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van Rijn, Patrick; Wang, HuiHui; Boeker, Alexander

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PAPER

Ultra-sound assisted formation of biodegradable double emulsion capsules from hen egg white†

Patrick van Rijn, HuiHui Wang and Alexander Böker*

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It was found that directed self-assembly of hen egg white proteins at oil–water interfaces in Pickering emulsions followed by acoustic denaturation of the proteins at the interface produces double emulsion capsules. The formation of capsules is dependent on the pH of the aqueous phase and on the concentration. At pH 5.5, the capsules are easily formed while at pH 8.5 the formation is difficult and only occurs at high concentration and in low quantity. The difference in capsule stability and interface stabilization at different pH was rationalized by competitive stabilization forces at the oil–water interface which was investigated by dynamic interfacial tension measurements. While the absolute interfacial stabilization does not differ much at different pH, when taking into account stabilization behavior of the pure buffer solutions without protein there is a significant difference. At neutral and alkaline conditions we observed that the pure buffer already stabilizes the interface which competes with the stabilization by the protein. This competitive stabilization resulted in less stable capsules formed at higher pH. Moreover, a lower pH enhances the ultra-sound assisted denaturation of the proteins, yielding an additional stabilization of the formed structures. In addition, the capsules are biodegradable. Upon digestion with trypsin, they were completely destroyed leaving behind small protein aggregates.

Introduction

Self-assembly of molecular- and nano-sized components has been used to stabilize emulsions, form membranes and prepare capsules.^{1,2} Especially the latter is of great interest as these structures are used by many different industries in a large variety of consumer goods *e.g.* drug delivery, perfumes, foods or inks.² Important properties of the capsules include stability, toxicity and biodegradability. The capsules need to be sufficiently tough to withstand mechanical force. When used for drug delivery or in food the toxicity should be kept to a minimum. Once active components are encapsulated, it should be possible to release them either by a special trigger or in a more natural fashion in the form of biodegradation.

The most convenient method for the preparation of capsules is the use of stabilized emulsions by means of self-assembling interfacially active components. In the case of Pickering emulsions (*i.e.* particle-stabilized emulsions) inorganic particles,³ proteins⁴ and polymer nanoparticles^{5,6} can be used to stabilize liquid–liquid and liquid–air interfaces. The variety of

nanoparticles employed in Pickering emulsions has led to diverse and interesting structures like polymersomes,⁷ colloidosomes,⁸ yeastosomes⁹ and capsules composed of microgels.^{10,11} In order to stabilize the nanoparticle assembly at the emulsion interface and thus the capsules, particles were functionalized with cross-linkers.^{12–14} Moreover, novel hybrid materials have been prepared *via* modification of bio-nanoparticles with small molecular components or cross-linkable polymers.¹⁵ Though all the different approaches have their advantages and disadvantages, before they will be applied in commercial products they all need advanced studies with respect to toxicity and biodegradability. One way to expedite this process is to make use of materials which are already known, preferably provided by nature.

Here we made use of hen egg white and the tendency of proteins to stabilize and interact with interfaces¹⁶ in combination with the strong tendency to form coagulates upon unfolding which is experienced in everyday food preparation. Hen egg white lysozyme has been investigated with respect to its interfacial behavior at the air–water interface¹⁷ and at solid interfaces¹⁸ as well as the use in emulsion preparation.¹⁹ It is known that proteins in egg white unfold upon heat treatment but it was also found that these—as well as other proteins—also unfold and aggregate when subjected to ultra-sound.²⁰ In the past, ultra-sound was used for the preparation of silica hybrid materials where a silica precursor (tetraethoxyorthosilicate) was employed

DWI an der RWTH Aachen e.V., Lehrstuhl für Makromolekulare Materialien und Oberflächen, RWTH Aachen University, Pauwelsstrasse 8, D-52056 Aachen, Germany. E-mail: boeker@dwf.rwth-aachen.de; Fax: +49 241-8023301; Tel: +49 241-8023304

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in combination with lysozyme for the creation of hollow silica capsules²¹ but also for microbubble formation with denatured lysozyme shells.²²

Results and discussion

As mentioned above, it is possible to prepare emulsions with the use of proteins as stabilizing agents. The tendency to accumulate at the interface was combined with high frequency radio wave treatment which allows for the formation of local fluctuations in temperature and pressure. While the proteins unfold at the interface, they also tend to coagulate. The combination results in a shell of protein aggregates around a hydrophobic liquid interface. The capsules formed from this procedure were found to contain capsules in capsules. A schematic representation is shown in Fig. 1 as well as a commonly observed capsule by fluorescence microscopy in which the oil-phase contains Nile Red (red) and the aqueous phase Fluorescein (green).

