A bioavailability study on microbeads and nanoliposomes fabricated by dense carbon dioxide technologies using human-primary monocytes and flow cytometry assay

E. Ciaglia\textsuperscript{a,1}, F. Montella\textsuperscript{a,1}, P. Trucillo\textsuperscript{b}, M.C. Ciardulli\textsuperscript{a}, P. Di Pietro\textsuperscript{a}, G. Amodio\textsuperscript{a}, P. Remondelli\textsuperscript{a}, C. Vecchione\textsuperscript{a}, E. Reverchon\textsuperscript{b}, N. Maffulli\textsuperscript{a}, A.A. Puca\textsuperscript{a}, G. Della Porta\textsuperscript{a,b,*}

\textsuperscript{a}Department of Medicine, Surgery and Dentistry “Scuola Medica Salernitana”, University of Salerno, Via S. Allende, 84081 Baronissi, SA, Italy

\textsuperscript{b}Department of Industrial Engineering, University of Salerno, Via Giovanni Paoli II, 84084 Fisciano, SA, Italy

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\textbf{ABSTRACT}

Supercritical Emulsion Extraction (SEE) and Supercritical assisted Liposome formation (SuperLip), use dense gases such as carbon dioxide (\textit{CO}_2) to fabricate advanced micro/nanocarriers.

SEE uses \textit{CO}_2 to extract solvent from the oily phase of an emulsion and obtain biopolymer microbead; For this study, poly-Lactic Acid (PLA) microbeads of 1 ± 0.2 \textmu m in mean size loaded at 1 \mu g/mg, with Rhodamine B (ROD) were prepared by SEE, the beads showed a solvent residue lower than 10 ppm and encapsulated the fluorochrome with an efficiency of 80%. SuperLip uses \textit{CO}_2 to enhance lipid/ethanol/water mixing and to promote the ethanol extraction from liposome suspension. In this case, phosphatidyl-choline (PC) vesicles with a mean size of 0.2 ± 0.05 \textmu m and loaded with Fluorescein Iso-ThioCyante (FITC) at 8 \mu g/mg, were prepared; small unilamellar structure was observed for all the vesicles with FITC encapsulation efficiency of 80%. Ethanol residue of 50 ppm was measured in all the liposome suspensions.

The bioavailability of microbeads and nanoliposomes was assessed through incubation with human monocytes previously isolated from healthy donors’ blood. A specifically optimized protocol that allowed their quenching on the cell surface was developed to monitor by flow cytometry assay only the cell population that effectively internalized the carriers. When microbeads were tested, the percentage of alive internalizing monocytes was of about 30%. An internalization of 96.1 ± 21% was, instead, obtained at dosage of 0.1 mg/mL for nanoliposomes. In this last case, monocytes showed a viability of almost 100% after vesicles internalization at all the concentrations studied; on the other hand, cell apoptosis progressively increased in a dose/response manner, after polymer microbeads phagocytosis.

The proposed data suggested that \textit{CO}_2 technologies can be reliably used to fabricate intracellular carriers.

1. Introduction

Nanotechnology for treatment and diagnosis of biological systems has been referred to “nanomedicine”. Nanomedicine trends are transforming the healthcare, allowing new approaches to drug targeting (Pautler and Brenner, 2010), and non-invasive diagnosis and treatment with exciting opportunities to monitor drug release and distribution, validating the effectiveness of the therapy (Kevadiya et al., 2018).

Mononuclear phagocytes, dendritic cells, endothelial and cancers cells are their key targets (Moghimi et al., 2005). Polymeric or lipid-based micro/nano-carriers have been described for the controlled or targeted delivery of the therapeutic agents in a wide range of nanomedicine applications (Horch et al., 2013; Daglar et al., 2014; Fontana et al., 2017; De Marco et al., 2018; Amodio et al. 2018). Biopolymer

\textbf{Abbreviations:} ROD, Rhodamine; PC, phosphatidylcholine; IgG, Immunoglobulin G; SEE, Supercritical Emulsion Extraction; SuperLip, Supercritical assisted Liposome formation; FITC, Fluorescein Iso-ThioCyante; PLA, Poly-Lactic Acid; EE, Encapsulation Efficiency; MD, Mean Diameter; SD, Standard Deviation; PSD, Particle Size Distribution; PPM, Part per million; PACS, Fluorescent Activated Cell Sorter; FBS, Fetal Bovine serum; BSA, Bovine Serum Albumin; MPS, Monocyte-Macrophage Phagocytic System

\textsuperscript{*} Corresponding author at: Translational Medicine Lab. at Department of Medicine, Surgery and Dentistry of University of Salerno, Via S. Allende, Baronissi SA, 84084, Italy.

