Uptake and intracellular fate of biocompatible nanocarriers in cycling and noncycling cells

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

M. Costanzo,[1] F. Vurro,[1] B. Cisterna,[1] F. Boschi,[2] A. Marengo,[3] E. Montanari,[4] C. Di Meo,[4] P. Matricardi,[4] G. Berlier,[5] B. Stella,[3] S. Arpicco,[3] M. Malatesta[1]

[1]Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Italy

[2]Department of Computer Science, University of Verona, Italy

[3]Department of Drug Science and Technology, University of Torino, Italy

[4]Department of Drug Chemistry and Technologies, Sapienza University of Rome, Italy

[5]Department of Chemistry and NIS Centre, University of Torino, Italy

Corresponding author:

Manuela Costanzo, Tel. +39.045.8027272; Fax: 39.045.8027163; e-mail: manuela.costanzo@univr.it

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 are 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 (Grottkau 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

97 (Akiyoshi et al., 1993) and/or synthetic (Vinogradov et al., 1999) polymers and, based on
98 the type of bonds present in the polymer network, they are subdivided into groups based
99 on either physical (Di Meo et al., 2015) or chemical (Pedrosa et al., 2014; Montanari et al.,
100 2016) cross-linking. A peculiar characteristic of NHs is their swelling properties in aqueous
101 media; control over the swelling of the polymer network is useful for the controlled release
102 of bioactive compounds. Moreover, as NHs are highly solvated, they display both liquid-
103 and solid-like behavior: usually, these viscoelastic properties allow NHs to deform in the
104 presence of a flow, enabling them to more easily travel through the extracellular matrix,
105 thus enhancing the permeation, binding and retention within the tissues.
106 The cytotoxicity of these different nanocarriers has been previously evaluated *in vitro* using
107 various established cancer cell lines (e.g. Slowing et al., 2006; Arpicco et al., 2013;
108 D'Arrigo et al., 2014; Costanzo et al., 2016; Jonderian and Maalouf, 2016; Quagliariello et
109 al., 2017). However, it has been reported that the effects induced by nanocarriers may
110 depend on cell metabolic activity and doubling time (Chang et al, 2007): this suggests that
111 the proliferation characteristics of the cell system used should be taken into account when
112 testing the biocompatibility of nanosystems designed for systemic administration, since the
113 organisms are composed of different tissues and cells with peculiar kinetic and metabolic
114 features.
115 The aim of this study was to elucidate whether different cytokinetic features may influence
116 the cell tolerance to different biocompatible nanocarriers. To do this, the cell uptake and
117 intracellular fate of liposomes, MSN, PLGA NPs, and NHs have been investigated by
118 confocal fluorescence microscopy and transmission electron microscopy (TEM) in C2C12
119 cells under cycling and non-cycling conditions. C2C12 cells are an immortalized murine
120 myoblast cell line, able to rapidly proliferate as myoblasts under high serum conditions,
121 and to efficiently fuse and differentiate into myotubes under low serum conditions: they
122 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-ethanesulforic 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

174 measurement was made after dilution of the nanoparticle suspension in MilliQ® water.
175 Each measure was performed in triplicate.
176 Particle size of MSN was determined by transmission electron microscopy measurements
177 with a JEM 3010-UHR microscope (JEOL Ltd.) operating at 300 kV. Powders were
178 dispersed on a copper grid coated with a perforated carbon film. The size distribution of
179 the samples was obtained by measuring a statistically representative number of particles
180 (ca. 250 particles). The results are indicated as mean particle diameter (dm) ± standard
181 deviation (SD).
182 The particle surface charge of all formulations was investigated by zeta potential
183 measurements at 25°C applying the Smoluchowski equation and using the Nanosizer
184 Nano Z. Measurements were carried out in triplicate.
185
186 ***In vitro cell culture***
187 C2C12 myoblasts (1-2x10^3 cells/cm^2) were grown in Dulbecco's modified Eagle medium
188 (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) glutamine, 0.5%
189 (v/v) amphotericin B, 100 units/ml of penicillin and 100 µg/mL of streptomycin (Gibco), at
190 37°C in a 5% CO_2 humidified atmosphere. Cells were trypsinized (0.25% trypsin in PBS
191 containing 0.05% EDTA) when subconfluent, and seeded either on 24 or 96 multi-well
192 plastic microplates for cell viability evaluation, or on glass coverslips in 12-multi-well plastic
193 microplates for fluorescence microscopy and TEM. All the experiments were performed
194 with cells at passage 7-10. For myogenic differentiation, when 80% confluency had been
195 reached, the growth medium was substituted with the differentiation medium containing
196 1% FBS.
197 Myoblasts were treated with the different nanocarriers one day after seeding, while
198 myotubes after six days in differentiation medium. The initial medium was replaced with a
199 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, $8x10^3$ 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% OsO_4 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 m^2/g and pore size about 3 nm (inner volume available to host drugs around 1.2 cm^3/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.

