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The quantitative detection of the uptake and intracellular fate of albumin nanoparticles†

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Little has been investigated about the intracellular fate of organic nanoparticles (NPs), which is important for the safety and drug delivery efficiency of NPs. In this study, to understand the NP cellular uptake and degradation characteristics, a quantitative method based on fluorescence resonance energy transfer (FRET) was developed and validated to detect the uptake and intracellular degradation of albumin NPs in MCF-7 cells. The effects of the crosslinking density and particle size on the intracellular uptake and degradation kinetics of albumin NPs were then systematically detected. The results indicated that the albumin NPs with higher crosslinking degrees could be internalized more quickly. With increasing NP diameter, the uptake number of NPs decreased, but the uptake NP weight increased due to the compensation of the increased weight of a single particle. The intracellular degradation results showed the NPs with a low crosslinking degree or a small diameter disassociated more quickly in cells, and the half-lives for the albumin NPs disassociation were in the range of 35-79h. These findings will provide fundamental but direct information for the optimal design and biomedical applications of NPs based on their intracellular fates, and the FRET method developed in this study can provide a novel and robust tool to track and monitor the NP intracellular fate.

25 Introduction

Due to the rapid development of material science and nanotechnology, biodegradable nano-sized drug delivery systems (NDDSs, such as nanoparticles, micelles, liposomes, etc.) have been extensively investigated for disease diagnosis1-3 and treatment4-6. Till now, large amounts of functional NDDSs7-13 have been proposed in labs or became pharmaceutical products, e.g. Abraxane8, Doxil9, Depocyt®, etc. but even the generally regarded safe carriers, such as the poly-ε-caprolactone (PCL), poly(DL-lactic acid), poly(lactide-co-caprolactone), and poly(lactide-co-glycide) (PLGA) nanoparticles (NPs), showed toxicity at high concentrations in vitro14,15. Therefore, it’s very important to obtain detailed information and develop quantitative tools about the cellular uptake and the intracellular fate of the biodegradable nanocarriers8.

Although the kinetics and mechanisms of cellular uptake of NDDSs have been well documented6,16,17, to our best knowledge, very few reports focused on the intracellular fate of these nanocarriers18-21. The lack of information on the intracellular fate, especially the intracellular degradation, not only made it difficult to design desired NDDSs, but also introduced excessive risk and sometimes even ethical problems for their future production and clinical application22,23.

Currently, the investigations of the NDDSs’ intracellular fate were limited due to the immature quantitative detection method. For example, the method established for determination of PLGA NPs degradation in mice by detecting the molecular weight was limited by its low detection sensitivity and sample extraction difficulty21, especially for the hydrophilic materials, which could hardly be extracted from the biological matrices efficiently. Another example is the coupled plasma mass spectrometry method developed for quantum dots intracellular elimination detection. This method may be applied only in the certain nano systems, which could release some elements or fragments with known molecular weights24. Therefore, a quantitative method with high sensitivity and widely applicable range is critical and highly needed for determining the NDDSs’ intracellular fate detection.
Fluorescence resonance energy transfer (FRET) involves the distance-dependent (<10nm) transfer of energy from a fluorescent donor in its excited state to another excitable acceptor through a long-range nonradioactive dipole-dipole interaction

As a powerful analytical technique for quantifying the distance between two fluorophores, it has been extensively and intensively utilized to interrogate changes in molecular conformation, interactions of biomolecular machinery, intracellular stability of polyplexes, etc.

In this report, the FRET theory was utilized and the NPs with FRET effect were designed to study the intracellular degradation of the NPs. In principle, in an intact NP, when the two dyes (the fluorescent donor and/or accepter labeled material), whose excitation and emission spectra overlap, are in sufficiently close proximity (<10nm), the excited-state energy of donor molecule is transferred to the neighboring acceptor molecule, resulting in a quenching of donor and enhancement of accepter. If the NP is disassociated, the donor-accepter distance is larger than 10nm, resulting the disappearance of FRET phenomenon. Therefore, a sensitive FRET-based method was developed and validated for the quantitative detection of NPs’ intracellular degradation and fluorescein isothiocyanate (FITC) and rhodamin B isothiocyanate (RBITC) were selected as the fluorescent donor and accepter, respectively.

