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(Article begins on next page)

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

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Abstract

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

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

45 **Introduction**

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

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

66 Silica is generally recognized as safe by FDA and used as excipient in tablet-form drug
67 formulations. MSN have recently attracted attention as promising components of
68 multimodal nanoparticle systems, owing to their straightforward synthesis and
69 functionalization, ordered mesoporous structure with tunable pore size, high surface are
70 and large pore volumes resulting in high drug loading capacity, good chemical stability,

71 and adequate biocompatibility (Slowing et al. 2008; Chen et al., 2014; Sapino et al., 2015).
72 MSN and, in particular, amino-MSN, can be used to deliver either small molecules or
73 oligonucleotides; moreover, they can be tailored with a variety of surface functional groups
74 to increase biocompatibility, delivery capability and targeting (Peng et al., 2006; Malfanti et
75 al., 2016; Ricci et al., 2018).
76 Polymeric NPs are solid submicron structures prepared from natural or synthetic polymers
77 in which drugs can be adsorbed, dissolved, entrapped or encapsulated. These NPs have
78 good encapsulation efficiency and high stability in plasma, and increase the solubility and
79 stability of hydrophobic drugs while lowering their toxicity, thus permitting a controlled
80 release at the target site at relatively low doses (Grottkau et al., 2013; Stella et al., 2000,
81 2007a,b; Lince et al., 2011). In particular, the safe, biocompatible and commercially
82 available poly(lactide-*co*-glycolide) (PLGA) is one of the most successfully used
83 biodegradable polymers because its hydrolysis leads to metabolite monomers, lactic acid
84 and glycolic acid, which are endogenous and easily metabolized by the body *via* the Krebs
85 cycle, thus leading to a minimal systemic toxicity. It is worth noting that PLGA is approved
86 by the US FDA and the European Medicine Agency (EMA) in various drug delivery
87 systems for humans (Danhier et al., 2012; Kapoor et al., 2015).
88 Nanohydrogels (NHs) are nano-sized three-dimensional networks able to absorb a high
89 amount of water, and to easily swell and de-swell in aqueous media (Kabanov and
90 Vinogradov, 2009; Soni et al., 2016). NHs are usually *soft*, hydrophilic, biocompatible and
91 represent a highly versatile nano-system able to deliver a variety of bioactive molecules
92 such as hydrophobic (Choi et al., 2012) as well as hydrophilic drugs (Montanari et al.,
93 2014), polypeptides (Montanari et al., 2013; Montanari et al., 2017) and genetic material
94 (Ganguly et al., 2014; Lallana et al., 2017). Indeed, the porosity of the NHs network
95 provides a reservoir for loading molecular and macromolecular therapeutics as well as
96 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-

123 nanocarrier interactions in cycling cells (myoblasts) and highly differentiated non-cycling
124 cells (myotubes).
125
126 **Materials and Methods**
127 ***Preparation and characterization of nanocarriers***
128 Liposomes were prepared by thin lipid film hydration and extrusion method. Briefly, a
129 chloroform solution of the lipid components (Avanti Polar-Lipids distributed by Spectra
130 2000 Rome, Italy) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol
131 (Chol), and L-α phosphatidyl-DL-glycerol sodium salt (PG) (70:30:3 molar ratios) was
132 evaporated and the resulting lipid film was dried under vacuum overnight. Lipid films were
133 hydrated with HEPES [4-(2-hydroxyethyl) piperazine-1-ethanesulforic acid] buffer (pH 7.4),
134 and the suspension was vortexed for 10 min and bath sonicated. The formulations were
135 extruded (Extruder, Lipex, Vancouver, Canada) at 60 °C passing the suspension 10 times
136 under nitrogen through a 400 and 200 nm polycarbonate membrane (Costar, Corning
137 Incorporated; NY). Fluorescently labeled liposomes (Fluo-Lipo) were prepared as
138 described above and a 10 mM solution of fluorescein-5-(and-6)-sulfonic acid trisodium salt
139 (Invitrogen, Life Technologies, Monza, Italy) in HEPES buffer was used during hydration.
140 Liposomes were then purified through chromatography on Sepharose CL-4B columns,
141 eluting with HEPES buffer at room temperature.
142 Amino-mesoporous silica NPs (NH_2-MSN) were prepared as previously described (Sapino
143 et al., 2015). Fluorescein isothiocyanate (FITC) labeled MSN were prepared as previously
144 reported (Yu et al., 2013) with minor modifications. Briefly, at a suspension of 1 mg of NH_2-
145 MSN in 150 μl of MilliQ® water 250 μl of FITC ethanol solution (0.3 mg/ml) were added.
146 The mixture was maintained for 5 h under stirring in the dark, and then the NPs were
147 centrifuged and washed with ethanol three times until the supernatants were colorless.

