ARTICLE TYPE

Encapsulation and release of aqueous components from sonochemically produced protein microspheres.

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Aqueous solutions of salts or dyes have been contained in sonochemically produced lysozyme micropsheres by encapsulating an inverse emulsion in tetradecane. Release can
10 **be triggered by chemically disrupting crosslinking in the protein shell or by mechanical disruption using high intensity ultrasound.**

The encapsulation, delivery and triggered release of active agents such as drugs, flavours, fragrances and agrochemicals
15 is an active area of research[1]. Micrometre sized capsules can be readily formed for this purpose by irradiating a solution of proteins at the air-water or oil-water interface with high intensity ultrasound. The microspheres form as a result of aggregation of the protein around a gas bubble or oil droplet
20 in solution during sonication[2] with stability conferred by sonochemically initiated crosslinking of thiol groups. The first example was presented by Suslick *et. al.*[3] who encapsulated a selection of hydrocarbons in albumin micospheres. Further examples include haemoglobin[4], avidin[5], α-amylase[6] and
25 lysozyme[2,7] being used to encapsulate fluorocarbon gases *e.g.* for use in medical imaging as ultrasound contrast agents[8] or oils such as tetradecane[7], cyclohexane[2], silicone oil[9] and perfluorohexane[7] as potential matrices for active organics. Effective encapsulation of Gemcitabine and Taxol was
30 described by Gedanken *et al.*[10] and Möhwald *et al.* reported[9] the encapsulation and release of Rifampicin, illustrating the potential for using sonochemically produced microspheres in drug delivery.

However, as yet, there has been no report of extending the
35 scope of this sonochemical protocol to encapsulate an aqueous phase with hydrophilic species. Currently, these can be delivered in an emulsion *e.g.* of perfluorocarbon-water[11] but such emulsions exhibit problems with long term stability. A range of microstructures can be assembled by the 'layer-by-
40 layer' technique[12] although this is only applicable to some materials and the assembly is quite time consuming. Development of a more readily produced, stable delivery system would facilitate the simultaneous delivery of both hydrophilic and hydrophobic species. This communication
45 presents the use of high intensity ultrasound (i.e. ultrasound with sufficient intensity to cause cavitation) to both prepare and encapsulate a water-in-oil (w/o) emulsion within a protein shell thus creating a stabilised water-in-oil-in-water (w/o/w)

system containing a hydrophilic species, thus broadening the
50 usefulness of the sonochemical synthesis method. For this proof of concept, we focussed on a model system employing disulfide crosslinked lysozyme.

Encapsulation of hydrophobic components dissolved in oil phases within the microspheres is accomplished by floating a
55 small amount of the oil solution on the surface of the protein solution and sonicating for a short time (30 s – 2 min) with the tip of the ultrasound horn at the phase boundary. This methodology was modified here by replacing the organic component with a water-in-oil emulsion, using a continuous
60 oil phase known to facilitate microsphere formation. The challenges were therefore; to prepare a stable w/o emulsion which could be layered on the surface of the lysozyme solution; to maximise the amount of aqueous phase within the emulsion; and to develop the methodology so that the
65 emulsion maintained its internal struture of dispersed water droplets during the mechanical stresses of the sonication step of microsphere preparation.

Fig. 1 demonstrates that these challenges have been overcome. Fig. 1(a) shows a confocal micrograph of the
70 formed microspheres clearly displaying the two-phase internal structure. The primary emulsion was formed by sonicating an aqueous phase with tetradecane for 5 min at 45 W cm^{-2} using a 23 kHz ultrasound probe with sorbitan monooleate (Span 80) as surfactant.[13] Microspheres were then prepared by
75 floating approx. 100 μL of this emulsion on the surface of 1 cm^3 of pH 8 Tris buffer containing 50 mg of lysozyme (see Elecronic Supplementary Information, ESI, for details). The tip of the ultrasound horn was placed at the interface and the system was sonicated for 30 s at 14 W cm^{-2}.
80 A surfactant concentration of 4 wt% in tetradecane was suffcient to form a stable emulsion which maintained an internal structure of dispersed water droplets during the microsphere preparation (see ESI Fig. S1). When using water as the aqueous phase, the as-formed emulsion had a mean
85 droplet diameter and standard deviation (SD) of 234 nm and 6 nm respectively (ESI Fig. S1). On forming microspheres, some coalescence ocurred giving significantly larger water droplets (mean diameter: 2292 nm, SD: 1537 nm). A high proportion of microspheres encapsulating just the organic
90 phase was also observed.
Addition of sodium chloride to the internal aqueous phase

