Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

"Specifiable biomimetic microsponges for timed release of crystal entrapped biomolecules useful in bone repair"

Supplementary Information

Microsponge synthesis

Microsponges were generated by a previously published method (**Figure 1**). In brief, a mixture of a metastable supersaturated calcium carbonate Kitano solution (Kitano 1962) was prepared with octodecane oil and SDS (Sigma-Aldrich). Changes were made to chemicals and the methods. Firstly, we experimented with alternatives to the oil phase and the surfactant because of their cell toxicities, although after drying, cleaning and sterilisation toxicity is extremely low. The alternative low cell toxicity oils tested were corn oil, *Zea mays*; C16:0[80%]/ C18:0[14%]/ C20:0[3%]/ C18:1 cis-9/C18:2 n-6) oil and (b) phospholipid source (from soybean, Glycine max; C57H98O12; C-18:3 [7%]/ C-18:2 [51%]/ C-18:1[23%]/ C-18:0 [4%]/ C-16:0 [10%]. The alternative analogue for SDS was a plant-based phospholipid (1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine; Sigma-Aldrich). The composition of the bio-based oil/SDS/water microemulsion compositions of ratios 81:4:15 wt.-%.

In effect a vinaigarette was created with water droplets in a vegetable oil additionally stabilised by plant-based phospholipids. Into the Kitano solution was added the different biological small molecules, RGD tripeptides (Arginylglycylaspartic acid; 25kDa), DNA plasmid (pcDNA3-myc-his lacZ; 20 kDa) and recombinant human rhBMP (28 kDa, homodimer, glycosylated) at higher than physiologically relevant concentrations. Unlike a vinagerette a permanent micodroplet (1-20 mm) emulsion was injected with the powerful energy from sonication to stabilise the entire system. The sonication in short bursts did not damage the biomolecule contents. In each 10 mL microemulsion 2.5- 3.3 million microsponges were generated on average.

