanced chemiluminescence.¹⁹ In addition, isoleucine²⁰, glycine²¹ and glutamic acids²² have also been exploited as carbon sources to synthesize bio-dots. Although a few selected amino acids have been studied, there is a lack of a comprehensive understanding of the relationship between the R groups of amino acid precursor and the photoluminescent properties of the as-synthesized bio-dots, mainly because the amino acid precursors were chosen arbitrarily under different experimental conditions previously.

Herein, we conducted the first systematic study to unravel the material design rule of bio-dots synthesis from the 20 different natural α -amino acids and their mixture of the best combination. The surface compositions and structural properties of the amino acids-derived bio-dots (AA-dots) were thoroughly characterised and compared to establish the correlation between the amino acid precursor and the resultant photoluminescent properties of the AA-dots. Several critical material-by-design rules were revealed through this comprehensive investigation. It was uncovered that the photoluminescent properties of the AA-dots were determined by the specific side chain functional groups (R group) of amino acid precursors. In addition, the carbon chain length of R group controls the final morphology of the AA-dots and consequently their photoluminescent properties. To further confirm these design rules, a set of

rationally designed mixed AA-dots with enhanced photo-stability, red-shifted emission, excellent biocompatibility and cell uptake were successfully synthesized.

2.2 Materials and Methods

Materials. 20 amino acids, Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich.

Instruments. The AA-dots were synthesized using Memmert UF55 Universal Oven. The photoluminescence (PL) images of AA-dots were taken under UV 365 nm irradiation using a High Performance 2UV™ Transilluminator (25 W). The UV-vis absorption spectrometry was conducted with Shimadzu UV-2450 UV-Visible Spectrophotometer. The PL spectrum was obtained using Tecan Infinition M200 Multimode Microplate Reader. The Fourier Transform Infra-red (FTIR) spectroscopic measurement was carried out using PerkinElmer Fourier transform infrared spectrometer. The X-ray Photoelectron Spectroscopy (XPS) was measured from Theta Probe X-ray Photoelectron Spectroscopy. The crystallinity of the AA-dots was investigated by X-ray Diffraction using Bruker D8 Advance X-ray Diffractometer. The Raman spectrum was recorded using Thermo Scientific™ DXR™ 2 Raman Microscope with 780 nm laser excitation. The high-resolution transmission electron microscope (HRTEM) and selected area electron diffraction (SAED) images of AA-dots were captured using Philips CM300 FEGTEM. The AFM images were recorded using ICON-PKG Atomic Force Microscopy and the height profiles were analysed by Gwyddion software.

Synthesis of Amino Acids Bio-dots. The amino acids were dissolved in 35 mL of deionized H₂O to achieve a final concentration of 0.15 M and then transferred into a Teflon-lined

autoclave to be heated at 180 °C for 12 hours. Upon completion of hydrothermal reaction, the solutions were centrifuged at 12, 000 rpm for 30 mins to remove large particles and the supernatant was filtered using 0.22 µm syringe filter and then dialyzed to obtain the final product.

Quantum Yield (QY) Measurement and Photo-stability study. The QY of the AA-dots was calculated by comparing the integrated photoluminescence (PL) intensity against the absorbance values of the samples when excited at 360 nm, using quinine sulfate as a standard reference.²³ The absorption of the samples was kept below 0.05 to prevent re-absorption effect. The QY was determined using the following equation:

$$\varphi_s = \varphi_R \frac{I_s}{I_R} \frac{A_R}{A_s} \left(\frac{n_s}{n_R}\right)^2 \quad (1)$$

where φ is the quantum yield, I represents the integrated PL intensity, A refers absorbance of the samples and n refers to the refractive index of the solvent. The subscript R denotes the reference fluorophore of known QY and the subscript s denotes the bio-dots sample. The quinine sulfate (QY = 54%) was dissolved in 0.1 M H₂SO₄ (refractive index n of 1.33) and the AA-dots were dissolved in distilled water (refractive index n of 1.33).

For photostability study, 1 mL of 5 mg mL⁻¹ AA-dots were prepared and exposed to continuous UV irradiation at wavelength 365 nm for 3 h. Controls were prepared similarly without UV treatment. Thereafter, the PL intensity was measured and calculated using the following equation:

$$\text{Photostability (\%)} = \frac{\text{PL intensity after UV exposure}}{\text{PL intensity before UV exposure}} \times 100 \quad (2)$$

Cytotoxicity Assay. HeLa cells were incubated in DMEM medium (High glucose, Invitrogen) with 10% fetal bovine serum and 1% penicillin-streptomycin (37 °C, 5% CO₂). The

viability of cells was evaluated using MTT assay. Briefly, HeLa cells were seeded into 96-well plates at a density of 1×10^4 per well in 200 μL of media for 24 h. The cells were then incubated with various concentrations of AA-dots for 24 h. Then, MTT solution (20 μL , 5 mg/mL) was added to each well for 4 h. Thereafter, the MTT solution was removed and the precipitated violet crystals were dissolved in 200 μL of DMSO. The absorbance at 570 nm was measured using a Tecan microplate reader.

Cell imaging. To investigate cell imaging capabilities, the cells were incubated with AA-dots samples (1 mg/mL). After 4 h incubation at 37 °C, the cells were washed three times with PBS buffer and the fluorescence images were acquired by confocal laser scanning microscopy (CLSM) (Fluoview 1000, Olympus, Japan) under 405, 488 and 564 nm excitation.

2.3 Results and Discussion

The 20 natural amino acids have a general molecular structure containing an amine and a carboxylic functional group, along with a specific R group. Each amino acid contains elements such as N, O and S (for Cys and Met) which would provide heteroatom doping on the surface of the AA-dots. This could potentially endow the AA-dots with trapping sites accompanied by different series of energy levels, allowing electronic transition among bonding (σ and π), antibonding (σ^* and π^*) and non-bonding (n) orbitals. As a result, the AA-dots are likely to emit photons with different excitation energy, thus providing them with unusual optical properties. We postulate that the R group is the main determining factor for the final morphologies and properties of AA-dots. Through in-depth multidimensional investigation, this study will be able to provide a set of materials-by-design rules for the bioinspired synthesis of AA-dots.

Figure 2.1. Photographs showing AA-dots under (a) white light and (b) UV at 365 nm; Left (Polar amino acid derived bio-dots): Arg-dot, His-dots, Lys-dots, Asp-dots, Glu-dots, Ser-dots, Thr-dots, Asn-dots, Gln-dots. Middle (Special amino acid derived bio-dots): Cys-dots, Gly-dots, Pro-dots. Right (Non-polar amino acid derived bio-dots): Ala-dots, Val-dots, Ile-dots, Leu-dots, Met-dots, Phe-dots, Tyr-dots, Trp-dots; (c) Photoluminescence spectra of AA-dots.

Figure 2.2. Photoluminescent excitation and emission spectra of (a) charged polar AA-dots, (b) neutral polar AA-dots and (c) non-polar AA-dots.

2.3.1 Structure-Properties Relationship and Formation Mechanism of Photoluminescent Bio-dots from Single Amino Acid Precursor. The photoluminescent properties of these heteroatom doped AA-dots were first investigated by measuring their respective photoluminescence spectra. It was observed that most of the AA-dots emit bright blue fluorescence with maximum emission at 450 nm when excited at 360 nm (**Figure 2.1 and 2.2**). Furthermore, it was revealed that the AA-dots prepared from polar amino acids generally exhibited higher photoluminescent intensities, as compared to their counterparts synthesized from non-polar precursors (**Figure 2.3a**). It was found that the six brightest samples generally exhibited quantum yield (QY) greater than 15% with Ser-dot displaying the highest QY of 30.44%. In stark contrast, the non-polar AA-dots displayed quantum yield less than 5%.

Figure 2.3b shows the proposed formation of the as-synthesized AA-dots in this study to better understand the differences in the QY among different AA-dots. As the formation mechanism of the carbon dots are still being investigated to date, many have postulated that the mechanism involves polymerisation, aromatization and nucleation.²⁴⁻²⁷ Likewise, it is likely that the amino acid molecules first polymerise to form a long chain polymer via amidation between the carboxyl group of one amino acid and the amine group of another amino acid. Subsequently, the unstable polymer would aromatize into a unique conformation consisting of layers of sp^2 carbon network systems. This results in the formation of carbon nucleus that facilitate the carbonization process, promoting the formulation of the AA-dots structure. Although the exact origin of photoluminescence of AA-dots remains unclear, it is most likely to correlate with the surface defective sites of the AA-dots (i.e., π -states of the sp^2 sites and sp^3 hybridised network).²⁸⁻²⁹ Upon absorbing near UV-vis light, the recombination of the electron-hole pairs in the strongly localised π and π^* electronic levels of the sp^2 sites and σ and σ^* states of the sp^3 matrix, allows the AA-dots to display strong photoluminescent emission in the visible region.³⁰⁻³¹ As such, the differences in the photoluminescent intensities between

the AA-dots prepared from polar and non-polar amino acids could be due to the inability of non-polar ones to form a photoluminescent carbon center. The AA-dots derived from non-polar amino acids could possess poor surface emissive sites of the AA-dots owing to their less reactive R groups, resulting in weak photoluminescent intensity. Unlike the non-polar amino acids, polar amino acids possess reactive R groups with amine, carboxyl and hydroxyl functional groups to render a better passivation, thus stabilising the surface defects on the AA-dots. Stable surface defects would mediate a more effective radiative recombination of surface confined electrons and holes, thus leading to enhanced photoluminescent emission.³²

Figure 2.3. (a) Quantum yield of each AA-dots and their respective amino acids side chain group. (b) Proposed mechanism illustrating the hydrothermal carbonization of amino acids precursor leading to the formation of AA-dots of different photoluminescent properties and

their chemical structures. (c) Summary table identifying important functional groups within AA-dots from the deconvolution of XPS measurements.

Figure 2.4. (a, b) C1s, (c, d) N1s and (e, f) O1s deconvolution of XPS spectrum of Ser-dots (a, c, e) and Thr-dots (b, d, f), respectively.

Figure 2.5. (a, b) C1s, (c, d) N1s and (e, f) O1s deconvolution of XPS spectrum of Leu-dots (a, c, e) and Ile-dots (b, d, f), respectively.

Thorough characterizations were performed to support and validate the formation mechanism of AA-dots proposed above. Firstly, the surface functional groups of the AA-dots were examined using X-ray Photoelectron Spectroscopy (XPS). The presence of C=O bond in all the AA-dots justified the initial polymerisation *via* amidation. Detailed deconvolution analysis of C1s, N1s and O1s in the AA-dots showed clear differences in their N- and O-related bonds with representative deconvolution for polar Ser-dot and Thr-dot (**Figure 2.4**) versus non-polar Leu-dot and Ile-dot (**Figure 2.5**). Most importantly, only the six brightest AA-dots (i.e., Arg-dot, Asn-dot, Asp-dot, His-dot, Ser-dot and Thr-dot) exhibit both C-OH/C-O-C bonds (285.9 - 286.6 eV) and N-H bonds (401 - 401.5 eV) whereas samples synthesized using non-polar precursors such as Ala, Gly, Leu, Ile, Phe, Pro and Val do not display such chemical bonds (**Figure 2.3b**). The XPS data were further supported by the Fourier Transform Infrared Spectroscopy (FTIR) results. Typically, the absorption band around 1630 cm⁻¹ represents the amide C=O stretch which agrees well with the XPS data. Specifically, the FTIR spectra of Ser-dot and Thr-dot show absorption peaks at 3400 - 3200 cm⁻¹ and 1150 - 1050 cm⁻¹, which signifies -OH and C-O stretches, respectively (**Figure 2.6a**). In comparison, these absorption peaks are almost negligible for Pro-dot and Val-dot. Trp-dot and Tyr-dot display the aromatic C=C stretch at around 1599 cm⁻¹, which indicate a conjugated π -system (**Figure 2.6b**). Furthermore, these findings were reinforced by the ¹³C Nuclear Magnetic Resonance (NMR) spectra (**Figure 2.7**). The chemical shift of 170 - 180 ppm present in all AA-dots indicates the presence of CO-NH bonds which reconfirms the successful polymerisation of amino acids *via* amidation. More interestingly, the six brightest AA-dots exhibit two peaks within 40 - 60 ppm indicating the presence of both C-O and C-N bonds, while poorly photoluminescent AA-dots (e.g., Ala-dot, Gly-dot) exhibit only a single peak in this region, which is attributed to the C-N bond only. The difference in bonding indicates the extent of surface passivation within the AA-dots which strengthens the stabilising effect on the surface defects.³³

Figure 2.6. (a) FTIR Spectrum of polar and non-polar AA-dots, i.e., Ser-dots and Val-dots respectively. (b) FTIR Spectrum of aromatic AA-dots, i.e., Trp-dots and Tyr-dots

Figure 2.7. ^{13}C NMR Spectrum of polar AA-dots (a) Ser-dots and (b) Thr-dots, and non-polar AA-dots (c) Ala-dots and (d) Glu-dots.

