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Uptake and intracellular fate of biocompatible nanocarriers in cycling and non-cycling cells

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Abstract
To elucidate whether different cytokinetic features may influence cell tolerance to biocompatible nanocarriers, cell uptake and intracellular fate of liposomes, mesoporous silica nanoparticles, poly(lactide-co-glycolide) nanoparticles and nanohydrogels were investigated by confocal fluorescence microscopy and transmission electron microscopy in C2C12 cells. These immortalized murine myoblast cells are able to proliferate as myoblasts and differentiate into myotubes, thus allowing comparative studies of cell-nanocarrier interactions in cycling and non-cycling cells. Nanocarrier internalisation and distribution was similar in myoblasts and myotubes: liposomes enter the cells by fusion with plasma membrane and undergo cytoplasmic degradation; MSN enter by endocytosis and persist enclosed in cytoplasmic vacuoles; poly(lactide-co-glycolide) nanoparticles and nanohydrogels enter by endocytosis, escape endosomes and then undergo autophagic process. However, the amount of nanocarriers internalized by myotubes is lower than in myoblasts, probably due to different plasma membrane composition. No cytological alteration has been found in both myoblasts and myotubes following nanocarrier uptake.

Keywords: C2C12 cells, liposomes, mesoporous silica nanoparticles, polymeric nanoparticles, nanohydrogels, confocal fluorescence microscopy, transmission electron microscopy
Introduction

Nanocarriers possess enormous potential as drug delivery systems for controlled and targeted drug release, and a wide range of nanosystems have been reported for the treatment of various diseases and disorders (Wicki et al., 2015, Dilnawaz et al., 2018). Nanocarriers are able to protect the encapsulated agents from enzymatic degradation and to allow drug delivery and sustained release inside the cells; they thus represent a promising approach to improve the administration of therapeutic agents while decreasing adverse systemic side effects. To play their therapeutic action without damaging the patient’s organism, these nanocarriers should be biocompatible and biodegradable. In recent years, liposomes, mesoporous silica nanoparticles (MSN), polymeric nanoparticles (NPs) and nanohydrogels (NHs) have received great attention as biocompatible and versatile systems to encapsulate active agents.

Liposomes are attractive vehicles for drug delivery thanks to their composition, which makes them biocompatible and biodegradable. They consist of an aqueous core entrapped by one or more bilayers composed of natural or synthetic phospholipids. Liposomes are biologically inert and weakly immunogenic, and possess low intrinsic toxicity. Further, drugs with different physico-chemical characteristics can be encapsulated into liposomes: lipophilic drugs are entrapped in the lipid bilayer, hydrophilic drugs are located exclusively in the aqueous compartment, and the amphiphilic ones are encapsulated both in the bilayer and in the aqueous core (Arpicco et al., 2013; Pedrini et al., 2014).

Silica is generally recognized as safe by FDA and used as excipient in tablet-form drug formulations. MSN have recently attracted attention as promising components of multimodal nanoparticle systems, owing to their straightforward synthesis and functionalization, ordered mesoporous structure with tunable pore size, high surface area and large pore volumes resulting in high drug loading capacity, good chemical stability,
and adequate biocompatibility (Slowing et al. 2008; Chen et al., 2014; Sapino et al., 2015).

MSN and, in particular, amino-MSN, can be used to deliver either small molecules or oligonucleotides; moreover, they can be tailored with a variety of surface functional groups to increase biocompatibility, delivery capability and targeting (Peng et al., 2006; Malfanti et al., 2016; Ricci et al., 2018).

Polymeric NPs are solid submicron structures prepared from natural or synthetic polymers in which drugs can be adsorbed, dissolved, entrapped or encapsulated. These NPs have good encapsulation efficiency and high stability in plasma, and increase the solubility and stability of hydrophobic drugs while lowering their toxicity, thus permitting a controlled release at the target site at relatively low doses (Grottkauf et al., 2013; Stella et al., 2000, 2007a,b; Lince et al., 2011). In particular, the safe, biocompatible and commercially available poly(lactide-co-glycolide) (PLGA) is one of the most successfully used biodegradable polymers because its hydrolysis leads to metabolite monomers, lactic acid and glycolic acid, which are endogenous and easily metabolized by the body via the Krebs cycle, thus leading to a minimal systemic toxicity. It is worth noting that PLGA is approved by the US FDA and the European Medicine Agency (EMA) in various drug delivery systems for humans (Danhier et al., 2012; Kapoor et al., 2015).

Nanohydrogels (NHs) are nano-sized three-dimensional networks able to absorb a high amount of water, and to easily swell and de-swell in aqueous media (Kabanov and Vinogradov, 2009; Soni et al., 2016). NHs are usually soft, hydrophilic, biocompatible and represent a highly versatile nano-system able to deliver a variety of bioactive molecules such as hydrophobic (Choi et al., 2012) as well as hydrophilic drugs (Montanari et al., 2014), polypeptides (Montanari et al., 2013; Montanari et al., 2017) and genetic material (Ganguly et al., 2014; Lallana et al., 2017). Indeed, the porosity of the NHs network provides a reservoir for loading molecular and macromolecular therapeutics as well as protecting them from the environmental degradation. NHs can be prepared from natural
(Akiyoshi et al., 1993) and/or synthetic (Vinogradov et al., 1999) polymers and, based on the type of bonds present in the polymer network, they are subdivided into groups based on either physical (Di Meo et al., 2015) or chemical (Pedrosa et al., 2014; Montanari et al., 2016) cross-linking. A peculiar characteristic of NHs is their swelling properties in aqueous media; control over the swelling of the polymer network is useful for the controlled release of bioactive compounds. Moreover, as NHs are highly solvated, they display both liquid- and solid-like behavior: usually, these viscoelastic properties allow NHs to deform in the presence of a flow, enabling them to more easily travel through the extracellular matrix, thus enhancing the permeation, binding and retention within the tissues.

