Rational Design of “Three-in-One” Ratiometric Nanoprobes: Protein-Caged Dityrosine, CdS Quantum Dots, and Gold Nanoclusters

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1. INTRODUCTION

Fluorescence nanomaterial-based probes (nanoprobes) with unique intrinsic optical and physicochemical properties, for example, controllably manipulated optical activities, large specific surface areas for conjugation of targeting molecules, carriers for various sensing, and signal-generating molecules, have found their wide applications in biology and medicine.1,2 Among them, luminescent gold nanoclusters (AuNCs), consisting of several to tens of gold atoms, have attracted increasing interest because of their intriguing aesthetic structures and exotic optical properties.3–12 Their molecular-level size (core size <2 nm) quantizes the electron energy of metal NCs, giving rise to highest occupied molecular orbital–lowest unoccupied molecular orbital transitions and then photoluminescence (PL).13,14 Their ultrasmall size also introduces considerable surface energy, and ligands are thus required to stabilize and maintain monodisperse AuNCs in solution. Recently, protein-directed AuNCs have emerged as eye-catching nanomaterials not only because of their much enhanced red fluorescence (quantum yields >6%) but also because of ready and green synthesis, excellent biocompatibility, and low cytotoxicity.15–17 Very recent progress in understanding the formation of protein-prototected AuNCs showed that the tyrosine residues in the protein ligands were oxidized and cross-linked, yielding brightly blue-emissive dityrosine (diTyr, quantum yields >57% at pH 8.5).16–18

Thus, a benign ratiometric probe can be facilely constructed with the fluorescent pair of diTyr residues and AuNCs in proteins. Specifically, such ratiometric nanoprobes are spacious enough to conjugate a third nanoprobe. Optically, the difference in the emission peak of diTyr residues and AuNCs is rather large (>200 nm); sterically, the large protein cage [e.g., bovine serum albumin (BSA) with a size of 14 × 3.8 × 3.8 nm] merely contains small-molecule diTyr residues and ultrasmall nanomaterial AuNCs.19,20

Ratiometric measurement is independent of the local probe concentration and various analyze-independent confounding factors, which can facilitate more accurate and reliable quantitation.21–28 Therefore, research for designing and fabricating ratiometric optical nanoprobes has attracted great interest. Recently, multiplexed ratiometric fluorescence sensors for detecting several targets have been very appealing because
of their convenience, multifunctionality, and lower cost.\textsuperscript{22,29–32} However, most of them are fabricated with small-molecule fluorophores, suffering from drawbacks including difficulty to be all excited with single wavelength because of their short Stokes shifts, photobleaching, tedious multistep synthesis, and sophisticated modification.\textsuperscript{23,32}

Here, we construct a novel trinity fluorescent nanoprobe in which one small-molecule fluorophore, diTyr residues, and two nanomaterial fluorophores, CdSQDs and AuNCs, are cocaged in a BSA molecule. The key to design the hybrid probe lies on the joint excitation wavelength and separate emission peaks. The differences of Stokes shifts among diTyr residues, CdSQDs, and AuNCs enable the complexing fluorescent probes to yield broad-spectrum emissions from blue to green to red at a single excitation wavelength. The fabrication of the hybrid probes follows a two-step intergradation process: first, diTyr residues and AuNCs are formed and tethered to the protein cage through the redox reaction between Au\textsuperscript{3+} and tyrosine residues of BSA; then, the CdSQDs are conjugated to the modified BSA cages through Cd\textsuperscript{2+} enrichment and the CdS combination reaction. Structural characterizations suggest that the two nanomaterials are embedded in a BSA molecule. The following fluorescence analysis unearth a novel internal fluorophore-quenching pair, that is, the emission of diTyr is partially quenched by CdSQDs through dual mechanisms, including static complexing quenching and fluorescence resonance energy transfer (FRET). The “three-in-one” fluorophore nanohybrids show their unique potentials in ratiometric molecule sensing and imaging. With established benign biocompatibility, the nanohybrids can sensitively detect ratiometric molecule sensing and imaging. With established fluorophores, su