E-mail address: gdellaporta@unisa.it (G. Della Porta).

The authors equally contributed as first author.

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carriers are often proposed for the sustained and controlled delivery over prolonged time intervals (Reis et al., 2017). On the other hands, liposomes and lipid vesicles are normally selected for a targeted delivery in one shot approach (Patra et al., 2018). The subsequent translation of these delivery systems from academic research to clinical practice is still extremely slow because of the insufficient production rate of the industrial preparation methods that often also have high batch-to-batch variations. A further limitation is the lack of technologies for their rapid screening with high correlation to the in vivo tests (Liu et al., 2018).

Recently, dense gases technologies have been described for polymeric or lipidic drug carrier’s preparation in the literature in the last decades. Solvent evaporation/extraction is the largely used for polymeric drug carrier’s production with drawback as high solvent residue and limited batch-to-batch reliability (Venditto and Szoka, 2013; Doshi and Mitragotri, 2009). For liposomes production, thin layer hydration method, microfluidic channel method and ethanol injection are the largely used techniques often characterized by low entrapment efficiencies, difficult control of vesicles size distribution and high solvent residue (Mufammadi et al., 2010).

Several conventional technologies have been described for the fabrication of these challenging carriers (Sandhya et al., 2009; Wang et al., 2011). Among them, Supercritical Emulsion Extraction (SEE) and Supercritical assisted Liposome formation (SuperLip) offer a higher control over carrier size, distributions and encapsulation efficiency (Della Porta et al., 2016; Cricchio et al., 2017; Campardelli et al., 2016; Trucillo et al., 2017). One of the main reasons of the enhanced performance of technologies that used dense gases is the improved mass transport properties involved in the processing steps. Indeed, dense gases such as carbon dioxide (dCO₂) can be used as a liquid organic solvent but with lower viscosity and higher diffusivity close to those of gases; additionally, the solvent residues within the product are minimized or absent, thus improving micro/nano carrier bioavailability and lowering its overall toxicity. SEE technology allows the continuous extraction of the oily phase of an emulsion by dCO₂ to fabricate polymer beads of specific size and distribution; several biopolymers were successfully processed, even though poly-lactic acid (PLA) and poly-lactic-co-glycolic acid (PLGA) are the most commonly described because of their use as injectable/implantable devices (Della Porta et al., 2017; 2018). SuperLip technology was also recently proposed for liposomes production with different compositions (Trucillo et al., 2019). This technology has been described as particularly innovative especially because it allowed the fabrication of Small Unilamellar Vesicles (SUVs) with a good control of their size and distribution as well as with high encapsulation efficiency (Espirito Santo et al., 2014; Trucillo et al., 2019). However, despite the attention given to these technologies in the recent literature, a study on the bioavailability and cytotoxicity of the prepared micro/nanodevices, as well as, their performances as intracellular carriers, is still missing.

The aim of the present study is to prepare microbeads and nanoliposomes using the two dense gas technologies described above and load them with fluorochromes to check their bioavailability by cellular uptake on human primary CD14 + monocytes using flow cytometry assay (Gu et al., 2014). These primary cells were selected because they are suitable cellular targets in several nanomedicine approaches (Underhill and Ozinsky, 2002; Gu et al., 2010); moreover, the phagocytosis of the described carriers may play a key role not only to enhance immunity but as a smart strategy of drug delivery (Fiala et al., 2007; Dale et al., 2008) in several tissues.

In more detail, microbeads of poly-Lactic Acid were loaded with Rhodamine B (PLA-ROD) and Phosphatidyl-Choline vesicles were loaded with Fluorescein Iso-Thiocyanate (PC/FITC) by using SEE and SuperLip technologies, respectively. The carriers were characterized for their size and morphology by a Dynamic Laser Scattering (DLS) and Scanning and/or Transmission Electron Microscopy; fluorochromes loadings were measured by UV-Vis spectrophotometry. Empty PLA microbeads and FC vesicles, respectively, were also fabricated as a negative control. Monocytes were isolated from peripheral blood of healthy donors and incubated with the different carriers’ types at several concentrations. The intracellular uptake and cytotoxicity were then assessed by specific protocol and cytometry assay. The study may open industrial perspectives for the production of a specific drug carrier system by the described technologies.

