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COMMUNICATION

Pickering emulsion templated soft capsules by self-assembling cross-linkable ferritin–polymer conjugates†

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Oil-in-water (o/w) and water-in-oil (w/o) Pickering emulsions were prepared using ferritin–polymer conjugates. UV cross-linking stabilised the particle assembly around the fluid droplets. The resulting soft protein–polymer capsules were transferred to a medium of equal polarity as the inside of the capsule, creating water-in-water (w/w) and oil-in-oil (o/o) capsules.

Self-assembly of molecular components and nanoparticles has been used to stabilise emulsions, form membranes and prepare capsules.^{1,2} Especially the latter is of great interest as these structures are used by many different industrial branches in a large variety of consumer goods, *e.g.* drug delivery, perfumes, foods or inks.² Over the years, many different approaches have been devised to prepare capsules, in particular ones with liquid interior. A natural approach is using phospholipids which are able to form spherical bilayers with aqueous cores. Their structure can be stabilised by addition of small molecules,³ inorganic components⁴ or by polymers.⁵ Other variations on the self-assembled hollow spherical structure have been designed based on polymers⁶ by using *e.g.* amphiphilic polymers,⁷ polymer microgels,⁸ layer-by-layer deposition of interacting polymers,⁹ surface polymerisation¹⁰ or interfacial polymerisation by polycondensation.¹¹ Even though interesting capsules can be formed with polymers, the liquid core is often organic, and although aqueous cores have been reported, the preparation is laborious.²

These days, emulsion stabilisation and capsule formation using nanoparticles, so called Pickering emulsions, is seen as a highly promising approach. These particles owe their stabilising effect to high attachment energies at liquid–liquid interfaces and range from inorganic particles¹² to proteins¹³ and polymer nanoparticles^{14,15} and led to interesting architectures like colloidosomes and yeastosomes.¹⁶ The use of particles as stabiliser results in oil-in-water (o/w) as well as water-in-oil (w/o) emulsions depending on the polarity of the particle¹⁷ and therefore both a polar and an apolar liquid core can

be obtained. Though the formed emulsions might be very stable, addition of co-solvents or transferring isolated emulsion droplets to a phase with equal polarity as the inner compartment will break the emulsion. In order to circumvent this loss in stability, nanoparticles be it biological (proteins, viral), metallic or polymer have been combined with cross-linkers.¹⁸ These cross-linkers are able to connect the particles in the emulsion shell and stabilise the emulsion to such an extent that they also can be used as stable capsules or even membranes.¹⁹ Stabilised interfaces have been produced by combining biotin-functionalised viral particles in combination with streptavidin,²⁰ CdSe-nanoparticles cross-linked with vinylbenzene moieties attached to the surface¹⁹ or by attaching polymerisable groups suitable for ring-opening metathesis polymerisation using Grubbs' catalyst.²¹ Though these structures are very interesting and yield insights into the use of nanoparticles for capsule formation, still they lack additional function to make them suitable for applications with respect to controlled release, carrier for catalysts or make them responsive to external stimuli like pH, temperature or ionic strength of the medium, which is still considered a major challenge in the preparation of responsive and well defined capsules.^{8,9,22}

Here we report on a bionanoparticle–polymer hybrid material recently developed by our group.²³ It consists of a ferritin protein cage which is decorated on the outside with PNIPAAm/DMIAAm co-polymers (poly(*N*-isopropyl acrylamide)/2-(dimethyl maleinimido)-*N*-ethyl-acrylamide) *via* ATRP (atom transfer radical polymerisation). Due to their high affinity towards polar–apolar interfaces, the ferritin–PNIPAAm conjugates were found to stabilise emulsions very efficiently even at low concentrations.²³ These Pickering emulsions, in which the stabilisers are particles instead of surfactants, are stabilised further *via* cross-linking the self-assembled protein–polymer shell (S1, ESI†). Therefore, in addition to the well-known PNIPAAm, a photo-responsive cross-linker moiety²⁴ (DMIAAm) was copolymerised.

