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# Fluorescent nanocarriers targeting VCAM-1 for early detection of senescent endothelial cells

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#### 11 Abstract

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Endothelial senescence has been identified as an early event in the development of endothelial dysfunction, a hallmark of cardiovascular 12 disease. This study developed theranostic nanocarriers (NC) decorated with VCAM-1 antibodies (NC-VCAM-1) in order to target cell 13 surface VCAM-1, which is overexpressed in senescent endothelial cells (ECs) for diagnostic and therapeutic purposes. Incubation of Ang II-14 15 induced premature senescent ECs or replicative senescent ECs with NC-VCAM-1 loaded with lipophilic fluorescent dyes showed higher fluorescence signals than healthy EC, which was dependent on the NC size and VCAM-1 antibodies concentration, and not observed 16 following masking of VCAM-1. NC loaded with omega 3 polyunsaturated fatty acid (NC-EPA:DHA 6:1) were more effective than native 17 EPA:DHA 6:1 to prevent Ang II-induced VCAM-1 and p53 upregulation, and SA-β-galactosidase activity in coronary artery segments. 18 19 These theranostic NC might be of interest to evaluate the extent and localization of endothelial senescence and to prevent pro-senescent 20 endothelial responses.

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22 Key words: Nanocarriers; Endothelial senescence and dysfunction; VCAM-1; Omega 3 polyunsaturated fatty acid

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Cardiovascular diseases (CVDs) are the leading cause of death in the developed world and represent an immense clinical burden.<sup>1</sup> Atherothrombosis, a chronic and progressive inflammatory disease,<sup>2</sup> has been identified as the major contributor to the pathogenesis of CVDs including acute coronary syndrome and stroke.<sup>3</sup>

Although clinical scores, such as that derived from the Framingham Heart Study, are useful to evaluate the cardiovascular risk of patients, they may lose predictive value in the large segment of the population at intermediate risk.<sup>4</sup> Thus, there is a

https://doi.org/10.1016/j.nano.2021.102379 1549-9634/© 2021 Elsevier Inc. All rights reserved. crucial need for novel diagnostic tools to identify early molecular 34 changes associated with atherogenesis and to assess noninva-35 sively the benefit of therapies, and also for new targeted and 36 specific therapies. Recent findings have emphasized the potential 37 role of nanoparticles, as promising tools for treatment of vascular 38 disease, including atherothrombosis and its associated 39 complications.<sup>5</sup> The recent development of ultrabright theranos-40 tic nanoparticles that may simultaneously act as carriers of both 41 imaging payloads and therapeutic agents, has great promises for 42 the future of personalized medicine.<sup>6</sup> 43

The atherogenesis process starts before childhood with the 44 appearance of early lesions of atherosclerosis, which can progress 45 into mature plaques impeding blood flow and optimal perfusion of 46 target organs, and, ultimately, trigger an atherothrombotic event. 47 Interestingly, the atherogenesis process is not a generalized 48 alteration of the cardiovascular system but is targeting well-49 defined arterial sites at risk such as bifurcations and curvatures 50 including the carotid and aorta-renal bifurcations.<sup>7,8</sup> Such 51

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atheroprone areas are characterized by an early development of 52 53 endothelial dysfunction most likely as a consequence of their particular local flow behavior involving disturbed flow and low 54 shear versus the atheroprotective areas characterized by laminar 55 flow and high shear.<sup>3,9</sup> Indeed, high shear stress is a pivotal trigger 56 of endothelial cell (ECs) protective mechanisms especially the 57 58 formation of nitric oxide (NO), a potent vasodilator and inhibitor of 59 atherothrombotic responses. Thus, ECs at athero-susceptible arterial sites are unlikely to protect adequately the arterial wall 60 thereby accelerating the development of atherosclerotic lesions.<sup>3</sup> 61

Recent investigations support the concept that endothelial 62 senescence is a determinant event leading to the development of 63 endothelial dysfunction.<sup>10,11</sup> Cellular senescence is character-64 ized by the hallmark marker senescence-associated β galactosi-65 dase activity, an irreversible cell cycle arrest, <sup>12</sup> and the induction 66 of a pro-inflammatory senescence-associated secretory pheno-67 type (SASP)<sup>13,14,15</sup> with increased expression of VCAM-1, 68 MCP-1 and tissue factor, and down-regulation of the endothelial 69 formation of NO, and is induced by pro-oxidant stimuli such as 70 angiotensin II (Ang II) and elevated glucose concentration.<sup>16,17</sup> 71 Increased numbers of senescent ECs have been observed 72 overlying plaques in the human coronary artery<sup>18</sup> and the aortic 73 arch.<sup>19</sup> The fact that overexpression of the senescence marker 74 p53 selectively in the endothelium blunted NO formation and 75 promoted endothelial dysfunction in rat aortic rings,<sup>20</sup> suggests 76 that endothelial senescence leads to endothelial dysfunction. 77 78 Among vasoprotective treatments, omega 3 polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA) and 79 docosahexaenoic acid (DHA) have been shown to protect the 80 cardiovascular system, in part, by causing a sustained endothelial 81 formation of NO. Indeed, our previous studies indicated that 82 EPA:DHA 6:1 is a superior omega 3 PUFA formulation to 83 induce endothelium-dependent vasorelaxation in coronary artery 84 rings, to cause eNOS activation in ECs, and to improve aging-85 related endothelial dysfunction.<sup>21,22</sup> 86