2. RESULTS AND DISCUSSION

Protein cages with reductive amino acid residues, for example, tyrosine, are desired biominerilization matrices to reduce metal ions in the high oxidation state and then allow metal atoms for crystallization and growth to particles with controlled sizes. The synthesis of fluorescence AuNCs using BSA, a reductive ellipsoidal protein (14 × 3.8 × 3.8 nm), as templates exactly follows the reduction-induced biominerilization pathway.\textsuperscript{20} Our previous study has demonstrated that the tyrosine residues in BSA (21 residues in one molecule) acted as reductants and were oxidized to dityrosine in the synthesis of AuNCs.\textsuperscript{16,18} Thus, we here qualitatively describe the BSA-directed synthesis of AuNCs using the following formula

\[
\text{Au}^{3+} + \text{Tyr-BSA} \rightarrow \text{AuNCs@diTyr-BSA}
\]

where Au\textsuperscript{3+} is reduced to Au\textsuperscript{3+}/Au\textsuperscript{0}, finally yielding AuNCs, and tyrosine residues of BSA are oxidized to dityrosine residues. The as-synthesized AuNCs with an average size of ~2 nm could be seen via transmission electron microscopy (TEM) (Figure S1). Their ultrasmall size leads to electron energy quantization, giving rising to fluorescence. As shown in Figure S2, the brown AuNC solutions emitted bright red fluorescence under ultraviolet (UV) irradiation (365 nm). The optical spectra showed their broad absorption from 600 nm to the UV region and a typical emission peak at 630 nm (excited at 365 nm, Figure S3). On the other hand, the dityrosine residues are strong blue-emitted fluorophores with an emissive peak at 410 nm, when excited at 340 nm. However, the emission of dityrosine residues in AuNCs@BSA is obscure because of inner filter effects (IFEs) of AuNCs. After removal of AuNCs by the etching reaction, the blue emission of dityrosine residues in the “hollow” BSA could be observed under 340 nm UV light, and the corresponding spectrum peak appeared at 410 nm (Figure S4). The IFE of AuNCs on diTyr residues could be well circumvented by dilution. As shown in Figure S5, the 20-fold dilution could maximize the emission intensity of dityrosine residues of AuNCs@diTyr-BSA. Additionally, in comparison with the emission spectrum of AuNCs@diTyr-BSA excited at 365 nm, the 340 nm excitation dramatically elevated the blue emission while maintaining the red emission (Figure S6). Therefore, the synthetic redox reaction using Au\textsuperscript{3+} as oxidants and BSA as reductants can integrate two relatively independent components of blue-emissive dityrosine residues and red-emissive AuNCs into the BSA cage, resulting in the formation of a dual emission system with proper dilution and excitation.

When we closely observed the fluorescence spectra of AuNCs@diTyr-BSA (Figure S6), a broad gap between the two emission peaks located at 410 and 630 nm attracted our attention, suggesting a window of 220 nm to grow a third fluorophore. Considering the least mutual emissive interferences, the fluorophore with an emission of an arithmetic mean peak of 520 nm is desired. Among yellow-emitted fluorophores, CdSQDs should be the best candidate. They can be readily obtained using BSA as templates and have relatively broad excitation wavelength.\textsuperscript{33} As shown in Figure S7, CdSQDs@BSA yielded a yellow-green fluorescence with an emission peak located at 520 nm when excited at 340 nm. Next, we investigated the synthesis of CdSQDs using AuNCs@diTyr-BSA as templates. We first confirmed that the direct mixing of Cd\textsuperscript{2+} and S\textsuperscript{2−} only generated nonemissive brown precipitates (Figure S8), indicating the necessity of templates. Then, the ratios of Cd\textsuperscript{2+} to S\textsuperscript{2−} for CdSQD formation were investigated. As can be seen in Figure S9, the optimized synthetic ratio was 2:1. Overdose of Cd\textsuperscript{2+} would introduce massive aggregates, whereas less Cd\textsuperscript{2+} led to a deficit in emissive CdSQD production. Finally, with constant synthetic ratios of Cd\textsuperscript{2+} to S\textsuperscript{2−}, the ratios of CdSQD precursors to AuNCs@diTyr-BSA were optimized. As can be seen in Figure S10, the increasing ratios of precursors to proteins promoted the emission intensity at 520 nm. Maximum emission of the CdSQDs was achieved at 13.3, and a further increase would cause precipitations, resulting in a decrease of the fluorescence intensity of CdSQDs. In short, Cd\textsuperscript{2+} to S\textsuperscript{2−} and CdSQD precursors to AuNCs@diTyr-BSA at molar ratios of 2:1 and 13.3, respectively, can be applied to prepare the desired CdSQD-loaded AuNCs@diTyr-BSA (CdSQDs/AuNCs@diTyr-BSA).

