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Preliminary Communication

Characterization of horse spleen apoferritin reactive lysines by MALDI-TOF mass spectrometry combined with enzymatic digestion

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ABSTRACT

Ferritins are a class of iron storage protein spheres found mainly in the liver and spleen, which have attracted many research interests due to their unique structural features and biological properties. Recently, ferritin and apoferritin (ferritin devoid of the iron core), have been employed as chemically addressable nanoscale building blocks for functional materials development. However, the reactive residues of apoferritin or ferritin have never been specified and it is still unclear about the chemoselectivity of apoferritin towards different kinds of bioconjugation reagents. In this work, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry combined with enzymatic digestion analysis was used to identify the reactive lysine residues of horse spleen apoferritin when conjugated with *N*-hydroxysuccinimide reagents. The result demonstrated that among all the lysine residues, K97, K83, K104, K67 and K143 are the reactive ones that can be addressed.

1. Introduction

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 [1–6]. Recent reports have demonstrated that these protein cages can be utilized as templates for constrained nanomaterial synthesis through the addition of new functionality on the exterior or interior surfaces, and through the self-assembly processes [1–4,7,8]. Therefore, the characterization of chemoaddressable residues of protein cages is critical for the development of more efficient, versatile and chemoselective bioconjugation methods. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [9,10], electrospray ionization time-of-flight MS (ESI-MS) [11] and tandem MS [12], have been broadly used in the determination of amino acid sequences and the elucidation of the modified structures of peptides and proteins. In literature, MS techniques have been used to determine the modification sites of various protein cages like viral proteins. For example, Siuzdak and coworkers reported the usage of mass spectrometry in analyzing the mass, structure and viability of viral particles [13–16]. MS was also employed to identify the reactive residues of cowpea mosaic virus [17], tobacco mosaic virus [18], turnip yellow mosaic virus [19,20] and bacteriophage MS-2 [21].

Ferritins are a family of iron storage protein spheres found mainly in the liver and spleen, which have attracted many research interests due to their fascinating structural features and biological properties [3,22,23]. Ferritin devoid of the iron core, providing a cage-like structure, is named as apoferritin. Horse spleen apoferritin (apo-HSF) cage contains 24 subunits arranged with 432 point symmetry (a dodecahedron). It is a hollow, roughly spherical shell with the inner diameter of 8 nm and the outer diameter of 12.5 nm (Fig. 1). The 24 subunits in the apoferritin complex are made up of 80–90% of L- (light chain) and 10–20% of H-chain (heavy chain) subunits, which share 55% sequence homology. The most striking differences between the L-chain and H-chain are found on the outer surface, in the cavity, and on the hydrophobic channel sequences; whereas the hydrophilic channel sequence is identical [24,25]. Electron density maps show clearly that each subunit consists of a bundle of four long helices lying parallel or anti-parallel to one another, together with a much shorter helix which lies perpendicular to the bundle and a loop on the outer surface [26–28]. According to the structural information shown in Fig. 1 (right), regions of secondary structure of L-chain are as follows: residues 1–9, *N*-terminal loop; residues 10–39, A helix; residues 40–44, AB turn; residues 45–72, B helix; residues 73–91, L loop; residues 92–120, C helix; residues 121–123, CD turn; residues 124–155, D helix; residues 156–159, DE turn; residues 160–169, E helix; and residues 170–174, C-terminal tail. There are nine Lysine residues on each L-chain subunit: K58, K67, K83, K97, K104, K139, K142,