Albumin, a biodegradable, nontoxic and non-immunogenic protein, is an attractive material for NDDSs preparation, was selected as the building material for NP. Plenty of albumin nanocarriers have developed to improve their pharmacokinetic and target behaviors, and the commercial albumin-bound paclitaxel (Abraxane®) had already shown good safety and efficiency in the clinical application. It has been reported that the both clathrin- and caveolae-mediated pathway could contribute to the intracellular endocytosis of albumin NPs, but the intracellular fate and the factors influencing the uptake and degradation of them were still unclear. In this report, as a model platform, a FITC or RBITC labeled bovine serum albumin (BSA) NP library was developed with different crosslinking degrees and diameters to investigate the uptake and intracellular degradation kinetics of NPs. Furthermore, the NPs’ uptake pathway was also determined in this study.

**Results and discussion**

**Albumin NPs preparation and characterization**

To investigate the effects of physicochemical properties on the uptake and intracellular fate of BSA NPs, a BSA NP library was developed with a series of crosslinking degrees and diameters, but comparable zeta potentials (-20 ~ -30mV). These physicochemical properties are summarized in Table 1. The NPs with a series of crosslinking degrees from 41.9% to 91.1% (C40S70, C70S70 and C90S70) were obtained by reacting with different amounts of glutaraldehyde. The NPs (C40S70, C40S160 and C40S260) formulated with different albumin concentrations had comparable crosslinking degrees of about 42% but different diameters (71.8nm, 157.4nm and 261.9nm). The fluorescence emission spectra of these NPs were shown in Fig.2, and the highly quenching of donor and enhancement of accepter indicated the NPs possessed evident FRET effects, which guaranteed a significant fluorescence change even if only a tiny NPs disassociation occurred, thus providing a high sensitivity for the
NPs disassociation detection. The FRET indices of NPs with various diameters were about 23%; those of NPs with different crosslinking degrees were in the range of 23%~32% with larger indices being possessed by NPs of bigger crosslinking degrees, and this was perhaps because the chemical crosslink decreased the albumin molecular distances.

Fig. 2 The fluorescence emission spectra of FITC-BSA (A), RBITC-BSA (B) and the albumin NPs with various crosslinking degrees (C) and diameters (D); A, C and D: excited at 488 nm; B: excited at 548 nm. The quenching of fluorescence donor and enhancement of acceptor indicated the albumin NPs possessed evident FRET effects.

Table 1. Physicochemical properties of FRET-based BSA NPs

<table>
<thead>
<tr>
<th>Crosslinking Degree(%)</th>
<th>Diameter (nm)</th>
<th>Zeta Potential(mV)</th>
<th>FRET Index(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C40S70</td>
<td>41.9±1.5</td>
<td>71.8±0.1</td>
<td>23.6±0.9</td>
</tr>
<tr>
<td>C70S70</td>
<td>66.2±2.1</td>
<td>72.0±0.1</td>
<td>27.0±2.8</td>
</tr>
<tr>
<td>C90S70</td>
<td>91.1±0.8</td>
<td>71.7±1.4</td>
<td>31.9±0.4</td>
</tr>
<tr>
<td>C40S160</td>
<td>42.3±1.0</td>
<td>157.4±2.3</td>
<td>23.1±0.3</td>
</tr>
<tr>
<td>C40S260</td>
<td>41.8±1.1</td>
<td>261.9±1.2</td>
<td>23.4±1.3</td>
</tr>
</tbody>
</table>

Validation of the NPs degradation detection method

To validate the method developed for the NPs degradation determination, the NPs with various crosslinking degrees and diameters were incubated in trypsin buffers, and the disassociation fractions and hydrolysis degrees of NPs detected by the developed FRET-based method were compared with the results detected by the photometrical method and the pH-stat method. Different trypsin concentrations (6.6, 33, 66 and 100 µg/mL, pH 8.0) were applied in this study, and the disassociation rates of C40S70 increased with the trypsin concentration as shown in Fig.3A1-A4. The first order disassociation rate constants were calculated as shown in Table 2. When the trypsin concentration was set at 66 and 100 µg/mL, no significant difference of the disassociation rate constants were observed between the two methods. As shown in Fig.3B1-B4, similar results for the degree of hydrolysis (DH) were obtained by the FRET-based method and the pH-stat method: during the degradation process the values of protein DH reached the plateau rapidly, and the hydrolysis behaviors were independent on the trypsin concentration. These results indicate the FRET-based method works well for the NPs degradation detection, and this could also be concluded from the results of the other NPs (C70S70, C90S70, C40S160 and C40S260), which were shown in Fig.S1.
Cellular Uptake pathway

