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*Original*

Biomimetic mesoporous vectors enabling the efficient inhibition of wild-type isocitrate dehydrogenase in multiple myeloma cells / Cauda, V.; Xu, T. T.; Nunes, I.; Mereu, E.; Villata, S.; Bergaggio, E.; Labrador, M.; Limongi, T.; Susa, F.; Chiodoni, A.; Cumerlato, M.; Rosso, G.; Stefania, R.; Piva, R.. - In: MICROPOROUS AND MESOPOROUS MATERIALS. - ISSN 1387-1811. - ELETTRONICO. - 325:(2021), p. 111320. [10.1016/j.micromeso.2021.111320]

*Availability:*

This version is available at: 11583/2922494 since: 2021-09-09T11:36:32Z

*Publisher:*

Elsevier B.V.

*Published*

DOI:10.1016/j.micromeso.2021.111320

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<http://dx.doi.org/10.1016/j.micromeso.2021.111320>

(Article begins on next page)

# 1 **Biomimetic mesoporous vectors enabling the efficient inhibition** 2 **of wild-type isocitrate dehydrogenase in multiple myeloma cells**

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14 Received: date; Accepted: date; Published: date

## 15 **Abstract:**

16 The discovery of isocitrate dehydrogenases (IDHs) mutations in several malignancies has brought  
17 to the approval of drugs targeting IDH1/2 mutants in cancers. More recently it has been suggested  
18 that the enzymatic inhibition of IDHs may have therapeutic potentials also for wild-type IDH  
19 cancers. Specifically, IDH2 inhibition can sensitize multiple myeloma cells to proteasome  
20 inhibitors. However, inhibitors directed against native IDHs are not present on the market. Here, we  
21 exploited an allosteric inhibitor of mutant IDH2 (AGI-6780), known to also decrease the activity of  
22 wild-type IDH2. Since AGI-6780 effectiveness in vivo is limited by its high hydrophobicity and  
23 very low bioavailability, the drug was loaded into mesoporous silica nanoparticles (MSNs) with the  
24 aim to enhance its efficacy. Furthermore, to enable high drug retention into the silica pores, improve  
25 biocompatibility, and reduce the off-target delivery of the drug, a Supported phosphoLipidic Bilayer  
26 (SLB) was self-assembled on the outer MSN surface. The silica nanoparticles were thus coated with

27 three different lipid formulations and characterized in terms of structure, size, and morphology. We  
28 demonstrated that MSN@SLB nanoparticles have improved colloidal stability and  
29 hemocompatibility with respect to pristine MSN. We showed that MSN@SLB formulation displays  
30 an excellent loading and retention of the IDH2 inhibitor AGI-6780, with a limited drug leakage  
31 depending on the lipid formulation. Finally, we proved that AGI-6780-loaded MSN@SLB  
32 nanoparticles efficaciously inhibited the IDH2 enzymatic activity of multiple myeloma cells.  
33 Overall, this study provides a proof of concept of drug delivery to multiple myeloma cells by  
34 repurposing a neglected/dismissed drug (AGI-6780) with the use of smart nanoparticles and  
35 enabling the sensitization of multiple myeloma cells towards other possible treatments.

36

37 **Keywords:** mesoporous silica; supported lipid bilayers; isocitrate dehydrogenases; enzymatic  
38 inhibition; wild-type IDH inhibitors; nanoparticle drug delivery; multiple myeloma.

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## 41 **1. Introduction**

42 The enzymes isocitrate dehydrogenases (IDHs) catalyze the oxidative decarboxylation of  
43 isocitrate, producing alpha-ketoglutarate ( $\alpha$ KG) and  $\text{CO}_2$  [1]. Human cells express three IDH paralogs  
44 (IDH1, IDH2, and IDH3), which differ in the catalytic mechanism, cofactor requirement, and  
45 subcellular localization. Specifically, IDH1 is situated in the cytosol and peroxisomes, whereas IDH2  
46 and IDH3 are in the mitochondria. In recent years, IDHs mutations have been discovered in several  
47 malignancies. The mutant proteins display a new enzymatic activity, able to catalyze the NADPH-  
48 dependent reduction of  $\alpha$ KG to D-2-hydroxyglutarate (D-2HG). The production of the  
49 oncometabolite D-2HG has a critical impact on the epigenetic cell status, affecting differentiation,  
50 metabolism, redox state, and DNA repair mechanisms [2, 3]. The discovery of the role of IDH1 and  
51 IDH2 mutations in oncogenesis has brought to the approval of drugs able to target such mutants in  
52 cancers. As a prominent example, the US Food and Drug Administration has approved the use of  
53 enasidenib (AG-221) and ivosidenib (AG-120), inhibitors of mutated IDH2 and IDH1, respectively,  
54 for the treatment of refractory or relapsed acute myeloid leukemia [4, 5].

55 While the role of mutated IDH1/2 genes has been deeply studied in cancers, the implication of  
56 aberrant expression of the wild-type IDHs enzymes and their role in carcinogenesis have been only  
57 partially investigated. In a recent work, aberrant expression of IDH1, IDH2, and IDH3 has been  
58 reviewed [6]. Concerning the focus of this paper, IDH2 plays a key role in cellular metabolism and  
59 acts in the tricarboxylic acid (TCA) cycle catalyzing the reversible oxidative decarboxylation of  
60 isocitrate to  $\alpha$ KG, NADPH, and CO<sub>2</sub>. In addition, by providing mitochondrial NADPH, IDH2 protects  
61 cells from ROS-mediated oxidative damage, avoiding lipid peroxidation and DNA damage [1].  
62 Therefore, given the critical role of IDH2 in mitochondrial bioenergetics, cellular stress responses  
63 and macromolecular synthesis, its inhibition is expected to impair the growth and survival of cancer  
64 cells [3]. Interestingly, aberrant expression of wild-type IDH2 has been recognized in several cancers  
65 [6-8]. Moreover, it has been reported that the inhibition of either mutated or wild-type IDH2  
66 expressions could increase the efficacy of conventional cancer therapies, such as chemotherapy,  
67 radiotherapy, photodynamic therapy, and small molecule inhibitors [6, 9]. However, inhibitors  
68 specifically directed against wild-type IDHs are not available. Thus, one can exploit the mutant IDH2  
69 inhibitor AGI-6780, which is known also to decrease the activity of wild-type IDH2, although less  
70 potently. The present challenge to face, however, is that AGI-6780 has previously failed the clinical  
71 translation as a free drug, owing to its very low bioavailability and high hydrophobicity *in vivo* [10].  
72 Therefore, here we propose to use hybrid nanoparticles to encapsulate and deliver IDH2 inhibitors to  
73 tumor cells, and thus develop future combination therapies.

74

75 Among the broad scenario of drug-delivery systems, mesoporous silica nanoparticles (MSNs)  
76 offer unique properties, such as a very high porosity (1 cm<sup>3</sup>/g) and a huge surface area (up to 1200  
77 m<sup>2</sup>/g), with very uniform and easily tunable pore sizes (ranging from 2 up to 15 nm) [11], which can  
78 be obtained in a hexagonal symmetry or in a worm-like structure [12-14]. MSNs, with their excellent  
79 biocompatibility and a size from 20 to 200 nm, have been broadly shown to be an ideal smart platform  
80 [15] for carrying and releasing drugs in a site-selective, controlled, and mechanized manner [16].  
81 Importantly, the silica surface, rich of hydroxyl groups, offers not only a highly-hydrophilic nature,  
82 but also efficacious chemical anchoring sites to functional molecules [17], polymers [18], nucleic

83 acids [19], lipid bilayers [20], or other nanoparticles [21] able to improve the therapeutic or diagnostic  
84 capabilities of MSNs. The colloidal stability of MSN in biological media has to be strictly preserved  
85 to avoid aggregation and degradation phenomena [22], as it can negatively affect the endocytosis  
86 process and the further molecule delivery inside the tumor cells. A recent strategy was proposed by  
87 enveloping the MSN in lipidic bilayers. This nanoconstruct, also called “protocell” [23], has several  
88 advantages: besides guaranteeing the colloidal stability of MSNs, it increases the biocompatibility  
89 toward the cell surface, improves the uptake by endocytosis inside the tumor cell, and reduces off-  
90 target delivery of drugs. The lipidic bilayer offers an actionable gate-keeper [24, 25], avoiding the  
91 loss of drug loaded into the mesopores in the extracellular medium, while guaranteeing the drug  
92 delivery only intracellularly. Previous results reported so far in the literature have shown the proficient  
93 use of lipid bilayer-coated silica nanoparticles as smart nanocarriers for anticancer drugs, such as  
94 doxorubicin, paclitaxel, curcumin, irinotecan [26-28], or nucleic acids, such as mRNA or dsDNA [29,  
95 30].

