



Production and characterization of engineered alginate-based microparticles containing ECM powder for cell/tissue engineering applications

Stefania Mazzitelli^{a,e}, Giovanni Luca^b, Francesca Mancuso^b, Mario Calvitti^b, Riccardo Calafiore^c, Claudio Nastruzzi^{a,e,*}, Scott Johnson^d, Stephen F. Badylak^d

^a Department of Chemistry and Technology of Drugs, University of Perugia, Perugia, Italy

^b Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy

^c Department of Internal Medicine, University of Perugia, Perugia, Italy

^d McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA

^e Caps-it Technologies, Ferrara, Italy

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ABSTRACT

A method for the production of engineered alginate-based microparticles, containing extracellular matrix and neonatal porcine Sertoli cells (SCs), is described. As a source for extracellular matrix, a powder form of isolated and purified urinary bladder matrix (UBM) was employed. We demonstrated that the incorporation of UBM does not significantly alter the morphological and dimensional characteristics of the microparticles. The alginate microparticles were used for SC encapsulation as an immunoprotective barrier for transplant purposes, while the co-entrapped UBM promoted retention of cell viability and function. These engineered microparticles could represent a novel approach to enhancing immunological acceptance and increasing the functional life-span of the entrapped cells for cell/tissue engineering applications. In this respect, it is noteworthy that isolated neonatal porcine SCs, administered alone in highly biocompatible microparticles, led to diabetes prevention and reversion in nonobese diabetic (NOD) mice.

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1. Introduction

A variety of hydrogels made of natural biopolymers have recently been proposed as artificial matrices for cell immobilization, cell transplantation and tissue engineering [1]. Biomaterials based on polysaccharides are usually employed to create an immunoprotected environment that, while preventing the grafted cell-directed immune destruction, allows diffusion of nutrients, oxygen, secreted factors and catabolic products [2].

Alginate occupies a prominent position among the different biopolymers suitable for the production of immunoisolating scaffolds, since it complies with the need for biocompatibility and long-term mechanical integrity/stability; in addition, alginate does not interfere with the cellular function of the immobilized cells; and finally the encapsulation procedure can be conducted under very mild environmental conditions, such as body temperature, physiological pH and isotonic solutions [3].

In spite of these favourable properties, alginate-based scaffolds present a number of limitations that require further improvement before they can routinely be used in clinical applications. For in-

stance, it should be considered that alginate-based hydrogels are extremely different (from a molecular point of view) from the cell's microenvironment, which largely is composed of extracellular matrix (ECM).

ECM, which usually isolated from a variety of natural sources, including dermis, submucosa of the small intestine and urinary bladder, has been proposed as a biologic scaffold for tissue engineering applications. It is known to play an important role in modulating cell adhesion, signalling, migration, proliferation and three-dimensional array [4].

On the other hand, ECM-based scaffolds do not offer the opportunity to create an immunoisolated environment. Furthermore, ECM scaffolds are inherently constrained by the material properties of the tissue from which they are derived, including shape, mechanical properties and oxygen diffusion. The material properties of ECM scaffolds can also be affected by the manufacturing process (i.e. mechanical decellularization vs. chemical decellularization), the age and health status of the animal from which the ECM is harvested, and inherent biological variability. There are limited ways in which the material and mechanical properties of ECM scaffolds can be manipulated to tailor the functional properties of the device for specific applications.

Highly purified and standardized scaffold materials (i.e. alginates) are an alternative to naturally derived ECM scaffolds, hallmarked by uniform and reproducible mechanical and material

* Corresponding author at: Department of Chemistry and Technology of Drugs, University of Perugia, Perugia, Italy. Tel.: +39 53229159.

E-mail address: nas@unipg.it (C. Nastruzzi).

properties. However, as reported above, alginates lack the bioactivity and constructive host tissue response characteristics of ECM-derived scaffolds.

Therefore, combining alginates with ECM may capture the advantages of both types of materials, i.e. mechanical and material properties, which can be manipulated with the “on-demand” gelling behaviour of alginates and bioactivity, which is provided by the ECM.

The idea of combining a polysaccharidic polymer with collagen/gelatin-based materials in a microparticle-based device for cell entrapment has been partially investigated by other research groups. For instance, Tsai et al. [5], in a short communication, described the production of gel beads containing a soluble (purified and reconstituted) form of collagen in alginate, which in this respect does not offer a solid anchorage to the entrapped cells. In a second paper, Del Guerra and co-workers [6] exploited the possibility of cultivating bovine pancreatic islets into commercially available macroporous gelatin-based microcarriers (Culti-Spher-S) before encapsulation in alginate-based microcapsules. In this respect, it is important to underline that commercial products are not easily distinguished by composition: manufacturers only say that the microparticles are produced by cross-linked gelatin, giving no technical details about the cross-linking procedure employed or the cross-linker residues ultimately present. In addition, the authors stated that they encountered serious problems with the microcarrier loading procedure. In fact, only about 50% of the dispersed islet cells were embodied within the microcarriers. In addition, the islets within microcarriers-in-alginate-microcapsules released an amount of insulin which was only about 60% of that secreted by islets directly within alginate microcapsules.

In an attempt to reconstitute the cell environment within an immunoisulatory device for cell immobilization in a much more controlled, biocompatible and cell-friendly environment, we tested the feasibility of entrapping a powder form of ECM, isolated and purified from urinary bladder (urinary bladder matrix; UBM) [7,8], together with living Sertoli cells (SCs), in alginate-based microparticles. This approach is therefore completely different, in terms of innovation, design and production strategy, from those cited and commented on above.

The specific aims of the study were to demonstrate that the inclusion of a powdered form of UBM: (i) does not alter the morphological and dimensional characteristics of the fabricated microparticles; and (ii) results in an optimal substrate for *in vitro* culture that predictably ameliorates the viability and function of the co-entrapped cells. Alginate microparticles were applied for SC encapsulation, while extracellular matrix (UBM) was embodied within these constructs to enhance cell survival and function.

SCs were selected as the cell model since they have been recognized as playing important roles at different levels, including regulation of synthesis of androgen binding protein and cross-talk with other non-Sertoli testicular cell populations, like peritubular and Leydig cells. Moreover, SCs provide an appropriate microenvironment for the development of germ cells. Recent reports have also shown that these cells may provide nutrients and immunomodulatory and trophic factors that are able to improve the development, survival and functional competence of different cell types [9,10]. In addition, it has recently been reported that isolated neonatal porcine SCs, administered alone in highly biocompatible microcapsules, led to diabetes prevention (88% of cases) and reversal (81% of cases) in overtly diabetic (nonobese diabetic [NOD]) mice, with no need for additional β cell or insulin therapy. The curative effects of SCs were strictly dependent on efficient tryptophan metabolism in the xenografts, leading to the transforming growth factor (TGF)- β -dependent emergence of autoantigen-specific regulatory T cells and the recovery of beta cells function in the diabetic recipients [11].

2. Materials and methods

2.1. Isolation of SCs

SCs were isolated from the testes of neonatal pre-pubertal “Large-White” pigs, aged 7–15 days, according to previously reported methods and as modified in our laboratory [12]. Briefly, decapsulated testicular tissue was minced and then digested with trypsin (2 mg ml^{-1}) and DNase I (Sigma) to obtain a clean fraction of seminiferous tubules devoid of other testicular cells such as Leydig cells and peritubular cells. The isolated SC aggregates were spun for 5 min at 800 rpm, washed twice with Hanks’ balanced salt solution, plated in HAM F12 (Euroclone) supplemented with 0.166 nM retinoic acid (Sigma) and 5 ml/500 ml of insulin-transforming selenium (Becton Dickinson #354352), and incubated in 95% air/ CO_2 at 37°C . After 3 days of *in vitro* culture maintenance, the SCs were incubated with 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer (Sigma) at pH 7.2 for 5 min in order to eliminate any residual germinal cells. After washing the Tris off, the SCs were cultured under the same conditions, in 75 cm^2 cell culture flasks. After isolation, purified SC monolayers were identified by anti-Mullerian inhibiting substance (MIS) immunostaining, according to previous reports [12]. Cell viability was assessed, before and after encapsulation, by double staining with PI and Calcein-AM according to the manufacturer’s instructions. For the viability analysis, the double-stained cells were examined under a fluorescence microscope (Nikon, Optiphot-2; Nikon Corporation, Tokyo, Japan) using the filter block for fluorescein; dead cells were stained in red, while viable ones appeared in green. Cell isolation was performed in compliance with national (Italian Approved Animal Welfare Assurance A-3143-01) and Perugia University Animal Care and Use Committee guidelines.