148 For the preparation of PLGA (50:50 or 75:25, Sigma-Aldrich) NPs, the nanoprecipitation
149 technique was employed (Fessi et al., 1989). Practically, for each preparation, 12 mg of
150 PLGA 50:50 or 75:25 were dissolved in 2 ml of acetone. This organic solution was then
151 poured into 4 ml of MilliQ® water under magnetic stirring. Precipitation of particles occurred
152 spontaneously. After solvent evaporation under reduced pressure, an aqueous suspension
153 of NPs was obtained. Fluorescently labelled PLGA NPs were prepared by
154 nanoprecipitation of PLGA 50:50 or 75:25 (12 mg) in the presence of 16.8 μg of Nile red
155 (9-diethylamino-5H-benzo[α]phenoxazine-5-one, Sigma-Aldrich) dissolved in acetone; this
156 solution was then added to 4 ml of MilliQ® water under magnetic stirring, as previously
157 described for non labelled NPs. Fluorescent NPs were purified from non-incorporated dye
158 by gel filtration on a Sepharose CL-4B column.
159 Hyaluronan-cholesterol (HA-CH) polymer was synthesized as previously reported
160 (Montanari et al., 2013). For NHs preparation, 5 mg of HA-CH were dispersed in 2.5 ml of
161 MilliQ® water (2 mg/ml) overnight with magnetic stirring at 25°C; 2.5 ml PBS (pH=7.4) were
162 then added. Samples were autoclaved for 20 min at 121°C, leading to the NHs formation
163 (Montanari et al., 2015). For the synthesis of fluorescent NHs (Rhod-NHs), rhodamine B-
164 isothiocyanate (Rhod), previously solubilized in DMSO (9 mg/mL), was added to NHs
165 aqueous suspension (8 μl for 1 mg of polymer, corresponding to a degree of
166 functionalisation (DF) of 6.3%; % mol/mol). The reaction mixture was left for 5 h at 25°C in
167 the dark under magnetic stirring, followed by extensive dialysis and freeze-drying. The final
168 DF% was assessed through UV-Vis analysis in DMSO solution at 550 nm by using a rhod
169 calibration curve (8.5-125 μg/ml), resulting 1.3% mol/mol (mol of rhod per mol of HA-CH
170 repeating unit).
171 The mean particle size and the polydispersity index (PI) of liposomes, polymeric NPs and
172 NHs were determined at 25°C by quasi-elastic light scattering (QELS) using a nanosizer
173 (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