96 In this paper, we analyze for the first time the efficiency of MSN, coated by three different types  
97 of lipidic bilayers (MSN@SLB), to encapsulate a specific inhibitor of IDH2, i.e. the above-mentioned  
98 drug AGI-6780, with the clear purpose to reposition it for future therapeutic treatments, as this  
99 molecule has previously failed the clinical translation as a free drug. MSN@SLB nanoconstructs were  
100 fully characterized in terms of morphology, pore size, colloidal stability, and finally proposed to  
101 shuttle the IDH2 inhibitor to multiple myeloma (MM) cancer cells. MM is indeed a hematological  
102 tumor for which high doses of chemotherapeutics are requested and the risk of disease progression,  
103 drug resistance establishment and relapse are very common [31, 32]. MSN@SLB nanoconstructs  
104 were loaded with AGI-6780 and characterized for their drug uptake and retention ability, with very  
105 limited drug loss depending on the lipid formulation. Then, we demonstrated that the molecular  
106 delivery of AGI-6780 mediated by biomimetic mesoporous vectors can enable the efficient enzymatic  
107 inhibition of wild-type IDH2 in MM cells. The obtained results can thus pave the way for smart drug  
108 delivery [20] of dismissed drugs and propose their future use in combination with other anticancer  
109 molecules [9]. Specifically, we previously demonstrated that the IDH2 inhibitor AGI-6780 triggers  
110 synergistic cytotoxicity with proteasome inhibitors in MM, mantle cell lymphoma, Burkitt

111 lymphoma, and diffuse large B cell lymphoma cell lines, as well as in primary cells from MM patients  
112 and in mouse models of MM [9].

113 In this broader context, the present study provides the first proof of concept on the possibility to  
114 repurpose already developed drugs, i.e. AGI-6780, thanks to the efficient drug shuttling capability  
115 operated by smart porous and biomimetic nanoparticles, thus paving the way to new drug combination  
116 strategies to treat B-cell malignancies.

117

## 118 **2. Materials and Methods**

### 119 *2.1 Synthesis of the Mesoporous Silica Nanoparticles (MSN)*

120 To obtain MSNs, a template-assisted sol-gel self-assembly process was applied. A first solution  
121 was obtained by heating at 90 °C for 20 minutes a mixture of 14.3 g of triethanolamine (TEA, 99%,  
122 Sigma-Aldrich) and 1.92 g of tetraethyl orthosilicate (TEOS, 98%, Sigma-Aldrich) in a 100 mL  
123 polypropylene reactor without stirring. A second solution was prepared by heating at 60 °C a mixture  
124 of 2.41 mL of cetyltrimethylammonium chloride (CTAC, Sigma-Aldrich) and 21.7 mL of milli-Q  
125 water. Then, the two solutions were rapidly combined together and stirred at 500 rpm for 30 minutes.  
126 To obtain an outer surface of the silica nanoparticles functionalized with amino-propyl groups (-NH<sub>2</sub>)  
127 for further dye labelling purposes, a 1:1 molar mixture of 21 µL of TEOS and 16 µL of (3-  
128 aminopropyl)trimethoxysilane (APTES, 98%, Aldrich) was prepared, where the molar amount  
129 corresponds to the 1% of the starting TEOS in the first solution mixture. The 1:1 mixture of TEOS  
130 and APTES was added to the reaction solution after exactly 30 minutes. The reaction was then left to  
131 stir overnight at 500 rpm at room temperature (RT) leading to a white suspension.

132 After that, 100 mL of ethanol (EtOH, 96%, Sigma-Aldrich) was added to the solution and then  
133 centrifuged at 10000 RCF (Relative Centrifugal Force) for 10 minutes.

134 Extraction of the organic template from the MSNs was performed by dispersing the pellet from  
135 the previous centrifugation in a solution containing 2 g of ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>, 98%, Sigma-  
136 Aldrich) in 100 mL of EtOH and heating at 90 °C for 45 minutes under reflux. After that, the MSN  
137 solution was centrifuged at 10000 RCF for 10 minutes. Then the pellet was dispersed in a solution of  
138 10 mL of concentrated hydrochloric acid and 90 mL of ethanol and heated at 90 °C for 45 minutes

139 under reflux. The MSN were then separated by centrifugation at 10000 RCF for 10 minutes, washed  
140 thoroughly with ethanol and centrifuged (10000 RCF for 10 minutes) for at least three times. The  
141 pelleted MSN were finally resuspended in 15 mL ethanol and an aliquot of 500  $\mu$ L was dried to  
142 measure the final concentration, which was between 13.6 mg/mL (as minimum) to maximum 18.7  
143 mg/mL, depending on the synthesis batch, with a yeald of  $18.5\% \pm 0.7$  for all the synthesis performed.

144 Only in case of fluorescence microscopy, the MSNs, thanks to the presence of the amino-propyl  
145 functional groups, were labelled with Atto-647 NHS ester or with Atto-550 NHS ester by reacting 1  
146 mg of MSN particles overnight in dark with 2  $\mu$ g of dye in dimethylformamide, DMF.

## 147 2.2 Drug loading

148 To monitor the drug loading and release tests, UV-Vis absorbance spectra were collected in the  
149 range 200–800 nm by means of a microplate reader (Multiskan™ FC Microplate Photometer, from  
150 ThermoFischer Scientific, interfaced to a PC with the software SkanIt RE) using a 96-well plate  
151 quartz-glass (Hellma™, Hellma Optiks, Jena, Germany). All of the UV spectra were background-  
152 subtracted using the respective medium. AGI-6780 drug was obtained from Selleckchem (Munich,  
153 Germany). Calibration curves of AGI-6780 drug in DMSO, water and cell culture medium (RPMI-  
154 1640, Rosewell Park Memorial Institute) were first collected at 5 different concentrations (0.01  $\mu$ M,  
155 0.1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M and 1 mM) considering the absorption peak at 283 nm. All these solutions  
156 were prepared starting from a stock solution of 10 mM AGI-6780 in DMSO. The calibration curve  
157 was built with Origin 8.5 software and fitted linearly.

158 For uploading the AGI-6780 drug (obtaned from Selleck Chem, München, Deutschland), 1 mg  
159 of MSNs, separated from ethanol by centrifugation, were combined with 0.4 mL of a solution AGI-  
160 6780 at 1 mM solution of drug in DMSO at 350 rpm under magnetic stirring for 1 hour. At the end  
161 of the loading time, each sample was centrifuged at 10000 RCF for 10 minutes and the supernatant  
162 was analysed in triplicates by the UV-Vis Spectrophotometer. A control AGI-6780 sample (in  
163 triplicate) containing the starting amount of drug, i.e. 1 mM in the same volume of DMSO, was used  
164 as Control Sample (CS). From the collected absorbance values, the residual drug concentration (in  
165 terms of  $\mu$ M) in the supernatant was calculated with the calibration curve of AGI-6780 in DMSO.  
166 The adsorbed amount in the MSN sample was evaluated as a subtraction between the absorption of

167 the control sample (CS) and that of the solution after drug loading, using the calibration curve to  
168 calculate the molar amount and thus the amount of uploaded drug, in terms of  $\mu\text{g}$  drug per mg of silica  
169 nanoparticles.

170 At the end of the test, the pelleted MSNs have been stored at  $-20\text{ }^{\circ}\text{C}$  or immediately used.

171 Only for the specific formulation of MSN coated with DOPC-chol-DSPE-PEG, after AGI-6780  
172 loading in pristine MSN, the collected supernatants were also analysed in triplicates and quantified  
173 by High Performance Liquid Chromatography (HPLC) in order to confirm the % encapsulation  
174 efficiency of drug. A Waters 2695 Alliance Separations Module with 2998 PDA detector was used.  
175 Signals were processed by Empower<sup>TM</sup> software (Waters, Milford, MA, USA). Separation was  
176 performed on a SunFire C18  $3.5\mu\text{m}$   $4.6\times 150\text{mm}$  Waters column and 0.1% TFA in water (A) and  
177 0.1% TFA in acetonitrile (B) as solvents, elution initial condition 40% B, isocratic elution 40 % B  
178 over 2min, gradient elution 40–100% B over 23 min, flow rate 1 mL/min and UV detection at 254  
179 nm ( $t_{\text{R}}$ , retention time = 14 min). The injection volume was 50  $\mu\text{L}$ . Calibration curves for AGI-6780  
180 were constructed over the range from  $0.6\ \mu\text{g ml}^{-1}$  to  $5\ \mu\text{g ml}^{-1}$  of AGI-6780 in water and acetonitrile  
181 1:1 (v/v). The limit of quantification was  $0.05\ \mu\text{g ml}^{-1}$ .

### 182 *2.3 Biomimetic lipid bilayer formation*

183 Three different types of lipids were prepared as biomimetic shielding of the nanoparticles. The  
184 first one was composed by DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine, 2.5 mg  
185 Avanti Polar). The second one was formulated by mixing DOPC and DOTAP (N-[1-(2,3-  
186 Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate, Avanti Polar) at a mass percentage  
187 ratio of 70 : 30 (resulting in 1.75 mg DOPC and 0.75 mg DOTAP, i.e. a molar ratio of 2 : 1), as  
188 previously reported [20]. The third lipid composition was prepared mixing DOPC, DSPE-PEG2000-  
189 amine (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000]-amine,  
190 Avanti Polar) and cholesterol (Sigma Aldrich) at a mass percentage ratio of 65.3: 8.6 : 26.1 (resulting  
191 in 2.5 mg DOPC, 0.33 mg DSPE-PEG2000-amine, 1.0 mg cholesterol, i.e. a molar ratio of 55 : 2 :  
192 44, as previously reported [23]). The respective amounts of lipids were mixed in chloroform in glass  
193 vials and dried overnight in dark conditions. Afterward, the dried lipid mixtures were dissolved in 1

194 mL of a solution composed by 600  $\mu\text{L}$  of MilliQ water and 400  $\mu\text{L}$  of 99% ethanol, EtOH (where  
195  $\text{H}_2\text{O}:\text{EtOH}$  are as 60:40 in volume). This mixture guarantees that the lipids are still dispersed as single  
196 macromolecules, preventing their assembly in liposomes. 1 mg of MSNs loaded with AGI-6780 drug  
197 or labeled by ATTO-dyes were separated by the solvent (either DMSO after drug loading or EtOH  
198 after labelling) by centrifugation at 10000 RCF for 5 minutes. The coupling between the lipids and  
199 the MSN was carried out by a solvent exchange method, as also reported in [20]. In particular, 100  
200  $\mu\text{L}$  of lipid solution, prepared as above, was added to each MSN sample, once pelleted after  
201 centrifugation. After slight pipetting, 900  $\mu\text{L}$  of MilliQ water was added, obtaining the samples  
202 MSN@DOPC, MSN@DOPC:DOTAP or MSN@DOPC-choI-DSPE-PEG. In the case of fluorescent  
203 labelling, the lipophilic dye DiOC<sub>18</sub>(3) (3,3'-dioctadecyloxycarbocyanine perchlorate) (DiO;  $\lambda_{\text{ex}} =$   
204 484 nm, Invitrogen, CA, USA) was also added (0.5  $\mu\text{l}$  from a stock dye solution of 10  $\mu\text{M}$  in DMSO)  
205 to each sample.

