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UPTAKE AND INTRACELLULAR DISTRIBUTION OF DIFFERENT TYPES OF NANOPARTICLES IN PRIMARY HUMAN MYOBLASTS AND MYOTUBES

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ABSTRACT

The use of nanoparticles as drug carriers in the field of skeletal muscle diseases has been poorly addressed and the interaction of nanoparticles with skeletal muscle cells has been investigated almost exclusively on C2C12 murine myoblasts. In this study we investigated the effects poly(lactide-*co*-glycolide) nanoparticles, mesoporous silica nanoparticles and liposomes, on the viability of primary human myoblasts and analyzed their cellular uptake and intracellular distribution in both primary human myoblasts and myotubes. Our data demonstrate that poly(lactide-*co*-glycolide) nanoparticles do not negatively affect myoblasts viability, contrarily to mesoporous silica nanoparticles and liposomes that induce a decrease in cell viability at the highest doses and longest incubation time. Poly(lactide-*co*-glycolide) nanoparticles and mesoporous silica nanoparticles are internalized by endocytosis, poly(lactide-*co*-glycolide) nanoparticles undergo endosomal escape whereas mesoporous silica nanoparticles always occur within vacuoles. Liposomes were rarely

observed within the cells. The uptake of all tested nanoparticles was less prominent in primary human myotubes as compared to myoblasts.
Our findings represent the first step toward the characterization of the interaction between nanoparticles and primary human muscle cells and suggest that poly(lactide-*co*-glycolide) nanoparticles might find an application for drug delivery to skeletal muscle.

Key words: primary human myoblast; primary human myotube; nanoparticle; fluorescence microscopy; electron microscopy.

Abbreviations: nanoparticles (NPs); poly(lactide-co-glycolide) (PLGA); mesoporous silica nanoparticles (MSNs); tetraethyl orthosilicate (TEOS); cetyltrimethylammonium bromide (CTAB); Structure Directing Agent (SDA); fluorescein isothiocyanate (FITC); 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC); 1,2-distearoyl-sn-glycero-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG); polydispersity index (PI); quasi-elastic light scattering (QELS); transmission electron microscopy (TEM).

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Declaration of interest: none.

1. INTRODUCTION

The continuous expansion of nanotechnology has led to a great interest in the potential applications of nanomaterials in the biomedical field. Nanomaterials are, by definition, materials with size ranging between 1 and 100 nm, at least in one dimension (Su et al. 2017). The interest in nanotechnology is due to the unique properties that the matter acquires at the nano-scale size that allow to overcome some limitations displayed by the corresponding material in its larger state (Su et al. 2017). Several nanodevices have been designed for different biomedical purposes including drug delivery, gene therapy, disease diagnosis and for the design of advanced protheses and implants (Su et al. 2017; Kunzmann et al. 2011). Nanomedicine is intensively exploring the use of nanoparticles (NPs) for drug delivery because it provides many advantages compared to the administration of the free drug including increased bioavailability, reduced toxicity and side effects as well as prolonged half-life and efficacy of the drug (Su et al. 2017). Moreover, NPs can be modified to target specific cells or tissues allowing to control the delivery of therapeutics at the site of action while reducing the dosage and unwanted side effects due to the interaction of the pharmacological agent with off-targets (Su et al. 2017).

When NPs are to be used for medical purposes it is mandatory to characterize their physicochemical properties, to exclude their intrinsic toxicity, and to elucidate their interactions with the biological systems (e.g., their ability to be internalized, persist and be degraded by the cell) (Kunzmann et al. 2011). Evaluating the effects of NPs on *in vitro* cell systems represents the first step to assess the biosafety and biocompatibility of nanoconstructs (Schrand et al. 2012).

Poly(lactide-*co*-glycolide) (PLGA) NPs, mesoporous silica nanoparticles (MSNs) and liposomes have been widely investigated in many *in vitro* and *in vivo* experimental systems. These studies demonstrated that PLGA NPs, MSNs and liposomes are biodegradable and biocompatible, creating the basis for their current use in the biomedical, pre-clinical or clinical setting (Wang et al. 2015; Danhier et al. 2012; Sercombe et al. 2015).

PLGA NPs are particularly attractive for biomedical uses because PLGA polymer is approved for medical application by both FDA and EMA, is biodegradable and is already widely employed in the biomedical field for sutures, drug delivery and tissue engineering (Ulery et al. 2011; Danhier et al. 2012).

MSNs have been drawing the attention in many fields including the medical one due to their unique honeycomb-like porous structure that allows to carry relatively high amount of molecules and to their unique properties such as tunable size, shape and pore dimension, large surface area and high reactive surface which makes their functionalization easy (Slowing et al. 2008; Asefa and Tao 2012).