2. Materials and methods

2.1. Biopolymer beads fabrication by SEE

Rhodamine B (ROD, Sigma-Aldrich, Milan, IT) loaded Poly-Lactic Acid (PLA, RESOMER R 203H, Boehringer Ingelheim, DE) microbeads were fabricated using SEE patented technology starting from water-oil-water (w-o-w) emulsions (Reverchon and Della Porta, 2014). Briefly, ROD (9 mg) was dissolved into 1 mL of water with poly-vinyl alcohol (PVA, 0.04% mass, Sigma-Aldrich, Milan, IT) and added to the oily phase (19 mL) of Ethyl Acetate (EA, Sigma-Aldrich, Milan, IT) with 1 g of PLA. The w-o-w phase was stabilized after 30 s of sonication with an ultrasonic probe at 30% of amplitude (Branson Ultrasonics Corporation, Danbury, CT) and then mixed with a aqueous Tween 80 solution (0.6% mass) to obtain the w-o-w using a high-forcer homogenizer (Silverton Machines, Chesham Bucks, UK) for 6 min at 10 °C at 2800 rpm. The oily phase of emulsion was extracted by dense-Carbon dioxide (dCO₂) purity 99% Merlindo Group, IT) in a packed column operating in a countertemperate mode at 8 MPa and 38°C, using a liquid/gas flow rate ratio of 0.1; further details on SEE apparatus layout are provided elsewhere (Della Porta et al., 2013).

Beads suspension was recovered at the bottom of the apparatus in a sterile vessel. The sterile operation protocol involves the sterilization of whole piping system by steam at 120 °C and the filtration of all water solution before their use with 0.2 μm filters (Merck Millipore, Milan IT). The suspension was washed by centrifugation at 1200 rpm for 20 min at 4 °C to remove the surfactant, and beads were recovered by lyophilization (Lab Freeze Dryer Lyophilizer, Baxter, USA). Empty beads were fabricated as a control, following the same protocol but without loading the fluorescent tracker in the internal water phase of the emulsion.

2.2. Liposomes fabrication by SuperLip

α-Phosphatidylcholine from egg yolk (PC, 99% pure, Sigma Aldrich, Milan, IT) and cholesterol (99% pure, Sigma Aldrich, Milan, IT) were dissolved in Ethanol (<99% pure, Sigma Aldrich, Milan, IT), the solution was then pumped in a static mixer with dCO₂ in a co-current mode at the operating pressure of 10 MPa and 40 °C with liquid/gas ratio of 0.4 to obtain a gas-expanded solution. The expanded ethanol solution is then pumped in a co-current mode with a water phase into a high pressure chamber; meanwhile, the water phase is sprayed through a nozzle (diameter 80 μm) to enhance the two phases mixing. Ethanol is then separated from vesicles/water suspension by CO₂ flushing out continuously from the top of the chamber and recovered in a further separator operating at 1 MPa; further details on the SuperLip apparatus layout are provided elsewhere (Campardelli et al., 2016). Liposomes were collected at the bottom of the high-pressure chamber, in a sterile vessel at concentration of 2 mg/mL (lipid in water). The protocol was optimized for sterile production including apparatus sterilization by steam at 120 °C before its use, as well as, ultrafiltration of all the water solution used. Empty vesicles were always fabricated as a control, following the same protocol but without loading the fluorescent tracker in the water phase.

2.3. Carrier characterization: Size and distribution, charge, morphology

Dynamic Light Scattering analyses were carried out (granulometer mod. Zetasizer Nano S, Worcestershire, UK) for carriers’ size and distribution characterization. Each measurement was repeated in triplicate.
using the carrier's suspension (beads or vesicles) recovered at the exit of the apparatuses without any other sample dilution.