Capsule formation

In principle, the double emulsion capsule structure was not anticipated. However, the preparation uses a method which introduces a high amount of energy into the system and most likely during the formation of the outer denatured protein shell, part of this shell is pushed inwards while the outer shell quickly reforms, trapping the newly formed smaller water-containing capsule in the interior (Fig. 1).

Even though it is known that proteins are able to stabilize interfaces, the stabilization is not always inert against external influences. When mixing 1 vol% benzotrifluoride (10 μ l) into a 1 wt% protein (dry weight) solution in PBS-buffer (1 ml) without the sonication treatment, at first sight a stable emulsion was formed. However, upon investigation of the emulsion with (fluorescence-) microscopy, it was found that the emulsion displayed large droplets of millimetre-size (ESI†, Fig. S1) and when placed between two microscope-slides, the emulsion was

Fig. 1 A schematic representation of the preparation method for the double emulsion capsule structure with the use of hen egg white in combination with interfacial self-assembly followed by acoustic denaturation. The double emulsion capsule structure shown is typically obtained by this method where the initial aqueous-phase contains Fluorescein and the oil-phase Nile Red, in order to prove that the formed internal capsules in the oil-phase originate from the surrounding water.

Fig. 2 (Fluorescence-) Microscopy images of a 1 wt% (dry weight) egg white solution in PBS at pH 7.4, which were not subjected to an ultrasound treatment (A and B) where initially droplets were observed but rapidly destroyed by mechanical forces of wedging between two microscope slides. Large domains of the oil-phase are seen (red) as well as non-fluorescent aggregates. The emulsion which was treated with ultrasound (C and D) displays besides aggregates as seen in (A), also capsules-in-capsules, resistant to the mechanical forces.

destroyed and large smeared structures were observed (Fig. 2A and B). After treatment of an identical emulsion in an ultrasound bath for 5 minutes, various smaller structures were collected which remained unaffected by the mechanical forces exerted by the microscope slides. The structures were mostly composed of stabilized double emulsion capsules with fewer single capsules. Besides the capsules, also precipitated aggregates were observed with no specific structure. The formation of precipitate can be explained by the known fact that proteins in solution subjected to ultra-sound form insoluble aggregates.

There are several factors that influence the composition of the resulting structures when forming the capsules of which the most important ones are concentration and pH. The concentration of protein will affect the amount of additional precipitated protein aggregates as well as the size and amount of capsules produced. As shown in Fig. 2, a 1 wt% protein solution gives rise to large amounts of additional precipitate. It is anticipated that a lower concentration of protein gives less of these aggregates. However, a too low concentration will not provide sufficient stabilization for the water–oil interface. A second important factor will be the pH. It is a well-known phenomenon that when cooking with hen egg white, a small amount of citric acid will give a more stable and stiffer foam of egg compared to no addition *i.e.* more alkaline conditions. Therefore, in addition to the 1 wt% protein solution in PBS-buffer of pH 7.4, lower concentrations (0.5 and 0.05 wt%) were investigated with PBS-buffer with different pH of 5.5, 7.4 and 8.5, respectively (Fig. 3 and 4).

Comparing the preparation of the capsules at different pH-values, we observed in all cases additional precipitation of aggregated structures which was inversely related to the number of formed capsules. At pH 5.5 and 7.4 (Fig. 3A and B), double emulsion capsules as well as normal capsules were observed in the range of a few to about 20 μ m and seemingly more precipitate

Fig. 3 (Fluorescent-) Microscopy images of ultrasound treated emulsions of 0.5 wt% (dry weight) of egg white in PBS at pH 5.5 (A), 7.4 (B) and 8.5 (C). Capsules-in-capsules are seen at low pH (5.5, A) while at high pH (8.5, C) hardly any capsules are observed but more precipitated structures. The intermediate pH (7.4, B) shows capsules as well as aggregates.

