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In-Situ Encapsulation of Protein into Nanoscale Hydrogen-Bonded Organic Frameworks for Intracellular Biocatalysis

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Abstract: Hydrogen-bonded organic frameworks (HOFs) are a novel class of porous materials that showing a great potential for biological applications. The self-assembly of HOFs and biomacromolecules, however, challenging the interface of HOFs with biological settings. We report herein the self-assembly of nanoscale HOFs (nHOFs) to encapsulate protein for intracellular biocatalysis. We show that the self-assembly of tetakis(4-aminophenyl)methane and azobenzene dicarboxylate can encapsulate protein in-situ to form protein@nHOFs under mild condition. This strategy is generally applicable to proteins with different surface charge and molecular weight, showing a high protein encapsulation efficiency and minimal effect on protein activity. Cellular delivery study demonstrates that the protein@TA-HOFs can efficiently enter cells and remain enzyme activity for biochemical catalysis in living cells for neuroprotection. Collectively, our strategy paves new avenues for interfacing nHOFs with biological settings, it will shed light on expanding nHOFs as a novel platform for biomacromolecule delivery and disease treatment.

Introduction

Hydrogen-bonded organic frameworks (HOFs) are a novel class of porous materials composed of organic building blocks that are connected via intermolecular hydrogen-bonding interactions[1-3]. Over the past decade, by selecting complementary H-bonding motifs, a large number of HOFs with varied morphologies and topological structures have been constructed[4,5], further enabling their functional applications[6-9], including molecular catalysis[10], fluorescence sensing[11] and gas separation[12,13]. Compared to metal-organic frameworks (MOFs) and covalent organic frameworks (COFs) that rely on metal coordination and covalent bonding to form porous structures respectively[14-16], the H-bonding interaction of HOFs is much weaker and dynamic, providing HOFs with flexibility and unique features that are highly desirable for biomedical applications[17]. For example, HOFs could be self-assembled in aqueous solutions at ambient temperature[18,19], allowing the immobilization of protein into HOFs to enhance its stability in harsh environments[20]. Also, the metal-free nature of HOFs may avoid the potential detrimental effect of metal ions on biological settings[21], enabling small molecule drug delivery[22] and antimicrobial application[23] using HOFs. Interfacing HOFs with living cells, particularly exploring the intracellular function of HOFs for potential biomedical applications, however, remains very limited mostly due to the challenge of controlling the self-assembly of HOFs with biomacromolecules, for instance, proteins into nanoscale composites for cellular internalization and cell fate manipulation.

As the fundamental components and building blocks of virtually every aspect of life process, proteins, especially enzymes, play essential roles in regulating the physiological function of cells[24]. Enzyme catalysis in living cells not only enables in-situ monitoring of cellular metabolism for disease diagnosis[25], but also offers promising therapeutic benefits for defective enzyme-induced diseases[26,27]. Native enzymes, however, tend to be unable to spontaneously enter cells and are also prone to degradation in complicated environment[28,29], making the development of delivery system to facilitate the cellular internalization of proteins especially crucial for enzyme catalysis-associated disease diagnosis and therapy[30,31]. One attractive strategy to enhance the cellular uptake of enzymes is to immobilize them within nanoscale porous materials[32]. For instance, infiltration of proteins into pre-synthesized nanoscale porous materials, such as metal-organic frameworks (MOFs) [33-38] and flexible organic materials[39,40] enhances the cellular uptake of proteins. Nevertheless, this strategy usually limits to the infiltration of proteins smaller than the inner pore size of porous materials. On the other hand, biominalerization of protein by self-assembling porous material precursors with proteins can overcome the inherent pore size limitation, enabling a generally applicable strategy for protein encapsulation and cellular delivery[41]. The delicate nature of protein, however necessitates the biominalerization to be performed under mild conditions, such as room temperature and aqueous solution to maintain the bioactivity of proteins. Moreover, a strong non-covalent interaction between protein and the precursors is usually required to assemble nanoscale bio-composites. As a consequence, to date, only very limited types of MOFs, such as zeolitic imidazole framework (ZIFs) have been used to incorporate proteins in situ for intracellular protein
However, the biomineralization of protein by ZIFs depends on the chemical property and residue sequence of protein, such as the surface charge of proteins, limiting the scope of this approach for protein encapsulation. In this regard, uncovering novel nanoscale HOFs for efficient and general protein encapsulation is of vital importance for enabling enzyme catalysis in living cells.

