Electronic Supplementary Information

High throughput fabrication of cell spheroids by templating water-in-water Pickering emulsions

Anupam A. K. Das,[a] Daniel Geddes, [a] Deborah Legrande, [a] Benjamin Filby [a] and Vesselin N. Paunov*, [a]

[a] *School of Mathematics and Physical Sciences (Chemistry), University of Hull, Hull, HU67RX, United Kingdom.*

**Corresponding author: Tel: +44 (0)1482 465660; E-mail: v.n.paunov@hull.ac.uk;*

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Materials

Saccharomyces cerevisiae cells (lyophilised), yeast extract, peptone, β-lactoglobulin (≥95%), FITC dextran (≥95%), polyoxyethylene oxide (PEO, MW 200 kDa) (≥95%), ethanol (≥99.97%) and fluorescein diacetate (FDA) (≥95%) were purchased from Sigma-Aldrich; dextran (≥95%) MW 500k Da, glucose (≥95%), NaCl (≥99.5 %), $CaCl_2$ ((≥95%) were supplied by Fisher Scientific. Whey protein isolate was obtained from Simpleorigin, UK. Human Embryonic Kidney cells (HEK 293 cell line) was sourced from ATTC (LGC Standards, UK). Deionised water was obtained by a Milli-Q purification system (Millipore) and used in all experiments.

Methods

Preparation of protein particles

We used two types of protein particles as water-in-water emulsion stabilised:

(i) For preparation of β-lactoglobulin particles we followed a protocol similar to Ref.1. β-lactoglobulin was dispersed in a 0.6 mM $CaCl_2$ aqueous solution and incubated in an oil bath at 85°C for 18 hrs. Subsequently, the solution was removed from the oil bath and allowed to cool for the formation of the protein particles. The average particle size of the protein particles measured using dynamic light scattering was found to be 200 nm (see Figures S1 and S2).

(ii) For preparation of whey protein particles the protocol was as follows. Whey protein isolate (2 wt%) was dissolved in Poly ethylene oxide (PEO, 5.5 wt%) fused Dulbecco's Modified Eagle's medium (DMEM) media for 2 hours, both procured from Sigma Aldrich, UK. The solution was then incubated at 1-4°C for 12 hours, to have complete hydration of the whey proteins. Subsequently, the solution was centrifuged (Thermo Scientific, Heraus multifuge X1) at 10800 g for 1 hour in order to separate the unwanted additives from the protein. The pH of the extracted supernatant was adjusted to 5.8, by drop-wise addition of filtered HCl (1 M), and mixed with equal amount of salt (NaCl) of the same adjusted pH value. The WPI/salt solution was then heated at 85°C for 15 minutes in an oil bath, and cooled to 4°C. It was also important to centrifuge the Poly ethylene oxide (5.5 wt%) fused Dulbecco's Modified Eagle's medium (DMEM) media at 5000 g for 10 mins in order remove the silica particles. The average particle size of the protein particles measured using dynamic light scattering was found to be 300 nm (see Figure S3 and S4).

		Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 201.4	Peak 1:	225.1	100.0	78.17
PdI: 0.097	Peak 2:	0.000	0.0	0.000
Intercept: 0.961	Peak 3:	0.000	0.0	0.000
Result quality Good				

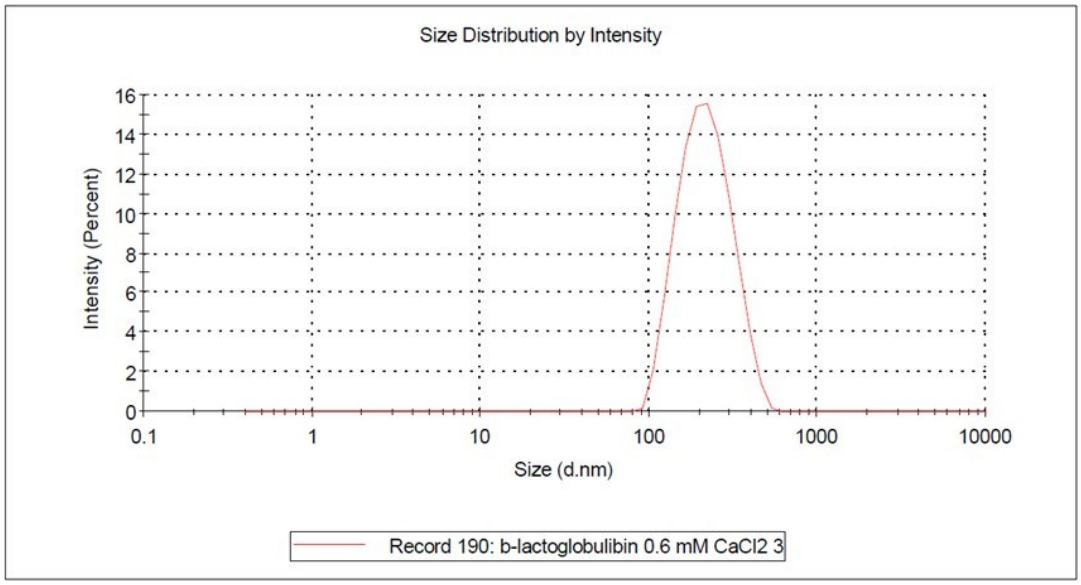

Figure S1. Size distribution of the produced β-lactoglobulin particles for stabilization of dextran-PEO water-in-water Pickering emulsions.