The cytotoxicity of these different nanocarriers has been previously evaluated in vitro using various established cancer cell lines (e.g. Slowing et al., 2006; Arpicco et al., 2013; D’Arrigo et al., 2014; Costanzo et al., 2016; Jonderian and Maalouf, 2016; Quagliariello et al., 2017). However, it has been reported that the effects induced by nanocarriers may depend on cell metabolic activity and doubling time (Chang et al, 2007): this suggests that the proliferation characteristics of the cell system used should be taken into account when testing the biocompatibility of nanosystems designed for systemic administration, since the organisms are composed of different tissues and cells with peculiar kinetic and metabolic features.

The aim of this study was to elucidate whether different cytokinetic features may influence the cell tolerance to different biocompatible nanocarriers. To do this, the cell uptake and intracellular fate of liposomes, MSN, PLGA NPs, and NHs have been investigated by confocal fluorescence microscopy and transmission electron microscopy (TEM) in C2C12 cells under cycling and non-cycling conditions. C2C12 cells are an immortalized murine myoblast cell line, able to rapidly proliferate as myoblasts under high serum conditions, and to efficiently fuse and differentiate into myotubes under low serum conditions: they thus represent a suitable cell system in vitro to perform comparative studies on the cell-
nanocarrier interactions in cycling cells (myoblasts) and highly differentiated non-cycling cells (myotubes).

**Materials and Methods**

**Preparation and characterization of nanocarriers**

Liposomes were prepared by thin lipid film hydration and extrusion method. Briefly, a chloroform solution of the lipid components (Avanti Polar-Lipids distributed by Spectra 2000 Rome, Italy) 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (Chol), and L-α phosphatidyl-DL-glycerol sodium salt (PG) (70:30:3 molar ratios) was evaporated and the resulting lipid film was dried under vacuum overnight. Lipid films were hydrated with HEPES [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid] buffer (pH 7.4), and the suspension was vortexed for 10 min and bath sonicated. The formulations were extruded (Extruder, Lipex, Vancouver, Canada) at 60 °C passing the suspension 10 times under nitrogen through a 400 and 200 nm polycarbonate membrane (Costar, Corning Incorporated; NY). Fluorescently labeled liposomes (Fluo-Lipo) were prepared as described above and a 10 mM solution of fluorescein-5-(and-6)-sulfonic acid trisodium salt (Invitrogen, Life Technologies, Monza, Italy) in HEPES buffer was used during hydration. Liposomes were then purified through chromatography on Sepharose CL-4B columns, eluting with HEPES buffer at room temperature.

Amino-mesoporous silica NPs (NH$_2$-MSN) were prepared as previously described (Sapino et al., 2015). Fluorescein isothiocyanate (FITC) labeled MSN were prepared as previously reported (Yu et al., 2013) with minor modifications. Briefly, at a suspension of 1 mg of NH$_2$-MSN in 150 µl of MilliQ® water 250 µl of FITC ethanol solution (0.3 mg/ml) were added. The mixture was maintained for 5 h under stirring in the dark, and then the NPs were centrifuged and washed with ethanol three times until the supernatants were colorless.
For the preparation of PLGA (50:50 or 75:25, Sigma-Aldrich) NPs, the nanoprecipitation technique was employed (Fessi et al., 1989). Practically, for each preparation, 12 mg of PLGA 50:50 or 75:25 were dissolved in 2 ml of acetone. This organic solution was then poured into 4 ml of MilliQ® water under magnetic stirring. Precipitation of particles occurred spontaneously. After solvent evaporation under reduced pressure, an aqueous suspension of NPs was obtained. Fluorescently labelled PLGA NPs were prepared by nanoprecipitation of PLGA 50:50 or 75:25 (12 mg) in the presence of 16.8 μg of Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one, Sigma-Aldrich) dissolved in acetone; this solution was then added to 4 ml of MilliQ® water under magnetic stirring, as previously described for non labelled NPs. Fluorescent NPs were purified from non-incorporated dye by gel filtration on a Sepharose CL-4B column.

Hyaluronan-cholesterol (HA-CH) polymer was synthesized as previously reported (Montanari et al., 2013). For NHs preparation, 5 mg of HA-CH were dispersed in 2.5 ml of MilliQ® water (2 mg/ml) overnight with magnetic stirring at 25°C; 2.5 ml PBS (pH=7.4) were then added. Samples were autoclaved for 20 min at 121°C, leading to the NHs formation (Montanari et al., 2015). For the synthesis of fluorescent NHs (Rhod-NHs), rhodamine B-isothiocyanate (Rhod), previously solubilized in DMSO (9 mg/mL), was added to NHs aqueous suspension (8 μl for 1 mg of polymer, corresponding to a degree of functionalisation (DF) of 6.3%; % mol/mol). The reaction mixture was left for 5 h at 25°C in the dark under magnetic stirring, followed by extensive dialysis and freeze-drying. The final DF% was assessed through UV-Vis analysis in DMSO solution at 550 nm by using a rhod calibration curve (8.5-125 μg/ml), resulting 1.3% mol/mol (mol of rhod per mol of HA-CH repeating unit).