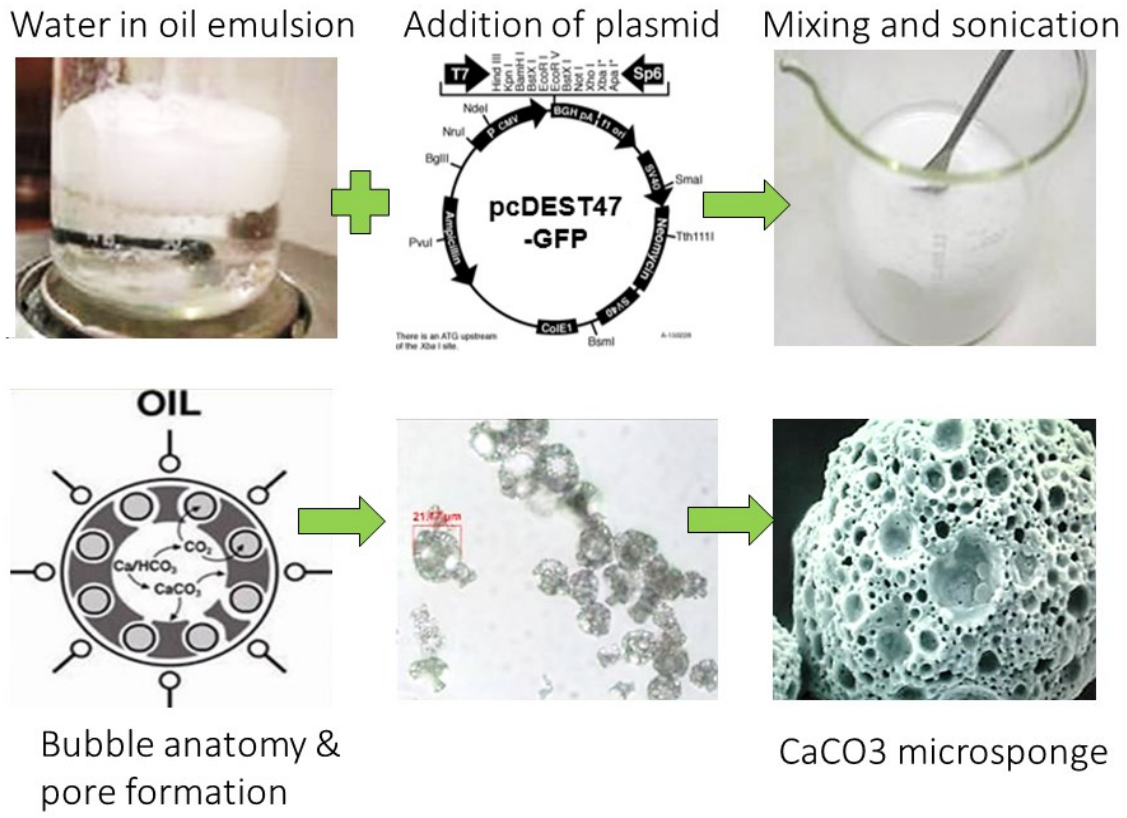

Figure 1: *Basic method of generating porous microsponges from a reverse microemulsion. The Kitano solution is prepared with admixed small biological molecules of 3 types, BMP, Plasmid and RGD.*

Cell Culture

Human osteoprogenitor cells

Bone marrow samples were obtained from haematologically normal patients (n=4) undergoing routine total hip replacement surgery. Primary cultures of bone marrow cells were established as previously described.[1] In brief, marrow cells were harvested using αMEM from trabecular bone marrow samples and pelleted by centrifugation at 1100 rpm for 4 min at 4°C. The cell pellet was resuspended in αMEM and passaged through nylon mesh (70 mm pore size; Lockertex, Warrington, UK). Nucleated marrow cells were cultured at a cell density of 2 x 10^7 in 80 cm^2 plastic culture flasks in αMEM supplemented with 10% FCS and in osteogenic media (supplemented with dexamethasone (10^{-8} M), ascorbate 2-phosphate (10^{-4}M)). Cultures were maintained at 37^0C and 5% CO_2 for up to 28 days. At confluence (12-14 days from extraction) cells were passaged into 25ml sterile universals in preparation for centrifugation and pellet formation with and without microsponges.

RGD and rhBMP-2 microsponge/ hBMSC pellets

The quantities of RGD and rhBMP-2 added to the Kitano solutions were set according to published accounts of optimised quantities to maximise cell activity relating to adhesivity for the RGD (1mM or 10 μg/ mL) set and osteogenesis for the rhBMP-2 (200ng/ mL of Kitano) set of microsponges. Human osteoprogenitor cells were centrifuged at 1500 rpm into either RGD or rhBMP-2 microsponges into three-dimensional culturable spheroidal pellets measuring 2 mm-5 mm in diameter. The pellets were cultured and suspended in plain non-osteogenic media for 14 to 21 days. At these two time points the pellets were removed, fixed, paraffin embedded, sectioned and stained with bone specific dyes and antibody colormetric labels to determine the presence and extent of bone matrix formation.

Timed release profiling

Release of entrapped biomolecules was measured by the quantities released into PBS. The concentration of the DNA biomolecules and PNPP dye were measured using a spectrophotometer. The spheroids were immersed in PBS for up to 7-14 days. On each day the PBS solution was removed and measured for the quantities of Plasmid using a spectrophotometer reading at an absorbance of 320nm to determine the A260 value. The increments each day of measurement produced the data for a cumulative plot of DNA concentration from the released 2.5 million microsponges over time. The plotted data was transformed into a percentage of the DNA in the solution as a fraction of the total loaded DNA at the start. The starting concentration was measured by crushing the microsponges straight after their fabrication, immersing into PBS and measuring the quantity of DNA in the solution. Suitable offset controls included measurements made of the solution from microsponges without the entrapped and occluded biomolecules.

Nacre water soluble matrix extraction

The water-soluble matrix of *Pinctada maxima* was extracted using the method of Rousseau *et al.* (**Rousseau M, Pereira-Mouriès L, Almeida MJ, Milet C, Lopez E. The water-soluble matrix fraction from the nacre of Pinctada maxima produces earlier mineralization of MC3T3-E1 mouse pre-osteoblasts. *Comp Biochem Physiol B Biochem Mol Biol*. 2003;135(1):1–7**). The nacreous layer was scraped from the inner surface of the seashell. The strips of nacre were then pulverised with a ceramic pestle in a mortar into fine grains with sizes between 100- 150 μm. 200g of nacre powder was suspended in distilled water for 24 hours under continuous stirring. The nacre powder suspension was centrifuged at 2,000 rpm to form a pellet and the supernatant was removed. The supernatant contained a water-soluble matrix (WSM) from the nacre made up of essentially glycoproteins (**Lopez *et al.* 1995; French Patent No FR 9515650, WO 97/24133-10/07/97**).

