Permeability of silk microcapsules made by the interfacial adsorption of protein†

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The assembly of colloidal particles at a liquid/liquid interface is a useful technique for the formation of a large variety of structures. Recently, we created a new method which uses liquid/liquid interfaces to assemble recombinant silk proteins into thin-shelled microcapsules. These microcapsules are mechanically stable and well suited to applications such as enzyme therapy and artificial cells. In this paper the permeability properties of these microcapsules are investigated using a novel measurement technique. It is found that the microcapsules are polydisperse in their permeabilities, but for all measured microcapsules the permeability is in the range required to protect encapsulants from immunoglobulin proteins, while allowing small molecules to enter the capsule freely.

1. Introduction

The controlled assembly at emulsion interfaces is a new technique which allows for the formation of structures using a wide range of colloidal materials. Interfacial assembly has been used to produce structures from colloidal polymers,[1–3] CdSe quantum dots,[4,5] nanoparticles,[6,7] polymeric microrods,[8] heterodimer particles,[9] and viruses.[10] The generality of interfacial assembly makes it a powerful tool for the formation of a large variety of structures. Among the structures which can be formed through interfacial adsorption, one of the most ecumenical is that of the thin-shelled vesicle. In applications such as drug delivery, flavor encapsulation, catalysis and artificial organs, thin-shelled vesicle microcapsules are of interest due to their ability to protect an active ingredient from the surrounding environment. Proteinaceous and polymeric materials are most often explored for microcapsule constructs because of their biocompatibility and functionalizability.

Recently, the assembly of recombinant spider-silk proteins at an emulsion interface has been used to form a thin-shelled microcapsule, 3 to 80 microns in size.[11] In this technique the emulsion interface is used to orient peptide sequences in the adsorbed polypeptide and subsequently immobilize the peptide chains by non-covalent interactions. Because a stable polypeptide film is formed, the structures can be removed from the emulsion and inserted into a single-phase solution. The final spider-silk shell has an aqueous core and is dispersed in an aqueous medium. High-molecular-weight dextrans can be encapsulated within the silk shell, while small molecules permeate freely.

Micron-size capsules, such as the ones produced through this method, are often explored as a way to encapsulate enzymes or even entire microorganisms.[12] For these applications, it is usually desirable to protect the encapsulated materials from antibodies (IgG M_W = 146 kDa) and white blood cells, while enabling small molecules such as nutrients, hormones and small peptides to permeate the capsule freely. Protecting the encapsulated material in this manner requires that the microcapsules are selectively permeable to molecules of a certain size. Therefore, microcapsule membranes are often characterized with a molecular-weight cutoff (MWCO) which defines the largest size of molecules which can pass through the membrane.

A number of methods have been developed to measure capsule permeability. In the most common method a marker is first encapsulated by the microcapsules. The capsules are then removed and the amount of marker released into the surrounding medium is measured.[13,14] This technique is a direct measure of the molecular size which can be encapsulated, but it is too slow to test a large number of different markers and provides no information on inhomogeneities between the capsules in the dispersion. Size-exclusion chromatography (SEC) can also be used to measure capsule permeability by using the capsules for column packing and measuring the retention time of differently-sized polymers as they pass through the column.[15,16] This method allows for a rapid screening of different permeates and is capable of measuring the distribution of pores within a sample. However, this determination is often complicated by artifacts caused by dead volume within the column, and the measured pore size is more representative of the average microcapsule pore size and not the maximum pore size. A third method which has been employed is to expose a capsule dispersion to differently-sized fluorescent polymers and observe with a confocal microscope which polymers permeate the membrane.[17] Until now this method has been used as a binary determination of capsule permeability, where each fluorescent marker was observed to

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either completely permeate a capsule or be completely excluded. One disadvantage of this method is that it can be slow, because a large number of different markers must be used for a precise determination of capsule permeability.