The mean particle size and the polydispersity index (PI) of liposomes, polymeric NPs and NHs were determined at 25°C by quasi-elastic light scattering (QELS) using a nanosizer (Nanosizer Nano Z, Malvern Inst., Malvern, UK). The selected angle was 173° and the
measurement was made after dilution of the nanoparticle suspension in MilliQ® water. Each measure was performed in triplicate.

Particle size of MSN was determined by transmission electron microscopy measurements with a JEM 3010-UHR microscope (JEOL Ltd.) operating at 300 kV. Powders were dispersed on a copper grid coated with a perforated carbon film. The size distribution of the samples was obtained by measuring a statistically representative number of particles (ca. 250 particles). The results are indicated as mean particle diameter (dm) ± standard deviation (SD).

The particle surface charge of all formulations was investigated by zeta potential measurements at 25°C applying the Smoluchowski equation and using the Nanosizer Nano Z. Measurements were carried out in triplicate.

**In vitro cell culture**

C2C12 myoblasts (1-2x10³ cells/cm²) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) glutamine, 0.5% (v/v) amphotericin B, 100 units/ml of penicillin and 100 µg/mL of streptomycin (Gibco), at 37°C in a 5% CO₂ humidified atmosphere. Cells were trypsinized (0.25% trypsin in PBS containing 0.05% EDTA) when subconfluent, and seeded either on 24 or 96 multi-well plastic microplates for cell viability evaluation, or on glass coverslips in 12-multi-well plastic microplates for fluorescence microscopy and TEM. All the experiments were performed with cells at passage 7-10. For myogenic differentiation, when 80% confluency had been reached, the growth medium was substituted with the differentiation medium containing 1% FBS.

Myoblasts were treated with the different nanocarriers one day after seeding, while myotubes after six days in differentiation medium. The initial medium was replaced with a fresh one containing either liposomes or MSN or PLGA NPs or NHs (see below); the cells
were then incubated for 2 h, 24 h and 48 h. At the end of each incubation time, the cells were processed as described below; in parallel, untreated cells were used as control. C2C12 are highly proliferating cells with a cell cycle of about 20 h (https://www.dsmz.de/catalogues/details/culture/ACC-565.html?tx_dsmzresources_pi5%5BReturnPid%5D=192), therefore a 48 h incubation time allows the completion of two cycles.

**C2C12 myoblast viability assay**

To estimate the effect on cell viability and on cell growth, three concentrations of each nanocarriers were tested in cultured cells: liposomes were administered at the concentrations of 125 µg/ml, 250 µg/ml, 500 µg/ml; MSN at 12.5 µg/ml, 25 µg/mL, 50 µg/ml; PLGA NPs at 100 µg/ml, 200 µg/ml, 400 µg/ml; NHs at 50 µg/ml, 100 µg/ml, 200 µg/ml. The chosen concentrations previously proved to be non-cytotoxic for various cultured cells (Slowing et al., 2006; Arpicco et al., 2013; D’Arrigo et al., 2014; Costanzo et al., 2016; Jonderian and Maalouf, 2016; Quagliariello et al., 2017). Nanocarrier suspensions were prepared by diluting the stock suspensions into DMEM with 200 units/mL of penicillin and 200 µg/ml of streptomycin, immediately before the administration. According to Thomas et al. (2015), at the end of each incubation time, 100 µl of medium was removed and the release of the cytosolic enzyme lactate dehydrogenase (LDH) upon cell lysis was estimated with CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega). Optical density was measured with a microplate reader (Tecan) at 490 nm. The relative amount of released LDH was normalized (as a percentage) to the total amount of LDH release in control cells, which were not exposed to nanocarriers and were completely lysed with lysis buffer provided in the kit. Results were expressed as the mean ± standard error (S.E.) of 5 independent experiments.
To evaluate cell population size, $8 \times 10^3$ cells/well were seeded on 24 multi-well plastic microplates. After the different incubation times, the cells were detached by mild trypsinization and their total number estimated by counting in a Burker Turk hemocytometer; data were expressed as the mean ± S.E. of three independent experiments.