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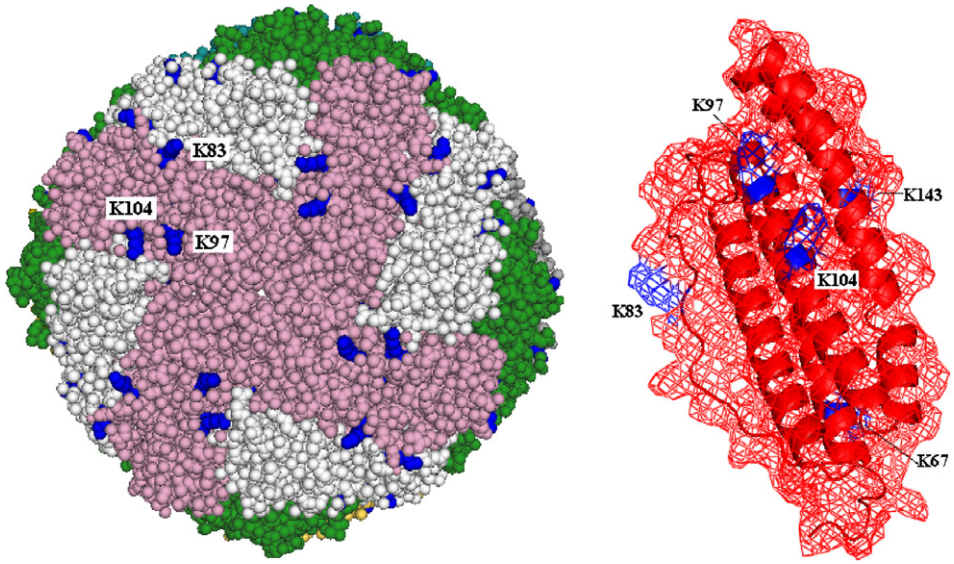


Fig. 1. (left) Structural model of HSF, with four subunits around the 4-fold axis highlighted in pink, exposed lysine residues highlighted in blue. (right) Structural model of one subunit of HSF with reactive lysines shown in blue color. It consists of a bundle of four long helices lying parallel or anti-parallel to one another, together with a much shorter helix which lies perpendicular to the bundle and a loop of extended chain on the outer surface of the cages [26–28]. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

K143 and K172 [26–28]. Among them, K97, K83 and K104 are exposed to the surface.

Apoferritin can be employed as a nanoscale building block for new materials development. For example, Mann and coworkers developed synthetic routes, which mimic the biosynthetic pathway, to generate non-native inorganic nanoparticles such as Fe_3O_4 [29,30], Mn [29], FeS [3] and CdS [31] using apo-HSF. Douglas and Stark have used apo-HSF as a constrained nano-size reactor for the oxidative hydrolysis of Co(I) to achieve site-specific mineralization within the protein cage [32]. In addition, peptide sequences with high specificity towards particular inorganic materials or reactive cysteines have been genetically inserted on apoferritin [33,34]. Moreover, the self-assembly of ferritin at the air-liquid or liquid–liquid interface has been studied extensively [35–40]. For example, 2D arrays of ferritins were produced via self-assembly at the air-water interface, which was then transferred on to a silicon surface. Upon calcination, scanning electron microscopy revealed a well-ordered array of nanosize iron crystals on the silicon surface, which can potentially be used in quantum electronic applications [41,42].

As part of our initial study in hierarchical assemblies of biological nanoparticles, polymer tailored HSF dispersed into diblock copolymer matrix led to phase segregation and self-assembly upon annealing [8,43]. To continue this work, more efficient, versatile and chemoselective bioconjugation methods are essential for altering the surface properties of HSF. In literature, Wetz and Crichton reported a general reactivity screening that targeted amino, carboxylate and thiol groups on apo-HSF [44]. Mann and coworkers modified lysine and carboxyl groups of apo-HSF to control the assembly or to modulate the surface lipophilicity [45–47]. However, in previous studies the reactive residues have never been identified. Our preliminary results demonstrated that apo-HSF can be employed as a robust scaffold for a variety of chemical reactions [48]. In this paper, we report the use of MALDI-TOF MS techniques, in combination of enzymatic digestions, to identify the reactivity and selectivity of the lysine residue of apo-HSF. The conventional *N*-hydroxysuccinimde (NHS)-mediated amidation reaction, which is selective for reaction with amino groups of proteins, was utilized for the chemical modification of the lysine groups. Two succinimide derivatives, i.e. fluorescein NHS ester **1** and 5-(propargylamino)-5-oxopentanoic acid NHS ester **2**, were chosen in our study to screen the reactivity of apo-HSF. The alkyne modified apoferritin was further derivatized using Cu(I) catalyzed 1,3-dipolar cycloaddition reactions [49–52].

