University of Groningen

Hierarchical structures via self-assembling protein-polymer hybrid building blocks

van Rijn, Patrick; Mougin, Nathalie C.; Boeker, Alexander

Published in:
Polymer

DOI:
[10.1016/j.polymer.2012.10.054](https://doi.org/10.1016/j.polymer.2012.10.054)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van Rijn, P., Mougin, N. C., & Boeker, A. (2012). Hierarchical structures via self-assembling protein-polymer hybrid building blocks. *Polymer*, 53(26), 6045-6052. <https://doi.org/10.1016/j.polymer.2012.10.054>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Hierarchical structures via self-assembling protein-polymer hybrid building blocks

Patrick van Rijn, Nathalie C. Mougin, Alexander Böker*

DWI an der RWTH Aachen e.V., Lehrstuhl für Makromolekulare Materialien und Oberflächen, RWTH Aachen University, Forckenbeckstrasse 50, D-52056 Aachen, Germany

ARTICLE INFO

Article history:

Received 3 August 2012

Received in revised form

25 October 2012

Accepted 31 October 2012

Available online 7 November 2012

Keywords:

Protein-polymer hybrid particles

Self-assembly

Interfaces

ABSTRACT

In this study we demonstrate that different hierarchically self-assembled materials can be formed by recently developed ferritin-polymer hybrid conjugates. Via interfacial self-assembly in combination with small-pore extrusion followed by inter-particle UV cross-linking, different states of capsule structures were obtained ranging from single capsules to capsules entrapped in fibers and high density capsule assemblies. The release of the capsule contents is regulated using different solvent combinations and visualized by entrapment of a lipophilic molecular dye. The structures and release properties were investigated using (fluorescence) microscopy and UV–Vis spectroscopy.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Self-assembly is an important tool to construct soft matter (nano-)structures with a variety of morphologies for the development of new materials [1,2]. These structures can be formed by small molecular components, *e.g.* surfactants [3] and low-molecular-weight gelators [4], but also by macromolecular structures like block-copolymers [5,6], dendrimers [7] and modified (bio-)nanoparticles [8]. In order to create novel functional systems, it becomes increasingly important for soft materials to be responsive and to obtain structures of various dimensions and complexity with respect to supra-molecular architectures, especially in combination with biological structures like proteins. In nature as well as in synthetic systems various types of (macro-)molecular components allow the system to respond to different stimuli [9–13] like for example temperature [14], light [15] and external fields [16]. For soft materials the ability to form complex multi-architectural systems which are able to coexist in a single system is also of great interest especially because nature derives its function from this: complex self-assembling systems coexist inside the cell, proteins and lipids coexist in the cellular membranes and the confinement of the cells themselves inside the extracellular matrix. This form of self-assembly allows for controlled formation of confined spaces [17,18] and the build-up of complex

superstructures [19] providing new material properties and functions. Very successful attempts have been made towards the formation of complex systems that show hierarchical structures in a single system with the use of different small molecular components [20] but also in combination with block-copolymers [12,21,22]. For these systems, in order to achieve different complexity and hierarchy, two or more molecular components are needed, each with its own driving force for self-assembly. The formation of hierarchical structures would be more convenient when only one component is required. In fact, not many systems are known to show this kind of behavior. Exceptions are block-copolymers in thin films [23] or subject to external fields like during the electro-spinning process [24]. However, both examples are far from being functional. Here we present a system of a protein-polymer hybrid which is able to form different types of structures by adjusting the preparation method. It is shown that this system is able to release a lipophilic dye in a controlled manner by using different solvent combinations. Here horse spleen ferritin (HSF) is used as a model protein structure. HSF has a very well characterized structure consisting of a dodecameric protein cage of 12 nm in diameter with a 6 nm central cavity containing a phosphate ferrihydrite core and is active as an iron storage protein in mammals. The protein cage is stable over a large pH and temperature range in water and tolerates organic co-solvents. The 72 chemically addressable primary amino-groups on the outside were heretofore used for bioconjugation of dyes [25–30]. With respect to the increasing interest in bionanoparticles, polymer-protein conjugates are a versatile way of modifying proteins especially, in

* Corresponding author. Tel.: +49 (0)241 80 233 04; fax: +49 (0)241 80 233 17.
E-mail address: boeker@dwf.rwth-aachen.de (A. Böker).

combination with a *grafting-from* approach via e.g. ATRP [8,31–38], a protein template gives access to a large variety of structures and ferritin is an excellent candidate in this regard [39].

2. Results and discussion

2.1. Capsule formation

We previously reported on the successful synthesis of thermo-responsive ferritin-PNIPAAm and ferritin-PNIPAAm-DMIAAm conjugates via ATRP [8]. Additionally we presented the thermo-responsiveness and interfacial behavior of these conjugates in aqueous solution. It was found that the newly formed bio-nanoparticles display a high surface activity at polar/apolar interfaces and can be used for stabilization of oil-in-water emulsions which allows for the formation of stable semi-permeable capsules by means of photo-cross-linking [40]. In addition to the use of the particles as interface stabilizers, the polymer-conjugated ferritin particles may also find possible applications as functional conjugates with different cores for therapeutic and diagnostic purposes and with non-natural cores in the field of biomedical devices as delivery systems for anti-cancer, anti-microbial or MRI contrast agents [41–45].

