

Protein–Polyphenol Networks: From Fundamentals to Biomedical Applications

Yiyuan Han

ORCID ID: [orcid.org/ 0000-0002-4547-3368](https://orcid.org/0000-0002-4547-3368)

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Abstract

Naturally occurring building blocks have attracted scientific interest for the assembly of functional materials due to their intrinsic biocompatibility and biodegradability. Proteins are a particularly crucial class of functional biomacromolecules involved in most fundamental processes of living organisms that can be assembled into nanomaterials for various biomedical applications. Another ubiquitous class of biomacromolecules are polyphenols, which have traditionally been referred to as “vegetable tannins”, have recently been employed in engineering advanced materials, owing to their available physicochemical and biological properties and capability of assembly through diverse interactions.

This thesis aims to introduce protein–polyphenol networks (PPNs), namely interconnected networks of proteins and polyphenols that can be deposited on a wide array of substrates. The polyphenol-mediated protein assembly of materials such as films, capsules, or nanoparticles (NPs) are introduced in this thesis because self-assembly approaches allow for the rapid generation of tailorable materials under mild conditions. This thesis also focuses on exploring the fundamentals of the interactions between proteins and polyphenols, which helps in understanding the assembly mechanism of PPNs.

The binding affinity between polypeptides and polyphenols is studied by analytical chemistry techniques, focusing on the interactions between side chains of proteins and polyphenols, which is crucial for the controllable design of protein-based materials. Then, a straightforward and versatile strategy through interfacial polyphenol-mediated protein assembly is introduced to create a library of functional PPN materials, including bioactive surface coatings and functional capsules. Moreover, the PPN capsules not only can be used to clarify the governing interaction(s) between different proteins and polyphenols, but also can be employed in various applications (*e.g.*, enzymatic catalysis, fluorescence imaging, and cell targeting).

Next, a template-mediated supramolecular assembly method is developed to synthesize PPN NPs capable of endosomal escape and subsequent protein release in the cytosol. The versatility of this strategy in terms of NP size and protein type makes this a promising platform for potential applications in protein therapeutics. Finally, the protein–polyphenol interactions related to actual biological environments are investigated by the studying protein corona formed around different polyphenol-modified gold NPs (AuNPs). Protein corona compositional analysis demonstrates the binding preference of serum proteins with various

polyphenols, and cellular uptake behaviors of polyphenol–AuNPs can elucidate the role of polyphenols in bio–nano interactions, which can act as reference works for the future implementation of polyphenols in biomedical applications.

Declaration

This is to certify that:

- i. The thesis comprises only my original work towards PhD except where indicated in the Preface;
- ii. Due acknowledgement has been made in the text to all other material used;
- iii. The thesis is less than 100,000 words in length, exclusive of tables, bibliographies, appendices, and footnotes.

Yiyuan Han

Preface

Chapters 3 and 4 of this thesis have been previously published in the following articles:

Han, Y.; Lin, Z.; Zhou, J.; Yun, G.; Guo, R.; Richardson, J. J., Caruso, F.* Polyphenol–Mediated Assembly of Proteins for Engineering Functional Materials. *Angew. Chem. Int. Ed.* **2020**, 59, 15618–15625.

Han, Y.; Zhou, J.; Hu, Y.; Lin, Z.; Ma Y.; Richardson, J. J., Caruso, F.* Polyphenol-Based Nanoparticles for Intracellular Protein Delivery *via* Competing Supramolecular Interactions. *ACS Nano* **2020**, 14, 12972–12981.

In Chapter 4, Dr. Joseph J. Richardson and Prof. Frank Caruso assisted with the design of the project. Zhixing Lin assisted with the TEM and SEM measurements and helped with the enzyme catalysis experiments. Dr. Jiajing Zhou assisted with material synthesis and helped with the interaction mechanism. Dr. Gyeongwon Yun assisted with the AFM measurements and pH-responsive experiments. Rui Guo assisted with both the synthesis of the templates and the planar substrate coating.

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Publications

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2. **Han, Y.**; Lin, Z.; Zhou, J.; Yun, G.; Guo, R.; Richardson, J. J., Caruso, F.* Polyphenol-Mediated Assembly of Proteins for Engineering Functional Materials. *Angew. Chem. Int. Ed.* **2020**, *59*, 15618–15625.
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4. Lin, Z.; Zhou, J.; Cortez-Jugo, C.; **Han, Y.**; Ma, Y.; Pan, S.; Hanssen, E.; Richardson, J. J., Caruso, F.* Ordered Mesoporous Metal-Phenolic Network Particles. *J. Am. Chem. Soc.* **2019**, *142*, 335–341.
5. Ma, Y.; Cortez-Jugo, C.; Li, J.; Lin, Z.; Richardson, R. T.; **Han, Y.**; Zhou, J.; Björnalm, M.; Feeney, O. M., Zhong, Q.-Z.; Porter, C. J.; Wise, A. K.*, Caruso, F.* Engineering Biocoatings to Prolong Drug Release from Supraparticles. *Biomacromolecules* **2019**, *20*, 3425–3434.
6. Zhong, Q.-Z.; Pan, S.; Rahim, M. A.; Yun, G.; Li, J.; Ju, Y.; Lin, Z.; **Han, Y.**; Ma, Y., Richardson, J. J.; Caruso, F.* Spray Assembly of Metal-Phenolic Networks: Formation, Growth, and Applications. *ACS Appl. Mater. Interfaces* **2018**, *10*, 33721–33729.

Oral Presentations

The following oral presentations were delivered during this PhD candidature:

1. **Han, Y.** “Protein–Polyphenol Networks: From Fundamentals to Biomedical Applications” *PhD Completion Seminar, August 2020*, The University of Melbourne, Australia.
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4. **Han, Y.** “Polyphenol-Mediated Functional Protein Assembly” *Nanostructured Interfaces and Materials Science (NIMS) Group Meeting, July 2019*, The University of Melbourne, Australia.
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7. **Han, Y.** “Engineering Macromolecule-Polyphenol Networks for Biomedical Applications” *PFPC Seminar, October 2019*, The University of Melbourne, Australia.
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1.1 Introduction

The development of self-assembly techniques involving small building blocks has attracted much attention over the last several decades, ranging from fundamental research to various technological applications.¹⁻⁴ Owing to vast array of building blocks available, diverse functional complex architectures such as polymer micelles or nanoparticles, liposomes, nanosheets or nanotubes, and metal–organic frameworks have been developed through direct interactions or indirect self-assembly using templates or externally applied fields (*e.g.*, magnetic or electric). Moreover, such structures can be constructed with controllable size, shape, rigidity, and surface properties.⁵ The existing toolbox for self-assembly methods includes layer-by-layer (LbL) assembly, interfacial assembly, ligand-driven assembly, evaporation-induced assembly and antibody–antigen specificity.⁴ The formation of molecular self-assembly involved in these methods is mainly driven by noncovalent interactions such as hydrophobic interactions, hydrogen bonding, coordination bonding, electrostatic interactions, and DNA–RNA hybridization.^{3,6} Therefore, most self-assembly materials have stimuli-responsive properties such as pH, light, temperature, and redox, which are useful properties in biomedical applications such as therapeutics, diagnostics, immune modulation, and regenerative medicine.⁷⁻⁹

Naturally occurring building blocks have attracted more scientific interest owing to their inherent biocompatibility and biodegradability. Moreover, they are renewable resources and abundant in nature. A wide variety of natural resources ranging from small molecules, biopolymers to complex substrates, such as plant- or animal-derived materials, have been successfully introduced as building blocks for self-assembly into elegant structures and functional materials. Polyphenols, traditionally referred to as “vegetable tannins”, have been recently used for engineering advanced materials owing to their physical, chemical, and biological properties and capability of assembly through diverse interactions.¹⁰ Proteins, as functional biomacromolecules, are involved in almost every fundamental process of living organisms such as structural and mechanical functions, hormone regulation, enzymatic catalysis, and immune responses.

This chapter will introduce two naturally occurring building blocks—proteins and polyphenols—including their structure, properties, and interactions, followed by an outline of protein assembly methods and polyphenol-mediated assembly approaches. The use of protein-

based functional materials and polyphenol-based particles for biomedical applications is also underlined based on the available properties of both building blocks.

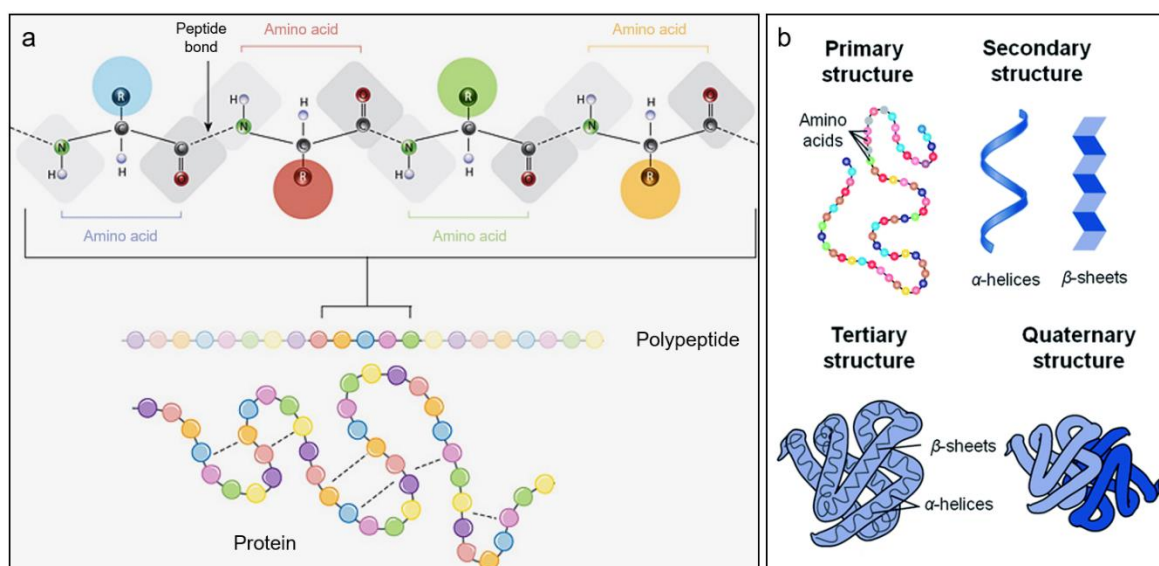
1.2 Proteins as Building Blocks: Assembly and Biomedical Applications

Proteins are essential components of living organisms and participate in nearly all necessary processes of body balance and metabolism. Many proteins can recognize specific proteins or bind with other substrates and form well-organized structures that exhibit unique properties. Research in proteins in the biological fields has ranged from the basic understanding of the molecular structure to in-depth investigations of protein–protein interactions in the past few decades.¹¹⁻¹³ Therefore, understanding the assembly mechanism of protein nanostructures and engineering bioinspired self-assembled protein materials with desirable properties have gained increasing interest in the biomedical fields.^{14,15}

1.2.1 Structures and Properties of Proteins

Proteins constitute of amino acid residues joined by peptide bonds. Specifically, a protein is obtained from the folding of one or more linear chains (polypeptides) into one or more specific spatial conformations (**Scheme 1.1a**). The linear chains constitute amino acids that are linked by condensation reactions.¹⁶ The amino acid sequence of proteins is coded by genes and determines the structures, properties, and functions of proteins.¹⁷ The protein structure typically refers to the three-dimensional arrangement of atoms in amino acid molecules. There are four distinct characteristics and levels of protein structures categorized into primary, secondary, tertiary, and quaternary structures (**Scheme 1.1b**).¹⁸ The primary structure of a protein describes the sequence of amino acids in the polypeptide chain held together with peptide bonds. The secondary structure, such as α -helix and β -sheet, refers to the folding of polypeptide chains by hydrogen bonds between different residues. The tertiary structure describes the three-dimensional geometry of protein molecules driven by noncovalent interactions. The quaternary structure consists of multiple polypeptide chains and different subunits that operate as a multimer.¹⁹ A protein generally undergoes reversible structural conformation when it performs its biological function. Thus, studying the protein structure is fundamental to understand the function of a protein at the molecular level.

Proteins can also be classified according to their distinct physicochemical properties. The number of amino acids in proteins typically ranges from tens to several thousand, resulting in proteins with sizes ranging between 1 and 100 nm and molecular weights ranging between thousand Dalton and million Dalton.²⁰ Proteins are also recognized according to their distinct shape pattern owing to the specific folding or crumpling of the polypeptide chains such as simple crystalloid spherical shape (globular proteins) or long thread-like shape (fibrillar proteins). The isoelectric point of proteins, which is determined by the composition of zwitterionic amino acids, not only affects their solubility or surface charge but also can be employed in applications like protein identification, purification, or precipitation.²¹ Besides, the hydrophobicity and hydrophilicity of proteins are typical characteristics of different types of proteins, such as membrane proteins or soluble proteins, owing to the different arrangement of hydrophobic residues and hydrophilic residues.²² Moreover, proteins consist of various functional groups, such as carboxyl groups ($-\text{COOH}$), amino groups ($-\text{NH}_2$), and thiol groups ($-\text{SH}$), which are chemically active and can therefore react with many substances to modify surfaces or can be modified with other molecules such as fluorescent dyes and chemical probes.²³



Scheme 1.1. (a) Formation of proteins from amino acids and polypeptides. (b) Structures of proteins. Reproduced from references [16] and [18].

The biological properties (or function) of proteins play essential roles in the natural processes of living organisms and cell metabolism.²⁴ Enzymes, referred to as biological catalysts, facilitate biochemical reactions in the body. For example, pepsin is a digestive enzyme in the stomach. Antibodies produced by the immune system show specific recognition to unique

antigens that allows the identification and neutralization of pathogenic bacteria and viruses. Structural proteins, such as collagen and elastin, provide essential support to connective tissues and hormone proteins, such as insulin, coordinate body functions and regulate cell metabolisms. Transport proteins can move molecules through the body. For example, hemoglobin in red blood cells transports oxygen in the blood.

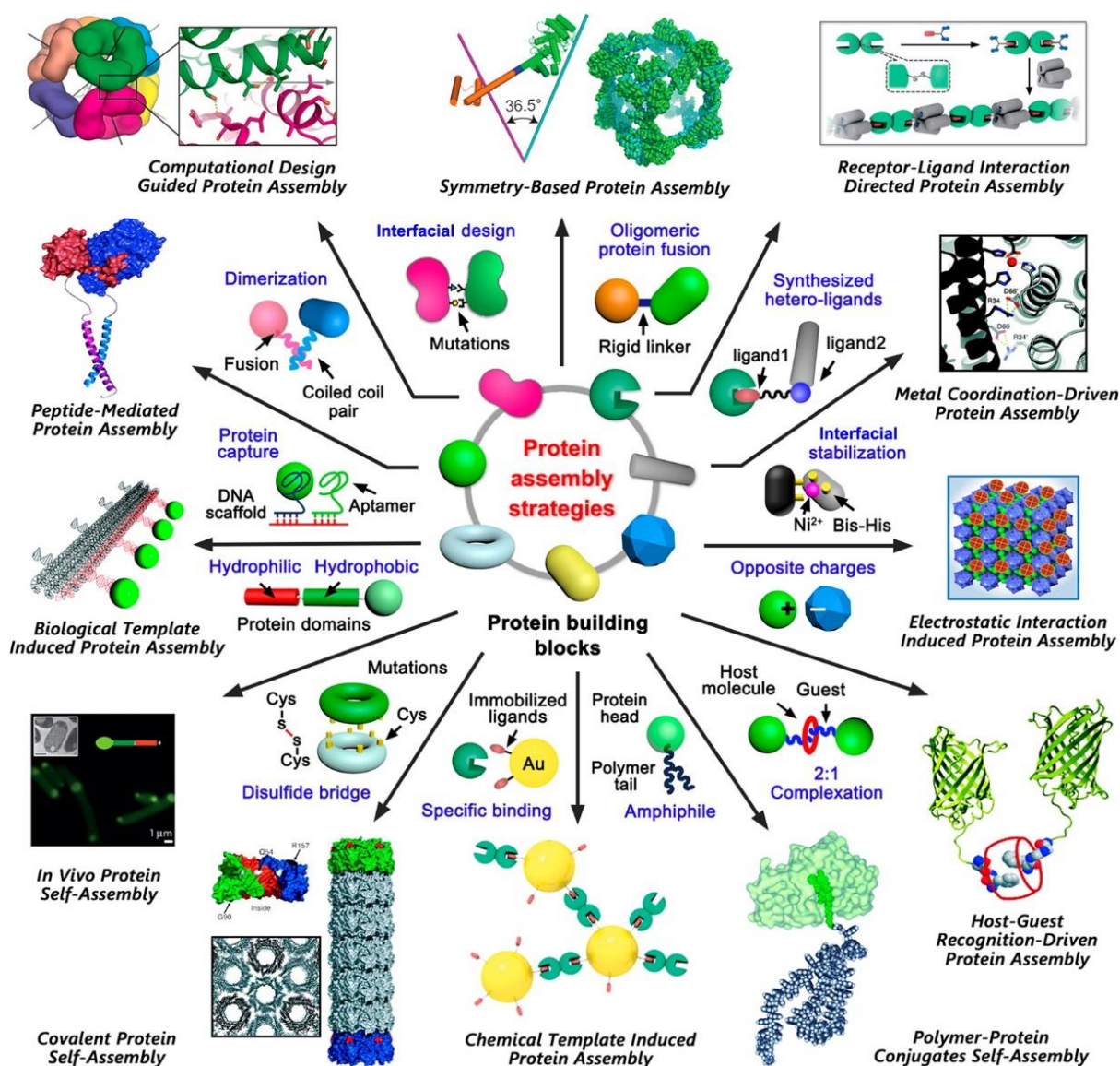
1.2.2 Protein Assembly Techniques

Nature exploits well-defined protein assemblies of different types such as linear amyloid fibrils, ferritin protein cages, ring-shaped clamp proteins, and tubular bacteriophage tail proteins.²⁵ Inspired by the “bottom-up” self-assembly approach, there are many synthetic strategies based on chemical and biological technologies that have been applied to achieve the assembly of proteins into nanostructures (**Scheme 1.2**).²⁶ Manipulating proteins into various nanostructures not only can provide a powerful tool to understand the natural protein assembly process but also offers the opportunity to develop advanced biomaterials.

Chemical strategies use chemical interactions, including supramolecular interactions, such as metal coordination, electrostatic interactions, receptor–ligand interactions, and covalent cross-linking, to construct protein assemblies. Metal coordination-driven self-assembly approaches commonly use amino acid residues on protein surfaces, such as histidine or cysteine, as ligands for metal chelation.²⁷ The electrostatic interaction-induced protein assembly approach is based on attractive forces between oppositely charged amino acids (*e.g.*, arginine and glutamic acid) to control and regulate the assembly.²⁸ Receptor–ligand interactions, including avidin–biotin and lectin–sugar, have also been employed in protein assembly, which enriches the toolbox for chemically directed protein assembly strategies.²⁶ Both the aforementioned supramolecular interactions-based protein assembly and some direct chemical cross-linking techniques, such as polymer–protein conjugation and covalent protein self-assembly through cysteine residues, have expanded the scope of protein assembly by various interactive mechanisms.²⁹

Biotechnological strategies include some traditional technologies such as peptide-mediated or DNA-induced protein assembly and various emerging technologies such as symmetric fusion-based protein assembly or computational design-guided protein assembly.^{30–32} Short peptide motifs, such as amyloid-like β -sheets and coiled coils, have often been used to construct large protein fibers. Protein assembly based on DNA nanotechnology, which involves DNA nanolattices, DNA nanogrids, aptamer-containing DNA, or DNA–peptide nanoarrays,²⁶ takes advantage of the self-assembly nature of DNA to achieve a high degree of accuracy and

complexity for protein assembly.³³ In addition, a wide range of symmetric architectures can be accomplished by using the genetically oligomeric fusion method for protein assembly in an accurate and predictable way.³² Computational approaches that combine existing protein design approaches or perform a theoretical investigation before actual experiments open up new avenues for the modular assembly of sorted building blocks into custom-designed assemblies.³¹ Additionally, *in vivo* self-assembly of genetically encoded synthetic proteins into fluorescent artificial organelles within cellular environments has led to new trends in the area of protein assembly.³⁴



Scheme 1.2. Schematic representation of various chemical and biological strategies from protein building blocks. Reproduced from reference [26].

synthetic multienzyme supramolecular sheet was developed by the self-assembly of multiple oligomeric enzymes (**Figure 1.1**).³⁶ These multienzyme assemblies demonstrated higher coenzyme regeneration efficiency and improved structural stability of enzymes than those observed for unassembled structures. In addition, protein-based nanocarriers for drug delivery have recently attracted increasing interest owing to the intrinsic biocompatibility and biodegradability of proteins.³⁷ Protein-based assemblies not only can directly deliver the functional proteins to cells but also can carry other guest molecules for biomedical diagnosis or therapy.³⁸ Various biological properties of proteins, such as specific cell targeting and therapeutics, have been integrated into protein assemblies to develop multifunctional nanocarriers, which could accelerate future clinical translation.³⁹

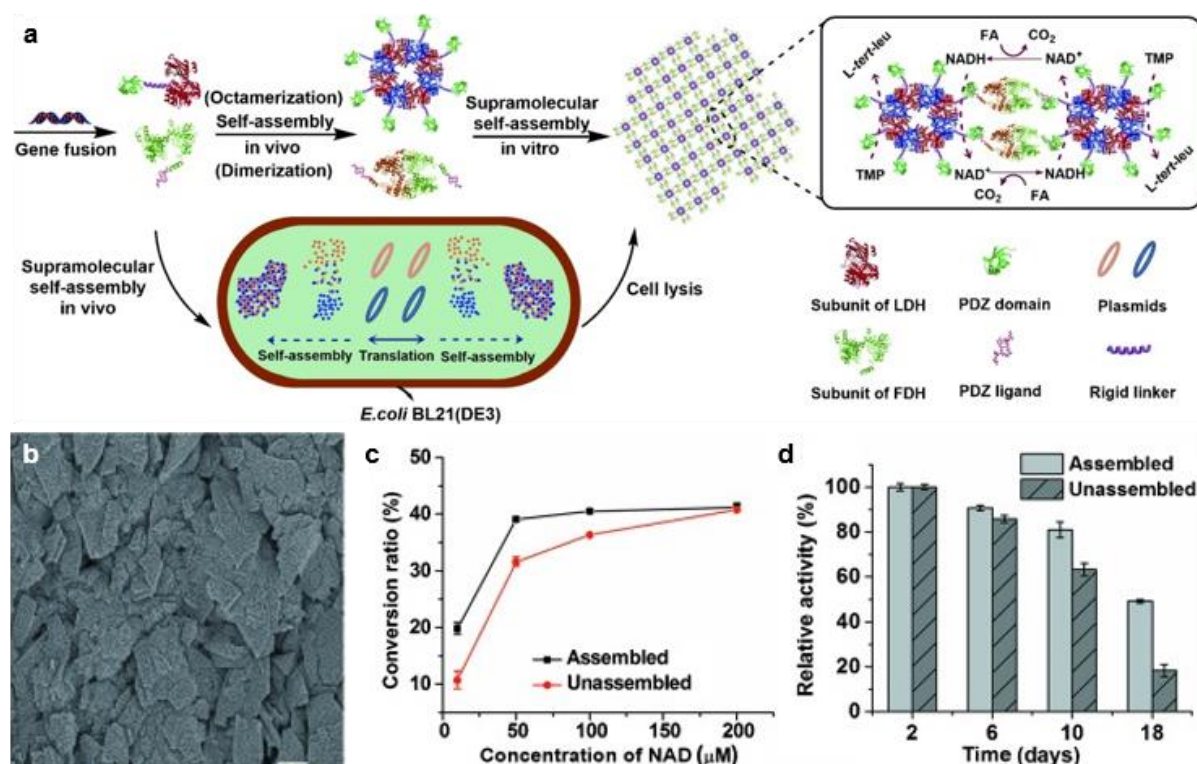


Figure 1.1. Engineered multienzyme supramolecular device. (a) Scheme of the self-assembly of multiple oligomeric enzymes (LDH, leucine dehydrogenase; FDH, formate dehydrogenase; PDZ, PSD95/Dlg1/zo-1; TMP, trimethylpyruvic acid; FA, ammonium formate; and NAD⁺/NADH, nicotinamide adenine dinucleotide oxidized/reduced). (b) Field-emission scanning electron microscopy image of the morphology and structure of the nanosheet. Scale bar: 200 nm. (c) Coenzyme regeneration efficiency and (d) stability of multienzyme assemblies. Reproduced from reference [36].

The design and construction of spontaneously assembled compartments using proteins as structural and functional building blocks has recently been developed in diverse research areas. Specifically, Huang et al. prepared micro-compartments through the interfacial assembly of protein–polymer nanoconjugates, which exhibited biomimetic protocell properties such as guest molecule encapsulation and protein synthesis *via* gene expression (**Figure 1.2**).⁴⁰ The encapsulation of a wide range of functional guest molecules, such as fluorescent dyes (calcein) and proteins (ferritin), was readily achieved in protocells (proteinosomes). In addition, cell-free gene expression was undertaken by loading a DNA plasmid in the proteinosomes to synthesize enhanced green fluorescent protein (eGFP). Interestingly, the proteinosomes with the selective permeability attributed to the thermo-responsiveness of the grafted polymer induced membrane-gated internalized enzyme catalysis. Except for the protocell functions, protein-based vesicles and capsules also can be expanded to other biomedical applications, including targeted drug delivery, imaging, or vaccination.⁴¹

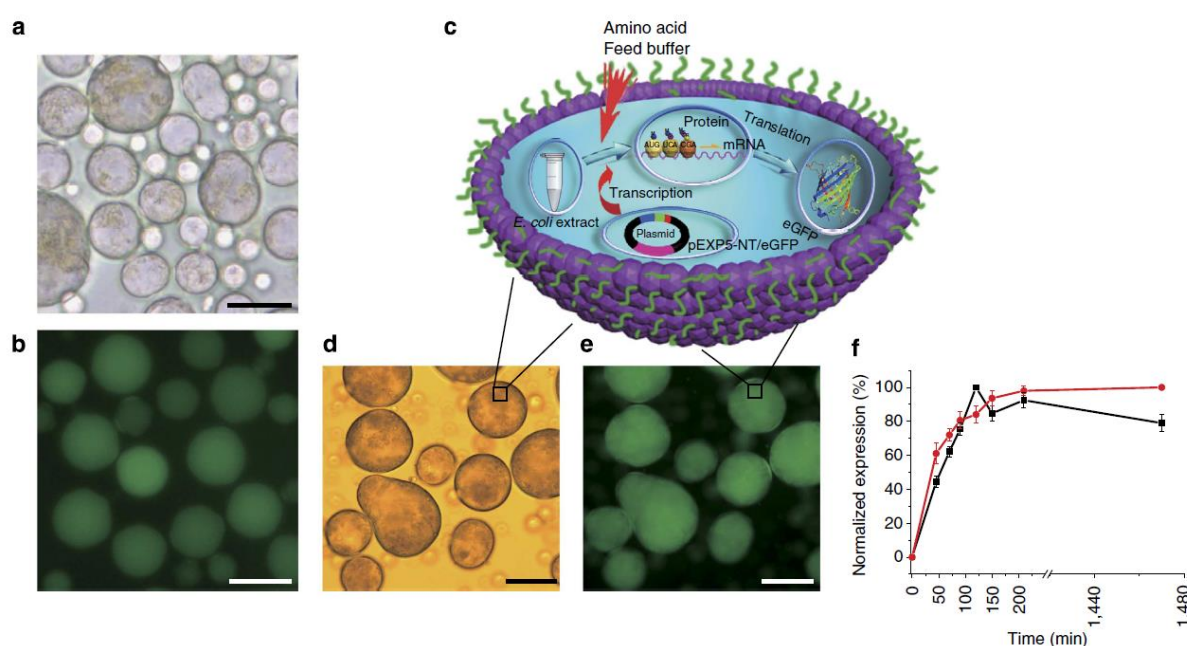
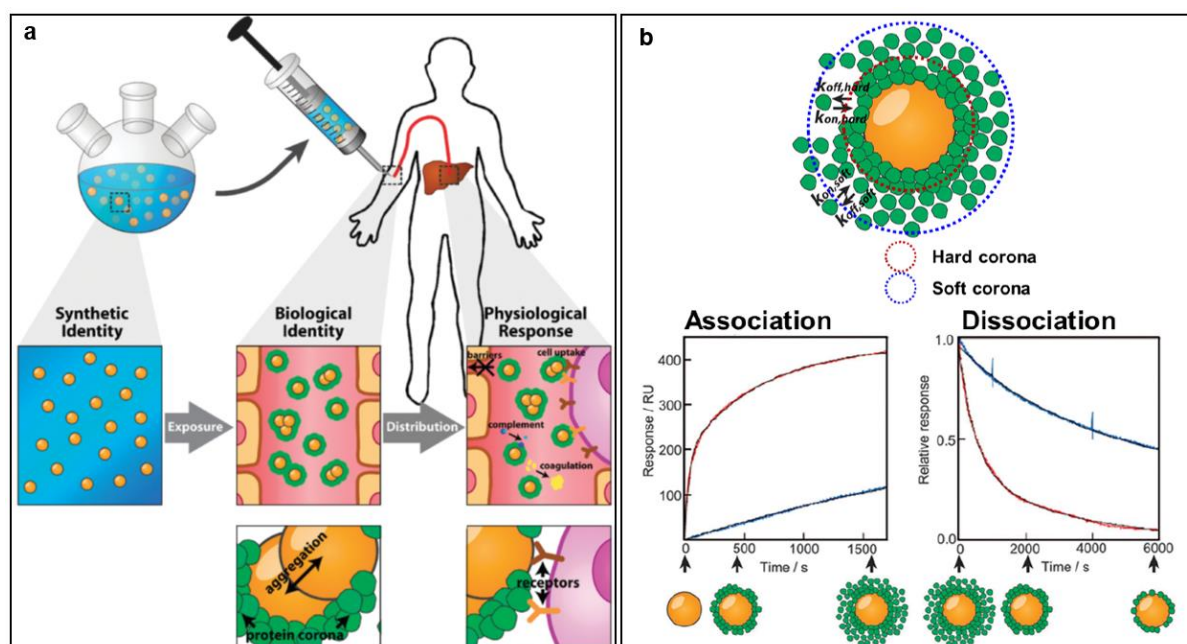


Figure 1.2. Encapsulation and gene expression in biomimetic protocells (proteinosomes). (a) Optical and (b) fluorescence microscopy images of proteinosomes containing ferritin (a) or calcein (b). Scale bars: 50 μm . (c) Schematic illustration of gene expression of protein (eGFP) synthesis in biomimetic protocells. (d) Optical and (e) fluorescence microscopy images of proteinosomes showing successful synthesis of eGFP. Scale bars: 100 μm . (f) Plots showing expression kinetic profiles for eGFP in bulk solution (red) and biomimetic protocells (black). Reproduced from reference [40].

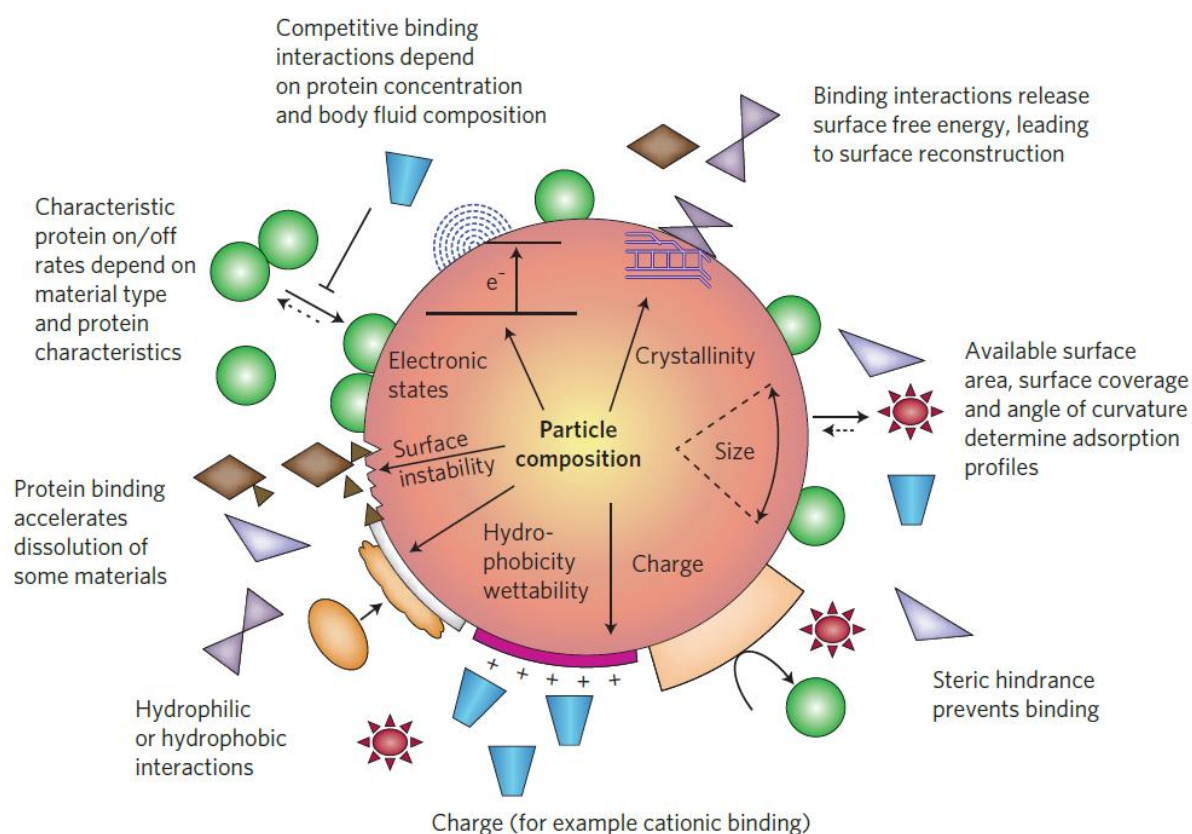
1.2.4 Protein Corona in Biological Environments

Most engineered particles (including those assembled from proteins) applied in the biomedical fields are administered to natural living environments, and many biomolecules (mainly proteins) readily adsorb on the surface of the particles to form a protein corona.⁴² The protein corona therefore gives the nanomaterial a biological identity that is distinct from its synthetic identity. This new biological identity can influence subsequent physiological responses by mediating particle–cell interaction (**Scheme 1.4a**).^{43,44} Uncontrolled physiological responses may trigger biological effects, thereby delaying translation of the nanomaterials to clinic applications.⁴⁵ The protein corona is considered to be divided into a hard corona (inner layer) and a soft corona (outer layer) (**Scheme 1.4b**). The hard corona is regarded as the near-monolayer of proteins that is tightly bound but not completely irreversibly to the surface of the nanoparticles. The soft corona, which is the outer layer, constitutes proteins that loosely and dynamically associate with the nanoparticles.⁴⁶ The formation of a protein corona has been reported to be fast (<0.5 min) on the nanoparticles and highly dynamic in which protein adsorption and desorption happen continually.⁴⁷

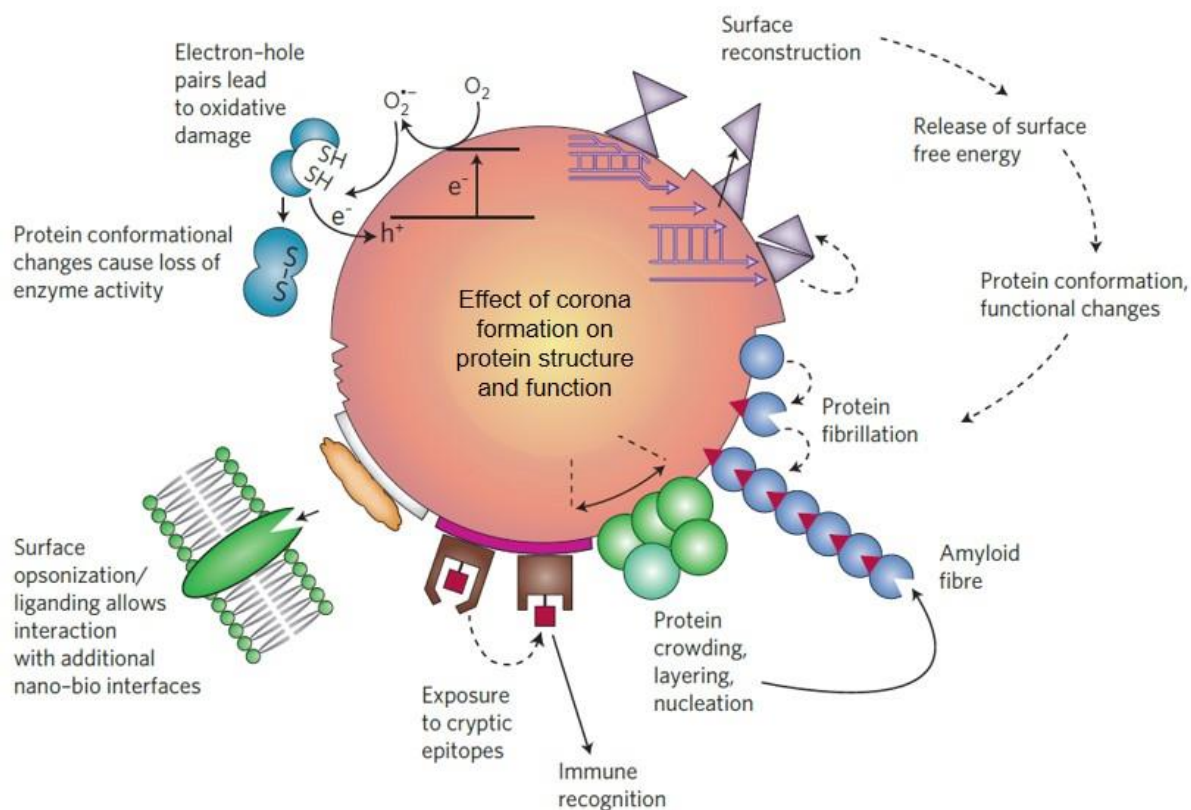


Scheme 1.4. Schematic illustration of protein corona formation in biological environments. (a) Relationship between synthetic or biological identity of nanomaterials and physiological response. (b) Conceptualized model for the hard corona (red circle) and soft corona (blue circle) and the kinetics of plasma adsorption and desorption from copolymer particles with different copolymer ratio 50:50 (red) and 85:15 (blue). Reproduced from reference [44].

Cellular interactions with particles are mediated by the protein corona, of which the formation can be influenced by the physicochemical properties of the particles, such as hydrophobicity, size, or charge, as these could affect which proteins interact with the particles (**Scheme 1.5**).⁴⁸ Conversely, the structure and function of the proteins can be disrupted after attaching to or detaching from the particles. These potential molecular changes can cause function loss or even contribute to autoimmune disease (**Scheme 1.6**). Thus, considering the influence of protein corona on the engineered particles is important when designing particles as carriers into biological environments.⁴⁹ Many approaches, including tuning of the particle properties to reduce protein adsorption or balancing the low-fouling property and particle functionality, can help eliminate the adverse effects of the protein corona. Conversely, the protein corona can enhance colloidal stability, improve the loading capacity of guest cargo, or reduce cytotoxicity. Altogether, these benefits could boost the performance of the engineered nanoparticles and further promote their clinical translation.⁵⁰



Scheme 1.5. Schematic illustration of the effects of the physicochemical properties of particles on the formation of protein coronas. Adapted from reference [48].



Scheme 1.6. Schematic illustration of the effects of corona formation on protein structure and function. Adapted from reference [48].

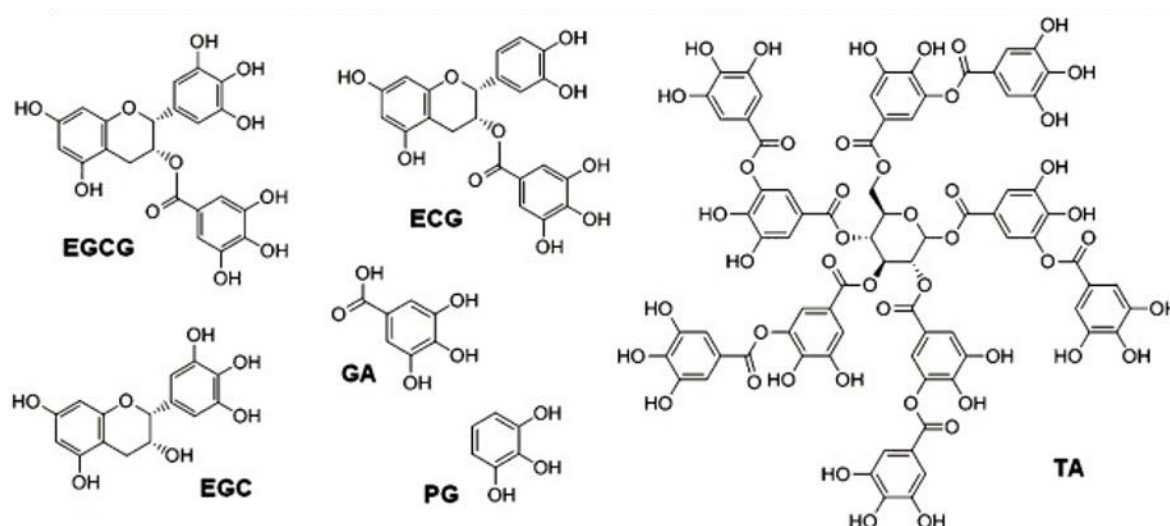
1.3 Polyphenols as Building Blocks: Assembly and Biomedical Applications

Polyphenols are naturally occurring compounds widely found in plants that have potential pharmacological benefits for human health.⁵¹ Recently, the demand for replacing petroleum-derived materials with green and renewable plant-derived materials has become increasingly important from both the social and environmental aspects.⁵² Among various plant-derived materials, polyphenols have gained growing attention from material scientists.⁵³

1.3.1 Structures and Properties of Polyphenols

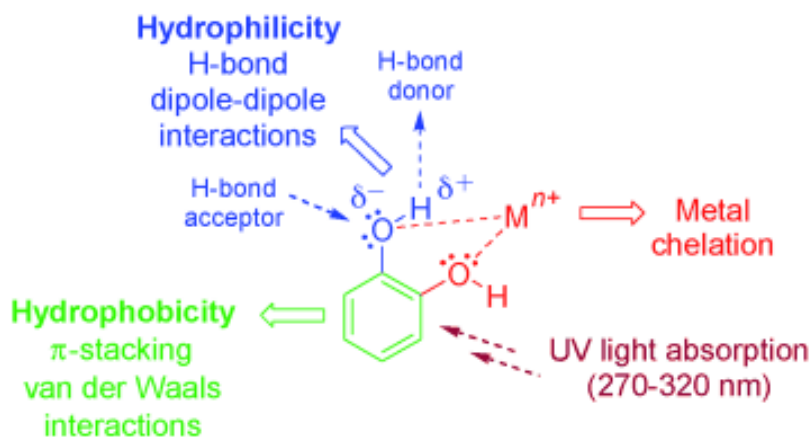
Most polyphenols contain repeating phenolic groups such as catechol, pyrogallol, resorcinol, and phloroglucinol coupled with stable C–C bonds (condensed tannins) or ester derivatives (hydrolyzable tannins). Common polyphenols include epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), and tannic acid (TA). In addition, simple phenolic mimics such as gallic acid (GA) and pyrogallol (PG) are commonly applied, as basic

models, to study the polyphenols or serve as building blocks to construct complex structures (**Scheme 1.7**).^{54,55} TA is a dendritic polyphenol that contains a glucose core and five digalloyl ester groups. This particular structure affords numerous possibilities of reaction with other molecules to form stable networks.⁵¹ Moreover, it is considered safe by the US Food and Drug Administration.



Scheme 1.7. Chemical structures of common polyphenols and phenolic mimics. Adapted from reference [54].

The basic physicochemical properties of polyphenols are based on their molecular structure (**Scheme 1.8**) that each contains phenolic functional groups.⁵³ The phenyl ring with hydroxyl groups combines the hydrophobic character derived from the aromatic benzene and the hydrophilic nature of abundant hydroxyl groups, thereby showing an amphiphilic property. In addition, the phenyl ring in the polyphenols can display hydrophobic interactions with other aromatic benzene through π - π stacking interactions. The hydroxyl groups can act as donors and/or acceptors to form hydrogen bonds. In addition, catechol (bearing two hydroxyl groups) and galloyl (bearing three hydroxyl groups) groups can form coordination bonds with various metal ions, in which higher pHs facilitate such coordination/complexation owing to the higher propensity of deprotonation of the hydroxyl groups. The additional third hydroxyl groups in galloyl groups can lower the pK_a of the other hydroxyl groups; thus, galloyl groups exhibit a higher metal-binding ability than catechol groups. Besides the interesting properties mentioned above, polyphenols show high UV absorption capacity because the hydroxyl groups on the aromatic ring can cause a shift of the maximum of π - π absorption of benzene at 254 nm to the phenol absorption at 280 nm.

