Chemoselective derivatization of a bionanoparticle by click reaction and ATRP reaction

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Horse spleen apoferritin, the hollow protein shell derived from ferritin, a special biological nanoparticle, can be chemoselectively modified at the lysine residues, which affords a robust scaffold for further chemical reactions including Cu(I)-catalyzed azide–alkyne cycloaddition reaction and atom transfer radical polymerization reaction.

Nature provides many kinds of protein cages in a wide variety of sizes and shapes that are attractive scaffolds for the construction of new materials at nanometer scale.[1] Recent reports have demonstrated that these protein cages can be utilized as templates for constrained nanomaterials synthesis through the addition of new functionality on the exterior and interior surfaces, and through the self-assembly process.[1,2]

Ferritins are a family of iron storage protein spheres found mainly in the liver and spleen, which have attracted many researchers due to their fascinating structural features and biological properties.[1c,3] Ferritin devoid of iron core, providing a cage-like structure, is named as apoferritin. Each horse spleen apoferritin (apo-HSF) cage contains 24 structurally equivalent subunits arranged into a hollow, roughly spherical shell with an inner diameter of 8 nm and an outer diameter of 12.5 nm (Fig. 1).[4] Mann and coworkers developed strategies to mimic the biosynthetic pathway for preparing non-native inorganic nanoparticles such as Fe_3O_4,[5] Mn,[6a] FeS[6b] and CdS[6c] using apo-HSF. Douglas and Stark have used horse spleen ferritin (HSF) as a constrained nanoreactor for the oxidative hydrolysis of Co(II) to achieve site-specific mineralization within the protein cage.[7] In addition, peptide sequences with high specificity towards particular inorganic materials or reactive cysteines have been genetically inserted on apoferritin.[8] The self-assembly of ferritin at the air–liquid or liquid–liquid interface has been studied extensively.[9]

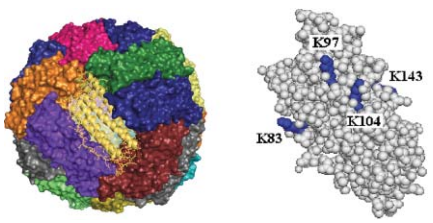

Fig. 1 (*left*) X-ray structural diagram of HSF with subunits presented in different colours. One subunit is represented in ribbon diagram form. (*right*) CPK model of one subunit of HSF with all exposed lysines shown in blue.[4]

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For example, 2-D arrays of ferritins were produced *via* self-assembly at the air–water interface, which was transferred onto a silicon surface. Upon calcination, scanning electron microscopy revealed a well-ordered array of nano iron crystals on the silicon surface that can be used in quantum electronic applications.[3b,10]

As part of our study in hierarchical assemblies of biological nanoparticles,[11] the polymer tailored HSF has been dispersed into diblock copolymer matrix that led to phase segregation and self-assembly upon annealing.[11a] To continue this work, more efficient, versatile, and chemoselective bioconjugation methods are essential for altering the surface properties of HSF. Wetz and Crichton reported a general reactivity screening to target amino, carboxylic acid, and thiol groups on the (apo)ferritin.[12] Mann *et al* have modified lysine and carboxyl groups of apoferritin for the purpose of controlled assembly or surface properties modification.[13] However, the reactive residues were never identified so that it is still unclear about the chemoselectivity of those modifications. In this paper, we report the identification of reactive lysines of horse spleen apoferritin (apo-HSF) using MALDI-TOF analysis. In addition, alkynes and bromoisobutyrates have been attached on the surface of apoferritin, which can be further derivatized by Cu(I) mediated azide–alkyne 1,3-dipolar cycloaddition (CuAAC) and atom transfer radical polymerization (ATRP) reactions.