Ferritin can be easily transformed into a macro-initiator by attaching 2-bromo-isobutyric acid (BIBA) to the amino groups of

the protein shell under aqueous conditions (phosphate buffer saline (PBS), pH 8.4/DMF; 5:1) (Fig. 1). Additionally, an atom transfer radical polymerization (ATRP) can be performed in aqueous solution (PBS pH 8.4) to produce a surface grafted random copolymer of *N*-isopropyl acrylamide, 2-(dimethyl maleinimido)-*N*-ethyl-acrylamide and *N*-5-Fluorescein-acrylamide. For preparing capsules free in solution first an oil-in-water emulsion was prepared by mixing Benzotrifluoride (BTF), with the aqueous solution containing the ferritin-PNIPAAm-DMIAAm-Fluorescein conjugate. The formed emulsion was then irradiated for 20 min with UV light which initiates a [2 + 2]-photocyclization of the DMIAAm moieties. The formed capsules are clearly visible due to the fluorescent nature of the polymers surrounding the conjugate. From fluorescent microscopy overlay images, it is clearly seen that the oil phase is surrounded by a green fluorescent layer of the ferritin-polymer conjugate due to the presence of the fluorescein-acrylate comonomer (Fig. 1).

2.2. Hierarchically addressable capsule/fiber structures

The protein-polymer hybrid system is able to form different hierarchically self-assembled structures by altering the concentration and preparation method. Using high oil content in combination with extrusion, various structures are obtained like well-dispersed capsules in solution, capsules entrapped in a fiber

Fig. 1. Schematic representation of the synthesis of the ferritin-polymer hybrid by ATRP forming a Ferritin-p(NIPAAm-DMIAAm-Fluorescein)-random copolymer conjugate, ratio:90/10/2. Synthesized by first forming a macro-initiator (A) phosphate buffer saline (PBS), pH 8.4/DMF; 5:1, with the activated carboxylic acid initiator, followed by ATRP (B) phosphate buffer saline (PBS), pH 8.4, monomers (NIPAAm:DMIAAm:Fluorescein; 90:10:2)/initiator/Cu(I)/Cu(II)/ligand = 100/1/0.7/0.3/2. When used as an emulsion stabilizer in a Pickering emulsion followed by cross-linking (mechanism shown in inset) with UV-irradiation capsules are formed of which a schematic representation is shown on the left and on the right an optical image of a Fer-PNIPAAm₉₀-DIMIAAm₁₀-Fluorescein₂ (~10 mg ml⁻¹) stabilized benzotrifluoride in water emulsion (10 μl benzotrifluoride in 1.0 ml conjugate solution) as well as a fluorescence image (scale-bars depicts 100 μm). The fluorescent properties arise from the fluorescein-acrylate monomer.

network and highly densely packed macroscopic capsule structures. Each different non-equilibrium hierarchical structure holds potential application as drug-delivery vessels, coverage of damaged tissue for regenerative purposes or as components in controlled local delivery systems seen in bandages and patches but also as different obtainable textures in foods and cosmetics. The hierarchical structures were obtained by interfacial self-assembly of a protein-polymer conjugate composed of a ferritin protein cage decorated on the outside with p(NIPAAm/DMIAAm/Fluorescein) random copolymers (poly(*N*-isopropyl acrylamide)-2-(dimethyl maleinimido)-*N*-ethyl-acrylamide/*N*-5-Fluorescein-acrylamide) (Fer-NDF) in combination with intense mixing by extrusion of an oil-water mixture through a track-etched membrane with 100 nm pore-diameter. Conventionally extrusion is used to produce monodisperse capsules when bigger pore-sizes are used rather than intense mixing [46]. The photo-cross-linkable polymer-protein hybrid tends to self-assemble at polar–apolar interfaces, reducing the interfacial tension significantly and thereby stabilizing the emulsion which is also known for native ferritin [8,47]. Recently self-assembled soft matter thin layer structures gained increasing interest [48,49] especially in combination with nanoparticle systems [50–52]. Here we found that the extrusion of an aqueous solution of the ferritin-polymer hybrid structure through a small pore polycarbonate membrane with relatively high oil content leads, amongst others, to complex superstructures of stable soft matter micro-compartments embedded in a cross-linked fiber structure (Fig. 2).

The superstructure can be directed by variation of the protein-polymer-conjugate concentration either towards single capsules or to highly concentrated macroscopic capsule structures in which a macroscopic precipitate displays closely packed covalently connected microcapsules. The dispersed capsules, fiber-capsule superstructures as well as the high density covalently bound capsules are easily isolated (Figs. 1–3). The release of active components from the oil-phase, here mimicked with the use of a fluorescent dye, was controlled by introducing isopropanol as

a co-solvent. By the addition of this secondary alcohol which is used in many forms of disinfectants and formulations, the release-rate of the lipophilic dye as well as the quantity was controlled.

In order to obtain an intensely mixed system, the oil-water mixture was passed 13 times through a polycarbonate filter with a pore-diameter of 100 nm (Fig. 2). The aqueous phase contains the Fer-NDF stabilizing agent. UV-irradiation induced a [2 + 2]-cyclo-addition between the cross-linker moieties which adds stability to the interface. The formed cloudy emulsion changed to a transparent solution with large precipitated structures which could be easily collected from the solution by means of a pipette. Upon analysis of the bulk precipitate, a network of fibers was found with capsules embedded inside the network (Fig. 2). The relatively high oil content produces large amounts of capsules and it is most likely that the cross-linking process during irradiation not only occurs between the particles in the capsule shell but also between particles confined in different capsules upon capsule collision. As a high concentration of capsules also leads to a high number of collisions, many reactions between particles from different capsules occur during the crosslinking process. Subsequent motion of connected capsules produces the fibers between them. The cross-linking process only takes place during UV-irradiation of sufficient intensity and the cross-linking between particles or capsules is not induced by the extrusion process as shown in Fig. 3D (inset). However, extrusion seems to foster fiber formation since fiber-like structures are also formed in the absence of an oil phase, although in very low quantity (Fig. 3A).