Carrier morphology was investigated by Field Emission-Scanning Electron microscopy (FE-SEM, mod. Leo 1525, Carl Zeiss SMT AG, Oberkochen, DE); samples were placed on a double-sided adhesive carbon tape previously stuck to an aluminum stub and coated with a thin gold film (layer thickness 250 Å) using a sputter coater (mod. 108 A, Agar Scientific, Stansted, UK). Lipid vesicles were dried by critical point drier (mod. R850, Quorum Technologies Ltd, East Sussex, UK) and also observed using Scanning Electron Microscope equipped with a STEM detector (mod. Zeiss Ultra Plus, Carl Zeiss SMT AG, Oberkochen, DE) after trapping them on specific STEM stub holder.

2.4. Solvent residue analysis

Solvents contents in water suspensions recovered from SEE and SuperLip, respectively, were monitored to check the efficiency of solvent extraction by dCO2. The solvent residue was measured using a head space sampler (mod. 50 Scan, Hewlett & Packard, Palo Alto, CA, USA) coupled to a Gas Chromatograph interfaced with a Flame Ionization Detector (GC-FID, mod. 6890, Agilent Techn., Wilmington, DE). Solvents were separated using a fused-silica capillary column 30 m length, 0.25 mm internal diameter, 0.25 μm film thickness (mod. DB-1, J&W, Folsom, CA, USA). GC conditions were oven temperature at 40 °C for 8 min. The injector was maintained at 180 °C (split mode, ratio 1:1) and Helium was used as the carrier gas (7 mL/min). Head space conditions were equilibration time 60 min at 100 °C, pressurization time 2 min, loop fill time 1 min. Head space samples were prepared in 10 mL vials filled with 3 mL of suspension. Analyses were performed on each sample as recovered from the apparatuses in three replicates, without any further dilution.

2.5. Carrier loading and encapsulation efficiency

ROD loading was measured by dissolving a known mass (5 mg) of beads in 2 mL of water at 1 N of Sodium Hydroxide. Samples were vortexed for 3 min to ensure the complete dissolution of the biopolymer and, then, centrifuged (5 min at 1500 rpm); ROD concentration in water was measured by UV–Vis probe at 554 nm (mod. Cary 50; Varian, Palo Alto, California).

Encapsulation Efficiency (EE) was expressed as percentage and calculated as the ratio between amount of molecule measured and the amount loaded, as in eq. (1):

$$EE_{\text{measured}} = \frac{\mu g_{\text{ROD loaded}}}{100}$$

FITC loading was measured after samples ultracentrifugation at 150,000 rpm for 6 h (mod. Optima MAX-XP, Beckman Coulter, Milian, IT); the supernatant was collected to measure the FITC concentration by UV–Vis probe at 490 nm (mod. Cary 50; Varian, Palo Alto, California).

Encapsulation Efficiency (EE) was expressed as percentage and it was calculated using eq. (2):

$$EE_{\text{FITC measured}} = \frac{\mu g_{\text{FITC loaded}}}{\mu g_{\text{FITC measured}}} \times 100$$

where μg FITC loaded is the amount loaded in water and μg FITC measured represents the amount found in the supernatant, therefore, not entrapped within the vesicles. The method is described as indirect method for loading measurement (Yan et al., 2013).

2.6. Isolation and treatment of human monocytes.

CD14+ monocytes were positively selected from peripheral blood mononuclear cells (PBMCs) of healthy donors by immuno-magnetic procedure (Miltenyi Biotec, Bergisch Gladbach, DE) and cultured at 10^6 cells/ml in RPMI 1640 free medium (Invitrogen, Milan, IT) as previously described (Montecucco et al., 2014; Giardino Torchia et al., 2010) All donors gave written informed consent in accordance with the Declaration of Helsinki to the use of their residual buffy coats for research purposes, with approval from the University Hospital of Salerno Ethics Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated over Ficoll-Hypaque gradients (lymphocyte separation medium; MP Biomedicals, Aurora, OH, USA). Soon after their isolation, CD14+ monocytes were incubated with the different indicated concentrations of PLA/ROD beads and PC/FITC vesicles for 2 h at 37 °C in humidified 5% CO2 atmosphere. Empty beads and vesicles were also used as negative control. After incubation, cells were collected and then assayed through flow cytometry.

