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## Supramolecular organization and biological interaction of the squalenoyl siRNA nanoparticles

Marie Caillaud, Frédéric Gobeaux, Miryana Hémadi, Suzan Boutary, Patrick Guenoun, Didier Desmaële, Patrick Couvreur, Frank Wien, Fabienne Testard, Liliane Massaad-Massade

### ► To cite this version:

Marie Caillaud, Frédéric Gobeaux, Miryana Hémadi, Suzan Boutary, Patrick Guenoun, et al.. Supramolecular organization and biological interaction of the squalenoyl siRNA nanoparticles. International Journal of Pharmaceutics, 2021, 609, pp.121117. 10.1016/j.ijpharm.2021.121117. hal-03379142

**HAL Id: hal-03379142**

**<https://hal.science/hal-03379142>**

Submitted on 14 Oct 2021

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1 **Supramolecular organization and biological interaction of the squalenoyl siRNA**  
2 **nanoparticles**

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21  
22 **Funding:** This work is supported by a public grant overseen by the French National Research  
23 Agency (ANR) as part of the “Investissements d’Avenir” program (Labex NanoSaclay,  
24 reference: ANR-10-LABX-0035).

25  
26 **Key words:** siRNA, nanotechnologies, structural studies, biological interaction

27 **Chemical keywords:** Azido-Squalene (N<sub>3</sub>-SQ), cationic squalenaldehyde  
28 hydrazinoguanidinium acetate salt (SQ<sup>+</sup>), dibenzylcyclooctyne (DBCO), N-(hexamethylenyl)-  
29 6-oxohexanamide spacer (C6), Bovine serum albumin (BSA), Low density lipoprotein (LDL),  
30 Lipofectamine 3:1 DOSPA (2,3- dioleoyloxy- N-  
31 [2(sperminocarboxamido)ethyl]- N,N- dimethyl- 1- propaniminium trifluoroacetate) :  
32 DOPE (1,2-dioléoyl-sn-glycéro-3-phosphoéthanolamine), Sybr Green C<sub>32</sub>H<sub>37</sub>N<sub>4</sub>S  
33

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## 81           **Abstract**

82           Small interfering RNAs (siRNA) are attractive and powerful tools to inhibit the  
83 expression of a targeted gene. However, their extreme hydrophilicities combined with a  
84 negative charge and short plasma half-life counteract their use as therapeutics. Previously, we  
85 chemically linked siRNA to squalene (SQ) which self-assembled as nanoparticles (NPs) with  
86 pharmacological efficiency in cancers and recently in a hereditary neuropathy. In order to  
87 understand the siRNA-SQ NP assembly and fate once intravenously injected, the present  
88 study detailed characterization of siRNA-SQ NP structure and its interaction with serum  
89 components.

90 We discovered that the siRNA-SQ bioconjugate self-assembled as 11-nm diameter core/shell  
91 supramolecular assemblies, which are connected one to another to form spherical  
92 nanoparticles of around 130-nm diameter. The siRNA-SQ NPs were stable in biological  
93 media and interacted with serum components, notably with albumin and LDL. The high  
94 specificity of siRNA to decrease or normalize gene expression and the high colloidal stability  
95 when encapsulated into squalene nanoparticles offer promising targeted therapy with wide  
96 applications for pathologies with gene expression dysregulation.

## 97           **1. Introduction**

98           Small interfering RNAs (siRNA) discovered by Fire and Mello at the end of the 90s  
99 (Fire, Xu et al. 1998), are small double-stranded RNAs composed of 19-21 bases pairs with  
100 3'-cohesive ends. The antisense strand is complementary to a targeted messenger RNA  
101 (mRNA), leading to its degradation and gene silencing (Elbashir 2001, Elbashir, Martinez et  
102 al. 2001). Despite their specificity and efficiency at low doses, their intrinsic parameters  
103 constitute the main hurdles for *in vivo* delivery (Caillaud, El Madani et al. 2020). Indeed, in  
104 blood, naked siRNAs are rapidly degraded by nucleases and cleared by glomerular filtration,  
105 resulting in short plasmatic half-life (less than 15 mins) (Thompson, Kornbrust et al. 2012,  
106 Ozpolat, Sood et al. 2014). Moreover, their hydrophilic and polyanionic nature prevents  
107 efficient cellular uptake (Whitehead, Langer et al. 2009, Kanasty, Dorkin et al. 2013).

108           We previously developed an original approach for the conjugation of siRNA to  
109 squalene (SQ), a precursor of the cholesterol's biosynthesis. The so-called "Squalenylation"  
110 technology consists of the bioconjugation of this lipid to a biologically active molecule,  
111 adopting a compact molecular conformation in aqueous media (Couvreux, Stella et al. 2006,

112 Couvreur, Reddy et al. 2008, Desmaele, Gref et al. 2012). Squalene was initially covalently  
113 coupled to siRNA *via* maleimide-sulfhydryl chemistry and the resulting siRNA-SQ  
114 nanoparticles (siRNA-SQ NPs) showed significant results in reducing tumor growth in two  
115 cancer models with fusion oncogenes: RET/PTC1 and RET/PTC3 (present in papillary  
116 thyroid carcinoma) and TMPRSS2-ERG (found in prostate cancer) (Raouane, Desmaele et al.  
117 2011, Ali, Maksimenko et al. 2014, Ali, Urbinati et al. 2014, Urbinati, Ali et al. 2015,  
118 Urbinati, de Waziers et al. 2016). The conjugation method has been further improved,  
119 implementing strain-promoted azide-alkyne cycloaddition. This copper-free (Cu-free) click  
120 chemistry approach increased the bioconjugation yield while preserving the antineoplastic  
121 activity of siRNA TMPRSS2-ERG-SQ NPs in a xenografted cancer model (Massaad-  
122 Massade, Boutary et al. 2018). Recently, we succeeded in delivering squalenoyl siRNA  
123 targeting PMP22, an overexpressed gene causing hereditary neuropathy, *i.e.* the Charcot-  
124 Marie-Tooth 1A (CMT1A) disease. The intravenous administration of siRNA PMP22-SQ  
125 NPs restored both motor and neuromuscular activities in transgenic mouse models (Boutary,  
126 Caillaud et al. 2021). Moreover, the targeted gene expression normalization indicated that the  
127 NPs successfully entered into the targeted tissue, the sciatic nerve and more specifically inside  
128 Schwann cells.

129 To better understand the siRNA-SQ NPs fate, their structure, as well as their  
130 biological behavior once intravenously injected into the circulating bloodstream should be  
131 investigated. Indeed, characterization of size, shape, surface properties and structure of  
132 siRNA-SQ NPs is important because these parameters may dramatically affect their biological  
133 outcome such as tissue accumulation and cell uptake (Kang, Kim et al. 2016, Dormont,  
134 Rouquette et al. 2019). In our previous studies, basic physicochemical parameters, like overall  
135 size and surface charge, were determined. However, their supramolecular organization,  
136 another key issue for the therapeutic efficiency in terms of availability and release of the  
137 active molecule (Lepeltier, Bourgaux et al. 2014, Kang, Kim et al. 2016), had never been  
138 investigated. Therefore, in the present study, high-resolution small-angle scattering has been  
139 performed to obtain new insights on the supramolecular organization of these nanoparticles.

140 *In vivo* interactions of nanoparticles with blood components represent another key  
141 point for biomedical applications. The interaction with serum biomolecules has already been  
142 investigated with squalenoyl conjugates bearing a small active functional group, like  
143 gemcitabine, doxorubicin (Sobot, Mura et al. 2017, Sobot, Mura et al. 2017, Yesylevskyy,  
144 Ramseyer et al. 2018) or adenosine (Gobeaux, Bizeau et al. 2020) but, never so far with  
145 macromolecules such as siRNA. Consequently, understanding the interaction between

146 siRNA-SQ NPs and blood proteins is mandatory for further development towards clinical  
147 applications. Among serum components interacting with NPs, we have focused our attention  
148 on albumin and lipoproteins (LPs).

149         Albumin is the most abundant serum protein (55%) and acts as carrier for several other  
150 endogenous proteins, such as steroids and fatty acids (Sleep 2015, Mariam, Sivakami et al.  
151 2016, Lamichhane and Lee 2020). Human albumin has been investigated and approved in  
152 clinics to deliver paclitaxel, a chemotherapy drug, to cancer cells (Abraxane®) or to extend  
153 the plasmatic half-life of insulin hormone linked to a fatty acid (Levemir®), which allows for  
154 98% binding to this protein (Kratz 2008). On the other hand, lipoproteins (LPs) are the third  
155 most abundant plasma molecules in humans. They consist of an assembly of lipids and  
156 proteins. Low-density lipoproteins (LDL) are the main carriers for cholesterol (75%) and fatty  
157 acids in mammals (Schumaker and Adams 1969). Thanks to their apolipoproteins  
158 (amphiphilic protein) embedded in the outer membrane, LPs bind to lipoprotein receptors  
159 resulting in cellular uptake by clathrin-mediated endocytosis (Chaudhary, Bower et al. 2019).  
160 These macromolecules interact in the bloodstream with lipophilic drugs and deliver them to  
161 tissues, such as the liver and tumors (Wolfrum, Shi et al. 2007, Wasan, Brocks et al. 2008,  
162 Chaudhary, Bower et al. 2019).

163         In the present study, we first fully characterized the external and internal structure of  
164 siRNA-SQ NPs at the supramolecular level and we assessed the interactions between siRNA-  
165 SQ NPs and serum components, notably albumin and LDL.

166

## 167 **2. Materials and Methods**

### 168 **2.1. Chemicals**

169  
170 All chemicals used were of highest analytical grade and purchased from Sigma–Aldrich. The  
171 anhydrous solvents were dried and distilled before use (tetrahydrofuran (THF) on  
172 Na/benzophenone) and reactions sensitive to air or humidity were performed under nitrogen  
173 pressure. D<sub>2</sub>O (99.85% D) for small angle neutron scattering (SANS) experiments was  
174 purchased from Eurisotop (France).

175 Modified siRNAs for the Cu-free click chemistry reactions were purchased from Eurogentec  
176 (France). Azido-Squalene and cationic squalenaldehyde hydrazinoguanidinium acetate salt  
177 (SQ<sup>+</sup>) were synthesized according to our previous studies (Bertrand, Lucas et al. 2015,  
178 Massaad-Massade, Boutary et al. 2018). Dulbecco's modified Eagle medium (DMEM), Opti-  
179 MEM, fetal bovine serum (FBS) were purchased from Fisher Scientific (Illkirch, France).  
180 UltraPure™ distilled water (Dnase and Rnase free water) purchased from Invitrogen. Water  
181 was purified using a Milli-Q system.

182 LDLs from human plasma [dissolved in 10 mM Tris, 150 mM NaCl, 0.3 mM EDTA pH 8.3  
183 and 2 mM NaN<sub>3</sub>] were purchased from Invitrogen (Thermo Fisher Scientific). For small-angle  
184 scattering and fluorescence experiments, lyophilized BSA powder was dissolved to a 200 μM  
185 concentration in 0.2 M Phosphate Buffer Saline (PBS) then in H<sub>2</sub>O for standard experiments  
186 and in D<sub>2</sub>O for SANS experiments. BSA solutions were dialyzed overnight against at least a  
187 10-fold volume PBS/D<sub>2</sub>O (or PBS/H<sub>2</sub>O) solution, then stored at 4 °C. FBS was also dialyzed  
188 overnight against PBS/D<sub>2</sub>O just after bottle opening.

### 189 **2.2. siRNAs used and chemical modifications**

190  
191 The siRNA targeting RET/PTC1 and PMP22 used in the experiments were previously  
192 described (Ali, Maksimenko et al. 2014, Massaad-Massade, Boutary et al. 2018, Boutary,  
193 Caillaud et al. 2021). All single-stranded RNAs were synthesized by Eurogentec (France) and  
194 purified by RP-HPLC. Single-strand RNAs were synthesized as 19-mers with two 3'-  
195 overhanging 2'-deoxynucleotide residues to provide stabilization against nucleases, as  
196 described by Tuschl *et al.* (Tuschl 2002). To allow conjugation with squalene, a  
197 dibenzocyclooctyne (DBCO) reactive group and a linker N-(hexamethylenyl)-6-  
198 oxohexanamide spacer (C6) were introduced at the 5'-end of the sense strand of each siRNA  
199 (Massaad-Massade, Boutary et al. 2018).

200 To anneal naked siRNA (*i.e.* siRNA not coupled to SQ), equimolar amounts of sense and  
201 antisense strands were mixed with the hybridization buffer [30 mM HEPES-KOH (pH 7.4), 2  
202 mM Mg acetate, 100 mM K acetate]. The mixture was incubated for 3 min at 95°C and then  
203 for 45 min at room temperature before being stored at -80°C.

