Protein Encapsulation

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A Facile Method To Encapsulate Proteins in Silica Nanoparticles: Encapsulated Green Fluorescent Protein as a Robust Fluorescence Probe**

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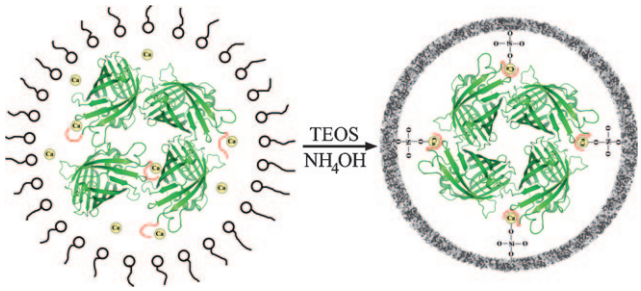
The encapsulation of proteins in biocompatible silica nanoparticles (NPs) has been extensively studied for many applications, such as biosensors, bioreactors, imaging, and drug delivery.[1–12] Unfortunately, a recent study has shown that the encapsulation of a protein in silica NPs is critically dependent on the pI value of the protein.[3] Negatively charged proteins (pI < 7) are difficult to encapsulate and escape easily because of repulsion with the negative charges on the silica NPs. Herein, we report a facile method to encapsulate proteins, including negatively charged proteins. We show that His-tagged enhanced green fluorescent protein (EGFP; His tag = polyhistidine; pI = 5.99) can be easily and stably encapsulated in silica NPs by using the widely used reverse-microemulsion method[7] with a small amount of additional calcium ions. The remarkably improved fluorescence properties and stability make this EGFP-encapsulated silica NP a robust and safe fluorescence probe.

Scheme 1 shows the modified reverse-microemulsion method for encapsulating His-tagged proteins. The added Ca^{2+} ions form ionic bonds with the oxygen atoms on the silica NPs, and provide anchors to link His-tagged proteins to the silica shell through coordinate bonds between the Ca^{2+} ions and the histidine residues of the His-tagged proteins.

In the presence of Ca^{2+} ions, a significant amount of EGFP can be encapsulated in silica NPs. The TEM image of EGFP-encapsulated silica NPs in the presence of Ca^{2+} ions (denoted as EGFP-Ca@SiO_2) shows an apparently hollow center (Figure 1a, and see the Supporting Information). However, in the absence of Ca^{2+} ions, the silica NPs appear as solid spheres (Figure 1b). X-ray diffraction (XRD) analysis indicated that silica NPs, which were prepared either in the presence or absence of Ca^{2+} ions, are amorphous (see the Supporting Information). Elemental analysis shows that there is 4.195% nitrogen in EGFP-Ca@SiO_2, which corresponds to 24.8% EGFP (nitrogen content in EGFP is 16.9%) in the NPs. The calcium content is about 0.4%, as determined by Zeeman atomic absorption spectrometery. The coordinate bonds between the Ca^{2+} ions and the His tags are essential for encapsulating negatively charged proteins in the silica NPs. A control experiment with FITC-labeled (FITC = fluorescein isothiocyanate) bovine serum albumin (BSA; pI = 4.7) without the His tag showed little encapsulation in the silica NPs, even in the presence of Ca^{2+} ions (data not shown). When



Scheme 1. Reverse-microemulsion procedure for encapsulating His-tagged proteins. EGFPs (green) with His tags (red) are anchored to the silica shell through coordinate bonds between Ca^{2+} ions (yellow) and the histidine residues of the His tags. TEOS = tetraethoxysilane.

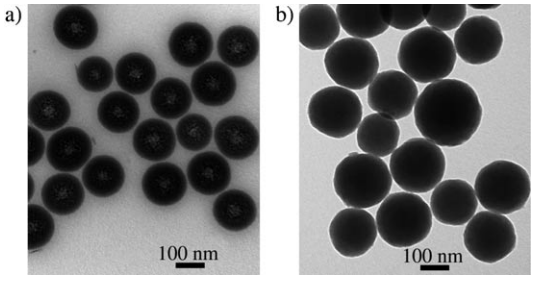


Figure 1. TEM images of EGFP-encapsulated silica NPs that were prepared in the presence (a) or in the absence of Ca^{2+} ions (b).