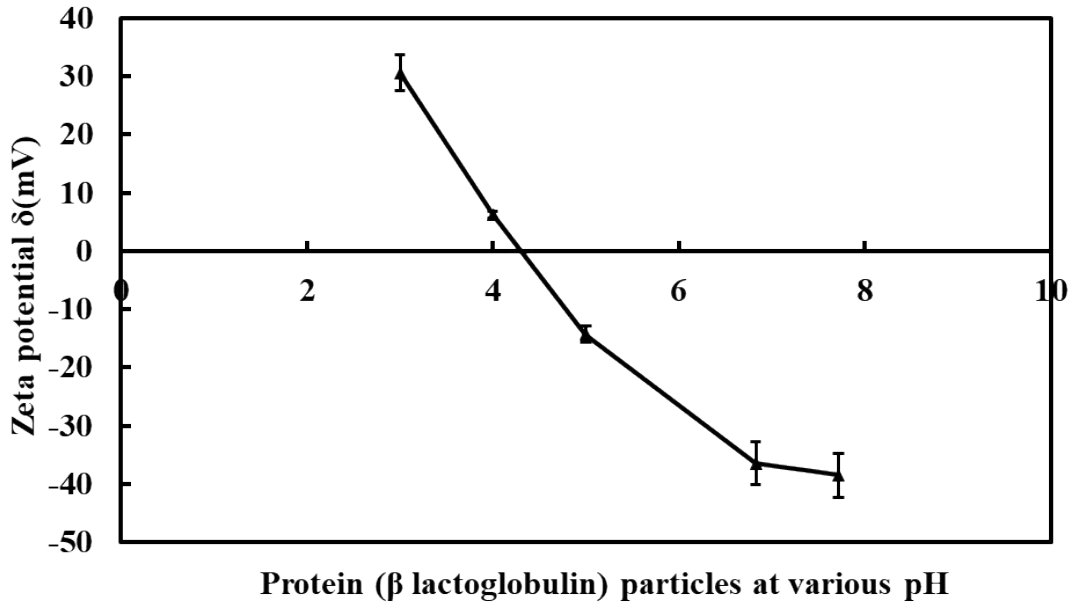

Figure S2. Zeta potential vs pH of the β-lactoglobulin particles produced by precipitation. The particles have an IEP of 4.3 and a zeta potential of -37 ± 3 mV at pH 7.

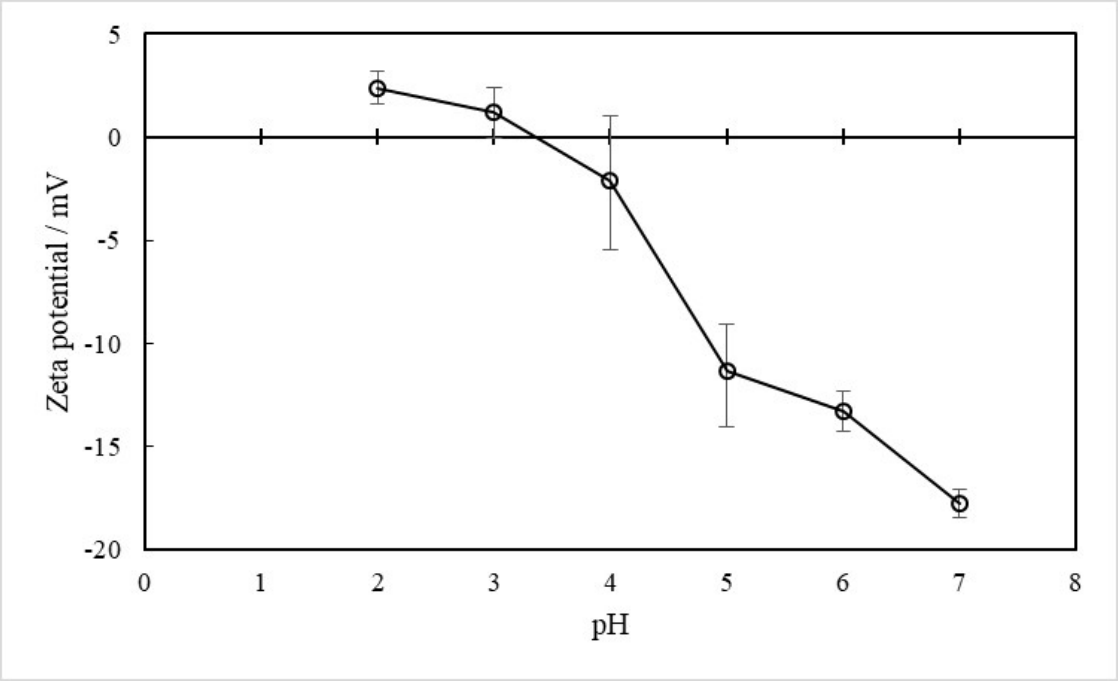

Figure S3. Zeta potential vs the pH of the protein particles produced by precipitation of whey protein with $CaCl_2$. The particles have an IEP 3.4 and a zeta potential of -18 ± 2 mV at pH 7.

