

1 **Peptide and nucleic acid-directed self-assembly of cationic nanovehicles through giant**
2 **unilamellar vesicle modification: targetable nanocomplexes for *in vivo* nucleic acid delivery**

3
4 AD Tagalakis^{a*}, R Maeshima^a, C Yu-Wai-Man^b, J Meng^a, F Syed^a, L-P Wu^c, AM Aldossary^a, D
5 McCarthy^d, SM Moghimi^{c, e} & SL Hart^a

6
7
8 ^aExperimental and Personalised Medicine Section, UCL Great Ormond Street Institute of Child
9 Health, 30 Guilford Street, London, WC1N 1EH, UK

10 ^bNational Institute for Health Research (NIHR) Biomedical Research Centre at Moorfields Eye
11 Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, 11-43 Bath Street,
12 London, EC1V 9EL, UK

13 ^cCentre for Pharmaceutical Nanotechnology and Nanotoxicology, Faculty of Health and Medical
14 Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark

15 ^dUCL School of Pharmacy, 29–39 Brunswick Square, London, WC1N 1AX, UK

16 ^e School of Medicine, Pharmacy and Health, Durham University, Stockton-on-Tees TS17 6BH,
17 UK

18
19
20 *Corresponding author: a.tagalakis@ucl.ac.uk

21
22
23
24
25
26
27
28
29
30 **Keywords: GUV; vesicles; non-viral vectors; liposomes; peptide; lipopolyplexes; DNA;**
31 **siRNA**

32 **ABSTRACT**

33 One of the greatest challenges for the development of genetic therapies is the efficient targeted
34 delivery of therapeutic nucleic acids. Towards this goal, we have introduced a new engineering
35 initiative in self-assembly of biologically safe and stable nanovesicle complexes (~90-140 nm)
36 derived from giant unilamellar vesicle (GUV) precursors and comprising plasmid DNA or
37 siRNA and targeting peptide ligands. The biological performance of the engineered nanovesicle
38 complexes were studied both *in vitro* and *in vivo* and compared with cationic liposome-based
39 lipopolyplexes. Compared with cationic lipopolyplexes, nanovesicle complexes did not show
40 advantages in transfection and cell uptake. However, nanovesicle complexes neither displayed
41 significant cytotoxicity nor activated the complement system, which are advantageous for
42 intravenous injection and tumour therapy. On intravenous administration into a neuroblastoma
43 xenograft mouse model, nanovesicle complexes were found to distribute throughout the tumour
44 interstitium, thus providing an alternative safer approach for future development of tumour-
45 specific therapeutic nucleic acid interventions. On oropharyngeal instillation, nanovesicle
46 complexes displayed better transfection efficiency than cationic lipopolyplexes. The
47 technological advantages of nanovesicle complexes, originating from GUVs, over traditional
48 cationic liposome-based lipopolyplexes are discussed.

49
50
51
52
53
54
55
56
57
58
59
60
61
62
63

64 1. Introduction

65 Formulations of cationic lipids that self-assemble into lipoplexes upon mixing with
66 nucleic acids have received considerable attention. These non-viral vectors have recently become
67 more popular with the development of small interfering RNA (siRNA)-mediated silencing and
68 chemically-modified mRNA [1-4]. Nucleic acid therapy has great potential for the treatment of a
69 wide range of diseases [5], however, only a small number of formulations used *in vitro*, make it
70 to clinical trials as there are a number of barriers to *in vivo* delivery and transfection [4, 6].
71 Previously, we described the use of liposome-peptide receptor-targeted nanoparticles (RTNs) for
72 both *in vitro* [7-11] and *in vivo* [12-18] nucleic acid delivery to various sites and targets in the
73 body. These lipopolyplexes are capable of inducing nucleic acid compaction and their protection
74 against premature degradation in biological fluids.

75 There is always a need to improve the performance of delivery vehicles and giant
76 unilamellar vesicles (GUVs) have some interesting properties for the development of functional
77 nucleic acid delivery systems with tunable properties [19, 20]. Earlier, DNA-directed self-
78 assembly of GUVs has been shown, where DNA was introduced to vesicular surface by covalent
79 conjugation [21-24]. These GUVs, also proved to be invaluable *in vitro* tools for the mechanistic
80 understanding of complex and integrated biophysical and biomembrane processes [25-32]. Here,
81 we exploit the bilayer properties of GUVs as the starting platform for self-assembly of a new
82 generation of safe and stable lipid-peptide-nucleic acid transfectants with improved biological
83 performance through the introduction of sugars for improved stabilization as well as targeting
84 peptide ligands [20]. Indeed, the difference in density between the equiosmolar monosaccharidic
85 intervesicular (external) and the disaccharidic intravesicular (internal) aqueous solutions offer
86 vesicular stabilization and shape uniformity [33] as well as optical contrast. In addition, it is
87 known that cationic transfectants can induce bioenergetic crisis, which dependent on cell type
88 and mitochondrial polarization state it may initiate cell death [34]. The sugars associated with the
89 engineered GUVs (e.g. glucose) could potentially help in re-establishing homeostasis with
90 increased ATP synthesis, thereby overcoming cytotoxicity pertaining to cationic lipoplexes and
91 lipopolyplexes.