2.2. UBM powder preparation

The preparation of UBM has been previously described [13,14]. In brief, porcine urinary bladders were harvested from 6-month-old 108–118 kg pigs immediately following euthanasia. The excess connective tissue and residual urine were removed. The tunica serosa, tunica muscularis externa, tunica submucosa and the majority of the tunica muscularis mucosae were mechanically removed. The urothelial cells of the tunica mucosa were dissociated from the luminal surface by soaking the tissue in 1.0 N saline solution. The resulting material, which was composed of the basement membrane of the urothelial cells plus the subjacent lamina propria, was referred to as urinary bladder matrix (UBM). UBM sheets were placed in a solution containing $0.1 \text{ vol.}\%$ peracetic acid (Sigma), $4 \text{ vol.}\%$ ethanol (Sigma) and $95.9 \text{ vol.}\%$ sterile water for 2 h. Peracetic acid residue was then removed with two 15 min phosphate-buffered saline (pH 7.4) washes, followed by two washes with sterile water for 15 min each. The decellularized UBM sheets were then lyophilized using an FTS Systems Bulk Freeze Dryer Model 8-54 and comminuted to a particulate form using a Wiley Mini Mill.

2.3. Preparation of engineered alginate microparticles

The preparation of engineered alginate microparticles was performed with minor modification of a previously published method, by a gas-driven monojet device [12]. The encapsulation system (schematized in Fig. 1) comprises a gas-driven monojet device connected to a precision peristaltic pump (for the feeding of the different alginate dispersions) and a gas flask (usually nitrogen) equipped with a flow meter (providing the gas for the atomization of the alginate dispersion). Depending on microparticle type, the gas-driven monojet device was fed with: (i) alginate dispersions

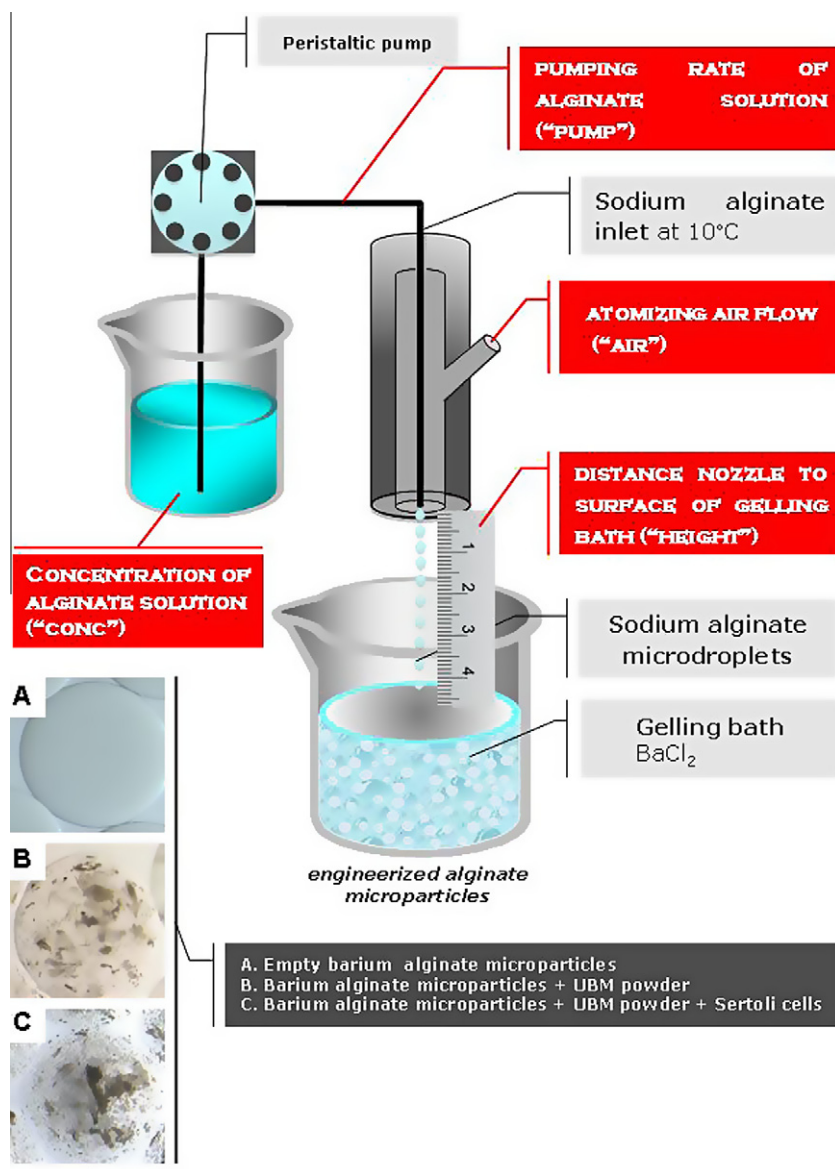


Fig. 1. General scheme depicting the preparation of barium alginate microparticles by a gas-driven monojet device. The insets present bright-field stereophotomicrographs of empty alginate microparticles (A), alginate microparticles containing UBM powder (B) and alginate microparticles containing UBM powder plus SCs (C); the bar corresponds to 260 μm . In the red boxes, the optimized experimental parameters considered for the microparticle preparation are indicated.

in water (0.5–3.0%, w/v); (ii) alginate dispersions containing SC ($4\text{--}12 \times 10^6$ cells mL^{-1}); (iii) alginate dispersions containing UBM powder (0.5–1.5%, w/v); and (iv) alginate dispersions containing SCs plus UBM powder. In the case of microparticles containing SCs, the cell suspension was continuously mixed by a magnetic stirrer to prevent cell clumping. Different alginate microparticle batches were prepared by changing the following reported experimental parameters: nozzle-to-gelling bath distance “height”, atomizing gas “gas”, feeding rate “pump” and alginate concentration “conc” (see Table 1). The microdroplets generated by the gas-driven monojet device were transformed into microparticles by an ionic gelation procedure using a gelling bath containing a barium chloride solution (1.2%; w/v).

2.4. Dimensional and morphological characterization of engineered alginate microparticles

The morphology of engineered alginate microparticles was evaluated by optical stereomicroscopy (Nikon SMZ 1500 stereo

microscope, Tokyo, Japan) and fluorescence microscopy (Nikon, Optiphot-2; Nikon Corporation, Tokyo, Japan). Microparticle size and size distribution were determined by photomicrograph analyses (Eclipsenet version 1.16.5, Laboratory Imaging s.r.o. for Nikon B.V.). Microparticles samples were deposited onto a microscope slide and examined microscopically. Samples of at least 300 microparticles were examined and the mean size $\pm$ SD was determined.

2.5. Optimization of the preparation procedure of engineered alginate microparticles by design of experiment (DoE) approach

To study the effect and the influence of different experimental parameters (i.e. “height”, “gas” and “pump”) on the dimensional and size distribution characteristics of alginate microbeads, a randomized central composite face-centered (CCF) design consisting of 17 runs was used. The experimental design and the evaluation of the experiments were performed using the PC software MODDE 8.0 (Umetrics AB, Sweden), followed by multiple linear regression (MLR) algorithms.

Table 1

Production of barium alginate microparticles by a gas-driven monojet device: investigated experimental parameters and their range of variation.

Parameter	Abbreviation	Meaning	Range
Nozzle-to-gelling bath distance	Height	The distance between the nozzle of the gas-driven monojet device and the surface of the aqueous barium chloride solution used to promote the ionotropic gelation of alginate	32.0–44.0 mm
Atomizing gas	Gas	The flow of atomizing gas causing the transformation of sodium alginate solution into microdroplets	3.8–5.8 l min ⁻¹
Feeding rate	Pump	The pumping rate of the alginate solution to the inlet of the gas-driven monojet device	0.38–0.52 ml min ⁻¹
Alginate concentration	Conc	The concentration of sodium alginate solution employed for the encapsulation procedure	0.5–3.0 (% w/v)

Table 2

Production of alginate microparticles by a gas-driven monojet device: experimental parameters used for the COST approach.

