Available online at www.sciencedirect.com
ScienceDirect
Polymer 48 (2007) 3632–3640
polymer
www.elsevier.com/locate/polymer

Surface modification of protein nanocontainers and their self-directing character in polymer blends

Merih Sengonul [a], Josef Ruzicka [b], Athula B. Attygalle [b], Matthew Libera [a,*]

[a] *Department of Chemical, Biomedical, and Materials Engineering, Stevens Institute of Technology, Hoboken, NJ 07030, USA*
[b] *Department of Chemistry and Chemical Biology, Stevens Institute of Technology, Hoboken, NJ 07030, USA*

Received 17 November 2006; received in revised form 4 April 2007; accepted 5 April 2007
Available online 19 April 2007

Abstract

Tailoring the surfaces of a nanocontainer with polymer brushes that have different affinities to the components of a phase-separating polymer blend should impart self-directing properties to the nanocontainers. Such nanocontainers could then be used to deliver a variety of functional species in tunable amounts and in a site-specific manner to polymer systems. This paper describes the surface modification, subsequent characterization of nanocontainers derived from ferritin, and the effects of surface modification on their self-directing properties in a binary phase-separating homopolymer blend. Wild ferritin was either PEGylated or alkylated by zero-length cross-linking to its surface carboxylate groups that were activated by carbodiimide. Modification was confirmed by ion-exchange chromatography, ζ-potential measurement, and electrospray ionization mass spectrometry. FT-IR spectrometry was used to quantify the extent of PEGylation by ratioing the intensity of the C–O–C asymmetric stretching vibration from the grafted PEG to that of the carbonyl stretching vibration (amide I band) from the protein. Importantly, modified ferritin was soluble in the organic solvent dichloromethane (DCM). Modified ferritin was introduced into a polymer blend of hydrophobic and hydrophilic polymers made up of poly(desaminotyrosyl tyrosine dodecyl ester carbonate) (PDTD) and PEG by solvent casting from solution in the common solvent DCM. Polymer thin films with an average thickness of ~200 μm were obtained upon evaporation of the solvent. Transmission electron micrographs of microtomed polymer films demonstrated remarkable selectivity of PEGylated ferritin to PEG domains, while alkylated ferritin self-directs to the PDTD matrix.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Polymer blend; Nanocontainer; PEGylation

1. Introduction

Many new polymeric materials with functional properties have historically been developed either by chemically or physically modifying existing polymer systems. For example, copolymerization of a passive polymer with a functional group or grafting of a functional molecule to a polymer backbone are common chemical modification methods [1]. Alternatively, blending functional species, such as bioactive peptides or colloidal particles with nano to micro-dimensions, in a host polymer are physical approaches for introducing functionality [2]. However, in addition to bringing desirable new properties and capabilities, these and related techniques can simultaneously affect the base polymer morphology, thermodynamic compatibility, crystallization behavior, glass transition temperature and melt viscosity. Consequently, time and resources must then often be invested to re-optimize polymer processing protocols and re-characterize the properties of the modified polymer.

Importantly, nanocontainers can separate the interactions between the container surface and the surrounding polymer medium from the functionality brought by the container interior. Changes to the container contents to impart different properties or functions can be made without altering the interactions between the container surface and the surrounding polymer. Modifications to sets of polymers can thus be made without the need to re-characterize many important properties. Furthermore, controlling the nanocontainer solubility within a

* Corresponding author. Tel.: +1 201 216 5259; fax: +1 201 216 8306.
E-mail address: mlibera@stevens.edu (M. Libera).

0032-3861/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.polymer.2007.04.017

particular phase of a multiphase polymeric solvent can enable the nanocontainer to self-direct so that new functionality is selectively brought to one phase rather than to the entire polymer. For example, nanocontainers loaded with therapeutic agents can in principle be designed to self-direct to different phases in a blend or copolymer to control multistage drug release.

Nanoscale core-shell structures are currently the focus of substantial research, especially in the pharmaceutical industry where they can host therapeutic peptides in drug delivery and carry contrast agents in diagnostic imaging, among other applications [3,4]. However, micelles and vesicles formed from amphiphiles present certain intrinsic problems when considered for use as additives in polymers. They are, for example largely restricted for use in aqueous environments, and they have relatively weak structural stability due to the non-covalent interactions responsible for their formation [5]. A challenge when trying to introduce these container systems into an organic polymer is thus their lack of solubility and their inability to maintain structural integrity in organic solvents or under melt-processing conditions. Alternatively, shell cross-linked assemblies of these amphiphilic molecules are emerging as potential candidates, since they are promising to retain size and shape persistency of these assemblies under harsh conditions [6].

Nature has already been using robust container systems based on self-assembled proteins for the transport and delivery of vital species such as trace elements, nucleic acids and hormones [4]. Natural containers such as cowpea chlorotic mottle virus (CCMV) and tobacco mosaic virus (TMV) are currently being explored for packaging of functional molecules with potential applications in drug delivery [7–9]. Ferritin is another example of a container system. Wild ferritin protects cells from potentially toxic effects by sequestering iron as a hydrous ferric oxide-phosphate mineral inside a protein coat for later use by the cell in the synthesis of heme. In the wild state, ferritin's spherical protein shell accommodates up to 4500 Fe(III) atoms as an inorganic complex [10]. Horse spleen ferritin is a particularly well-known and well-characterized protein with 24 polypeptide subunits which self assemble through non-covalent interactions to give a spherical shell with an 8-nm diameter cavity and a shell thickness of approximately 2 nm [11,12]. Its quaternary structure is stable to temperatures as high as 85 °C and over wide pH variations (2.0–9.0) [13]. Compared to many synthetic container systems, ferritin has remarkable stability due to the lack of dynamic exchange between ferritin subunits [14].

Significantly, ferritin can be demineralized by reductive dissolution to form apoferritin, a hollow capsule. The cavity of apoferritin can subsequently be filled with nanoparticles of minerals such as magnetite, manganese oxide, uranyl oxide, and cadmium sulfide quantum dots [15]. Magnetoferritin has already shown good performance as an MRI contrast enhancement agent [16], and ferritins loaded with CoPt magnetic particles are promising for ultrahigh density data recording [17]. Hainfeld has also shown that apoferritin loaded with uranium atoms has potential as a delivery system in antibody-mediated neutron-capture cancer therapy [18].

Wild ferritin is hydrophilic and its surface must thus be modified in order to solubilize it in hydrophobic small-molecule or macromolecular solvents. Significantly, the ferritin surface presents a well-defined array of chemically functional groups that can be modified. Wong et al., for example, have demonstrated that the external ferritin surface can be derivatized with short-chain alkanes to make the protein soluble in a number of organic solvents such as dichloromethane (DCM), ethyl acetate and toluene [19].

