



Functional protein–organic/inorganic hybrid nanomaterials

Rui Wang,¹ Yifei Zhang,¹ Diannan Lu,¹ Jun Ge,^{1*} Zheng Liu^{1*} and Richard N. Zare^{2*}

Nanotechnology offers immense opportunities for regulating and improving biological functions of proteins *in vitro*. Recent years have witnessed growing efforts to develop protein-incorporated hybrid nanostructured materials with potential applications in functional materials, enzymatic catalysis, drug delivery, and analytical sciences. In this review, recent advances in functional protein–organic/inorganic hybrid nanomaterials are discussed with an emphasis on the novel preparation methods, resulting nanostructures, and their potential applications in drug delivery and enzymatic catalysis. Future directions toward the rational design of these bionanomaterials are suggested. © 2013 Wiley Periodicals, Inc.

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INTRODUCTION

Nanomaterials with controllable chemical compositions and structures, large surface-to-volume ratios, various surface properties, and functionalities offer many opportunities for regulating the biological function of incorporated protein^{1–5} with interesting potential applications in catalysis,^{6–9} drug delivery,^{10–13} and biosensors.^{14–17} By far, immobilization of protein onto nanomaterials, chemical conjugation of protein with synthetic polymers, *in situ* crosslinking of protein with polymers, and self-assembly of protein with organic/inorganic components represent commonly used methods to prepare protein-incorporated hybrid bionanomaterials, as discussed in a number of reviews.^{1,9,18–22} The design and synthesis of such hybrid bionanomaterials remain a challenge in terms of tailoring the structures of the bionanomaterials in response to their applications. This review focuses on the recent advances of the preparation methods and structures of functional protein–organic/inorganic hybrid nanomaterials with

potential applications to biocatalysis and drug delivery. These newly developed methods are grouped into categories in terms of protein–polymer conjugates, protein–polymer nanogels, and protein-incorporated complex hybrid nanomaterials. The design and synthesis of these materials for drug delivery and enzymatic catalysis are also discussed with reference to the conventionally modified biological molecules.

AN OVERVIEW OF THE PROTEIN-INCORPORATED HYBRID NANOMATERIALS

As shown in Table 1, the preparation methods for protein–organic/inorganic hybrid nanomaterials developed to date can be grouped into four categories in terms of immobilization, conjugation, crosslinking, and self-assembly. Some typical nanostructures of the protein-incorporated nanomaterials are given in Figure 1. Immobilization is the most extensively used method, which gives reusable biocatalysts with enhanced stabilities of enzymes, as reviewed elsewhere.^{22–24} Chemical conjugation and *in situ* crosslinking of proteins with polymers are effective for preparing protein–polymer hybrid structures with integrated properties of both proteins and synthetic polymers. As exemplified by PEGylation, the

*Correspondence to: zare@stanford.edu; liuzheng@mail.tsinghua.edu.cn; junge@mail.tsinghua.edu.cn

¹Department of Chemical Engineering, Tsinghua University, Beijing, China

²Department of Chemistry, Stanford University, Stanford, CA, USA

TABLE 1 | Methods and Some Examples of Preparing Protein–Organic/Inorganic Hybrid Nanomaterials

Methods	Materials	Protein
Immobilization	Silica nanoparticles	Butyryl cholinesterase, ²⁵ α -chymotrypsin, ²⁶ catalase, peroxidase, and lysozyme ²⁷
	γ -Fe ₂ O ₃ magnetic nanoparticles	Lipase ²⁸
	Al ₂ O ₃ nanoparticles	Pepsin ²⁹
	Pickering emulsions	Lipase, benzaldehyde ligase, and α -chymotrypsin ³⁰
	Carbon nanotubes	Peroxidase ³¹ and lipase ³²
	Polystyrene and poly(methyl methacrylate) nanoparticles	Lipase ³³
	Peptide nanotubes	Lipase ³⁴
Conjugation	Polyethylene glycol	Bovine liver catalase, ³⁵ nerve growth factor, ³⁶ insulin, ³⁷ and chymotrypsin ³⁸
	Poly(<i>N</i> -isopropylacrylamide)	Bovine serum albumin ³⁹ and lysozyme ⁴⁰
	Hyperbranched or dendronized polymers	Lipase, ⁴¹ superoxide dismutase, and horseradish peroxidase ⁴²
Crosslinking	Polymeric nanogels	Horseradish peroxidase, ⁴³ lipase, ^{44,45} carbonic anhydrase, ⁴⁶ urate oxidase, ⁴⁷ green fluorescence protein, superoxide dismutase, ⁴⁸ and caspase-3 ⁴⁹
Self-assembly	Magnetic nanogels	Lipase, horseradish peroxidase, and trypsin ⁵⁰
	Copper phosphate nanoflowers	Laccase and carbonic anhydrase ⁵¹
	ZnS and CdS ordered nanocrystal arrays	P22 coat protein ⁵²