A more efficient way to measure permeability using fluorescent markers is to expose a capsule dispersion to a polydisperse sample of dextran and observe what fraction of the dextran permeates the capsule. The molecules which permeate the capsule will be the fraction of the dextran which is lower in molecular weight than the MWCO. If the molecular-weight distribution of the polymer is known, an accurate determination of the MWCO can be made. This technique has the advantage that it can be done quickly and can be used to observe differences between capsules within the same dispersion. Here, a polydisperse fluorescently-labeled dextran is added to dispersions of microcapsules made from spider silk. The MWCO of the capsule membranes and the distribution of MWCOs in a capsule dispersion are determined by measuring the fraction of dextran which permeates each capsule.

2. Experimental

2.1 Protein synthesis

Dragline-silk protein ADF-4 from the garden spider *Araneus diadematus* has been used as a template for the spider-silk construct, C_{16}, which was engineered for bacterial expression.[18–20] The repetitive part of ADF-4 is generally composed of a single conserved repeat unit displaying only slight variations. These variations were combined to from one consensus module termed C (GSSAAAAAAAASGPGGYGPENQ-GPSGPGGYGPGGP), which was multimerized to obtain the repetitive protein C_{16}. The resulting C_{16} protein has a molecular mass of 48 kDa.[19]

The C_{16} silk gene was expressed in the *Escherichia coli* strain BLR [DE3] (Novagen). Cells were grown at 37 °C in Luria–Bertani medium to an optical density of 0.5 measured at a wavelength of 600 nm. Before induction with 1 mM IPTG (isopropyl-ß-D-thiogalactoside), cells were shifted to 25 °C. Cells were harvested after 3–4 hours of induction. C_{16} protein was purified and protein identity and purity were assessed as described by Huemmerich *et al.*[19]

2.2 Microcapsule preparation

Microcapsules were made from recombinantly produced spider-silk protein C_{16} in a manner similar to those made previously.[11] The protein solution from which the microcapsules were formed was prepared by first dissolving lyophilized C_{16}-silk protein at a concentration of 10 mg mL^{-1} in 6 M guanidine thiocyanate. The protein solution was then cooled to 4 °C and the protein solution was dialyzed against 10 mM Tris (hydroxymethyl) aminomethane–HCl, pH 8.0, overnight using dialysis tubing from Carl Roth GmbH & Co. with a molecular-weight cutoff of 14 kDa. Any protein which was not dispersed was removed by centrifuging the dialyzed solution for 30 minutes at 100 000g at 4 °C. The final protein concentration was determined using UV adsorption. The recombinant protein has a molar-extinction coefficient of 46 400 M^{-1} cm^{-1} at a wavelength of 276 nm.

The microcapsules were formed by emulsifying 5 μL of dialyzed protein solution in 300 μL toluene for 90 seconds. Silk microcapsules were formed using protein solutions with concentrations ranging from 2 to 3 mg mL^{-1} and with emulsification times as short as 30 seconds. The size of the microcapsules depends on the size of the emulsion droplets. Once formed, the polymer shells surrounding the emulsion droplets were transferred from the two-phase emulsion into a one-phase solution. To transfer the capsules into a one-phase solution, 300 μL of water was added to the toluene to form an aqueous sublayer. The polymer shells surrounding the water droplets were then centrifuged from the toluene layer into the aqueous sublayer at a force of 100g for 4 minutes. After the microcapsules were transferred to a one-phase solution, the microcapsules were investigated with a Zeiss Axiovert 200 M optical microscope.