Liposomes are vesicles constituted of a bilayer of phospholipids and an internal aqueous cavity (Sercombe et al. 2015). They offer many advantages including high-loading capacity, biocompatibility, low toxicity and a great versatility allowing the incorporation of both hydrophobic and hydrophilic drugs, that are entrapped

in the membrane bilayer or in the aqueous cavity, respectively (Sercombe et al. 2015; Abu Lila and Ishida 2017). Liposomes have a long history of successful applications in the medical field, specifically in drug delivery, that testify their ability to prevent early degradation of the encapsulated compound, to reduce toxicity and improve biodistribution and drug delivery (Bulbake et al. 2017; Sercombe et al. 2015). The ultimate goal of our research is to develop a NP-based drug delivery system to deliver pentamidine to skeletal muscle for the treatment of myotonic dystrophy type 1 (DM1), the most common muscular dystrophy in adults (LoRusso et al. 2018). The main clinical symptoms of DM1 include myotonia, muscle weakness and cardiomyopathy (LoRusso et al. 2018). Currently, there is no treatment for the disease that leads to severe disability. Pentamidine is a Food and Drug Administration (FDA)-approved compound already used in the clinical practice to treat various parasitic infections (Lopez-Morato et al. 2018). Although *in vitro* studies performed on DM1 patients' cells and on cellular models of the disease demonstrated that pentamidine mitigates the pathological hallmarks of DM1, studies performed on the murine model (HSA^{LR} mice) highlighted its toxicity therefore limiting its therapeutic application (Warf et al. 2009; Lopez-Morato et al. 2018). NP-based drug delivery systems may represent a suitable approach to maintain therapeutic dose of the drug at target tissue, while reducing toxicity. In this context, understanding the interaction between NPs and skeletal muscle cells is a mandatory initial step in the development of a NP-based drug delivery system. Therefore, in the present study, we investigated the effects on cell viability, cellular uptake and intracellular distribution of PLGA NPs, MSNs and liposomes in primary human myoblasts and myotubes, which have been used as an *in vitro* model of skeletal muscle tissue.

2. MATERIALS AND METHODS

2.1 Preparation and characterization of nanoparticles

For the preparation of PLGA (50:50 - Resomer® RG 502 H - or 75:25 - Resomer® RG 752 H, 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. MSNs were prepared by using tetraethyl orthosilicate (TEOS) as silica source and cetyltrimethylammonium bromide (CTAB) as Structure Directing Agent (SDA), in a basic aqueous solution (NaOH, Sigma-Aldrich) at 80 °C, as described elsewhere (Sapino et al. 2015; Musso et al. 2015). SDA was

removed from the inner porosity by calcination at 550°C in nitrogen and oxygen, followed by surface functionalization using 3-aminopropyltriethoxysilane (APTS, Sigma-Aldrich) as grafting agent (Sapino et al. 2015). The resulting material, after drying, was suspended in MilliQ® water (1 mg MSNs in 150 µl) before adding 250 µl of fluorescein isothiocyanate (FITC) ethanol solution (0.3 mg/ml), adapting a previously proposed procedure (Yu et al. 2013). After 5 h under stirring in the dark, FITC labeled MSNs were centrifuged and washed with ethanol three times to obtain colorless supernatants. Liposomes were prepared by the thin lipid film hydration and extrusion method. Chloroform solution of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar-Lipids), cholesterol (Chol, Avanti Polar-Lipids) and 1,2-distearoyl-sn-glycero-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG, ammonium salt, Avanti Polar-Lipids) in a molar ratio 65:30:5 were mixed and evaporated under reduce pressure to obtain a thin lipid film. The resulting lipid film was hydrated with a 20 mM HEPES buffer (pH 7.4) and vortexed for 10 min to obtain a suspension of multilamellar liposomes. The resulting suspension was then extruded 10 times under nitrogen through 200 nm polycarbonate filter at 60°C. Fluorescently labeled liposomes were prepared as described above by adding 10 mM solution of fluorescein-5-(and-6)-sulfonic acid trisodium salts (Invitrogen) in HEPES buffer during the hydration of the lipid film. The unentrapped fluorescein was removed by gel filtration using Sepharose CL-4B column eluting with HEPES buffer.
The mean particle size and the polydispersity index (PI) of liposomes and PLGA NPs 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.

2.2 Primary human myoblasts and myotubes cultures

Primary human myoblasts were established from a portion of diagnostic *vastus lateralis* of two different subjects who underwent muscle biopsy for diagnostic purpose and that, after all tests had been performed, were deemed to be free of muscle diseases (the samples were conventionally named ctr1 and ctr2). Myoblasts were isolated and grown in a cell culture incubator with saturating humidity in a mixture of 5% CO_2 and air at 37°C, as previously described (Askanas and Engel 1975). For the differentiation into myotubes, myoblasts were grown at confluence and then shifted to a medium without growth factors for 8 days (Guglielmi et al. 2017). The study was approved by the local ethical board.

