Production of polymeric micelles by microfluidic technology for combined drug delivery: Application to osteogenic differentiation of human periodontal ligament mesenchymal stem cells (hPDLSCs)

L. Capretto a, S. Mazzitelli b, G. Colombo b, R. Piva c, L. Penolazzi c, R. Vecchiatini c, X. Zhang a, C. Nastruzzi b, c

a Engineering Sciences, Faculty of Engineering and the Environment, University of Southampton, University Road, Southampton SO17 1BJ, UK
b Department of Life Sciences and Biotechnology, University of Ferrara, Via Fossato di Mortara 17/19, 44100 Ferrara, Italy
c Department of Biochemistry and Molecular Biology, University of Ferrara, Via Fossato di Mortara 74, 44100 Ferrara, Italy

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A B S T R A C T

The current paper reports the production of polymeric micelles (PMs), based on pluronic block-copolymer, as drug carriers, precisely controlling the cellular delivery of drugs with various physico-chemical characteristics. PMs were produced with a microfluidic platform to exploit further control on the size characteristic of the PMs.

PMs were designed for the co-delivery of dexamethasone (Dex) and ascorbyl-palmitate (AP) to in vitro cultured human periodontal ligament mesenchymal stem cells (hPDLSCs) for the combined induction of osteogenic differentiation.

Mixtures of block-copolymer and drugs in organic, water miscible solvent, were conveniently converted in PMs within microfluidic channel leveraging the fast mixing at the microscale. Our results demonstrated that the drugs can be efficiently co-encapsulated in PMs and that different production parameters can be adjusted in order to modulate the PM characteristics. The comparative analysis of PM produced by microfluidic and conventional procedures confirmed that the use of microfluidics platforms allowed the production of PMs in a robust way with improved controllability, reproducibility, smaller size and polydispersity.

Finally, the analysis of the effect of PMs, containing Dex and AP, on the osteogenic differentiation of hPDLSCs is reported. The data demonstrated the effectiveness and safety of PM treatment on hPDLSC.

In conclusion, this report indicates that microfluidic approach represents an innovative and useful method for PM controlled preparation, warrant further evaluation as general methodology for the production of colloidal systems for the simultaneous drug delivery.

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1. Introduction

Periodontal diseases are infectious pathologies characterized by the destruction of tooth supporting tissue which includes periodontium, cementum, alveolar bone and gingiva (Andriankaja et al., 2006; Seo et al., 2004; Pihlstrom et al., 2005).

The ultimate goal of periodontal therapy is the regeneration of the tissue lost caused by periodontal diseases. Unfortunately, this process is particularly complicated and can be theoretically achieved by employing specific mesenchymal stem cells, only if many conditions, both local and systemic, are met during the healing process, resulting in a successful therapeutic treatment (Iwata et al., 2010; Yang et al., 2010).

In all organisms small stem cell clusters are present in adult tissues, called “niches”. Dental tissues have been also investigated to assess the presence of stem cell niches with potential application in tissue regeneration (Shi and Gronthos, 2003), with the finding of small clusters of cells in the extravascular region of the periodontal ligament (PDL) (Chen et al., 2006; Bartold et al., 2006; Seo et al., 2004; Pitaru et al., 1994).

As the MSCs present in the bone marrow, human PDL mesenchymal stem cells (hPDLSCs) display a multidifferentiation potential, with the ability to give rise, when properly stimulated, to at least 3 distinct cell lineages: osteo/odontogenic, adipogenic and neurogenic lineages (COURA et al., 2008; Huang et al., 2009; Park et al., 2011). Notably, PDLSCs can re-constitute their native tissue if transplanted in an ectopic site (Seo et al., 2004; Gronthos et al., 2006), for instance, when transplanted...
into immunocompromised mice, PDLCs form the typical alveolar bone/cementum/periodontal ligament-like structure, if combined with an adequate inducer/scaffold (Gay et al., 2007; Seo et al., 2004).

The therapeutical use of stem cells, isolated from dental tissues, could thus offer new opportunities, especially for patients suffering from tooth loss or periodontal destruction (Arnold et al., 2010).

The identification of factors and conditions capable of promoting osteogenesis of MSCs in general and hPDLCs in particular is therefore critical to promote and enhance the osteogenic potential of osteochondroprogenitors. Using different experimental models, it was demonstrated that bone formation/regeneration can be induced by (i) synthetic compounds able to activate or increase the osteogenic signaling, (ii) suppressing the activity of bone formation inhibitors, and (iii) specific molecules, including growth factors, cytokines, transcription factors and hormones. These molecules can be potentially added within bone defects both alone (to reactivate the endogenous MSC niches) or in combination with MSC/biomaterial constructs (Ardivson et al., 2011; Prabhakaran et al., 2012). For these reasons, there is a great interest in the scientific community for the identification/selection of optimal in vitro cell culture conditions (i.e. culture medium supplements) being able to stimulate the osteogenic process in different cellular precursors. The elaboration of a simple, effective and reproducible culture medium for differentiation protocols remains therefore a “pre-implant” crucial matter for any therapeutic approach aimed at the in vivo regeneration of bone tissue.

Many studies focusing on the control of MSC differentiation by osteogenic cocktails have shown that glucocorticoids such as dexamethasone (Dex) have an essential role for both osteogenesis and mineralization processes (generally evaluated by Alizarin Red or Von Kossa staining); those processes depended on the degree of cellular differentiation, the donor species, dose duration and dosing regimen (Song et al., 2009). In addition to Dex, it has been suggested that the presence of ascorbic acid plays a crucial role in osteogenic differentiation together with organic phosphates (in particular beta-glycerophosphate) that serve as a source of inorganic phosphates when hydrolyzed by alkaline phosphatase (Song et al., 2009).