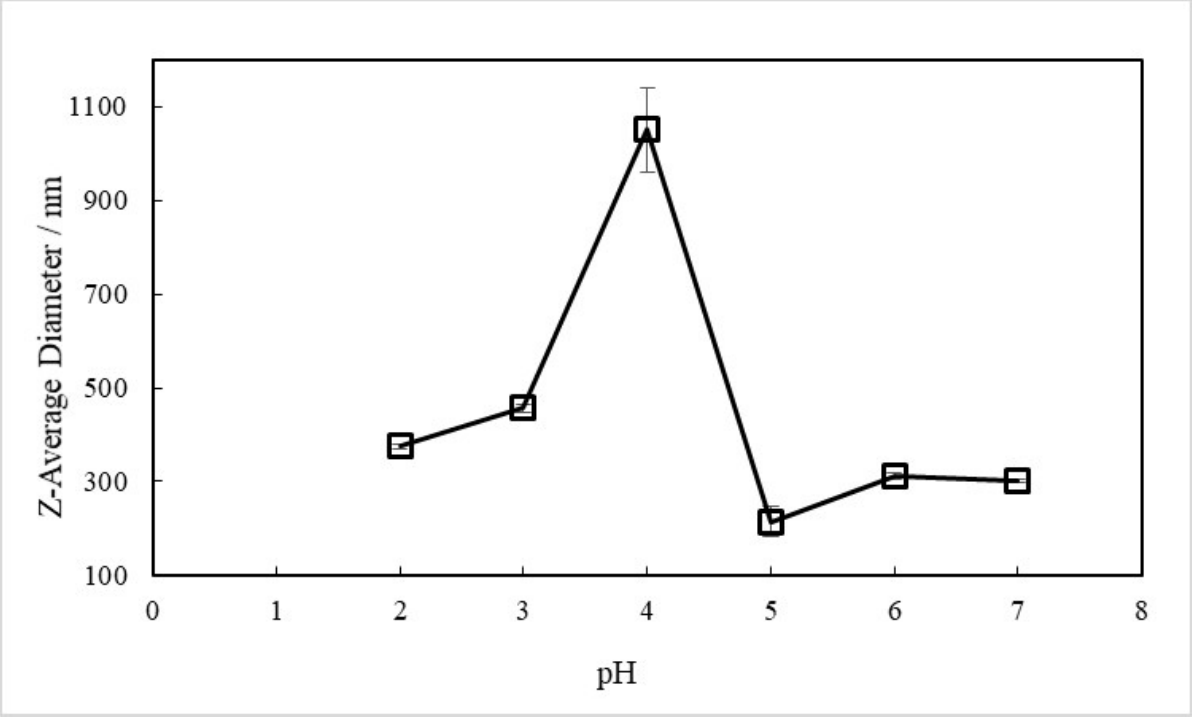

Figure S4. Average particle size of the whey protein particles produced by precipitation with $CaCl_2$ vs the pH of the solution. The whey protein particles are unstable and are aggregated at pH near the IEP 3.4 but maintain an average diameter of about 300 nm at pH 7.

Production of a w/w emulsion using β-lactoglobulin and whey protein particles

β-lactoglobulin particles (0.6 wt%) were dispersed in the dextran phase (2 wt %), along with 50 µl of FITC dextran (1×10^{-5} M) for 10 ml total solution, both obtained from Sigma Aldrich, UK. This solution was then added to the PEO (4 wt%) slowly over the course of 1 min, while homogenising at 9000 rpm for 10 mins.

In case of the whey protein particles and fibroblast spheroid production, the PEO phase (volume fraction ϕ=0.8181 of PEO concentration 5.5 wt%) was dissolved into the heat-treated solution of whey protein particles and centrifuged at 5000 g for 10 minutes to remove the manufacturer-added silica particles. Dextran aqueous solution in DMEM media (volume fraction ϕ=0.0909, dextran concentration 5.5 wt%) was then dispersed into the solution and homogenised with a syringe (21 gauge, 0.8 mm) with 10 pumps.

In case of cell spheroid production, the viable cells were dispersed in the dextran phase before homogenisation using the syringe method (see Figure S5). The concentration of the PEO phase was increased by ~2-3 wt% by the several fold dilution of the emulsion with pre-centrifuged more concentrated PEO aqueous solution. This resulted in an increase in the osmotic pressure of the continuous phase, which caused the droplets of dextran phase to shrink, as water leaves the dextran droplets to re-establish the equilibrium with the more concentrated PEO phase (see Figures S6, S7 and S8). The interfacial tension of the shrinking dextran drops compresses the encapsulated cells and packs them into cell spheroids where the cells adhere with each other within the drops. The water-in-water emulsion was then broken by diluting the sample with culture media, by a factor of 10, and the spheroids were allowed to sediment under gravity and were extracted for further analysis (see Figure S9). We avoided centrifuging the cell spheroids as they irreversibly aggregate during this procedure due to being made from adherent cells.

The emulsions made using 2 wt% Dextran and 4 wt% PEO were stabilised using 0.6 wt% β-lactoglobulin particles i.e., the droplets did not merge but they did sediment at various rate. The droplets were stable for at least a period of one week. The emulsions made using 5.5 wt% Dextran and 5.5 wt% PEO were stabilised using 2 wt% whey protein isolates particles and was found to be stable for at least 1 day. The emulsion produced doesn't need long term stability as the spheroids are made almost immediately and the emulsion is broken for the efficient extraction of the produced cell aggregates.

Cell viability analysis in cell spheroids

The isolated cell spheroid (1 mL solution) were treated with a 10 μL of 5 g/L solution of fluorescein diacetate (FDA) in acetone. The sample was incubated at room temperature in a dark container with gentle shaking for 10 minutes. The FDA is taken by the viable cells and converted to fluorescein in presence of the intracellular esterase, hence serves as an indicator for viable cells with intact membranes. The cell viability was tested after breaking the emulsion and the production of the spheroids. The viability of the cells were monitored by fluorescein diacetate (FDA) live/dead cell assay using a BX-51 fluorescence microscope equipped with a DP70 digital camera. FDA is a non-fluorescent compound which can easily diffuse through the cell membranes. The uptake and hydrolysis of the non-fluorescent FDA by intracellular esterase leads to the accumulation of fluorescein within the morphologically intact cells. Most cells mammalian, yeast, gram positive or negative contains esterase and can hydrolyse FDA.[2] The presence of the important mitochondrial enzyme Esterase in the cells is a proof of its biochemical activities which is breaking down ester into acids and alcohol. In some cases it has also shown to have an activity towards breaking down lipids.[3] Hence the efficient conversion of the non-fluorescent FDA to fluorescent Fluorescein by the nonspecific activity of intracellular enzyme esterase in HEK293 shows that the cells are highly active and have the capability to grow and proliferate."