Fig. 4 (Fluorescent-) Microscopy images of ultrasound treated emulsions of 0.05 wt% (dry weight) of egg white in PBS at pH 5.5 (A), 7.4 (B) and 8.5 (C). Capsules-in-capsules are seen at low pH (5.5, A) and intermediate pH (7.4, B) while at high pH (8.5, C) no capsules are observed but more precipitated structures and large or ill-defined oil-phases.

was found at pH 7.4. At pH 8.5, hardly any capsules could be found and mostly just precipitated structures appeared (Fig. 3C). Those were smaller and more needle-like than in the cases of

lower pH. Apparently, pH influences the formation of precipitated aggregates which in turn most likely also affects the capsule formation.

Lowering the protein content by a factor of 10 (0.05 wt%) still provided small stable capsules and less of the precipitated aggregates at pH 5.5 and 7.4 (Fig. 4A and B). For the more alkaline environment at pH 8.5 (Fig. 4C), apparently the interface is insufficiently stabilized and no capsules were observed anymore which was to be expected since at 0.5 wt% the capsule formation already proved difficult. Comparing the sizes of the capsules, we found that the average size of capsules prepared at pH 5.5 and 7.4 with 0.05 wt% concentration is $4.7 \pm 1.7 \mu\text{m}$ and $5.2 \pm 2.2 \mu\text{m}$, respectively. This is considerably smaller than the capsules found for pH 8.5 which is $32.0 \pm 14.7 \mu\text{m}$ (prepared with 0.5% concentration) (detailed analysis and size histogram, ESI†, Fig. S2).

From the micrographs it becomes clear that the type of structures formed depends on the concentration and pH. Also it is seen that in addition to multi-core structures and capsules with a single aqueous core inside the oil phase, also conventional capsules are seen. In order to get more information about the composition of the formed capsule system, a more detailed analysis of the various structures was performed concerning the quantity of each individual structure as well as the difference in size of these structures. In addition, also sonication times for the preparation of the samples were varied to investigate if the relative amounts of the different capsule structures can be influenced. For these experiments, a 0.5 wt% hen egg white solution was used at pH 5.5 with the addition of 1 vol% of BTF, containing Nile Red (0.01 mM). The samples were sonicated for the duration of 1, 5, 20 and 60 minutes, respectively, and the composition analyzed with respect to relative amounts and size distribution of each structure (Table 1) (overview micrographs and histogram are given in the ESI†, Fig. S3).

It was found that varying the sonication times influences the composition of the system and changes the relative amounts of the different types of capsules. At very short sonication times of 60 seconds, about 52% of the found structures are oil capsules without an additional aqueous core (N_C) and the other capsules with additional single core (S_C) (35%) and multi-core (M_C) (13%) form the other part. The individual size of the structures varies and while the multi-core structures have an average diameter of $21.9 \mu\text{m}$, the single core and no core structures are significantly smaller, 8.9 and $5.3 \mu\text{m}$, respectively. Increasing the sonication time influences the relative composition as well as the average diameter of the multi-core capsules. Increasing the sonication time to 5 or 20 minutes shows a decreasing amount of capsules

without additional aqueous core and an increase in the oil capsules with additional core(s). When the sonication time was increased even further to 60 minutes, the opposite was observed in the sense that more capsules without additional cores were observed. This suggests that there is an optimum time for sonication and a further input of energy provided by the sonication process has a destructive effect of the multi-core structures. An additional effect was seen with respect to the diameter of the capsules. While the diameter of N_C and S_C remained relatively constant, the diameter of M_C decreased with increasing sonication times which relates in accordance to the observed changes in composition that mainly affects the amount of M_C .

While the contents of the individual proteins in hen egg white can be considered to be relatively constant, the egg white of different types of hen eggs was used to validate if variation in the egg type influences the capsule structure. While for the experiments described so far, white eggs have been used, the experiment was repeated with egg white collected from brown hen egg. The same concentrations and a sonication time of 5 minutes were used. It was observed that also with this type of egg white, the same structures are obtained, however, the composition and size distribution differ (Table 1, ESI†, Fig. S4), indicating that the type of egg white plays a significant role.