The as-prepared CdSQDs/AuNCs@diTyr-BSA solution with zeta potentials of −18.3 were yellowish-brown (Figure
1A inset) under visible light with a broad absorption from 600 to 400 nm (Figure 1A). Compared to the equally molar AuNCs@diTyr-BSA, the incorporation of CdSQDs broadly promoted their absorption, yielding a dramatic absorbance increase starting at 450 nm. These changes agree with the absorption of the pristine BSA modified by CdSQDs alone (Figure S11). The size changes induced by CdSQD embedment were first investigated using dynamic light scattering (DLS). As shown in Figure 1B, the hydrodynamic diameter of CdSQDs/AuNCs@diTyr-BSA was around 20 nm, almost 10 nm larger than that of AuNCs@diTyr-BSA. The increasing size should be ascribed to the conjugation of CdSQDs. TEM technology was then used to characterize the core size of the inorganic nanoparticles. The TEM image of the obtained CdSQDs/AuNCs@diTyr-BSA nanohybrids (Figure 1C) displays a variety of monodisperse black regions with a size of 8.8 ± 1.9 nm (Figure 1C inset). The following high-resolution TEM imaging (Figure 1D) shows that the black regions are detailed by abundant lattice fringes spaced at 3.16 and 2.45 Å assigned to the (101) and (102) planes of the hexagonal phase CdS [JCPDS no. 65-3414], respectively. The measurement using TEM technology indicates that the particle sizes of CdSQDs are ca. 10 nm, which significantly match the DLS size increase induced by CdSQD incorporation. A structure mode, as schematically described in Scheme 1, is thus suggested in which one molecule of AuNCs@diTyr-BSA is loaded with one CdSQD. That is, three functional moieties, CdSQDs, AuNCs, and diTyr, are imprisoned inside one BSA cage. Additionally, as shown in Figure 1D, a region (~2 nm) with a lattice fringe spacing of 2.35 Å, denoted as Au(111) of AuNCs, could be seen around the polycrystalline structure area. The gap (ca. 5 nm) between AuNCs and CdSQDs is suggested to be bridged by the BSA cage.

Next, we investigated the composition of the “three-in-one” nanohybrids. As an informative tool, X-ray photoelectron spectroscopy (XPS) can characterize the elemental composition and oxidation states of elements. After loading CdSQDs, the characteristic binding energies of AuNCs which peaked at 87.5 and 83.8 eV, assigned to Au 4f5/2 and Au 4f3/2, respectively, were almost unchanged, indicating negligible impacts of CdSQD incorporation on AuNCs (Figure S12). Additionally, as shown in Figure S13, a new pair of peaks located at 405.3 and 412.3 eV, denoted as the featured Cd 3d5/2 and Cd 3d3/2, respectively, occurred. The effects of...
CdSQD embedment on AuNCs@diTyr-BSA were further investigated using Fourier transform infrared (FTIR) spectra. As shown in Figure 2, in comparison with AuNCs@diTyr-BSA, the CdSQD conjugation had insignificant impacts on the secondary structure of acylamino in proteins, with supportive evidences of intactness of the amide I band (−C=O) at 1651 cm$^{-1}$, which can be assigned to the α-helix, the amide II band at 1531 cm$^{-1}$ (−N–H bending coupled with −C–N stretching), and the amide III band at 1242 cm$^{-1}$, an integration of C–N stretching, C=O in plane bending, and C–C and −C–N stretching.\(^36\) Differently, the intensity of the characteristic S–H stretching band of nanohybrids at ∼2358 cm$^{-1}$ (red line) is much higher than that of AuNCs@diTyr-BSA (black line), suggesting the increase of thiol groups resulting from S$^{2–}$ binding in the synthesis of CdSQDs.\(^37\) The significant intensity and position changes of characteristic −CH$_3$ deformation and O–H stretching band peaks of the protein cage at ∼2900 and ∼3500 cm$^{-1}$, respectively, after conjugating CdSQDs indicated the possible interactions of amino acid residues in proteins with the surface of CdSQDs.\(^38\) Additionally, the typical tyrosine/dityrosine residue band at 1043 cm$^{-1}$ (phenol-OH or phenol-O$^–$) disappeared after loading CdSQDs.\(^37\) This suggests that there were interactions between phenol groups and CdSQDs, probably affecting the optical properties of diTyr residues.