Encapsulation of cells in Dextran-in-PEO water-in-warer emulsions

Example with yeast cells: 2 wt% dextran was dissolved in the YPD media containing 2.5 wt% (wet cell weight) yeast cells. Approximately 0.1 volume fraction of this suspension was dispersed in the 4 wt% PEO phase and homogenised at 9000 rpm for 10 mins using an Ultra Turrax 8 mm homogeniser. The YPD media composition was: yeast extract (10 g, 1 %), peptone (20 g, 2 %) and D-glucose (20 g, 2 %) all dispersed in Milli Q water.

Additional results and discussion

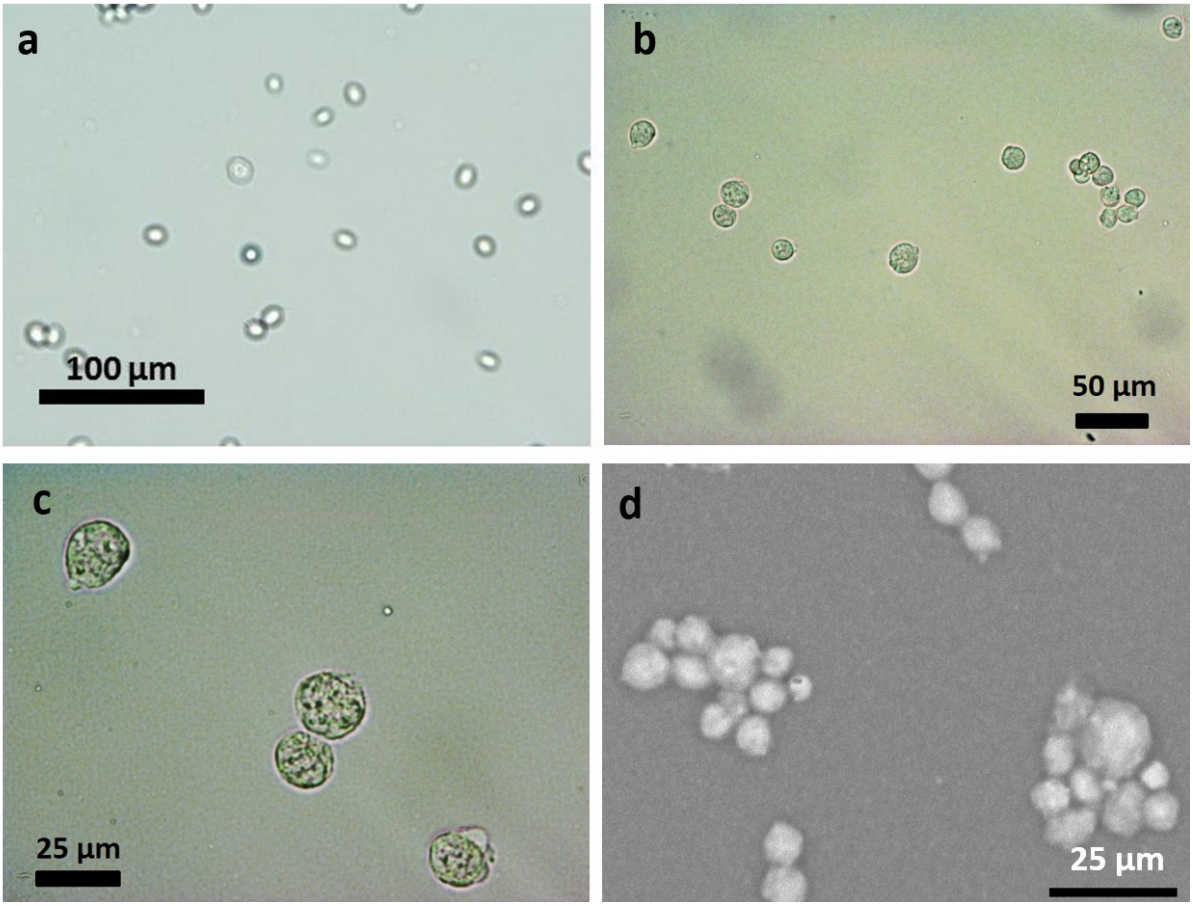

Figure S5. (a) Optical microscope image of yeast cells dispersed in YPD media. (b)-(d) Optical microscope images of HEK293 cells after detachment from the growth flask surface by using trypsin and dispersed in DMEM growth media; (d) SEM images of HEK293 cells after fixing with 2% glutaraldehyde.