2.3 Cell viability assay

The effect of two different formulations of PLGA NPs (PLGA 50:50 and PLGA75:25), MSNs and liposomes on the viability of primary human myoblasts and myotubes was evaluated by the MTT assay (Mosmann 1983). Cells were seeded in flat-bottom 96 multiwell plates at the density of $3x10^3$ cells/well. Four wells for each condition were seeded. After 24 h, the medium was removed and replaced with medium containing the NPs. PLGA NPs and liposomes were administered at the final concentrations of 0.1, 0.2 or 0.4 mg/ml

whereas MSNs were supplied at the final doses of 0.01, 0.05 or 0.1 mg/ml. MTT assay was performed after 2 h, 24 h and 72 h treatment, as previously reported (Denizot and Lang 1986). Briefly, after incubation with NPs, the medium was replaced with 100 µl of 0.5 mg/ml MTT in culture medium and incubated for 4 h at 37°C in a cell culture incubator. Then, MTT solution was removed, formazan crystals were dissolved in 100 ul of DMSO and the absorbance was measured at 570 nm.

2.3 Statistical analysis

Statistically significant differences ($p<0.05$) in cell viability were determined by one-way ANOVA followed by Bonferroni's post-hoc test. Cell viability was expressed as percentage of control (sham-treated) cells (mean ± SE).

2.4 Uptake and intracellular distribution of nanoparticles

Uptake and intracellular distribution of NPs were investigated by confocal fluorescence microscopy and transmission electron microscopy (TEM) in both primary human myoblasts and myotubes.

Cells were seeded on glass coverslips in 24 multiwell plates. Twenty-thousand cells were seeded in each well. Twenty-four hours after seeding, the medium was replaced with fresh medium containing the NPs. Myotubes were treated after 8 days of differentiation. PLGA NPs and liposomes were administered at the final concentration of 0.2 mg/ml whereas MSNs were used at the final dose of 0.01 mg/ml. After 2 h incubation with NPs, cells were either fixed or moved to fresh medium without NPs and further grown for 24 h or 72 h.

For fluorescence microscopy, cells were fixed with 1% paraformaldehyde and 0.01% glutaraldehyde in PBS, pH 7.4, for 30 min at 4°C. Myoblasts and myotubes treated with Nile red labeled-PLGA NPs were permeabilized with 0.1% Triton X100 in PBS at room temperature (RT) for 8 min and incubated with phalloidin conjugated with Alexa488 (1:20 in PBS) for 1 h at RT. Myoblasts exposed to either FITC-labeled liposomes or FITC-labeled MSNs were counterstained with 0.004% Trypan Blue in PBS for 1 min at RT. Myotubes incubated with FITC-labeled liposomes or FITC-labeled MSNs were counterstained with phalloidin conjugated with Atto 594 (1:20 in PBS) for 1 h at RT. Cells nuclei were stained with Hoechst 33258 (1 mg/ml in PBS for 5 min). After washes with PBS, the samples were mounted in 50% glycerol in PBS. Imaging by confocal laser scanning microscopy was performed with a Leica TCS SP5 AOBS system (Leica Microsystems Italia). For fluorescence excitation, a diode laser at 405 nm for Hoechst 33342, an Ar laser at 488 nm for FITC and PKH67 Green Fluorescent Cell Linker, and a He/Ne laser at 543 nm for Trypan blue, Nile Red, PHK26 Red Fluorescent Cell Linker and phalloidin conjugated with Atto 594 were employed. To access whether NPs enter the cells via endocytosis, myoblasts were incubated with either the PKH67 Green Fluorescent Cell Linker or the PHK26 Red Fluorescent Cell Linker at the final concentration of 2 uM for 5 min in the incubator, to stain the plasma membrane, and then treated with Nile red-labeled or FITC-labeled NPs, respectively. This allowed to track the endocytic vesicles originated for the plasma membrane,

and to detect their possible co-localization with the fluorescent NPs internalized by the cell. Then myoblasts were fixed, stained for DNA with Hoechst 33258, and analyzed by confocal fluorescence microscopy as described above.

For TEM, cells were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 1 h, post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide at RT for 1 h, dehydrated with acetone and embedded in Epon. Ultrathin sections were observed with a Philips Morgagni transmission electron microscope (FEI Company Italia Srl, Milan, Italy) operating at 80 kV and equipped with a Megaview II camera for digital image acquisition.