Poly(ethylene glycol) (PEG) is often used to modify proteins because of its exceptional solubility in water and in many organic solvents, lack of toxicity, and biocompatibility. Its amphiphilic character can render PEGylated peptides and proteins less antigenic and less susceptible to clearance by the immune system, while at the same time enhancing their solubility in an organic environment [20,21]. By expanding Wong et al.'s surface modification approach [22,23], we have covalently attached poly(ethylene glycol) (PEG) to the ferritin surface by zero-length cross-linking [24] and attained both organic and aqueous solubility. Subsequently, taking advantage of this solubility and the colloidal stability provided by PEG against denaturation in organic solvents [20,25], we incorporated PEGylated ferritin into synthetic homopolymer matrices via organic solvent casting.

It has also been well known that creation of nanocomposites via introduction of nanoparticles modified with different block components or chemically similar ligands can provide control over spatial and orientation distribution of the nanocomponents in the block copolymers and affect their properties [26,27]. Recently, Lin et al. demonstrated the effect of incorporated nanoparticles on the crystallization of poly(ethylene oxide) domains of a block copolymer by using PEG tagged ferritin nanoparticles [28]. Chiu et al. also showed that they can selectively locate gold nanoparticles in a diblock copolymer poly(styrene-*b*-2-vinyl pyridine) (PS-PVP) matrix by coating their surfaces with energetically similar block units [29]. Tadd et al. described the selective phase separation of cobalt metal nanoclusters within a block copolymer PS-PMMA due to different reactivities of the functional groups in the blocks toward the metal fragments [30]. We adopted a similar approach based on energetically similar polymer brushes and matrices to self-direct ferritin nanocontainers in a phase-separating homopolymer blend.

Here we first assess the degree of PEGylation and resulting solubility of PEGylated ferritin in a small-molecule organic solvent and then study the solubility and selectivity of modified ferritin in macromolecular solvents, namely, synthetic homopolymers. We used a tyrosine-derived poly(desaminotyrosyl tyrosine dodecyl ester carbonate) [poly(DTD carbonate) or PDTD] and poly(ethylene glycol) (PEG) as the components of a homopolymer blend with which to test the self-directing properties of modified ferritin. PDTD is a hydrophobic pseudo-poly(amino acid) being developed mainly for biomedical device applications [31,32]. We used PEG as the blend's minor phase. As shown in this paper, modified ferritin was soluble in DCM which is a common solvent for both polymeric components. PEGylated ferritin was self-directed to the

PEG domains while the alkylated ferritin selectively located in the hydrophobic PDTD of the binary polymer blend system.

2. Experimental

2.1. Preparation of purified ferritin

Horse spleen ferritin solution containing at least 90% ferritin was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). The as-received solution was further purified with a HiTrap Q Sepharose 5-ml anion exchange column (Amersham Pharmacia Biotech) using a Biorad Duoflow ion-exchange chromatography system with a 0–1.0 M NaCl gradient in 25 mM Tris buffer at pH 7.5. This column binds the ferritin until a critical NaCl concentration is reached while the impurities are immediately eluted. The purified ferritin solution was subsequently desalted via dialysis using regenerated cellulose tubing with a molecular weight cutoff of 14 kDa (Fisher Scientific) against 2 l of Type I (18.2 Mohm cm, pH: 5.6) Millipore water for 2 periods of 4 h.

2.2. Preparation of PEGylated and alkylated ferritin

In a typical PEGylation experiment, *O*-(2-aminoethyl)-*O*′-methyl poly(ethylene glycol) [PEG 750] (250 mg, 0.33 mmol) with an average molecular weight 750 Da (Fluka) and polydispersity of 1.1 was dissolved in 10 ml deionized water/THF (50% v/v) solution. The pH was adjusted to 5.5 by gradual addition of HCl solution. Purified and desalted protein (7.5 mg) was added to this solution, and the pH was readjusted to 5.5. Subsequently, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride [EDC] (Aldrich) (0.33 mmol) was added to the reaction mixture while the pH was maintained at 5.5. In order to overcome the low yield of carbodiimide-mediated conjugation due to fast hydrolysis of active carboxylate groups, *N*-hydroxysuccinimide (NHS) (Aldrich) (5 mM) was introduced to the reaction mixture [25]. Horse spleen ferritin has 7.0 ± 0.7 modifiable COOH groups per subunit on its exterior surface [33], which gives an average of 170 surface COOH groups for the 24 ferritin subunits. PEG 750 was used at an excess of 175 times that of the available carboxylic acid groups. Likewise, wild ferritin was alkylated as described by Wong et al. [19]. For both the alkylation and PEGylation reactions, the pH was maintained at 5.5 by the dropwise addition of dilute HCl for approximately 2 h. After 2 h, pH fluctuations were minimal. After mixing overnight at room temperature, the reaction mixtures were centrifuged to separate any coagulated proteins from soluble ones. Then the supernatant was dialyzed (14 kDa cutoff) overnight against 2.5 l Type I water. Some dialyzed samples were freeze dried and subsequently reconstituted in dichloromethane (DCM) under a fume hood.

2.3. Ion-exchange chromatography

Samples of modified ferritin were analyzed by ion-exchange chromatography. Anion exchange chromatography runs were performed using Amersham Hi-trap Q column (1 ml) with a mobile phase of 25 mM Tris buffer at pH 7.2 and an increasing linear gradient of NaCl (0 to 1.0 M). Phosphate buffer at the same molarity and pH was used with Amersham HiTrap Sp column (1 ml) for cation exchange chromatography. Runs were followed using a QuadTec UV/Vis detector at a wavelength of 280 nm for aromatic groups and 405 nm for the heme groups.

2.4. Zeta potential measurements

A Malvern Nano Zetasizer (Malvern Instruments Ltd.) was used for zeta potential measurements. PEGylated ferritin was dialyzed as described previously against Type I water. It was subsequently freeze dried and reconstituted in DCM. The portion soluble in DCM was left in a fume hood to allow for solvent evaporation overnight. This isolated fraction of DCM-soluble ferritin was then dissolved in DI water. Both wild and PEGylated ferritin samples were prepared by mixing the protein in 1 ml of phosphate buffer ranging over a series of pH values to produce 0.2 mg/ml solutions.