The H-bonding interaction between protein residues, such as glutamic acid and arginine, and the building blocks of HOFs may enable a general strategy for protein integration into HOFs regardless of the physiochemical property and residue of proteins. Herein, we report the self-assembly of nanoscale HOFs (nHOFs) as a general approach for in-situ protein encapsulation and intracellular delivery for enzyme catalysis in living cells. We demonstrated that the H-bonding interaction between tetraakis(4-amidiniumphenyl)methane (TAM) and azobenzenedicarboxylate (AZB) is able to self-assemble TA-HOFs (Scheme 1). In addition, the self-assembly process was not affected in the presence of proteins, and further enables in situ encapsulation of proteins into nHOFs regardless of protein surface charge and molecular weight. We found that the protein encapsulation has minimal negative effect on protein activity. Moreover, the nanoscale protein-encapsulated HOFs can be efficiently internalized by cells, remaining the enzyme activity to catalyze chemical conversion in living cells. The efficient encapsulation and cellular delivery of catalase (CAT) using nHOFs can significantly alleviate the oxidative stress for neuroprotection by degrading the reactive oxygen species of neural cells. Our results illuminate the great potential of interfacing nanoscale HOFs with biomacromolecules for cellular function manipulation and biomedical applications.

Results and Discussion

Synthesis and characterization of nanoscale protein@TA-HOFs

The synthesis of nHOFs and the in-situ encapsulation of protein into nHOFs were depicted in Scheme 1. The H-bonding interaction between the amidinium group of TAM and the carboxylate group of AZB resulted in the formation of TA-HOFs when mixing TAM (2 mM) and AZB (8 mM) in an aqueous solution under ambient conditions. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) imaging indicated a nanorod morphology of TA-HOFs with length around 200 nm (Figure 1a and 1b). In addition, it was found that the size and morphology of TA-HOFs were not greatly affected by the self-assembly ratio between TAM and AZB (Figure S1). We hypothesized that the convenience of self-assembling TAM and AZB into nHOFs may allow an in-situ encapsulation of protein into TA-HOFs by making use the H-bonding between protein residue and TA-HOFs. To verify this potential, green fluorescent protein (GFP) was selected as a model protein and was added to the self-assembly mixture of TAM (2 mM) and AZB (8 mM). We found with the fixed concentration of GFP (5 µM) and molar ratio of TAM and AZB at 1:4, the concentration of TAM and AZB in the mixture affected the size of TA-HOFs greatly. With the increased concentration of TAM and AZB, the size of GFP@TA-HOFs particle was decreased from ~2 µm down to ~500 nm (Figure 1c-1e). This may be due to the faster nucleation of protein@TA-HOFs at a higher concentration of HOFs motifs, generating a large number of HOFs nuclei to assemble nanoscale HOFs(Figure 1e and 1f). In addition, there was a minimal change of the fluorescence spectra of GFP before and after TA-HOFs encapsulation, indicating the minimal effect of HOFs encapsulation on the function of GFP (Figure S2). The CLSM images of GFP@TA-HOFs confirmed the successfully encapsulation and homogeneously distribution of GFP inside TA-HOFs(Figure S3).

The shift of C=O stretching vibration at ~1691 cm⁻¹ and C=N stretching vibration at ~1681 cm⁻¹ in the Fourier-Transformed Infrared (FT-IR) spectrum of TA-HOFs indicated the restriction of stretching movements of C=O and C=N owing to the formation of a rigid structure connected by hydrogen bonds in TA-HOFs and GFP@TA-HOFs (Figure S4). Thermogravimetric analysis (TGA) indicated that TA-HOFs and GFP@TA-HOFs remained thermally stable up to ~300 °C (Figure S5). Also, the difference of TGA curves between TA-HOFs and GFP@TA-HOFs suggested the successful encapsulation of GFP into TA-HOFs. Moreover, powder X-ray diffraction (PXRD) analysis confirmed that the encapsulation of GFP into TA-HOFs had minor impact on the crystalline structure of TA-HOFs (Figure S6). In addition, protein release study indicated that minimal GFP was released from GFP@TA-HOFs under acidic condition or in cell culture medium (Figure S7), suggesting the stability of protein@TA-HOFs for potential intracellular catalysis. Lastly, we evaluated the accessible porosity of TA-HOFs and
GFP@TA-HOFs using a guest inclusion method as described recently\(^\text{49}\). It was found that both TA-HOFs and GFP@TA-HOFs can include resorufin (~0.4 nm in width) and fluorescein (~0.7 nm in width) (Figure S8), suggesting the porosity of these HOFs. The pore size of TA-HOFs was estimated to be around 1 nm in diameter according to a report that using similar ligands to self-assemble HOFs\(^\text{15}\). In addition, both TA-HOFs and GFP@TA-HOFs showed 4-fold higher inclusion ability toward small sized resorufin than that of fluorescein with a larger size, indicating the higher inclusion of TA-HOFs to access small molecules even after protein encapsulation.