In order to evaluate the effect of nanocarrier administration on cell proliferation, the S-phase cells fraction was estimated after 24 h and 48 h incubation with nanocarrier concentrations that did not induce decrease in cell population i.e., 125 µg/mL liposomes, 50 µg/ml MSN, 100 µg/ml PLGA NPs and 100 µg/ml NHs. Cells grown on coverslips were pulse-labelled with 20 µM bromodeoxyuridine (BrdU, Sigma) for 30 min at 37°C, fixed with 70% ethanol and treated for 20 min at room temperature in 2 N HCl, to denature DNA partially. After neutralization with 0.1 M sodium tetraborate (pH 8.2) for 3 min, samples were washed in PBS, permeabilized for 15 min in PBS containing 0.1% bovine serum albumin and 0.05% Tween-20, and incubated for 1 h with a mouse monoclonal antibody recognizing BrdU (BD) diluted 1:20 in PBS. After two washings with PBS, samples were incubated for 1 h with an Alexafluor 488-conjugated anti-mouse secondary antibody (Life Techonologies), diluted 1:200 in PBS. The cell samples were washed with PBS, stained for 5 min with 1 µg/mL Hoechst 33342 (Sigma) in PBS, and finally mounted in PBS:glycerol (1:1) to be observed and scored in fluorescence microscopy (see below). Data were expressed as the mean ± S.E. of three independent experiments (number of counted cells: 1000 per sample). All statistical comparisons between treated and control samples were carried out by the Mann Whitney U test.

An Olympus BX51 microscope equipped with a 100W mercury lamp (Olympus Italia Srl, Milan, Italy) was used under the following conditions: 450-480 nm excitation filter (excf), 500 nm dichroic mirror (dm), and 515 nm barrier filter (bf), for FITC; 330-385 nm excf, 400 nm dm, and 420 nm bf, for Hoechst 33342. Images were recorded with an QICAM Fast
1394 digital camera (QImaging) and processed using Image-Pro Plus 7.0 software (Media Cybernetics Inc.).

**Analysis of nanocarrier distribution in C2C12 myoblasts and myotubes**

Confocal fluorescence microscopy

C2C12 myoblasts and myotubes were incubated for 2 h and 24 h with Fluo-Lipo, FITC-labelled MSN, Nile Red-labelled PLGA NPs or Rhod-labelled NHs at the concentrations found to be non-cytotoxic by cell viability and proliferation tests. At each incubation time, cells were fixed with 4% (v/v) paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature.

To visualize the intracellular distribution of fluorescent nanocarriers, the cells were permeabilized with 0.05% PBS Tween, washed in PBS, incubated with either 0.1% Trypan blue (Gibco) or Phalloidin-Atto 594 or Phalloidin-Atto 488 (Sigma) diluted 1:20 in PBS, stained for DNA with Hoechst 33342 (1 μg/ml in PBS), rinsed in PBS, and finally mounted in 1:1 mixture of glycerol:PBS.

To investigate nanocarrier cellular uptake, myoblasts were pre-incubated with either PKH26 Red Fluorescent Cell Linker or PKH67 Green Fluorescent Cell Linker (Sigma) to stain the plasma membrane, then incubated with the different fluorescently-labelled nanocarriers for 30 min (the short incubation time is necessary to label early endosomes only, Grecchi and Malatesta, 2014) and finally fixed and processed for fluorescence microscopy, as described above. This procedure allowed detecting possible co-localization of the fluorescence signals of endocytotic vesicles and nanocarriers.

For confocal laser scanning microscopy (CLSM), a Leica TCS SP5 AOBS system (Leica Microsystems Italia) was used: for fluorescence excitation, a diode laser at 405 nm for Hoechst 33342, an Ar laser at 488 nm for FITC, and a He/Ne laser at 543 for Trypan blue, Nile Red and Rhod were employed. Z-stack of 1 μm step sized images (each image in the
1024x1024 pixel format) were collected using a 40x oil immersion objective, and processed by the Leica confocal software.

**Transmission electron microscopy**

C2C12 myoblasts and myotubes were incubated for 2 h, 24 h and 48 h with liposomes, MSN, PLGA NPs or NHs. At each incubation time, cells were fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 2 h, post-fixed with 1% OsO₄ and 1.5% potassium ferrocyanide at room temperature for 1 h, dehydrated with acetone and embedded in Epon. Ultrathin sections were observed unstained or after weak staining with UAR-EMS Uranyl acetate replacement stain (Electron Microscopy Science). Observations were made in a Philips Morgagni transmission electron microscope (FEI Company Italia Srl), operating at 80 kV and equipped with a Megaview II camera for digital image acquisition.

**Results**

**Characterization of nanocarriers**

Liposomes either blank and fluorescent showed diameters around 180 nm (PI < 0.1) and a negative zeta potential (around -15 mV). Blank and fluorescent PLGA NP mean diameter was around 110-120 nm (PI < 0.1) and the zeta potential was always around -30 mV; NHs and Rhod-NHs showed mean hydrodynamic diameters of around 200 nm (PI < 0.2) and 250 nm (PI < 0.2), and negative zeta potentials, -38 ± 2 mV and -35 ± 3 mV, respectively. MSN are characterized by spherical particles with size of 100 ± 23 nm in diameter. The ordered mesoporous structure with MCM-41-like hexagonal array results in high specific surface area of around 800 m²/g and pore size about 3 nm (inner volume available to host drugs around 1.2 cm³/g) and a zeta potential of +35.0 ± 0.90 mV in water. The positive
charge results from the presence of the amino groups used for functionalization.

Nanocarrier features are summarized in Table 1.

**C2C12 myoblasts: cell viability and proliferation**

Cell viability was evaluated after 2 h, 24 h, 48 h after treatment with nanocarriers at different concentrations; the LDH test demonstrated values ranging from 2.09 to 12.71% for all nanocarriers, at no variance with the control samples for any incubation time (*data not shown*).