By using a cross-linkable comonomer, no additional cross-linker moieties need to be added as seen in other systems,^{18,25} giving a more controlled system which does not rely on the solubility of a second component in one of the liquid phases. Upon irradiation, DMIAAm undergoes a [2 + 2]-photocyclisation reaction and thereby connecting the stabilising particles covalently. This stabilisation allows not only the preparation of highly stabilised o/w and w/o emulsions

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(S1, ESI†), but also, due to the high stability and presumably shell elasticity, it is possible to transfer the minority phase to an environment with the same polarity, *i.e.* oil to oil and water to water. Normally, the capsule would be destroyed due to the loss of a stabilising interface. The presence of PNIPAAm in the newly formed capsules renders the system responsive towards temperature, pH and ionic strength. Also, the protein cage ferritin is an integral part of the shell and gives the possibility to selectively remove it by denaturation, creating 12 nm sized pores inside the shell structure. It can also potentially act as an additional container²⁶ to hold other active materials.

At first, emulsions were prepared by addition of oil to water or water to oil of which the water-phase contains ~ 10 and ~ 400 mg ml⁻¹ of ferritin-PNIPAAm₉₅-DMIAAm₅, respectively. (The subscripts denote the average degree of polymerisation of each component.) The higher concentration for the w/o is necessary because of the reduced amount of available conjugate as the water phase constitutes the minority component. After prolonged agitation, the emulsions were cooled to 0 °C and subsequently irradiated by UV-light (S1, ESI†). This initiates the cross-linking of the polymer chains and forms a covalently bound protein-polymer shell around the droplet surface. The emulsions were subsequently analysed by (fluorescence) microscopy. In order to observe these in fluorescence mode, different fluorescent dyes were added to the oil- and water-phase. To the oil-phase Nile Red was added which is detected in the red upon irradiation and to the water-phase amino-fluorescein was added which is detected in the green. Two different types of emulsions were prepared, an o/w, in which benzotrifluoride (BTF) was added to water and a w/o where water was added to toluene (Fig. 1). Two different types of oil were used because it was found that the emulsion was best prepared and the transfer of the emulsion droplets from one phase to the other was easier when the minority phase had a higher density than the majority phase. In Fig. 1 overlay fluorescence micrographs of the two emulsions are shown (the non-overlay fluorescence micrographs are shown in S2 and S3, ESI†). Micrographs were taken of the same area while selectively exciting either Nile Red (oil-phase, red) or fluorescein (aqueous phase, green). The two fluorescence micrographs were projected on top of one another to display the location of the different phases. The o/w emulsion displays droplets between 10–50 μ m where the centre is coloured red and the outer-phase green (Fig. 1A). At first sight, the rim of the droplet displays a more intense green colouration than the main phase. This was confirmed by analysis of the emission intensity (S4, ESI†) along the equator of the droplet. Indeed the rim displays a much higher intensity at the edges of the droplets than on the outside. This suggests that the cross-linked protein-polymer shell takes up the hydrophilic fluorescent dye. Also it is clearly seen that the increase in fluorescence of the Nile Red begins at the edge of the droplet and is strongest in the centre, showing that it is nicely located inside the capsule.

The same data evaluation was performed for the w/o emulsion. The emulsion showed a larger size distribution of about 10–100 μ m and here the bulk oil-phase is coloured red and the aqueous droplets green (Fig. 1B). The emission profile

Fig. 1 Fluorescence microscopy (overlay) images of emulsion of (A) 10 μ L BTF + Nile Red (0.01 mM) in 1000 μ L water + fluorescein (0.01 mM) with ~ 10 mg ml⁻¹ Fer-PNIPAAm-DMIAAm, cross-linked; inset: the emission intensity profile across a capsule (black dotted line), the red line corresponds to the Nile Red emission and the green to fluorescein; (B) 10 μ L water + fluorescein (0.01 mM) with ~ 400 mg ml⁻¹ Fer-PNIPAAm-DMIAAm, in 1000 μ L toluene + Nile Red (0.01 mM), cross-linked; inset: the emission intensity profile across a capsule (black dotted line). (C) Capsules of A, collected and re-dispersed in water without fluorescein, collected and re-dispersed in BTF. (D) Capsules of B, collected and re-dispersed in toluene, collected and re-dispersed in water without fluorescein. The inset shows the bright field image of the w/w capsules. Measurements were performed at room temperature, scale bars represent 100 μ m.

across a droplet shows that the emission intensity of the Nile Red is strongly reduced once the edge of the droplet is reached and that the inside of the droplet is coloured brightly green which is nearly the same intensity over the entire area of the droplet (S4, ESI†). No significant uptake of fluorescent dye by the shell was observed here.