2. Experimental

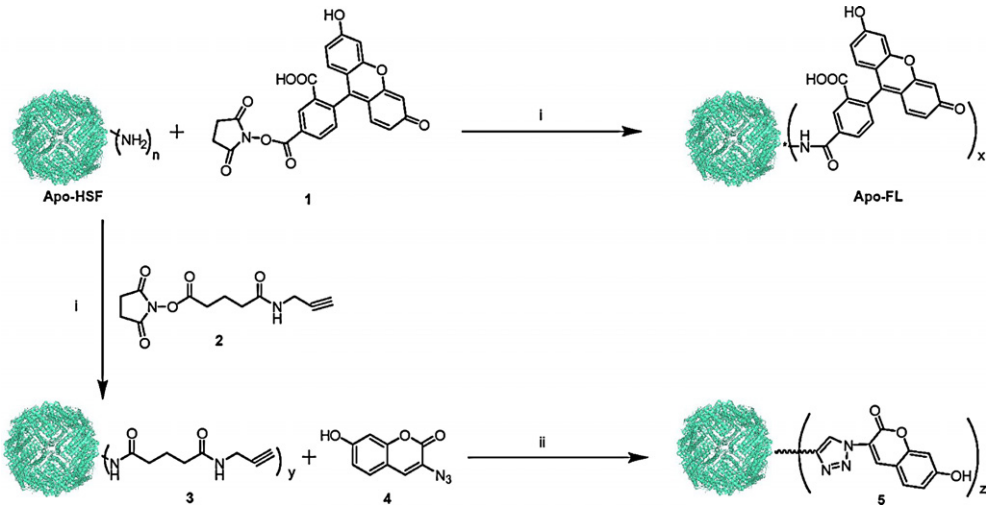
2.1. Chemicals and materials

α-Cyano-4-hydroxycinammic acid (CHCA) and sinapic acid were obtained from Fluka (St. Louis, MO). Sequencing Grade Modified Trypsin was purchased from Promega (Madison, WI). Endoproteinase Glu-C from *Staphylococcus aureus* V8 was from Sigma (St. Louis, MO). Ziptip Pipette Tips were obtained from Millipore (Billerica, MA). Stock solution of ferritin was purchased from Sigma stored at 4 °C. Dye reagent fluorescein-NHS **1**, was purchased from Molecular Probes; and 5-(propargylamino)-5-oxopentanoic acid NHS ester **2** was synthesized via ester chemistry [50,53].

2.2. Horse spleen apoferritin lysine residues bioconjugate with succinimide derivatives

Before covalent modification, the iron core of native horse spleen ferritin was removed by reductive dissolution of the mineral phase under anaerobic conditions according to the method mentioned by Stephen Mann et al. to give apo-HSF [47]. To probe the reactivity of apo-HSF lysine residues, 5-carboxyfluorescein *N*-hydroxysuccinimidyl ester (FL-NHS) **1** and NHS alkyne **2** were employed (Scheme 1). Upon incubation with 200-fold excess of **1** or **2** (to an apoferritin subunit) in a solution of pH 7.8 K-phosphate buffer and DMSO (4:1) for 24 h, apo-HSF was derivatized to give **apo-FL** or **3**, which were purified by size exclusion chromatography over P-100 resin (Bio-Rad).