The total number of cells (Figure 1) was similar in control samples and in samples exposed to 75:25 PLGA NPs at all times considered. Conversely, cell populations exposed to liposomes, MSN, 50:50 PLGA NPs and NHs underwent significant modification in comparison to control samples; in detail, liposomes and NHs induced a significant decrease after 48 h incubation, while 50:50 PLGA NPs induced a decrease after both 24 h and 48 h. Cell population exposed to MSN showed a significant increase in comparison to control samples after 48 h incubation.

The S-phase fraction did not significantly change after 24 h incubation with all nanocarriers except for 50:50 PLGA NPs and for NHs; in detail, 50:50 PLGA NPs showed a significant increase in BrdU incorporation at the concentration of 100 µg/ml, while BrdU incorporation significantly decreased in cells incubated with 100 µg/ml NHs. After 48 h incubation, no change was found in any samples (Figure 2).

**Nanocarrier distribution in C2C12 myoblasts and myotubes**

**Liposomes**

Confocal fluorescence microscopy showed that in myoblasts liposomes were mostly found in the peripheral region of the cytoplasm, never entering the nucleus. They always appeared as isolated fluorescing spots which never formed aggregates nor markedly
accumulated in the cell; their intracellular amount was evidently larger after 24 h incubation (Figure 3 a,b). In myotubes, only few liposomes were found in the peripheral and perinuclear region of the cytoplasm after all incubation times (Figure 3 c). The green fluorescence of liposomes was never found to co-locate with red fluorescing intracellular (i.e., endosomal) membranes (Figure 3 d).

At TEM, liposomes were strongly electron dense due to the lipid staining by osmium tetroxide: in both myoblasts and myotubes their number was very low and they occurred both at the cell periphery, just beneath the cell membrane (Figure 3 e) or in the perinuclear area (Figure 3 g). No internalization processes such as endocytosis or phagocytosis were observed. In myoblasts, an electron dense fine granular material was freely distributed in the cytosol as well as in close proximity to lipid droplets (Figure 3 f). Cell nuclei and cytoplasmic organelles of both myoblasts and myotubes never showed morphological alterations.

MSN

Confocal fluorescence microscopy revealed that, in both myoblasts and myotubes, after 2 h incubation, MSN mostly occurred as aggregates at the cell surface and only a few small clusters were observed inside the cytoplasm. After 24 h incubation, many internalized MSN clusters were distributed in the cytoplasm, preferentially located around, but never inside, the cell nuclei (Figure 4 a-c).

The green fluorescence of MSN was found to co-locate with red-fluorescing intracellular membranes, thus suggesting that MSN are internalized via endocytosis (Figure 4 d).

At TEM, the MSN were roundish and highly electron dense. According to the observations in fluorescence microscopy, aggregates of MSN were found adhering to the cell surface and to be internalized by endocytosis (Figure 4 e). In the cytoplasm, MSN were always found inside ubiquitously distributed vacuoles of various sizes, but were never observed
inside the cell nuclei (Figure 4 e, f). After 24 h and 48 h incubation, MSN accumulated inside large vacuoles (probably secondary lysosomes) and sometimes appeared as loosened (Figure 4 g, h). MSN always remained confined inside vacuoles and did not contact any cell organelle. No sign of subcellular alteration or organelle damage was observed at any incubation time in both myoblasts and myotubes.

**PLGA NPs**

Observations at fluorescence and electron microscopy were similar for the two formulations of PLGA NPs. Confocal fluorescence microscopy showed that, in both myoblasts and myotubes, after 2 h incubation, a few PLGA NPs were present in the cytoplasm; after 24 h incubation, PLGA NPs accumulated in large amount in the cytoplasm, often forming aggregates preferentially located in the perinuclear area, but never entering the cell nucleus (Figure 5 a-c).

Overlapping of red fluorescing PLGA NPs and green fluorescing membrane marker suggested the occurrence of endocytotic processes (Figure 5 d).

At TEM, PLGA NPs showed a regular roundish shape and moderate electron density. After 2 h incubation, single NPs were seldom observed inside endosomes at the periphery of the cell (Figure 5 e) and some NPs were found to escape from endosomes (Figure 5 f), but most of PLGA NPs were found free in the cytosol (Figure 5 g). After 24 h and 48 h incubation, numerous residual bodies containing roundish moderately electron dense structures (likely remnants of PLGA NPs) accumulated in the cytoplasm (Figure 5 h, i); these particular residual bodies were never found in control cells or in samples treated with the other nanocarriers. PLGA NPs were never found inside the cell nucleus, nor making contact with cytoplasmic organelles. No cell alteration or damage was observed in myoblasts and myotubes at any incubation time.
Confocal fluorescence microscopy showed that only low amounts of NHs were internalized in myoblasts after 2 h incubation, while after 24 h NHs were present in large quantity in the cytoplasm, especially in the perinuclear region (Figure 6 a,b). They were never found inside the nucleus. In myotubes, NHs were observed in the cytoplasm only after 24 h incubation, but their amount was always very low (Figure 6 c).

The red fluorescence of NHs was found to co-locate with the green fluorescing membrane marker (Figure 6 d), thus suggesting that internalization occurs via endocytosis.