NHs

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

406 times, when the number of cells was significantly lower than in controls likely due to
407 intracellular accumulation of nanocarriers perturbing cell proliferation. Indeed, MSN
408 administration led to a significant increase in cell population, probably related to the silica-
409 NP-induced activation of MAPK signaling and the down-regulation of p53, which in turn
410 inhibit apoptosis and induce cell proliferation (Christen et al., 2014). The S-phase cell
411 fraction was also found to be unaffected after liposomes, MSN and 75:25 PLGA NP, and
412 even increased after 50:50 PLGA NP administration, thus definitely demonstrating that
413 liposomes, MSN and both PLGA NP formulations do not negatively affects cell cycle
414 progression and proliferation of C2C12 myoblasts. On the other hand, NHs administration
415 induced a decrease of S phase cell fraction after 24 h, followed by a recovery after 48 h,
416 thus suggesting an only transitory slowing down of myoblasts proliferation, without
417 negative effects on cell population at longer incubation times. This phenomenon could be
418 due to cell overloading after 24 h incubation, as suggested by the evidence at
419 fluorescence microscopy.
420 All together, our results confirm and provide additional evidence that all the tested
421 nanocarriers are highly biocompatible to C2C12 myoblasts. However, biocompatibility is
422 here attained at lower nanocarrier concentrations than those reported as safe for other cell
423 types, such as breast, ovarian, pancreatic, and prostate cancer cell lines, (e.g. Slowing et
424 al., 2006; Arpicco et al., 2013; D'Arrigo et al., 2014; Costanzo et al., 2016; Jonderian and
425 Maalouf, 2016; Quagliariello et al., 2017). It is actually known that different cell types may
426 differently react to nanocarrier administration; in particular, a comparative *in vitro* study
427 demonstrated that myoblasts are much more sensitive than cells of fibroblastic, hepatic or
428 endodermic origin (Nie et al., 2012). In addition, although our results demonstrate that
429 C2C12 myoblasts are able to internalize all the nanocarriers tested, this uptake occurs
430 more slowly than in other cell types (e.g. Pan et al., 2012; Arpicco et al., 2013; D'Arrigo et
431 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

