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1 **Degradable and Biocompatible Nanoparticles Decorated with Cyclic RGD Peptide for Efficient**
2 **Drug Delivery to Hepatoma Cells *In Vitro***

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12

13 *Keywords:* Biotinylated nanoparticles; site-specific targeting; anti-cancer drug encapsulation;
14 degradable poly(benzyl malate) derivatives; cyclic RGD peptide; HepaRG hepatoma cells.

15

16 **ABSTRACT**

17 Amphiphilic derivatives of poly(benzyl malate) were synthesized and characterized with the aim of
18 being used as degradable and biocompatible building blocks for the design of functional nanoparticles
19 (NPs). An anti-cancer model drug, doxorubicin, has been successfully encapsulated into the prepared
20 NPs and its release profile has been evaluated in water and in culture medium. NPs bearing biotin
21 molecules were prepared either for site-specific drug delivery via the targeting of biotin receptors
22 overexpressed on the surface of several cancer cells, or for grafting biotinylated cyclic RGD peptide
23 onto their surface using the strong and highly specific interactions between biotin and the streptavidin
24 protein. We have shown that this binding did not affect dramatically the physico-chemical properties
25 of the corresponding NPs. Cyclic RGD grafted fluorescent NPs were more efficiently uptaken by the

26 HepaRG hepatoma cells than biotinylated fluorescent NPs. Furthermore, the targeting of HepaRG
27 hepatoma cells with NPs bearing cyclic RGD was very efficient and much weaker for HeLa and HT29
28 cell lines confirming that cyclic RGD is a suitable targeting agent for liver cells. Our results also
29 provide a new mean for rapid screening of short hepatotropic peptides in order to design NPs showing
30 specific liver targeting properties.

31

32 **1. Introduction**

33 Nanotechnology, especially nanomedicine corresponding to the use of nanoparticles (NPs) in
34 biomedicine, is currently an ever growing scientific and technological domain [Psimidas et al., 2012;
35 Garanger et al., 2012]. The main reason for this unprecedented development relies on the aim to
36 improve both early detection and treatment of numerous pathologies such as cancers. The
37 encapsulation of a selected biologically active molecule into NPs might result in an increased drug
38 bioavailability within solid tumors, arising from a decrease in its non-specific recognition by the
39 reticuloendothelial system (RES) and an improvement of its *in vivo* specific biodistribution, as well as
40 a minimized toxicity against healthy tissues and organs [Yan et al., 2012; Elsaesser et al., 2012;
41 Lamprecht, 2008]. Knowing that cancer, characterized by an abnormal and anarchical cell proliferation
42 within normal tissue, is a very complex disease and a major cause of mortality [Misra et al., 2010], the
43 development of efficient nanomedicine is thus a major challenge for public health [Reddy et al., 2011].
44 In this context, several anti-cancer drug loaded NPs such as Doxyl® and Abraxane® have been
45 approved by the Food and Drug Administration (FDA) for clinical uses [Yan et al., 2012; Wang et al.,
46 2012; Jain et al., 2010]. However besides these encouraging results, several challenges have to be
47 overcome in order to obtain NPs allowing highly efficient site-specific drug delivery.

48 The materials constituting the NPs have to respect very strict specifications: they must be (i)
49 biocompatible and non-toxic, (ii) (bio)degradable into non-toxic low molecular weight molecules or, at
50 least, bioassimilable after releasing the encapsulated drug, (iii) undetectable by the RES meaning
51 having stealth properties, (iv) adapted for carrying large amounts of drug that should be released in a

52 controlled manner at its site of action (targeting). Within this context, we have recently developed a
53 family of degradable non-toxic polymers derived from poly(malic acid), PMLA, which are able to
54 form well-defined NPs [Huang et al., 2012]. We have selected PMLA as macromolecular backbone
55 because this polymer, originally synthesized for application in the biomedical field, has been
56 successfully used as a platform in the synthesis of nanovectors [Huang et al., 2012; Cammas et al.,
57 2000; Cammas-Marion et al., 2000; Osanai et al., 2000; Martinez Barbosa et al., 2004; Abdellaoui et
58 al., 1998] and macromolecular conjugates [Ding et al., 2010; Ljubimova et al., 2008; Fujita et al.,
59 2007; Fujita et al., 2006]. PMLA is known to be non-toxic and degradable into malic acid under
60 physiological conditions [Vert et al., 1979] and its derivatives are accessible from naturally occurring
61 PMLA [Ljubimova et al., 2008] or by anionic ring-opening polymerization (ROP) of β -substituted β -
62 lactones [Cammass et al., 1996; Cammas et al., 1993]. PMLA derivatives used for the formulation of
63 NPs were obtained by ROP of benzyl malolactonate (MLABe) in presence of either
64 tetraethylammonium benzoate, α -methoxy ω -carboxy poly(ethylene glycol) -PEG₄₂-CO₂H- or α -biotin
65 ω -carboxy poly(ethylene glycol) -Biot-PEG₆₂-CO₂H- as initiator [Huang et al., 2012]. Starting from
66 these three PMLA derivatives, we were able to obtain well-defined non-toxic NPs in which the
67 doxorubicin (Dox), and a fluorescent probe, the DiD oil, have been successfully encapsulated for *in*
68 *vitro* assays [Huang et al., 2012]. It is worth noting that PEG has been selected as hydrophilic block
69 because it is a well-known polymer conferring stealth properties at nanoparticles on which it is grafted
70 [Romberg et al., 2008]. On the other hand, biotin has been chosen firstly because it is a targeting agent
71 of certain cancer cells [Le Droumaguet et al., 2012; Patil et al., 2009; Kim et al., 2007] and secondly
72 because it is able to interact strongly with streptavidin [Yang et al., 2009] which is an important
73 property for our study as it will be explained afterwards in this paper.

74 Hepatocellular carcinoma (HCC) is the main primary malignant tumor of the liver representing 80 to
75 90% of liver tumors. It is the fifth most common tumor worldwide (5.4% of new cancer cases per year)
76 and the third in term of mortality (8.2% of all cancer death) [Parkin et al., 2005]. Early detection and
77 classification of HCC are crucial for the choice and effectiveness of therapeutic strategy. For small size

78 HCC, surgical treatment (resection and liver transplantation) is the most effective treatment [Hasegawa
79 et al., 2009; Mazzeferro et al., 2008; Ishikawa et al., 1992]. Palliative treatments such as
80 chemoembolization [Bernades-Genisson et al., 2003] and, more recently, chemotherapy using an
81 inhibitor of tyrosine kinase, Sorafenib (Nexavar®) are proposed to patients with advanced HCC.
82 Despite these advances, the therapeutic options for the treatment of HCC remains limited partly due
83 to the chemoresistance of liver tumors to conventional anti-tumor agents. Therefore, the use of
84 nanocarriers containing anti-tumor drugs has been envisaged for the treatment of HCC in order to
85 increase the intra-hepatic drug concentration while limiting the exposure of healthy tissues and side
86 effects [Reddy et al., 2011]. Several formulations are currently undergoing clinical trials in phase II
87 and III such as NPs of poly(alkyl cyanoacrylate) loaded with doxorubicin (Trandrug®) for HCC
88 treatment [Barraud et al., 2005]. In a first step, the passive accumulation of nanocarriers in the RES
89 cells (endothelial and Kupffer cells) [Lanaerts et al., 1984] was utilized for liver targeting with a real
90 relevance for diseases involving liver Kupffer cells such as parasitic diseases [Alving et al., 1978].
91 Conversely, liver targeting based on the nanocarrier's uptake by Kupffer cells has the major drawback
92 of allowing only a low hepatic accumulation of nanocarriers since Kupffer cells represent only a few
93 percent of liver cell volume against 90% for hepatocytes. In addition, the accumulation of nanocarriers
94 in Kupffer cells does not target the cells responsible for HCC thus limiting the use of this approach in
95 this case. Therefore, to overcome this drawback, active hepatocyte targeting has been studied. Most of
96 the proposed strategies are based on the binding of NPs to the asialoglycoprotein receptors [Wu et al.,
97 2002]. These NPs are usually liposomes incorporating glycosylated proteins or galactose/lactose linked
98 to lipophilic anchors [Wu et al., 2002]. To date, very few nanovectors, especially polymer-based NPs,
99 carrying peptides with high tropism for the liver have been developed [Reddy et al., 2011].