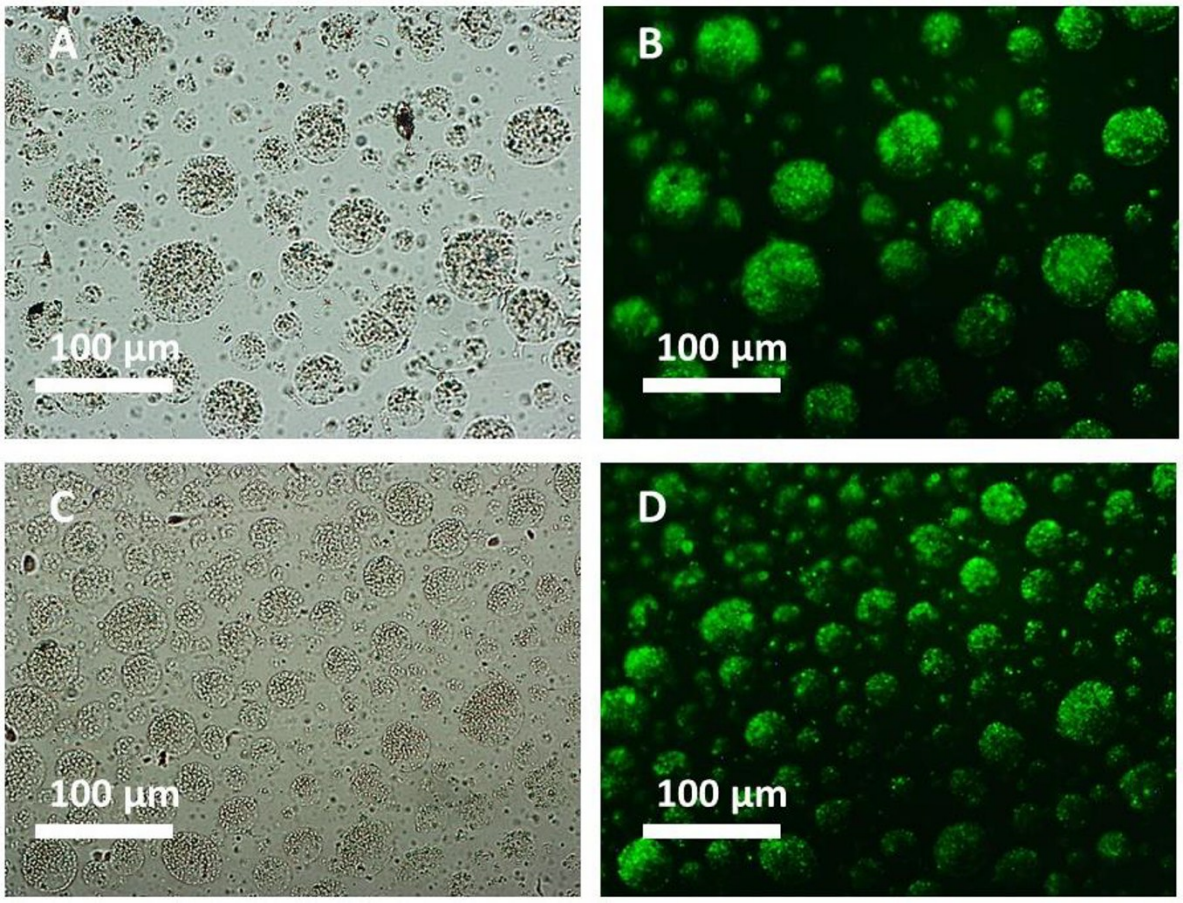

Figure S6. (A) and (C) Optical microscopy images showing a Dextran-in-PEO emulsion stabilised by 0.6 wt% (β-lactoglobulin) protein particles introduced in the PEO phase. FITC-dextran is used to show the dextran phase. The dextran phase drops are packed with yeast cells. (B) and (D) Fluorescence microscopy images of the same system. Images (A) and (B) were taken after homogenisation, (C) and (D) were taken after 30 mins.