### 204 **2.3. Preparation of siRNA-SQ nanoparticles**

205  
206 Cu-free click chemistry, a 1,3-dipolar cycloaddition was developed by our lab as previously  
207 described (Massaad-Massade, Boutary et al. 2018, Boutary, Caillaud et al. 2021). Then, the  
208 siRNA-SQ NPs were prepared by inverse nanoprecipitation in acetone/water as sketched in  
209 Figure S1. Practically, annealed siRNA-SQ bioconjugates in UltraPure™ distilled water  
210 (Dnase and Rnase free water) were added drop wisely to a stirred organic phase (acetone)  
211 with a volume/volume ratio of 1 [organic phase]: 2 [aqueous phase]. Then acetone was  
212 evaporated using nitrogen flux for 30 min to obtain an aqueous suspension of pure siRNA-SQ  
213 NPs.

### 214 215 **2.4. Physicochemical characterization of siRNA-SQ nanoparticles**

216  
217 Hydrodynamic diameter, PDI and  $\zeta$ -potential were measured by dynamic light scattering  
218 (DLS) using the Malvern Zetasizer Nano ZSP (Malvern Instrument) with a scattering angle of  
219 173° for size measurements. Samples were analyzed at 10  $\mu$ M concentration in RNase-free  
220 water.

221 Cryogenic Transmission Electron Microscopy (cryo-TEM) was performed with a JEOL 2100  
222 electron microscope at the Electronic Microscopy Platform (IBPS/Institut de Biologie Paris-  
223 Seine, Université P. et M. Curie, Paris, France). A 4  $\mu$ L drop of the concentrated siRNA-SQ  
224 NPs (166  $\mu$ M) was deposited on a carbon-coated copper grid. Excess liquid was removed  
225 with blotting filter paper, and the samples were quickly vitrified by plunging them into liquid  
226 ethane using a guillotine-like frame. The samples were then transferred to a cryo-sample  
227 holder. Observations were made at an acceleration voltage of 200 kV under a low electron  
228 dose. Analysis was performed with Image J software.

### 229 **2.5. Preparation of siRNA-SQ/SQ<sup>+</sup> NPs**

230  
231 Cationic squalenaldehyde hydrazinoguanidinium acetate salt (SQ<sup>+</sup>) was dissolved in acetone  
232 at a concentration of 1 mg/mL and added dropwisely to a suspension of already prepared  
233 siRNA-SQ NPs at a molar ratio of 1 [NPs]:100 [SQ<sup>+</sup>]. The organic solvent was then



234 evaporated for 30 min under stirring using the nitrogen flow to obtain siRNA-SQ/SQ<sup>+</sup> NPs in  
235 aqueous solution. Then size and ζ-potential were measured.

## 236 **2.6. Cell lines and culture**

237  
238 BHP 10-3 is a papillary thyroid carcinoma cell line harboring the fusion oncogene *RET/PTC1*  
239 and MSC80 is a mouse Schwann cell line expressing *PMP22*. Both cell lines were maintained  
240 at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% humidity in complete DMEM supplemented  
241 with 10% heat-inactivated FBS, with 100 units/mL penicillin, and 100 µg/mL streptomycin.

## 242 **2.7. In vitro cell transfection**

243  
244 One feature of siRNA-SQ NPs is that they cannot spontaneously enter into cells *in vitro*  
245 without the help of a transfection agent (Raouane, Desmaele et al. 2011, Ali, Maksimenko et  
246 al. 2014, Urbinati, Ali et al. 2015, Massaad-Massade, Boutary et al. 2018, Boutary, Caillaud  
247 et al. 2021). Therefore, Lipofectamine 2000 was used to assess naked siRNA and siRNA-SQ  
248 NPs efficiency *in vitro*.

249 To study the effect of surface charge, 3 x 10<sup>5</sup> cells of BHP 10-3 and MSC80 were seeded, 24  
250 h before transfection, in six-well plates in DMEM supplemented with 10% FBS, penicillin  
251 (100 U/mL), and streptomycin (10 µg/mL). The next day, the cell medium was replaced with  
252 2mL of Opti-MEM medium (Gibco Life technologies, ref. 11058021). The siRNA-SQ NPs  
253 and naked siRNA were transfected using Lipofectamine 2000, according to the supplier's  
254 recommendations, in the reduced Opti-MEM medium for a final concentration of 50 nM/well.  
255 Similarly, siRNA-SQ/SQ<sup>+</sup> NPs and siRNA-SQ NPs alone (as a negative control) were added  
256 to Opti-MEM and incubated with the cells. 4h later, the medium was replaced by complete  
257 DMEM and 48 h later the experiments were stopped, and RNA was extracted to analyze  
258 silencing of targeted genes *RET/PTC1* and *PMP22*. Each experiment was performed at least  
259 twice in duplicate.

## 260 **2.8. mRNA Extraction and Real-Time PCR (RT-qPCR) experiments**

261  
262 Total RNA was extracted from BHP 10-3 and MSC80 cells using the RNeasy mini-kit  
263 (Qiagen, Courtaboeuf, France). Reverse transcription was performed using the M-MLV RT  
264 buffer pack (Invitrogen, Charbonnières-les-Bains, France). Then, real-time PCR (qPCR) was  
265 carried out with the CFX96TM Real-time system (Biorad) using the Maxima Syber Green  
266 Rox qPCR master Mix (Thermo Scientific, Villebon-sur-Yvette, France), according to the  
267 manufacturer's instructions. Samples were run in duplicate; gene expression was determined

268 by the  $2^{-\Delta\Delta Ct}$  method. Relative mRNA levels of targeted genes (*RET/PTC1* and *PMP22*) were  
269 compared to non-treated (NT) cells after normalization regarding control gene levels in at  
270 least 2 independent experiments.

## 271 **2.9. Small-Angle Neutron Scattering (SANS)**

272  
273 SANS experiments were carried out on the D33 spectrometer at ILL (doi: 10.5291/ILL-  
274 DATA.9-13-781). Samples prepared in D<sub>2</sub>O were placed in 1-mm size Hellma quartz cells  
275 and thermostated at 20°C. The raw data were radially averaged, corrected for electronic  
276 background and normalized by water scattering using the LAMP software (Richard, Ferrand  
277 et al. 1996). Three configurations were used to cover a  $q$  range going from 0.0013 to 0.27 Å<sup>-1</sup>:  
278 1) wavelength:  $\lambda = 13$  Å, sample detector distance  $D = 12$  m, collimation:  $\Phi = 12.8$ , 2)  $\lambda = 4.6$   
279 Å,  $D = 12$  m, collimation:  $\Phi = 7.8$  and 3)  $\lambda = 4.6$  Å,  $D = 2$  m, collimation  $\Phi = 7.8$ , for small,  
280 medium and large angles, respectively.

## 281 **2.10. Small-Angle X-ray Scattering (SAXS)**

282  
283 Small-angle X-ray scattering patterns (SAXS) were obtained on the SWING beamline of the  
284 SOLEIL synchrotron (Saint-Aubin, France). The scattering vector range  $q$  of  $2 \times 10^{-3}$  Å<sup>-1</sup> to 2.5  
285 Å<sup>-1</sup> was obtained with an energy of 16 keV and two sample-to-detector distances (6 and 0.50  
286 m for the SAXS and the wide-angle X-ray scattering ranges, respectively). The data were  
287 recorded by a two-dimensional Eiger 4M (Dectris) detector. Data reduction was performed  
288 using Foxtrot software, an application developed at SOLEIL. The sample was placed in a 1.5-  
289 mm diameter quartz capillary sealed with kerosene wax.

## 290 **2.11. siRNA-SQ NPs internal structure characterization**

291  
292 Analysis of the SANS and SAXS patterns of siRNA PMP22-SQ NPs in aqueous solution  
293 allows to draw a first sketch of the shape and internal structure of the NPs. The scattering  
294 curves were fitted by SASview software ([www.sasview.org/](http://www.sasview.org/)) to a lognormal sphere  
295 distribution model for the siRNA-SQ particles and a cylinder model for the siRNA moiety.  
296 The fitting of the experimental curves of siRNA led to a cylindrical shape whose volume was  
297 given by the following formula:  $V(\text{siRNA}) = \pi \times \text{radius}^2 \times \text{height}$ . Fitting of the curves of  
298 siRNA-SQ NPs gave a spherical distribution. The nanoparticle volume corresponded to:  $(4/3)$   
299  $\times \pi \times \text{radius}^3$ . Consequently, the ratio between the volume of the siRNA-SQ nanoparticle and  
300 the volume of the siRNA made it possible to determine the number of siRNAs linked to SQ in  
301 a nanoparticle.

302

303

### **2.12. Electrophoresis of siRNA PMP22-SQ NPs incubated in FBS or with serum components**

304

305

306 Interactions between siRNA PMP22-SQ NPs and FBS or with one of its main components  
307 (BSA or LDL) were performed by incubating increasing amounts of FBS, BSA (molar ratio)  
308 or LDL (mass ratio) with 5  $\mu$ M siRNA PMP22-SQ NPs for 30 min at 37°C. The same volume  
309 of each aliquot was loaded into 4% agarose electrophoresis gel stained with Midori Green  
310 Advance (Nippon Genetic Europe, Germany) that only reveals the oligonucleotides. The  
311 molecular weight (MW) size marker served as an evidence to testify the migration of siRNA  
312 PMP22-SQ NPs. Bands were revealed by camera under UV lamp illumination and recorded  
313 by Visio-capt 14.2 software.

314

315

### **2.13. BSA Fluorescence quenching**

316

317 A series of BSA solutions at a fixed concentration (5  $\mu$ M) incubated with an increasing  
318 amount of siRNA PMP22-SQ NPs (BSA/siRNA PMP22-SQ molar ratios = 0.5, 1, 2, 4 and 8 )  
319 were analyzed. Solutions were filled into Hellma quartz Ultra-Micro cells and placed in a  
320 Cary Eclipse fluorimeter (Varian, Oxford, UK) for measurements. The samples were excited  
321 at 280 nm and emission scans were recorded between 300 and 550 nm. Spectra were averaged  
322 over 5 measurements. Quenching experiments could be affected by the inner filter effect, where  
323 the sample could attenuate both the excitation beam and the emission spectrum. However, in  
324 our case, we worked with rather low concentrations of both BSA and siRNA PMP22-SQ NPs,  
325 and the optical density of the solutions was below 0.1; thus, this effect could be ruled out.

326

327

### **2.14. Steady-state fluorescence**

328

329 To a fixed concentration of siRNA PMP22-SQ NPs (5  $\mu$ M, *i.e.* 0.065 mg/mL), the BSA (100  
330  $\mu$ M stock solution) or LDL (2.5 mg/mL stock solution) were gradually added to achieve a  
331 final molar ratio of 1 [siRNA PMP22-SQ]:1 [BSA 5  $\mu$ M] or mass ratio of 1 [siRNA PMP22-  
332 SQ]:2 [LDL 0.12 mg/mL]. Briefly, 1 mL of a 5  $\mu$ M or 0.065 mg/mL siRNA PMP22-SQ NPs  
333 solution was filled into a Hellma Ultra-Micro quartz cell and 2  $\mu$ L of BSA or LDL were  
334 progressively added until reaching the final ratio. In parallel, BSA and LDL were added to  
335 water to reach the same final concentration. After each addition, samples were excited at 280

336 nm and the fluorescence emission spectra were recorded from 300 to 400 nm using a Horiba  
337 Jobin Yvon Fluorolog 3 spectrometer.

### 338 **2.15. Synchrotron radiation circular dichroism (SRCD)** 339

340 Synchrotron radiation circular dichroism (SRCD) experiments were performed on the DISCO  
341 beamline at the SOLEIL Synchrotron (Saint Aubin, France). The conformation of naked  
342 siRNA PMP22 at two stages of NPs preparation (before and after the nanoprecipitation) was  
343 performed, as well as, the study of the interaction with BSA by mixing siRNA PMP22-SQ  
344 NPs and BSA at a volume/volume ratio of 1 [siRNA PMP22-SQ]:1 [BSA]. Four  $\mu\text{L}$  droplets  
345 of the samples were placed in 3.7  $\mu\text{m}$  path length  $\text{CaF}_2$  cells (Hellma). The raw spectra were  
346 acquired with a 1 nm spectral resolution. The spectra presented in this study were treated with  
347 CDTool software that can be freely downloaded at <http://www.cdtools.cryst.bbk.ac.uk/> (Miles  
348 and Wallace 2018). Each is the average of three spectra. A background (water or  
349 corresponding buffer spectrum recorded under the same conditions) was subtracted. Intensity  
350 calibration was obtained with a camphor sulfonic acid CSA scale standard sample. Intensities  
351 were converted from millidegrees ( $\theta$  machine units) into  $\Delta\epsilon$  using the formula  $\Delta\epsilon =$   
352  $\theta \times (0.1 \times \text{MRW}) / (3298 \times l \times C)$ , where MRW is the mean residue weight of the BSA (protein  
353 weight/number of residues = 114 Da),  $l$  is the path length of the  $\text{CaF}_2$  cell in cm and  $C$  is the  
354 protein concentration in mg/ml. In some cases, we have added a smoothing function.