To investigate whether the overall morphology can be controlled, the preparation process was varied systematically, shown in Fig. 3. An aqueous solution of Fer-NDF was mixed with 10 vol% of benzotrifluoride (BTF) containing Nile Red as a fluorescent dye (0.01 mM). This mixture was extruded through a polycarbonate membrane with a pore-diameter of 100 nm. The resulting emulsion was then used as prepared as well as diluted 4 and 20 times, respectively. The different mixtures were irradiated as well as a solution which did not contain any oil but also extruded as control sample to gain insight into the cross-linking process between particles which are not confined to an interface. All samples were analyzed by fluorescence microscopy and revealed distinct morphological differences (Fig. 3). Here, it was most surprising that, though present in low numbers, the irradiated sample without oil contained fiber-like structures, indicating that cross-linking between Fer-NDF particles also occurs without being confined at interfaces and that the initial concentration is sufficiently high to produce larger cross-linked structures (Fig. 3A). The sample which was 20 times diluted showed small capsules dispersed in solution, slightly clustered (Fig. 3B). When the 4 times diluted sample displays large precipitated structures composed of a fiber network with capsules embedded within (Fig. 3C). The undiluted sample also displayed bulk precipitation of high density covalently connected capsules (Fig. 3D). From non-cross-linked samples shown in the inset in Fig. 3D, a distinctly different morphology is observed, indicating that the capsules are connected exclusively after the cross-linking procedure and not during the extrusion process. Apparently, the density of capsules present in solution is the most important factor for the formation of the observed superstructures. While a low capsule concentration results in less contacts between the capsules giving single capsules and small clusters, a high capsule concentration leads to larger superstructures in the form of densely connected capsules. More collisions result in a higher probability for the capsules to become connected, although fibers were only observed for the semi-diluted state. Most likely, a high capsule concentration allows for a high collision probability but not for a high mobility of the capsules and the diffusion distance of the capsules is limited. In the semi-diluted state sufficient collisions

Fig. 2. Extrusion process of an oil-water (1:9) mixture with Ferritin-p(NIPAAm-DMIAAm-Fluorescein-random copolymer, ratio:90/10/2)(green) through a 100 nm pore-size track-etch polycarbonate membrane followed by cross-linking with UV-irradiation (oil contains Nile Red). In a semi-diluted state, the process produces a network of fibers that entrap the stabilized capsules seen in the fluorescence microscopy overlay image, scale bar depicts 100 μm. The bottom left shows an enlarged section with on top a schematic interpretation of how the protein-polymer hybrids are able to form such a capsule-fiber network (size in drawing is highly exaggerated for clarity).

Fig. 3. A) Fer-NDF (green), ~ 10 mg/ml in MilliQ water (pH 9.0, adjusted with 1 M NaOH), extruded and subsequently irradiated, shows the formation of small amounts of large fiber like structures; B) Fer-NDF, ~ 10 mg/ml in MilliQ water (pH 9.0) with 10 vol%, 0.01 mM Nile Red (red) solution in BTF, extruded, diluted 20 times, irradiated. The micrograph shows a low concentration of individual capsules. C) and D) have the same preparation as B, only with a dilution of 4 times and undiluted, respectively. The 4 times dilution shows a network of Fer-NDF fibers with entrapped Fer-NDF capsules filled with oil while the undiluted sample reveals a high density of covalently connected capsules. The samples were irradiated while cooling with ice and measurements were performed at R.T., the scale bars depict 100 μm . The inset in D shows an overlay image of the situation before irradiation, and clearly free-floating capsules are observed indicating that there is no cross-linking during extrusion (scale bar inset, 20 μm).

occur but since the concentration is lower, the capsules have a larger free diffusion path before the next collision allowing fibers to be formed between the capsules. The fiber formation is probably facilitated by residual Fer-NDF particles in solution which is suggested by the control-sample (Fig. 3A).

The reasoning for the collision-induced cross-linking followed by fiber formation via diffusion of the capsules is supported by the observed deformation of the capsules inside the network. If the capsules were simply entrapped in the fiber-network which is formed in a separate process, the capsules would remain spherical. However, from Fig. 4 it is seen that the capsules are deformed to a great extent. In addition to spherical capsules, Fig. 4 shows an elongated capsule of about 70 μm in length and only a fraction of that wide as seen from the embedded oil-phase (red) on the right of Fig. 4. From this image it can be deduced that the capsules are an integrated part of the fibers. In addition to this highly elastic behavior, the structures also are able to endure mechanical force during the isolation of the structures without losing their basic integrity (SI 1).