In addition, the UV-vis absorption spectra of AA-dots (**Figure 2.8**) revealed that the AA-dots prepared from polar amino acids displayed absorption peaks at 240 nm and 270 nm, which could be attributed to $\pi-\pi^*$ and $n-\pi^*$ transition, respectively.³⁴ Whereas, the AA-dots derived from non-polar amino acids exhibited only one blue-shifted absorption peak of $\pi-\pi^*$ transition at 195 nm. This phenomenon could also help to explain the differences in the PL intensity of AA-dots. According to Yan *et al.*, presence of C-O-C and C-OH functional groups could promote surface distortion and generate different energy gaps.³⁵ These new energy gaps could locate between $\pi-\pi^*$ level, creating a number of $n-\pi^*$ transition possibilities.³⁶ Since the C-O-C and C-OH functional groups are more prominent in the AA-dots prepared from polar amino acids, various types of radiative recombination could occur, leading to the possibility of excitation dependent emission in the visible region. Conversely, the lack of C-O-C and C-OH functional groups in the non-polar group could limit the number of pathways for $\pi-\pi^*$ transition, leading to the blue-shift of excitation and emission wavelengths (330/400nm).

Figure 2.8. (a) UV-vis absorption spectra of polar AA-dots: Arg-dots, Asp-dots, Asn-dots, His-dots, Ser-dots and Thr-dots. (b) UV-vis absorption spectrum of non-polar AA-dots: Ala-dots, Gly-dots, Leu-dots, Ile-dots and Val-dots.

Figure 2.9. TEM images of (a) Asp-dot vs (b) Glu-dot, (c) Asn-dot vs (d) Gln-dot and (e) Ser-dot vs (f) Thr-dot. Inset (top right): Photo images of AA-dots under white light (left) and UV365 nm (right) excitation. Inset (top left): SAED images for Figure 2.9 (a, c, e & f).

As discussed earlier, the formation of the AA-dots possibly results from the aromatization of unstable long chain polymers formed from individual amino acids (**Figure 2.3b**). The different chain length of the R group may play a significant role in determining the final morphology (i.e., size and shape) of the as-synthesized AA-dots. Thus, the morphology of the as-synthesized AA-dots with similar R groups, i.e., Asp-dot vs. Glu-dot, Asn-dot vs. Gln-dot, Ser-dot vs. Thr-dot., was first studied by using Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM). It was observed that the brighter AA-dots such as Asp-dot and Asn-dot are highly monodispersed (**Figure 2.9a-b**) in size with an average diameter of 2.62 ± 0.42 nm and 4.56 ± 0.89 (**Figure 2.10a-b**). Interestingly, the individual height profile of these bright photoluminescent AA-dots as obtained by AFM (**Figure 2.11**) were found to be

smaller than their diameter, exhibiting average height of only 0.65 ± 0.36 nm (**Figure 2.11a**) and 1.13 ± 0.50 nm (**Figure 2.11b**), respectively.

Figure 2.10. Statistical size distribution of (a) Asp-dot, (b) Asn-dot, (c) Ser-dot and (d) Thr-dot measured from at least 100 dots (Insets show their respective TEM images).

Considering the diameters measured from their TEM images previously (**Figure 2.10**), these nanosized AA-dots actually exhibit distinct disc-like structures. On the other hand, their counterparts, Glu-dot and Gln-dot exhibited a large aggregated structure with an overall length of $> 1 \mu\text{m}$ (**Figure 2.9d-e**). It is postulated that the additional carbon in the side chain R group of Glu and Gln could have reduced reactivity, thus deterring the aromatization reaction and hindering the carbonization process to form a well-carbonized sp^2 network structure of AA-dots (as suggested in **Figure 2.3b**). As a result, an aggregated nanostructure was formed, leading to the poor photoluminescent properties as observed. Similarly, Ser-dot also displayed a nanodisc-like structure (**Figure 2.9c**) with a diameter of 19.04 ± 3.27 nm (**Figure 2.10c**) with

a much smaller average height of only 2.2 ± 0.84 nm (**Figure 2.11c**). Different from Ser-dot, the Thr-dot was found to be slightly aggregated (**Figure 2.9f**), having a diameter of 27.66 ± 1.76 nm (**Figure 2.10d**) and average height of 3.11 ± 0.68 nm (**Figure 2.11d**).

Figure 2.11. AFM images and their respective height profiles of (a) Asp-dot, (b) Asn-dot, (c) Ser-dot and (d) Thr-dot.

Although Thr has an additional methyl group in its R group compared to Ser, it is worthy to note that the hydroxyl groups within both amino acids remain reactive that could undergo

reaction, leading to the formation of AA-dots with bright photoluminescence. This indicates a successful carbonization process, leading to the formation of highly photoluminescent AA-dots. Furthermore, the crystallinity of the AA-dots was investigated using X-ray Diffraction (XRD). It was found that the four brightest AA-dots, i.e. Asp-dot, Asn-dot, , Ser-dot and Thr-dot, and some of the less bright dots such as Glu-dots and Gln-dots all display amorphous characters with broad peak centred around $2\theta = 25^\circ$ (**Figure 2.12**) which is attributed to highly disordered carbon atoms.³⁷ Likewise, the Raman spectra of Asp-dot, Asn-dot, Ser-dot and Thr-dot show a distinct D-band peak at around 1375 cm^{-1} and a weak shoulder G-band peak at around 1580 cm^{-1} (**Figure 2.13**). The relative intensity ratio of the D-band and G-band (ID/IG) of Asp-dot, Asn-dot, Ser-dot and Thr-dot were determined to be 0.69, 0.74, 0.59 and 0.71 respectively, indicating a high degree of defect in the carbon structure.³⁸⁻³⁹ In addition, the HRTEM of the AA-dots did not reveal any discernible lattice. Further Selected Area Electron Diffraction (SAED) analysis of Asp-dots, Asn-dots, Ser-dots and Thr-dots also suggested an amorphous state, which agree well with the XRD findings. Hence, the crystallinity is not critical to achieve high photoluminescence for AA-dots in this study.

Figure 2.12. X-ray Diffraction pattern of Asn-dot, Asp-dot, Glu-dot, Gln-dot, Ser-dot and Thr-dot.

Figure 2.13. Raman spectrum of Asp-dot, Asn-dot, Ser-dot and Thr-dot, and their respective I_D/I_G ratio.

2.3.2 Rational Design of Photoluminescent Bio-dots from Mixed Amino Acids Precursors with Enhanced Photostability and Tunable Color Emission Properties. The fluorescence property of a material is governed by its QY and photostability, also known as resistance towards photo-degradation. We found that Ser-dot and Thr-dot exhibit higher photostability (i.e., > 90% intensity conserved) while Arg-dot, Asn-dot, Asp-dot and His-dot have poorer photostability (< 75% intensity conserved), upon constant irradiation of UV light for 3 hours (**Figure 2.14a**). The higher photostability of Ser-dot and Thr-dot could be due to their reactive hydroxyl groups which promote an extensive and rapid dehydration reaction, enabling the establishment of carbon-core state. Hence, they are less susceptible towards the high oxidation potential from OH radicals generated by UV exposure.⁴⁰ In contrast, Arg-dot, Asn-dot, Asp-dot and His-dot which do not possess reactive hydroxyl groups, have a slower rate of dehydration thus are unable to achieve a perfect carbon-core state. Instead, they may form a molecular state whereby the structure consists of several fluorophore molecules connected to the carbon polymer backbone.⁴¹ As such, these AA-dots are not able to withstand the strong oxidation potential, thus, destroying the molecular state resulting in a decreased in the PL intensity upon UV exposure.⁴²

Figure 2.14. (a) Photostability of AA-dots after continuous UV irradiation for 3 h. Comparison of (b) green, (c) red fluorescence emissions of mixed AA-dots produced from single amino acids ($x = \text{Arg, His, Asp, Asn, Ser, Thr}$), and their selective mixture ($\text{Ser}+x$ and $\text{Thr}+x$).

In order to further validate this hypothesis, a series of mixed AA-dots were synthesized by mixing either Ser or Thr with one of Arg, Asn, Asp and His. Upon pyrolysis, the mixed AA-dots, Ser+Arg, Ser+Asn, Ser+His, Thr+Arg, Thr+Asn and Thr+Asp were obtained. All mixed AA-dots displayed outstanding photostability (i.e. > 90% intensity conserved). The improved photostability confirmed our postulation that the introduction of reactive hydroxyl group by Ser and Thr could indeed accelerate the dehydration reaction and essentially facilitate the formation of stable carbon-core state. Besides, the incorporation of Ser and Thr also promotes the red-shifting of the emission wavelength (**Figure 2.14b-c**). This enables the AA-dots to emit green and red fluorescence with enhanced intensity. The red-shifting is possibly due to the increase in new energy gaps with the introduction of C-OH/C-O-C bonds.⁴³⁻⁴⁴ As a result, this allows different forms of radiative recombination, producing fluorescence of various wavelengths.

2.3.3 Study of Biocompatibility and Multicolour Fluorescence Imaging Capabilities of Cell Mixed AA-dots. Upon establishing the structure-property relationship of the AA-dots, we further evaluated their biocompatibility and cellular imaging capabilities for multicolour bioimaging application based on the unique excitation dependent photoluminescence properties of AA-dots as discussed previously. The cytotoxicity of Arg-dot, Asn-dot, Asp-dot, His-dot, Ser-dot and Thr-dot were first studied with the MTT cell proliferation assay using HeLa cells as the model cell line. It was observed that these AA-dots showed good cell viability (i.e. > 90%) after 24 h incubation, even at a high concentration of 1.5 mg mL^{-1} (**Figure 2.15**). This could be due to the utilization of both the natural amino acid precursors and green

hydrothermal synthesis without the addition of any toxic solvents or harsh treatment processes, giving rise to AA-dots with high biocompatibility. Subsequently, the cell imaging ability of the AA-dots was investigated by Confocal Laser Scanning Microscopy (CLSM). Both Ser-dot and Thr-dot treated HeLa cells displayed bright fluorescence in the entire cell including cytoplasm and nucleus, which indicates the successful uptake of the AA-dots (**Figure 2.16a-b**). On the other hand, Arg-dot, His-dot, Asp-dot and Asn-dot treated cells showed much weaker fluorescence, suggesting their poor bio-imaging ability which could be partially due to their lower QYs (**Figure 2.16c-f**). Interestingly, it was observed that Thr-dot stained cells exhibited green fluorescence which corresponds to their excitation dependent PL emission characteristics. It is noteworthy to mention that by co-incubating Ser and Thr AA-dots in Hela cells with LysoTracker Red, it was found from the overlay images that both AA-dots were capable of lysosomal escape (**Figure 2.17**) which indicates their potential as efficient nanocarriers in therapeutic delivery in addition to bioimaging.

Figure 2.15. MTT cytotoxicity test showing at least 90% cell viability after 1.5 mg mL⁻¹ of AA-dots were incubated with HeLa cells for 24 h.

Figure 2.16. CLSM images of HeLa cells stained with (a) Ser-dots, (b) Thr-dots, (c) Arg-dots, (d) His-dots, (e) Asp-dots, (f) Asn-dots at 405 (left), 488 nm (middle) or 564 nm (right) laser excitation (Scale Bar = 100 μm).

Figure 2.17. Co-incubating the HeLa cells with (a, b) Ser-dots, (c-f) Thr-dots and LysoTracker red reveals that these dots are capable of lysosomal escape (Scale Bar = 20 μm).

Ser+x

x = Arg

His

Asp

Asn

Thr+x

Arg

His

Asp

Asn

Ser-Thr

Figure 2.18. Fluorescence images of HeLa cells stained with mixed AA-dots (a) Ser+Arg, (b) Ser+His, (c) Ser+Asp, (d) Ser+Asn, (e) Thr+Arg, (f) Thr+His, (g) Thr+Asp, (h) Thr+Asn and (i) Ser+Thr dots under 405 nm (top), 488 nm (middle) or 564 nm (bottom) laser excitation (Scale Bar = 20 μ m).

