Photo-Decomposable Sub-Micrometer Albumin Particles Cross-Linked by ortho-Nitrobenzyl Derivatives

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Stimuli-responsive particles are widely used as carriers for on-demand delivery of drugs, genes, proteins, etc., due to their response to environmental stimuli. In this study, photoresponsive albumin sub-micrometer particles are fabricated by using ortho-nitrobenzyl derivative 4-bromomethyl-3-nitrobenzoic acid (BNBA) as a cross-linker. Bovine serum albumin (BSA)-doped MnCO₃ particles are used as the sacrificial template, in which the BSA molecules are cross-linked by BNBA under the activation of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride. After removal of MnCO₃ particles by ethylene diamine tetraacetic acid, photoreactive albumin sub-micrometer particles are obtained. Since the C–N bond formed via reaction of benzyl bromide and amine is photocleavable, the particles can be decomposed upon UV irradiation at 365 nm under acidic environment. The loaded macromolecules can be released upon UV irradiation, exhibiting great potential applications of the particles in the field of controlled release.

1. Introduction

Colloidal particles that can respond to stimuli on demand at the targeted sites or at specific time points have received extensive attention due to the versatility in biomedical fields, including gene/drug delivery, biosensors, and bioreactors.[1–3] The stimuli-response can be divided into internal stimuli (e.g., redox,[4] pH,[5] and enzyme)[6] and external stimuli (e.g., light,[7,8] magnetic,[9] and ultrasound)[10] and the most common approach to obtain stimuli-response is the incorporation of various functional components into the colloidal particles.

With the explosive development of nanotechnology, requirement for more precise and complex control of materials is emerged. In this regard, photoresponsive hydrogels, micelles, nanoparticles, as well as capsules[21–26] have been fabricated in past decades. Compared with particles fabricated from synthetic polymers, protein particles show low toxicity and are biodegradable and biocompatible. Albumin, the most abundant protein in serum, is suitable for controlled release application due to its excellent biocompatibility and high stability in blood.[27] Carbonates have been widely used as template for the fabrication of microcarriers and microcapsules because of their high loading capacity and the mild fabrication conditions.[28] In our previous studies,[29,30] we found that different proteins could be doped into carbonates with very high efficiency.

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In this study, bovine serum albumin (BSA) doped-MnCO_{3} sub-micrometer particles are used as sacrificial template. The carboxyl and benzyl bromide groups of ortho-nitrobenzyl derivative 4-bromomethyl-3-nitrobenzoic acid (BNBA) can react with the amine groups of BSA to form the cross-linking points. After MnCO_{3} removal, the BSA–BNBA sub-micrometer particles are obtained. Irradiation with UV light (365 nm) cleaves the cross-linking points, resulting in the decomposition of particles in acid environment (Scheme 1). Rhodamine B isothiocyanate labeled (RBITC)-dextran can be loaded into the particles and released upon UV irradiation. Compared with those larger microcapsules composed of synthetic polymers in our previous work,[24] such sub-micrometer protein particles are more biocompatible and easier for cellular uptake, thus may find more extensive applications in biomedical fields.

2. Experimental Section

2.1. Materials

BSA, RBITC, and rhodamine B isothiocyanate-labeled dextran (RBITC-dextran, M_{w} = 70 kDa) were purchased from Sigma-Aldrich. BNBA was purchased from Acros. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was obtained from Shanghai Aladdin Co., Ltd. Sodium carbonate anhydrous (Na_{2}CO_{3}), manganese chloride (MnCl_{2}), sodium chloride (NaCl), sodium bicarbonate (NaHCO_{3}), and ethylene diamine tetraacetic acid (EDTA) were purchased from Sinopharm Chemical Reagent Co., Ltd. The water used in all experiments was prepared via a Millipore Milli-Q purification system and had a resistivity higher than 18 M\Omega cm\(^{-1}\). BSA-doped MnCO_{3} sub-micrometer particles were prepared by mixing Na_{2}CO_{3} and MnCl_{2} solutions containing BSA according to literature.[29] Briefly, BSA (100 mg) was dissolved in 20 mL 0.25 \(\text{m}\) MnCl_{2} solution and mixed with an equal volume of 0.25 \(\text{m}\) Na_{2}CO_{3} solution under magnetic agitation (1000 rpm) for 1 min at room temperature. Then the particles were washed 3 times with 0.9% NaCl and stored in dark at 4 °C.