**Figure 1** The structural characterization of TA-HOFs and GFP@TA-HOFs. a) Scanning electron microscopy (SEM) images and b) Transmission electron microscopy (TEM) images of TA-HOFs (TAM/AZB=1/4). c-e) SEM images of GFP@TA-HOFs prepared with different TAM and AZB concentrations. (f) TEM image of nanoscale GFP@TA-HOFs self-assembled with 2 mM TAM and 8 mM AZB.

**General encapsulation of protein into TA-HOFs** To verify the encapsulation of protein into nHOFs is a general approach and to investigate the effect of surface charge of protein on its encapsulation efficiency by TA-HOFs, two additional GFP variants bearing different surface charge, (+36)GFP and (-30)GFP that are positively and negatively supercharged respectively, were co-assembled with TAM and AZB under the same condition to that of wide-type GFP(wtGFP). (+36)GFP and (-30)GFP are glutamic acid and arginine-rich protein respectively, we hypothesized the potential H-bonding interaction between these two residues and TA-HOFs precursors may lead to efficient protein encapsulation. Indeed, it was found that all three GFP variants could be encapsulated into TA-HOFs, though the efficiency of (-30)GFP encapsulation was slightly lower (around 60%) (Table S1). Also, both SEM and TEM imaging study clearly confirmed the nanorod morphology of (-30)GFP@TA-HOFs and (+36)GFP@TA-HOFs (Figure S9 and S10). These findings indicated that the in situ protein encapsulation using TA-HOFs is less affected by the surface charge of protein than the biominalization of protein by MOFs\(^\text{10}\), suggesting the potential of TA-HOFs as a general protein encapsulation strategy, providing an intrinsic advantage over other porous materials for protein loading.

Next, the encapsulation of proteins and enzymes with different molecular weight by TA-HOFs was explored as well to demonstrate the general use of TA-HOFs for protein encapsulation(Table S2). To this end, horseradish peroxidase (F-HRP, labeled with fluorescein, ~44 KD), catalase (CAT, 240 KD) or β-galactosidase (β-Gal, ~465 KD) was co-assembled with TAM and AZB under the same condition to that of GFP@TA-HOFs. It was found that mixing all three proteins with HOFs precursors formed nanoscale protein@TA-HOFs biocomposites, as confirmed by the nanorod morphology in the SEM images of protein@TA-HOFs (Figure S11). Dynamic light scattering (DLS) analysis indicated the monodispersity of protein@TA-HOFs in aqueous solutions, while the surface charge of enzyme-encapsulated TA-HOFs was varied and dependent on the protein nature (Figure S12 and Table S3). The general yet efficient encapsulation of different enzymes by TA-HOFs paved the way for further intracellular delivery, as to be discussed below.

**Figure 2** (a-b) Cytotoxicity of TA-HOFs and GFP@TA-HOFs against HeLa cells. The cells were treated with a) TA-HOFs and b) GFP@TA-HOFs at indicated concentrations (calculated based on the concentration of TAM motifs) for 18 h, followed by cell viability measurement using MTT assay. (c) Cellular uptake study of GFP@TA-HOFs by treating HeLa cells with different concentration of HOFs for 18 h, followed by flow cytometry analysis to quantify GFP-positive cells. (d) Cellular uptake pathway study of GFP@TA-HOFs. HeLa cells were pretreated with endocytosis inhibitors as indicated for 1 h or incubated at 4 °C before cellular delivery of GFP@TA-HOFs containing 100 nM GFP.