100 In this paper, we report, first, the monitoring of Dox release from NPs prepared from PEG₄₂-*b*-
101 PMLABe or Biot-PEG₆₂-*b*-PMLABe in water and in culture medium at 37°C. Second, we have grafted
102 fluorescein amine (FA) molecule at the free end of the PMLABe block of PEG₄₂-*b*-PMLABe or Biot-
103 PEG₆₂-*b*-PMLABe block copolymers in order to obtain fluorescent NPs for *in vitro* cell uptake assays.

104 Starting from the corresponding fluorescent NPs, we have studied the influence of the nature of
105 molecules localized at NPs' surfaces [PEG₄₂, Biot-PEG₆₂ or Arginine-Glycine-Aspartic acid (RGD)
106 peptide-Biot-Streptavidin-Biot-PEG₆₂] on their internalization into cells *in vitro* and demonstrated that
107 the uptake by HepaRG hepatoma cells is considerably enhanced by the grafting of the RGD peptide
108 onto NPs. These results also show that these biotinylated NPs can be useful tools for rapid *in vitro*
109 screen and selection of highly hepatotropic peptides with the ultimate goal to design nanomedicine
110 targeting hepatocytes from HCC *in vivo*.

111

112 **2. Materials and methods**

113 *2.1. Materials*

114 All chemicals were used as received. Anhydrous THF was obtained by distillation over
115 sodium/benzophenone under N₂ atmosphere.

116 Three cell lines have been selected within the frame of this project: the HepaRG hepatoma cells
117 [Gripon et al., 2002; Laurent et al., 2010;], the colorectal adenocarcinoma cell line HT29 [Fogh et al.,
118 1975] and the cervical cancer cell line HeLa [Rahbari et al., 2009].

119

120 *2.2. Apparatus*

121 Nuclear magnetic resonance spectra (¹H NMR) were recorded on a Bruker ARX 400 instrument (¹H
122 at 400 MHz). Data are reported as follows: chemical shift (multiplicity, number of hydrogen). The
123 chemical shifts (δ) are reported as parts per million (ppm) referenced to the appropriate residual solvent
124 peak. Abbreviations are as follows: *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *dd* (doublet of
125 doublet), *m* (multiplet).

126 The size (average diameter obtained by the cumulant result method), polydispersity and zeta potential
127 of the formulations were measured by dynamic light scattering using a Delsa™ Nano Beckman
128 Coulter apparatus at 25°C.

129 UV spectra were recorded on a Secoman apparatus at 485 nm.

130 2.3. *Synthesis of PEG₄₂-b-PMLABe and Biot-PEG₆₂-b-PMLABe*

131 The monomer, the benzyl malolactonate (MLABe), was synthesized from DL-aspartic acid according
132 to the previously reported synthesis [Cammass et al., 1996]. The PEG₄₂-b-PMLABe and Biot-PEG₆₂-b-
133 PMLABe block copolymers were obtained by anionic ring opening polymerization of MLABe in
134 presence of, respectively, α -methoxy ω -carboxy poly(ethylene glycol), PEG₄₂-CO₂H, and α -biotin ω -
135 carboxy poly(ethylene glycol), Biot-PEG₆₂-CO₂H, as initiators following a protocol described
136 elsewhere [Huang et al., 2012].

137

138 2.4. *Grafting of fluorescein amine (FA)*

139 2.4.1 Grafting of FA on the PEG₄₂-b-PMLABe block copolymer (Scheme 1)

140 The PEG₄₂-b-PMLABe block copolymer (500 mg) was dissolved into 1 mL of anhydrous CH₂Cl₂
141 under nitrogen atmosphere. To this solution were added 5 mg (1eq.) of *N,N'*-dichlorohexylcarbodiimide
142 (DCC) dissolved in 1 mL of anhydrous CH₂Cl₂ followed by 7.5 mg (1eq.) of *N*-hydroxysuccinimide
143 (NSH) dissolved in 1 mL of anhydrous CH₂Cl₂. The mixture was stirred under nitrogen atmosphere for
144 24 hours at room temperature (RT). The resulting PEG₄₂-b-PMLABe-NHS was precipitated in cold
145 heptane. After the elimination of the supernatant, the precipitate was dissolved in CH₂Cl₂ and the
146 solution was filtrated on celite. The CH₂Cl₂ was eliminated under vacuum and the activated block
147 copolymer polymer (480 mg) was obtained with 96% yield. The activated block copolymer (480 mg)
148 was then dissolved in 1 mL of anhydrous CH₂Cl₂. To this solution was added 12 mg of FA (1eq.)
149 solubilised into a mixture of 1 mL of anhydrous CH₂Cl₂ and 1 mL of acetone HPLC grade under
150 nitrogen atmosphere. After stirring at RT for 24 hours, the solution containing the PEG₄₂-b-PMLABe-
151 FA block copolymer was precipitated into cold heptane. After removing the supernatant, the
152 precipitate was dissolved into DMSO. This DMSO solution was poured into a dialysis bag (MWCO
153 3,500 Da) and the dialysis was conducted during 8 hours against DMSO. The solution contained into
154 the dialysis bag was lyophilized and the PEG₄₂-b-PMLABe-FA block copolymer was recovered with
155 60% yield. The polymer was characterized by ¹H NMR in deuterated DMSO.

156 $^1\text{H NMR}$ ($d_6\text{-DMSO}$, δ ppm): 2.92 (s, 2nH, $\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5$), 3.32 (m, 4mH ($m=42$), $(\text{CH}_2\text{CH}_2\text{O})_{42}$), 5.10
157 (m, 2nH, CHCH_2CO_2), 5.42 (m, 1nH, CHCH_2CO_2), 6.50-7.00 (m, 9H, FA); 7.31 (m, 5nH,
158 $\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5$).

159 $M_{\text{NMR}} = 11,000$ g/mol for the PMLABe block

160

161 2.4.2 Grafting of FA on the Biot-PEG₆₂-b-PMLABe block copolymer (Scheme 1)

162 The grafting of FA on the Biot-PEG₆₂-b-PMLABe block copolymer was realized as described above.

163 The Biot-PEG₆₂-b-PMLABe-FA block copolymer was obtained with 58% yield and characterized by

164 $^1\text{H NMR}$ in DMSO.

165 $^1\text{H NMR}$ ($d_6\text{-DMSO}$, δ ppm): 2.92 (s, 2nH, $\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5$), 3.37 (m, 4mH ($m=62$), $(\text{CH}_2\text{CH}_2\text{O})_{62}$), 5.07
166 (m, 2nH, CHCH_2CO_2), 5.41 (m, 1nH, CHCH_2CO_2), 6.50-7.00 (m, 9H, FA); 7.26 (m, 5nH,
167 $\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5$). Peaks corresponding to the biotin are either under peaks corresponding to the PEG
168 and PMLABe blocks or too small to be detectable on the NMR spectrum.

169 $M_{\text{NMR}} = 6,000$ g/mol for the PMLABe block.