At TEM, NHs appeared as roundish homogeneously electron-dense structures. They were found to adhere to the cell surface inside invaginations of the plasma membrane (Figure 6 e) and, in the cytoplasm, a few of them were observed inside endosomes (Figure 6 f). However, most of NHs occurred free in the cytosol, and were often partially surrounded by double membranes, as it typically occurs during autophagic processes (Figure 6 g). Some NH remnants were still recognizable inside secondary lysosomes (Figure 6 h). NHs were never found to make contact with cell organelles or to occur inside the nucleus; moreover, no sign of cell structural alteration was observed in both myoblasts and myotubes.

Discussion

This study aimed at investigating the possible influence of cytokinetic features on the cellular response to different nanocarriers (liposomes, MSN, PLGA NPs and NHs) previously demonstrated to be safe for various established cancer cell lines (e.g. Slowing et al., 2006; Arpicco et al., 2013; D'Arrigo et al., 2014; Costanzo et al., 2016; Jonderian and Maalouf, 2016; Quagliariello et al., 2017).

Under our experimental conditions, myoblast viability was unaffected by the exposure to all the tested nanocarriers. Consistently, no nanocarrier induced quantitative reduction of myoblast population, apart from the highest concentrations tested at the longest incubation
times, when the number of cells was significantly lower than in controls likely due to intracellular accumulation of nanocarriers perturbing cell proliferation. Indeed, MSN administration led to a significant increase in cell population, probably related to the silica-NP-induced activation of MAPK signaling and the down-regulation of p53, which in turn inhibit apoptosis and induce cell proliferation (Christen et al., 2014). The S-phase cell fraction was also found to be unaffected after liposomes, MSN and 75:25 PLGA NP, and even increased after 50:50 PLGA NP administration, thus definitely demonstrating that liposomes, MSN and both PLGA NP formulations do not negatively affects cell cycle progression and proliferation of C2C12 myoblasts. On the other hand, NHs administration induced a decrease of S phase cell fraction after 24 h, followed by a recovery after 48 h, thus suggesting an only transitory slowing down of myoblasts proliferation, without negative effects on cell population at longer incubation times. This phenomenon could be due to cell overloading after 24 h incubation, as suggested by the evidence at fluorescence microscopy.

All together, our results confirm and provide additional evidence that all the tested nanocarriers are highly biocompatible to C2C12 myoblasts. However, biocompatibility is here attained at lower nanocarrier concentrations than those reported as safe for other cell types, such as breast, ovarian, pancreatic, and prostate cancer cell lines, (e.g. Slowing et al., 2006; Arpicco et al., 2013; D'Arrigo et al., 2014; Costanzo et al., 2016; Jonderian and Maalouf, 2016; Quagliariello et al., 2017). It is actually known that different cell types may differently react to nanocarrier administration; in particular, a comparative in vitro study demonstrated that myoblasts are much more sensitive than cells of fibroblastic, hepatic or endodermic origin (Nie et al., 2012). In addition, although our results demonstrate that C2C12 myoblasts are able to internalize all the nanocarriers tested, this uptake occurs more slowly than in other cell types (e.g. Pan et al., 2012; Arpicco et al., 2013; D'Arrigo et al., 2014; Costanzo et al., 2016; Ricci et al., 2018, Freichels et al., 2011), as demonstrated
by the very low amounts of nanocarriers observed in the intracellular milieu after 2 h incubation. We may speculate that these differences in cellular uptake may depend on different metabolic rates or peculiar cell features, such as dissimilar membrane composition or endocytotic capability.

Anyway, the uptake mechanisms and intracellular fate observed in C2C12 myoblasts correspond to those reported in the literature for other cell types.

Liposomes enter the cells by mechanisms different from typical endocytosis, as demonstrated by both fluorescence microscopy (fluorescing liposomes never overlap endosome staining) and TEM (liposomes never occur inside endosomes). Liposomes probably enter the cell by fusing with the plasma membrane (Verma and Stellacci, 2010; Nazarenus et al., 2014); in particular, their internalization may take place by the process of lipid raft-mediated endocytosis (Lanza et al., 2011). Once inside the cytoplasm, as previously observed in HeLa cells (Costanzo et al., 2016), liposomes undergo rapid degradation and migrate in the cytosol towards lipid droplets, probably for chemical affinity: this prevents the intracellular accumulation of liposomes and explains their preferential occurrence at the periphery of the cell. However, in HeLa cells lipid droplets became so numerous to be extruded from the cell (Costanzo et al., 2016) whereas, in C2C12 myoblasts, no accumulation and/or extrusion of lipid droplet was observed, probably due to the lower concentrations of liposomes administered.

Consistent with previous observations (Poussard et al., 2015; Costanzo et al., 2016; Ricci et al., 2018), fluorescence microscopy and TEM confirmed that MSN enter the myoblasts by endocytosis and follow the endolytic pathway, always remaining confined inside membrane-bounded vacuoles and never entering the cell nucleus. This is probably the reason for the absence of cell injury even after long term exposure (7 days), as reported by Poussard et al. (2015) in this cell line; moreover, the same authors demonstrated that
MSN uptake in C2C12 myoblasts enhances their differentiation into myotubes, opening interesting perspectives for the use of this nanocarrier for muscle tissue therapy.