protein–polymer conjugate possesses the application potential for drug delivery. Self-assembly that does not involve chemical modification or reaction of protein presents another way to construct the protein–organic/inorganic hybrid nanostructures. One essential advantage of this method is that it is free of chemical reactions that are sometimes unfavorable for the stabilization of the target protein.

PROTEIN–POLYMER CONJUGATES

Synthetic polymers may have useful properties such as stimulus-responsive functionality, biocompatibility, biodegradability, and desired mechanical strength. These properties could be used to design various types of protein–polymer conjugates, which have interesting applications in drug delivery, biosensing, and enzymatic catalysis. In addition, protein–polymer conjugates could also be employed as macromolecular building blocks to construct nanostructured materials for biomedical devices and systems.

Hoffman and coworkers⁵⁴ have shown a temperature-controlled ligand binding by linking the protein with poly(*N*-isopropylacrylamide). The conjugate showed an 84% increase in biotin-binding affinity at 37°C compared with that of at 4°C. They have also applied photoresponsive polymers for a controlled display of the activity of a protein conjugate,⁵⁵ using the copolymer of *N,N*-dimethyl

acrylamide and 4-phenylazophenyl acrylate. The conjugated endoglucanase existed as a soluble, extended coil structure under far UV illumination and showed a 60% activity of its native counterpart in free form. Under visible illumination, the conjugate collapsed into a compact, hydrophobic conformation, resulting in the turning off of the activity. By covalently attaching the protein to a photoresponsive copolymer that carried spiropyran groups, Ito et al.⁵⁶ synthesized a subtilisin conjugate whose solubility was tuned by photoirradiation (Figure 2). The conjugate was perfectly soluble in toluene and efficiently catalyzed transesterification reaction. Owing to the increased solubility of the conjugate in toluene, it exhibited more than 100 times higher transesterification activity than that of native subtilisin. Upon ultraviolet irradiation, the conjugate was precipitated, and thus easily recovered. The activity of the conjugate was almost unchanged after three cycles of reuse.

Polyethylene glycol (PEG), as approved by FDA, is one of the most extensively tested polymers for preparing protein pharmaceuticals. In addition, poly(methyl methacrylate) (PMMA) has also been examined for preparing a serum albumin (bovine serum albumin, BSA) conjugate⁵⁷ that served as building blocks to form uniform spherical nanoparticles for drug delivery. The BSA–PMMA nanoparticles could be obtained with a smooth surface or a surface with small protein ‘islands’, as a function of the ratio of