To further explore the potential of post-functionalization of apo-HSF, the Cu(I) catalyzed 1,3-dipolar cycloaddition reaction of alkyne and azide was performed using the alkyne derivatized apoferritin **3**, 200-fold excess (to each apoferritin subunit) of the 3-azido-7-hydroxycoumarin **4** [54] under the catalysis of CuBr and BCDS (Scheme 1). Triazolylcoumarin derivatized apoferritin **5** were purified by size exclusion chromatography over P-100 resin.



Scheme 1. Bioconjuagtion reaction on apoferritin lysine residues (i) PBS/DMSO = 4/1, pH 7.8, 4 °C, 24 h; (ii) CuBr, 2 mM BCDS, HEPES buffer/DMF = 4/1, pH 8.5, 4 °C. BCDS, Bathocuproniedisulfonic acid disodium salt hydrate).

2.3. Proteolytic digestion

Apo-HSF and derivatives were denatured in a solution of 6.0 M urea and 10 mM DTT, and then digested in 0.1 M Tris Buffer with either trypsin or V8 protease for 12 h at 37 °C. Digestion was stopped by adding concentrated acetic acid to adjust the pH of solution to <6.0. The samples were concentrated using C18 Zip-Tips and eluted with 50% acetonitrile/0.1% trifluroacetic acid following the manufactures protocol.

2.4. Mass spectrometry

MALDI-TOF MS analysis were conducted using a Bruker Ultra-Flex II (Berman, Germany) equipped with solid state smart beam. In linear mode, the instrument was calibrated with a protein mixture consisting of: Ubiquitin, Cytochrome C, and Myoglobin. In reflection mode, the instrument was calibrated with peptide calibration standard II (Bruker).

The purified apoferritin subunit was spotted by the dried droplet method using a 0.75 μL of a saturated sinapinic acid matrix in 50% acetonitrile/0.1% TFA and 0.75 μL of the purified protein samples. The samples were run in linear mode with an accelerating voltage of 25 kV. The protein subunit mass data indicated in this paper are all average masses.

The Zip-Tips-purified peptide digests were spotted with a saturated CHCA or sinapinic acid (50% Acetonitrile/0.1% TFA) matrix and run in reflection mode at an accelerating voltage of 25 kV. The peptide mass data indicated in this paper are all average masses. The peptide mass data indicated in this paper are all monoisotopic masses.

3. Results and discussion

3.1. Subunit analysis by MALDI-TOF MS

To determine the molecular weight of the apo-HSF subunit and modified subunits MALDI-TOF MS was used. The study was focused on the L-chain since it is the major components of apo-HSF and there is only the L-chain structure information of HSF available from the PDB database. The MALDI-TOF MS of the apo-HSF subunit was significantly broader than expected, which could result from the sample being an undetermined mixture of 80–90% L-chain and 10–20% H-chain. However, there is no way to differentiate them, or separate them using MALDI MS. The mass of the broadened apo-HSF subunit peak corresponds to the molecular weight of L-chain, i.e. 19,846 Da (Fig. 2A). Upon incubation with 200-fold excess of FL-NHS, the mass of the modified protein **apo-FL** was 20,071 Da (Fig. 2B). If all subunits were modified, an increase of 358 Da would be expected; however there was only an increase of 255 Da as shown here. This suggests an incomplete modification, where only a portion of the subunits were derivatized by the FL-moiety. This result is consistent with the reaction stoichiometry determined by measuring the protein concentration using Lowry's assay and the dye concentration by the absorption intensities of the dye (495 nm for FL) reported in our previous study [48].

When apo-HSF was modified with the NHS alkyne reagent **2**, the mass of each subunit of **3** was 20,416 Da determined by MALDI-TOF MS, an increase of 570 Da (Fig. 2C). This suggests three or four alkyne moieties were attached to each apo-HSF subunit since the molecular weight of the alkyne molecule is 151 Da. The much higher reactivity of the alkyne molecule might be attributed to the smaller size, as compared to the larger FL-NHS moiety, allowing an easier access to the reactive lysine residues on the protein.