355

### 356 **2.16. Statistical analysis** 357

358 Statistics were computed with GraphPad Prism 8.3.0 software. When we had two groups to  
359 compare, the Mann-Whitney analysis was performed to assess the statistical difference. For  
360 studies requiring grouped analyses, a one-way ANOVA followed by Bonferroni multiple  
361 comparison test was performed. A value of  $p < 0.05$  was considered significant.

362

### 363 **3. Results**

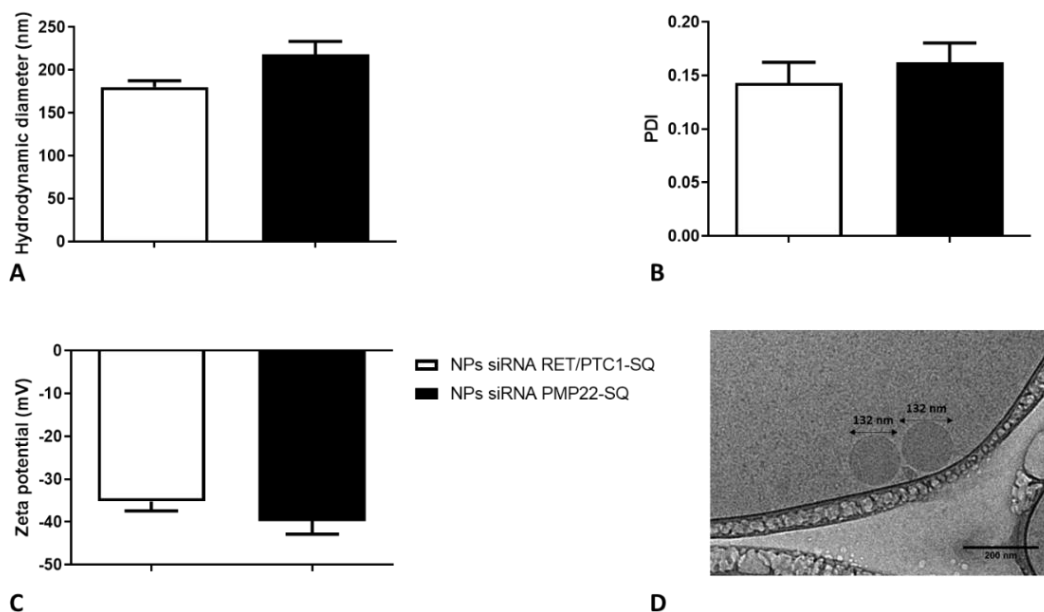
#### 364 **3.1. Structure determination of siRNA-SQ NPs**

365 3.1.1. The physico-chemical characteristics of squalenoyl siRNA  
366 nanoparticles are nucleoside sequence-independent

367

368 To obtain nanoparticles, an inverse nanoprecipitation method (Bilati, Allémann et al.  
369 2005) was performed by the addition of the aqueous solution containing the siRNA-SQ  
370 bioconjugates into acetone as an organic solvent, where siRNA-SQs were not soluble. The  
371 size and polydispersity of two resulting nanoformulations with siRNA RET/PTC1 or siRNA  
372 PMP22 were measured by DLS (**Figures 1A and 1B**). For the siRNA RET/PTC1,  
373 conjugation to SQ led to 180 nm NPs with a homogeneous distribution as indicated by the  
374 polydispersity index (PDI; less than 0.2). Surface charge of NPs was negative, around -35  
375 mV, which favors colloidal stability of the NP suspension (**Figure 1C**). For siRNA PMP22,  
376 the results were similar: NPs size was 218 nm, PDI < 0.2 and surface charge -40 mV. No  
377 significant difference was observed between siRNA RET/PTC1-SQ and siRNA PMP22-SQ  
378 NPs in terms of size, polydispersity index and  $\zeta$ -potential (**Figures 1A, 1B and 1C**). As no  
379 significant difference was found concerning the physicochemical parameters of both siRNAs  
380 (PMP22 or RET/PTC1) and previously with the siRNA Tmprss2-ERG (Massaad-Massade,  
381 Boutary et al. 2018), it was decided to continue further experiments with the siRNA PMP22-  
382 SQ NPs. This resolution is also supported by our recent findings showing that the siRNA  
383 PMP22-SQ NPs could lead to a targeted therapy for CMT1A neuropathy (Boutary, Caillaud  
384 et al. 2021).

385 Then, the siRNA PMP22-SQ NPs were observed by cryo-TEM (**Figure 1D**). Spherical  
386 nano-objects of around 130 nm in size were observed. Interestingly, the shape and size were  
387 in accordance with our previous observations (Massaad-Massade, Boutary et al. 2018).



388 **Figure 1: Physicochemical characterization of siRNA-SQ NPs.** After inverse  
 389 nanoprecipitation of siRNA RET/PTC1-SQ NPs and siRNA PMP22-SQ NPs the size (A) and  
 390 polydispersity index (B) were measured by dynamic light scattering and the surface charge  
 391 (C) by electrophoretic light scattering. Three measurements were performed and the average  
 392 diameter  $\pm$  standard error of the mean (SEM) of more than 5 independent nanoformulations,  
 393 was calculated. Using the Mann and Whitney test, no significant difference between the two  
 394 nanoformulations was found. D) Micrography of siRNA PMP22-SQ NPs by cryoTEM. The  
 395 siRNA-SQ NPs are spherical with a diameter of around 130 nm.

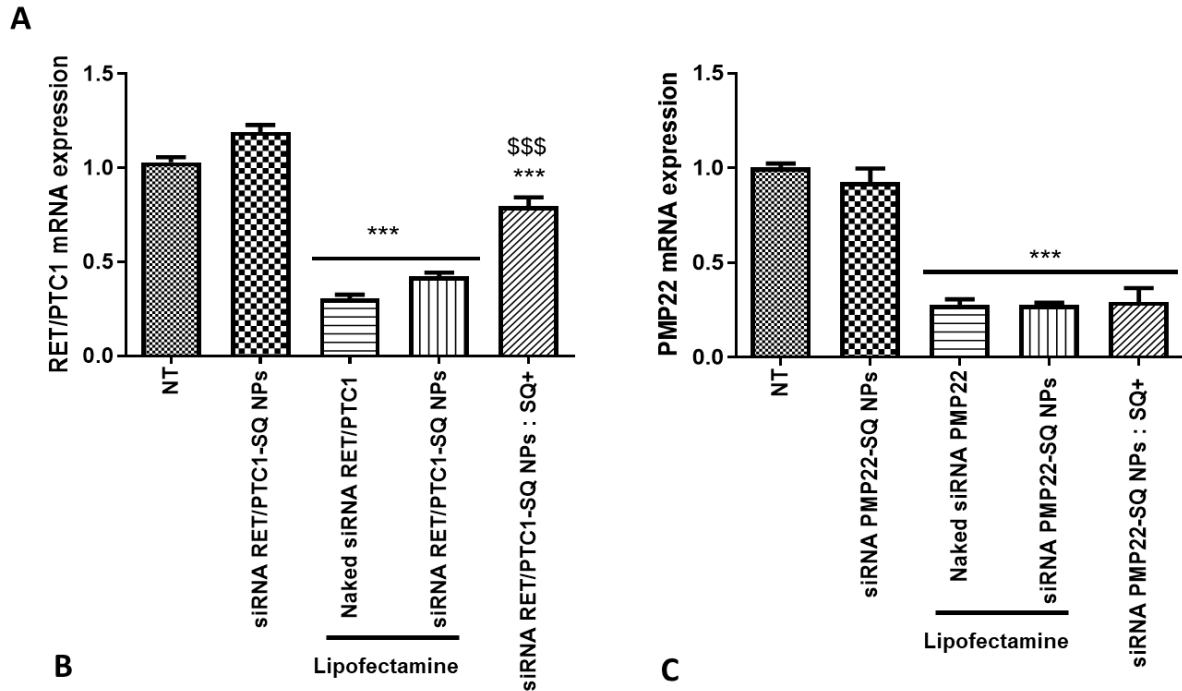
396

397 3.1.2. Inversion from negative to positive charge of siRNA-SQ NPs improves their  
 398 transfection  
 399

400 To understand the impact of surface charge on the internalization of siRNA-SQ NPs,  
 401 several experiments were conducted *in vitro*. Previously, we showed that the siRNA-SQ NPs  
 402 were unable to enter cultured cells spontaneously without any cationic transfection agent  
 403 (Raouane, Desmaele et al. 2011, Ali, Maksimenko et al. 2014, Boutary, Caillaud et al. 2021).  
 404 We postulated that the negative surface charge of siRNA-SQ NPs prevented cell uptake due  
 405 to electrostatic repulsion with the negative plasma membrane. It is well known that cationic  
 406 nanoparticles had a higher internalization rate than neutral and negative nanoparticles. This  
 407 difference can be explained by Coulomb's law, where opposite charges are attracted to each  
 408 other. Thus, the cationic nanoparticles can be delivered to the cytoplasm by electrostatic  
 409 attraction with the negatively charged cell membranes. To confirm this assumption, we  
 410 modified the nanoparticle charge by addition of cationic guanidium-squalene to the siRNA-  
 411 SQ NPs (**Figure 2A**). The resulting positive nanoparticles were then incubated with BHP 10-  
 412 3 and MSC80, two cell lines of different origins (tumoral and normal). Interestingly, the

413 inhibition of the targeted gene expression without using lipofectamine testified that the uptake  
 414 of the nanoparticle is facilitated by the use of a cationic compound (**Figures 2B and 2C**).

	Size	PDI	Zeta potential
siRNA-SQ NPs	171.3 ± 12.3 nm	0.127 ± 0.019	-37.1 ± 2.4 mV
siRNA-SQ NPs : SQ <sup>+</sup>	225.5 ± 8.5 nm	0.119 ± 0.014	39.6 ± 4.3 mV **



415 **Figure 2: Positively charged siRNA-SQ NPs are successfully cell internalized.** A)  
 416 Physicochemical characteristics of NPs. No statistical difference was observed between  
 417 siRNA-SQ NPs and siRNA-SQ NPs: SQ<sup>+</sup> for their size and PDI. Using the Mann-Whitney  
 418 test, a statistical difference was found concerning the zeta potential between the siRNA-SQ  
 419 NPs and siRNA-SQ NPs:SQ<sup>+</sup> ( $p < 0.001$ ). **B and C**) mRNA inhibition. BHP 10-3 **B**) and  
 420 MSC80 **C**) cells were transfected with 50 nM siRNA RET/PTC1 or PMP22-SQ NPs with or  
 421 without SQ<sup>+</sup>. NT: non-treated cells. Lipofectamine was also used to transfect the naked  
 422 siRNA and siRNA RET/PTC1 or PMP22-SQ NPs (positive controls). 48 hours later, cells  
 423 were harvested, RNA was extracted, and RT-qPCR performed. One-way Anova followed by a  
 424 Bonferroni test was used to assess the significant difference between treatments: \*\*\*  $p <$   
 425  $0.0001$ , compared to NT and untransfected targeted gene-SQ NPs ; \$\$\$  $p < 0.0001$  when  
 426 compared to transfected naked siRNA RET/PTC1 and siRNA RET/PTC1-SQ NPs by  
 427 lipofectamine.  
 428