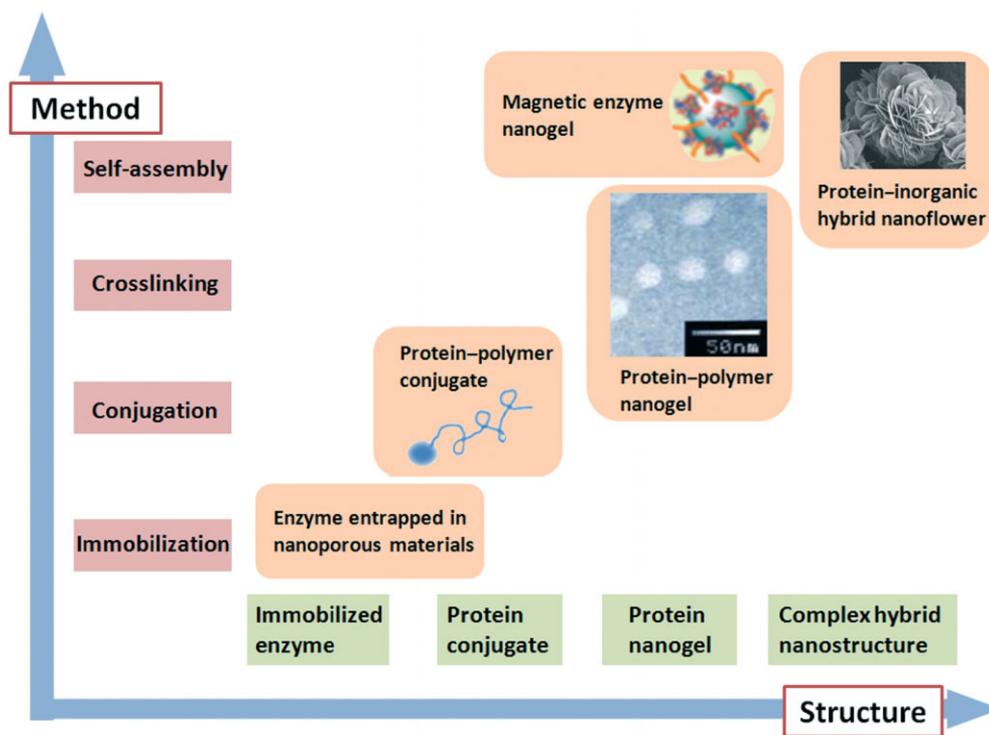


FIGURE 1 | Preparation methods and structures of protein-based bionanomaterials. Examples shown in the figure are that of an immobilized enzyme,⁵³ a protein–polymer conjugate,⁴⁰ a protein–polymer nanogel,⁴³ and a complex protein-based nanostructures.^{50,51}

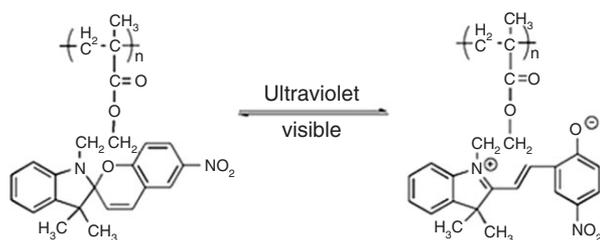


FIGURE 2 | The copolymer carries spiropyran groups regulated by photoirradiation. (Reprinted with permission from Ref 56. Copyright 1999 Nature Publishing Group)

BSA to PMMA. Further investigation illustrated that the ‘islands’ were formed as a result of a unique frustrated phase separation between BSA and PMMA components in the nanoparticles (Figure 3). The encapsulation of camptothecin (CPT), a hydrophobic anticancer drug in the BSA–PMMA nanoparticle, was accomplished by precipitation, showing an enhanced antitumor activity both *in vitro* and in animals.⁵⁸

Living radical polymerization is effective for synthesizing uniform polymer–enzyme conjugates caused by the advantages of control over molecular weight and polydiversity. Both atom transfer radical polymerization (ATRP) and reversible addition-fragment chain transfer polymerization

(RAFT) techniques have been employed,^{38–40,59,60} in which ATRP initiators or RAFT agents were first attached to protein surface, followed by living radical polymerization of vinyl monomers such as *N,N'*-dimethylisopropylacrylamide⁴⁰ or monomethoxy poly(ethylene glycol) methacrylate.^{38,60} Liu and coworkers⁶¹ synthesized uniform horseradish peroxidase (HRP)–polyacrylamide conjugates by aqueous ATRP with activators generated by electron transfer (AGET ATRP) in air using CuBr/PMDTA (1,1,4,7,7-pentamethyldiethylenetriamine) as the catalyst and ascorbic acid as the reducing agent. This modified procedure avoided the use of an inert gas to prevent the CuBr catalyst from being oxidized and was easily carried out under ambient conditions of exposure to air. In comparison with the conventional ‘grafting onto’ strategy of synthesizing polymer–enzyme conjugates, which often exhibits a low conjugation efficiency because of the spatial hindrance encountered during the macromolecular reactions, the ‘growing from’ method ensures a higher yield of conjugation.