at concentrations 0.5 mol dm^{-3} or greater allowed the formation of microspheres which contained a fine, well dispersed structure of internal water droplets (Fig. 1(a) and ESI Fig. S1). Additionally, the number of solely oil-filled microspheres formed was negligible. These observations indicate that the addition of salt stabilizes the w/o emulsion towards breakdown during the microsphere synthesis. This may be due to the salt increasing the surfactant head group stability reducing the likelihood of droplet coalescence.[14] The microspheres formed containing an emulsion with 1 mol dm^{-3} NaCl had a mean diameter of 5 μm (SD: 1.5 μm) which is not significantly different from that for tetradecane filled lysozyme micropsheres (4.5 μm, SD: 1.5 μm) formed under the same conditions. The internal droplet structure of these microspheres remains intact over five sequential washings and is stable to storage for several weeks immersed in water at room temperature.

In considering the composition of the internal emulsion, microsphere synthesis was attempted with a range of w/o emulsion compositions ranging from 20-80% v/v aqueous phase (ESI Fig. S3). Stable microspheres were formed only when at least 60% of the organic component was used. Large protein aggregates and solely oil-filled microspheres were formed when higher proportions of aqueous phase were used, indicating that there is a theshold below which a stable w/o/w emulsion cannot be formed and hence microspheres cannot form. Systems involving a 40% w/o emulsion gave the most consistent results and so were used for the remainder of the work reported here.

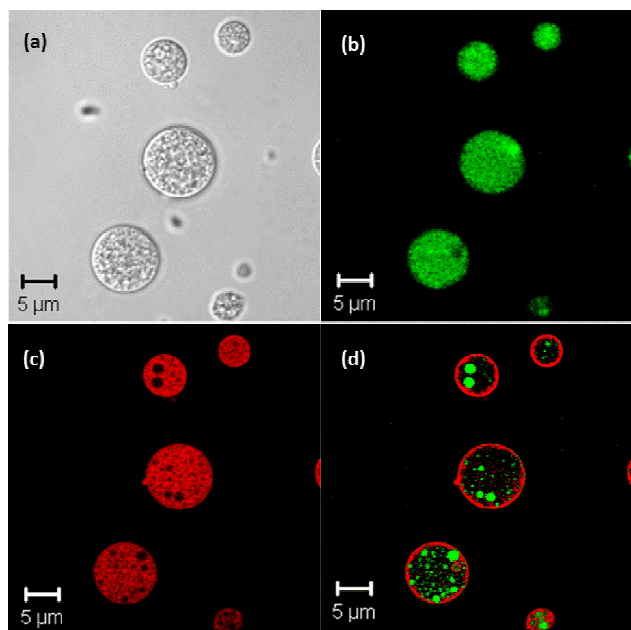Further characterization of the internal structure as well as demonstration of the potential for encapsulating hydrophilic as well as hydrophobic species is provided in Fig. 1. The components comprising the internal emulsion contain 5(6)-carboxyfluorescein in the aqueous phase and Nile Red in the oil phase. These were visualised using dual-wavelength laser scanning confocal microscopy (LSCM). A 1 μm optical slice through the microspheres was imaged using both channels simultaneously. In the resulting micrographs the encapsulated water droplets appear as green under illumination from the 488 nm laser (Fig. 1(b)). The oil phase appears red under 543 nm illumination and can clearly be seen surrounding the water droplets (Fig. 1(c)). When the two channels are overlaid and colocalised pixels are removed from the image (Fig. 1(d)) the water droplets can be clearly observed.

Fig. 1 provides strong evidence for the encapsulation of both hydrophilic and hydrophobic species within protein microspheres. The final challenge is to demonstrate triggered release. Lysozyme microspheres were prepared with the aqueous phase containing 50 mmol dm^{-3} 5(6)-carboxyfluorescein. At this concentration, the dye undergoes self-quenching of fluorescence,[15] thereby allowing release to be monitored by measuring the increase in fluorescence as the 5(6)-carboxyfluorescein is diluted on release.