With the prominent red-shifting of emission wavelength for both Ser and Thr mixed AA-dots, the cell imaging capabilities of these mixed AA-dots were further assessed. The green and red fluorescence staining were observed for incubating HeLa cells with Ser-mixed AA-dots (**Figure 2.18a-d**) while Thr-mixed AA-dots displayed negligible intracellular green and red fluorescence staining (**Figure 2.18e-i**). The extra carbon chain on Thr could have altered the surface of Thr-mixed AA-dots, reducing their intracellular uptake and hence poorer fluorescence labelling of the cells. On the other hand, the surface of Ser-mixed AA-dots may contain certain unique functionalities, which enhance the cellular uptake and consequently provide excellent fluorescence cell imaging. Taking advantage of the bright photoluminescence and low toxicity, the photostability of selected AA-dots with high brightness such as Asn-dot, Ser-dot and Ser+Asn-dot were assessed in cellular environment. Although Asn-dot exhibited a slight decrease in fluorescence intensity after 10 min of imaging experiment, both Ser-dot and Ser-Asn-dot displayed bright and stable fluorescence signals even after 10 min of continuous observation (**Figure 2.19**). These findings agree with the in vitro photostability results, which suggest the high potential of Ser-dot and Ser mixed AA-dots for long-term cellular imaging applications.

Figure 2.19. Evolution of photoluminescent signals of HeLa cells stained with (a) Asn-dot, (b) Ser-dot and (c) Ser+Asn-dot.

2.4 Conclusion

In summary, the PL properties for amino acids derived bio-dots (AA-dots) are primarily governed by the unique R groups of the precursor molecules. The presence of reactive R groups such as amine, hydroxyl and carboxyl R groups could lead to the formation of unique C-O-C/C-OH and N-H bonds, which subsequently improve the stability of surface defects within AA-dots, thus enhancing their PL intensity and increasing QY. In addition, the length of the carbon chain in the R groups plays a critical role in determining the final morphologies of the AA-dots, which in turn influences their PL properties. An additional carbon in the R group could result in an incomplete carbonization which consequently produces AA-dots with nanorod, nanowire or nanomesh unusual structures and also poor PL characteristics. Moreover, it was observed that the amino acids with reactive hydroxyl groups such as Ser and Thr could promote the dehydration process, facilitating the carbonization process, which improves the photostability of the AA-dots. In general, both Ser-dot and Thr-dot displayed excellent photostability, high quantum yield, biocompatibility and good intracellular uptake. By combining Ser and Thr with four other selected amino acids, the photostability of the mixed AA-dots improves with a clear red-shift in PL emission. This study unravels a unique set of the material-by-design rules to synthesize AA-dots. The finding revealed in this study could serve as good guidelines which will provide important insights towards the bioinspired synthesis of bio-dots using different types of programmable biomolecular precursors to achieve customizable optical and biological features.

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Chapter 3

Bioinspired Antimicrobial Nanodots (Ser-PEI dot) with Amphiphilic and Zwitterionic-like Characteristics for Combating Multi-Drug Resistant Bacteria and Biofilm Removal

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Abstract

Inspired by the natural antimicrobial peptides, we have developed a benign antimicrobial nanodot (BAM dot) via a simple one-step hydrothermal synthesis. The BAM dot possesses both the unique properties of nanoparticles and biomaterials, leading to excellent antibacterial properties with high therapeutic index. Specifically, this BAM dot has ultra-small size (3.83 ± 0.73 nm) with charged neutral surface. They also exhibited amphiphilic and zwitterionic-like properties, which enabled effective bacteria membrane permeabilization, leading to rapid bactericidal effect and significant biofilm removal. This study opens up new opportunity to design effective bioinspired nanomaterials for combating superbugs against drug resistance and their related applications.

3.1 Introduction

Bacterial infection has been a concerning issue that troubles the healthcare industry owing to its ability to spread and progress rapidly.¹ Recently, this has been made worse by the emergence of multi-drug resistance (MDR) bacteria.²⁻³ The root of this emergence is mainly due to the prolonged misuse of antibiotics. This allows the bacterial genes to adapt and mutate, gradually developing drug resistance.⁴ As a result, this renders many conventional antibiotics ineffective.⁵⁻⁶ In addition to MDR bacteria, the bacterial biofilms consists of a network of bacteria cells surrounded with a matrix of extracellular components, rendering it more difficult to remove than its planktonic counterparts.⁷⁻⁸ These biofilms are often highly resistant towards conventional antibiotics and emerge in clinical implants causing various types of chronic inflammations.⁹⁻¹⁰

Recently, discoveries of human antimicrobial peptides (AMP) such as Human Cathelicidin LL-37 and Histatin 5 have been receiving a lot of attention for antibacterial application owing to their unique antibacterial mechanism.¹¹⁻¹² Unlike conventional antibiotics, most of the natural AMPs exert antibacterial effect by disrupting the bacteria cell membrane and damaging the bacteria morphology. However, due to the difficulty in extracting and purifying the natural AMPs, many have used biomimetic approaches to create synthetic AMPs.¹³⁻¹⁴ These synthetic AMPs were often designed to have cationic and amphiphilic characteristics to achieve similar antibacterial functions.¹⁵⁻¹⁶ Nonetheless, most synthetic AMPs suffered from many setbacks such as high cytotoxicity resulting from their cationic nature, highly labile to proteases leading to short half-lives, and high manufacturing costs, which largely restricted their applications. On the other hand, nanotechnology offers distinctive features such as tunable particle size, large surface-to-volume ratio and facile surface engineering, which could potentially overcome these limitations.¹⁷⁻¹⁸ Through nanoscale delivery, it could positively alter the biodistribution

and improve interaction with the bacteria, achieving better efficacy in antibacterial therapy.¹⁹⁻

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Herein, inspired by the natural AMPs and coupled with nanotechnology, a unique bio-inspired antimicrobial nanodot (BAM dot), derived from a biomolecule precursor, i.e., serine and a cationic polymer - polyethylenimine (PEI, molecular weight = 1800 Da), was constructed via facile one-step hydrothermal synthesis. The BAM dot was observed to have ultrasmall size (3.83 ± 0.73 nm), while having zwitterionic (0.515 ± 0.089 mV) and amphiphilic character. Endowed these unique properties, the BAM dot demonstrated superior antibacterial activity and rapid bactericidal rate against a range of bacteria, including both gram-positive, gram-negative and MDR bacteria. Critically, the BAM dot displayed an excellent biocompatibility towards mammalian cells, NIH/3T3 Fibroblast cells, providing an excellent therapeutic index. The antibacterial mechanism was found to be via membrane lysis involving permeabilizing cell membrane and leakage of cytoplasmic content, leading to cell death. This prevented the bacteria from developing any resistance towards the BAM dot. Interestingly, the BAM dot also illustrated the ability to disperse persistent biofilms, revealing itself as a potential antibacterial agent for treatment against bacterial infection.

3.2 Materials and Methods

Materials

Serine and PEI agents were purchased from Sigma-Aldrich. Bacteria, *E. coli*, ATCC 25922 strain, *S. aureus*, ATCC 6538 strain, *E. faecalis*, ATCC 29212 strain, *S. epidermidis*, ATCC 12228 strain and *P. aeruginosa*, ATCC® MP-23 BAA-2114™ were selected as representatives for gram-negative, gram-positive and multi-drug resistant bacteria respectively.

Synthesis of BAM dot

The BAM dot was prepared using one-step hydrothermal method. Briefly, 0.72 g Serine and 0.36 g PEI (branched, MW: 1800) were dissolved in 35 mL of deionized H₂O and transferred into a Teflon-lined autoclave to be heated at 180 °C for 24 hours. Upon completion of hydrothermal reaction, the solutions were centrifuged at 12 000 rpm for 30 mins to remove large particles and the supernatant was filtered using 0.22 μ m syringe filter and then dialyzed using dialysis bag (MWCO: 3 kDa) to obtain the final product.

Similarly, PEI dot was synthesized by dissolving 0.36 g PEI (branched, MW: 1800) in 35 mL of deionized H₂O and transferred into a Teflon-lined autoclave to be heated at 180 °C for 24 hours. The as-obtained PEI dot was then purified in the same way as BAM dot stated above.

Characterization of BAM dot

The BAM dot was synthesized using Memmert UF55 Universal Oven. The photoluminescence (PL) images of BAM dot were taken under UV 365 nm irradiation using a High Performance 2UV™ Transilluminator (25 W). The UV-vis absorption spectrometry was conducted with Shimadzu UV-2450 UV-Visible Spectrophotometer. The PL spectrum was obtained using Tecan Infinition M200 Multimode Microplate Reader. The Fourier Transform Infra-red (FTIR) spectroscopic measurement was carried out using PerkinElmer Fourier transform infrared spectrometer. The X-ray Photoelectron Spectroscopy (XPS) was measured from Theta Probe X-ray Photoelectron Spectroscopy. The high-resolution transmission electron microscope (HRTEM) images of amino acids bio-dot were captured using Philips CM300 FEGTEM.

Bacterial Growth and Assay

Bacteria was grown separately in Tryptic Soy (TS) media. A single colony of each strain was lifted from the Tryptic agar plates and inoculated in 10 mL TS media. The cultures were grown at 37 °C with shaking at 150 rpm until the absorbance at 600 nm (OD₆₀₀) reached 1.0 (optical path length: 1.0 cm). The cell mixture was then diluted to respective OD which gives 1×10^6 cfu mL⁻¹.

Determination of Minimum Inhibition Concentration (MIC) of BAM dot

MICs of BAM dot were determined via broth microdilution methods.²¹ Typically, BAM dot with concentration ranging from 500 to 62.5 µg mL⁻¹ were prepared using PBS buffer pH 7.24. Overnight cultured gram-negative and gram-positive bacteria, *E. Coli* and *S. Aureus*, were diluted using tryptic soy broth respectively to achieve 1×10^6 cfu mL⁻¹. 100 µL as-prepared BAM dot was added to 100 µL bacteria suspension in a 96-wells plate and incubated at 37 °C over 24 h. The growth inhibition was determined by measuring the OD₆₀₀ using a Tecan Infinition M200 Multimode Microplate Reader. The MICs were reported as the minimum concentration capable of inhibiting > 90% growth of each bacteria strain. Broth containing untreated bacteria was used as the negative control.

Kinetics of antimicrobial activities

For time-kill studies, 100 µL BAM dot at MIC value was added to 100 µL diluted inoculum from bacteria suspension (1×10^6 cfu mL⁻¹) and incubated at 37 °C over 100 min. Untreated bacteria were used as control. The sample were pipetted out at 20 min interval and serially diluted by 200 times using TS broth. Subsequently, 50 µL diluted samples were plated onto Tryptic Agar Plate at incubated at 37 °C over 24 h. The number of viable colonies were counted and compared across different time intervals to determine the killing kinetics of BAM dot.

For time-course analysis, 100 μL bacteria suspension ($1 \times 10^6 \text{ cfu mL}^{-1}$) were added to 96-wells plate and incubated at 37 °C over 2 h. Then after, 100 μL BAM dot at MIC value was added to the bacteria suspension in a 96-wells plate and incubated at 37 °C over 24 h. The growth rate was determined by measuring OD₆₀₀ at every 60th min interval.

Antibacterial Mechanism Study

Membrane Potential Measurements

The membrane sensitive fluorophore, 3,3'-Dipropylthiadicarbocyanine iodide (DiSC3(5)), was selected to determine the membrane potential of the bacteria cells. The red-emitting fluorescence probe, DiSC3(5), localises to the cell membrane and the fluorescence is quenched in presence of polarised membrane.²²⁻²³ Briefly, the overnight cultured bacteria, *E. coli* and *S. aureus*, were harvested, washed and adjusted using PBS buffer to obtain $1 \times 10^6 \text{ cfu mL}^{-1}$. 5 μL 0.3 mM DiSC3(5) in ethanol was added to 3 mL bacteria suspension and incubated at room temperature until a stable reduction in fluorescence intensity was achieved. Subsequently, 100 μL BAM dot at MIC value was added to 100 μL bacteria and incubated at 37 °C for 1 h. The fluorescence intensity was then monitored using excitation and emission wavelength, 622 nm and 670 nm respectively.

Protein Release Analysis

100 μL bacteria suspension ($1 \times 10^6 \text{ cfu mL}^{-1}$), *E. coli* and *S. aureus*, were treated with 100 μL BAM dot at MIC value and incubated at 37 °C for 1 h. The bacteria samples were then centrifuged, supernatants removed, and the pellet was resuspended in 20 μL 1x SDS loading buffer. Untreated bacteria were employed as negative control while positive control is achieved by boiling bacteria at 100 °C for 2 h. The samples were then subjected to SDS-PAGE followed

by staining with coomassie brilliant blue solution. The photo images were captured with a digital camera.