Besides macropinocytosis, which was the major pathway for microparticle internalization\(^40\), there are three types of pinocytosis for NPs: clathrin-mediated pathway, caveola-mediated pathway and clathrin- and caveola-independent pathway\(^16\,\,41\). To detect the mechanisms involved in the uptake of albumin NPs in the MCF-7 cells, the uptake of albumin NPs in the presence of chemical inhibitors was measured as shown in Fig.3. A significant, but not complete, inhibition of uptake was observed in the presence of hypertonic medium, but no significant inhibition could be observed for all the NPs when using nystatin as the caveola-mediated pathway inhibitor. The results showed that the uptake of the albumin NPs applied in this study was mainly via the clathrin-mediated endocytosis, and the uptake pathway was not influenced by the crosslinking degree and diameter of the albumin NPs.

Cellular uptake kinetics of albumin NPs

The membrane binding and intracellular uptake kinetics of the albumin NPs with different crosslinking degrees and diameters were detected in MCF-7 cells.

The effect of crosslinking degree on membrane binding and intracellular uptake of the albumin NPs are shown in Fig.5. The results showed that the membrane binding was almost saturated within 1h incubation, and NPs with higher crosslinking degree demonstrated a more efficient membrane binding. Compared with the intact NPs, the disassociated NPs in the membranes were negligible perhaps due to the lack of enzymes in the cell membranes. The intracellular uptake kinetics showed that the amount of intact albumin NPs increased within 6h, and a slight decrease was observed from 6 to 12h. Within the total incubation time of 12h, the amount of disassociated NPs inside the cells increased gradually. The total amounts of albumin NPs in the MCF-7 cells increased within 6h, at which time the uptake of C40S70, C70S70 and C90S70 plateaued, indicating saturation of uptake. The intracellular uptake kinetics showed the albumin NPs with higher crosslinking degrees (C90S70 and C70S70) were internalized more efficiently than those with a lower crosslinking degree (C40S70). A rapid hydrolysis of proteins disassociated from the NPs could be observed inside the cells as shown in Fig.5.

The influences of diameter on the albumin NP intracellular uptake kinetics were shown in Fig.6. For NPs with comparable crosslinking degrees but various diameters, a rapid saturation of the cell membrane-binding was observed, and with increasing diameter, a significant increase of binding could be obtained. As shown in the figure, a gradual increase of the intact NPs and the total amount of the albumin NPs in MCF-7 cells could be observed during the first 6h, and the internalization increased with the increasing of particle size. The disassociated NPs inside the cells increased gradually within the period of 12h. When the number of particles instead of their weight was used as the unit of measurement, it was interesting to find that compared with C40S160 and C40S260 the NPs with a smaller diameter (71.8 nm, C40S70) had the most efficient uptake (Fig.7), concluding that the increase in diameter reduced the intracellular uptake particle number, but the weight of the intracellular particles was compensated by the increased weight of a single particle.

The cellular uptake kinetics of albumin NPs indicated the internalization of albumin NPs was controlled by the crosslinking degree and diameter of the NPs. As the nanoparticle-cell interface was the primary factor governing the interaction between particle and cell\(^52\) and the NPs with various crosslinking degrees offered comparable diameter and zeta potential, the deviations in the uptake behaviors were thought to be due to the different surface chemistry caused by the various crosslinking degrees. As shown, the uptake of albumin NPs was mediated by the clathrin-mediated pathway, which involves the ligand-receptor interaction and receptor-mediated endocytosis. It was thought the crosslink reaction reduced the ligand density of the albumin NPs and
Fig. 5 The membrane binding and cellular uptake of the albumin NPs with different crosslinking degrees (C40S70, C70S70 and C90S70). The MCF-7 cells were incubated with albumin NPs (37.5 µg/mL) at 37°C and 4°C to detect the total cell-interacting and membrane-binding amounts of NPs. The intracellular uptake amounts were obtained from the differences between the total cell-interacting and the membrane-binding data. (n=3)