#### 206 *2.4 Samples characterization*

207 The pristine MSNs were analysed by Fourier-Transformed infraRed (FTIR)-Spectroscopy in  
208 transmission mode to reveal their correct functionalization with amine-groups. Spectra were acquired  
209 with 4  $\text{cm}^{-1}$  resolution and 16 scans accumulation, with a Nicolet 5700 FTIR Spectrometer  
210 (ThermoFisher, Waltham, MA, USA equipped with a RT working DLaTGS detector), and  
211 background subtracted.

212 The specific surface area of the pristine MSN was measured by nitrogen adsorption and desorption  
213 isotherms by using a QUADRASORB evo<sup>TM</sup> Gas Sorption Surface Area and Pore Size Analyzer  
214 instrument from Quantachrome.

215 Transmission Electron Microscopy (TEM) of the pristine MSN, to allow visualization of the  
216 mesopores at high resolution, was performed by a Tecnai F20ST from FEI operating at 200 kV. The  
217 samples were prepared by diluting 6  $\mu\text{L}$  of MSN suspension in ultrapure absolute ethanol (99%,  
218 Sigma) with a final concentration of 100  $\mu\text{g}/\text{mL}$  and drying a drop of the resulting suspension on a  
219 holey carbon-coated copper grid for TEM. With the same instrument, a Scanning Transmission  
220 Electron Microscopy (STEM) analysis was also carried out.

221 To analyse the MSNs size and their Zeta potential, before and after lipid bilayer formation, the  
222 nanoparticles were characterized by Dynamic Light Scattering (DLS) and Z-potential analysis with a  
223 Zetasizer Nano ZS90 (laser source He-Ne of 633 nm). Samples were measured in polystyrene  
224 disposable cuvettes for DLS and electrode-equipped and folded capillary cell cuvettes for Z-potential  
225 measurements. A volume of 1 mL was used for each samples at a concentration of 500  $\mu\text{g}/\text{mL}$  either  
226 in water and ethanol, for DLS measurement, and in water for Z-potential ones.

### 227 *2.5 Nanoconstruct stability over time*

228 To investigate the effective residence time of the AGI-6780 drug in the MSN pores during the  
229 lipid bilayer formation, as well as the possible sealing effect of the different lipid bilayers over time,  
230 the nanoconstruct stability was also tested in water medium only, i.e. the starting water used for the  
231 self-assembly of the lipid bilayer. The drug-loaded MSNs (0.5 mg) coated by lipids were centrifuged  
232 and transferred in 500  $\mu\text{L}$  water solution, as used during lipid self-assembly in static conditions at  
233 either 4  $^{\circ}\text{C}$  or 37  $^{\circ}\text{C}$ . At selected time points, i.e. 30 min, 4 h, 24 h and 48 h, the drug-loaded particles  
234 were processed as in the drug-release experiments (see Paragraph 2.6) and the water supernatant  
235 analysed by microplate reader to collect the UV-Vis absorbance spectra of the drug. With the use of  
236 the calibration curve of AGI-6780 in water, the amount of leaked AGI-6780 was then calculated.

### 237 *2.6 Drug release*

238 The release of AGI-6780 drug was first performed in acellular media, using aliquots of 100  $\mu\text{g}$   
239 each of drug-loaded MSN@SLB nanoparticles and drug-loaded pristine MSN without lipids as  
240 control. As all these samples were prepared in water medium, they were first centrifuged at 10000  
241 RCF for 5 minutes. The water supernatant was then removed and the 100  $\mu\text{g}$  sample aliquots were  
242 dispersed in 1 mL of RPMI 1640 (Pan Biotech), thus having a final concentration of MSN@SLB in  
243 RPMI of 100  $\mu\text{g}/\text{mL}$  (five replicates were used for each sample type), and then placed in the orbital  
244 shaker at 37  $^{\circ}\text{C}$  and speed 200 rpm. After selected time points of 2h, 4h, 6h, 24h, 48h, 72h, 96h (4d)  
245 and 168h (7d), the samples were centrifuged (10000 RCF for 5 minutes), the supernatant was  
246 separated from the MSN@SLB to stop the drug release and three aliquots of 100  $\mu\text{L}$  from the RPMI  
247 supernatant were analysed by a microplate reader. Thus, the total amount of aliquots analysed per  
248 each sample type at each time point of the release was in total 15, allowing to calculate the standard

249 deviation as error bar. The UV-Vis absorbance was acquired at 283 nm, according to the previous  
250 calibration curve of AGI-6780 in RPMI-1640, which was used to estimate the delivered concentration  
251 of drug in RPMI medium from the NPs.

252 After the reading, the aliquots were recovered and combined with their remaining supernatant  
253 and then back to each respective sample. Then the MSN@SLB were dispersed by a vortex mixer and  
254 then placed into the orbital shaker at 37 °C to further proceed with the drug release.

255 As controls, three samples of RPMI only (1 mL for each one) were processed in the same way as the  
256 samples containing the drug-loaded NPs and used for the background subtraction at each microplate  
257 reading.

### 258 *2.7 Addition of Triton X in Drug release from MSN@SLB*

259 To prove that the lipid coating hinders like a barrier to the drug release from the MSN, 100 µL  
260 of the surfactant Triton X-100 (Sigma-Aldrich) were added in each drug-loaded MSN@SLB at the  
261 end of the drug release experiments in RPMI-1640 medium, as already described above. It is actually  
262 expected that the surfactant would disassemble the lipid bilayer constituted on the MSN, thus  
263 promoting the release of the encapsulated drug. The analysis of the supernatants after the Triton X-  
264 100 addition was done 48 hours later, to lead enough time to the drug to out-diffuse. Then  
265 centrifugation to separate the MSN particles was operated and the analysis on the supernatant again  
266 performed by UV-Vis spectrometry at 283 nm.

### 267 *2.8 Fluorescence microscopy imaging*

268 Each sample of MSNs coupled with lipids ready after preparation (at the concentration of 1  
269 mg/mL) was characterized through fluorescence microscopy with a co-localization method to  
270 evaluate the percentage of coupling between lipids and MSNs. Samples were prepared by  
271 withdrawing 10 µL of the lipid-coated MSN solution and depositing them on the microscope slide;  
272 then the drops were covered with a cover-glass slip and this was fixed with a common nail polish.  
273 The images were acquired using a wide-field optical fluorescence microscope Nikon Eclipse Ti,  
274 equipped with a super-bright wide spectrum Shutter Lambda XL source with a collection of four filter  
275 cubes. The images were acquired with 60x and 100x PLAN-APO immersion oil objectives and the  
276 data analyzed by the NIS-element software. MSNs were labelled with Atto-647 NHS ester or with

277 Atto-550 NHS ester and the lipids with DiOC<sub>18</sub>, as described above. Images were thus acquired by  
278 exciting the dyes at two different wavelength channels: 647 nm (far-red channel) and 488 nm (green  
279 channel). The colocalization tool of NIS-Element software (NIS-Elements AR 4.5, Nikon) was used  
280 to evaluate the coupling percentages, as previously reported [20, 33, 34]: after setting a threshold  
281 between 0.1 and 1  $\mu\text{m}$  to disregard larger aggregates, the spots in the red channel (identifying the  
282 MSNs) and green channels (corresponding to the lipid bilayer vesicles) were counted and an overlay  
283 of the two images was performed, counting only the spots in which the two fluorescences were  
284 colocalized. At least 10 fields of view of each sample were analyzed by this automatic routine by  
285 applying a dimensional threshold. The percentage of colocalization was then calculated with respect  
286 to the MSN channel with the following formula, Equation 2:

$$287 \quad \% \text{MSNs colocalized} = (n^\circ \text{MSNs colocalized}) / (n^\circ \text{MSNs}) \quad [\text{Eq. 2}]$$