Thus, the main objective of the present study was to target 87 endothelial senescence by taking advantage of a unique feature 88 of the cell membrane of senescent ECs, the increased cell surface 89 90 expression of VCAM-1. For this purpose, a core-shell nanoe-91 mulsions carrier decorated with antibodies (Abs) targeting 92 VCAM-1 was generated and loaded with either lipophilic red 93 or near-infrared (NIR) fluorescent dyes to enable detection of 94 senescent ECs using fluorescence imaging or EPA:DHA 6:1 for delivery and regeneration of the protective endothelial function. 95

#### 96 Methods

A detailed description of the experimental protocols andchemicals is provided in the Supplementary material.

Preparation of maleimide-decorated nanoemulsions: grafting ofanti-VCAM-1 antibody and fluorescent dye loading

Oil and aqueous phases were prepared separately and heated at 90 °C for 30 min. Oil phase consists of polymer and 1% of dye based on Nile Red (NR668)<sup>23</sup> or NIR cyanine with a bulky counterion tetraphenylborate (Cy5.5-TPB)<sup>24</sup> dissolved in oil (Labrafac WL®), while the aqueous phase used was distilled water. 100 mg of maleimide modified polymer (synthesis 106 described in supporting information) and 25 mg of dye/Labrafac 107 WL® were mixed in a vial (4 ml). Then, 800 µl of hot distilled 108 water was added and this mixture was homogenized by vortex 109 for 30 s to obtain a primary emulsion and finally homogenized 110 by ultrasonication to produce nanoemulsions (technical details 111 are reported in the Supplementary information section). In some 112 experiments, the non-functionalized nanocarriers were formu- 113 lated with EPA:DHA 6:1 instead of Labrafac WL® as dispersed 114 phase and distilled water as continuous phase, without any 115 fluorescent probe. Furthermore, different amounts of the anti- 116 VCAM-1 Ab were added into the diluted nanoemulsions to study 117 the effect of increasing concentrations of the antibody on the 118 extent of grafting. After Ab addition, the nanoemulsions were 119 incubated for 24 h at 4-6 °C to cause attachment of the Ab onto 120 the maleimide functions at the surface of the nanocarriers (NC- 121 VCAM-1). The maleimide-decorated nanoemulsions, without 122 grafting of the antibody at the surface of the nanocarriers (NC), 123 were also used as a control formulation for the study. 124

#### Endothelial cell culture and preparation of coronary artery rings 125

ECs were isolated from freshly harvested porcine left 126 circumflex coronary arteries using type I collagenase as 127 described previously,<sup>25</sup> and cultured in MCDB 131 medium 128 containing 15% fetal calf serum, fungizone (2.5  $\mu$ g/ml), 129 penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and 5 L- 130 glutamine (2 mM). ECs at passage 1 were exposed to serum- 131 free culture medium for 2 h before the addition of Ang II to 132 induce premature senescence. ECs passaged at a ratio of 1:3 until 133 passage 3 to induce replicative senescence were also studied.<sup>26</sup> 134 Coronary artery segments were incubated in RPMI without 135 serum for 1 h before the addition of Ang II. Thereafter, they were 136 embedded in FSC22 Frozen section medium and frozen. 137

Detection of senescence-associated β-galactosidase activity 138 (SA-β-gal) 139

Senescence-associated  $\beta$ -galactosidase activity was assessed 140 in porcine coronary artery segments by staining with X-gal 141 solution as previously reported.<sup>27</sup> 142

#### *Immunofluorescence studies and physicochemical characterization* 143 *of nanocarriers* 144

For immunofluorescence histochemistry, ECs and isolated 145 healthy porcine coronary artery rings were evaluated using a 146 Leica SP2 UV DM Irbe confocal laser-scanning microscope with 147  $20 \times$  or  $63 \times /OIL$  CS objectives. Quantification of fluorescence 148 levels was performed with macros developed with Image J 149 software. Nanocarrier physicochemical characteristics were 150 evaluated for the chemical optimization, such as the nanocarrier 151 average size, the different amounts of Abs, the PEG/PEG- 152 antibody ratio, the temperature stability, the use of different 153 fluorescent probes and the targeting efficiency. 154