Chitin nanofibril fabrication

High aspect ratio alpha chitin nanofibrils 3-4 nm by 200 nm were generated by solubilising the crab shell in 3M HCl at 109°C for 1 h (15 g of chitin in 300 ml) and treating the remainder to 5% NaOH at 100°C removing the proteins (**Murray SB, Neville AC. The role of pH, temperature and nucleation in the formation of cholesteric liquid crystal spherulites from chitin and chitosan. *Int J Biol Macromol*. 1998;22(2):137–144**). The chitin nanocolloid was centrifuged at 2500 rpm and the overlying solvent was replaced with distilled water. It was in this form that chitin, as nanofibrils, was mixed into the Kitano solution.

Statistical analysis

Values are expressed as mean +/- SD. Experiments were performed at least three times and results of representative experiments are presented except where otherwise indicated. Statistical analysis was performed using Student's t-test using GRAPHPAD INSTAT software (Graphpad Software, Inc., San Diego CA).

***In vivo* chick defect model to test *in situ* bone formation**

DNA plasmid containing microsponges were prepared (**Figure 2**). The microsponges were mixed into alginate solutions composited with Chinese hamster ovary (CHO) cells and the LacZ expression vectors (**Figure 3**). Two million to 2.5 million microsponges were added into 2 mL of alginate, which was excreted into 6 individual chick defect locations and spontaneously gelled by the short covering in chitosan solution (2% w/v) injected over the alginate (2% w/v) filling the defect. Chitosan treatment leads to gelling within 30-60 seconds. Small chick bone femurs were subject to a non-union defect by cutting into the bone midway along the bone using a sharp scalpel blade under a dissecting microscope. Chick bones were implanted subcutaneously in nude, immunocompromised mice for 7 days (athymic MF1-nu/nu mice (28–32 g, 8–10 weeks old, Harlan UK Ltd). The mouse was sacrificed and the chick bone removed prior to fixing, embedded in paraffin wax and subsequently sectioned. The sections were labelled with anti-LacZ GFP conjugated antibodies to visualise the expression of LacZ by CHO cells at the defect site.