170

171 2.5. Preparation of NPs

172 2.5.1. Dox encapsulation and release from NPs

173 The protocol for Dox encapsulation and for the monitoring of its release was the same whatever the
174 nature of the block copolymer constituted the NPs. The encapsulation of Dox into the NPs was realised
175 as described previously [Huang et al., 2012]. Briefly, the Dox hydrochloride (Dox,HCl, Sigma) was
176 encapsulated into the two kinds of NPs during the nanoprecipitation procedure. The selected polymer
177 (5 mg) was dissolved in acetone (1 mL). Two hundred μL of a Dox solution [1.5 mg of Dox,HCl
178 solubilised in 0.6 mL of a mixture of chloroform (6 mL) and NEt_3 (23 μL)] were added to the polymer
179 solution. This mixture was then nanoprecipitated into 2 mL of water under vigorous stirring. After
180 organic solvent evaporation, the unloaded Dox was removed by ultracentrifugation at 15,000 g at 15°C
181 for 7 min using filter with an exclusion limit 10,000 Da. The filters were returned and centrifuged for 1

182 min at 1,000 g at 15°C. The volume of the recovered solutions was completed to 2 mL with distilled
183 water in order to obtain a final concentration in NPs of 2.5 g/L. The concentration of loaded Dox was
184 evaluated by UV at 485 nm, as described elsewhere [Huang et al., 2012; Cammas et al., 1995]. Briefly,
185 200 µL of Dox-loaded NPs were dissolved into 800 µL of DMF and the resulting solutions were
186 analyzed by UV at 485 nm. The absorbance of Dox encapsulated into NPs was converted into a
187 concentration using a calibration curve and the encapsulation efficiency (e.e.) was calculated using the
188 following equation:

$$e.e. = \frac{[\text{Total drug}] - [\text{Free drug}]}{[\text{Total drug}]} \times 100$$

191 The characteristics of the Dox-loaded NPs (diameter, polydispersity index and zeta potential) were
192 measured using the Delsa™ Nano Beckman Coulter apparatus (Table 1).

193 The Dox release from both kinds of NPs were monitoring by dialysis in water and in culture medium
194 at 37°C. The protocol used in both cases was identical. Two mL of the Dox-loaded NPs solution were
195 placed into a dialysis bag (MWCO = 3,500 Da); this bag was then incubated into 40 mL of water or
196 culture medium maintained at 37°C. After different incubation time, from 30 min up to 72 hours, 2 mL
197 of the outside solution are taken and replaced by 2 mL of fresh water or culture medium. For each
198 sample, 200 µL of the collected outside solution were analyzed by UV at 485 nm and the quantity of
199 Dox was determined thanks to calibration curves previously realized in water and in culture medium
200 by UV measurements at 485 nm.

202 2.5.2. Preparation of fluorescent NPs

203 The fluorescent NPs were prepared by the nanoprecipitation technique as previously described
204 [Thioune et al., 1997; Huang et al., 2012]. Briefly, the mixture of PEG₄₂-*b*-PMLABe-FA (2.5 mg) and
205 PEG₄₂-*b*-PMLABe (2.5 mg) or Biot-PEG₆₂-*b*-PMLABe-FA (2.5 mg) and Biot-PEG₆₂-*b*-PMLABe (2.5
206 mg) were dissolved in 1 mL of acetone. This solution is added to 2 mL of water under vigorous
207 stirring. The organic solvent (acetone) was then evaporated under vacuum and the final volume was

208 completed to 2 mL with fresh water. The final concentration in block copolymers under NPs' form was
209 2.5 g/L. The solutions containing the fluorescent NPs were characterized by dynamic light scattering
210 using a Delsa™ Nano Beckman Coulter apparatus at 25°C (Table 2).

211

212 2.5.3. Grafting of the cyclic RGD peptide

213 The selected biotinylated peptide, the cyclic Biot-RGD peptide (Eurogentec, Belgium), was grafted
214 onto the biotinylated NP's surfaces via the streptavidin (Strept, AnaSpect, Eurogentec, Belgium)
215 protein. An aqueous solution of Biot-RGD peptide was prepared at a final concentration of 2.9 mM. In
216 parallel, an aqueous solution of streptavidin was also prepared with a final concentration of 178 μM.
217 The Biot-RGD peptide (2.7 μL) and the streptavidin (22 μL) solutions were mixed in a final volume of
218 50 μL (H₂O qsp) and incubated for 1 hour at 4°C. Then, the Biot-PEG₆₂-*b*-PMLABe-FA or Biot-
219 PEG₆₂-*b*-PMLABe based NP's solution (35 μL), previously prepared, was added to a final volume of
220 100 μL (H₂O qsp). This mixture was incubated for 1 hour and diluted to a final volume of 1 mL in
221 culture medium for final concentrations of the Biot-RGD peptide at 8μM, the streptavidin at 4μM and
222 polymers at 4μM. In order to demonstrate that both the Biot-RGD peptide grafting and the dilution
223 have no influence on NP's characteristics, we analyzed the NPs formed in the conditions described
224 above by DLS (Table 3).

225

226 2.6. Cell uptake assays

227 The cell lines, HT29 [Fogh et al., 1975] and HeLa [Rahbari et al., 2009], were cultured as described in
228 the literature. The HepaRG cell line was cultured in the medium William's E (Lonza) supplemented
229 with 2mM of glutamine (Gibco), 5 mg/L of insulin (Sigma), 10⁻⁵ M hydrocortisone hemisuccinate and
230 10% of fetal calf serum (Lonza) [Gripon at al., 2002; Laurent et al., 2010]. During the sub-culturing,
231 2.10⁶ cells were seeded in a 75 cm³ flask. The medium was renewed every 48 hours. The sub-culturing
232 was realized by trypsinization every 2 weeks in order to maintain the progenitor phenotype. For an

233 optimal differentiation, the cells were maintained at confluence after the two weeks and the medium
234 was supplemented with 2% of dimethylsulfoxide (DMSO) [Laurent et al., 2013].

235 For the cell uptake assays, the 24 wells culture plates were seeded with the selected cell line (HepaRG,
236 HT29 or HeLa) with 10^5 cells per well. Then the NPs' preparations ($4\mu\text{M}$ of block copolymer under
237 NPs' form $\pm 4\mu\text{M}$ streptavidin and $8\mu\text{M}$ Biot-RGD) or a negative control (buffer without NPs or non
238 fluorescent NPs) were added to the wells. For the competitive experiments, the cells were pre-treated
239 with an excess of free RGD peptide ($32\mu\text{M}$).

240 The cells were incubated from 1 to 24 hours. After incubation, the culture medium was removed; the
241 cell monolayers were washed with PBS before the observation by fluorescence microscopy (Zeiss
242 inverted microscope, analysis software AxioVision). Then the cells were detached with trypsin and
243 analyzed by flow cytometry (FACSCalibur Becton Dickinson) to quantify the fluorescence (Channel
244 FL1H) emitted by the fluorescent NPs captured by the cells. Cytometry data were analyzed using
245 CellQuest software (Becton Dickinson).

246

247 **3. Results and Discussion**

248 In order to further characterize the PMLABe based NPs as drug nanocarriers for applications in
249 nanomedicine, we (i) studied the release of Dox in water and culture medium at 37°C , (ii) grafted a
250 fluorescent probe at the free end of the hydrophobic PMLABe block for studying *in vitro* cellular
251 uptake and (iii) evaluated the possibility to build a molecular scaffold by grafting the cyclic RGD-
252 biotinylated peptide onto biotinylated NPs via the streptavidin as a bridging factor and determine the
253 impact of the RGD peptide addition on cell uptake.

254

255 **Figure 1**

256

257

258

259 3.1. *Dox encapsulation and release from NPs*

260 Both Dox-loaded PEG₄₂-*b*-PMLABe and Biot-PEG₆₂-*b*-PMLABe based NPs have been prepared by
261 the nanoprecipitation technique and have been characterized by DLS. Table 1 collects the results
262 obtained for PEG₄₂-*b*-PMLABe and Biot-PEG₆₂-*b*-PMLABe based NPs. Initial Dox content in both
263 NPs determined by UV at 485 nm as described previously [Huang et al., 2012] showed an
264 encapsulation efficiency ranging from 32 to 36%.