#### Statistical analysis

Data are expressed as means  $\pm$  SEM. Statistical analysis was 156 assessed by one-way analysis of variance followed by Bonferro- 157 ni's Multiple Comparison post hoc test using GraphPad Prism 158

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Figure 1. Design and chemical development of targeting lipid nanocarriers. (A) Synthesis of the maleimide decorated polymer, starting by a reaction between PMAO and Boc-NH-PEG3000-NH<sub>2</sub> on 20% of the anhydride functions of the polymer (1); the remaining 80% of the anhydride functions of the polymer were then reacted with Jeffamine-2000 (2); afterwards, ethanolamine was used to neutralize the unreacted carboxylic groups (3); then the deprotection of the amino functions of the Polymer-Boc<sub>20</sub> was performed (4) followed by coupling of amino groups with the maleimidoacetic acid (5) resulting in the formation of Polymer-Mal<sub>20</sub>. (B) Formation of nanoemulsions by ultrasonication method and (C) grafting of VCAM-1 Abs with the available maleimide functions on the surface of the nanocarriers. (D) TEM characterization of nanoemulsions formed by using polymer dissolved in 1% Cy5.5-TPB solution in Labrafac® as oil phase and distilled water as aqueous phase with spherical shape.

159 (Version 5). Group differences were considered statistically 160 significant at P < 0.05.

#### 161 **Results**

#### 162 Synthesis of the amphiphilic polymer and evaluation of the 163 structure by IH NMR analysis

164 As a polymeric platform, PMAO is an alternated polymer (average  $M_n$  30,000-50,000) with a repeating unit composed of a 165 hydrophobic hydrocarbon chain  $(C_{18})$  and a succinic anhydride 166 function. As illustrated, an amphiphilic polymer (Polymer-167 168 Boc<sub>20</sub>) was first synthesized through a reaction of PMAO with an amino-PEG<sub>3000</sub> bearing a protected amino function (Boc-NH-169 PEG<sub>3000</sub>-NH<sub>2</sub>) on 20% of the anhydride functions (Figure 1, A). 170 The remaining 80% of anhydride functions were then reacted 171 with Jeffamine®2000, an amino/methoxy terminated PEG/PPG 172 (poly(ethylene glycol)/poly(propylene glycol)) copolymer, to 173 reduce the polymer hydrophobicity. Then the deprotection of the 174 amino functions of Polymer-Boc20 was performed followed by 175 coupling of the amino groups with the maleimidoacetic acid to 176 result in the formation of Polymer-Mal<sub>20</sub> (Figure 1, A). The Q7 progress of the reaction, the structure of the polymer was studied 178 by performing NMR at each step of the synthesis of this 179 180 amphiphilic polymer derivative (reported in Supplementary 181 Information Figure S1). In Figure S1, ethanol is visible (not dry enough after dialysis) as well as the peak of NHBoc. The 182 integration and comparison to the CH<sub>3</sub> of PEG show 183 functionalization with PEG-NHBoc close to 20% (equal to 184 1.72 while 20% would correspond to a value of 1.8). This result 185 proves that the ratio expected between non-functional and 186 functional PEG chains is obtained. This value of 80% is 187 confirmed with the integration of the NMR peak of the Me 188

function (terminating the Jeffamines chains), as indicated in 189 Figure S1. Then, the disappearance of the NHBoc peak in Figure 190 S2 clearly confirmed the deprotection has been efficiently done. 191 Comparison of two spectra is reported in Figure S3. On the other 192 hand, NMR spectra with maleimide addition appear to be 193 identical to the those after deprotection of Boc (Figure S4), likely 194 due to screening of the numerous PEG chains present in these 195 macromolecules. To summarize, NMR results have confirmed 196 the attachment of Jeffamine® 2000 (80%) and PEG-NH<sub>2</sub> (20%) 197 with the polymer network and confirmed the gradual building of 198 the functional polymer. 199