2.7. Flow cytometry

Before acquisition, the cells were washed with PBS to remove exceeding not engulfed particles and suspended in staining buffer (PBS 2% Fetal Bovine Serum, PBS). The fluorescent intensity of the cells was measured using FACSVersa Flow Cytometer (BD FACSVerse, BD Biosciences, IT), as previously described (Ciglioli et al., 2014). For each analysis, about 10^6 cells were collected. To measure the percentage of viable cells and to distinguish engulfment of particles from simple adhesion, cells were pre-incubated with trypan blue solution 0.4% (Sigma-Aldrich, IT) for 10 min before acquisition.

2.8. Statistical analysis

Statistical analysis was performed by using the GraphPad Prism 6.0 software for Windows (GraphPad Software). For each type of assay or phenotypic analysis, data obtained from multiple experiments are calculated as mean ± SD and analysed for statistical significance using the two-tailed Student's t-test, for independent groups.
Fig. 2. (a) SEE technology layout is obtained by a high pressure (HP) packed column for the countercurrent contact of the emulsion and CO$_2$, during this contact the oily phase solvent is extracted and recovered by a separator on the top of the column; beads in water suspension are continuously recovered at the bottom of the column thanks to a depressurization step obtained with a needle valve. (b) SuperLip technology uses dense CO$_2$ mainly to improve the mixing between ethanol/lipid solution (gas-expanded solution) and water phase within a high pressure (HP) static mixer; CO$_2$ is also involved in removing ethanol from liposomes suspension flushing continuously from the top of the liposome preparation chamber. Liposome in water are collected at the bottom of the same chamber.

Fig. 3. (a) Example of optical microscope image of w-o-w emulsion. (b) Scanning Electron Microscope image of PLA/ROD microbeads obtained after emulsion processing by SEE. (c) Confocal Microscope image of microbeads loaded with 1 μg/mg of ROD. (d) Carrier size distribution measured by laser scattering.
3. Results and discussion

3.1. dcO2 technologies

The dcO2 involvement in both processes is schematically illustrated in Fig. 1a, b, respectively. In detail, SEE uses dcO2 to extract the oily phase of a water–oil–water emulsion, inducing droplet hardening and biopolymer beads formation. The size of the bead is related to the droplet size by mean of a shrinking factor (defined as the ratio between the particles and droplets mean sizes) that in our case was measured at 0.6. At the operative condition selected (see methods section), the oily phase extraction was assured within 4 min of residence time in the packed tower of the SEE apparatus, achieving a solvent residue of 10 ppm and an encapsulation efficiency of 90%. More important, in these conditions the shrinkage factor was maintained almost constant for all the droplets, assuring a high batch-to-batch reproducibility.

SuperLip uses dcO2 to enhance the mixing between the lipid and water phase that allows the preparation of vesicles with a controlled size and distribution (see methods section for the operative pressure and temperature and flow rates ratio); dcO2 also improves ethanol flushing out from the liposome suspension, reducing the solvent residue that, in our case, was measured at 30 ppm in almost all the batches produced. SEE layout is obtained by a high pressure packed column for the countercurrent contact of liquid emulsion and dcO2. Thanks to this enhanced contact, the organic solvent of the oily phase is rapidly extracted and, then, recovered into a separator on the top of the column by depressurization. The polymer beads, in water suspension, are continuously recovered at the bottom of the column by depressurization achieved with a needle valve. SuperLip technology uses dcO2 first to reduce the viscosity and improve diffusivity of lipid phase using a high-pressure static mixer; this “gas-expanded” solution is, then, injected into the main chamber to be mixed with the water-phase to obtain liposomes. Liposomes encapsulate any water-soluble molecules, previously solubilized in the water-phase. Ethanol used in SuperLip is further washed out by CO2 from liposome water suspension that is meanwhile continuously recovered at the bottom of the chamber by depressurization obtained with a needle valve. Both process layouts are schematically described in Fig. 2a, b.

PLA micro-beads were prepared with ROD loading of 1 μg/mg. An optical microscopy image of a typical w1–e–w2 emulsion used is reported in Fig. 3a; a FE-SEM image of PLA/ROD microbeads obtained is illustrated in Fig. 3b. The carriers showed a spherical morphology with a smooth surface. Red fluorochrome was clearly monitored in the confocal microscope image reported in Fig. 3c, illustrating the beads dispersed in a PBS medium. Fluorochrome intensity is plotted in Fig. 3d and indicated a mean size of 1 ± 0.2 μm. FE-SEM of dried liposomes reported in Fig. 4a confirmed the uniformity of the sample; the STEM image in Fig. 4b clearly suggested the unilamellar structure of the vesicles produced by SuperLip. The carrier mean size was of 0.2 ± 0.03 μm (see Fig. 4d) loaded with 8 μg/mg of FITC; the green fluorochrome was encapsulated with an efficiency of 80% and it was clearly tracked by confocal microscopy (see Fig. 4c).