**Intracellular delivery of protein@TA-HOFs** Cytotoxicity study revealed that both TA-HOFs and GFP@TA-HOFs showed minor inhibition effect on HeLa (human cervical carcinoma) cell growth (Figure 2a and 2b). More than 90% of HeLa cells remained alive after the treatment of TA-HOFs or GFP@TA-HOFs with concentration increased up to 120 μM, indicating the high biocompatibility of TA-HOFs for cellular protein delivery and enzyme catalysis study. To demonstrate the capability of GFP@TA-HOFs to deliver protein into cells, HeLa cells were incubated with varied concentrations of GFP@TA-HOFs, followed by flow cytometry analysis to quantify GFP-positive cells. It was found that the cellular uptake efficiency of GFP@TA-HOFs exhibited a positive correlation with the concentration of the GFP added to cells (Figure 2c and S13). When the concentration of GFP added to cells reached 100 nM, 92% of HeLa cells were transfected with GFP@TA-HOFs, exhibiting a higher cellular delivery efficiency compared to the delivery of same concentration of GFP (100 nM) using other porous biocomposites(Table S4), such as ZIF-8 and ZIF-90\(^\text{40}\) under the same condition, highlighting the advantage of harnessing TA-HOFs for intracellular protein delivery. However, we did not observe an
efficient internalization of free GFP by HeLa cells under the same condition, indicating the effectiveness of GFP@TA-HOFs to deliver GFP into cells.

We next studied the cellular internalization pathway of GFP@TA-HOFs by pre-treating cells with endocytosis inhibitors before GFP@TA-HOFs delivery. To this end, HeLa cells were pretreated with five different endocytosis inhibitors, including rottlerin (micropinocytosis inhibitor), chlorpromazine and sucrose (clathrin-mediated endocytosis inhibitor), nystatin (caveolae-mediated endocytosis inhibitor) and methyl-β-cyclodextrin (Me-β-CD, clathrin-independent endocytosis inhibitor) before GFP@TA-HOFs delivery. By comparing the efficiency of GFP@TA-HOFs internalization into cells with and without endocytosis inhibitor pretreatment, it was found that among the five endocytosis inhibitors, only Me-β-CD pre-treatment significantly reduced GFP@TA-HOFs uptake, with the percentage of GFP-positive cells decreased down to 15% of that without endocytosis inhibitor pre-treatment, though sucrose could also slightly inhibited the intracellular delivery of GFP@TA-HOFs. This result suggested that clathrin-independent endocytosis is mostly involved in the cellular uptake of GFP@TA-HOFs. Additionally, the cellular internalization of GFP@TA-HOFs was significantly inhibited when the cells were treated with TA-HOFs at a low temperature (4 °C), indicating an energy-dependent endocytosis of GFP@TA-HOFs. Therefore, an energy-dependent, clathrin-independent endocytosis was confirmed for the cellular uptake of GFP@TA-HOFs. We further monitored the intracellular localization of GFP@TA-HOFs using confocal laser scanning microscopy (CLSM) imaging. The accumulation of GFP in the cytosol was observed after treating HeLa cells with GFP@TA-HOFs (containing 100 nM GFP) for 18 h (Figure 3a). Moreover, it was shown that GFP@TA-HOFs was efficiently escaped from endosome by staining of the endo-lysosomes before CLSM imaging, the colocalization coefficient (Pearson’s correlation coefficient) was calculated to be 0.46. The efficient endo-lysosome escape could be assigned to the overflow of ions and water and destruction of the membrane induced by the proton sponge effect of the TAM moieties [42,48].