3. RESULTS

3.1 Characterization of NPs

NPs mean particle size and zeta potential values are reported in Table 1. In particular, all the systems showed a mean diameter under 200 nm. The zeta potential was negative for PLGA NPs and liposomes while for MSNs a positive value was observed in relation to the functionalization with aminopropyl groups (Musso et al. 2015) necessary for preparation of FITC labeled MSNs through a covalent link. Fluorescent labeling did not appreciably affect both particle size and zeta potential.

3.2 Cell viability

A statistically significant increase in cell viability was observed after 24 h of treatment with 0.4 mg/ml PLGA 50:50 NPs in ctr1 myoblasts and after 24 h incubation with 0.4 mg/ml PLGA 75:25 NPs in both ctr1 and ctr2 myoblasts (Fig. 1).

A statistically significant decrease in cell viability was reported after 72 h treatment with MSNs at the final concentration of 0.1 mg/ml in both ctr1 and ctr2 myoblasts (Fig. 1).

A statistically significant decrease in cell viability was observed after 24 h and 72 h incubation with 0.4 mg/ml liposomes in ctr2 myoblasts and after 72 h treatment with 0.2 mg/ml and 0.4 mg/ml liposomes in ctr1 myoblasts (Fig. 1).

A statistically significant increase in cell viability was reported after 2 h treatment with PLGA 50:50 NPs at the final concentration of 0.4 mg/ml in ctr1 myotubes (Fig. 2).

Statistically significant differences were not observed for any of the other tested conditions.

3.3 Uptake and intracellular distribution of nanoparticles

NPs uptake and intracellular distribution were investigated in primary human myoblasts and myotubes treated with fluorescent or unlabeled NPs using confocal fluorescence microscopy and TEM, respectively.

3.3.1 PLGA NPs

By confocal fluorescence microscopy and TEM, the two formulations of PLGA NPs, i.e. PLGA 50:50 and PLGA 75:25, did not display any difference in term of cellular uptake, intracellular localization and ultrastructural

features in both primary human myoblasts and myotubes. By confocal fluorescence microscopy PLGA NPs were found in the cytoplasm and close to cell nuclei in cells treated for 2 h, 24 h and 72 h (Fig. 3a-c, g-i). A partial co-localization between PLGA NPs and fluorescent-labeled membrane derived vesicles was observed in proximity of cell surface and in the cytoplasm of a few treated myoblasts (Fig. 3d-f). The uptake of PLGA NPs was less prominent in primary human myotubes as compared to myoblasts (Fig. 3a-c, g-i). At TEM, PLGA NPs showed a regular round shape and a moderate electron density (Fig. 3j-m). In myoblasts, after 2 h incubation, single NPs were rarely found adhering to the plasma membrane or inside endosomes, whereas they mostly occurred free in the cytosol (Fig. 3j-l). Accordingly, some PLGA NPs were found escaping from endosomes (Fig. 3k). Some PLGA NPs occurring free in the cytosol were partially surrounded by a double membrane as typical of autophagy (Fig. 3l). After 24 h incubation, a high number of small vacuoles containing roundish electron dense structures accumulated in the cytoplasm (Fig. 3m); these particular residual bodies were never found in untreated cells or in samples treated with MSNs or liposomes. In myotubes, PLGA NPs were less frequent than in myoblasts, but their intracellular distribution was similar (not shown). No contact between PLGA NPs and cytoplasmic organelles was observed; moreover, NPs never occurred inside the nucleus. Neither myoblasts nor myotubes showed alteration or damage of their structural components at any incubation time.

3.3.2 MSNs

Confocal fluorescence microscopy revealed MSNs aggregates in the cytoplasm of a few myoblasts and myotubes that were treated for 24 h and 72 h (Fig. 4a-g). MSNs co-localizing with fluorescent-labeled membrane derived vesicles were occasionally observed (Fig. 4e).

At TEM, MSNs appeared roundish and showed a finely granular highly electron dense content (Fig. 4h-i). In myoblasts, clusters of MSNs were found to adhere to the cell surface and to be internalized by endocytosis (Fig. 4h). In the cytoplasm, MSNs always occurred inside heterogeneous vacuoles ubiquitously distributed in the cytoplasm at all analyzed incubation times (Fig. 4h). In myotubes, MSNs were less frequent than in myoblasts and were usually enclosed in vacuoles (Fig. 4i). MSNs neither get in contact with organelles nor enter the cell nucleus. No signs of subcellular alteration or damage were observed in both myoblasts and myotubes at short incubation time (2 h, Fig. 4h, i), whereas starting form 24 h incubation the cells contained large amounts of vesicular and membranous structures while organelles were morphologically unrecognizable (not shown).

3.3.3 Liposomes

By confocal fluorescence microscopy, liposomes were rarely observed inside primary human myoblasts and myotubes (Fig. 5a-d). A co-localization between liposomes and fluorescent-labeled membrane derived vesicles was never observed (Fig. 5a, b). A few myotubes displayed liposomes in their cytoplasm after 24 h and 72 h treatment (Fig. 5c, d).