265

266

Table 1

267

268 The release of Dox from both PEG₄₂-*b*-PMLABe and Biot-PEG₆₂-*b*-PMLABe NPs in water and in
269 culture medium was realized at 37°C by dialysis. Aliquots of the external solution (outside the dialysis
270 bag) were collected after various incubation times and analyzed by UV at 485 nm and confirmed that
271 the Dox was encapsulated with an efficiency of nearly 35% (Table 1). As shown in figure 2, Dox
272 release profiles from both types of NPs in water (Figure 2, A) and in the culture medium (Figure 2, B)
273 are quite similar.

274

275

Figure 2

276

277 The release, expressed as a percentage of the total amount of encapsulated Dox, is nearly 5% after one
278 hour of incubation. This release accelerates after the second hour to reach 40% after 6 hours of
279 incubation in both water and culture medium. Then the release reaches a plateau around 55 to 60%
280 from 24 to 72 hours of incubation. The fast release of Dox within the first hours is probably due to the
281 Dox absorbed in the hydrophilic PEG corona. Indeed, Dox is known to be an amphiphilic molecule
282 which is therefore spread from the hydrophilic corona to the surface of the hydrophobic core of the
283 nanoparticles. However to conclude regarding the exact location of the Dox in the PEG-*b*-PMLABe
284 forming NPs, it will be necessary to realize further experiments such as X-ray measurements.

285 Nevertheless, these results are quite encouraging because we are able to encapsulate substantial
286 amount of this drug and the release profile is in agreement with the Dox amphiphilic nature.

287

288 *3.2. Grafting of fluorescein amine and cellular uptake assays*

289 Besides the use of biotin as a well-known targeting agent of cancer cells [Yang et al., 2009], we aimed
290 at developing a versatile procedure to screen for more specific targeting agents, especially short
291 peptides, towards transformed hepatocytes from hepatocellular carcinoma (HCC).

292 Our goal is to select peptides exhibiting a remarkably high tropism for the hepatocytes as targeting
293 agents and to graft them at the surface of PMLA derivatives-based NPs in order to achieve an
294 optimized uptake of NPs by the hepatocytes. In a first step, we wish to screen, rapidly and in a simple
295 manner without engaging more organic chemistry, a large number of peptides which potentially show
296 a strong hepatotropism to select the most efficient ones. For that purpose, we used the non-covalent
297 binding of selected peptides via the strong biotin-streptavidin affinity as shown by Figure 3 [Yang et
298 al., 2009].

299

300 *Figure 3*

301

302 In a first step to determine whether such a molecular scaffold could allow the production of NPs and
303 could be used for cell uptake *in vitro*, we have selected biotinylated cyclic RDG (Arg-Gly-Asp- DTyr-
304 Lys-Biotin) peptide, known for interacting with integrin proteins well expressed in the liver and even
305 more in tumors rich in extracellular matrix [Jiang et al., 2011]. This biotinylated RGD peptide has been
306 introduced after the formation of biotinylated NPs through streptavidin interactions [Yang et al., 2009].
307 Moreover, in order to follow the *in vitro* cellular uptake of the NPs, we have synthesized fluorescein
308 amine grafted PEG₄₂-*b*-PMLA_{Be} and Biot-PEG₆₂-*b*-PMLA_{Be} block copolymers. As shown by scheme
309 1, the fluorescein amine (FA) was successfully grafted at the free carboxylic acid end of the PMLA_{Be}

310 block activated with N-hydroxysuccinimide (NHS) without modifying the structure of block
311 copolymers.

312

313

Scheme 1

314

315 After purification by dialysis allowing the elimination of unreacted FA and low molecular weight side
316 products, both block copolymers were characterized by ¹H NMR in deuterated DMSO. The ¹H NMR
317 spectra allowed us to conclude that the structures of FA-modified block copolymers were in agreement
318 with the expected ones and that the molecular weights of PMLABe blocks calculated from the ¹H
319 NMR spectra were identical to the ones of initial materials.

320 Starting from a mixture of the fluorescent block copolymers and the non-fluorescent ones (50/50 wt%),
321 we have then prepared the corresponding fluorescent NPs using the nanoprecipitation method [Huang
322 et al., 2012; Thioune et al., 1997]. The obtained NPs were characterized by dynamic light scattering.
323 As shown by results gathered in table 2, the presence of FA molecules at the end of the PMLABe
324 block has no significant influence on the NP's diameters and polydispersity indices.

325

326

Table 2

327

328 Biotinylated cyclic RGD peptide was then associated with NPs formed by a mixture of Biot-PEG₆₂-b-
329 PMLABe-FA and Biot-PEG₆₂-b-PMLABe (50/50 wt%) using streptavidin, a tetrameric protein of 56
330 KDa purified from the bacterium *Streptomyces avidinii*, as an intermediate link between the biotin
331 localized on the surface of preformed polymeric NPs and the biotinylated peptide (Figure 3). The
332 dissociation constant (Kd) of the biotin/streptavidin complex is on the order of 10⁻¹⁵ mol/L, ranking
333 among the strongest known non-covalent interactions [Yang et al., 2009]. The non-covalent binding of
334 RGD modified fluorescent NPs was realized by first mixing the biotinylated RGD peptide with the
335 streptavidin followed by the addition of this complex to biotinylated fluorescent NPs. The relative

336 amounts of block copolymers constituting the NPs, RGD peptide and streptavidin can have a
337 significant influence on the cell capture. Therefore, different amounts of block copolymer constituting
338 the NPs, RGD peptide and streptavidin were tested and the cell uptake was measured by fluorescent
339 microscopy and flow cytometry (Data not shown); the best results were obtained with the following
340 conditions: 4 μ M of block copolymers under NP's form, 4 μ M of streptavidin and 8 μ M of
341 biotinylated RGD peptide.

342 The diameter and polydispersity index of unmodified and modified NPs were measured by DLS in
343 order to demonstrate that the addition of RGD-Biot-streptavidin construct and the dilution had no
344 influence on the properties of the corresponding NPs. As shown by results gathered in table 3, the
345 dilution of Biot-PEG₆₂-b-PMLABe NPs either in PBS or in culture medium with or without serum
346 does not have a significant influence on both the diameter and the polydispersity index values,
347 meaning that the polymers constituting the NPs are still associated under NP's form.

348

349

Table 3

350

351 The addition of the Strep-Biot-RGD construct on the biotinylated NPs has led to a moderated increase
352 in the NPs' diameter and polydispersity indices. For NPs resuspended either in PBS or culture medium
353 with serum the emergence of a second peak centered at 600 nm and 970 nm, respectively, was
354 observed. Such results indicate that addition of Strep-Biot-RGD construct does not lead to the
355 destabilization of the NPs but rather a moderate formation of aggregates. However, such limited
356 modifications were considered acceptable for cell uptake *in vitro* assays. It is important to note that
357 RGD modified NPs were stable for at least 48 hours when stored at room temperature as shown by
358 values of diameters and polydispersity indices unchanged (measures realized by DLS, data not shown).
359 We then realized *in vitro* assays of cell captation using the human hepatoma HepaRG cell line [Laurent
360 et al., 2010; Gripon et al., 2002]. HepaRG cells were incubated for 24 hours in the presence of
361 biotinylated NPs modified or not with the RGD-Biot-Streptavidin construct with final concentrations

362 in culture media of 4 μ M of block copolymers, 4 μ M of streptavidin and 8 μ M of RGD peptide. The
363 cell uptake was studied by FACS and fluorescent microscopy (Figure 4).

364

365 ***Figure 4***

366

367 The FACS analysis showed that the peak of fluorescence in cells incubated with the Biot-PEG₆₂-*b*-
368 PMLABe-FA NPs had significantly shifted on the right compared to fluorescence in control cells
369 incubated with non fluorescent NPs demonstrating that most of the cells contained fluorescent NPs.
370 However, the mean of fluorescence remained low (~30 arbitrary units) for Biot-PEG₆₂-*b*-PMLABe
371 NPs and Biot-PEG₆₂-*b*-PMLABe-FA NPs, respectively, compared to the mean for cells incubated with
372 non-fluorescent NPs. The fluorescent microscopy confirmed that HepaRG cell uptake of Biot-PEG₆₂-
373 *b*-PMLABe-FA NPs was very limited compared to negative control cells exposed to the non-
374 fluorescent NPs demonstrating that the biotin did not trigger a strong captation. Addition of the RGD
375 peptide led to strong increase in the fluorescence level within HepaRG cells reaching a mean of ~1500
376 U.A and with over 90% of positive cells (Figure 4). These data demonstrate that the addition of the
377 RGD peptide onto NPs has considerably enhanced the cell uptake of the NPs.