Upon subsequent derivatization of the alkyne groups with azidocoumarin **4**, the molecular weight was found to be 20,737 Da (protein **5**, Fig. 2D), an increase of 321 Da. This suggests the attachment of one coumarin unit per apoferritin subunit via the formation of triazole rings. Considering there were four alkyne units per subunit, the incomplete conjugation of alkynes may be due to the steric hindrance caused by the attachment of the first coumarin units, which prevents the further cycloaddition reaction. Since the fluorescence of 3-azido-7-hydroxycoumarin **4** can be triggered by the formation of the triazole ring upon conjugation, [54] the bioconjugation efficiencies on apo-HSF after the cycloaddition reaction can be reported by the fluorescent emission assays. The fluorescence of the triazolylcoumarin-apo-HSF **5** revealed ~20 courmarin-triazoles formed per apo-HSF particle, which was consistent with the result of the MALDI analysis [48].

3.2. Tryptic digestion and analysis by MALDI-TOF MS

Native apo-HSF and derivatives were subjected to proteolytic digestion using both trypsin and V8 protease to determine the reactive lysines. To identify a suitable matrix compound for MALDI MS experiments, α-cyano-4-hydroxycinnamic acid was used first. However, no peptides over 3000 Da were observed. Upon switch-

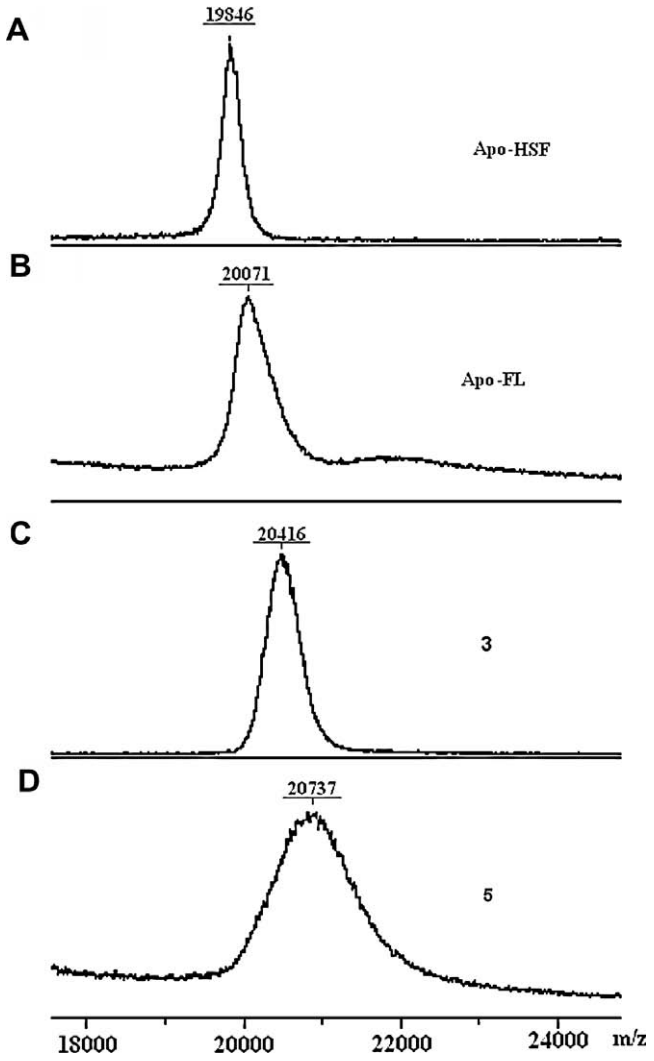


Fig. 2. Subunit analysis of apo-HSF and its derivatives by MALDI-TOF MS. (A) Native unmodified apo-HSF, (B) **apo-FL**, (C) alkyne derivatized apoferritin **3** and (D) triazolylcoumarin derivatized apoferritin **5**. Due to the incomplete click reaction, the MS peak in (D) is substantially broader than in (A) and (C).

ing to sinapinic acid, the larger peptides could be observed although the resolution of the spectra was decreased. Therefore, in all MALDI MS experiments reported in this paper, sinapinic acid was used as the matrix.