### 429 3.1.3 Spherical nanoparticles contain core/shell assemblies

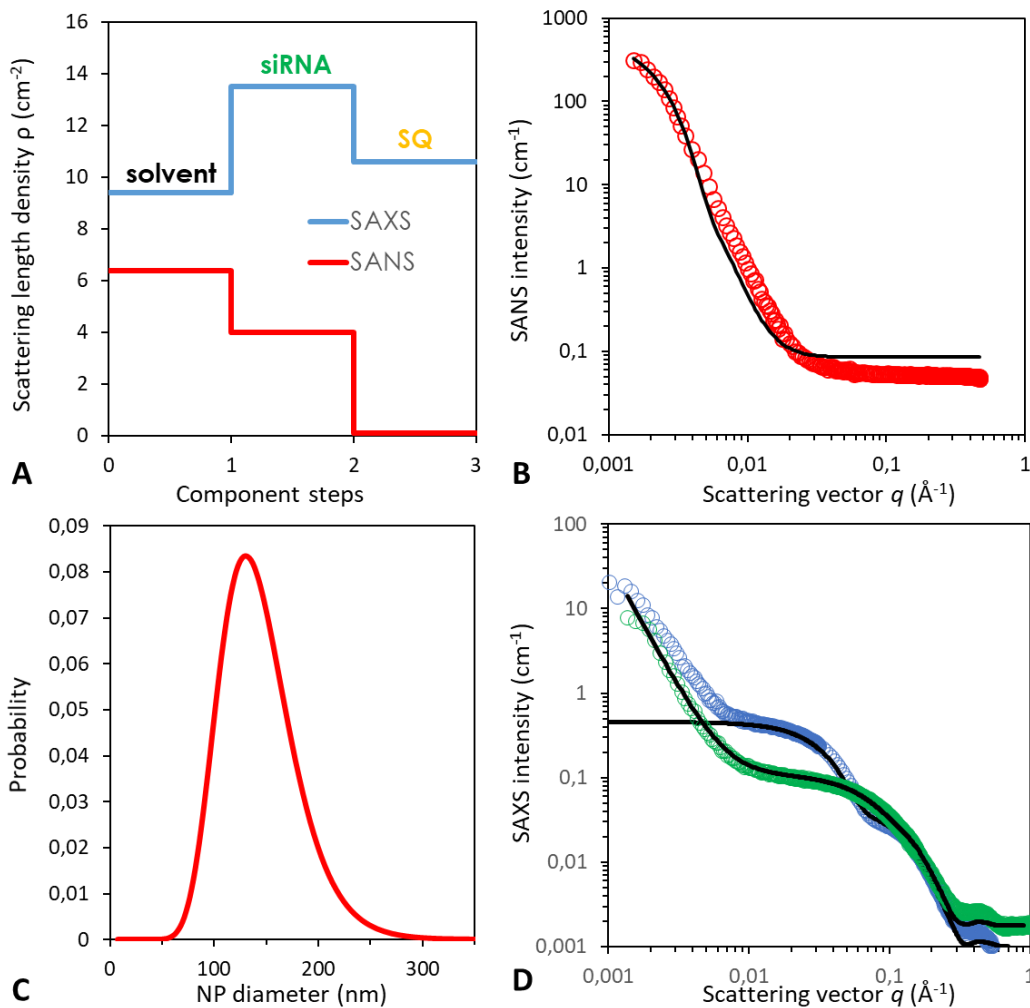
430

431 Based on our previous results showing impressive therapeutic results in several tumor  
 432 models (thyroid and prostate cancers (Ali, Urbinati et al. 2012, Urbinati, Hafiz et al. 2013,  
 433 Ali, Maksimenko et al. 2014, Ali, Urbinati et al. 2014, Urbinati, Ali et al. 2015, Urbinati, de  
 434 Waziers et al. 2016) as well as in monogenic neuropathy (CMT1A (Boutary, Caillaud et al.  
 435 2021)) and considering that cationic lipid have a higher *in vivo* toxicity (Caillaud, El Madani  
 436 et al. 2020), we decided to deeply characterize the conjugated siRNA-SQ nanoparticles.

437 To verify that the conjugation to SQ and the formulation as nanoparticle did not induce  
438 changes in siRNA conformation, circular dichroism measurements were performed. As shown  
439 in **Figure S2A**, naked siRNA PMP22 and siRNA PMP22-SQ both before and after  
440 nanoprecipitation displayed the same spectrum type, confirming that the  $\alpha$ -helix structure of  
441 the siRNA was kept intact after chemical conjugation to SQ and formulation as nanoparticles.  
442 Small-angle scattering methods are prominent tools for characterizing such nanometric  
443 colloidal suspensions. Taking advantage of the high difference of the scattering length density  
444 between hydrogen and deuterium, small-angle neutron scattering (SANS) represents, indeed,  
445 a method of choice to characterize the size distribution of hydrogenated colloids in a  
446 deuterated solvent and it has been proven previously to be a good way of characterizing the  
447 size of the squalene-based nanoparticles (Saha, Testard et al. 2015, Rouquette, Ser-Le Roux et  
448 al. 2019, Gobeaux, Bizeau et al. 2020). On the other hand, small-angle X-ray scattering  
449 (SAXS), which is sensitive to contrasts in electron densities, offered a description of the  
450 squalenoyl siRNA nanoparticles with deeper access to their internal structure. In the case of  
451 siRNA PMP22-SQ NPs, it was relevant to combine SANS and SAXS analysis since the  
452 former would be capable of detecting a larger contrast between the solvent and the full  
453 squalene derivative, and the latter between the solvent and the siRNA moiety (**Figure 3A**).  
454 The SANS of a 45  $\mu$ M suspension of siRNA PMP22-SQ NPs is shown in **Figure 3B**. This  
455 pattern was not sensitive to dilution and the nanoparticle size remained stable under a dilution  
456 factor of two (**Figure S2B**). The absence of structural peaks in the wide-angle region ( $q >$   
457  $0.04 \text{ \AA}^{-1}$ ) indicated that the nanoparticle core displayed no regular crystal-like structure, in  
458 accordance with cryo-TEM images (see **Figure 1**). In the medium  $q$ -range ( $0.006$  to  $0.02 \text{ \AA}^{-1}$ ),  
459 the scattering intensity followed a power-law in  $q^{-3.44}$  slightly deviating from the  $q^{-4}$  expected  
460 for sharp interfaces (Porod's Law (Guinier, Fournet et al. 1955)) between particle surfaces  
461 and solvent. If such observation may eventually indicate a slightly diffuse or fractal interface,  
462 it was however unlikely, given that the siRNA PMP22-SQ NPs surface appeared rather  
463 smooth in the cryo-TEM images. At the lowest wavevectors, the scattering intensity reached  
464 the onset of a plateau indicating a finite size. A simple Guinier analysis gave a gyration radius  
465 ( $R_g$ ) of 80 nm that corresponded to a hydrodynamic radius (as it would be determined by  
466 DLS) of 103 nm for a spherical shape. Overall, this scattering pattern could be fitted by  
467 considering a lognormal distribution of spheres (black line on red pattern in **Figure 3B**), with  
468 a median radius of 61.9 nm and a polydispersity index (PDI) of 0.25. The corresponding  
469 diameter distribution resulting from this fit is presented in **Figure 3C**.



470 The SAXS pattern of a 263  $\mu\text{M}$  solution of siRNA PMP22-SQ NPs (blue pattern) is  
471 presented in **Figure 3D**. Again, no specific structural peak could be observed in the large  $q$   
472 region ( $q > 0.3 \text{ \AA}^{-1}$ ). On the contrary to the SANS pattern, two large oscillations were visible  
473 in the middle  $q$ -range ( $0.007 < q < 0.3 \text{ \AA}^{-1}$ ), that could be attributed to siRNA moieties in the  
474 nanoparticle or at least to electronic density heterogeneities in the particle. As a matter of  
475 comparison, the SAXS pattern of the siRNA PMP22 moiety (224  $\mu\text{M}$ ) was also plotted (green  
476 pattern). The scattering pattern originating from the naked siRNA PMP22 dispersion  
477 exhibited an oscillation in the same  $q$ -range and could be fitted by a cylindrical form factor ( $L$   
478 = 7.1 nm,  $R = 1.1$  nm), in accordance with the SANS analysis of similar siRNA strands  
479 reported by Ristori *et al.* (Ristori, Ciani et al. 2012) (**Table S1**). This characteristic shape was  
480 found again in the oscillation, visible in the large  $q$  of the siRNA PMP22-SQ pattern,  
481 yielding the following dimensions:  $L = 4$  nm and  $R = 1.1$  nm from the fitting by a cylindrical form  
482 factor. The other oscillation (at lower  $q$ ) could be fitted by spheres with a radius of 5.5 nm.  
483 Since these spheres could accommodate the siRNA strands, we suggested that the siRNA  
484 PMP22 moieties were segregated and surrounded by the squalene moiety in core/shell  
485 assemblies with interconnections of the squalene moieties within the NPs. The obtained  
486 dimensions indicated a heterogeneity in electronic density inside the NPs, but without long  
487 range ordering.



489

### 490 **Figure 3: Structural characterization of siRNA PMP22-SQ NPs by small-angle** 491 **scattering.**

492 **A)** Scattering length densities show that the SANS approach allows to characterize the overall  
493 structure of the nanoparticles and the SAXS analysis to characterize the siRNA encapsulated  
494 within the NPs. The solvent used are H<sub>2</sub>O and D<sub>2</sub>O for SAXS and SANS, respectively. **B)**  
495 SANS pattern for siRNA PMP22-SQ NPs (open circles) depicting a fit with a lognormal  
496 distribution of spheres (black trace). **C)** SANS view software analysis of the fit B showed that  
497 the corresponding siRNA PMP22-SQ NPs diameters are 124 nm. **D)** SAXS patterns of the  
498 naked siRNA PMP22 (green circles) and of siRNA PMP22-SQ NPs (blue circles) with their  
499 corresponding fits (black traces). The naked siRNA PMP22 can be fitted by a 7 nm-long  
500 cylindrical shape and a scattering in  $q^{-3}$  in the low  $q$  range (a signature of aggregation). A  
501 combination of 4 nm cylindrical and 11 nm spherical shapes was found for the internal  
502 organization of the siRNA PMP22-SQ NPs.

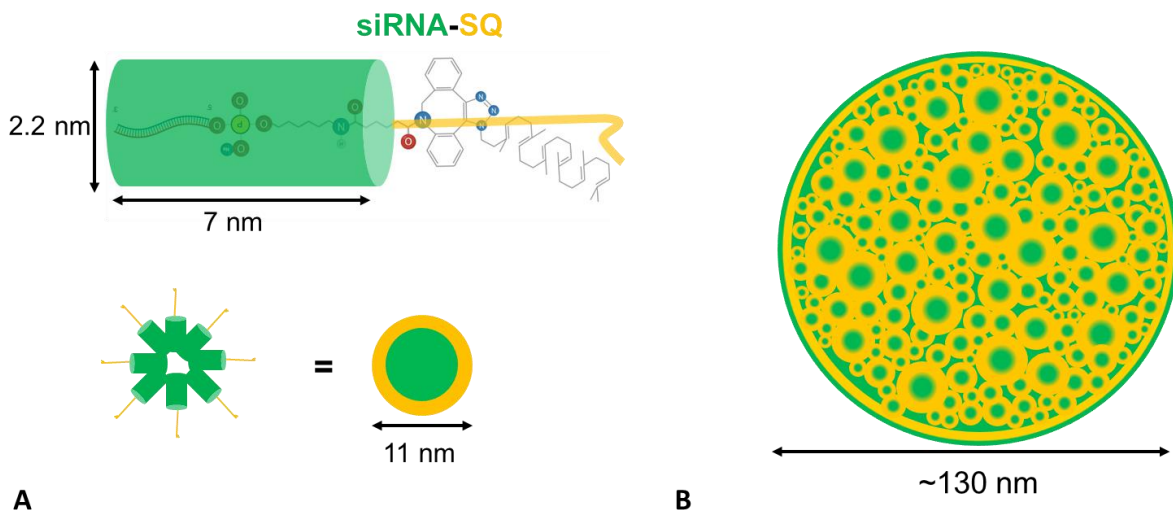
503

#### 504 3.1.4. A 130 nm nanoparticle can encapsulate around 60 000 siRNAs

505

506 The structural model of siRNA PMP22-SQ NPs is illustrated by the sketch in **Figure**  
507 **4**. The siRNA PMP22-SQ bioconjugate monomer is represented by a green cylinder for the  
508 siRNA moiety and a yellow lipid tail for the squalene moiety (**Figure 4A**). The

509 supramolecular organization inside the nanoparticle is represented by 11-nm core/shell  
 510 spheres with the lipidic moieties surrounding the hydrophilic siRNAs (**Figure 4A**). As no  
 511 specific inner structure was apparent in the cryo-TEM images, the core/shell structures were  
 512 supposed to be randomly distributed inside the whole nanoparticle volume. Therefore,  
 513 representation of a top view nanoparticle cross-section depicts a random pattern of circular  
 514 motifs (**Figure 4B**). Since the formation of squalene-based nanoparticles was clearly driven  
 515 by hydrophobic interactions (Lepeltier, Bourgaux et al. 2014), we assumed that the squalene  
 516 shells around the hydrophilic siRNA core could be interpenetrated and formed a continuous  
 517 lipophilic network and it is most likely that some charged hydrophilic siRNA strands point  
 518 towards the solvent (represented by the green circle around the yellow one in **Figure 4B**). By  
 519 a simple geometrical calculation (volume of nanoparticle/volume of siRNA), we could  
 520 estimate that one nanoparticle contained on average 60,000 siRNA PMP22-SQ bioconjugates.



521  
 522 **Figure 4: Graphical representation of a siRNA PMP22-SQ nanoparticle as determined**  
 523 **by the fit of the scattering patterns. A)** Sketch of siRNA PMP22-SQ monomer. The siRNA  
 524 moiety (green) of the bioconjugate can be viewed as a cylinder (siRNA not to scale in  
 525 comparison to others chemical entities). The squalene moiety is depicted in yellow. The  
 526 SAXS pattern reveals 11-nm spherical structures inside the nanoparticle that we view as self-  
 527 assembled core/shell domains with a siRNA core surrounded by squalene which could  
 528 interpenetrate with the other squalene moieties. **B)** Representation of a cross-section of 2D  
 529 siRNA PMP22-SQ nanoparticle. The core/shell spheres of 11-nm each are randomly arranged  
 530 inside the nanoparticle. The 2D cross section is the cause of the difference in the core/shell  
 531 sphere size in the sketch.

