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# Hybrid extracellular matrix microspheres for development of complex multicellular architectures

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**Supporting Information**

## 1. Materials and Methods

### 1.1. Preparation of hybrid microparticles

Spherical microparticles of $CaCO_3$ were prepared by wet chemical process in presence of gelatin type A. Briefly, 10mL of $Na_2CO_3$ (3 M) solution were rapidly added into an equal volume of 3 M $CaCl_2$ solution with 2.30 mg.mL$^{-1}$ of gelatin type A (Sigma), the final solution is agitated at 700 rpm on a magnetic stirrer for 40 seconds and centrifuged at 1000 rpm for 10 minutes. These microparticles are resuspended in a 14% aqueous gelatin and 1% of hyaluronic acid (207 kPa, CONTIPRO - Czech Republic) solution and incubated at 50°C overnight for the gelatin penetration into the pores of the $CaCO_3$. The process was stopped by the addition of 100 mL of water at 4°C and the microparticles harvested by centrifugation (1000 rpm during 5 min at 37°C), they were washed with phosphate buffered saline (PBS) until the elimination of the gelatin solution. For crosslinking, the microparticles were incubated for 12 hours in a solution of transglutaminase [1] (100 mg.mL$^{-1}$) in PBS at room temperature, overnight (Gelatin-HA-$CaCO_3$). The microparticles were then washed 2 times with water and harvested by centrifugation (1000 rpm during 5 min at 25°C).

### 1.2. Characterization of hybrid microparticles

The morphology of the particles was characterized by SEM (FEI, Quant 250 FEG) and TEM (Jeol ARM-200F instrument working at 200 kV). Thermogravimetric analysis of microparticles (TGA) was conducted on a TGA 851 (Mettler-Toledo) thermogravimeter by heating the sample under air (100 mL.min$^{-1}$) up to 900 °C with a 5 °C.min$^{-1}$ heating rate.

Fourier transform infra-red spectroscopy (FTIR) was used to analyze the microparticles before and after the gelatin penetration on the $CaCO_3$ particles powder at 25 °C using a Nicolet 8700 spectrometer. The spectra were acquired at a resolution of 2 cm$^{-1}$ by the accumulation of 16 interferograms. XRD of microparticles was conducted using a Bruker D8 diffractometer with a Cu Kα radiation source (1.54 Å).

The porosity of the microparticles were determined from the study of the adsorption/desorption isotherms of $N_2$ at 77 K using a Micromeritics ASAP 2020 instrument. Prior to the analysis, the microparticles were out-gassed overnight under vacuum at 37 °C during 24h. The BET surface area was calculated from the adsorption isotherm in the partial pressure range of 0.05–0.3 for $N_2$ adsorption. The total pore volume Vp was obtained from the amount of $N_2$ adsorbed at a relative pressure of 0.95. The pore size distribution was assessed by the BJH adsorption model. Calcium release from gelatin-HA-$CaCO_3$ crosslinked microparticles was determined colorimetrically using Calcium Colorimetric Assay kit (BioVision, UK) as described by the manufacturer.

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**1.3. Cells-Microparticles system construction**

The cells-microparticles systems were obtained by alternate deposition of Gelatin-HA-$CaCO_3$ crosslinked microparticles dispersed in 0.75 $mg.mL^{-1}$ of fibronectin (FN), $5\times10^5$ NIH 3T3 fibroblast and a second layer of microparticles/FN. The fibronectin was purified as described previously.[2] Briefly, the fibronectin was purified from cryoprecipitated or fresh citrated human blood plasma (the *Agence Franc¸aise du Sang*, Les Ulis,France) by a three-step combination of gelatin and heparin– cellufine affinity chromatography. The microparticles were sterilized 15 minutes under UV.

NIH-3T3 fibroblast cell line (mouse, ATCC®,CRL-1658™) was cultured in RPMI 1640 medium (Gibco) with 10% Fetal bovine serum (Dutscher S1810-50) and 1% Penicillin/Streptomycin (Dutscher, L0022-100). Confluent cells were removed with trypsin (Dutscher, 0940-100), counted with a haemocytometer and seed on 96 well plates with glass bottom (Grenier bio-one, Germany) at a concentration of $5\times10^5$ cells/well (control).

**1.4. Biological analysis**

After 3 days of culture, the samples were fixed with 3.7% paraformaldehyde and observed with confocal laser scanning microscopy. The microparticles were previously stained with HA-FITC (Fluorescein isothiocyanate - HA, Mw = 207 kDa, Contipro, Czech Republic), and the cells were fixed/permeabilized in 3.7% (w/v) paraformaldehyde (PFA) in 1× PBS containing 0.1% Triton X-100 for 15 min and blocked with 10% decomplemented FBS (Invitrogen) DNA was revealed with Hoechst 33258 (20 µg $mL^{-1}$, Sigma) and actin staining with rhodamine (rho)-phalloidin (2 $\mu g.mL^{-1}$, Sigma).