378 We then followed the time course of uptake by HepaRG cells of NPs formed by Biot-PEG₆₂-*b*-
379 PMLABe-FA or Biot-RGD-Strept-Biot-PEG₆₂-*b*-PMLABe-FA by flow cytometry analysis at 2, 6, 14
380 and 24 hours of incubation. As shown by figure 5, the results of mean of fluorescence intensity
381 indicated a very rapid uptake within the first 6 hours which continued slower until 24 hours for RGD-
382 Biot-Strept-Biot-PEG₆₂-*b*-PMLABe-FA based NPs (Figure 5, red curve), while no change in the
383 fluorescence intensity has been observed for the uptake of Biot-PEG₆₂-*b*-PMLABe-FA based NPs
384 (Figure 5, blue curve).

385

386 ***Figure 5***

387

388 Furthermore, we have evaluated the specificity of the uptake of RGD modified fluorescent NPs by
389 HepaRG cells by comparing the efficiency of the uptake in absence or presence of an excess of free
390 RGD peptide at a concentration of 34 μ M (Figure 6). The results of fluorescence intensity measured by
391 flow cytometry indicated that the pre-incubation of HepaRG cells with free RGD peptide before the
392 addition of RGD modified fluorescent NPs strongly inhibited the cell uptake. Therefore, we can
393 conclude that the presence of the RGD peptide at the NP's surfaces is responsible for the increase in
394 HepaRG cell uptake of NPs.

395

396

Figure 6

397

398 In final experiment, we compared the efficiency of the uptake of RGD modified fluorescent NPs by
399 three different cell lines: the hepatoma HepaRG, the colon HT29 and the cervical HeLa cancer cells
400 (Figure 7).

401

402

Figure 7

403

404 The three cell lines showed low levels of fluorescence following incubation with Biot-PEG₆₂-b-
405 PMLABe-FA NPs for 24 hours demonstrating that these biotinylated NPs are poorly uptaken by cells
406 from different tissue origin. Importantly, the three cell types exhibited very different uptake of the
407 RGD-Biot-Strept-Biot-PEG₆₂-b-PMLABe-FA formed NPs. The number of positive HT29 cells is very
408 low (< 5%) while about 90% of HeLa cells captured the RGD modified fluorescent NPs (Figure 7).
409 However, the degree of uptake (fluorescence intensity) in HeLa cells is much lower than the one
410 observed for HepaRG cells (Figure 7). This result suggested that the number of membrane receptors
411 binding RGD peptide (integrin family members) on HeLa cells is sufficient to trigger the uptake of
412 NPs by most of the cells. However, the density of these receptors onto HeLa cells might be lower than

413 onto HepaRG cells leading to a higher degree of NPs captation by the hepatoma cells than the HeLa
414 cells.

415

416 **4. Conclusions**

417 In this study, we have demonstrated a rapid release of the Dox from PEG₄₂-*b*-PMLABe and Biot-
418 PEG₆₂-*b*-PMLABe NPs over the first 6 hours. However, the NPs, which are stable in water and culture
419 medium for several days, entrapped a fraction of the drug that is slowly released over several days.
420 Then, we characterized the cell uptake of PEG₄₂-*b*-PMLABe and Biot-PEG₆₂-*b*-PMLABe NPs using
421 fluorescein amine modified polymers. We demonstrated that biotinylated NPs modified with the cyclic
422 RGD peptide significantly increase the uptake by HepaRG cells in comparison to NPs formed by Biot-
423 PEG₆₂-*b*-PMLABe-FA without peptide. We have also proved that this capture was dependent on the
424 presence of the RGD peptide because the addition of an excess of free RGD peptide strongly inhibited
425 the uptake. In addition, our results indicate that the RGD peptide presents a real tropism for liver cells
426 since its uptake by HeLa and HT29 cells was much lower than the uptake by HepaRG cells. These
427 results should be complemented by a larger study including other liver and non-hepatic cell lines as
428 well as primary cells such as endothelial cells and normal hepatocytes. Our study also provides a proof
429 of concept for the use of the versatile molecular scaffold presented in Figure 3 in order to screen for
430 other short peptides targeting hepatocytes and HCC. Since we have shown that it was possible to use
431 the streptavidin to graft a biotinylated peptide onto the biotin present at the surface of Biot-PEG₆₂-*b*-
432 PMLABe based NPs, we are evaluating a number of peptides without having to engage additional
433 chemistry. The most efficient peptides will be then grafted at the end of the hydrophilic PEG block to
434 avoid the use of immunogenic streptavidin for further *in vivo* biodistribution assays.

435

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597 Yang, W., Cheng, Y., Xu, T., Wang, X., Wen, L.P. 2009. Targeting cancer cells with biotin-dendrimer
598 conjugates., Eur. J. Med. Chem. 44, 862-868.

599

600 Table 1. Characteristics of Dox-loaded NPs.

NPs	Diameter (nm) ^a	Polydispersity index (Ip) ^a	Zeta potential (mV) ^a	Encapsulation efficiency (%) ^b
PEG ₄₂ - <i>b</i> -PMLABe	66 ± 7	0.22	- 8 ± 2	36 ± 3
Biot-PEG ₆₂ - <i>b</i> -PMLABe	74 ± 4	0.22	- 6 ± 1	32 ± 2

601

a. Measured by DLS (Delsa™ Nano Beckman Coulter); b. Measured by UV at 485 nm.

602

603 Table 2. Characteristics of fluorescent NPs measured by DLS (Delsa™ Nano Beckman Coulter).

NPs	Diameter (nm)	Polydispersity index
PEG ₄₂ - <i>b</i> -PMLABe-FA + PEG ₄₂ - <i>b</i> -PMLABe (50wt%)	64 ± 14	0.16
Biot-PEG ₆₂ - <i>b</i> -PMLABe-FA + Biot-PEG ₆₂ - <i>b</i> -PMLABe (50wt%)	70 ± 13	0.22

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618 Table 3. Characteristics of NPs modified or not by the strep-Biot-RGD construct measured by DLS
 619 (Delsa™ Nano Beckman Coulter).

Conditions	NPs Biot-PEG ₆₂ -b-PMLABe		NPs Biot-PEG ₆₂ -b-PMLABe + strep-Biot-RGD	
	Diameter (nm)	Ip	Diameter (nm)	Ip
Initial (2.5 g/L)	53 ± 12	0.17	--	--
Solution in PBS (0.107 g/L)	79 ± 15	0.37	208 ± 30 ^(a)	0.35 ^(a)
Solution in culture medium without serum (0.107 g/L)	78 ± 15	0.29	191 ± 21	0.28
Solution in culture medium with serum (0.107 g/L)	106 ± 30	0.15	185 ± 30 ^(b)	0.23 ^(b)

620 (a). Presence of a second peak centered at 600 ± 100 nm; (b). Presence of a second peak centered at

621 970 ± 220 nm

622

623 **Figure captions:**

624 Figure 1. Uses of PMLA derivatives as building blocks for the design of versatile NPs.

625

626 Figure 2. Release profile of Dox in water (A) and in culture medium (B) at 37°C.

627

628 Figure 3. Schematic representation of the molecular scaffold following grafting of biotinylated peptide
629 on the biotinylated NPs via streptavidin as a bridging factor.