Upon a more detailed analysis of the individual stages of sample preparation, the effect of the sample treatment on the size and shape of the structures can be derived (Fig. 5A). First of all, when an emulsion is prepared via simple vortexing, the average diameter obtained is about 36.9 μm (± 10.8) with a large size distribution. When extruded through a 100 nm filter, the average size becomes much smaller 4.2 μm (± 1.4) and more monodisperse. The smaller size means that with the same volume ratio of water:oil, there are more capsules present. When the sample is diluted (20 $\times$) and the shell of the capsule is cross-linked via irradiation, isolated capsules and a few clusters are formed. It can be seen from the average

diameter that the cross-linking induces some changes to the system in the form of stress and likely also induces some coalescence of capsules since the average size increases to 10.0 μm (± 3.8). The semi-diluted sample (4 $\times$) contains capsules with a much larger average diameter in combination with a very large size distribution. Comparing the fluorescence microscope images in Fig. 3 and as stated earlier, this is due to the formation of highly deformed capsules which occurs during the incorporation and formation of the capsule-network. The capsules in the 20 $\times$ diluted- and the non-diluted state still display spherical isotropic capsules and to illustrate the overall deformation of the capsules inside the network the anisotropy of the particles was investigated by measuring the length and the width of the capsules (Fig. 5B). In a perfect isotropic (spherical) system the length divided by the width yields a value of "1" (shown as the gray line in the distribution plot, Fig. 5B). Each point in Fig. 5B represents a capsule and most of the points are above the line indicating that most capsules are anisotropic. While most capsules have a value between 1 and 5, there are a few extremes with a value of up to 25, meaning that the length of the capsule is 25 times larger than the average width (elongated capsule shown in Fig. 4).

2.3. Controlled release

The release of the lipophilic fluorescent dye Nile Red from the oil-phase from covalently connected capsules into the aqueous phase was investigated by UV–Vis spectroscopy. A 90:10 mixture (vol%) of aqueous buffer Fer-NDF solution/BTF Nile Red (1.0 mM) was extruded and irradiated. The supernatant was removed and exchanged for 1.0 ml of fresh PBS buffer (pH 8.4), pure isopropanol

Fig. 4. Fluorescent microscope images of a Fer-NDF network holding capsules that contain an oil phase. On the left the Fer-NDF is selectively imaged and on the right, the oil-phase, scale bars depict 100 μm . It is shown that the capsules are very elastic since elongated non-spherical capsules are observed (inset: overlay image, scale bar 10 μm).

or a 50:50 vol% mixture. The solutions were analyzed over time by absorption measurements and compared to the maximum release of dye which was achieved by completely destroying all capsules by prolonged ultra-sonication treatment. Prolonged treatment with ultra-sound will completely destroy the capsules and therefore release the contents. From these samples, the maximum amount of measurable dye was determined and used as a calibration for calculating the release percentage of the capsule contents.

From Fig. 6A, it is seen that with pure buffer hardly any release is observed over a period of 24 h probably due to the low solubility of the Nile Red in water and therefore the capsule system remains almost unaffected. In a mixture of buffer:isopropanol (50 v/v%), a quick initial release was observed followed by a steady increase towards 90% release after 24 h. In pure isopropanol, an initial fast release was observed, reaching full release of the dye after about 240 min. The fact that in all cases a fast initial release is observed may be explained by incomplete removal of the supernatant. However, the data clearly indicates that the release behavior can be regulated via the polarity of the external medium. The precipitate was still present after 24 h (Fig. 7). Upon investigating the precipitates by fluorescence microscopy, the relative amount of release by the different solvent compositions was confirmed (Fig. 7). In buffer, the system still shows a large amount of densely packed and connected capsules containing high amounts of Nile Red. The system at 50 v/v% buffer:isopropanol displays capsules as well as Fer-NDF precipitate around it (colored green). Finally, the 100% isopropanol system

displays a large precipitate of empty threadlike Fer-NDF structures and hardly any Nile Red fluorescence, confirming the near 100% release. When looking at the average diameter of the capsules still present after 24 h under the different solvent conditions, it is observed that the capsules in buffer are reasonably equal to the freshly prepared structures (Fig. 6B). However, the capsules stored in water/isopropanol (50 v/v%) are significantly smaller as well as fewer in numbers which relates to the incomplete release of the capsule contents. In pure isopropanol no capsules are observed as a complete release has been achieved. The average size of the remaining capsules was obtained by fluorescence microscopy analysis.

3. Conclusions

We have shown that superstructures are easily prepared from a single self-assembling protein-polymer conjugate in combination with a two phase solvent system. Different superstructures are formed by changing the capsule concentration producing small capsules in solution, capsules trapped in a fibrous network and high density covalently connected capsules in a bulk precipitated state. The release of dye from the capsules was controlled by using different buffer/isopropanol mixtures and after a 24 h exposure to the solvent, mostly intact, semi-intact and completely emptied left-over networks of ferritin-polymer compositions were observed, depending on the solvent ratio.

Fig. 5. A) Average diameters of the capsules in different stages of the preparation process. B) Display of the anisotropy of the capsules when embedded inside the protein-polymer network. The gray line displays length/width = 1 which describes a perfect spherical capsule, anything above this line is non-spherical. The inset shows a magnification of the area around the gray line i.e. the isotropic indicator. Two extreme anisotropic capsules are highlighted in the plot indicating that for those the length is 18 and 25 times larger than the width.

Fig. 6. A) Release of hydrophobic compound from the oil-phase into the surrounding medium followed in time. The release is depicted as the percentage of absorption with respect to the maximum absorption upon complete destruction of the capsule system by prolonged treatment with ultra-sound. B) The average diameter of the capsules which still exist after 24 h under different solvent conditions. The actual structures are shown in Fig. 7.