#### Preparation of targeted fluorescent lipid nanocarriers 200

The multifunctional lipid nanocarriers were formulated by 201 ultrasonication method. To render them fluorescent, their core was 202 loaded with highly lipophilic dyes (NR668 or Cy5.5-TPB).<sup>22,23</sup> 203 While NR668, operating in the red spectral range, is well adapted for 204 fluorescence microscopy studies, the NIR dye Cy5.5-TPB is more 205 suitable for in vivo and tissue imaging and they both display high 206 stability against dye leakage in biological media.<sup>28</sup> The amphiphilic 207 polymer derivative described above was used to stabilize the 208 nanoemulsions and also to provide the facility to functionalize the 209 nanocarriers after their formulation. During the formation of 210 nanodroplets the amphiphilic polymer is expected to reorganize in 211 such a way that the hydrophobic hydrocarbon chains remain in the 212 apolar interior of the nanodroplets (oil phase) whereas the 213 hydrophilic part of the polymer (J-2000 and maleimide-decorated 214 PEG chains) is exposed towards the interface of the droplets 215 (towards aqueous phase), as previously described.<sup>29</sup> This provides 216 active maleimide functions at the surface of the nanodroplets which 217 will ensure an anchorage of the VCAM-Abs, because the polymer is 218 a part of the nanocarrier "shell" (Figure 1, B). Antibody decoration is 219 performed by incubation of the functional maleimide-decorated 220

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Figure 2. Optimization and evaluation of the theranostic potential of nanocarriers targeting surface VCAM-1 in a model of premature senescence. ECs are incubated with Ang II for 24 h to induce premature senescence and subsequently with either nanocarriers (NC) or nanocarriers decorated with VCAM-1 Abs (NC-VCAM-1) for 1 h. Experimental conditions tested NC with different diameters (A), different concentrations of the anti-VCAM-1 Abs (B), different storage temperatures (C), and containing a specific designed lipophilic dye (NR668 or Cy5.5-TPB) (D). NC and NC-VCAM-1 fluorescence staining appears in red and nuclei are stained with DAPI (blue). Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean  $\pm$  SEM of n = 3-9 D. \*P < 0.05 vs respective NC, \*P < 0.05 vs respective NC-VCAM-1, \*P < 0.05 vs Ang II NC-VCAM-1 4.56 µg/ml. Original magnification, 63×. Scale bar = 20 µm.

nanoemulsions with the solution of anti-VCAM-1 Abs attaching the nanodroplets surface (Figure 1, *C*), giving rise to functionalized nanocarriers. As a control formulation, nanoemulsions with a polymer without having the Boc-NH-PEG<sub>3000</sub>-NH<sub>2</sub> component, i.e., PMAO:J-2000, 1:1 respectively were prepared. This formulation was prepared to compare the average hydrodynamic diameter as well as the surface charge on the droplets with the ones obtained with 227 maleimide decorated nanocarriers. 228

Characterization of size and surface charge 229

The nanocarriers loaded with fluorescent probes (NR668 or 230 Cy5.5-TPB) were characterized by DLS analysis. The average 231

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D NR668 Cy 5.5-TPB DAPI NC-VCAM-1 MERGED Fluorescence Intensity (% of Basal NC) 600 \*# \*# 400 200-0 NC-VCAM-1 NC NC-VCAM-1 ŃĊ NC-VCAM-1 ŃС NC-VCAM-1 NC Basal Ang II Basal Ang II (100 nM, 24 h) (100 nM, 24 h)

Figure 2. (continued).

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hydrodynamic diameter of the maleimide-decorated nanoemul-232 sions was  $96 \pm 7$  nm (n = 10). The hydrodynamic diameters 233 obtained for nanocarriers developed by using Labrafac®, omega 234 3 EPA:DHA 6:1 oil and corn oil as dispersed phase were 96 nm. 235 236 80 nm and 190 nm, respectively. This difference is likely related to the nature of this oil impacting on the spontaneous 237 emulsification process.<sup>30</sup> In the other two cases the average 238 hydrodynamic diameters of the nanodroplets were below 239 100 nm, the optimal size for theranostic applications.<sup>31</sup> The 240 average size of the nanodroplets obtained in the case of control 241 formulation (PMAO: J2000 1:1) was considerably larger (around 242 160 nm) as compared to the maleimide-decorated nanodroplets. 243 According to zeta-potential measurements, the surface charge on 244 the nanoemulsion droplets, in the control formulation, was 245 negative (-39 mV $\pm$ 1, n = 3) and the surface charge on the 246 maleimide-decorated nanodroplets was positive and ranged from 247 248 10 to 15 mV, regardless of the nature of the oil used to formulate the nanocarriers (n = 8 for NR668/Cy5.5 TPB in Labrafac®, 249 n = 3 for omega 3 EPA:DHA 6:1, n = 3 for corn oil). This 250 251 change in the surface charge from negative to positive is most likely due to complete saturation of the carboxylic groups, 252 253 followed by grafting of maleimide on the available sites.