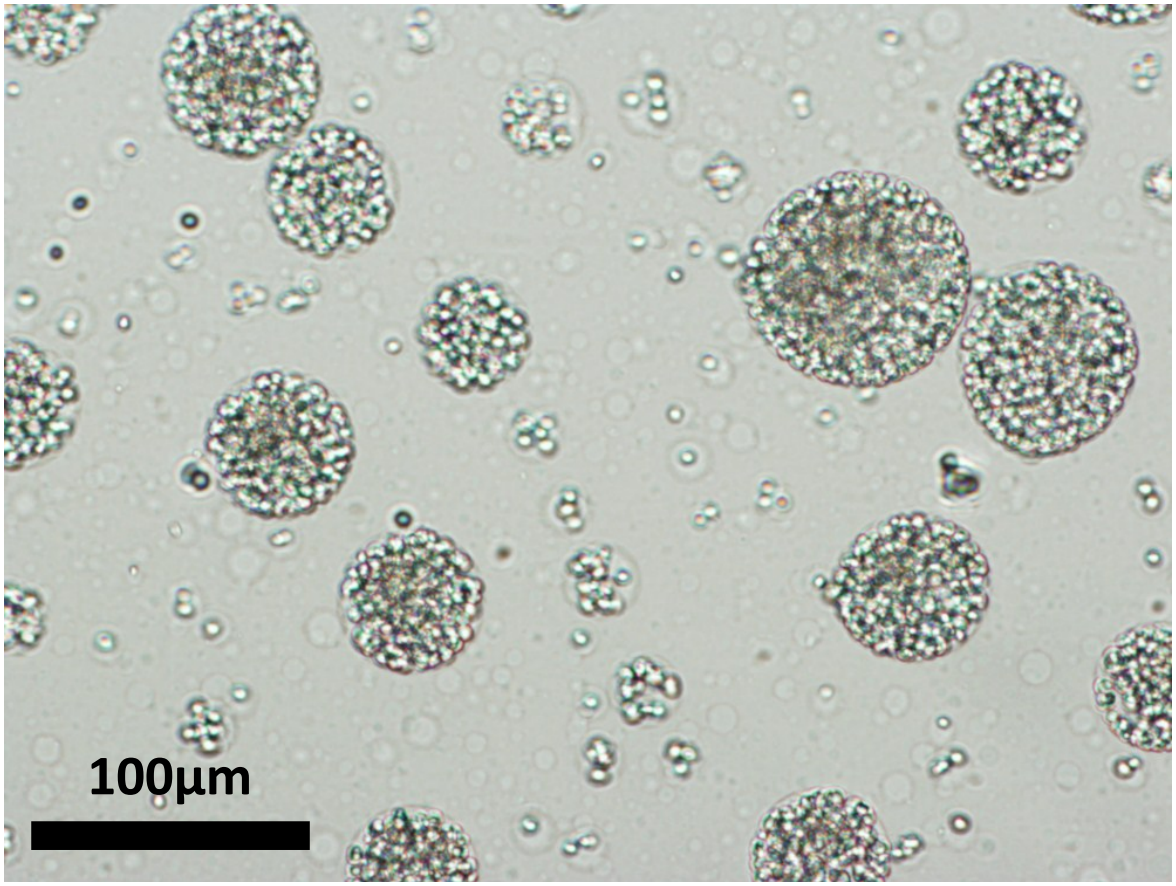

Figure S7. Microscope image of yeast cells packed in spheroid structures in Dextran-in-PEO emulsion stabilised with (β-lactoglobulin) protein particles. The image show the sample just after homogenisation. The droplets are filled with yeast cells due to the high concentration of cells. Similar result can be obtained by using lower cell concentration and further adding more concentrated PEO phase. Yeast cells are used here due to the ease of observation of the structure of the cell aggregates.

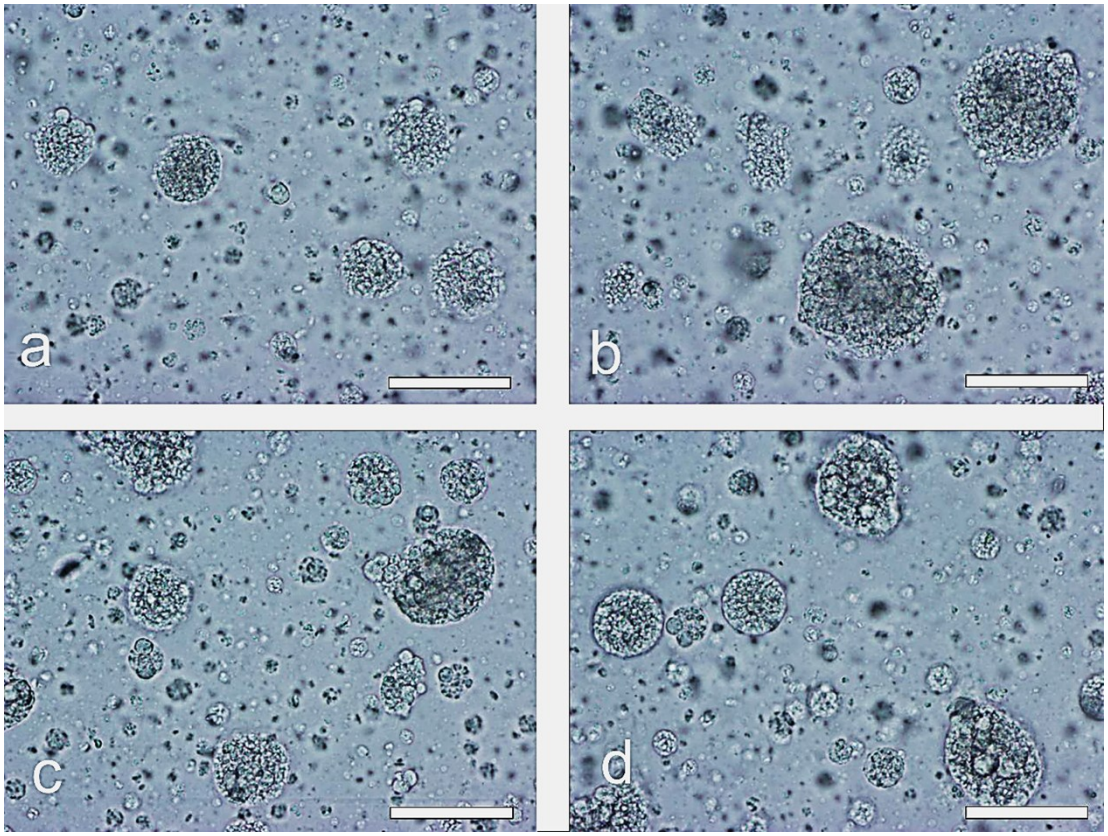

Figure S8. Optical microscope images of dextran-in-PEO water-in-water emulsion stabilised with whey protein particles with encapsulated HEK293 cells in the dextran phase. Scale bars are 100 μm. The emulsions contain 5.5 wt% dextran solution encapsulating the cells as a disperse phase of volume fraction ϕ=0.091 and 5.5 wt% PEO phase. Images (a) and (b) are taken immediately after emulsification, while (c) and (d) are taken 30 min after emulsification.

Although the obtained tissue spheroids are not completely uniform and monodisperse, they can find have various applications from the reconstruction of organs and tissues by the bioprinting method to the generation of various kinds of tumour models.[4] In this case the generated spheroids can be used for the fabricating a living organ construct. The fairly similarly sized spheroids can be used for bio fabrication of living organs by populating a biodegradable scaffold and the resultant tissue fusion process for the generation of a full organ. The method could be improved further if a method for preparation of monodisperse water-in-water emulsion is used. Currently this is achievable by microfluidic techniques, similar to Ref. 5 but the yield would be very small which is not practical for tissue engineering applications as it is not a high-throughput method.