630

631 Figure 4. Flow cytometry (right) and *in situ* fluorescence microscopy (left) analysis of HepaRG cells
632 incubated with Biot-PEG₆₂-*b*-PMLABe NPs, Biot-PEG₆₂-*b*-PMLABe-FA NPs and RGD-Biot-Strept-
633 Biot-PEG₆₂-*b*-PMLABe-FA NPs. For Facs analysis, size (FSC-H) and granularity (SSC-H) were
634 visualized to select the R1 gate corresponding to living cells. Detection on fluorescent cells in the R1
635 gate was performed using the FL1-H channel: negative cells incubated with Biot-PEG₆₂-*b*-PMLABe
636 NPs were set in the M1 window and positive cells were detected in the M2 window. For cells
637 incubated with Biot-PEG₆₂-*b*-PMLABe-FA and RGD-Biot-Strept-Biot-PEG₆₂-*b*-PMLABe-FA NPs (\geq)
638 50 and (\geq) 90% of cells were fluorescent, respectively; white bar : 50 μ m.

639

640 Figure 5. Time course of the uptake of NPs by HepaRG cells. Red curve: RGD-Biot-Strept-Biot-
641 PEG₆₂-*b*-PMLABe-FA NPs; Blue curve: Biot-PEG₆₂-*b*-PMLABe-FA NPs; white bar : 50 μ m.

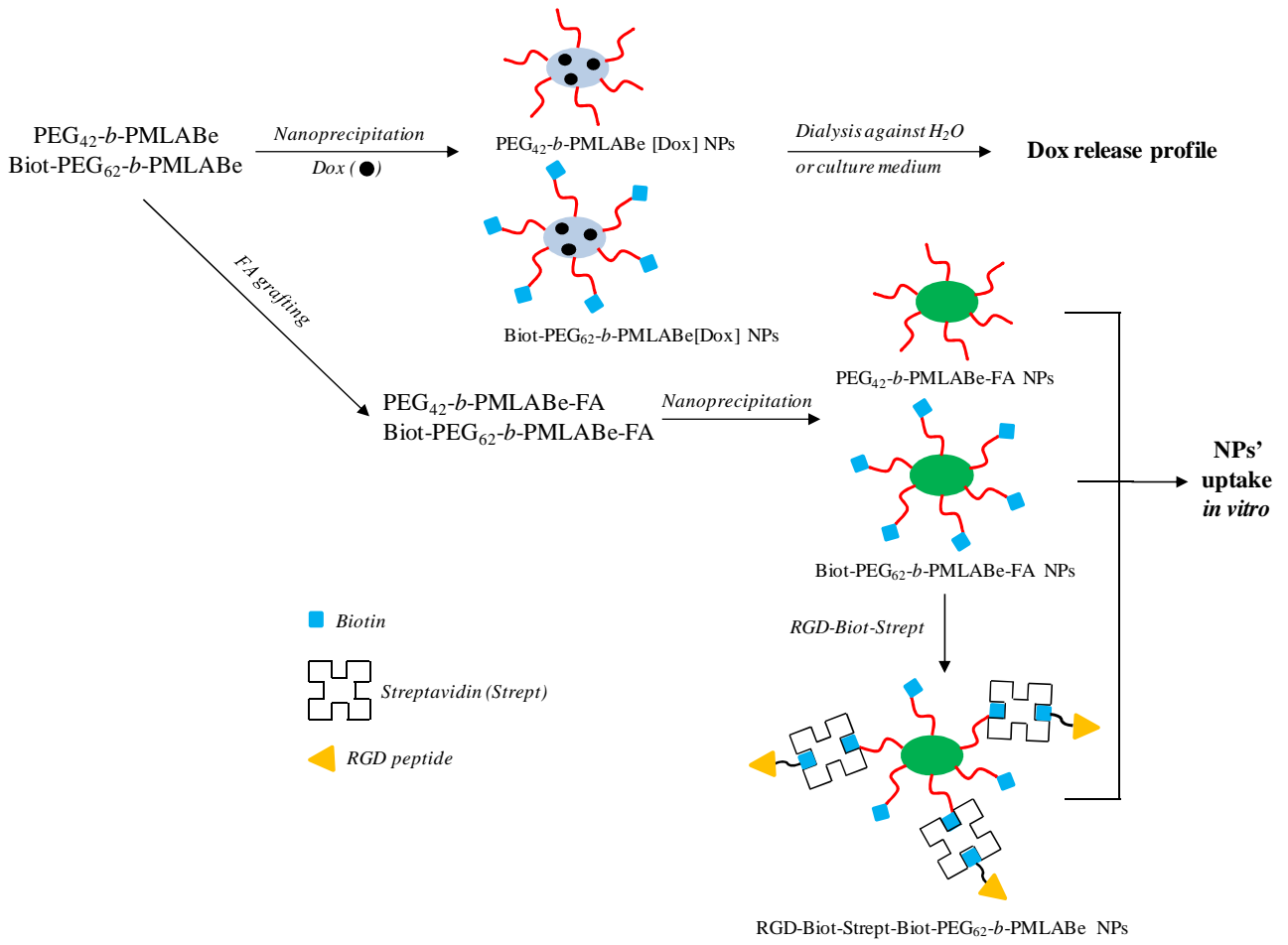
642

643 Figure 6. Captation of fluorescent nanoparticles (24 hour incubation) by HepaRG cells in absence or in
644 presence of free RGD peptide; Flow cytometry (Left) and fluorescence microscopy (Rigth) analyses
645 with Biot-PEG₆₂-*b*-PMLABe-FA NPs, RGD-Biot-Strept-Biot-PEG₆₂-*b*-PMLABe-FA NPs and RGD-
646 Biot-Strept-Biot-PEG₆₂-*b*-PMLABe-FA NPs + 34 μ M of free RGD peptide. Bottom chart: Mean of
647 fluorescence under the various tested conditions.

648 Figure 7. Flow cytometry measurements of fluorescence in HepaRG, HT29 and HeLa cells incubated
649 with Biot-PEG₆₂-*b*-PMLABe-FA or RGD-Biot-Strept-Biot-PEG₆₂-*b*-PMLABe-FA formed NPs for 24
650 hours. Left chart: Number of positive cells; right chart: Mean of fluorescence.

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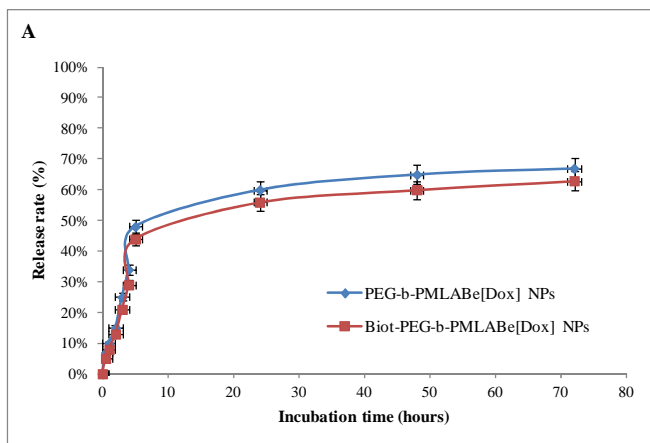
652 Figure 1.



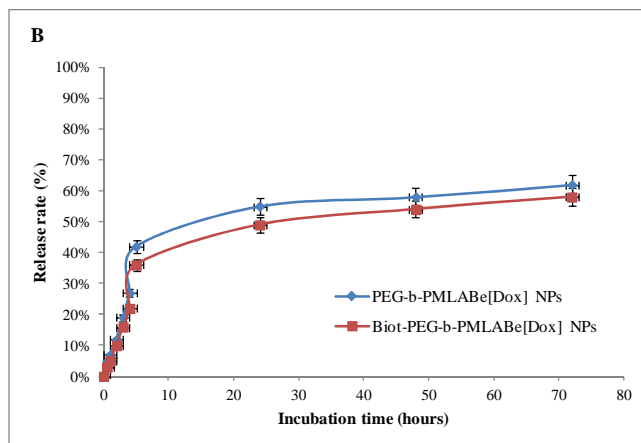
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655 Figure 2.



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658 Figure 3.

