In vitro cell delivery by gelatin microspheres prepared in water-in-oil emulsion

Nicola Contessi Negrini[1, 2], Maria Veronica Lipreri[1], Maria Cristina Tanzi[2], Silvia Farè[1, 2]

[1]Department of Chemistry, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milan, Italy

[2]INSTM, National Interuniversity Consortium of Materials Science and Technology, Local Unit Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milan, Italy

Corresponding author:

Nicola Contessi Negrini, PhD

E-mail: nicola.contessi@polimi.it

Tel: 0039 0223994741

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Abstract

The regeneration of injured or damaged tissues by cell delivery approaches require the fabrication of cell carriers (e.g., microspheres, MS) that allow for cell delivery to limit cells spreading from the injection site. Ideal MS for cell delivery should allow for cells adhesion and proliferation on the MS before the injection, while they should allow for viable cells release after the injection to promote the damaged tissue regeneration. We optimized a water-in-oil emulsion method to obtain gelatin MS crosslinked by methylenebisacrylamide (MBA). The method we propose allowed obtaining spherical, chemically crosslinked MS characterized by a percentage crosslinking degree of 74.5 ± 2.1%. The chemically crosslinked gelatin MS are characterized by a diameter of 70.9 ± 17.2 μm in the dry state and, at swelling plateau in culture medium at 37 °C, by a diameter of 169.3 ± 41.3 μm. The MS show dimensional stability up to 28 days, after which they undergo complete degradation. Moreover, during their degradation, MS release gelatin that can improve the engraftment of cells in the injured site. The produced MS did not induce any cytotoxic effect *in vitro* and they supported viable L929 fibroblasts adhesion and proliferation. The MS released viable cells able to colonize and proliferate on the tissue culture plastic, used as release substrate, potentially proving their ability in supporting a simplified *in vitro* wound healing process, thus representing an optimal tool for cell delivery applications.

1. Introduction

Among the approaches nowadays under investigation or clinically used in regenerative medicine [1], a possible strategy is based on the transplantation of cells to be injected in the damaged site to promote the tissue regeneration (i.e., cell therapy). Typically, cells are isolated from a biopsy of the biological tissue of a donor (i.e., autologous or allogenic cells), they are manipulated *in vitro* (i.e., expansion and/or differentiation), and finally injected into the tissue target site [2]. Although direct injection is a commonly used clinical practice thanks to the easy procedure [3], this method is affected by some drawbacks, including a low percentage of viable cells post injection (i.e., viability is as low as 1 – 32%), a time-consuming cells *in vitro* culture to obtain the required number of cells for the injection, and a tendency of cells to move from the injection site to the surrounding tissues, causing low cell retention and engraftment in the injured site [4–6].

Cell carriers have been proposed as vehicles for controlled cell delivery. Cell carriers act as a temporary substrate for cell adhesion before, during and after injection, thus promoting cell survival (e.g., anchorage-dependent cells), viability and proliferation compared to the direct injection approach. In fact, in the former case, cells anchored or encapsulated in a carrier may also develop cell-cell interconnection and produce/deposit extracellular matrix biomolecules, avoiding possible cells dispersion in the surrounding tissues, favoring a controlled cell delivery in the target site and increasing the efficiency of cells transplantation, with a higher number of viable cells delivered in the target tissue site, compared to the direct injection procedure [7]. For instance, the engraftment in the myocardium of cardiac progenitor cells was improved by gelatin microspheres delivery, compared to direct cells injection [8]. Other studies demonstrated, by *in vitro* models, that the presence of microcarriers facilitated both the survival and differentiation of osteo-progenitor cells, especially when cell-adhesive motives are present on the microcarrier [9].

Different cell microcarriers and delivery strategies have been proposed, including porous microspheres [10,11], microcapsules with encapsulated cells [5,12–16], and microspheres (MS) with cells anchored on the MS surface [17–20]. The choice of the microcarrier structure is strictly