Interfacial stabilization at different pH

From pendant-drop interfacial tension (IFT) measurements, it is rationalized that at higher pH values the stabilization and capsule formation become less likely. Fig. 5 shows the dynamic IFT-plots of buffer solutions at different pH, with and without 0.5 wt% hen egg white, *versus* benzotrifluoride. In all cases, a large decrease in IFT was found resulting from aggregation of protein at the interface, thus stabilizing the interface *via* the Pickering effect which is well-known for proteins and colloidal nanoparticles.²³ At first sight, under basic conditions (pH 8.5), the stabilization seems to proceed fastest with the IFT dropping instantaneously from 38.5 mN m^{-1} for pure buffer to 19.7 mN m^{-1} when egg white is introduced. When compared to the other pH values (5.5 and 7.4) where the reduction in IFT proceeds from 40.5 and 37.7 mN m^{-1} to 23.3 and 24.6 mN m^{-1} for pH 7.4 and 5.5, respectively, it seems that the stabilization is better at higher pH reflected in the lower IFT. Over a period of approximately 1000 seconds, the IFT reaches a steady state for the stabilization of the interface at values of 13.7 , 15.2 and 14.9 mN m^{-1} for pH

Table 1 Capsule composition of the differently prepared samples in which the sonication time is varied

Composition/prep ^a	% ($M_C/S_C/N_C$) ^b	$\bar{\phi}$ (μm) M_C (S.D.) ^c	$\bar{\phi}$ (μm) S_C (S.D.) ^c	$\bar{\phi}$ (μm) N_C (S.D.) ^c
(W) 0.5 wt%/1 min	13/35/52	21.9 (4.2)	8.9 (2.4)	5.3 (2.1)
(W) 0.5 wt%/5 min	12/47/41	19.1 (7.3)	7.4 (2.9)	3.6 (1.4)
(W) 0.5 wt%/20 min	30/39/31	16.9 (3.4)	10.4 (3.2)	6.8 (3.8)
(W) 0.5 wt%/60 min	17/35/48	13.2 (1.9)	8.4 (2.1)	5.0 (1.9)
(B) 0.5 wt%/5 min	21/36/43	20.1 (4.3)	9.3 (2.5)	4.9 (1.5)

^a Preparation was done according to the procedure described in the Experimental section with (W) depicting egg white collected from a white egg and (B) from a brown egg. ^b Percentage (%) of the structures that were observed; M_C = multi-core structure, S_C = single aqueous core in oil capsule and N_C = no aqueous core inside oil capsule. ^c Diameter ($\bar{\phi}$) was determined from averaging the size of individual structures of the same type on various micrographs with S.D. as the standard deviation.

Fig. 5 Dynamic interfacial surface tension measurements of PBS with different pH with and without 0.5 wt% (dry weight) of egg white (denoted as ew) (left). It shows that the stabilization is rapid and initially better for high pH than for low pH. It also displays a contribution in stabilization of the buffer itself without protein. The graph on the right shows the absolute interfacial tension where the stabilizing effect of pure buffer is compensated. A competitive behavior between two stabilizing forces is observed for pH 7.4 and 8.5, which first shows a fast and high stabilization while after an initial period, the system becomes less stable. For pH 5.5, this behavior is not observed and an initial increase in absolute stabilization is found which equilibrates after 400 s. Error is typically below 5%.