To probe the reactivity of the amino groups of apo-HSF, 5-carboxyfluorescein (FL) *N*-hydroxysuccinimidyl ester (NHS) **1** was employed. Upon incubation with **1** at pH 7.8 for 24 h, the fluorescently tailored apoferritins were purified by size exclusion chromatography over P-100 resin (BioRad). The stoichiometries of the labeling were determined by measuring the protein concentration using Lowry's assay and the dye concentration by the absorption intensities of dyes (495 nm for FL). Fig. 2a shows a plot of the number of FL motifs attached to apoferritin as a function of increasing concentrations of reagent **1** in the reaction mixture. The curve reached a plateau at approximately 22–24 dyes per particle, suggesting that a single lysine residue per subunit has been modified by FLNHS. The MALDI-MS also confirmed a single FL moiety has been attached per protein subunit (Fig. 2b). The FL-labeled apo-HSF was characterized by sucrose gradient ultra-sedimentation showing a single fluorescent band, indicating that fluorescein tailored apoferritin particles remained intact (Fig. 2c). The integrity of the particles was also confirmed with size-exclusion FPLC, (Fig. 2d) and transmission electron microscopy (TEM) (Fig. 2e).

In order to identify the reactive lysines, the coat protein of apoferritin and FL-labeled apoferritin were digested with trypsin. MALDI-TOF MS of the tryptic digests revealed three residues, *i.e.* K83, K97 and K143 were modified, which was further confirmed

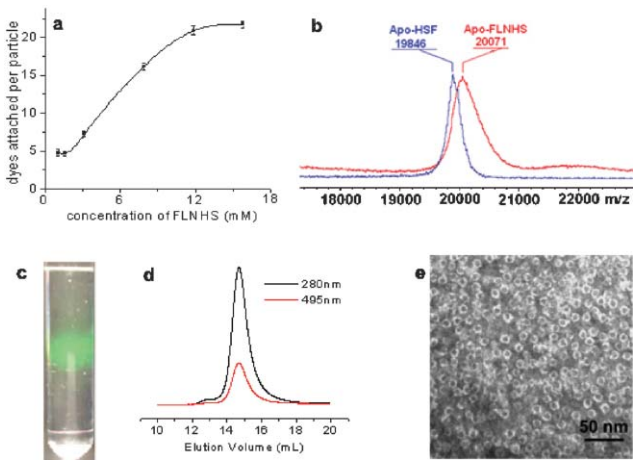
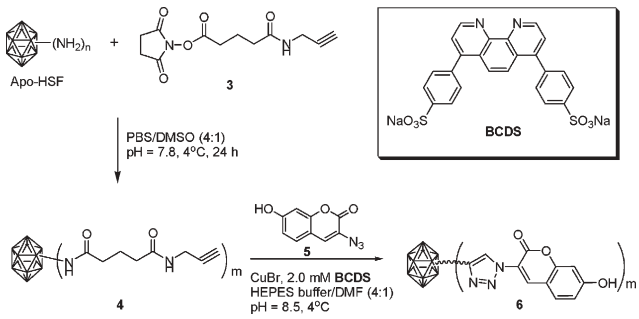


Fig. 2 (a) UV-vis analysis of covalently attached FL to apo-HSF as a function of increasing concentration of reagent FLNHS. (b) MALDI-MS spectra of native and FL modified apo-HSF, showing a *ca.* 225 Dalton molecular weight increase for a single FL unit (theoretical MS for apo-HSF-FL: 20204 *m*/*z*). (c) Ultra-sedimentation analysis of FL-labelled apo-HSF through sucrose gradient column. (d) Size-exclusion chromatogram of apo-HSF-FL conjugate showed a single peak with strong absorption at 495 nm, indicating the integrity of the particles and the dye attachment. (e) TEM image of apo-HSF-FL.

by V8 protease digestion results. All three lysines are highly exposed to the exterior surface (Fig. 1). Using trypsin and V8 protease digestion, more than 80% of the peptide sequences of apo-HSF were identified in the MALDI-TOF MS analyses. The proteomics result was consistent with the literature report[12] as well as the conventional wisdom that their accessibility often modulates the reactivity of amino acids on the surface of biological particles.[14] When one of the residues among K83, K97 and K143 is derivatized with FL, which brings in considerable steric hindrance, it is nearly impossible for FL-NHS to attack other residues. Therefore, only one FL can be attached per protein subunit. When much smaller reagents, acetyl NHS ester **2** and alkyne reagent **3**, were employed in the reaction, trypsin and V8 digestions and sequential MS analyses indicated that four lysines of apo-HSF, *i.e.* K83, K97, K104 and K143, were derivatized. MALDI-MS spectra of the undigested protein showed four acetyl groups or four alkyne moieties were attached to each subunit.