PROTEIN–POLYMER NANOGELS

An aqueous two-step, *in situ* polymerization that encapsulates protein into nanogel was developed by

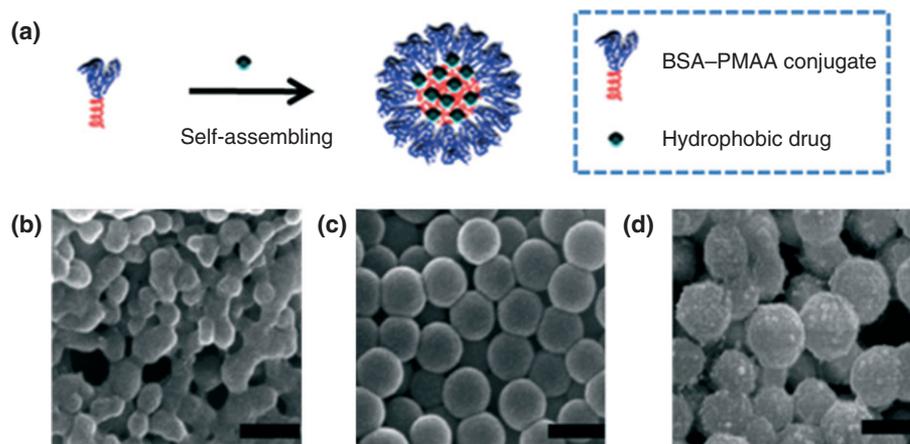


FIGURE 3 | (a) Self-assembly of drug-encapsulated bovine serum albumin (BSA)–poly(methyl methacrylate) (PMMA) nanoparticles. Scanning electron microscope (SEM) images of BSA–PMMA nanoparticles made with (b) 4 wt% BSA, (c) 69 wt% BSA, and (d) 82 wt% BSA (scale bars are 100 nm). (Reprinted with permission from Ref 57. Copyright 2011 American Chemical Society; Reprinted with permission from Ref 58. Copyright 2012 John Wiley & Sons)

Liu and coworkers (Figure 4).⁴³ After encapsulation in the crosslinked polymer network, which is nanometers in depth, enzymes preserved most of their catalytic activities and showed increased stability at high temperature and in the presence of organic solvents. The effectiveness of this method has been proven by using different proteins such as HRP,⁴³ bovine carbonic anhydrase,⁴⁶ *Candida Rugosa* lipase,^{44,45} and urate oxidase.⁴⁷ As an example, when HRP was chosen as the model protein, the Michaelis–Menten parameters, K_m and k_{cat} of the enzyme encapsulated in nanogel were 0.307 mM and 2037 s⁻¹, respectively, very close to the values of free enzyme, which are 0.297 mM and 2187 s⁻¹, respectively. This result indicates that by controlled synthesis of the hydrophilic polymer shell to a depth of several nanometers around the enzyme, the transport of the substrate to the active site of the encapsulated enzyme was hardly affected and the encapsulated enzyme retains most of its activity.⁴³

Yan et al.⁴⁸ have shown that the size, surface charge, and degradability of the nanocapsules can be precisely controlled by varying the concentration of monomers, the ratios of the cationic and neutral monomers, and the degradability of crosslinkers. In this manner, they made the protein nanogel a promising delivery platform. Gu et al.⁴⁹ reported an enzymatically degradable protein–polymer nanogel by using a short peptide, which was specifically cleaved by protease, as the crosslinker in the preparation of the protein nanogel. After polymerization, a protective polymer shell was formed around protein, which can be digested in the presence of protease to release encapsulated protein in a functional form.

SELF-ASSEMBLED COMPLEX HYBRID NANOSTRUCTURES

Recently, Lin et al.⁵⁰ proposed magnetic enzyme nanoparticle that was easily operated and recovered. The Fe₃O₄ nanoparticles were first modified with amine groups, followed by acryloylation that generates vinyl groups on the nanoparticle surface. The remaining amine groups may also be converted to carboxyl groups in order to bind the target enzyme of positive charge. Finally, *in situ* polymerization of the acrylamide generated a polymer network that encapsulated the enzymes around the magnetic nanoparticles. The magnetic enzyme nanoparticles can be readily recovered by means of a magnetic field.