dependent on the desired application and on the materials used for the microcarriers production (i.e., possibility of embedding cells during the carrier preparation). Among them, microcapsules and microspheres have been investigated as cell delivery vehicles. In particular, microcapsules allow for cells encapsulation. Microcapsules are generally hydrogel-based, since hydrogels provide a high degree of permeability for nutrient and metabolites, thus allowing for encapsulated cells survival [7]. Typically, alginate [12,13], pectin [14], gelatin [5], agarose [16] or hyaluronic acid [15] can be used for microcapsules fabrication. A strict requirement for microcapsules fabrication is that the gelation process used to stabilize the hydrogel is cytocompatible, to avoid cytotoxic effects on the encapsulated cells. Such processes include, for instance, divalent ions for alginate and pectin crosslinking [12,13] and UV for gelatin methacryloyl photo-crosslinking [5]. The advantage in using microcapsules is the possible protection offered to encapsulated cells from the host's immunoresponse, which allows avoiding allo- and xeno-rejection after cells administration; however, diffusion of oxygen, carbon dioxide and nutrients from and into the microcapsules has to be finely optimized to avoid possible cells suffering [16]. Alternatively, microspheres (MS) can be selected and used by first fabricating the spherical MS and by subsequently seeding cells on the MS surface. Materials proposed for MS production include both natural and synthetic polymers, such as collagen [17], gelatin [19] chitosan [20], and poly(lactide-co-glycolide) (PLGA) [18]. The successful use of MS as cell delivery systems is strictly dependent on the efficient anchorage of cells to the MS surface and to the controlled release of viable cells from the MS, to allow the colonization of the injured site and the tissue regeneration.

In the present work, we fabricated and characterized gelatin MS crosslinked during the fabrication by an ongoing Michael-type crosslinking reaction by using a water-in-oil emulsion process. We then used the produced MS as cell carriers to prove their ability to support *in vitro* cells adhesion and proliferation as well as potential as cell delivery vehicles.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma Aldrich, unless differently specified. The culture medium used for *in vitro* experiments was composed by Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) 10% v/v, penicillin/streptomycin 1% v/v, glutamine 2 mM, Hepes 10 mM and sodium pyruvate 1 mM.

2.2. Preparation of gelatin microspheres (MS) by water-in-oil emulsion

Chemically crosslinked gelatin MS were prepared by a non-zero length chemical crosslinking reaction described elsewhere, based on a Michael-type addition [21,22]. Briefly, a 15% w/v gelatin solution (type A, from porcine skin) was dissolved in water at 50 °C. Then, the pH was adjusted at 10.5 by adding triethylamine (TEA) to the solution under stirring. After reaching the desired pH, the solution was allowed to stir for further 10 min and 23.3 mg of methylenebisacrylamide (MBA, crosslinker) were added. After 20 min, the gelatin solution was loaded in a polypropylene (PP) syringe (Terumo®) mounted on a syringe pump (KD Scientific). The gelatin solution (4 mL) was added dropwise (11 mL h^{-1}) in 100 mL of soybean oil (preheated at 50 °C, distance oil surface – syringe = 10 cm) under stirring at 400 rpm to obtain the water-in-oil emulsion (Figure 1). The emulsion was kept under stirring (50 °C, 400 rpm) for 16 h to allow the complete gelatin crosslinking reaction. MS were then collected (Fig. S1) by first filtering by gravity with a metal sieve (400 μm pores), to eliminate possible MS aggregates. Then, the MS suspended in oil were inserted in a PP syringe equipped with a 35 μm pore size filter (Torviq). Oil was discharged by pushing the syringe plug, while MS remained in the syringe due to the filter. Acetone was sucked in the syringe and subsequently removed to wash the MS from the oil; the procedure was repeated three times. Finally, 10 mL of acetone were sucked in the syringe, the syringe was opened, MS collected in a poly-ethylene-terephthalate container (Ø = 13 cm) and allowed to dry at air overnight. MS were then disinfected by immersion in 70% v/v solution overnight; the ethanol solution was allowed to completely evaporate in sterile condition and, finally, MS were collected and stored in sterile Eppendorf before further use and characterization.

2.3. Morphological and physical analysis

The morphology of the crosslinked gelatin MS was evaluated immediately after dehydration by scanning electron microscopy (SEM, Stereoscan 360 Cambridge instruments), to prove the obtainment of spherical MS and the absence of aggregates. Anhydrous MS were collected on aluminum stubs using adhesive carbon tape, sputter-coated with palladium and observed at 75 and 250X magnification. The average MS diameter was calculated by measuring the diameter of 900 MS (i.e., 300 MS from three independently prepared MS batches) using Image J software (NIH, version 1.51).