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EDTA (EDTA = ethylenediaminetetraacetate) was added to chelate the Ca^{2+} ions, the encapsulation of the His-tagged EGFP decreased and eventually became completely inhibited as the concentration of EDTA increased (Figure 2a).

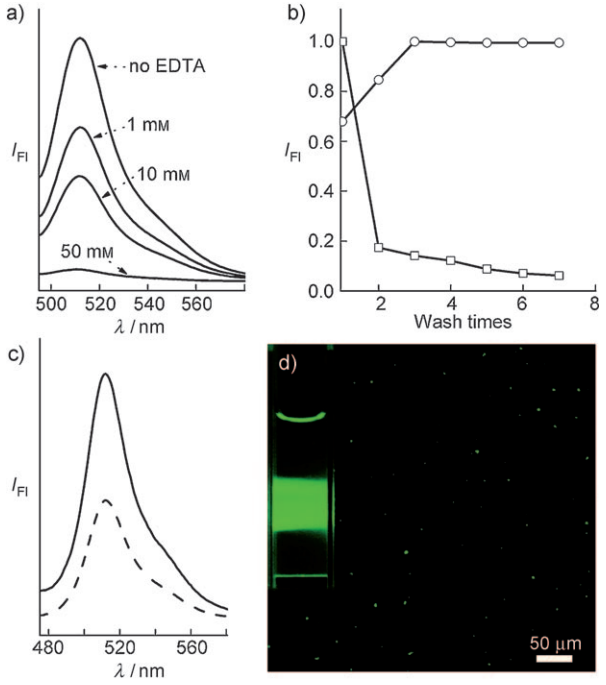

Figure 2. a) Influence of EDTA on the encapsulation of EGFP in silica NPs prepared in the presence of Ca^{2+} ions. b) Leakage of EGFP from silica NPs that were prepared in the presence (○) and absence (□) of Ca^{2+} ions. c) The fluorescence spectra of EGFP-Ca@SiO_2 (solid line) and free EGFP (dashed line) at the EGFP-equivalent concentration of 0.25 mg mL^{-1}. d) Confocal microscopy image of EGFP-Ca@SiO_2, excited by a laser operating at 488 nm. Inset: Photo of an EGFP-Ca@SiO_2 suspension under irradiation at 350 nm (UV irradiation was used to prevent the interference of scattered light on the picture).

The addition of Ca^{2+} ions also prevented leakage of the encapsulated EGFP (Figure 2b). The increase in the relative fluorescence intensity of EGFP-Ca@SiO_2 after the first two washing cycles is due to the slow change in the pH value in the core of the NPs from the reaction pH value of 11.5 to the washing pH value of 7.5, which is consistent with the effect of the pH value on the free EGFP (see the Supporting Information). However, without Ca^{2+} ions, the encapsulated EGFPs quickly leaked out from the silica NPs after a few washing cycles (Figure 2b).

To further test the general applicability of this encapsulation method for His-tagged proteins, His-tagged yellow fluorescent protein (YFP, pI = 5.99), FITC-labeled His-tagged BSA (pI = 4.7), FITC-labeled His tag (FITC-HHHHHH, pI = 7.21; H = histidine), and an FITC-labeled His-tagged aspartic acid and glycine polypeptide (FITC-DDDDDGHHHHHH pI = 5.39; D = aspartic acid, G = glycine) were also stably encapsulated in silica NPs in the presence of Ca^{2+} ions (see the Supporting Information). In all cases, the absence of Ca^{2+} ions during the encapsulation process led to rapid leeching of the encapsulated proteins or peptides from the NPs. His tags are a widely used, powerful, molecular biological technique for protein purification.[13,14] Many industrial and biomedical protein products, such as enzymes and antibodies, are His-tagged proteins. Therefore, our method provides a general, simple encapsulation route to these products.