50 μL of a microsphere suspension was shaken into 2 cm^3 of HEPES buffer and incubated at 37 °C with DL-Dithiothreitol (DTT), a disulfide cleavage agent which weakens the lysozyme shell leading to rupture and release of the contents. Fig. 2 shows that incubation in the absence of DTT does not result in an increase in fluorescence. In contrast, the presence of DTT increases the fluorescence signal at a rate dependent on its concentration. The implications of this are twofold; firstly that the aqueous soluble dye, once encapsulated, can be effectively released on rupture of the microsphere shell and secondly that the dependence of release upon the concentration of cleavage agent demonstrates that disulfide linkages are crucial to the stability of the lysozyme shell. It further demonstrates that the small amount of tetradecane present does not prevent release into the bulk aqueous phase.
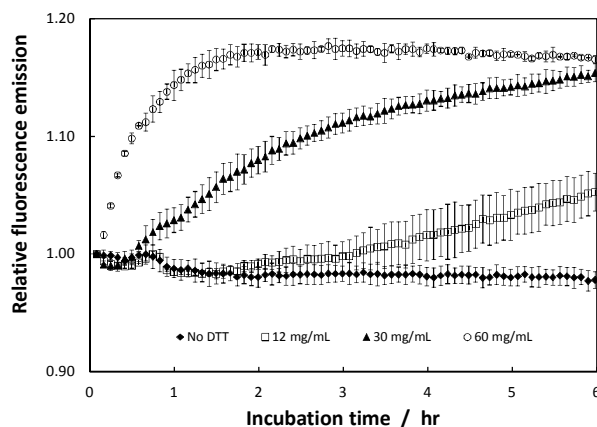

Fig. 1: Confocal micrographs of lysozyme microspheres containing a 40% aqueous emulsion of 1 mM 5(6)-carboxyfluorescein in 1M NaCl solution and saturated solution of nile red in tetradecane. (a) white light transmission micrograph, (b) green channel showing carboxyfluorescein fluorescence, (c) red channel showing nile red fluorescence, (d) Overlaid image with co-localised pixels removed

Fig. 2: Change in solution fluorescence on breakdown of lysozyme microspheres containing 50 mM 5(6)-carboxyfluorescein dispersed in tetradecane on incubation with DTT. ○ 60 mg cm^{-3} DTT, ▲ 30 mg cm^{-3} DTT, □ 12 mg cm^{-3} DTT, ◆ no DTT.

Release can also be triggered by mechanical rupture by exposing the microspheres to 45 W cm⁻², 20 kHz ultrasound (ESI Fig. S4). The maximum fluorescence signal was achieved after 60 s sonication giving a fluorescence emission comparable to that observed on incubation for 2 h with 60 mg cm⁻³ DTT.

In addition to studying the encapsulation and release of large organic molecules such as 5(6)-carboxyfluorescein, we are interested in whether other aqueous species can be encapsulated and released. Potential targets are water soluble polymers, nanoparticle suspensions and ionic species. As a first example and since some sodium chloride must be present in the aqueous phase, it was of interest to establish to what extent salt could be incorporated and released.