The gelatin hydrogel MS crosslinking degree was evaluated by ninhydrin assay, by comparing the number of free amino groups before (i.e., gelatin powder) and after (i.e., crosslinked gelatin hydrogel MS) the MBA-gelatin amino groups crosslinking reaction. The ninhydrin assay was performed to quantify the free amino groups [23,24], following a previously optimized protocol [25]. Briefly, the ninhydrin working solution was prepared by mixing two solutions for 45 min: the first solution was obtained by adding 1.05 g citric acid, 0.4 g NaOH and 0.04 g $SnCl \cdot H_2O$ in 25 mL of distilled water, the second solution by dissolving 1 g ninhydrin powder in 25 mL ethylene glycol monomethyl ether. Three samples of 10 mg of gelatin MS were put in different glass tubes and 1 mL of ninhydrin working solution was added to each tube; the samples were then incubated at 100 °C for 20 min to promote the ninhydrin reaction. Then, 5 mL of 50% v/v isopropanol was added to dilute the samples and, after mixing, the surnatants was read by using a spectrophotometer (λ = 570 nm, 6705 UV/Vis Spectrophotometer Jenway). A calibration curve was used by preparing glycine standards at known concentrations (i.e., 8, 4, 2 and 1 mM); the number of amino groups of the glycine solutions was measured as performed for the gelatin samples. The number of free amino groups in the gelatin samples was then related to the measured absorbance by using the obtained calibration curve. Then, the crosslinking degree (CD%) of the gelatin hydrogel MS was calculated following Equation (1):

$$CD\% = \frac{[NH_2]_{gelatin} - [NH_2]_{microspheres}}{[NH_2]_{gelatin}} \times 100 \qquad (1)$$

where $[NH_2]_{gelatin}$ is the number of free amino groups of non-crosslinked gelatin and $[NH_2]_{microsphere}$ is the number of free amino groups measured for crosslinked MS samples. As control, bulk hydrogels were prepared with the same gelatin hydrogel formulation (i.e., same gelatin concentration and crosslinker amount, Figure 1) [22].

The swelling properties of the crosslinked gelatin hydrogel MS were evaluated to investigate the MS stability and their dimensional variation. MS samples (weight = 10 mg per sample, n = 9 samples obtained by three independent MS batches) were put in 12-multiwell tissue culture plastic (TCPS) and immersed in 3 mL per well of DMEM culture medium and incubated at 37 °C, 5% CO_2, to replicate the cell culture conditions. At established time points (t = 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 24, 48, 72 h and twice per week up to 32 days), the MS were observed by optical microscopy and the diameter of the MS was measured (n = 5 images per well, Image J software). The dimensional variation Δd [%] at the considered time points was calculated following Equation (2):

$$\Delta d\% = \frac{d_{swollen} - d_{anhydrous}}{d_{anhydrous}} \times 100 \qquad (2)$$

where $d_{swollen}$ is the average diameter of the MS immersed for a defined time t, $d_{anhydrous}$ is the average diameter of the MS before immersion (i.e., anhydrous state).

The release of gelatin from MS during their immersion in aqueous environment was investigated by a previously described protocol [26]. Briefly, 30 mg of MS were collected in Cell Strainers (pore diameter Ø = 35 µm), lodged in 6-multiwell TCPS, immersed in 5 mL of Hank's Balanced Salt Solution (HBSS) and stored at 37 °C. At established time points (t = 1, 2, 4, 8, 24, 36, 48 and 72 h), the strainers containing the MS were removed from the wells, the solutions were collected for released gelatin quantification and refreshed with new HBSS. Then, the gelatin released in the supernatant was quantified by Bicinchoninic Acid Assay (Pierce BCA Protein Assay Kit - Thermo Fisher Scientific), following manufacturer's guidelines, by using bovine serum albumin (BSA)

standards. The cumulative release of gelatin was represented as percentage of released mass of gelatin ($m_{released\ gelatin}$), in time, to the mass of initial MS ($m_{total\ gelatin}$) used for the test (3):

$$\text{Gelatin release}[\%] = \frac{m_{released\ gelatin}}{m_{total\ gelatin}} \times 100 \qquad (3)$$