8.5, 7.4 and 5.5, respectively. Though there are significant differences during the initial stage of stabilization, the IFT-values hardly deviate after 1000 seconds. However, this is deceptive, since the blank measurement of buffer against BTF already shows better interface stabilization, seen as a lower value for the IFT, than pH 5.5 and 7.4. It is known that basic aqueous solutions are able to influence interfaces due to the adsorption of hydroxyl-ions (OH^-), especially in degassed solution as obtained by sonication.²⁴ When, instead of looking at the absolute stabilization, the difference in interface stabilization is investigated between the pure buffer and the buffer containing protein (ΔIFT), it seems that there is a competitive behavior in the stabilization of the interface (Fig. 5, right). At first there is still a better stabilization observed for pH 8.5 with a ΔIFT of 18.8 mN m^{-1} which is larger than the ΔIFT of 17.3 mN m^{-1} for pH 7.4 which in turn is larger than for pH 5.5 with an ΔIFT of 13.1 mN m^{-1} . While ΔIFT increases initially for pH 8.5 and 7.4, it quickly decreases after approximately 100 seconds and the emulsion becomes less stable again. For pH 5.5, the increase continues longer and levels off slowly but does not decrease. After about 500–600 seconds, the absolute stabilization becomes better for pH 5.5 than for pH 8.5. For pH 7.4 this occurs at about 700 seconds. From Fig. 5, it is clearly observed that after 1000 seconds the steady state has not yet been reached for pH 8.5 and 7.4, while for pH 5.5 the value for ΔIFT remains stable after 400 seconds. That the IFT first increases and then decreases would suggest that competitive forces are indeed present. Since the behavior affects the higher pH values and to a much lesser extend the lower pH of 5.5, the adsorption of OH^- would be a possible explanation. The interference from the competitive component starts to significantly influence the IFT after about 50–60 seconds which is shown as a decrease in IFT for the pure buffers *versus* benzotrifluoride (Fig. 5) (ESI, Fig. S5† which shows a more detailed plot of the IFT in time for the pure buffers and of the ΔIFT). The absolute decrease at 1000 s after the initiation of the dynamic IFT measurement in pure buffer is largest for pH 8.5 (ΔIFT : 5.7 mN m^{-1}) and 7.4 (ΔIFT : 5.8 mN m^{-1}) as for pH 5.5 it is only 2.2 mN m^{-1} , indicating a larger contribution to the surface stabilization by pure buffer at higher pH. This competition means that protein is replaced by the other component, most probably the OH^- -ions, and therefore upon denaturation of the proteins in order to form the shell, there is not enough protein left

to create a completely closed shell around the emulsion droplets or one which is sufficiently thick to be stable.

The IFT-measurements concern the case of stabilization before the protein is denatured. A second contribution to the stability is that at lower pH, the protein denatures more rapidly. From previous studies with hen egg white lysozyme, it was known that a reduced pH, especially at higher ionic strengths, may result in a facilitated denaturation of the proteins, depending on the protein used.²⁵ A combination of both factors will contribute to an easier formation of more stable capsules at lower pH.

Scanning force microscopy analysis

The structures were further analyzed using Scanning Force Microscopy (SFM). The emulsion prepared at pH 5.5 was drop cast in diluted form onto a silicon wafer and allowed to dry in air for several hours. The cast film was extensively washed by submersing it in MilliQ-water. Analyzing a large area, structures were displayed ranging from 1 to about $7 \mu\text{m}$ in diameter with a height profile up to 500 nm (Fig. 6A and B). A more detailed image of a representative structure shows an overall round shape with rough and irregular edges (Fig. 6B). The dried capsule takes a doughnut-shape in the sense that the middle part is lower than the surrounding structure but still contains a thick layer of material. From the cross-section it can be seen that the diameter is in the low micrometre regime and that the thickest part of the capsule is roughly between 300 and 500 nm thick, depending on the individual structures. The collapsed middle part of the capsule is about 200 nm thick (Fig. 6C). The edges are contracted inwards showing a slope from the edge of the capsule towards the middle instead of an abrupt increase as soon as the capsule structure is reached upon scanning. This steady increase in height suggests that there is a strong interaction with the silicon wafer surface and that the contact area is not retracted upon drying like the rest of the capsule. It was found that the whole area was covered with an ultrathin film of residual protein which remained even after washing. This also indicates a strong interaction with the substrate. An overview optical microscope image of the silica surface with the dried capsules is shown in ESI†, Fig. S6, depicting typical structures analyzed by SFM.

Fig. 6 Scanning Force Microscopy images of dried capsules on a silicon wafer in which (A) a 3D height image and (B) a top-view of $72 \times 72 \mu\text{m}$ surface with an enlargement of a single capsule are shown. (C) shows the height profile across 2 capsules marked with the white dotted line in (A) and (B).