Fig. 6 The membrane binding and cellular uptake of the albumin NPs with different diameters (C40S70, C40S160 and C40S260). The MCF-7 cells were incubated with albumin NPs (37.5 µg/mL) at 37°C and 4°C to detect the total cell-interacting and membrane-binding amounts of particles. The intracellular uptake amounts were obtained from the differences between the total cell-interacting and the membrane-binding data. (n=3)

consequently affected their cellular uptake. Yuan and Zhang\(^ {43} \) have theoretically elucidated the effects of ligand density and diameter on the kinetics of receptor-mediated endocytosis of NPs from thermodynamic aspect. It was revealed that because of the entropic penalty involved in ligand-receptor binding there existed an optimal condition (in terms of ligand density and diameter) at which the endocytic time is minimized. The time scale of full wrapping of NP was defined as \( \frac{I^2}{D} \), where \( I \) was a characteristic length, and \( D \) was the diffusivity of the receptor. Here, the values of \( I \) as the function of ligand density and diameter of the albumin NPs were calculated as shown in Fig. 8. As shown, when the ligand densities were larger than the optimal one, the increasing of crosslinking density could decrease the ligand density and consequently decrease the wrapping time of the albumin NP. When the diameter of albumin NPs were all larger than the optimal one, the increasing diameter increased the wrapping time and consequently decreases the NP’s cellular uptake.

The kinetics of disassociated NPs inside the MCF-7 cells was governed not only by the disassociation rate but also by the uptake of the NPs. Therefore, the degradation behaviors of these
NPs were determined in absence of uptake in the following studies to elucidate the effects of crosslinking degree and diameter of albumin NPs on their intracellular degradation behaviors.

**Intracellular degradation of albumin NPs**

The intracellular degradation of albumin NPs was observed and detected based on the FRET phenomenon in absence of cellular uptake. As shown in Fig.9A, the signal of channel D could hardly be observed within the uptake of 2h. After 2h, the NP-containing medium was replaced by fresh NP-free medium to terminate the particle uptake, and after the subsequent incubation for 8h. Then a significant signal increasing from channel D (green fluorescence) could be observed for all the NPs (Fig.9B), indicating the disassociation of particles occurred within the subsequent 8h incubation.

The quantitative results of the NP’s intracellular degradation were shown in Fig.10. For different amounts of NPs that were absorbed at the starting time of the degradation detection, the disassociated NPs were normalized by the initial NP content, and their disassociation fractions were obtained in the degradation detection. As shown in Fig.10A1 and B1, no significant change in the amount of total cell-associated NPs could be observed during the degradation detection, indicating the exocytosis of the albumin NPs was negligible within the time period of 8h. As shown in Fig.10A2, a decrease of the disassociation fraction were observed when the crosslinking degree increased, indicating that the albumin NPs with a lower crosslinking degree are disassociated more efficiently. With comparable crosslinking degrees, the NPs with a small diameter of 71.8 nm (C40S70) were disassociated more quickly than those with diameters of 157.4nm and 261.9nm (C40S160 and C40S260), indicating the intracellular disassociation rate of the particles was inversely related to the particle size (Fig.10B2). Quick and similar albumin hydrolysis behaviors of the NPs with different crosslinking degrees and diameters were observed in Fig.10A3 and B3.

The quantitative detection of NP degradation showed a gradual disassociation and quick hydrolysis of the particles in MCF-7 cells. The NPs with lower crosslinking degrees were disassociated more quickly in MCF-7 cells, indicating the extent of chemical crosslinking among the proteins was an important factor governing the NP’s disassociation efficiency. The quick disassociation of the particles with smaller diameters may have resulted from the larger specific surface area and the consequently increased opportunities for the particle-enzyme interaction. Quick hydrolysis behaviors of the protein disassociated from NPs were observed in this study, indicating the disassociation was the rate-limiting step for the intracellular degradation of albumin NPs. The disassociation constants (k) of albumin NPs were obtained by fitting NPs’ disassociation process using a first-order kinetic. The values of k and the half-lives of disassociation are shown in Table 3. When taking 5 times of half-lives as the washout period, after which more than 95% of NPs could be disassociated, the washout periods of albumin NPs applied in this study were in the range of 1-2.5 weeks. Although the data obtained in cell experiments could hardly represent in vivo results, it at least indicated the physicochemical properties of albumin NPs, such as their crosslinking degree and diameter, had an important impact on the clearance rate of NPs from the body and consequently on the safety of NPs.