TEM analysis of these nanocarriers (containing Cv5.5-TPB) 254 255 is reported in Figure 1, D. The results confirmed the morphology and average size of the nanoemulsions given by DLS, i.e. below 256 257 100 nm. In addition, micrographs disclosed structural information, indeed coherent with the one expected: oil core (arrow 1 in 258 Figure 1, D) is surrounded by a polymer shell (arrow 2). In some 259 particles the presence of amorphous polymer can be observed 260 (arrow 3). Interestingly, the bright aspect of the polymer shell is 261 related to a pure and concentrated material, and this can be 262 explained by the process itself. Initially the polymer is 263 homogeneously solubilized in the oil phase, and then the 264 emulsification process induces its concentration at the interface, 265 making a concentrated shell. In some cases, excess of polymer 266 (arrow 3) has induced amorphous domains. TEM pictures have 267 confirmed a nanocarrier structure in line with the one eventually 268 expected. 269

#### 270 Optimization and evaluation of theranostics nanocarriers in in 271 vitro senescence models

272 Size

In order to determine the importance of the size of VCAM-1 273 Ab-decorated nanocarriers, lipid nanocarriers with hydrodynam-274 ic diameters below 100 nm. 100-150 nm and 150-200 nm 275 ranges were prepared by changing the time of formulation 276 exposition to the ultrasonication process, and tested using control 277 and Ang II-induced premature endothelial cell senescence. The 278 279 highest fluorescence signal in cells was observed with the 280 nanocarriers having sizes below 100 nm compared to those 281 having sizes ranging from 100 to 150 nm and 150-200 nm 282 (Figure 2, A). Thus, the targeting efficiency increased with the decrease in the size of the VCAM-1 Ab-decorated nanocarriers. 283 Therefore, the lipid nanocarriers having diameter size below 284 100 nm were selected and used for further experiments. It is 285 interesting to note that a better targeting efficiency is obtained for 286 smaller particles. Indeed, for a similar amount of polymer and 287

oils forming the nanocarriers, two different sizes mean a 288 difference in particle number (concentration) and in the particle 289 surface area. Thus, for the smaller particles, the particle 290 concentration and surface area exposing specific ligands should 291 be higher, which should favor targeting properties. In addition, 292 smaller particles diffuse faster, allowing faster ligand/receptor 293 interaction.

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#### Different concentrations of anti-VCAM-1 antibodies

To find the optimum concentration of the Abs linked to the 296 maleimide active sites at the surface of the nanocarriers for 297 targeting, different concentrations of the anti-VCAM-1 Abs were 298 tested and analyzed. Higher fluorescence signals were obtained 299 with the formulation with the highest antibody concentration 300 (4.56  $\mu$ g/ml) in Ang II-induced endothelial cell senescence 301 whereas low levels were observed in control cells (Figure 2, *B*), 302 indicating that the targeting was directly dependent on the 303 amount of VCAM-1 Abs decorating the nanocarriers. All further 304 experiments were performed with NC-VCAM-1 generated with 305 anti-VCAM-1 Abs at a concentration of 4.56  $\mu$ g/ml. 306

#### Different temperature and encapsulated dye

To further characterize the targeting of the formulations and 308 validate their stability, the role of the temperature was assessed. 309 The fluorescent VCAM-1 Abs decorated nanocarriers targeted 310 Ang II-induced senescent ECs within 1 h, at both 4 °C and 311 37 °C (body temperature) with minimal off-target cell surface 312 interactions (Figure 2, *C*). Nanocarriers labeled with specially 313 designed lipophilic derivatives of dyes either NR668 or Cy5.5- 314 TPB were bright enough to allow the fine tracking of interactions 315 with biological systems (Figure 2, *D*). Altogether, these results 316 indicate that the different temperatures and fluorescent probes 317 can be used to image active targeting of cells by the lipid 318 nanocarriers. 319

#### Specific targeting of NC-VCAM-1 in premature and replicative 320 senescence ECs 321

The targeting specificity of fluorescent VCAM-1 Abs 322 decorated nanocarriers at 37 °C was evaluated by competition 323 experiments using a VCAM-1 Ab to mask the VCAM-1 antigen. 324 Nanocarriers decorated with VCAM-1 Abs strongly stained Ang 325 II-induced senescent ECs and replicative senescence ECs 326 (passage 3, Figure 3. *A*, *B*) whereas no such effects were 327 observed following pretreatment of the cells with a VCAM-1 Ab 328 demonstrating their uniqueness to selectively target membrane 329 surface VCAM-1. 330

#### Protective effect of theranostics NC-EPA:DHA 6:1 in native 331 endothelium of coronary artery segments 332

Since all investigations were performed with cultured ECs, 333 experiments evaluated the protective effect of the omega 3 EPA: 334 DHA 6:1 formulation and corn oil on Ang II-induced premature 335 senescence in coronary artery segments, and to assess the impact of 336 nanoencapsulation of the two oils. Exposure of coronary artery 337 segments to Ang II upregulated the expression level of VCAM-1 338 and the senescence marker p53, and increased SA- $\beta$ -gal activity 339 predominantly in the endothelium (Figure. 4, *A*, *B*, *C*). The EPA: 340 DHA 6:1 treatment significantly reduced the stimulatory effect of 341