288

289

290

### 291 *2.9 Haemocompatibility tests*

292 To assess nanoparticles' hemocompatibility, the plasma re-calcification time was measured  
293 following the activation of prothrombin in the presence of calcium cations ( $\text{Ca}^{2+}$ ), as previously  
294 described [35-37]. Here, two identical sets of samples were prepared in citrated plasma analyzed  
295 contextually: one was treated with calcium chloride and showed coagulation, the other was left  
296 without calcium chloride and used as control. Pristine MSN and MSN@DOPC-cholesterol-DSPE-PEG after  
297 overnight dialysis were washed twice with water and then centrifuged (10000 RCF for 5 min). Under  
298 sterile conditions, each pellet was resuspended in 1 mL of a 0.1  $\mu\text{m}$  filtered physiological solution  
299 (water with 0.9% NaCl). Samples were then diluted with physiological solution to obtain two  
300 concentrations each: 100  $\mu\text{g}/\text{mL}$  and 50  $\mu\text{g}/\text{mL}$ . In details, a 96-well plate was prepared with 75  $\mu\text{L}$   
301 of human citrated plasma (Human Recovered Plasma Pooled-frozen – NaCitate from ZenBio) per  
302 each well. Then, 75  $\mu\text{L}$  of pure physiological solution or two different sample concentrations were  
303 added. The plate was first incubated at 37°C for 5 min, 150  $\mu\text{L}$  calcium chloride (25 mM) were

304 quickly added and the plate was immediately read with an UV-Vis spectrometer (pre-heated at 37°C).  
305 Specifically, samples were divided in two groups: the samples (and related control solutions) treated  
306 with calcium chloride (CaCl<sub>2</sub>, 25 mM) and untreated samples (each of them in triplicate). The plasma  
307 clotting protocol consists in the periodical reading of the UV absorbance of the samples at 405 nm,  
308 as a change of turbidity of the solutions of recalcified plasma due to the formation of fibrins: a measure  
309 was collected every 30 s for 45 minutes at 37 °C. The coagulation time ( $t_c$ ) was calculated as the time  
310 point corresponding to the central absorbance point ( $a_c$ ), calculated as follows:  $a_c = \min(a) + [\max(a)$   
311  $- \min(a)]/2$ , where  $a$  is the vector of the UV absorbance values of the sample. Two independent  
312 experiments were carried out.

### 313 *2.10 Cell cultures*

314 Human multiple myeloma (MM) cell line KMS-28 was kindly provided by Prof. Antonino Neri  
315 (University of Milan) and authenticated by DNA fingerprinting using GenePrint system (Promega,  
316 Madison, Wisconsin, USA). Cells were maintained in RPMI 1640 medium (EuroClone, Pero, Italy),  
317 supplemented with 2 mM of L-glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin  
318 (Gibco), 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, Missouri, USA), and grown at 37°C  
319 in a humidified atmosphere with 5% CO<sub>2</sub>.

320

### 321 *2.11 IDH enzymatic activity*

322 To test the ability of lipid-coated nanoparticles to intracellularly release AGI-6780, IDH2  
323 enzymatic activity was measured after 6 hours and 24 hours post treatment in mitochondrial extracts  
324 derived from KMS-28 cells previously incubated with empty and AGI-6780-loaded MSN@SLB  
325 nanoconstructs. Controls were performed treating cells with soluble AGI-6780 (5 µM), used as  
326 positive control, or left untreated (UT).

327 Isocitrate Dehydrogenase Activity was measured using the IDH assay kit (Sigma-Aldrich, St.  
328 Louis, Missouri, USA), according to the manufacturer's protocol. IDH activity was determined using  
329 isocitrate as a substrate of the reaction, which results in a colorimetric (450 nm) product proportional  
330 to the enzymatic activity present. One unit of IDH is the amount of enzyme that generates 1.0 µmole

331 of NADH or NADP per minute at pH 8.0 at 37 °C. IDH2 activity is reported as milliunit per milligram  
332 of extracted protein (mU/mg).

333 AGI-6780 was loaded into MSN and the lipid bilayer formulations DOPC or DOPC-cholesterol-DSPE-  
334 PEG were self-assembled on drug loaded MSN (as reported above). Only for the MSN@ DOPC-  
335 cholesterol-DSPE-PEG, this sample was dialyzed in 1 L Phosphate Buffer Saline (PBS) by magnetic stirring  
336 (200 rpm) for 20 hours at room temperature. The dialysis membrane had a cut-off of 3.5 K MWCO  
337 (SnakeSkin Dialysis Tubing, Thermo Scientific). MSN@SLB was then collected from the dialysis  
338 bag, centrifuged (10'000 RCF, 5 minutes), and the supernatant was analyzed by HPLC to exclude  
339 drug leakage from nanoparticles. Finally, all the drug-loaded MSN@SLB were pelleted by  
340 centrifugation and dissolved in cell culture medium and aliquoted (at 25 or 100 µg/ml) for further  
341 incubation with KMS-28 cells.

342

343

### 344 **3. Results**

#### 345 *3.1. MSNs design and characterization*

346 To efficiently load and deliver the drug in a controlled manner, without off-target release, MSNs  
347 with a size of around 50 nm and mesoporous pore size of about 3 nm were designed and then  
348 produced. As described in the Materials and Methods section, a template-assisted sol-gel chemical  
349 synthesis was applied to obtain MSNs displaying amine-functional groups at the external nanoparticle  
350 surface. Amine groups enable the labelling of the MSNs with fluorescent dye for fluorescence  
351 microscopy studies, and allow a positively charged surface, thus increasing their z-potential, which  
352 is beneficial for the nanoparticle stability in water media.

353 TEM and STEM show the highly porous MSN, nanoparticles diameter of around 50 nm and  
354 pores of around 3 nm (Figure 1a and b). Mesopores have a worm-like structure, asymmetrical but  
355 highly interconnected, ideal to store high amount of drug molecules. The nitrogen sorption isotherm  
356 in Figure 1c is of Type IV, which is typical of mesoporous materials. It further confirms that the  
357 obtained MSNs have both a specific surface area (912.7 m<sup>2</sup>/g), as evaluated by BET model, as well

358 as a high pore volume (around 1.185 cm<sup>3</sup>/g) with a uniform pore size of 3 nm, as evaluated by DFT  
359 model. Such properties may guarantee a high adsorption level of the drug. FTIR in Figure 1d shows  
360 the fingerprint of silica and confirms the chemical surface functionalization with amino-propyl  
361 groups. In particular, at 1063 cm<sup>-1</sup> and at 1080 cm<sup>-1</sup> the -OH bond bending vibration and the  
362 characteristic peak of the silicon oxide are observed, respectively. The broad band from 3000 to 3600  
363 cm<sup>-1</sup> is related to the stretching vibrations of -OH groups, while those from 3000 to 2800 cm<sup>-1</sup> are  
364 ascribed to the alkyl groups (-CH<sub>x</sub>) related to the propyl chain of the APTES functional moiety.  
365 Finally, at 3700 cm<sup>-1</sup> the stretching vibration related to the amine groups, grafted at the nanoparticles  
366 surface, is observed.

367 The MSNs size distributions in both ethanol (EtOH) and water were measured by DLS and Z-  
368 potential measurements. As reported in Figure 2.a and Table 1 (first row), these amine-functionalized  
369 nanoparticles show a moderate agglomeration, as compared with the TEM results, both in ethanol  
370 and water solutions, with a hydrodynamic diameter of 220 nm and 190 nm, respectively. They also  
371 have a polydispersed size distribution in both media, with PDI (PolyDispersive Index) of 0.83 in  
372 ethanol and 0.91 in water. The Zeta potential value is of +26 mV, accounting to the amine group  
373 protonation.

374

### 375 *3.2 MSNs drug loading and lipid coating*

376 Drug loading was performed on the MSN before self-assembling the lipidic bilayer, obtaining  
377 22.2 ± 1.4 µg of loaded drug per 1 mg of silica (n=8, see Table 2, first row). Considering the molecular  
378 structure of the drug AGI-6780 (as provided by the producer, Selleckchem), the molecule displays  
379 three amine groups which can potentially establish hydrogen interactions with hydroxyl group present  
380 on the silica pore surface, thus allowing an efficient drug physisorption.

381 After drug adsorption, the AGI-loaded MSNs were coated by three different lipid mixtures (as  
382 detailed in the Material and Method section) to form lipid bilayers, obtaining AGI-MSN@SLB. The  
383 aim of this Supported phosphoLipidic Bilayer (SLB), self-assembled on the outer MSN surface, is to  
384 induce high drug retention of the drug molecule loaded in the silica pores and further improve the  
385 MSNs stability in water-based media. The three different lipid formulations, i.e. DOPC, DOPC-

386 DOTAP, and DOPC-chol-DSPE-PEG, where selected according to the previous literature related to  
387 our group [20, 33, 38, 39] for the first two formulations, while the third one was explored according  
388 to similar formulations reported in [23]. The process used to self-assemble the phospholipidic bilayers  
389 on the silica outer surface is based on the solvent exchange method, as previously reported [20, 33].  
390 Briefly, the driving idea is to have lipid mixtures in a solution of 60 %vol. water and 40 %vol. EtOH,  
391 which guarantees that the lipids are still dispersed as single macromolecules, preventing their  
392 assembly in liposomes. Then, after being in contact with silica nanoparticles, the water content is  
393 dramatically increased, forcing the phospholipids to self-assemble as bilayers on the most  
394 energetically favoured conditions, i.e. the surface offered by the silica nanoparticles.

395 To prove the effective coating of SLB on the MSN, dynamic light scattering (DLS), Z-potential  
396 measurement, and co-localization fluorescence microscopy experiments were firstly run using the  
397 unloaded MSNs.