Interest exists in using protein as templates to construct highly ordered nanostructures and arrays.^{62,63} Shen et al.⁵² reported the nucleation and growth of nanocrystals of ZnS and CdS on a self-assembled template made of genetically engineered P22 coat protein. The protein-directed crystal growth exhibited different structures according to the differences in reaction time and reactant concentration. The structures varied from ordered spherical nanocrystal assemblies in the early stage to spherical hollow nanostructures for longer growth periods.

A recent study by Zare and coworkers⁵¹ showed that proteins can be incorporated *in situ* into the nanostructures during inorganic material growth and integrated as part of organic–inorganic hierarchical nanostructures. They obtained flower-like structures made of protein and inorganic Cu₃(PO₄)₂·3H₂O crystals by adding CuSO₄ solution to a protein solution prepared with phosphate-buffered saline

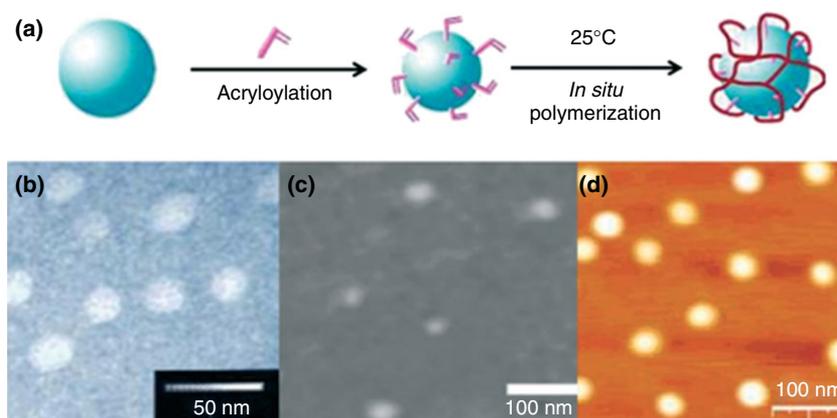


FIGURE 4 | (a) The procedure for encapsulating a single protein into a nanogel. (b) Transmission electron microscope (TEM) image of a horseradish peroxidase-encapsulated nanogel. (c) Scanning electron microscope (SEM) image of a *Candida rugosa* lipase-encapsulated nanogel. (d) Atomic force microscope (AFM) image of a bovine carbonic anhydrase-encapsulated nanogel. (Reprinted with permission from Ref 43. Copyright 2006 American Chemical Society; Reprinted with permission from Ref 45. Copyright 2009 American Chemical Society; Reprinted with permission from Ref 46. Copyright 2007 American Chemical Society)

(Figure 5). The authors proved that the protein molecules are primarily located in the core of the nanoflowers. After a careful study of the mechanism of the formation of hybrid nanoflowers, the authors proposed that in the self-assembly process, the protein induced the nucleation of the copper phosphate crystals, which served as ‘glue’ binding together the petals of the nanocrystals. Nanoflowers made with BSA, α -lactalbumin, laccase, carbonic anhydrase, and lipase were obtained. An increase of laccase activity by fivefold to sevenfold was found when it was incorporated into nanoflowers, in addition to an enhanced stability in solution.

POSSIBLE FUTURE APPLICATIONS OF PROTEIN-INCORPORATED BIONANOMATERIALS

Examples of utilizing some of the protein-incorporated bionanomaterials discussed above show a promising future of such bionanomaterials for applications in enzymatic catalysis and drug delivery. Many methods have been reported for encapsulating or immobilizing enzymes in nanostructures. Owing to the properties of high surface area, noninferior mass transfer, and confined space, enzymes usually exhibit high activity and stability once entrapped in these nanostructures. This property provides many opportunities to prepare nanobiocatalysts that can be used under harsh conditions in industrial processes. The work of using lipase-polymer nanogel for catalysis in anhydrous dimethyl sulfoxide (DMSO) represents one example of utilizing an enzyme in nanostructures for

biocatalysis at harsh conditions for which the native enzyme would fail.⁴⁵ The lipase nanogel was used to catalyze the transesterification between dextran and vinyl decanoate in DMSO at 60°C for 10 days. The substitution degree of dextran reached 23% and the enzyme nanogel had remarkable regioselectivity by discriminating between the three available secondary hydroxyl groups in the glucopyranoside unit. The product of hydrophobic group-modified dextran could be used as a biocompatible, enzyme-responsive material for preparing smart nanoparticles for drug delivery.⁶⁴