532  
 533  
 534

535

### 3.2. siRNA PMP22-SQ NP interactions with serum and its components

536

#### 3.2.1. Serum components stabilize siRNA PMP22-SQ NPs

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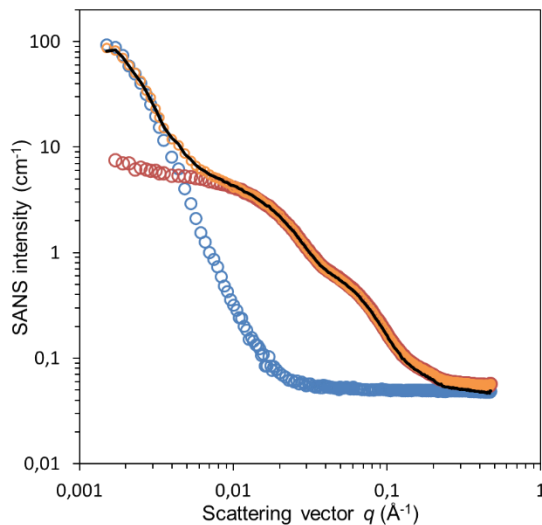
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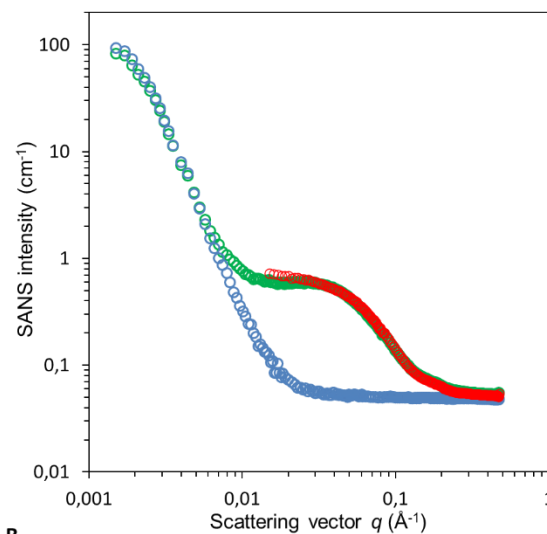
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SANS was performed to determine the influence of fetal bovine serum (FBS), a biological medium with high ionic strength containing several proteins, on the structure of siRNA PMP22-SQ NPs. In the low  $q$  range ( $q < 0.003 \text{ \AA}^{-1}$ ), the resulting SANS patterns did not show strong deviation from the scattering intensity of the siRNA PMP22-SQ suspension (at similar concentration) (**Figure 5A**). No intensity increase was observed, as it could be expected when particles are growing or when attractive interactions appear between particles. It demonstrates that siRNA PMP22-SQ NPs were colloidally stable and did not aggregate in this complex mixture. On the contrary, the scattering intensity arising from the siRNA PMP22-SQ NPs slightly decreased. As confirmed by the fitting curve (black line on **Figure 5A**), the SANS pattern of the mixture appeared to be a linear combination of the two reference patterns. If we consider the formula:  $I_{\text{fit}} = A \times I_{\text{single}}(\text{siRNA PMP22-SQ NPs})_{\text{ini}} + B \times I_{\text{single}}(\text{FBS})_{\text{ini}}$ , the best fit was obtained for  $A = 0.87$  and  $B = 0.98$ , demonstrating that only 13% of the siRNA PMP22-SQ signal had disappeared. The same result has been observed after incubation of siRNA PMP22-SQ NPs with a high concentration of bovine serum albumin (BSA) (200  $\mu\text{M}$ , green pattern in **Figure 5B**). As with FBS, SANS patterns of BSA alone (red pattern) or siRNA PMP22-SQ NPs alone (blue pattern) were also recorded. Likewise, the scattering intensity was not much modified at small  $q$ . Thus, neither siRNA PMP22-SQ NPs nor FBS or BSA were significantly affected by the presence of each other. Therefore, it was concluded that siRNA PMP22-SQ NPs had a high colloidal stability in both biological media.



A



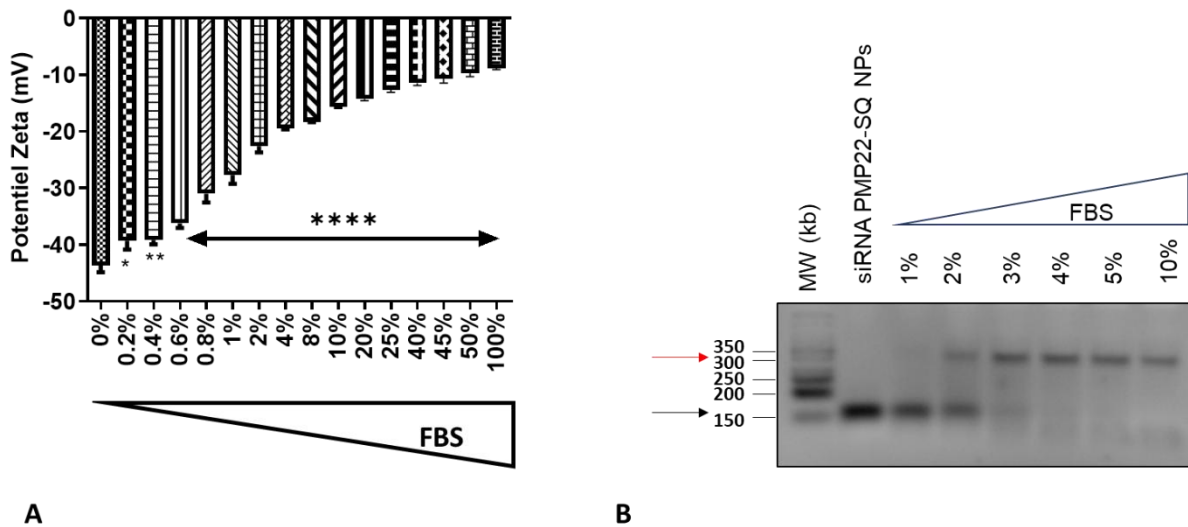
B

557

558 **Figure 5: The siRNA PMP22-SQ NPs are stable in FBS and in the presence of high BSA**  
 559 **concentration.** A) SANS pattern of siRNA PMP22-SQ NPs/FBS mixture (yellow circles).  
 560 Blue circles: 23  $\mu$ M [siRNA PMP22-SQ] in D<sub>2</sub>O, red circles: FBS dialyzed against D<sub>2</sub>O and  
 561 diluted by a factor of 2 to reach the same concentration as in siRNA PMP22-SQ NPs/FBS  
 562 mixture. The smooth black curve corresponds to a linear combination of the FBS and siRNA  
 563 PMP22-SQ NPs curves. B) SANS pattern of siRNA PMP22-SQ NPs/BSA mixture (green  
 564 circles). Blue circles: 23  $\mu$ M [siRNA PMP22-SQ] in D<sub>2</sub>O, red circles: 200  $\mu$ M [BSA] in D<sub>2</sub>O.  
 565

3.2.2. Serum neutralizes the siRNA-SQ NPs surface charge

566  
 567  
 568 The  $\zeta$ -potential increased progressively towards neutrality when siRNA PMP22-SQ  
 569 NPs were incubated with 0.2 % to 100 % of FBS (**Figure 6A**). The 100% of FBS represent its  
 570  $\zeta$ -potential and is used as a control to discriminate the  $\zeta$ -potential of the nanoparticles from  
 571 the undiluted FBS. These results highlighted the strong interaction occurring between some  
 572 FBS components and siRNA PMP22-SQ NPs. Interaction with serum components was also  
 573 confirmed by electrophoresis (**Figure 6B**). A progressive slowdown of migration reflected by  
 574 a delay in their electrophoretic mobility was observed when siRNA PMP22-SQ NPs were  
 575 incubated in FBS (respectively around 350 kb for NPs with FBS as against around 150 kb for  
 576 NPs in H<sub>2</sub>O). Interestingly, this phenomenon was observed at very low FBS concentration  
 577 (2%). Taken together, these results showed that FBS modified the surface charge and  
 578 electrophoretic mobility of siRNA-SQ NPs.



579  
 580 **Figure 6: siRNA PMP22-SQ NPs interact with serum components.** A) Measurements of  
 581 the siRNA-PMP22-SQ NPs  $\zeta$ -potential in the presence of increasing concentrations of FBS  
 582 (from pure water (0% FBS) to pure FBS (100%)). \*  $p < 0.05$ , \*\* $p < 0.001$  and \*\*\*\* $p < 0.00001$   
 583 by using One-way Anova followed by a Bonferroni test. B) The siRNA PMP22-SQ NPs were  
 584 incubated with increased concentration of FBS and loaded onto 4% agarose electrophoresis  
 585 gel, then stained by Midori, a nucleotide maker. Migration slows down when FBS is added to

586 the siRNA PMP22-SQ NPs (red arrow, wells 3 to 8), compared to siRNA PMP22-SQ NPs in  
587 water (black arrow, well 1).

### 588 3.2.3. siRNA PMP22-SQ NPs interact with BSA

589 Albumin is the main serum component, representing around 55% of total protein  
590 content. BSA has intrinsic fluorescence, due to its amino acid, tryptophan, which can be  
591 recorded by fluorescence emission spectroscopy. Thus, the interaction between BSA and  
592 siRNA PMP22-SQ NPs was investigated by two fluorescence spectroscopic approach: Stern-  
593 Volmer quenching and steady-state fluorescence. First, by using the Stern-Volmer quenching  
594 experiment, we showed an interaction between siRNA PMP22-SQ NPs and BSA (**Figure**  
595 **7A**). Indeed, when BSA was incubated with increasing siRNA PMP22-SQ concentrations, the  
596 intrinsic fluorescence of BSA was quenched, represented by a reduction of fluorescence  
597 emission compared to BSA alone at 5  $\mu\text{M}$  (red curve in **Figure 7A**). These results were  
598 determined according to the Stern-Volmer equation described in **Supplementary**  
599 **information, equation A**. The value obtained for the quenching rate constant,  $K_q$ , was higher  
600 than  $10^{10} \text{ mol.L}^{-1}.\text{s}^{-1}$ , suggesting a static mechanism with the formation of a complex between  
601 BSA and siRNA PMP22-SQ NPs (**Table 1**). The equivalent of the affinity constant for the  
602 kinetic intermediate, the Stern-Volmer constant, was equal to  $2.60 \pm 0.80 \times 10^5 \text{ L.mol}^{-1}$ . All  
603 the obtained values are presented in **Table 1**.

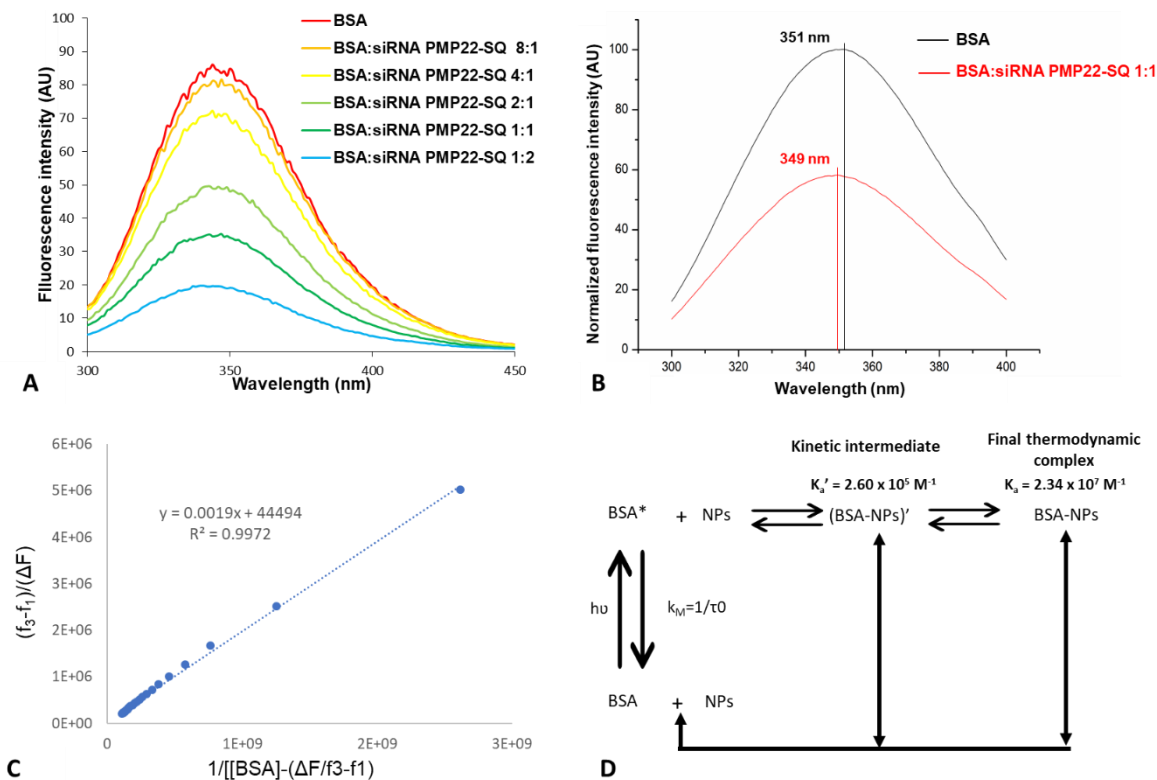
604 **Table 1: Numerical values based on linear fit of Stern-Volmer and Steady-state**  
605 **equations**