In solution digestion of apo-HSF and its derivatives were carried out using proteomics grade trypsin, which can cleave the lysine and arginine residues as summarized in supporting information. In our experiments, about 82% sequence coverage was obtained by trypsin digestion (Fig. 3A and supporting information). If a lysine has been modified, trypsin can no longer cut its C-terminus. In order to identify the reactive residue, a missed cleavage at lysine must be observed in addition to the mass of newly tailored functionality.

Upon digestion of **Apo-FL**, three lysines residues, i.e. K83, K97 and K143, showed the observable modification as comparison with the digestion of unmodified apo-HSF. For example, Fig. 3B. shows a peak at *m/z* 3604.6, not found in the control, which corresponds to K97 modification caused by a missed cleavage at K97 plus the addition of 358.06 Da ([76–104] + 358 Da). Similarly, the peak at *m/z* 2880.3 corresponding to K83 bioconjugation was also observed ([76–97] + 358 Da). Furthermore, a peak at *m/z* 1670.7 which correspond to K143 modification ([143–153] + 358 Da) was also observed, which was not seen in the unmodified sample.

Tryptic digestion of alkyne derivatized apoferritin **3** gave very similar results (Fig. 3C). Peptides were identified at *m/z* 2673.3 and *m/z* 3397.7, corresponding to modification at K83 ([76–97] + 151 Da) and K97 ([76–104] + 151 Da), respectively. The peak corresponding to K143 modification (*m/z* 1463.7) was overlapped with an unknown peptide peak of the unmodified apo-HSF. Nevertheless, the enhanced relative intensity of the peak of *m/z* 1463.7 suggests the possible modification (Fig. 3C). The sequential cycloaddition product **5** showed a consistent digestion pattern with a further mass increment of 203 Da, corresponding to the addition of the coumarin moiety (Fig. 3D). Fig. 4 shows the amino acid sequence of the L-chain of apo-HSF, which outlines the impact of lysine modification on the trypsin digestion.

3.3. V8 protease digestion and analysis by MALDI-TOF MS

In order to confirm the trypsin digestion result, another protease, V8, was employed to perform digestion of apo-HSF and its derivatives. As shown in Fig. 5A and Supporting information, the MALDI MS of the V8 digestion solution cover ~87% of the L-chain