457 MSN uptake in C2C12 myoblasts enhances their differentiation into myotubes, opening
458 interesting perspectives for the use of this nanocarrier for muscle tissue therapy.
459 At both formulations, PLGA NPs enter the cell as single units by endocytosis; however,
460 they rapidly escape from the endosomes, as already observed for other polymeric NPs
461 (Varkouhi et al., 2011; Malatesta et al., 2012), and occur free in the cytosol without
462 making contact with any organelle. Afterwards, PLGA NPs re-enter the lytic pathway by
463 autophagic process (Malatesta et al., 2015; Zhang et al., 2017; Panyam and Labhasetwar,
464 2003), thus undergoing enzymatic degradation and giving rise to the numerous residual
465 bodies observed after 24 h incubation. Their presence as free NPs in the cytosol is
466 therefore transient and the observations at TEM suggest that most of the fluorescing spots
467 detectable at confocal microscopy are likely remnants of PLGA NPs inside residual bodies.
468 Similarly to other polymeric NPs (Kim et al., 2007; Brambilla et al., 2010; Costanzo et al.,
469 2016), PLGA NPs do not enter the cell nucleus.
470 NHs also enter the cells *via* endocytosis, as already reported for cancer cells (Ossipov,
471 2010; Pan et al., 2012; D'Arrigo et al., 2014; Palvai and Kuman, 2017; Quagliarello e al.,
472 2017), but our data provide the first ultrastructural evidence of their intracellular fate. NH
473 uptake in C2C12 appears to be less efficient than in cancer cells. It has been reported that
474 NHs show a CD44 dependent endocytosis (Quagliarello e al., 2017) whose efficiency
475 could be related to expression levels of this transmembrane protein: consistently, C2C12
476 cells do express CD44 (Kaneko et al., 2015), but cancer cells are often characterized by a
477 very high expression of this receptor (recent reviews in Morath et al., 2016; Senbanjo and
478 Chellaiah, 2017). Once in the cytoplasm, NHs seem to rapidly escape from endosomes in
479 C2C12 cells; in fact, findings of NHs occurring free in the cytosol are very frequent
480 whereas endosomes containing NHs are quite scarce. However, free NHs re-enter the lytic
481 pathway by the autophagic process, which finally led to the enzymatic degradation of
482 these nanocarriers. Evidence of intracellular degradation by lysosomal enzymes has been

483 also reported in HaCaT keratinocytes, where NHs were found to co-locate with acidic
484 organelles up to 24 h from incubation (Montanari et al., submitted manuscript).
485 C2C12 myotubes, treated with nanocarrier concentrations found to be safe for myoblasts,
486 did not show any sign of cell stress as clearly demonstrated by TEM, thus extending the
487 biocompatibility of all tested nanocarriers to the non-cycling, differentiated muscle cells.
488 However, it is evident that the amount of nanocarriers internalized by myotubes is lower
489 than in myoblasts. Similarly, other NPs easily entering myoblasts were found to be unable
490 to penetrate myotubes (Salova et al., 2011). One of the reasons for such a difference may
491 reside in the higher metabolic rate of a cycling cell compared to its differentiated non-
492 cycling counterpart (Chang et al., 2007); however, it is worth noting that the differentiation
493 process of a C2C12 myoblast into a myotube entails a differential expression of numerous
494 proteins among which those related to cell adhesion, transmembrane transport, and
495 cytoskeleton composition and dynamics (Kislinger et al., 2005; Casadei et al., 2009;
496 Forterre et al., 2014). In addition, the lipid and fatty acid composition of cell membranes
497 significantly changes during the myogenic process (Briolay et al., 2013) when the plasma
498 membrane composition undergoes marked modifications that could more or less markedly
499 affect nanocarrier uptake depending on the internalization mechanisms involved.
500 Once internalized in the myotubes, all nanovectors undergo a fate similar to that observed
501 in myoblasts, without perturbing cell organelles.
502 In conclusion, our results demonstrate that all the tested nanocarriers are suitably
503 biocompatible for both cycling myoblasts and non-cycling differentiated myotubes,
504 although the differentiation stage markedly affects the uptake efficiency (and this should
505 be taken into consideration when designing nanocostructs for therapeutic or diagnostic
506 purposes). At the concentrations used in our experiments, all the tested NPs enter the
507 intracellular environment and undergo degradation through the physiological pathways
508 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.

Acknowledgements

Funding from Italian Ministry for University and Research (MIUR)-University of Turin, "Fondi Ricerca Locale (ex-60%)" are kindly acknowledged.

References

Akiyoshi, Kazunari Deguchi, Shigeru Moriguchi, Nobuhiro Yamaguchi, Shigehiko Sunamoto, JunzoSelf-aggregates of hydrophobized polysaccharides in water. Formation and characteristics of nanoparticles. Macromolecules, 1993. 26(12): p. 3062-3068.