Microspheres were prepared using 6.1 mol dm⁻³ sodium chloride as the aqeous phase of the internal emulsion and suspended by shaking in 10 cm³ water (see ESI). Fig. 3 shows the change in ionic conductivity of the supernatant solution after sonicating the microsphere suspensions for 2 min at 45 W cm⁻². As expected, a significant increase in conductivity is observed (Fig. 3(a)) due to the release of the salt when the microspheres are ruptured. Sonication of microspheres filled only with tetradecane (b) results in a much smaller increase in conductivity, presumably due to release of some small ionic protein fragments. To ensure that the conductivity change was not simply ocurring due to sonication, tetradecane containing microspheres were suspended in a solution with a concentration of sodium chloride equivalent to that if all the salt contained in (a) were released. In this case (c) the conductivity decreases somewhat upon sonication, most likely due to adsorption of salt onto the protein fragments that form when the microspheres are broken. These experiments confirm that the conductivity increase in 3(a) is due to salt release from ruptured microspheres and open up the possibility of using ionic species as both the emulsion stabiliser and the active agent to be delivered.
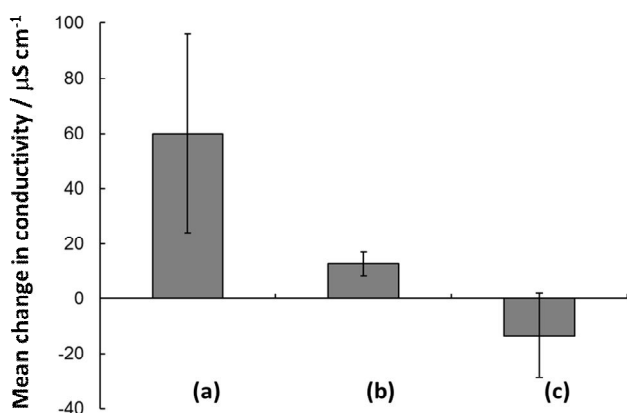

Fig. 3: Mean change in solution conductivity at 25 °C upon ultrasonic breakdown of microsphere suspensions: (a) microspheres containing an emulsion of tetradecane and 6.1 M NaCl solution suspended in water; (b) microspheres containing only tetradecane suspended in water; (c) microspheres containing only tetradecane suspended in NaCl solution.

In conclusion, we have extended the scope of sonochemically produced protein microspheres to include the encapsulation of aqueous soluble components such as dyes and salts through the use of a pre-formed w/o emulsion. This method also facilitates the simultaneous encapsulation and delivery of both hydrophobic and hydrophilic species. In addition, the ability to stimulate release of hydrophilic species after mechanical or chemical triggering has been demonstrated. Further work is currently under way to extend the range of species that can be encapsulated and released as well as to conduct longer term storage and release studies to further characterise the microspheres formed. Other potential release mechanisms involving changes of temperature or ionic strength and photochemical mechanisms are also under investigation. The development of synthetic polymer analogues of the proteins is also underway and will be reported in in forthcoming manuscripts.

Notes and references

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Supplementary Information:

Encapsulation and release of aqueous components from sonochemically produced protein microspheres.

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Experimental:

All chemicals were purchased from Sigma Aldrich and were used as received. A Sonics and Materials Vibra Cell VC600 generator fitted with a 20 kHz horn and a microtip attachment was used for the microsphere synthesis and release studies. Ultrasound intensities were measured calorimetrically. Dynamic light scattering measurements were performed using a Malvern Nano-S instrument. Optical micrographs were captured using a GX optical L3001 microscope fitted with an Infinity 2 camera. Laser scanning confocal microscopy experiments were carried out using a Zeiss LSM 510 META microscope. Fluorescence experiments were carried out using a Spectrostar Omega plate reader (BMG Labtech) with NUNC 12 plates, the cycle time was 299s, 20 flashes per cycle with double orbital shaking at a frequency of 300 rpm for 289 s after each cycle, excitation filter; 485 nm, emission filter; 520 nm. A Mettler Toledo Seven Easy conductivity meter was used to carry out the conductivity measurements together with a Techne Tempette Junior TE-8J water bath.

Emulsion preparation: An oil phase of tetradecane containing span 80 and an aqueous phase of pure water or sodium chloride solution were sonicated with the horn tip at the oil : water interface using an intensity of 45 Wcm^{-2}. Placement of the tip in other positions e.g. in the bulk oil or aqueous phase results in less effective mixing and reduced formation of microspheres. After initial experiments covering a variety of conditions, the following optimised conditions were used for later experiments: oil phase: 4 % w/w Span 80 in tetradecane, aqueous phase: 1 M NaCl$_{(aq)}$, emulsion: 40% aqueous phase, sonication time: 5 minutes. The water droplet size distribution in the emulsion was characterised using dynamic light scattering after dilution with bulk tetradecane/span 80 (matching the composition of the continuous phase) to achieve 4 % w/w of aqueous phase in the sample.