2.4. *In vitro* cytotoxicity

In vitro indirect cytotoxicity tests were performed to investigate the possible release of toxic compounds from the crosslinked gelatin MS, according to ISO 10993 standard. L929 murine fibroblast cell line (ECACC, n 85011425) was selected. MS samples (15 mg, n = 3) were put in Cell Strainers, in 6-multiwell TCPS and immersed in 5 mL of culture medium for 1, 3, 7 and 14 days. As control, wells (n = 3) were filled with complete DMEM culture medium, without MS. Cells were seeded in 96-multiwell (cell density = 1×10^4 cells per well) and cultured with 150 µL of DMEM culture medium until 70% confluence was reached. Then, DMEM was replaced with eluates (150 µl, in triplicate) or medium controls (150 µl, in triplicate) and cells metabolic activity measured by Alamar Blue assay [22]. Fluorescence was measured (Tecan Genios Plus) for cells cultured in culture medium eluates (f_{sample}) and in culture medium controls ($f_{control}$), after subtracting the background fluorescence of the Alamar Blue solution incubated without cells ($f_{Alamar\ Blue}$); finally, percentage cell viability was calculated following Equation (4):

$$\text{Viability}\% = \frac{f_{sample} - f_{Alamar\ Blue}}{f_{control} - f_{Alamar\ Blue}} \times 100 \qquad (4)$$

2.5. *In vitro* cell delivery

The seeding procedure of cells on the MS was optimized to maximize the number of cells adhered to MS. L929 cells (5×10^3 per well) were seeded on 24-multiwell (TCPS) or on 24-multiwell previously coated with polydimethylsiloxane (PDMS, ELASTOSIL®RT, poured and allowed to crosslink in the wells), used to prevent cells adhesion to the bottom of the well. After 4 h, cells adhered to TCPS and PDMS were detached by trypsin and viable cells counted by Trypan Blue exclusion method; similarly, cells in the supernatant of TCPS (TCPS_supernatant) and of PDMS (PDMS_supernatant) were counted (i.e., cells not attached to the well that can adhere to MS).

Direct cytocompatibility tests were then performed to investigate the suitability of the MS in supporting viable cells adhesion and proliferation onto MS surface, and to test the possible *in vitro* release of viable cells from the MS. For cells seeding, 15 mg of MS (n = 3) were put in 24-multiwell TCPS coated with PDMS, to prevent cell attachment on the bottom of the well, contemporarily promoting the cell adhesion to the MS. Then, 100 µl of cell suspension (cell density = 5×10^4 cell mL^{-1}) were seeded dropwise on the anhydrous MS samples and incubated (37 °C, 5% CO_2) for 40 min to allow cells attachment onto the MS. The percentage seeding efficiency was calculated by measuring the metabolic activity of cells adhered to the MS and cells adhered to the bottom of the well after one day of culture by Alamar Blue assay (see below). Then, cell-seeded MS were transferred in a Cell Strainer, lodged in 6-multiwell TCPS and immersed in 5 mL of culture medium; culture medium was replaced every three days, as schematically shown in Fig. S2. The capability of MS in supporting viable cells adhesion and proliferation was investigated by Alamar Blue assay and LIVE/DEAD staining. After 1, 3, 7, 14 and 21 days, the Cell Strainers containing the MS were moved from the wells and placed into new 6-multiwell TCPS and the metabolic activity was read by Alamar Blue assay both on the bottom of the well (i.e., cells delivered by MS) and on the transferred MS (i.e., cells still adhered onto the MS surface). At the same time points, LIVE/DEAD staining was performed to visualize viable (green) and dead (red) cells attached onto the MS surface. The staining was performed by removing the culture medium from wells containing the MS; then, a calcein-AM 2 µm and propidium iodide 10 µM in PBS was used to stain cells adhered to MS for 40 min (37 °C, 5% CO_2). Then, cells adhered to MS were washed twice with PBS and observed by fluorescent microscopy (Zeiss Axioplan).

2.6. Statistical analysis

Data are presented as mean ± standard deviation. ANOVA test was performed to compare data groups; $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Crosslinked gelatin hydrogels microspheres

The optimized water-in-oil emulsion process allowed processing the chemically crosslinked gelatin hydrogel to successfully obtain gelatin hydrogel MS. In particular, from each synthesis, 0.313 ± 0.127 mg MS were obtained, thus reaching a 47% process yield (i.e., ratio of the mass of collected MS to the mass of gelatin dropped in the stirring oil). The optimized production process allowed obtaining MS without aggregates, as observed by SEM (Fig. 2a*i*), characterized by a spherical shape (Fig. 2a*ii*). The average measured diameter of the MS in the anhydrous state (i.e., MS collected after the production) was equal to 70.9 ± 17.2 µm. Considering the size distribution (Fig. 2b), the measured diameters in the anhydrous state (i.e., at the end of the fabrication process) ranged from 35 to 155 µm (Fig. 2b), with more than 80% of MS included in the 45 – 95 µm range, thus proving the possibility of obtaining MS with a well-controlled dimensional distribution range with the set-up here optimized.