Confocal Microscopy

The membrane integrity studies were conducted using Confocal Laser Scanning Microscopy (CLSM). The bacteria, *E. coli* and *S. aureus*, were cultured overnight, washed and adjusted to washed and adjusted using PBS buffer to obtain 1×10^6 cfu mL⁻¹. 100 μ L bacteria suspension was treated with 100 μ L BAM dot at MIC value and incubated at 37 °C for 1 h. Untreated bacteria was used as control. The mixture was then centrifuge and suspended in 100 μ L PBS buffer. The fluorescence working solution was prepared by addition of 50 μ L 5 mg fluorescein isothiocyanate (FITC) in absolute ethanol and 50 μ L 1 mg propidium iodide (PI) in PBS buffer to 1900 μ L PBS buffer. 10 μ L fluorescence working solution was then added the bacteria mixture and incubated at 37 °C for 30 min. This is followed by washing thrice with PBS buffer to reduce background signals. The fluorescence images were acquired by confocal laser scanning microscopy (CLSM) (Fluoview 1000, Olympus, Japan) under 405, 488 and 564 nm excitation.

3.3 Result and Discussion

3.3.1 Synthesis and Characterization of BAM dot

The BAM dot, derived from a biomolecule precursor, i.e., natural amino acids, serine and a cationic polymer - polyethylenimine (PEI, molecular weight = 1800 Da), was constructed via facile one-step hydrothermal synthesis for broad spectrum antibacterial resistant application and biofilm removal. In order to achieve excellent biocompatibility while maintaining effective antibacterial activity, the BAM dot was systematically engineered to possess amphiphilic and

zwitterionic-like characteristics that closely mimic that of the natural AMPs as illustrated in the schematic diagram in **Figure 3.1a**. By combining with serine with PEI, amidation could occur between carboxyl groups of serine molecule and amino groups of the PEI polymer, neutralising the high positive charges on PEI and forming a long-chained polymer. Subsequently, the unstable polymer would aromatise into a unique conformation consisting of layers of conjugated π -systems interacting via π - π stacking, forming an ultra-small-sized BAM dot. Figure 1a shows that the as-synthesized BAM dot is highly monodisperse with a mean size of 3.83 ± 0.73 nm (**Figure 3.2**). High Resolution Transmission Electron Microscope (HRTEM) image (inset of **Figure 3.1a**) also revealed a crystalline structure of BAM dot with a lattice spacing of 0.21 nm, which may be attributable to the (102) diffraction planes of graphitic (sp²) carbon (JCPDS 26-1076).

To ascertain the amphiphilic properties of the BAM dot, the surface properties of the BAM dot was characterized using X-ray Photoelectron Spectroscopy (XPS) and Fourier Transform Infrared Spectroscopy (FTIR). From the XPS analysis, it disclosed the presence of C-C/C=C bonds (284.7 eV), N-H bonds (401.9 eV), O=C bonds (531.1 eV) and C-O-C/C-OH bonds (532.8 eV) (**Figure 3.1b**).^{24, 25} Similarly, the FTIR spectrum revealed absorption bands around 1642 cm⁻¹, 3156 cm⁻¹ and 3466 cm⁻¹ which represent the presence of C=C bonds (aromatic hydrocarbon), N-H bonds (amine) and O-H bonds (carboxylic acids) respectively (**Figure 3.1c**).²⁶ In addition, ¹³C nuclear magnetic resonance (NMR) spectrum of the as-synthesized BAM dot also revealed the presence of C-N bonds (42.01 ppm), aromatic carbon (160.64 ppm), O=C-N bonds (171.08 ppm) and O=C-O bonds (174.66 ppm) (**Figure 3.3**). All of these findings suggest an amphiphilic characteristic of the BAM dot whereby layers of sp² carbon network systems within BAM dot served as a hydrophobic segment surrounded by the hydrophilic groups such as amine, carboxyl and hydroxyl groups on the surface/edge acting as the hydrophilic segment of the BAM dot.

Figure 3.1. (a) Schematic diagram and HRTEM images of bioinspired antimicrobial nanodots (BAM dot). Inset of Figure 1a shows the lattice spacing, average size and zeta potential of BAM dot. (b) Deconvolution of XPS spectrum with the C1s (left), N1s (middle) and O1s (right) scans, (c) FTIR spectrum and (d) absorbance and emission spectrum of as-synthesized BAM dot.

Figure 3.2. Size distribution of BAM dot (Right) and additional TEM images (Left) for particle counting (> 100 BAM dots).

Figure 3.3. ^{13}C NMR spectrum of the as-synthesized BAM dot.

Figure 3.4. Excitation dependent PL emission spectrum of BAM dot (inset: photo image of BAM dot under UV360 irradiation (Right) and under white light (Left)).

Besides, the BAM dot was also found to have zwitterionic-like characteristics. The zeta potential was determined to be 0.515 ± 0.089 mV at pH 7.0 which further reveals that the amino groups on PEI have been neutralised by the addition of serine molecules, resulting in near neutral charge. These characteristics signify the successful integration of the serine molecules onto the PEI polymer backbone, forming BAM dot with zwitterionic-like characteristics and rich chemical functionalities. In addition, it was found that BAM dot exhibits a distinctive absorption bands at 210 nm with a shoulder peak at 325 nm (**Figure 3.1d**) which could be attributed to the π - π^* and n - π^* transition of C=O and C=N bonds²⁷, respectively. This results further indicated the successful formation of the BAM dot. Interestingly, the BAM dot was observed to display bright photoluminescence with quantum yield of 11.4% against quinine sulfate. The BAM dot displayed an excitation-dependent emission, with maximum emission intensity at 450 nm when excited at 360 nm (**Figure 3.4**). Such phenomenon could be a result

of the recombination of electron-hole pairs in the strongly localised π and π^* electronic levels of the sp^2 sites and σ and σ^* states of the sp^3 matrix within the BAM dot, thus allowing the BAM dot to display strong emission in the visible light region.²⁸⁻²⁹

3.3.2 Antibacterial Activity of BAM dot

Figure 3.5. (a) Growth of bacteria against various concentration of BAM dot ranging from 0 to 500 $\mu\text{g mL}^{-1}$. (b) MTT cell proliferation assay of the BAM dot after 24 h incubation with NIH/3T3 Fibroblast cells. Time-kill kinetic study of the BAM dot against (c) gram-negative bacteria *E. coli* and (d) gram-positive bacteria *S. aureus*. Inset: Bactericidal effect of BAM dot treated bacteria (Right) and untreated bacteria (Left) at 40 min and 60 min treatment respectively.

Endowed with these properties, the BAM dot was applied as an antibacterial agent against representative gram-positive (*Staphylococcus aureus*), gram-negative (*Escherichia coli*) and

MDR (*Pseudomonas aeruginosa*) bacteria. It was found that the BAM dot could lead to significant inhibition of bacterial growth for all the bacteria strains tested. The MIC values were determined to be 125 $\mu\text{g mL}^{-1}$ for both *E. coli* and *S. aureus*, and 250 $\mu\text{g mL}^{-1}$ for MDR *P. aeruginosa* respectively (**Figure 3.5a**). Moreover, the BAM dot eliminated the bacteria at their respective MIC values, demonstrating effective bactericidal effect. To show the generic efficacy of BAM dot against broad spectrum of bacteria, we have also tested the other species which include *E. faecalis* and *S. epidermidis*. Similar antibacterial effect of BAM dot was observed for both the *E. faecalis* and *S. epidermidis* bacteria at 250 $\mu\text{g mL}^{-1}$ (**Figure 3.6**).

Figure 3.6. Antibacterial effect of BAM dot *E. faecalis* (left) and *S. epidermidis* (right) bacteria species. Inset: Photo images of untreated *E. faecalis* against BAM dot treated *E. faecalis* (Top) and untreated *S. epidermidis* against BAM dot treated *S. epidermidis* (Bottom).

To assess the therapeutic efficacy of the BAM dot, the biocompatibility of the BAM dot was evaluated via MTT cell proliferation assay using NIH/3T3 Fibroblast cells as the model cell line. The IC_{50} of BAM dot was determined to be $1000 \mu\text{g mL}^{-1}$ (**Figure 3.5b**) which is greater than their respective MIC values, giving an excellent therapeutic index value of 8 and 4 for both non-MDR and MDR bacteria strains respectively. To establish a comparison, control experiment was conducted using PEI dot synthesized from PEI polymer (MW: 1800 Da, branched) and their charge property, antibacterial activity and biocompatibility was compared with the BAM dot as shown in the **Figure 3.7**. In term of charge property, the PEI dot was found to be positively charged with zeta potential of $6.42 \pm 0.65 \text{ mV}$ (**Figure 3.7a**) owing to the abundance of amine group on the PEI polymer. This is in contrary to the BAM dot which exhibits a charged neutral characteristic with zeta potential of $0.52 \pm 0.09 \text{ mV}$ as the addition of serine as the precursor in the synthesis introduces functional group such as carboxyl and hydroxyl groups, which could condense the positive charge on PEI, thus enabling the formation of BAM dot with charged neutral properties.

Despite the differences in their surface charge, both BAM dot and PEI dot demonstrated excellent antibacterial effect on both *E. coli* and *S. aureus*. The PEI dot was capable of inhibiting both *E. coli* and *S. aureus* with MIC values of $250 \mu\text{g mL}^{-1}$ and $125 \mu\text{g mL}^{-1}$ respectively (**Figure 3.7b-c**). However, the PEI dot exhibit poor biocompatibility, inducing significant toxicity towards NIH/3T3 Fibroblast cells. The IC_{50} of PEI dot was determined to be $500 \mu\text{g mL}^{-1}$ (**Figure 3.7d**) which resulted in a poor therapeutic index value of 1 and 2 for *E. coli* and *S. aureus* respectively. Unlike PEI dot, BAM dot demonstrated a greater IC_{50} value of $1000 \mu\text{g mL}^{-1}$, resulted in a better therapeutic index of 12 against both *E. coli* and *S. aureus*. Such differences could likely be due to the charged nature of the nanodots. It has been reported that the positively charged species such as PEI polymer, could destabilise the membrane and inducing toxicity.²³ Similarly, it is possible that the positively charge PEI dot which could

enhance non-specific interaction with the negatively charged NIH/3T3 Fibroblast cell membrane via electrostatic interaction, causing adverse effect to the cell. Conversely, BAM dot as reported herein displayed near neutral charge which reduces the non-specific interaction with cell membrane of NIH/3T3 Fibroblast cell, hence, improving its biocompatibility.

In addition to the effective antibacterial efficacy and excellent biocompatibility, it is essential that bactericidal effect occurs rapidly to limit the circulation of bacterial endotoxins and exotoxins to avert any undesired complications such as septic shock.³⁰ Henceforth, the time-kill kinetics of the BAM dot was evaluated. Intriguingly, at MIC value, the BAM dot could achieve 98.5% eradication (1.69 log reduction) of *E. coli* within a short treatment period of 40 min (**Figure 3.5c**). On the other hand, the BAM dot eradicated 97.4% (1.58 log reduction) of *S. aureus* within 60 min treatment (**Figure 3.5d**). The slight difference could be a result of the different cell membrane structure of gram-negative and gram-positive bacteria. In comparison, the gram-negative bacteria comprise of a lipid bilayer on the outer membrane whereas the outermost layer of the gram-positive bacteria such as *S. aureus* consist of a thick peptidoglycan layer.³¹ Therefore, the thick peptidoglycan layer may hinder the interaction between the BAM dot and the bacteria, resulting in a slightly slower bactericidal rate. Combined with effective therapeutic efficacy and rapid time-time kinetics, the BAM dot signifies a promising therapeutic modality against bacterial infection.

Figure 3.7. (a) Table summarising the zeta potential, MIC values against *E. coli* and *S. aureus*, IC₅₀ and therapeutic index of both BAM dot and PEI dot (* $p < 0.05$). Percentage growth of (b) *E. coli* and (c) *S. aureus* in the presence of varying concentration of BAM dot and PEI dot respectively. (d) MTT cell proliferation assay of BAM dot and PEI dot against 3T3 Fibroblast cells.