2.5. Electrospray ionization mass spectrometry

Electrospray ionization mass spectra were recorded on a Waters-Micromass Q-ToF API-US (Manchester UK) mass spectrometer equipped with a nanospray ionization source. Samples were introduced as acetonitrile/water/formic acid (50:50:0.2) solutions at a flow rate of 1 μl min^{-1}. To minimize background interference, we acquired blank mass spectra under similar instrument settings used for the wild and PEGylated protein. The instrument was operated under positive polarity to detect positively charged molecules accelerated and allowed to follow V-mode trajectory within the TOF analyzer. The source temperature was held at 80 °C, cone gas flows at 50 l h^{-1}, and TOF voltage at 9.1 kV. The capillary voltage, argon gas pressure in the collision cell, and cone voltage were set for each compound individually to obtain optimal output signals.

2.6. Estimation of grafting density by fourier transform infrared spectrometry

Fourier transform infrared spectrometry was used to estimate the number of grafted PEG chains on the protein surface. The dimensionless grafting density corresponds to the number of chains grafted in an area equal to the square of the Kuhn statistical segment length, *b*. This density were calculated by

$$\sigma = \frac{N_H b^2}{4\pi r^2} \tag{1}$$

where *r* is the ferritin radius (6.0 nm) and N_H is the average number of grafted chains per protein [34]. The Kuhn statistical segment length for PEG is 0.7 nm [35].

A Perkin Elmer FT-IR spectrometer (Paragon 1000) was used for estimating the grafting density of PEG onto ferritin. First, a calibration curve was prepared using various

concentrations of wild ferritin and homopolymer PEG mixtures freeze dried from aqueous solutions. For these experiments, we used hydroxy-terminated PEG homopolymer with an average molecular weight of 750. The resulting powders were ground with KBr on an agate mortar and pelletized under 15,000 psi pressure. A background subtraction was performed on each spectrum using a pure KBr reference. After baseline subtraction, the ratio of the area intensity of the ferritin amide I band due to carbonyl stretching vibrations 1750–1590 cm^{-1} to that of the PEG ether asymmetric stretching vibration at 1200–1000 cm^{-1} was calculated for a series of specimens each having a different ratio of ferritin to homopolymer PEG. Then, the FT-IR spectrum of PEGylated ferritin dialyzed against Type I water for 14 days was used to determine N_H by using the calibration curve.

2.7. Polymer thin film preparation and transmission electron microscopy

Using DCM as a common solvent, modified ferritin was introduced into a homopolymer blend of hydroxy-terminated poly(ethylene glycol) with a molecular weight of 6800 Da [PEG 6800] (Scientific Polymer Products), and 130 kDa PDTD [31]. Solutions of these polymers dissolved in DCM at a concentration of 50 mg/ml were prepared from 80% (w/w) PDTD/PEG 6800. Modified ferritin was added to create a final composition after solvent evaporation of 0.5–1.0 wt% in the polymer blend. Films were cast in aluminum pans (1 cm dia.) and kept under a fume hood overnight to allow for DCM evaporation. The resultant thickness of the films was approximately 200 μm. Later, the cast samples were vacuum ($\sim 10^{-2}$ Torr) dried overnight at ambient temperature. Thin films ($\sim$100 nm) for TEM were cut at −60 °C using a Leica Ultracut S Cryo Ultra-Microtome with a 35° Diatome diamond knife and collected on copper TEM support grids. Electron micrographs were recorded using a Philips CM 20 Field Emission Gun (FEG) Transmission Electron Microscope (TEM) at a 200 kV accelerating voltage equipped with a Gatan Multi-Scan digital CCD camera. Because of its mineral core, modified ferritin could be easily detected by traditional bright-field TEM imaging methods without heavy element staining to enhance the contrast.

3. Results and discussion

One important piece of evidence indicating that the ferritin surface was modified by the PEGylation process is the different retention and elution behaviors of PEGylated ferritin during ion-exchange chromatography (Fig. 1). Wild ferritin has an isoelectric point (pI) of about 4.5–4.8 [36], and, because of its overall negative charge at pH 7.5, it is not retained by the negatively charged cation exchange column. PEGylated ferritin, on the other hand, is retained and elutes only after a gradual increase in the NaCl concentration. The fact that the PEGylated ferritin sticks to the cation exchange column indicates that consumption of surface COOH groups shifts the isoelectric point to higher pH levels, rendering modified ferritin less negative at pH 7.5. Anion exchange chromotography verifies the modification. Because of the inversion of the column's charge, modified ferritin does not stick whereas wild ferritin is retained and elutes only after a gradual increase in the NaCl concentration (Fig. 2). The shift of the pI point to ~pH 8.0 was confirmed with zeta potential measurements (Fig. 3) and reflects the fact that protonated amine groups remain on the ferritin surface.

A second indication that the ferritin is modified by PEGylation comes from electrospray ionization mass spectroscopy. A mass spectrum recorded from wild ferritin (Fig. 4) shows a peak pattern typical for proteins ionized under electrospray conditions [37]. Each spectral peak represents multiply charged ferritin subunits with the same effective charge. The most intense peaks in the spectrum represent a charged ferritin

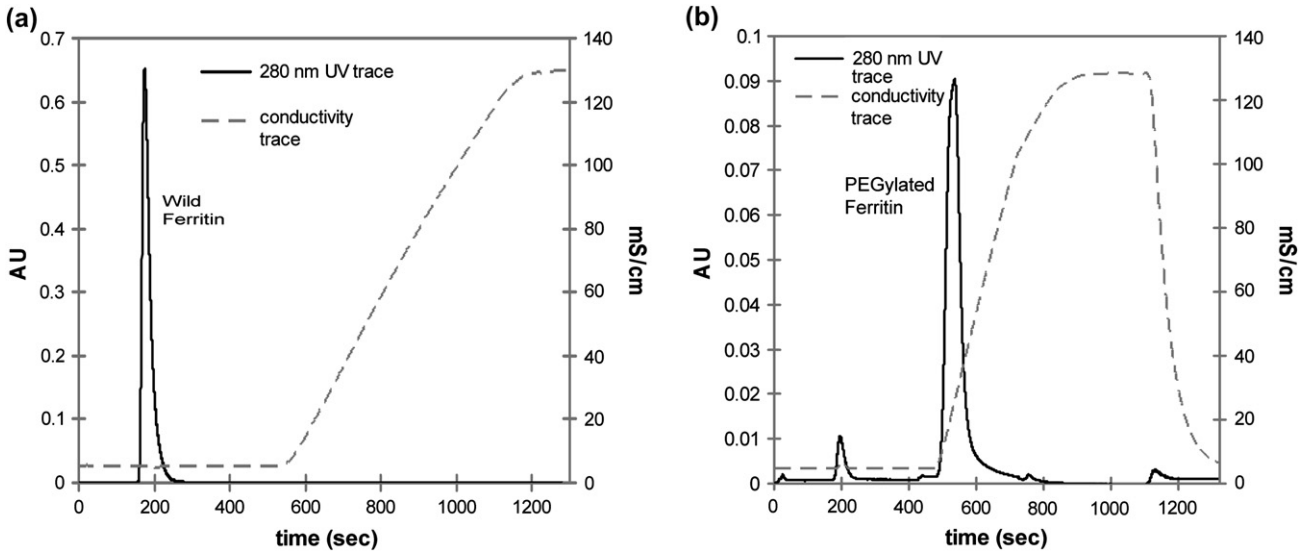

Fig. 1. Wild ferritin is not retained during cation exchange (a) while PEGylated ferritin is retained and elutes by increasing the NaCl concentration (b).