398 The DLS results are reported in Figure 2, showing a comparison of the hydrodynamic size  
399 distributions among the pristine MSN in water (black curves) versus the MSN@SLB (in Figures from  
400 2b to 2d). As it can be observed, both the size distributions and the related PDIs of each MSN@SLB  
401 are lower than the pristine MSN, pointing out a fairly well-dispersed distribution peaking at 142 nm  
402 for MSN@DOPC (red curve), at 106 nm for MSN@DOPC-DOTAP (pink curve), and at 106 nm  
403 MSN@DOPC-chol-DSPE-PEG (orange curve). In contrast, both the hydrodynamic size and the  
404 related PDI of pristine MSNs show that the sample easily aggregates.

405 The improved stabilization of lipid-coated silica nanoparticles is also supported by the variation  
406 of the Z-Potential values (Table 1). These data confirm the coating of the nanoparticles by lipid  
407 bilayers and supporting their role as efficient steric stabilizer preventing the aggregation of MSN in  
408 water, as also previously reported [20]. In particular, the use of cationic lipids like DOTAP and DSPE-  
409 PEG, having indeed an amine terminal (DSPE-PEG2000-amine, as reported in the Materials and  
410 Method section) account for a positive value of Z-potential in water. To predict the behavior in cell  
411 culture medium, Z-potential measurements were also performed in RPMI-1640 supplemented with  
412 10% of FBS at 37°C, which is actually the same conditions used to culture the KMS-28 cancer cells.  
413 The values (see Table 1, second column) show that all MSN@SLB nanoconstructs report a negative

414 Z-potential value, attributed to the adsorption of proteins on the lipid bilayer surface once immersed  
415 in the cell culture medium, also in fair agreement with the previous literature [39, 40]. The z-potential  
416 of pristine MSN was not recorded, as the material aggregates and precipitates almost immediately  
417 when in contact with the medium at 37 °C.

418 Further wide-field fluorescence microscopy images in Figure 3 show in the green channel (left  
419 panels) the DiOC<sub>18</sub>-labelled phospholipids, in the red channel (middle panels) the Atto 550-labelled  
420 MSNs, and the merged channels in the right panels, demonstrating a broad level of colocalization of  
421 the dyes in all the three MSN@SLB formulations. It has to be noted that the optical resolution of  
422 wide-field fluorescence microscopy is not high enough to allow for a resolution of single MSN@SLB  
423 nanoparticles. Here, the colocalization technique is just used to support the previous characterizations  
424 and provide an estimation of the colocalization % of the two dyes. Such estimation leads to values of  
425 24% for MSN@DOPC, 60% for MSN@DOPC-DOTAP, and 55% MSN@DOPC-choI-DSPE-PEG.  
426 These data, together with the other characterizations, in particular DLS and Z-Potential, confirm with  
427 good confidence that most of MSNs are encapsulated by the phospholipidic bilayers. The possible  
428 empty lipids nanoparticles are not relevant for the further evaluation in terms of drug encapsulation,  
429 release and cell viability experiments, as they are empty vesicles, while the drug is sealed inside the  
430 silica nanoparticles.

431 It has to be noted that, during the lipid self-assembly process, drug-loaded MSNs are thoroughly  
432 mixed with the lipids solution (made of 60% water and 40% EtOH, as mentioned above) for few  
433 seconds, thus a certain amount of drug is leaked out from the silica pores and dissolved in the medium  
434 before the lipid self-assembly on the silica surface. Actually, by analyzing with UV-Vis absorption  
435 the solution after the lipid self-assembly, various amounts of drug were found in solution, depending  
436 on the lipid bilayer type. The drug loss in water after immediate constitution of the lipidic bilayer was  
437 found to be relatively high, i.e. up to 21 µg per mg of silica for the sample MSN@DOPC, while less  
438 in MSN@DOPC-DOTAP samples (up to 10 µg of AGI-6780 per mg of silica) and almost zero in  
439 MSN@DOPC-choI-DSPE-PEG. The remaining amount estimated in each MSN@SLB is reported in  
440 Table 2. We hypothesize that the lipid formulation containing DOPC only is not sufficiently stable  
441 and compact to prevent the drug to leak out from the silica surface.

442

### 443 3.3 MSN@SLB stability over time and release

444 The previous results motivated us to further investigate the stability over time of the MSN@SLB  
445 in water at 4 °C and 37 °C, in order to understand how long and at which conditions they can be  
446 eventually stored prior to biological tests. In particular, the stability of the three nanoconstructs after  
447 24 hours in water was evaluated, calculating the amount of leaked drug in water media as a function  
448 of storage temperatures, i.e. 4 °C and 37 °C, before getting the nanoconstructs in contact with cell  
449 cultures. Figure 4a show that the highest leakage is for sample MSN@DOPC at both temperatures,  
450 followed by MSN@DOPC-DOTAP, in line with what obtained above when analyzing the solution  
451 immediately after the lipid self-assembly. We observed that the DOPC-chol-DSPE-PEG lipid  
452 formulation shows the best drug retention, most likely as a consequence of the fluidity impaired to  
453 the lipid membrane by cholesterol, thus it can be the preferred formulation for MSN@SLB.

454

455 Preliminary drug release tests in cell culture medium in absence of cells were then conducted on  
456 the AGI-loaded silica (i.e. MSN@DOPC-DOTAP and MSN@DOPC-chol-DSPE-PEG) at 37 °C in  
457 RPMI-1640 medium and, as a reference, on the uncoated AGI-loaded silica nanoparticles. It has to  
458 be noted that the possible drug leaked in water medium after the lipid self-assembly was discarded  
459 by centrifugation and the pelleted nanoconstructs resuspended in fresh RPMI-1640 medium.

460 Figure 4b reports the progressive release of the hydrophobic drug AGI-6780 out from the pores  
461 of MSN as percentage of drug released with respect to the total adsorbed amount. Concerning the  
462 uncoated MSN, it is noticeable that the drug release trend increases in the first 24 hours up to 26 %  
463 of the total adsorbed drug in the silica (corresponding to  $6\pm 2$   $\mu\text{g}/\text{mg}$ ), then it remains constant until  
464 72 hours of release. At last, after 5 days, the release increases sharply. At the end of the test, i.e. after  
465 7 days, the release curve has not reached a plateau yet, corresponding to around 55% of the total  
466 loaded drug, i.e.  $12\pm 2$   $\mu\text{g}/\text{mg}$ .

467 In contrast, when a lipidic bilayer is self-assembled on the MSN surface after the loading of AGI,  
468 almost no drug release is observed in the cell culture medium, except for a first small burst release  
469 probably due to a partial retention of drug in the outer lipid bilayer (see more details in the inset of

470 Figure 4b). This absence of drug release is persistent up to 7 days of daily monitoring, reaching in the  
471 worst case around 2.2% of released drug with respect to the total adsorbed amount, which corresponds  
472 to 0.3  $\mu\text{g}/\text{mg}$  of AGI-6780. This result actually demonstrates that the lipid bilayer, irrespectively from  
473 its composition (DOPC-DOTAP or DOPC-chol-DSPE-PEG), is able to tightly seal the AGI-6780  
474 inhibitor inside the silica mesopores, providing possibly a successful drug release only once  
475 internalized into cancer cells, as shown below.

476 After the above-reported drug release, an artificial breakage of the lipidic membrane is operated  
477 by adding Triton X-100, a surfactant well-known for its ability to disrupt the self-assembly of lipidic  
478 bilayers, as reported in [20]. Punctual release of AGI-6780 is observed reaching the average  
479 concentration of  $14.6\pm 4$   $\mu\text{g}$  per mg of nanoparticles in 48 hours in the releasing solution for all  
480 MSN@SLB lipid formulations yet corresponding almost to 66% of the total AGI-6780 incorporated  
481 initially in the MSN pores, or almost completely when considering the residual amount after lipid  
482 self-assembly (Table 2). Therefore, this triton-triggered release demonstrated, as previously reported  
483 in the literature [20], the effective sealing operated by the lipids and thus the ability of the whole  
484 MSN@SLB to reach intact and without drug loss the target cells.

485

486

487

#### 488 *3.4 Haemocompatibility of MSN@SLB*

489 The haemocompatibility test consists in the evaluation of the time necessary for plasma to clot, in  
490 presence of nanoparticles. Normally, plasma is treated with anticoagulants in order to prevent clotting  
491 and allow its proper storage and employment. In the present case, we therefore used plasma citrate.  
492 The anticoagulant effect of plasma citrate was overridden by calcium chloride ( $\text{CaCl}_2$ ) which typically  
493 induces a rapid calcification and thus clotting in about 10 minutes, with increase of turbidity. The  
494 obtained results of coagulation time in presence of pristine MSN and MSN@DOPC-chol-DSPE-PEG  
495 at two different concentrations (50 and 100  $\mu\text{g}/\text{ml}$ , without drug) are reported in Figure 5 and  
496 compared to the control solutions, i.e. citrate plasma, citrate plasma with physiologic solution, and all  
497 the samples without the coagulating agent, calcium chloride. Two independent tests were conducted

498 (here only one is shown as representative result for both) showing notably similar results, suggesting  
499 a good repeatability of the test.

500 The data show a clear difference between all the control samples (not clotted, see dashed lines)  
501 and samples treated with  $\text{CaCl}_2$  (clotted plasma, solid lines). Furthermore, it is worth to note that the  
502 plasma containing the MSN@SLB nanoparticles coagulates at nearly the same time (around 9.5  
503 minutes) of the pure citrate plasma and citrate plasma together with physiological solution. In  
504 contrast, the plasma containing the uncoated MSNs samples at both concentrations coagulate earlier  
505 than the other samples, i.e. after about 4 minutes. More in details, there is a statistical significant  
506 difference between the uncoated MSNs and the lipid-coated ones at both concentrations, i.e. at 50  
507  $\mu\text{g/mL}$  (with P value equals to 0.0019) and at 100  $\mu\text{g/mL}$  ( $P = 0.0225$ ).