Emulsion-containing microsphere synthesis: In a 15 mL plastic centrifuge tube, 50 mg of lysozyme was dissolved in 1 mL of 50 mM pH 8 Tris buffer and left to stand for 1 hour. 30 mg of DTT was charged to the lysozyme solution and left to stand for 2 minutes. 100 μL of freshly prepared emulsion was layered on the surface of the lysozyme solution. The horn tip was placed at the oil water interface and the system was sonicated for 30 s at 14 W cm^{-2}. After sonication the suspension was diluted to 15 mL with deionised water and left to stand overnight. If required, the suspension of microspheres was washed, to remove excess oil and protein fragments, by sequential re-dilution of the creamed suspension in clean deionised water. Samples for confocal microscopy were prepared using a w/o emulsion in which the tetradecane phase was saturated with nile red and the aqueous phase contained 1 mM 5(6)-carboxyfluorescein.

Release of 5(6)-carboxyfluorescein: Microsphere samples were prepared according to the protocol detailed above using an aqueous phase containing 50 mM 5(6)-carboxyfluorescein. Release was monitored using a

fluorescence plate reader; each well contained 50 μL of microsphere suspension in 2.0 mL of HEPES buffer with the prescribed amount of DTT. Fluorescence was measured at 5 minute intervals over a 6 hour time period, during which time the plate was incubated at 37 °C. Each DTT concentration was run in triplicate. In the case of mechanical breakdown by ultrasound, triplicate samples were taken from the microsphere suspension at chosen time points and diluted for measurement on the plate reader as described above.

Release of Sodium Chloride: Microsphere samples were prepared according to the protocol detailed above using a w/o emulsion containing a 6.1 M solution of NaCl. For each experiment the microsphere suspension was diluted to exactly 10 mL using MilliQ water (18.2 MΩ cm). The suspension temperature was equilibrated to 25 ± 0.5 °C using a water bath prior to each conductivity measurement. The conductivity of the freshly prepared suspension was measured, the microspheres were then sonicated for 2 minutes at 45 Wcm^{-2} to cause rupture. The conductivity of the degraded suspension was then measured. Control samples were treated in an identical manner. Experiments were carried out on each sample type in triplicate.
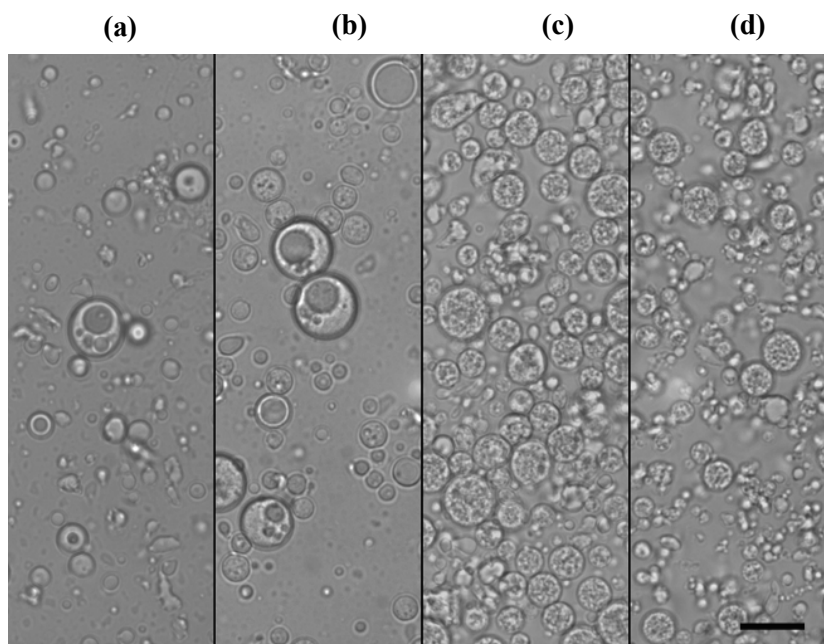
Results:

Figure S1: Emulsion containing microspheres formed using different salt concentrations in the internal phase; (a) pure water, (b) 0.06 M, (c) 0.5 M, (d) 3 M aqueous sodium chloride respectively. Scale bar: 10 μm.
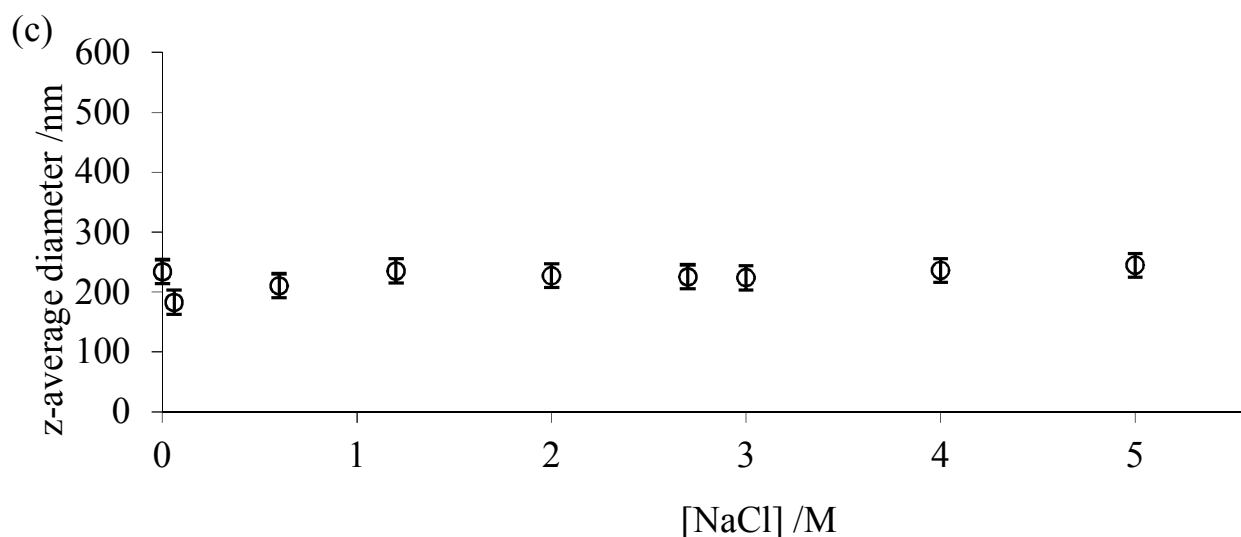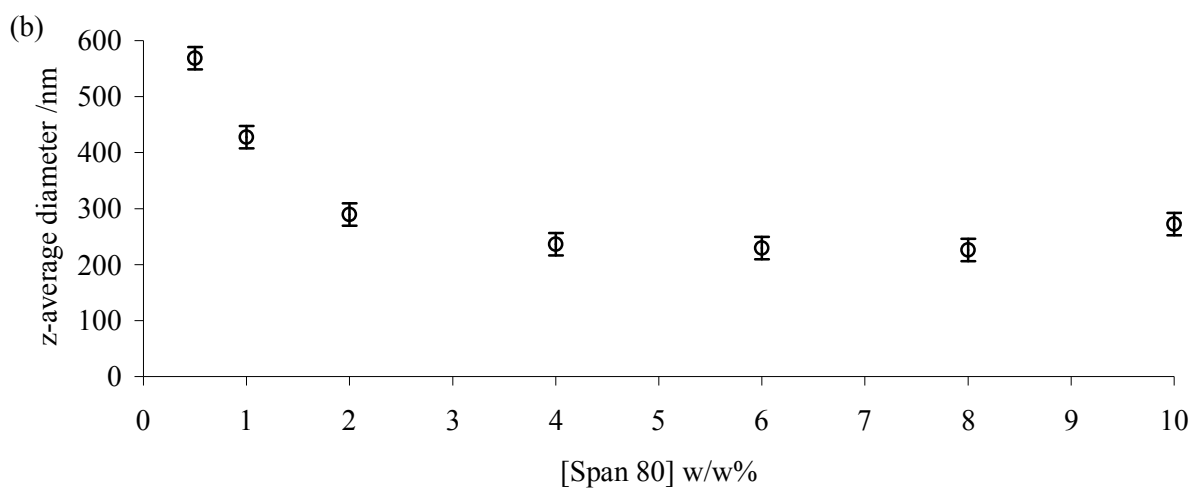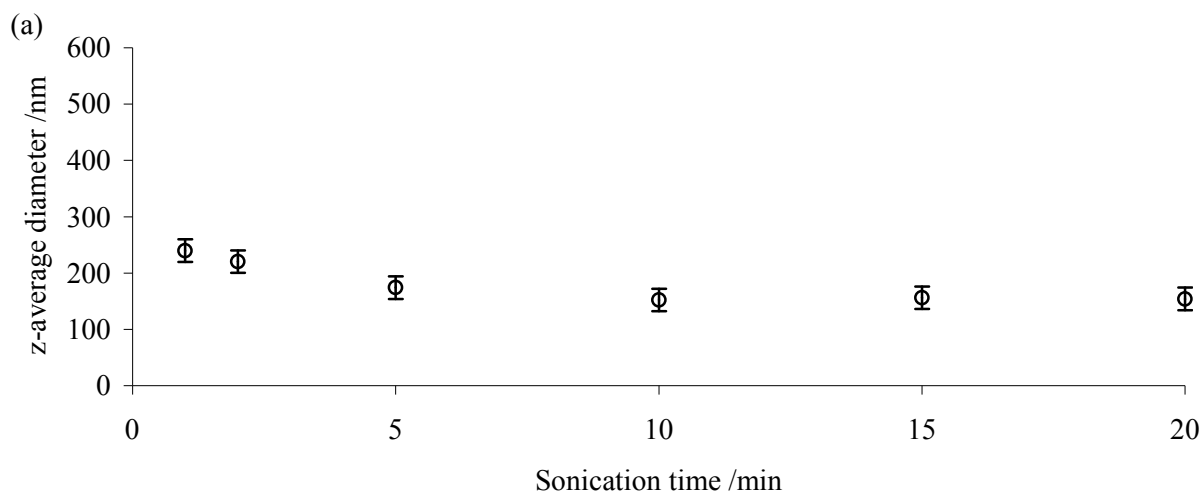