Constants		Value	Unit
Quenching rate constant	$K_q$	$2.60 \pm 0.80$	$\times 10^{13} \text{ L.mol}^{-1}.\text{s}^{-1}$
Stern-Volmer constant (kinetic intermediate)	$K_{sv}$	$2.60 \pm 0.80$	$\times 10^5 \text{ M}^{-1}$
Overall affinity constant (final thermodynamic complex)	$K_a$	$2.34 \pm 0.59$	$\times 10^7 \text{ M}^{-1}$

606 In the steady-state fluorescence approach, BSA was progressively titrated against  
607 siRNA PMP22-SQ NPs suspension, to a final molar ratio of 1 [siRNA PMP22-SQ]:1 [BSA].  
608 In parallel, BSA was added to distilled water at the same concentration and the final spectra  
609 were compared. The presence of siRNA PMP22-SQ NPs led to a significant modification of  
610 the BSA emission spectrum (red curve in **Figure 7B**) compared to that of BSA alone in water  
611 (black curve in **Figure 7B**). Indeed, we observed a smaller fluorescence intensity under the  
612 final experimental conditions and a shift in the maximum fluorescence intensity (from 351 nm

613 for BSA alone to 349 nm for NPs-BSA), which indicated the formation of a complex between  
 614 the two species (*i.e.*, siRNA PMP22-SQ NPs and BSA). The interaction mechanism could be  
 615 probed by several equations according to the species in solution that are dependent on pH and  
 616 temperature (**Supplementary information, equation B**). The linear least-squares regression  
 617 showed good correlation between siRNA PMP22-SQ NPs and BSA (**Figure 7C**). The  
 618 calculated overall apparent dissociation constant,  $K_{obs}$ , was equal to  $4.95 \pm 1.38 \times 10^{-8}$  M ( $n =$   
 619  $3$ ; pH 7) with an overall affinity constant,  $K_a$ , of  $2.34 \pm 0.59 \times 10^7$  M<sup>-1</sup>.

620 Using both spectroscopic approaches, we showed the formation of a complex between  
 621 siRNA PMP22-SQ NPs and BSA. However, there was a disparity between the affinity  
 622 constants obtained. This could be explained by the experimental conditions, especially the  
 623 time (**Figure 7D**). Indeed, with Stern-Volmer quenching, a kinetic intermediate (BSA-NPs)<sup>'</sup>  
 624 was obtained with  $K_a'$  of  $2.6 \times 10^5$  M<sup>-1</sup>. The slow titration of the steady-state fluorescence led  
 625 to the final thermodynamic complex between BSA and NPs: BSA-NPs ( $K_a = 2.34 \pm 0.59 \times$   
 626  $10^7$  M<sup>-1</sup>). Therefore, the intermediate complex underwent a conformational change which  
 627 allowed to attempt its final thermodynamic state by increasing even more the stability of the  
 628 complex by a factor of 100.

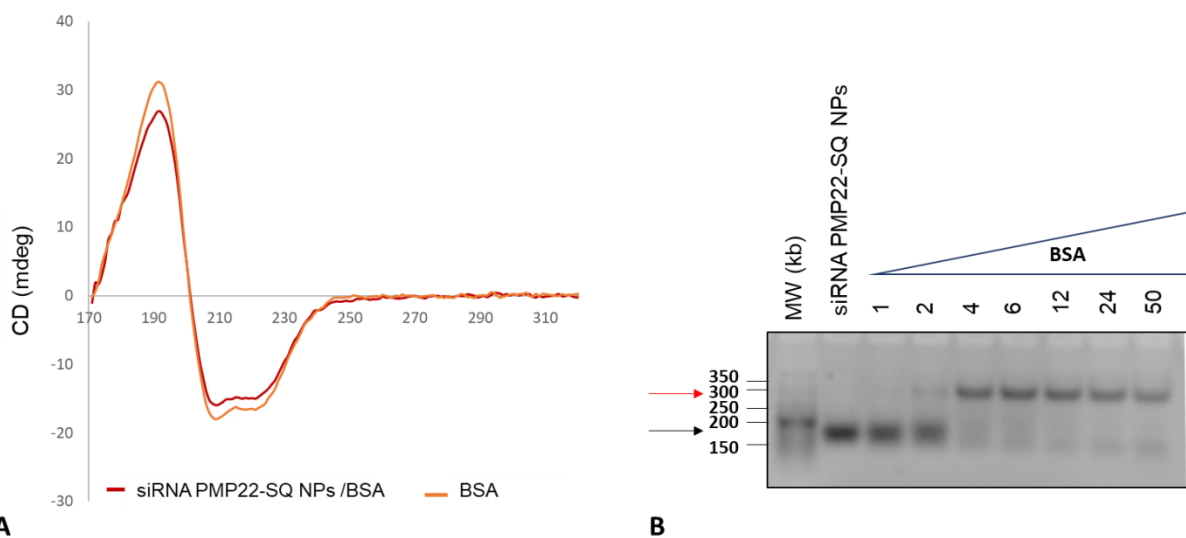


629  
 630 **Figure 7: Spectroscopic analysis of siRNA PMP22-SQ NPs/BSA mixtures.** A) Stern-  
 631 Volmer quenching of BSA in the presence of siRNA PMP22-SQ NPs. The fluorescence  
 632 intensity of BSA decreases progressively in the presence of siRNA PMP22-SQ NPs. B)  
 633 Steady-state experiment showing the emission fluorescence spectra at the end of the

634 experiment, excitation wavelength ( $\lambda_{\text{ex}} = 280 \text{ nm}$ ), pH 7.4 at 37°C: BSA (5  $\mu\text{M}$ , black curve)  
635 and BSA (5  $\mu\text{M}$ ) + siRNA PMP22-SQ (5  $\mu\text{M}$ ) in red. The maximal fluorescence intensity  
636 decreases, and the emission wavelength shifts by 2 nm (from 351 nm to 349 nm) when BSA  
637 interacts with siRNA PMP22-SQ NPs. Fluorescence intensity has been normalized on BSA  
638 maximum peak. **C)** Linear least-squares regression of the formula given in the  
639 **Supplementary information, equation 2** ( $R^2 = 0.9972$ ). **D)** Graphical representation  
640 showing the relationship between Stern-Volmer and steady-state approaches, showing the  
641 affinity constants,  $K_a'$  and  $K_a$

642 The interaction between BSA and siRNA PMP22-SQ NPs was further investigated by  
643 circular dichroism spectroscopy (**Figure 8A**). Incubation of BSA with siRNA PMP22-SQ  
644 NPs resulted in a decrease of 8% to 10% in the absolute signal intensity. Secondary structure  
645 quantifications of BSA was performed by using BestSel software, showing a slight decrease  
646 in the number of  $\alpha$ -helices, similarly to concentrated BSA (**Figure S3**). This interaction  
647 between BSA and siRNA PMP22-SQ NPs was also tested by electrophoresis gel. Migration  
648 of siRNA PMP22-SQ NPs was slowed down after incubation with increasing concentrations  
649 of BSA (**Figure 8B**). At molar ratio [siRNA PMP22-SQ] 1: 4 [BSA], migration delay was  
650 maximum and we estimated that around 10 BSA molecules surrounded 1 nanoparticle  
651 (**Supplementary information, equation C**). Taken together, both results highlighted the  
652 formation of a NPs-BSA complex. However, this complex was not able to modify the BSA  
653 secondary structure.

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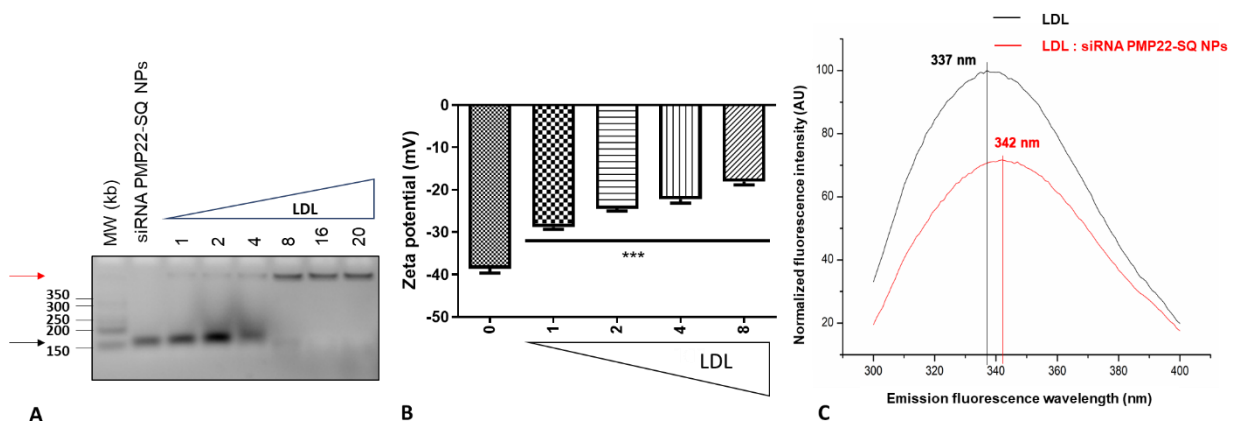
655 **Figure 8: siRNA PMP22-SQ NPs interact with BSA.** **A)** Circular dichroism spectrum of  
656 BSA alone (orange curve) and in the presence of siRNA PMP22-SQ NPs (red curve). In  
657 water, BSA spectra exhibits one positive peak at 192 nm and two negative bands at 210 and  
658 224 nm, both characteristic of the  $\alpha$ -helical structure. A decrease in the BSA signal in the  
659 presence of siRNA PMP22-SQ NPs is observed. **B)** Gel electrophoresis representing the  
660 migration of the siRNA PMP22-SQ NPs (5  $\mu\text{M}$ ) incubated at 37°C with increasing BSA  
661



662 concentration loaded on 4% agarose gel. Migration progressively slows down (red arrow,  
663 wells 4 to 8), compared to siRNA PMP22-SQ NPs in water (black arrow, well 1).  
664  
665

### 666 3.2.4. siRNA-SQ NPs interact with LDL.

667 Finally, we focused on the low-density lipoproteins (LDL), that carry 75% of blood  
668 cholesterol in humans. Incubation of siRNA PMP22-SQ NPs with increasing amounts of LDL  
669 showed a slowdown of the gel electrophoresis migration testifying an interaction between the  
670 two species (**Figure 9A**). In addition,  $\zeta$ -potential measurements revealed a significant  
671 progressive increase of the NPs surface charge with increasing LDL concentration (**Figure**  
672 **9B**). These results were confirmed by the fluorescence spectrum of the LDL (**Figure 9C**),  
673 which weakened in the presence of the NPs and was accompanied by a 5-nm shift in the peak  
674 emission wavelength. This clearly confirmed the formation of a siRNA PMP22-SQ NPs -  
675 LDL complex.  
676

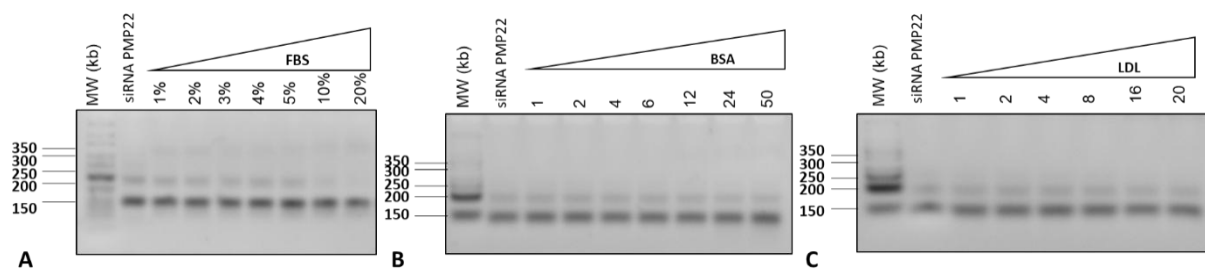


677 **Figure 9: siRNA-SQ NPs interact with LDLs.** **A)** Gel electrophoresis showing the  
678 migration of siRNA PMP22-SQ NPs incubated at 37°C with increasing LDL concentration  
679 loaded on 4% agarose gel. Migration progressively slows down (red arrow, wells 5 to 7),  
680 compared to siRNA PMP22-SQ NPs in water (black arrow, well 1). **B)**  $\zeta$ -potential  
681 measurement after incubation with growing concentrations of LDL. \*\*\*  $p < 0.0001$  vs siRNA  
682 PMP22-SQ NPs (One-way Anova followed by a Bonferroni test). **C)** Emitted fluorescence  
683 spectrum of LDL in the absence (black curve) or presence of siRNA-SQ NPs (red curve). The  
684 maximal fluorescence intensity decreases and there is a 5 nm shift of emission peak (from 337  
685 nm to 342 nm) when LDL interacts with siRNA PMP22-SQ NPs. Fluorescence intensity has  
686 been normalized to LDL peak maximum.  
687

### 688 3.2.5. siRNA PMP22-SQ NPs interact via the squalene moiety with serum 689 components 690

691 NPs made from small molecules linked to squalene, including nucleoside analogs,  
692 interact with serum components *via* the SQ moiety (Sobot, Mura et al. 2017, Sobot, Mura et  
693 al. 2017, Yesylevskyy, Ramseyer et al. 2018). To confirm that similar interaction could occur

694 with siRNA PMP22-SQ NPs and FBS or its components (*i.e.*, BSA and LDL), naked siRNA  
695 PMP22 (*i.e.* not conjugated with SQ) was incubated with increasing amounts of whole serum,  
696 BSA or LDL, before electrophoresis were performed. Contrary to siRNA PMP22-SQ NPs,  
697 migration of serum and serum components did not slow down (**Figure 10**). This confirmed  
698 that the interaction of nanoparticles with serum components occurred well *via* the squalene  
699 moiety and not *via* the oligonucleotide moiety.