**Experimental**

**Materials**

BSA (96%), fluorescein isothiocyanate (FITC, 90%), rhodamin B isothiocyanate (RBITC, labeling efficiency with bovine albumin >70%) and cysteine (97%) were purchased from Sigma-Aldrich. Trypsin was purchased from Hualan Chemical. Paraformaldehyde (99%) was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. RPMI-1640 medium, fetal calf serum and antibiotics (penicillin 10,000 units/mL, streptomycin 10,000 µg/mL) were purchased from Hyclone Laboratories Inc.. All other reagents were analytical grade and were purchased from Nanjing Chemical Reagent Co., Ltd.
The uptake (A) and the degradation (B) of albumin NPs observed by the confocal laser scanning microscope (Leica TCS S P5, Leica, Germany). The uptake was observed after incubation of MCF-7 cells with albumin NPs for 2h, and the degradation was observed after the subsequent incubation of cells with the NPs-free medium for 8h.

**Fig. 9**

**Fig. 10** The degradation kinetics of albumin NPs with the series of crosslinking degrees (A) and diameters (B) within the uptake of 2h and the subsequent degradation of 8h in NPs-free medium. During the degradation of 8h, the total amounts of cell-interacting NPs (M+N) had no significant changes, indicating the exocytosis of the albumin NPs was negligible within 8h. The gradually increased dissociation fraction, which was calculated from N/(M+N), indicated the albumin NPs were degraded gradually in MCF-7 cells within the period of 8h. (n=3)

**Table 4** The formulation of albumin NPs prepared using the disulfide bond reducing method

<table>
<thead>
<tr>
<th>Protein Concentration* (mg/mL)</th>
<th>Glutaraldehyde: Protein (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C40S70</td>
<td>1</td>
</tr>
<tr>
<td>C70S70</td>
<td>1</td>
</tr>
<tr>
<td>C70S90</td>
<td>1</td>
</tr>
<tr>
<td>C40S160</td>
<td>2</td>
</tr>
<tr>
<td>C40S260</td>
<td>4</td>
</tr>
</tbody>
</table>

*The protein is the mixture of \textit{RBITC}_{9.5}-BSA and \textit{FITC}-BSA with a weight ratio of 6:4.

**NP characterization**

**Particle size and zeta potential measurement**

The particle size and zeta potential of the NPs were determined using laser light scattering and a Zetaplus zeta potential analyzer (Brookhaven Instrument, USA), respectively.

**Crosslinking degree determination**

After adding the same volume of acetic acid-sodium acetate buffer (pH 3.8) to the crosslinking reaction system, the NPs suspension was centrifuged at 8,000rpm for 30min at 4°C, and the supernatant was collected for glutaraldehyde detection. The crosslinking degrees of BSA NPs were determined using Eq. (1).

\[
\text{Crosslinking Degree} = \frac{(m_{\text{gluadd}} - m_{\text{glusupernatant}}) 	imes M_{\text{BSA}} 	imes 2}{(m_{\text{FITC-BSA}} 	imes m_{\text{RBITC}_{9.5}-\text{BSA}} + m_{\text{RBITC}_{9.5}-\text{BSA}} 	imes m_{\text{FITC-BSA}}) 	imes M_{\text{glu}}} \tag{1}
\]

Where \(m_{\text{gluadd}}, m_{\text{FITC-BSA}}\), and \(m_{\text{RBITC}_{9.5}-\text{BSA}}\) were the amounts (g) of glutaraldehyde, \textit{FITC}-BSA and \textit{RBITC}_{9.5}-BSA added during the NPs preparation, respectively, and \(m_{\text{glusupernatant}}\) was the amount (g) of glutaraldehyde in the supernatant after centrifugation at 8,000rpm for 30min at 4°C. \(M_{\text{glu}}\) and \(M_{\text{BSA}}\) are the molecular weight of glutaraldehyde and BSA, respectively.
where \( n \) was the percentage of FITC-BSA in the NPs.