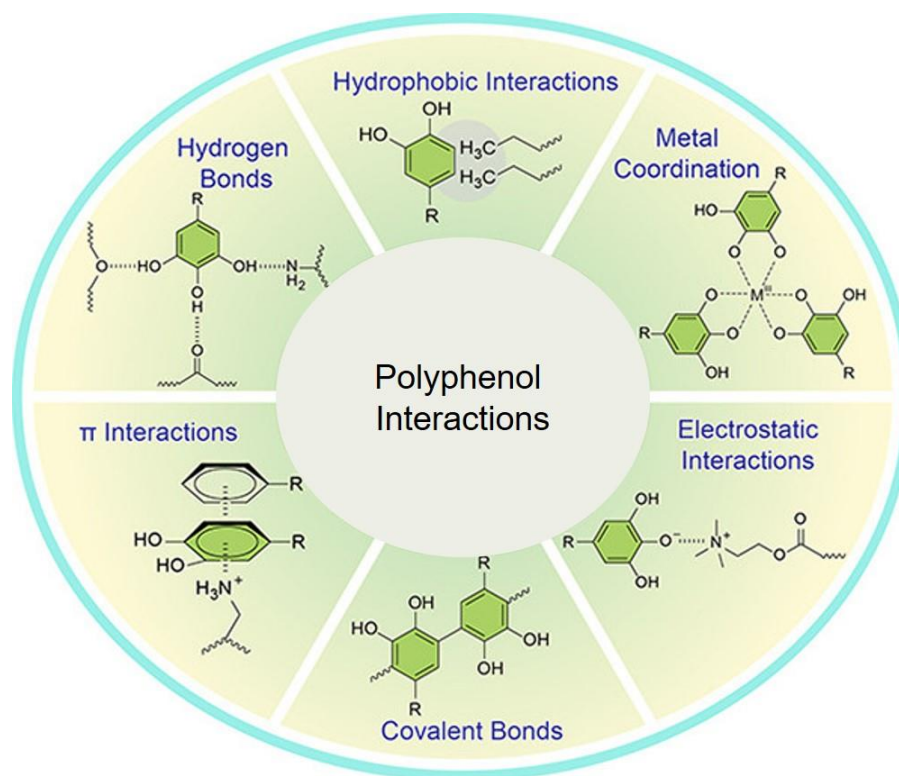


Scheme 1.8. Physicochemical properties of polyphenols arising from the different phenolic functional groups constituting polyphenols. Reproduced from reference [53].

1.3.2 Polyphenol Interactions

Naturally occurring polyphenols have been used to build wide-ranging functional materials because of their capability of assembling various building blocks through diverse interactions (**Scheme 1.9**).⁵⁶ The metal coordination affords the formation of complexes between the polyphenols and diverse metal ions, metal oxides, or metal nanoparticles. Other noncovalent bonds, such as hydrogen bonds, hydrophobic or electrostatic interactions, offer the potential for interaction with many biomacromolecules such as proteins or DNA. Additionally, polyphenols can undergo oxidative coupling to form polymeric assemblies by covalent bonds, which can be induced by oxidants or enzymes. These various types of interactions offer opportunities for the engineering of advanced functional materials and their subsequent application in the biomedical fields.

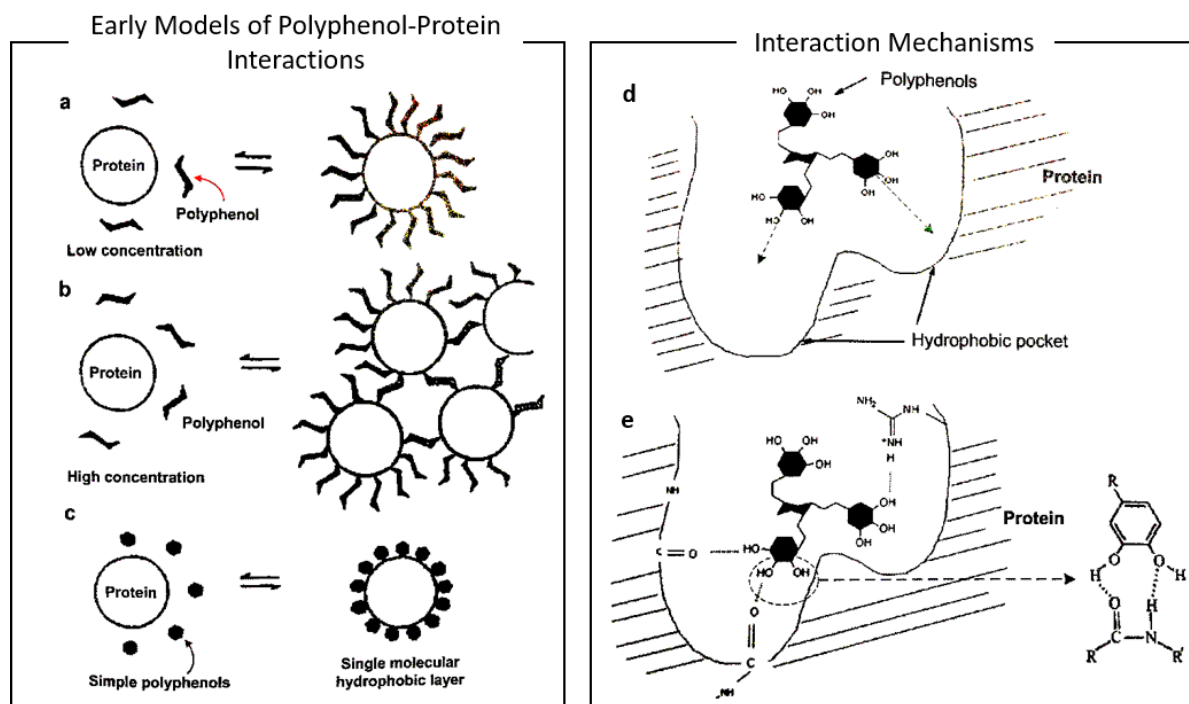
Supramolecular interactions, also referred to as noncovalent interactions, have been extensively employed in material design for biomedical applications owing to their instinct stimuli-responsiveness. Among these diverse interactions, the coordinating bonds between polyphenols and metal ions have been extensively studied, and the complexes formed between the metal ions and polyphenols have different stoichiometries (*e.g.*, mono-, bis-, tris-complex). In contrast, noncovalent interactions between polyphenols and biomacromolecules have been less comprehensively studied by modern analytic chemistry or molecular simulations to exhibit the interaction mechanism. The complexity of structures and physicochemical properties of biomacromolecules leads the research in this direction more difficult.



Scheme 1.9. Polyphenols can form various interactions with different materials. Reproduced from reference [56].

To date, the most acceptable theoretical mechanism of polyphenol–protein interactions is based on a combination of hydrophobic interactions and hydrogen bonding.⁵⁷ The major factors that influence the formation or strength of these binding forces including the structure and ratio of the polyphenol molecules and proteins (**Scheme 1.10a–c**). In proteins with a secondary structure, there are abundant “hydrophobic pockets” such as aromatic rings or aliphatic residues. In the early stages of the intermolecular forces, the hydrophobic moieties of polyphenols, *i.e.*, catechol or galloyl groups, are caught in the hydrophobic pockets of the protein (**Scheme 1.10d**). Subsequently, the hydroxyl and carboxyl groups of the polyphenols form hydrogen bonding with the protein. These two driving forces work together to strengthen the polyphenol–protein interactions, and most of the interactions are dynamically reversible processes (**Scheme 1.10e**).

The structural units of DNA are nucleotides, and polyphenols also show the potentials to form the hydrogen bonding along the DNA phosphate backbone. However, the understanding of polyphenol–DNA interactions is lacked and in demand. Moreover, the intermolecular forces between polyphenols and DNA, whether disturbing the complementary base pairing within DNA assemblies, need more investigation.



Scheme 1.10. Early models of polyphenol–protein interactions and the interaction mechanisms based on hydrophobic interaction–hydrogen bonding theory. Reproduced from reference [57].

1.3.3 Polyphenol-Mediated Assembly

As discussed above, polyphenols can form coordination bonds with different metal ions and interact with macromolecules, such as proteins and polymers, through hydrogen bonding and/or hydrophobic interactions. Such types of noncovalent bond interactions have been successfully employed in the self-assembly nanomaterials. There are extensive studies on polyphenol-based self-assembly involving metal ions, proteins, polymers, and DNA, as exemplified in this section.

Polyphenols can display coordination interactions with metal ions, which is being exploited as an emerging and versatile tool to prepare pH-responsive films owing to the reversibility of coordination bonds.⁵⁸ Recently, Caruso and co-workers reported a simple and rapid coating method, applicable to a range of substrates, based on the coordination interactions between TA and ferric iron (Fe^{III}) ions (**Figure 1.3**).⁵⁹ The materials prepared from this approach have many useful advantages such as the use of naturally occurring compounds providing films with biocompatibility, and the rapid disassembly of films in acid conditions, which can be employed to tailored drug release.

The versatility of this coating strategy using TA was demonstrated by the fabrication of functional metal–phenolic network (MPNs) films and capsules with 18 different metal ions.⁶⁰ This established system of MPNs represents a new area of metal–organic coordination materials and offers pathways for the facile and rapid engineering of nanostructured films and capsules. The MPN coating strategy also proved to be versatile in terms of the choice of the phenolic building block—besides TA, simple phenolic ligands with a single aromatic ring, and monotopic or ditopic chelating groups were successfully applied in the construction of films and capsules. For instance, GA, PG, and pyrocatechol all displayed coordination interactions with metal ions (*e.g.*, Fe^{III}).⁶¹

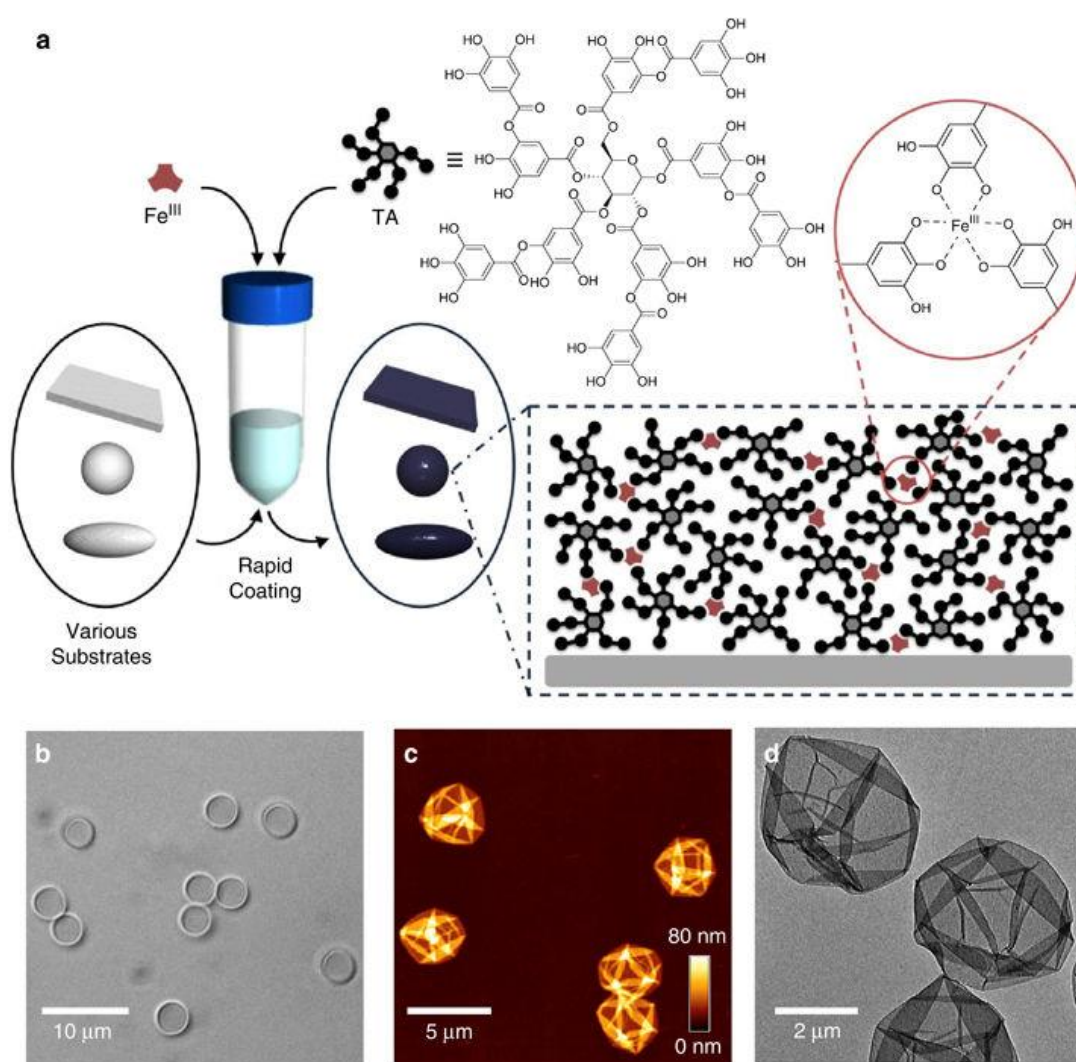


Figure 1.3. One-step assembly of Fe^{III} –TA capsules. (a) Schematic of the Fe^{III} –TA film assembly process. (b) Differential interference contrast, (c) atomic force microscopy, (d) transmission electron microscopy (TEM) images of Fe^{III} –TA capsules. Adapted from reference [59].

Polyphenols have also been demonstrated to interact with macromolecules through the various interactions between their functional groups.⁵³ The most common macromolecules in biochemistry are proteins, DNA or lipids, and synthetic macromolecules, such as polymers.⁶²⁻⁶⁴ These macromolecules have different useful functions. Thus, studying the interactions and assembly methods between polyphenols and macromolecules is important for developing more biocompatible applications.⁶⁵⁻⁶⁷

In recent years, a few methods have been reported that polyphenol-mediated protein assembly. For instance, in a study by Kurisawa and co-workers, the authors demonstrated the formation of stable micellar nanocomplexes (MNCs) between oligomerized EGCG (OEGCG) and anticancer protein (Herceptin). Specifically, the MNCs formed upon the first complexation of OEGCG with Herceptin (core), followed by complexation of poly(ethylene glycol)(PEG)-EGCG around the pre-formed core to form the outer shell. The MNCs exerted better anticancer effects *in vitro* and *in vivo* than free Herceptin (**Figure 1.4**).⁶⁸ Most importantly, the anticancer activities of the protein Herceptin were retained following complexation with OEGCG and fully reacted after disassembly.

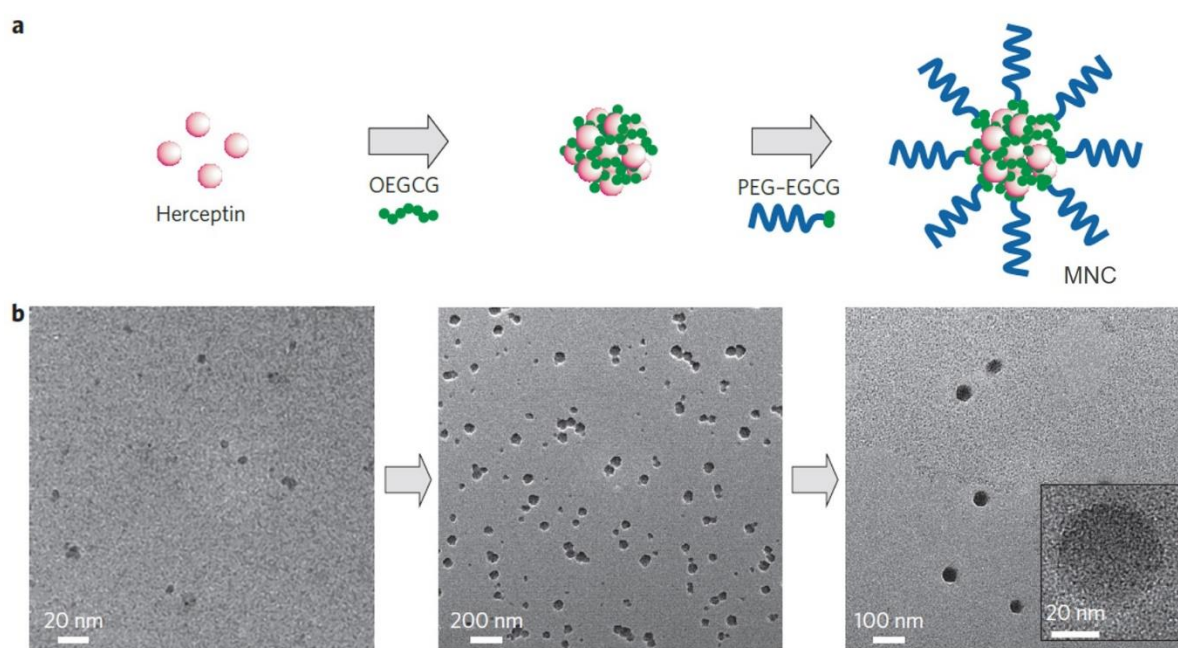


Figure 1.4. Self-assembly of OEGCG with Herceptin into micellar MNCs and their anticancer effects. (a) Schematic of the self-assembly of the MNCs and (b) TEM images of the corresponding materials observed at each step of the self-assembly process. Adapted from reference [68].

In another recent study by Lee and co-workers, the authors demonstrated the adherence of tannic-acid-modified (TANNylated) proteins to extracellular matrix proteins such as elastin and collagens for improved targeting to the heart (**Figure 1.5**).⁶⁹ A model protein, such as green fluorescent protein (GFP), was modified with TA and then chose other therapeutic peptides (substance P) or viruses (adeno-associated virus serotype 9) to improve the heart disease treatment. The TANNylated proteins showed long blood circulation time in body, thereby showing potential for increasing the drug residence time. Collectively, the emergence and development of these polyphenol-mediated protein assembly methods indicate that more and more material scientists have paid attention to this field.

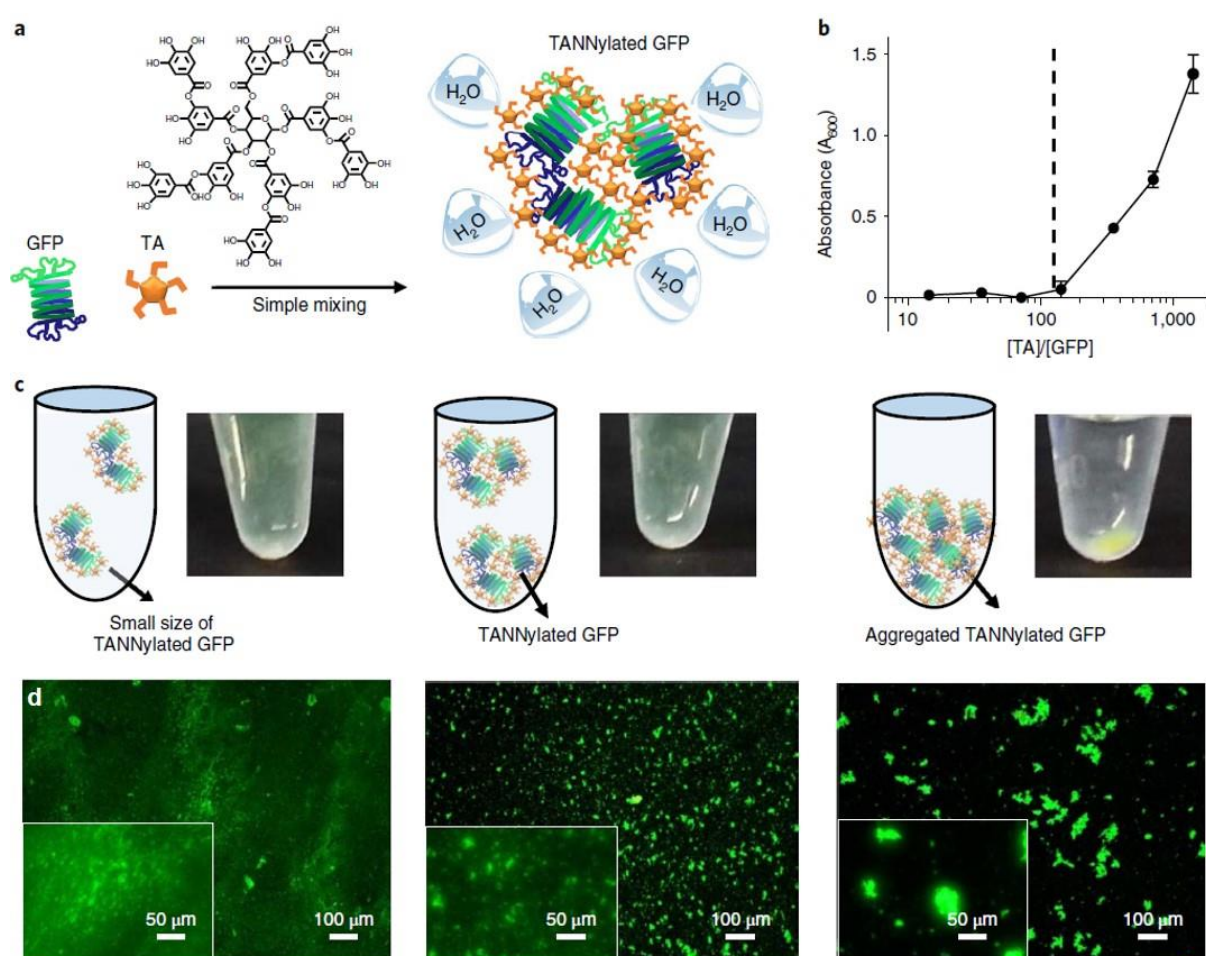
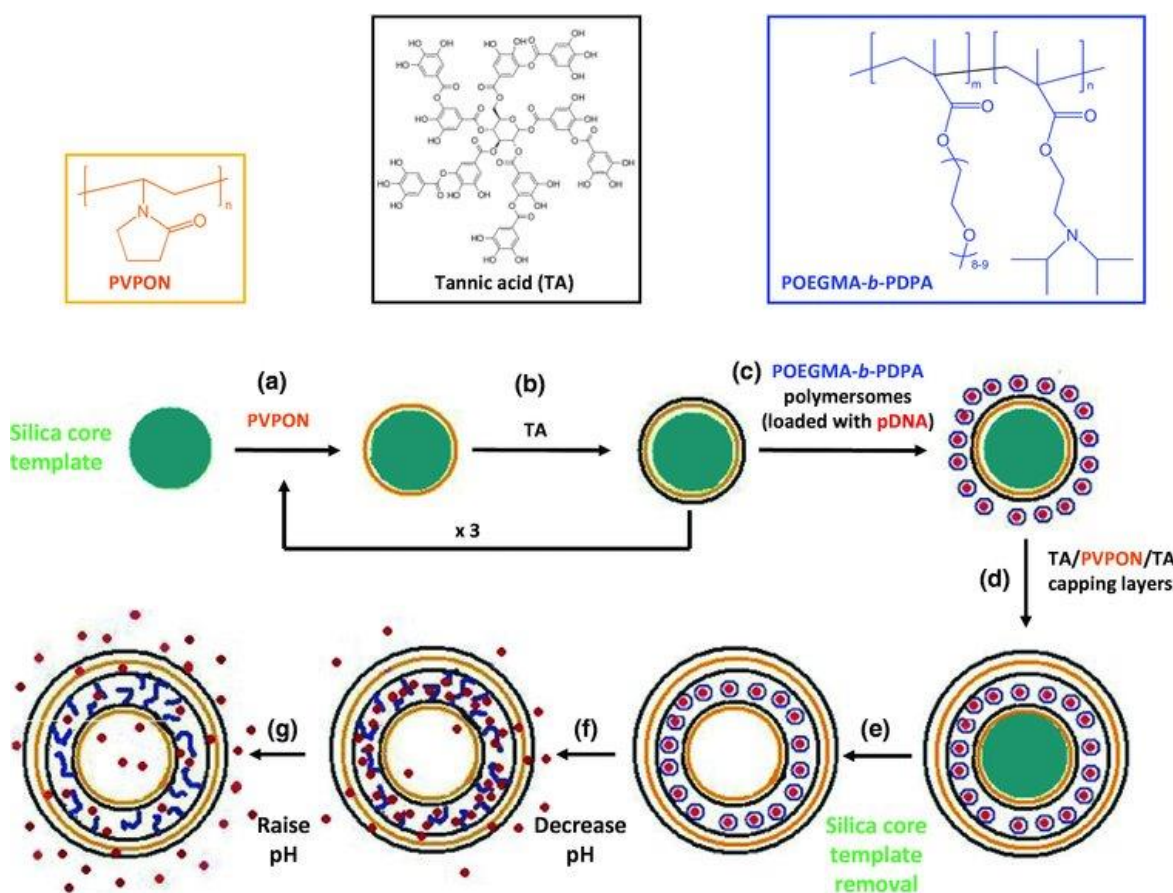


Figure 1.5. Preparation and physicochemical characterization of TANNylated protein complexes. (a) Schematic illustration of the preparation process of TANNylated GFP complexes. (b) Turbidimetric assay (UV absorbance at 600 nm) for determining the critical stoichiometric ratio of [TA]/[GFP]. (c) Schematic representation and associated photographs and (d) corresponding fluorescence images of TANNylated GFP prepared with increasing [TA]/[GFP] stoichiometric ratios from left to right. Reproduced from reference [69].

Unlike protein as biopolymers are natural compounds, and synthetic polymers are with controllable structures and designable functional groups. TA has shown intermolecular bindings with various polymers such as PEG, poly(N-vinylpyrrolidone) (PVPON), poly(sodium 4-styrenesulfonate) (PSS), and poly(N-isopropylacrylamide) (PNIPAM).^{70,71} Besides, TA has been demonstrated the assembly *via* the LbL method with polymers through electrostatic interactions or hydrogen interactions to form thin films and capsules. For example, Caruso and co-workers previously reported the incorporation of polymersomes loaded with plasmid DNA (pDNA) into multilayers of TA and PVPON (**Scheme 1.11**). TA served as a hydrogen bond donor for both polymer layers and the polymersomes. The polymersome-loaded capsules disintegrated at low endocytic pH, which triggered the release of pDNA.^{72,73} Besides, TA has been successfully applied in the self-assembly of polyphenol–polymer nanoparticles; the polymers examined were PEG, poloxamer 407 (Pluronic F-127), and poloxamer 188 (Pluronic F-68). Furthermore, the nanoparticles loaded with dexamethasone showed esterase-responsive release behaviors.⁷⁴



Scheme 1.11. Preparation of polymersome-loaded LbL capsules and pH-responsive pDNA release. Adapted from reference [73].

In addition to particulate functional materials, TA can be used as a molecular glue to connect polymers to form three-dimensional intermolecular networks.^{72,75} Lee and co-workers reported a novel medical adhesive termed TAPE obtained through intermolecular hydrogen bonding between TA and PEG (**Figure 1.6**).⁷⁶ The preparation process is simple, involving the mixing of TA and PEGs without the need for chemical cross-linking. TAPE showed up to a 250% increase in adhesive ability compared with the surgical fibrin glue. In addition, the adhesion strength of TAPE could be controlled by varying the terminal functional groups of PEGs. TAPE was demonstrated to be stable in aqueous environments, easily scalable in preparation, and biodegradable *in vivo*, suggesting a new adhesive platform.

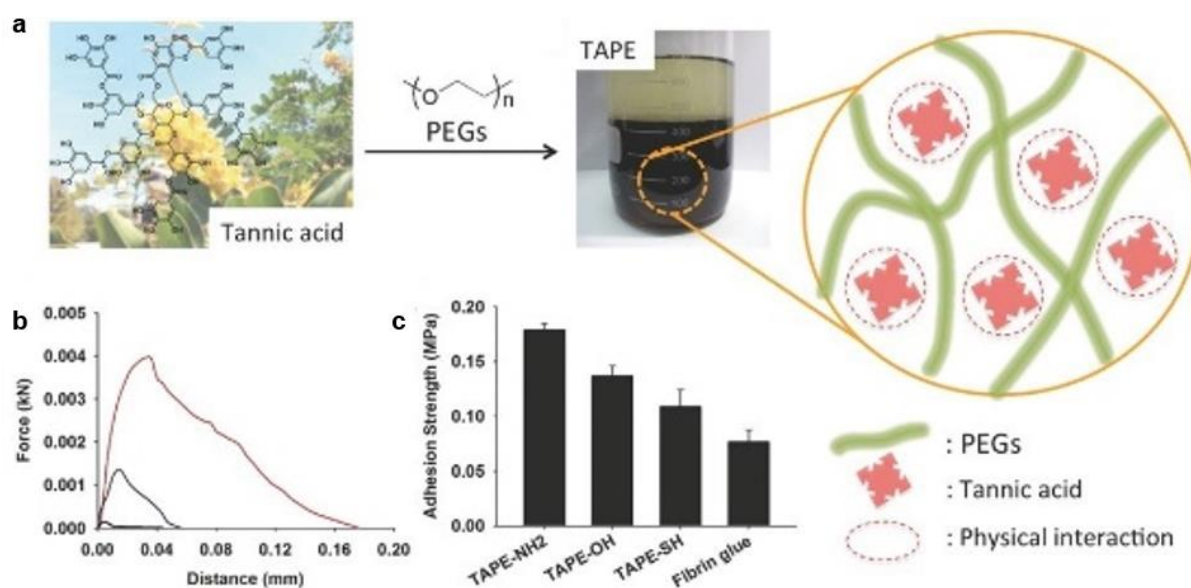
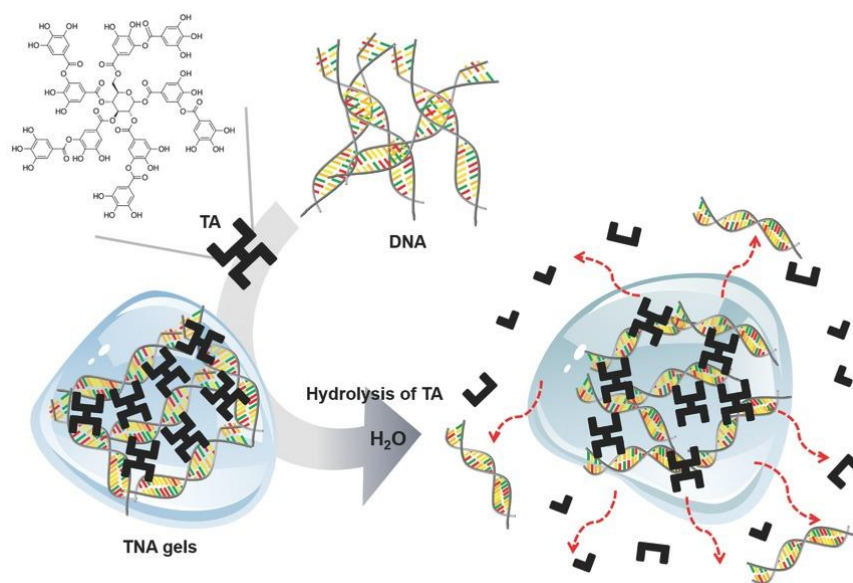


Figure 1.6. Formation and adhesion strength of TA-mediated adhesive: TAPE. (a) Schematic illustration of the TAPE synthesis by simply mixing TA with PEGs. (b) Tensile test measurements of TAPE-NH₂ (red), TA (blue), and PEG-NH₂ (black). (c) Adhesion strength of different TAPE and commercial fibrin glue. Reproduced from reference [76].

DNA has recently emerged as a novel and popular material in materials science because of its programmability. Therefore, studies on polyphenol–DNA interactions have attracted are important for successful application in material engineering and biomedical applications. For instance, studies on polyphenol–DNA interactions have demonstrated that EGCG can inhibit the DNA methyltransferase activity and that polyphenols can prevent human cells against UV-induced DNA damage.⁷⁷ Lee and co-workers have demonstrated the use of TA and DNA to form DNA hydrogels named as TNA hydrogels (**Scheme 1.12**).⁷⁸ TA reversibly cross-linked with the phosphodiester bonds in DNA, which is different from widely used complementary

base pairing in DNA assemblies. Moreover, the TNA hydrogels showed degradability upon exposure to physiological buffers owing to the intrinsic hydrolysis of TA. In another study, Cheng and co-workers prepared core-shell-structured nanoparticles by assembling EGCG, low-molecular-weight cationic polymers, and small-interfering RNAs (siRNA).⁷⁹ The supramolecular nanoparticles successfully delivered siRNA and specifically down-regulated target gene, thus showing high efficiency in gene silencing.



Scheme 1.12. Schematic representation of the molecular structure of TA and DNA, and the formation and degradation of TNA hydrogels. Reproduced from reference [78].

1.3.4 Polyphenol-Based Functional Materials for Biomedical Applications

Polyphenol-based materials have been traditionally used as pigments, antioxidants, and inks. Recently, their functional chemical and biological properties have been exploited further for constructing advanced materials for biomedical applications. This section focuses on examples of different biomedical applications, including theranostic sensing or imaging, anticancer, and artificial sporulation, as relevant to polyphenol-based materials.

Through coordination with different metal ions, MPNs have provided a platform for the engineering of phenolic particles with theranostic sensing or imaging functions (**Figure 1.7**).⁸⁰ The bioimaging function of polyphenol-based materials was first demonstrated by incorporating lanthanides (Eu^{III} or Tb^{III}) with co-ligands (2-thenoyltrifluoroacetone (TTA) or acetylacetone (AA)) into MPN capsules for enhanced fluorescent imaging.⁶⁰ Radioactive $^{64}\text{Cu}^{\text{II}}$ -TA capsules have also been used for positron emission tomography (PET) imaging, and

Fe^{III} -, Mn^{II} -, and Gd^{III} -based MPN capsules have been employed as magnetic resonance imaging (MRI) contrast agent (**Figure 1.7a**). Liu et al. reported a versatile platform of photothermal (PT) MPN-based materials by the incorporation of Fe^{III} , V^{III} , and Ru^{III} , respectively. These materials were successfully employed in tumor targeting near-infrared (NIR) PT therapy, photoacoustic (PA) imaging, and T_1 -weighted MRI (**Figure 1.7b**).⁸¹ Moreover, the catalytic activity displayed by Fe^{III} in the MPN material was exploited to generate microbubbles that could be detected by ultrasound imaging for diagnostics (**Figure 1.7c**).⁸²

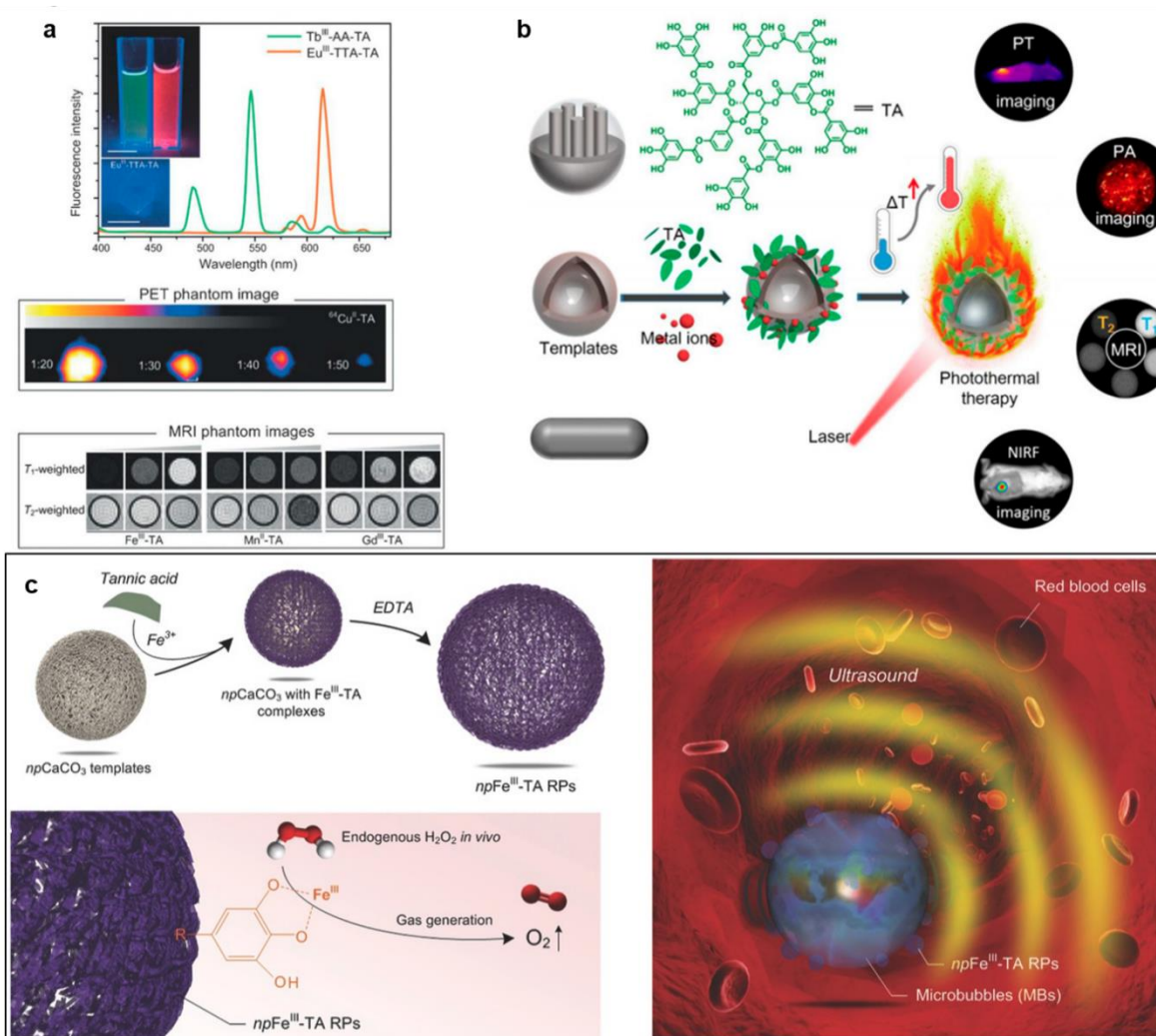


Figure 1.7. Polyphenol-mediated particle systems for theranostic sensing or imaging applications. (a) Various metal ions coordinated in MPN systems for biomedical imaging functions including fluorescence imaging, PET imaging, and MRI relaxation. (b) PT MPN-based nanoparticles for tumor targeting NIR PT therapy coupled with PA imaging and MRI. (c) MPN nanoparticles with Fe^{III} catalytically generating microbubbles for ultrasound imaging. Reproduced from reference [80].

As discussed in Section 1.3.3., the anticancer effects displayed by Herceptin–MNCs were due to Herceptin (trastuzumab), which is a humanized monoclonal antibody against breast cancer tumors.⁶⁸ Herceptin–MNCs showed higher inhibition effects for cancer cell growth and tumor growth than free Herceptin (**Figure 1.8a and b**). The *in vivo* biodistributions of Herceptin–MNCs at 24 h post-injection exhibited higher accumulation in the tumor site and lower accumulation in the healthy organs (**Figure 1.8c**). The intratumoral microdistribution of Herceptin–MNCs showed extravasation and deep tumor penetration (**Figure 1.8d**).

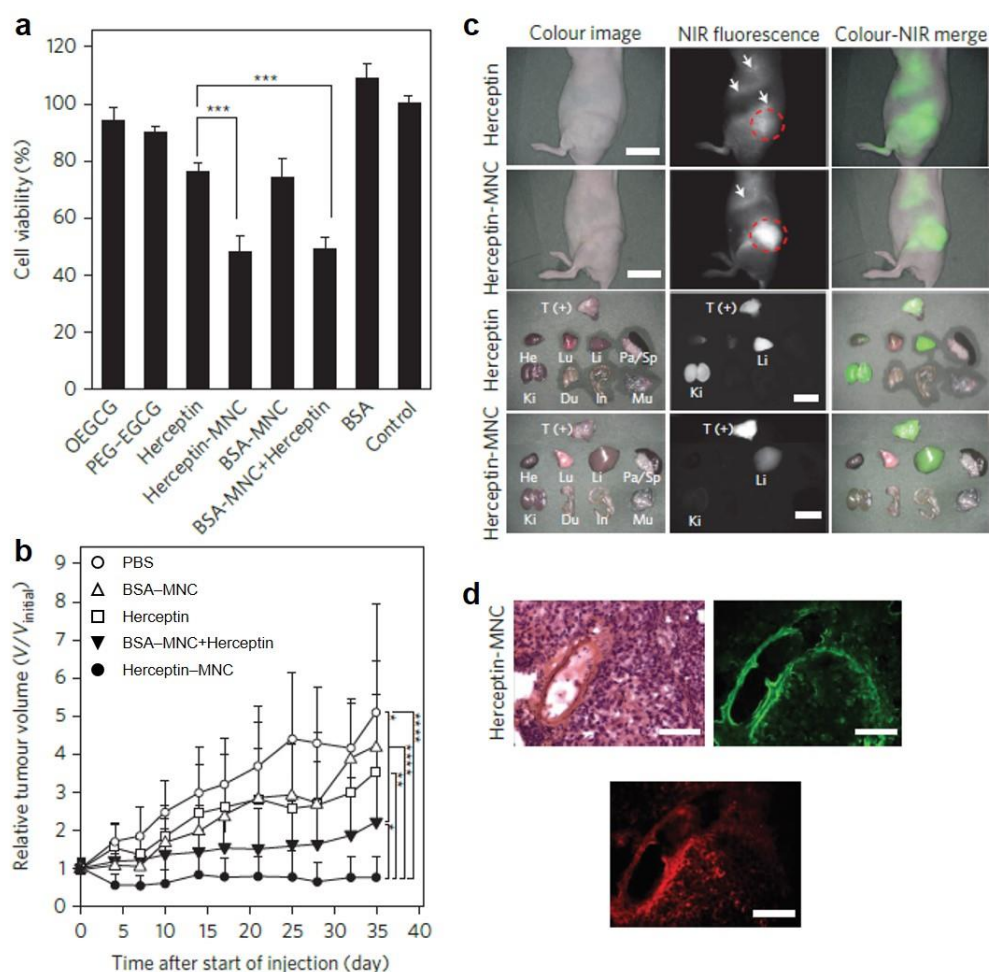


Figure 1.8. Anticancer effect and biodistribution of polyphenol-mediated MNCs. (a) Growth inhibitory effects of Herceptin, Herceptin–MNC, and other drug-free carriers or carrier components groups on BT-474 cells. BSA, bovine serum albumin. (b) Anticancer effect on BT-474-xenografted nude mouse model. PBS, phosphate buffer saline. (c) Biodistribution of Herceptin–MNCs at 24 h post-injection. T (+), positive tumor; He, heart; Lu, lung; Li, liver; Pa, pancreas; Sp, spleen; Ki, kidneys; Du, duodenum; In, intestine; and Mu, muscle. Scale bars: 1 cm. (d) Intratumoral microdistribution of Herceptin–MNCs in BT-474 xenograft tumor. Scale bars: 100 μ m. Adapted from reference [68].

TA-Fe^{III} MPNs were used to encapsulate single-cell native yeasts (**Figure 1.9**).⁸³ Yeast cell division was suppressed after coating with MPN and was restored after disassembly of the coating layer. Moreover, the MPN coatings could prevent the yeast cells from multiple external stimuli such as UV and silver nanoparticles, mimicking the natural sporulation process.

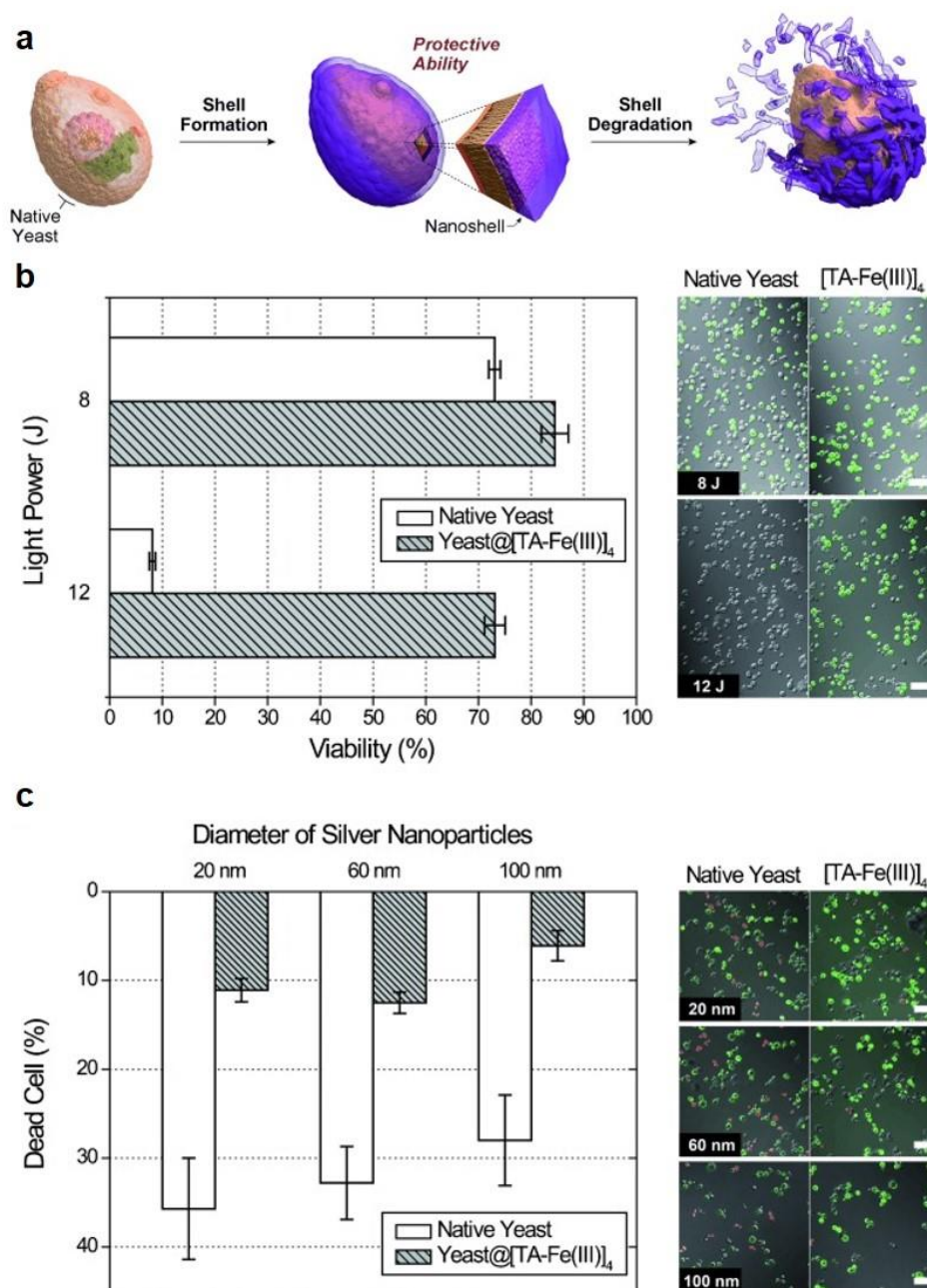


Figure 1.9. Encapsulation of single cell by MPN and artificial sporulation mimicking. (a) Schematic representation of the controlled formation and degradation of a TA-Fe^{III} nanoshell on yeast. Enhanced tolerance of yeast@TA-Fe^{III} against external (b) UV stressors and (c) silver nanoparticles. 8J and 12 J represent light power; 20 nm, 60 nm, and 100 nm represent the diameter of silver NPs. Scale bars: 20 μm. Adapted from reference [83].