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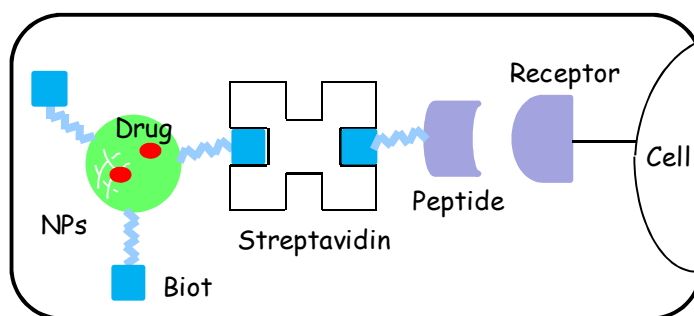
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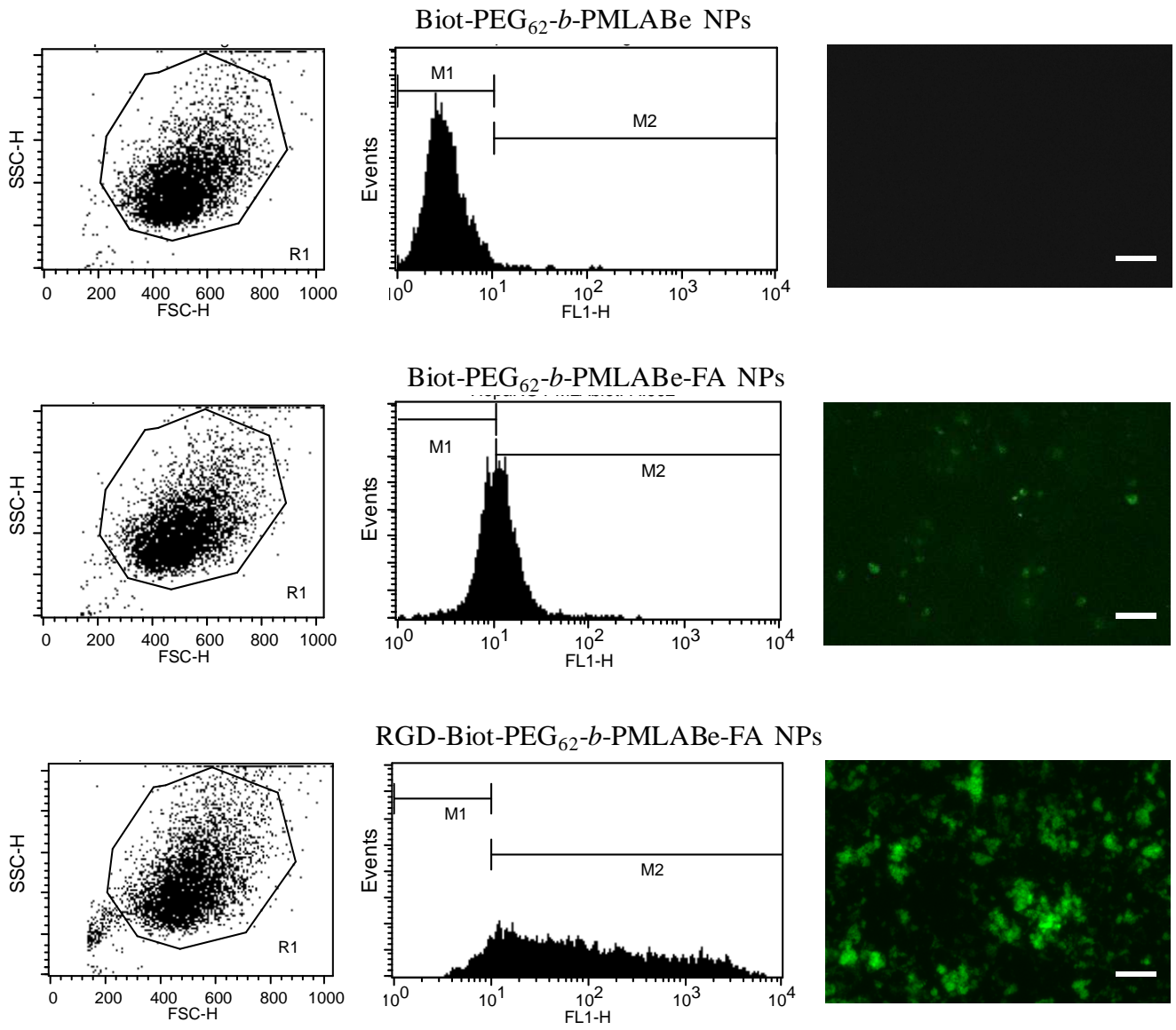
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666 Figure 4.



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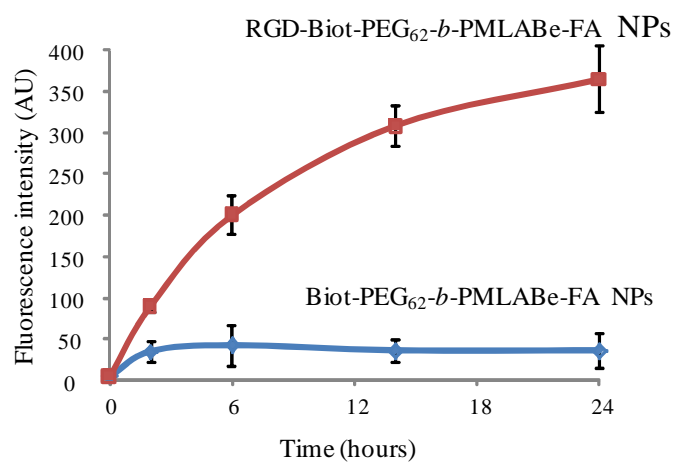
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678 Figure 6.