Batch	Height	Gas	Pump	Conc	Microparticle size (μm) ± SD
#1-μcaps-E	32.0	4.8	0.46	1.5	606.0 ± 53.89
#2-μcaps-E	38.0	4.8	0.46	1.5	547.0 ± 16.70
#3-μcaps-E	44.0	4.8	0.46	1.5	530.0 ± 56.34
#4-μcaps-E	38.0	3.8	0.46	1.5	644.0 ± 52.33
#5-μcaps-E	38.0	5.8	0.46	1.5	438.0 ± 92.30
#6-μcaps-E	38.0	4.8	0.38	1.5	522.0 ± 83.72
#7-μcaps-E	38.0	4.8	0.52	1.5	488.0 ± 99.82
#8-μcaps-E	38.0	4.8	0.46	0.5	Broken
#9-μcaps-E	38.0	4.8	0.46	1.0	Partially fused
#10-μcaps-E	38.0	4.8	0.46	2.0	567.0 ± 46.70
#11-μcaps-E	38.0	4.8	0.46	2.5	664.0 ± 22.33
#12-μcaps-E	38.0	4.8	0.46	3.0	688.0 ± 52.33

For definitions of height, gas, pump and conc see Table 1.

2.6. Reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from SCs by Invisorb Spin-cell RNA mini-kit (Invitex GmbH, Berlin, Germany). Reverse transcription was performed using a RevertAid™ H Minus First-Strand cDNA Synthesis kit (Fermentas, Canada). The oligos sequences used are listed as forward then reverse, 5' to 3': β-actin 5'-ATGGTGGGTATGGGTCAGAA-3' and 5'-CTTCTCCATGTCGTCCAGT-3' amplify a product of 123 bp; MIS 5'-GCCAACTTAGCGTGGACCTG-3' and 5'-CTTGGCAGTTGTTGGCTTGATATG-3' amplify a product of 75 bp; indoleamine 2,3 dioxygenase (IDO) 5'-ATGAAGCGTTTGGGACACC-3' and 5'-GAGGAATCCAGCAGCAGAGC-3' amplify a product of 140 bp; TGF-β2 5'-AATGTTGTTGCCCTCCTACG-3' and 5'-TGCTATGCTGG GTGTCAGAG-3' amplify a product of 140 bp; c-kit

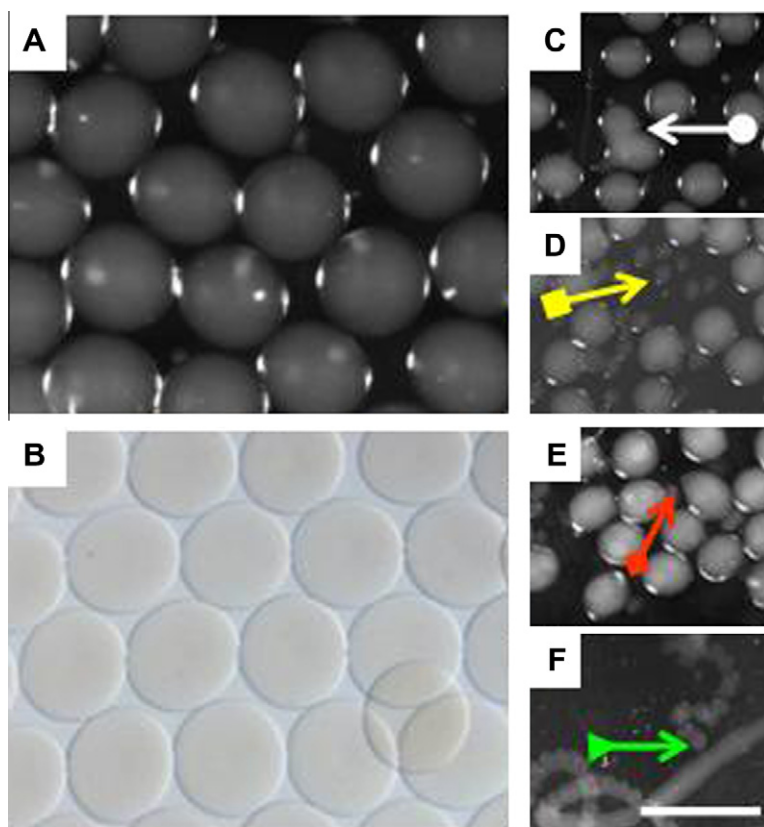


Fig. 2. Effect of the experimental parameters on alginate microparticles. Dark-field (A) and bright-field (B) stereomicrophotographs of microparticles produced with selected experimental parameters (see Table 2). (C–F) Stereomicrographs of alginate microparticles showing evident morphological (geometrical) defects, namely: coalescences (white arrow, circle), resulting from high value of the “height” parameter (C); bimodal size distribution (yellow arrow, square), with a large proportion of small irregular microparticles, resulting from a high value of “gas” (D); tear-shaped microparticles (red arrow, diamond), resulting from a low level of the “pump” parameter (E); and finally partially fused microparticles with an irregular shape, resulting from a low alginate concentration (green arrow, triangle) (F). The bar corresponds to 520 μm in panels A and B and 1500 μm in panels C–F, respectively.

receptor, also called stem cell factor receptor (c-kit), 5'-ACA AATCCATGCCACACCT-3' and 5'-TTAAGCCGTATGCAGTGGCCTC-3' amplify a product of 293 bp; laminin 5'-CCGCTTCTGGATGA ACTG-3' and 5'-CCGCCACACTCTGTCTCG-3' amplify a product of 114 bp; laminin receptor 5'-ATGCCTGACCTCTACTCTAC-3' and 5'-ACCTTCGGACCAATCTGC-3' amplify a product of 162 bp; integrin $\alpha 6$ 5'-CGGCTGTGGCTGCTCTAC-3' and 5'-TCTCTGGTGTCCTCA GTGAAGG-3' amplify a product of 74 bp; integrin $\beta 1$ 5'-CAAGC GAGCCGAGGACTATC-3' and 5'-GCTGGTGTGTGCTAATGTAAGG-3' amplify a product of 198 bp; clusterin 5'-ATCTCGGACAAAGAGCTC-CA-3' and 5'-TTGCGCTCTTCATTGATTG-3' amplify a product of 128 bp; transferrin 5'-CAGCAGAACTGACGAAA-3' and 5'-CTCCTCAGCAACACAGGTCA-3' amplify a product of 186 bp; c-kit ligand, also called stem cell factor (SCF), 5'-GATGCCTCAAGGAT TTGA-3' and 5'-ATGGAATCTGAGGCCTTCCT-3' amplify a product of 182 bp; type IV collagen 5'-CACACGCTTCCCTCCTG-3' and 5'-GAACCAGACGCTTTGC-3' amplify a product of 90 bp. RT-PCR analyses were performed in the Mx3000P® Instruments (Stratagene, CA) in a total volume of 20 μ l reaction mixture following the manufacturer's recommendations, using the Brilliant® SYBR Green QPCR Master Mix 2x (Stratagene, CA) and 10 μ M of each primer using the dissociation protocol. Negative controls contained

water instead of first-strand cDNA. Each sample was normalized on the basis of its housekeeping gene (β -actin). The relative gene expression levels were normalized to a calibrator that was chosen to be the control sample (untreated). Final results, expressed as relative expression, were calculated by MxPro™ software (Stratagene, CA).

3. Results and discussion

3.1. Production of engineered alginate microparticles: general considerations

Encapsulation scaffolds (i.e. microparticles) for cell transplantation protocols would require a number of specific requisites, such as a spherical shape, a dimensional range usually between 200 and 600 μ m, good mechanical stability and a suitable extracellular microenvironment, with care being taken ensure the viability of the embodied cell (eventually by also including extracellular adhesion molecules and growth factors) [15,16].