1.4 Thesis scope

Throughout history, a broad spectrum of naturally occurring compounds ranging from small molecules, biopolymers to complex substances, such as plant- or animal-derived materials, have been successfully introduced as building blocks for assembly into sophisticated structures and functional materials. This thesis focuses on two sets of naturally occurring building blocks (proteins and polyphenols) and their development into protein–polyphenol networks (PPNs).

The early studies on protein–polyphenol interactions have been largely limited to theoretical research or food science. Chapter 2 presents an extension of these studies to discuss the binding affinity between polypeptides and polyphenols by modern analytical chemistry techniques, primarily focusing on the interactions between the side chains of proteins and polyphenols. The discussion presented is essential in the context of achieving controllable design of protein-based materials.

Versatile and straightforward approaches for protein assembly are in demand with the development of functional protein materials. Chapter 3 reports a universal strategy through the interfacial polyphenol-mediated protein assembly to create a library of PPN materials, including bioactive surface coatings and functional capsules. The engineered PPN capsules are also employed as models to elucidate the dominant interaction(s) between different proteins and polyphenols, as well as being applied in various biomedical applications (*e.g.*, catalysis, fluorescence imaging, and cell targeting).

Intracellular protein delivery by nanoparticles (NPs) has emerged as an attractive strategy in biomedicine. In Chapter 4, a template-mediated supramolecular assembly method is demonstrated to synthesize PPN NPs that are capable of endosomal escape and subsequent protein release in the cytosol. The versatility of this strategy in relation to the nanoparticle size and protein type shows potential in protein therapeutics.

Studying the protein–polyphenol interactions related to actual biological environments is necessary for the future implementation of polyphenols in biomedical applications. Chapter 5 presents a protein corona study on different polyphenol-modified gold nanoparticles (AuNPs). Protein corona composition analysis is used to demonstrate the binding preference of serum proteins with various polyphenols, and cellular uptake behaviors of polyphenol–AuNP have been studied to clarify the role of polyphenols in bio–nano interactions.

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Chapter 2

Interactions between Polypeptides and Polyphenols

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2.1 Aim

The multiple levels of protein structure have complicated the interactions between proteins and polyphenols. Herein, this chapter aims to study the binding affinity between polypeptides and polyphenols to circumvent the 3D structures of proteins and focus on the side chains of proteins. Homopolypeptides with different side chains (positively charged, negatively charged, uncharged, hydrophobic, and special case) are used as models to clarify the different types of interactions with polyphenols. Thus, this chapter is a fundamental study of protein–polyphenol networks, which is critical for controllable design of biomaterials and development of bioapplications.

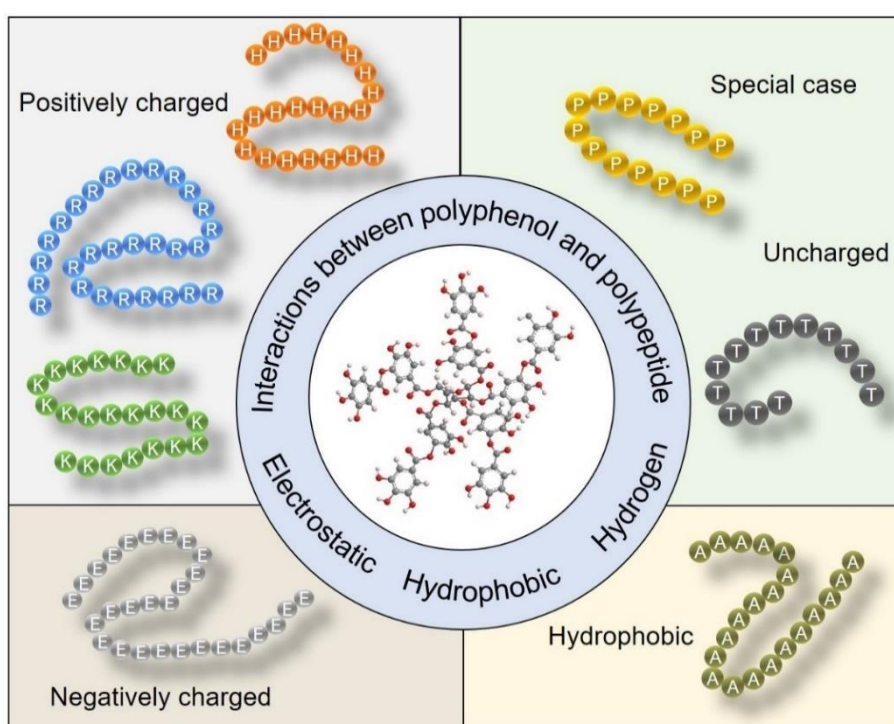
2.2 Introduction

The research interest of protein assembly materials has ranged from the basic understanding of the molecular structure to in-depth investigation of protein interactions.¹ Studying interaction mechanism not only can clearly show the protein assembly process but also can help to customize or upgrade properties of materials. Polyphenols have recently demonstrated their capability of assembling proteins through various noncovalent interactions.² Protein-precipitation was traditionally recognized as the defining characteristic of polyphenols in the field of food science.³ The most acceptable mechanism about protein–polyphenol interactions up to now is based on a combination of hydrophobic interactions and hydrogen bonds.⁴ However, the interactions between protein and polyphenols have rarely been extensively studied by analytic chemistry or molecular simulations due to the complexity of protein structure.⁵

Protein structures can be categorized into four levels (primary, secondary, tertiary, and quaternary) and the physicochemical properties of proteins also vary with different proteins.⁶ Although polyphenols show effective bindings with diverse proteins,⁷ which one of those noncovalent interactions is the dominant interaction and whether all the proteins can bind with polyphenols are still unclear. A polypeptide, a linear chain of amino acids, is the primary constituent of proteins, and it can act as a simple model to help clarify protein interactions. To study the interactions between side chains of proteins and polyphenol is a prerequisite for a comprehensive understanding of protein–polyphenol interactions.

Herein, this chapter studied the binding affinity between polypeptides and polyphenols to clarify the interactions between functional groups of proteins and polyphenols (**Scheme 2.1**).

Seven homopolypeptides with different side chains (*e.g.*, polylysine, polyarginine, polyhistidine, polyglutamic acid, polythreonine, polyproline, and polyalanine) were chosen as model polypeptides and tannic acid (TA) was as a model polyphenol. The binding affinity was tested on quartz crystal microbalance (QCM) by measuring frequency changes of the quartz crystal.⁸ Then, the binding ratios of different polypeptides with TA were calculated to find which side chains show higher bindings with TA. Next, the strong binding affinity between the polypeptides and TA offered free-standing hollow microcapsules through a sacrificial template method.⁹ Thus, this chapter can provide valuable information for the subsequent development of protein–polyphenol networks in this thesis.



Scheme 2.1. Schematic representation of interactions between different polypeptides and polyphenol.

2.3 Experimental Section

2.3.1 Materials

TA, Poly-L-lysine (15–30 kDa), Poly-L-arginine (5–15 kDa), Poly-L-histidine (5–25 kDa), Poly-L-glutamic acid (15–50 kDa), Poly-L-threonine (5–15 kDa), Poly-L-proline (1–10 kDa), Poly-DL-alanine (1–5 kDa), sodium acetate (NaOAc), ethylenediaminetetraacetic acid (EDTA), sodium carbonate (Na_2CO_3), calcium chloride (CaCl_2), and poly(sodium 4-styrenesulfonate) (PSS, $M_w \sim 70$ kDa) were purchased from Sigma-Aldrich. Dulbecco's phosphate-buffered

saline (DPBS) was obtained from Life Technologies. High-purity Milli-Q water with a resistivity of 18.2 M Ω cm was obtained from an inline Millipore RiOs/Origin water purification system.

2.3.2 Characterization

Differential interference contrast (DIC) microscopy images of capsules were acquired with an inverted Olympus IX71 microscope. QCM was performed using a Q-Sense E4 quartz crystal microbalance from Biolin Scientific (Västra Frölunda, Sweden), and gold-coated QCM crystals were purchased from ATA Scientific (Taren Point, Australia). Zeta-potential measurements of capsules were performed in different pH buffers at a concentration of 5 mM on a Malvern Zetasizer Nano ZS instrument (Malvern Instrument, Worcestershire, UK).

2.3.3 Quartz Crystal Microbalance Analysis

Gold-coated QCM crystals were cleaned with Piranha solution (7:3 (v/v) H₂SO₄/H₂O₂) before use. (*Caution! Piranha solution is extremely corrosive and reacts violently with organic compounds! Extreme care should be taken during solution preparation and use!*) The crystals were then extensively rinsed with Milli-Q water and successively rinsed with ethanol and finally dried under a nitrogen stream. Experiments were performed at 25 °C. The pre-cleaned gold-coated sensor crystals were mounted in the certain sample chambers for each measurement, and the baseline was stabilized using Milli-Q water for 10 mins. The frequency values quoted in data analysis are for the fifth overtone only. All sample injection and washing steps were performed at a speed of 100 μ L min⁻¹ by a peristaltic pump. After initially depositing a layer of TA (10 mg mL⁻¹ in Milli-Q water, ~45 min), the crystals were sequentially washed with pH 4.0 buffer (10 mM NaOAc) or pH 7.4 buffer (10 mM PBS) for 20–30 min. Different types of polypeptide solution (1 mg mL⁻¹ in pH 4.0 or pH 7.4 buffer) was sequentially injected and filled the chamber and then incubated ~40 mins until the frequency values stabilized.

2.3.4 Synthesis of Calcium Carbonate Particles

Calcium carbonate (CaCO₃) particles were synthesized as literature.¹⁰ Typically, 20 mL of PSS solution (20 mg mL⁻¹) and 180 mL Milli-Q water were added to a beaker and then stirred vigorously at 2,500 rpm. Then, 2.4 mL of 1 M Na₂CO₃ solution was added to the beaker at room temperature (20 °C) and stirred for 1 min. Subsequently, 4.8 mL of 1 M CaCl₂ solution was added, and the solution was mixed at 2,500 rpm for 60 s. The solution was then incubated for 90 s to achieve the desirable particle size (~3 μ m). The CaCO₃ particles were washed with

Milli-Q water three times to remove excess PSS, Na₂CO₃, and CaCl₂. In the washing step, the particles were spun down by centrifugation (1,000 *g*, 1 min) and the supernatant was removed. PSS was removed by calcination at 450 °C for 2 h.

2.3.5 Formation of Polypeptide–Polyphenol Capsules

CaCO₃ particles (5 mg; 3 µm) were dispersed in Milli-Q water (470 µL). Then, TA (5 µL; 40 mg mL⁻¹, Milli-Q water) was added and incubated with the particles for 10 min under gentle shaking. A polypeptide solution in Milli-Q water (25 µL; 12 mg mL⁻¹) was then added, followed by NaOAc buffer (500 µL; 50 mM, pH 4.0) to a total of 1 mL. The CaCO₃-templated protein/TA mixture was then incubated in an Eppendorf thermomixer at 37 °C and 1,400 rpm for 4 h. The mixture was centrifuged at 1,000 *g* for 60 s and washed with Milli-Q water three times to remove excess protein and TA. Polypeptide–TA capsules were obtained after CaCO₃ core removal with EDTA (100 mM, pH 4.0) and subsequent washing *via* centrifugation (3,000 *g*, 5 min) three times with Milli-Q water.

2.4 Results and Discussion

2.4.1 Homopolypeptides as Models

The chemical properties of seven homopolypeptides are listed in **Table 2.1**, and they are polylysine (PLys), polyarginine (PArg), polyhistidine (PHis), polyglutamic acid (PGlu), polythreonine (PThr), polyproline (PPro), and polyalanine (PAla). PLys, PArg and PHis are positively charged polypeptides, and PGlu is a negatively charged polypeptide. PThr is one uncharged polypeptide with polar side chains. PPro is one special case polypeptide with pyrrolidine moieties, and PAla is one hydrophobic polypeptide. The detailed chemical structures of their constituent amino acid are listed in **Scheme S2.1**.

Table 2.1. Chemical properties of polypeptides.

Polypeptide name	Side chain type	Mw (kDa)	Est. length (aa)	Avg. Mw of amino acid (g mol ⁻¹)
Poly-L-lysine	Positively charged	15–30	120–240	128
Poly-L-arginine		5–15	40–100	156
Poly-L-histidine		5–25	40–180	137
Poly-L-glutamic acid	Negatively charged	15–50	120–400	129
Poly-L-threonine	Uncharged	5–15	50–150	101
Poly-L-proline	Special case	1–10	10–100	98
Poly-DL-alanine	Hydrophobic	1–5	15–70	72

First, the mass concentration ratios of polypeptides (*i.e.*, PThr, PAla, and PPro) and TA were changed to test the size distribution of their mixture by dynamic light scattering (DLS). The size distribution results from DLS represent all different states (from nm to μm) of polypeptide–TA complexation (**Figure S2.1**). PThr and PAla can form aggregates with TA, and PPro shows the potential to create self-assembly nanoparticles with TA, suggesting their different complexation behaviors.

2.4.2 Binding Affinity Test by Quartz Crystal Microbalance

QCM was then used to detect and monitor molecular interactions in real-time. QCM measures the change in frequency of a quartz crystal resonator to reflect the mass variation per unit area. Frequency changes can represent molecular adsorption, binding, and detachment. First, the binding kinetics of TA with charged polypeptides (*i.e.*, PLys, PArg, PGlu and PHis) were examined under pH 7.4 and pH 4. TA solution was first injected into the chamber of QCM and then incubated a longer time to deposit a layer of TA. After washing with the corresponding buffers, different polypeptides were sequentially injected until the frequency values stabilized.

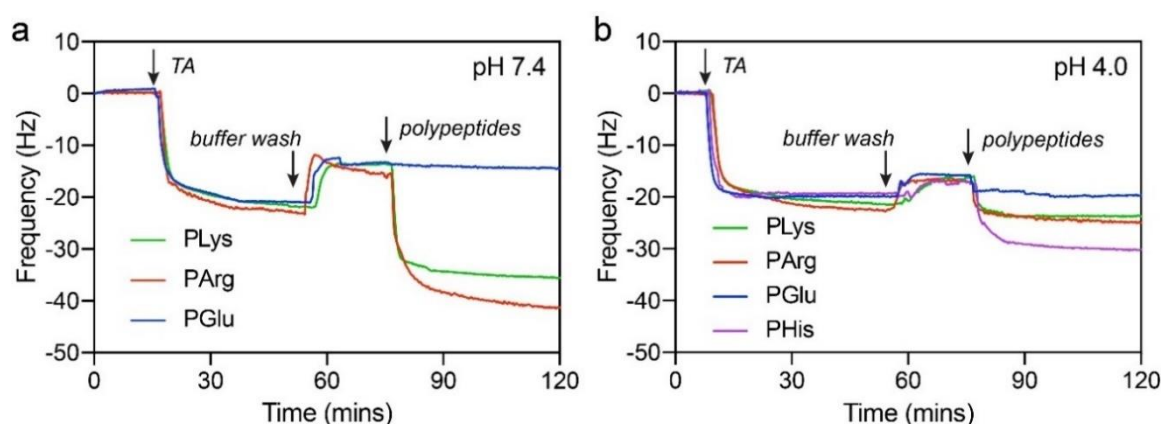


Figure 2.1. The binding kinetics of TA with charged polypeptides under pH 7.4 (a) and pH 4.0 (b) were monitored by QCM.

The binding kinetics exhibited that the positively charged polypeptides showed high binding with TA, and the negatively charged polypeptide showed low binding with TA (**Figure 2.1**). One theoretical principle of QCM is Sauerbrey equation that shows the linear relation between frequency change and mass change of the quartz crystal.^{11,12} The binding ratios (mass) of the charged polypeptides with TA that were calculated upon the frequency change observed on QCM revealed a clear distinction of the binding strength among different polypeptides (**Figure S2.2**). Interestingly, charged polypeptides showed pH-responsive binding with TA. Positively charged side chains showed increased binding to TA from low pH to high pH, probably owing

to the deprotonation of TA under high pH.¹³ Negatively charged side chains showed slightly increased binding under low pH because the protonation of P_{Glu} under pH 4.0 may reduce its electrostatic repulsion with TA.

Not just the charged side chains, uncharged side chains were utilized by the same method to study with TA under different pH (**Figure 2.2**). P_{Pro} with pyrrolidine moieties showed the highest binding with TA, and hydrophobic P_{Ala} offered the weak binding with TA. And all uncharged side chains showed pH-nonresponsive binding behavior (**Figure S2.3**), which is attributed to the incapability of protonation or deprotonation of their functional side chains.

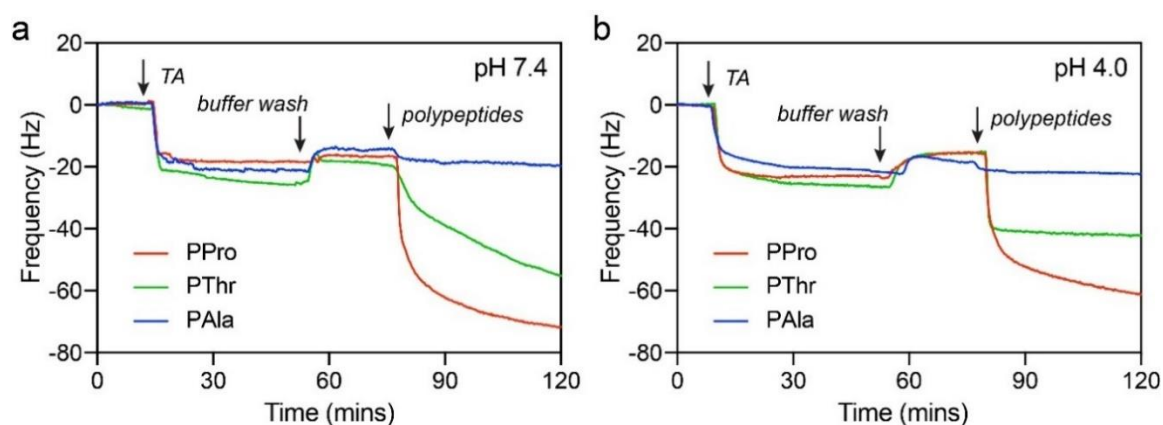


Figure 2.2. The binding kinetics of TA with uncharged, hydrophobic, and special case polypeptides under pH 7.4 (a) and pH 4.0 (b) were monitored by QCM.

Due to the variation of molecular weight of polypeptides, the mass ratio cannot be directly compared across different polypeptides. Then, the molar binding ratios of amino acid with TA were calculated, in which Pro showed the highest binding affinity owing to the strong aromatic–proline interactions,¹⁴ and Glu offered the weakest binding.

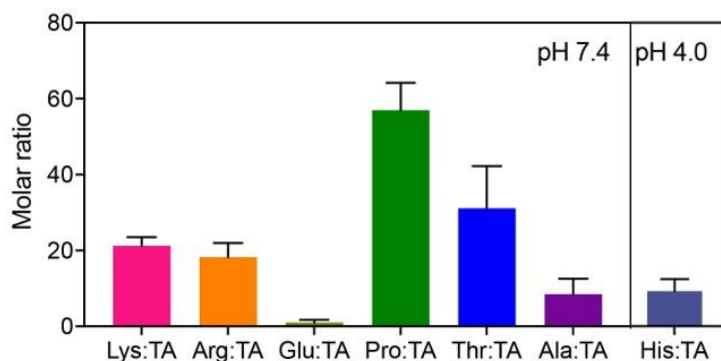


Figure 2.3. The molar binding ratio of amino acids from polypeptides with TA calculated from their frequency changes on QCM and their molecular weights. Data are represented as mean $\pm$ standard error ($n = 3$).

2.4.3 Formation of Polypeptide–Polyphenol Capsules

Next, the possibility of whether these bindings can be utilized to assemble nanostructures was considered. Five polypeptides including charged or uncharged side chains were assembled with TA to successfully form hollow capsules, which indicates the interactions are strong enough to hold the free-standing structures (**Figure 2.4a** and **2.4b**). The zeta-potential results showed that the surface charge of all formed capsules varied with different polypeptides (**Figure 2.4c**), demonstrating the potential for customizable design of polypeptide-based materials.

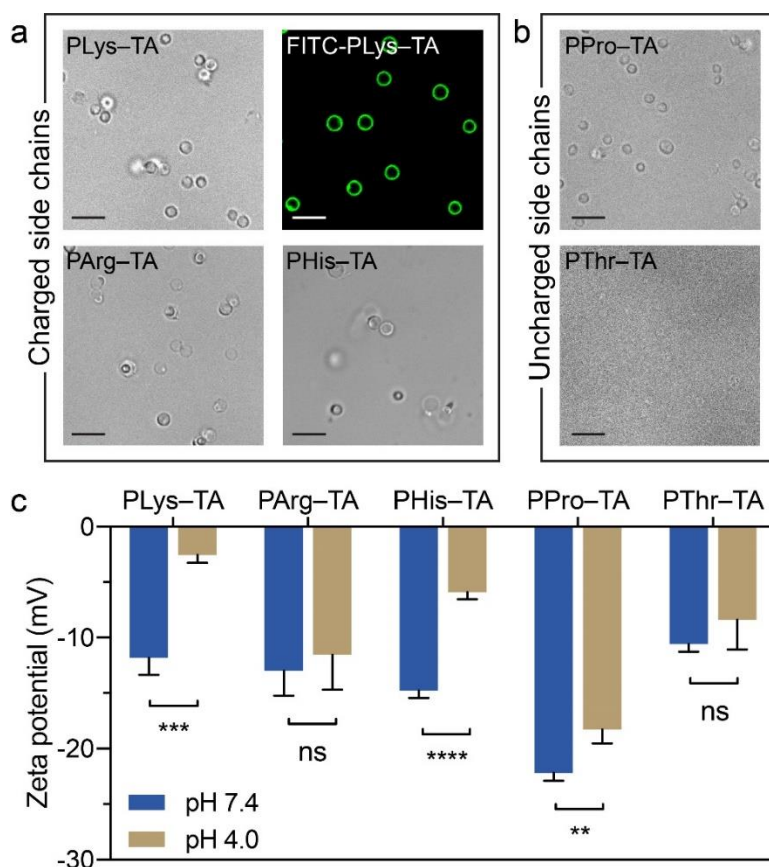


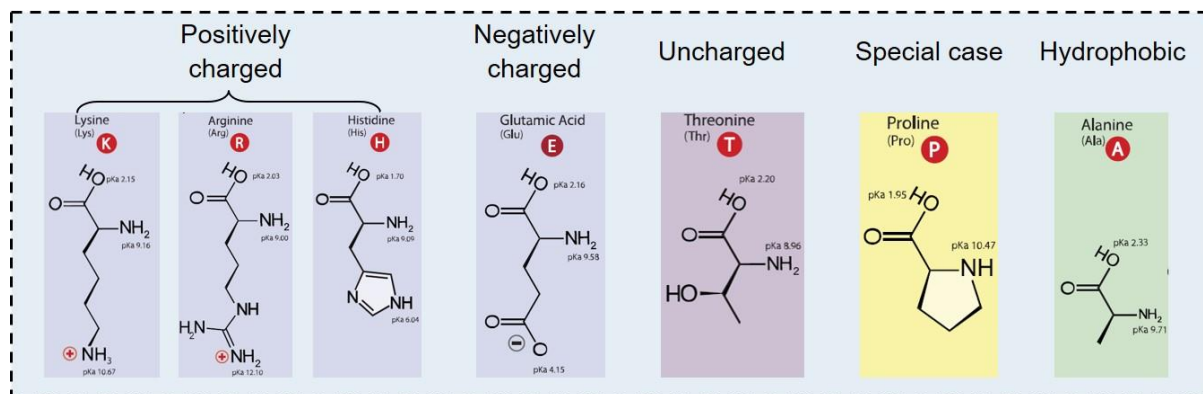
Figure 2.4. Formation of polypeptide–TA capsules. (a, b) Differential interference contrast microscopy and fluorescence microscopy images of different polypeptide–TA capsules. Scale bars: 10 μm . (c) Zeta-potential results of different polypeptide–TA capsules. Data are represented as mean $\pm$ standard error ($n = 3$). Statistical significance was determined by a two-tailed t -test and is reported as ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.5 Conclusion

In conclusion, the binding affinity of various polypeptides with polyphenol (TA) was studied by QCM. Positively charged side chains showed high binding relationships to TA compared to

negatively charged side chains due to the electrostatic interactions. Uncharged side chains and hydrophobic side chains also bound with TA by hydrogen bonding and hydrophobic interactions, respectively. Polyproline shows the highest binding with TA among all tested polypeptides. Moreover, polypeptides exhibiting strong binding with TA can form hollow capsules with TA, suggesting the interactions are stable enough to hold the free-standing structure. Overall, this chapter indicates that polyphenol–protein networks could be formed from nearly any protein, as at least one of those amino acids will be present in any protein. Besides, there are many functional polypeptides available to be developed into smart polypeptide–polyphenol nanocarriers to deliver active polypeptides in the future.¹⁵ Stimuli-responsive polypeptide–polyphenol nanomaterials can also be developed by exploiting their interactive forces, which will afford smarter nanocarriers for bio-applications. And in this chapter, only 7 specific polypeptides were examined, and later more polypeptides can be tested to compare their binding strength with polyphenols to get a comprehensive understanding of protein–polyphenol interactions.

2.6 Supporting Information



Scheme S2.1. Chemical structure of seven polypeptides.

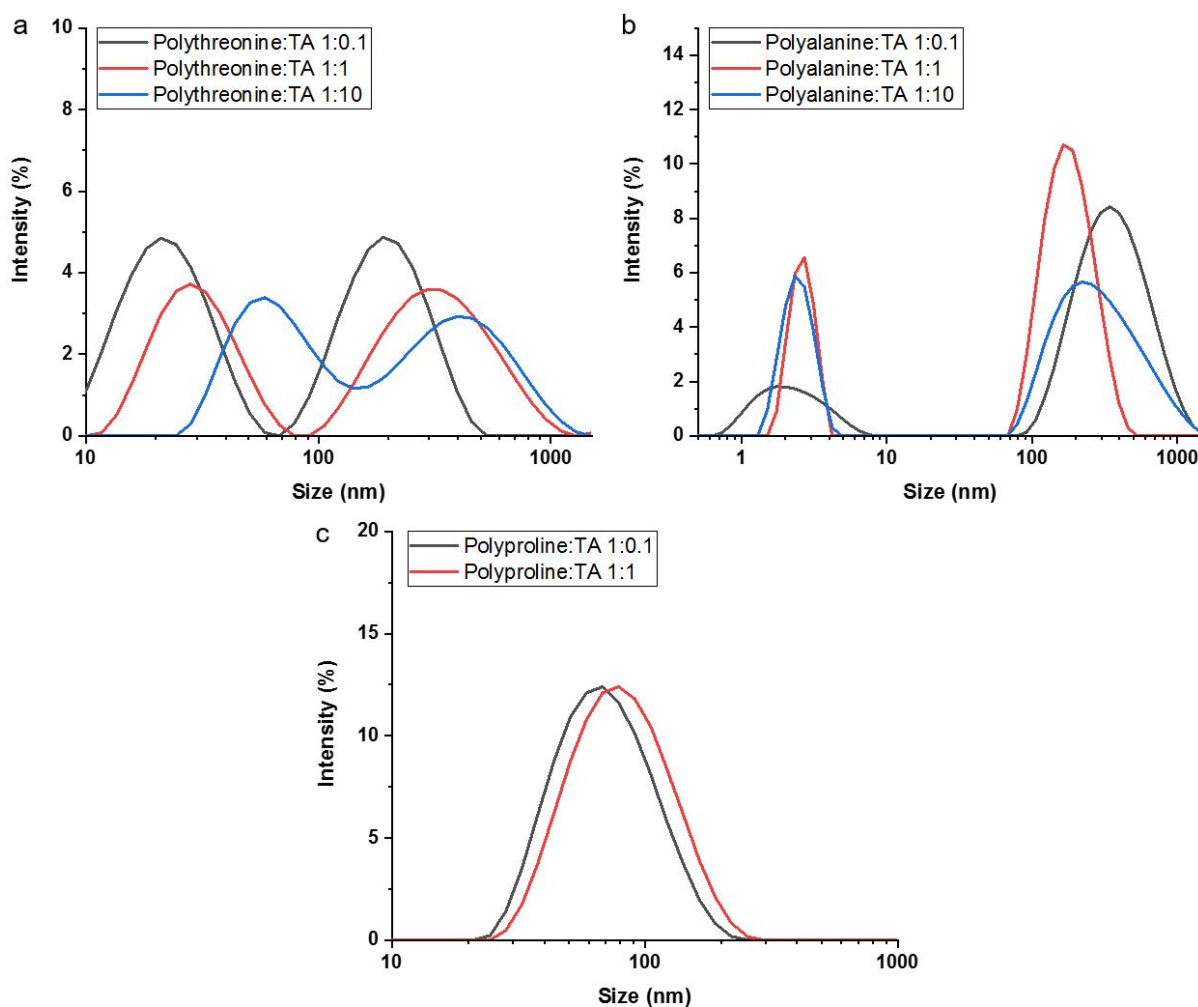


Figure S2.1. Size distribution (intensity) of different polypeptide-TA complexes formed at different mass concentrations as measured by DLS. (a) Polythreonine (b) Polyalanine (c) Polyproline. All three polypeptides concentration is 0.5 mg mL^{-1} .

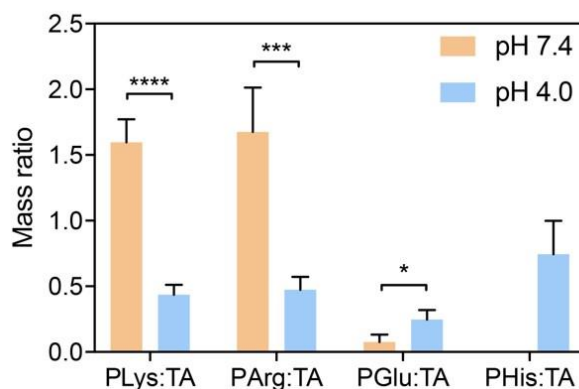


Figure S2.2. The binding ratio (mass) of the charged polypeptides with TA calculated from their frequency change on QCM. Data are represented as mean $\pm$ standard error ($n = 3$). Statistical significance was determined by a two-tailed t -test and is reported as * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

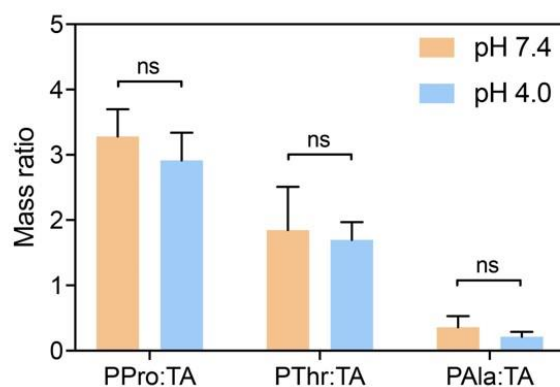


Figure S2.3. The binding ratio (mass) of the uncharged polypeptides with TA calculated from their frequency change on QCM. Data are represented as mean $\pm$ standard error ($n = 3$). Statistical significance was determined by a two-tailed t -test and is reported as ns $p > 0.05$.

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Chapter 3

Polyphenol-Mediated Assembly of Proteins for Engineering Functional Materials

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3.1 Manuscript



Research Articles

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Polyphenol-Mediated Assembly of Proteins for Engineering Functional Materials

Yiyuan Han, Zhixing Lin, Jiajing Zhou, Gyeongwon Yun, Rui Guo, Joseph J. Richardson, and Frank Caruso*

Abstract: Functional materials composed of proteins have attracted much interest owing to the inherent and diverse functionality of proteins. However, establishing general techniques for assembling proteins into nanomaterials is challenging owing to the complex physicochemical nature and potential denaturation of proteins. Here, a simple, versatile strategy is introduced to fabricate functional protein assemblies through the interfacial assembly of proteins and polyphenols (e.g., tannic acid) on various substrates (organic, inorganic, and biological). The dominant interactions (hydrogen-bonding, hydrophobic, and ionic) between the proteins and tannic acid were elucidated; most proteins undergo multiple noncovalent stabilizing interactions with polyphenols, which can be used to engineer responsiveness into the assemblies. The proteins retain their structure and function within the assemblies, thereby enabling their use in various applications (e.g., catalysis, fluorescence imaging, and cell targeting).

Introduction

Naturally occurring building blocks that can assemble into functional materials have attracted considerable scientific interest.^[1] Proteins, as biologically functional macromolecules involved in many fundamental processes of living organisms, can mediate specific interactions with diverse macromolecules for various applications, including catalysis, biosensing, diagnostics, and therapy.^[2] However, protein assembly is often achieved on a case-by-case basis owing to the sensitivity of proteins to denaturation and the chemical specificity of assembly routes.^[3] In addition, simple and versatile strategies for assembling proteins on substrates are limited because the noncovalent interactions between proteins and substrates are often insufficiently strong to effectively tether them to surfaces.^[4] Therefore, developing versatile protein-assembly strategies are important to: 1) deposit proteins onto various surfaces of different sizes, aspect ratios, and material classes; 2) assemble a range of proteins; 3) preserve the structure and function of the proteins after assembly; and 4) allow various

physicochemical properties to be introduced into the assemblies.^[5]

In the present study, we developed a general platform for assembling functional protein materials through a simple, versatile polyphenol-mediated coating strategy. Polyphenols [e.g., tannic acid (TA)] are highly adherent natural compounds that can form conjugates with various macromolecules, particularly proteins.^[6] Various proteins (more than 10 types, including enzymes, antibodies, transport proteins, and fluorescent proteins) were assembled onto diverse substrates of different composition, size, and shape (Figure 1a) with different phenolics (e.g., TA, gallic acid, and epigallocatechin gallate; Figure S1 in the Supporting Information). Polyphenols exhibit multiple interactions with proteins, including hydrogen-bonding, hydrophobic, and ionic interactions (Figure 1b).^[7] These interactions allow robust and responsive protein–polyphenol networks to be deposited, for example, free-standing protein–polyphenol capsules are obtained after coating and dissolution of spherical sacrificial templates (Figure 1c).^[8] Owing to their purely organic nature, these protein-based systems can be exploited for various biomedical applications.^[9] We demonstrate that their bioactive functions can be applied to enzyme catalysis, cell targeting, and tandem reactions (involving multicomponent functional materials; Figure 1d). Moreover, these protein–polyphenol capsules enable elucidation of the dominant interactions between the different proteins and TA, which is not readily possible by examining unstructured polyphenol–protein complexes in solution, because individual proteins in solution can be difficult to handle.^[10] By applying this knowledge, pH-responsive capsules with different shrinking–swelling behaviors, which are dependent on the isoelectric point (pI) of the proteins, can be engineered. This work introduces 1) a toolbox for protein assembly, 2) a model to study the interactions between proteins and other macromolecules, and 3) a versatile platform for diverse applications in biotechnology.^[4,11]

Results and Discussion

To demonstrate the versatility of the present method, protein–polyphenol assemblies were deposited on substrates of different composition, size, and shape (Figure 1e–h). Bovine serum albumin (BSA), a globular protein commonly found in blood plasma, was used as a model protein for protein–polyphenol assembly. Fluorescein isothiocyanate (FITC)-labeled BSA (FITC-BSA) was also used to visualize

*Y. Han, Z. Lin, Dr. J. Zhou, Dr. G. Yun, R. Guo, Dr. J. J. Richardson, Prof. F. Caruso

ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Department of Chemical Engineering
The University of Melbourne, Parkville, Victoria 3010 (Australia)
E-mail: fcaruso@unimelb.edu.au

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
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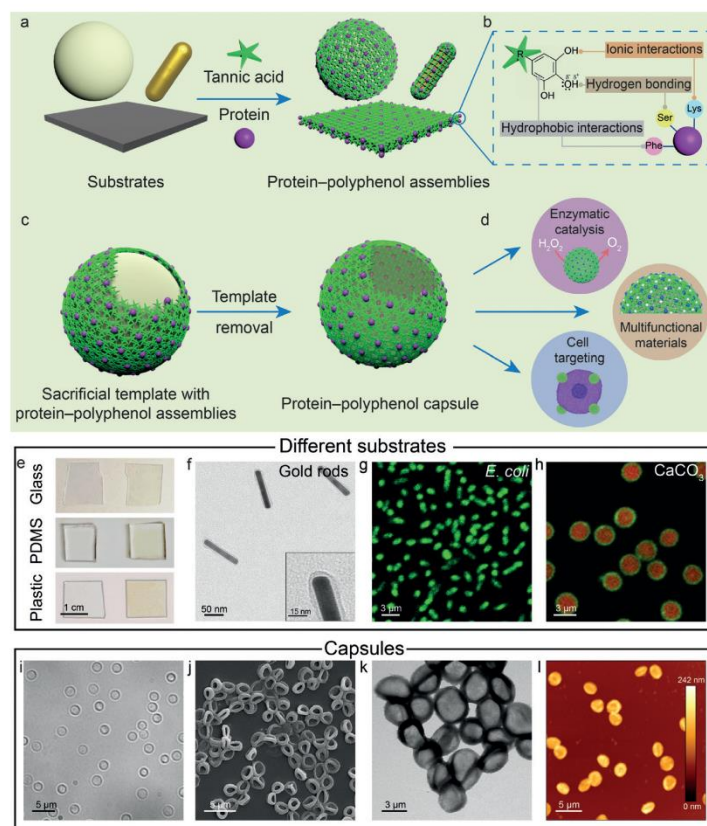


Figure 1. a) The interfacial assembly process on different substrates. b) Different interactions between proteins and polyphenols exemplified for the representative amino acids Lys (lysine), Ser (serine), and Phe (phenylalanine). c) Coating of sacrificial template and subsequent capsule formation. d) Examples of biotechnological applications of protein-polyphenol capsules. e) Photographs of planar substrates before (left) and after (right) coating with FITC-BSA-TA assemblies. PDMS = polydimethylsiloxane. f–h) Microscopy images of BSA-TA assemblies on various substrates: TEM image for gold nanorods (f); CLSM image for *Escherichia coli* (g); and TRITC-dextran-loaded CaCO₃ particles (h; particles in red, FITC-BSA-TA coating in green). i–l) Microscopy images of BSA-TA capsules formed using sacrificial CaCO₃ templates: DIC (i), SEM (j), TEM (k), and AFM (l) images.

the assemblies. Simply, by depositing TA on a planar substrate (e.g., glass, polydimethylsiloxane (PDMS), or plastic on a centimeter scale) and subsequently adding FITC-BSA in one pot without washing, protein-polyphenol complexes (FITC-BSA-TA) were rapidly deposited, as visualized by a change in the color of the substrate after coating (Figure 1e). BSA-TA was also deposited on micro- and nanoparticles. As observed from the transmission electron microscopy (TEM) image in Figure 1f, the gold nanorods featured a distinct shell after coating. From the confocal laser scanning microscopy (CLSM) image in Figure 1g, h, FITC-BSA-TA coatings were observed after deposition on live *Escherichia coli* (*E. coli*) bacteria or tetramethylrhodamine (TRITC)-dextran-loaded calcium carbonate (CaCO₃) particles. Other organic and inorganic particles were also coated with FITC-BSA-TA, including polystyrene and silica (Figure S2). Collectively, these results suggest that the present protein-assembly strategy is applicable to diverse substrates across multiple length scales (20 nm to 1 cm).

To study the physicochemical properties of the assemblies, CaCO₃ particles (3 μ m in diameter) were used as sacrificial templates for preparing free-standing protein-polyphenol capsules.^[12] The surface of the uncoated CaCO₃ particles was rough and porous. In contrast, after BSA-TA assembly, the particle surfaces were relatively smooth and appeared less porous, thus demonstrating that the interfacial assembly process was successful (Figure S3). Removal of the CaCO₃ template with ethylenediaminetetraacetic acid (EDTA) generated spherical and monodisperse BSA-TA microcapsules, as observed from the differential interference contrast (DIC) microscopy image in Figure 1i, thus highlighting the robust nature of the assemblies. Compared with the BSA-TA-coated CaCO₃ core-shell particles (before template removal), the BSA-TA capsules demonstrated lower light scattering, thus indicating the successful removal of the solid CaCO₃ core (Figure S4a). Both the FITC-BSA-TA-coated CaCO₃ core-shell particles and FITC-BSA-TA capsules showed comparable fluorescence, thus suggesting that the template-removal

process did not cause significant loss of BSA (Figure S4b). Scanning electron microscopy (SEM), TEM, and atomic force microscopy (AFM) showed collapsed (air-dried) capsules, and excess polyphenol–protein aggregates, unbound or attached to the BSA–TA capsules, were not detected (Figure 1j–l). The shell thickness of the BSA–TA capsules was approximately 40 nm, as measured by AFM (Figure S5), and circular dichroism demonstrated that the conformation of BSA did not change after assembly^[13] (Figure S6), thus indicating no loss of protein functionality.

A library of functional protein–polyphenol assemblies was subsequently established using TA and a variety of proteins with different pI, molecular weight (M_w), and aliphatic index (Figure 2a and Table S1),^[14] which allowed in-depth characterization of the influence of the physicochemical properties of the proteins on the properties of the protein–polyphenol assemblies. The functional proteins studied include lysozyme (LYZ), glucose oxidase (GOx), pepsin, horseradish peroxidase (HRP), hemoglobin (Hgb), cytochrome C (CYC), immunoglobulin G (IgG), fibrinogen (FGN), insulin, and green fluorescent protein (GFP; Figure 2b). LYZ, GOx, HRP, and pepsin are enzymes. CYC and Hgb are essential transport proteins. IgG is a class of antibodies and FGN is responsible for blood clotting (protection proteins). Insulin is a peptide hormone and GFP is a fluorescent protein that is often used as a gene expression marker (regulatory proteins). Hemoproteins (e.g., CYC and HRP) were first

chosen to characterize the possible interactions between TA and the metal centers of the heme group because polyphenols are known to chelate metals.^[15] Interestingly, the mixture of hemoproteins with TA did not show the characteristic ligand-to-metal charge-transfer band for polyphenol/ Fe^{III} complexes by UV/Vis spectroscopy (Figure S7), and the CYC–TA capsules featured a heme group peak at around 410 nm, similar to free CYC (Figure S8a), thus suggesting that TA did not competitively chelate the bound iron.^[16] The Fourier-transform infrared spectrum of the CYC–TA capsules demonstrated peak overlaps with the spectra of free TA and free CYC (Figure S8b). Energy-dispersive X-ray mapping results demonstrated that the CYC–TA capsules comprised four elements making up CYC and TA, namely C, N, O, and Fe (Figure S9). The loading efficiency of HRP in the HRP–TA capsules was around 80 % (Figure S10 and Table S2), as determined from the iron content in HRP measured by inductively-coupled-plasma optical emission spectrometry. The thicknesses of the protein–TA capsules prepared from proteins other than BSA were between 50 and 90 nm (Figure S11). Importantly, all the proteins studied herein successfully assembled at interfaces and formed conformational and robust films, likely due to diverse interactions with TA.^[17]

The structural units of proteins are amino acids, which possess different electrically charged, polar uncharged, and hydrophobic side chains. These functional side chains of amino acids can potentially interact with polyphenols through

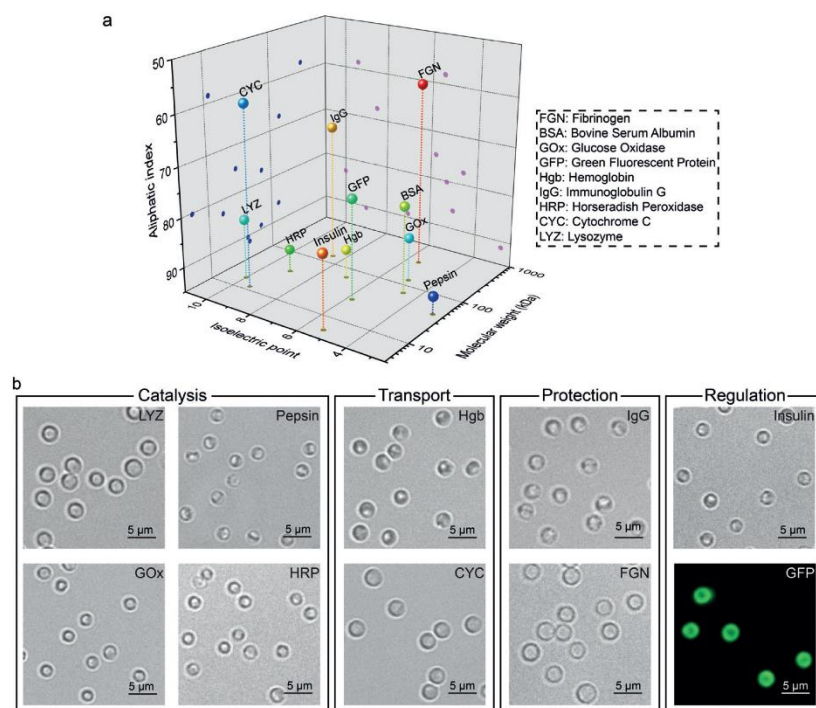


Figure 2. a) Physicochemical properties of different proteins. (x axis: isoelectric point; y axis: molecular weight; and z axis: aliphatic index). b) DIC microscopy images of different protein–TA capsules and a fluorescence microscopy image of GFP–TA capsules.

hydrogen bonding, hydrophobic interactions, and/or ionic interactions (Figure 3a). To investigate how these three interactions govern the stability of different protein-TA assemblies, urea, Tween 20, or sodium chloride (NaCl) was added to each of the five model protein-polyphenol capsules (LYZ-TA, IgG-TA, Hgb-TA, GOx-TA, and CYC-TA) to study disassembly over time.^[6b] These proteins were chosen as model systems because they have different pI, M_w , and aliphatic index values. Urea, which can participate in the formation of strong hydrogen bonds (and therefore break weaker hydrogen bonds),^[18] effectively disassembled the IgG-TA and GOx-TA capsules (within 2 h), thus indicating that these two types of protein-polyphenol assemblies are stabilized by hydrogen bonds (Figure S12a). In contrast, 90, 89, and 69% of the LYZ-TA, CYC-TA, and Hgb-TA capsules, respectively, remained after incubation with 100 mM urea for 42 h, thus suggesting that different or additional intermolecular forces stabilize these systems. Tween 20 is a nonionic surfactant that can form hydrophobic interactions.^[19] CYC-TA, LYZ-TA, and IgG-TA capsules were completely disassembled by 100 mM Tween 20 within

5 min of incubation as a result of the hydrophobic interactions being disrupted. In contrast, 50 and 75% of the GOx-TA and Hgb-TA capsules remained intact in Tween 20 solution over 40 h of incubation (Figure S12b). Incubation of the capsules in 100 mM NaCl resulted in the disassembly of all five protein-polyphenol capsules to varying extents over 90 h, owing to ionic shielding; among the capsules studied, the GOx-TA capsules showed the highest disassembly rate, that is, the lowest percentage of capsules remaining (37%), after 90 h (Figure S12c). Generally, the type of protein determines which interactions are present in the assemblies (Figure 3b and Figure S13). For instance, hydrophobic interactions are the dominant stabilizers for LYZ-TA and CYC-TA, whereas they are the least dominant interactions between Hgb and TA. In IgG-TA, the most dominant interactions are hydrogen bonding and hydrophobic interactions. In GOx-TA, the interactions are governed by all three types of interactions.