Innovative therapeutic tools for bone diseases must take into account that the long culturing time and long time chemical agent exposure are major drawbacks of these strategies and may not be the most feasible to satisfactorily meet clinical demands. This encourages the development of alternative strategies to induce human MSC osteogenic differentiation and to specifically optimize the drug delivery to cells for differentiation protocols.

Drug delivery of bioactive factors to target cells or tissues is a very critical aspect in tissue engineering (Richardson et al., 2001). Many studies have focused on the development of innovative formulations in the form of nano- and microparticles. In this context, polymeric micelles (PMs) possess a prominent position within nanotechnology based formulations (Civiale et al., 2009); they are self-assembling colloidal systems obtained by the aggregation block or graft amphiphilic copolymers.

With respect to the application of PMs for differentiation induction they could result in a series of advantages including simultaneous controlled delivery, increase of drug availability, changes in cellular distribution and a general increase in amount of drug uptake by the target cells. In addition, considering the easy accessibility to the anatomical site, this approach is suitable for application to the regenerative treatment of periodontal defects.

In this respect, PMs are ideal drug delivery candidates since: (a) their nanoscale dimension permits the local administration and (b) the presence of the polyethylene glycol (PEG) shell prolongs the in vivo bioavailability (Harada et al., 2011). The PEG chains create indeed a highly water-bound barrier on the micellar surface that impairs the adhesion of opsonins and therefore the recognition and phagocytosis by mammalian immune cells.

Regarding the presented application, PMs are an appealing formulation for Dex since the drug molecule presents specific attributes fitting the PMs’ nanoenvironment, which is characterized by a hydrophobic core and an outer hydrophilic shell (Gaucher et al., 2005). Dex possess indeed a relevant lipophilic portion constituted by the cyclopentaphenanthren-3-one moiety and a hydrophilic part represented by the 11,17 dihydroxy and 17-(2-hydroxyacetyl) functional groups. In this respect, Dex is practically insoluble in water whether it is soluble in ethanol, methanol, acetone, dioxane and slightly soluble in chloroform.

The present paper describes the development of nanosized formulations based on PMs, produced by microfluidic technology, for the combined delivery of Dex and ascorbyl-palmitate (AP). In this respect, it should be taken into account that micellar systems can solubilize different compounds, from the hydrophilic drugs to the completely insoluble hydrophobic ones, even if micelles are most typically employed to encapsulate sparingly soluble or insoluble substances in water. In this context, for the solubilization into PMs ascorbic acid was replaced by AP that is the ester formed from ascorbic acid and palmitic acid creating a fat-soluble (water insoluble) form of vitamin C which is suitable for the solubilization within the micellar core. For comparison, PMs were also produced by a conventional method. The production parameters and their effects on PM characteristics were analyzed, and the effects of Dex and AP association in PMs on the osteogenic differentiation of PDLCs were investigated in terms of mineral matrix deposition.

2. Materials and methods

2.1. Materials

Amphiphilic block copolymer Pluronic F127 (average molecular weight 12,600) was provided as a gift by BASF Chem Trade GmbH. Dexamethasone (Dex) (BioReagent ≥97.0%, molecular weight 392.46), ascorbic acid 6-palmitate (AP) (USP testing, molecular weight 414.53), and dimethyl sulfoxide (DMSO) (purity, ≥99.5%, molecular weight 78.13) were obtained from Sigma–Aldrich, UK, and used as supplied. Unless otherwise stated all other chemicals were obtained from Sigma–Aldrich, UK, and used without further purification.

2.2. Device fabrication

A photolithography/wet etching procedure was used for the fabrication of glass microreactors based on the protocol previously developed (Fletcher et al., 2001) with minor adaptations. Briefly, the channel network was designed using AutoCAD drawing software first. A film negative of the desired final size was then prepared by a commercial photo mask producer (J.D. Photo Tools, UK) to form the optical mask. Crown white glass (B-270) plates (thickness of 1.5 mm) coated with a thin layer of chromium metal mask plus an upper layer of positive photoresist (AZ1500), supplied by Telic (Telic, USA), were used for channel network fabrication. By exposing to UV light, the pattern of interconnecting channels was transferred from the negative film to the photoresist layer on glass, which was then developed and removed, together with the chromium layer. The exposure and development processes revealed the channel areas of glass to be etched. Once the pattern had been transferred, the glass plate was baked in an oven at 80 °C overnight to dry and harden the mask on glass. The channels were then etched using 1% hydrofluoric acid buffered with 5% ammonium fluoride solution at 65 °C, under ultrasonic agitation (Ultrasonic Bath, VWR, UK). Finally, the etched glass was thermally...
bonded (595 °C for 3 h) with a top plate of the same material into which outlet and inlet ports had been previously drilled to link the channels. Before the bonding step, the cross section profile of the etched microchannel was measured by a surface profiler (P-16+ stylus profiler, KLA Tencor, USA). The microreactor fabricated for this study consisted of three inlets and one main mixing/reaction channel with an outlet (Fig. 1). The channel depth and width were 30 μm and 80 μm, respectively. The length of the main channel was 30 mm. TEFLOH tubes (Upchurch Scientific, USA) with an inner diameter of 500 μm were used to connect the microreactor to gastight glass syringes (Hamilton, Reno, USA) where a KDS syringe pump (KD100, KD scientific Inc., USA) was used to control the flow rate.