Delivering proteins into cells to replace dysfunctional protein is considered a direct and safe approach for treating disease. However, the effectiveness of this method has been limited by low delivery efficiency and poor stability against proteases in the cell, which digest the protein. To improve the stability and efficiency of delivery of protein, therapeutic proteins can be encapsulated in nanoparticles, which protect them from adverse conditions. The intracellular delivery platform based on nanocapsules containing a protein and a thin layer of permeable polymeric shell represents one example of using protein–polymer hybrids for protein delivery.^{48,49} The cell transduction efficiency was studied using a nondegradable polymeric nanocapsule containing enhanced green fluorescent protein (EGFP) and HeLa cells. Cells carrying nanocapsules of EGFP showed significantly higher fluorescence intensity than those with native EGFP. Compared with cell-penetrating peptide-assisted delivery, cells incubated with nanocapsules showed two to three orders higher fluorescence intensities than those with

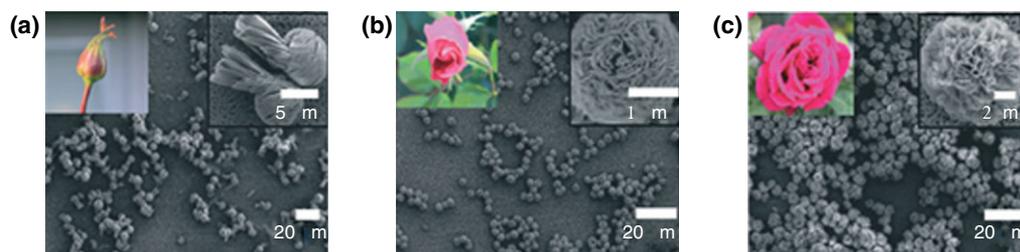


FIGURE 5 | Scanning electron microscope (SEM) images showing the 'growth process' of the nanoflowers achieved with bovine serum albumin at concentrations of (a) 0.5 mg/mL, (b) 0.1 mg/mL, and (c) 0.02 mg/mL. Insets to the right show high-magnification images of the nanoflowers. Insets to the left show flowers in different stages of development in nature. (Reprinted with permission from Ref 51. Copyright 2012 Nature Publishing Group)

TAT-EGFP fusion proteins or antennapedia-EGFP conjugates.⁴⁸ *In vivo* studies showed that the nondegradable nanocapsules have good stability and activity in animals. The *in vivo* activity of the nanocapsule containing HRP was assessed within the organ sections of mice using dihydroethidium, a fluorogenic substrate for HRP. The tissues of the nanocapsule-injected mice showed intense red fluorescence 8 h after injection.⁴⁸ For a therapeutic protein with macromolecular substrate, a type of acid-degradable polymeric nanocapsules was used in the study. The nanocapsule maintained stable in the circulation system, and was degraded in the acidic environment in endosome to release the encapsulated protein.⁴⁸ Using protease-digestible peptides as the crosslinkers, the peptides were cleaved by the protease and the polymeric nanocapsule was degraded to release its contents. The released protein showed good internalization and apoptosis characteristics.⁴⁹

In addition to protein delivery, protein-polymer conjugates could be used as building blocks to make nanoparticles for small molecular drug delivery. Developed by Zare and coworkers,⁵⁸ the nanoparticles made of BSA-PMMA conjugates have been used to encapsulate hydrophobic anticancer drugs and deliver the drugs in animals for chemotherapy treatment. CPT was encapsulated as a model chemotherapy drug. Results of an *in vitro* experiment showed that CPT-encapsulated BSA-PMMA nanoparticles improved antitumor activity over human colon cells (HCT116), compared with a free drug formulation in solution. The dose-dependent cytotoxic effect of the CPT solution was evident culminating in approximately 50% HCT116 survival at 500 ng/mL after 48 h. For the HCT116 cells treated with CPT-encapsulated nanoparticles, after 48 h, 50% survival was observed at 125 ng/mL. In addition, the empty nanoparticles showed excellent biocompatibility. Around 100% cell survival was observed for cells treated with different concentrations of empty nanoparticles. More interesting is that a

slight promotion of cell growth (~120% cell survival) was observed after 48 h, which might be caused by the empty nanoparticles that contain BSA, a nutrient for cells. *In vivo* studies in mice were applied to prove the antitumor activity of the nanoparticle formulation. After the injection of CPT nanoparticles at 9 mg/kg, the median tumor volume of the CPT nanoparticle-treated mice was 40% increased at day 21 compared with that of at day 1. In contrast, for the free CPT-treated group, the median tumor volume increased over 300%. The authors suggested that two effects are responsible for the increased uptake of the drug-loaded nanoparticles by cancerous cells: the enhanced permeability and retention effect and the binding of albumin on cancerous cells with specific receptors.