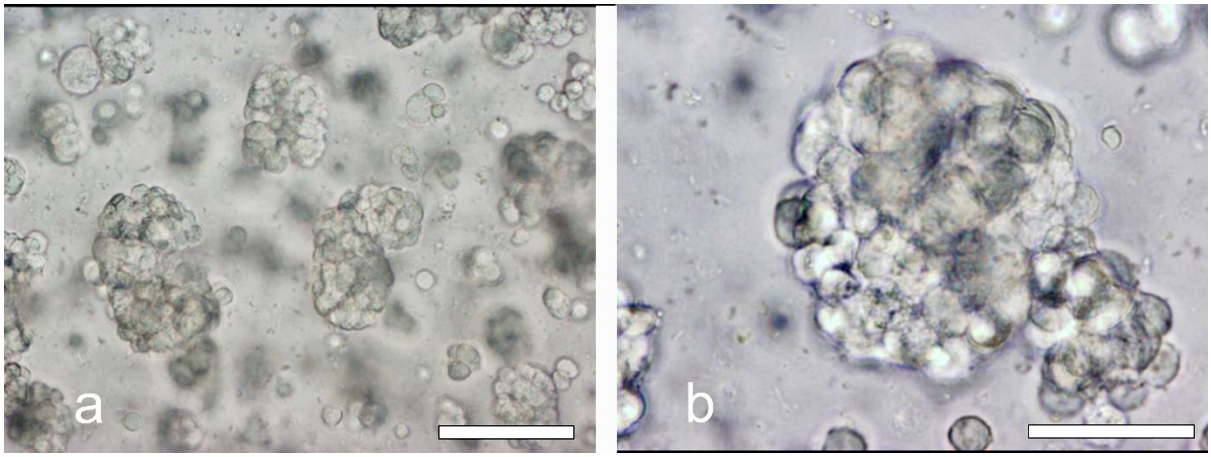

Figure S9. Optical microscope images of isolated HEK293 cell spheroids after breaking down of the dextran-in-PEO water-in-water emulsion stabilised with whey protein particles. Images (a) and (b) correspond to different magnifications with scale bars 100 μm and 50 μm, respectively.

There is a small fraction of cells that did not form cell spheroids but they can be efficiently extracted by the process of separation of the spheroids and reintroduced in fresh media based emulsions for gradual spheroid generation. Further optimisation of the method can minimize the need for recycling of these cells. The advantage of this method is that it can pack large amounts of adherent cells into spheroids.

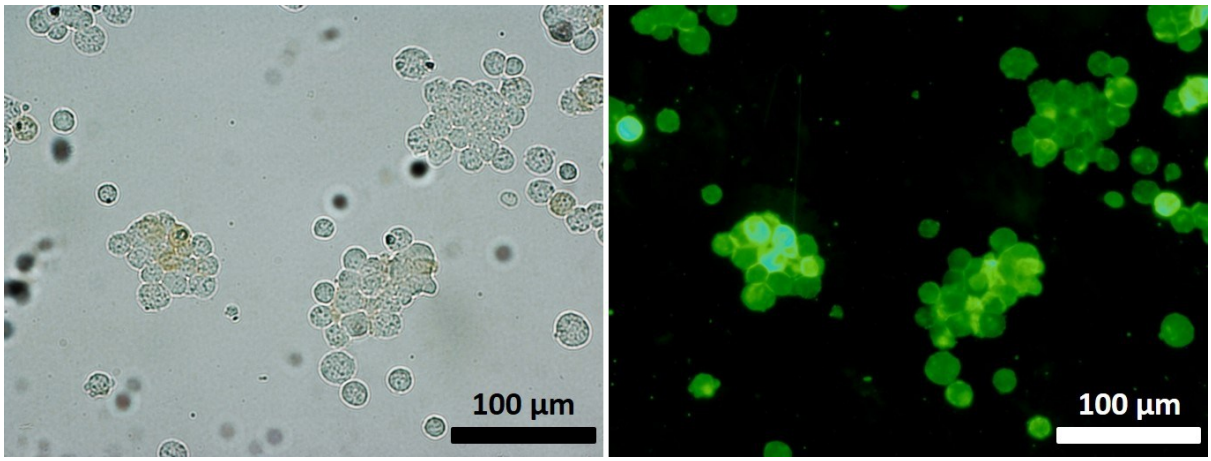

Figure S10. Optical microscopic image of (a) HEK293 spheroids, and (d) florescence microscope images of HEK293 cell spheroids isolated from the DEX/PEO emulsion, showing the viability of the fibroblast cells by using FDA live/dead assay.

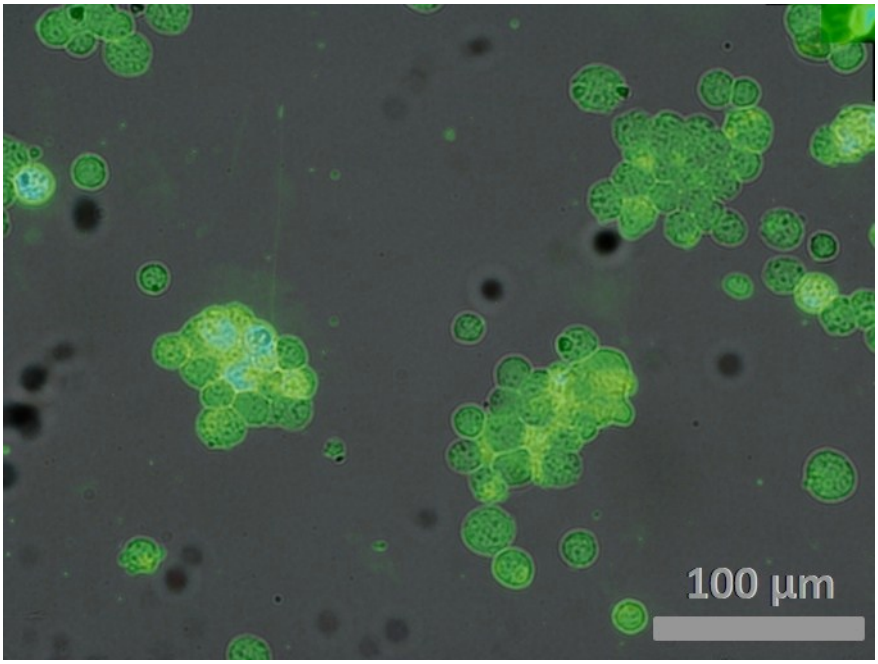

Figure S11. Combined optical and fluorescence microscopic image of HEK293 cell spheroids isolated from the DEX/PEO emulsion, showing the viability of the fibroblast cells by using FDA live/dead assay.