The system has great potential to act as a delivery system of lipophilic compounds embedded in an aqueous system which would be a useful addition to existing epidermal delivery systems but also in formulations where texture and consistency

of encapsulated materials are of great importance *e.g.* in foods and cosmetics [53,54]. Additionally, these PNIPAAm-based hierarchical structures could potentially serve as interesting cell culture systems which has recently been shown [55].

Fig. 7. Structures which remain after 24 h of incubation in solvents of different compositions. The initial states of the samples are the same and are collected from the same bulk sample preparation. After the preparation, the bulk water was removed and exchanged for (A) PBS buffer (pH: 7.4); (B) PBS/Isopropanol 50/50 (vol%); (C) Isopropanol. The release of Nile Red was monitored for 24 h and the remaining structures investigated by (fluorescence-)microscopy. Green (top) shows micrographs of the Fer-NDF structures, Red displays the position of the remaining oil-phase colored with Nile Red and the bottom shows a conventional optical micrograph. In 100% buffer, most structures remain intact and this coincides with the low amount of release of Nile Red. In the 50/50 buffer-isopropanol, few capsules remain together with an empty collapsed Fer-NDF structure. In 100% isopropanol, no capsules are visible and only a network of empty and collapsed Fer-NDF can be found. The coloration of the structures seen in the bright field image originates from the color of the fluorescein-cononomer which by itself has yellow/orange color. Scale bar represents 100 μm.

4. Experimental section

4.1. Materials and characterization

The release experiments were performed on a Thermo scientific Evolution 300 UV–Visible spectrophotometer and the maximum wavelength of absorption was monitored. Destruction of the control-sample which represents the maximum release from the capsule structure was done by placing the glass vial inside an ultrasound bath, Bandelin Sonorex operating at 35 kHz for 60 min at ambient temperature.

For extrusion of the mixtures, an Avanti's mini-extruder device was used in combination with Whatmann track-etch polycarbonate membranes 100 nm pore-size. The number of times of the mixture was passed through the filter was 13 times and was maintained for each sample preparation.

Emulsions were prepared from milli-pore water (pH 9.0, adjusted with NaOH) and Benzotrifluoride (98%) purchased from Sigma–Aldrich. The pH 9.0 was used to guarantee that the Fluorescein-comonomer was kept in one molecular structure since it changes on pH and actually possesses different structures at neutral pH.

UV irradiation of the emulsions was carried out with a 400 W UV lamp (Panacol 400F), emitting light of λ : 315–400 nm and cooling the sample with an ice-bath.

The fluorescent dye Nile Red was purchased from Sigma–Aldrich and used without further purification.

For the optical and fluorescence microscope images a Keyence BZ-8100E was used with excitation-mode for Texas Red ($\lambda_{\text{excitation}}$: 589 nm) and GFP ($\lambda_{\text{excitation}}$: 475 nm) for visualization of the Nile Red and Fluorescein-co-monomer, respectively.

The Fer-p(NIPAAm-DMIAAm-Fluorescein) conjugate was characterized by size exclusion chromatography (SEC) using UV detection ($d = 280$ nm) in Potassium Phosphate buffer (pH 7.4) as eluent with an elution rate of 0.25 ml min^{-1} . UV–Vis analysis of the bulk Fer-NDF conjugate was performed on a Thermo scientific Evolution 300 UV–Visible spectrophotometer.

4.2. Synthesis

Synthesis of the Ferritin-macroinitiator and procedure for polymerization was described in reference 8, similar reaction conditions were used and therefore it is assumed that the same distribution is obtained with the sole exception that a small amount of fluorescent co-monomer was added to the polymerization reaction in slightly different monomer ratio's, though the relative amounts of monomers:catalyst:ligand was maintained as before. *N*-5-Fluorescein-acrylamide was prepared according to a previously reported procedure Ref. [56].

Ferritin-p(NIPAAm₉₀-DMIAAm₁₀-Fluorescein₂) conjugate (Fer-NDF): ATRP (random copolymerization) of NIPAAm-DMIAAm-Fluorescein was performed with a ratio of: monomers (NIPAAm:DMIAAm:Fluorescein; 90:10:2)/initiator/Cu(I)/Cu(II)/ligand = 100/1/0.7/0.3/2. The dialyzed Ferritin macro-initiator was shaken with NIPAAm, DMIAAm and *N*-5-Fluorescein-acrylamide until complete dissolution. In a second flask, CuBr, CuBr₂ and Me₆TREN were mixed, and dissolved in Millipore water. Both solutions were then degassed for 15 min in an ice bath. 0.5 ml of the copper/ligand solution was added to the Ferritin solution under agitation. The polymerization was performed over 24 h to ensure a higher degree of polymerization. The reaction mixture was extensively dialyzed against water (14 kDa cut-off dialysis-tubing) to remove the catalyst and unreacted monomer. The solution was analyzed by UV–Vis and SEC (SI 2).

Acknowledgment

The Alexander von Humboldt-Stiftung (PvR) and the Lichtenberg program of the VolkswagenStiftung (AB) are kindly acknowledged for financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.polymer.2012.10.054>.