2.3 Permeability measurement

Microcapsule permeability was measured by adding 20, 40 and 70 kDa FITC-labeled dextran (FD20, FD40, FD70, Sigma-Aldrich) at a concentration of 0.67 wt% to the aqueous microcapsule dispersion. In order to eliminate any charge effects resulting from trapped, unabsorbed spider silk, NaCl was added to the silk-microcapsule suspension until a concentration of 0.1 M NaCl was reached. Before observation with a confocal microscope the dextran concentration in the microcapsules was equilibrated for twelve hours at room temperature. A Zeiss LSM 510 confocal microscope was used for measuring the dextran concentration inside and outside the microcapsules. A zeta stack of each microcapsule was first performed at a scan rate of 1.6 μs per pixel, where each line was averaged 16 times, and a frame distance between 0.4 and 0.6 μm. Close to the top and bottom of the microcapsule the measured fluorescent intensity was equal to that of the external medium. At confocal slices closer to the center of the capsule, the fluorescent intensity was observed to decrease quickly to a constant value. To determine the amount of dextran which permeated the capsule, the fluorescent intensity at the center of the capsule was compared to the fluorescent intensity of the surrounding medium at the same focal depth. The dextran molecular weight which permeates the capsule is determined by comparing the fluorescence measured by the confocal with the cumulative fluorescence molecular-weight distribution as measured by gel-permeation chromatography. The fraction of dextran which permeates the capsules was measured for 59 different capsules from 16 different samples, all prepared in the same manner.

2.4 Molecular-weight measurement

The dextran molecular weight was measured using an Agilent 1100 gel-permeation chromatograph (GPC) with both ultraviolet-adsorption and refractive-index detectors. The GPC was equipped with one precolumn (Polymer Standards Suprema SUA080520) and three analytical columns (Polymer Standards Suprema SUA0830101E2, SUA0830101E3 and SUA0830103E3) which allow the GPC to separate polymers with sizes between 100 and 1.6×10^6 Da. The GPC molecular-

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weight calibration was done using 14 calibration standards ranging in molecular weight from 180 to 4.01×10^{-4} Da.

For sample measurements 3.0 g L^{-1} of the FITC-labeled dextran was dissolved in 0.1 M NaNO$_3$ overnight. The sample solution was filtered through a 1.0 μm filter to remove any undissolved dextran and dust particles, and then eluted through the column with 0.1 M NaNO$_3$ at a flow rate of 1.0 mL min^{-1}. The dextran molecular weight was measured using a refractive-index detector, and the amount of fluorescent labeling at each molecular weight was measured using the ultraviolet-adsorption detector by observing FITC adsorption at 488 nm.

3. Results and discussion

Proteins would be the most appropriate solute for measuring microcapsule permeability. However, the characterization of capsules with differently-sized proteins is difficult because a number of additional factors including protein shape, charge and hydrophobicity affect protein permeability. Therefore, polymers of different lengths are often used for microcapsule permeability measurements.[15,21,22] Polymers such as dextran are ideal because they are uniform in chemical composition and shape, and form a random coil structure in solution. Polysaccharides also have the additional advantage that the size can be continually varied by increasing molecular weight. Polysaccharides are commonly used to assess microcapsule permeability. It has been shown that in order to exclude biological molecules of the same size as immunoglobulin antibodies ($\sim$150 kDa) the capsules should be able to exclude 70 kDa dextran.[22]

Most microcapsule permeability measurements which use dextran are made with a series of dextrans of narrow polydispersity. Capsules will typically exclude a dextran with a molecular weight higher than the MWCO and allow the permeation of a dextran lower than the MWCO. These methods are used to determine the range of the MWCO. If a polydisperse dextran with a molecular weight close to the MWCO is used for permeability measurements, the fraction of the dextran which is less than the MWCO will permeate the capsule, and the fraction of the dextran which is greater than the MWCO will be excluded.