700  
701 **Figure 10: Naked siRNA PMP22 do not interact with total serum, BSA and LDL.** 4%  
702 non-denaturated agarose electrophoresis were performed after incubation of naked siRNA  
703 PMP22 with increasing amounts of FBS (A), BSA (B, molar ratio) and LDL (C, mass ratio).  
704 No slowdown of migration was observed.

#### 705 4. Discussion

706 Conjugation of siRNA to squalene by Cu-free click chemistry, has been previously  
707 demonstrated to protect the double-strand oligonucleotide from degradation and to treat  
708 different experimental diseases such as cancer with fusion oncogene or peripheral neuropathy  
709 (Massaad-Massade, Boutary et al. 2018, Boutary, Caillaud et al. 2021). To be injectable and  
710 efficient *in vivo*, squalenoyl siRNAs needed to be formulated as nanoparticles. However, due  
711 to the different molecular weight of the two bioconjugate moieties (*i.e.*, ~ 13,000 Da for  
712 siRNA / ~ 494 Da for SQ forming a ratio of 26/1 between hydrophilic siRNA and lipophilic  
713 squalene) and because the siRNA-SQ bioconjugates were not soluble in organic solvents but  
714 totally soluble in water, an inverse nanoprecipitation protocol has been optimized by adding  
715 the aqueous phase to the organic one, before solvent evaporation to obtain the final siRNA-  
716 SQ NPs suspension (Bilati, Allémann et al. 2005).

717 The size and size distribution of the nanoparticles are important parameters for drug  
718 delivery systems. Using DLS, a rapid method for determination of nanoparticles size, we  
719 found that the diameter of squalenoyl siRNA nanoparticles were nucleoside sequence  
720 independent. Indeed, both siRNA PMP22-SQ and RET/PTC1-SQ nanoparticles displayed  
721 similar dimension, around 200 nm with a negative charge less than -30 mV; they were stable  
722 for a month at 4 °C (Massaad-Massade, Boutary et al. 2018, Boutary, Caillaud et al. 2021). As  
723 no significant difference was found concerning their physicochemical parameters, it was

724 decided to use the siRNA PMP22-SQ NPs for all further experiments. This resolution was  
725 also supported by our recent findings concerning the therapeutic efficiency of siRNA PMP22-  
726 SQ NPs as a targeted therapy for CMT1A neuropathy (Boutary, Caillaud et al. 2021).

727 Then, the siRNA PMP22-SQ NPs were observed by cryo-TEM. Spherical nanoobjects of  
728 around 130 nm in size with no specific internal structure were observed similar to our  
729 previous observation on siRNA Tmprss2-ERG-SQ NPs, which confirmed once again that  
730 the size was independent of the nucleoside sequence (Massaad-Massade, Boutary et al. 2018).  
731 Of note, cryoTEM is more specific than DLS, since it provides additional information on  
732 shape and morphology. The smaller nanoparticles size observed by cryoTEM was explained  
733 by the fact that laser light scattering measured the hydrodynamic size when cryoTEM allowed  
734 to visualize the nanoparticle core only. Small-angle neutron scattering (SANS) confirmed the  
735 observed size and shape of the siRNA PMP22-SQ nanoparticles. Altogether DLS, cryo-TEM  
736 and SANS analysis represented complementary approaches, giving an ensemble picture of the  
737 siRNA PMP22-SQ NPs.

738 To describe more precisely the internal composition and the siRNA organization inside of  
739 the NPs, SAXS analysis have been performed, which depicted that the siRNA PMP22-SQ  
740 bioconjugates self-organized in water into small core/shell domains of 11-nm size with the  
741 lipidic moiety exposed to the outside and the siRNA located in the core, without modification  
742 of the  $\alpha$ -helix structure of the nucleic acid. We assume that hydrophobic interactions are the  
743 main driving force leading to the self-assembly of the nanoparticle into a 130-nm size  
744 spherical random structure with some hydrophilic siRNA moieties being exposed onto the  
745 nanoparticle surface for solubility reasons. To our knowledge, this unique random  
746 supramolecular structure, composed of typical 11-nm size spheres with internal electronic  
747 density heterogeneities, has only been observed with siRNA PMP22-SQ NPs. It confirms the  
748 diversity of the squalenoyl nanostructures and the flexibility of the hydrophobic squalene  
749 chain which allows to self-assemble into nanoparticles, even when a hydrophilic  
750 macromolecule of much higher molecular weight was attached to the SQ.

751 We then assessed the interaction of siRNA PMP22-SQ NPs with blood components,  
752 specifically albumin and lipoproteins. Indeed, once administered intravenously, siRNA-SQ  
753 NPs encounter different blood components that can adsorb to the NPs surface, either  
754 stabilizing or destabilizing their structure and this may dramatically impact their *in vivo* fate  
755 in terms of biodistribution, pharmacokinetics, toxicity and therapeutic efficiency (Aggarwal,  
756 Hall et al. 2009, Lesniak, Fenaroli et al. 2012, Monopoli, Aberg et al. 2012, Gao and He 2014,  
757 Marichal, Giraudon-Colas et al. 2019, Marichal, Degrouard et al. 2020).

758 It was observed that siRNA PMP22-SQ nanoparticles interacted hydrophobically, *via* the  
759 squalene moiety, with serum components, especially BSA and LDL. Interestingly, it has been  
760 reported that BSA may display some dysopsonin-like activity (Ogawara, Furumoto et al.  
761 2004). These interactions with serum components can undergo conformational changes on the  
762 NPs surface which could also affect their biological fates (Gunawan, Lim et al. 2014). This  
763 hypothesis was assessed by SANS experiments performed in FBS and in presence of high  
764 BSA concentrations. No significant modifications in the SANS pattern of the nanoparticles  
765 could be observed. This proved that the siRNA PMP22-SQ NPs kept high colloidal stability  
766 in biological media, contrarily to previous studies performed with small chemical molecules  
767 conjugated to squalene showing a destabilization of the squalenoyl NPs in the presence of  
768 blood components, with an insertion of these bioconjugates, as molecular entities, into the  
769 LDL and BSA fractions (Ambike, Rosilio et al. 2011, Sobot, Mura et al. 2017, Yesylevskyy,  
770 Ramseyer et al. 2018, Gobeaux, Bizeau et al. 2020). Thus, the present study clearly suggests  
771 that the biological behavior of squalene-based nanoparticles may depend on the nature of the  
772 associated biologically active molecule. Such different behavior of squalenoyl NPs may be  
773 explained by: i) the respective molecular weight of the linked drug *versus* the squalene (*i.e.*, a  
774 ratio of 1 or 2 *vs* 26 with siRNA-SQ), ii) the different physicochemical parameters (siRNA-  
775 SQ is highly negatively charged) and, iii) the supramolecular organization.

776 Since LDL and HDL are the main transporters of cholesterol, an important lipid for tumor  
777 cell proliferation (Chaudhary, Bower et al. 2019) and myelin synthesis (Zhou, Bazick et al.  
778 2019) , we hypothesize that the interaction of LDL with siRNA PMP22-SQ NPs may trigger a  
779 natural nanoparticles functionalization for targeting these pathological tissues. Additionally,  
780 such specific biodistribution may be amplified by the inflammation of the pathological  
781 tissues, resulting in histological modifications such as defective endothelial cells with wide  
782 fenestrations in cancer tissues (Tatiparti, Sau et al. 2017, Hoogenboezem and Duvall 2018)  
783 and edema with lymphocyte infiltration in neurodegenerative diseases (Weis, Claeys et al.  
784 2017). Especially, albumin accumulate preferentially in these type of tissues (Hoogenboezem  
785 and Duvall 2018). Thus, siRNA-SQ NPs are expected to extravasate through damaged tissues,  
786 as supported by our previous studies for cancers and a disease of the peripheral nervous  
787 system (CMT1A), where in both pathologies a significant inhibition of the targeted gene was  
788 observed in the tissues concerned (Massaad-Massade, Boutary et al. 2018, Boutary, Caillaud  
789 et al. 2021).

## 790 **5. Conclusions**

791 The present study has provided an important contribution to better understand the  
792 physico-chemical structures of siRNA squalene-based nanoparticles, as well as their  
793 interactions with some biological components encountered after intravenous administration.  
794 The high specificity of siRNA to decrease or normalize gene expression offers new outlooks  
795 to cure pathologies with gene expression dysregulation such as fusion oncogene cancer or  
796 hereditary monogenic peripheral neuropathy. In this area, siRNA-SQ NPs are promising new  
797 potential drug candidates with wide therapeutic applications thanks to their high colloidal  
798 stability.  
799

## 800 **6. Credit author statement**

801 M.C. designed and performed the experiments and wrote the manuscript, F.G. contributed  
802 and performed the experiments of SAXS and SANS studies, M.H. developed the  
803 Fluorescence quenching experiments, S.B. contributed to the synthesis and characterization of  
804 the siRNA-SQ NPs, P.G. contributed to study design and discussion, D.D developed the  
805 azido-squalene synthesis, P.C. contribute on the discussion of the results and to the writing of  
806 the manuscript, F.W. and I.G. performed with MC and FG the SRCD experiments, F.T.  
807 contributed and performed the experiments of SAXS and SANS studies and, L.M. is the PI of  
808 the study, she supervised, designed the study and contributed to the writing of the manuscript.

## 809 **7. Declaration of Competing Interest**

810 The authors declare that they have no known competing financial interests or personal  
811 relationships that could have appeared to influence the work reported in this paper.

## 812 **8. Acknowledgment**

813 This paper is dedicated to Ms. Isabelle Grillo† (Institut Laue Langevin, 71 avenue des  
814 Martyrs, B.P. 156, 38042 Grenoble Cedex 9, France) who passed away. Ms Grillo performed  
815 the SRCD experiments.

816 We thank for Julien Loisel Duwattez and Alexandre Rodrigues for their technical  
817 assistance, David Carrière for SAXS-WAXS measurements performed on the SWING  
818 beamline (SOLEIL Synchrotron) during the BAG 20181790 and 20201118. SRCD  
819 experiments were performed on the DISCO Beamline at the SOLEIL Synchrotron (Saint  
820 Aubin, France). This work benefited from the use of the SasView application  
821 (<http://www.sasview.org>), originally developed under NSF award DMR-0520547. SasView  
822 contains code developed with funding from the European Union's Horizon 2020 research and  
823 innovation program under the SINE2020 project, grant agreement no. 654000.

824 The authors also thank Ghislaine Frebourg for Cryo-TEM observations (Electronic  
825 Microscopy Platform, IBPS/Institut de Biologie Paris-Seine, Université P. et M. Curie, Paris,  
826 France) and John S. Lomas (Interfaces, Traitements, Organisation et DYnamique des  
827 Systèmes (ITODYYS), UFR de Chimie 75013 Paris, France) for the English editing. Marie  
828 Caillaud is grateful for the financial support of the Ile-de-France region within the framework  
829 of the "ARDoC" program (doctoral grants in targeted fields) during her doctoral fellowship.