Keeping in mind these points, we started a series of experiments aimed at fabricating engineered alginate microparticles for tissue engineering applications, by an approach based on

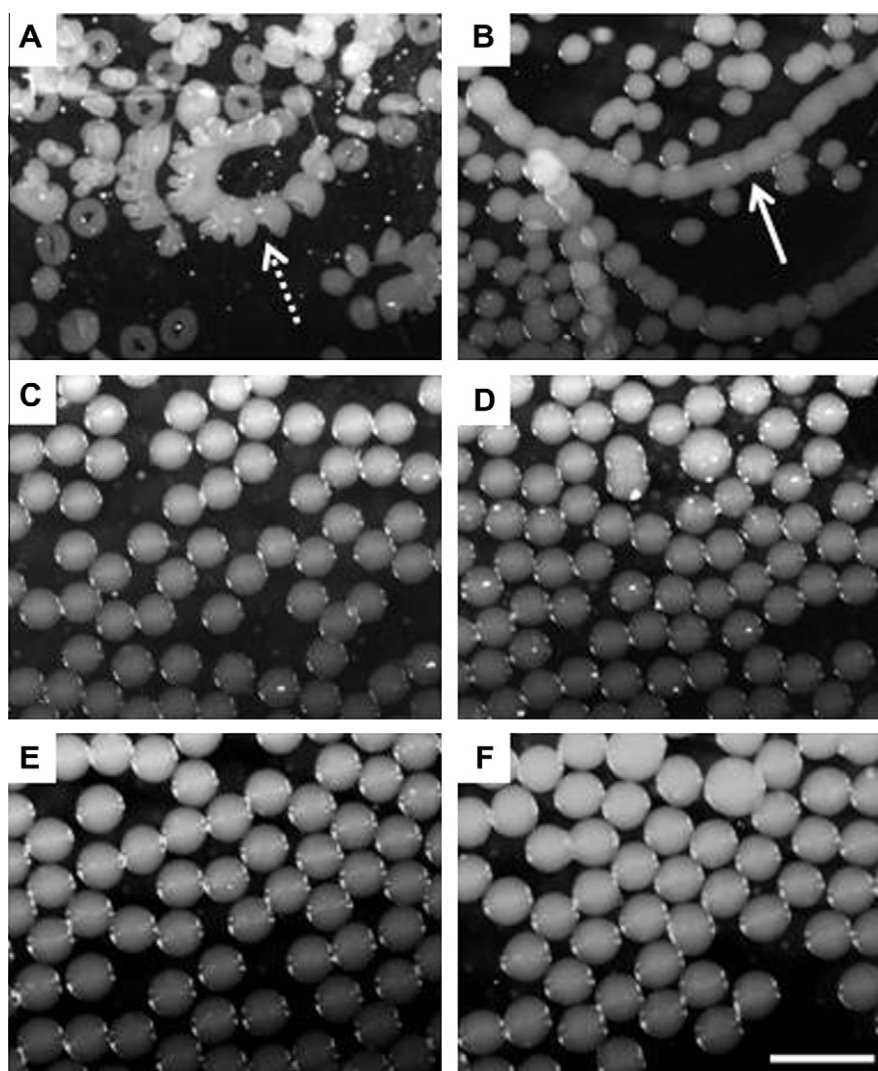


Fig. 3. Optical stereophotomicrographs of empty alginate microparticles prepared by a gas-driven monojet device, showing the effect of alginate concentration on the morphological and dimensional characteristics of the microparticles. Samples were prepared with alginate concentrations of 0.5 (A), 1.0 (B), 1.5 (C), 2.0 (D), 2.5 (E) and 3.0% (w/v) (F). The solid arrow indicates clusters of partially fused microcapsules (partial coalescences); the dotted arrow indicates broken microparticles. The bar corresponds to 1500 μ m.

a gas-driven monojet device developed in our laboratory [12]. In Fig. 1, the general scheme depicting the preparation of alginate microparticles is reported. We designed engineered microparticles that simultaneously contained living cells (i.e. SCs) and an extracellular matrix component, namely a particulate ECM. This form of powdered ECM, consisting of UBM, could be combined advantageously with natural or synthetic polymers to create hybrid biomaterials [4].

3.2. Selection of the operating conditions for microparticle production: the intuitive changing-one-separate-factor-at-a-time (COST) approach

The first part of the study was aimed at the optimization of empty barium alginate microparticles. In this respect, the classic intuitive COST approach was performed. This strategy allowed the selection of the most critical parameters influencing the production process, especially considering those affecting microparticle morphology, dimensions and surface characteristics. With respect to morphology, we focused our attention on two main aspects: the presence of fused or partially fused microparticles (a phenomenon named “coalescence”) and the presence of tear-shaped microparticles (“tail”). Following this approach, a number of experimental parameters (listed in Table 1) were analysed and varied one by one, while all the others were maintained constant. The parameters considered were “height”, “gas”, “pump” and “conc” (see Section 2.3 above). In Table 2 the entire set of alginate microparticles batches produced including the experimental parameters used for the COST approach and their variations is indicated. In Fig. 2, a number of stereophotomicrographs of alginate microparticles are reported that summarize the results of the COST study. For instance, in Fig. 2A and B are depicted the microparticles with the best morphological characteristics: (i) an extremely homogeneous size distribution; (ii) a regular spherical geometry; (iii) a smooth surface; and finally (iv) the complete absence of “coalescences” or “tails”. For comparison, the microphotographs presented in Fig. 2C–F show how different experimental settings,

including variations of “height” (Fig. 2C), “gas” (Fig. 2D), “pump” (Fig. 2E) and “conc” (Fig. 2F), led to the production of dimensionally or morphologically unacceptable microparticles. Specifically, high values of “height” (44 mm) and “gas” (5.8 l min^{-1}) result in a great number of “coalescences” (Fig. 2C), or microparticles with a large number of different diameters with at least a bimodal size distribution (Fig. 2D), respectively. Finally, when “pump” was set at a low level (0.38 ml min^{-1}) it caused the formation of a large amount of tear-shaped microparticles (Fig. 2E).

3.3. Effects of alginate concentration on microparticle characteristics

After investigating the main effects of the instrumental parameters, we focused our attention on the concentration of sodium alginate (range between 0.5% and 3.0%, w/v), analysing its effect on the produced microparticles. Using the gas-driven monojet device, we succeeded in obtaining microparticles at all the tested alginate concentrations. Nevertheless, it should be underlined that high concentrations of alginate ($>2.0\%$, w/v) resulted in very viscous solutions that caused an inefficient atomization and a considerable percentage ($>10\%$) of large, inhomogeneous droplets. On the other hand, lowering the alginate concentration below 1.0% (w/v) resulted in the formation of partially broken microparticles. This behaviour was attributed to the mechanical stress caused by landing in the hardening solution (see the arrow in Fig. 3A). In addition, the use of alginate below 1.0% causes the formation of long chains of partially fused microparticles with an irregular shape (see the arrow in Fig. 3B). As shown in Fig. 3C and in Table 2, using an alginate concentration of 1.5% (w/v), microparticles with a small mean diameter ($547.0 \mu\text{m}$) and homogeneous dimensional distribution were obtained.

3.4. Production of engineered alginate microparticles containing UBM powder and SCs

In an attempt to prepare alginate microparticles allowing/promoting adhesion of the encapsulated cells, we tested the possibility

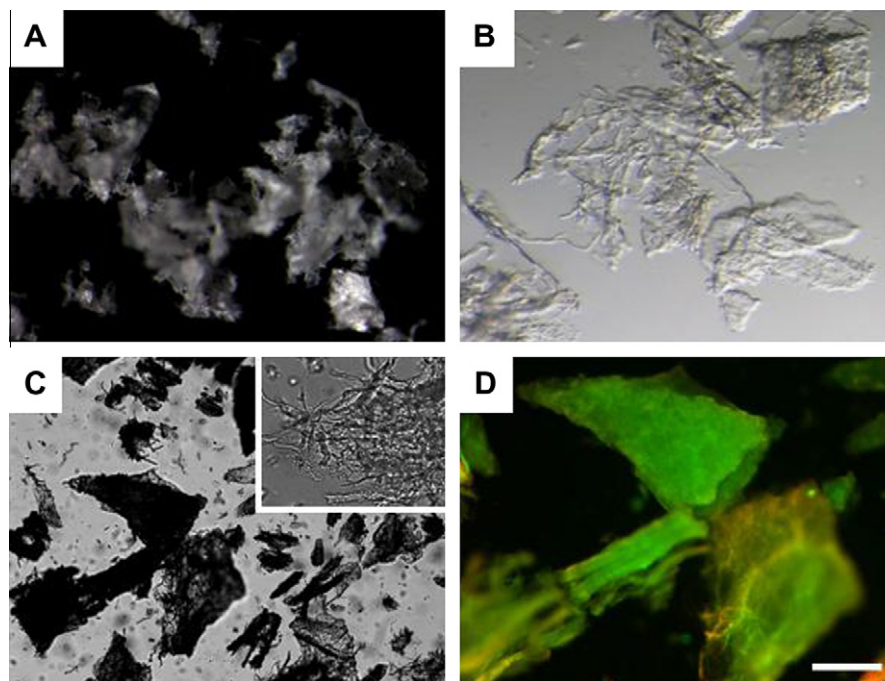


Fig. 4. Morphological characterization of UBM particles by stereomicroscopy: dark (A) and bright fields (B); by optical microscopy: bright field (C); and by fluorescence microscopy, after staining with fluorescein (D). The inset of panel C shows in detail the microfibrillar structure typical of the UBM flakes. The bar corresponds to $50 \mu\text{m}$ for panels A–D and $10 \mu\text{m}$ for the inset of panel C, respectively.

of producing engineered alginate microparticles containing UBM powder.