200 were then incubated for 2 h, 24 h and 48 h. At the end of each incubation time, the cells
201 were processed as described below; in parallel, untreated cells were used as control.
202 C2C12 are highly proliferating cells with a cell cycle of about 20 h
203 (https://www.dsmz.de/catalogues/details/culture/ACC-
204 565.html?tx_dsmzresources_pi5%5BreturnPid%5D=192), therefore a 48 h incubation time
205 allows the completion of two cycles.
206
207 ***C2C12 myoblast viability assay***
208 To estimate the effect on cell viability and on cell growth, three concentrations of each
209 nanocarriers were tested in cultured cells: liposomes were administered at the
210 concentrations of 125 µg/ml, 250 µg/ml, 500 µg/ml; MSN at 12.5 µg/ml, 25 µg/mL, 50
211 µg/ml; PLGA NPs at 100 µg/ml, 200 µg/ml, 400 µg/ml; NHs at 50 µg/ml, 100 µg/ml, 200
212 µg/ml. The chosen concentrations previously proved to be non-cytotoxic for various
213 cultured cells (Slowing et al., 2006; Arpicco et al., 2013; D'Arrigo et al., 2014; Costanzo et
214 al., 2016; Jonderian and Maalouf, 2016; Quagliariello et al., 2017). Nanocarrier
215 suspensions were prepared by diluting the stock suspensions into DMEM with 200
216 units/mL of penicillin and 200 µg/ml of streptomycin, immediately before the administration.
217 According to Thomas et al. (2015), at the end of each incubation time, 100 µl of medium
218 was removed and the release of the cytosolic enzyme lactate dehydrogenase (LDH) upon
219 cell lysis was estimated with CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega).
220 Optical density was measured with a microplate reader (Tecan) at 490 nm. The relative
221 amount of released LDH was normalized (as a percentage) to the total amount of LDH
222 release in control cells, which were not exposed to nanocarriers and were completely
223 lysed with lysis buffer provided in the kit. Results were expressed as the mean ± standard
224 error (S.E.) of 5 independent experiments.

225 To evaluate cell population size, $8x10^3$ cells/well were seeded on 24 multi-well plastic
226 microplates. After the different incubation times, the cells were detached by mild
227 trypsinization and their total number estimated by counting in a Burker Turk
228 hemocytometer; data were expressed as the mean ± S.E. of three independent
229 experiments.
230 In order to evaluate the effect of nanocarrier administration on cell proliferation, the S-
231 phase cells fraction was estimated after 24 h and 48 h incubation with nanocarrier
232 concentrations that did not induce decrease in cell population i.e., 125 µg/mL liposomes,
233 50 µg/ml MSN, 100 µg/ml PLGA NPs and 100 µg/ml NHs. Cells grown on coverslips were
234 pulse-labelled with 20 µM bromodeoxyuridine (BrdU, Sigma) for 30 min at 37°C, fixed with
235 70% ethanol and treated for 20 min at room temperature in 2 N HCl, to denature DNA
236 partially. After neutralization with 0.1 M sodium tetraborate (pH 8.2) for 3 min, samples
237 were washed in PBS, permeabilized for 15 min in PBS containing 0.1% bovine serum
238 albumin and 0.05% Tween-20, and incubated for 1 h with a mouse monoclonal antibody
239 recognizing BrdU (BD) diluted 1:20 in PBS. After two washings with PBS, samples were
240 incubated for 1 h with an Alexafluor 488-conjugated anti-mouse secondary antibody (Life
241 Techonologies), diluted 1:200 in PBS. The cell samples were washed with PBS, stained
242 for 5 min with 1 µg/mL Hoechst 33342 (Sigma) in PBS, and finally mounted in
243 PBS:glycerol (1:1) to be observed and scored in fluorescence microscopy (see below).
244 Data were expressed as the mean ± S.E. of three independent experiments (number of
245 counted cells: 1000 per sample). All statistical comparisons between treated and control
246 samples were carried out by the Mann Whitney U test.
247 An Olympus BX51 microscope equipped with a 100W mercury lamp (Olympus Italia Srl,
248 Milan, Italy) was used under the following conditions: 450-480 nm excitation filter (excf),
249 500 nm dichroic mirror (dm), and 515 nm barrier filter (bf), for FITC; 330-385 nm excf, 400
250 nm dm, and 420 nm bf, for Hoechst 33342. Images were recorded with an QICAM Fast

251 1394 digital camera (QImaging) and processed using Image-Pro Plus 7.0 software (Media
252 Cybernetics Inc.).
253

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

255 Confocal fluorescence microscopy

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

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

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

273 For confocal laser scanning microscopy (CLSM), a Leica TCS SP5 AOBS system (Leica
274 Microsystems Italia) was used: for fluorescence excitation, a diode laser at 405 nm for
275 Hoechst 33342, an Ar laser at 488 nm for FITC, and a He/Ne laser at 543 for Trypan blue,
276 Nile Red and Rhod were employed. Z-stack of 1 μm step sized images (each image in the