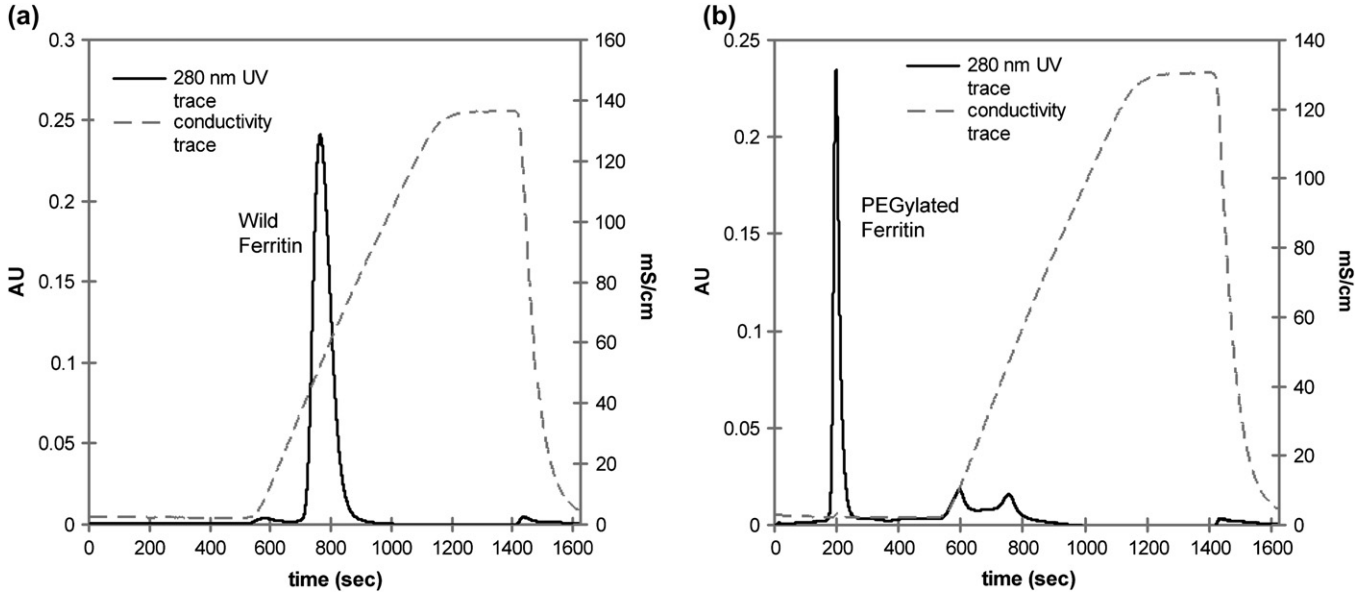

Fig. 2. Wild ferritin is retained by anion exchange and elutes with increasing NaCl concentration (a), whereas PEGylated ferritin is not retained by anion exchange (b).

subunit with an average mass of 20,005 Da. On the other hand, a mass spectrum recorded from PEGylated ferritin (Fig. 4) lacks any typical protein signals and shows only a large broad peak between 700 and 2500 *m*/*z*. This observation indicates that there is a wide mass distribution of modified ferritin subunits. We attribute this to the fact that different ferritin subunits can have widely varying degrees of PEGylation [38,39]. Thus, the mass spectrum for the final product (Fig. 4) confirms successful chemical modification of ferritin, but, due to the wide mass distribution, it does not give clear information concerning the degree to which the ferritin is modified.

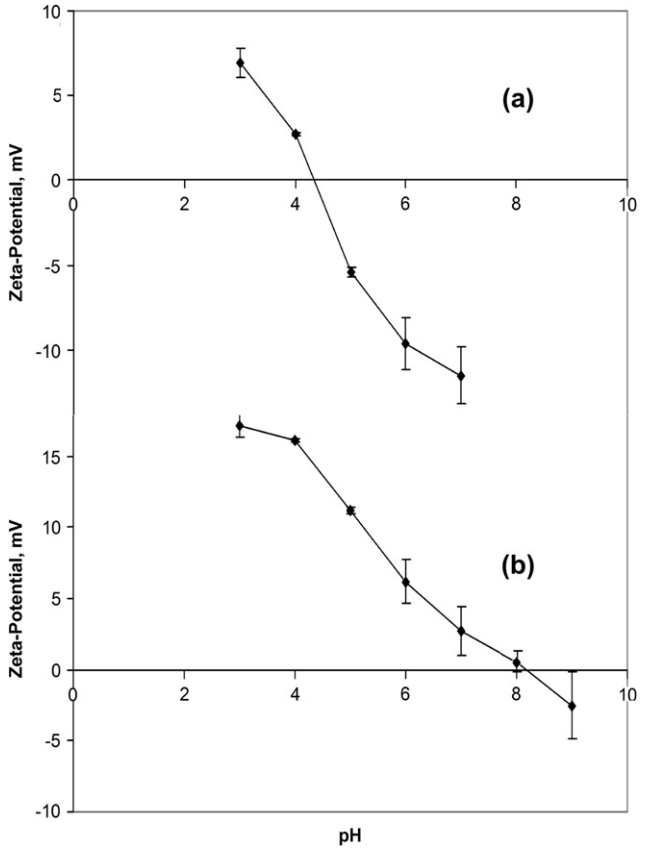

Fig. 3. ζ-Potential measurements of wild ferritin (a) and PEGylated ferritin (b) show the change in the net surface charge of the protein and the shift in the pI point from pH 4.8 to 8.0 after modification of its surface COOH groups.