References

- [1] a) Hamley IW. Nanotechnology with soft materials. *Angew Chem Int Ed* 2003; 42:1692–712;
b) Nayak S, Lyon LA. Soft nanotechnology with soft nanoparticles. *Angew Chem Int Ed* 2005;44:7686–708;
c) Hendrickson GR, Smith MH, South AB, Lyon LA. Design of multiresponsive hydrogel particles and assemblies. *Adv Funct Mater* 2010;20:1697–712.
- [2] a) Whitesides GM, Grzybowski B. Self-assembly at all scales. *Science* 2002; 295:2418–21;
b) Cohen Stuart MA, Huck WTS, Genzer J, Müller M, Ober C, Stamm M, et al. Emerging applications of stimuli-responsive polymer materials. *Nat Mater* 2010;9:101–13;
c) Fernández-Barbero A, Suárez IJ, Sierra-Martín B, Fernández-Nieves A, de las Nieves FJ, Marquez M, et al. Gels and microgels for nanotechnological applications. *Adv Colloid Interface Sci* 2009;147–148:88–108.
- [3] a) Israelachvili J. *Intermolecular & surface forces*. 2nd ed. London: Academic Press; 1991;
b) Faul CFJ, Antonietti M. Ionic self-assembly: facile synthesis of supramolecular materials. *Adv Mater* 2003;15(9):673–83;
c) Kirby AJ, Camilleri P, Engberts JBFN, Feiters MC, Nolte RJM, Söderman O, et al. Gemini surfactants: new synthetic vectors for gene transfection. *Angew Chem Int Ed* 2003;42:1448–57.
- [4] a) Estroff LA, Hamilton AD. Water gelation by small organic molecules. *Chem Rev* 2004;104:1201–17;
b) Sangeetha NM, Maitra U. Supramolecular gels: functions and uses. *Chem Soc Rev* 2005;34:821–36.
- [5] a) Jain S, Bates FS. On the origins of morphological complexity in block copolymer surfactants. *Science* 2003;300:460–4;
b) Matyjaszewski K, Tsarevsky NV. Nanostructured functional materials prepared by atom transfer radical polymerization. *Nat Chem* 2009;1:276–88.
- [6] Ikkala O, ten Brinke G. Hierarchical self-assembly in polymeric complexes: towards functional materials. *Chem Commun* 2004:2131–7.
- [7] Zeng F, Zimmerman SC. Dendrimers in supramolecular chemistry: from molecular recognition to self-assembly. *Chem Rev* 1997;97:1681–712.
- [8] Mougin NC, van Rijn P, Park H, Müller AHE, Böker A. Hybrid capsules via self-assembly of thermo-responsive and interfacially active bionanoparticle-polymer conjugates. *Adv Funct Mater* 2011;21:2470–6;
Pester CW, Konradi A, Varnholt B, van Rijn P, Böker A. Responsive macroscopic materials from self-assembled cross-linked SiO₂-PNIPAAm core-shell structures. *Adv. Funct. Mater* 2012;22:1724–31.
- [9] Hamley IW, Castelletto V. Biological soft materials. *Angew Chem Int Ed* 2007; 46:4442–55.
- [10] Percec V, Ahn C-H, Ungar G, Yeardley DJP, Möller M, Sheiko SS. Controlling polymer shape through the self-assembly of dendritic side-groups. *Nature* 1998;391:161–4.
- [11] Volodkin DV, Madaboosi N, Blacklock J, Skirtach AG, Möhwald H. Surface-supported multilayers decorated with bioactive materials aiming at light-triggered drug delivery. *Langmuir* 2009;25(24):14037–43.
- [12] a) Liang Y-Z, Li Z-C, Li F-M. Multiple morphologies of molecular assemblies formed by polystyrene-block-poly[2-(β -D-glucopyranosyloxy)ethyl acrylate] in water. *New J Chem* 2000;24:323–8;
b) Bellomo EG, Wyrsta MD, Pakstis L, Pochan DJ, Deming TJ. Stimuli responsive polypeptide vesicles via conformation specific assembly. *Nat Mater* 2004;3(4): 244–8.
- [13] a) Meng F, Zhong Z, Feijen J. Stimuli-responsive polymersomes for programmed drug delivery. *Biomacromolecules* 2009;10(2):197–209;
b) Minkenberg CB, Florusse L, Elkekema R, Koper GJM, van Esch JH. Triggered self assembly of simple dynamic covalent surfactants. *J Am Chem Soc* 2009; 131:11274–5.
- [14] a) Muñoz-Bonilla A, Fernández-García M, Haddleton DM. Synthesis and aqueous solution properties of stimuli-responsive triblock copolymers. *Soft Matter* 2007;3:725–31;
b) van Rijn P, Janeliunas D, Brizard AMA, Stuart MCA, Koper GJM, Elkekema R, et al. Self-assembly behaviour of conjugated terthiophene surfactants in water. *New J Chem* 2011;35:558–67;
c) Kuckling D, Vo CD, Adler H-JP, Völkel A, Cölfen H. Preparation and characterization of photo-crosslinked thermo-sensitive PNIPAAm nanogels. *Macromolecules* 2006;39:1585–91.