The UBM–ECM powder was characterized by stereomicroscopic and fluorescence microscopic analysis. The micrographs in Fig. 4 depict the fibrous, flake-like aspect typical of the UBM powder, with an average particle size usually between 50 and 200 μm . It is noteworthy that UBM particles, even in the larger comminuted form, retain the ultrastructural 3-D surface characteristics of the parent UBM sheets. Therefore, suspensions made from this powdered form of lyophilized UBM into biopolymer dispersions can be successfully used in mixture with cells for the direct fabrication of engineered alginate microparticles.

Alginate microparticles containing different amounts of UBM powders (Fig. 5A–C) were prepared and the effects of the presence of the powder on microparticles morphology were determined.

Fig. 5A–C shows that use of UBM powder at 2.5–7.5 mg ml^{-1} (in alginate suspension) did not cause any appreciable alteration in the microparticle geometry. In addition, the microparticle surface remained smooth, with no signs of particle protrusion from the microparticle body.

Pre-pubertal Large-White piglets were used as SC animal donors [12]. SC preparations were grown as monolayers (Fig. 5D). After isolation, SC purity was assessed by immunostaining for MIS, as presented in Fig. 5E. The presence of MIS has been detected in SCs of various mammals, including humans and immature mice [17,18]. This cytoplasmic staining is essential to identify SC cells because, currently, there are no definitive function markers for this cell type [19]. In addition, viability of the final culture was excellent (>95%), as demonstrated by the fluorescence photomicrograph shown in Fig. 5F.

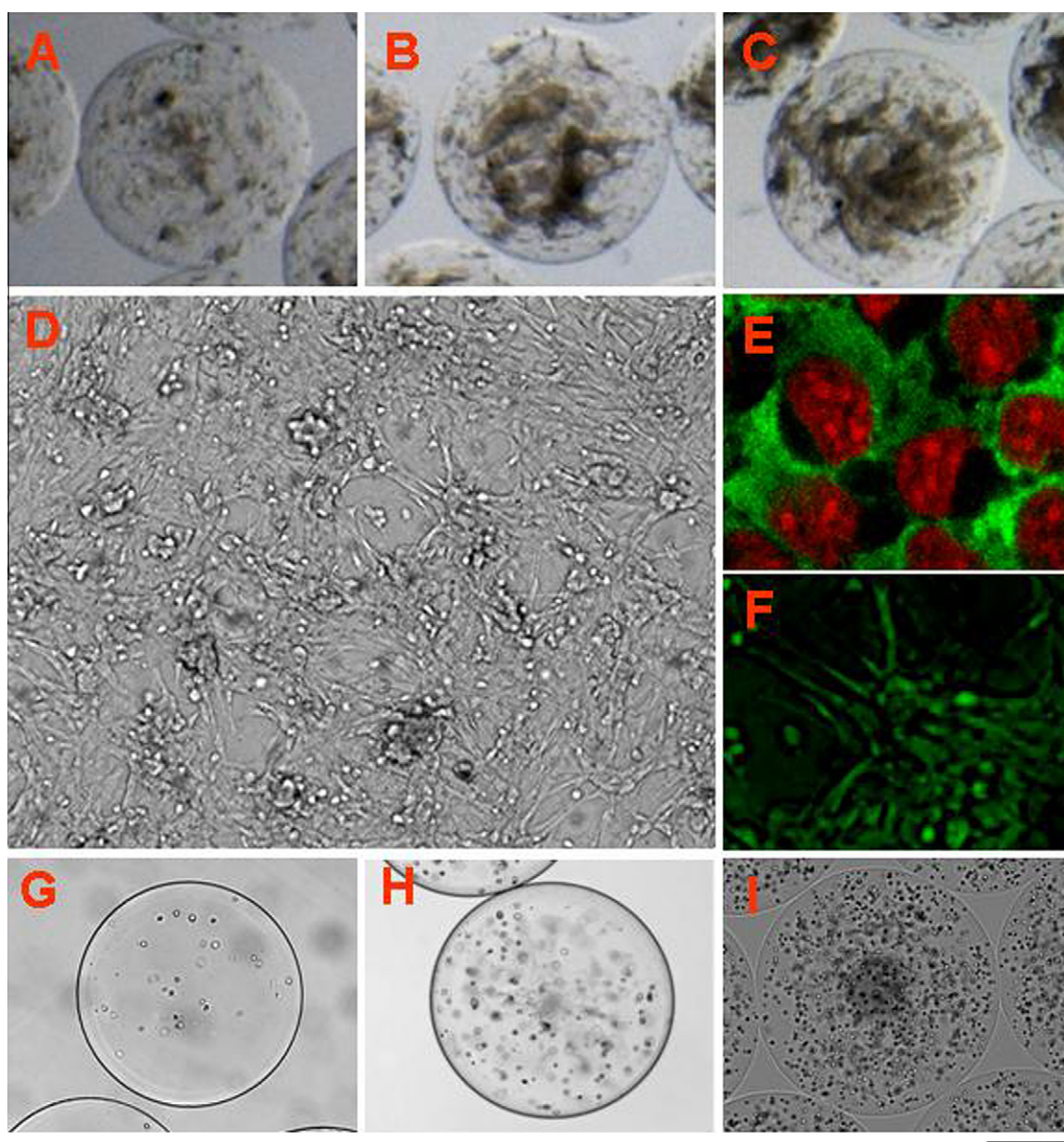


Fig. 5. Optical stereophotomicrographs of alginate microparticles containing UBM powder (A–C), prepared by a gas-driven monojet device. The microparticles contain 2.5, 5.0 and 7.5 mg of UBM powder per ml of alginate. Optical photomicrographs of SC monolayers: (D) before encapsulation; (E) SCs after immunostaining with specific antibody for MIS; and (F) the viability of SCs after double staining with Calcein-AM and propidium bromide. (G–I) Optical stereophotomicrographs of alginate microparticles containing SCs at (G) 4.0, (H) 8.0 and (I) 12.0×10^6 cells ml^{-1} . The bar corresponds to 180, 90, 10, 40 and 180 μm in panels A–C, D, E, F and G–I, respectively.

For UBM powder, we tested the effects of different amounts of cells ($4\text{--}12 \times 10^6 \text{ cells ml}^{-1}$) on microparticle morphology. Fig. 5G–I shows that in the presence of cells the geometry and surface of the microparticles remained unaltered.

Finally, alginate microparticles containing both UBM powder and SCs were prepared (Fig. 6A) and characterized for size distribution (Fig. 6B). The photomicrographs presented in Fig. 6 clearly indicated that the combined presence of UBM and SCs at the tested concentrations did not cause any morphological alterations of the microparticles, which presented with smooth and regular surfaces. Likewise, as indicated in Fig. 6, SCs were uniformly distributed throughout the entire microparticles volume.

Nevertheless, in order to gather further information about the possible effects of UBM powder and SCs on microparticle production, a statistical experiment design was also planned.

3.5. Effects of the operating conditions on microparticle characteristics: DoE approach

Statistical experimental design, also called design of experiments (DoE), is indeed a well-established concept for planning and executing informative experiments.

As previously described, the parameters “height”, “gas” and “pump” were chosen as variables and tested at three levels, resulting in 27 possible combinations, to give a full factorial design. In our case, we selected fewer experiments by a randomized CCF design, which requires fewer trials (namely, 14) [20]. We also added three centre points in order to get estimation figures of the experimental error. In Table 3 the complete list of experiments for the CCF study is reported. In this way, we obtained a response surface modelling.