At both formulations, PLGA NPs enter the cell as single units by endocytosis; however, they rapidly escape from the endosomes, as already observed for other polymeric NPs (Varkouhi et al., 2011; Malatesta et al., 2012), and occur free in the cytosol without making contact with any organelle. Afterwards, PLGA NPs re-enter the lytic pathway by autophagic process (Malatesta et al., 2015; Zhang et al., 2017; Panyam and Labhasetwar, 2003), thus undergoing enzymatic degradation and giving rise to the numerous residual bodies observed after 24 h incubation. Their presence as free NPs in the cytosol is therefore transient and the observations at TEM suggest that most of the fluorescing spots detectable at confocal microscopy are likely remnants of PLGA NPs inside residual bodies. Similarly to other polymeric NPs (Kim et al., 2007; Brambilla et al., 2010; Costanzo et al., 2016), PLGA NPs do not enter the cell nucleus.

NHs also enter the cells via endocytosis, as already reported for cancer cells (Ossipov, 2010; Pan et al., 2012; D’Arrigo et al., 2014; Palvai and Kuman, 2017; Quagliarello e al., 2017), but our data provide the first ultrastructural evidence of their intracellular fate. NH uptake in C2C12 appears to be less efficient than in cancer cells. It has been reported that NHs show a CD44 dependent endocytosis (Quagliarello e al., 2017) whose efficiency could be related to expression levels of this transmembrane protein: consistently, C2C12 cells do express CD44 (Kaneko et al., 2015), but cancer cells are often characterized by a very high expression of this receptor (recent reviews in Morath et al., 2016; Senbanjo and Chellaiah, 2017). Once in the cytoplasm, NHs seem to rapidly escape from endosomes in C2C12 cells; in fact, findings of NHs occurring free in the cytosol are very frequent whereas endosomes containing NHs are quite scarce. However, free NHs re-enter the lytic pathway by the autophagic process, which finally led to the enzymatic degradation of these nanocarriers. Evidence of intracellular degradation by lysosomal enzymes has been
also reported in HaCaT keratinocytes, where NHs were found to co-locate with acidic organelles up to 24 h from incubation (Montanari et al., submitted manuscript).

C2C12 myotubes, treated with nanocarrier concentrations found to be safe for myoblasts, did not show any sign of cell stress as clearly demonstrated by TEM, thus extending the biocompatibility of all tested nanocarriers to the non-cycling, differentiated muscle cells. However, it is evident that the amount of nanocarriers internalized by myotubes is lower than in myoblasts. Similarly, other NPs easily entering myoblasts were found to be unable to penetrate myotubes (Salova et al., 2011). One of the reasons for such a difference may reside in the higher metabolic rate of a cycling cell compared to its differentiated non-cycling counterpart (Chang et al., 2007); however, it is worth noting that the differentiation process of a C2C12 myoblast into a myotube entails a differential expression of numerous proteins among which those related to cell adhesion, transmembrane transport, and cytoskeleton composition and dynamics (Kislinger et al., 2005; Casadei et al., 2009; Forterre et al., 2014). In addition, the lipid and fatty acid composition of cell membranes significantly changes during the myogenic process (Briolay et al., 2013) when the plasma membrane composition undergoes marked modifications that could more or less markedly affect nanocarrier uptake depending on the internalization mechanisms involved.

Once internalized in the myotubes, all nanovectors undergo a fate similar to that observed in myoblasts, without perturbing cell organelles.

In conclusion, our results demonstrate that all the tested nanocarriers are suitably biocompatible for both cycling myoblasts and non-cycling differentiated myotubes, although the differentiation stage markedly affects the uptake efficiency (and this should be taken into consideration when designing nanoconstructs for therapeutic or diagnostic purposes). At the concentrations used in our experiments, all the tested NPs enter the intracellular environment and undergo degradation through the physiological pathways without inducing microscopically detectable cytological alterations. The high
biocompatibility of these nanoconstructs is also supported by their inability to enter the cell nucleus, thus avoiding the unpredictable long-term risks of possible interactions between nanomaterials and nucleic acids and/or nuclear protein factors. All these features make these nanocarriers potential candidates for delivering therapeutic agents in vivo for treating also diseased differentiated cells which are to be preserved, such as muscle cells in dystrophic patients.

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<table>
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<th></th>
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\(^a\) determined by transmission electron microscopy analysis.
Legends

Figure 1. Effect of nanocarrier administration on cell population. Mean values±SE of cell number after 2h, 24h and 48h incubation with the different nanocarriers. Values identified with asterisks are significantly different from the control (untreated) cells at the same incubation time.

Figure 2. Effect of nanocarrier administration on cell proliferation. Mean values±SE of BrdU-positive cell percentage after 24h and 48h incubation with the different nanocarriers. Values identified with asterisks are significantly different from the control (untreated) cells at the same incubation time.