- [15] a) de Jong JJD, Lucas LN, Kellogg RM, van Esch JH, Feringa BL. Reversible, light-driven, transcription of supramolecular into molecular chirality. *Science* 2004;304:278; b) de Jong JJD, van Rijn P, Tiemersma-Wegeman TD, Lucas LN, Browne WR, Kellogg RM, et al. Dynamic chirality, chirality transfer and aggregation behavior of dithienylethene switches. *Tetrahedron* 2008;64:8324–35; c) Peng K, Tomatsu I, Kros A. Light controlled protein release from a supramolecular hydrogel. *Chem Commun* 2010;46:4094–6.
- [16] a) Shklyarevskiy IO, Jonkheijm P, Christianen PCM, Schenning APHJ, Meijer EW, Henze O, et al. Magnetic deformation of self-assembled sexithiophene spherical nanocapsules. *J Am Chem Soc* 2005;127:1112–3; b) Schmidt K, Schobert HG, Ruppel M, Zettl H, Hänsel H, Weiss TM, et al. Reversible tuning of a block copolymer nanostructure via electric fields. *Nat Mater* 2008;7:142–5; c) Pester CW, Ruppel M, Schobert HG, Schmidt K, Liedel C, van Rijn P, et al. Piezoelectric properties of non-polar block copolymers. *Adv Mater* 2011;23:4047–52.
- [17] Chen H, Zhao Y, Song Y, Jiang L. One-Step multicomponent encapsulation by compound-fluidic electrospray. *J Am Chem Soc* 2008;130:7800–1.
- [18] Li Z, Kesselman E, Talmon Y, Hillmyer MA, Lodge TP. Multicompartment micelles from ABC miktoarm stars in water. *Science* 2004;306:98–101.
- [19] Cölfen H, Mann S. Higher-order organization by mesoscale self-assembly and transformation of hybrid nanostructures. *Angew Chem Int Ed* 2003;42:2350–65.
- [20] a) Hirst AR, Smith DK. Two-component gel-phase materials – highly tunable self-assembling systems. *Chem Eur J* 2005;11:5496–508; b) Brizard A, Stuart M, van Bommel K, Friggeri A, de Jong M, van Esch JH. Preparation of nanostructures by orthogonal self-assembly of hydrogelators and surfactants. *Angew Chem Int Ed* 2008;11:2063–6.
- [21] Cayre OJ, Noble PF, Paunov VN. Fabrication of novel colloidosomes microcapsules with gelled aqueous cores. *J Mater Chem* 2004;14:3351–5.
- [22] Boal AK, Ilhan F, DeRouchey JE, Thurn-Albrecht T, Russell TP, Rotello VM. Self-assembly of nanoparticles into structured spherical and network aggregates. *Nature* 2000;404:746–8.
- [23] Segalman RA. Patterning with block copolymer thin films. *Mater Sci Eng* 2005;R48:191–226.
- [24] Ye J, Wang C, Su P, Wu H, Zhou Y, Yao K, et al. Biochemically active hydrosol as a means of collecting electrosprayed microcapsules for drug delivery. *J Mater Chem* 2010;20:9025–8.
- [25] Wetz K, Crichton R. Chemical modification as a probe of the topography and reactivity of horse-spleen apoferritin. *Eur J Biochem* 1976;61:545–50.
- [26] Farrant JL. An electron microscopic study of ferritin. *Biochim Biophys Acta* 1954;13:569–76.
- [27] Silk S, Breslow E. Hydrogen ion interactions of horse spleen ferritin and apoferritin. *J Biol Chem* 1976;251:6963–73.
- [28] Mainwari W, Hoffmann T. Horse spleen apoferritin: N-terminal and C-terminal residues. *Arch Biochem Biophys* 1968;125:975–80.
- [29] a) Hannink JM, Cornelissen JJLM, Farrera JA, Foubert P, De Schryver FC, Sommerdijk NAJM, et al. Protein–polymer hybrid amphiphiles. *Angew Chem Int Ed* 2001;40:4732–4; b) Meldrum FC, Heywood BR, Mann S. Magnetoferritin: in vitro synthesis of a novel magnetic protein. *Science* 1992;257:522–3; c) Aime S, Frullano L, Geninatti Crich S. Compartmentalization of a gadolinium complex in the apoferritin cavity: a route to obtain high relaxivity contrast agents for magnetic resonance imaging. *Angew Chem* 2002;114(6):1059–61; d) Kasyutich O, Ilari A, Fiorillo A, Tatchev D, Hoell A, Ceci P. Silver ion incorporation and nanoparticle formation inside the cavity of *Pyrococcus furiosus* ferritin: structural and size-distribution analyses. *J Am Chem Soc* 2010;132:3621–7.
- [30] Zeng Q, Reuther R, Oxshar J, Wang Q. Characterization of horse spleen apoferritin reactive lysines by MALDI-TOF mass spectrometry combined with enzymatic digestion. *Bioorg Chem* 2008;36:255–60.
- [31] Bontempo D, Heredia KL, Fish BA, Maynard HD. Cysteine-reactive polymers synthesized by atom transfer radical polymerization for conjugation to proteins. *J Am Chem Soc* 2004;126:15372–3.
- [32] Lutz J-F, Börner HG, Weichenhan K. Combining ATRP and “click” chemistry: a promising platform toward functional biocompatible polymers and polymer bioconjugates. *Macromolecules* 2006;39:6376–83.
- [33] Wang Q, Raja KS, Janda KD, Lin T, Finn MG. Blue fluorescent antibodies as reporters of steric accessibility in virus conjugates. *Bioconjug Chem* 2003;14:38–43.