3.2. Physical properties and morphology during swelling

The dimensional variation of MS immersed in culture medium at 37 °C (Figure 3a.*i*) evidenced a quick swelling (i.e., increase in diameter in the first hours after immersion) and a swelling plateau phase after 1 h of immersion in culture medium (Figure 3a.*ii*). In particular, the average diameter of MS increased from 70.9 ± 17.2 µm (i.e., anhydrous state) to 169.3 ± 41.3 µm after 6 h of immersion in culture medium, thus increasing their dimension by 140%. The MS are characterized by a spherical shape during the test performed in culture medium at 37 °C, up to 21 days (Fig. 3b*i*, *ii*, *iii* and *iv*), thus confirming the stability of the crosslinked gelatin hydrogel MS. After 21 days of immersion, MS started degrading (Fig. 3b*v*, at 28 days of incubation) until completely degraded after 32 days of immersion (Fig. 3b*vi*), where only small gelatin residues were visible. The stability of the MS in culture medium at 37 °C, up to 21 days, was given by the successful crosslinking reaction confirmed by the ninhydrin assay. In fact, the crosslinking degree of the gelatin hydrogel MS and that of the bulk gelatin hydrogel, as control, were 74.5 ± 2.1% and 79.3 ± 2.8%, respectively ($p > 0.05$). The comparable crosslinking degree obtained for the MS and the bulk

gelatin hydrogel ($p > 0.05$) confirms that the method used for the preparation of MS (i.e., water-in-oil emulsion) did not affect the chemical crosslinking reaction used to crosslink the gelatin hydrogel. Moreover, comparable crosslinking degrees were obtained for gelatin microspheres crosslinked either by using similar (i.e., non-zero length crosslinking, such as genipin [27]) or different crosslinking strategies (i.e., zero length crosslinking, such as EDC [28]). After their immersion in aqueous environment at 37 °C, gelatin MS start releasing gelatin (Fig. 5a), probably due to occurring hydrolytic degradation and release or not crosslinked percentage. In particular, a quick release of gelatin is observed in the first 12 h (i.e., approximately 20% of gelatin mass released), while a slower release kinetic of gelatin is observed for the subsequent time points, with similar release profile compared to other gelatin-based MS [26].

3.3. *In vitro* cytocompatibility and cell delivery

The percentage viability of cells cultured in culture medium eluates (i.e., medium incubated with MS) normalized on the viability of cells cultured in culture medium controls (i.e., medium incubated without MS) is shown in Fig. 4. For all the considered time points (i.e., 1, 3, 7 and 14 days), the percentage cell viability is close to 100%, thus proving the absence of released toxic compounds from the MS when incubated in cultured medium at 37 °C.

Once the absence of indirect cytotoxic effects was proved, cells were directly seeded on the MS to investigate their suitability in supporting cells adhesion and proliferation. The seeding procedure was optimized by coating the TCPS with PDMS to prevent the adhesion of cells on the bottom of the well, thus promoting the adhesion to the MS. In fact, when TCPS are coated with PDMS, the number of viable cells that do not adhere to the bottom of the well significantly increases ($p < 0.05$), thus increasing the number of cells that can potentially adhere to the MS (Fig. S3).