2.2. Fabrication of BSA–BNBA Sub-Micrometer Particles

The as-prepared BSA-doped MnCO_{3} particles were reacted with BNBA to prepare sub-micrometer particles. Five milliliter concentrated particles (27 mg mL\(^{-1}\)) were centrifuged to remove the 0.9% NaCl solvent, and then redispersed in 5 mL BNBA dimethyl sulfoxide (DMSO) solution with required concentration according to the designed ratio of amine groups to BNBA (to avoid the reaction between carboxyl and amine groups of BSA itself, BNBA was mixed with DMT-MM for 1 h in advance to activate the carboxyl groups of BNBA only, the molar feeding ratio was BNBA:DMT-MM = 1:1.1).[31] After mild agitation for 24 h at room temperature, the sample was washed by DMSO to remove free BNBA. Due to the reaction between benzyl bromide and carboxyl groups of BNBA with amine groups of BSA, BSA was cross-linked by BNBA. Centrifugation and washing by water was conducted several times. For better observation, the particles were labeled by RBITC. In brief, after cross-linked with BNBA, the particles were incubated in 1 mg mL\(^{-1}\) RBITC Na_{2}CO_{3}/NaHCO_{3} buffer solution (pH 9.4), and the system was maintained in dark overnight. Finally, the as-prepared particles were incubated in 0.2 \(\text{m}\) EDTA (pH 7.4) solution for 15 min under shaking to remove the MnCO_{3} template. The obtained BSA–BNBA particles were further washed with fresh EDTA for three times and then washed with water until neutral, and finally dispersed in water in dark at 4 °C.

2.3. Photo-Decomposition of BSA–BNBA Sub-Micrometer Particles

The photo-decomposition process of BSA–BNBA particles was observed by fluorescence microscopy in situ. The
particles were dispersed in solutions with pH values ranging from 2 to 7 (5.0 × 10⁶ particles mL⁻¹). 20 μL particle suspension was placed into the tiny well on a perforated glass slide, and then the photo-decomposition was observed in situ by fluorescence microscopy (Zeiss Axiovert 200) equipped with a 100 × oil immersion objective. During irradiation, the light of microscopy was cut off and the UV lamp (365 nm, UVEC-4 II system) was placed vertically above the sample with a distance of 40 mm (450 mw cm⁻²). The fluorescence images were captured under the excitation of 546 ± 12 nm after UV irradiation for different time.

2.4. Loading and Photocontrolled Release

Unlabeled BSA–BNBA particles were incubated in 2 mg mL⁻¹ RBITC-dextran solution for at least 2 h. The solution was then centrifuged and washed until free RBITC-dextran was completely removed (no visible color from the supernatant). The supernatant was collected and used to quantify the unloaded RBITC-dextran by fluorescence spectroscopy (FL-55 fluorometer, Perkin Elmer, Japan) at an excitation wavelength of 543 nm according to a calibration curve.

The RBITC-dextran encapsulation efficiency (EE) was calculated according to

\[
EE = \frac{w_{\text{total RBITC-dextran}} - w_{\text{free RBITC-dextran}}}{w_{\text{total RBITC-dextran}}} \tag{1}
\]

where \(w\) is the weight of the corresponding substance.

Dispersed in a buffer solution (pH 3.5), the release of RBITC-dextran from BSA–BNBA particles under UV irradiation was measured. In brief, 300 μL particles suspension (the concentration of particles was about 8.0 × 10⁶ particles mL⁻¹) was placed in a well of a 96-well culture plate and irradiated for 30 min (450 mw cm⁻²). The control group was not irradiated. Fluorescence microscopy images of the particles before and after irradiation for 30 min were captured under the excitation of 546 ± 12 nm. The particles suspension with and without irradiation were collected and centrifuged (11 000 rpm, 15 min) to obtain the supernatant, respectively, and then fluorescence spectra were measured.

2.5. Characterizations

2.5.1. Scanning Electron Microscopy (SEM)

A drop of particles suspension was placed on a silicon wafer. After dried in air overnight, the samples were sputtered with gold and observed with HITACHI S-4800 instrument at an operation voltage of 3 kV.

2.5.2. Transmission Electron Microscopy (TEM)

TEM images were recorded by a JEM-1230 TEM instrument at an acceleration voltage of 120 kV. A drop of sample suspension was placed onto a carbon film-coated copper grid, and dried naturally. The cross-sectional (ultramicrotomy) samples were obtained by microtoming the embedded particles in polymerized epoxy resin into thin sections, which were transferred onto a carbon film-coated copper grid and observed.