TOWARD THE RATIONAL DESIGN OF PROTEIN-INCORPORATED HYBRID NANOMATERIALS

Advances in molecular simulation offer a tool for the design of protein-incorporated hybrid nanomaterials. An atom-level molecular dynamics simulation⁴⁴ showed that hydrogen bonding was the driving force for the assembly of acrylamide around lipase in aqueous phase, which was evidenced by a fluorescence resonance energy transfer study. The multipoint linkage within the polyacrylamide network not only strengthened the thermal stability of the lipase but also inhibited the stripping of the essential water by polar solvent such as DMSO. This increased stability thus enabled a lipase-catalyzed transesterification reaction in DMSO at 60°C.

Recently, Yang et al.⁶⁵ have shown by all-atom molecular dynamics simulation that PEG became entangled around the protein surface through hydrophobic interaction and concurrently formed hydrogen bonds with the surrounding water molecules. This interaction thus accounts for the improved hydrophilicity and enlarged molecular

volume of the conjugate while reduced solvent access to the protein molecule. Moreover, the simulation results suggested that an optimal chain length exists that could maximize drug potency of the protein–PEG conjugate. Molecular insight, generated from the supplementary input of molecular simulation, will help the development of the protein-incorporated hybrid nanomaterials.

CONCLUSIONS AND FUTURE PROSPECTS

Protein–organic/inorganic hybrid nanomaterials, though in its infancy, have shown unprecedented opportunities for improving biological functions of proteins and expanded potential applications in areas such as drug delivery, biosensors, bioanalytical devices, and industrial biocatalysis. It is interesting to note that at the beginning of the development of enzyme immobilization technology in the 1960s, the stability of an enzyme could be enhanced after immobilization, whereas the loss of activity is obvious. In the 1990s and thereafter, enzymes immobilized on nanostructured materials could preserve most of the enzymatic activity compared with free enzymes. The increased stability and highly retained activity usually arise from the physically stabilized configuration of protein and insignificant mass-transfer restriction of the enzymatic reaction in nanostructured materials. The nanoflowers made of enzymes and inorganic crystals have much higher activity than free enzymes, arising from the synergistic effect of the chemical interactions between protein and inorganic nanostructures. This fact suggests that, to further improve the performance of protein in a given environment, much

more attention should be paid to the chemical interactions between proteins and nanomaterials, which might have synergistic effects to altering the biological functions of proteins. For example, when designing enzyme–nanomaterial hybrids, attention could be paid to the allosteric effect, which is an interesting phenomenon that binding of a ligand to one site on a protein molecule indirectly manipulates the properties of another specific site on the same protein as a consequence of conformational changes. This allosteric effect could be utilized to regulate and sometimes increase the biological activity of proteins. The design of nanostructured materials containing an effector to an allosteric site of enzyme would improve the performance of the enzyme in such nanostructures.

As for applications in industrial biocatalysis, enzymes are often subjected to organic media because of the increased solubility of substrates and the minimized negative impact of water molecules in organic solvents. In this case, the extremely low solubility of proteins in organic solvent is one of the major reasons for the low activity of an enzyme, being four to five orders of magnitude lower than that in aqueous solution.^{66–68} A highly dispersing or even solubilizing of enzyme-incorporated nanomaterials as catalysts in organic solvents remains a challenge for industrial biocatalysis.

In general, the research on functional protein–organic/inorganic hybrid nanomaterials requires the input of efforts from interdisciplinary areas including chemistry, materials science, biology, and engineering. Future development of such bionanomaterials with more sophisticated, well-controlled structures, and high performance of the biological functions will have a significant impact on the research field of biotechnology and biomedicine.

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