The cell seeding efficiency on the MS, calculates 24 h after cells seeding on the MS, was 23.8 ± 5.0%, thus confirming the possibility of directly seeding viable cells on the produced MS. The increasing fluorescent values measured for cells adhered on the MS after 3, 7 and 14 days of culture (Fig. 5b) proved an increasing metabolic activity of cells adhered to the MS ($p < 0.05$, comparing

the fluorescence value at each time point with the previous measured time point), demonstrating their viability and proliferation in time. The adhesion of cells and their proliferation on MS surface was also proved by optical microscopy images acquired after 3 and 14 days of culture (Fig. 5c). In fact, randomly distributed cells colonizing the MS surface and characterized by an elongated shape can be observed after 3 days of culture (Fig. 5c*i*), while the MS surface appears completely colonized by L929 cells after 14 days of culture (Fig. 5c*ii*), thus demonstrating the suitability of the produced MS in supporting cells adhesion and MS surface colonization. The LIVE/DEAD staining confirmed the presence of viable cells (i.e., green cells) adhered to the MS surface, compared to the few dead cells (i.e., red cells) qualitatively visible, and the colonization of the MS surface over time. The Alamar Blue test performed on the well (i.e., substrate where the cells are released from the MS surface, Fig. 5b) confirmed the possibility of delivering viable cells using the here produced MS as cell carriers. In fact, starting after 3 days of culture, there is an increase of metabolic activity of cells on the well plate, thus confirming the presence of cells released from the MS that subsequently adhered and proliferated on the well bottom. Cells released on the TCPS well bottom showed a healthy and elongated morphology, thus confirming their ability in adhering and colonizing the release substrate (Fig. S4).

4. Discussion

Regeneration of damaged tissues by cell delivery approach offers some advantages compared to scaffold-based approaches, such as the use of cells as therapeutic tools without the need of a structural support for the tissue regeneration and minimally invasive clinical procedures. However, for an efficient cell delivery and successful tissue regeneration, an appropriate cell delivery system must be designed and fabricated to avoid cells dispersion in the injection site. Two different strategies can be adopted to efficiently deliver cells to a damaged tissue site by microcarriers: cells encapsulated into microcapsules and cells seeded on the surface of the microspheres [7]. In this work, we optimized the production of crosslinked gelatin MS, obtained by a water-in-oil emulsion

procedure during the ongoing crosslinking reaction of the gelatin MS, to be used as cells carriers with cells seeded on the MS surface.

MS offer an optimal alternative to deliver cells in damaged sites; in fact, compared to microcapsules, cell cultured onto the surface of the 3D carrier ensures a large surface available for cells adhesion and proliferation, and an ease access for cells to oxygen and nutrient supply, since cells adhered to the MS surface are directly exposed to culture medium, compared to cells embedded in microcapsules where an adequate diffusion of oxygen and nutrients must be guaranteed by correctly designing the materials used for the capsules fabrication [7]. However, two requirements must be met to ensure a successful cells delivery by using MS: (1) cells must adhere to the MS surface and proliferate onto it to ensure cells adhesion during the pre-injection *in vitro* culture and during the injection procedure and, subsequently, (2) viable cells must be released in the site of interest to promote the tissue regeneration, with absence of cytotoxic effects during the MS degradation.

To achieve these goals, different synthetic and natural-derived materials have been proposed for MS fabrication, including poly(D,L-lactic-co- glycolic acid) (PLGA) [29–31], gellan gum [32], chitosan [20], collagen [17,33], and gelatin [8,34]. Despite the use of synthetic polymers allows for a reproducible, well-controlled size and shape of the microcarriers, these polymers generally require a surface functionalization (e.g., by cell-adhesive molecules [29] or by coating, such as with gelatin [31]) to promote an adequate anchorage-dependent cell adhesion on the MS surface. Moreover, during the MS degradation, an inflammatory response can locally occur caused by the synthetic polymer degradation products (e.g., PLGA [29,35]). On the contrary, these drawbacks do not affect the gelatin MS described in this work. In fact, cell-adhesive motifs (i.e., Arg-Gly-Asp, RGD motifs) are intrinsically available in the gelatin polymer chain, thus allowing for an anchorage-dependent cell adhesion on the MS surface. Moreover, thanks to the presence of metalloproteinase-sensitive sites, gelatin can physiologically undergo *in vivo* enzymatic degradation [36] and its degradation products are well tolerated by the human organism without stimulating any adverse reaction [37].

Moreover, the choice of gelatin compared to collagen, described as alternative materials for the production of MS [17,33], is advantageous thanks to the lower gelatin immunogenicity and antigenicity, lower cost and its readily availability and reproducibility [38].