TA-HOFs are a general platform to encapsulate and deliver protein with different surface charge into cells. When HeLa cells were treated with (+36)GFP@TA-HOFs and (-30)GFP@TA-HOFs containing 100 nM protein, it was found that the uptake efficiency of (-30)GFP@TA-HOFs was decreased down to 80% of the other two GFP variants@TA-HOFs (Figure 3b and S15). Also, the treatment of cells with 40 μM TA-HOFs encapsulating different amount of GFP variants, the cellular uptake of (-30)GFP@TA-HOFs was only 50% of the other two two GFP variants@TA-HOFs. This could be mostly ascribed to the negatively charged surface of (-30)GFP@TA-HOFs that might decrease the cellular uptake efficiency of nHOFs, as well ascribed to the overflow of ions and water and destruction of the membrane induced by the proton sponge effect of the TAM moieties [42,48].
Figure 5 Intracellular delivery of β-Gal for enzyme catalysis in living cells. (a) Enzyme activity assay of β-Gal@TA-HOFs (5 nM β-Gal) determined by measuring the catalytic hydrolysis of O-nitrophenyl-β-D-galactopyranoside (2 mM). (b) The schematic outline for fluorescein di(β-D-galactopyranoside) (FDG) hydrolysis into fluorescein catalyzed by β-Gal. (c) CLSM images of HeLa cells treated with β-Gal@TA-HOFs (9.4 μg/mL β-Gal, 80 μM TA-HOFs) for 18 h, followed by another 4 h of incubation with 33.3 μM FDG. Scale bar: 20 μm.

Intracellular biocatalysis using enzyme@TA-HOFs Having demonstrated the efficient cellular uptake of GFP variants into cells using TA-HOFs (Figure S16-S17), we subsequently studied whether enzyme-encapsulated TA-HOFs remain its activity for intracellular biocatalysis. To this end, we first fluorescently labeled horseradish peroxidase with fluorescein (FHRP) to better study its intracellular delivery using TA-HOFs. HRP is an enzyme known to catalyze the oxidation of non-fluorescent Amplex Red into red fluorescent and membrane-impermeable resorufin in the presence of H₂O₂ (Figure 4a). FHRP could be assembled into TA-HOFs along with TAM and AZB. FHRP@HOFs showed a comparable enzyme activity compared to that of FHRP without encapsulation (Figure 4b), indicating again the minimal effect of TA-HOFs encapsulation on enzyme activity. When HeLa cells were co-incubated with free FHRP and Amplex Red, we did not observe cellular resorufin fluorescence, suggesting that HRP alone is not able to enter cells to oxidize Amplex Red (Figure S18). FHRP@TA-HOFs treated cells, however, showed significantly increased green and red fluorescence, which could be ascribed to FHRP and resorufin respectively (Figure 4c). Additionally, using flow cytometry analysis, the percentage of red fluorescent cells was higher than 90% after FHRP@TA-HOFs treatment (Figure S19 and S20), demonstrating the high efficacy of FHRP@TA-HOFs delivery to facilitate enzyme catalysis inside cells. Additionally, we found that FHRP@TA-HOFs delivery exhibited 1.5-fold higher intracellular catalysis efficiency compared with the delivery of FHRP using