To gain deeper insight into the nature of the protein-polyphenol interactions in the assemblies, we further analyzed the relationship between the dominant interactions and the physicochemical properties of the proteins such as the size

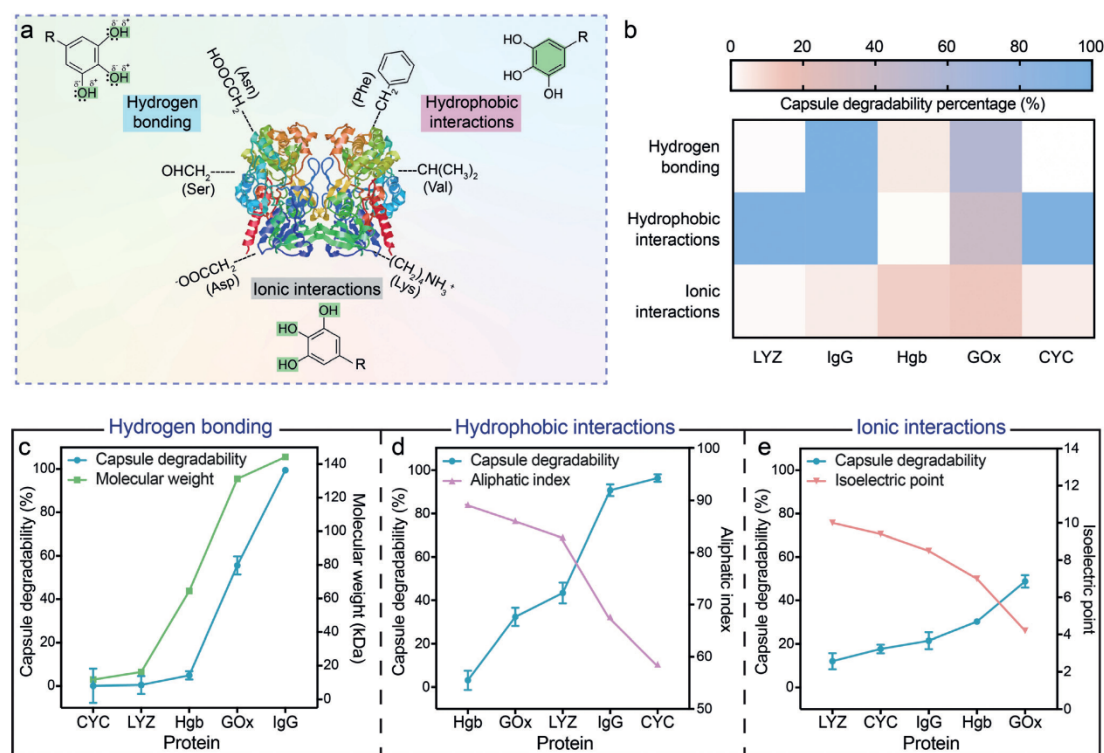


Figure 3. a) Schematic diagram of the possible interactions between the functional groups of polyphenols and proteins. Asn = asparagine, Val = valine, Asp = aspartic acid. b) Percentage of protein-polyphenol capsules (LYZ, IgG, Hgb, GOx, and CYC) degraded after 1 h of incubation with 100 mM of urea, Tween 20, or NaCl, highlighting the dominant interactions between the different proteins and TA. c-e) Relationship between the dominant interactions and physicochemical properties of the proteins, as analyzed from the capsule degradability of CYC-TA, LYZ-TA, Hgb-TA, GOx-TA, and IgG-TA: hydrogen bonding versus molecular weight, incubation for 1 h with 100 mM urea (c); hydrophobic interactions versus aliphatic index, incubation for 2 min with 100 mM Tween 20 (d); and ionic interactions versus isoelectric point, incubation for 19 h with 100 mM NaCl at pH $\approx$ 7 (e). Data are shown as the mean $\pm$ SD ($N = 3$).

(M_w), aliphatic index, and pI. The three key findings are as follows. 1) The strength of hydrogen bonding between polyphenols and proteins is likely based on the M_w or size of the proteins because the peptide backbone and the majority of the exposed protein surface (polar amino acids) are hydrophilic. For example, the molecular size of the proteins increases in the order $CYC < LYZ < Hgb < GOx < IgG$, and the disassembly rate of the respective protein-TA capsules also increases in the same order as hydrogen bonds between the proteins and hydroxy groups of the catechol/gallol moieties in the polyphenols are disrupted (Figure 3c). 2) Proteins with high aliphatic indexes, that is, those containing more aliphatic and hydrophobic side chains (such as alanine, valine, isoleucine, and leucine), can form stronger hydrophobic interactions with the aromatic groups of polyphenols compared with proteins with low aliphatic indexes (low hydrophobicity). For example, the interactions of polyphenols with high-aliphatic-index proteins, such as Hgb and GOx, are more difficult to disrupt using surfactants when compared with those of polyphenols with low-aliphatic-index proteins such as CYC and IgG (Figure 3d). 3) The strength of ionic interactions between proteins and polyphenols depends on the charge of the protein. For instance, the pI of the proteins increases in the order of $GOx < Hgb < IgG < CYC < LYZ$, which correlates to the relatively more positively charged side chains in the protein and thus enhanced ionic interactions with the deprotonated hydroxy groups of the catechol/gallol moieties of the polyphenols (Figure 3c). These results highlight the complex nature of the interaction between polyphenols and different proteins at the molecular level.

The protein-polyphenol assemblies exhibited distinct and tunable pH-responsive properties, owing to the various pK_a values of the hydroxy groups of TA and the pIs of the different proteins. For example, BSA-TA capsules swelled from 2.1 to 4.2 μm when the environmental pH increased from 4.0 to 7.4 and shrank to their original size when the pH was lowered to 4.0 (Figure 4a). This swelling/shrinking process could be repeated reproducibly over multiple cycles (Figure 4b). TA and BSA both possess a net negative charge when the pH rises above the pI of BSA (4.6), which leads to electrostatic repulsion, and thus swelling and eventual dissociation. The other protein-TA capsules also showed reversible pH-responsive swelling and shrinking behaviors when the pI values of the proteins (pepsin: 2.9, CYC: 9.6) were within the swelling/shrinking values (pepsin: pH 2.8–6.0, CYC: pH 4.0–10.0), which supports our proposed mechanism (Figure 4b and Figure S14). In addition, the zeta potential of the pepsin-TA, BSA-TA, and CYC-TA capsules changed under different pH, further suggesting the importance of the protonation state of the proteins (Figure 4c). The thickness of the BSA-TA capsules decreased from around 50 nm at pH 4.0 to around 30 nm at pH 7.4, which demonstrates that the swelling behavior expands the films to accommodate the increase in volume of the capsule (Figure 4d and Figure S15). Given that such pH-responsiveness can be desirable for drug-delivery applications, the molecular permeability of the CYC-TA capsules was assessed using FITC-dextran of different molecular weights as the model cargo. As observed in Figure 4e, the permeability of the CYC-TA capsules was pH dependent, thus suggesting that these assemblies could be

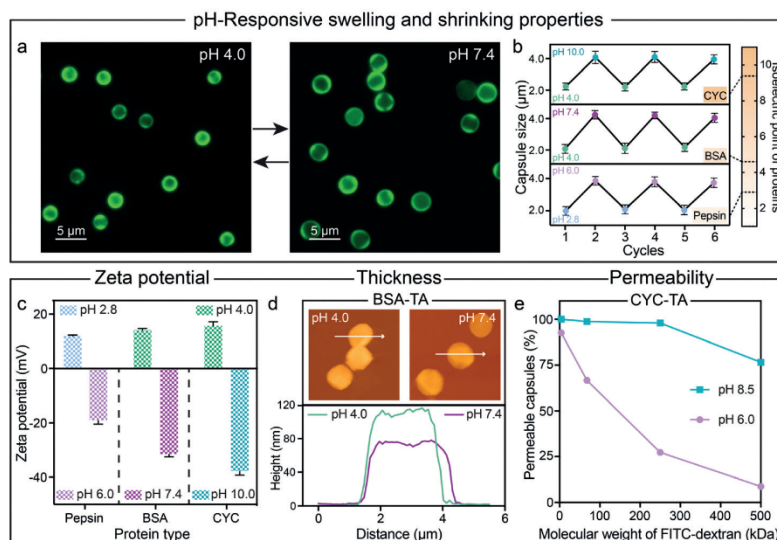


Figure 4. pH-Responsiveness of protein-polyphenol assemblies. a) Fluorescence microscopy images of the BSA-TA capsules at pH 4.0 and pH 7.4. b) Changes in the diameters of the pepsin-TA, BSA-TA, and CYC-TA capsules as a function of pH wash cycle. The diameters of 20 capsules were measured using DIC microscopy, and data are shown as the mean $\pm$ SD. c) Zeta potential values of the pepsin-TA, BSA-TA, and CYC-TA capsules at different pH values. Data are shown as the mean $\pm$ SD. d) AFM images and corresponding height measurements of the BSA-TA capsules at pH 4.0 and 7.4. e) Percentage of permeable capsules plotted against FITC-dextran of different molecular weight at pH 8.5 and 6.0. The permeability of 100 capsules was measured using CLSM.

used to load and release drugs under specific pH environments. For instance, around 25 % of the CYC-TA capsules were permeable to 250 kDa FITC-dextran at pH 6.0, whereas nearly 100 % of the capsules were permeable at pH 8.5 (Figure 4f and Figure S16), which corresponds to higher permeability when the capsules are swollen. When considering physiological pH changes, such as the stomach (pH 1.0–3.0), blood (pH 7.4), or duodenum (pH 4.8–8.2),^[20] we envision that our polyphenol–protein assemblies could serve as smart delivery systems for various biomedical applications.

The potential biotechnological applications of the protein–polyphenol assemblies were examined. The functionality of the protein–polyphenol assemblies for catalysis was first investigated. HRP is a common enzyme that catalyzes the oxidation of various organic substrates in the presence of H_2O_2 .^[21] Specifically, the conversion of amplex red (AR) into resorufin (RS), which has a strong absorbance at 560 nm,^[22] in the presence of the HRP-TA assemblies (core-shell particles and capsules) was examined (Figure 5a). The HRP-TA capsules showed comparable catalytic activity to free HRP, thus suggesting that the enzymatic activity was not significantly affected after assembly (Figure 5b and Figure S17).^[23] Moreover, these capsules showed higher catalytic activity (ca. 25 %) than the HRP-TA core-shell particles, which is attributed to the likely improved mass transfer in the hollow (capsule) system. In addition, the enzymes in the HRP-TA capsules displayed excellent recyclability, where more than

68 % of the initial HRP activity was retained after five cycles of catalysis and collection (Figure 5c).

Multicomponent systems composed of GOx and HRP (GOx&HRP-TA) were engineered and investigated to conduct a cascade reaction.^[24] Specifically, GOx can catalyze the oxidation and hydrolysis of β -D-glucose into gluconic acid and H_2O_2 , and HRP can catalyze AR oxidation to RS in the presence of the generated H_2O_2 (Figure 5d). The GOx&HRP-TA capsules showed successful colorimetric reaction with glucose in contrast to the (single-component) GOx-TA and HRP-TA capsules (Figure 5e). Therefore, the current approach adopted to prepare the multicomponent and multifunctional protein–polyphenol assemblies presents a simple and versatile strategy to design intricate biomimetic systems.^[25]

Proteins also play a significant role in recognition, such as cell targeting by antibodies.^[26] Antibody-TA capsules were thus synthesized to study their cell binding and targeting ability. CD44, a cell-surface glycoprotein involved in cell–cell interactions, is overexpressed on many types of cancer cell membranes,^[27] and anti-CD44 antibodies can specifically target CD44 overexpressing (CD44+) cells, but do not readily interact with CD44 minimal expressing (CD44–) cells (Figure 5f). The cell binding and targeting ability of antiCD44-TA capsules were investigated using the two human breast cancer cell lines MDA-MB-231 (CD44+) cells and BT-474 (CD44–) cells; IgG-TA (anti-Human IgG) capsules were

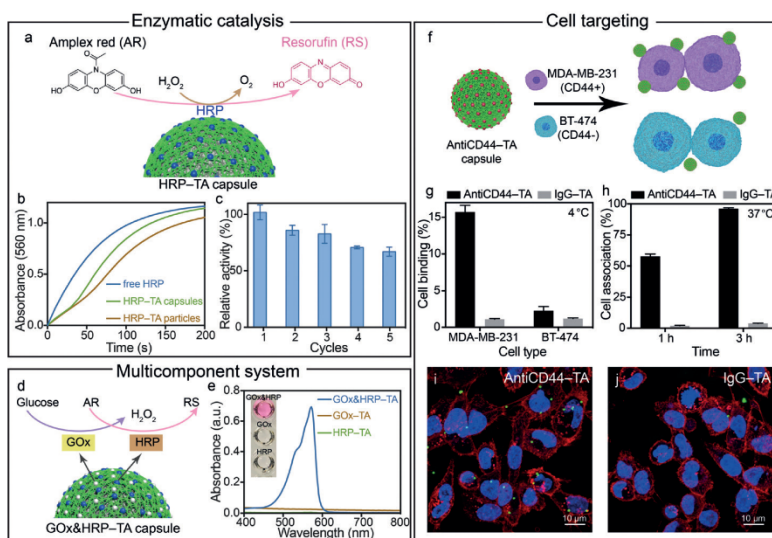


Figure 5. a–c) Enzymatic activity of the HRP-TA capsules: HRP-catalyzed oxidation of AR to RS over HRP-TA capsules (a); time-dependent absorbance changes as a result of the oxidation of AR by different catalytic systems: free HRP, HRP-TA capsules, and HRP-TA core-shell particles (b); and changes in the relative catalytic activity of the HRP-TA capsules as a function of cycle number (c). d, e) Cascade reaction using multicomponent GOx&HRP-TA capsules: glucose-triggered AR oxidation to produce RS in the presence of GOx and HRP (d) and UV/Vis spectra of the cascade reaction using different single- and multicomponent capsule systems (e). Inset: color changes using the different systems. f–j) Cell targeting of antibody-TA capsules: targeting specificity of antiCD44-TA capsules toward CD44 overexpressing (CD44+) MDA-MB-231 cells and the CD44 minimal expressing (CD44–) BT-474 cells (f); flow cytometry analysis of the antiCD44-TA and IgG-TA capsules binding to MDA-MB-231 and BT-474 cells after incubation for 1 h at 4 °C (g); percentage of MDA-MB-231 cells associated with antiCD44-TA and IgG-TA capsules after 1 or 3 h incubation at 37 °C (h); and CLSM images of antiCD44-TA (i) and IgG-TA capsules (j) incubated with MDA-MB-231 cells for 3 h at 37 °C. The capsule-to-cell ratio was 50:1 in all cell experiments. Data are shown as the mean $\pm$ SD ($N=3$).

used as the negative control. Cell binding was performed at 4 °C after 1 h incubation to prevent internalization.^[28] The antiCD44–TA capsules showed high binding specificity toward CD44+ cells over CD44– cells, whereas the IgG–TA capsules demonstrated nonspecific cell binding toward both cells (Figure 5g and Figure S18). In addition, cell-association experiments with CD44+ cells were performed at 37 °C after 1 and 3 h incubation to compare the antiCD44–TA capsules with control IgG–TA capsules. The flow cytometry results showed that after 1 h incubation, 58 % of the MDA-MB-231 (CD44+) cells associated with the antiCD44–TA capsules, whereas only 2 % of the MDA-MB-231 cells associated with the IgG–TA capsules (Figure 5h). After 3 h, the difference in cell association became more significant: 96 % of the MDA-MB-231 cells associated with the antiCD44–TA capsules, whereas only 4 % of the MDA-MB-231 cells associated with the IgG–TA capsules (Figure 5h and Figure S18), as supported visually by CLSM (Figure 5i,j). These above results reveal that the formation of antibody–TA assemblies does not negatively affect the inherent targeting ability of the antibodies. Additionally, the BSA–TA and LYZ–TA capsules displayed excellent stability in serum (Figure S19), negligible cytotoxicity and were internalized in cells (ca. 100 % capsules internalized by cells after 14 h; Figure S20), thus highlighting that this assembly strategy shows potential for intracellular protein-delivery applications.^[29] Moreover, protein–TA capsules did not have significant interactions with live *E. coli*, thus indicating that these coatings have the potential to endow materials with low-fouling properties (Figure S21).^[30] To improve reproducibility, reporting, and re-analysis, this study conforms to the Minimum Information Reporting in Bio-Nano Experimental Literature (MIRIBEL) standard,^[31] and a companion checklist is provided in the Supporting Information.

Conclusion

We have introduced interfacial polyphenol-mediated protein assembly as a versatile strategy to create a library of functional protein-based materials. Protein–polyphenol assemblies can be deposited on various substrates of different composition, size, and shape, affording new possibilities for surface modification. This technique is applicable to a wide range of proteins with diverse physicochemical properties and biological functions, thus enabling bioactive surface coating and functional capsule formation. Moreover, the protein–polyphenol capsules can be used to elucidate the dominant interaction(s) between different proteins and polyphenols; the results reveal that the molecular weight (size), hydrophobicity (aliphatic index), and isoelectric point (electrical charge) of the protein determine the strength of hydrogen bonding, hydrophobic interactions, and ionic interactions, respectively. These various noncovalent stabilizing interactions between proteins and polyphenols offer possibilities to engineer assemblies with desirable responsiveness to multiple stimuli such as changes in pH, as exemplified. Finally, we have demonstrated various applications, from catalysis to fluorescence imaging and cell targeting, for the protein–polyphenol

assemblies, as a range of functionalities can be engineered into the assemblies through judicious choice of the constituent proteins. This platform is expected to advance both fundamental and applied research owing to its simplicity and versatility.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biomaterials · particle surface engineering · polyphenols · protein assembly · proteins

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3.2 Supporting Information



Supporting Information

Polyphenol-Mediated Assembly of Proteins for Engineering Functional Materials

*Yiyuan Han, Zhixing Lin, Jiajing Zhou, Gyeongwon Yun, Rui Guo, Joseph J. Richardson, and Frank Caruso**

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Materials

Tannic acid (TA, ACS reagent), gallic acid (GA), epigallocatechin gallate (EGCG), sodium phosphate-buffered saline (PBS), glycine, hydrogen chloride (HCl), sodium carbonate (Na_2CO_3), calcium chloride (CaCl_2), sodium acetate, poly(sodium 4-styrenesulfonate) (PSS, $M_w \sim 70$ kDa), fluorescein isothiocyanate (FITC), FITC-dextran of various average molecular weights (4, 63, 250, and 500 kDa), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), insulin, lysozyme (LYZ), glucose oxidase (GOx), hemoglobin (Hgb), immunoglobulin G (IgG), pepsin, fibrinogen (FGN), horseradish peroxidase (HRP), cytochrome C (CYC), green fluorescent protein (GFP), urea, and Tween 20 were purchased from Sigma-Aldrich. Anti-CD44 antibody [IM7] (FITC) was purchased from Abcam. Polystyrene (PS) particles ($D = 3.55 \pm 0.07 \mu\text{m}$) and silica particles ($D = 3.08 \pm 0.13 \mu\text{m}$) were purchased from microParticles GmbH. *Escherichia coli* (ATCC number 14948) were obtained from the American Type Culture Collection (ATCC). Dulbecco's modified eagle medium (DMEM) and Dulbecco's phosphate-buffered Saline (DPBS) were obtained from Life Technologies. Wheat germ agglutinin (WGA) and Hoechst 33342 were purchased from Invitrogen. High-purity Milli-Q water with a resistivity of $18.2 \text{ M}\Omega \text{ cm}$ was obtained from an inline Millipore RiOs/Origin water purification system.

Characterization

Differential interference contrast (DIC) microscopy images of capsules were acquired with an inverted Olympus IX71 microscope. CLSM images were taken with a Nikon A1R+ laser scanning confocal microscope (Nikon Corporation, Japan). Atomic force microscopy (AFM) experiments were conducted on a JPK NanoWizard II BioAFM instrument. Scanning electron microscopy (SEM) images of capsules were captured on an FEI Quanta 200 field-emission scanning electron microscope, operating at an accelerating voltage of 10 kV. For the SEM experiments, dried samples (gold-sputtered) were used. Transmission electron microscopy (TEM) and energy-dispersive X-ray (EDX) mapping analysis of capsules were performed on an FEI Tecnai TF20 instrument (USA) with an operation voltage of 200 kV. The TEM samples were prepared by dropping $5 \mu\text{L}$ of diluted samples on formvar carbon-coated copper grids. UV-Visible absorption spectra were recorded on a Specord 250 Plus spectrophotometer (Analytik Jena AG). Fourier transform infrared (FTIR) spectroscopy analysis was conducted on a Tensor-II- FTIR spectrometer. The metal content in the capsules was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using an ICP Varian 710-ES instrument. Zeta potential measurements of capsules were performed in water and in different pH buffers at a concentration of 5 mM on a Malvern Zetasizer Nano ZS instrument (Malvern

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Instrument, Worcestershire, UK). Circular dichroism spectra were recorded using a model 410 circular dichroism spectrometer (Aviv Biomedical, Inc.)

Cell culture

Human mammary gland cancer cell lines MDA-MB-231 and BT-474 were purchased from ATCC (USA). MDA-MB-231 cells and BT-474 cells were maintained in complete DMEM media containing 10% fetal bovine serum (FBS) and GlutaMAX. Both cell lines were maintained at 37 °C in a cell culture incubator with 5% CO₂ and 95% relative humidity.

Protein–polyphenol coating on planar substrates

Glass, PDMS, or plastic substrates (~1 cm × 1 cm) were placed in Milli-Q water (850 µL), followed by the addition of solutions of TA (50 µL, 10 mg mL⁻¹) and FITC-BSA (100 µL, 10 mg mL⁻¹). The resulting mixture, containing the protein, TA, and substrate, was vigorously mixed using a vortex mixer for 5 s and then incubated in an Eppendorf thermomixer at 37 °C and 1,400 rpm for 4 h. The coating process was repeated three times to enhance the color difference seen in Figure 1e.

Protein–polyphenol coating on particle substrates

Aliquots (5 µL) of TA (40 mg mL⁻¹) and BSA (25 µL, 12 mg mL⁻¹) were sequentially added to an aqueous particle solution (10 mg mL⁻¹). The resulting suspension was vigorously mixed using a vortex mixer for 5 s and then incubated in an Eppendorf thermomixer at 37 °C and 1,400 rpm for 4 h. The particles were retrieved and washed with water three times to remove excess TA and BSA. In the washing step, the particles were spun down by centrifugation (2,000 g, 1 min for the micrometer-sized particles; 4000 g, 5 min for the gold nanorods), and the supernatant was removed. The particles were then resuspended with vigorous mixing prior to the next wash step.

Formation of protein–polyphenol capsules using sacrificial templates

CaCO₃ particles (5 mg; 3 µm) were dispersed in Milli-Q water (470 µL). Then, TA (5 µL; 40 mg mL⁻¹, Milli-Q water) was added and incubated with the particles for 10 min under gentle shaking. A protein solution in Milli-Q water (25 µL; 12 mg mL⁻¹) was then added, followed by glycine-HCl buffer (500 µL; 50 mM, pH 4.0) to a total of 1 mL. The CaCO₃-templated protein/TA mixture was then incubated in an Eppendorf thermomixer at 37 °C and 1,400 rpm for 4 h. To remove excess protein and TA, the mixture was centrifuged at 1,000 g for 60 s and washed with Milli-Q water. The centrifugation/wash cycle was repeated three times. Protein–TA capsules were obtained after CaCO₃

core removal with EDTA (100 mM, pH 4.0) and subsequent washing via centrifugation (3,000 g, 5 min) three times with Milli-Q water.

Capsule disassembly experiments

FITC-labeled protein–TA capsule suspensions (1.0×10^5 capsule μL^{-1}) in 100 mM urea, 100 mM Tween 20, or 100 mM NaCl solution were incubated in an Eppendorf thermomixer at 37 °C and 500 rpm for the desired time. The suspensions were diluted with water and subjected to flow cytometry assays to determine the number of fluorescent capsules.

Permeability tests

CYC–TA capsules were dispersed in FITC-dextran solution (1 mg mL^{-1}) of different molecular weights. Confocal laser scanning microscopy (CLSM) images of the capsules were taken within 20 min of mixing the capsules with FITC-dextran. Dark capsule interiors were considered to be impermeable, whereas capsules with interiors of similar fluorescence intensity as the outer environment were considered to be permeable; 100 capsules were examined.

Enzyme activity tests

The H_2O_2 –amplex red colorimetric reaction was used to characterize and compare the activities of the HRP–TA capsules, free HRP, and HRP–TA core–shell particles. In the presence of H_2O_2 , HRP can oxidize amplex red (AR) to resorufin (RS), characterized by a strong absorbance at 560 nm. For the activity study, H_2O_2 (40 μL ; 20 mM) and AR (2 μL ; 1 mg mL^{-1}) were added to phosphate-buffered saline (PBS; 10 mM, pH 7.4), followed by HRP. The final volume of the reaction solution was 400 μL . The kinetic behavior of HRP was studied by monitoring the absorbance at 560 nm in 10 s intervals by UV–vis spectroscopy. HRP activity was quantified by adding various amounts of HRP to the AR– H_2O_2 solution. For the cascade reaction involving GOx&HRP capsules, glucose (10 mg mL^{-1}) was used to initiate the two tandem reactions in PBS (10 mM, pH 7.4) and the total volume of the reaction solution was 200 μL . After completion of the reaction (20 min at 37 °C), the UV–vis absorption spectra were recorded.

Synthesis of calcium carbonate particles

CaCO_3 particles were synthesized as follows. Typically, 20 mL of 20 mg mL^{-1} PSS solution and 180 mL Milli-Q water were added to a beaker and then stirred vigorously at 2,500 rpm. Then, 2.4 mL of 1 M Na_2CO_3 solution was added to the beaker at room temperature (20 °C) and stirred for 1 min. Subsequently, 4.8 mL of 1 M CaCl_2 solution was added, and the solution was mixed at 2,500 rpm for

60 s. The solution was then incubated for 60 s or 90 s to achieve the desirable particle size (i.e., $\sim 1\ \mu\text{m}$ or $\sim 3\ \mu\text{m}$, respectively). The CaCO_3 particles were washed with Milli-Q water three times to remove excess PSS, Na_2CO_3 , and CaCl_2 . In the washing step, the particles were spun down by centrifugation (1,000 g, 1 min) and the supernatant was removed. PSS was removed by calcination at $450\ ^\circ\text{C}$ for 2 h. The resulting CaCO_3 particles powder was stored in a dry solid state and dispersed in the desired solvents when needed.

Cell targeting studies of antibody–TA capsules via flow cytometry

MDA-MB-231 cells and BT-474 cells were seeded in a 24-well plate at a cell density of 8×10^4 cells per well and then cultured in complete media at $37\ ^\circ\text{C}$ for 24 h to allow cellular adhesion on substrates. Following this, FITC-labeled antiCD44–TA and IgG–TA capsules were added to the cells at a capsule-to-cell ratio of 50:1 and then incubated for 1 h at $4\ ^\circ\text{C}$ for the cell binding study or 1 and 3 h at $37\ ^\circ\text{C}$ for the cell association study. After incubation, the cells were gently washed three times with DPBS, dissociated using trypsin solution, and analyzed on an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK). The degree of cell binding or association of the capsules was evaluated by using the percentage of cells that exhibited stronger fluorescence intensity than the control untreated cells.

Cell targeting studies of antibody–TA capsules via confocal microscopy

MDA-MB-231 cells were seeded into 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, USA) at a cell density of 4×10^4 cells per well and then cultured in complete media at $37\ ^\circ\text{C}$ for 24 h to allow cellular adhesion on substrates. Then, FITC-labeled antiCD44–TA and IgG–TA capsules were added to the cells at a capsule-to-cell ratio of 50:1 and then incubated for 3 h at $37\ ^\circ\text{C}$. After incubation, the cells were gently washed three times with DPBS, fixed with paraformaldehyde (4% in DPBS) for 30 min at $37\ ^\circ\text{C}$, then stained with WGA-594 ($5\ \mu\text{g mL}^{-1}$) at room temperature for 15 min, followed by Hoechst 33342 ($2\ \mu\text{g mL}^{-1}$) staining at room temperature for 10 min. CLSM imaging was performed to monitor the cell association of the capsules on a microscope equipped with a Plan Apo $\lambda\ 60\times 1.4$ NA oil immersion objective, 405, 488, 561, and 640 nm lasers, and 450/50, 525/50, 595/50, and 700/75 nm bandpass emission filters. The images were processed by Fiji software using the maximum intensity projection.

Cytotoxicity assay of protein–TA capsules

The (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) (XTT)-based *in vitro* cytotoxicity assay was performed to assess the biocompatibility of the protein–TA capsules. XTT was dissolved in the complete DMEM (with 10% FBS) to form 0.2

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mg mL⁻¹ solution, and phenazine methosulfate (PMS) was dissolved in DPBS to form 1 mM solution. The XTT reagent was activated by mixing with PMS solution at a volume ratio of 400:1. MDA-MB-231 cells were seeded on a 96-well plate at a cell density of 2×10^4 cells per well. After incubation with the capsules at various capsule-to-cell ratios for 48 h, the media in the 96-well plate was aspirated and replaced with 100 μ L fresh activated XTT media. The cells were further incubated for 4 h and the absorption at 475 nm was measured relative to non-treated cells.

Minimum information reporting in bio–nano experimental literature (MIRIBEL). The studies conducted herein, including material characterization, biological characterization, and experimental details, conform to the MIRIBEL reporting standard for bio–nano research,^[1] and we include a companion checklist of these components herein.

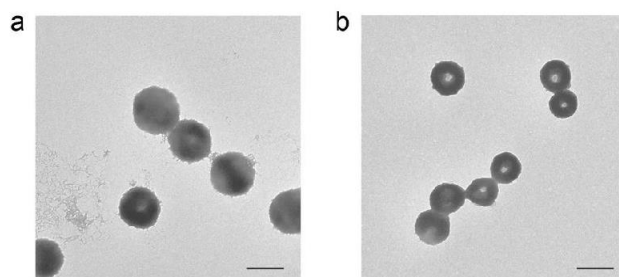


Figure S1. TEM images of BSA–GA capsules (a) and BSA–EGCG capsules (b) after the removal of sacrificial template (CaCO_3). Scale bars: 2 μm .

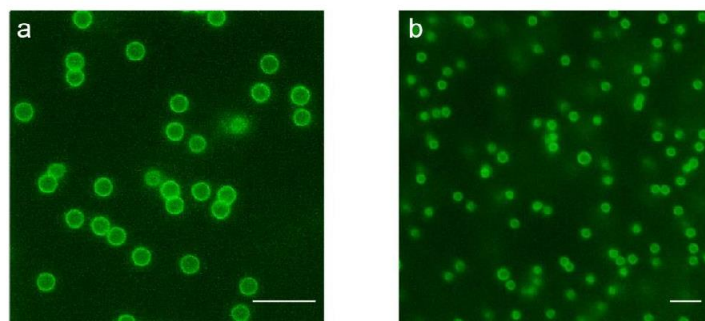


Figure S2. Fluorescence images of polystyrene (a) and silica (b) particles after FITC-BSA–TA coating. Scale bars: 10 μm .

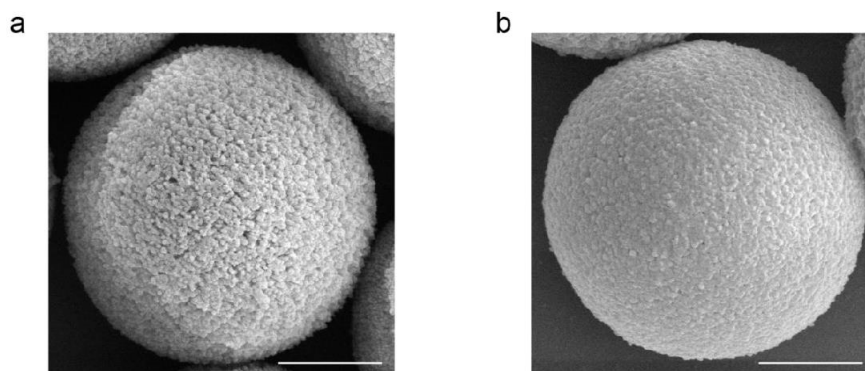


Figure S3. SEM images of CaCO_3 before (a) and after (b) BSA–TA coating. Scale bars: 1 μm .

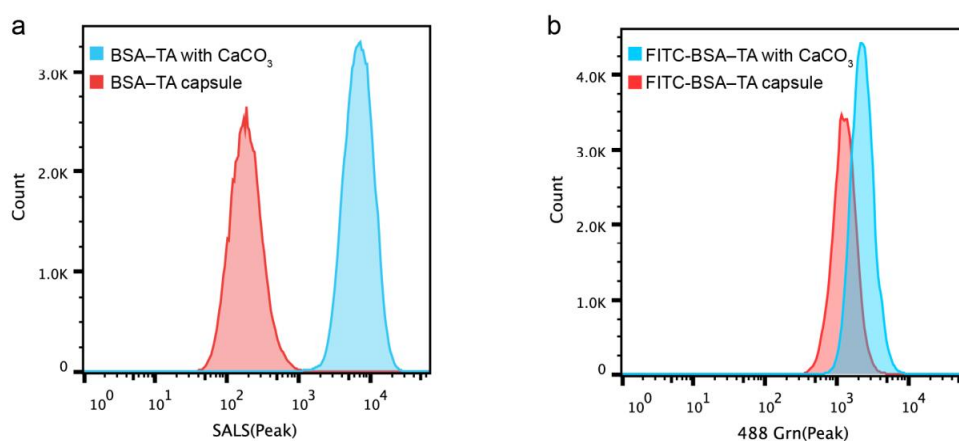


Figure S4. Histograms of BSA–TA and FITC-BSA–TA core–shell particles (prior to core CaCO_3 removal) and capsules (after core removal) determined by flow cytometry at a small-angle light scattering (SALS) signal (a) and 488 nm green (488 Grn) signal (b).

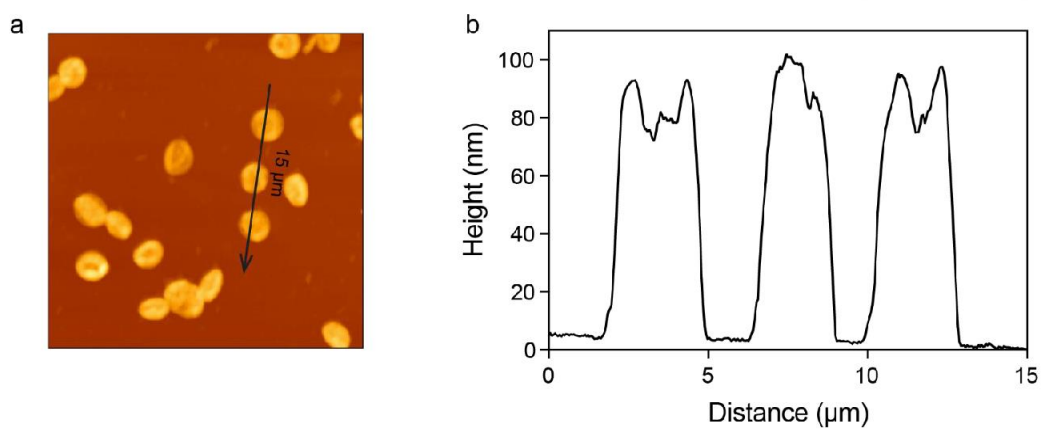


Figure S5. AFM image (a) and corresponding height profile (b) of the BSA-TA capsules.

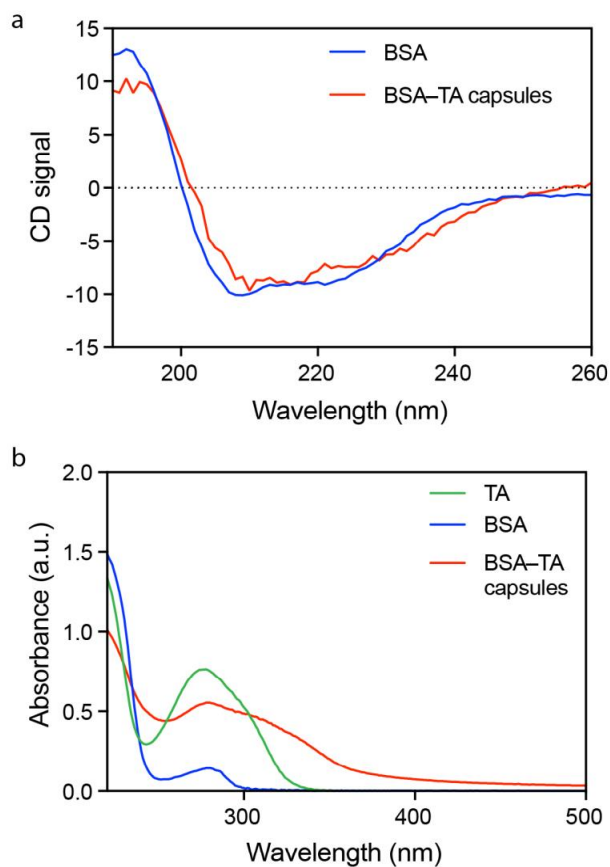


Figure S6. Circular dichroism (CD) spectra of BSA and BSA-TA capsules (a) and UV-vis spectra of TA, BSA, and BSA-TA capsules (b).

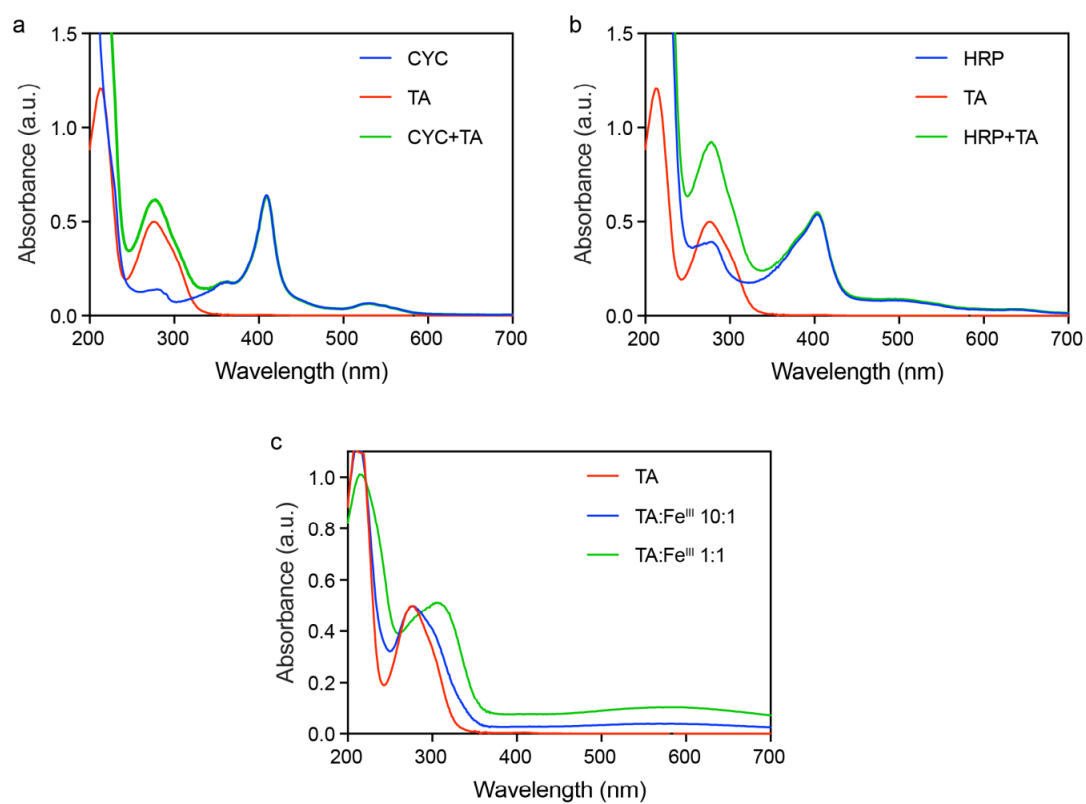


Figure S7. UV-vis spectra of CYC, TA, and mixture of CYC and TA (CYC+TA) (a) and HRP, TA, and mixture of HRP and TA (HRP+TA) (b); CYC: 0.1 mg mL⁻¹; HRP: 0.5 mg mL⁻¹; TA: 0.01 mg mL⁻¹. UV-vis spectra of TA, mixtures of TA and Fe^{III} at different ratios (mass ratio: 10:1 and 1:1) (c).

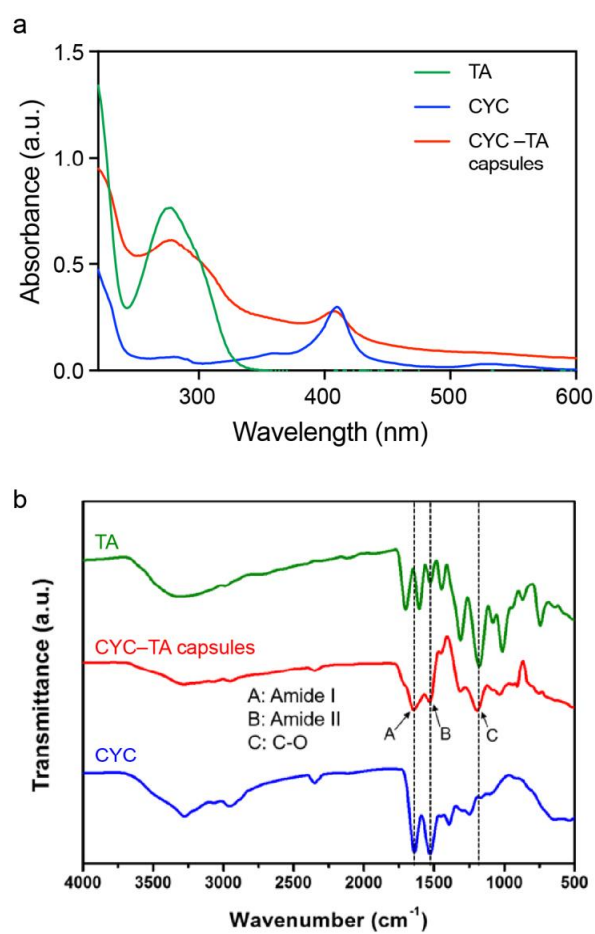


Figure S8. UV-vis spectra (a) and FTIR spectra (b) of TA, CYC, and CYC-TA capsules.

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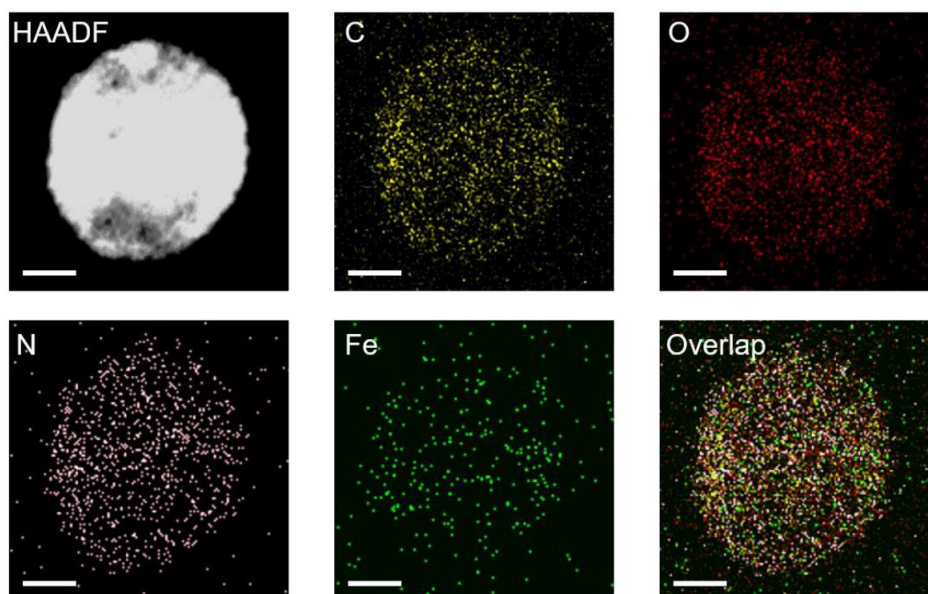


Figure S9. High-angle annular dark-field (HAADF) image and EDX elemental mapping of the CYC-TA capsules. Scale bars: 1 μm .