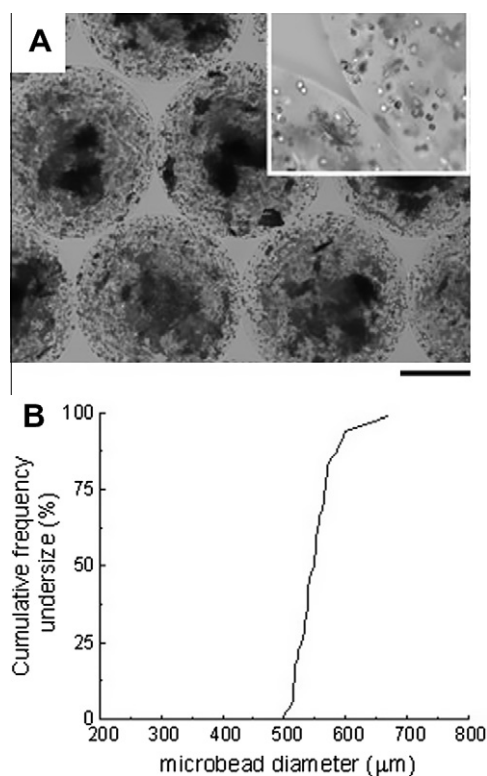


Fig. 6. Optical micrographs of alginate microparticles containing UBM powder plus SC (A). The inset presents an image at higher magnification showing the detail of the smooth microparticle surface. (B) Cumulative size distribution analysis of optical micrographs of alginate microparticles containing UBM powder plus SCs. The bar corresponds to 220 μm in A and 70 μm in the inset, respectively.

Table 3

Experimental design matrix and results of the DoE (design of experiments) approach for engineered alginate microparticles containing ECM powder.

Run	Factors			Responses	
	Air (l min^{-1})	Pump (ml min^{-1})	Height (cm)	Mean diameter (μm)	SD
#1	3.5	0.45	3.2	606.0	43.8
#2	4.5	0.45	3.2	459.0	17.2
#3	3.5	0.55	3.2	606.0	48.6
#4	4.5	0.55	3.2	455.0	23.7
#5	3.5	0.45	4.0	650.0	22.8
#6	4.5	0.45	4.0	454.0	18.5
#7	3.5	0.55	4.0	638.0	23.2
#8	4.5	0.55	4.0	442.0	36.2
#9	3.5	0.50	3.6	644.0	22.3
#10	4.5	0.50	3.6	438.0	23.3
#11	4.0	0.45	3.6	552.0	33.7
#12	4.0	0.55	3.6	488.0	39.8
#13	4.0	0.50	3.2	524.0	21.8
#14	4.0	0.50	4.0	538.0	36.3
#15	4.0	0.50	3.6	547.0	26.7
#16	4.0	0.50	3.6	551.0	33.8
#17	4.0	0.50	3.6	553.0	32.8

The MLR equation (Eq. (1)) for the responses of the chosen model is:

$$y_i = \text{Constant} + A_1F + A_2P + A_3H + A_4F_2 + A_5P_2 + A_6H_2 + A_7FP + A_8FH + A_9PH \quad (1)$$

where Constant is the mean of the seventeen runs and A_i are the regression coefficients of the factors and their interactions.

The three-dimensional graphs of the investigated factors are presented in Fig. 7, which shows the influence of factors on “mean diameter” (panels A–C) and “SD” (panels D–F).

By examining the results presented in Table 3 and Fig. 7, the main finding was that a change in the factor “gas” from a low to a high level (from 3.5 to 4.5 l min^{-1}) resulted in a strong decrease of response “mean diameter”, which decreased from over 600 to around 450 μm . In addition, the increase in “gas” was beneficial for the response “SD”, which was progressively lowered.

The factor “pump” was associated with only marginal influence on the response “mean diameter”, which declined slightly with increasing pump values. In contrast, this factor plays an important role in the microparticle size distribution, its high and low levels causing a marked increase in the response “SD”.

Finally, the factor “height” appeared to exert minor effects on both responses, showing an effect at its upper level, on the microparticle size.

In order to assess the validity and the significance of the DoE model, an analysis of variance (ANOVA), was performed. This demonstrated that all the obtained experimental data fitted well the model, indicating its good reproducibility. In addition, in Table 4 are reported the results obtained for R^2 and Q^2 , which respectively represent the percentage variation of the response explained by the model and that predicted by the model. The high values of both coefficients indicate that the model has good validity (R^2) and a significant power of prediction for new data (Q^2).

3.6. Effect of UBM powder on the viability of SCs entrapped in engineered, alginate-based microparticles

An important issue that should be investigated in the case of cell embedding/seeding procedures involving cell/scaffold interactions concerns the possible effects of the encapsulation/seeding procedure on the growth as well as the functional activity of cells.

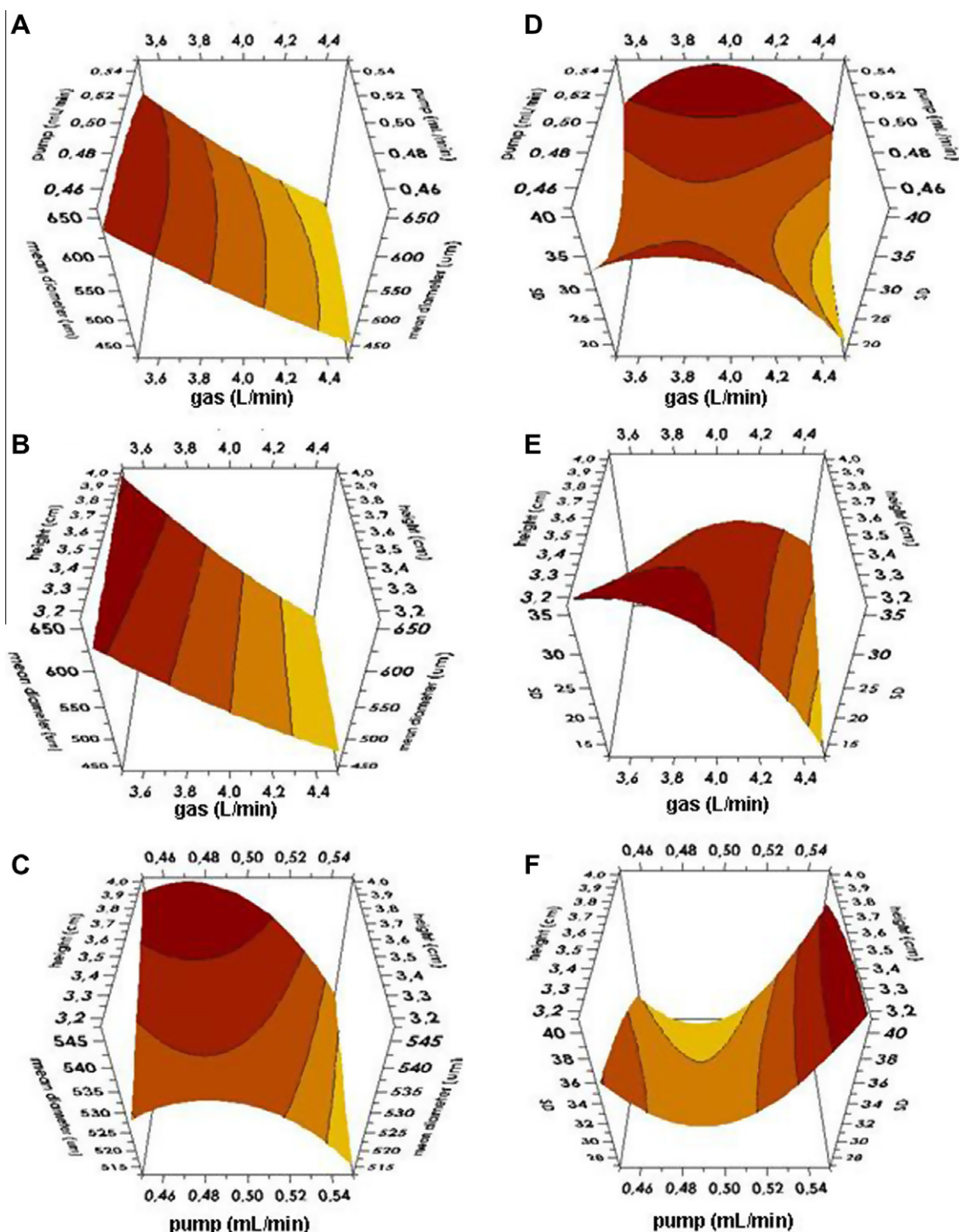


Fig. 7. DoE analysis for alginate microparticles containing UBM powder. Surface plots of the responses “mean diameter” (A–C) and “standard deviation” (D–F) for the interactions between gas and pump (A, D), gas and height (B, E), and pump and height (C, F). Factors, levels and responses employed for the DoE analysis are presented in Table 3.