An important operational indication of the extent of modification is that the PEGylated ferritin exhibits different solubility behavior than the wild ferritin. We know that ferritin has to be sufficiently modified to be soluble in DCM. For example, freeze-dried samples of PEGylated ferritin readily dissolve in DCM. Furthermore, PEGylated ferritin is preferentially soluble in DCM when simultaneously exposed to water (Fig. 5). DCM and water are immiscible and, due to their different densities, they form phase-separated layers when mixed in a vial. In Fig. 5, PEGylated ferritin dissolved in DCM can be easily differentiated from the upper water layer because of the rust color characteristic of the ferritin FeOOH mineral core. This experiment indicates that a sufficient amount of grafting took place during the conjugation reaction so that PEGylated ferritin can maintain its colloidal stability in DCM. Our FT-IR measurements, to be discussed later, address the degree of this grafting. Conversely, wild ferritin is soluble in water, but it coagulates in DCM and precipitates out of solution. We found that PEGylated ferritin remains stable in DCM for several months.

In addition to working with PEGylated ferritin, we also examined the organic solvent solubility and modification of alkylated ferritin. We followed the synthesis protocol described by Wong et al. [19] and found that dodecylamine modified ferritin showed a retention and elution pattern in ion-exchange chromatography similar to that of the PEGylated ferritin. Alkylated ferritin also displayed a single broad peak similar to that of PEGylated ferritin in mass spectrograms,

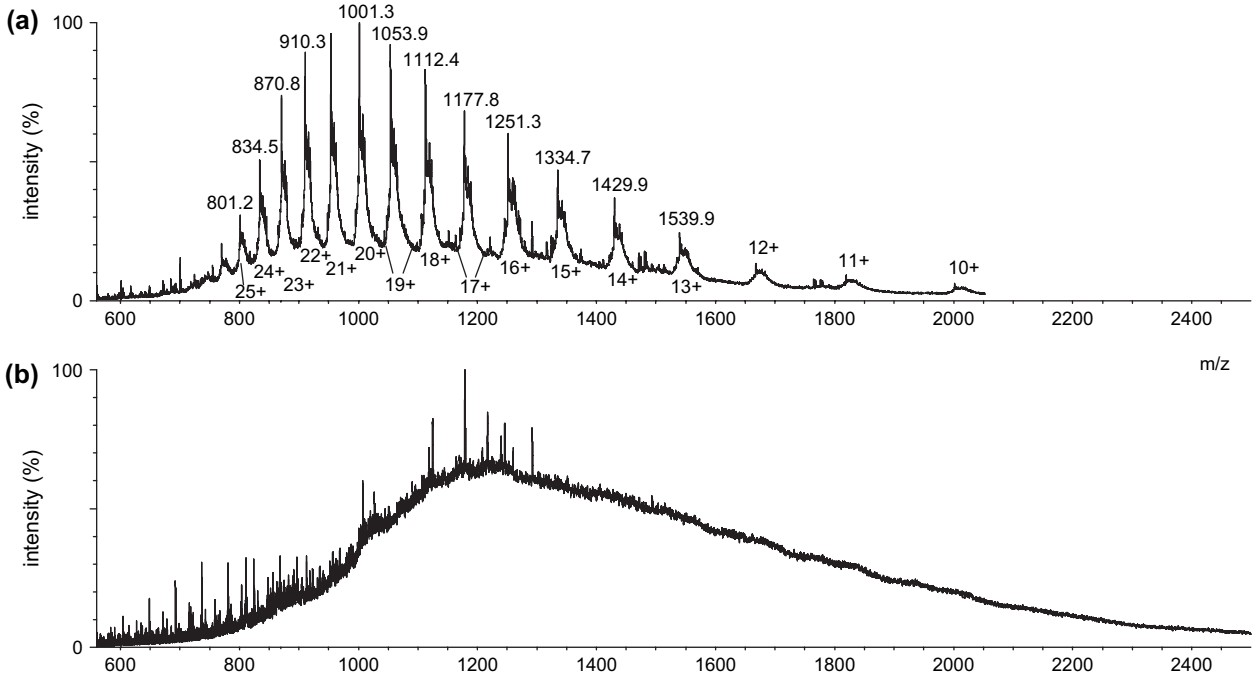

Fig. 4. (a) Electrospray ionization mass spectrum of wild ferritin. Each peak represents a ferritin subunit charged with a different number of protons (number of charges are indicated by the "+" suffix). The most intense peak represents a charged ferritin subunit with an average mass of 20,005 Da. (b) ESI mass spectrum of PEGylated ferritin.

and this is a good indication that different levels of modification occurred during the carbodiimide-mediated alkylation reaction. We also witnessed the passage of dodecylamine modified ferritin from the reaction mixture of THF/H_2O (50% v/v) to DCM upon addition of a small amount of NaCl consistent with observations by Wong et al. [19]. After dialysis and freeze drying, the alkylated ferritin which most probably did not have enough surface modification or went through coalescence during the reaction adhered to the surface of the beaker, whereas the rest was readily soluble in DCM. We subsequently worked with the soluble fraction.

Significantly, transmission electron microscopy of solid polymer films of modified ferritin mixed with a homopolymer blend of PEG and PDTD shows that these modified protein nanocontainers have a self-directing character. Bright-field TEM images from such specimens are given in Figs. 6 and 7.

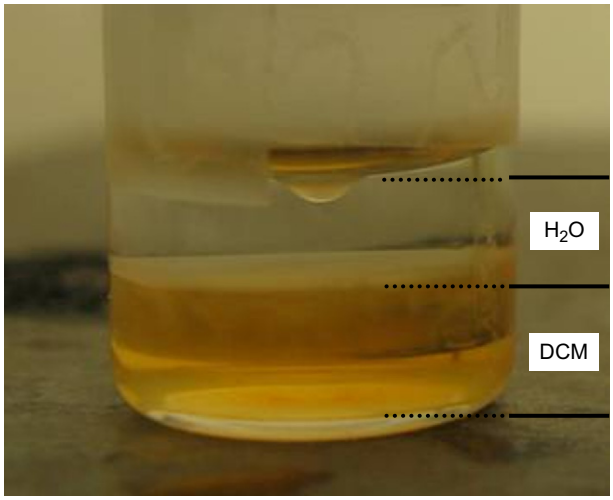

Fig. 5. Photograph illustrating the stability of PEGylated ferritin in DCM after addition of water. Water and DCM are not miscible, and DCM with its higher density, sinks to the bottom of the vial. The DCM layer has a reddish yellow color due to the iron core of PEGylated ferritin.