Cell organization in Gelatin-HA-$CaCO_3$ microparticles were obtained by confocal laser analysis. For that, a Zeiss confocal microscope (LSM, 710) using a 10, 20 and 40x (Zeiss Achroplan) objective was used.

The filter settings were as follows: excitation at λ = 488 nm and an emission band-pass filter of 489-556 nm (green) for the FITC dye, an excitation at λ = 568 nm and an emission band-pass filter of 563-663 nm (red) for rhodamine dye and an excitation at λ = 470 nm for Hoechst (blue).

Seeding efficacy was measured by a MTT assay. It is based on the ratio between the number of adhering cells (after 4 h in culture) and the total number of adhered cells on the control. The control was the NIH-3T3 fibroblast cells cultured on 96 well plates with glass bottom. For that, 100 µL of 0.2 $mg.mL^{-1}$ MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) were added to the attached cells and incubated for 3 h at 37 °C under a humidified atmosphere of 95% air and 5% $CO_2$. Afterwards, MTT was removed and the cells were immersed in 300 µL of dimethyl sulfoxide to dissolve intracellular formazan crystals produced by viable cells. Absorbance was determined at 570 nm using a spectrofluorimeter (Xenius, SAFAS, Monaco).

The metabolic activity of NIH-3T3 fibroblast cells seeded Gelatin-HA-$CaCO_3$ crosslinked microparticles dispersed in fibronectin (FN) was evaluated with the in vitro Toxicology Assay Kit (Sigma Aldrich). This test is based on the reduction of resazurin dye which will become fluorescent (red) when incubated with viable cells. After 2 hours of incubation, the solution was read with the spectrofluorimeter (λex/ λem = 560 nm / 590 nm).

The seeding efficacy and metabolic activity results were averaged from three individual experiments with 3 samples for each condition (control and with microparticles).

**1.5. Statistical Analysis**

The statistical significance of the obtained data was assessed using the t-test. Differences at $P \geq 0.05$ were considered statistically not significant.

## 2. Results

 

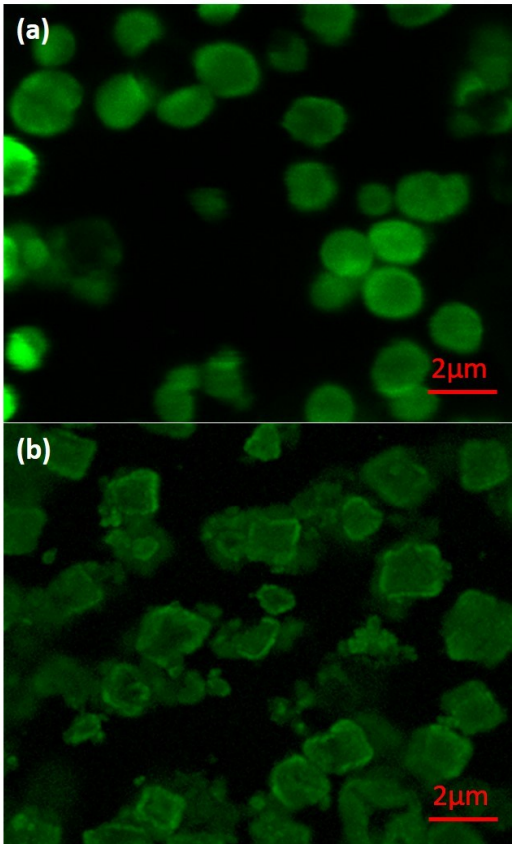


**Figure S.1.** $CaCO_3$ precipitation in presence or not of gelatin: **(a)** vaterite and **(b)** calcite formation, respectively.

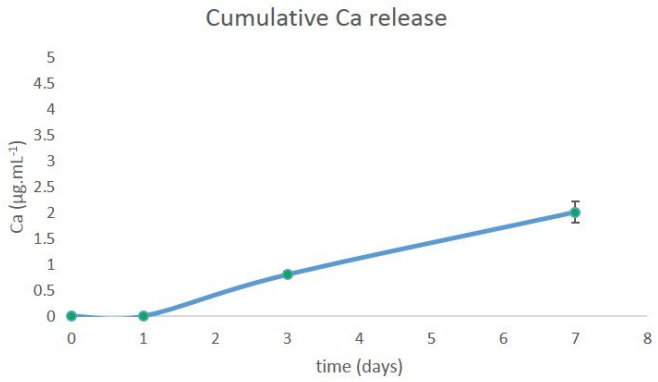


**Figure S.2.** Ca release kinetics from Gelatin-HA-$CaCO_3$ once immersed in the RPMI 1640 medium (Gibco) with 10% Fetal bovine serum and 1% penicillin/Streptomycin at 37°C.

**References**

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