3.3.3 Antibacterial Mechanism of BAM dot

The antibacterial mechanism was investigated further using various characterisation techniques. The membrane integrity was examined using membrane potential sensitive fluorophore, 3,3'-Dipropylthiadicarbocyanine iodide (DiSC3(5)). The red-emitting fluorescence probe, DiSC3(5), localises to the cell membrane and the fluorescence is quenched in presence of polarised membrane.²³ As shown in **Figure 3.8a**, the untreated *E. coli* and *S. aureus* displayed weak fluorescence while the BAM dot treatment enhanced the fluorescent

intensity. This indicates that the membrane potential of the BAM dot treated bacteria was dissipated, suggesting a possible interaction between BAM dot and bacteria cell membrane. Furthermore, the surface morphology of both untreated and BAM dot treated bacteria was assessed using SEM. The surface of untreated *E. coli* and *S. aureus* remained smooth and intact (**Figure 3.8b-c**) whereas the surface of both BAM dot treated *E. coli* and *S. aureus* appeared to be rough and uneven (**Figure 3.8d-e**), indicating the ability of the BAM dot in destabilising the cell membrane. The leakage of cytoplasmic contents was evaluated using SDS-PAGE. Both BAM dot treated *E. coli* and *S. aureus* demonstrated more intense bands than untreated *E. coli* and *S. aureus* (**Figure 3.8f**), indicating that significant amount of cytoplasmic proteins has been released.

Besides, the bacteria cell death was ascertained by Confocal Laser Scanning Microscopy (CLSM). Upon staining red with both membrane permeable FITC and membrane impermeable PI dyes, both BAM dot treated *E. coli* exhibited obvious green and red fluorescence compared to the absence of fluorescence in untreated *E. coli* (**Figure 3.8g**). Similar observation was recorded for *S. aureus* (**Figure 3.9**). This implies that the cell membrane was compromised by BAM dot treatment which may allow the otherwise membrane impermeable PI dyes to enter the cells, displaying bright red fluorescence.

Figure 3.8. (a) Fluorescence intensity of DiSC3(5) in presence of untreated and BAM dot treated *E. coli* and *S. aureus*. SEM images of untreated (b) *E. coli* and (c) *S. aureus*, and BAM dot treated (d) *E. coli* and (e) *S. aureus* for 1 hour at MIC. (f) SDS-PAGE showing the release of proteins by *E. coli* and *S. aureus*, with and without treatment of BAM dot. (+) boiled bacteria, (-) untreated bacteria. (g) CLSM images of fluorescently stained untreated BAM dot treated *E. coli*. Green channel: FITC dye which stains all bacteria, Red channel: PI dye which stains the dead bacteria. (h) Possible antibacterial mechanism of BAM dot via membrane permeabilization strategy.

Figure 3.9. CLSM images of fluorescently stained untreated *S. aureus*, and BAM dot treated *S. aureus*. Green channel: FITC dye which stains all bacteria, Red channel: PI dye which stains the dead bacteria.

With these findings, it provides strong evidence that the antibacterial mechanism of the BAM dot involves membrane permeabilization. It was postulated that the amphiphilic-like BAM dot could interact with the surface species such as lipopolysaccharides (LPA) or lipoteichoic acids (LTA) on the bacteria membrane via non-covalent interactions such as electrostatic interaction, hydrogen bonding or Van der Waals attraction. Subsequently, the ultra-small-sized BAM dot could accumulate within the lipid bilayer through hydrophobic interaction, resulting in membrane permeabilization (**Figure 3.8h**). This causes the leakage of cytoplasmic content, resulting in cell death.

3.3.4 Resistance Development Studies of Bacteria and Biofilm Dispersal Study

Since MDR bacteria are often developed through prolonged sub-lethal dosage of antibiotics³², it is of great interest to assess any resistance development of the bacteria against the BAM dot. Hence, the resistance development study of bacteria was conducted by serial passaging of bacteria cells in presence of sub-lethal dosage of the BAM dot and conventional antibiotics, kanamycin as a control (ie. 0.5x MIC). Indeed, after 14 days of serial passaging, there was 8-fold increase in MIC observed for kanamycin (**Figure 3.10a**). The observation is anticipated, as kanamycin inhibits the translocation during protein synthesis by interacting with the ribosome.³³ However, the sub-lethal dosages treatment selects bacterial cells with gene mutation to compensate protein synthesis inhibition, eventually leading to the development of antibiotics resistance. In contrast, the MIC value of the BAM dot remained relatively the same (**Figure 3.10b**). This is likely the results of the antibacterial mechanism of the BAM dot which involve physical disruption and permeabilization the bacteria membrane, causing a change in the surface morphology of the bacteria and rendering any resistance development within the bacteria more difficult.

Likewise, the BAM dot could potentially remove the highly resistive bacterial biofilms. Unlike planktonic cells, biofilms consisting of densely packed communities of bacterial cells within its extracellular matrix which enable it to adhere strongly to surfaces, causing it difficult to be removed. Henceforth, the biofilm removal ability of the BAM dot was evaluated against both *E. coli* and *S. aureus* biofilms. The formation and removal of the biofilm was verified using crystal violet staining (**Figure 3.11**). Upon treatment with BAM dot at 2x MIC, the *E. coli* biofilm was observed to have 46.2 ± 5.3 % reduction in biomass within 1 h and up to 68.5 ± 7.6 % biomass, within 24 h treatment (**Figure 3.10c**). Similarly, for *S. aureus* biofilm, the

BAM dot was able to achieve a 31.7 ± 4.3 % reduction in biomass within 1 h, and up to 61.3 ± 4.8 % biomass within 24 h treatment (**Figure 3.10d**).

Figure 3.10. Resistance development assay showing the change in MIC of (a) Kanamycin and (b) BAM dot after 14 days of serial passaging against MDR *P. aeruginosa*. Inset: Formation of bacteria colonies on agar plate after serial passaging. Biofilm removal study of the BAM dot against (c) *E. coli* and (d) *S. aureus* at 2x MIC. Inset: Photo images of untreated and BAM dot treated bacteria stained with crystal violet.

Figure 3.11. Formation of biofilm by bacteria *E. coli* and *S. aureus* as evidenced by crystal violet staining.

3.4 Conclusion

In summary, a unique bioinspired antimicrobial nanodot, BAM dot, has been carefully designed to achieve both effective antibacterial properties and excellent biocompatibility. Owing to the amphiphilic and zwitterionic-like characteristics, the BAM dot was bestowed with superb antibacterial activity and rapid bactericidal kinetics against a broad spectrum of bacteria including MDR bacteria. In addition, the charge neutral design of the BAM dot ensured an improved biocompatibility, providing an excellent therapeutic index of 8. Owing to its nano-scale properties, the BAM dot demonstrated a new antibacterial mechanism involving membrane permeabilization, which prohibited the bacteria from developing resistance. Remarkably, the BAM dot was also able to exert significant removal effect on persistent bacterial biofilms, potentially improving the efficacy of conventional antibiotic treatment. This study opens up new avenue on using bioinspired nanomaterials against multi-drug resistant bacteria for their relevant biomedical applications.

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Chapter 4

Nanodot-directed Formation of Plasmonic-Fluorescent Nanohybrid (AgNP@Ser-Hist dot) towards Dual Optical Detection of Glucose and Cholesterol via Hydrogen Peroxide Sensing

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Abstract

Hybrid nanoparticles have emerged as an important class of nanomaterials owing to its integrated enhanced properties and functionality. In this study, we have developed an effective nanodot templating strategy for the *in-situ* formation of surfactant-free nanohybrid with unique plasmonic-photoluminescent properties. A bright photoluminescent (PL) biodot synthesized from serine and histamine biomolecular precursors (Ser-Hist dot), was first engineered to have rich functional group on the nanosurface capable of anchoring Ag^+ ions via electrostatic interaction. Upon UV irradiation, the free electron could transfer from photoexcited Ser-Hist dot to the Ag^+ ions, facilitating the in-situ growth of AgNPs. The resulting nanohybrid system (Bio@AgNPs) exhibit distinct characteristic SPR absorbance and highly quenched photoluminescent due to inner filter effect. Furthermore, the Bio@AgNPs nanohybrid retains its redox capability, enabling hydrogen peroxide sensing via AgNPs etching which in turn, empowers a dual colorimetric and photoluminescence detection of glucose and cholesterol in complex biological samples (i.e., synthetic urine and human plasma) with high selectivity and sensitivity. This finding reveals a new effective and facile method for the preparation of highly functional hybrid nanomaterials for dual mode detection of hydrogen peroxide-producing species and/or reactions.

4.1 Introduction

With the rapid advancing nanoscience and technology, it has enabled the preparation of nanomaterials with superior features. One example includes the hybrid nanomaterials which comprises of different component within the system. Unlike conventional single-component nanomaterials, the hybrid nanomaterials possess integrated enhanced functionality and properties which empowers great performance in their application.¹⁻² Specifically, carbon-based nanohybrids system such as carbon dot/gold nanoparticles mixture³, carbon dot/gold nanoclusters mixture⁴ and ferric dithiocarbamate complex functionalized carbon dots⁵, have been reported to exhibit unique optical properties such as dual emissive fluorescence for the detection of small molecules, ions and antibiotic drugs. However, facile synthesis of these multifunctional hybrid nanomaterials has been extremely challenging. Most of these methods require time-consuming preparation of two different components and/or involves tedious multistep conjugations with precise control to form a single nanohybrid entity. Furthermore, the use of harsh conditions and toxic reagents in some of the reactions may pose potential hazard to both human and environmental health, which greatly limits the usefulness in their applications.

The recent emergence of “bioinspired” synthesis of nanomaterials has gathered significant attention owing to the numerous benefits associated with the utilization of green processes or biocompatible reagents. In particular, biomimetic approach that utilises biomolecules as template to direct the synthesis and assembly of inorganic nanoparticles have resulted in the formation of various bio-hybrid nanomaterials useful for biomedical applications such as therapy, sensing or even theranostics.⁶⁻¹⁰ Unlike conventional synthesis method, the biotemplating synthesis offers both green and environmental friendly approach to fabricate bio-hybrid nanomaterials.¹¹⁻¹² For instance, biomolecules such as protein¹³⁻¹⁴, peptide¹⁵⁻¹⁶ and

nucleic acids¹⁷⁻¹⁸ which possess distinctive three dimensional structure, specific functional group/complementary base pair, could be employed to sequester metal ions such as Au⁺ and Ag⁺ ions, and directing the formation of their respective nanomaterials ranging from nanoparticles^{10, 19-24} to nanoclusters^{8, 25-31} without additional chemical reagents. On the other hand, biomolecule-derived nanodot (biodot), has been a rising star among the carbonaceous nanomaterial and presents a potential template for the formation of hybrid nanomaterials. The biodot has zero dimension with ultra-small sizes ranging from 1 to 10 nm, which could effectively facilitate the growth of nanomaterials.^{11-12, 32} Derived from biomolecular precursor, it inherits unique properties from the biomolecule which include good aqueous solubility and excellent biocompatibility.³³⁻³⁵ Moreover, the use of biomolecule dopes the nanodot with different elements such as nitrogen, oxygen, sulphur, and/or phosphorous which bestows them with bright photoluminescent.³⁶⁻³⁸ Besides, the surface chemistry of the biodot are highly tunable and could be enriched with specific chemical functionality through careful design and selection of the biomolecular precursor.³⁹⁻⁴⁰ Therefore, these fascinating features not only enable the biodot as potential template to effectively direct the growth of nanomaterials, but also equipped the resulting nanomaterials with advantageous properties for the desired applications.

Herein, a unique biodot synthesized from the biomolecular precursors, i.e., serine and histamine, (Ser-Hist dot) has been engineered via a simple one-step hydrothermal synthesis. The resulting biodot was decorated with abundance of chemical functional groups on the surface such as amine, carboxyl and hydroxyl groups which are beneficial for the subsequent nano-templating synthesis. Besides, the Ser-Hist dot displayed bright photoluminescent with high quantum yield of 12.8%, while having excellent stability against photobleaching. By utilizing the nanodot templating strategy, a facile synthesis method has been successfully developed to form biodot@silver nanoparticles (AgNPs) hybrids, which employed Ser-Hist

dots as templates to anchor Ag^+ ions for the *in-situ* reduction reaction in forming AgNPs via UV irradiation under mild condition, without the need of strong base/reducing agent and surfactants. The AgNPs formed has a mean size of 14.08 ± 1.30 nm and could serve as an excellent “nano-quencher”, considerably quenching the photoluminescent of the biodot via inner filter effect. The resulting hybrid nanomaterial, Bio@AgNPs, displayed a yellowish-brown colored solution with a distinct SPR characteristic absorption band at 430 nm. As such, these unique features empowered the Bio@AgNPs hybrid to act as a unique plasmonic and photoluminescent sensing probe to detect glucose and cholesterol via a dual mode detection. The sensing mechanism involved specific enzymatic oxidation of glucose/cholesterol to produce hydrogen peroxide (H_2O_2), which would etch the nanosilver surface leading to distinct solution color changes and diminishing the SPR characteristics absorption band of AgNPs. On the other hand, the photoluminescent signal of Ser-Hist dot was also recovered simultaneously due to the etching of AgNPs by the as-produced H_2O_2 . Based on this sensing principle, we have successfully employed the Bio@AgNPs for dual mode detection of glucose and cholesterol in artificial urine and human plasma sample respectively with higher sensitivity and selectivity as compared to other detection methods using different type of nanohybrid sensing materials, such as platinum-carbon core-shell nanoparticles⁴¹, poly(thymine)-templated copper nanoparticles⁴² and platinum-graphene oxide nanocomposite⁴³ (Table S1). Unlike other assays that involve tedious material/sample preparation steps or require sophisticated and expensive analytical devices⁴⁴⁻⁴⁸, our approach presents a facile method for sensing materials preparation with versatile assay design, which results can be obtained *via* naked eye (colorimetric) or simple analytical tools (fluorescent) for rapid sensing and accurate analysis, towards practical applications in clinical diagnostics.