When 40 kDa FITC-dextran at a concentration of 0.67 wt% is added to the outside of the silk microcapsule dispersion, the dextran undergoes partial permeation (Fig. 1). To observe and measure the fluorescence intensity inside the microcapsules accurately, a confocal microscope was used to reduce the
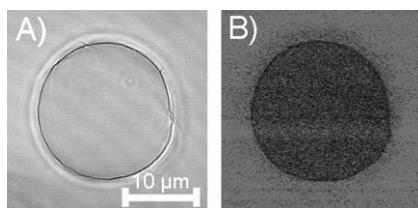

Fig. 1 Microcapsule after the addition of 40 kDa FITC-dextran to the outside of the dispersion. (a) Brightfield microscopy image of microcapsule. (b) Confocal-laser scanning microscopy image of internal microcapsule fluorescence.
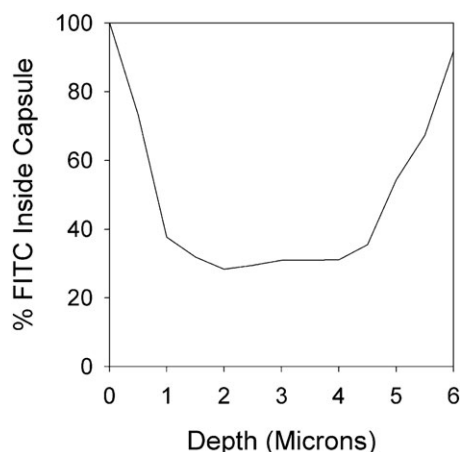

Fig. 2 The percentage of added FITC-dextran observed as a function of capsule depth. Fluorescence at the center of the capsule is constant.

focal-plane depth below the capsule size and eliminate out-of-focus light from the surrounding media. Fig. 2 shows the observed fluorescent intensity within a microcapsule as a function of microcapsule depth. At focal planes close to the capsule surface the measured intensity is affected by fluorescence from the surrounding media, while at focal planes close to the center of the capsule the measured intensity is constant, indicating that out-of-focus fluorescence from the surrounding media is negligible. To further verify that measured intensities were not affected by background fluorescence and ambient light, measurements were also made on solid 5 μm silica particles. When 0.67 wt% 40 kDa dextran was added to a dispersion of silica particles, the measured intensity inside the silica particles was less than 4% of the fluorescence of the surrounding media.

In order to measure the MWCO, three different-molecular-weight dextrans were added to the outside of different capsule dispersions. The fraction of the dextran which permeated the capsules increased with decreasing molecular weight. For all three of the dextrans the permeability varied between capsules. On average 12% of the 70 kDa dextran permeated the capsule, while for the 40 and 20 kDa dextrans, 34 and 66% of the dextran permeated on average, respectively. After scanning each capsule multiple times, and changing the confocal scan rate, there was no change in the measured internal capsule fluorescence, demonstrating that the lower internal fluorescence is not caused by photobleaching of the FITC-labeled dextran. Of the three dextrans, only the 40 kDa dextran could be used to measure the permeability accurately. For the 20 kDa dextran capsule permeabilities often exceeded 75%, and for the 70 kDa dextran capsule permeabilities were often less than 10%. Because measurement errors for permeabilities above 75 and below 10% were too large, only the 40 kDa dextran was used to determine the MWCO.

The exclusion of FITC-labeled dextran has also been observed in microcapsules created by the layer-by-layer technique that encapsulate polyelectrolytes.[23–25] In this case, the partial permeation of the dextran into the microcapsules was attributed to like-charge repulsion between the encapsulated polyelectrolyte and the charged dye molecules on the FITC-dextran. In the case of the layer-by-layer technique, the

amount of dextran permeating the capsules could be adjusted through the addition of salt which screened the electrostatic charge of the encapsulated polyelectrolyte. In the measurements shown here, enough salt has been added to screen the charge of any trapped, unadsorbed silk protein. When additional NaCl was added to increase the concentration in the dispersion to 0.5 M, no change in dextran permeation was observed. As further proof that the partial permeation is not caused by charge repulsion, fluorescein sodium salt (M_W = 376 Da) was also added, at the same concentration as the fluorescein which labels the 40 kDa dextran. After the dispersion came to equilibrium, the concentration of fluorescein sodium salt inside and outside the capsule was identical. Neither the concentration of the spider-silk protein used during capsule formation nor the concentration of dextran was observed to affect the measured permeability significantly. To test whether dextran adsorption to the capsule affects the measured permeabilities, the capsules were first incubated with dextran. Then, after incubation, the dextran was removed and the capsules were tested for residual fluorescence. For the 40 kDa dextran no measurable adsorption was observed.