Figure S2: Effect of sonication time, surfactant concentration and salt concentration on the droplet diameter in a
40% v/v tetradecane emulsion measured by dynamic light scattering. (a) 4 w/w% Span 80, 0.06 M NaCl. (b) 6
M NaCl, 5 min sonication (45 Wcm^{-2}) (c) 4 wt% Span 80, 5 min sonication (45 Wcm^{-2}).
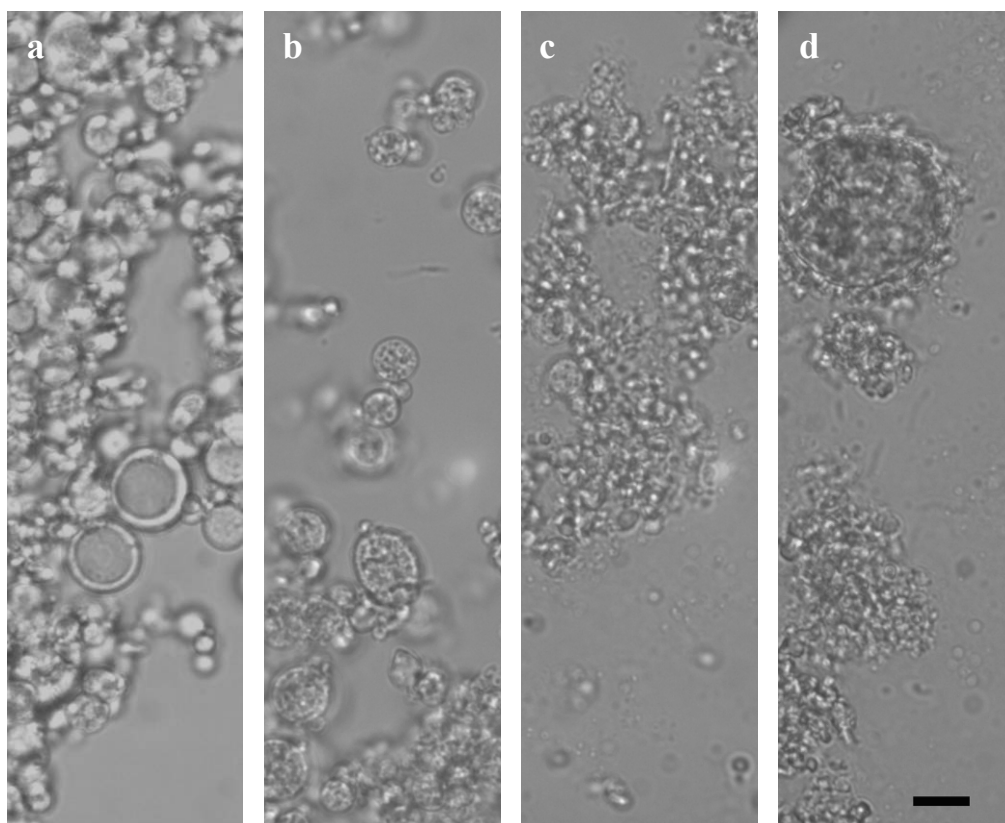