2.3. Preparation of polymeric micelles

Pluronic F127 (15.0 mM), Dex (3.3 μM) and AP (4.1–33.0 mM) were dissolved in DMSO and subsequently filtered through 0.22 μm filters before use. Dynamic viscosity (η) measurements of various Pluronic F127 solutions in DMSO were performed using a Cannon–Fenske viscometer. All of the measurements were performed at a constant temperature of 25 °C. The viscosity was found to be 0.02819 Pa.s. The value was an average value of five different measurements for each sample. The effect of Dex and AP on η was found to be negligible. The production of polymeric micelles (PMs) was carried out in a continuous flow format within microreactors where nanoprecipitation was achieved in a hydrodynamic focusing flow configuration. The organic solution containing Dex, AP and co-polymer flowed in the central focused stream, and water, acting as a non-solvent, in the adjacent lateral streams. The flow rate of each stream was controlled by the syringe pumps to achieve a desired volumetric flow rate (R) of organic solution to water. R was set to range between 0.03 and 0.13. Total volumetric flow rate was set at 0.65 mL h−1. An inverted microscope (Olympus IX71, Japan) was used to monitor the flow within the microchannel during nanoprecipitation. To prepare PMs by bulk mixing conditions, Dex/AP/pluronic solution (prepared as previously described) was added to 2–10 mL of deionized water in a glass round-bottom flask and thoroughly mixed by vortexing for 5 min. Different volumetric ratios (R) of organic solution to water were selected varying between 0.03 and 0.13. For both, microfluidic and bulk mixing procedures, the preparation of PMs was performed at environmental temperature, typically ranging between 21 and 23 °C.

2.4. Characterization of polymeric micelles

Typically, 2 mL of the produced PMs was collected and used for further analysis and size characterization. Immediately after the preparation of PMs, their size and size distribution were determined by Dynamic Light Scattering (DLS) (Zetamizer Nano ZS, Malvern Instruments Ltd., UK). For each measurement, 300 μL of PM suspension was loaded in the detection cell and five measurements were performed to give an average value using Zetasizer software 6.12 (Malvern Instruments Ltd., UK).

PMs were also characterized by surface topography images by means of Atomic Force Microscopy (AFM) (MFP3D – Asylum Research) as described (Bourogiopoulos et al., 2012). In brief, samples were prepared by depositing 10 μL of PM suspension onto microscopy glass-slide and then dried under reduced pressure to allow PMs absorption onto the glass surface. Observations were performed directly following grid preparation. The dispersed PMs were imaged in AC mode (scan rate: 1 Hz) in air using Olympus AC200TS silicon probes (k = 9.7 N/m; Freq = 115 kHz; tip radius 9 nm).

2.5. Computational fluid dynamics (CFD) study

Mass fraction profiles of Pluronic F127 within injected polymer stream sheathed by two adjacent water (non-solvent) streams were numerically simulated with a three-dimensional model using Ansys Fluent 12.1.4 (ANSYS Inc., Canonsburg, PA). Computational fluid dynamics simulations based on the finite volume method
(FVM) were performed on a geometry applying 9670768 cubic hexahedra (3D) mesh elements accounting for the fluid domain comprised between channel junctions and channel outlet. The flow field and mixing dynamics within the device were modeled using single phase three-dimensional continuity and full Navier–Stokes equations for incompressible flow coupled with the convection–diffusion equations for DMSO and Pluronic F127 mass transfers. The governing conservation equations of mass, momentum and species are given respectively by

\[ \nabla \cdot (\rho \bar{v}) = 0, \]  
\[ \rho \left( \frac{\partial}{\partial t} + \bar{v} \cdot \nabla \right) \bar{v} = -\nabla p + \mu \nabla^2 \bar{v} + \bar{g}. \]  
\[ \frac{\partial}{\partial t} (\rho Y_i) + \nabla \cdot (\rho \bar{v} Y_i) = -\nabla \cdot \bar{j}_i \] 

where \( \bar{v} \) is the fluid velocity, \( \rho \) is the density, \( \mu \) is the dynamic viscosity, \( D_{m,i} \) is the diffusivity, \( \rho \) is the pressure, \( Y_i \) and \( \bar{j}_i \) represent the mass fraction and the diffusion flux due to concentration gradient of the \( i \)th species, respectively (DMSO and Pluronic F127). Constants \( \rho \) and \( \eta \) of 998 kg m\(^{-3}\) and 8.94 \times 10\(^{-3}\) Pa s, respectively, were set for water, while for organic solutions they were assumed according to the experimental measurements performed on the Pluronic F127 solutions, \( \rho \) and \( \eta \) of fluid mixture forming within the microfluidic channel were assumed to vary according to a volume-weighted mixing law and mass-weighted mixing law, respectively (Sahu et al., 2009; Sullivan et al., 2007).

To investigate the effect of viscosity variation on the microfluidic mixing process, a user defined function was implemented in order to describe the effect of the variation of \( \mu \) on the diffusion coefficient (D) of the species under investigation. From the assumption that temperature and density were constant, an approximate dependence of the diffusion coefficient on viscosity of the medium could be written as (Kamholz et al., 1999),

\[ \frac{D_{\mu_1}}{D_{\mu_2}} = \frac{\mu_2}{\mu_1} \]  

then, knowing the value of diffusion coefficient (\( D_{\mu_1} \)) at a certain viscosity \( \mu_1 \), Eq. (5) could be used to estimate \( D_{\mu_2} \) at any other viscosity. The binary diffusion coefficient for DMSO/water and Pluronic F127/water was assumed to be 10\(^{-4}\) and 5.22 \times 10\(^{-11}\) m\(^2\) s\(^{-1}\) (Luk et al., 2008), respectively, at viscosity \( \mu_1 \) equal to 0.894 \times 10\(^{-3}\) Pa s (viscosity of water at 25°C).

The velocity and the species concentration at the flow inlet boundary were assumed to have uniform profiles, while a constant pressure (101325 Pa) was imposed at the outlet boundary. Finally, no-slip conditions and zero species concentration flux were applied at the solid walls of the device.