Arpicco S, Lerda C, Dalla Pozza E, Costanzo C, Tsapis N, Stella B, et al. Hyaluronic acid-coated liposomes for active targeting of gemcitabine. Eur J Pharm Biopharm 2013;85:373-80.

Brambilla D, Nicolas J, Le Droumaguet B, Andrieux K, Marsaud V, Couraud PO, et al. Design of fluorescently tagged poly(alkyl cyanoacrylate) nanoparticles for human brain endothelial cell imaging. Chem Commun (Camb) 2010;46:2602-4.

Briolay A, Jaafar R, Nemoz G, Bessueille L. Myogenic differentiation and lipid-raft composition of L6 skeletal muscle cells are modulated by PUFAs.Biochim Biophys Acta. 2013;1828(2):602-13. doi: 10.1016/j.bbamem.2012.10.006.

Casadei L, Vallorani L, Gioacchini AM, Guescini M, Burattini S, D'Emilio A, Biagiotti L, Falcieri E, Stocchi V. Proteomics-based investigation in C2C12 myoblast differentiation. Eur J Histochem. 2009 Dec 29;53(4):e31. doi: 10.4081/ejh.2009.e31

Chang JS, Chang KL, Hwang DF, Kong ZL. In vitro cytotoxicitiy of silica nanoparticles at high concentrations strongly depends on the metabolic activity type of the cell line. Environ Sci Technol. 2007; 15;41(6):2064-8.

Chen Y, Chen H, Shi J. Drug delivery/imaging multifunctionality of mesoporous silica-based composite nanostructures. Expert Opin Drug Deliv 2014;11:917-30.

Choi, K.Y., Choi, Ki Young Jeon, Eun Jung Yoon, Hong Yeol Lee, Beom Suk Na, Jin Hee Min, Kyung Hyun Kim, Sang Yoon Myung, Seung-Jae Lee, Seulki Chen, Xiaoyuan Kwon, Ick Chan Choi, Kuiwon Jeong, Seo Young Kim, Kwangmeyung, Park, Jae

Hyunget, Theranostic nanoparticles based on PEGylated hyaluronic acid for the diagnosis, therapy and monitoring of colon cancer. Biomaterials, 2012. 33(26): p. 6186-6193.

Christen V, Camenzind M, Fent K. Silica nanoparticles induce endoplasmic reticulum stress response, oxidative stress and activate the mitogen-activated protein kinase (MAPK) signaling pathway. Toxicology Reports 2014;1:1143-51

Costanzo M, Carton F, Marengo A, Berlier G, Stella B, Arpicco S, Malatesta M (2016). Fluorescence and electron microscopy to visualize the intracellular fate of nanoparticles for drug delivery. Eur J Histochem. 60, 2640.

Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V (2012). PLGA-based nanoparticles: An overview of biomedical applications. J Control Release. 161, 505-522.

D'Arrigo G, Navarro G, Di Meo C, Matricardi P, Torchilin V. Gellan gum nanohydrogel containing anti-inflammatory and anti-cancer drugs: a multi-drug delivery system for a combination therapy in cancer treatment. Eur J Pharm Biopharm. 2014;87(1):208-16. doi: 10.1016/j.ejpb.2013.11.001.

Di Meo, Chiara Montanari, Elita Manzi, Lucio Villani, Claudio Coviello, Tommasina Matricardi, Pietro, Highly versatile nanohydrogel platform based on riboflavin-polysaccharide derivatives useful in the development of intrinsically fluorescent and cytocompatible drug carriers. Carbohydrate Polymers, 2015. 115: p. 502-509.

Fessi H, Puisieux F, Devissaguet JPh, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm 1989;55: R1–R4.

Dilnawaz F, Acharya S, Sahoo SK. Recent trends of nanomedicinal approaches in clinics. Int J Pharm. 2018;538(1-2):263-278. doi: 10.1016/j.ijpharm.2018.01.016.