Table S1. Comparison of various hybrid nanomaterials and their analytical performance for glucose (blue) and cholesterol detection (green)

Materials	Synthesis Method	Linear Range / μM	LOD / μM	Mode of Detection	Reference
PtNP – Mesoporous Carbon Shell	Multistep synthesis to separately prepare PtNP and mesoporous carbon shell, followed by loading	300 – 10000	100 (Glu)	Amperometry	28
Poly(thymine)-Templated CuNP	Poly(thymine)-templated synthesis	50 – 1600	50 (Glu)	Turn off Fluorescence using electrophoresis	29
MnO ₂ -Nanosheet-Modified UCNP	Multistep preparation involving core-shell synthesis and chemical conjugation	0 – 250	3.7 (Glu)	Upconversion Fluorescence	31
Pt ₇₄ Ag ₂₆ nanoparticle-decorated ultrathin MoS ₂ nanosheets	Liquid exfoliation method followed by hydrothermal treatment	1 – 10	0.8 (Glu)	Colorimetry	32
Pt/reduced GO/poly(3-aminobenzoic acid) nanocomposite film	Multistep synthesis involving electrodeposition	250 – 4000	40.5 (Cho)	Amperometry	30
Boron nitride nanosheet-Copper sulfide nanohybrids	Multistep preparation involving exfoliation and attachment	10 – 100	2.9 (Cho)	Colorimetry	33
Luminol and AuNP-co-precipitated reduced GO hybrids	Multistep synthesis involving separate preparation of AuNP, reduced GO and luminol	0.71 – 11.43	0.55 (Cho)	Chemiluminescence	34
AgNC/MoS ₂ -NS nanocomposite	Multistep preparation involving exfoliation and reduction	0.06 – 15	0.03 (Cho)	Turn on Fluorescence	35
Bio@AgNPs Nanohybrid System	In-situ nanodot templating synthesis by UV irradiation	10 – 400 (Glu) 0.5 – 40 (Cho)	Glu Abs = 53.39 PL = 41.90 Cho Abs = 8.16 PL = 5.50	Dual optical detection Colorimetry and Turn on Fluorescence	Current study

4.2 Materials and Methods

Materials

Serine, histamine, silver nitrate, hydrogen peroxide, glucose, cholesterol, glucose oxidase, cholesterol oxidase, surineTM negative urine control and plasma from human were purchase from Sigma-Aldrich.

Instruments and Characterizations

The Ser-Hist dot was synthesized using Memmert UF55 Universal Oven. The PL images of the biodot were captured under UV365 illumination using a High Performance 2UV Transilluminator (25 W). Both UV-vis absorption and PL spectrum were measured using Tecan

Infinite M200 Multimode Microplate Reader. The PL lifetime was recorded using FluoroLog-3 Spectrofluorometer. The high-resolution transmission electron microscope (HRTEM) images were taken using Philips CM300 FEGTEM. The Fourier transform infrared (FTIR) spectrum was obtained using a PerkinElmer Fourier transform infrared spectrometer. The X-ray photoelectron spectroscopy (XPS) was performed using Theta probe XPS. The ^{13}C nuclear magnetic resonance (NMR) spectrum was recorded using JEOL 500 MHz NMR. The zeta potential measurement was carried out using Malvern Zetasizer Nano S.

Synthesis of Serine-Histamine derived (Ser-Hist) Biodot

Ser-Hist dot was prepared via a one-step hydrothermal treatment. Briefly, 0.4 g serine was mixed with 0.2 g histamine in 20 mL distilled water and stirred until complete dissolution. The mixture was then transferred into a Teflon-lined autoclave and heated at 180 °C for 12 h. Thereafter, the solution was centrifuged at 12 000 k rpm for 30 min and filtered using a 0.22 μm syringe filter to remove bulky precipitate, followed by dialysis (molecular weight cutoff = 3000 Da) over 48 h to obtain the final product.

Synthesis of Biodot-Silver Nanoparticles Hybrids (Bio@AgNPs)

Solution of 50 $\mu\text{g mL}^{-1}$ Ser-Hist dot was mixed with 4 mg mL^{-1} AgNO_3 at equal volume. The mixture was irradiated with UV light at wavelength of 365 nm for 3 min using a High Performance 2UV Transilluminator (25 W). The formation of Bio@AgNPs hybrid can be monitored by the changes in the color of the solution from clear to yellowish-brown and the diminishing of PL from Ser-Hist dot.

Detection of H_2O_2 , Glucose and Cholesterol in Buffer

For H_2O_2 detection, 60 μL Bio@AgNPs was added to 60 μL of various concentration of H_2O_2 in 5 mM phosphate buffer pH 7.24, in a 96-well plate. The mixture was incubated for 30

min at room temperature with continuous shaking. Upon completion, the absorbance and photoluminescent spectrum were recorded.

For glucose detection in buffer, 1 mg mL⁻¹ GOx was first incubated with various concentration of glucose respectively in 5 mM phosphate buffer pH 7.24, at equal volume in a 96-well plate for 30 min at 37 °C to yield H₂O₂. Thereafter, an equal volume of Bio@AgNPs was added to the reaction mixture. After incubating for 30 min at 37 °C, the absorbance and photoluminescent spectrum of the mixture were measured.

Likewise, for cholesterol detection in buffer, 1 mg mL⁻¹ ChOx was first incubated with various concentration of cholesterol in 5 mM phosphate buffer pH 7.24, at equal volume in a 96-well plate for 30 min at 37 °C to yield H₂O₂. Afterward, an equal volume of 3 times diluted Bio@AgNPs was added to the reaction mixture. The absorbance and photoluminescent spectrum of the mixture were measured after incubating for 30 min at 37 °C.

Detection of Glucose and Cholesterol in Complex Sample Matrixes

SurineTM negative urine control and human plasma were selected as complex matrix samples for glucose and cholesterol detection respectively. Prior to analysis, 1 mg mL⁻¹ GOx was first incubated with 190 μM glucose, in a 50x diluted artificial urine at equal volume in a 96-well plate for 30 min at 37 °C to yield H₂O₂. Afterwards, an equal volume of Bio@AgNPs was added to the reaction mixture and incubated for 30 min at 37 °C. Upon completion, the absorbance and photoluminescent spectrum of the mixture were measured.

Similarly, 1 mg mL⁻¹ ChOx was incubated with 18 μM cholesterol in a 100x diluted human plasma at equal volume in a 96-well plate for 30 min at 37 °C to yield H₂O₂. Later, an equal volume of 3 times diluted Bio@AgNPs was added and incubated for 30 min at 37 °C. Finally, the absorbance and photoluminescent spectrum of the mixture were measured.

4.3 Results and Discussion

Scheme 1. Schematic illustration of the synthetic route of plasmonic-photoluminescent Bio@AgNPs nanohybrid via the nanodot templating strategy.

In this study, a unique nanohybrid (Bio@AgNPs) containing the photoluminescent biomolecules-derived nanodots (Biodots) and plasmonic silver nanoparticles (AgNPs) were prepared via a simple 2-steps templating strategy as shown in Scheme 1. Briefly, the biodots derived from the serine and histamine precursors were prepared and used as a template to guide the formation of AgNPs under UV irradiation without exogenous addition of other reagents. The details of each synthesis step and the characteristic of each component of the hybrid nanoparticles will be discussed in the subsequent sections. It was found that the as-prepared Bio@AgNPs hybrid nanomaterials possess distinctive fluorescent and plasmonic properties and in the presence of hydrogen peroxide (H_2O_2), the nanohybrid can be used as a dual mode sensing probe for glucose and cholesterol detection in complex biological samples as demonstrated in this work.

4.3.1 Synthesis and characterization of Ser-Hist Dot

A naturally occurring amino acid, i.e., serine, and a nitrogenous biomolecule, i.e., histamine were used as the biomolecular precursors for the synthesis of biodot (Ser-Hist) via a simple hydrothermal synthesis (**Figure 4.1a**). According to previous studies⁴⁹⁻⁵⁰, it is likely that amidation could occur between the carboxyl groups of serine and amine groups of histamine,

neutralizing the positive charges on the histamine and forming a long-chained polymer. Subsequently, the unstable polymer could rearrange and aromatized into a unique stable conformation consisting of layers of sp^2 carbon interacting via π - π interaction with unique surface chemical functionality, forming the resulting O and N doped Ser-Hist dot. As shown in the HRTEM images (**Figure 4.1b**), the as-prepared biodot, Ser-Hist dot, are well dispersed with mean size of 4.63 ± 0.89 nm (**Figure 4.2**) and a lattice spacing of 0.21 nm, which is similar to the (102) diffraction planes of graphitic (sp^2) carbon (JCPDS 26-1076). This indicated the successful formation of Ser-Hist dot.

Figure 4.1. (a) Schematics illustration of formation of Ser-Hist dot. (b) HRTEM images and lattice spacing of Ser-Hist dot. (c) Deconvolution of C1s (left), N1s (middle) and O1s (right) scans from XPS spectrum of Ser-Hist dot. (d) Absorbance, (e) PL spectrum of Ser-Hist dot (inset: photo images of Ser-Hist dot under (left) white light and (right) UV light at 365 nm

wavelength). (f) PL intensity of Ser-Hist dot before and after continuous UV irradiation for 3 h.

Figure 4.2. Size distribution, additional HRTEM images and lattice spacing of SER-Hist dot.

To better understand the surface properties and composition of Ser-Hist dot, Fourier transform infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS) and ^{13}C nuclear magnetic resonance (NMR) were used for the biodot characterization. From the FTIR spectrum, Ser-Hist dot showed distinct absorption bands at 1207 cm^{-1} , 1611 cm^{-1} , and a broad adsorption band from 2637 to 3496 cm^{-1} (**Figure 4.3**), which corresponds to the characteristics of C-O (carboxylic acids), C=C (aromatic) and O-H (carboxylic acids/alcohols) bonds respectively.⁵¹ This is further supported by the XPS analysis as shown in **Figure 4.1c**. From the deconvolution of C1s scan in XPS spectrum, it disclosed the presence of C=C (284.9 eV), C-N (286 eV) and C=O (287.2 eV) bonds.⁵² Similarly, the deconvolution of N1s revealed the existence of C=N-C (398.2 eV) and C-N-C (400 eV) bonds⁵³, while O1s scans unveiled the C=O (531.4 eV) and HO-C (532.5 eV) bonds within Ser-Hist dot.⁵² In addition, the presence of C=C (158.27 ppm), O=C-N (172.34 ppm) and O=C-O (174.33 ppm) bonds were also confirmed by ^{13}C NMR spectroscopy (**Figure 4.4**). These findings indicate that the Ser-Hist dot consists of a sp^2 carbon network surrounded with rich chemical functionality including

amine, carboxyl and hydroxyl groups, which confirms the formation of the Ser-Hist dot and enabling it as a potential templating agent.

Figure 4.3. FTIR spectrum of Ser-Hist dot.

Besides, the optical properties of Ser-Hist dot was also investigated. The UV-vis spectrum of Ser-Hist dot shows two absorption bands at 206 nm and 321 nm (**Figure 4.1d**), which could be attributed to π - π^* and n - π^* transition of carbon respectively.⁵⁴ This result concurs with the above findings which supports the formation of Ser-Hist dot. Intriguingly, Ser-Hist dot was also observed to displayed bright photoluminescent (PL). It is found to have excitation dependent emission (**Figure 4.5**), with maximum emission at 450 nm when excited at 365 nm (**Figure 4.1e**). Furthermore, the quantum yield of Ser-Hist dot was calculated to be 12.8% against quinine sulfate and exhibited excellent photostability ($94.96 \pm 0.55\%$) whereby negligible decay in PL intensity was observed upon continuous UV exposure for 3 h (**Figure 4.1f**). This phenomenon could be due to the abundance functional group presents on Ser-Hist dot which stabilises the surface defects. As a results, it mediated an effective radiative recombination of surface-confined electrons and holes in the strongly localized π and π^* electronic levels of the sp^2 sites, and σ and σ^* states of the sp^3 matrix within the Ser-Hist dot, enabling Ser-Hist dot to emit bright PL in the visible-light region.⁴⁹⁻⁵⁰

Figure 4.4. ¹³C NMR spectrum of Ser-Hist dot.