Biodegradation by trypsin

Since the capsules are made from a biological material which is already vastly used in foods and consumer products, it should also be biodegradable. A common protein which is able to degrade peptide sequences is trypsin, belonging to the serine proteases.²⁶ This class of proteolytic enzymes contains a reactive serine side chain and trypsin is able to cleave the carboxylic sides of lysine and arginine residues by coupling of the cleavable peptide sequence in a catalytic triad and by that activating the peptide bond making it susceptible for hydrolysis.²⁷ The hen egg white protein mixture contains amongst others ~ 54 wt% of ovalbumin (dry weight), ovotransferrin (~ 12 wt%), ovomucoid (~ 11 wt%) and the mostly studied one, lysozyme (3.4 wt%). All of these proteins are digested by trypsin to a certain degree²⁸ and in order to investigate the biodegradability of the denatured protein capsules, trypsin was added in 1 mg ml^{-1} to a capsule solution prepared from a 1 wt% egg white solution in PBS, pH 7.4, with $10 \mu\text{l}$ BTF. The trypsin/capsule mixture was incubated overnight at room temperature and analyzed by optical microscopy (Fig. 7). The control emulsion obtained from the initial homogenized capsule solution used for the trypsin experiment showed the double emulsion capsules (Fig. 7A) as mentioned before while the solution containing the trypsin did not display

Fig. 7 Microscope images of egg white capsules (A) without trypsin and (B) after the addition of trypsin and equilibrating overnight. Both initial solutions were obtained from the same initial sample and analyzed after identical time intervals. Scale bar depicts $100 \mu\text{m}$. The insets show an enlarged image where in (A) the capsules and in (B) only small protein aggregates are clearly seen, scale bar depicts $10 \mu\text{m}$.

any capsules but only small undefined globular and fibrous aggregates (Fig. 7B). This observation shows that the formed capsules are not only easily formed but also easily digested when introducing specific peptide-digesting proteins.

Conclusions

The ultra-sound assisted formation of the hen egg white double emulsion capsule structures not only offers a simple tool to encapsulate hydrophobic phases but also to prepare structures that potentially have several complementary active components stored inside the water-filled capsules in the encapsulated oil-phase. The raw material is easily obtained by splitting the egg white and the yolk and removing the water from the egg white solution by freeze-drying. Since egg white is a commonly used ingredient in foods it would be possible to directly use this approach in different kinds of food emulsions like *e.g.* mayonnaise but also as a delivery vessel for medical components. Here, not only one hydrophobic component would be able to be delivered but different combinations of hydrophilic and hydrophobic components as well since both types of capsules are located in the same overall superstructure. This offers the possibility of administering more complex medical cocktails. We have shown that the hen egg white capsules are biodegradable by the addition of trypsin. This protein is commonly occurring in organisms and used for the digestion of peptide strands and therefore disposal of as well as the release from the capsules will occur in a natural fashion.

Experimental section

Synthesis

Hen egg white was isolated from commercially available hen eggs by separating the whites and the yolk followed by freeze-drying. A 1 wt% solution was obtained by mixing 10 mg of freeze-dried egg white in 1 ml of phosphate buffer saline, pH 7.4, with adjustment of the pH by addition of 1 M solution of HCl or NaOH in order to reach pH 5.5 and 8.5, respectively. After the

dissolution of the egg white, non-solubilized remaining egg white was removed by decantation. Dilutions for further experiments were made from this concentration. For the oil-phase, 1 vol% benzotrifluoride was added to the egg white solution and subsequently shaken by hand as well as extensive vortexing at 2500 rpm. The acoustic denaturation of the proteins around the oil-droplets was done by placing the glass vial inside an ultrasound bath, Bandelin Sonorex operating at 35 kHz for 1, 5, 20 or 60 minutes at an ambient temperature.

Materials

For the optical and fluorescence microscope images a Keyence BZ-8100E was used with excitation-mode for GFP for visualization of Fluorescein and Texas Red for the visualization of Nile Red. Both fluorescent dyes were purchased from Sigma Aldrich and used without further purification and in concentrations of 0.01 mM. The changes in the interfacial tension between the protein solution and the oil phase were determined at room temperature and monitored with a DSA100 pendant-drop tensiometer with a CCD-camera for drop-image processing, which allows rapid drop-image acquisition, edge detection and fitting of the Young–Laplace equation. Small blunt-end cannulas of 0.5 mm in diameter (NE44 Krüss) were used with 100 $\mu\text{L min}^{-1}$ for the speed of drop-formation. Scanning Force Microscopy images were taken on a Veeco Instruments Scanning Force Microscope operating on Nanoscope software. For the biodegradability experiments trypsin from the Hogg pancreas was used as purchased from Sigma Aldrich. All measurements were performed at ambient conditions unless stated otherwise.

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