Encapsulation in the silica can also significantly enhance the fluorescence intensity of the encapsulated EGFP. Figure 2c shows the fluorescence spectra of free EGFP and EGFP-Ca@SiO_2 at the same EGFP concentration. A much higher fluorescence intensity of EGFP-Ca@SiO_2 is observed compared with free EGFP. The confocal fluorescence microscopy image of EGFP-Ca@SiO_2 also shows this strong fluorescence response (Figure 2d), which suggests that these compounds have a promising application in bioimaging. Such fluorescence enhancements have been reported for other NP-encapsulated dyes, which may be a general phenomenon because of the nano-confinement effect and the separation of the fluorophore from the solvents.[3,8]

Moreover, we also observed that encapsulation in silica NPs increased the stability of EGFP against denaturants, protease, and heat. Free EGFP quickly became denatured in 6 M guanidine hydrochloride (GdHCl), within 10 minutes, whereas EGFP-Ca@SiO_2 maintained more than 60% of its fluorescence intensity in the same solution after 20 minutes (Figure 3a). This result suggests the possibility of using EGFP-Ca@SiO_2 as a fluorescence probe in extreme protein-denaturing conditions.

EGFP-Ca@SiO_2 is also stable against protease K digestion. Free EGFP was readily degraded by protease K in 6 M urea at 58 °C, whilst the majority (about 70%) of EGFPs in EGFP-Ca@SiO_2 were not degraded following treatment with the protease (Figure 3b). The slow decline in the I_{FI} value after 20 minutes is mainly due to the partial unfolding of the encapsulated EGFP by the 6 M urea that diffuses into the silica NPs.

Encapsulation in silica also improved the thermal stability of EGFP (see the Supporting Information), which may be due to a similar confinement effect on the thermal stability of RNase A by mesoporous silica NPs.[15] After drying the solid EGFP-Ca@SiO_2 at 40 °C under vacuum it was stored at room temperature for several months. During this time the green color of EGFP-Ca@SiO_2 gradually turned pale, thus indicating the unfolding of the EGFPs. However, the unfolded EGFPs were refolded after resuspension in water, and the green color returned. About 70% of the fluorescence of EGFP-Ca@SiO_2 remained after resuspension in solution following storage for 70 days (see Figure 3c).

GFP and its variants are powerful tools widely used in cell and molecular biology for in vivo imaging and probing because of their efficient bioluminescence.[16–18] However, GFPs are generally used as gene-expression markers.[16,17] The purified GFPs are not suitable for bioimaging because of the unstable nature of the proteins. Here, the encapsulation in silica NPs significantly improves various properties of the EGFP, which suggests that the encapsulated EGFP could act as a robust fluorescence probe. We expect that other GFP