The governing equations and their boundary conditions were solved iteratively until steady-state was reached using an implicit technique. Note that under-relaxation technique was adopted to avoid divergence during the iterative solution procedure.

In order to minimize the effect of numerical diffusion in the current numerical analysis, a third-order QUICK spatial discretization scheme was used for momentum and species mass fraction (Hardt and Schönfeld, 2003; Hou et al., 2009; Tsai et al., 2008). Moreover, during the meshing step, care was taken in order to have a large number of elements in the grid, and to have edges parallel to flow direction (Hardt and Schönfeld, 2003; Hou et al., 2009; Tsai et al., 2008). The accuracy and reliability of the code were validated by comparing the numerical outcome with experimental data of the width of the focused stream (Fu et al., 2008). The focused/sheath stream interface calculated numerically was defined as the contours that have a Pluronic F127 mass fraction equal to 0.5.

2.6. Periodontal ligament samples recruitment

Following informed consent, teeth with healthy PDL were obtained as a result of tooth extraction for orthodontic purposes or removal of totally impacted lower third molars. All patients selected were healthy patients with clinically healthy PDL. The extent of experiments was explained to all patients and informed consent was obtained according to the Helsinki Declaration.

Human PDLs were collected, within 4 h from extraction, with a gentle separation by dental scaler from the mid-third of roots of extracted teeth. After several washes, PDL tissue was cut into small pieces (approximately 2 mm\(^2\)) and placed in 6-well culture dishes with 1 ml Dulbecco’s Modified Essential Medium Low Glucose (DMEM LG, Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 mg/ml) and streptomycin (10 mg/ml). The culture medium was changed twice a week, until PDL cells were subconfluent (70–80%), thereafter cells were scraped off by 0.05% trypsin/EDTA (Gibco, Grand Island, NE)(2 min), washed, counted by hemocytometric analysis, assayed for viability before all experiments.

2.7. Viability assay

Before and after all experiments, the viability of the cells was determined after double staining with propidium iodide (PI) and Calcein-AM according to the manufacturer’s instructions. For the PI and Calcein-AM analysis, cells were visualized under a fluorescence microscope (Nikon, Optiphot-2; Nikon Corporation, Tokyo, Japan) using the filter block for fluorescein. Dead cells stained red, while viable ones appeared green.

2.8. Flow cytometric analysis

Homogeneous populations of hPDLSCs were isolated according to the particular markers expressed on the cell surface. Cells were analyzed for expression of MSC surface marker molecules, by direct immunofluorescent staining, as reported in the literature (Stewart et al., 2003). Cell pellets were resuspended in PBS and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human antibodies CD45-PE, CD34-FITC, CD90-FITC, CD105-PE and Stro-1-FITC (DakoCytomation; Dako, Glostrup, Denmark) for 15 min at 4 °C. Monoclonal antibodies with no specificity were used as negative control.

2.9. Cytotoxicity analysis

The cytotoxicity analysis was done on in vitro cultured hPDLSCs before, during and after experiments. Determination of viable cells was done with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) colorimetric assay (thiazolyl blue). MTT assay is based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals in the mitochondria of living cells (Denizot and Lang, 1986). MTT provides a quantitative determination of viable cells. After 72 h of treatments in triplicate, 200 μL of a solution of MTT (5 mg/ml) was added to each well of cells, and the plate was incubated for 2 h at 37 °C. The medium was removed, and the MTT crystals were solubilized with 50% dimethylformamide (DMF). Spectrophotometric absorbance of each sample was measured at 570 nm.

2.10. Osteogenic differentiation

Confluent hPDLSCs, from the 2nd to 4th passages, were trypsinized and placed inside 6-, 12- and 24-well plates. Cells were maintained in the basal medium for 1–2 days until they reached confluence, and then the culture was continued with the
osteogenic induction supplement-containing medium. hPDLCs were induced by employing three different conditions, namely: (i) standard osteogenic induction medium consisting of Dulbecco’s Modified Essential Medium High Glucose (DMEME HG, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and with 100 nM Dex, 280 μM ascorbic acid and 10 mM β-glycerophosphate; (ii) as before apart from the fact that Dex and ascorbic acid added to the culture medium as PM dispersion prepared by batch procedure (PMs-B); (iii) as before but Dex and ascorbic acid added in the culture medium as PM dispersion prepared by microfluidics (PMs-M). Notably, the final concentration of Dex was the same for all the conditions; ascorbic acid was present at 280 μM in condition (i) and in the form of AP at 280 and 100 μM in conditions (ii) and (iii), respectively. For condition (i) DMSO was added to the culture medium at the same concentration with respect to condition (ii) and (iii) (in which it is already present in the PMs-B and PMs-M), namely: 2 μM/L. Medium changes were performed every three days. The osteogenic differentiation of cells was determined after 21 days of in vitro culture. The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (alizarin (AR-S, Sigma) at different time, and before and after osteogenic induction. Cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS, and stained with 40 mM AR-S (pH 4.2) for 10 min at room temperature. Next, cell preparations were washed five times with deionized water and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was observed at different magnification using a Leitz microscope.

2.11. Adipogenic differentiation

Confluent hPDLCs, from the 2nd to 4th passages, were trypsinized then maintained in basal medium for 1–2 days until they reached confluence, and then the culture was continued with the adipogenic medium. Cells were treated with adipogenic medium for three weeks with medium changes twice a week. The adipogenic medium consisted Dulbecco’s Modified Essential Medium High Glucose (DMEME HG, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and with 1 μM Dex (Sigma–Aldrich, St. Louis, MO, USA), 5 μg/mL bovine insulin (Sigma–Aldrich, St. Louis, MO, USA), 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma–Aldrich, St. Louis, MO, USA), and 60 μM indomethacin (Sigma–Aldrich, St Louis, MO, USA). On the 21st day cells were fixed in 10% formalin neutral solution (Merck, Darmstadt, Germany) for 30 min and stained with fresh 0.6% oil red O solution to show lipid droplets in induced cells.