Figure 6 Intracellular delivery of CAT for ROS scavenging. (a) Fluorescent images of SY-SY5Y cells pretreated with CAT or, TA-HOFs alone, or CAT@TA-HOFs (containing 11.2 μg/mL CAT, 80 μM TA-HOFs) for 18 h before 6-OHDA incubation (320 μM). The intracellular ROS level following above different treatments were imaged using 20 μM DCFH-DA before CLSM imaging. Scale bar: 20 μm. (b) Normalized fluorescence intensity of SY-SY5Y cells incubated following above different treatments imaged using 20 μM DCFH-DA before flow cytometry analysis to quantify the fluorescence intensity change. The fluorescent intensity was normalized to cells treated with CAT@TA-HOFs. (c) Viability of SH-SY5Y cells incubated with free CAT, TA-HOFs alone (40, 80 or 120 μM TA-HOFs) and CAT@TA-HOFs for 18 h followed by 6-OHDA (320 μM) treatment for 12 h. The cell viability was measured by MTT assay. *** represents p<0.001.
porous ZIF-8 or ZIF-90 that has been previously used for protein delivery\(^{[42-44]}\) (Figure S21), confirming again the high efficiency of TA-HOFs delivery for intracellular biocatalysis. Similarly, the encapsulation of β-galactosidase (β-Gal, 465 kD) into TA-HOFs showed partially preserved enzyme activity to catalyze the hydrolysis of O-nitrophényl-β-D-galactopyranoside (ONPG) (Figure 5a), which might be owing to the size effect and the limited ONPG diffusion into TA-HOFs for β-Gal catalysis compared to that of Amplex Red diffusion for HRP catalysis. \(\beta\)-Gal@TA-HOFs can efficiently enter HeLa cells (Figure S22) to catalyze the hydrolysis of fluorescein di(β-D-galactopyranoside) (FDG) to form a strong green fluorescent product, fluorescein, in a significantly enhanced intracellular catalysis efficiency than free \(\beta\)-Gal-treated cells (Figure 5b and 5c). Lastly, to demonstrate the potential of protein@TA-HOFs for enzyme catalysis in living cells for therapeutic promise, we selected catalase (CAT), an enzyme that catalyzes the decomposition of hydrogen peroxide to study its potential to alleviate intracellular oxidative stress for neuroprotection. Scavenging reactive oxygen species (ROS) is closely associated with the treatment of neurodegenerative diseases, such as Alzheimer and Parkinson’s disease\(^{[46]}\). Catalase has been proposed as a promising anti-oxidative agent for protection of cells from severe oxidative stress\(^{[23,56]}\), while its intracellular delivery and catalysis inside cells challenge this potential. Therefore, as a proof of concept study, the delivery of CAT@TA-HOFs was performed to study its potential to protect neural cells from oxidative stress, which is one of main causes of neurodegenerative diseases\(^{[49,51]}\). CAT could be effectively encapsulated into TA-HOFs following the general procedure used for protein encapsulation. CAT@TA-HOFs can be internalized into SH-SY5Y neuroblastoma cells in a same pathway to that by HeLa cells through clathrin-independent endocytosis (Figure S23 and Figure S24), while remaining its catalytic activity within TA-HOFs to catalyze the decomposition of hydrogen peroxide. To verify the delivery of CAT@TA-HOFs into neural cells for catalytic ROS degradation, SH-SY5Y neuroblastoma cells were incubated with 6-hydroxydopamine hydrobromide (6-OHDA), a catecholaminergic neurotoxin related with Parkinson’s disease\(^{[42]}\) in the presence and absence of CAT@TA-HOFs. It was found that SH-SY5Y cells treated with 6-OHDA (320 nM) showed significantly enhanced cellular ROS level, as measured by 2’,7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining and imaging (Figure 6a), as well as flow cytometry analysis as shown in Figure 6b. The treatment of SH-SY5Y cells with free CAT or TA-HOFs did not show an effect to decrease cellular ROS level. In a sharp contrast, CAT@TA-HOFs treatment reduced the cellular oxidative stress significantly (Figure 6a and 6b). Cellular growth measurement indicated that 6-OHDA (320 nM) treatment resulted in apparent decreased cell viability down to 35% compared to that of untreated cells (Figure 6c). CAT@TA-HOFs pre-treatment, however, reduced the cellular oxidative stress, and the cell viability was increased up to 60% compared to untreated cells. Meanwhile, cells pretreated with free CAT and TA-HOFs alone hardly led to notable improvement of cell survival with 6-OHDA incubation, confirming the great potential to harness the catalysis of CAT@TA-HOFs inside cells to alleviate cellular oxidative stress for potential neurodegenerative disease treatment.

Conclusion

In summary, we report herein a novel approach for the self-assembly and in situ encapsulation of protein into nanoscale HOFs. This strategy was generally applicable for a variety of proteins regardless of their surface charge and molecular weight. Furthermore, the nanoscale protein@TA-HOFs could be internalized by cells, maintaining the enzyme activity to catalyze chemical conversions in living cells. We showed that the cellular delivery of CAT@TA-HOFs was able to protect neural cells from oxidative stress damage. Therefore, we believe our strategy of assembly protein@TA-HOFs for enzyme catalysis in living cells will shed light on expanding nanoscale HOFs as a novel platform for biomacromolecule delivery and potential disease treatment.

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Keywords:
hydrogen-bonded organic frameworks • self-assembly • in-situ protein encapsulation • protein delivery • enzyme catalysis in living cells
The *in-situ* encapsulation of protein by self-assembly of hydrogen-bonded organic frameworks (HOFs) enables the formation of nanoscale protein@TA-HOFs under mild conditions. Moreover, this strategy is generally applicable to proteins with different surface charge and molecular weight, facilitating the cellular internalization of proteins for efficient enzyme catalysis inside living cells.