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Figure 3. Selective targeting of the VCAM-1 decorated nanocarriers to premature and replicative senescent ECs. Nanocarriers decorated with VCAM-1 Abs (NC-VCAM-1) strongly stain ECs following their exposed to Ang II for 24 h to induce premature senescence (**A**), and ECs at passage 3 (P3, replicative senescence) (**B**). The targeting potential of NC-VCAM-1 is prevented by the previous exposure of ECs to the VCAM-1 Ab. NC and NC-VCAM-1 fluorescence staining appears in red and nuclei are stained with DAPI (blue). Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean  $\pm$  SEM of n = 3-4. \*P < 0.05 vs respective NC, \*P < 0.05 vs Basal NC-VCAM-1, \*P < 0.05 vs respective NC-VCAM-1. Original magnification, 63×. Scale bar = 20 µm.

Ang II on VCAM-1 and SA- $\beta$ -gal activity and reduced that on p53, 342 which, however, did not reach statistical significance (Figure 4, A, 343 B, C). The corn oil treatment blunted the stimulatory effect of Ang 344 II on SA-β-gal activity and did not significantly affect that on 345 VCAM-1and p53 (Figure 4, A, B, C). NC-EPA:DHA 6:1 markedly 346 347 and significantly prevented Ang II-induced VCAM-1 and p53 348 upregulation in the endothelium of coronary artery segments, 349 compared to the NC-CORN OIL (Figure 5, A, B). NC-EPA:DHA but not NC-CORN OIL also slightly but significantly reduced the 350 VCAM-1 and p53 expression levels in control coronary artery 351 segments (Figure 5, A, B). 352

#### 353 Discussion

Atherosclerosis underlying major cardiovascular diseases, is 354 355 a silent killer, which cannot be easily detected at an early stage by current imaging methods. Since the induction of endothelial 356 senescence is thought to promote the development of endothelial 357 dysfunction, an early event in the atherogenesis process, 358 endothelial senescence appears to be an interesting target for 359 both preventive and therapeutic interventions.9,26,32,33 The 360 361 major findings of the present study indicate the ability of nanocarriers decorated with VCAM-1 Abs and loaded with 362 363 lipophilic fluorescent dyes to selectively and specifically target VCAM-1 overexpressed at the surface membrane of cultured 364 365 senescent ECs compared to healthy ECs. They further show that nanoencapsulation of the vasoprotective omega 3 formulation 366 EPA:DHA 6:1 is associated with enhanced inhibition of pro-367 atherosclerotic and pro-senescence responses in the native 368

endothelium of isolated coronary arteries. Thus, fluorescent 369 nanocarriers targeting a senescence-associated cell surface 370 protein appear to be an attractive strategy to restore the protective 371 endothelial function on the vascular system. 372

A low-grade inflammatory response is involved in the 373 atherogenesis process affecting initially the endothelium with 374 the appearance at the cell surface of several pro-atherothrombotic 375 molecules including adhesion molecules that are tightly 376 regulated both spatially and temporally.<sup>3,34</sup> Thus, these novel 377 cell surface molecules are interesting targets for innovative 378 diagnostics and therapeutics approaches in atherogenesis. 379 Among potential candidates, VCAM-1 appears to be of 380 particular interest since it is involved in the development of 381 early atherosclerotic lesions<sup>35,36</sup> and has been observed on the 382 endothelial cell surface of atheroprone areas before the onset of 383 visible structural changes,<sup>34</sup> and increased levels of VCAM-1 384 expression have been associated with senescent ECs overlaying 385 human atherosclerotic plaques.<sup>18</sup>

To date, several approaches have been described to image 387 VCAM-1 and other cell adhesion molecules expression using 388 radiolabeled antibodies,.<sup>37,38</sup> However, these agents usually 389 result in modest target-to-background ratios,<sup>37,38</sup> limiting their 390 use for in vivo cardiovascular imaging. Therefore, there is a need 391 to develop alternative methods for imaging, ideally with 392 improved sensitivity and resolution. Recently, nanoparticles 393 have attracted a lot of interest as potential therapeutics, 394 diagnostics, and theranostics due to their ability to target in a 395 specific manner to the site of disease and to reduce the 396 therapeutic load and, hence, side effects. The nanometric size 397 of these materials and their surface functionalization 398

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characteristics preclude them from being readily cleared through
 the kidneys and decelerate the opsonization mechanism, thereby
 extending circulation in blood.<sup>39</sup>