2.5.3. Elemental Analysis

Elemental analysis was performed on Vario MICRO cube (Elementar, Germany). Each specimen with ~6 mg was examined in triplicate. The samples were freeze-dried before measurement.

2.5.4. Dynamic Light Scattering Measurement (DLS)

To determine the size and surface charge of particles, the particles (8.0 × 10⁶ particles mL⁻¹) were dispersed in pH 3.5 0.9% NaCl solution, and then measured by Beckman Delsa TM Nano (Beckman Coulter) at 25 °C. 300 μL particles suspension (the concentration of particles was about 8.0 × 10⁶ particles mL⁻¹) was placed in a well of a 96-well culture plate and irradiated for 60 min (450 mw cm⁻²). The particles suspension with and without irradiation were collected, respectively, and then measured. Each value was averaged from 3 parallel measurements.

3. Results and Discussion

3.1. Fabrication of BSA–BNBA Sub-Micrometer Particles

To prepare the photodegradable albumin particles, the BSA-doped MnCO₃ particles were used as template as shown in Scheme 1. The particles were prepared by coprecipitation of BSA with MnCO₃.[29] By this way the MnCO₃ particles with high protein entrapment efficiency can be obtained. Here, according to a calibration curve, the entrapment efficiency of BSA was calculated as about 91.2%, which was much higher than that of adsorption on preformed MnCO₃ particles. After centrifugation and washing, the BSA-doped MnCO₃ particles were characterized by SEM. As shown in Figure 1A, these particles showed a peanut-like shape. The hydrated diameter was 1034.2 ± 57.7 nm, measured by DLS. Their surface was quite rough, and was built from smaller crystallites. Then, via the reaction of amine groups of BSA with the carboxyl and benzyl bromide groups of BNBA, the BSA molecules were cross-linked. Since the C–N bond formed by amine groups and benzyl bromide groups was photocleavable, photodegradable albumin particles were obtained after removal of MnCO₃ particles by EDTA (0.2 m, pH 7.4). By adjusting the feeding ratios of cross-linkers, the properties of the obtained BSA–BNBA particles could be adjusted. With the decrease of feeding ratio of cross-linkers, the BSA–BNBA particles changed from compact (Figure 1B) to relative incompact (Figure 1C), although their apparent diameter and surface zeta potential were kept almost unchanged (Table 1). With a lower cross-linking degree (BB-0.15), the obtained BSA particles had a hollow inner
structure with more BSA molecules near the periphery and a loose network structure in the middle (Figure 1C, inset), which may result from the inhomogeneous distribution of BSA inside the MnCO₃ particles. Such a structure also can facilitate the loading of macromolecular cargos. According to the result of elemental analysis, the C/N molar ratio of BSA and BSA–BNBA particles was 3.64 and 3.73, respectively. As the C/N molar ratio of BNBA was 8, the cross-linking ratio was calculated as about 4.1%.

3.2. Photo-Decomposition of BSA–BNBA Sub-Micrometer Particles

Since the C–N bond formed by benzyl bromide and amine groups is photocleavable,[23] the BSA–BNBA particles are expected to decompose as the cleavage of cross-linking points upon UV irradiation (365 nm). To investigate the photoresponse of the particles, the dissociation behavior was initially observed by microscopy in situ. However, no obvious change of the particles was observed after irradiation in water. As shown in Scheme 1, the cleavage of the C–N bond under UV irradiation (365 nm) is accompanied with the generation of aldehyde groups,[19,20] which may react with remaining amino groups of BSA to form Schiff base bonds, serving as new cross-linking points and preventing the decomposition of the BSA particles. Since the hydrolysis rate of Schiff base bonds increases with the decrease of pH value,[32,33] the particles are expected to be decomposed more easily at lower pH. Moreover, electrostatic repulsion between protonated amino groups in acidic condition may also contribute to the expansion and thereby facilitate the decomposition. Indeed, the particles were decomposed completely after sufficient UV irradiation (60 min) in solutions with a pH range of 2–4. However, in the pH range of 5–7, almost no decomposition of particles was observed even with prolonging irradiation time and intensity. The photoresponsive decomposition behavior under acidic condition can be used to trigger the release of loaded cargos from the particles only after they are internalized into cells and in acidic compartments, such as in lysosomes.