277 1024x1024 pixel format) were collected using a 40x oil immersion objective, and
278 processed by the Leica confocal software.
279
280 Transmission electron microscopy
281 C2C12 myoblasts and myotubes were incubated for 2 h, 24 h and 48 h with liposomes,
282 MSN, PLGA NPs or NHs. At each incubation time, cells were fixed with 2.5% (v/v)
283 glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C
284 for 2 h, post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide at room temperature for
285 1 h, dehydrated with acetone and embedded in Epon. Ultrathin sections were observed
286 unstained or after weak staining with UAR-EMS Uranyl acetate replacement stain
287 (Electron Microscopy Science). Observations were made in a Philips Morgagni
288 transmission electron microscope (FEI Company Italia Srl), operating at 80 kV and
289 equipped with a Megaview II camera for digital image acquisition.
290
291 **Results**
292 ***Characterization of nanocarriers***
293 Liposomes either blank and fluorescent showed diameters around 180 nm (PI < 0.1) and a
294 negative zeta potential (around -15 mV). Blank and fluorescent PLGA NP mean diameter
295 was around 110-120 nm (PI < 0.1) and the zeta potential was always around -30 mV; NHs
296 and Rhod-NHs showed mean hydrodynamic diameters of around 200 nm (PI < 0.2) and
297 250 nm (PI < 0.2), and negative zeta potentials, -38 ± 2 mV and -35 ± 3 mV, respectively.
298 MSN are characterized by spherical particles with size of 100 ± 23 nm in diameter. The
299 ordered mesoporous structure with MCM-41-like hexagonal array results in high specific
300 surface area of around 800 m^2/g and pore size about 3 nm (inner volume available to host
301 drugs around 1.2 cm^3/g) and a zeta potential of +35.0 ± 0.90 mV in water. The positive

302 charge results from the presence of the amino groups used for functionalization.
303 Nanocarrier features are summarized in Table 1.
304
305 ***C2C12 myoblasts: cell viability and proliferation***
306 Cell viability was evaluated after 2 h, 24 h, 48 h after treatment with nanocarriers at
307 different concentrations; the LDH test demonstrated values ranging from 2.09 to 12.71%
308 for all nanocarriers, at no variance with the control samples for any incubation time (*data*
309 *not shown*).
310 The total number of cells (Figure 1) was similar in control samples and in samples
311 exposed to 75:25 PLGA NPs at all times considered. Conversely, cell populations exposed
312 to liposomes, MSN, 50:50 PLGA NPs and NHs underwent significant modification in
313 comparison to control samples; in detail, liposomes and NHs induced a significant
314 decrease after 48 h incubation, while 50:50 PLGA NPs induced a decrease after both 24 h
315 and 48 h. Cell population exposed to MSN showed a significant increase in comparison to
316 control samples after 48 h incubation.
317 The S-phase fraction did not significantly change after 24 h incubation with all nanocarriers
318 except for 50:50 PLGA NPs and for NHs; in detail, 50:50 PLGA NPs showed a significant
319 increase in BrdU incorporation at the concentration of 100 µg/ml, while BrdU incorporation
320 significantly decreased in cells incubated with 100 µg/ml NHs. After 48 h incubation, no
321 change was found in any samples (Figure 2).
322
323 ***Nanocarrier distribution in C2C12 myoblasts and myotubes***
324 Liposomes
325 Confocal fluorescence microscopy showed that in myoblasts liposomes were mostly found
326 in the peripheral region of the cytoplasm, never entering the nucleus. They always
327 appeared as isolated fluorescing spots which never formed aggregates nor markedly