Figure 4.5. Excitation dependent emission wavelength of Ser-Hist dot.

4.3.2 *In-situ* formation of Bio@AgNPs and their characterizations

Bestowed with the unique properties such as bright photoluminescent and rich surface functional groups, Ser-Hist biodot was deployed as a template to facilitate the growth of silver nanoparticles (AgNPs) in forming the hybrid nanomaterial (Bio@AgNPs). It was postulated

that the negatively charged Ser-Hist dot could interact with the Ag^+ ions via electrostatic attraction, and subsequent UV exposure could result in transfer of free electrons from photoexcited Ser-Hist dot towards the anchored Ag^+ ions. This could promote the growth of AgNPs which lead to the formation of a unique Bio@AgNPs nanohybrid as illustrated in **Figure 4.6a**. It is worth noting that this approach demonstrates several advantages over conventional synthesis. For instance, most of the reported methods are often either a simple mixture of carbon nanodot and silver nanoparticles⁵⁵⁻⁵⁶, or requiring the use of strong base/reducing agents and surfactants⁵⁷⁻⁶⁰. In most cases, tedious multistep synthesis routes with precise control and sophisticated synthesis conditions such as reflux and electrochemical processes were also necessary to prepare the hybrid nanomaterials.⁶¹⁻⁶⁴ In comparison, the nanodot-directed synthesis offers a facile *in-situ* synthesis of carbon@silver nanohybrid that take places in mild conditions such as aqueous solution, neutral pH and room temperature reduction under UV irradiation, without exogenous reducing and stabilizing agents.

Figure 4.6. (a) UV-vis absorbance spectrum of Ser-Hist dot and Bio@AgNPs. (b) Quenching of PL upon formation of Bio@AgNPs. (c) Average PL lifetime of Ser-Hist dot and Bio@AgNPs. (d) HRTEM images of Bio@AgNPs, showing lattice spacing of both AgNPs and Ser-Hist dot. (e) Zeta potential of Ser-Hist dot and Bio@AgNPs. (f) FTIR spectrum of Ser-

Hist dot and Bio@AgNPs. Deconvolution of (g) O1s and (h) Ag3d scan from XPS spectrum of Bio@AgNPs. (i) Schematic illustration of the formation of Bio@AgNPs.

The as-formulated nanohybrid, Bio@AgNPs, exhibited a yellowish-brown colored solution with a distinct absorption peak at 430 nm which is attributed to the SPR of AgNPs (**Figure 4.6b**). Conversely, the PL intensity of nanohybrids was considerably reduced when excited at 365 nm, with a PL quenching efficiency of 71.6 % (**Figure 4.6c**). Such phenomenon could be due to the inner filter effect where energy emitted from excited Ser-Hist dot was re-absorbed by AgNPs, leading to PL quenching. In order to verify the dominant quenching process, the average PL lifetime of Ser-Hist dot and Bio@AgNPs were subsequently measured. Since the Stern-Volmer equation can be written as $(I_0/I) = (K_d + 1)(K_s + 1)$, where K_d and K_s represent the dynamic constant and the static quenching constant respectively, and $\tau_0/\tau = 1 + K_d$, where τ represents the PL lifetime of the fluorophore⁶⁵⁻⁶⁶, the average PL lifetime decreases from 5.83 ns (Ser-Hist dot) to 4.73 ns (Bio@AgNPs) (**Figure 4.6d**), implying that dynamic quenching is the dominant mechanism.

To ascertain the formation mechanism of the nanohybrid, various characterization techniques were employed. Firstly, HRTEM was conducted to examine the structure of the Bio@AgNPs hybrid. **Figure 4.7a** clearly shows the formation of AgNPs on the surface of the Ser-Hist dot, with an average size of 14.08 ± 1.30 nm (**Figure 4.8**), forming a nanohybrid system. The lattice spacing of the hybrid were determined to be 0.21 and 0.24 nm which is likely to be attributed to the (102) diffraction planes of graphitic (sp^2) carbon (JCPDS 26-1076) and (111) lattice space of metallic Ag (JCPDS 04-0783) respectively. This is further supported by EDX analysis (**Figure 4.9**), suggesting that AgNPs was anchored onto the surface of the Ser-Hist dot. Hence, these results indicate the successful synthesis of the Bio@AgNPs hybrid.

Figure 4.7. (a) HRTEM images of Bio@AgNPs, showing lattice spacing of both AgNPs and Ser-Hist dot. Deconvolution of (b) O1s and (c) Ag3d scan from XPS spectrum of Bio@AgNPs. (d) FTIR spectrum of Ser-Hist dot and Bio@AgNPs. (e) Zeta potential of Ser-Hist dot and Bio@AgNPs.

Figure 4.8. HRTEM images and lattice spacing of Bio@AgNPs, and size distribution of AgNPs grown on Ser-Hist dot.

Figure 4.9. EDX analysis of Bio@AgNPs (Inset: Table of various elements and their respective weight and atomic percentage).

Subsequently, the surface conformation of the hybrid was studied using XPS and FTIR spectroscopy. From the XPS analysis, the deconvolution of O1s scan disclosed a considerable reduction in the content of HO-C bonds while the content of C=O bonds increased after formation of the hybrid (**Figure 4.7b**). On the other hand, the deconvolution of Ag 3d scan revealed Ag 3d_{5/2} (369.0 eV) and Ag 3d_{3/2} (375.1 eV), which well indicate the formation of metallic Ag (**Figure 4.7c**).⁶⁷ Similar observation was also made from FTIR analysis. In comparison to Ser-Hist dot, the broad O-H absorption band has been significantly diminished with C=O stretching (1776 cm⁻¹) becoming more prominent in the case of Bio@AgNPs hybrid (**Figure 4.7d**). Furthermore, the zeta potential was also found to have drastically increased from -2.83 ± 0.81 mV (Ser-Hist dot) to 34.40 ± 3.55 mV (Bio@AgNPs) (**Figure 4.7e**), which

strongly suggested that the carboxyl and hydroxyl groups could have been consumed during the formation of Bio@AgNPs. Hence, through these findings, it is highly possible that the Ag^+ ions could be anchored onto Ser-Hist dot via non-covalent interactions such as electrostatic attraction between the positively charged Ag^+ ions and negatively charged carboxyl groups of the Ser-Hist dot. Upon exposure to UV irradiation, it could induce the photoexcited Ser-Hist dot to transfer free electrons towards the anchored Ag^+ ions, thereby oxidizing hydroxyl groups into carbonyl groups and reducing Ag^+ to Ag^0 . Intrinsically, this would create conducive nucleation sites to engage more Ag^+ ions, eventually leading to the growth of AgNPs and formation of Bio@AgNPs nanohybrid (**Figure 4.6a**).

Figure 4.10. PL spectrum of Ser-Hist dot and mixture containing (a) Ser-Hist dot and citrate-capped AgNPs, and (b) Ser-Hist dot and PEI-functionalised AgNPs. Changes in PL intensity of mixture containing (c) Ser-Hist dot and citrate-AgNPs, and (d) Ser-Hist dot and PEI-AgNPs in presence of varying concentration of H_2O_2 over a time period of 45 min.

To further verify that Bio@AgNPs was not a simple mixture of Ser-Hist dot and AgNPs, control experiments involving either the citrate-capped AgNPs (negatively charged) or polyethylenimine (PEI) functionalized AgNPs (positively charged), and Ser-Hist dot were conducted. It was found that the physical mixture of Ser-Hist dot with either citrate-AgNPs or PEI-AgNPs could only quench the PL intensity of the biodots in less than half of the efficiency that Bio@AgNPs could achieve (**Figure 4.10a-b**). This could be due to the inefficient chemical interactions between Ser-Hist dot and citrate- or PEI-capped AgNPs, causing ineffective-quenching of the PL intensity of biodots. On the other hand, a similar fluorescent recovery experiment as the Bio@AgNPs has been conducted in the presence of H₂O₂. Result shows that both the physical mixture of Ser-Hist dot and molecule-capped AgNPs were unable to produce significant and linear-dependent PL changes at different H₂O₂ concentration (**Figure 4.10c-d**). Hence, this study clearly indicates the uniqueness of Bio@AgNPs as a H₂O₂-dependent hybrid system, which optical response cannot be achieved by a simple mixture of the two components.

4.3.3 Photoluminescent Recovery and Color Change of Bio@AgNPs Hybrid via H₂O₂ Oxidation

Being in a hybrid state, Bio@AgNPs exhibited unique plasmonic characteristics with a localized SPR absorption peak indicating the formation of AgNPs at 430 nm wavelength in the UV-vis region, while the photoluminescence of Ser-Hist dot was quenched in the presence of AgNPs grown on the biodot surface. It is noteworthy to mention that the surfactantless synthesis of AgNPs open up an opportunity to recover the photoluminescent properties of Ser-Hist through oxidative etching in the presence of hydrogen peroxide (H₂O₂). On the other hand, the broken down of Bio@AgNPs hybrid structure by H₂O₂ dependent etching of AgNPs also enable a simultaneous solution color change observable by naked eyes from yellowish-brown to colourless under day light. This grants the possibility of Bio@AgNPs hybrid to serve as a

unique dual optical detection probe to sense a wide range of H_2O_2 producing reactions and/or species.

Figure 4.11. Changes in (a) absorbance and (b) photoluminescent of Bio@AgNPs in presence of 300 μM H_2O_2 over a time period of 45 min.

By introducing strong oxidants such as H_2O_2 into the system, the Ag^0 would be oxidized back to Ag^+ , etching the AgNPs and weakening its SPR absorbance. Correspondingly, the PL of Ser-Hist dot would be “turn-on” due to the disappearance of AgNPs from the Bio@AgNPs surface as evidence from the decrease in SPR absorbance peak of AgNPs and the recovered PL of Ser-Hist dot when excited at 365 nm. To improve the sensitivity of the reaction system, the effect of reaction time was first examined. It was observed that both absorbance and PL changed gradually as the reaction time proceed until reaching a maximum reading at 30 min (**Figure 4.11**). In addition, we have optimized both the reaction time required for UV irradiation and the concentration of Ag^+ ions to form suitable amount of AgNPs capable of achieving both the maximum PL quenching efficiency of biodot ($> 70\%$), as well as good recovery of the ‘quenched’ PL in the presence of H_2O_2 for assay development. Besides, the effect of pH on the reaction system was studied using three different buffers, namely, acetate buffer (AB) pH 5.5, phosphate buffer (PB) pH 7.24 and tris buffer pH 8.0 at 5 mM. It was found that PB pH 7.24 produced the highest absorbance and PL intensity (**Figure 4.12**) among

others, indicating that Bio@AgNPs is more stable at near neutral pH, and hence, pH adjustment would be required during sample preparation prior to the sensing process. Thus, PB pH 7.24 was selected as the optimal buffer for further sensing studies.

Figure 4.12. Changes in (a) absorbance and (b) photoluminescent of Bio@AgNPs in presence of different buffers at 5 mM.

Under the optimized conditions, H₂O₂ of varying concentration from 30 to 1000 μ M was tested against Bio@AgNPs hybrid to assess the linear response range of the sensing system. Predictably, the characteristic SPR absorbance of AgNPs decreases, accompanying the distinct solution color change from yellowish-brown to colorless while the PL intensity increases with increasing H₂O₂ concentration (**Figure 4.13**). Both the absorbance and PL signals of Bio@AgNPs demonstrated good linear relationship with varying H₂O₂ concentration. The regression lines of the above reactions could be expressed as $Abs = 0.56 - (3.85 \times 10^{-4}) [H_2O_2]$ ($R^2 = 0.980$) and $F/F_0 = (6.97 \times 10^{-4}) [H_2O_2] + 0.98$ ($R^2 = 0.995$) respectively. The limit of detection (LOD) was defined as $3\sigma_{blank}/\text{slope}$, where σ_{blank} is the standard deviation of the blank samples. Based on the equations, it was determined that the LOD of absorbance and PL detection mode are 73.50 and 93.50 μ M respectively, with a relative standard deviation (RSD) of less than 5% for absorbance and PL detection respectively. Henceforth, by exploiting the redox function of Bio@AgNPs hybrid, the dual mode detection assay could be extended to

detect different species capable of generating H_2O_2 -dependent signals as demonstrated in the following application studies.