FRET index determination

The efficiency of the donor-accepter energy transfer of the NPs was reflected using a FRET index, which was calculated by Eq. (2).

\[
\text{FRET Index} = \frac{F_b - F_d}{D_b - D_d}
\]

Eq (2)

Where \( F_b \) and \( D_b \) are the fluorescence intensity of NPs in the channel F and D; \( F_d \) and \( D_d \) are the fluorescence intensity of FITC-BSA in the channel F and D. The detection of signals in different channels was described in details in Section “Sample fluorescence detection and data processing”.

FRET-based method development

In the FRET-based method, the fluorescence intensity was detected in different channels (D, F and A), and the excitation and emission of these channels were listed in Table 5.

Table 5 The excitation and emission measurements of signals in channel D, F and A.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Excitation of d (488 nm)</td>
<td>Emission of d (517 nm)</td>
</tr>
<tr>
<td>F</td>
<td>Excitation of d (488 nm)</td>
<td>Emission of a (576 nm)</td>
</tr>
<tr>
<td>A</td>
<td>Excitation of a (548 nm)</td>
<td>Emission of a (576 nm)</td>
</tr>
</tbody>
</table>

The samples of FITC-BSA (donor), RBITC\(_{9.5}\)-BSA (accepter) and NPs were termed \( d \), \( a \) and \( b \), respectively. The fluorescence intensity of samples was termed using the letters describing the sample and the channel. For example, \( D_b \) was the fluorescence of the donor in the channel D.

In this study, the degradation of NPs was divided into two steps as shown in Fig 1B: the disassociation of NPs and the hydrolysis of the protein disassociated from NPs. In the degradation process, the fluorescence intensity of samples was contributed by the intact NPs (M), the disassociated NPs (N) and the degree of hydrolysis of protein. According to the contribution of M, N and DH, signals in channel D, F and A (\( F_d \), \( F_F \) and \( F_A \)) can be expressed using the following equations.

\[
F_D(M, N, DH) = F_{Dd} + F_{Dd}(N) \times f_d
\]

Eq (3)

\[
F_F(M, N, DH) = F_{Ff} + F_{Ff}(N) \times f_f
\]

Eq (4)

\[
F_A(M, N, DH) = F_{Af} + F_{Af}(N) \times f_a
\]

Eq (5)

Where \( F_{Dd} \), \( F_{Ff} \) and \( F_{Af} \) were the fluorescence contribution of the intact NPs in channel D, F and A, and their standard curves were described by Eq. (6)-Eq. (8) using \( \alpha \) and \( \beta \) as the slopes and intercepts of the standard curves. \( f_d \), \( f_f \) and \( f_a \) were the fluorescence intensities of the disassociated NPs in channel D, F and A and their standard curves are described by Eq. (9)-Eq. (11), where \( n \) was the percentage of FITC-BSA in the NPs.

\[
F_{Dd}(M) = \alpha_{Dd} M + \beta_{Dd}
\]

Eq (6)

\[
F_{Ff}(M) = \alpha_{Ff} M + \beta_{Ff}
\]

Eq (7)

\[
F_{Af}(M) = \alpha_{Af} M + \beta_{Af}
\]

Eq (8)

\[
F_{Dd}(N) = \alpha_{Dd} N + \beta_{Dd}
\]

Eq (9)

\[
F_{Ff}(N) = \alpha_{Ff} N + \beta_{Ff}
\]

Eq (10)

\[
F_{Af}(N) = \alpha_{Af} N + \beta_{Af}
\]

Eq (11)

Where \( \alpha_{Dd} \) and \( \beta_{Dd} \) are the concentrations of the intact NPs and the disassociated NPs. The slopes and intercepts in Eq. (6)-Eq. (11) could be obtained by plotting the fluorescence intensities in the corresponding channel against the concentrations of the samples.