Targeted nanoparticles like polymeric nanoparticles (e.g., 402 403 PLGA), liposomes and nanoemulsions, have been shown to specifically accumulate in ECs, and suggested to be used for 404 imaging or drug delivery applications.<sup>5</sup> In comparison to the 405 other nanocarriers, nanoemulsions (the system used in the 406 current study) are particularly attractive as drug delivery vehicles 407 because of their non-toxic components, high loading capacity, 408 biodegradability, good stability, low manufacturing cost and 409 their potential to encapsulate hydrophilic, lipophilic, as well as 410 amphiphilic drugs.<sup>40-44</sup> Such a system is a dispersion of two 411 immiscible liquids (water-in-oil or oil-in-water), stabilized by a 412 surfactant, which is a isotropically clear and thermo-dynamically 413 stable liquid solution, usually with droplet diameter within the 414 range of 10-500 nm.<sup>30,45,46</sup> However, it is not easy to 415 chemically modify the surface of the nanoemulsions due to the 416 challenges involved in the stabilization of the interface. Recent 417 418 findings demonstrate that when the oil phase containing the polymer is in contact with the aqueous phase, the polymer 419 420 PMAO as well as its derivatives with Jeffamine polyetheramines (J-1000 & J-2000), shows the ability to migrate at the oil-water 421 interface and produce stable oil-in-water nanoemulsions in the 422 absence of an organic solvent as well as surfactant.<sup>29</sup> In the 423 current study, a strategy to functionalize nanoemulsions with 424 antibodies based on PMAO polymer has been developed. 425 Chemical modification of PMAO, by performing a systematic 426 reaction of Jeffamine polyetheramine (Jeffamine® M2070) and a 427 polyethylene glycol (PEG)-containing compound terminated by 428 a reactive maleimide on each anhydride function of the polymer 429 has been made. The nanoemulsions are then developed using this 430 newly synthesized polymer, with some PEG presenting 431 maleimide at the surface of the nanodroplets to react with the 432 cysteine function of the anti-VCAM-1 antibody, by ensuring a 433 strong anchorage of the ligands since it will be a part of the 434 nanocarriers as a "shell". The use of Jeffamine®2000 and PEG in 435 this system, has great significance because the PEG-chains not 436 only create steric repulsion among the neighboring nanodroplets 437 438 and improve the interfacial stabilization but also have a wellrecognized ability to i) reduce the uptake of the nanoemulsions by 439 the immune system (after in vivo administration) and ii) inhibit the 440 441 opsonization and phagocytosis processes, normally observed with conventional nanoemulsions, which in turn increases the circula-442 tion time of nanoemulsions in the body.<sup>47,48</sup> Another important 443

Figure 4. Native EPA:DHA 6:1 prevents the Ang II-induced up-regulation of VCAM-1 and SA- $\beta$ -gal activity in the endothelium of coronary artery segments. Segments were either untreated, or exposed to CORN OIL or EPA: DHA 6:1 for 30 min before the addition of Ang II for 24 h. Thereafter, VCAM-1 and p53 signals were assessed by immunofluorescence, and SA- $\beta$ -gal activity using X-gal. Representative images show VCAM-1 (**A**) and p53 (**B**) staining in red, nuclei in blue and autofluorescence in green (upper panels), and SA- $\beta$ -gal activity (**C**, upper panels) and corresponding cumulative data of the endothelial signal (lower panels). Data are expressed as mean ± SEM of n = 3-4. \*P < 0.05 vs basal,  ${}^{\$}P < 0.05$  vs Ang II. Original magnification, 20×. Scale bar = 50 µm (**A**, **B**). Original magnification, 10×. Scale bar = 200 µm (**C**).



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Figure 5. NC-EPA:DHA 6:1 strongly prevent the Ang II-induced upregulation of VCAM-1 and p53 immunofluorescence signals in the endothelium of coronary artery segments. Segments were either untreated, or exposed to NC-CORN OIL or NC-EPA:DHA 6:1 for 30 min before the addition of Ang II for 24 h. Thereafter, the expression levels of VCAM-1 and p53 were assessed by immunofluorescence. Representative confocal immunofluorescence images showing VCAM-1 (**A**) and p53 (**B**) staining in red, nuclei in blue and autofluorescence in green (upper panels), and corresponding cumulative data of the endothelial signal (lower panels). Data are expressed as mean  $\pm$  SEM of n = 3. \*P < 0.05 vs respective basal, \*P < 0.05 vs untreated basal,  $^{\$}P < 0.05$  vs Ang II. Original magnification, 20x. Scale bar = 50 µm.