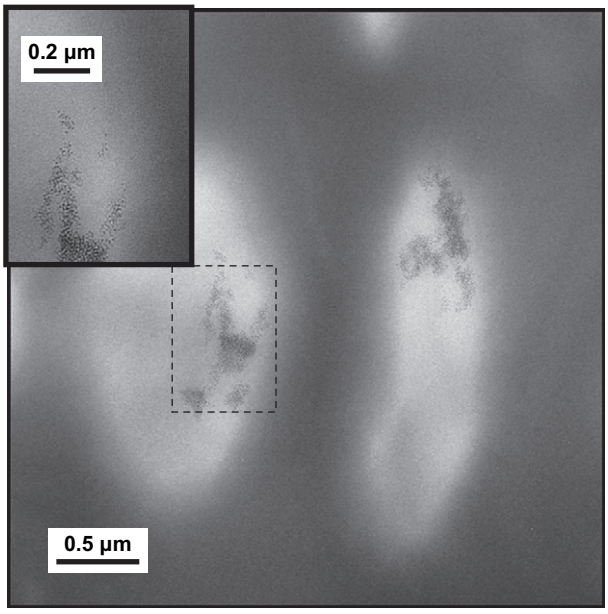

Fig. 6. Bright-field TEM micrographs showing PEGylated ferritin in the hydrophilic PEG minor phase of an 80 wt% PDTD/20 wt% PEG homopolymer blend.

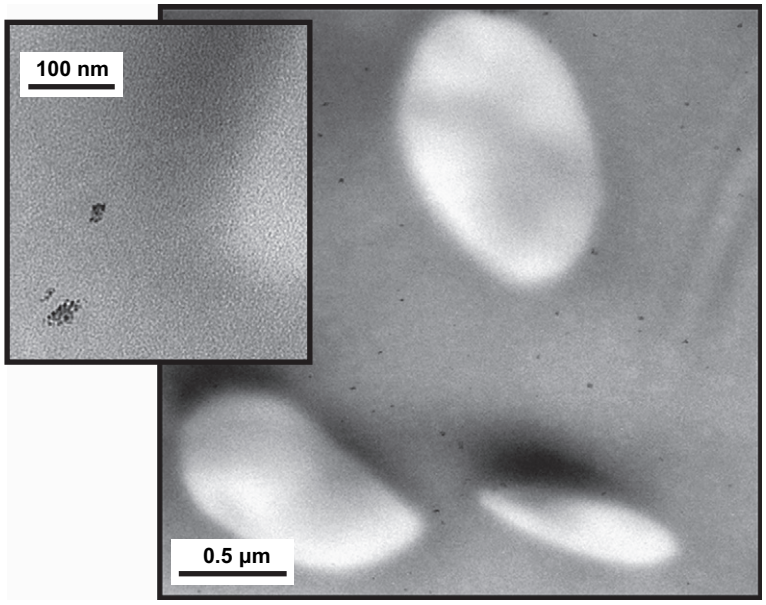

Fig. 7. Bright-field TEM micrographs showing alkylated ferritin in the hydrophobic PDTD major phase of an 80 wt% PDTD/20 wt% PEG homopolymer blend.

For a typical 80% (w/w) PDTD/PEG 6800 blend system, the micron-sized round domains with light contrast correspond to the minor PEG 6800 phase, and the dark contrast corresponds to the PDTD major phase. Modified ferritin manifests itself as tiny black dots and is readily detectable in the TEM images, because its Fe-based core scatters electrons more strongly than the surrounding polymer. In Figs. 6 and 7, one can see that modified protein nanocontainers in a binary polymer blend system show a remarkable selectivity. PEGylated ferritin selectively locates itself in the hydrophilic PEG 6800 domains (Fig. 6), while alkylated ferritin selectively locates itself in the hydrophobic PDTD phase (Fig. 7).

We used Fourier transform infrared spectroscopy to experimentally estimate the extent of PEGylation on the modified ferritin. Fig. 8 shows FT-IR spectra characteristic of wild ferritin, pure homopolymer PEG, and PEGylated ferritin. The protein and the PEG can be differentiated from each other based on the amide I and II bands (1750–1500 cm^{-1}) and the ether band (1200–1000 cm^{-1}). The calibration curve (Fig. 9) was created by taking the ratio of the 1750–1590 cm^{-1} vibration band due to carbonyl stretching of the amide group (I) on the protein shell to the 1200–1000 cm^{-1} vibration band due to asymmetric ether stretching on PEG (A_{PEG}/A_{Fn}). We obtained a linear calibration plot in which A_{PEG}/A_{Fn} corresponds to a particular number of PEG chains per ferritin.

In order to determine the grafting amount of PEG on ferritin, we dialyzed PEGylated ferritin for 14 days and collected samples daily to measure the PEG-protein ratio using FT-IR and the calibration curve in Fig. 9. We found that this ratio decreased with increasing dialysis time indicating that noncovalently attached PEG was released from the PEGylated ferritin. Fig. 10 shows that the curve reaches an asymptote after 4 days of dialysis. Using the calibration curve (Fig. 9), IR spectra of this exhaustively dialyzed PEGylated ferritin indicate that the average number of covalently attached PEG molecules per ferritin nanocontainer, N_H, is 120. This corresponds to a 70% substitution of the modifiable 170 acidic residues on the ferritin surface. This amount of surface coverage corresponds to an average dimensionless grafting density of $\sigma \sim 0.13$ (Eq. (1)). The grafting degree (g_d = weight of grafted chains/weight of ferritin) is 18.9%. The average distance between 2 anchor sites, D was calculated as the square root of the area occupied by the single PEG molecule on the nanocontainer surface [40]. PEG surface concentration with a 43.9 pmol cm^{-2} gave a D value equal to 1.94 nm. The average anchoring distance is at the range of Flory radius for PEG in ideal solution conditions (1.98 nm), which suggests that our system is at the mushroom/brush border in good solvent like water.

The TEM images in Figs. 6 and 7 indicate that both the PEGylated ferritin and alkylated ferritin flocculate in their respective host phase. This effect may be due to the fact that the ferritin surface is not fully modified or to the fact that the molecular weight of the grafted chains is substantially less than that of the homopolymer PEG 6800 and PDTD used in the blend. Hasegawa et al. [41] demonstrated experimentally that there is an optimum grafting density in dispersing steric stabilized particles in a polymer matrix. When the grafting density is too high or too low, there are interparticle strong attractive interactions and the particles tend to form clusters inside the polymer matrix [41]. A rough estimate using Hasegawa et al.'s approach, $\sigma \sim N^{-1/2}$ suggests that the optimum graft density to disperse the nanocontainers would be 0.242. However, this grafting density corresponds to 220 average chains which is higher than the 170 accessible COOH surface groups. Achieving it would require either the disruption of the intersubunit bonding or a second chemistry to conjugate to other functional groups on the ferritin surface.