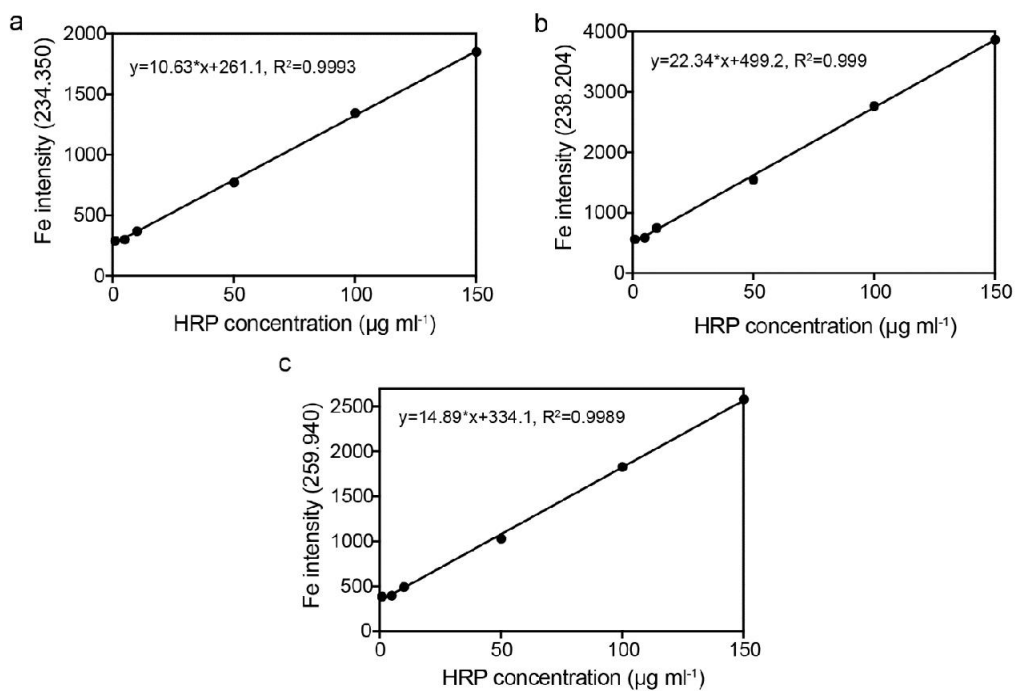


Figure S10. ICP-OES standard curves of HRP concentration and Fe intensity at different wavelengths: 234.350 nm (a); 238.204 nm (b); and 259.940 nm (c).

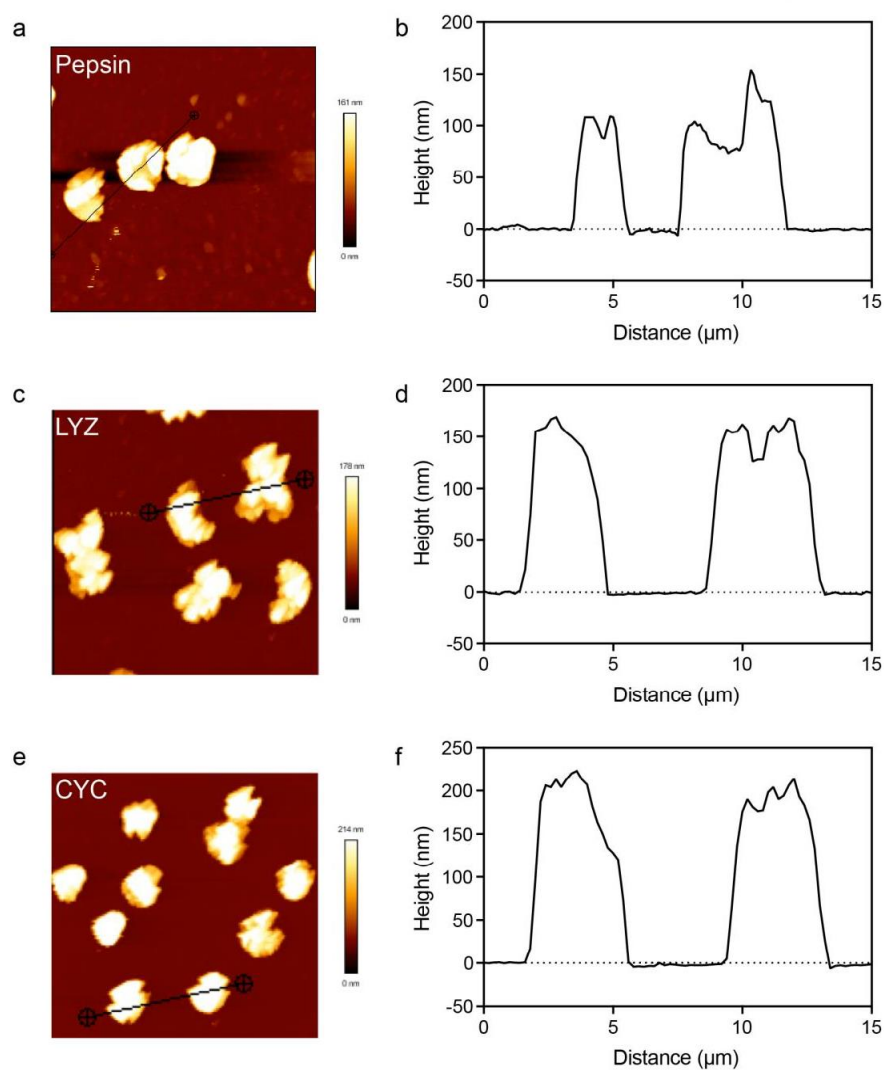


Figure S11. AFM images (a, c and e) and corresponding height profiles (b, d and f) of the Pepsin–TA (a, b), LYZ–TA (c, d), and CYC–TA (e, f) capsules, respectively. The black cross section is 15 μm in length.

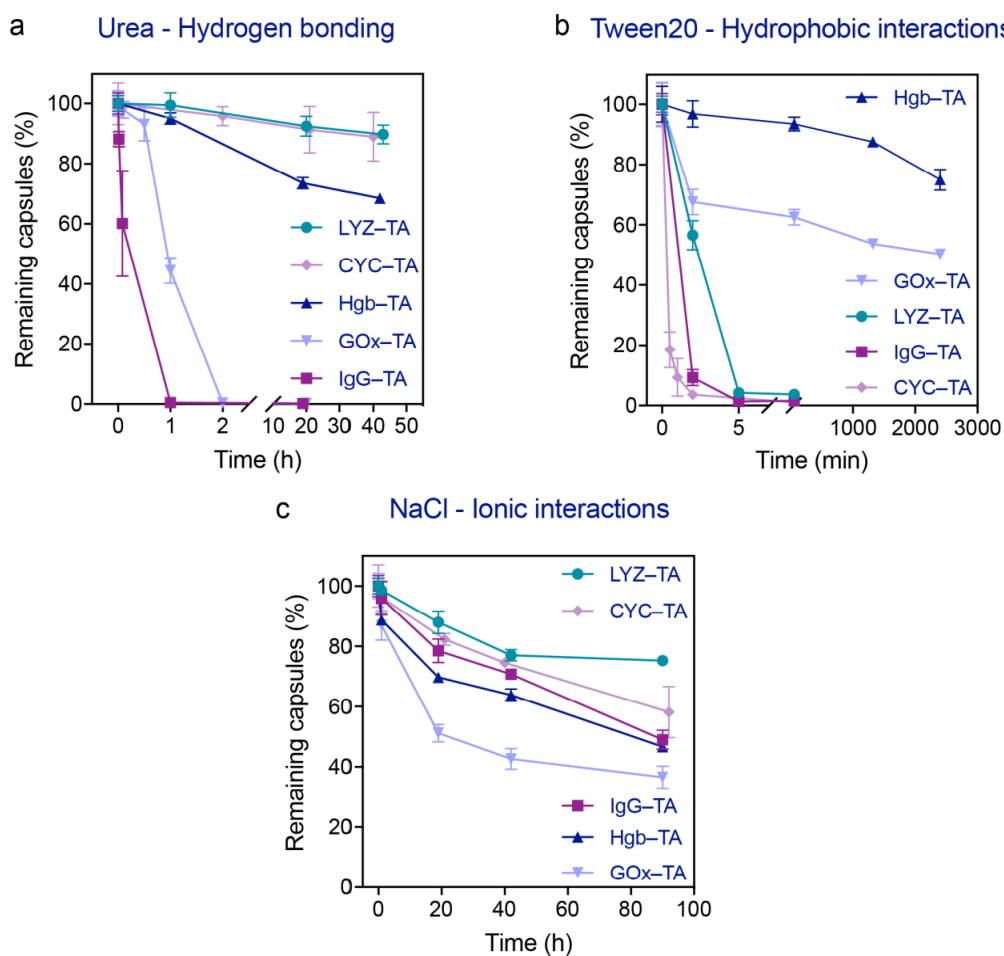


Figure S12. Interaction-dependent disassembly kinetics of LYZ-TA, IgG-TA, Hgb-TA, GOx-TA, and CYC-TA capsules in the presence of 100 mM of urea (a), 100 mM Tween 20 (b), or 100 mM NaCl (c), as assessed by flow cytometry. Data are shown as the mean $\pm$ standard deviation ($N = 3$).

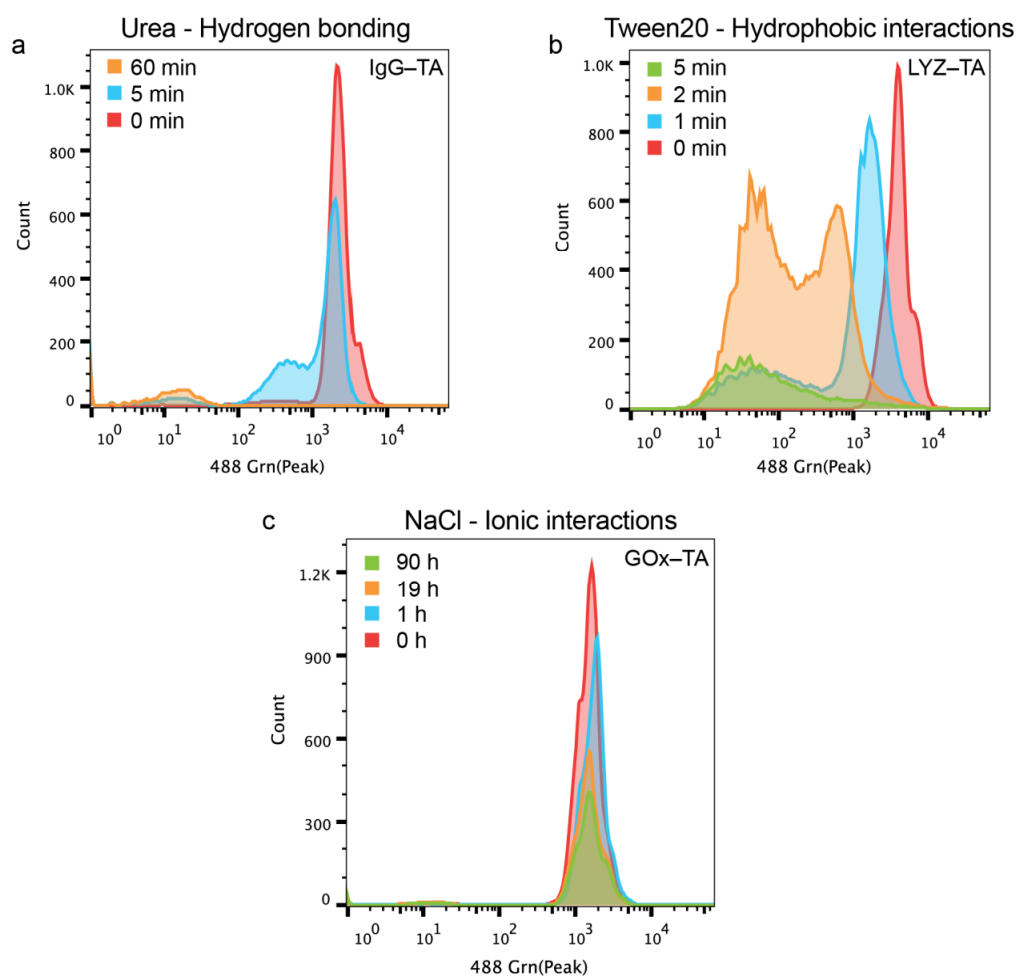


Figure S13. Histograms of the disassembly of different protein-TA capsules in 100 mM urea (a), 100 mM Tween 20 (b), or 100 mM NaCl (c) determined by flow cytometry at 488 nm.

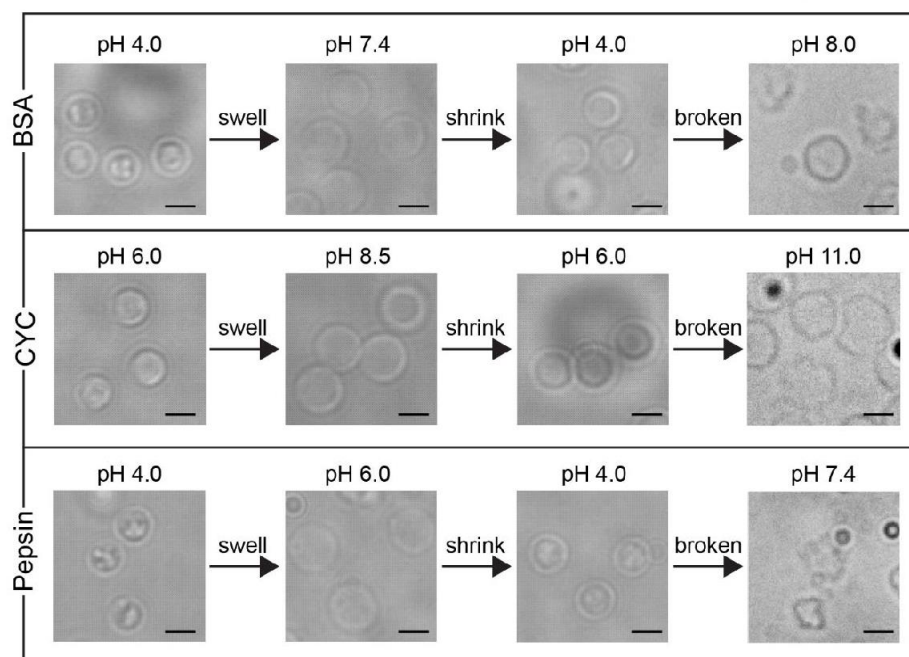


Figure S14. DIC images showing the swelling and shrinking, and dissociation of BSA-TA, CYC-TA, and pepsin-TA capsules under different pHs. Scale bars: 2 μm .

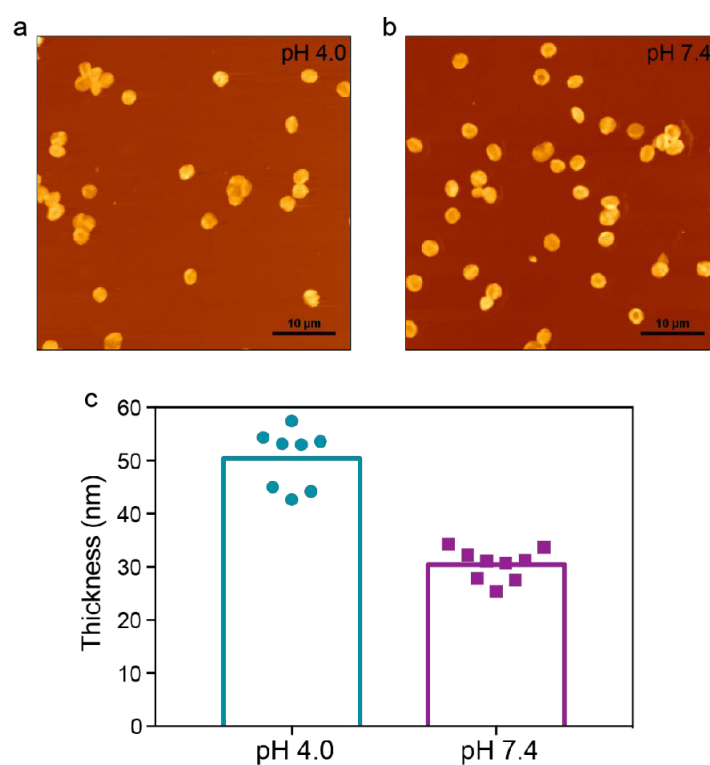


Figure S15. AFM images of the BSA-TA capsules at pH 4.0 (a) and pH 7.4 (b). Thickness of the BSA-TA capsules at pH 4.0 and pH 7.4 (c).

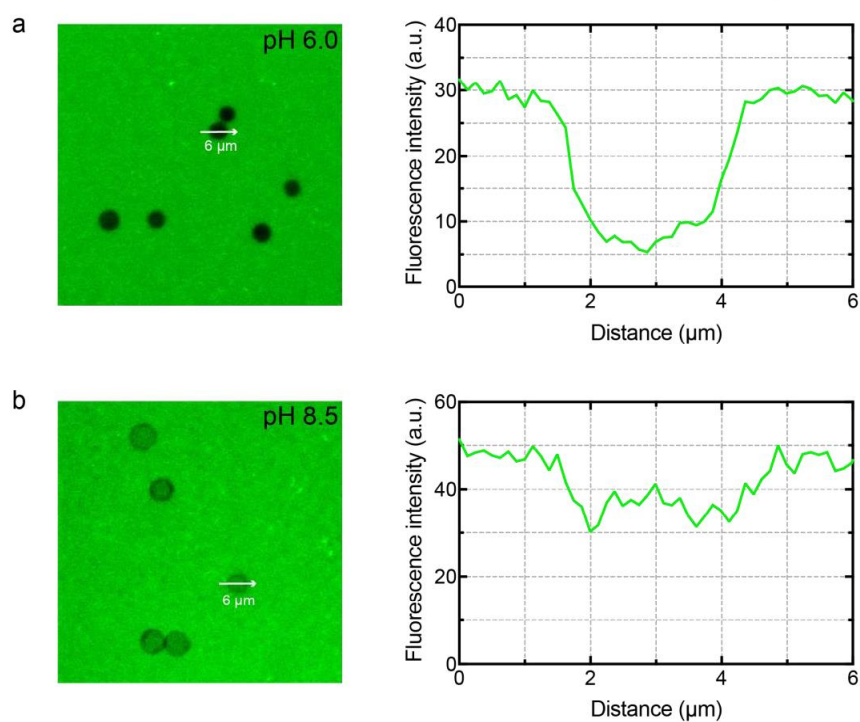


Figure S16. CLSM images and corresponding fluorescence intensity profiles of the CYC-TA capsules incubated with 250 kDa at pH 6.0 (a) and pH 8.5 (b).

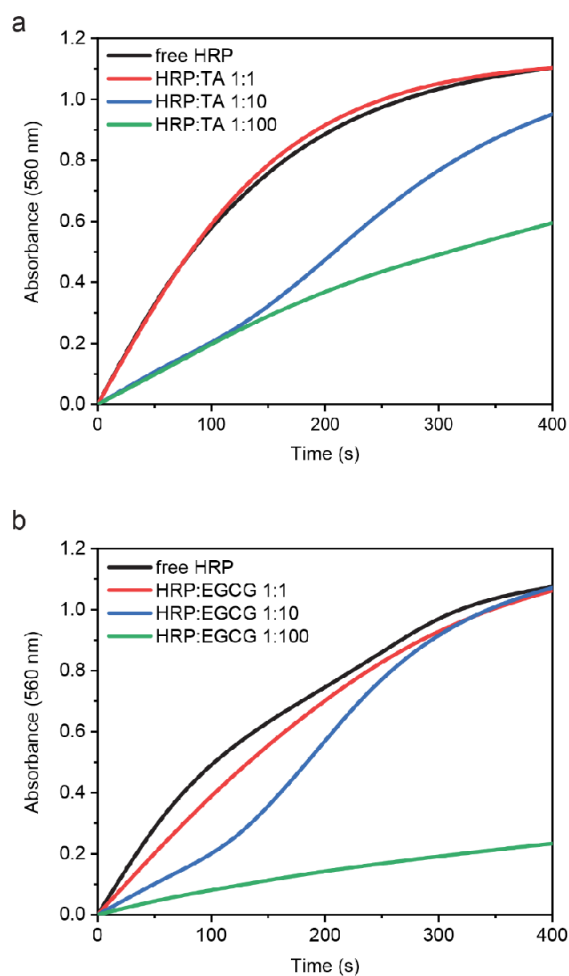


Figure S17. Time-dependent absorbance changes as a result of the oxidation of AR by different catalytic systems: free HRP and HRP+TA mixtures at different ratios (mass ratio: 1:1, 1:10, and 1:100; HRP concentration: $0.1 \mu\text{g ml}^{-1}$) (a) and free HRP and HRP+EGCG mixtures at different ratios (mass ratio: 1:1, 1:10, and 1:100; HRP concentration: $0.1 \mu\text{g ml}^{-1}$) (b).

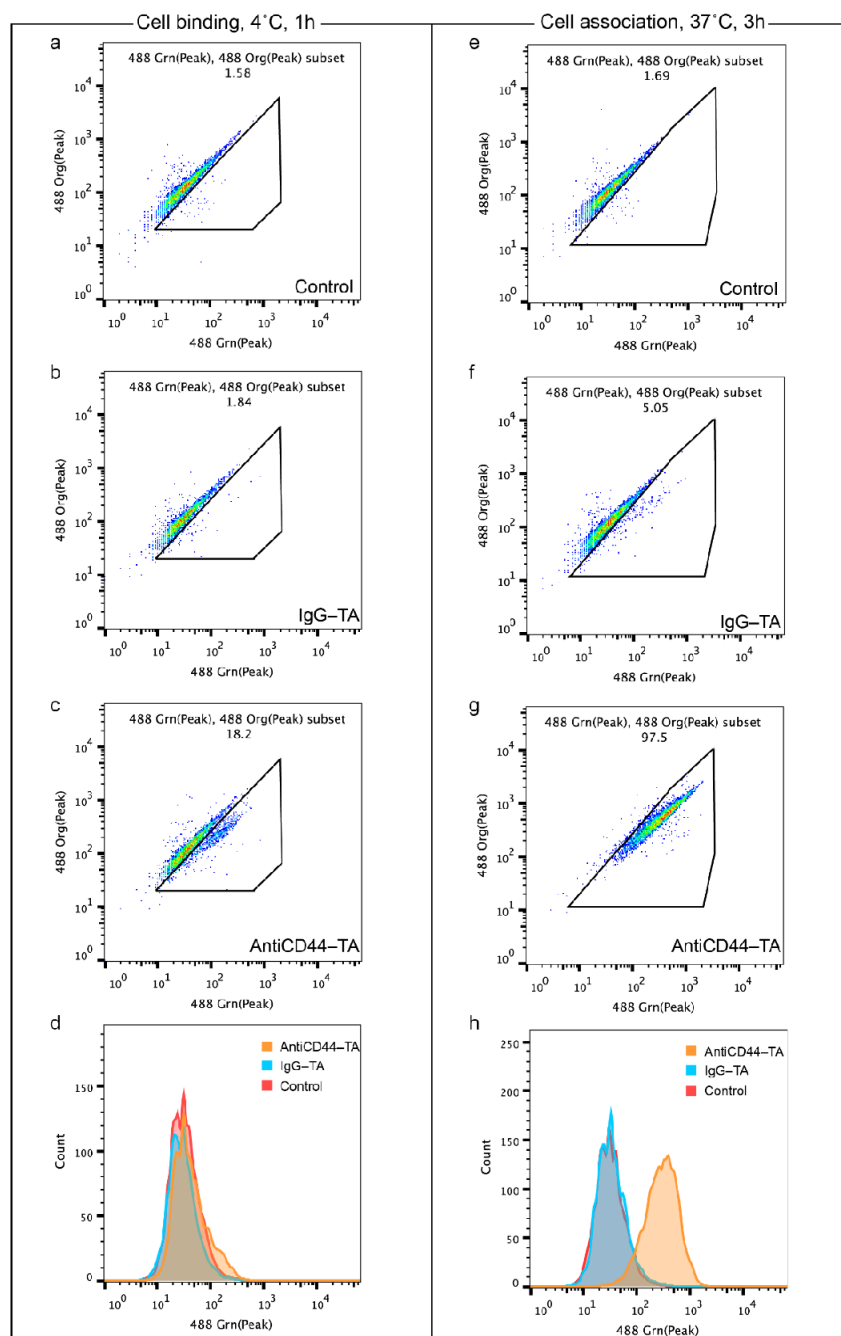


Figure S18. Flow cytograms showing the cell binding (a–c) or association degree (e–g) of antiCD44–TA and IgG–TA capsules with MDA-MB-231 cells. For the cell binding studies, the untreated (a) and treated (b,c) cells were incubated at 4 °C for 1 h incubation. For the cell association study, the untreated (e) and treated (f,g) cells were incubated at 37 °C for 3 h. Histograms of MDA-MB-231 cells treated with different antibody–TA capsules under different conditions at 488 green signal (d,h).

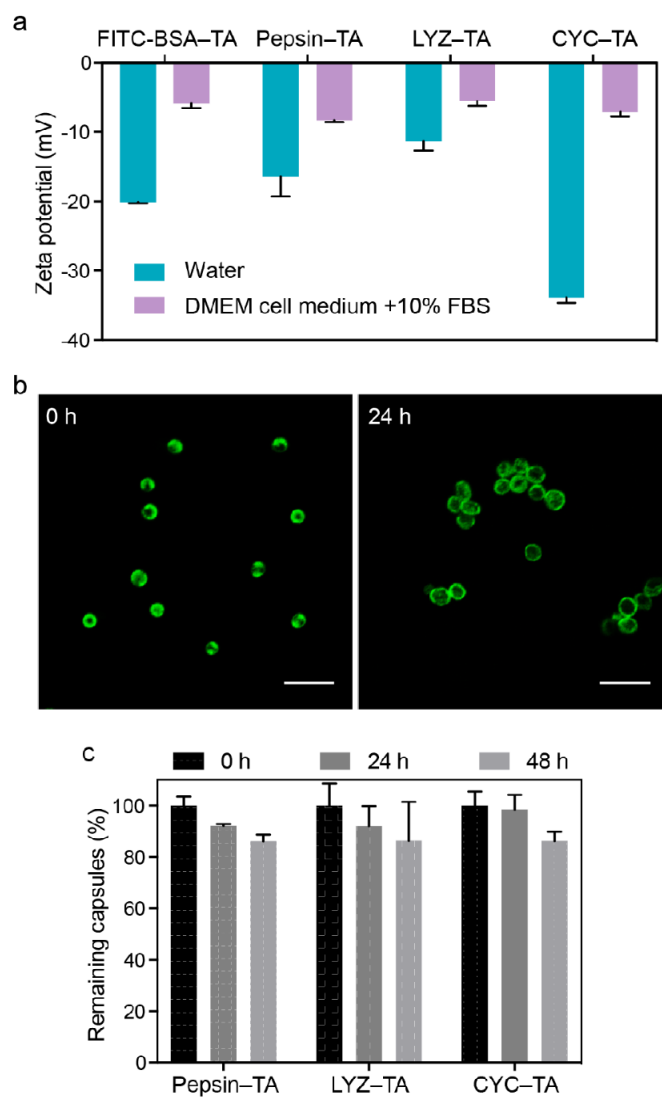


Figure S19. Stability of protein-TA capsules in cell culture medium with serum. Zeta potential values of the FITC-BSA-TA, pepsin-TA, LYZ-TA, and CYC-TA capsules in water and DMEM cell medium with 10% FBS (a). Data are shown as the mean $\pm$ SD. CLSM images of FITC-BSA-TA incubated in DMEM cell medium with 10% FBS for 0 and 24 h at 37 °C (b); scale bars: 10 μ m. Protein-TA capsule stability in DMEM cell medium with 10% FBS for 0, 24 and 48 h (c).

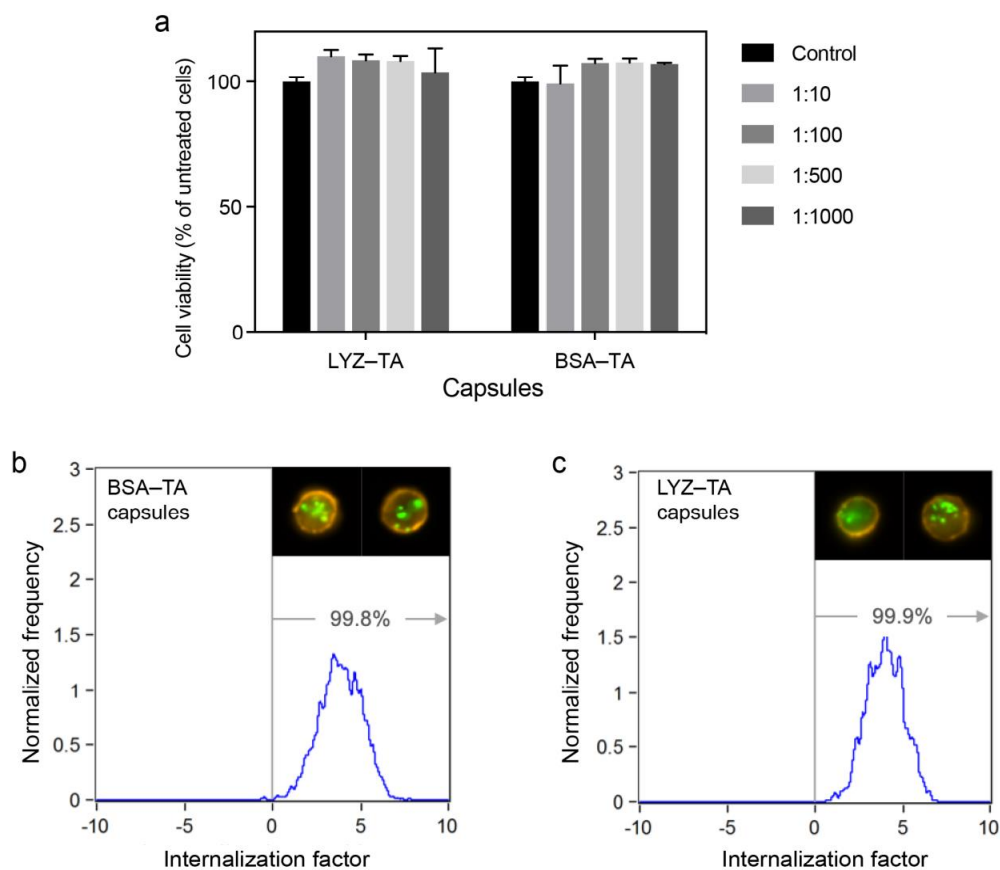


Figure S20. Cell viability assay in the presence of LYZ-TA and BSA-TA capsules at different dosages (a). Cell viability was measured by XTT assays after incubation for 48 h at 37 °C. Experiments were performed in triplicate. Cell internalization assay performed on MDA-MB-231 cells in the presence of BSA-TA capsules (b) and LYZ-TA capsules (c). Experiments were monitored by imaging flow after incubation for 14 h at 37 °C.

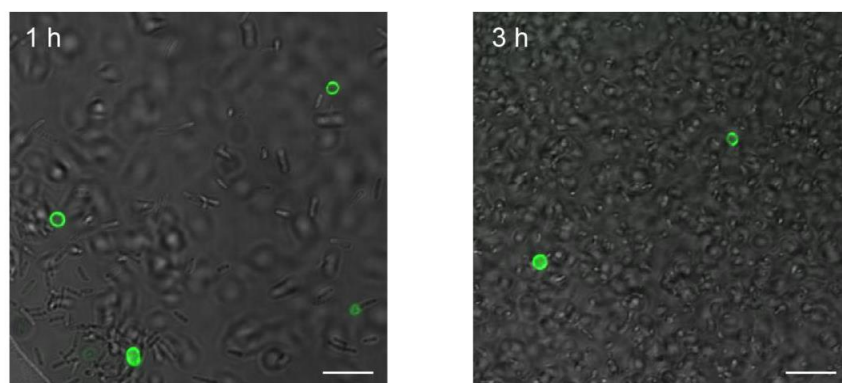


Figure S21. CLSM images of FITC-BSA-TA capsules incubated with live *Escherichia coli* bacteria for 1 and 3 h at 37 °C. Scale bars: 10 μm .

Table S1. Molecular weight, isoelectric point, and aliphatic index of different proteins

Name	Molecular Weight (kDa) ^a	Isoelectric Point (pI) ^b	Aliphatic Index ^a
Pepsin	41.262	2.9	91.38
Glucose Oxidase (GOx)	131.280	4.2	86.12
Insulin	5.741	5.3	80.2
Green Fluorescent Protein (GFP)	26.886	5.8	74.87
Hemoglobin (Hgb)	64.479	7.0	89.18
Horseradish Peroxidase (HRP)	38.825	7.2	90.34
Immunoglobulin G (IgG)	144.263	8.5	67.64
Cytochrome C (CYC)	11.703	9.4	58.57
Lysozyme (LYZ)	16.317	10	82.93
Fibrinogen (FGN)	353.241	5.5	58.08
Bovine Serum Albumin (BSA)	69.293	4.6	77.46

^aThe molecular weight and aliphatic index are calculated based on the amino acid sequence of each protein using ProtParam tool (<https://web.expasy.org/protparam/>). Amino acid sequence is collected from protein data bank (<https://www.rcsb.org/>).

^bThe isoelectric point of each protein was obtained from Reference.^[2]

Table S2. HRP encapsulation rate in HRP–TA capsules based on ICP-OES results and standard curve from Figure S9

Element (wavelength)	Peak Intensity (a.u.)	HRP Concentration ($\mu\text{g mL}^{-1}$)
Fe (234.350)	593.44	31.255
Fe (238.204)	1227	32.578
Fe (259.940)	809.598	31.934
Average HRP concentration in HRP–TA ($\mu\text{g mL}^{-1}$) in HRP–TA capsules (dilution ratio, 1:5)		159.61
HRP encapsulation rate (%) (feed HRP concentration, $200 \mu\text{g mL}^{-1}$)		79.8

References

- [1] M. Faria, M. Björnmalm, K. J. Thurecht, S. J. Kent, R. G. Parton, M. Kavallaris, A. P. Johnston, J. J. Gooding, S. R. Corrie, B. J. Boyd, P. Thordarson, A. K. Whittaker, M. M. Stevens, C. A. Prestidge, C. J. H. Porter, W. J. Parak, T. P. Davis, E. J. Crampin, F. Caruso, *Nat. Nanotechnol.* **2018**, *13*, 777.
- [2] P. G. Righetti, G. Tudor, K. Ek, *J. Chromatogr. A* **1981**, *220*, 115.

Checklist

Minimum Information Reporting in Bio–Nano Experimental Literature

The MIRIBEL guidelines were introduced here: <https://doi.org/10.1038/s41565-018-0246-4>

The development of these guidelines was led by the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology: <https://www.cbns.org.au/>. Any updates or revisions to this document will be made available here: <http://doi.org/10.17605/OSF.IO/SMVTF>. This document is made available under a CC-BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>.

The MIRIBEL guidelines were developed to facilitate reporting and dissemination of research in bio–nano science. Their development was inspired by various similar efforts:

- MIAME (microarray experiments): *Nat. Genet.* **29** (2001), 365; <http://doi.org/10.1038/ng1201-365>
- MIRIAM (biochemical models): *Nat. Biotechnol.* **23** (2005) 1509; <http://doi.org/10.1038/nbt1156>
- MIBBI (biology/biomedicine): *Nat. Biotechnol.* **26** (2008) 889; <http://doi.org/10.1038/nbt.1411>
- MIGS (genome sequencing): *Nat. Biotechnol.* **26** (2008) 541; <http://doi.org/10.1038/nbt1360>
- MIQE (quantitative PCR): *Clin. Chem.* **55** (2009) 611; <http://doi.org/10.1373/clinchem.2008.112797>
- ARRIVE (animal research): *PLOS Biol.* **8** (2010) e1000412; <http://doi.org/10.1371/journal.pbio.1000412>
- *Nature's* reporting standards:
 - Life science: <https://www.nature.com/authors/policies/reporting.pdf>; e.g., *Nat. Nanotechnol.* **9** (2014) 949; <http://doi.org/10.1038/nnano.2014.287>
 - Solar cells: <https://www.nature.com/authors/policies/solarchecklist.pdf>; e.g., *Nat. Photonics* **9** (2015) 703; <http://doi.org/10.1038/nphoton.2015.233>
 - Lasers: <https://www.nature.com/authors/policies/laserchecklist.pdf>; e.g., *Nat. Photonics* **11** (2017) 139; <http://doi.org/10.1038/nphoton.2017.28>
- The “TOP guidelines”: e.g., *Science* **352** (2016) 1147; <http://doi.org/10.1126/science.aag2359>

Similar to many of the efforts listed above, the parameters included in this checklist are **not** intended to be definitive requirements; instead they are intended as ‘points to be considered’, with authors themselves deciding which parameters are—and which are not—appropriate for their specific study.

This document is intended to be a living document, which we propose is revisited and amended annually by interested members of the community, who are encouraged to contact the authors of this document. Parts of this document were developed at the annual International Nanomedicine Conference in Sydney, Australia: <http://www.oznanomed.org/>, which will continue to act as a venue for their review and development, and interested members of the community are encouraged to attend.

After filling out the following pages, this checklist document can be attached as a “Supporting Information” document during submission of a manuscript to inform Editors and Reviewers (and eventually readers) that all points of MIRIBEL have been considered.

Supplementary Table 1. Material characterization*

Question	Yes	No
1.1 Are “best reporting practices” available for the nanomaterial used? For examples, see <i>Chem. Mater.</i> 28 (2016) 3535; http://doi.org/10.1021/acs.chemmater.6b01854 and <i>Chem. Mater.</i> 29 (2017) 1; http://doi.org/10.1021/acs.chemmater.6b05235		√
1.2 If they are available, are they used? If not available, ignore this question and proceed to the next one.		
1.3 Are extensive and clear instructions reported detailing all steps of synthesis and the resulting composition of the nanomaterial? For examples, see <i>Chem. Mater.</i> 26 (2014) 1765; http://doi.org/10.1021/cm500632c , and <i>Chem. Mater.</i> 26 (2014) 2211; http://doi.org/10.1021/cm5010449 . Extensive use of photos, images, and videos are strongly encouraged. For example, see <i>Chem. Mater.</i> 28 (2016) 8441; http://doi.org/10.1021/acs.chemmater.6b04639	√	
1.4 Is the size (or dimensions, if non-spherical) and shape of the nanomaterial reported?	√	
1.5 Is the size dispersity or aggregation of the nanomaterial reported?	√	
1.6 Is the zeta potential of the nanomaterial reported?	√	
1.7 Is the density (mass/volume) of the nanomaterial reported?		√
1.8 Is the amount of any drug loaded reported? ‘Drug’ here broadly refers to functional cargos (e.g., proteins, small molecules, nucleic acids).	√	
1.9 Is the targeting performance of the nanomaterial reported, including amount of ligand bound to the nanomaterial if the material has been functionalised through addition of targeting ligands?		√
1.10 Is the label signal per nanomaterial/particle reported? For example, fluorescence signal per particle for fluorescently labelled nanomaterials.	√	
1.11 If a material property not listed here is varied, has it been quantified ?		√
1.12 Were characterizations performed in a fluid mimicking biological conditions ?		√
1.13 Are details of how these parameters were measured/estimated provided?	√	
Explanation for No (if needed):		

*Ideally, material characterization should be performed in the same biological environment as that in which the study will be conducted. For example, for cell culture studies with nanoparticles, characterization steps would ideally be performed on nanoparticles dispersed in cell culture media. If this is not possible, then characteristics of the dispersant used (e.g., pH, ionic strength) should mimic as much as possible the biological environment being studied.

Supplementary Table 2. Biological characterization*

Question	Yes	No
2.1 Are cell seeding details, including number of cells plated, confluency at start of experiment, and time between seeding and experiment reported?	✓	
2.2 If a standardised cell line is used, are the designation and source provided?	✓	
2.3 Is the passage number (total number of times a cell culture has been subcultured) known and reported?	✓	
2.4 Is the last instance of verification of cell line reported? If no verification has been performed, is the time passed and passage number since acquisition from trusted source (e.g., ATCC or ECACC) reported? For information, see <i>Science</i> 347 (2015) 938; http://doi.org/10.1126/science.347.6225.938	✓	
2.5 Are the results from mycoplasma testing of cell cultures reported?	✓	
2.6 Is the background signal of cells/tissue reported? (E.g., the fluorescence signal of cells without particles in the case of a flow cytometry experiment.)	✓	
2.7 Are toxicity studies provided to demonstrate that the material has the expected toxicity, and that the experimental protocol followed does not?	✓	
2.8 Are details of media preparation (type of media, serum, any added antibiotics) provided?	✓	
2.9 Is a justification of the biological model used provided? For examples for cancer models, see <i>Cancer Res.</i> 75 (2015) 4016; http://doi.org/10.1158/0008-5472.CAN-15-1558 , and <i>Mol. Ther.</i> 20 (2012) 882; http://doi.org/10.1038/mt.2012.73 , and <i>ACS Nano</i> 11 (2017) 9594; http://doi.org/10.1021/acsnano.7b04855		✓
2.10 Is characterization of the biological fluid (<i>ex vivo/in vitro</i>) reported? For example, when investigating protein adsorption onto nanoparticles dispersed in blood serum, pertinent aspects of the blood serum should be characterised (e.g., protein concentrations and differences between donors used in study).		NA
2.11 For animal experiments, are the ARRIVE guidelines followed? For details, see <i>PLOS Biol.</i> 8 (2010) e1000412; http://doi.org/10.1371/journal.pbio.1000412		NA
Explanation for No (if needed):		

*For *in vitro* experiments (e.g., cell culture), *ex vivo* experiments (e.g., in blood samples), and *in vivo* experiments (e.g., animal models). The questions above that are appropriate depend on the type of experiment conducted.

Supplementary Table 3. Experimental details*

Question	Yes	No
3.1 For cell culture experiments: are cell culture dimensions including type of well , volume of added media , reported? Are cell types (i.e.; adherent vs suspension) and orientation (if non-standard) reported?	✓	
3.2 Is the dose of material administered reported? This is typically provided in nanomaterial mass, volume, number, or surface area added. Is sufficient information reported so that regardless of which one is provided, the other dosage metrics can be calculated (i.e. using the dimensions and density of the nanomaterial)?	✓	
3.3 For each type of imaging performed, are details of how imaging was performed provided, including details of shielding , non-uniform image processing , and any contrast agents added?	✓	
3.4 Are details of how the dose was administered provided, including method of administration , injection location , rate of administration , and details of multiple injections ?		✓
3.5 Is the methodology used to equalise dosage provided?	✓	
3.6 Is the delivered dose to tissues and/or organs (in vivo) reported, as % injected dose per gram of tissue (%ID g ⁻¹)?		✓
3.7 Is mass of each organ/tissue measured and mass of material reported?		NA
3.8 Are the signals of cells/tissues with nanomaterials reported? For instance, for fluorescently labelled nanoparticles, the total number of particles per cell or the fluorescence intensity of particles + cells, at each assessed timepoint.	✓	
3.9 Are data analysis details , including code used for analysis provided?	✓	
3.10 Is the raw data or distribution of values underlying the reported results provided? For examples, see <i>R. Soc. Open Sci.</i> 3 (2016) 150547; http://doi.org/10.1098/rsos.150547 , https://opennessinitiative.org/making-your-data-public/ , http://journals.plos.org/plosone/s/data-availability , and https://www.nature.com/sdata/policies/repositories	✓	
Explanation for No (if needed):		

* The use of protocol repositories (e.g., *Protocol Exchange* <http://www.nature.com/protocolexchange/>) and published standard methods and protocols (e.g., *Chem. Mater.* **29** (2017) 1; <http://doi.org/10.1021/acs.chemmater.6b05235>, and *Chem. Mater.* **29** (2017) 475; <http://doi.org/10.1021/acs.chemmater.6b05481>) are encouraged.

Chapter 4

Polyphenol-Based Nanoparticles for Intracellular Protein Delivery *via* Competing Supramolecular Interactions

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4.1 Manuscript



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Polyphenol-Based Nanoparticles for Intracellular Protein Delivery *via* Competing Supramolecular Interactions

Yiyuan Han, Jiajing Zhou, Yingjie Hu, Zhixing Lin, Yutian Ma, Joseph J. Richardson, and Frank Caruso*

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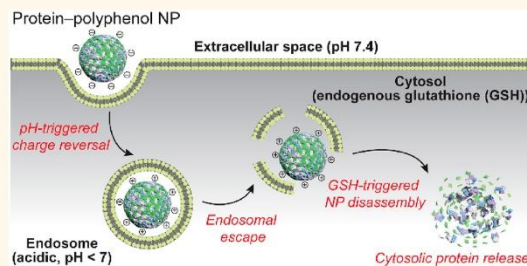
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ABSTRACT: Intracellular delivery of proteins is a promising strategy for regulating cellular behavior and therefore has attracted interest for biomedical applications. Despite the emergence of various nanoparticle-based intracellular delivery approaches, it remains challenging to engineer a versatile delivery system capable of responding to various physiological triggers without the need for complex chemical synthesis of the delivery system. Herein, we develop a template-mediated supramolecular assembly strategy to synthesize protein–polyphenol nanoparticles (NPs) capable of endosomal escape and subsequent protein release in the cytosol. These NPs are stable in serum and undergo surface charge reversal from negative to positive in acidic environments, leading to spontaneous endosomal escape. In the cytosol, endogenous small peptides and amino acids with relatively high charge densities, such as glutathione, trigger NP disassembly through competitive supramolecular interactions, thereby releasing functional bioactive proteins, as validated using cytochrome C and β -galactosidase. The versatility of the present strategy in terms of nanoparticle size, protein type, and functional protein delivery makes this a promising platform for potential application in the field of protein therapeutics.