508 These results indicate that the lipid bilayer coating is fundamental for haematological compatibility  
509 of the proposed silica nanoparticles, as also previously reported by some of us with similar tests on  
510 polymer-coated MSNs [41]. These data can be also considered as a demonstration of the efficient and  
511 stable lipidic coating on the MSNs surface.

512

513

### 514 *3.5 IDH2 enzymatic inhibition tests of AGI-6780-loaded MSN@SLB*

515 To test the ability of the lipid-coated nanoparticles to efficiently release the IDH2 inhibitor to  
516 multiple myeloma cells, AGI-6780-loaded MSN@SLB nanoconstructs were incubated with KMS-28  
517 cells. The effects on the mitochondrial enzyme IDH2 was then evaluated. It was decided to work with  
518 the MSN@SLB particles with either the lowest amount of loaded drug (i.e. MSN@DOPC, at a high  
519 concentration 100  $\mu\text{g/mL}$ , where thus the amount of 0.3  $\mu\text{g}$  of drug is calculated, according to Table  
520 2), to look at the possible most critical or even inefficient conditions, as well as with the optimized  
521 nanoconstruct (i.e. MSN@DOPC-chol-DSPE-PEG, lowering the concentration to 25  $\mu\text{g/mL}$ ), which  
522 have shown the highest colloidal stability and drug retention over time. For this sample, the average  
523 amount of AGI loaded in 1 mg of MSN is estimated as 2.5  $\mu\text{g}$  after the dialysis process with HPLC  
524 (see Materials and Method section), corresponding to a drug concentration in 1 mL of cell medium

525 of 0.13  $\mu$ M (considering the nanoconstruct concentration of 25  $\mu$ g/mL used in this enzymatic  
526 inhibition test) .

527 Table 3 shows the results of enzymatic inhibition after 6 and 24 hours calculated as the inhibition  
528 percentage of drug-loaded MSN@SLB with respect to the control, i.e. MSN@SLB constructs without  
529 drug. The data also displays the enzymatic activity inhibition in cells treated with the drug dissolved  
530 at a concentration of 5  $\mu$ M in the cell medium with respect to the untreated cells.

531 While untreated and MSN@SLB-treated cells did not display any enzymatic activity inhibition, both  
532 AGI-6780-loaded MSN@SLB nanoconstructs inhibited IDH2 activity in a time-dependent manner.  
533 It is worth noting that drug-loaded MSN@DOPC and MSN@DOPC-chol-DSPE-PEG slightly affect  
534 the endogenous IDH2 activity at 6 hours, while this inhibition increases after 24 hours. As a positive  
535 control, 5  $\mu$ M AGI-6780 dissolved in the cell culture medium showed a comparable but higher  
536 enzymatic inhibition. This is known to occur in in-vitro tests [9], while in-vivo data or further clinical  
537 translation for the free AGI-6780 could not be obtained for its low solubility and short half-life in  
538 liver microsomes [10]. Therefore, the contribution of the here proposed nanoconstructs, able to carry  
539 efficiently the drug and deliver it intracellularly, is a valuable option for further in-vivo and clinical  
540 translations.

541 It is worth to note that these experiments are just a proof of the principle, but they already confirm  
542 not only the efficacy of AGI-6780 as a wild-type IDH2 inhibitor, but also that the drug is efficiently  
543 delivered to cells by the formulated nanoconstructs and a low amount of nanocarried drug is thus  
544 sufficient to induce a consistent enzymatic inhibition.

545

546 It has to be also pointed out that AGI-6780 is not per se cytotoxic at concentrations that  
547 specifically inhibit IDH2 activity. Therefore, its clinical relevance could be detectable only in  
548 combinations with other drugs, such as chemotherapeutics or proteasome inhibitors [6]. In particular,  
549 we have previously shown that wild type IDH2 inhibition sensitizes B-cell malignancies to  
550 proteasome inhibitors [9]. Based on this previous knowledge, the current results open the way to new  
551 possible nanotechnological strategies, enabling the synergistic treatment of nanocarried IDH2  
552 inhibitor with chemotherapeutics or proteasome inhibitors.

553

## 554 **5. Conclusions**

555 In the present study, we have described the preparation of mesoporous silica nanoparticles  
556 shielded by various formulations of lipid bilayer coatings to be used efficiently as drug delivery  
557 vehicles. The colloidal stability, the haemocompatibility, the efficient drug loading and release  
558 capabilities of such nanoconstruct were demonstrated. Specifically, we have shown that porous  
559 nanocarriers loaded with the IDH2 inhibitor AGI-6780 are suitable vectors for efficient drug retention  
560 and cancer cell delivery. Furthermore, we have demonstrated that such drug-loaded nanoconstruct  
561 could successfully inhibit wild-type IDH2 enzymatic activity in multiple myeloma cells.

562 Our study provides a proof-of-concept instrumental to reposition unapproved drugs and to  
563 propose their therapeutic use through nanocarrier administration. We suggest that inhibition of wild-  
564 type IDH2 can become a potential therapeutic option for synergistic treatments in multiple myeloma  
565 aimed at sensitizing drug-resistant cancer cells. Future studies will focus on the design of nanoparticle  
566 delivery systems equipped with high-affinity ligands capable of targeting drug combinations towards  
567 cancer cells while sparing healthy tissues. In conclusion, in this paper we report a preliminary but  
568 important step toward in-vivo translation and the application of nanosized weapons in the fight against  
569 cancer and, possibly, toward other diseases.

570

## 571 **6. Patents**

572 A patent is already filed with research report leading to part of the work reported in this  
573 manuscript. Title: "A biomimetic nanoporous carrier comprising an inhibitor directed towards the  
574 native form of IDH2 protein" PCT: IB2020/050401 of 20<sup>th</sup> Jan. 2020 (First priority: "Vettore  
575 nanoporoso biomimetico comprendente un inibitore diretto verso la forma nativa della proteina  
576 IDH2" Italian Patent N. IT102019000001009, priority on 23<sup>rd</sup> January 2019) Inventors: V. Cauda, R.  
577 Piva, T. Limongi, L. Racca, M. Canta, F. Susa, E. Bergaggio, N. Vitale, E. Mereu.

578

579 **Funding:** This research was funded by AIRC Investigator Grant N. IG 21587 from the Italian  
580 Association of the Cancer Research to Prof. Roberto Piva and was carried out in the framework of  
581 the project PoC Instrument granted to Prof. Valentina Cauda by LINKS, LIFTT and Compagnia di  
582 San Paolo.

583

584 **Conflicts of Interest:** The authors declare no conflict of interest.

585

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704 23-25, 27-29, 35-41

## 705 TABLES

706 **Table 1.** Z-Potential measurements of the pristine and lipid-coated MSNs in MilliQ water at room  
707 temperature or cell culture medium (RPMI-1640 completed with 10% of Fetal Bovine Serum, FBS)  
708 at 37 °C, which is actually the same conditions used to culture the KMS-28 cancer cells.

709

Sample	Z-Potential in water (mV)	Z-Potential in cell culture medium (mV)
MSN	26±3	n.a. *
MSN@DOPC	0.8±0.2	-8.1±1.6
MSN@DOPC-DOTAP	32±3	-10±3
MSN@DOPC-choI-DSPE-PEG	29.0±1.3	-6.0±0.9

710

711 \*This data is not available due to the imminent aggregation and precipitation of the pristine MSN  
712 in cell culture media.

713

714 **Table 2.** Amount of drug adsorbed in the MSN (calculated for 1 mg of silica) and remaining after  
715 the lipid self-assembly.

716

Sample	Drug loading amount [ $\mu\text{g}$ drug per 1 mg of silica]
MSN	$22.2 \pm 1.4 \mu\text{g}/\text{mg}$
<b>Residual amount after lipid self-assembly</b>	
MSN@DOPC	$1 \pm 1 \mu\text{g}/\text{mg}$
MSN@DOPC-DOTAP	$12 \pm 4 \mu\text{g}/\text{mg}$
MSN@DOPC-chol-DSPE- PEG	$21.91 \pm 0.05 \mu\text{g}/\text{mg}$

717

718 **Table 3.** Mitochondria-extracted IDH2 enzymatic activities using AGI-6780 loaded in  
719 MSNs@DOPC ( $100 \mu\text{g}/\text{mL}$ ), MSN@DOPC-chol-DSPE-PEG ( $25 \mu\text{g}/\text{mL}$ ) nanoconstructs and  
720 referred to the enzymatic activity of empty nanoconstructs counterparts. KMS-28 cells were also  
721 treated with  $5 \mu\text{M}$  AGI-6780 as positive control and its value reported to untreated cells. The IDH2  
722 activity was monitored at 6 hours and 24 hours post-treatment.