chemical feature of this formulation is the type of fluorescent probe
used. The probes studied, namely NR668 and Cy5.5-TPB, are
specially designed to be highly lipophilic, resistant to dye leakage

in biological media<sup>28</sup> and can be highly concentrated without 447 excessive decrease of the fluorescence quantum yield.<sup>23,24,49–52</sup> 448 As a result, nanocarriers are ultrabright and allow fine tracking of 449 their interactions with biological systems.<sup>23,24,49,50</sup> 450

The biological evaluation of the fluorescent nanocarriers 451 targeting VCAM-1 was assessed in vitro in premature and 452 replicative senescent cultured ECs<sup>26,33</sup> and ex vivo in senescent 453 native endothelium of isolated porcine coronary arteries. The 454 nanoemulsion formulation has been optimized in relation to the 455 size of the nanodroplets, which has an important impact on their 456 interaction with living cells and also on the number of functional 457 sites, <sup>31,52</sup> and the amount of the antibody to be attached to the 458 maleimide groups available at the surface of the nanodroplets, 459 which influences the efficient targeting of the targets.<sup>39</sup> These 460 studies indicate that nanocarriers with average size below 461 100 nm and the highest antibody concentration tested 462 (4.56 µg/ml) showed maximum fluorescence signals in senes- 463 cent compared to healthy ECs, indicating that the targeting was 464 directly dependent on the amount of VCAM-1 Abs decorating 465 the nanocarriers. The targeting of senescent ECs by the 466 nanocarriers is observed rapidly within 1 h, pronounced at 467 body temperature and with minimal off-target cell surface 468 interactions, and similar with the two fluorescent probes. 469

Among vasoprotective treatments, the omega 3 PUFAs, 470 including EPA and DHA, have emerged as an attractive therapy 471 to protect the cardiovascular system, by improving the 472 endothelial function.<sup>21</sup> The beneficial effect of the omega 3 473 treatment is critically dependent on the purity level of the EPA 474 and DHA, the EPA:DHA ratio, and also on the ability to protect 475 the highly sensitive double bonds within their structures against 476 oxidization.<sup>21</sup> Indeed, highly purified EPA:DHA 6:1 is a 477 superior formulation than pure EPA, pure DHA and other 478 EPA:DHA ratio including 1:1, causing a sustained endothelial 479 formation of NO in cultured ECs and isolated artery rings 480 including the human mammary artery.<sup>21,53</sup> The oral daily intake 481 of EPA:DHA 6:1 has shown antihypertensive and improvement 482 of the endothelial function in Ang II-treated rats, and has 483 improved aging-related endothelial dysfunction by normalizing 484 vascular levels of oxidative stress and restoring the endothelial 485 formation of NO.<sup>22</sup> Such findings are in good agreement with 486 those of the REDUCE-IT clinical trial indicating that the intake 487 of pure EPA (4 g) provided about a 25% additional reduction in 488 major adverse cardiovascular events in patients with elevated 489 cardiovascular risk treated with a statin.<sup>54</sup> Therefore, the present 490 study has evaluated the effect of EPA:DHA 6:1 compared to that 491 of corn oil, an isocaloric "control" lipid, both in the native form 492 and after loading in non-functionalized nanocarriers on the Ang 493 II-induced activation of the native endothelium using coronary 494 artery segments. The Ang II treatment caused the appearance of 495 pronounced VCAM-1 and p53 signals and increased SA-β-gal 496 activity predominantly in the endothelium highlighting the 497 particular susceptibility of the endothelium in the arterial wall 498 towards pathological stimuli.<sup>55</sup> The stimulatory effect of Ang II 499 on the endothelial VCAM-1 and p53 signals was reduced to a 500 greater extent by the nanoencapsulated EPA:DHA 6:1 compared 501 to the native omega-3 form, and also compared to nanoencapsu- 502 lated and native corn oil. Thus these ex vivo investigations 503 indicate a therapeutic potential of nanocarriers containing EPA: 504

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505 DHA 6:1 in the core to reach sufficient levels to restore the key 506 protective endothelial function.

In conclusion, the present findings indicate the efficiency of 507 fluorescent nanocarriers decorated with VCAM-1 Abs to 508 509 selectively and specifically target VCAM-1 overexpressed at 510 the membrane surface of cultured senescent ECs. They further 511 indicate that nanoencapsulation of the vasoprotective and highly 512 sensitive to oxidation EPA:DHA 6:1 leads to a greater beneficial effect towards the Ang II-induced dysfunction of native 513 endothelium ex vivo. Additional in vivo studies are required to 514 provide evidence of their monitoring and regenerative potentials 515 of pathological endothelium overlying areas at risk. Ultimately, 516 the developed platform could be applied to other targets, making 517 it a useful scaffold for the development of novel targeted 518 therapeutics and/or diagnostic tools. 519

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#### 526 Appendix A. Supplementary data

527 Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2021.102379.

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