Forterre A, Jalabert A, Berger E, Baudet M, Chikh K, Errazuriz E, De Larichaudy J, Chanon S, Weiss-Gayet M, Hesse AM, Record M, Geloen A, Lefai E, Vidal H, Couté Y, Rome S. Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk? PLoS One. 2014;9(1):e84153. doi: 10.1371/journal.pone.0084153.

Freichels H, Danhier F, Préat V, Lecomte P, Jérôme C. Fluorescent labeling of degradable poly(lactide-*co*-glycolide) for cellular nanoparticles tracking in living cells. Int J Artif Organs 2011;34(2):152-160.

Ganguly, Kuntal Chaturvedi, Kiran More, Uttam A. Nadagouda, Mallikarjuna N. Aminabhavi Tejraj M. Polysaccharide-based micro/nanohydrogels for delivering macromolecular therapeutics. Journal of Controlled Release, 2014. 193: p. 162-173.

Grecchi S, Malatesta M. Visualizing endocytotic pathways at transmission electron microscopy via diaminobenzidine photo-oxidation by a fluorescent cell-membrane dye. Eur J Histochem. 2014 Oct 22;58(4):2449. doi: 10.4081/ejh.2014.2449.

Grottkau BE, Cai X, Wang J, Yang X, Lin Y. Polymeric nanoparticles for a drug delivery system. Curr Drug Metab 2013;14:840-6.

Jonderian A, Maalouf R (2016): Formulation and In vitro Interaction of Rhodamine-B Loaded PLGA Nanoparticles with Cardiac Myocytes. Front Pharmacol 7:458. doi: 10.3389/fphar.2016.00458.

589 Kabanov, A.V. and S.V. Vinogradov, Nanogels as Pharmaceutical Carriers: Finite
590 Networks of Infinite Capabilities. Angewandte Chemie International Edition, 2009.
591 48(30): p. 5418-5429.
592 Kaneko K, Higuchi C, Kunugiza Y, Yoshida K, Sakai T, Yoshikawa H, Nakata K.
593 Hyaluronan inhibits BMP-induced osteoblast differentiation. FEBS Lett.
594 2015;589(4):447-54. doi: 10.1016/j.febslet.2014.12.031.
595 Kapoor DN, Bhatia A, Kaur R, Sharma R, Kaur G, Dhawan S (2015). PLGA: a unique
596 polymer for drug delivery. Ther Deliv. 6, 41-58.
597 Kim HR, Gil S, Andrieux K, Nicolas V, Appel M, Chacun H, et al. Low-density lipoprotein
598 receptor-mediated endocytosis of PEGylated nanoparticles in rat brain endothelial
599 cells. Cell Mol Life Sci 2007;64:356-64.
600 Kislinger T, Gramolini AO, Pan Y, Rahman K, MacLennan DH, Emili A. Proteome
601 dynamics during C2C12 myoblast differentiation. Mol Cell Proteomics. 2005;4(7):887-
602 901.
603 Lallana, Enrique Rios de la Rosa, Julio M. Tirella, Annalisa Pelliccia, Maria Gennari,
604 Arianna Stratford, Ian J. Puri, Sanyogitta Ashford, Marianne Tirelli, Nicola,
605 Chitosan/Hyaluronic Acid Nanoparticles: Rational Design Revisited for RNA Delivery.
606 Molecular Pharmaceutics, 2017. 14(7): p. 2422-2436.
607 Lanza, R., Langer, R., Vacanti, J.P., 2011. Principles of tissue engineering. fourth ed.
608 Academic Press, New York.
609 Lince F, Bolognesi S, Stella B, Marchisio DL, Dosio F. Preparation of polymer
610 nanoparticles loaded with doxorubicin for controlled drug delivery. Chem Eng Res Des
611 2011;89:2410-9.
612 Malatesta M, Giagnacovo M, Costanzo M, Conti B, Genta I, Dorati R, et al.
613 Diaminobenzidine photoconversion is a suitable tool for tracking the intracellular
614 location of fluorescently labelled nanoparticles at transmission electron microscopy.
615 Eur J Histochem 2012;56:e20.
616 Malatesta M, Grecchi S, Chiesa E, Cisterna B, Costanzo M, Zancanaro C. Internalized
617 chitosan nanoparticles persist for long time in cultured cells. Eur J Histochem
618 2015;59:2492.
619 Malfanti A, Miletto I, Bottinelli E, Zonari D, Blandino G, Berlier G, Arpicco S. 2016. Delivery
620 of gemcitabine prodrugs employing mesoporous silica nanoparticles. Molecules
621 21:522.
622 Montanari E, Capece S, Di Meo C, Meringolo M, Coviello T, Agostinelli E, Matricardi P.
623 Hyaluronic acid nanohydrogels as a useful tool for BSAO immobilization in the
624 treatment of melanoma cancer cells. Macromol Biosci. 2013;13(9):1185-94. doi:
625 10.1002/mabi.201300114.
626 Montanari E, D'Arrigo G, Di Meo C, Virga A, Coviello T, Passariello C, Matricardi P.
627 Chasing bacteria within the cells using levofloxacin-loaded hyaluronic acid
628 nanohydrogels. Eur J Pharm Biopharm. 2014;87(3):518-23. doi:
629 10.1016/j.ejpb.2014.03.003.
630 Montanari, Elita De Rugeriis, Maria Cristina Di Meo, Chiara Censi, Roberta Coviello,
631 Tommasina Alhaique, Franco Matricardi, Pietro, One-step formation and sterilization of
632 gellan and hyaluronan nanohydrogels using autoclave. Journal of Materials Science:
633 Materials in Medicine, 2015. 26(1): p. 32.