To this end, in a first set of experiments, the viability of SCs encapsulated in standard vs. engineered alginate microparticles containing UBM powder was investigated.

SC embedded in alginate microparticles were double-stained (after different times of in vitro culture) with Calcein-AM (a marker of living cells; the fluorescent signal was monitored using 485 nm excitation wavelength and 530 nm emission wavelength) and with PI (a marker of cell death; excitation, 535 nm; emission, >610 nm), as described in Section 2.

The fluorescent photomicrographs recorded immediately after the encapsulation (presented in Fig. 8A and B) indicated that the cells were highly viable (>95%) for both standard and engineered microparticles (containing UBM). As indicated also by the bar plot presented in Fig. 8G, after 24 h of cell culture (Fig. 8C and D), the viability of SCs remain very high (>85%). Significant differences between the viability of SCs encapsulated in standard vs. engineered microparticles became evident at day 7 (Fig. 8E and F) and even more so at day 14 (Fig. 8G and H) of encapsulation, assumed as

Table 4

ANOVA analysis of variance of the model for DoE approach.

	<i>N</i>	<i>R</i> ²	<i>R</i> ² adj.	<i>Q</i> ²	Model validity	Reproducibility
Mean diameter	17	0.97	0.93	0.81	0.62	0.99
SD	17	0.76	0.41	0.61	0.81	0.72

N is the number of experiments; *R*² is the percentage variation of the response explained by the model; *R*² adj. is the fraction of the variation of the response explained by the model adjusted for the degree of freedom; *Q*² is the percentage variation of the response predicted by the model.

day 0. The viability of SCs in standard vs. engineered microparticles is indeed significantly different being 65 vs. 79 and 45 vs. 65%, respectively, at days 7 and 14 (*p* < 0.0001).

These data clearly indicate that the viability of SCs encapsulated in engineered microparticles (containing UBM) increased. This effect was possibly due to the presence of individual UBM components in the same relative amounts existing in nature and in an ultrastructural milieu similar to that of native UBM. Likely, it is the diverse combination of both the structural and functional components present within UBM scaffolds that accounts for the success of UBM-based biomaterials [4]. For instance, UBM has been shown to contain adhesion molecules such as fibronectin and laminin, the proteoglycan decorin and the glycoproteins biglycan and entactin [4].

3.7. Effect of UBM powder on the functions of SC entrapped in engineered alginate microparticles

In order to investigate whether the presence of UBM components, in powder form, in alginate microparticles could influence the cell function of the embedded SCs, expression levels of a number of selected genes were analysed by quantitative real-time PCR. The gene expression of SCs encapsulated into plain alginate microparticles was compared to SCs encapsulated into engineered microparticles (containing UBM) (Fig. 9).

To collect general evidence about the effect of UBM on SC function, different genes were studied, with special regard to those related to biomedical applications of SCs. Three different categories were considered, namely: (i) genes that support the maintenance of differentiated phenotype (MIS, IDO); (ii) genes related to cell growth and viability (TGF-β2, c-kit, clusterin, transferrin, SCF; and finally (iii) genes involved in the adhesion to extracellular matrix constituents (type IV collagen, laminin, laminin receptor, integrin β1, integrin α6).

The expression levels of the above-listed genes was quantified by real-time RT-PCR on reverse-transcribed mRNA isolated from SCs that were embedded in standard and engineered microparticles after 7 and 14 days of in vitro cell culture.

The obtained result levels were normalized to mRNA levels of the reference gene β-actin.

3.8. Genes that support the maintenance of differentiated cell phenotypes

SC preparations were analysed for the expression of MIS. The presence of MIS has in fact been detected in SCs of various mammals, including humans [17]. The obtained data indicated that MIS expression was not decreased by the presence of UBM (Fig. 9A); this finding is particularly important since it clearly indicates that the embedding into alginate microparticles does not alter the typical SC phenotype and thus the differentiated status of cells. It should be remembered that MIS marker is currently the only marker used to identify SC cells because there are no other exclusive markers for this cell type [19].

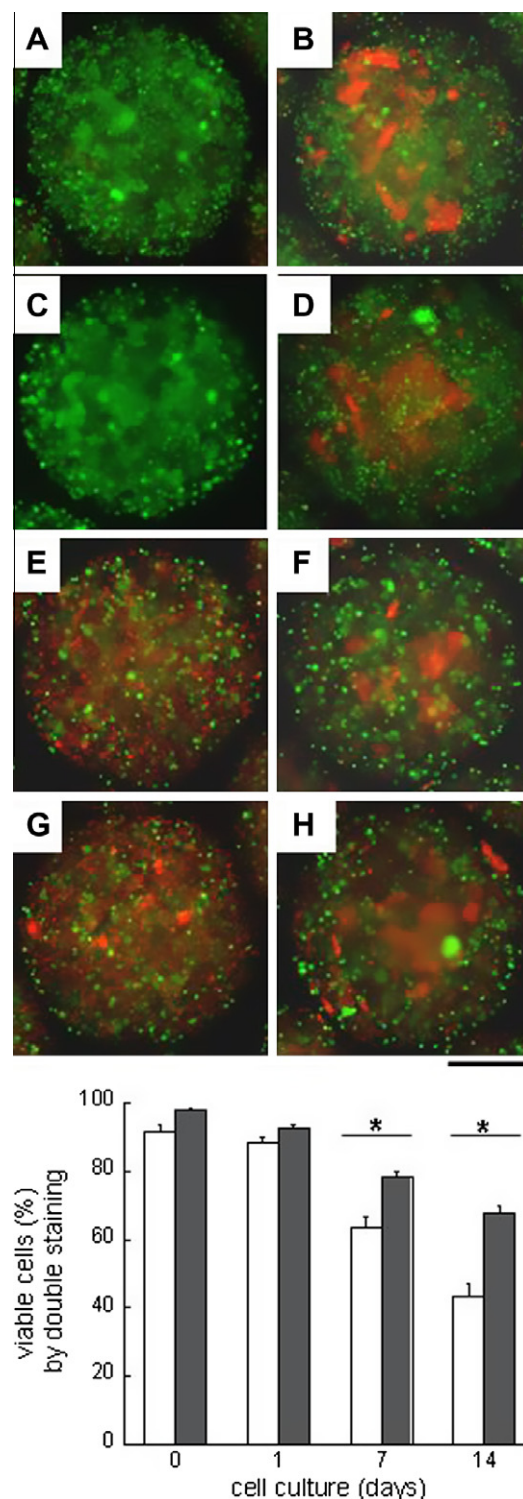


Fig. 8. Fluorescence micrographs of SCs encapsulated in alginate microparticles (A, C, E, G) and in alginate microparticles containing UBM powder (B, D, F, H) after a double-staining assay with Calcein-AM and propidium bromide, determined at days 0 (A, B), 1 (C, D), 7 (E, F) and 14 (G, H) of cell culture. The bar corresponds to 180 μm. The histogram plot of panel I reports the percentage of viable cells (by double staining) determined at days 0, 1, 7 and 14 of cell culture, for SCs encapsulated in alginate microparticles (open bars) and SCs encapsulated in alginate microparticles containing UBM powder (closed bars). Values are expressed as percentage of viable cells with respect to day 0 (rated to 100%), and represent the mean of five independent samples analysed in quadruplicate ±SD (**p* < 0.0001).

To further characterize the effects of UBM on SC function, we analysed the expression of the IDO gene, since it represents a

crucial enzyme. Recently, we reported that isolated neonatal porcine SC, administered alone in highly biocompatible microcapsules, led to diabetes prevention (88% of cases) and reversion (81% of cases) in overtly diabetic NOD (nonobese diabetic) mice, with no need for additional beta cells or insulin therapy. The effect was associated with the restoration of systemic immune tolerance and detection of functional pancreatic islets that consisted of glucose-responsive and insulin-secreting cells. The curative effects by SCs were strictly dependent on efficient tryptophan metabolism in the xenografts, which is regulated by IDO activity [11].