It is well known both theoretically and experimentally that high-molecular-weight solvent polymer may destabilize the

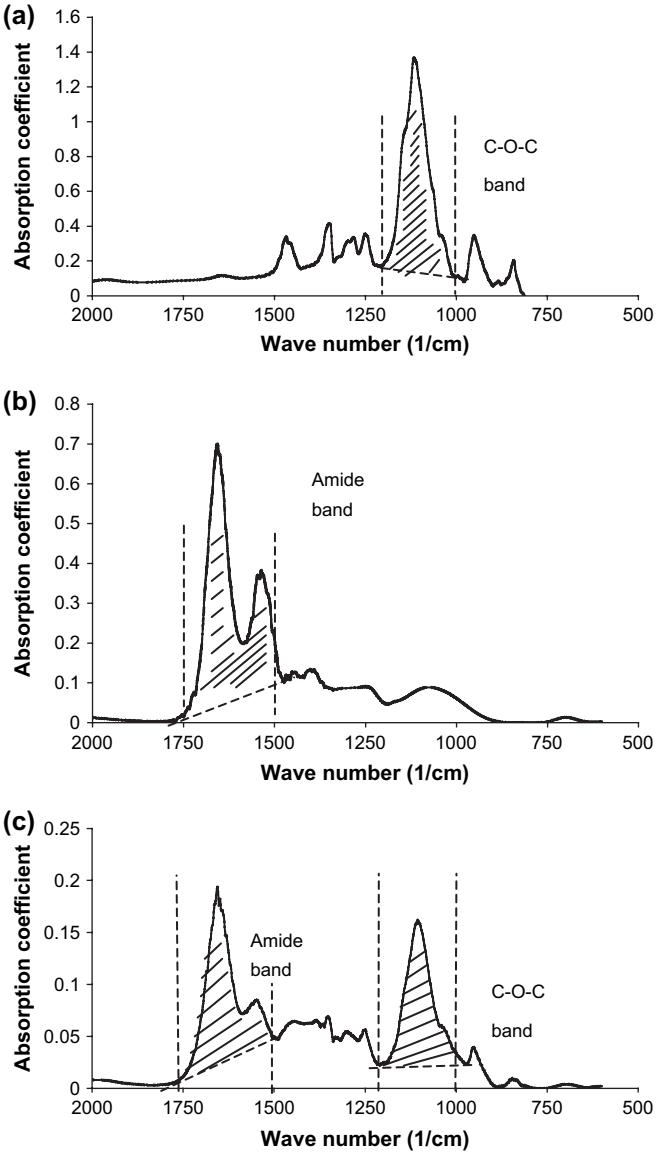

Fig. 8. Regions of FT-IR spectra of (a) pure PEG (b) wild ferritin; and (c) PEGylated ferritin.

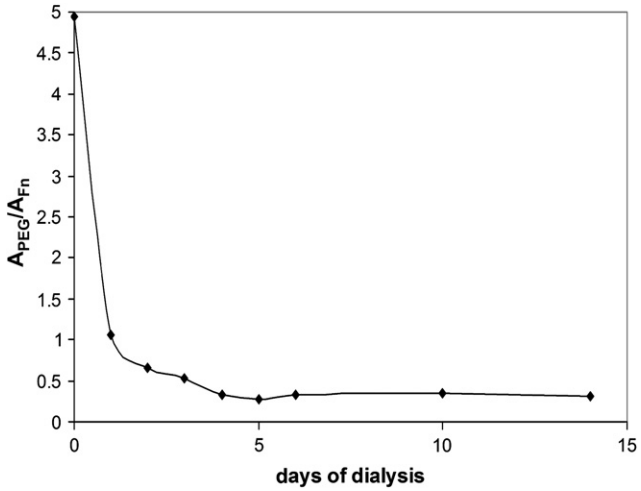

Fig. 10. PEGylated ferritin was dialyzed for consecutive 14 days against DI water at pH 6.0 through a cellulose membrane with a MW cutoff of 14,000. The baseline corrected area ratio of PEG to ferritin gathered from FT-IR shows that, after 4 days of dialysis amount of PEG reaches an asymptote which signifies the net conjugated PEG.

colloid, because the solvent molecules are excluded from the brush (dry brush regime) [42]. Though our grafted chains are on a curved surface, we used the diagram of states developed for chains grafted on a flat surface [43] as a guide to illustrate how the PEGylated ferritin might interact with a polymeric PEG homopolymer solvent. Our particular system is defined by tethered PEG chains with a degree of polymerization of 17 inside a PEG 6800 matrix with a degree of polymerization of 154 and a dimensionless grafting density of $\sigma \sim 0.13$. According to the diagram of states, such a system with a medium grafting density is in the unstretched brush regime where the neighboring grafting chains can overlap, yet the brushes are unswollen and characterized by ideal random walk dimensions [44]. We believe that this effect is more influential in flocculation behavior of the ferritin containers in high molecular weight matrices.

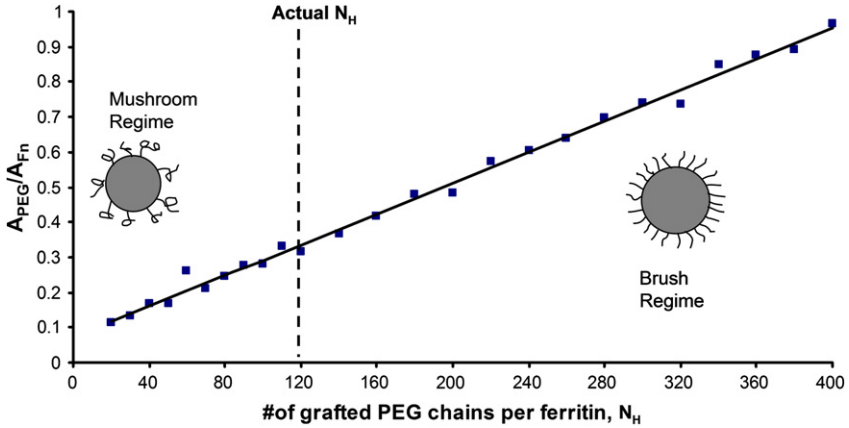

Fig. 9. Calibration curve obtained by ratioing the intensity of the ether stretching vibration band (1200–1000 cm^{-1}) of homopolymer PEG to that from the amide I group stretching vibration band (1750–1590 cm^{-1}) of wild ferritin.