Figure 3. Microscopical analysis of cell-liposome interactions. Confocal optical sections of myoblasts (a,b) and myotubes (c) after 2h (a) and 24h (b, c) of liposome incubation. DNA is stained with Hoechst 33342 (blue fluorescence), cytoplasm is counterstained with trypan blue (red fluorescence). d) A myoblast incubated with the PKH26 red-fluorescing dye to visualise endocytotic vesicles, and then incubated for 30 min with green fluorescent liposomes: the two signals never co-locate (the inset shows a 2x magnification of the detail indicated by the small arrows). DNA was stained with Hoechst 33342 (blue fluorescence). Bars: 20 µm (a-c), 10 µm (d). Transmission electron microscopy analysis of liposomes intracellular distribution in myoblasts (e, f) and myotubes (g) after 24 h incubation. e) A liposome (arrow) occurs free in the cytoplasm at the cell periphery. f) Electron-dense fine granular material (arrowheads) is located in close proximity of lipid droplets (L). g) A liposome (arrow) occurs in perinuclear position. Note the good structural preservation of cell organelles in both myoblasts and myotubes:
nucleus (N), Golgi complex (G), mitochondria (M), endoplasmic reticulum (ER). Bars: 500 nm.

**Figure 4. Microscopical analysis of cell-MSN interactions.** Confocal optical sections of myoblasts (a, b) and myotubes (c) 2h (a) and 24h (b, c) after incubation with MSN. DNA was stained with Hoechst 33342 (blue fluorescence) and the cytoplasm counterstained for actin with phalloidin (red fluorescence). d) A myoblast incubated with the PKH26 red-fluorescing dye to visualise endocytotic vesicles, and then incubated for 30 min with green fluorescent MSN: the two signals co-locate giving rise to yellow fluorescence (the inset shows a 2x magnification of the detail indicated by the small arrows). DNA was stained with Hoechst 33342 (blue fluorescence). Bars: 20 µm (a-c), 10 µm (d). Transmission electron microscopy analysis of MSN intracellular distribution after 2 h (e, f) and 24 h (g) incubation in myoblasts, and after 24 h incubation in myotubes (h). e) Clusters of MSN occur at the cell surface; some of them are enclosed in a membrane invagination (arrow). Internalised MSN occur inside a vacuole (arrowhead). f) Vacuoles containing MSN (arrowheads) occur very close to the nucleus (N). g) After 24 h incubation, in myoblasts MSN accumulate in various vacuolar structures, while the cytoplasm contains many residual bodies (R). h) In myotubes, after 24 h incubation, MSN occur in large vacuoles without perturbing the typical structural organization of cytoplasmic organelles: bundles of myofibrils (asterisks), mitochondria (M), endoplasmic reticulum (ER). Bars: 500 nm.

**Figure 5. Microscopical analysis of cell-PLGA NPs interactions.** Confocal optical sections of myoblasts (a, b) and myotubes (c) after 2h (a) and 24h (b, c) of PLGA NPs incubation. DNA was stained with Hoechst 33342 (blue fluorescence) and the cytoplasm counterstained for actin with phalloidin (green fluorescence). d) A myoblast incubated with the PKH67 green-fluorescing dye to visualise endocytotic vesicles, and then incubated for
30 min with red fluorescent PLGA NPs: the two signals co-locate giving rise to yellow fluorescence (the inset shows a 2x magnification of the detail indicated by the small arrows). DNA was stained with Hoechst 33342 (blue fluorescence). Bars: 20 µm (a-c), 10 µm (d). Transmission electron microscopy analysis of PLGA NPs intracellular distribution after 2 h (e, f, g) and 24 h (h) incubation in myoblasts, and after 24 h incubation in myotubes (i). e) A PLGA NP enclosed in an endosome (arrow) occurs just beneath the cell surface. f) A PLGA NP (arrow) is escaping from an endosome. g) Two PLGA NPs (arrows) occur free in the cytosol. h) After 24 h incubation, the cytoplasm contains large amounts of peculiar vacuolated residual bodies where it is sometimes possible to recognize NP remnants (arrowhead). i) In myotubes, after 24 h incubation, the same residual bodies (arrowheads) are frequently present. Nucleus (N), Golgi complex (G), mitochondria (M), endoplasmic reticulum (ER), bundles of myofibrils (asterisk). Bars: 500 nm.

Figure 6. Microscopical analysis of cell-NHs interactions. Confocal optical sections of myoblasts (a, b) and myotubes (c) after 2h (a) and 24h (b, c) of NHs incubation. DNA was stained with Hoechst 33342 (blue fluorescence) and the cytoplasm counterstained for actin with phalloidin (green fluorescence). d) A myoblast incubated with the PKH67 green-fluorescing dye to visualise endocytotic vesicles, and then incubated for 30 min with red fluorescent NHs: the two signals co-locate giving rise to yellow fluorescence (the inset shows a 2x magnification of the detail indicated by the small arrow). Due to the slow NHs uptake, co-locations are quite scarce after this short time. DNA was stained with Hoechst 33342 (blue fluorescence). Bars: 20 µm (a-c), 10 µm (d). Transmission electron microscopy analysis of NHs intracellular distribution after 2 h (e, f) and 24 h (g, h) incubation in myoblasts. e) An NH (arrow) occurs at the cell surface. f) An NH is enclosed in an endosome (arrow). g) Two NH (arrows) occur free in the cytosol and are partially...
enclosed by cisternae of the endoplasmic reticulum (small arrows). h) A residual bodies with an NH remnant (arrowhead). Mitochondria (M). Bars: 500 nm.