328 accumulated in the cell; their intracellular amount was evidently larger after 24 h incubation
329 (Figure 3 a,b). In myotubes, only few liposomes were found in the peripheral and
330 perinuclear region of the cytoplasm after all incubation times (Figure 3 c). The green
331 fluorescence of liposomes was never found to co-locate with red fluorescing intracellular
332 (i.e., endosomal) membranes (Figure 3 d).
333 At TEM, liposomes were strongly electron dense due to the lipid staining by osmium
334 tetroxide: in both myoblasts and myotubes their number was very low and they occurred
335 both at the cell periphery, just beneath the cell membrane (Figure 3 e) or in the perinuclear
336 area (Figure 3 g). No internalization processes such as endocytosis or phagocytosis were
337 observed. In myoblasts, an electron dense fine granular material was freely distributed in
338 the cytosol as well as in close proximity to lipid droplets (Figure 3 f). Cell nuclei and
339 cytoplasmic organelles of both myoblasts and myotubes never showed morphological
340 alterations.
341
342 <u>MSN</u>
343 Confocal fluorescence microscopy revealed that, in both myoblasts and myotubes, after 2
344 h incubation, MSN mostly occurred as aggregates at the cell surface and only a few small
345 clusters were observed inside the cytoplasm. After 24 h incubation, many internalized
346 MSN clusters were distributed in the cytoplasm, preferentially located around, but never
347 inside, the cell nuclei (Figure 4 a-c).
348 The green fluorescence of MSN was found to co-locate with red-fluorescing intracellular
349 membranes, thus suggesting that MSN are internalized *via* endocytosis (Figure 4 d).
350 At TEM, the MSN were roundish and highly electron dense. According to the observations
351 in fluorescence microscopy, aggregates of MSN were found adhering to the cell surface
352 and to be internalized by endocytosis (Figure 4 e). In the cytoplasm, MSN were always
353 found inside ubiquitously distributed vacuoles of various sizes, but were never observed

354 inside the cell nuclei (Figure 4 e, f). After 24 h and 48 h incubation, MSN accumulated
355 inside large vacuoles (probably secondary lysosomes) and sometimes appeared as
356 loosened (Figure 4 g, h). MSN always remained confined inside vacuoles and did not
357 contact any cell organelle. No sign of subcellular alteration or organelle damage was
358 observed at any incubation time in both myoblasts and myotubes.
359
360 PLGA NPs
361 Observations at fluorescence and electron microscopy were similar for the two
362 formulations of PLGA NPs. Confocal fluorescence microscopy showed that, in both
363 myoblasts and myotubes, after 2 h incubation, a few PLGA NPs were present in the
364 cytoplasm; after 24 h incubation, PLGA NPs accumulated in large amount in the
365 cytoplasm, often forming aggregates preferentially located in the perinuclear area, but
366 never entering the cell nucleus (Figure 5 a-c).
367 Overlapping of red fluorescing PLGA NPs and green fluorescing membrane marker
368 suggested the occurrence of endocytotic processes (Figure 5 d).
369 At TEM, PLGA NPs showed a regular roundish shape and moderate electron density.
370 After 2 h incubation, single NPs were seldom observed inside endosomes at the periphery
371 of the cell (Figure 5 e) and some NPs were found to escape from endosomes (Figure 5 f),
372 but most of PLGA NPs were found free in the cytosol (Figure 5 g). After 24 h and 48 h
373 incubation, numerous residual bodies containing roundish moderately electron dense
374 structures (likely remnants of PLGA NPs) accumulated in the cytoplasm (Figure 5 h, i);
375 these particular residual bodies were never found in control cells or in samples treated with
376 the other nanocarriers. PLGA NPs were never found inside the cell nucleus, nor making
377 contact with cytoplasmic organelles. No cell alteration or damage was observed in
378 myoblasts and myotubes at any incubation time.
379

380 NHs

381 Confocal fluorescence microscopy showed that only low amounts of NHs were internalized
382 in myoblasts after 2 h incubation, while after 24 h NHs were present in large quantity in the
383 cytoplasm, especially in the perinuclear region (Figure 6 a,b). They were never found
384 inside the nucleus. In myotubes, NHs were observed in the cytoplasm only after 24 h
385 incubation, but their amount was always very low (Figure 6 c).
386 The red fluorescence of NHs was found to co-locate with the green fluorescing membrane
387 marker (Figure 6 d), thus suggesting that internalization occurs *via* endocytosis.
388 At TEM, NHs appeared as roundish homogeneously electron-dense structures. They were
389 found to adhere to the cell surface inside invaginations of the plasma membrane (Figure 6
390 e) and, in the cytoplasm, a few of them were observed inside endosomes (Figure 6 f).
391 However, most of NHs occurred free in the cytosol, and were often partially surrounded by
392 double membranes, as it typically occurs during autophagic processes (Figure 6 g). Some
393 NH remnants were still recognizable inside secondary lysosomes (Figure 6 h). NHs were
394 never found to make contact with cell organelles or to occur inside the nucleus; moreover,
395 no sign of cell structural alteration was observed in both myoblasts and myotubes.
396