For this reason, the level of IDO, which is an immune regulatory enzyme, was carefully considered [21,22].

Interestingly, IDO expression was remarkably up-regulated in SCs co-entrapped with UBM powder, which displayed a 5.5-fold increase (at day 14, Fig. 9A) in IDO mRNA with respect to control SCs (encapsulated in plain alginate). In order to confirm that IDO mRNA levels are correlated with IDO protein levels, we show in Fig. S1 a plot that shows good correlation ($R = 0.9938$) between the IDO-encoding mRNA analysed by real-time PCR and the IDO protein determined by immunoblot analysis.

The positive effect of UBM on IDO suggests that SCs embedded in engineered alginate microparticles containing UBM could exert an increased ability to prevent/revert diabetes, at least in experimental animals. We plan extending the application of new SC-containing microparticles to other autoimmune disorders, e.g. experimental encephalomyelitis and cholangitis, using rodent animal models.

3.9. Genes related to cell growth and viability

In addition to IDO, SCs secrete a number of proteins that are involved in the ability of SCs to inducing acquired tolerance to transplantation with no interference from the host's systemic immune surveillance system. Therefore we extended the study of the UBM activity on SC gene expression to TGF- β 2 and c-kit (Fig. 9B), clusterin and transferrin (Fig. 9C) and SCF (Fig. 9D). All are involved in the physiological proliferation and differentiation of germ cells [23,24] and influence the functional activity of SCs upon their transplantation. In particular, TGF- β 2 was shown to modulate mesenchymal–epithelial interactions between peritubular cells and SCs [25]. The comparative mRNA expression levels for the

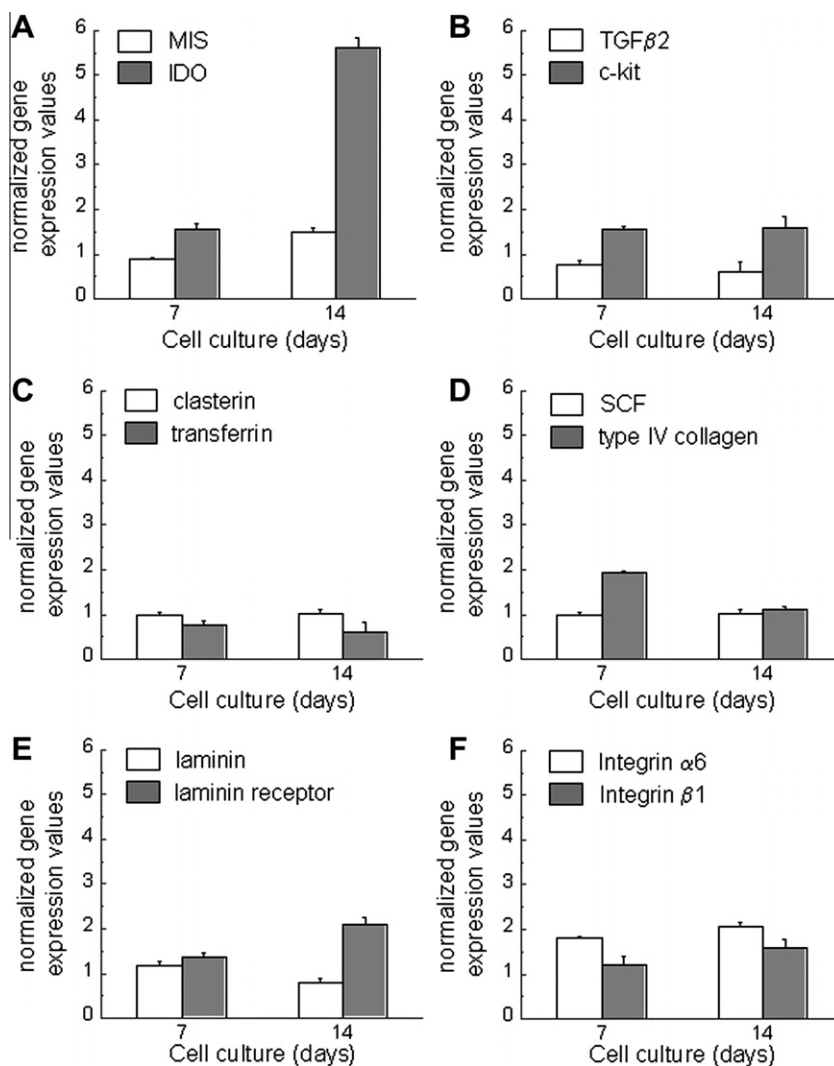


Fig. 9. Effect of UBM powder on mRNA expression levels of SCs entrapped in engineered alginate microparticles, as determined by real-time PCR. (A) MIS (open bar); IDO (closed bar); (B) TGF- β 2 (open bar); c-kit proto-oncogene (closed bar); (C) clusterin (open bar); transferrin (closed bar); (D) SCF (open bar); type IV collagen (closed bar); (E) laminin (open bar), laminin receptor (closed bar); (F) integrin α 6 (open bar), integrin β 1 (closed bar). Data represent the mean of three determinations performed on three independent samples $\pm$ SD. The reported values were obtained by normalization, on the basis of the housekeeping gene (β -actin). The relative gene expression levels were normalized to a calibrator that was chosen to be the control samples (SCs encapsulated in alginate microbeads without UBM powder).

above-reported secretion proteins was only marginally affected by the presence of UBM except for c-kit, which was slightly increased at both 7 and 14 days within engineered microparticles containing SCs.

3.10. Genes involved in the adhesion to extracellular matrix constituents

SCs are also known to produce ECM macromolecules. In vitro studies have demonstrated that SCs synthesize laminin and type IV collagen, but not fibronectin [26]. In vivo, while the 1- and 3-laminin isoforms [27] and type IV collagen are immunolocalized in the basement membrane underlying the seminiferous tubular epithelium, upon direct contact with SCs, fibronectin is localized in the connective tissue external to the peritubular cells, and appears to be secreted by this cell type in a polarized manner [28]. Stem cell factor receptor (c-kit or CD117) is secreted by fibroblasts, always localized in the connective tissue. Like many other ECM adhesion molecules, laminin binds to cell surface receptors of the integrin superfamily. There is an increasing number of laminin receptors, including the $\beta 1$ subunit, that may be associated with $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$ or $\alpha 7$ and the $\beta 4$ subunits, associated with either $\gamma 5$ or $\gamma 6$. Integrin receptors not only serve as structural receptors connecting the extracellular matrix to the cytoskeleton in order to attach the cell to their substrate, but also function as signaling receptors that lead to changes at the second messenger molecule level [29]. Thus, the ability of ECM to influence growth, differentiation and other cell functions is likely related to its effects on signaling pathways inside the cells [30].

Interestingly, mRNA expression levels for type IV collagen (Fig. 9D), laminin and laminin receptor (Fig. 9E), and integrins $\alpha 6$ and $\beta 1$ (Fig. 9F) were increased remarkably in SCs encapsulated in UBM, suggesting a role of the adhesion molecules present in the ECM powder form.

4. Conclusions

This paper confirms that the production of alginate microparticles, with highly controlled morphological and dimensional properties, by a gas-driven monojet device-based-procedure, is an important procedure for the preparation of novel hybrid microparticles intended for cell encapsulation and tissue engineering applications.

In particular, we have demonstrated that the combined use of alginate and urinary bladder matrix resulted in a synergistic activity of both materials. While the engineered microparticles offer the mechanical and material properties of alginates, which can be varied through “on-demand” gelling procedures, UBM provides an array of bioactive functions that seem to improve the viability and function of the co-entrapped SCs.

UBM is constituted by an epithelial basement membrane on one surface and the connective tissue of the underlying tunica propria on the opposite surface [31]. This bimodal microstructure provides an ideal environment for the attachment and growth of cells. Moreover, UBM contains a variety of bioactive molecules, including cytokines and growth factors, that act as potent modulators of cell behaviour. The list of growth factors is large, and includes vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor beta (TGF- β), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) [32].

In conclusion, the engineered microparticle concept presented here could be used to improve both the encapsulated cell survival and function of cells micro- and macro-encapsulated in devices fabricated for tissue engineering and cell transplantation purposes.

Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 2, 4, 5, 7 and 8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at ([doi:10.1016/j.actbio.2010.10.005](https://doi.org/10.1016/j.actbio.2010.10.005)).

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