The cross-linking of the protein-polymer shell around the formed droplet allows it to endure harsher conditions compared to ordinary emulsions. The emulsion droplets shown in Fig. 1 were allowed to sediment after which the top phase was removed and the emulsion was washed once with the pure solvent without any fluorescent dye. Afterwards the procedure was repeated using the pure phase of which the droplets are composed, also without fluorescent dye. This transfer resulted in dye filled oil droplets encapsulated by the protein-polymer shell in oil (Fig. 1C) and encapsulated water droplets in water (Fig. 1D). The oil capsules in oil nicely show a homogeneous coloration across the droplets while this is not the case for the water droplets. Here the dye is located on the inside as well as the outside of the droplet with a slightly increased concentration in the rim of the droplets, again displaying that there is an affinity for the uptake of the dye by the shell as was mentioned earlier. The coloration of the outer phase in the w/w system indicates that the capsule membrane allows for diffusion. This diffusion past the capsule shell is not observed in the o/o system. PNIPAAm is often used in micro-gel structures meaning that it is able to hold a significant amount of water. The water entrapped in the shell obviously acts as an efficient barrier preventing the coloured oil-phase or the hydrophobic Nile Red from diffusion past the

Fig. 2 (A) shows an overlay of an optical and fluorescence image of a large dried capsule with distinct features of a soft capsule obtained from a w/o emulsion as described earlier only containing fluorescein in the aqueous phase, scale bar 100 μm . A dried capsule from an emulsion of 10 μL BTF in 1000 μL water with $\sim 10 \text{ mg ml}^{-1}$ Fer–NIPAAm–DMIAAm, cross-linked and imaged by (B) SFM height image $32 \times 32 \mu\text{m}^2$. Measurements were performed at room temperature.

capsule shell. The fact that the capsules remain visible after exchanging the medium with opposite polarity again points to the fact that they are highly stable.

In solution, the capsules are spherical (Fig. 1) and stable when transferred to a medium which has the same polarity as the inside of the capsule. When the capsules are dried and sufficiently large, the contours of the ferritin–polymer shell could be visualised by optical microscopy. Fig. 2A shows an overlay image of an optical and fluorescence micrograph of a large dried capsule from a w/o emulsion of which the aqueous phase contains fluorescein. The contour is clearly visible and in the middle of the capsule wrinkles are seen which is typical for a soft matter capsule. The soft matter composition of the capsules was further investigated by Scanning Force Microscopy (SFM) and Transmission Electron Microscopy (TEM) (Fig. 2B/S5, ESI†). An air-dried cross-linked emulsion of BTF in water, analysed by SFM, displayed a more detailed image of the collapsed surface of the capsule (Fig. 2B). A cross-section of the height image shows large deviations in thickness due to the wrinkled structure. While at the top of a wrinkle the thickness may increase to 150 nm, the relatively homogeneous flat areas are as thin as 15 nm, corresponding to a shell thickness of about 7.5 nm (S5, ESI†) which is an average over the ferritin and polymer. Analysis by TEM shows a similar collapsed structure with variations in contrast at the wrinkles where a high contrast is observed and low contrast in the flat area, indicating only a very thin layer. Closer inspection shows the iron-cores of the ferritin embedded in the capsule shell (S6, ESI†) providing evidence that the protein cages remain intact during capsule formation. Many are seen on top of the wrinkles where the shell is collapsed and dense while a low number of iron-cores are observed at the flat regions, indicating that long polymer chains are attached to the protein structures.

We have shown a versatile and straightforward approach for the formation of stable capsules. All possible combinations are accessible: polar/apolar, apolar/polar, polar/polar, apolar/apolar. These combinations allow for any type of active

component to be encapsulated be it hydrophobic or hydrophilic. The different release mechanisms are currently being investigated.

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