Montanari, Elita Gennari, Arianna Pelliccia, Maria Gourmel, Charlotte Lallana, Enrique Matricardi, Pietro McBain, Andrew J. Tirelli, Nicola, Hyaluronan/Tannic Acid Nanoparticles Via Catechol/Boronate Complexation as a Smart Antibacterial System. Macromolecular Bioscience, 2016. 16(12): p. 1815-1823.

Montanari, Elita Di Meo, Chiara Sennato, Simona Francioso, Antonio Marinelli, Anna Laura Ranzo, Francesca Schippa, Serena Coviello, Tommasina Bordi, Federico Matricardi, Pietro, Hyaluronan-cholesterol nanohydrogels: Characterisation and effectiveness in carrying alginate lyase. New Biotechnology, 2017. 37: p. 80-89.

Morath I, Hartmann TN, Orian-Rousseau V. CD44: More than a mere stem cell marker. Int J Biochem Cell Biol. 2016;81(Pt A):166-173. doi: 10.1016/j.biocel.2016.09.009.

Nazarenus M, Zhang Q, Soliman MG, del Pino P, Pelaz B, Carregal-Romero S, et al. In vitro interaction of colloidal nanoparticles with mammalian cells: What have we learned thus far? Beilstein J Nanotechnol 2014;5:1477-90.

Nie Y, Zhang ZR, He B, Gu Z. Investigation of PEG-PLGA-PEG nanoparticles-based multipolyplexes for IL-18 gene delivery. J Biomater Appl. 2012;26(8):893-916. doi: 10.1177/0885328210384889.

Ossipov, D.A. Nanostructured hyaluronic acid-based materials for active delivery to cancer. Exp. Opin. Drug Deliv. 2010, 7, 681-703.

Palvai, S, Kuman, M.M. Hyaluronic acid cloaked oleic acid nanoparticles inhibit MAPK signaling with sub-cellular DNA damage in colon cancer cells. J. Mat. Chem. B. 2017, 5,
3658-3666.

Pan YJ, Chen YY, Wang DR, Wei C, Guo J, Lu DR, Chu CC, Wang CC. Redox/pH dual stimuli-responsive biodegradable nanohydrogels with varying responses to dithiothreitol and glutathione for controlled drug release. Biomaterials. 2012;33(27):6570-9. doi: 10.1016/j.biomaterials.2012.05.062.

Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev 2003;55:329–347.