Figure 3A shows the excitation spectra at 410, 520, and 630 nm, referred to the featured emission peaks of diTyr residues, CdSQDs, and AuNCs, respectively. All of them were able to generate relatively strong emission at 340 nm excitation [also to meet the need to obtain right images under the available 340 nm light-emitting diode (LED)-light]. When the solution was exposed to UV LED-light (340 nm), an intense orange emission was observed (Figure 3B inset). As expected, the three-fluorophore-containing nanohybrids displayed a spectrum with two fluorescence peaks at 610 and 410 nm (Figure 3B), with a nearly filled valley around 520 nm. As suggested by the abovementioned FTIR analysis, the introduction of CdSQDs imparted impacts to the optical properties of diTyr residues. Owing to the interaction between CdSQDs and phenol groups of diTyr residues, the resultant quenching of diTyr residue emission is in accordance with static quenching emission. However, because diTyr is known as a benign energy transfer donor,\(^39\) FRET from the diTyr residues to CdSQDs should be also considered. FRET affects the excited donor,
whereas static quenching generally blocks the electron transition of the donor in the ground state. Fluorescence lifetime decay thus can be used to distinguish the two mechanisms. In this study, the average lifetime of diTyr-BSA emission before and after grafting CdSQDs was measured to be 3.77 and 3.12 ns, respectively (Figure 3C,D). Accordingly, the transfer efficiency according to eq 2 was calculated to be 0.17, which made up 27.4% of the total quenching efficiency (0.62, calculated using the steady-state emission data according to eq 3). These results suggest that the static complexing interaction and FRET simultaneously play essential roles in CdSQD-induced emission quenching of the diTyr residues.

The triple-emissive systems of one small-molecule fluorophore and two nanomaterials contain three responsive fluorophores, exhibiting their promising potentials as multifunctional ratiometric fluorescent nanoprobes. Thiols were reported to be the benign stabilizer to protect CdSQDs, inspiring us initially to investigate the responsiveness of the "three-in-one" nanohybrids toward biothiols, where the incorporated CdSQDs were used as probes.\(^\text{40}\) We added GSH at different concentrations to the nanohybrid solution. As anticipated, fluorescence in the green spectra region, referred to the emission of CdSQDs, was gradually enhanced with increasing amounts of GSH (Figure 4A), together with the corresponding color changes from orange to white (Figure 4A).
The possible mechanism of the fluorescence enhancement was ascribed to the passivation of trap states of CdSQDs via the coordination of the thiol group of GSH with Cd$^{2+}$. A significant 4.5-fold enhancement of emission can be observed in the green spectra region after augmenting 600 μM of GSH, whereas there were 1.2-fold and 0.4-fold fluorescence enhancements in the blue and red spectra regions, respectively. Thus, AuNCs should be better signal references. As shown in Figure 4B, the ratio values of $F_{\text{green}}/F_{\text{red}}$ increase as the GSH concentration increases, and two linear regions of the GSH concentration are from 1 to 100 μM ($R^2 = 0.9803$) and from 100 to 600 μM ($R^2 = 0.9920$). This method has a low detection limit (limit of detection (LOD)) of 0.46 μM. With established ratiometric fluorescence probes, we further investigated the capability of CdSQDs/AuNCs@dTyr-BSA for monitoring intracellular GSH levels in cancer cells. Before investigation, we first tested the anti-interference and biocompatibility of the nanohybrids. As can be seen in Figure 4C, non-thiol-containing amino acids and culture medium have no effects on the emission of CdSQDs. Although cysteine can also stabilize CdSQDs and significantly enhance their fluorescence, its intracellular concentration is typically 100-fold lower than that of GSH, and thus there are insignificant effects of cysteine on the performance of the fluorescence probes for detecting intracellular GSH. On the other hand, the cellular toxicity of CdSQDs is circumvented by the detoxification function from the BSA stabilizer, as supported by the slight increase of MCF-7 cell viability after incubating with varied amounts of nanohybrids (Figure 4D). Next, the hybrid nanoprobes were employed in imaging and sensing of intracellular GSH. As shown in Figure 4F, MCF-7 cells incubated with the nanohybrids (100 μg/mL) for 24 h exhibited a strong green fluorescence emission with detestable red remission, indicating a high level of GSH in MCF-7 cells. Correspondingly, the average emission ratio $F_{\text{green}}/F_{\text{red}}$ was calculated to be 7.458 ± 1.015 (Figure 4E). However, the addition of buthionine sulfoximine (BSO, a GSH synthesis inhibitor) significantly suppressed the green fluorescence intensity, while the red emission remained constant. Likewise, the ratio significantly decreased to 1.286 ± 0.358 when the cells were incubated with BSO-supplemented culture medium (Figure 4E). Taken together, these results indicate the feasibility of intracellular GSH detection by the CdSQDs/AuNCs@dTyr-BSA nanoprobes.