723

Time of inhibition assay	% of enzymatic activity with respect to controls		
	AGI-6780 loaded in MSNs@DOPC	AGI-6780 loaded in MSNs@DOPC- chol-DSPE-PEG	Free AGI-6780
After 6 hours	$103.57 \pm 0.14$	$87.32 \pm 0.10$	$50.80 \pm 0.11$
After 24 hours	$47.49 \pm 0.08$	$66.28 \pm 0.07$	$25.65 \pm 0.08$

724

725

726 **FIGURE CAPTIONS**

727 **Figure 1.** (a) Transmission Electron Microscopy (TEM) and (b) Scanning Transmission Electron  
728 Microscopy (STEM) of the mesoporous silica nanoparticles (MSN). Scale bar is 20 nm; (c) Nitrogen  
729 sorption isotherms with DFT pore size distribution in the inset; (d) Fourier-Transform infrared  
730 Spectroscopy (FTIR).

731

732 **Figure 2.** Particle size distribution measured by dynamic light scattering (DLS), comparing the  
733 pristine MSN with MSN coated by the various SLB.

734

735 **Figure 3.** Fluorescence co-localization microscope images of the various lipid-coated MSN. (a-c)  
736 MSN@DOPC; (d-f) MSN@DOPC-DOTAP; (g-i) MSN@DOPC-chol-DSPE-PEG. Figures on the  
737 left column refer to the emission in the green channel ( $\lambda_{\text{ex}} = 488$  nm); those in the central column to  
738 the red channel ( $\lambda_{\text{ex}} = 550$  nm), and the right column refers to the merged channel for colocalization  
739 evaluation (yellow indicates colocalization of MSN with lipids). Scale bars are 10  $\mu\text{m}$ .

740

741 **Figure 4.** (a) Drug leakage at 24 hours evaluated in percentage with respect to the total adsorbed drug  
742 evaluating the stability of the various nanoconstructs (0.5 mg of sample) in water at two different  
743 storage temperatures, 4 °C (filled bars) and 37 °C (dashed bars). (b) Release of AGI drug from pristine  
744 MSN (in grey) and from two lipid-coated mesoporous silica, i.e. MSN@DOPC-DOTAP (in red),  
745 MSN@DOPC-chol-DSPE-PEG (in black). Y-axis reports the percentage of the released drug AGI-  
746 6780 in RPMI-1640 with respect to the loaded amount. The inset shows a detail of the drug release  
747 from the MSN@SLB only. Error bars are S.E.M.

748

749 **Figure 5.** Results of the haemocompatibility tests comparing samples in citrate plasma and evaluating  
750 the coagulation time ( $t_c$ ) after addition of calcium chloride ( $\text{CaCl}_2$ ). The UV-vis absorption at 405 nm  
751 is recorded over time for 45 minutes on samples (upper panel): plasma; plasma with physiological

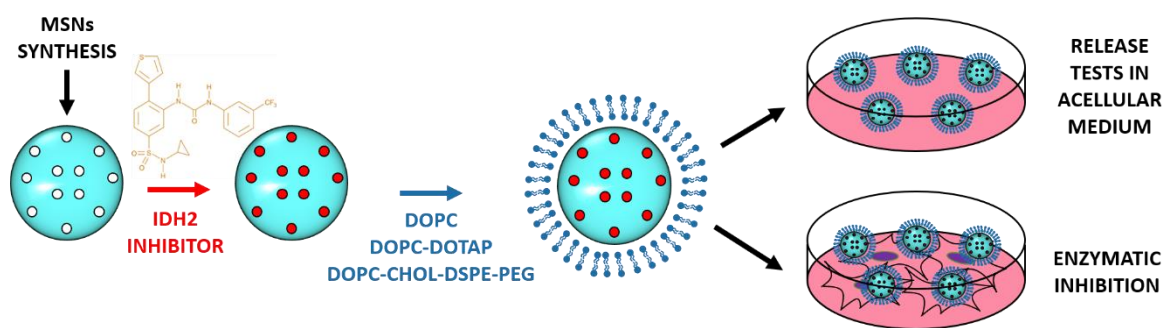
752 solution (0.9% wt NaCl); pristine MSNs at two concentrations (50 and 100  $\mu\text{g/mL}$ ); MSN@DOPC-  
753 chol-DSPE-PEG at two concentrations (50 and 100  $\mu\text{g/mL}$ ); and the coagulation time recorded, as  
754 reported in the table (lower panel).

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756

## 757 FIGURES

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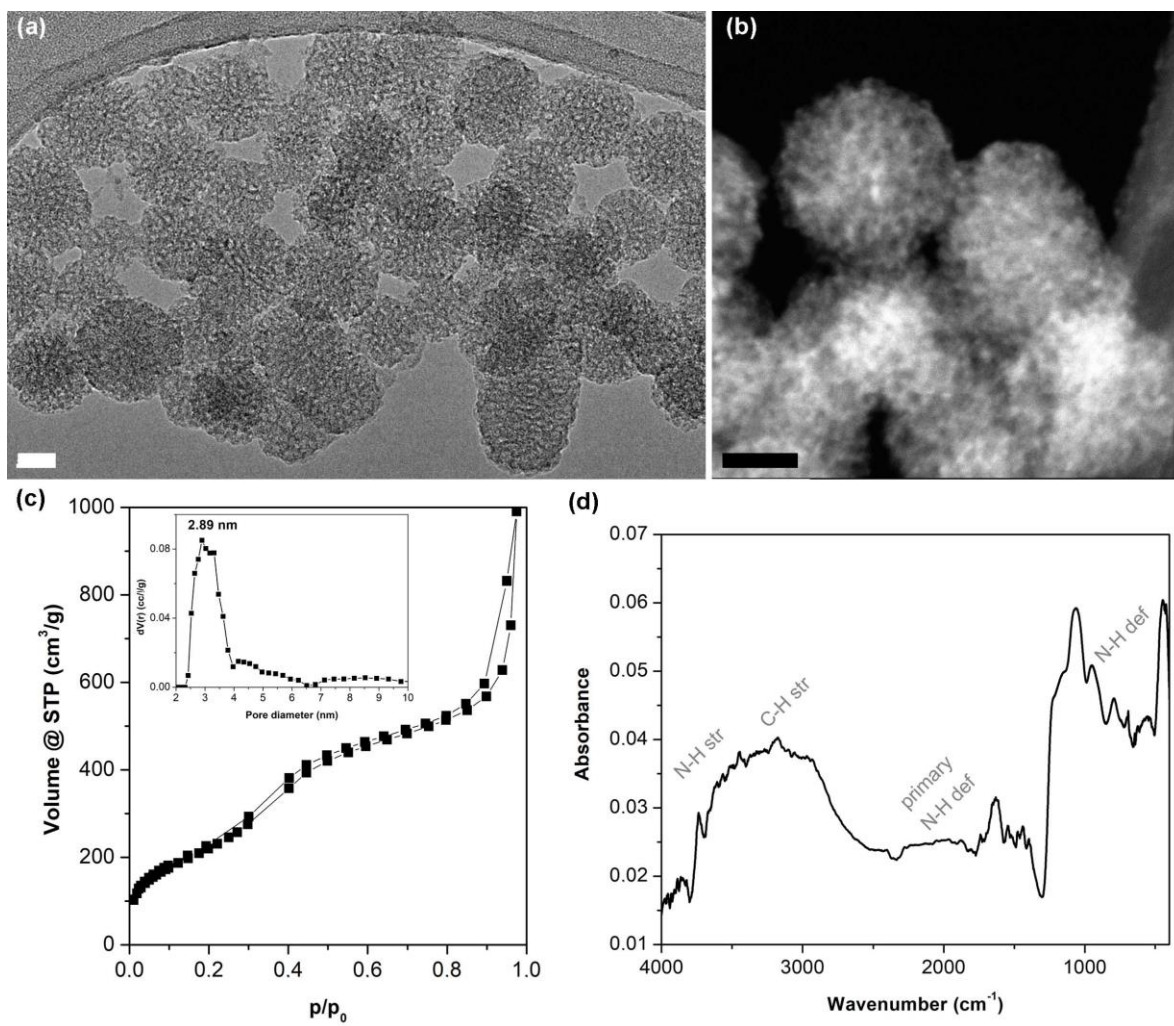