In this work, the successful delivery of cells was demonstrated by a simple method that proved the possible release from the MS of viable cells that were subsequently able to colonize and proliferate on the release substrate (i.e., TCPS). To stabilize gelatin at physiological temperature (i.e., 37 °C), we selected a non-zero length chemical crosslinking reaction; in this reaction, MBA is used as crosslinker to form crosslinking bonds between gelatin amino groups (Figure 1). The proposed crosslinking strategy is simple, cost-effective and quick (i.e., approximately 24 h required to fabricate gelatin MS). The crosslinking reaction occurs simultaneously to the water-in-oil emulsion step, thus allowing to skip the production step generally performed when MS are immersed in a crosslinking bath once completed the water-in-oil emulsion procedure, such as immersion in genipin [27] or glutaraldehyde solution [39]. Moreover, no gelatin pre-modification (e.g., methacrylation of gelatin) is required [40]. This crosslinking reaction also allows for the loading of drugs and biomolecules, as previously demonstrated for bulk hydrogels crosslinked using this mechanism [41], thus potentially allowing for drug loading and release from the produced MS. By using this reaction, coupled with the water-in-oil emulsion technique, we developed a very simple, cost-effective and quick (i.e., 1 day required for the MS production) procedure to obtain gelatin MS stable in physiological-like environment (i.e., culture medium at 37 °C). Moreover, no cytotoxic effects were detected and related to the crosslinking method used (Figure 4).

MS where characterized by spherical geometry immediately after their preparation, as observed by SEM micrographs; this morphology, characterizing anhydrous MS after their preparation, is the one experienced by cells during the cell seeding procedure. Then, the maintenance of the spherical shape during the swelling was confirmed by the optical microscopy images, that proved the spherical shape is maintained by the MS until their degradation. By using the procedure here optimized, >80% of fabricated MS were characterized by a diameter in the 45 – 95 μm range, that

increased after 6 h of swelling to an average diameter of 169.3 ± 41.3 μm. The diameter range in the swollen state is comparable, or lower, to those of other MS used as cell delivery systems with cells seeded on the MS surface, including MS produced by crosslinking gelatin by genipin (i.e., 50 – 450 μm) [25], that acts similarly to the MBA used in this work, or comparable to those of collagen (i.e., 75 – 180 μm) [17] and lower that other obtain using chitosan (i.e., ≈300 μm) [20]. Potentially, the diameter ranges obtained for the MS in the swollen state are suitable for injection of MS for hypodermal needle sizes routinely used in clinics (i.e., up to 27 G needle size), thus proving the potential use of the here proposed MS as cell carriers. In this approach, the choice of gelatin has a double advantage. In fact, cells seeded on the gelatin MS surface can adhere and proliferate, thanks to the intrinsic presence of cell-adhesive motifs in the gelatin polymer chain. At the same time, during gelatin degradation and release in the injection site, cells can detach from the MS surface and colonize the site of interest.

Despite other gelatin types with lower isoelectric points (e.g., gelatin type B) were proved to be less cytotoxic compared to type A gelatin used in this work [42], the MS here fabricated did not show any cytotoxic effect and were demonstrated to be suitable for the here described application. To prove the potential of the gelatin MS produced in this work, we developed a simplified *in vitro* wound model by scratching a confluent layer of L929 cell line fibroblast (Figure 6), to investigate the potential use of the here fabricated MS for wound healing application [43]. After the scratch, we manually applied by a spatula [34] MS previously seeded with cells on the *in vitro* wound model, leaving an untreated wound as control. We qualitatively evidenced by LIVE/DEAD staining that viable cells can be delivered from the microspheres on the simplified *in vitro* wound model (i.e., scratched area). Moreover, cell metabolic activity (measured by Alamar Blue assay) was lower after the applied scratch, due to the removal of cells after the scratch was performed. Then, when viable cells were delivered by MS, we evidenced an increased cells viability on the simplified wound treated with the MS, compared to the wound without MS, used as control (Figure 6). Thus, we can demonstrate that the MS here developed are suitable microcarriers to deliver viable cells in an

injured site and potentially improve the tissue regeneration. Moreover, the release of gelatin during the MS degradation represents a potential advantage for the colonization of the injured site from the delivered cells.

5. Conclusions

Gelatin hydrogel MS crosslinked by MBA were here successfully fabricated in a water-in-oil emulsion, allowing to quickly obtain MS with a spherical shape and controlled range dimension. The MS swelled in culture medium and maintained stable dimensions for more than 2 weeks, thus confirming the successful crosslinking reaction coupled with the water-in-oil emulsion method used for the MS preparation. The MS evidenced to be non-cytotoxic; moreover, they were suitable in sustaining cells adhesion and proliferation, as well as in delivering cells, thus representing optimal candidates as cell delivery systems.