Ionization of phenolic group ($pK_a = 7.1$) residues activates the fluorescence emission of dTyr, enabling dTyr as an optical probe for alkalinity. We thus evaluated the fluorescence responsiveness of CdSQDs/AuNCs@dTyr-BSA toward basic pH values. With increasing pH values, the emission intensity of dTyr residues in the blue spectra region increases, whereas the emissions from CdSQDs and AuNCs in the green and red spectra regions remain constant (Figure 5A). The stabilities of the emission of CdSQDs and AuNCs toward alkalinity render both of them suitable to be the reference in ratiometric optical nanoprobes. Here, we selected the AuNCs as the reference signal. As shown in Figure 5B, the ratiometric probes exhibit a wide dynamic range of the ratiometric linear response against pH values from 7.9 to 12.0 ($R^2 = 0.9937$). By consecutive additions of NaOH followed by HCl, the ratios were tuned back and forth from pH 12.0 to 10.0 to 7.9 without any signal loss, indicating good working stability for alkalinity sensing (Figure S14). Additionally, the optionality of the reference signal can endow the pH probe with improved robustness in the case when one of them is disabled.
TCEP, a biochemically/pathologically important, odorless disulfide bond cleaver and free sulphydryl group maintainer, plays an important role in the retinal therapy as the neuroprotective agent for retinal ganglion cells. Previous report demonstrated that it could quench the red fluorescent emission of AuNCs@BSA because of chemical etching of TCEP toward AuNCs. Here, we applied this chemistry to test the responsiveness of the “three-in-one” nanoprobes to TCEP. As can be seen in Figure 5C, after adding increasing concentrations of TCEP, besides the anticipated quenching of red emission, there is a wide equivalent fluorescence enhancement from the blue to green emission region. Thus, the ratiometry for TCEP with three reversible signal changes could be performed on the basis of TCEP-induced triple response of CdSQDs/AuNCs@diTyr-BSA to detect TCEP. Considering the application scenario in body fluids where there is a strong blue-emissive background noise, we selected the green emission of CdSQDs and the red emission of AuNCs as two interrelated detection signals. The ratio values of $F_{green}$/$F_{red}$ display a good linear relationship with the TCEP concentration in the range of 1–80 μM ($R^2=0.9823$) with the LOD of 270 nM (signal-to-noise ratio = 3) (Figure 5D).

3. CONCLUSIONS

In summary, BSA cages simultaneously imprisoned one small-molecule fluorescent probe, diTyr residues, and two fluorescent nanoparticles, CdQDs and AuNCs, with a two-step synthetic reaction. With optimized synthetic conditions, the satisfied nanohybrids were characterized and suggested that one diTyr-modified protein cage captures a CdSQD and a AuNC. The conjugation of CdSQDs was found to quench the emission of diTyr residues through multiple mechanisms, including complexing-induced static quenching, FRET, and IFE, which are favorable to design sensors based on such diTyr-CdSQDs fluorescent-quencher pair. The three linked fluorescent probes were used to develop ratiometric methods for varied analytes. The trinity probes not only are capable of ratiometric detecting and imaging intracellular GSH contents for varied analytes. The trinity probes not only are capable of ratiometric detecting and imaging intracellular GSH contents but also provide optionality of the reference signal or the probe pair with reversal signal change, which can improve the robustness of probes in the case when one of reference signals is disabled. This study addressed a novel concept of trinity fluorescence ratiometric sensing system with multiple targets and optional references, which should be a promising pathway to meet the challenges from complexing biochemical environments and multivariate analysis.

4. EXPERIMENTS AND METHODS

4.1. Chemicals. HAuCl₄, BSA, and GSH were purchased from Sigma-Aldrich. CdCl₂, Na₂S, and other agents with analytical purity at least were purchased from Beijing Chemical Corporation (China) and used as received. Water used in all experiments was deionized by the Millipore purification system (resistivity >18.2 MΩ cm).