Pedrini I, Gazzano E, Chegaev K, Rolando B, Marengo A, Kopecka J, et al. Liposomal nitrooxy-doxorubicin: one step over Caelyx® in drug-resistant human cancer cells. Mol Pharm 2014;11:3068-79.

Pedrosa, Sílvia Santos Gonçalves, Catarina David, Laurent Gama, Miguel, A novel crosslinked hyaluronic acid nanogel for drug delivery. Macromolecular Bioscience, 2014. 14(11): p. 1556-1568.

Peng J, He X, Wang K, Tan W, Li H, Xing X, et al. An antisense oligonucleotide carrier based on amino silica nanoparticles for antisense inhibition of cancer cells. Nanomedicine 2006;2:113-20.

Poussard S, Decossas M, Le Bihan O, Mornet S, Naudin G, Lambert O. Internalization and fate of silica nanoparticles in C2C12 skeletal muscle cells: evidence of a beneficial effect on myoblast fusion. Int J Nanomedicine 2015;10:1479-92.

Quagliariello V, Iaffaioli RV, Armenia E, Clemente O, Barbarisi M, Nasti G, Berretta M, Ottaiano A, Barbarisi A (2017): Hyaluronic Acid Nanohydrogel Loaded With Quercetin Alone or in Combination to a Macrolide Derivative of Rapamycin RAD001 (Everolimus) as a New Treatment for Hormone-Responsive Human Breast Cancer. J Cell Physiol 232:2063-2074. doi: 10.1002/jcp.25587.

679 Ricci V, Zonari D, Cannito S, Marengo A, Scupoli MT, Malatesta M, Carton F, Boschi F,
680 Berlier G, Arpicco S. Hyaluronated mesoporous silica nanoparticles for active
681 targeting: Influence of conjugation method and hyaluronic acid molecular weight on the
682 nanovector properties. J. Colloid Interface Sci. 2018 516, 484–497
683 Salova AV, Leont'eva EA, Mozhenok TP, Kornilova ES, Krolenko SA, Beliaeva TN.
684 Change in localization of cellular vesicular apparatus during differentiation of
685 myoblasts into myotubules in cell culture. Cell Tissue Biol., 2011;5(3) 255–263.
686 Sapino S, Ugazio E, Gastaldi L, Miletto I, Berlier G, Zonari D, et al. Mesoporous silica as
687 topical nanocarriers for quercetin: characterization and in vitro studies. Eur J Pharm
688 Biopharm 2015;89:116-25.
689 Senbanjo LT, Chellaiah MA CD44: A Multifunctional cell surface adhesion receptor is a
690 regulator of progression and metastasis of cancer cells.Front Cell Dev Biol. 2017 Mar
691 7;5:18.
692 Slowing I, Trewyn BG, Lin VS (2006): Effect of surface functionalization of MCM-41-type
693 mesoporous silica nanoparticles on the endocytosis by human cancer cells. J Am
694 Chem Soc 128:14792-3.
695 Slowing I., Vivero-Escoto J. L., WuVictor C.-W., Lin S.-Y.L. (2008) Mesoporous silica
696 nanoparticles as controlled release drug delivery and gene transfection carriers, Adv.
697 Drug Deliv. Rev. 60:278-1288
698 Soni, K.S., S.S. Desale, and T.K. Bronich, Nanogels: An overview of properties,
699 biomedical applications and obstacles to clinical translation. Journal of Controlled
700 Release, 2016. 240: p. 109-126.
701 Stella B, Arpicco S, Peracchia MT, Desmaële D, Hoebeke J, Renoir JM, et al. Design of
702 folic acid-conjugated nanoparticles for drug targeting. J Pharm Sci 2000;89:1452-64.
703 Stella B, Arpicco S, Rocco F, Marsaud V, Renoir JM, Cattel L, et al. Encapsulation of
704 gemcitabine lipophilic derivatives into polycyanoacrylate nanospheres and
705 nanocapsules. Int J Pharm 2007a;344:71-7.
706 Stella B, Marsaud V, Arpicco S, Géraud G, Cattel L, Couvreur P, et al. Biological
707 characterization of folic acid-conjugated poly(H2NPEGCA-co-HDCA) nanoparticles in
708 cellular models. J Drug Target 2007b;15:146-53.
709 Thomas MG, Marwood RM, Parsons AE, Parsons RB. The effect of foetal bovine serum
710 supplementation upon the lactate dehydrogenase cytotoxicity assay: Important
711 considerations for in vitro toxicity analysis. Toxicol In Vitro. 2015;30(1 Pt B):300-8. doi:
712 10.1016/j.tiv.2015.10.007.
713 Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of
714 biologicals. J Control Release 2011;151:220-8.
715 Verma A, Stellacci F. Effect of surface properties on nanoparticle-cell interactions. Small
716 2010;6:12-21.
717 Vinogradov, S., E. Batrakova, and A. Kabanov, Poly(ethylene glycol)–polyethyleneimine
718 NanoGel™ particles: novel drug delivery systems for antisense oligonucleotides.
719 Colloids and Surfaces B: Biointerfaces, 1999. 16(1): p. 291-304.
720 Wicki, A; Witzigmann, D; Balasubramanian, V; Huwyler, J 2015, Nanomedicine in cancer
721 therapy: Challenges, opportunities, and clinical applications, J CONTROLLED
722 RELEASE, 200, 138-157