Figure 4.13. (a) Changes in absorbance and (b) photoluminescent of Bio@AgNPs in presence of varying concentration of H_2O_2 .

4.3.4 Applications of Bio@AgNPs for Dual Optical Detection of Glucose and Cholesterol

Biomarkers including glucose and cholesterol are of great interest owing to their association in chronic diseases such as diabetes mellitus and atherosclerosis. Precise monitoring of these biomarkers is essential as it could help to lower the risk of complications. Since both glucose and cholesterol could undergo oxidation catalyzed by their respective oxidase to produce H_2O_2 as their by-product, it is possible for Bio@AgNPs hybrid to generate H_2O_2 - dependent signals which can be translated into the amount of glucose and cholesterol presents respectively (Figure 4.14a).

In the first assay design, we have demonstrated the detection of glucose using Bio@AgNPs through the glucose oxidation reaction. Glucose of varying concentration ranging from 10 to 400 μM was incubated with glucose oxidase (GOx) for 30 min in 5 mM PB buffer pH 7.24 to yield H_2O_2 . The as-produced H_2O_2 was then used to treat the Bio@AgNPs for 30 min to

produce analytical signals. It was observed that the characteristic SPR absorbance of AgNPs weakens progressively while the PL intensity improved gradually as the concentration of glucose increases. The responses are linear with regression equation of $Abs = 1.140 - (2.71 \times 10^{-3}) [H_2O_2]$ ($R^2 = 0.985$) (**Figure 4.14b**), and $F/F_o = (1.71 \times 10^{-3}) [H_2O_2] + 1.02$ ($R^2 = 0.986$) (**Figure 4.14d**) respectively. The LOD was determined to be 53.39 μM for the absorbance-based colorimetric detection (insert of **Figure 4.14b**) and 41.90 μM for the photoluminescent recovery-based detection, with $RSD < 5\%$ for absorbance and PL detection mode correspondingly. The LOD obtained under both detection modes were much lower than the amount of glucose (> 2.8 mM) that can be found in healthy human urine.⁶⁸

To demonstrate the versatility of the Bio@AgNPs sensing probe, detection of cholesterol was also conducted using a similar assay principle and aforementioned method. In the presence of cholesterol oxidase (ChOx), cholesterol would be oxidized to produce H_2O_2 that could etch the AgNPs component from the Bio@AgNPs hybrid. Thus, it results in diminishing of the characteristic SPR absorbance of AgNPs (**Figure 4.14c**) and the PL recovery of Ser-Hist dot (**Figure 4.14e**) as the concentration of cholesterol increases. The linear regression equations can be expressed as $Abs = 0.36 - (3.16 \times 10^{-3}) [H_2O_2]$ ($R^2 = 0.985$), and $F/F_o = (6.68 \times 10^{-3}) [H_2O_2] + 1.00$ ($R^2 = 0.999$) respectively. The LOD was determined to be 8.16 μM and 5.50 μM , with $RSD < 5\%$ for absorbance and PL detection correspondingly, which were also much lower cholesterol (~ 5 mM) found in human blood.⁶⁹

Figure 4.14. (a) Schematic illustration of glucose/cholesterol sensing using Bio@AgNPs. Changes in absorbance of Bio@AgNPs in presence of (b) glucose and (c) cholesterol, and their respective oxidases (Inset: Photo images depicting colour changes of Bio@AgNPs in presence of increasing concentration of glucose and cholesterol respectively). Changes in PL intensity of Bio@AgNPs in presence of (d) glucose and (e) cholesterol, and their respective oxidases (Inset: PL spectrum of Bio@AgNPs depicting recovery of PL intensity with increasing concentration of glucose and cholesterol respectively).

Figure 4.15. (a) Changes in absorbance and (b) photoluminescent intensity of Bio@AgNPs against 300 μ M of several potential interferences (i.e., Na⁺, K⁺, Mannose, Fructose, Sucrose, Lactose, Arginine, Glutamic Acid, Glycine and Lysine) using glucose sensing protocol.

In addition, the selectivity of the detection assay was investigated. Several glucose analogues including mannose, fructose, sucrose and lactose, and potential interfering ions and molecules such as Na⁺, K⁺, arginine, glycine, lysine and glutamic acid were analyzed using the Bio@AgNPs hybrid assay. As shown in (Figure 4.15a-b and 4.16a-b), the interferential species illustrated negligible influence on the absorbance and PL intensity in comparison to the result of glucose and cholesterol. This indicate that the assay is not only highly sensitive, but also exhibit excellent selectivity towards glucose and cholesterol, highlighting the potential of this Bio@AgNPs sensing probe for complex biological samples detection.

Figure 4.16. (a) Changes in absorbance and (b) photoluminescent intensity of Bio@AgNPs against 40 μ M of several potential interferences (i.e., Na⁺, K⁺, Mannose, Fructose, Sucrose, Lactose, Arginine, Glutamic Acid, Glycine and Lysine) using cholesterol sensing protocol.

Since both dynamic range of detection obtained from glucose and cholesterol are much lower than the amount presents in the human urine and blood, this allow samples to be diluted further which could effectively reduce potential interfering and background signals within these complex matrix, and further enhance sensitivity of the assay.⁷⁰ Henceforth, the detection of glucose and cholesterol were conducted in artificial urine and human plasma respectively, using standard addition method.⁷¹ Both glucose and cholesterol were spiked into their respective complex samples and sensing was carried out using the above proposed method. As shown in **Figure 4.17**, the recovery for glucose were determined to be (Abs) $96.65 \pm 3.01\%$ and (PL) $105.94 \pm 3.06\%$, while for cholesterol, the recovery obtained were (Abs) $93.56 \pm 4.10\%$ and (PL) $104.83 \pm 10.60\%$. This demonstrated the potential use of the sensing platform for accurate glucose and cholesterol detection in practical clinical diagnostics.

	Spiked	Recovery (Abs)	Recovery (Abs) / %	Recovery (PL)	Recovery (PL) / %
Glucose (μM)	190.02	183.64 $\pm$ 5.53	96.66 $\pm$ 3.01	201.3 $\pm$ 6.17	105.94 $\pm$ 3.06
Cholesterol (μM)	18.04	16.84 $\pm$ 0.69	93.35 $\pm$ 4.10	18.87 $\pm$ 2.00	104.60 $\pm$ 10.60

Figure 4.17. Absorbance of (a) glucose and (b) cholesterol detection in artificial urine and human plasma respectively (Inset: Changes in absorbance of Bio@AgNPs during glucose and cholesterol detection in complex samples). PL intensity of (c) glucose and (d) cholesterol detection in artificial urine and human plasma respectively (Inset: Changes in PL of Bio@AgNPs during glucose and cholesterol detection in complex samples). (e) Percentage recovery of glucose and cholesterol by Biodot@AgNPs assay after spiking respective analytes into artificial urine and human plasma respectively.

4.4 Conclusion

In summary, an effective nanodot templating strategy has been exploited for the formation of a unique nanohybrid system containing photoluminescent biodot (Ser-Hist dot) and plasmonic silver nanoparticles (AgNPs). The Ser-Hist dot was first synthesized and used as a template to anchor the Ag^+ ions and form AgNPs on the biodots spontaneously under UV irradiation without exogenous reagents. The as-prepared Bio@AgNPs nanohybrids displayed interesting optical properties inheriting from both the biodots and AgNPs components and their molecular interactions. This include the plasmonic characteristics of AgNPs which gave rise to a distinctive yellowish-brown colored solution, while effectively quenched the photoluminescent of biodots owing to its inner filter effect. Furthermore, *in-situ* formation of Bio@AgNPs enabled a close proximity between the two hybrid components, permitting a unique H_2O_2 dependent optical signal generation which could not be achieved by physical mixing of the two components. This equipped the plasmonic-photoluminescent hybrid with the unique ability to serve as a dual optical sensing probe for H_2O_2 detection via oxidative AgNPs etching that trigger 1) distinctive solution color changes from yellowish-brown to colorless and 2) ‘turn-on’ the previously quenched photoluminescent signal of biodots. Based on this sensing principle, we have successfully applied the Bio@AgNPs for detecting glucose and cholesterol in synthetic urine and human plasma, respectively. More importantly, the rich surface chemical functionality of the nanohybrid may enable opportunity to attach useful ligands such as coumarin dye that can be selectively enhanced by hydroxyl radical⁷² potentially improving the sensitivity of the assay and fluorescent signal. All in all, these findings demonstrate an effective and facile nanodot templating method to fabricate highly functional Bio@AgNPs hybrid nanomaterials towards practical application in clinical diagnostic and detecting other related H_2O_2 -producing species or reactions.

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Chapter 5

Conclusion and Future Outlook

5.1 Conclusion

In this thesis, we have explored on the programmability of biomolecule in the synthesis of biodots to achieve distinctive properties. Particularly, each biodot was designed through careful selection of biomolecular precursor, and engineered with unique features suitable for specific biomedical application such as intracellular imaging, antimicrobial therapy, and biosensing.

In Chapter 2, we first conducted a systematic study to investigate the material design rule of biodot synthesis from 20 naturally occurring amino acids. Through various comprehensive characterisation of the biodot, a structure-property relationship between the amino acids precursor and the corresponding biodot was established. Ser and Thr biodots were found to exhibit the brightest photoluminescent with high quantum yield and excellent photostability among other biodots. Intriguingly, the combination of Ser or Thr with another amino acid precursor could result enhancement of photostability and red shifting in their emission wavelength. In addition, the biodots demonstrated superb biocompatibility, excellent intracellular uptake activity and imaging capability which are highly suitable for *in vivo* bioimaging application.

In Chapter 3, we developed a unique biodot for antimicrobial application. The as-synthesized biodot possess unique amphiphilic and zwitterionic-like characteristics which were made possible through careful design and selection of the biomolecular precursors. Coupled with the ultrasmall size of the biodot, it improved the biocompatibility of the biodot while

empowering an effective antimicrobial activity. Through an effective bacteria membrane permeabilization, the biodot could exert the rapid bactericidal effect and significant biofilm removal while prohibiting resistance development within the bacteria. Thus, an excellent therapeutic efficacy was achieved, demonstrating the biodot potential as antimicrobial agent.

In Chapter 4, we explored the bio-templating ability of biodot in the synthesis of hybrid nanomaterials. The biodot was prepared with unique surface properties which could anchor Ag^+ ions and directing the formation of AgNPs on the surface of the biodot upon photoexcitation. The resulting nanohybrid displays a distinct SPR AgNP characteristic absorption band while significantly quenching the luminescent of biodot. This enabled the selective and sensitive detection of glucose and cholesterol via a dual mode detection including fluorometric and colorimetric sensing. The practicability of the detection was also evaluated using artificial urine and human plasma sample.

In summary, the emergence of bioinspired synthesis has presented a new benign and facile approach to prepare nanomaterials. Unlike conventional synthesis, the bioinspired synthesis offers a green and inexpensive fabrication technique as it utilises cheap and readily available biomolecules as reagents while reduces the usage of harsh and toxic reagents. In addition, the resulting nanomaterials are often exhibit superior properties such as rich chemical functionality, good aqueous solubility and excellent biocompatibility which are extremely difficult to achieve in the conventional nanomaterials. Moreover, the bioinspired synthesis is highly versatile owing to the programmability of the biomolecule which enables the ability to fine-tune the properties of the nanomaterials for desired application.

5.2 Future Outlook

Bioinspired synthesis of nanomaterials is an evolution from convention synthesis method. The strategy offers high versatility in the synthesis by exploiting the programmability of the biomolecule. This in turn enables the ability to fine-tune the properties of the nanomaterial for desired application. Despite the advantages of the strategy, there still remains plenty of room to explore owing to the extensive amount of biomolecules and its combination. Principally, the interaction of the biomolecule precursors should be thorough studied and investigate to ensure desired properties can be instilled into the final nanomaterial. Only through understanding the programmability and interaction of the biomolecules, it would then provide essential insights towards more the formation of higher ordered materials. This could involve the understanding of the cooperation of multiple intermolecular interactions including non-covalent interactions such as π - π stacking, hydrogen bonding, electrostatic attraction, hydrophobic interaction and van der Waals' forces. By manipulating their interplay, it could give rise to dynamic and responsive changes, allowing the construction of a multitude of structures and morphologies from 1D, 2D to 3D over a range of length scales. Only by doing so, we will be able to engineer next generation biomimetic nanomaterials with responsive and multifunctional nanostructure for complex applications such as theranostic, deep tissue diagnosis and nanorobots for surgery.