These results suggested that it is possible to selectively functionalize lysines side chains with NHS ester reagents. To further explore the potential of post-functionalization of apo-HSF, CuAAC reaction was pursued, which was recently developed by Fokin and Sharpless and termed as one kind of "click reaction",[15] and used in a broad list of bioconjugative applications including the functionalization of bionanoparticles.[16] As shown in Scheme 1, the alkyne derivatized apoferritin **4** was synthesized by reaction with **3**. A recently reported 3-azidocoumarin derivative **5** was used as the azide reagent. The reaction can be easily monitored without complicated separation steps due to its fluorogenic characteristic.[17] Initial attempts using $CuSO_4$–ascorbic acid or $CuSO_4$–phosphine as catalyst systems were unsuccessful. Apoferritin was either aggregated or denatured by introducing the reducing reagents. Thus, as an alternative protocol developed by Finn and coworkers, a Cu(I) source and bathophenanthroline ligand (**BCDS**),[16b] which has shown high efficiency for the derivatization of biological



Scheme 1

particles, was examined. After stirring a suspension of CuBr (99.999% in purity) in a solution of **4**, **BCDS** and **5** (3.0 mg mL^{-1}) for 24 h (Scheme 1), fluorescence spectra of a diluted sample of the reaction mixture showed a strong fluorescent emission at 474 nm upon exciting at 340 nm. A control sample of a mixture of **5** and **4** without catalysts showed little fluorescence (Fig. 3a). These results clearly indicated the formation of **6** under the catalysis of CuBr and **BCDS**.

Upon purification by size exclusion chromatography, the UV-vis spectrum of conjugate **6** gave absorption peaks of protein at 280 nm and coumarin at 340 nm (Fig. 3b). The intact nature of derivatized particles was verified by size-exclusion FPLC as well as TEM (data not shown). As shown in Fig. 3c and 3d, the formation of triazolylcoumarins on apoferritin was confirmed by mass spectroscopic analyses of the V8 protease digests. Although all three lysines were accessed by the reactions, only one coumarin-triazole per subunit was detected by the whole protein MALDI-MS analysis, which could also result from steric effects. In addition, quantification with the fluorescent signal arising from the newly formed triazolylcoumarin revealed about 20 courmarin-triazoles formed per apoferritin particle, *i.e.* about one per protein subunit.

The reactive amino groups of lysine residues were analogously derivatized to tertiary bromide with NHS ester **7** (Scheme 2). The

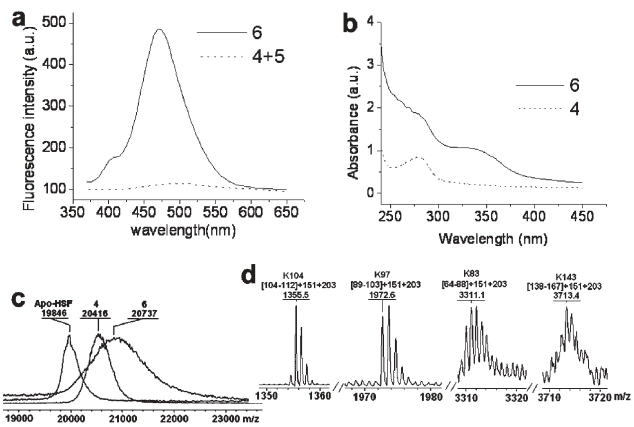
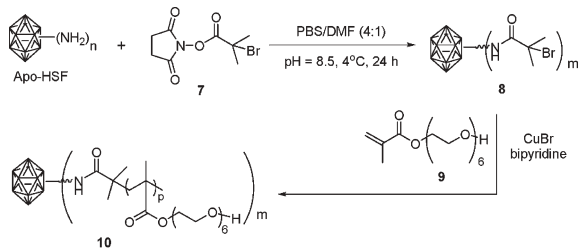


Fig. 3 (a) Fluorescence spectra of coumarin functionalized apoferritin **6** and the mixture (without reaction) of **4** and **5** . (b) UV-vis spectra of **4** and **6**, the latter showed a distinctive peak at 340 nm that belonged to the coumarin. (c) MALDI MS of **4** and **6**, showing a 570 and a 891 Da increase of molecular weight compared with the original apo-HSF, indicating the attachment of about three alkyne units in **4** and about one coumarin in **6**. Due to the incomplete click reaction, the MS peak of **6** is substantially broader than apo-HSF and **4**. (d) V8 digests of **6** showed four reactive sites: K97, K83, K104 and K143.