Due to the better decomposition ability, here the particles suspension at pH 3.5 was selected for further study. As shown in Figure 2, upon UV irradiation, the particles began to swell slightly, and the size increased by about 16% after 30 min. The fluorescence intensity of particles was also weakened to some extent (Figure 2B) as a consequence of cleavage of cross-linking points and release of RBITC-labeled BSA chains. After 60 min, the particles almost disappeared (Figure 2C). The TEM images of original particles (Figure 2D) showed a typical hollow inner structure. Though the fluorescence microscopy showed that the particles were decomposed thoroughly after 60 min (Figure 2C), some small aggregates were found in the decomposition products by TEM (Figure 2E), which was further confirmed by DLS (Figure 2F). Even though the cross-linking points formed by benzyl bromide and amine groups can be cleaved under UV irradiation, the hydrophobic BNBA are still linked to the side chain of BSA by the amide groups, and the incomplete cleavage of the cross-linking points may induce the aggregation of the BNBA-linked BSA in aqueous solution, forming nanoparticles with a size about 200 nm in wet and 30 nm in dry states, respectively.

3.3. Photocontrolled Release of Loaded RBITC-Dextran

Since the decomposition of the BSA–BNBA particles can be tailored by irradiation, their ability as intelligent carriers for delivery of cargos was demonstrated by loading and release of RBITC-dextran. First, RBITC-dextran was entrapped inside the particles via diffusion after incubation for 2 h. The network-like structure inside the BSA–BNBA particles as well as the hydrogen bonding interaction between dextran and the network facilitated the entrapment of RBITC-dextran. As shown in Figure 3A, before irradiation the particles showed obvious red fluorescence which derived from RBITC-dextran, indicating the successful loading of RBITC-dextran into the particles. Quantitative analysis found the encapsulation efficiency (EE) of 48%. Upon irradiation, the particles

Table 1. Size of BSA–BNBA particles with different cross-linker feeding ratios.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Feeding ratio (−NH₂:BNBA)</th>
<th>Sizea) (after removal of MnCO₃) [nm]</th>
<th>PDI</th>
<th>Zeta potential [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB-0.5</td>
<td>1:0.5</td>
<td>841.4 ± 26.1</td>
<td>0.095</td>
<td>−45.6 ± 3.2</td>
</tr>
<tr>
<td>BB-0.4</td>
<td>1:0.4</td>
<td>863.8 ± 25.3</td>
<td>0.092</td>
<td>−44.3 ± 1.4</td>
</tr>
<tr>
<td>BB-0.25</td>
<td>1:0.25</td>
<td>887.1 ± 22.4</td>
<td>0.091</td>
<td>−42.0 ± 2.2</td>
</tr>
<tr>
<td>BB-0.15</td>
<td>1:0.15</td>
<td>863.3 ± 28.2</td>
<td>0.123</td>
<td>−41.7 ± 2.7</td>
</tr>
</tbody>
</table>

a)Size of BSA-doped MnCO₃ particles: 1034.2 ± 57.7 nm.
were slightly expanded by 16% as a result of cleavage of the cross-linking points, resulting in more permeable shells. Furthermore, the network-like structure inside particles may be destroyed and BSA molecules may be detached. Due to the electrostatic repulsion, the detached BSA and RBITC-dextran molecules will diffuse into the bulk solution, resulting in the significant decrease of fluorescence intensity of the particles (Figure 3B). The release of RBITC-dextran was further confirmed by the fluorescence spectra recorded for the supernatants, showing that the irradiated one had significantly stronger emission than the original one (Figure 3C).

Calculation found that nearly 87.5% loaded RBITC-dextran was released.

4. Conclusions

Photoresponsive sub-micrometer albumin particles were fabricated by utilizing BSA doped-MnCO₃ sub-micrometer particles as the template and ortho-nitrobenzyl derivate BNBA as the cross-linker. After removal of the MnCO₃ particles, photoresponsive particles were obtained, which could be decomposed under UV irradiation in acidic environment. During the irradiation, the particles were slightly swollen, and finally only small aggregates were remained in solution. The decomposition of particles in response to UV irradiation resulted in the photocontrolled release of RBITC-dextran. Such a system can be used as potential carriers to realize stimuli-responsive release of loaded cargos.

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Conflict of Interest

The authors declare no conflict of interest.
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