830

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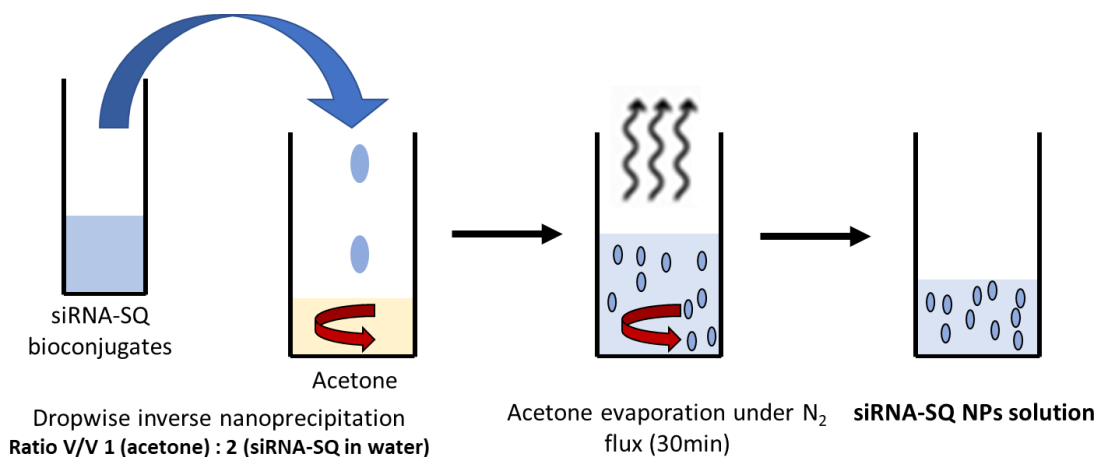
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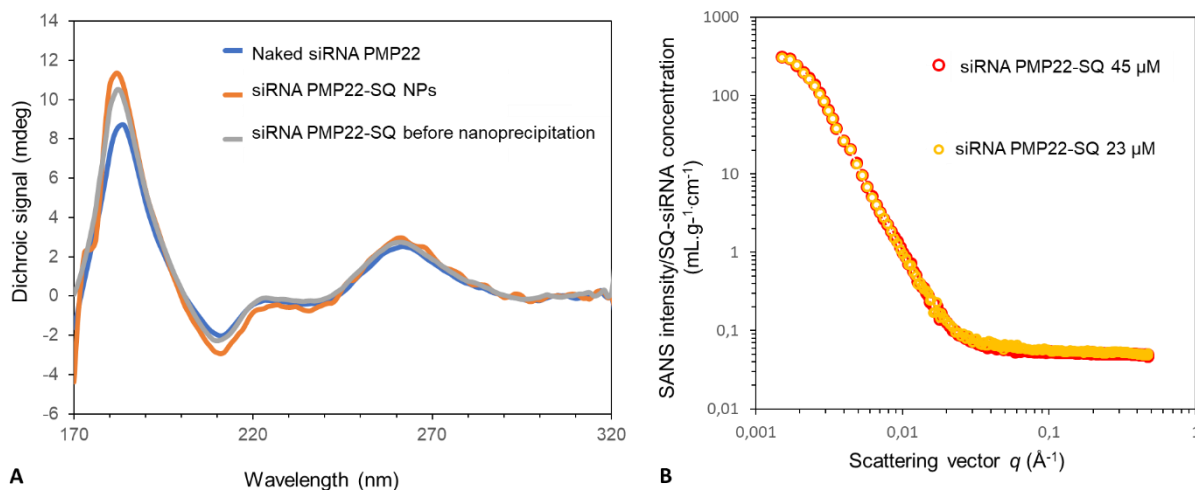
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## Appendix A. Supplementary material

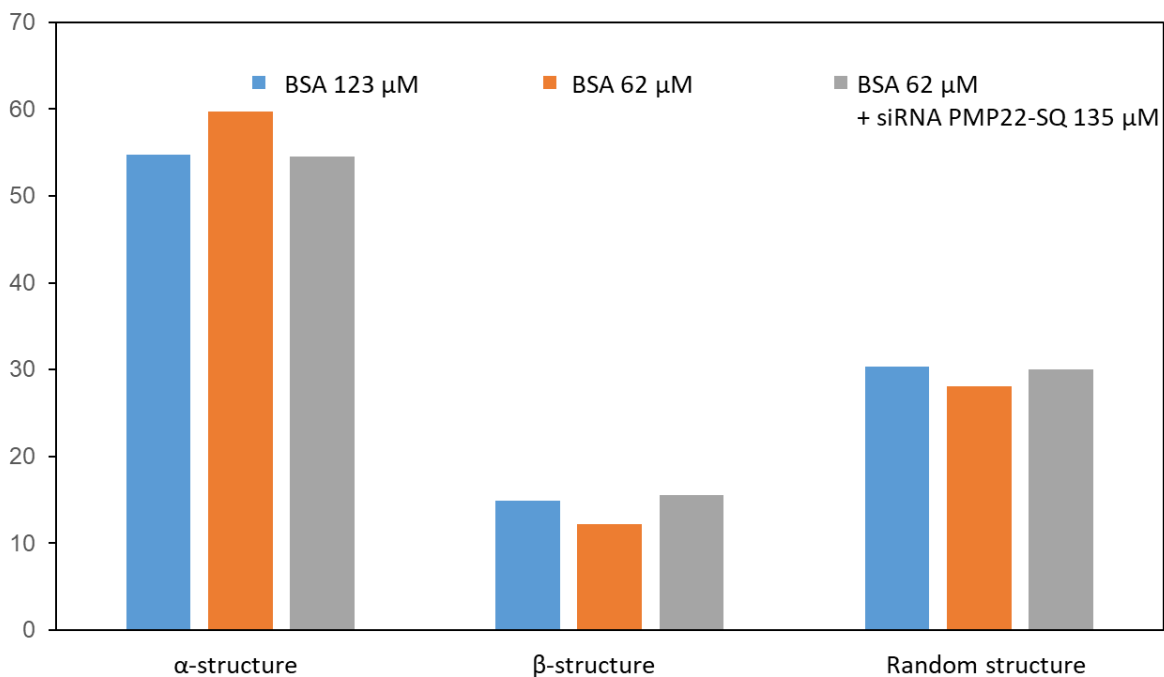


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Figure S1: Sketch of siRNA-SQ nanoparticles preparation. The siRNA-SQ bioconjugates were obtained after Cu-free click chemistry and annealing to the antisense strand. Then, the siRNA-SQ NPs were prepared by inverse nanoprecipitation : siRNA-SQ bioconjugates in water were added drop wisely to a stirred organic phase (acetone) with a volume/volume ratio of 1 [organic phase, acetone]: 2 [aqueous phase, siRNA-SQ bioconjugate]. Then acetone was evaporated using nitrogen (N<sub>2</sub>) flux to obtain an aqueous suspension of pure siRNA-SQ NPs.



1007 **A**  
 1008 Figure S2: Circular dichroism spectrum of siRNA. **A**) Naked siRNA (blue curve); siRNA-SQ  
 1009 NPs (orange) siRNA-SQ bioconjugates before nanoprecipitation (grey). Intensity difference  
 1010 (y-axis) is due to the use of different sample concentrations. **B**) Small-angle neutron scattering  
 1011 patterns of two dilutions of siRNA-SQ suspensions. Red circles: 45  $\mu\text{M}$ ; yellow circles: 23  
 1012  $\mu\text{M}$ . The small-angle region corresponds to values of  $q < 0.002 \text{ \AA}^{-1}$ , mean angles 0.002 to  
 1013  $0.04 \text{ \AA}^{-1}$  and the large-angle  $q > 0.04 \text{ \AA}^{-1}$ .  
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 1017 Figure S3: Quantification of the secondary structure of BSA. After circular dichroism  
 1018 analysis, quantifications of BSA were performed by BestSel program  
 1019 (<http://bestsel.elte.hu/index.php>). The histogram represents the comparison of two BSA  
 1020 concentrations (123  $\mu\text{M}$  in blue and 62  $\mu\text{M}$  in orange) alone or in presence of siRNA-SQ NPs  
 1021 (in grey) with BSA at 62  $\mu\text{M}$ . No difference was observed for the different conditions.  
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**Table S1:** Table comparing siRNA size estimation

<b>“Source”</b>	<b>Dimensions (nm)</b>
<b>22 base pairs x 3.5 Å</b>	L = 7 nm
<b>SANS pattern Ristori <i>et al.</i></b>	Cylindrical form factor L = 5.2 nm R = 1.1 nm
<b>SAXS pattern of siRNA strands</b>	Cylindrical form factor L = 7 nm R = 1.1 nm
<b>SAXS pattern of siRNA-SQ</b>	Cylindrical form factor L = 4 nm R = 1.1 nm

1025 The scattering pattern originating from the naked siRNA PMP22 dispersion exhibited an  
1026 oscillation in the same  $q$ -range in accordance with the SANS analysis of similar siRNA  
1027 strands reported by Ristori *et al.* (Ristori, Ciani et al. 2012).  
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**Supplementary information, equation A**

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$$\frac{F_0}{F} = 1 + K_{sv}[\text{siRNA PMP22-SQ}] = 1 + K_q\tau_0[\text{siRNA PMP22-SQ}]$$

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1033 **Equation A. Stern-Volmer equation:**  $F_0$  and  $F$  are fluorescence intensities of BSA in  
1034 absence and presence of quencher (siRNA-SQ NPs),  $[\text{siRNA PMP22-SQ}]$  the molar  
1035 concentration of siRNA PMP22-SQ (in M),  $K_{sv}$  ( $M^{-1}$ ) the Stern-Volmer constant which  
1036 depends on  $K_q$  ( $M^{-1}.s^{-1}$ ) and  $\tau_0$  (s) the average lifetime of tryptophan fluorescence in the  
1037 absence of quencher, equals  $10^{-8}$  s.  
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### Supplementary information, equation B

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Interaction between the siRNA-SQ nanoparticles (NPs) and BSA can be probed in the following formula:



According to this equation we can determine the apparent dissociation constant ( $K_{\text{obs}}$ ) at pH 7 and at 25°C.

$$(B2) \quad K_{\text{obs}} = \frac{[\text{siRNA PMP22-SQ}][\text{BSA}]}{[\text{siRNA PMP22-SQ-(BSA)}]}$$

Based on equation (1), the intrinsic fluorescence intensity of the mixture is expressed as a function of the concentrations of all species in solution, weighted by experimental factors  $f_1$ ,  $f_2$  and  $f_3$  for [siRNA PMP22-SQ], [BSA] and [siRNA PMP22-SQ-BSA] respectively:

$$(B3) \quad F = f_1 [\text{siRNA PMP22-SQ}] + f_2 [\text{BSA}] + f_3 [\text{siRNA PMP22-SQ-(BSA)}_n]$$

In absence of BSA, the initial fluorescence intensity is given by:  $F_0 = f_1 [\text{siRNA PMP22-SQ}]_{\text{initial}}$

The experimental conditions are as follow: 1) ratio [BSA]: [siRNA-SQ] < 1; 2) the fluorescence intensity emitted by the siRNA PMP22-SQ NPs + BSA mixture should be completely adsorbed BSA, therefore 3) the intensity emitted from free BSA ( $f_2[\text{BSA}]$ ) can be neglected.

The experimental factor  $f_3$  is calculated based on raw data. From the variation in fluorescence emission  $\Delta F = F - F_0$  of equation (3) the  $K_{\text{obs}}$  (2) can be calculated from the linear least-squares regression:

$$(B4) \quad \frac{f_3 - f_1}{\Delta F} = \frac{1}{[\text{siRNA PMP22-SQ}]_{\text{initial}}} + \frac{K_{\text{obs}}}{[\text{siRNA PMP22-SQ}]_{\text{initial}} \left( [\text{BSA}] - \frac{\Delta F}{f_3 - f_1} \right)}$$



## Supplementary information, equation C

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### 1) Mass calculation of one nanoparticle

As serum components will interact with the squalene moiety which surround the nanoparticle, we postulated that the mass of one nanoparticle is based on its density ( $\rho_{SQ} = 858 \text{ kg/m}^3$ ) and the nanoparticle volume:

$$(C1) \quad m_{NP} = \rho_{SQ} V_{NP}$$

Number of NPs can be calculated as follow:

$$(C2) \quad N_{NP} = \frac{C_m(\text{siRNA PMP22-SQ})}{m_{NP}}$$

### 2) Determination of the number of proteins

Based on electrophoresis experimental conditions and molecular weight of the BSA, we can calculate the total mass of BSA:

$$(C3) \quad m_{\text{total BSA}} = [\text{BSA}] \text{MM}_{\text{BSA}} V$$

Where [BSA] is the molar concentration of BSA,  $\text{MM}_{\text{BSA}}$  the BSA molecular weight (65,000 g/mol) and V the volume used for incubation before electrophoresis.

Number of BSA proteins can be determined as follow:

$$(C4) \quad N_{\text{BSA}} = \frac{m_{\text{total BSA}}}{\text{MM}_{\text{BSA}}} N_A$$

Where N is the BSA protein number and  $N_A$  the Avogadro number (number of elementary entities contained in a mole of matter, equal to  $6.023 \times 10^{23}$ ).

### 3) Determination of BSA proteins per nanoparticle

Based on (2) and (4), we can calculate the number of BSA interacting with one nanoparticle:

$$(C5) \quad \text{Number BSA}/_{NP} = \frac{N_{\text{BSA}}}{m_{NP}}$$