At TEM, liposomes showed a roundish shape with a fine irregular profile and strong electron density (likely due to the lipid staining by osmium tetroxide, Fig. 5e, f). A few liposomes were found inside myoblasts and they were even scarcer in myotubes. In both cell types, liposomes were found adhering to the cell surface and in the peripheral region of the cytoplasm but never occurred inside the cell nuclei (Fig. 5e, f). No internalization processes such as endocytosis or phagocytosis, and no contact with any cell organelles were observed. No evident structural alterations were observed in myoblasts at short and long incubation time and in myotubes after 2 h incubation, whereas in myotubes incubated with liposomes for longer times the organelles were morphologically unrecognizable (not shown).

4. DISCUSSION

In the last decades, many efforts have been directed to develop NPs-based drug formulations for treating cancer and other pathologies but, so far, the use of NPs has been poorly investigated in the field of skeletal muscle diseases (Su et al. 2017). Only recently NPs have been explored as a possible drug delivery system for antisense oligonucleotides (AONs) in the exon-skipping treatment of Duchenne muscular dystrophy (DMD) (Falzarano et al. 2014): these studies have been performed on the murine model of DMD (*mdx* mice) and aimed at improving the delivery of AONs and reducing the effective therapeutic dose (Falzarano et al. 2014). However, the interaction between NPs and skeletal muscle cells has been poorly investigated and the current knowledge is limited to *in vitro* studies performed almost exclusively on C2C12 immortalized murine myoblasts and only a few papers described the impact of NPs on human primary myoblasts and myotubes (Nie et al. 2012; Leite et al. 2015; Lojk et al. 2015; Poussard et al. 2015; Ramachandran et al. 2017).

It has been demonstrated that the extent of the NPs cellular uptake depends on the intrinsic chemical and physical properties of the NPs as well as on the target cell type (Akinc and Battaglia 2013; Sahay et al. 2010; Frohlich et al. 2012). NPs size and shape as well as the chemical properties of their surface dictate the ability of the NPs to interact with the cell membrane, the extent and pathway of internalization and, once inside the cells, their intracellular fate (Akinc and Battaglia 2013). On the other hand, cell type, proliferation rate and cell-membrane characteristics have a major influence on the effect that a certain type of NPs might have on a cell (Lojk et al. 2015; Kim et al. 2011; Tang et al. 2015; Wang et al. 2016; Kettler et al. 2014; Frohlich et al. 2012). Therefore, it is of extreme importance to study the impact of NPs on the appropriate *in vitro* cellular model especially when the final goal is to translate the findings to the medical field (Kunzmann et al. 2011).

In the present study, the effects of PLGA NPs, MSNs and liposomes have been investigated on primary human myoblasts and myotubes that have been used as an *in vitro* model of human skeletal muscle. PLGA NPs, MSNs and liposomes have been chosen because they are biodegradable and biocompatible, as

previously demonstrated in several studies performed on other cell types, and already have a biomedical, pre-clinical or clinical application (Wang et al. 2015; Danhier et al. 2012; Sercombe et al. 2015). Two formulations of PLGA NPs with a different composition of lactic acid and glycolic acid (50:50 *vs* 75:25) have been synthetized and applied to primary human muscle cells; indeed, PLGA composition affects some properties of the copolymer such as degradation time that increases with increasing lactide:glycolide ratio (Ulery et al. 2011). We reported that PLGA NPs do not negatively affect myoblasts viability at any of the tested doses and duration of treatment, contrarily to MSNs and liposomes, which both reduce cell viability when administered at the highest tested doses for long incubation time. A slight but statistically significant increase in cell viability was observed after 24 h incubation at the highest tested dose (i.e. 0.4 mg/ml) with both PLGA NPs formulations. This data could be due to an increased mitochondrial dehydrogenase activity, measured by MTT assay, due to high levels of glycolic acid and lactic acid resulting from PLGA degradation that enter the Krebs cycle bursting oxidative metabolism in the mitochondria (Danhier et al. 2012). Interestingly, the effect of PLGA 50:50 NPs and MSNs on cell viability was similar in myoblasts from different donors, namely increasing (24 h with 0.4 mg/ml) *vs* decreasing (72 h with 0.1 mg/ml) cell viability, respectively. Similarly, a reduction in cells viability after 72 h incubation with 0.4 mg/ml liposomes was reported in myoblasts derived from both donors. On the contrary, treatment with 0.4 mg/ml liposomes for 24 h negatively affected the viability of cells derived from only one of the two healthy subjects. Moreover, the increase in cell viability after 24 h of incubation with 0.4 mg/ml PLGA 75:25 was found only in myoblasts derived from ctr1 donor whereas PLGA 75:25 did not influence the viability of ctr2 myoblasts. Due to limited availability we could investigate the effect of NPs on viability of myotubes obtained from only one control subject (ctr1). A statistically significant increase in myotubes viability was detected after incubation with PLGA 50:50 NPs (2h, 0.4 mg/ml), whereas cell viability was unchanged after treatment with the other NPs.