3.2. Intracellular uptake on human monocytes

The internalization of microbeads (PLA/ROD) and nanoliposomes (PC/FITC) by human monocytes was performed incubating monocytes with increasing concentration of PLA/ROD of 0.1, 0.5 and 1 mg/mL.
Fig. 5. Human primary monocytes internalize ROD/PLA (microbeads) and PC/FITC (nanoliposomes). (a) CD14+ monocytes, isolated through immune-magnetic procedure from human healthy PBMCs, were incubated at 1x10^6 cells/ml density with ROD/PLA (upper panel) and PC/FITC (lower panel) in RPMI free medium at 37°C in humidified 5% CO₂ atmosphere. The concentrations used are indicated. After 2 h of CO₂ incubation, cells were collected and analyzed by flow cytometry in basal condition or after TB addition. The panel shows a representative cytometric FCS vs SSC (cell size vs granularity index) cells scatter of 3 experiments performed with similar results. Autofluorescence of untreated control cells was reported in histograms on the right. (b) Schematic diagram of membrane quenching effect by Trypan Blue (TB) addition. (c) Representative fluorescence intensity histogram of ROD/PLA from CD14+ internalizing monocytes coming from flow cytometric analysis. Analysis of total FL2 signal from membrane and internalized microbeads (left panels) and that of the quenched signal after TB 0.4% addition (right panel) is shown. (d) Representative fluorescence intensity histogram of PC/FITC from CD14+ internalizing monocytes coming from flow cytometric analysis. Analysis of total FL1 signal from membrane and internalized nano-vesicles (left panels) and that of the quenched signal after TB 0.4% addition (right panel) is shown.

(see Fig. 5a, upper panel). PC/FITC was used at concentrations of 0.01, 0.1 and 0.5 mg/ml (see Fig. 5a, lower panel). Forward versus side scatter (FSC vs SSC) gating, which is commonly used to identify cells of interest based on size and granularity (complexity), showed the increasing titration of the particles used (black dots). Moreover, the acquisition of a more granular appearance of gated monocytes (red dots) can reflect the ongoing uptake process by the immune cells (see also Fig. 5). To distinguish the engulfment of microbeads from simple adhesion, trypan blue (TB) quenching of extracellular microcarriers was performed. A schematic representation of TB quenching principle is shown in Fig. 5b. TB can enter only in cells with damaged membrane; thus, in live cells, its quenching effect only occurs on the extracellular space. The protocol developed gives the opportunity to detect only internal signals, such as those cells linked to fluorescent particles internalization (see Fig. 5b).

Then, fluorescence signals of PLA/ROD or, alternatively, of PC/FITC carriers were analyzed before and after the addition of TB (Fig. 5c and d). Panel 5D shows a representative flow cytometric analysis of the internalization process: in gated population of CD14+ monocytes we quantified the FL2 channel signal intensity as a measurement of fluorescence PLA/ROD microbeads uptake (Fig. 5e), or alternatively, the FL1 channel signal intensity as a measurement of fluorescence PC/FITC vesicles uptake (Fig. 5d). Specifically, we observed that, while ROD fluorescence was partly eliminated after TB addition, FITC signal was almost entirely maintained, suggesting a better rate of internalization of nanoliposomes compared to microbeads. Since TB is a viability marker, we then searched for dead phagocytes which emitted red fluorescence in FL5 channel because of the presence of TB in their cytoplasm. After incubation with increasing doses of PLA/ROD microbeads, the percentage of live internalizing monocytes was of about 30%; on the contrary the percentage of internalizing dead cells progressively increase in a dose/response manner (Fig. 6a-b). Compared to PLA/ROD microbeads, we observed that nanoliposomes were internalized with a 3-fold higher efficiency which reached the 96.1 ± 21% for the concentration of 0.1 mg/mL. Moreover, nanoliposomes internalizing monocytes were almost completely alive, except at the highest dose of 0.5 mg/ml, for which a percentage of dead internalizing cells of 17.1 ± 4.2% was observed (Fig. 6d, d). These results highlighted that even though with different uptake capabilities, both carrier systems were efficiently internalized by human primary cells. A better performance was monitored for the nanoliposomes.