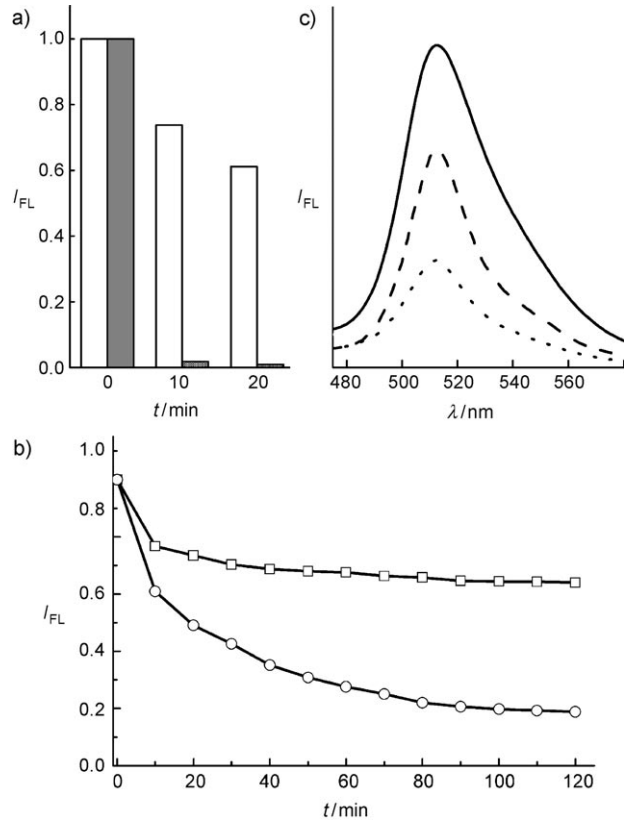

Figure 3. a) Decay in the relative fluorescence intensity (I_{FI}) for free EGFP (filled bar) and silica-NP-encapsulated EGFP (empty bar) in 6 M GdHCl. b) The relative I_{FI} decay for free EGFP (○) and silica-NP-encapsulated EGFP (□) on treatment with protease K. c) The fluorescence spectra of EGFP-Ca@SiO_2 at a EGFP-equivalent concentration of 0.25 mg mL^{-1}: newly prepared (solid line), suspension of dried EGFP-Ca@SiO_2 after storage for 70 days at room temperature (dotted line), the above suspension recorded after 96 h (dashed line).

variants could be encapsulated by the same methods. Furthermore, the potential abundant chemistry of the silica nanoparticle shell provides a variety of opportunities for surface functionalization, which could be used to incorporate additional functional groups, such as therapeutic and biotargeting molecules.[12] Furthermore, this new type of fluorescence probe is safer than the extensively studied quantum dot probes and NP-encapsulated organic/inorganic dyes, which contain toxic metallic elements or molecules.[19–24]

In summary, we have developed a facile general method to encapsulate His-tagged proteins. The key features in this method include taking advantage of the widely used His tag for protein purification and using Ca^{2+} ions to bind the His-tagged protein onto the silica shell. Since the His-tag technique has been widely used for proteomics research and many industrial and biomedical products, we believe that this method could be used to encapsulate many proteins for enhanced industrial and biomedical applications. As an example, the fluorescence intensity and the stabilities of encapsulated EGFP against denaturants, protease, and heat were significantly increased, thus making the EGFP-encapsulated silica NPs potentially robust fluorescence probes.

Experimental Section

Synthesis of EGFP-encapsulated silica NPs: A water-in-oil microemulsion was prepared by mixing cyclohexane (7.50 mL), Triton X-100 (1.77 mL), *n*-hexanol (1.80 mL), EGFP (300 μL) in an aqueous solution of $CaCl_2$ (0.2–2.0 mg mL^{-1}) containing TEOS (100 μL). Then, 25% ammonia (60 μL) was added to initiate the polymerization. After stirring the mixture for 24 hours, acetone (20 mL) was added to break-up the microemulsion and precipitate the EGFP-encapsulated silica NPs. The NPs were obtained by centrifugation at 12000 rpm and washed several times with ethanol and deionized water. Other proteins and peptides were also encapsulated following the same procedure.

Characterization of EGFP-encapsulated silica NPs: X-ray diffraction (XRD) patterns were recorded on a D/MAX-2550 diffractometer (Rigaku, Japan) with a $Cu_{K\alpha}$ radiation source (λ = 1.54178 Å). TEM images were obtained with a JEM 200CX microscope (JEOL, Japan). HRTEM images and EDX spectra were obtaiened with a JEM-2010F microscope (JEOL, Japan). Confocal images were obtained with an FV1000 confocal fluorescence microscope (Olympus, Japan) with an excitation laser operating at 488 nm. The nitrogen content of the NPs was determined with a Vario EL-III elemental analyzer (Elementar, German); the calcium content was determined with a Zeeman atomic absorption spectrometer (Perkin–Elmer 5100PC, USA). Fluorescence spectra were recorded on a U-3010 spectrophotometer (Hitachi, Japan) at 25 °C. See the Supporting Information for more detailed experimental procedures.

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