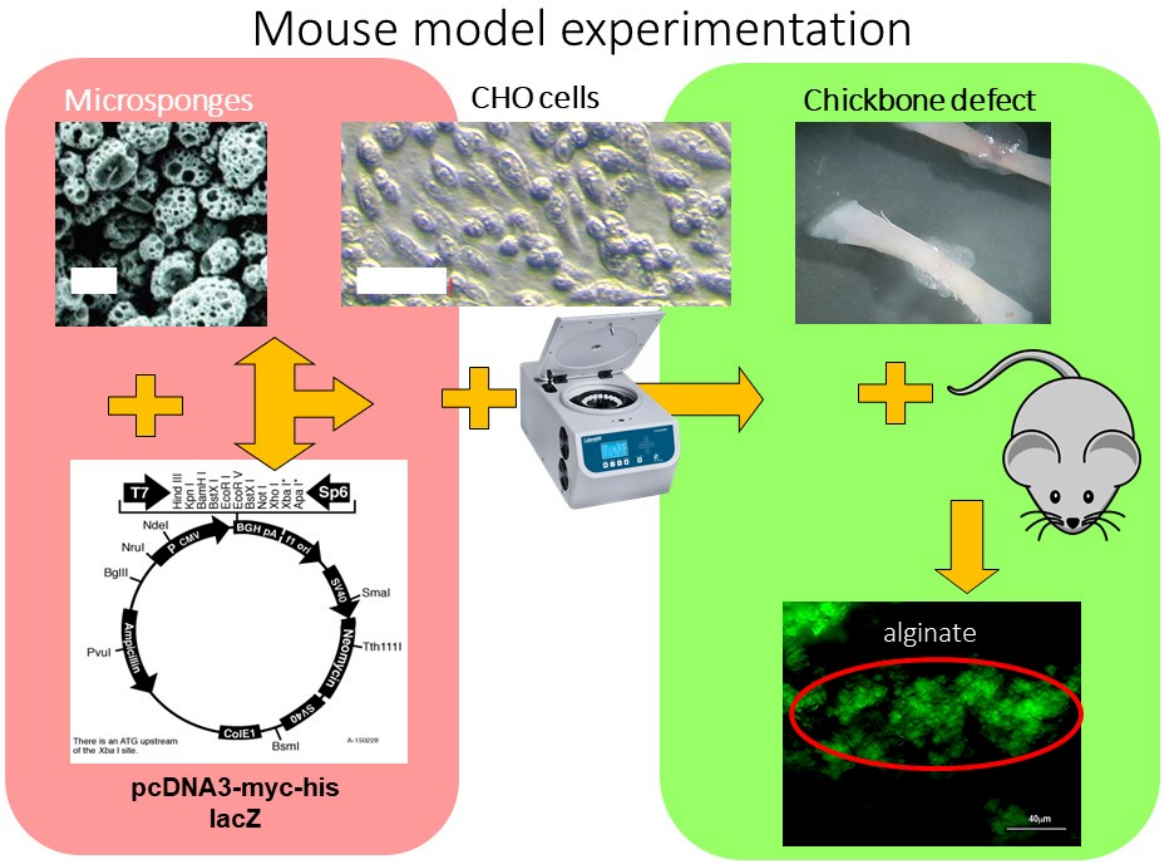

Figure 2: *Scheme of the microsponge in vivo experiment conveyed inside a chick bone defect to demonstrate the functionality of microsponges.*

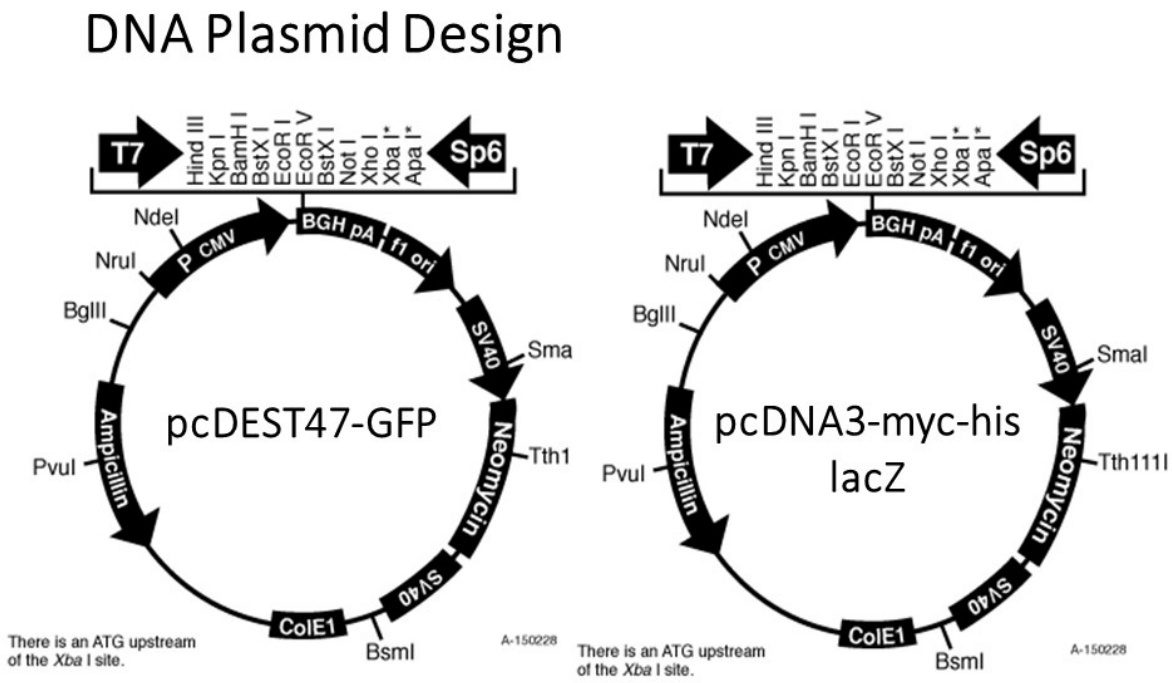

Figure 3: *The two plasmid constructs/ vectors used in this study. One containing an insertion for expressing GFP and the other for the expression of LacZ inside the transfected cell.*

Basic histochemistry and Immunocytochemistry

Microsponge pellets were fixed in 4% paraformaldehyde and dehydrated in an alcohol series prior to embedding in paraffin wax for thin sectioning. Sectioned pellets were stained histologically using a standard bone specific colormetric staining and immunolabelling to determine the presence of bone matrix substances. Alcian blue and sirius red *staining:* Samples stained using Weigert's Haemotaxylin A & B and with Alcian blue stain (1% in 1% $^{v}/_{v}$ acetic acid) and Sirius red. *Type I collagen labelling of the hBMSC secreted matrix*: expression of the classic bone marker, type I collagen was detected using the monoclonal antibody LF 67 conjugated to horseradish peroxidase giving a rusty red colour.