4.2. Synthesis of AuNCs@diTyr-BSA. AuNCs@diTyr-BSA was synthesized according to a previous method with some modifications. In a typical experiment, 20 mL of HAuCl₄ solution (37 °C, 10 mM) was added to equal volumes of BSA solutions (37 °C, 50 mg/mL). Upon vigorous stirring at 37 °C for 2 min, NaOH (1 M) was introduced to maintain the pH value at 11.5, and the mixture was incubated at 37 °C overnight. The final solution was collected and stored at 4 °C.

4.3. Synthesis of CdSQDs/AuNCs@diTyr-BSA. CdCl₂ solution (2 mL, 4 mM) was mixed with 1 mL of aqueous AuNCs@diTyr-BSA complexes under stirring at room temperature for 2 min. The mixed solution was then added to 1 mL of Na₂S solution (4 mM), following a supplement of 6 mL of Tris-HCl buffer solution (50 mM, pH = 8). The resultant solution was ultrafiltrated using Millipore filters (cutoff: 10 kD). The retentates were collected and suspended in 10 mL of Tris-HCl buffer solution. This process was repeated twice, and the final solution was stored at 4 °C.

4.4. FRET Study. AuNCs@diTyr-BSA solution and CdSQDs/AuNCs@diTyr-BSA solution (3 mg/mL) were prepared using Tris-HCl solution (50 mM, pH 8.0). The testing samples were added into a 1 cm path length quartz cuvette. Measurements were recorded using a 340 nm excitation wavelength while monitoring the emission at 410 nm. The ratio between the emission intensities ($F$) or the average decayed lifetimes ($\tau_{ave}$) of the diTyr residues (photons donor, D) in the BSA with ($F_{D\lambda}$, $\tau_{aveD}$) respectively and without ($F_{D}$, $\tau_{aved}$) respectively) CdSQDs (photons acceptor, A) was used to calculate the efficiency of the energy transfer ($E$), according to eqs 2 and 3:

$$E = 1 - \frac{\tau_{aved}}{\tau_{aveD}}$$  \hspace{1cm} (2)

$$E = 1 - \frac{F_{D\lambda}}{F_{D}}$$  \hspace{1cm} (3)

4.5. Cytotoxicity of CdSQDs/AuNCs@diTyr-BSA. Cytotoxicity of CdSQDs/AuNCs@diTyr-BSA nanohybrids in vitro was evaluated utilizing the thiazolyl blue tetrazolium bromide (MTT) assay and a breast cancer cell line (MCF-7). MCF-7 cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, streptomycin at 37 °C under 5% CO₂. The cells were then seeded into a 96-well plate (1 x 10⁴ cells/well) and incubated for 24 h. Afterward, MCF-7 cells were incubated with different concentrations of CdSQDs/AuNCs@diTyr-BSA hybrid microgels for 24 h. After they were washed with phosphate-buffered saline (PBS), the cells were supplemented with 100 μL of DMEM and exposed to MTT (20 μL in PBS, 5 mg/mL) for 4 h. Subsequently, after removal of the supernatant solution from each well, 150 μL of dimethyl sulfoxide was added, and the absorbance of the solution in each well was recorded by using a multimode microplate reader. Eight replicates were prepared for each treatment group.

4.6. Cell Imaging. MCF-7 cells (cancerous breast cells) were selected for this study. The cells were seeded in two confocal Petri dishes. BSO treatment was conducted through preincubating cells in BSO (1 mM) culture solutions for 4 h. Then, the cells are cultured for 24 h to grow to desired density. Fresh CdSQDs/AuNCs@diTyr-BSA (100 μg/mL) was added to the cell medium for another 24 h incubation. The cells were washed with PBS and observed under a confocal laser scanning microscope (LSM710META, Zeiss, Germany) with two different excitation wavelengths of 488 and 586 nm. The confocal fluorescence images were quantitatively analyzed using ImageJ software. The fluorescence intensity of CdSQDs/AuNCs@diTyr-BSA hybrid microgels within each cell was obtained by calculating the integrated intensity divided by the cell area.
nm. XPS spectra were recorded on a VG Scientific (United Kingdom) X-ray photoelectron spectrometer (model ESCALab 220i-XL). TEM images were taken on a Hitachi HT7700 microscope with an accelerating voltage of 200 kV. FTIR spectra were recorded on a Nicolet 400 Fourier transform infrared spectrometer (Madison, WI). All measurements were carried out at room temperature.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00711.

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**Author Contributions**

T.S. and Y.S. contributed equally to this publication.

**Notes**

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