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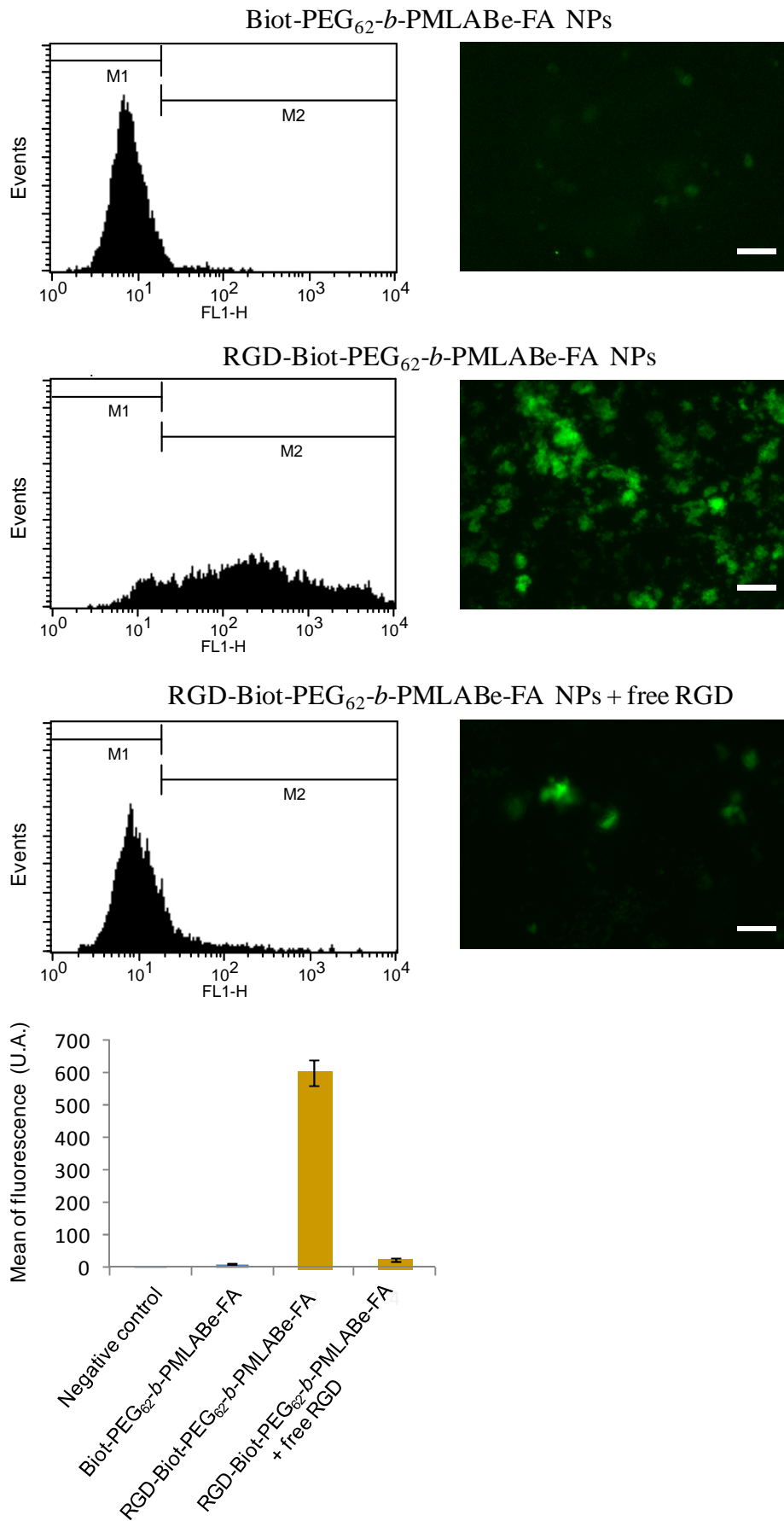
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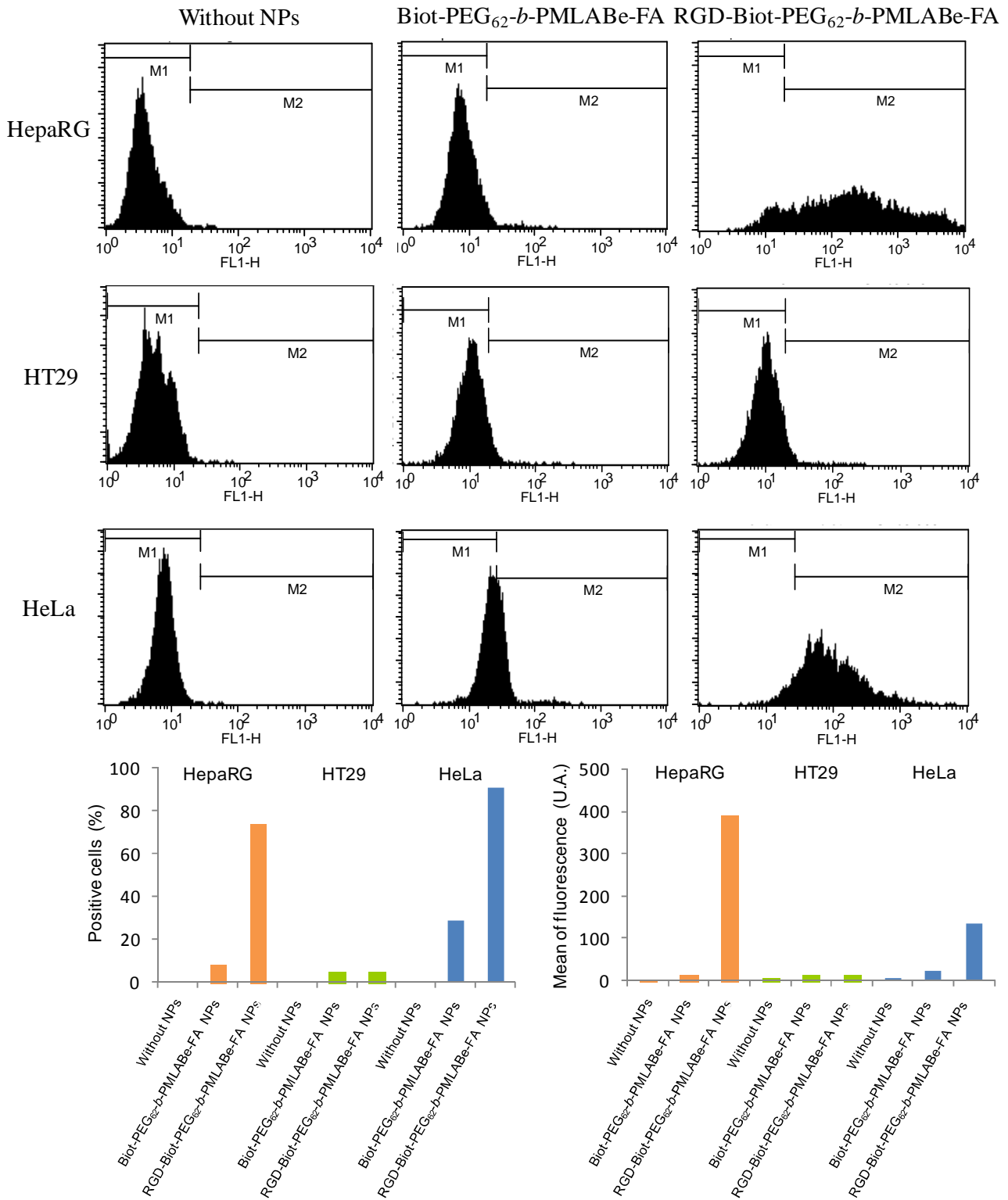
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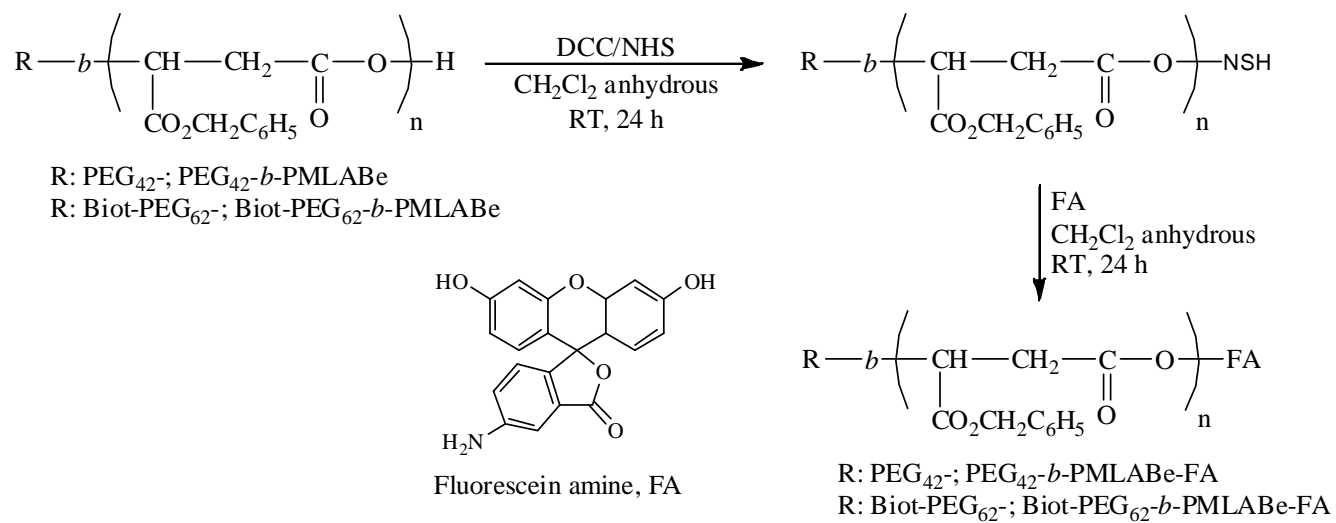
707 *Scheme captions*

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709 Scheme 1. Synthetic route to FA grafted PEG₄₂-b-PMLABe and Biot-PEG₆₂-b-PMLABe.

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711 Scheme 1.



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