Because of the self-quenching of FITC-BSA \( ^{37, 48} \) and RBITC\(_{9.5}\)-BSA as shown in Fig S2, an increase of fluorescence can be attributed to the protein hydrolysis, and therefore the fluorescence intensities of the disassociated NPs (\( F_{Dd} \), \( F_{Ff} \) and \( F_{Af} \)) in Eq (3)-Eq (5) were corrected by the DH terms (\( f_{Dd} \), \( f_{Ff} \) and \( f_{Af} \)). The DH terms could be determined by plotting the fluorescence intensities against the DH of FITC-BSA and RBITC\(_{9.5}\)-BSA. Samples with different values of DH were prepared by digesting FITC-BSA and RBITC\(_{9.5}\)-BSA (2.5 mg/mL) in trypsin buffers at various concentrations. The DH of samples were detected using a pH-stat method \( ^{49} \). The relationships between fluorescence increasing and DH of proteins were shown in Fig S3, and it was found the DH terms of \( f_{Dd} \), \( f_{Ff} \) and \( f_{Af} \) could be described using the linear equations (Eq. (12)-Eq. (13)). As shown in Fig S3, a burst fluorescence increase was induced by the initial hydrolysis of RBITC\(_{9.5}\)-BSA (DH of 0.49%), and after the burst increase, the fluorescence intensity reached a plateau. To keep consistent with the formats of Eq. (12)-Eq. (13), a linear equation (Eq. (14)) was still used to express \( f_{Dd} \), but the slope (\( \beta_{Dd} \)) was set at 0 in the calculation.

\[
f_{Dd} = a_{Dd} DH + b_{Dd}
\]

Eq (12)

\[
f_{Ff} = a_{Ff} DH + b_{Ff}
\]

Eq (13)

\[
f_{Af} = a_{Af} DH + b_{Af}
\]

Eq (14)

After substituting \( F_{Dd} \), \( F_{Ff} \), \( F_{Af} \), \( F_{Dd} \), \( f_{Dd} \) and \( f_{Af} \) into Eq (3)-Eq. (5), the unknown variables of \( M \), \( N \) and DH could be obtained by solving the equation group using a sequential scanning method. A software program written in Visual Basic 6.0 was applied for the calculation.

Sample fluorescence detection and data processing

For the fluorescence detection, the fluorescence intensity of each sample in channel D, F and A were detected according to the excitation and emission method (Table 5) using the fluorescence spectrometer (RF 5301PC, SHIMADZU, Japan).

The slit width of excitation and emission was set at 10 nm in this study. After substituting the fluorescence intensities into Eq (3)-Eq (5), the intact NPs content (M), the disassociated NPs content (N) and the hydrolysis degrees of proteins could be obtained by solving the equation group using the program provided in the Supporting Information.

Method validation

To validate the method developed in this study, the degradation of albumin NPs in trypsin buffer was measured, and the disassociation fractions and values of DH calculated using the FRET-based method were compared with the results obtained by the photometrical method and pH-stat method.

FRET-based method: NPs were suspended in the trypsin solution to a concentration of 500μg/mL at 37 ℃ under shaking, and the pH was adjusted to 8.0. After various time intervals (0.25, 0.5, 1, 2, 4, 6, 8 and 12h), 300μL of the sample was withdrawn and diluted with PBS buffer (pH8.0) to a volume of 3.0mL for the fluorescence analysis. The disassociation fractions and DH of the NPs were obtained by solving Eq (3)-Eq (5).

Photometrical method: \( ^{48} \) The disassociation fractions calculated from the FRET-based method were compared with the results detected by the photometrical method. After suspending...
NPs in the trypsin solution, at the same time points mentioned above, 3mL of the sample was withdrawn for photometrical detection at 565nm. The NPs concentration was calculated using the calibration curve, and the disassociation fraction (DF) of the sample was calculated by Eq.(15). The calibration curve was obtained by plotting the turbidity of the NPs at 565nm against the NP’s concentration.

\[
DF = 1 - \frac{\text{NPs Concentration}}{\text{Initial NPs Concentration}} \quad \text{Eq.(15)}
\]

pH-stat method: The values of DH calculated from the FRET-based method were compared with those detected by the pH-stat method. At the same time points mentioned above, 10 mL of the sample was withdrawn, and the pH of the sample was adjusted to 8.0 using NaOH solution (1 M). The DH of the sample was calculated using Eq.(16).

\[
DH = \frac{C_{\text{NaOH}} \times V_{\text{NaOH}}}{\alpha \times h \times C_N \times V} \quad \text{Eq.(16)}
\]

Where \(C_{\text{NaOH}}\) and \(V_{\text{NaOH}}\) are the concentration and the volume of NaOH solution consumed for pH adjustment; \(C_N\) is the disassociated NPs concentration calculated by the photometrical method; \(V\) is the volume of the sample detected; \(\alpha\) is the reciprocal of pK for amino groups of albumin (1.5) \(^{49, 50}\); \(h\) is the total number of peptide bonds in BSA (0.0088 mol/g protein).