Conflict of Interest

The authors declare that they have no conflict of interest.

Figure Captions

Fig. 1. Schematic representation of the water-in-oil emulsion process used for crosslinked gelatin microspheres (MS) fabrication: a gelatin solution (containing the crosslinker) is dropped in soy bean oil under stirring at 50 °C and allowed to crosslink for 16 h (left); after 16 h, the chemical crosslinking allows fixing the spherical shape of the MS that are subsequently washed by acetone to remove oil residues. Gelatin hydrogel composition and parameters used to fabricate the gelatin hydrogel MS (right).

Fig. 2. Chemically crosslinked gelatin hydrogel microspheres produced by water-in-oil emulsion. (a) Representative SEM images (scale bar *i* = 250 μm; scale bar *ii* = 100 μm) and (b) frequency of the diameter distribution of anhydrous microspheres obtained after the fabrication process.

Fig. 3. Morphology during the swelling/degradation and physical properties of crosslinked gelatin hydrogel microspheres. (a) Average diameter of microspheres immersed in culture medium (37 °C, 5% CO_2) for 28 days (*i*) and particular of the first 4 h of immersion (*ii*). (b) Representative images after (*i*) 1, (*ii*) 7, (*iii*) 14, (*iv*) 21, (*v*) 28 and (*vi*) 32 days of immersion (scale bar: 100 μm). (c) Average percentage crosslinking degree of gelatin hydrogel microspheres and bulk gelatin hydrogels produced using the same hydrogel formulation, as control ($p > 0.05$).

Fig. 4. *In vitro* indirect cytotoxicity of crosslinked gelatin hydrogel microspheres: percentage cell viability of cells cultured in culture medium eluates obtained in contact with MS for 1, 3, 7 and 14 days compared to the viability of cells cultured in culture medium controls (i.e., culture medium incubated for 1, 3, 7 and 14 days without MS).

Fig. 5. *In vitro* cytocompatibility and cell delivery tests. (a) Cumulative release of gelatin from gelatin MS in Hank's Balanced Salt Solution (HBSS) at 37 °C. (b) Metabolic activity (relative fluorescent unit, RFU) of cell adhered to microspheres and cells delivered form microspheres on the tissue culture plastic and schematic representation of the cell delivery process. (b) Optical and (c) fluorescent (LIVE/DEAD staining) images of cells cultured on the microspheres after (*i*) 3 and (*ii*) 14 days of culture.

Fig. 6. I*n vitro* model of wound healing by cells delivered by gelatin microspheres. LIVE/DEAD staining of a confluent layer of L929 cells and of cells affected by a scratch (wound simplified model). The wound model is then treated by delivering cells by gelatin microspheres or without the gelatin MS (as control); scale bar 150 μm. Metabolic activity of the cell confluent layer, of the layer affected by the wound and of the wound treated with or without microspheres (* $p < 0.05$ compared to the confluent cell layer).

Fig. S1. Schematic representation of the protocol optimized for microspheres preparation. (a) MS are collected in oil, in a syringe, at complete crosslinking reaction. (b) Oil is ejected from the syringe while MS are kept in the syringe thanks to the filter and (c) repeated acetone washes are performed. (d) Finally, MS are collected and allowed to dry.

Fig. S2. Schematic representation of the in vitro cell delivery test. Microspheres are seeded on a PDMS substrate to promote cells adhesion to the MS. After 1 day of culture in a Cell Strainer, the MS and adhered cells are moved to a new TCPS to discharge not attached cells on the TCPS plate. At established time points, the cell metabolic activity on the MS and the metabolic activity of cells released from the MS on the TCPS is measured by Alamar Blue Assay.

Fig. S3. Optimization of cells seeding procedure for MS. Number of viable cells adhered to traditional tissue culture plastic (TCPC) or silicon-coated TCPS (PDMS) 4 h after cell seeding,

compared to the number of viable cells counted in the supernatant of TCPS (TCPS_supernatant) and PDMS (PDMS_supernatant). * $p < 0.05$.

Fig. S4. Representative optical microscopy image of L929 cells adhered on the TCPS well bottom after being released from gelatin microspheres after 14 days of culture (scale bar: 150 μm).

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