These findings demonstrate that NPs might have a variable effect on the viability of cells derived from different subjects, and indicate that, despite the difficult access to human material and requirement of appropriate expertise, primary human cultures offer the important advantage to better reproduce the *in vivo* physiological conditions and variability between donors, highlighting the importance of using primary human cells to test the biocompatibility of NPs for biomedical applications.

Subsequently, the intracellular distribution of NPs was analyzed by confocal fluorescence microscopy and TEM. For this purpose, PLGA NPs, MSNs and liposomes were used at concentrations that did not show a negative effect on myoblasts viability. The two formulations of PLGA NPs did not display any remarkable difference in term of cell uptake and intracellular localization: PLGA NPs were observed in the cytoplasm inside membrane-derived vesicles (suggesting they enter the cell by endocytosis) and undergoing endosomal escape, in agreement with previous observations (Panyam et al. 2002; Danhier et al. 2012). Free

PLGA NPs were observed close to the nuclei in both myoblasts and myotubes but were never found within the nuclear compartment, making unlikely their interaction with genetic material. Autophagic processes led PLGA NPs inside the lysosome pathway, as demonstrated by the residual bodies likely made of PLGA NPs remnants starting from 24 h incubation. In agreement with data from the literature demonstrating their biocompatibility and biosafety, PLGA NPs did not affect the structural components of the cells neither in myoblasts nor in myotubes, and in PLGA NPs-treated cells all organelles appeared to be well preserved. Previous studies investigated the use of PLGA polymer in the production of scaffolds and matrices for myoblasts culture and differentiation as well as for the development of microcarriers for successful expansion of myoblasts *in vitro* and enhanced engraftment and survival of myoblasts after transplantation (Gu et al. 2013; Parmar and Day 2015; Shin et al. 2015). However, for the first-time, we focused on the interaction between PLGA NPs and primary human myoblasts and provided data supporting the biocompatibility of nanostructured PLGA with this cell type, opening the way to future studies on biomedical applications involving skeletal muscle tissue.

As regard MSNs, after 24 h from the treatment, both myoblasts and myotubes displayed numerous cytoplasmic vesicular and membranous structures, pointing to the over-activation of the lysosome pathway, and morphologically altered organelles even after exposure to NPs concentrations found to be safe by the viability assay. This highlights the key role of morphological studies in evaluating the biological impact of nanoconstructs, and suggests a poor biocompatibility of MSNs with primary human myoblasts and myotubes. MSNs clusters were observed on the cell surface as well as in large cytoplasmic vacuoles in both primary human myoblasts and myotubes, a distribution closely resembling the one reported in C2C12 myoblasts after treatment with silica-based NPs (Poussard et al. 2015). To our knowledge, the present study is the first investigating the interaction between MSNs and human myoblasts. Indeed, MSNs have been used to develop scaffolds for the delivery of γ-secretase inhibitors of Notch pathway to promote the differentiation of C2C12 myoblasts, but data on the effects of MSNs on cell viability and intracellular distribution have not been reported in primary human muscle cells (Bocking et al. 2014).

Even if treatment with liposomes did not affect the structural integrity of myoblasts, morphological alterations of organelles were detected in myotubes starting at 24 h from liposome exposure, suggesting that myotubes might be more sensitive to liposomes as compared to myoblasts. It is possible that the degradation of the internalized liposomes may cause an overload of lipid components that these highly differentiated non-cycling cells are unable to metabolize. Despite this difference, in both myoblasts and myotubes, liposomes were observed in the cytoplasm of only a few cells, pointing to their poor cellular uptake. The negative effect of PEGylation on liposome internalization by C2C12 reported in a previous study may provide another explanation of the poor uptake of our liposomes, which also harbor PEG on their surface, by primary human myoblasts and myotubes (Teo et al. 2013). Alternatively, liposomes might

have a short half-life within the cell and be subjected to rapid cytosolic degradation, as documented by the presence of liposomes with irregular profiles and undergoing dissolution, as previously observed in HeLa cells (Costanzo et al. 2016).