3. Results

3.1. Production of polymeric micelles by microfluidic technology

3.1.1. General consideration

PMs are usually prepared by different methods by taking into consideration of the physicochemical properties of drug(s) and polymeric surfactants (Gaucher et al., 2005). For instance, when both polymer and drug(s) are sparingly soluble or practically insoluble in water, an organic solvent, miscible in water (e.g. DMSO, acetonitrile, tetrahydrofuran and acetone), is required for their dissolution. Thereafter, the polymeric surfactant and the drug(s) solubilized in an organic solvent (named “solvent”) are appropriately diluted, by “bulk” mixing procedures, in water (named “non-solvent”), causing the formation of the supramolecular micellar aggregates.

As an alternative, a new and controllable procedure for PM preparation based on a microfluidic approach has been introduced (Karnik et al., 2008; Capretto et al., 2011, 2012). This method, based on a multi-fluid flow, allows to accomplish the mixing of “solvent” and “non-solvent” in a defined robust manner, by a rapid and adjustable process owing to the specific characteristics of the microfluidic environment.

Microfluidics represents indeed a reproducible approach for the production of PMs; it allows for the control of PM physicochemical characteristics, essential for their biopharmaceutical applications. Moreover, the PMs obtained by microfluidic reactors posses further positive characteristics such as narrow size distribution and high drug loading efficiency (Karnik et al., 2008). The first feature is particularly important since previous studies have demonstrated that PMs of small dimensions evade, more efficiently, the scavenging from macrophages, resulting in a prolonged plasma circulation (Alexis et al., 2008).

For the production of PMs, Pluronic F127 was employed as a model block copolymer, due to its well-studied characteristics and the impressive safety profile that resulted in the approval by Food and Drug Administration for pharmaceutical and medical applications, including parenteral administration (Croy and Kwon, 2004). Fig. 1A reports the general scheme of the microfluidic setup employed for PM production, together with the dimensional characteristics of the microfluidic mixer. The device was built in glass in order to: (i) reduce the well-known problem of polymer adsorption onto the channel's walls during the PM production process (Rhee et al., 2011) and (ii) overcome the possible microchannels swelling of polydimethylsiloxane (PDMS) microreactors.

The device consists of three inlet channels merging in one mixing channel. All channels have the same width and depth (80 μm × 30 μm); the mixing channel is 3 in. length and terminates with a single outlet. The particular geometry adopted for the micromixer allowed for the production of a hydrodynamic flow focusing mixing pattern. Namely, the central stream of polymer + drug(s) in “solvent” (i.e. DMSO) which flows along the central channel is hydrodynamically focused at the channel junction, when it meets the two lateral sheath streams of “non-solvent” (i.e. water), side by side (see Fig. 1B).

3.1.2. Effects of operational conditions

It has been demonstrated that the final active concentrations of the two substances required, in the cell culture medium, for cell osteogenic differentiation are profoundly different. Namely, Dex is employed at 100 nM while ascorbic acid is used at a three orders of magnitude higher concentration (i.e. 280 μM) (Franceschi, 1992). In this respect, we initially attempted to solubilize into the PMs, ten times higher amounts of both drugs, with respect to the final working concentrations, namely Dex 1.00 μM and AP 2.800 mM. This choice was made in order to cope with the dilution (1:10, v/v) of the micellar solution into the cell culture medium for the in vitro experiments.

It was found that the solubilization of the sole Dex, at 1.000 μM, resulted in minimal changes in the average micellar size, empty PMs were indeed 7 ± 4 nm whereas Dex loaded PMs were only slightly smaller, being 6 ± 5 nm in diameter. In contrast, when AP was tentatively loaded in PMs at the concentration of 2.800 mM, together with Dex, it caused severe problems including: (i) the formation of very large aggregates, in the micrometer range and (ii) an uncontrolled precipitation within the microfluidic device, resulting in clogging of the channels, therefore largely impairing the production of PMs.

In consideration of the uncontrolled precipitation of AP when it was used at 2.800 mM, the AP concentration was gradually reduced from 2.800 mM down to 0.125 mM, while keeping constant the Dex concentration at 1.000 μM. Fig. 2 summarizes the effect of such reduction in AP concentration. From the results is evident the strong role played by AP on PM mean size (Fig. 2A) and size distribution (Fig. 2B). When AP is present at a concentration of 1.000 μM,
the size of the produced PM is dramatically reduced to a mean diameter of 207 ± 28 nm. Notably, this concentration did not cause any precipitation/clogging issues inside the microreactor channels. A further reduction of AP concentration, down to 0.500, 0.250 and 0.125 mM led to a progressive reduction of the micellar size with mean diameter of 14 ± 6, 12 ± 4 and 6 ± 4 nm, respectively.

Based on the obtained results, we decided to select the AP concentration of 1.000 mM since it represents a good compromise between two contrastive aspects: the possibility to achieve as high as possible loading efficiency for AP into the PMs, maintaining, at the same time, the PM dimensions in the nanoscale range.