Figure S3: Lysozyme microspheres synthesised using: (a) 20% (b) 40% (c) 60% (d) 80% v/v aqueous phase of 1 M NaCl in tetradecane. (Scale bar 10 μm)
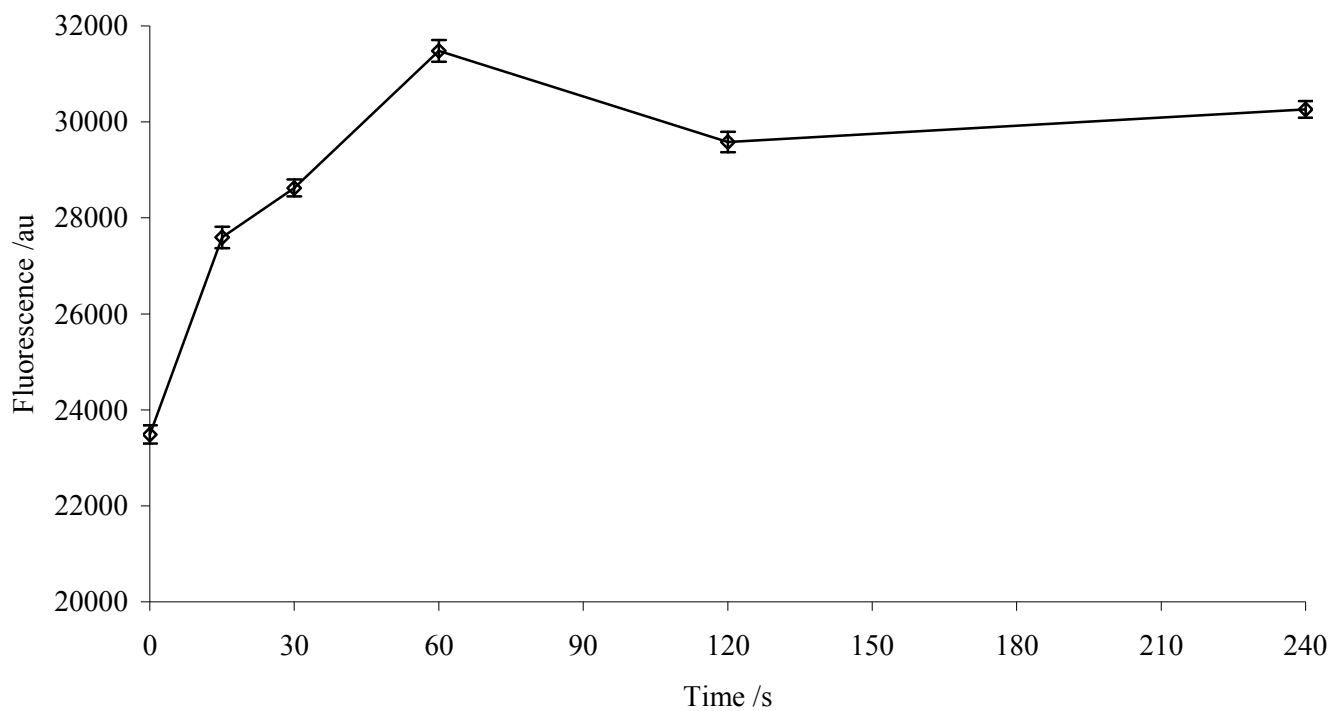
Figure S4: Change in fluorescence due to release of carboxyfluorescein on ultrasonic breakdown of emulsion containing microspheres with 50 mM 5,6-carboxyfluorescein as the internal aqueous phase.