Even though, in the past, liposomes have been tested on C2C12 myoblasts and on primary human myoblasts and myotubes, most of these studies evaluated liposomes prepared with cationic lipids to deliver nucleic acid to these low transfectable cells (Helbling-Leclerc et al. 1999; Vitiello et al. 1998; Dodds et al. 1998; Neuhuber et al. 2002; Pampinella et al. 2002). Up to now, data on the interaction of liposomes and primary human myoblasts and myotubes were missing; indeed, only a few other studies assessed the interaction between liposomes and murine myoblasts (Jeong and Conboy 2011; van der Westen et al. 2012; Teo et al. 2013). Our liposomes contain DSPC, a derivative of phosphatidylcholine (PC). It has been reported that PC-liposomes decrease primary murine myoblasts fusion and myotube elongation; however the uptake and the intracellular distribution of PC-liposomes were not evaluated (Jeong and Conboy 2011). The internalization of liposomes has been demonstrated also in C2C12 by flow cytometry and an increased uptake/association with the cells was reported for polydopamine (PDA)-coated liposomes (van der Westen et al. 2012). We report a poor internalization of PEGylated liposomes by primary human myoblasts and myotubes and their negative effect on cell viability, suggesting that PEGylated liposomes might not be suitable for drug delivery to this cell type.

5. CONCLUSIONS

The present study provides novel data about the interaction between PLGA NPs, MSNs and liposomes and primary human muscle cells, and suggests that nanocarriers previously demonstrated to be safe in various cell lines can pose biocompatibility issue in this cell type, as supported by the cell viability assay and by the ultrastructural analysis of MSNs and liposome treated myoblasts/myotubes. This further highlights the importance to use the appropriate cell type for biocompatibility studies. Our findings suggest that, among the tested NPs, PLGA NPs are the most promising nanocarriers for skeletal muscle cells, as they do not affect cell viability and structural organization; future *in vivo* experiments will allow to better understand the interaction between PLGA NPs and the skeletal muscle, and to develop functionalized nanocarriers for drug delivery to this specific tissue. The investigation *in vivo* will be crucial, since we also observed a difference in the uptake of all NPs investigated between myoblasts and myotubes. Even if we did not perform a quantitative evaluation, myotubes appeared to be less prone to internalize NPs suggesting that the interaction and uptake of NPs might differ between cycling and post-mitotic cells, probably because of modifications of cell membrane and cytoskeleton composition and dynamics occurring during the differentiation process. Elucidating *in vivo* the actual interaction of NPs with the myofibers will be crucial to

design the most appropriate approach for the NP-mediated administration of therapeutic agents to a diseased muscle tissue.

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TABLES

Table 1. Characteristics of NPs (*n*=3)

	mean diameter (nm±S.E.)	**polydispersity index**	**zeta potential (mV±S.E.)**
Liposomes	155±12	0.09	-10±2.10
Fluorescent liposomes	160±8	0.08	-11±1.75
MSNs	94±23[a]	-	+35±0.90
Fluorescent MSNs	105±24[a]	-	+22±0.60
PLGA 50:50 NPs	117±8	0.07	-31±0.92
PLGA 75:25 NPs	128±4	0.06	-27±0.84
Fluorescent PLGA 50:50 NPs	121±6	0.08	-29±1.18
Fluorescent PLGA 75:25 NPs	132±4	0.06	-25±2.01

[a] determined by TEM analysis

FIGURE LEGENDS

Fig. 1 Effect of NPs on cell viability of primary human myoblasts

Primary human myoblasts were obtained from two healthy subjects (Ctr1: a, c, e and g; Ctr2: b, d, f and h). The effect of PLGA 75:25 NPs (a-b), PLGA 50:50 NPs (c-d), MSNs (e-f) and liposomes (g-h) on cell viability was measured by MTT assay. Box-plots show the mean value±S.E. of percentage of cell viability after 2 h, 24 h and 72 h of incubation with NPs at different concentrations. *p<0.05; **p<0.01

Fig. 2 Effect of NPs on cell viability of primary human myotubes

Primary human myotubes were obtained from a healthy subject (Ctr1). The effect of PLGA 75:25 NPs (a), PLGA 50:50 NPs (b), MSNs (c) and liposomes (d) on cell viability was measured by MTT assay. Box-plots show the mean value±S.E. of percentage of cell viability after 2 h, 24 h and 72 h of incubation with NPs at different concentrations. *p<0.05; **p<0.01

Fig. 3 Intracellular distribution of PLGA NPs in primary human myoblasts and myotubes