In the following experiments the concentrations of Dex and AP were maintained constant in the feeding solution while changing the DMSO/water (“solvent”)/“non-solvent”) volumetric ratio (R). The obtained results, reported in Fig. 3A, show the significant effect of R on the mean size of PMs. At a low R value (i.e. 0.03), micelles had a mean diameter of 207 ± 28 while at a higher R value (i.e. 0.13) micelles became excessively large in size (1484 ± 235 nm) well exceeding the nanometers range. For comparison in Fig. 3A is also reported the size distribution analysis of micelles, with the same chemical composition, produced by a conventional bulk mixing procedure (mean diameter = 559 ± 63 nm). The results, in agreement with the current literature on PMs (Karnik et al., 2008; Capretto et al., 2011, 2012), confirmed the superior dimensional quality both in terms of size and size distribution offered by the microfluidic approach. Notably, in our case, the narrower size distribution of the PMs obtained by microfluidics with respect to that of PMs-conventional (at R = 0.03) is proved by the full width at half maximum (FWHM) values that are 70.35 and 185.60, respectively (Fig. 3A).

In order to confirm the dimensional data obtained by DLS and to gain information about the morphology of the PMs, an AFM analysis was performed. In particular, in Fig. 3B and C the AFM phase and height images for PMs are respectively reported. The image reported in panel B indicates that PMs have a spherical shape, in addition the image suggests a possible core–shell structure for PMs; this hypothesis is supported by the differences in physical
properties between the peripheral (purple) and the core (darker) regions. The difference in colors reflects the differences in friction and/or adhesion forces experienced by the cantilever when passing between the micellar regions, resulting in the phase shift during the imaging (Pang et al., 2000). Finally, the profile analysis, reported in panel C, confirms the dimensions obtained by DLS analysis.

3.2. Computational fluid dynamic analysis of microfluidic parameters

For the comprehension of the microfluidic process, it is important to consider that the high viscosity difference between the central focused stream and the two sheath fluids represents a crucial feature of the entire process. Multi-fluid flows have been characterized by the tendency for the lower viscous fluids (water) to place themselves where the shear stress is greater (near the channel walls) enveloping the high viscosity fluid (polymeric solution) (Cubaud and Mason, 2008). In addition, the diffusion between the two components induces complex viscosity gradients which affect velocity field and mixing (Cubaud and Mason, 2008).

Our previous investigation demonstrated that, during the nucleation (i.e., self assembling) dynamics, both drugs and polymer molecules tended to remain within the focused stream and they diffused only partially out of it (Capretto, 2011). As a result, drug and polymer molecules are exposed to the change of environment caused by diffusive mixing that takes place in the focused stream fluid domain. In reason of that, we focused our investigation on the kinetics of the DMSO mass fraction evolution without taking into consideration the negligible effect of drugs and drug diffusion. Nevertheless, it is recognized that the presence of Pluronic F127 could strongly affect the kinetics of diffusion by changing the viscosity of the fluid, as implemented by Eq. (5).

To take forward the results of the previous investigation (Capretto et al., 2011) and extend the knowledge on the effect of fluidic conditions on solvent diffusion in the frame of microfluidic nanoprecipitation, we present here how the focused stream shape affects the solvent diffusion and in turn the nanoprecipitation process. In this respect, Fig. 4 shows the numerical simulated mass fraction contours of DMSO at a distance from the channel junction of 5 mm for two different R, namely 0.03 (panel A) and 0.13 (panel B). It is shown that at small R (R = 0.03) the more viscous fluid is detached from the top and bottom walls and becomes ensheathed by the less viscous fluid leading to the formation of a viscous thread. This effect also occurs for larger R (R = 0.13) but at a significant less extent and decreasing R the focused stream becomes progressively more ensheathed by water. This behavior causes a progressive increase in the volume-to-surface ratio of the contact interface between the two phases, which is likely to result in faster mixing and thus smaller and less polydisperse PMs, as confirmed by the results of Fig. 3.

To further demonstrate the correlation between focused stream shape and mixing process Fig. 4C shows a typical computed DMSO mass fraction evolution along the mixing channel at two different R levels. The mass fraction profile was computed along three lines located in mid-sagittal plane of the mixing channel at three different distances from the bottom channel wall, namely 7.25 (lower), 14.5 (center) and 21.75 μm (upper). The graph shows that the mass fraction of DMSO evolves with different kinetics in the three regions. Specifically, the mass fraction tends to remain higher in the central region while

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Fig. 4. Effects of flow rate ratio on the mixing process. (A and B) CFD simulated mass fraction contours DMSO polymeric solution along the main channel for R = 0.03 (A) and R = 0.13 (B). The contours refer to a cross section 5 mm downstream the channel junction. CFD simulated mass fraction evolution of DMSO polymeric solution along the main for R = 0.03 (solid markers) and R = 0.13 (open markers). Panel D reports CFD simulated mass fraction evolution of DMSO as function of the residence time for R = 0.13 (open markers) and R = 0.03 (solid markers).
Fig. 5. (A) Phenotype characterization of hPDLSCs. Flow cytometric analysis showing the immunophenotype of hPDLSCs. The gated cells were negative for the hematopoietic markers CD45 and CD34, partially positive for CD105 and STRO-1, and positive for the mesenchymal stem cells marker CD90. (B) Adipogenic and (C) osteogenic differentiation of hPDLSC. Cultured periodontal ligament cells formed oil O red-positive lipid clusters (B) and Alizarin Red positive mineralized nodules (C); cells were cultured for 21 days in adipogenic or osteogenic medium, respectively.

decreases faster in upper and lower regions. Notably, the mass fraction in upper region decreases faster than in lower region, for smaller $R$ (0.03), while it decreases slower than in lower section for higher $R$ (0.13). In addition, at $R=0.13$, a larger difference in the value of the mass fraction in the three regions can be noted. The different kinetics of the evolution of DMSO mass fractions are likely to have an effect on the PM assembly resulting in the production of PMs with different sizes within the three different regions.