The cell viability of the spheroids in general were tested for different spheroid sizes. It was found that the cells in larger spheroids were equally viable compared to cells in smaller spheroids. We enclose in the ESI additional images of spheroids after their isolation from the w/w emulsion which were tested with the FDA assay and show high viability (note that practically all cells in the spheroids are fluorescent, i.e. their membrane is intact and they are viable" (see Figures S10 and S11).

Spheroid size distribution

Tissue spheroids of broad size distribution of were produced and used in various successful applications in tumour models analysis and tissue engineering (see Refs. 6-8). A novel cylindrical microwell platform was used by these authors to produce human bone marrow-derived mesenchymal stem cells (hMSCs) spheroids without any cell loss (see Ref.6). The size distribution of the (hMSCs) spheroids in their study shows that the generated spheroids are also far from being monodisperse and have a degree of polydispersity similar to the HEK293 cell spheroids generated by our new method in the present study. Another example of use of highly polydisperse spheroids was presented in Ref. 8 and is based on the human mesothelioma cell line H2052 generated on a microfluidic platform, with a size range of 100-220 µm which were

fabricated and used as tumour models. This shows that spheroids with different sizes has broad range of successful applications from tumour models to tissue engineering. The method presented in our study is not aimed to generate monodisperse spheroids of uniform shapes. A variation in the cell spheroid size is observed and in principle the spheroids size can be controlled by the volume fraction of the DEX phase and the power of the homogeniser. Alternatively, the water-in-water emulsion formation process can be modified and improved to produce monodisperse droplets of narrower spheroid generation by using microfluidics at the expense of becoming low throughput method. We intend to study these topics in more detail in a follow up publication. The method presented in this study generates similarly sized tissue spheroids and from this point of view is much better than the broadly used hanging drop method which suffers from very small yield of spheroids and long preparation times.

The HEK293 cell spheroids produced in the present study using the templating of water-in-water Pickering emulsions were observed using optical microscopy and the spheroids sizes were measured using the ImageJ software for the particle size distribution analysis. The spheroid sizes were measured over 10 images and were used for the analysis, and the distribution was presented by normal distribution curve as shown in Figure S12f.

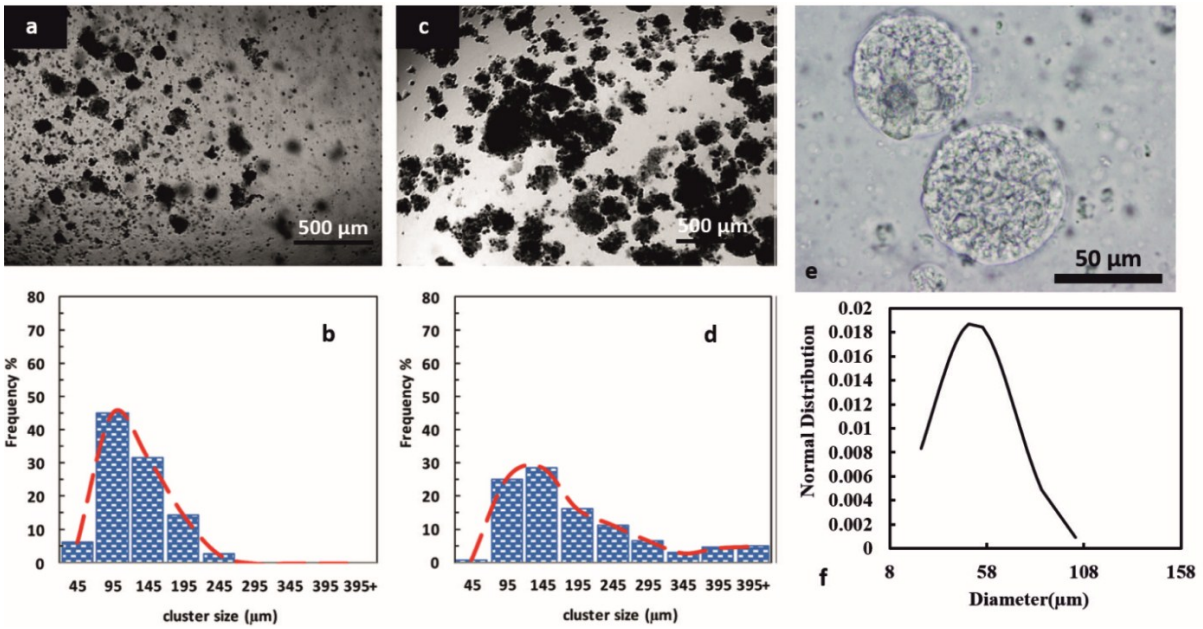

Figure S12 Optical microscope images and size distribution of cell spheroids (a) HeLa cells in microgel, (c) in suspension culture, (e) HEK293 cell spheroid and (b), (d) and (f) spheroid size distribution of HeLa cells in microgel, suspension culture and HEK293 spheroids respectively.[6] Reproduced with permission from Refs. 6. Copyright 2016 The Royal Society of Chemistry.

In Ref. 6, spheroids of the cervical tumour cell line HeLa were produced as tumour spheroid model in thermosensitive hydrogels and cell suspension as shown in Figure S12 (a) and (c) respectively. The size distribution showed that these spheroids are also not monodisperse and have similar distribution (Figure S12f) to the HEK293 cell spheroids shown in our present work as evident from comparing Figures S12b and S12d with Figure S12f.

References

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