4. Conclusion

We have explored the possibility of using naturally-derived ferritin as a nanocontainer that self-directs in a multiphase macromolecular solvent. Others have shown that ferritin's mineral core can be replaced with a number of different compounds of interest for their optical, magnetic, and mechanical properties. We have concentrated on modifying the ferritin surface to controllably incorporate it into a polymeric solvent. We found that ferritin can be modified to achieve solubility in dichloromethane by covalently conjugating poly(ethylene glycol) to its surface carboxyl groups. We also modified the ferritin surface by alkyl chains as described by Wong et al. [14], and such alkylated ferritin is also soluble in dichloromethane. TEM imaging confirmed our hypothesis that ferritin modified by different oligomers has different interactions with the polymer matrix. Hence, in a homopolymer blend of a hydrophilic polymer dispersed in a hydrophobic matrix, ferritin modified by hydrophobic alkanes prefers to be in the hydrophobic polymer phase, whereas, ferritin modified by poly(ethylene glycol) oligomers prefers to stay in the hydrophilic polymer phase.

Acknowledgments

The authors thank Prof. Trevor Douglas (Montana State University) for his early involvement in the ferritin derivatization experiments and Prof. Svetlana Sukhishvili (Stevens) for her many suggestions regarding characterization of the PEGylated ferritin. The authors are grateful for financial support from the Army Research Office (Grant DAAD 19-03-1-0271) and to RESBIO-*The National Resource For Polymeric Biomaterials* (NIH NIBIB Grant PH1 EB001046-01A1) for providing poly(DTD carbonate).

References

[1] West JL. Modification of materials with bioactive peptides. In: Hollander AP, Hatton PV, editors. Biopolymer methods in tissue engineering. Totowa: Humana Press Inc.; 2004. p. 113–21.
[2] Lee H, Archer LA. Polymer 2002;43:2721–8.
[3] Torchilin VP. Adv Drug Delivery Rev 2002;54:235–52.
[4] Sauer M, Meier W. Colloidal nanoreactors and nanocontainers. In: Caruso F, editor. Colloids and colloid assemblies synthesis, modification, organization and utilization of colloid particles. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2004. p. 150–74.
[5] Lasic DD. Liposomes: from physics to applications. Amsterdam: Elsevier; 1993.
[6] Thurmond KBI, Huang H, Clark CGJ, Kowalewski T, Wooley KL. Colloids Surf B 1999;16:45–54.
[7] Goldmann C, Stolte N, Nisslein T, Hunsmann G, Luke W, Petry H. J Virol Methods 2000;90:85–90.
[8] Douglas T, Young M. Nature 1998;393:152–5.
[9] Douglas T, Strable E, Willits D, Aitouchen A, Libera M, Young M. Adv Mater 2002;14:415–8.
[10] Proulx-curry PM, Chasteen ND. Coord Chem Rev 1995;144:347–68.
[11] Harrison PM, Arosio P. Biochim Biophys Acta 1996;1275:161–203.
[12] Theil EC, Takagi Hidenori, Small GWilliam, He L, Tipton AR, Danger D. Inorg Chim Acta 2000;297:242–51.
[13] Meldrum FC, Wade VJ, Nimmo DL, Heywood BR, Mann S. Nature 1991;349:684–5.
[14] Wong KKW, Whilton NT, Colfen H, Douglas T, Mann S. Chem Commun 1998;1621–2.
[15] Douglas T, Dickson DPE, Betteridge S, Charnock J, Garner CD, Mann S. Science 1995;269:54–7.
[16] Bulte JW, Douglas T, Mann S, Frankel RB, Moskowitz BM, Brooks RA, et al. J Magn Reson Imaging 1994;4:497–505.
[17] Nanobiotechnology, concepts, applications and perspectives. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2004.
[18] Hainfeld JF. Proc Natl Acad Sci USA 1992;89:11064–8.
[19] Wong KKW, Colfen H, Whilton NT, Douglas T, Mann S. J Inorg Biochem 1999;76:187–95.
[20] Inada Y, Takahashi K, Yoshimoto T, Ajima A, Matsushima A, Saito Y. TIBTECH 1986;190–4.
[21] Harris JM, editor. Poly(ethylene glycol) chemistry biotechnical and biomedical applications. New York: Plenum; 1992.
[22] Grabarek Z, Gergely J. Anal Biochem 1990;185:131–5.
[23] Danon D, Goldstein L, Marikovsky Y, Skutelsky E. J Ultra Mol Struct R 1972;38:500–10.
[24] Hermanson GT. Bioconjugate techniques. San Diego: Academic Press; 1996.
[25] Kynclova E, Elsner E, Kopf A, Hawa G, Schalkhammer T, Pittner F. J Mol Recognit 1996;9:644–51.
[26] Bockstaller MR, Mickiewicz RA, Thomas EL. Adv Mater 2005;17: 1331–49.
[27] Balazs AC. Curr Opin Colloid Interface Sci 2000;4:443–8.
[28] Lin Y, Boker A, He J, Sill K, Xiang H, Abetz C, et al. Nature 2005;434: 55–9.
[29] Chiu JJ, Kim BJ, Kramer EJ, Pine DJ. J Am Chem Soc 2005;127: 5036–7.
[30] Tadd EH, Bradley J, Tannenbaum R. Langmuir 2002;18:2378–84.
[31] Yu C, Kohn J. Biomaterials 1999;20:253–64.
[32] Tangpasuthadol V, Pendharkar SM, Peterson RC, Kohn J. Biomaterials 2000;21:2379–87.
[33] Wetz K, Crichton RR. Eur J Biochem 1976;61:545–50.
[34] Yezek L, Schartl W, Chen Y, Gohr K, Schmidt M. Macromolecules 2003; 36:4226–35.
[35] Oesterhelt F, Rief M, Gaub HE. New J Phys 1999;1:6.1–6.11.
[36] Nerurkar AV, Vaidya NR, Advani SH, Gothoskar BP. Neoplasma 1993; 40(1):31–4.
[37] Cole RB, editor. Electrospray ionization mass spectrometry. John Wiley; 1997.
[38] Veronese FM. Biomaterials 2001;22:405–17.
[39] Gbadamosi JK, Hunter AC, Moghimi SM. FEBS Lett 2002;532: 338–44.
[40] Meng F, Engbers GHM, Feijen J. J Biomed Mater Res 2004;70A:49–58.
[41] Hasegawa R, Aoki Y, Doi M. Macromolecules 1996;29:6656–62.
[42] Borukhov I, Leibler L. Macromolecules 2002;35:5171–82.
[43] Aubouy M, Fredrickson GH, Pincus P, Raphael E. Macromolecules 1995; 28:2979–81.
[44] Jones RAL, Richards RW. Polymers at surfaces and interfaces. Cambridge: Cambridge University Press; 1999.