KEYWORDS: nanoparticle, protein delivery, charge reversal, polyphenol, supramolecular interaction, glutathione



The delivery of proteins to intracellular targets has emerged as an attractive strategy in biomedicine and biotechnology because of the higher specificity and relatively lower side effects of proteins when compared with genetic or small-molecule therapeutics.¹ However, native proteins generally display limited access to cells, as well as high clearance and low stability in the bloodstream, which collectively reduce their potential for efficient intracellular delivery.² Conventional approaches for intracellular protein delivery include the chemical modification of proteins with cell-penetrating peptides or other ligands,^{3,4} the complexation of cargo proteins with carriers, such as polymers, liposomes, and nanoparticles,^{5–7} the genetic engineering of the protein systems themselves, for example, with protein transduction domains,^{8,9} or membrane disruption methods such as microinjection or electroporation.¹⁰ Among these techniques, polymer-mediated intracellular protein delivery is particularly promising because of the ease of protein functionalization and the overall effectiveness of intracellular delivery.^{6,11,12} For example, cationic polymers have been used as carriers for protein translocation¹³ and fluoropolymers have been used to encapsulate proteins for intracellular delivery.¹⁴ However,

concerns still remain regarding the binding affinity of polymers with diverse proteins and the overall safety of some polymer conjugates.^{6,13}

Advances in nanotechnology have enabled the development of a number of nanoparticle-based systems that can effectively deliver specific proteins, however, these often involve complex synthesis routes.^{15–17} Efficient and versatile intracellular protein delivery requires nanoparticles (NPs) to (i) accommodate various proteins, (ii) be stable in serum, (iii) enter cells and escape endosomal compartments, and (iv) release intact functional proteins in the cytosol.¹⁸ However, few approaches have emerged that can fulfill all of these requirements. Furthermore, an alternative to synthesizing NPs with increasing complexity would be to identify simple and readily

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available building blocks that are capable of assembling with proteins through various adaptable supramolecular interactions.¹⁹ Previous noncovalent strategies for intracellular delivery have been demonstrated, such as those based on the commercially available Chariot peptide (Pep-1, Pep-2) for protein transduction²⁰ or pyrenebutyrate treatment for membrane translocation of peptides or quantum dots.^{21,22} These examples demonstrate the promise of supramolecular interactions for engineering NPs as carriers for the intracellular delivery of native proteins.

We recently reported the templated assembly of microcapsules from various proteins with polyphenols *via* diverse supramolecular interactions and demonstrated that the assembly process did not inhibit the functionality of the proteins.²³ Devising future synthesis strategies based on the identification of suitable NP templates is expected to allow for the formation of protein-based NPs with suitable sizes and functionalities useful for intracellular protein delivery. Herein, three therapeutic proteins (*i.e.*, cytochrome C (CYC), immunoglobulin G (IgG), and β -galactosidase (β -Gal)) are examined and assembled with tannic acid (TA) on sacrificial mesoporous silica (MS) NP templates to form protein–polyphenol NPs, which are obtained after template dissolution (Scheme 1a). These protein–TA NPs are stable in both

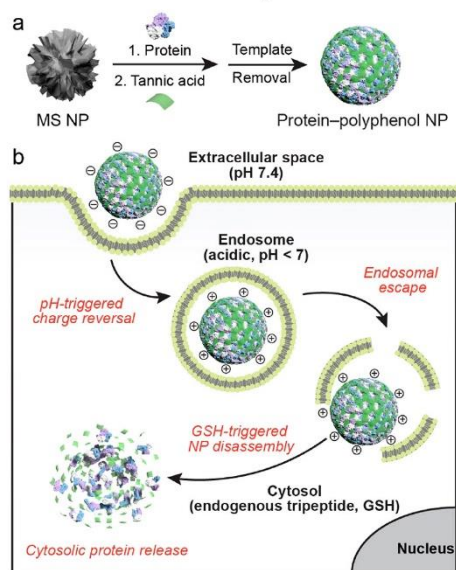
surface charge reversal on the NPs.²⁴ Finally, upon reaching the cytosol, functional protein is released when intracellular glutathione (GSH, 1–11 mM in the cytosol²⁵) triggers supramolecular disassembly of the NPs *via* the formation of competitive noncovalent interactions with both the protein and TA (Scheme 1b). Specifically, CYC–TA NPs can induce cell apoptosis after incubation for 48 h and β -Gal–TA NPs can induce catalytic hydrolysis inside cells. This robust protein–TA NP assembly strategy provides opportunities for the development of protein delivery vehicles.

RESULTS AND DISCUSSION

Protein–TA NPs were synthesized *via* the self-assembly of a protein and TA on sacrificial MS NPs (Scheme 1a and Figure S1). First, the protein was incubated with MS NPs for 12 h. Excess protein was then washed off before TA was used to “cross-link” the protein *via* noncovalent interactions. Stable protein–polyphenol NPs were obtained after template removal with hydrofluoric acid. CYC was chosen as a model protein for investigating the physicochemical parameters and therapeutic efficacy of the polyphenol–TA NPs because it can only induce cell apoptosis *via* mitochondrial affinity after entering the cytosol.²⁶ After template removal, CYC–TA NPs in solution displayed a hydrodynamic size distribution similar to that of the MS template (Figure 1a), centered at ~250 nm. Atomic force microscopy (AFM) measurements showed that the CYC–TA NPs were smaller (*i.e.*, ~120 nm) because of the air-drying process (Figure 1b and Figure S2), suggesting the soft nature of the NPs. A similar degree of shrinkage was observed from both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analyses (Figure 1c and Figure S3). The UV–vis spectrum of the CYC–TA NPs featured the heme group peak at 410 nm, similar to that observed in free CYC (Figure 1d), and the circular dichroism spectra of CYC–TA NPs and free CYC also indicated the preservation of protein structure after assembly (Figure S4). The present strategy was applicable to various proteins of different sizes and isoelectric points (pIs), as exemplified from the successful preparation of IgG–TA NPs and β -Gal–TA NPs (Figure 1e,f). Because of the higher molecular weights of IgG (~150 kDa, pI 8.0) and β -Gal (~430 kDa, pI 5.0) compared with that of CYC (~12 kDa, pI 9.8),²⁷ the air-dried IgG–TA NPs and β -Gal–TA NPs displayed denser morphologies and larger diameters (Figure S5), indicating that careful choice of the protein affords tuning of the physical properties of the protein–TA NPs. In addition, the size of the protein–TA NPs could be varied according to the size of the MS template (Figure S6), allowing for better size control of NPs in contrast with the template-free self-assembly of these similar protein–TA complexes (Figure S7). Collectively, the control afforded by the present strategy *via* tuning of the physicochemical properties of the NPs (*e.g.*, size, density) presents a viable route to modulate the bio–nano interactions of NPs.²⁸ Importantly, the various structures (tertiary or quaternary) and functional side chains of proteins not only affect the binding ratio of protein and TA (Figure S8) but also govern the different interactions between proteins with TA (Figure S9) which allow for the design of NPs capable of responding to diverse physiological environments.

Figure 2a shows the pH-responsive surface charge reversal nature of the CYC–TA NPs, transitioning from negative to positive as the pH is switched from pH 6 to pH 5. This charge reversal is likely due to the protonation of a single hydroxyl

Scheme 1. Supramolecular Assembly of Protein–TA NPs and Intracellular Protein Delivery^a



^a(a) Schematic of the supramolecular assembly of a protein–TA NP on a sacrificial template (mesoporous silica, MS). (b) Scheme depicting intracellular protein delivery from a protein–polyphenol NP.

serum-containing media and human plasma for more than 1 week and can be readily internalized in cells. The diameters of the NPs are tunable, as they are determined by the diameters of the template particles. Importantly, the protein–TA NPs can escape endosomal compartments, as acidic environments such as those present in endosomes (pH 4.5–7.0) trigger a

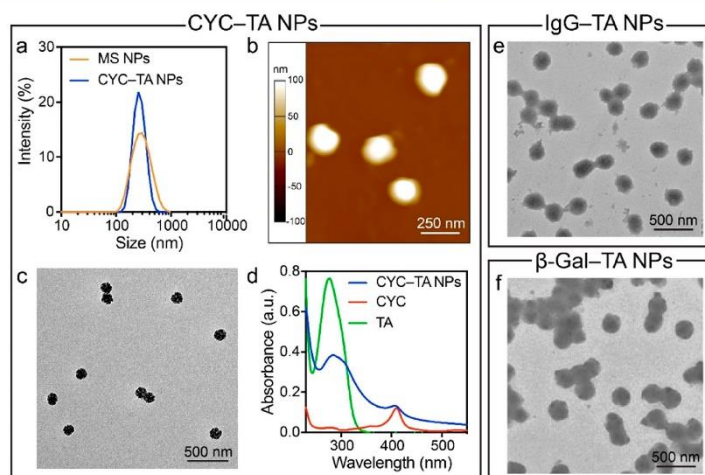


Figure 1. (a) Size distribution profiles of MS NPs (template) and CYC-TA NPs measured in aqueous solution. (b) AFM and (c) TEM analyses of CYC-TA NPs. (d) UV-vis spectra of CYC-TA NPs, CYC, and TA. (e,f) TEM images of IgG-TA and β-Gal-TA NPs.

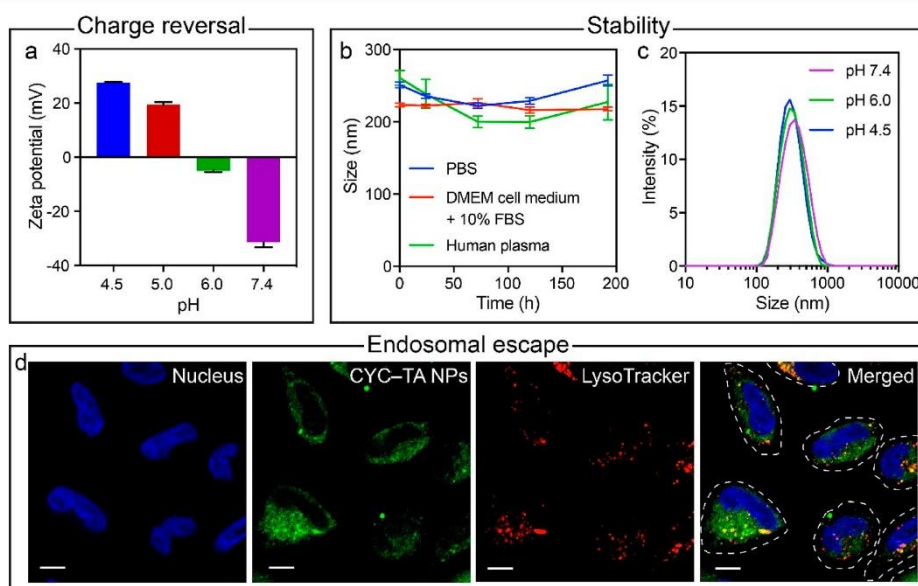


Figure 2. (a) Charge reversal of CYC-TA NPs assessed by microelectrophoresis (*i.e.*, zeta-potential) measurements at different pH. (b) Stability of CYC-TA NPs in different media assessed by monitoring the diameter of the NPs for 192 h by DLS. (c) Size distribution of CYC-TA NPs as a function of pH. (d) Confocal laser scanning microscopy imaging of a LysoTracker colocalization assay performed in HeLa cells after incubation for 24 h with FITC-labeled CYC-TA NPs. Scale bars: 10 μ m.

moiety in each galloyl group in TA as CYC (pI 9.8) is positively charged at the pH values examined (4.5–7.4).²⁹ Other protein-TA NPs (IgG and β-Gal) also exhibited charge reversal but this occurred at pHs related to their pIs (Figure S10). Negatively charged NPs are considered to perform better (*i.e.*, they display less nonspecific binding and clustering) in the extracellular environment (pH 7.4).³⁰ In contrast, positively charged materials have a higher tendency to escape the acidic intracellular compartments (pH 4.5–5.5) that internalize NPs.³¹ The surface charge of the CYC-TA NPs was negative

at pH 7.4, likely due to the presence of the phenolic groups on the surface of the NPs, which conferred colloidal stability to the NPs. As confirmed from the dynamic light scattering (DLS) analysis, aggregation or disassembly of the CYC-TA NPs was not observed following incubation for 192 h in diverse media, that is, phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), human plasma, and different pH solutions (Figure 2b,c). To validate endosomal escape of the NPs, fluorescein isothiocyanate (FITC)-labeled CYC-TA NPs

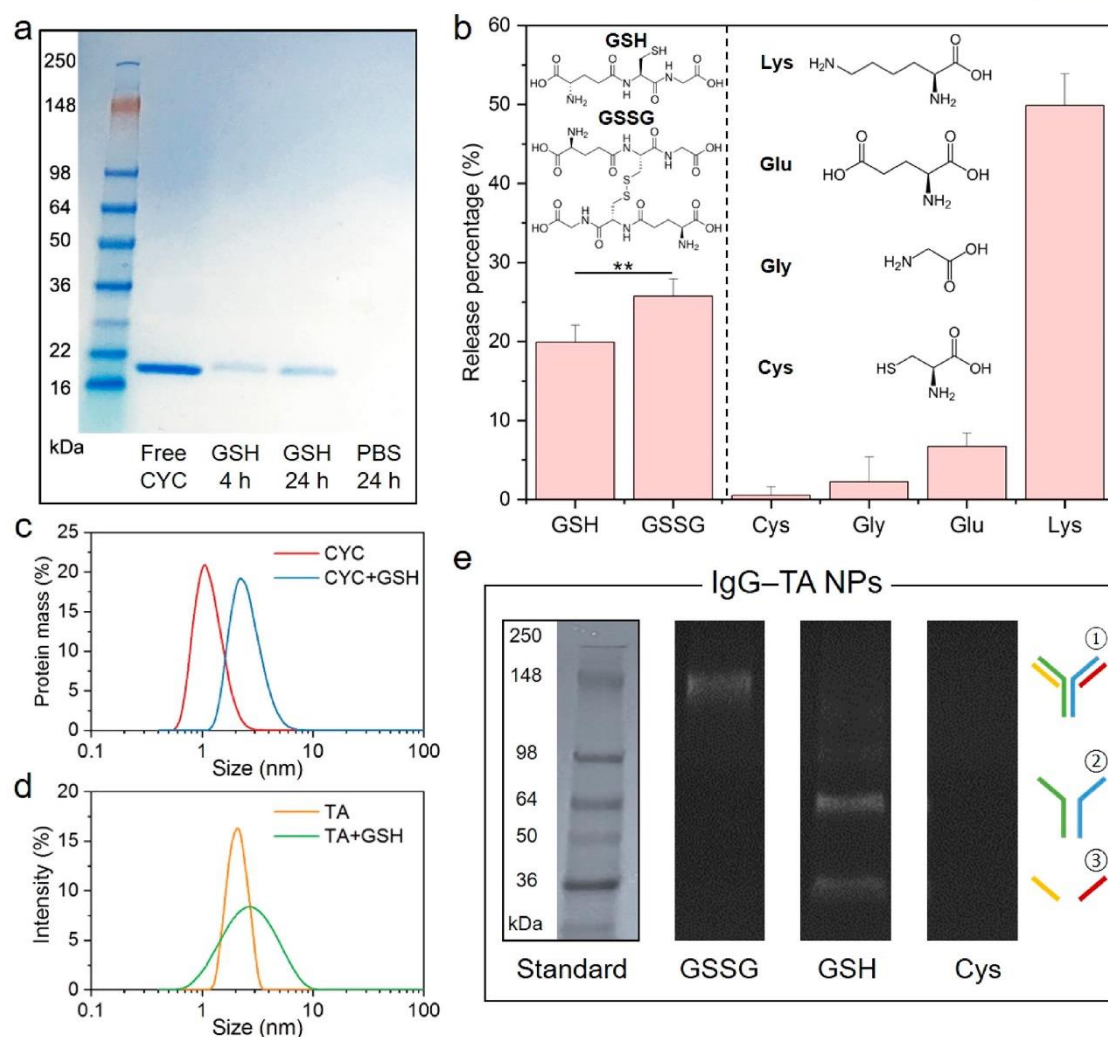


Figure 3. (a) SDS-PAGE analysis of supernatants released from the incubation of CYC-TA NPs with 10 mM GSH for 4 and 24 h or with 1x PBS for 24 h. The positive control was free CYC. (b) Percentage of CYC released from CYC-TA NPs after incubation for 4 h with GSH (10 mM), GSSG (5 mM), Lys (10 mM), Glu (10 mM), Gly (10 mM), or Cys (10 mM). The corresponding chemical structures are also shown. The experiments were performed in triplicate and expressed as mean $\pm$ standard deviation (SD) ($n = 3$). $**P < 0.01$ with 95% confidence level from the unpaired t -test. (c,d) Changes in the size distribution profiles of CYC protein or TA molecule after mixing with GSH. [CYC] = 0.5 mg mL⁻¹; [TA] = 0.5 mg mL⁻¹; [GSH] = 10 mM. (e) SDS-PAGE analysis of supernatants released from the incubation of IgG-TA NPs with 5 mM GSSG, 10 mM GSH, or 10 mM Cys for 24 h. The schematic on the right represents the structure of intact IgG (①), two heavy chains (②), and two light chains (③).

were incubated with HeLa cells for 4 or 24 h and their intracellular distribution was examined. Generally, a portion of the internalized CYC-TA NPs were not colocalized with acidic compartments such as the endosomes and lysosomes at 4 h (Figure S11), as indicated from the relatively low Pearson's correlation coefficient R value of 0.28 ± 0.03 (Figure S12) where the R value ranges from 1 when the green and red fluorescence signals are 100% linearly correlated (i.e., colocalization) to 0% when the signals have no correlation (i.e., endosomal escape).^{32,33} After 24 h incubation with NPs, the cells displayed diffuse green fluorescence, suggesting both

endosomal escape and intracellular disassembly of the NPs (Figure 2d), which are in agreement with electroporation results using free CYC (Figure S13). Additionally, calcein leakage assays of cells treated with CYC-TA NPs confirmed that the NPs could destabilize endo/lysosomal membranes (Figure S14).³⁴ In contrast, MS NPs loaded with FITC-CYC did not escape from endosomes even after incubation for 24 h, which demonstrated the important contributions of TA in regards to the endosomal escape of CYC-TA NPs (Figure S15). FITC-labeled IgG-TA NPs also escaped from endo/lysosomes as validated by CLSM images after 4 h (Figure S16).

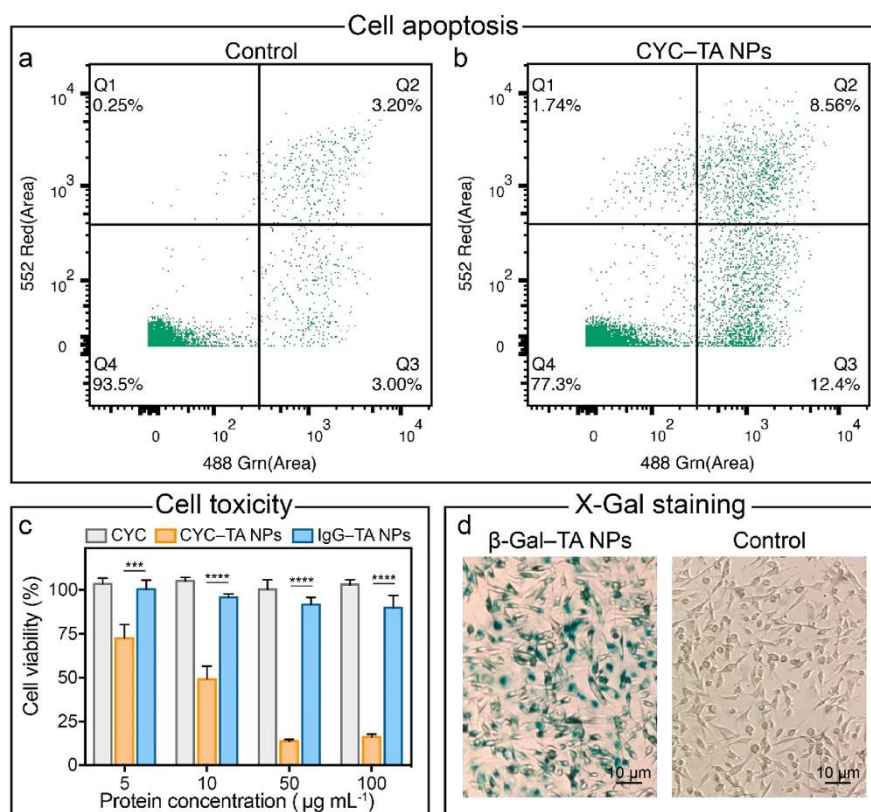


Figure 4. (a,b) Cell apoptosis assay by annexin V-FITC/PI staining performed on Jurkat cells that were incubated in the absence (control) or presence of CYC-TA NPs for 48 h at 37 °C. Q1–Q4 represent necrotic cells, late apoptotic cells, early apoptotic cells, and live cells, respectively. (c) Cell viability of MDA-MB-231 cells incubated in the presence of free CYC, CYC-TA NPs, or IgG-TA NPs at different protein dosages for 48 h at 37 °C. (Data are shown as the mean $\pm$ SD, $n = 6$, *** $P < 0.001$, **** $P < 0.0001$ from the unpaired t -test). (d) X-Gal staining of MDA-MB-231 cells treated with β -Gal-TA NPs for 24 h. The control group was treated with free β -Gal.

These results suggest that NPs self-assembled from simple and natural building blocks without complex chemical modification can successfully escape the endosome, a requirement for intracellular protein release.³⁵

We then investigated the release of the proteins from the NPs in the intracellular environment after endosomal escape. It was postulated that the noncovalent (supramolecular assembly)²³ and charge-shifting nature of the NPs may trigger, through competitive binding with cytosolic proteins, the release of the protein CYC. Because the NPs have been shown to be stable in various protein-containing media (Figure 2b), the trigger is expected to be a protein or peptide not readily present in the extracellular or endosomal environment. The cytosol contains GSH, present at a high concentration (1–11 mM),²⁵ which is widely used as a representative intracellular component to cause the disassembly of disulfide-based systems *via* cleavage of the cross-linker.^{26,36} Particularly, because of its zwitterionic nature, GSH can interact with both the positively charged CYC and negatively charged TA to instigate the disassembly of the protein NPs. To investigate this hypothesis, CYC-TA NPs were incubated with GSH (10 mM) for 4 and 24 h and sodium dodecyl sulfate and polyacrylamide gel electrophoresis (SDS-PAGE) was per-

formed. The findings revealed that CYC was gradually released from the NPs (following incubation for 24 h), which was not observed upon incubation in PBS (control) (Figure 3a and Figure S17). Additionally, lower concentrations of GSH (1 and 5 mM) can still induce the disassembly of NPs and gradual protein release (Figure S18). To examine the GSH-triggered release mechanism, CYC-TA NPs were treated for 4 h with GSH (10 mM), oxidized glutathione (GSSG, 5 mM), and individual amino acids (*i.e.*, glutamic acid (Glu), cysteine (Cys), glycine (Gly), lysine (Lys); 10 mM). The highest concentration at which GSH is found in the cytosol is $\sim$ 10 mM.^{37–39} To ensure that the molar content of the functional groups in GSSG and GSH is the same, a concentration of 5 mM was chosen for GSSG. The concentration of the amino acids was chosen to match that of GSH. The protein release amounts measured by UV-vis spectrophotometry at 410 nm demonstrated that GSSG triggered a slightly higher CYC release ($\sim$ 25%) than GSH ($\sim$ 20%) (Figure 3b and Figure S19). Individually, glutamic acid, glycine, and cysteine, the amino acids that make up GSH, released significantly less CYC, with Glu releasing $\sim$ 6% CYC, followed by Gly ($\sim$ 2%) and Cys ($\sim$ 0.2%). In contrast, the positively charged Lys, which features a flexible and long side-chain and has more

amino groups than GSH or the other individual amino acids (i.e., 2 in Lys vs 1 each in GSH, Gly, and Cys), triggered the most efficient CYC release ($\sim 50\%$) among the different molecules examined (Figure 3b). These results suggest that multiple carboxyl and amine groups may act as important triggering sites rather than the thiol groups of GSH, which is typically used to trigger the intracellular destabilization of nanocarriers.^{39,40} The multiple binding moieties of GSH and GSSG can offer multivalent interactions with a single CYC or TA that are collectively stronger than the corresponding monovalent interactions from individual amino acids.⁴¹ In addition, serum proteins such as FBS with globular structures and low charge densities only formed a protein corona around the surface of the NPs in comparison to the disassembly of the NPs caused by GSH and GSSG, as assessed by DLS (Figure S20). This suggests that intracellular disassembly of the NPs is triggered by endogenous small peptides and amino acids.

CYC contains several lysine and arginine moieties ($\sim 18\%$ of total amino acids) that are positively charged at physiological pH and readily accessible on the surface of the protein (Figure S21). Both lysine and arginine can complex with the carboxylate ions in GSH by forming salt bridges, and a combination of hydrogen bonding and ionic interactions.^{42–45} After the addition of GSH to a solution of free CYC, the size of CYC (assessed by relative mass composition distribution) increased by an order of magnitude (due to aggregation) relative to the size of free CYC (Figure 3c). This result suggests that competitive binding with CYC is one possible mechanism for protein release from the NPs (Figure S22).⁴⁶ However, GSH can also form noncovalent interactions (ionic interactions *via* the N-terminus or hydrogen bonding *via* the amide backbone and C-terminus) with TA, leading to molecular clustering as measured by DLS (Figure 3d), which suggests that this GSH-triggered protein release may be applicable to other protein–TA NPs.

IgG–TA NPs also demonstrated GSH/GSSG-triggered NP disassembly and protein release (Figure 3e). Intact IgG was released from the IgG–TA NPs following incubation with GSSG, whereas incubation with GSH led to IgG release and fragmentation into heavy and light chains *via* disulfide cleavage. Incubation with Lys also led to the release of intact IgG, whereas Cys could not induce significant protein release, further demonstrating that the presence of multivalent charges is more important than the presence of reducing conditions (Figure S23). Collectively, these results demonstrate that the dual-responsive endosomal escape and intracellular delivery achieved herein is an attribute of the delivery platform itself, rather than specific protein–polyphenol pairing.

The bioactivity of the protein released from the NPs was then evaluated. The apoptotic activity of CYC was first monitored by flow cytometry using an annexin V and propidium iodide (PI) staining kit. The results showed that CYC–TA NPs induced $\sim 12\%$ early apoptosis and $\sim 10\%$ late apoptosis toward model Jurkat cells (human T-cell leukemia cell line) after 48 h (Figure 4a,b) because of the low nonspecific association of T cells with NPs ($\sim 30\%$ cells were associated with NPs after 48 h, Figure S24). Furthermore, the cell viability results revealed that the CYC–TA NPs could cause cell death in two different breast cancer cell lines, MDA-MB-231 and BT-474, after 48 h of incubation (Figure 4c and Figure S25), demonstrating general cytotoxic effects for different types of cells including suspension and adherent cells. In contrast, free CYC showed negligible cell toxicity due

to a lack of uptake or trafficking to the cytosol. Note that the effective CYC concentrations ($5\text{--}100\text{ }\mu\text{g mL}^{-1}$) for our CYC–TA NPs to induce apoptosis are consistent with other literature reports of NP systems capable of escaping the endosome and delivering CYC to the cytosol.^{26,47} In addition, NPs containing a nontoxic protein (IgG–TA NPs) displayed negligible cytotoxicity toward both cancer cell types (Figure 4c and Figure S25) and were not capable of inducing cell apoptosis in Jurkat cells (Figure S26), highlighting the tunable, high biocompatibility of polyphenol-based NPs.

To confirm that the activity of the proteins was also maintained in other protein–polyphenol NPs, β -Gal–TA NPs were also studied. MDA-MB-231 cells were treated with β -Gal–TA NPs followed by the addition of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal). β -Gal catalyzes the hydrolysis of X-Gal into an insoluble blue dye, which can be used to determine the amount of bioactive β -Gal in the treated cells.⁴⁸ As observed from the results in Figure 4d, incubation of β -Gal–TA NPs with the cells resulted in a high accumulation of blue dye in the cells after X-Gal staining, confirming the intracellular delivery of active protein. Moreover, the cell targeting ability of the antibodies in the NPs was evaluated using an anti-CD44 antibody that can specifically target CD44-overexpressing cells (MDA-MB-231).⁴⁹ The anti-CD44–TA NPs showed high binding specificity toward MDA-MB-231 cells (46% cell association after 4 h) compared with the negative control of IgG–TA NPs (3% cell association after 4 h) (Figure S27), demonstrating preservation of the antibody function after the formation of NPs. To improve reproducibility, reporting, and reanalysis, this study conforms to the Minimum Information Reporting in Bio–Nano Experimental Literature (MIRIBEL) standard,⁵⁰ and a companion checklist is provided in the Supporting Information.

CONCLUSIONS

In summary, we have developed a versatile NP strategy for the intracellular release of proteins in response to multiple endogenous triggers. The supramolecular protein–TA NPs are stable in the extracellular environment but rapidly disassemble in the cytosol of cells due to competitive binding of small intracellular peptides such as GSH or GSSG. Importantly, the protein–TA NPs can escape the endosome after internalization through intrinsic charge shifting in acidic environments. The released proteins, that is, CYC or β -Gal, maintain their bioactivity after intracellular delivery. This simple and general strategy allows for protein delivery and is thus promising for a variety of therapeutic applications. Because of the universal adhesion properties of polyphenols,^{51,52} it is expected that other cargo, such as DNA⁵³ and RNA, could also be formulated with the NPs for therapeutic codelivery.

EXPERIMENTAL SECTION

Materials. TA, PBS, hydrogen chloride (HCl), FITC, CYC, IgG, β -Gal, GSH, GSSG, Glu, Cys, Gly, Lys, ethylene glycol-bis(2-aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), magnesium chloride (MgCl_2), glutaraldehyde, potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$), potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), X-Gal, cetyltrimethylammonium bromide (CTAB), sodium salicylate (NaSal), tetraethyl orthosilicate (TEOS), and triethylamine (TEA) were purchased from Sigma-Aldrich. DMEM, Roswell Park Memorial Institute (RPMI) 1640 medium, FBS, and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Life Technologies. SimplyBlue SafeStain, NuPAGE LDS sample buffer (4 $\times$), and

SeeBlue Plus2 prestained protein standard were purchased from Invitrogen. Mini-PROTEAN TGX precast gels and Tris/Glycine/SDS running buffer (10X) were purchased from Bio-RAD. High-purity Milli-Q water with a resistivity of 18.2 MΩ cm was obtained from an inline Millipore RiOs/Origin water purification system.

Characterization. TEM of the particles was performed on an FEI Tecnai TF20 instrument (U.S.A.) at an operation voltage of 200 kV. The TEM samples were prepared by dropping an aliquot (5 μL) of diluted samples (~0.05 mg mL⁻¹) on Formvar carbon-coated copper grids. SEM images of the particles were captured on an FEI Quanta 200 field-emission scanning electron microscope, operating at an accelerating voltage of 10 kV. For the SEM experiments, dried samples (gold-sputtered) were used. AFM experiments were conducted on an Asylum Research Cypher AFM instrument. Optical microscopy images of X-Gal-stained cells were acquired on an inverted Olympus IX71 microscope. Confocal laser scanning microscopy (CLSM) images were captured on a Nikon A1R+ confocal laser scanning microscope (Nikon Corporation, Japan). UV–visible absorption spectra were recorded on a Specord 250 Plus spectrophotometer (Analytik Jena AG). Size and zeta-potential measurements of the particles were performed in water and in different pH buffers at a concentration of 5 mM on a Malvern Zetasizer Nano ZS instrument (Malvern Instrument, Worcestershire, U.K.). Protein molecular size (assessed by relative mass composition distribution) and TA molecular size (assessed by intensity distribution) measurements were performed on this Zetasizer. Circular dichroism spectra were recorded on a Model 410 circular dichroism spectrometer (Aviv Biomedical, Inc.).

Cell Culture. HeLa, Jurkat, MDA-MB-231, and BT-474 cells were purchased from the American Type Culture Collection (U.S.A.). HeLa, MDA-MB-231, and BT-474 cells were maintained in complete DMEM media containing 10% FBS. Jurkat cells were maintained in complete RPMI-1640 medium supplemented with 10% FBS. All cell lines were maintained at 37 °C in a cell culture incubator with 5% CO₂ and 95% relative humidity.

Synthesis of MS Particle Templates. MS templates with large and interconnected pores were synthesized by the anion-assisted method and modified from the literature.⁵⁴ Briefly, TEA (68 mg, catalyst), CTAB (380 mg, cationic surfactant), and NaSal (168 mg, structure-directing agent) were successively added to Milli-Q water (25 mL) in a round-bottom flask, and the solution mixture was stirred at 80 °C in an oil bath for 1 h. Then, TEOS (4 mL, silica source) was added dropwise to the above mixture within 10 s and this solution was stirred (175 g) at 80 °C for 2 h. The reaction products were washed with ethanol three times and then purified three times by extraction with 1 M HCl in methanol at 60 °C for 6 h. The MS template particles were finally oven-dried at 80 °C overnight for future use.

Synthesis of Protein–TA NPs. Protein (50 μL, 10 mg mL⁻¹) was added to an aqueous MS particle solution (200 μL, 2.5 mg mL⁻¹). The resulting suspension was vigorously mixed in an ultrasonic bath for 2 min and then incubated in an Eppendorf thermomixer overnight at 37 °C and 1400 rpm. The particles were washed with Milli-Q water once to remove excess protein and resuspended in Milli-Q water (200 μL), after which an aliquot (5 μL) of TA (40 mg mL⁻¹) was added to the particle solution. The resulting suspension was vigorously mixed in an ultrasonic bath for 2 min and then incubated in an Eppendorf thermomixer at 37 °C and 1400 rpm for 4 h. The particles were retrieved and washed with Milli-Q water three times to remove excess TA. In the washing step, the particles were spun down by centrifugation (4000×g, 5 min) and the supernatant was removed. The particles were then resuspended by vigorously mixing prior to the next washing step. Protein–TA NPs were obtained after removal of the MS template with a 1 M HF/3 M NH₄F solution (pH ~ 5).⁵⁵ *Caution! HF is highly toxic and only small quantities should be prepared and handled with extreme care.* The resultant protein–TA NPs were washed three times with water and resuspended in water.

Extracellular Stability of Protein–TA NPs. The extracellular stability of the CYC–TA NPs was examined by monitoring changes in the particle size over 192 h using DLS. The samples were incubated

in 1× PBS, DMEM cell medium+10% FBS, or human plasma before being subjected to the DLS measurements. The experiments were performed in triplicate and the results are expressed as the mean ± SD (*n* = 3).

Endosomal Escape of Protein–TA NPs. HeLa cells were seeded into 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, U.S.A.) at a cell density of 4 × 10⁴ cells per well and then cultured in complete media at 37 °C for 24 h to allow cellular adhesion on substrates. Then, FITC-labeled CYC–TA NPs were added to the cells at a protein concentration of 50 μg mL⁻¹ and then incubated for 4 or 24 h at 37 °C. After incubation, the treated cells were gently washed three times with DPBS and stained with LysoTracker Red for endosome/lysosome labeling, and the colocalization of the green and red fluorescence signals were studied. Live cell imaging was performed by CLSM using a Plan Apo λ 60× 1.4 NA oil immersion objective, 405, 488, 561, and 640 nm lasers, and 450/50, 525/50, 595/50, and 700/75 nm bandpass emission filters. The images were processed by Fiji software.

Calcein Leakage Assay. MDA-MB-231 cells were seeded in 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, U.S.A.) at a cell density of 4 × 10⁴ cells per well in complete media (400 μL) at 37 °C for 24 h to allow cellular adhesion on substrates. Then, the culture media was aspirated and replaced with fresh media (180 μL), and calcein (20 μL, 1.5 mg mL⁻¹ in DPBS) was added to each well in the presence or absence of NPs at a protein concentration of 50 μg mL⁻¹ and incubated with cells for 4 h. Nonfluorescent CYC–TA NPs were used in this assay to avoid interference with the calcein signal. After incubation, the treated cells were gently washed four times with DPBS and live cells were imaged by CLSM.

Release Studies of Protein–TA NPs Assessed by SDS-PAGE.

For the protein release studies, the protein–TA NPs were incubated with 10 mM GSH, 5 mM GSSG, or 1× PBS at 37 °C for a designated time. Centrifugation (5000×g, 10 min) was then performed to remove the intact NPs and complexes before subjecting the supernatants to polyacrylamide gel electrophoresis. Aliquots (15 μL) of the supernatants containing the released proteins were mixed with NuPAGE LDS sample buffer (5 μL, 4×), after which aliquots (18 μL) of the samples were loaded into the gel lanes. The gel was run using 1× Tris/Glycine/SDS running buffer at 200 V until the dye front reached the reference line. Then, the gels were stained with SimplyBlue SafeStain for 1 h under mild shaking and washed with water for at least 1 h to visualize protein staining. The stain-free gels were activated in the stain-free-enabled imager (Gel Doc, BIO-RAD).

Cytotoxicity Assay of CYC–TA NPs. The (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate) (XTT)-based *in vitro* cytotoxicity assay was performed to assess the biocompatibility of the protein–TA NPs. XTT was dissolved in complete DMEM (with 10% FBS) to form a 0.2 mg mL⁻¹ solution, and phenazine methosulfate (PMS) was dissolved in DPBS to form a 1 mM solution. The XTT reagent was activated by mixing with PMS solution at a volume ratio of 400:1. MDA-MB-231 and BT-474 cells were seeded on a 96-well plate at a cell density of 2 × 10³ cells per well and cultured in complete DMEM media at 37 °C for 24 h to allow cellular adhesion on substrates. After incubation with the CYC–TA NPs or free CYC at various protein concentrations for 48 h, the media in the 96-well plate was aspirated and replaced with fresh activated XTT media (100 μL). The cells were further incubated for 4 h and the absorbance at 475 nm was measured relative to nontreated cells. The experiments were performed in sextuplicate and the results are expressed as the mean ± SD (*n* = 6).

Cell Apoptosis with CYC–TA NPs. Jurkat cells were seeded on a 6-well plate at a cell density of 10 × 10⁴ cells per well and cultured in complete media at 37 °C. CYC–TA NPs at a protein concentration of 50 μg mL⁻¹ were added to the cell medium and incubated for 48 h. The cells were harvested after the incubation period and washed in cold PBS buffer. The washed cells were then resuspended in 1× annexin-binding buffer (100 μL). Aliquots of Alexa Fluor 488 annexin V (5 μL) and PI working solution (1 μL) were added to the cell suspension (100 μL), and the cells were incubated at room temperature for 15

min. After the incubation period, 1× annexin-binding buffer (400 μ L) was added to the sample, and the stained cells were analyzed by flow cytometry by measuring the fluorescence emission at 530 and 575 nm using 488 nm excitation.

X-Gal Staining for Intracellular Delivery of β -Gal. MDA-MB-231 cells were treated with β -Gal-TA NPs for 24 h. Then, the cell media was aspirated and the treated cells were fixed with X-Gal fix buffer (100 mM phosphate buffer (pH 7.4) supplemented with 5 mM EGTA, pH 7.3, 2 mM MgCl_2 , and 0.2% glutaraldehyde) for 15 min at room temperature. The cells were washed twice (5 min each) with X-Gal wash buffer (100 mM phosphate buffer (pH 7.4) supplemented with 2 mM MgCl_2) and incubated with the X-Gal staining buffer (100 mM phosphate buffer (pH 7.4) supplemented with 2 mM MgCl_2 , 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, and 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$) containing 1 mg mL^{-1} X-Gal overnight at 37 $^\circ\text{C}$ inside an enclosed humidified chamber. The treated cells were further washed with X-Gal wash buffer and observed under an optical microscope.

MIRIBEL. The studies conducted herein, including material characterization, biological characterization, and experimental details, conform to the MIRIBEL reporting standard for bio-nano research,⁵⁰ and we include a companion checklist of these components herein.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.0c04197>.

TEM image of SiO_2 particles, AFM and SEM images of CYC-TA NPs, CD spectra of CYC-TA NPs, size distribution of air-dried protein-TA NPs, mass/molar ratio of TA and proteins inside NPs, interaction-dependent disassembly of protein-TA NPs, charge reversal of protein-TA NPs, CLSM images and corresponding Pearson's *R* values for endosomal escape, original SDS-PAGE of released protein solutions, cell viability, cell targeting of antibody-TA NPs, and MIRIBEL checklist for reporting research in bio-nano science (PDF)

AUTHOR INFORMATION

Corresponding Author

Frank Caruso – ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia; orcid.org/0000-0002-0197-497X; Email: fcarus@unimelb.edu.au

Authors

Yiyuan Han – ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

Jiajing Zhou – ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia; orcid.org/0000-0001-5203-4737

Yingjie Hu – ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

Zhixing Lin – ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia; orcid.org/0000-0001-9372-3424

Yutian Ma – ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical

Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

Joseph J. Richardson – ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acsnano.0c04197>

Notes

The authors declare no competing financial interest.

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4.2 Supporting Information

Supporting Information

Polyphenol-Based Nanoparticles for Intracellular Protein Delivery *via* Competing Supramolecular Interactions

Yiyuan Han, Jiajing Zhou, Yingjie Hu, Zhixing Lin, Yutian Ma, Joseph J. Richardson, and

*Frank Caruso**

ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the
Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria
3010, Australia

*Corresponding author. E-mail address: fcaruso@unimelb.edu.au

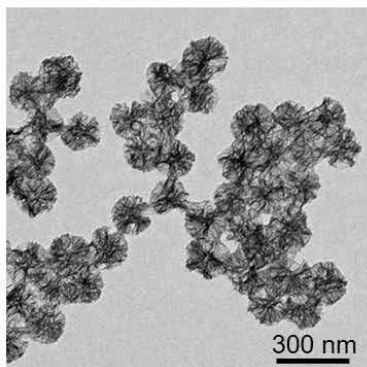


Figure S1. TEM image of mesoporous silica (MS) nanoparticles, with an average size of 259 ± 13 nm. An average of 20 particles were counted.

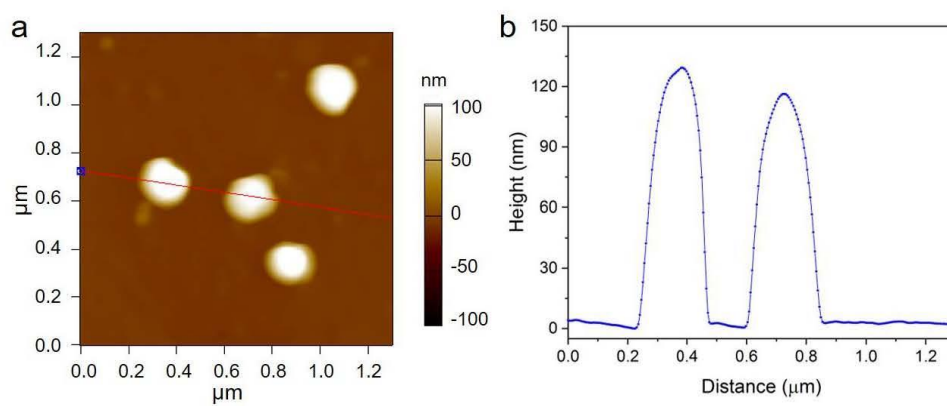


Figure S2. (a) AFM image and (b) corresponding height profile of CYC-TA NPs. The height of the NPs is ~ 120 nm.

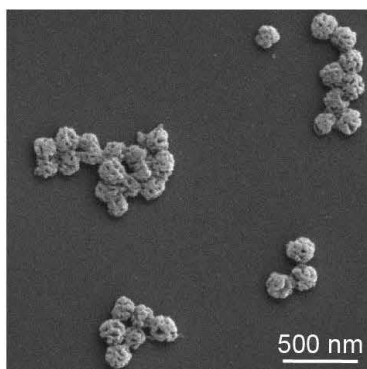


Figure S3. SEM image of CYC-TA NPs, with an average size of 161 ± 10 nm. An average of 20 particles were counted.

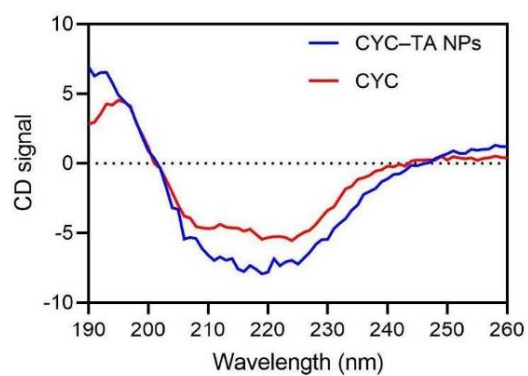


Figure S4. CD spectra of CYC and CYC-TA NPs.