Our previous studies have also shown that hydrodynamic and diffusive mixing in microchannels are highly coupled features (Capretto et al., 2011). Therefore, to investigate solely the effect of
mixing process, computed mass fraction evolutions along the mixing channel have been processed through an in-house developed Matlab-based code. The code was used to transform the domain of space (i.e. mixing channel longitudinal position) into the domain of time (i.e. residence time). Specifically the residence time \( t_{res} \) was calculated using the following equations,

\[
\begin{align*}
  t_{res} &= \frac{N}{p} \sum_{i=0}^{N} x_{i+1} - x_i \\
  \bar{v} &= \frac{v_{x_i} + v_{x_{i+1}}}{2}
\end{align*}
\]

where \( x_i \) is the distance from the channel junction of the node (i) in which the mass fraction of DMSO was computed, \( N \) represents the maximum number of nodes within the line in which mass fraction of DMSO was computed, and \( v_{x_i} \) is the velocity of the fluid at the node (i).

Fig. 4D shows the computed DMSO mass fraction evolution along the mixing channel as function of \( t_{res} \) for different flow focusing conditions. The mass fraction evolution was computed at the center of the focused stream equidistant from the top and bottom walls. The mass fraction time evolution clearly depicts the kinetics of the mixing process in a fashion that is not convoluted with the role of velocity field. Is therefore possible to investigate the role of mixing process and residual solvent content during the self-assembly of PMs.

As evidenced in Fig. 4D, at small \( R \), a much faster mixing kinetics is obtained, as demonstrated by the low DMSO mass fraction value, compared with that at \( R = 0.13 \). In particular, for small \( t_{res} \) (i.e. \( t_{res} \leq 10 \) ms), the mixing at \( R = 0.03 \) shows an abrupt reduction of the mass fraction due to the mixing in the arrowhead-shaped focusing region (Hertzog et al., 2004; Jahn et al., 2010). A faster rate characterizes the mixing at \( t_{res} > 10 \) ms, as suggested by the higher slope of the curve at \( R = 0.03 \). This is likely due to a series of factors including, higher diffusivity (Capretto et al., 2011), narrower focused stream width and fluid enveloping. For \( R = 0.03 \), less than 4 ms are needed to drop the DMSO concentration to below the critical level (Capretto et al., 2011), causing the nucleation of unimers. On the contrary, at \( R = 0.13 \), a much longer time (~80 ms) is necessary to trigger the nucleation within the entire domain of the focused stream. These computational results suggest that a fast nucleation rate and a low amount of DMSO during the self-assembly process result in smaller and more homogeneous PMs, as experimentally confirmed by the results reported in Fig. 3.

3.3. hPDLSC harvesting and cell characterization

For all the experiments a simplified protocol based uniquely on the capacities of MSCs to adhere to a plastic surface without enzymatic treatment or dissection was chosen. This particular protocol was adopted since it is known that the overdigestion of tissue may result in diminished cellular viability, degradation of cellular surface receptors, and altered cellular function (Huang and Mauck, 2004; De Bruyn et al., 2010). After isolation, cells showed the characteristic spindle-shape, fibroblastic morphology and formed adherent colonies that are typical of other stromal stem cell populations (Shi et al., 2005; Xu et al., 2009).

The isolated cells grew rapidly and were routinely subcultured after 14 days. As shown in Fig. 5 cell populations expressed the cell surface molecules STRO-1, CD90 and CD105, while they did not express CD45 and CD34, according to literature (Shi and Grnthsos, 2003; Shi et al., 2005). We demonstrated the capacity of our experimental model to undergo adiogenesis (Fig. 5B) and osteogenesis (Fig. 5C), according to literature (Du et al., 2012; Huang et al., 2009; Seo et al., 2004).

3.4. Effect of combined delivery of inducers by polymeric micelles on osteogenic differentiation of hPDLSC

Well-defined ex vivo protocols to expand mesenchymal stem cells (MSCs), as well as suitable assays to study their osteogenic potential, are needed for developing stem cell-based therapy for bone regeneration. In this respect, we were interested in investigating the role of biochemical osteogenic inducers, i.e. ascorbic acid, dexamethasone, and β-glycerophosphate, which are currently employed in protocols for osteogenic differentiation. In this context, we propose PMs as advanced formulation for the solubilization and delivery of osteogenic differentiating agents. hPDLSCs were cultured in adhesion conditions and exposed for 72 h to the following conditions: standard osteogenic medium, empty PMs, PMs–batch and PMs–microfluidics. All the experimental conditions were set up with the same Dex and ascorbic acid molar concentration present in standard osteogenic medium. In order to analyze mineral matrix deposition, β-glycerophosphate was always present in all the tested conditions. Cell viability was evaluated by MTT assay (Fig. 6A), demonstrating that all the different culture conditions exhibited no appreciable cytotoxicity.
measured as percentage of viable cells respect to control culture condition (rated to 100%). The differentiating agents present both in osteogenic medium and in PM-microfluidics affected cell viability at very low level, even after 8–10 days of cell exposure (Fig. 6B). Nevertheless, no significative differences were observed compared to control culture conditions \( p > 0.05 \). Thereafter, the osteogenic potential of hPDLSCs cultured in standard osteogenic medium or in the presence of differentiating agents delivered by PMs was compared in terms of ability to deposit mineral matrix. The osteogenic differentiation was evaluated after 7, 14 and 21 days of in vitro cell culture, by Alizarin Red that stains in red the calcified nodules. As shown in Fig. 7, the osteogenic differentiative event is anticipated in cells treated with PMs, as proved by the large number of stained nodules present since day 14. In addition, PMs-microfluidics induce a higher level of mineralization, compared to traditional osteogenic medium and PMs-batch, without affecting the viability of the cells that are not differentiated.