The choice of human monocytes studies for PLA microbeads and PC
nanoliposomes internalization was consequent to their ability to engulf and destroy apoptotic cells, pathogens, and other targets in the course of an immune response. Primary monocytes efficiently internalize both PLA beads of 1 μm and 0.1 μm size vesicles. Concerning this result, we were positively surprised to find a higher rate of uptake for 0.1 μm nanoliposomes. Indeed, even though these small-size vesicles are characterized by a lower fluorophore content (with respect the amount of ROD loaded in PLA), we registered, after treatment with these carriers, higher values of internalized signal by FACS analysis. This is a new interesting result, as phagocytosis has been widely studied by monitoring the uptake of fluorescent beads or bacteria of 1.0–3.0 μm in diameter or length, but not largely explored for nanoliposomes. In the same way, a better rapid engulfment of 1 μm beads compared to 0.1 μm vesicles by human monocytes was recently described (Dale et al., 2008); moreover, rapid phagocytosis of latex beads by alveolar macrophages with maximal phagocytic rate was also observed for 2–3 μm sized beads (Champion et al., 2008). However, in our opinion the better uptake efficiency of nanoliposomes vs microbeads might be partly explained by the specific size-dependent mechanisms of internalization used by primary monocytes (e.g. pinocytosis, endocytosis etc) (Rejman et al., 2004) or simply by surface properties of established carriers.

Regardless of the intracellular fate, loading drugs, cytokines or chemicals into microbeads and nanoliposomes can offer an efficient means of selectively targeting monocyte-macrophage phagocytic system (MPS). This is a promising field of study given the participation of MPS in a huge variety of inflammatory responses, tissue healing and tumor evolution (Champion et al., 2008). Indeed, the recruitment of monocytes is associated with pathological, but also with homeostatic response by differentiating into a broad spectrum of both inflammatory (M1) and resolving (M2) macrophages. Moreover, recent studies (Forbes and Rosenthal, 2014; Ogle et al., 2016; Julier et al., 2017; Pajarinen et al., 2018) reported the involvement of these cells in tissue healing and repair over a control of local mesenchymal stem cells actions, which constitutes a new interesting aspect of modern regenerative therapies. Indeed, an active control of the peripheral monocytes through the internalization of those factors and immune modulators capable to redirect the deleterious inflammatory response toward a protective one (switch macrophage M1 to M2) are challenging strategies.
4. Conclusions and perspectives

CD14 + monocytes display a surprising phagocytic activity, which stimulates both the innate and adaptive arms of an efficient immune response; on the other hand, micro/nano carriers have strong potential for pharmaceutical and biomedical applications. In this sense, the implementation of innovative and robust technologies and a better comprehension of their crosstalk with biological systems is required. dco2 technologies provide a fixed size carrier with a good loading as well as excellent solvent removal resulted in an advanced quality product. The two different micro and nano-carrier studied showed good and even excellent cells uptake and internalization that opens important perspectives for the use of dense gases technologies for fabrication of drug carriers in nanomedicine applications. The monocytes successfully internalized both carriers even though lower toxicity was monitored in the case of nano-vesicles.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Corrigendum

Corrigendum to “A bioavailability study on microbeads and nanoliposomes fabricated by dense carbon dioxide technologies using human-primary monocytes and flow cytometry assay” [Int. J. Pharm. 570 (2019) 118686]


a Department of Medicine, Surgery and Dentistry “Sicula Medica Salernitana”, University of Salerno, Via S. Allende, 84081 Baronissi, SA, Italy
b Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 84084 Fisciano, SA, Italy
c Vascular Physiopathology Unit, IRCCS NEROMED, 86077 Pozzilli, IS, Italy

The authors regret that they forgot to insert the second affiliation for the authors ‘C. Vecchione and A.A. Puca’ and updated as above.

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⁎ Corresponding author at: Translational Medicine Lab. at Department of Medicine, Surgery and Dentistry of University of Salerno, Via S. Allende, Baronissi, SA 84084, Italy.

E-mail address: gdellaporta@unisa.it (G. Della Porta).

The authors equally contributed as first author.

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