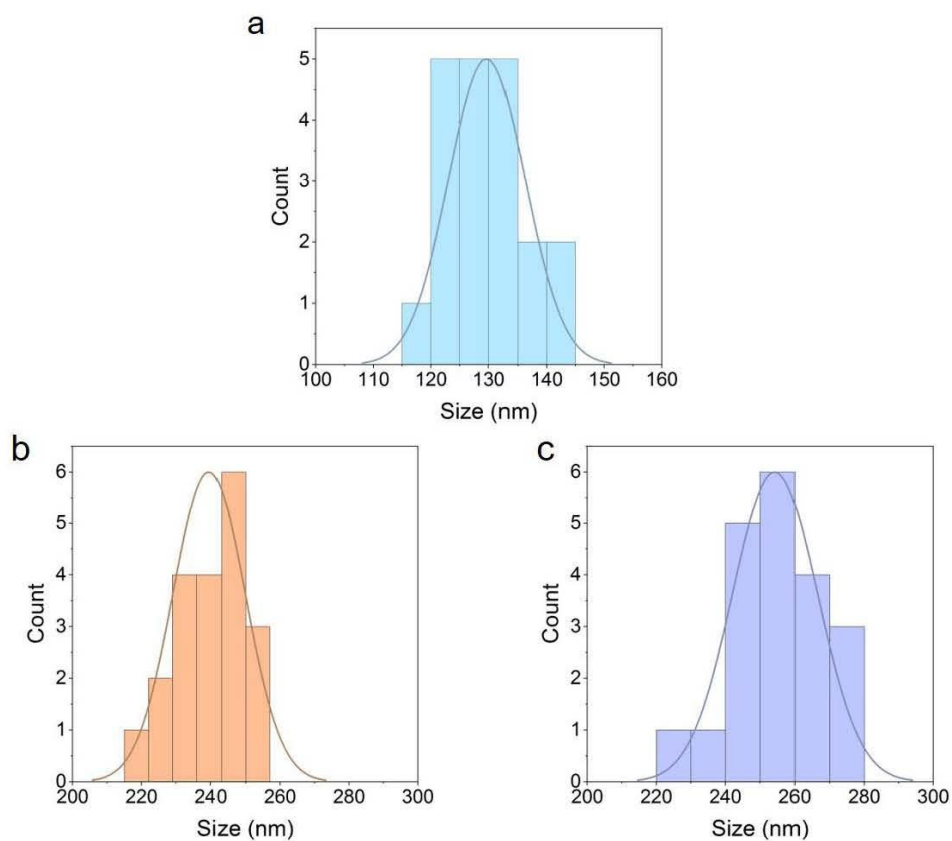


Figure S5. Size distributions of air-dried (a) CYC-TA NPs, (b) IgG-TA NPs, and (c) β -Gal-TA NPs, as measured from TEM images. Twenty particles were counted.

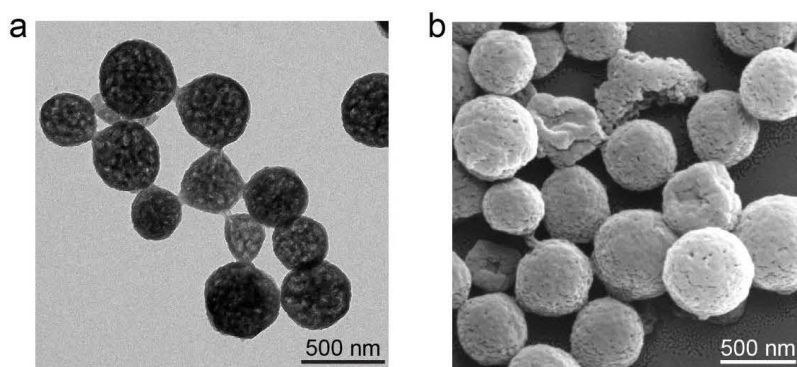


Figure S6. (a) TEM and (b) SEM images of CYC-TA NPs (~400–500 nm). MS particles with an average size of ~500 nm were used as the template. An average of 20 particles were counted.

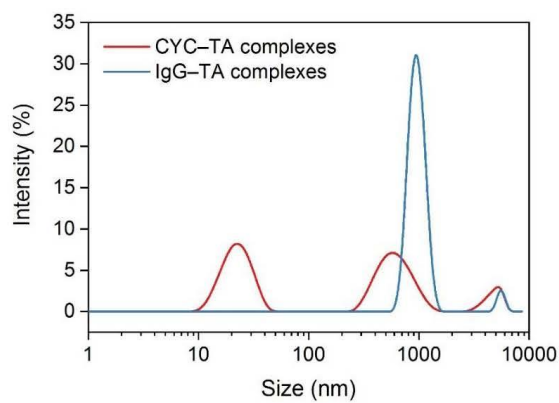


Figure S7. Size distribution of the protein–TA complexes without any template present. (CYC: 0.2 mg mL^{-1} , IgG: 0.2 mg mL^{-1} , TA: 0.2 mg mL^{-1} . Note that these concentrations are the same as those used for the templated NP synthesis.)

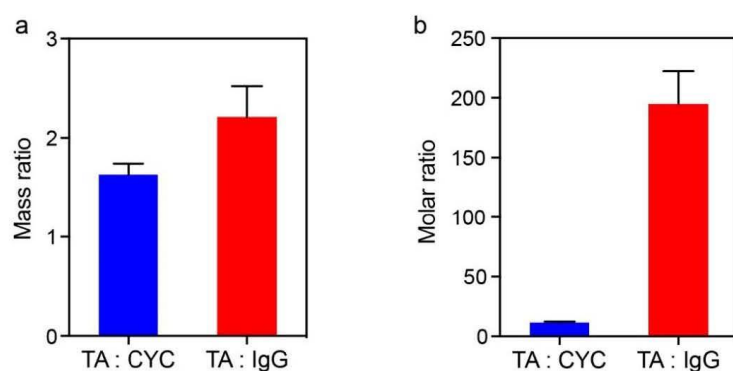


Figure S8. (a) Mass ratio and (b) molar ratio of TA and CYC, and TA and IgG, as assessed by the loaded amount of protein inside the NPs and the binding amount of TA with the protein. The concentrations of CYC and IgG were determined by UV-vis absorbance at wavelengths of 410 and 280 nm, respectively. The concentration of TA was determined by the Folin-Ciocalteu method. Data are shown as the mean $\pm$ standard deviation ($n = 4$).

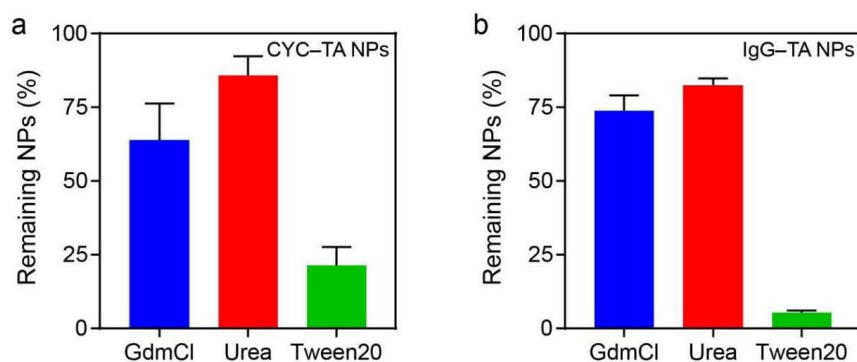


Figure S9. Interaction-dependent disassembly of (a) CYC-TA and (b) IgG-TA NPs in the presence of 100 mM of guanidinium chloride (GdmCl), urea, or tween 20 (representing ionic interactions, hydrogen bonding, and hydrophobic interactions, respectively) after 24 h, as assessed by UV-vis absorbance at a wavelength of 600 nm (OD600). Data are shown as the mean $\pm$ standard deviation ($n = 3$).

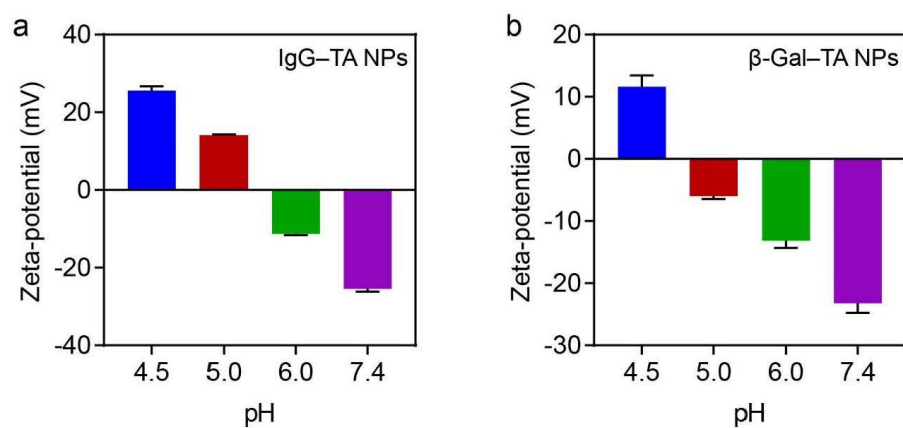


Figure S10. Charge reversal of protein-TA NPs assessed by microelectrophoresis (*i.e.*, zeta-potential) measurements at different pH: (a) IgG and (b) β-Gal.

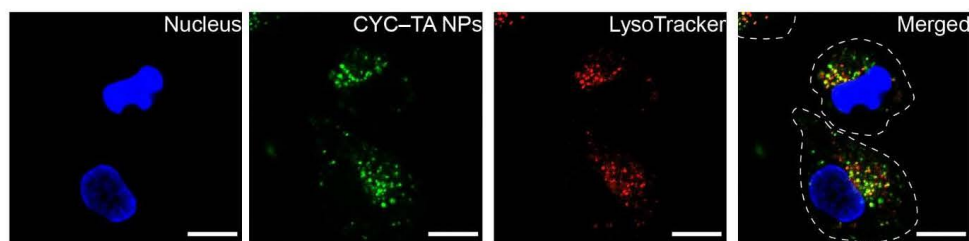


Figure S11. CLSM images of a LysoTracker colocalization assay performed in HeLa cells after 4 h incubation with FITC-labeled CYC-TA NPs. CYC concentration: $50 \mu\text{g mL}^{-1}$. Scale bars: $10 \mu\text{m}$.

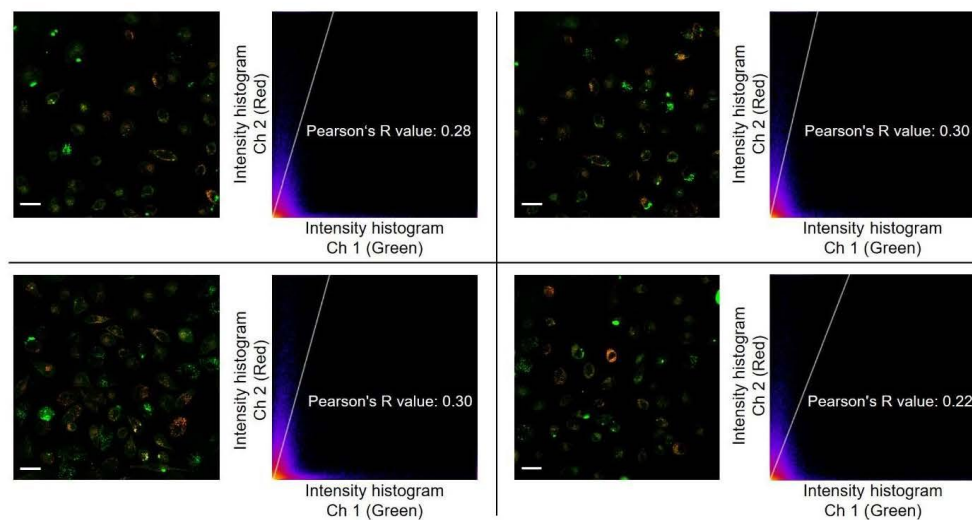


Figure S12. Endosomal escape studies of co-localization of LysoTracker Red (red channel) and FITC-labeled CYC-TA NPs (green channel). Four sets of CLSM images and corresponding Pearson's R values are shown. Scale bars: 20 μm .

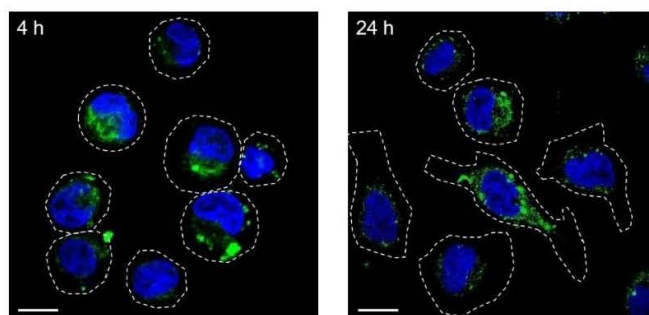


Figure S13. CLSM images of HeLa cells electroporated with FITC-CYC after 4 and 24 h incubation. Protein concentration: $50 \mu\text{g mL}^{-1}$. Scale bars: $20 \mu\text{m}$. Electroporation experiments were performed on Amaxa Nucleofector™ 2b Device, following the protocol from the Cell Line Nucleofector™ Kit R (Catalog number: VACA-100, Lonza).

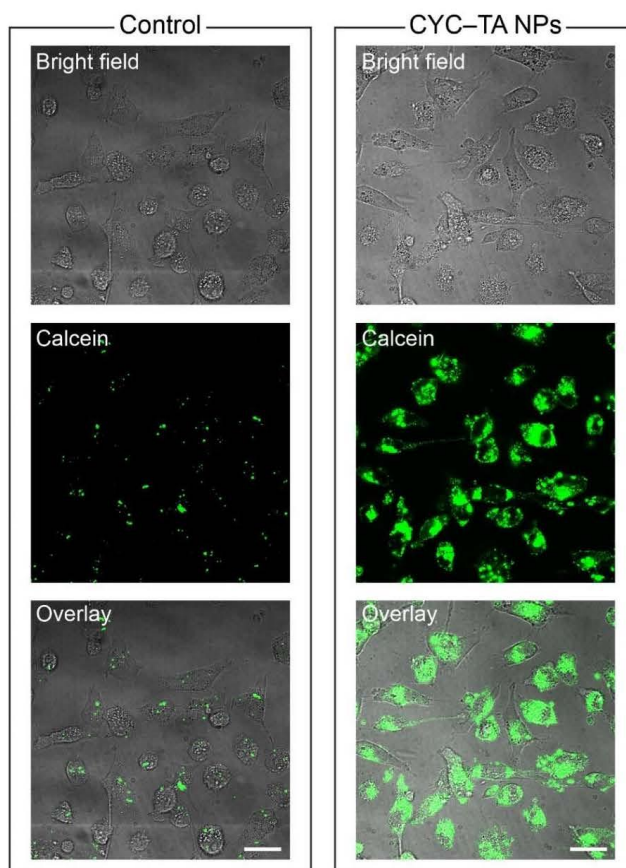


Figure S14. CLSM images of MDA-MB-231 cells incubated for 4 h with calcein (control) or with calcein and CYC-TA NPs. NPs were not fluorescently labeled to avoid interference with the calcein signal. CYC concentration: $50 \mu\text{g mL}^{-1}$. Calcein concentration: 0.15 mg mL^{-1} . Scale bars in all images: $50 \mu\text{m}$.

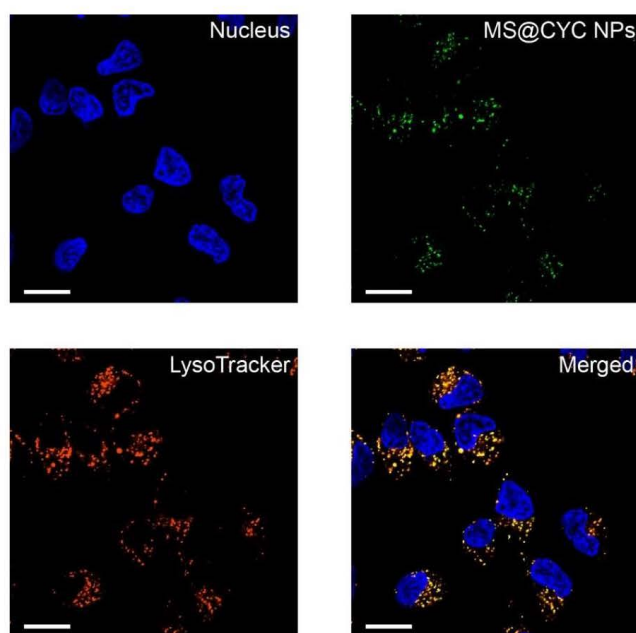


Figure S15. CLSM images of a LysoTracker colocalization assay performed in HeLa cells after 24 h incubation with FITC-labeled CYC-loaded MS NPs (MS@CYC NPs) without any TA. CYC concentration: $50 \mu\text{g mL}^{-1}$. Scale bars: $20 \mu\text{m}$.

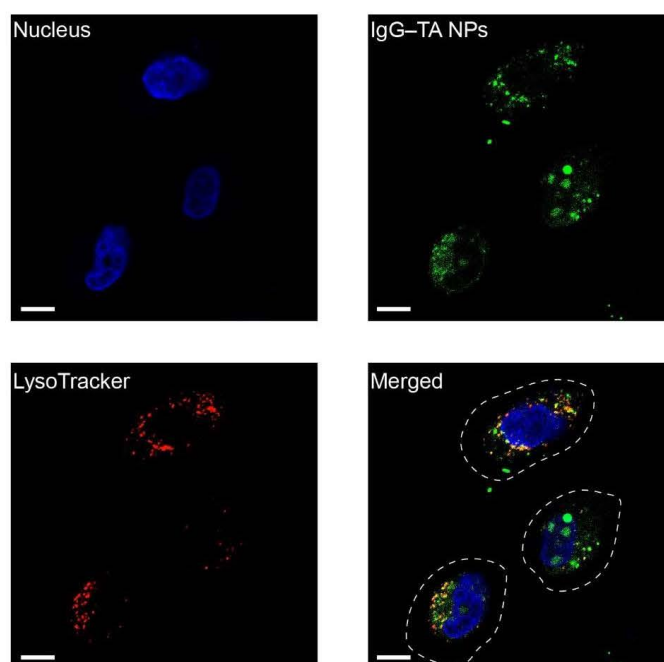


Figure S16. CLSM images of a LysoTracker colocalization assay performed in HeLa cells after incubation for 4 h with FITC-labeled IgG-TA NPs. IgG concentration: $50 \mu\text{g mL}^{-1}$. Scale bars: $10 \mu\text{m}$.

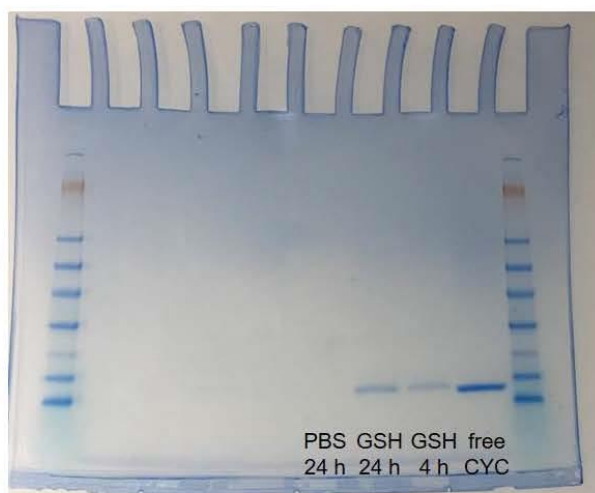


Figure S17. Original SDS-PAGE of the solutions of protein released from CYC-TA NPs following incubation with $1\times$ PBS for 24 h or 10 mM GSH for 4 and 24 h. The positive control was free CYC.

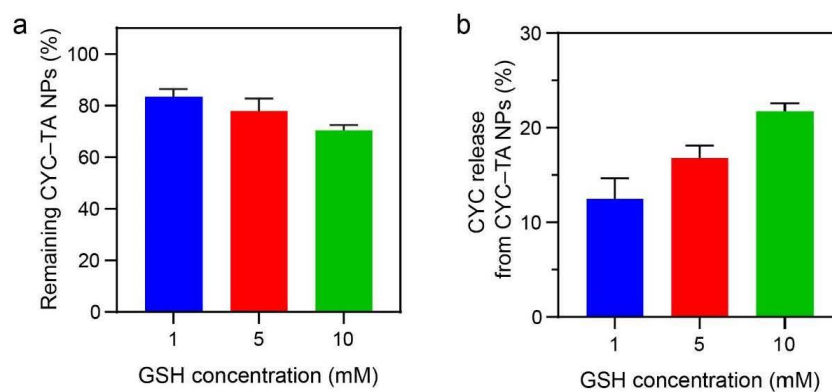


Figure S18. (a) Disassembly of CYC-TA NPs in the presence of different concentration of GSH after 4 h as assessed by UV-vis absorbance at a wavelength of 600 nm (OD600). (b) Percentage of CYC released from CYC-TA NPs after incubation for 4 h with different GSH concentrations. Data are shown as the mean $\pm$ standard deviation ($n = 4$).

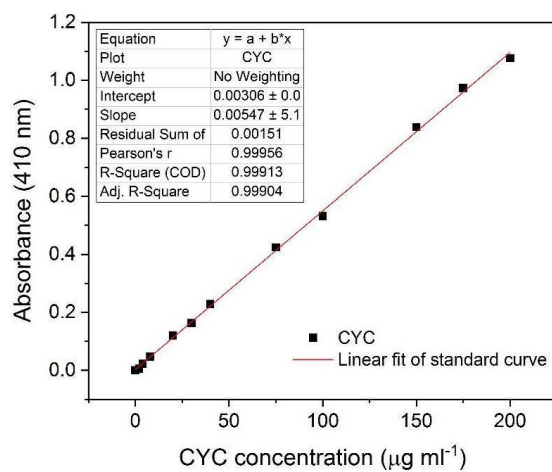


Figure S19. Standard calibration curve used for determining the concentration of CYC at 410 nm.

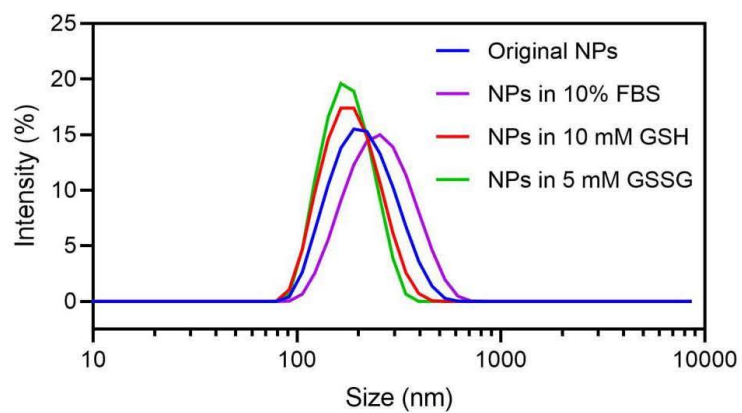


Figure S20. Size distribution of CYC-TA NPs before and after 1 h incubation with 10% FBS, 10 mM GSH, or 5 mM GSSG.

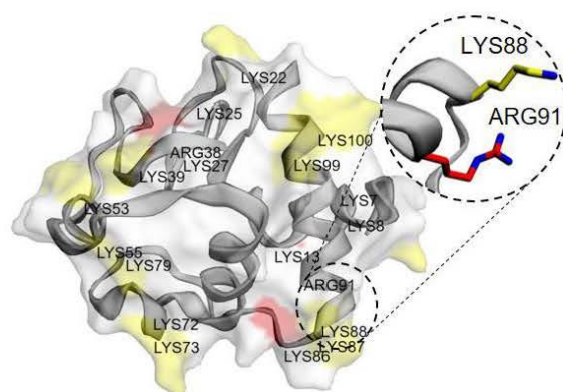


Figure S21. Three-dimensional structure of CYC (PDB: 6FF5). Lys residues (yellow) and Arg residues (red) were colored and labeled on the surface of CYC. A close-up view of LYS88 and ARG91 is also shown.

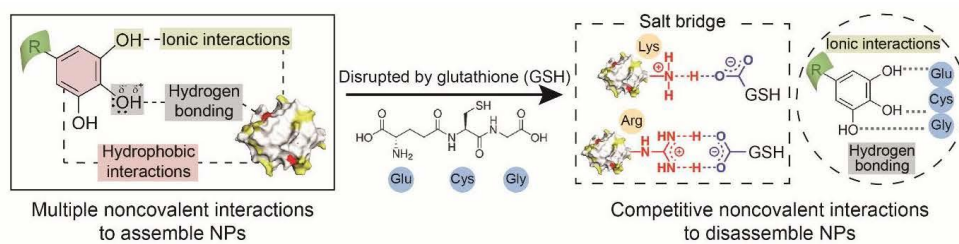


Figure S22. Schematic of GSH-triggered NP disassembly *via* competitive interactions.

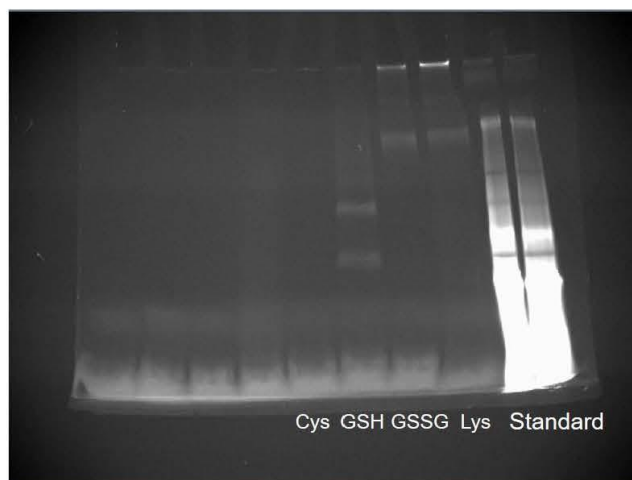


Figure S23. Original SDS-PAGE analysis of the solutions of protein released from IgG-TA NPs following incubation for 24 h with 10 mM GSH, 5 mM GSSG, 10 mM Cys, or 10 mM Lys.

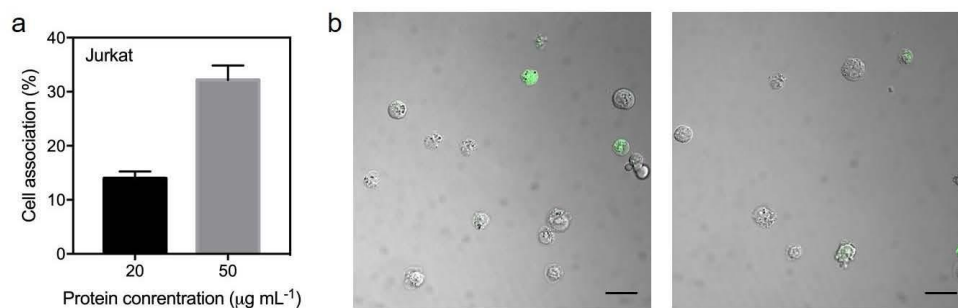


Figure S24. (a) Cell association percentage of Jurkat cells with FITC-labeled CYC-TA NPs at different protein dosages assessed by flow cytometry after 48 h incubation at 37 °C. (b) CLSM images of Jurkat cells with FITC-CYC-TA NPs at protein concentration of 50 µg mL⁻¹ after 48 h incubation at 37 °C; scale bars: 20 µm.

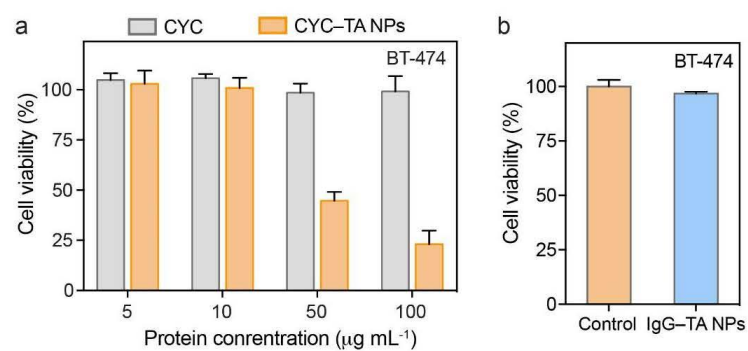


Figure S25. Cell viability assay performed on BT-474 cells following incubation for 48 h at 37 °C (a) in the presence of free CYC or CYC-TA NPs at different protein dosages and (b) in the absence (Control) and presence of IgG-TA NPs at a protein concentration of 20 $\mu\text{g mL}^{-1}$.

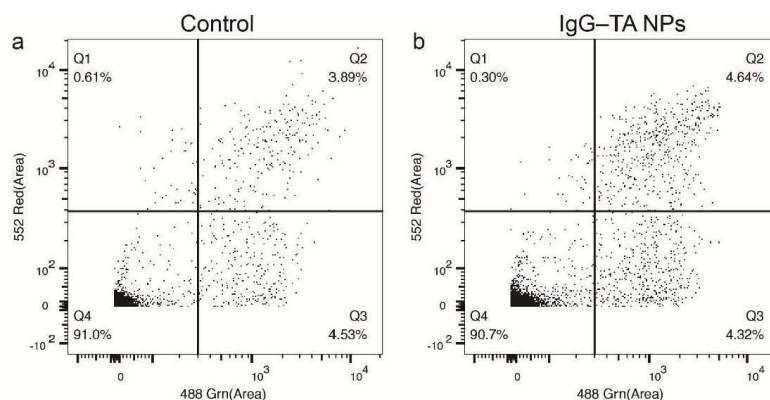


Figure S26. Cell apoptosis assay by annexin V-FITC/PI staining performed on Jurkat cells incubated in the absence (Control) or presence of IgG-TA NPs for 48 h at 37 °C. Protein concentration: 50 $\mu\text{g mL}^{-1}$. Q1–Q4 represent necrotic cells, late apoptotic cells, early apoptotic cells, and live cells, respectively.

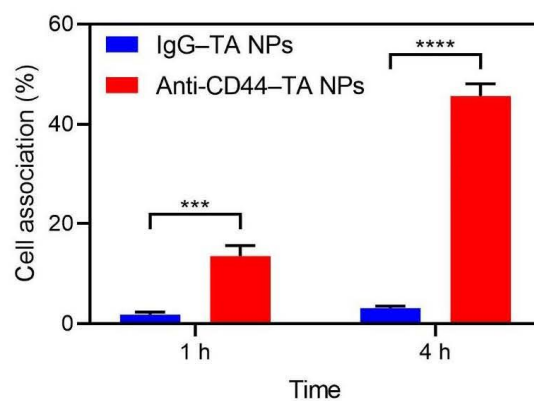


Figure S27. Cell targeting of antibody–TA NPs assessed by flow cytometry analysis of the percentage of MDA-MB-231 cells associated with anti-CD44–TA (targeted) and IgG–TA (control) NPs after 1 or 4 h incubation at 37 °C. Protein concentration (anti-CD44 and IgG): 10 $\mu\text{g mL}^{-1}$. *** $P < 0.001$, **** $P < 0.0001$ with 95% confidence level from the unpaired t -test.

Checklist

Minimum Information Reporting in Bio–Nano Experimental Literature

The MIRIBEL guidelines were introduced here: <https://doi.org/10.1038/s41565-018-0246-4>

The development of these guidelines was led by the ARC Centre of Excellence in Convergent Bio–Nano Science and Technology: <https://www.cbns.org.au/>. Any updates or revisions to this document will be made available here: <http://doi.org/10.17605/OSF.IO/SMVTF>. This document is made available under a CC-BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>.

The MIRIBEL guidelines were developed to facilitate reporting and dissemination of research in bio–nano science. Their development was inspired by various similar efforts:

- MIAME (microarray experiments): *Nat. Genet.* **29** (2001), 365; <http://doi.org/10.1038/ng1201-365>
- MIRIAM (biochemical models): *Nat. Biotechnol.* **23** (2005) 1509; <http://doi.org/10.1038/nbt1156>
- MIBBI (biology/biomedicine): *Nat. Biotechnol.* **26** (2008) 889; <http://doi.org/10.1038/nbt.1411>
- MIGS (genome sequencing): *Nat. Biotechnol.* **26** (2008) 541; <http://doi.org/10.1038/nbt1360>
- MIQE (quantitative PCR): *Clin. Chem.* **55** (2009) 611; <http://doi.org/10.1373/clinchem.2008.112797>
- ARRIVE (animal research): *PLOS Biol.* **8** (2010) e1000412; <http://doi.org/10.1371/journal.pbio.1000412>
- Nature’s reporting standards:
 - Life science: <https://www.nature.com/authors/policies/reporting.pdf>; e.g., *Nat. Nanotechnol.* **9** (2014) 949; <http://doi.org/10.1038/nnano.2014.287>
 - Solar cells: <https://www.nature.com/authors/policies/solarchecklist.pdf>; e.g., *Nat. Photonics* **9** (2015) 703; <http://doi.org/10.1038/nphoton.2015.233>
 - Lasers: <https://www.nature.com/authors/policies/laserchecklist.pdf>; e.g., *Nat. Photonics* **11** (2017) 139; <http://doi.org/10.1038/nphoton.2017.28>
- The “TOP guidelines”: e.g., *Science* **352** (2016) 1147; <http://doi.org/10.1126/science.aag2359>

Similar to many of the efforts listed above, the parameters included in this checklist are **not** intended to be definitive requirements; instead they are intended as ‘points to be considered’, with authors themselves deciding which parameters are—and which are not—appropriate for their specific study.

This document is intended to be a living document, which we propose is revisited and amended annually by interested members of the community, who are encouraged to contact the authors of this document. Parts of this document were developed at the annual International Nanomedicine Conference in Sydney, Australia: <http://www.oznanomed.org/>, which will continue to act as a venue for their review and development, and interested members of the community are encouraged to attend.

After filling out the following pages, this checklist document can be attached as a “Supporting Information” document during submission of a manuscript to inform Editors and Reviewers (and eventually readers) that all points of MIRIBEL have been considered.

Supplementary Table 1. Material characterization*

Question	Yes	No
1.1 Are “best reporting practices” available for the nanomaterial used? For examples, see <i>Chem. Mater.</i> 28 (2016) 3535; http://doi.org/10.1021/acs.chemmater.6b01854 and <i>Chem. Mater.</i> 29 (2017) 1; http://doi.org/10.1021/acs.chemmater.6b05235		√
1.2 If they are available, are they used? If not available, ignore this question and proceed to the next one.		
1.3 Are extensive and clear instructions reported detailing all steps of synthesis and the resulting composition of the nanomaterial? For examples, see <i>Chem. Mater.</i> 26 (2014) 1765; http://doi.org/10.1021/cm500632c , and <i>Chem. Mater.</i> 26 (2014) 2211; http://doi.org/10.1021/cm5010449 . Extensive use of photos, images, and videos are strongly encouraged. For example, see <i>Chem. Mater.</i> 28 (2016) 8441; http://doi.org/10.1021/acs.chemmater.6b04639	√	
1.4 Is the size (or dimensions , if non-spherical) and shape of the nanomaterial reported?	√	
1.5 Is the size dispersity or aggregation of the nanomaterial reported?	√	
1.6 Is the zeta potential of the nanomaterial reported?	√	
1.7 Is the density (mass/volume) of the nanomaterial reported?		√
1.8 Is the amount of any drug loaded reported? ‘Drug’ here broadly refers to functional cargos (<i>e.g.</i> , proteins, small molecules, nucleic acids).	√	
1.9 Is the targeting performance of the nanomaterial reported, including amount of ligand bound to the nanomaterial if the material has been functionalised through addition of targeting ligands?		√
1.10 Is the label signal per nanomaterial/particle reported? For example, fluorescence signal per particle for fluorescently labelled nanomaterials.		√
1.11 If a material property not listed here is varied, has it been quantified ?		√
1.12 Were characterizations performed in a fluid mimicking biological conditions ?		√
1.13 Are details of how these parameters were measured/estimated provided?	√	
Explanation for No (if needed):		

*Ideally, material characterization should be performed in the same biological environment as that in which the study will be conducted. For example, for cell culture studies with nanoparticles, characterization steps would ideally be performed on nanoparticles dispersed in cell culture media. If this is not possible, then characteristics of the dispersant used (*e.g.*, pH, ionic strength) should mimic as much as possible the biological environment being studied.

Supplementary Table 2. Biological characterization*

Question	Yes	No
2.1 Are cell seeding details, including number of cells plated, confluency at start of experiment, and time between seeding and experiment reported?	✓	
2.2 If a standardised cell line is used, are the designation and source provided?	✓	
2.3 Is the passage number (total number of times a cell culture has been subcultured) known and reported?		✓
2.4 Is the last instance of verification of cell line reported? If no verification has been performed, is the time passed and passage number since acquisition from trusted source (e.g., ATCC or ECACC) reported? For information, see <i>Science</i> 347 (2015) 938; http://doi.org/10.1126/science.347.6225.938	✓	
2.5 Are the results from mycoplasma testing of cell cultures reported?	✓	
2.6 Is the background signal of cells/tissue reported? (e.g., the fluorescence signal of cells without particles in the case of a flow cytometry experiment.)	✓	
2.7 Are toxicity studies provided to demonstrate that the material has the expected toxicity, and that the experimental protocol followed does not?	✓	
2.8 Are details of media preparation (type of media, serum, any added antibiotics) provided?	✓	
2.9 Is a justification of the biological model used provided? For examples for cancer models, see <i>Cancer Res.</i> 75 (2015) 4016; http://doi.org/10.1158/0008-5472.CAN-15-1558 , and <i>Mol. Ther.</i> 20 (2012) 882; http://doi.org/10.1038/mt.2012.73 , and <i>ACS Nano</i> 11 (2017) 9594; http://doi.org/10.1021/acsnano.7b04855		✓
2.10 Is characterization of the biological fluid (<i>ex vivo/in vitro</i>) reported? For example, when investigating protein adsorption onto nanoparticles dispersed in blood serum, pertinent aspects of the blood serum should be characterised (e.g., protein concentrations and differences between donors used in study).	Not applicable	
2.11 For animal experiments, are the ARRIVE guidelines followed? For details, see <i>PLOS Biol.</i> 8 (2010) e1000412; http://doi.org/10.1371/journal.pbio.1000412	Not applicable	
Explanation for No (if needed):		

*For *in vitro* experiments (e.g., cell culture), *ex vivo* experiments (e.g., in blood samples), and *in vivo* experiments (e.g., animal models). The questions above that are appropriate depend on the type of experiment conducted.

Supplementary Table 3. Experimental details*

Question	Yes	No
3.1 For cell culture experiments: are cell culture dimensions including type of well , volume of added media , reported? Are cell types (<i>i.e.</i> ; adherent <i>vs</i> suspension) and orientation (if non-standard) reported?	√	
3.2 Is the dose of material administered reported? This is typically provided in nanomaterial mass, volume, number, or surface area added. Is sufficient information reported so that regardless of which one is provided, the other dosage metrics can be calculated (<i>i.e.</i> using the dimensions and density of the nanomaterial)?	√	
3.3 For each type of imaging performed, are details of how imaging was performed provided, including details of shielding , non-uniform image processing , and any contrast agents added?	√	
3.4 Are details of how the dose was administered provided, including method of administration , injection location , rate of administration , and details of multiple injections ?	Not applicable	
3.5 Is the methodology used to equalise dosage provided?	√	
3.6 Is the delivered dose to tissues and/or organs (<i>in vivo</i>) reported, as % injected dose per gram of tissue (%ID g ⁻¹)?	Not applicable	
3.7 Is mass of each organ/tissue measured and mass of material reported?	Not applicable	
3.8 Are the signals of cells/tissues with nanomaterials reported? For instance, for fluorescently labelled nanoparticles, the total number of particles per cell or the fluorescence intensity of particles + cells, at each assessed timepoint.	√	
3.9 Are data analysis details , including code used for analysis provided?	√	
3.10 Is the raw data or distribution of values underlying the reported results provided? For examples, see <i>R. Soc. Open Sci.</i> 3 (2016) 150547; http://doi.org/10.1098/rsos.150547 , https://opennessinitiative.org/making-your-data-public/ , http://journals.plos.org/plosone/s/data-availability , and https://www.nature.com/sdata/policies/repositories	√	
Explanation for No (if needed):		

* The use of protocol repositories (*e.g.*, *Protocol Exchange* <http://www.nature.com/protocolexchange/>) and published standard methods and protocols (*e.g.*, *Chem. Mater.* **29** (2017) 1; <http://doi.org/10.1021/acs.chemmater.6b05235>, and *Chem. Mater.* **29** (2017) 475; <http://doi.org/10.1021/acs.chemmater.6b05481>) are encouraged.

Chapter 5

Protein Corona Analysis of Polyphenol-Modified Nanoparticles

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5.1 Aim

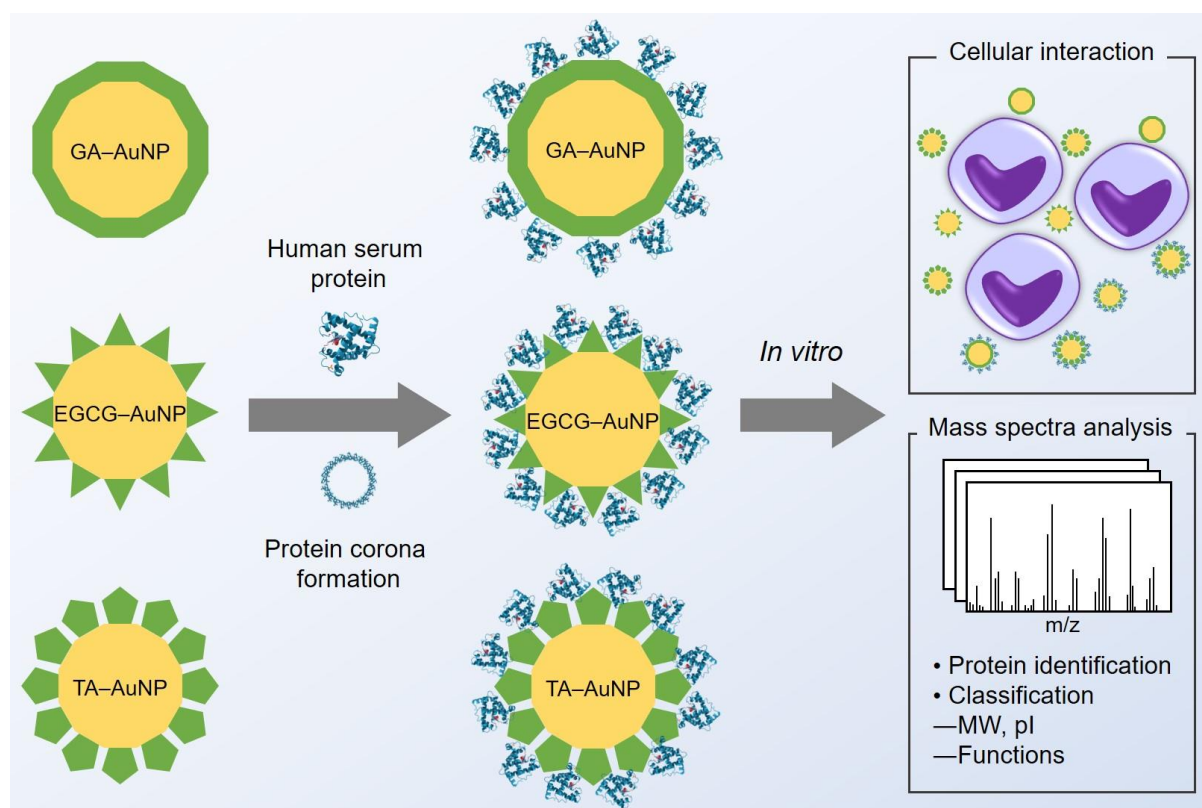
Protein coronas are formed by protein adsorption on engineered particles when the latter administered to physiological environments. This chapter aims to study the protein–polyphenol interactions related to biological environments by investigating the protein corona formation around different polyphenol-modified substrates (polyphenol-stabilized gold nanoparticles, polyphenol–AuNPs). The protein corona composition analysis was carried by mass spectrometry to demonstrate the binding preference of serum proteins. Cell association behaviors of polyphenol–AuNPs with macrophages in the absence and presence of a protein corona were also investigated to show the effect of protein corona on the polyphenol–cell interactions.

5.2 Introduction

Nanoengineered particles after entering physiological environments interact with biological molecules.^{1,2} The most abundant environmental components are proteins, and the protein adsorption layer forms on the surface of engineered nanoparticles (NPs) is defined as protein corona (PC).^{3,4} PCs have been recognized to play a vital role in their interactions with cells or their influence on the original molecular identity of the NPs.^{5,6} On the positive side, PCs can help stabilize NPs in physiological conditions or prolong the blood circulation of NPs, which is beneficial for engineered NPs to realize elaborate biological functions.^{7,8} In contrast, it has been shown that PCs can reduce cell membrane adhesion, which significantly inhibits the cellular uptake of NPs in the targeted delivery system.⁹ Moreover, PCs can influence the cellular internalization mechanisms of NPs or even lead to different cell signaling pathways, which may cause harmful inflammatory responses to the living organisms.¹⁰ Therefore, an in-depth investigation of the PCs is necessary for understanding how their exposure to NPs or specific functional ligands affects the bio–nano interactions of materials with cells.¹¹

Polyphenol-based materials have attracted increasing attention in biomedical research because of the various interactive mechanisms and desirable inherent biocompatibility of polyphenol building blocks.¹² Due to the universal adherence of polyphenols to diverse biomacromolecules, polyphenol-mediated particulate materials such as metal–phenolic networks (MPNs) capsules have shown nonspecific cellular internalization and rapid accumulation in normal organs,¹³ which may be attributed to the formation of PCs. To address these limitations of MPNs in biological applications, low-fouling synthetic polyphenol derivative, poly(ethylene glycol)

(PEG)-polyphenol, was used in MPN capsules to reduce protein adsorption and nonspecific cellular association.¹⁴ Interestingly, tannic acid (TA) has been reported to prefer targeting to live cardiac extracellular matrix (ECM) proteins because TA binds particularly well to proline-rich proteins such as elastin that is the major components of ECM proteins.¹⁵ Besides, unlike unmodified proteins, TA-modified proteins have been shown to increase the blood circulation time, which seemingly contradicts the previous findings with MPNs. This inconsistency raises some questions: i) Is the high-fouling property of MPNs due to the strong binding of metal ions with proteins rather than polyphenols with proteins? ii) How does the formation of PCs from interactions with polyphenols affect the cellular behavior of polyphenol-mediated materials?



Scheme 5.1. Schematic illustration of protein corona analysis of polyphenol-AuNPs prepared with different polyphenols.