4. Discussion

A microfluidic based protocol for the production of a micellar delivery system for the combined delivery of Dex and AP was developed and examined in comparison to micelles produced with conventional methods. Different production parameters were examined in relation to their effects on the size and size distribution, namely: the amount of AP present into the micelles and \( R \), the flow rate ratio between “solvent” and “non-solvent”.

In particular, the microfluidic arrangement allowed for a fast diffusive mixing between the “solvent” and “non-solvent” streams thanks to the reduced diffusive mixing length of the focused stream,
since in the laminar flow regime at low Reynolds numbers, mixing generally occurs as a result of diffusion. In such conditions, the reciprocal diffusive process of “solvent” and “non-solvent” molecules (i.e. water and DMSO) at the focused stream domain determines the mixing extent (Karnik et al., 2008; Capretto et al., 2011).

Following the work of Johnson et al., we tentatively propose for our micellar system a similar mechanism, in which the mixing time plays a central role in the PMs assembly when polymers and drug molecules are subjected to a rapid environmental switch caused by the fast change of solvent polarity (i.e. from “solvent” to “non-solvent”) (Johnson and Prud’homme, 2003a). The environmental switch triggers the mechanism of self-assembly that is at the base of PM formation.

Therefore, the tentative mechanism for PM formation could rely on the mutual “solvent”–“non-solvent” diffusion, inside the central channel, where the DMSO stream is brought in contact with the “non-solvent” water, causing a progressive increase of the microenvironment hydrophilicity. The environment change forces the unimers orientating themselves so that the hydrophobic blocks are removed from the aqueous environment in order to minimize free energy, through a reduction of the number of enthalpically unfavorable contacts between the hydrophobic moieties and water (Letchford and Burt, 2007; Rapoport, 2007).

This process causes the nucleation of unimers forming the primary PM’s nuclei (i.e. growing PMs) (Johnson and Prud’homme, 2003a). Thereafter, a growing phase occurs where other unimers and drug molecules insert into the growing PMs. This phase is halted by the formation of an insertion barrier when a critical size is reached that kinetically quench the PM growth (Johnson and Prud’homme, 2003a; Cui et al., 2007; Zhang and Eisenberg, 1999). In this respect, it has been demonstrated that the kinetics of solvent exchange affects the size of the nano-assembly by altering the kinetics of nucleation and growth (Johnson and Prud’homme, 2003b). Specifically, it has been demonstrated that by reducing the mixing time smaller and more homogenous PMs are obtained. This is further supported by the experimental results reported in Fig. 3A which are corroborated by the computational data reported in Fig. 4.

In addition to the effect of the variation of $R$, our data also suggested an important effect on PM size played by the AP concentration. We tentatively explain the effect of AP concentration on PM formation taking into account the concepts of micellar solubilization capacity and maximum loading capacity. The solubilization of a molecule by a micellar system can be defined by a descriptor that is the molar solubilization capacity, defined as the number of moles of the solute (drug) that can be solubilized by one mole of micellar surfactant, and characterizes the ability of the surfactant to solubilize the drug (Rangel-Yaguil et al., 2005; Dar et al., 2007).

Since the hydrophobic core of PMs serves as the loading space for the lipophilic drugs, given the nanometric size of the micelles, a limited space is available and therefore a maximum molar solubilization capacity (i.e. maximum loading capacity) can be identified (Lee et al., 2007; Allen et al., 2000). In this view, the encapsulation of drug in an amount below the maximum molar solubilization capacity might cause a modest increase of the micellar dimensions which still remain in the nanometer range as previously shown (Sezgin et al., 2006; Zhang et al., 2005). This is reflected in the formation of larger PMs when AP concentration was increased up to 0.500 mM (see Fig. 2). In contrast, if the maximum solubilization capacity is overpassed, the excess of drug cannot be solubilized in the core of the PMs and, is therefore exposed to a solvent which is prone to precipitate in the form of crystalline suspension leading to a large increase in size as in the case of AP concentration of 1.000 mM.

After preparation and characterization, PMs prepared both by microfluidic and bulk mixing protocols were in vitro tested for their possible cytotoxic activity and effectiveness as osteogenic inducers of hPDLCs.

Notably, the incorporation of drugs into PMs resulted in a lower in vitro cytotoxicity. Moreover, PMs-M showed a more pronounced osteogenic differentiative activity when compared to PMs prepared by a bulk method and also with respect to a conventional osteogenic medium. The smaller dimension of the PMs-M, as compared to those produced by bulk method (Fig. 3A), might be the reason for more efficient internalization within target cells, resulting in higher intracellular drugs concentration and therefore relatively higher differentiative activity. In this respect, it has been previously shown that particle size could significantly affects cellular and tissue uptake through non-phagocytic translocation. In particular, it has been shown that the extent of nanoparticle uptake is indirectly proportional to the particle size (Foged et al., 2005; Prokop and Davidson, 2008; Rejman et al., 2004; Win and Feng, 2005).

In addition, the current paper suggests that PMs might be of great interest as delivery systems for lipophilic drugs acting as inducers of osteoblastic differentiation that represents an essential part of bone formation. This funding represents an important first step toward the future development of micellar and more, in general, nanomedicine ex vivo protocols for the expansion, induction and treatment of mesenchymal stem cells.

Considering that an important issue for bone tissue regeneration is the employment of pre-differentiated osteogenot progenitor in suitable induction media, our study demonstrates the efficacy of PMs as delivery system and encourages the development of alternative strategies to induce efficient differentiation for clinical use of hMSCs in bone and cartilage tissue engineering and repair.

Finally, the simplicity and reproducibility of the presented microfluidic technology suggest that the production strategy might be suitable for personalized medicine applications, which require controllable PM characteristic and consistency from batch to batch.

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