Cell culture

Human breast cancer MCF-7 cells were cultured in RPMI 1640 medium containing 10% fetal calf serum and 1% antibiotics (penicillin 10,000 units/mL, streptomycin 10,000 µg/mL) at 37 °C and 5% CO2.

Cellular uptake pathway detection

For the uptake inhibition study, MCF-7 cells seeded in 24 well plates were treated with the chemical inhibitors for 0.5h at 37°C. Then the NPs were added to reach a concentration of 37.5µg/mL. After various incubation periods, the effects of the inhibitors on the particle uptake were obtained by both inverted fluorescence microscope observations and quantitative detection. The inhibitors applied in this study included nystatin (25µg/mL) \(^{37}\), and sucrose (0.45M) \(^{51}\), which inhibited the caveolae- and clathrin-mediated pathway of endocytosis, respectively.

Cellular uptake kinetic detection

For the uptake inhibition study, MCF-7 cells seeded in 24 well plates were treated with the chemical inhibitors for 0.5h at 37°C. Then the NPs were added to reach a concentration of 37.5µg/mL. After various incubation periods, the effects of the inhibitors on the particle uptake were obtained by both inverted fluorescence microscope observations and quantitative detection. The inhibitors applied in this study included nystatin (25µg/mL) \(^{37}\), and sucrose (0.45M) \(^{51}\), which inhibited the caveolae- and clathrin-mediated pathway of endocytosis, respectively.

Intracellular degradation of albumin NPs

To quantify the NPs intracellular degradation, cells seeded in 24-well plates were incubated with the NPs diluted in the serum-free medium (37.5µg/mL) for 2h. After incubation, the medium was replaced by the fresh RPMI 1640 medium, and this time point was set as the starting time (0h) for the degradation detection. At different time points (such as 2, 4, 6 and 8h), the medium was removed, and the cells were washed with cold PBS buffer (pH 8.0). After splitting through three freeze-thaw cycles and dilution with PBS buffer to a volume of 3mL, the samples were stored at -20°C until fluorescence detection as mentioned above.

Confocal laser microscope observation

The fluorescence signals of the cells in the channel D and channel A could be recorded using the confocal laser scanning microscope (Leica TCS SP5, Leica, Germany) after fixing the cells with 4% paraformaldehyde. The signals in the channel D and A were excited at 488 nm and 543 nm, respectively.

Conclusions

In this study, an albumin NP platform with controllable crosslinking degrees and diameters were prepared using FITC-BSA and RBITC\(_{0.5}\)-BSA. The cellular uptake and the intracellular fate these albumin NPs were detected quantitatively through a FRET-based method. It was found the albumin NP binding with the cell membrane is rapid and saturated within 1h. The albumin NPs entered MCF-7 cells via the clathrin-mediated endocytosis, which was not affected by the crosslinking degree and diameter of the NPs. However, these NP properties did affect the intracellular uptake of the albumin NPs. The cellular uptake of the albumin NPs increased with the increasing of crosslinking degree (in the range of 42%-92%). The NP number of uptake decreased with the increasing of diameter. However, the cellular uptake increased as diameter increased in terms of NP weight. The albumin NPs cellular degradation behaviour was investigated in the MCF-7 cells. The results indicated that the exocytosis of the NPs was negligible within the 8h. The disassociation rate of the albumin NPs were dependent on the crosslinking degree and diameter of the NPs: with the decreasing of crosslinking degrees and diameters, the disassociation rates increased. The disassociation half-lives of the albumin NPs were found in the range of 35-79h. In MCF-7 cells, the proteins disassociated from the NPs would be hydrolyzed quickly. All these findings provide fundamental information not only for the biological effects and safety of albumin NPs, but also for controlling their properties and intracellular fates which are important for their future drug delivery applications. Furthermore, the FRET-based method developed in this study could provide an applicable tool for the
investigation of NP’s intracellular behaviour.

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Notes and references