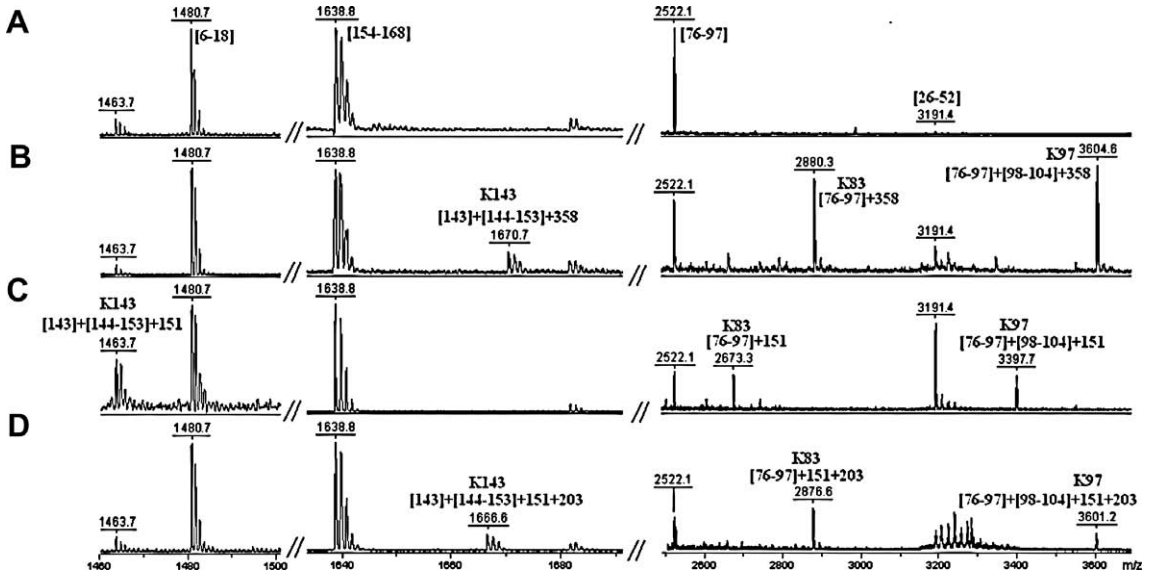


Fig. 3. MALDI-TOF spectra of in solution trypsin digestion of (A) apo-HSF; (B) **apo-FL**; (C) alkyne derivatized apoferritin **3**; and (D) triazolylcoumarin-derivatized apoferritin **5**.

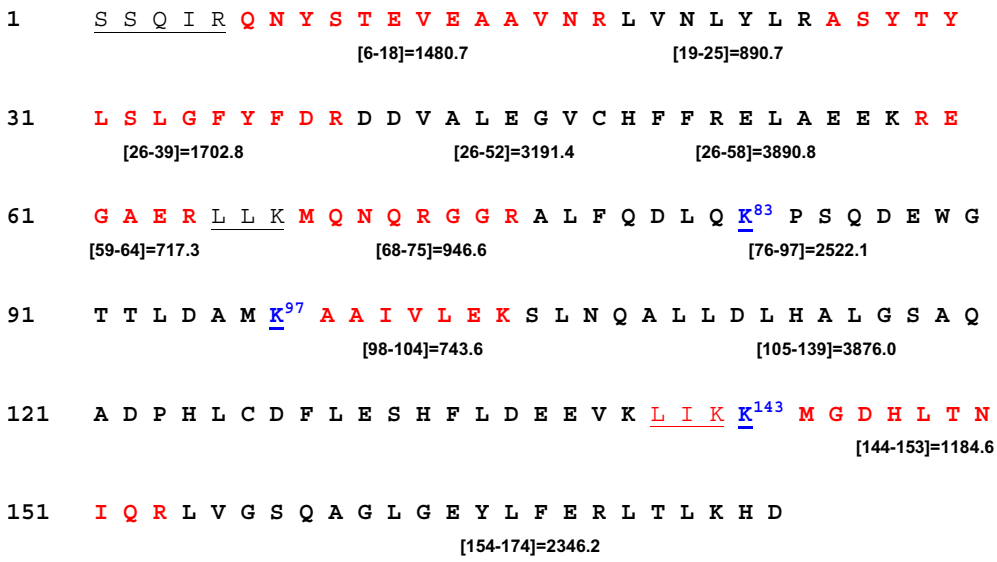


Fig. 4. Amino acid sequence of the L-chain of apo-HSF. The experimental MS data of peptide fragments of the in solution trypsin digestion of unmodified apo-HSF are listed. K83, K97 and K143 are the three reactive lysines that can be determined by tryptic digestion. The other underlined sequences are fragments not shown in MS spectra.