In this chapter, the analysis of PCs around polyphenol-modified substrates was performed to clarify the biological role of polyphenols (**Scheme 5.1**). The polyphenol-stabilized gold nanoparticles (AuNPs) were first synthesized with different phenolic ligands such as TA, gallic acid (GA), and epigallocatechin gallate (EGCG). Next, TA-AuNPs, GA-AuNPs, and EGCG-AuNPs were incubated with human plasma (HP) to form PCs. The protein composition of the PCs was analyzed by mass spectrometry (MS) to show whether different phenolic structures could bind particular proteins. In addition, the macrophage cell association with three types of

AuNPs was investigated in the presence and absence of pre-formed of PCs to examine the role of PCs in bio–nano interactions. This chapter not only underlines the formation preference of PCs around polyphenols but also offers a clearer understanding of protein–polyphenol interactions related to biological environments.

5.3 Experimental Section

5.3.1 Materials

GA, EGCG, TA, nitric acid (HNO₃), hydrochloric acid (HCl), and tetrachloroauric acid (HAuCl₄) were purchased from Sigma-Aldrich. Dulbecco's modified eagle medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Life Technologies. High-purity Milli-Q water with a resistivity of 18.2 MΩ cm was obtained from an in-line Millipore RiOs/Origin water purification system.

5.3.2 Characterization

UV–Visible absorption spectra were recorded on a Specord 250 Plus spectrophotometer (Analytik Jena AG). Size distribution and zeta potential measurements of NPs were performed in different pH buffers at a concentration of 5 mM on a Malvern Zetasizer Nano ZS instrument (Malvern Instrument, Worcestershire, UK). Transmission electron microscopy (TEM) of NPs was performed on an FEI Tecnai TF20 instrument (USA) with an operation voltage of 200 kV. Small-angle X-ray scattering (SAXS) data were collected at the SAXS/WAXS beamline at the Australian Synchrotron facility (Australian Nuclear Science and Technology Organisation). MS analysis was performed on liquid chromatography-tandem mass spectrometry (LC-MS/MS). Cell association with AuNPs was performed by cytometry by time-of-flight (CyTOF; Helios, Fluidigm).

5.3.3 Synthesis of Polyphenol–AuNPs

Vials and stirrer bars used for the synthesis of AuNPs were washed with aqua regia (HNO₃: HCl= 1:3) before use. First, 100 µL of polyphenol solution (GA: 10 mg mL⁻¹; EGCG: 40 mg mL⁻¹, TA: 40 mg mL⁻¹) was added to a solution of 4.82 mL of Milli-Q water with continuous stirring, and the mixture was further stirred for 5 min. Then 80 µL of HAuCl₄ solution (50 mM) was added dropwise (within 2 min) to the resulting suspension solution with continuous stirring and was further stirred for 15 min. The suspension was then centrifuged at 5, 000 *g* for 10 min.

The supernatant was carefully removed, and the resultant AuNPs were washed with Milli-Q water once.

5.3.4 Formation of Protein Corona-Coated Particles

GA–AuNPs, EGCG–AuNPs or TA–AuNPs were incubated in 500 μL of HP (10% - 50%) for 1 h at 37 °C with gentle shaking. The NPs were then washed three times with DPBS buffer to obtain the protein corona-coated NPs that were used directly for the cell association and proteomic experiments or other characterizations.

5.3.5 Synchrotron SAXS

SAXS patterns were recorded spanning a q -ranges from 0.01 to 0.5 nm^{-1} with a voltage of 16 keV and 7000 mm camera length using Pilatus 1 M and 200 K detectors, and in transmission mode. The measurements were performed at room temperature. Scatterbrain software was used for the spectrum analysis.

5.3.6 Mass Spectrometry Analysis of Protein Coronas

The PC coated AuNPs, obtained from the protocols described in Section 5.3.4, were prepared for MS analysis. The bound proteins on the AuNPs were eluted using the elution buffer (tetraethylammonium bromide and trifluoroethanol denaturation agent). The eluted proteins were reduced by tris(2-carboxyethyl)phosphine and then alkylated by iodoacetamide and finally digested by fresh sequencing grade trypsin. Four replicates of each protein sample were analyzed by LC-MS/MS using a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) connected to a nanoESI interface with reversed-phase nano high-performance liquid chromatography (Ultimate 3000, Dionex). The nanoLC system was equipped with an Acclaim PepMap nano-trap column (Dionex C18, 100 Å, 75 $\mu\text{m} \times 2$ cm) and an Acclaim PepMap RSLC analytical column (Dionex C18, 2 μm , 100 Å, 75 $\mu\text{m} \times 50$ cm). The tryptic peptides were injected at an isocratic flow of 5 $\mu\text{L min}^{-1}$ to the enrichment column of 2% v/v CH_3CN containing 0.1% v/v formic acid for 5 min. Then, the enrichment column was switched in-line with the analytical column. The eluents were 0.1% v/v formic acid (solvent A) and 100% v/v CH_3CN in 0.1% v/v formic acid (solvent B). The flow gradient was (i) 0–6 min at 3% B, (ii) 6–40 min, 3–25% B, (iii) 40–48 min, 25–45% B, (iv) 48–50 min, 40–80% B, (v) 50–53 min, 85–85% B, (vi) 53–54 min, 85–3%, and equilibrated at 3% B for 10 min before the next sample injection. The Q-Exactive plus mass spectrometer was operated in the data-dependent mode,

whereby full MS1 spectra were acquired in positive mode, 70 000 resolution, AGC target of $3e^6$, and maximum IT time of 50 ms. Fifteen of the most intense peptide ions with charge states ≥ 2 and an intensity threshold of $1.7e^4$ were isolated for MS/MS. The isolation window was set at 1.2 m/z and precursors fragmented using a normalized collision energy of 30 at 17,500 resolution, AGC target of $1e^5$, and maximum IT time of 100 ms. Dynamic exclusion was set to be 30 s. Data were analyzed by Perseus software (MaxQuant), and the predefined contaminants like Keratin from fingers were removed.¹⁶

5.3.7 *In Vitro* Cellular Association Assay

5.3.7.1 Cell Culture

RAW264.7 macrophages cells were purchased from the American Type Culture Collection. They were maintained in complete DMEM media containing 10% fetal bovine serum and GlutaMAX at 37 °C in a cell culture incubator with 5% CO₂ and 95% relative humidity. Cells with a passage number of 15–30 were used in the study.

5.3.7.2 Cellular Association of Particles by CyTOF

RAW264.7 cells were cultured into a 12-well plate with 2×10^5 cells per well. After 18 h, cells were attached to the well, and different AuNPs were added to the wells with a particle-to-cell ratio of 200:1 or 1000:1. After incubation of 4 h at 37 °C, cells were washed with DPBS twice and trypsinized. Cells were further washed with DPBS three times and then fixed in 400 μ L of 4% paraformaldehyde containing Ir-intercalator at 4 °C overnight. Cells were washed with 500 μ L of Milli-Q water three times (800 g , 10 min). Then the cell suspension was filtered through a 70 μ m mesh and analyzed by CyTOF.

5.4 Results and Discussion

5.4.1 Preparation and Characterization of Polyphenol–AuNPs

Investigating PC formation on polyphenol-modified AuNP substrates is ideal because of the simple synthesis involved and the ability to control the size of the particles and generate identical particle surface. Polyphenols (*i.e.*, GA, EGCG, or TA) served as reducing agents as well as capping agents to synthesize AuNPs. The similar size and morphology of AuNPs, reduced by different polyphenols, are advantageous because they can reduce the interference of the physical properties of AuNPs and only compare the protein adsorption induced by the

polyphenols. Previous literature has reported the well-established protocol to produce AuNPs in the presence of TA in the range of 2–30 nm.^{17,18} However, it is difficult to synthesize uniform and spherical AuNPs by GA and EGCG based on the reported literature.¹⁹ Here, the synthesis conditions of AuNPs reduced by different polyphenols were optimized and spherical, monodisperse GA–AuNPs (43 ± 4 nm), EGCG–AuNPs (27 ± 4 nm), and TA–AuNPs (28 ± 3 nm) were then obtained (**Figure 5.1a**). The hydrodynamic diameters of the polyphenol–AuNPs were measured by dynamic light scattering (DLS) analysis; the NPs all featured comparable diameters of around 40 nm (**Figure 5.1b**). Due to the differences in chemical structure and hydrophobicity of the polyphenols, the surface zeta-potential of the polyphenol–AuNPs varied (GA–AuNPs, ~ 55 mV; EGCG–AuNPs, ~ 35 mV; TA–AuNPs ~ 21 mV; **Figure 5.1c**). The UV–vis spectra of the three polyphenol–AuNPs all featured a localized surface plasmon resonance band peaking around 530 nm (**Figure 5.1d**), which is in accordance with reported AuNPs in the same size regime.¹⁷ These comprehensive analyses of the polyphenol–AuNPs suggest that the latter is a suitable platform to probe the effect of polyphenols on protein binding and macrophage uptake in biological environments, which will clarify and emphasize the functions of polyphenols in biomedical applications.

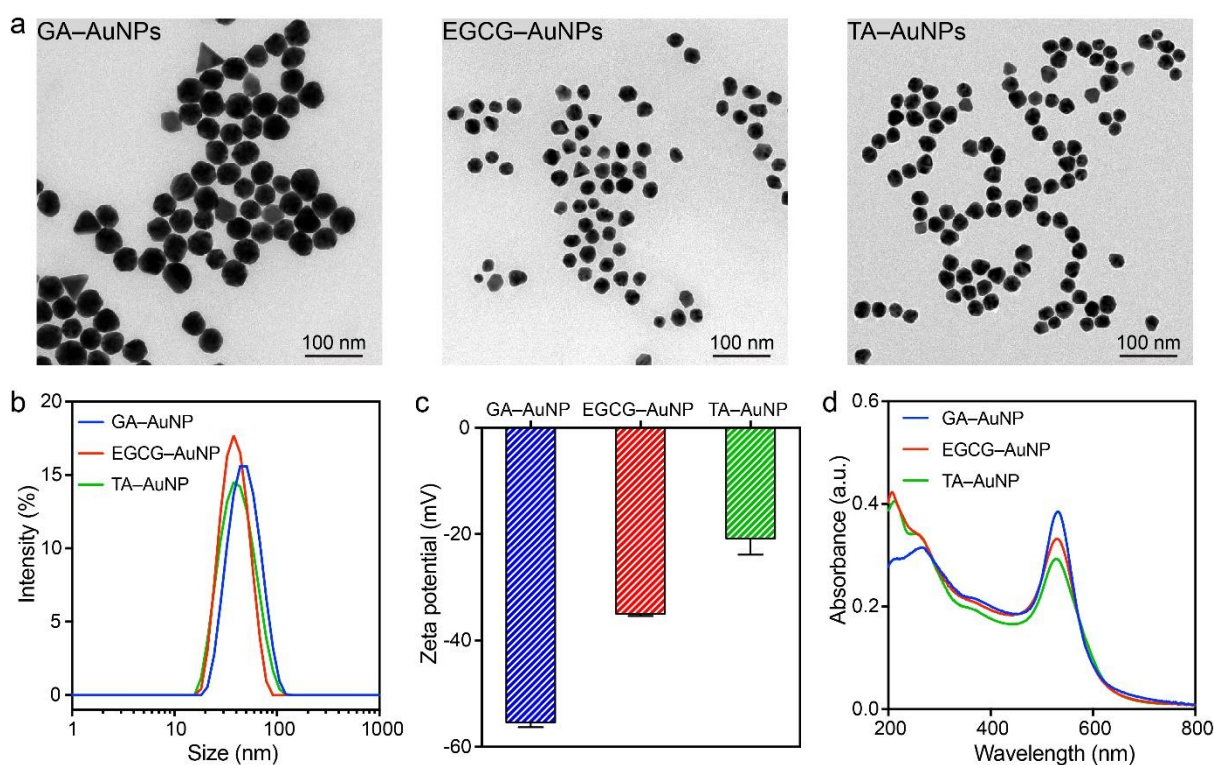


Figure 5.1. Structural and physicochemical characterization of polyphenol–AuNPs: TEM analysis (a), size distribution by DLS (b), zeta-potential measurements (c), and UV–vis spectroscopy analysis (d) of GA–AuNPs, EGCG–AuNPs and TA–AuNPs.

5.4.2 SAXS Monitoring of Protein Corona Formation

The PC formation around the polyphenol–AuNPs under different concentrations of HP was monitored and confirmed by SAXS (**Figure 5.2**).²⁰ The results suggested that the incubation of polyphenol–AuNPs in HP (from 5% to 50%) did not cause the aggregation of AuNPs based on the lack of shift of the peak derived from the small nano-size of AuNPs. Besides, the signal intensity that plateaued at high HP concentration indicated the enrichment of bound proteins and high surface coverage. The zeta-potential measurements of the PC-coated AuNPs demonstrated that protein adsorption led to the neutralization of the surface charge of the AuNPs (**Figure S5.1**), where the neutralizing effect of the PC on the particle surface was dependent on the amount and types of adsorbed proteins, as detailed in Section 5.4.3.

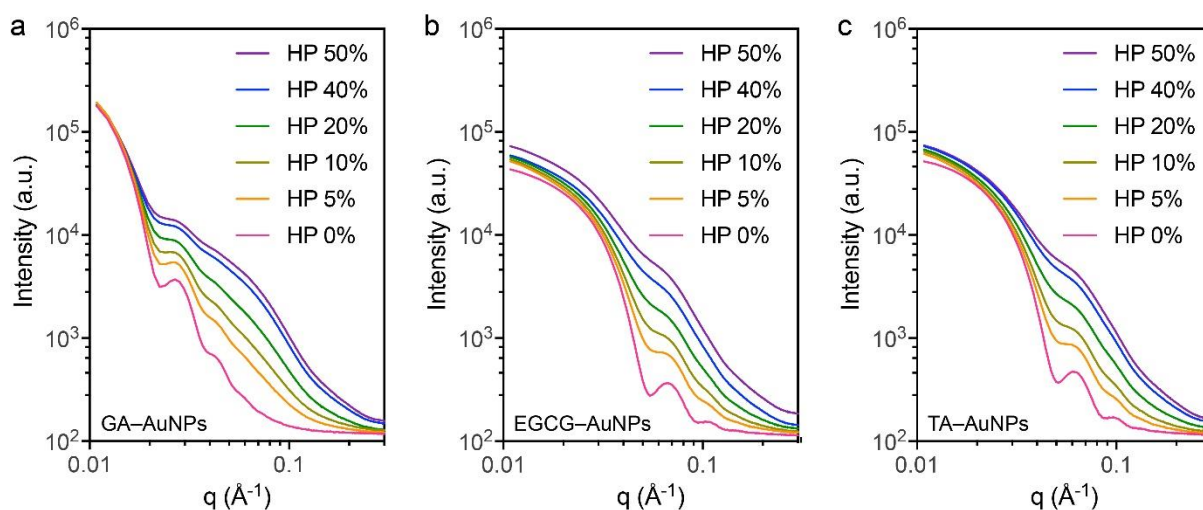


Figure 5.2. Synchrotron SAXS patterns of polyphenol–AuNPs and protein complexes under different HP concentrations: (a) GA–AuNPs, (b) EGCG–AuNPs and (c) TA–AuNPs.

5.4.3 Protein Corona Analysis by Mass Spectrometry

The “hard” PCs (irreversibly bound proteins) around the polyphenol–AuNPs were examined in 50% serum to mimic *in vivo* conditions, where hard corona proteins can also represent the proteins that have strong interactions with polyphenols.²¹ MS was used to identify the composition of the PCs—the number of total identified proteins on the three polyphenol–AuNPs was similar. The Venn diagram shows that among a total of 367 proteins, 329 proteins were present in the PCs of all three AuNPs, while 3 of the proteins were only found in the corona of EGCG–AuNPs, and 1 of the proteins was only found in the corona of GA–AuNPs (**Figure S5.2**). Twenty-nine of the total identified proteins existed in both the corona of GA–

AuNPs and EGCG–AuNPs but were not found on the TA–AuNPs, suggesting the similarity in the protein composition of corona with small phenolic ligands.

The AuNP surface-bound proteins were then classified according to their physicochemical properties such as molecular weight (MW) and isoelectric point (pI) to investigate the role of different polyphenols on corona formation.²² It is noted that the percentage of a specific protein from the total bound proteins on NPs was calculated by dividing a given protein's intensity-based absolute quantification (iBAQ) value by the sum of iBAQ value of all identified proteins. First, although there was no distinct difference in the protein binding profile in the three NPs (**Figure 5.3a**), an enrichment of proteins with low MWs was observed (~90% of proteins < 80 kDa) compared to the control serum (~70% of proteins < 80 kDa in reported literature²²). Proteins with MWs in the range of 10–20 kDa (~24% in three coronas) and 40–50 kDa (~23% in three coronas) were found to be the most enriched amount. Second, proteins (pI < 7) displaying a net negative charge at physiological pH 7.4 constitute ~60% of the corona components for all NPs regardless of the negative surface charge of NPs. (**Figure 5.3b**), indicating that the binding of proteins with polyphenols is not limited to electrostatic interactions. Specifically, GA–AuNPs and EGCG–AuNPs adsorbed more positively charged proteins at physiological pH 7.4 compared to TA–AuNPs. This observation is consistent with the zeta-potential results that show that GA–AuNPs and EGCG–AuNPs have a more negatively charged surface than TA–AuNPs.

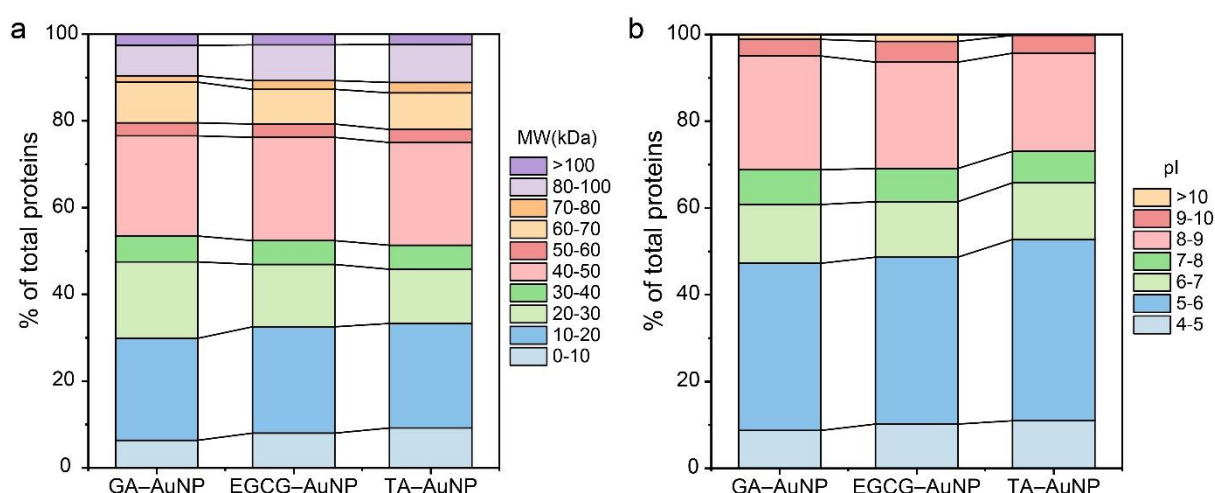


Figure 5.3. Classification of surface-bound proteins for polyphenol–AuNPs in 50% HP according to their (a) MW and (b) calculated pI.

To understand the subsequent effect of polyphenol–AuNPs in blood, the identified proteins were further bioinformatically classified according to their physiological molecular functions

in blood, namely apolipoproteins, immunoglobulins, complement proteins, coagulation proteins, acute phase proteins, tissue leakage proteins, and other proteins (**Figure 5.4a**).^{5,22} From our results, the amount of apolipoproteins adsorbed on the AuNPs (< 10% of total proteins) was lower than the amount of those in control serum (~30% of total proteins), suggesting the key role of the hydrophilicity of the polyphenols (**Figure 5.4b**). In addition, immunoglobulins were the major protein type (~38% of total proteins) found in corona composition across all three polyphenol–AuNPs due to the high enrichment of immunoglobulins in HP (**Figure 5.4c**). Notably, a 2–3-fold enrichment of complement proteins was observed for all AuNPs compared to the control (~5% of total proteins²²), and the amount of complement proteins adsorbed on the polyphenol–AuNPs decreased in the order of GA–AuNPs > EGCG–AuNPs > TA–AuNPs (**Figure 5.4d**). Similarly, a significant enrichment (~10×) of coagulation proteins was observed in all AuNPs compared to the control (~2% of total proteins²²) but the amount of coagulation proteins adsorbed on the polyphenol–AuNPs increased in the order of GA–AuNPs < EGCG–AuNPs < TA–AuNPs (**Figure 5.4e**). Collectively, these results indicate that the different chemical structures displayed by the three polyphenols may alter protein adsorption on the surface of NPs. Furthermore, no obvious enrichment of acute phase or tissue leakage proteins was found on either of the three polyphenol–AuNPs, and GA–AuNPs had the tendency to adsorb more these types of proteins compared to the other two NPs (**Figure 5.4f** and **5.4g**). Interestingly, the other components of serum including albumin (ALB) displayed a lower affinity for these NPs (**Figures 5.4h**).

The percentage of the top 25 most abundant proteins from the total adsorbed proteins on all three AuNPs is shown in **Figure S5.3**, which provides more details about the correlation of protein functions with different NPs. The complement proteins such as C1QB and C1QC showed higher affinity to GA–AuNPs than to EGCG–AuNPs or TA–AuNPs. On the contrary, the coagulation proteins such as FGA and FGB adsorbed to a greater amount on TA–AuNPs than on EGCG–AuNPs or GA–AuNPs. The enrichment of immunoglobulin proteins and serum ALB exhibited no obvious distinction with different AuNPs.

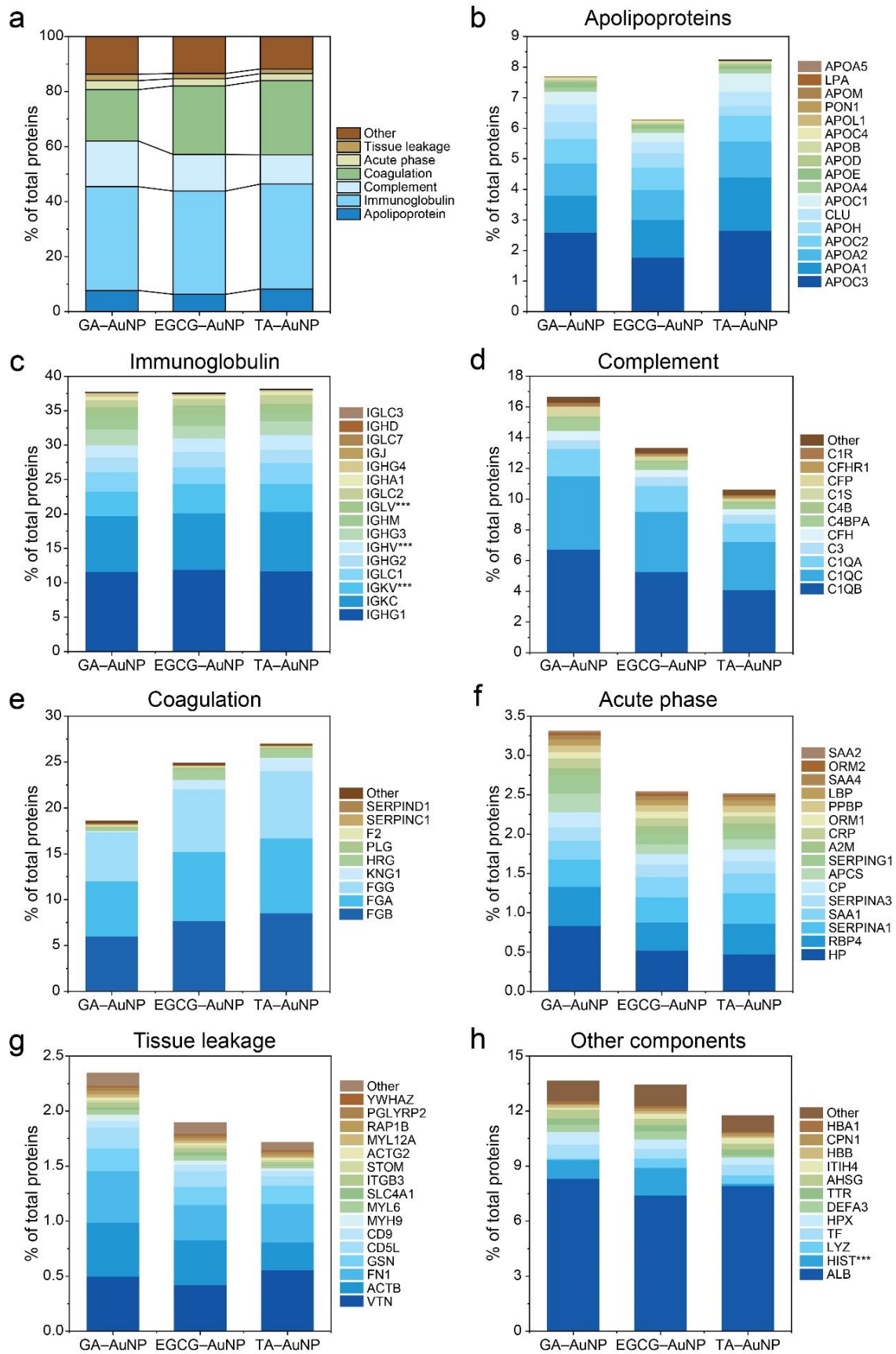


Figure 5.4. Classification of identified corona on polyphenol-AuNPs according to their physiological functions in 50% HP.

5.4.4 Effects of Protein Corona on Cell Association

The cellular uptake of the polyphenol–AuNPs by macrophages was investigated and the difference between bare AuNPs and PC-coated AuNPs was compared to clarify the role of polyphenols in cell association.²³ The polyphenol–AuNPs were incubated with Raw264.7 cells in the absence (–) or presence (+) of PC after 4 h in serum-free medium, and then the treated cells were analyzed by CyTOF using the of ¹⁹⁷Au signal. GA–AuNPs and EGCG–AuNPs showed lower cell association after pre-coating with PC compared to bare AuNPs, and TA–AuNPs displayed comparable cell association (~80%) after pre-coating with PC (**Figure 5.5a**). The cellular uptake of NPs can also be demonstrated by the median ¹⁹⁷Au intensity of treated cells, where TA–AuNPs and GA–AuNPs in the absence (–) of PC showed significantly higher median ¹⁹⁷Au intensity (TA: ~40-fold, GA: ~20-fold) compared to NPs in the presence (+) of PC, whereas EGCG–AuNPs showed ~4.4-fold median ¹⁹⁷Au intensity (**Figure 5.5b**). The median ¹⁹⁷Au intensity of bare polyphenol–AuNPs was also determined to calculate the number of associated or internalized NPs per cell (**Figure S5.4**).

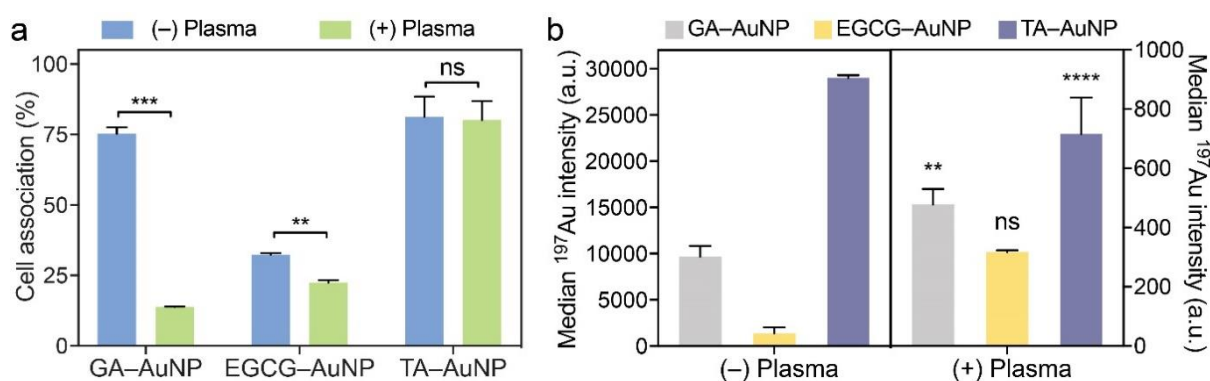


Figure 5.5. Effects of protein corona on cell association with polyphenol–AuNPs. (a) Cell association of polyphenol–AuNPs with Raw264.7 in the absence (–) and presence of PC (+). (b) Median ¹⁹⁷Au intensity of Raw264.7 cells treated with polyphenol–AuNPs in the absence (–) and presence of PC (+) after 4 h. The cell-to-particle ratio was 1:200. Data represent the mean value $\pm$ standard deviation ($n = 3$). Statistical significance was determined by a two-tailed t -test and is reported as ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In addition to the findings above, it was found that a higher cell-to-particle ratio (1:1000) resulted in an increased number of NPs per cell across all AuNPs with or without PC compared with the low cell-to-particle ratio (1:200) results, and the stealth properties of PC for NPs were obvious at different cell-to-particle ratios (**Figure S5.5**). Collectively, the above results indicate that the formation of PC can reduce cell association and cellular uptake of NPs and that the

different protein adsorption on the NPs depending on the polyphenols leads to differential macrophage recognition. Specifically, TA–AuNPs in the absence (–) and presence (+) of PC displayed similar macrophage association but vastly different cellular uptake numbers of NPs.

5.5 Conclusion

Different polyphenol–AuNPs were synthesized to provide a suitable platform to probe the effect of polyphenols in biological environments. There was no distinct difference in protein binding profiles in the polyphenol–AuNPs according to the physicochemical properties of the bound proteins. Cell association results not only showed the distinct cell binding ability of the polyphenol–AuNPs with or without pre-formed PC, but also revealed significantly different amounts of cell uptake. To obtain a correlation between macrophage uptake and the type of corona protein found on the NPs, a PC analysis under different concentrations of HPs is required.²² Overall, this chapter studied the effects of the protein corona on protein–polyphenol networks; the findings suggest that the biomedical applications developed in other chapters could be implemented in actual physiological environments. A future in-depth understanding of the interplay of different polyphenols and protein compositions is expected to allow the careful selection of specific polyphenols to potentially achieve desirable functions in biological applications. The latter comprehensive study is also expected to enable the development of specific human protein-functionalized polyphenol materials that can exhibit stealth properties, reach targeted sites or modulate immune responses.

5.6 Supporting Information

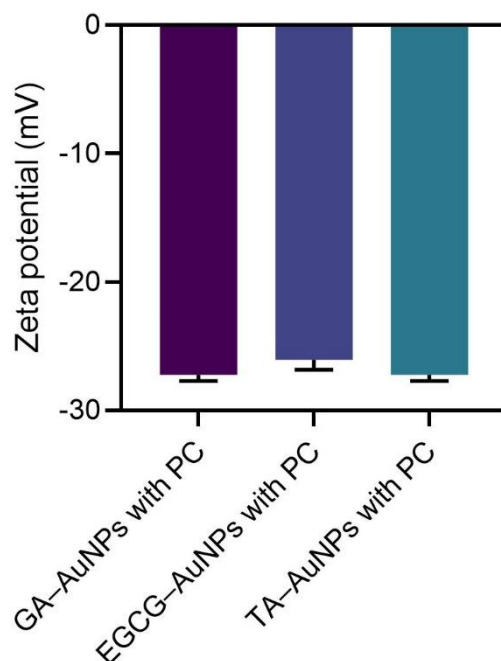


Figure S5.1. Zeta-potential results of polyphenol–AuNPs coated with a PC. Data represent the mean value $\pm$ standard deviation. ($n = 3$).

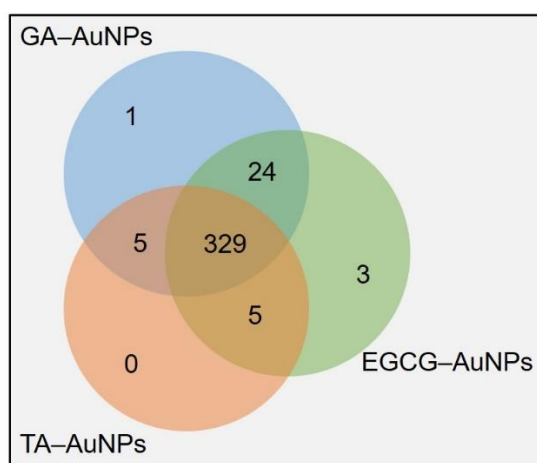


Figure S5.2. Venn diagram reporting the number of proteins identified in the PC formed on polyphenol–AuNPs.

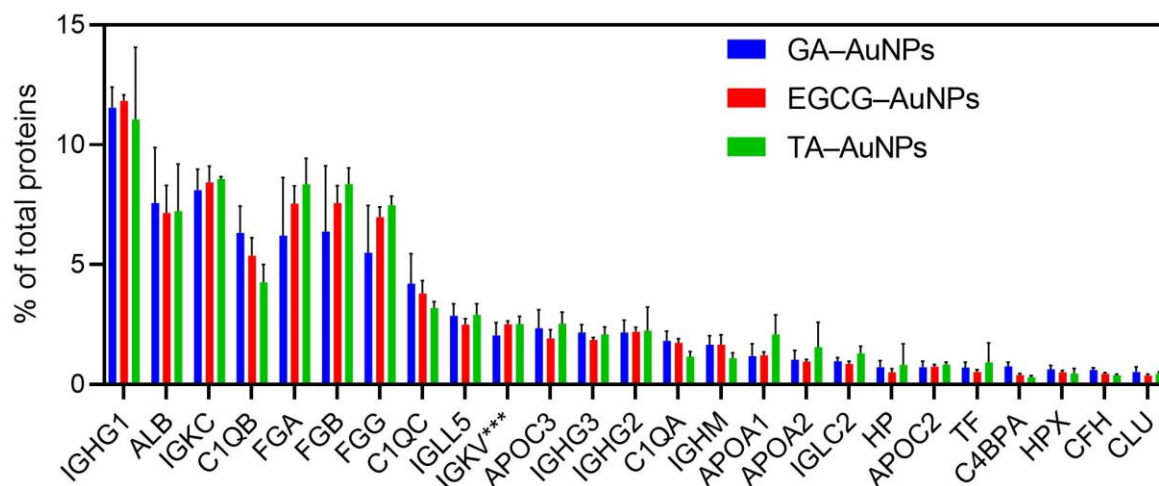


Figure S5.3.. Top 25 most abundant proteins found in the PC on different polyphenol-AuNPs. Data represent the mean value $\pm$ standard deviation ($n = 4$).

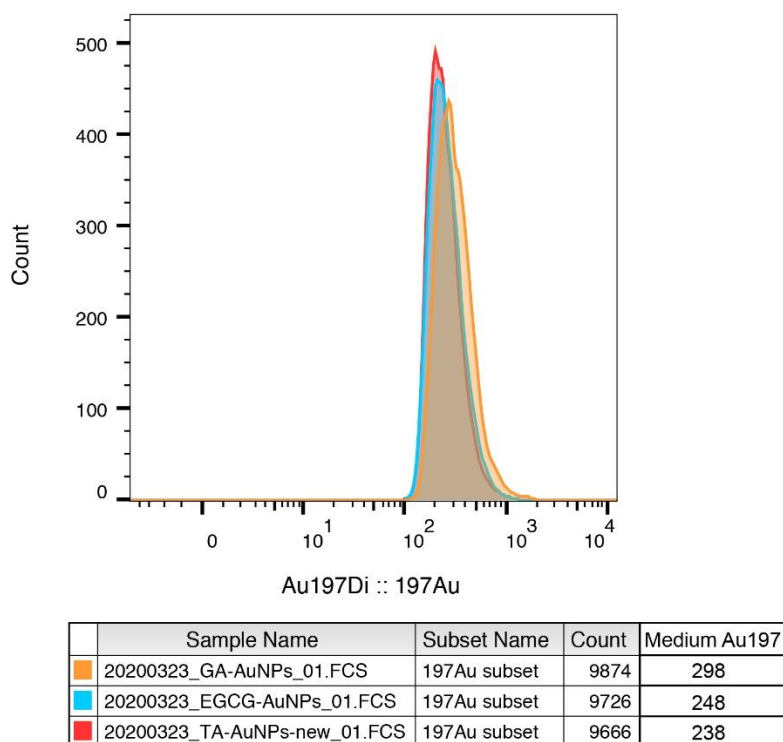


Figure S5.4. CyToF histograms of the polyphenol-AuNPs showing ^{197}Au intensity.

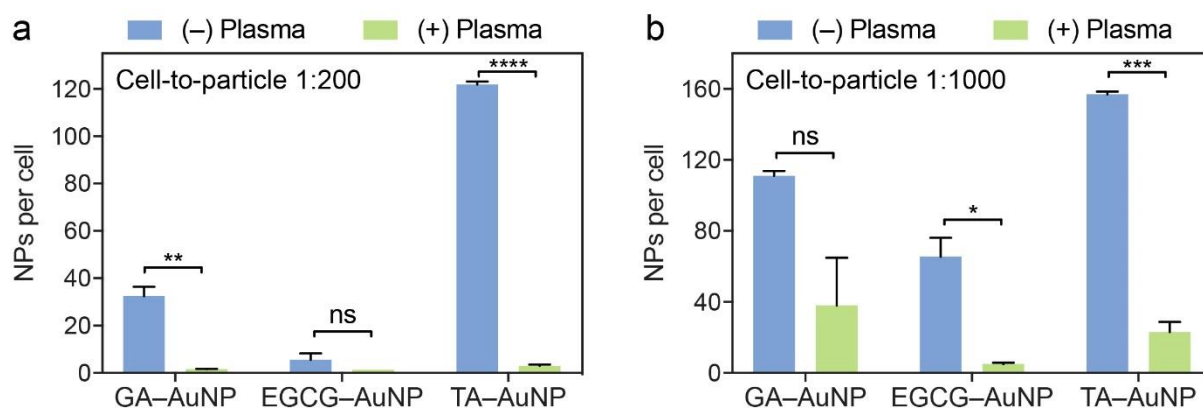


Figure S5.5. Quantitative analysis of the number of polyphenol–AuNPs per cell at different cell-to-particle ratios. Data represent the mean value $\pm$ standard deviation ($n = 3$). Statistical significance was determined by a two-tailed t -test and is reported as ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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Chapter 6

Conclusion and Perspectives

The development of self-assembly approaches from small building blocks has recently attracted much attention from fundamental research to various technological applications. Originating from many fundamental building blocks, diverse functional complex architectures have been developed through direct interactions or indirect self-assembly. The self-assembly of naturally occurring building blocks has developed rapidly due to their natural abundance and inherent biocompatibility. Proteins are involved in nearly all fundamental processes of living organisms and are therefore integral components of bioactive assemblies spanning diverse applications, including catalysis, diagnostics, and therapeutics. Polyphenols, readily found in fruits and vegetables, have been explored in materials design and bioapplications, owing to their capability of assembly through diverse interactions and instinctive biological properties. Therefore, in this thesis, investigations of protein–polyphenol networks (PPNs) were carried out to clarify the interactions between proteins and polyphenols and develop polyphenol-mediated protein assembly materials for biomedical applications.

In Chapter 2, the binding affinity between homopolypeptides and polyphenol was studied to clarify the dominant interactions between proteins and polyphenols. It was found that the positively charged side chains showed a high binding relationship with tannic acid (TA) due to the attractive electrostatic interactions. In contrast, the negatively charged side chains showed weak binding to TA due to the repulsive electrostatic interactions. Uncharged polar side chains interacted with TA through hydrogen bonding, and hydrophobic side chains bound with TA through hydrophobic interactions. Among all tested polypeptides, polyproline showed the highest binding strength with TA owing to the strong aromatic–proline interactions. In addition, the strong binding affinity between the polypeptides and TA offered free-standing films (hollow microcapsules) with TA. In summary, the strength and mechanism of interactions between the side chains of the proteins with polyphenols varied with the functional groups of the amino acid residues, which not only increases the complexity of protein–polyphenol interactions but also opens up more possibilities for polyphenol-mediated protein assembly.

In Chapter 3, a platform for assembling a diverse range of functional nanomaterials using proteins and polyphenols as building blocks was introduced. The assembly process involved simply mixing the two components in the presence of a template (organic, inorganic, or biological templates, ranging from nanoscale to macroscale), as the various interactive forces between the polyphenols and proteins enabled simultaneous cross-linking of the proteins and tethering of the proteins to the templates. The diverse range of functional nanomaterials produced herein from more than 10 functional/therapeutic proteins highlights the general

applicability of the assembly technique to nearly all classes of proteins. Specifically, protein–polyphenol capsules fabricated through sacrificial templates were used to clarify the governing interaction(s) between different proteins and polyphenols. The results indicate that the physicochemical properties (*i.e.*, size, hydrophobicity, and isoelectric point) of the proteins affect the strength of the hydrogen bonding and hydrophobic or electrostatic interactions. Notably, the proteins retained their structure and function within the nano-assemblies, thereby enabling their usage in a range of applications, such as biocatalysis (single and tandem reactions), fluorescence imaging, and cell targeting, depending on the functional protein(s) used, as exemplified in the study.

In Chapter 4, protein–polyphenol nanoparticles (NPs) were introduced as an effective platform for intracellular protein delivery in response to multiple endogenous triggers leading to, for example, endosomal escape and cytosolic protein release. The NPs were synthesized *via* a template-mediated supramolecular assembly approach, which applied to diverse proteins. This simple and versatile strategy, using natural building blocks, offered numerous beneficial properties suitable for protein delivery. The engineered NPs underwent pH-responsive surface charge reversal (from negative to positive) in acidic environments, which led to their high stability in the extracellular space (serum-containing media and human plasma) and spontaneous escape from acidic endosomes after cellular uptake. Subsequently, the abundance of glutathione in the cytosol triggered supramolecular disassembly of the NPs and protein release upon the formation of competitive noncovalent interactions with both the protein and polyphenol. The bioactivity of the released proteins was validated by the apoptotic activity of cytochrome C and catalytic hydrolysis by β -galactosidase.

In Chapter 5, protein–polyphenol interactions related to biological environments were studied by investigating the protein corona formed with different polyphenol–AuNPs (three phenolic ligands). There was no apparent distinction between the protein binding profiles of the polyphenol–AuNPs according to the physicochemical and biofunctional properties of the bound proteins. The cell association results showed significantly different cell binding ability of the polyphenol–AuNPs with or without pre-formation of the protein corona. However, the correlation between macrophage uptake and the type of corona proteins found in NPs still requires more exploration.

In summary, this thesis has demonstrated i) a fundamental study of the interactions between proteins or polypeptides with polyphenols; ii) a polyphenol-mediated protein assembly for the

development of functional materials in biomedical applications; iii) the application of protein–polyphenol NPs for effective intracellular protein delivery; and iv) the use of protein corona analysis around polyphenol–AuNPs to determine the influence of polyphenols on bio–nano interactions. It is expected that the application of PPNs could establish an ideal platform for readily engineering protein-based functional materials for diverse biotechnological applications.

Currently, more attention is being devoted to the development of functional particles, whereas the mechanisms surrounding the formation and disassembly of the designed materials have often been overlooked. Although the interactions between different side chains of proteins and polyphenols were studied in this thesis, a better understanding of the protein–polyphenol interactions, notably the consideration of protein structural conformation, is still needed. For example, molecular simulation, theoretical calculation, or thermodynamic measurements can be performed to study the interaction mechanisms further. Elucidating how the interplay of diverse interactions alters the stability and responsiveness of nanomaterials based on PPNs is an essential fundamental question to be addressed.

Polypeptides, as simple linear chains of proteins, also have useful physicochemical or biological functions such as self-assembly or antimicrobial properties. The preparation of polypeptide–TA capsules was successfully shown but has not been employed in any applications highlighted in Chapter 1. In the future, smart polypeptide–polyphenol nanocarriers or multiple polypeptide NPs are expected to be developed for the delivery of functional polypeptides. For example, cationic polyarginine can act as cell membrane peptides to facilitate high-efficiency transfection. Also, the interactions between homopolypeptides and polyphenols are relatively simple when compared with the interactions between proteins and polyphenols. Therefore, the self-assembled nanomaterials with a controllable structure derived from these components are worth exploring further.

Nanocarriers with multiple stimuli-responsive properties have shown great potential for various biomedical applications. In this thesis, PPN capsules displayed the pH-responsive swelling properties, which can be further used for tunable encapsulation or release of guest molecules (*e.g.*, drug, fluorescent dye, or gene). Additionally, the PPN NPs showed pH-responsive charge reversal and glutathione-triggered disassembly, which are useful properties for intracellular protein release. However, the stability or disassembly of PPN materials in more varied biological environments such as enzymatic, bacterial, *etc.*, has not been studied but can

be done in the future to dictate more possibilities of PPNs in certain environmental applications. This extensive investigation can broaden the toolbox of PPNs for engineering responsive materials.

Various structures and types of proteins have been employed for the construction of PPNs exhibiting different properties and functions. In addition to proteins, phenolic compounds such as catechol- and gallol-containing building blocks can be employed to synthesize materials, thereby expanding the field of PPNs. For example, phenolic lipids would allow for the incorporation of PPNs with membrane-disrupting properties, hyaluronic acid–catechol conjugates may offer PPNs with cell-targeting abilities, or poly(ethylene glycol)-polyphenol can endow PPNs with low-fouling properties.

This thesis focused on the interactions of proteins with polyphenols, while the universal adhesion of polyphenols with other biomolecules was introduced in Chapter 2. It is expected that the integrated nanocarriers consisting of proteins and DNA or RNA can be formulated for therapeutic co-delivery. Polymers can also be incorporated with PPNs to regulate the mechanical properties or enhance stability. Specifically, the introduction and development of PPNs in this thesis could serve as a reference or even a model for the subsequent studies on the interactions of polyphenols with other biomolecules. Hence, DNA–polyphenol networks, polysaccharide–polyphenol networks or lipid–polyphenol networks are up-and-coming research fields for the scientists who are interested in polyphenol-based materials.