The molecular-weight cutoff of each capsule in the dispersion can be determined by comparing the measured intensity inside the capsule to the amount of fluorescence at each molecular weight. Fig. 3 shows the cumulative fluorescence distribution for the 40 kDa dextran, as measured by adsorption at a wavelength of 488 nm with the GPC ultraviolet-adsorption detector. This figure shows the amount of fluorescence which occurs below a given dextran molecular weight. Because the fluorescence intensity within the capsules is produced by the fraction of dextran which is below the capsule MWCO, the cumulative fluorescence distribution can be used to relate the capsule MWCO to the internal capsule fluorescence.

When the dextran molecular-weight distribution is compared to the fluorescence observed inside each capsule, all of the MWCO are found to be between 16 and 47 kDa (Fig. 4). The average molecular-weight cutoff is 27 kDa, therefore molecules which are smaller than the radius of gyration of 27 kDa dextran (r_g = 53 Å)[26] are expected to pass freely through the membrane. The molecular-weight cutoff measured here is in the same range as those found for the more widely studied alginate/polylysine capsules, which have a dextran
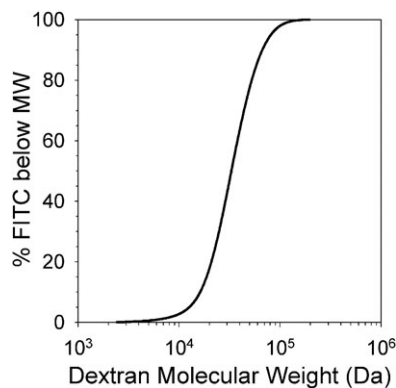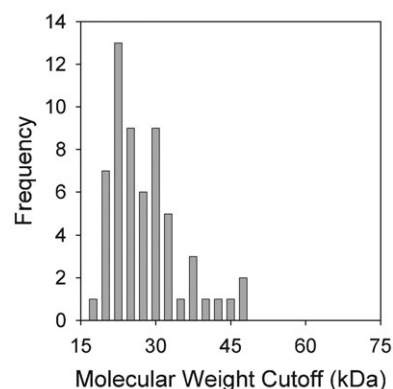

Fig. 4 Histogram of measured molecular weight cutoffs for 59 different silk microcapsules from 16 different samples. All samples were prepared in the same manner.

molecular-weight cutoff between 14.5–100 kDa depending on the concentration of polylysine used.[15,17,21]

These measurements are expected to be systematically affected by the small amount of ambient light and background fluorescence which is observed within the capsule, as well as random errors caused by laser fluctuations or photodiode noise. The random error of the technique was assessed by conducting multiple measurements on the same capsule. The error in measured fluorescence intensity was found to be 2%.

4. Conclusion

This paper reports on molecular-weight cutoff measurements of thin-shelled silk microcapsules. The capsules were found to have an average molecular-weight cutoff of 27 kDa. The permeability method used had the advantage that it allows for the investigation of variations within a capsule dispersion and requires only a single permeability marker, enabling the permeability to be measured quickly. For the capsule dispersions measured here the permeabilities varied between capsules but were always between 16 and 47 kDa. This is below the 70 kDa dextran MWCO required to exclude 150 kDa biological molecules, such as immunoglobulin antibodies.[22] With an average molecular-weight cutoff of only 27 kDa the membranes would provide good protection against the immunological system for applications such as enzyme therapy and artificial cells.

Fig. 3 Fraction of the FITC-fluorescence labeling which occurs below a given dextran molecular weight.

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