397 **Discussion**

398 This study aimed at investigating the possible influence of cytokinetic features on the
399 cellular response to different nanocarriers (liposomes, MSN, PLGA NPs and NHs)
400 previously demonstrated to be safe for various established cancer cell lines (e.g. Slowing
401 et al., 2006; Arpicco et al., 2013; D'Arrigo et al., 2014; Costanzo et al., 2016; Jonderian
402 and Maalouf, 2016; Quagliariello et al., 2017).
403 Under our experimental conditions, myoblast viability was unaffected by the exposure to all
404 the tested nanocarriers. Consistently, no nanocarrier induced quantitative reduction of
405 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

432 by the very low amounts of nanocarriers observed in the intracellular milieu after 2 h
433 incubation. We may speculate that these differences in cellular uptake may depend on
434 different metabolic rates or peculiar cell features, such as dissimilar membrane
435 composition or endocytotic capability.
436 Anyway, the uptake mechanisms and intracellular fate observed in C2C12 myoblasts
437 correspond to those reported in the literature for other cell types.
438 Liposomes enter the cells by mechanisms different from typical endocytosis, as
439 demonstrated by both fluorescence microscopy (fluorescing liposomes never overlap
440 endosome staining) and TEM (liposomes never occur inside endosomes). Liposomes
441 probably enter the cell by fusing with the plasma membrane (Verma and Stellacci, 2010;
442 Nazarenus et al., 2014); in particular, their internalization may take place by the process of
443 lipid raft-mediated endocytosis (Lanza et al., 2011). Once inside the cytoplasm, as
444 previously observed in HeLa cells (Costanzo et al., 2016), liposomes undergo rapid
445 degradation and migrate in the cytosol towards lipid droplets, probably for chemical affinity:
446 this prevents the intracellular accumulation of liposomes and explains their preferential
447 occurrence at the periphery of the cell. However, in HeLa cells lipid droplets became so
448 numerous to be extruded from the cell (Costanzo et al., 2016) whereas, in C2C12
449 myoblasts, no accumulation and/or extrusion of lipid droplet was observed, probably due
450 to the lower concentrations of liposomes administered.
451 Consistent with previous observations (Poussard et al., 2015; Costanzo et al., 2016; Ricci
452 et al., 2018), fluorescence microscopy and TEM confirmed that MSN enter the myoblasts
453 by endocytosis and follow the endolytic pathway, always remaining confined inside
454 membrane-bounded vacuoles and never entering the cell nucleus. This is probably the
455 reason for the absence of cell injury even after long term exposure (7 days), as reported
456 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

509 biocompatibility of these nanoconstructs is also supported by their inability to enter the cell
510 nucleus, thus avoiding the unpredictable long-term risks of possible interactions between
511 nanomaterials and nucleic acids and/or nuclear protein factors. All these features make
512 these nanocarriers potential candidates for delivering therapeutic agents *in vivo* for treating
513 also diseased differentiated cells which are to be preserved, such as muscle cells in
514 dystrophic patients.

515

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519

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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:

759 nucleus (N), Golgi complex G), mitochondria (M), endoplasmic reticulum (ER). Bars: 500
760 nm.
761
762 **Figure 4. Microscopical analysis of cell-MSN interactions.** Confocal optical sections of
763 myoblasts (a, b) and myotubes (c) 2h (a) and 24h (b, c) after incubation with MSN. DNA
764 was stained with Hoechst 33342 (blue fluorescence)n and the cytoplasm counterstained
765 for actin with phalloidin (red fluorescence). d) A myoblast incubated with the PKH26 red-
766 fluorescing dye to visualise endocytotic vesicles, and then incubated for 30 min with green
767 fluorescent MSN: the two signals co-locate giving rise to yellow fluorescence (the inset
768 shows a 2x magnification of the detail indicated by the small arrows). DNA was stained
769 with Hoechst 33342 (blue fluorescence). Bars: 20 µm (a-c), 10 µm (d). Transmission
770 electron microscopy analysis of MSN intracellular distribution after 2 h (e, f) and 24 h (g)
771 incubation in myoblasts, and after 24 h incubation in myotubes (h). e) Clusters of MSN
772 occur at the cell surface; some of them are enclosed in a membrane invagination (arrow).
773 Internalised MSN occur inside a vacuole (arrowhead). f) Vacuoles containing MSN
774 (arrowheads) occur very close to the nucleus (N). g) After 24 h incubation, in myoblasts
775 MSN accumulate in various vacuolar structures, while the cytoplasm contains many
776 residual bodies (R). h) In myotubes, after 24 h incubation, MSN occur in large vacuoles
777 without perturbing the typical structural organization of cytoplasmic organelles: bundles of
778 myofibrils (asterisks), mitochondria (M), endoplasmic reticulum (ER). Bars: 500 nm.
779
780 **Figure 5. Microscopical analysis of cell-PLGA NPs interactions.** Confocal optical
781 sections of myoblasts (a, b) and myotubes (c) after 2h (a) and 24h (b, c) of PLGA NPs
782 incubation. DNA was stained with Hoechst 33342 (blue fluorescence) and the cytoplasm
783 counterstained for actin with phalloidin (green fluorescence). d) A myoblast incubated with
784 the PKH67 green-fluorescing dye to visualise endocytotic vesicles, and then incubated for

785 30 min with red fluorescent PLGA NPs: the two signals co-locate giving rise to yellow
786 fluorescence (the inset shows a 2x magnification of the detail indicated by the small
787 arrows). DNA was stained with Hoechst 33342 (blue fluorescence). Bars: 20 μm (a-c), 10
788 μm (d). Transmission electron microscopy analysis of PLGA NPs intracellular distribution
789 after 2 h (e, f, g) and 24 h (h) incubation in myoblasts, and after 24 h incubation in
790 myotubes (i). e) A PLGA NP enclosed in an endosome (arrow) occurs just beneath the cell
791 surface. f) A PLGA NP (arrow) is escaping from an endosome. g) Two PLGA NPs (arrows)
792 occur free in the cytosol. h) After 24 h incubation, the cytoplasm contains large amounts of
793 peculiar vacuolated residual bodies where it is sometimes possible to recognize NP
794 remnants (arrowhead). i) In myotubes, after 24 h incubation, the same residual bodies
795 (arrowheads) are frequently present. Nucleus (N), Golgi complex G), mitochondria (M),
796 endoplasmic reticulum (ER), bundles of myofibrils (asterisk). Bars: 500 nm.
797
798 **Figure 6. Microscopical analysis of cell-NHs interactions.** Confocal optical sections of
799 myoblasts (a, b) and myotubes (c) after 2h (a) and 24h (b, c) of NHs incubation. DNA was
800 stained with Hoechst 33342 (blue fluorescence) and the cytoplasm counterstained for actin
801 with phalloidin (green fluorescence). d) A myoblast incubated with the PKH67 green-
802 fluorescing dye to visualise endocytotic vesicles, and then incubated for 30 min with red
803 fluorescent NHs: the two signals co-locate giving rise to yellow fluorescence (the inset
804 shows a 2x magnification of the detail indicated by the small arrow). Due to the slow NHs
805 uptake, co-locations are quite scarce after this short time. DNA was stained with Hoechst
806 33342 (blue fluorescence). Bars: 20 μm (a-c), 10 μm (d). Transmission electron
807 microscopy analysis of NHs intracellular distribution after 2 h (e, f) and 24 h (g, h)
808 incubation in myoblasts. e) An NH (arrow) occurs at the cell surface. f) An NH is enclosed
809 in an endosome (arrow). g) Two NH (arrows) occur free in the cytosol and are partially

810 enclosed by cisternae of the endoplasmic reticulum (small arrows). h) A residual bodies
811 with an NH remnant (arrowhead). Mitochondria (M). Bars: 500 nm.