723 Yu M.H., S. Jambhrunkar, P. Thorn, J.Z. Chen, W.Y. Gu, C.Z. Yu, Hyaluronic acid
724 modified mesoporous silica nanoparticles for targeted drug delivery to CD44-
725 overexpressing cancer cells, Nanoscale 5(1) (2013) 178-183.
726 Zhang J, Chang D, Yang Y, Zhang X, Tao W, Jiang L, Liang X, Tsai H, Huang L, Mei L.
727 Systematic investigation on the intracellular trafficking network of polymeric
728 nanoparticles. Nanoscale 2017:9:3269.

729 **Table 1. Characterisation of nanocarriers**

730

	mean diameter (nm±S.E.)	polydispersity index	zeta potential (mV±S.E.)
Liposomes	180±12	0.09	-15±2.10
Fluo-Lipo	177±8	0.08	-14±1.75
MSN	100±23[a]	-	+35±0.90
MSN fluo	100±23[a]	-	+22±0.60
NPs PLGA 50:50	109±6	0.06	-33±1.17
NPs PLGA 75:25	121±4	0.05	-25±0.64
NPs PLGA 50:50-Nile Red	115±4	0.06	-31±2.34
NPs PLGA 75:25-Nile Red	123±3	0.04	-23±1.72
NHs	200±15	0.12	-38±2.24
Rhod-NHs	250±35	0.18	-35±3.31

731 [a] determined by transmission electron microscopy analysis

732

733

734 **Legends**

735

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

740

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

745

746 **Figure 3. Microscopical analysis of cell-liposome interactions.** Confocal optical
747 sections of myoblasts (a,b) and myotubes (c) after 2h (a) and 24h (b, c) of liposome
748 incubation. DNA is stained with Hoechst 33342 (blue fluorescence), cytoplasm is
749 counterstained with trypan blue (red fluorescence). d) A myoblast incubated with the
750 PKH26 red-fluorescing dye to visualise endocytotic vesicles, and then incubated for 30 min
751 with green fluorescent liposomes: the two signals never co-locate (the inset shows a 2x
752 magnification of the detail indicated by the small arrows). DNA was stained with Hoechst
753 33342 (blue fluorescence). Bars: 20 μm (a-c), 10 μm (d). Transmission electron
754 microscopy analysis of liposomes intracellular distribution in myoblasts (e, f) and myotubes
755 (g) after 24 h incubation. e) A liposome (arrow) occurs free in the cytoplasm at the cell
756 periphery. f) Electron-dense fine granular material (arrowheads) is located in close
757 proximity of lipid droplets (L). g) A liposome (arrow) occurs in perinuclear position. Note
758 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)n 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.