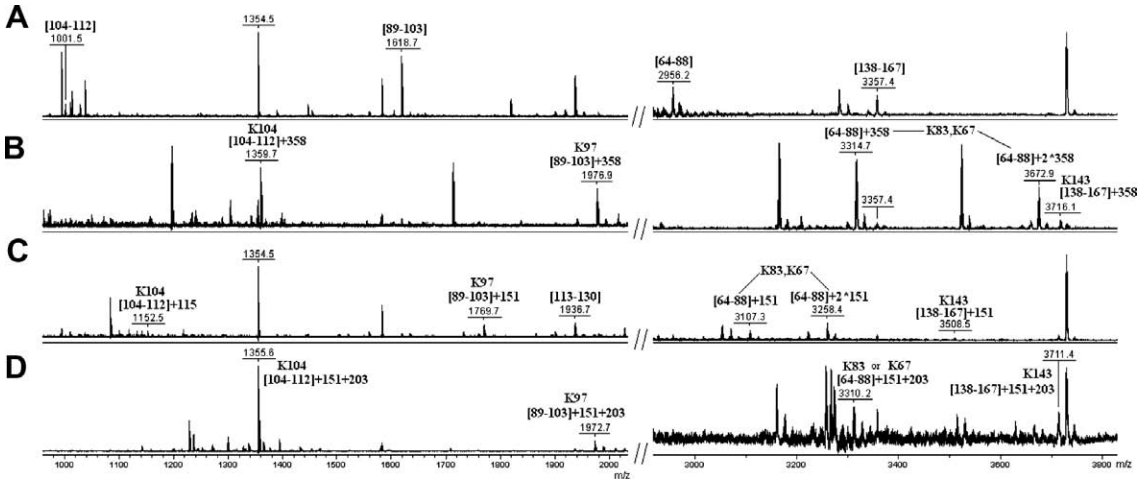


Fig. 5. MALDI-TOF spectra of in solution V8 digestion of (A) apo-HSF; (B) apo-FL; (C) alkyne derivatized apoferritin 3; and (D) triazolylcoumarin derivatized apoferritin 5.

of apo-HSF. Among all peptides, fragments at *m/z* 1618.7 ([89–103]), 2956.2 ([64–88]), 1001.5 ([104–112]) and 3357.4 ([138–167]) are corresponding to lysine residues K97, K67, K83, K104 and K143. Fig. 5B shows new peaks at *m/z* 1359.7, 1976.9, 3314.7, 3672.9 and 3716.1, which are corresponding to the FL derivatization of lysine residues K104, K97, K83, K67 and K143, respectively. Similarly, the MS spectra of V8 digestions of alkyne derivatized **3** spectra (Fig. 5C) show peaks at *m/z* 1152.5, 1769.7, 3107.3, 3258.4 and 3508.5 were observed, which indicate the modification at K104, K97, K83, K67 and K143. The MALDI spectra of the V8 digestion of **5** further confirmed above analysis (Fig. 5D).

Combined with the trypsin and V8 digestion and MALDI MS results in Figs. 3 and 5 we consider K97, K83, K104, K67 and K143 of apo-HSF (L-chain) are the accessible lysine residues to be derivatized by NHS reagents. The proteomics result is consistent with the literature report [44] as well as the conventional wisdom that accessibility often modulates the reactivity of amino acids on the surface of biological particles [55].

4. Conclusions

From the investigation of apoferritin lysine residues coupling reaction with succinimide derivatives and post-functionalization of apo-HSF by a Cu(I) catalyzed 1,3-dipolar cycloaddition reaction, the reactivity of apo-HSF lysine residues was evaluated with protease digestion and MALDI MS. Using FL-NHS **1**, a molecule with relative higher steric hindrance, in average only one lysine per apo-HSF subunit could be conjugated. When a smaller NHS reagent **2** was used, up to four alkyne moieties could be attached to each apo-HSF subunit. The products of apo-HSF modification reactions were subjected to trypsin and V8 digestions, and the digested fragments were identified by MALDI-TOF MS analysis: K83, K97, K104, K67 and K143 were shown to be reactive. Our study demonstrated that the combination of trypsin/V8 digestion with MALDI MS analysis is an effective method to study the reactivity and regioselectivity of biological nanoparticles towards chemical modifications.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2008.06.001.

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