- [34] Nicolas J, San Miguel V, Mantovani G, Haddleton DM. Fluorescently tagged polymer bioconjugates from protein derived macroinitiators. *Chem Commun* 2006;45:4697–9.
- [35] Venkataraman S, Wooley KL. ATRP from an amino acid-based initiator: a facile approach for α -functionalized polymers. *Macromolecules* 2006;39:9661–4.
- [36] Börner HG. Functional polymer-bioconjugates as molecular LEGO®-Bricks. *Macromol Chem Phys* 2007;208:124–30.
- [37] Albertin L, Stenzel MH, Barner-Kowollik C, Foster IJR, Davis TP. Well defined diblock-glycopolymers from RAFT polymerization in homogenous aqueous media. *Macromolecules* 2005;38:9075–84.
- [38] Millard P-E, Mougin NC, Böker A, Müller AHE. Fast ATRP of N-isopropylacrylamide in water and its application to bioconjugates. *Polym Prepr (Am Chem Soc Div Polym Chem)* 2008;49(2):121.
- [39] a) Edmondson S, Osborne VL, Huck WTS. Polymer brushes via surface-initiated polymerizations. *Chem Soc Rev* 2004;33:14–22; b) Nicolas J, Mantovani G, Haddleton DM. Living radical polymerization as a tool for the synthesis of polymer-protein/peptide bioconjugates. *Macromol Rapid Commun* 2007;28:1083–111; c) Hu Y, Samanta D, Parekar SS, Hong SW, Wang Q, Russell TP, et al. Ferritin–polymer conjugates: grafting chemistry and integration into nanoscale assemblies. *Adv Funct Mater* 2010;20:3603–12.
- [40] van Rijn P, Mougin NC, Franke D, Park H, Böker A. Pickering emulsion templated soft capsules by self-assembling cross-linkable ferritin–polymer conjugates. *Chem Commun* 2011;47:8376–8.
- [41] Raja KS, Wang Q, Gonzalez MJ, Manchester M, Johnson JE, Finn MG. Hybrid virus–polymer materials. 1. Synthesis and properties of PEG-decorated cowpea mosaic virus. *Biomacromolecules* 2003;4:472–6.
- [42] Lele B, Murata H, Matyjaszewski K, Russell AJ. Synthesis of uniform protein–polymer conjugates. *Biomacromolecules* 2005;6:3380–7.
- [43] Bontempo D, Li RC, Ly T, Brubaker C, Maynard HD. One-step synthesis of low polydispersity, biotinylated poly(N-isopropylacrylamide) by ATRP. *Chem Commun* 2005;4702–4.
- [44] Heredia KL, Bontempo D, Ly T, Byers JT, Halstenberg S, Maynard HD. In-situ preparation of protein-“smart” polymer conjugates with retention of bioactivity. *J Am Chem Soc* 2005;127:16955–60.
- [45] Broyer RM, Quaker GM, Maynard HD. Designed amino acid ATRP initiators for the synthesis of biohybrid materials. *J Am Chem Soc* 2008;130:1041–7.
- [46] Tangirala R, Revanur R, Russell TP, Emrick T. Sizing nanoparticle-covered droplets by extrusion through track-etch membranes. *Langmuir* 2007;23:965–9.
- [47] Fujii S, Aichi A, Muraoka M, Kishimoto N, Iwahori K, Nakamura Y, et al. Ferritin as a bionano-particulate emulsifier. *J Colloid Interface Sci* 2009;338:222–8.
- [48] Lin Y, Skaff H, Böker A, Dinsmore AD, Emrick T, Russell TP. Ultrathin cross-linked nanoparticle membranes. *J Am Chem Soc* 2003;125:12690–1.
- [49] Mueggenburg KE, Lin X-M, Goldsmith RH, Jaeger HM. Elastic membranes of close-packed nanoparticle arrays. *Nat Mater* 2007;6:656–60.
- [50] Thompson KL, Armes SP. From well-defined macromonomers to sterically-stabilised latexes to covalently cross-linkable colloidosomes: exerting control over multiple length scales. *Chem Commun* 2010;46:5274–6.
- [51] Böker A, He J, Emrick T, Russell TP. Self-assembly of nanoparticles at interfaces. *Soft Matter* 2007;3:1231–48.
- [52] a) Russell JT, Lin Y, Böker A, Su L, Carl P, Zettl H, et al. Self-assembly and cross-linking of bionanoparticles at liquid-liquid interfaces. *Angew Chem Int Ed* 2005;44:2420–6; b) Uchida M, Klem MT, Allen M, Suci P, Flenniken M, Gillitzer E, et al. Biological containers: protein cages as multifunctional nanoplateforms. *Adv Mater* 2007;19:1025–42; c) Kostiainen MA, Pietsch C, Hoogenboom R, Nolte RJM, Cornelissen JJLM. Temperature-switchable assembly of supramolecular virus–polymer complexes. *Adv Funct Mater* 2011;21:2012–9.
- [53] Augustin MA, Hemar Y. Nano- and micro-structured assemblies for encapsulation of food ingredients. *Chem Soc Rev* 2009;38:902–12.
- [54] Yow HN, Routh AF. Formation of liquid core–polymer shell microcapsules. *Soft Matter* 2006;2:940–9.
- [55] Nash ME, Carroll WM, Nikoloskya N, Yang R, O’Connell C, Gorelov AV, et al. Straightforward, one-step fabrication of ultrathin thermoresponsive films from commercially available pNIPAm for cell culture and recovery. *ACS Appl Mater Interfaces* 2011;3:1980–90.
- [56] Martin MM, Lindqvist L. pH-dependence of fluorescein fluorescence. *J Lumin* 1975;10:381–90.