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Graphical abstract

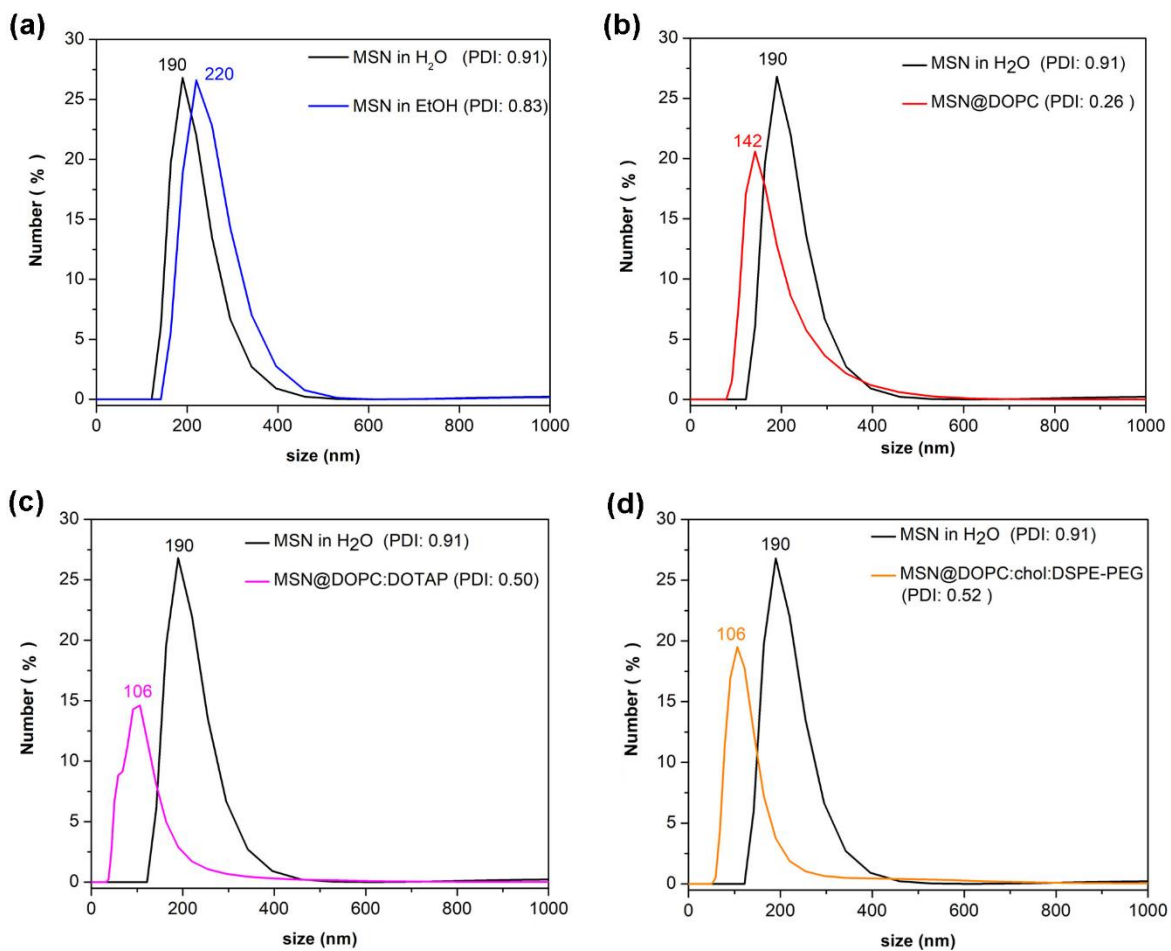


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Figure 1



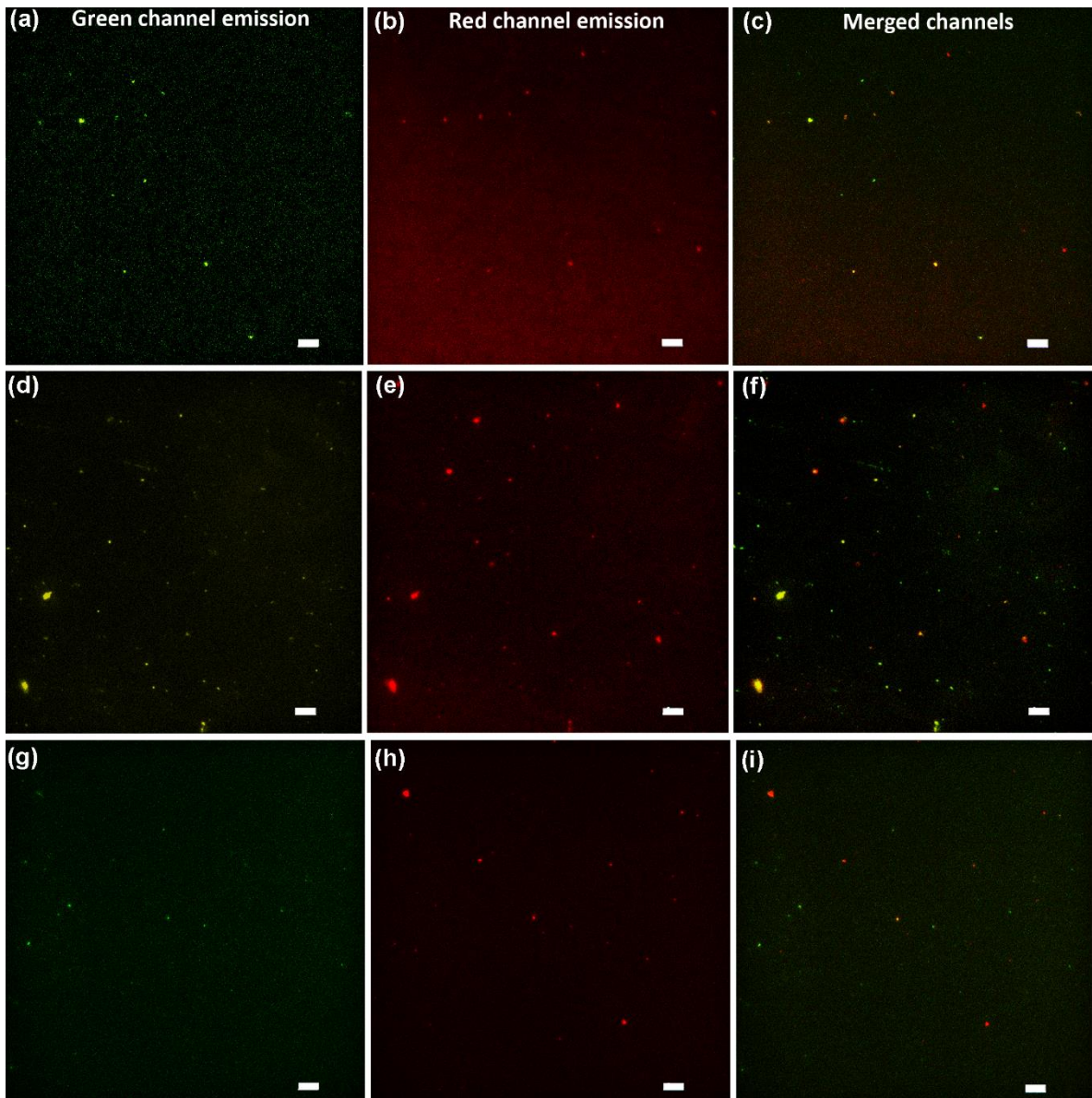
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Figure 2

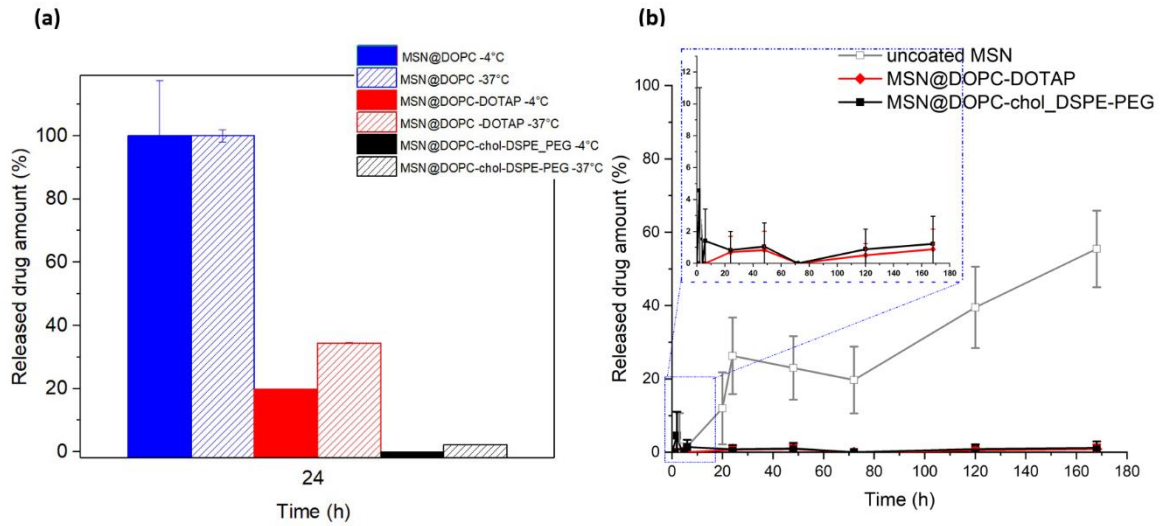


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**Figure 3**

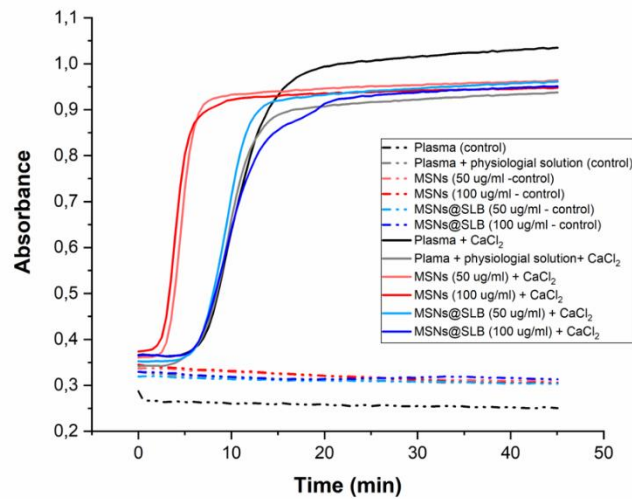
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Figure 4



Samples	Average $t_c \pm \text{st.dev}$ (min)
Plasma	10.2±0.8
Physiological solution	9.6±0.3
MSNs (50 ug/ml)	4.8±0.2
MSNs 100 ug/ml	4.4±0.4
MSNs@DOPC-cholesterol-DSPE-PEG (50 ug/ml)	9.39±0.19
MSNs@DOPC-cholesterol-DSPE-PEG (100 ug/ml)	9.5±1.1

774

775

Figure 5

776

777