Confocal fluorescence microscopy analysis of myoblasts (a-f) and myotubes (g-i) that have been incubated with PLGA NPs at the concentration of 0.2 mg/ml for 2 h, 24 h and 72 h. PLGA NPs are loaded with Nile red (red fluorescence) and cells are counterstained with either phalloidin conjugate with Alexa488 (green fluorescence) to highlight actin filaments (a-c, g-i) or PKH67 Green Fluorescent Cell Linker (green fluorescence, d-f) to label cell-membrane derived vesicles. Hoechst 33258 (blue fluorescence) staining depicts cell nuclei. Confocal fluorescence microscopy analysis revealed PLGA NPs in the cytoplasm after 2 h (a, arrows), 24 h (b, arrow) and 72 h treatment (c, arrow). PLGA NPs partially co-localizing with fluorescent-labelled membrane derived vesicles were visible on the surface of myoblasts that have been treated for 2 h (D, arrow) and on the cell surface and cytoplasm of myoblasts after 24 h (e, arrow) and 72 h (f, arrow) incubation. The insets in d, e and f show the partial co-localization between the green fluorescent cell membrane stain PKH67 and PLGA NPs. In myotubes, PLGA NPs were observed near the cell surface but not in the cytoplasm after 2 h incubation (g). PLGA NPs were detected in the cytoplasm in myotubes that have been treated for 24 h (h) and 72 h (i). PLGA NPs near cell nuclei were visible after 24 h (h, arrows and inset) incubation. Scale bars: 50 um. TEM analysis of PLGA NPs intracellular distribution after 2 h (j-l) and 24 h (m) incubation in myoblasts. A PLGA NP enclosed in an endosome (j, arrow) occurs just beneath the cell surface. A PLGA NP (k, arrow) is escaping from an endosome. Two PLGA NPs (l, arrows) occur free in the cytosol. After 24 h incubation (m), the cytoplasm contains large amounts of peculiar vacuoles containing NPs remnants (arrowhead). The inset shows a NPs partially enclosed by autophagic double-membranes (small arrows). Mitochondria (m), endoplasmic reticulum (er), Golgi complex (g). Bars: 250 nm

Fig. 4 Intracellular distribution of MSNs in primary human myoblasts and myotubes

Conventional (a, b) and confocal (c-g) fluorescence microscopy analysis of myoblasts (a-e) and myotubes (f and g) that have been incubated with MSNs at the final dose of 0.01 mg/ml for 24 h (a, c, e and f) and 72 h (b, d and g). MSNs are labelled with FITC (green fluorescence) whereas myoblasts and myotubes are counterstained with Trypan blue (red fluorescence, a-d) or with PHK26 Red Fluorescent Cell Linker (red fluorescence, e) or phalloidin conjugate with Atto 594 (red fluorescence, f, g), respectively. Hoechst 33258 (blue fluorescence) staining has been used to depict cell nuclei. MSNs aggregates occurred in the cytoplasm of a few myoblasts that have been treated with for 24 h (a, c) and 72 h (b, d). A partial co-localization between the red fluorescent cell membrane stain PKH26 and MSNs was observed in myoblasts (e, arrows). MSNs occurred in the cytoplasm of some myotubes that have been treated for 24 h (e) and 72 h (f). MSNs near cell nuclei have also been observed in both myoblasts (d, arrow) and myotubes (e and f, arrows). TEM analysis of MSNs intracellular distribution after 2 h incubation in myoblasts (h) and myotubes (i). Internalized MSNs occur inside various vacuoles (h, arrows), sometimes located very close to the nucleus. Inset: A cluster of MSNs at the cell surface. In myotubes (i), MSNs accumulate in vacuoles (arrows) without perturbing the structural organization: bundles of myofibrils (asterisks), mitochondria (m), endoplasmic reticulum (er), nucleus (n). Bars: 250 nm

Fig. 5 Intracellular distribution of liposomes in primary human myoblasts and myotubes

Confocal fluorescence microscopy analysis of myoblasts (a, b) and myotubes (c, d) that have been incubated with liposomes at the concentration of 0.2 mg/ml for 24 h (a, c) and 72 h (b, d). Liposomes are labelled with FITC (green fluorescence) whereas myoblasts and myotubes are counterstained PKH26 Red Fluorescent Cell Linker (red fluorescence), to label cell-membrane derived vesicles, or with phalloidin conjugated with Atto 594, respectively. Hoechst 33258 (blue fluorescence) staining has been used to depict cell nuclei.

Confocal fluorescence microscopy revealed rare liposomes inside primary human myoblasts located close but not within cell nuclei (a, b, arrows). A co-localization between liposomes and fluorescent-labelled membrane derived vesicles was never observed (a, b). A few myotubes displayed liposomes in their cytoplasm after 24 h (c) and 72 h (d) treatment. Scale bars 25 um. TEM analysis of liposome intracellular distribution in myoblasts after 2 h (e) and 24 h (f) incubation, and in myotubes (g) after 2 h incubation. Some liposomes (e, arrow) occur free in the cytoplasm at the cell periphery. The internalized liposomes (f, g, arrows) show fine irregular profiles. Mitochondria (m), endoplasmic reticulum (er). Bars: 250 nm