Scheme 2

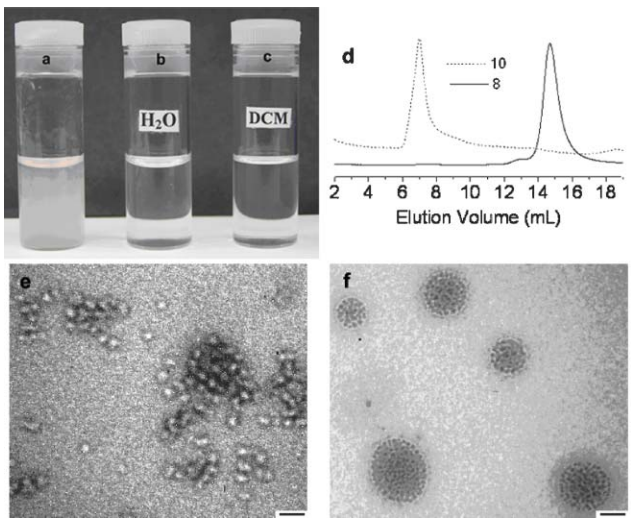


Fig. 4 Polymer tailored apo-HSF **10** appeared as a hydrogel (a), which could be redissolved in water (b) and DCM (c). (d) Size exclusion FPLC analysis of **10** in comparison with bromide modified apo-HSF **8**. (e, f) are TEM images of solutions (b) and (c).

resulting particle **8** acted as a macro-initiator to graft polymers on the surface of apoferritin, which allowed us to perform ATRP reactions using a similar protocol to that developed by Maynard and coworkers.[18] In our study, water soluble PEG methacrylate **9** was employed as the monomer, and CuBr–bipyridine complex was used as a catalyst to promote the ATRP reaction. The concentration of macro-initiator in our case was high enough so that non "sacrificial initiator" was necessary for the radical polymerization reaction.[19] A hydrogel was formed when the molar ratio of compound **8** : **9** was 1 : 1800. This could be redissolved with a large quantity of water or dichloromethane (DCM), revealing the amphiphilic properties of the graft polymers (Fig. 4a–c). Since both the original apoferritin and **8** can only be dissolved in aqueous solution, this demonstrated the possibility of altering the surface affinities of bioparticles *via in situ* polymerization. Upon dialysis over water, FPLC analysis of purified **10** showed an elution peak at 7 mL, which was the void volume of the column, indicating a substantial increase in the size of the particles (Fig. 4d). TEM showed that the polymer grafted apoferritin, **10**, formed clusters in DCM as well as in water (Fig. 4e–f). The diameter of the resulting particles was about 25 nm. The aggregation of particles may result from the non-covalent interactions of the polymer chains. No hydrogel was formed in a control experiment in which underivatized apo-HSF was exposed to identical polymerization conditions.

In conclusion, we have demonstrated that the chemoselective modification of apo-HSF can be achieved *via* acylation of lysine residues with NHS ester reagents. Furthermore, post-functionalization of apo-HSF can be realized by a CuAAC reaction, and an *in situ* ATRP reaction on the outer surface of apoferritin. These transformations afford versatile methods to alter the properties of apoferritin particles and can be extended to other bionanoparticles.

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19 Typical polymerization protocol: In a N_2 atmosphere, **8** (14.6 mg, 3.3×10^{-5} mmol) and **9** (518.4 mg, 1.44 mmol) were dissolved in degassed water (200 uL). 400 uL of a catalyst stock solution (obtained by mixing 14.3 mg CuBr, 27.5 mg 2,2′-bipyridine in 4 mL degassed water) was added to start the polymerization. The reaction proceeded under nitrogen for 90 min before the mixture was exposed to air and diluted with 200 uL of water to quench the reaction. The product was purified by dialysis against nanopure water.