92 GUVs carrying nucleic acids may exhibit limited cell uptake and transfection efficacy
93 due to their large size compared with conventional large unilamellar vesicles [35]. Accordingly,
94 we have introduced GUVs as precursors for generating vesicles in the nanoscale range

95 (hereinafter termed “nanovesicles”). Nanovesicles were complexed with nucleic acids (DNA or
96 siRNA) and functionalized with different targeting peptides. The latter have included: 1) ME27,
97 which contains the Arg-Gly-Asp (RGD) motif capable of targeting integrins and particularly
98 $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ classes expressed in a wide range of tumours, 2) YGLPHKF (which is
99 derived from peptide Y, a generic targeting peptide that works well across a range of tissues,
100 including cells of neuronal origin) [8, 9] and closely resembles part of a targeting protein
101 expressed by the intracellular pathogen *Legionella pneumophila* [36], and 3) peptide E, which
102 has the SERSMNF motif that displays close similarity to receptor binding proteins of two
103 intracellular pathogens, rhinovirus and *Listeria monocytogenes* [36]. Rhinoviruses bind the
104 intercellular adhesion molecule-1 (ICAM-1) [37]. ICAM-1 is present in the airway epithelium
105 and is upregulated in the inflamed epithelium as in cystic fibrosis [37, 38].

106 Collectively, our studies comprise biophysical characterization of targetable nanovesicle
107 complexes as well as their improved biosafety in relevant *in vitro* and *in vivo* models compared
108 with conventional cationic lipoplexes and lipopolyplexes.

109
110

111 **2. Experimental section**

112

113 **2.1. Materials**

114 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and 1,2-dioleoyl-*sn*-
115 glycerol-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids, Inc.
116 (Alabaster, AL, USA). Peptide Y (K16GACYGLPHKFCG) was synthesized by ChinaPeptides
117 Co., Ltd. (Shanghai, People’s Republic of China), peptide E (K16GACSERSMNFCEG) was
118 synthesized by Zinsser Analytics (Maidenhead, UK), peptide ME27 (K16RVRRGACRGDCLG)
119 was synthesized by Alta Bioscience (Birmingham, UK) and the linear lysine peptide K16 was
120 purchased from ImunnoKontakt (Abingdon, UK). Dy677 control siRNA (siRNA-Dy677) was
121 purchased from GE Healthcare (Amersham, UK). Cy3-labelled control plasmid DNA (DNA-
122 Cy3) was purchased from Cambridge Bioscience (Cambridge, UK). The plasmid pCI-Luc
123 consists of the luciferase gene from pGL3 (Thermo Fisher Scientific, Hemel Hempstead, UK)
124 sub-cloned into pCI (Promega Corporation, Fitchburg, WI, USA). The plasmid pEGFP-N1 (4.7

125 kb) containing the gene *GFP* was obtained from Clontech Laboratories, Inc. (Mountain View,
126 CA, USA).

127

128 ***2.2. Nanovesicle formation from GUV precursors***

129 DOTMA and DOPE were dissolved in chloroform to a concentration of 10 mg/mL. Lipids
130 were mixed at a 1:1 molar ratio. The chloroform was evaporated in a rotary evaporator (BÜCHI
131 Labortechnik AG, Flawil, Switzerland). The lipid film was dissolved in light mineral oil (catalog
132 number: M5310; Sigma-Aldrich, Poole, UK) to a final concentration of 1.7 mg/mL by heating up
133 to 50°C, sonicated for 30 min in an ultrasonic water bath (Jencons-PLS, Bedfordshire, UK) and
134 incubated overnight at room temperature (RT). The lipid solution in mineral oil was then stored
135 at -20°C prior to further use. For the DOTMA/DOPE (DD) liposome preparation, the lipid film
136 was dissolved in water followed by sonication. For the nanovesicle (DOTMA/DOPE_{ves} or DD_{ves})
137 preparation, we used a modified version of the water/oil (W/O) emulsion transfer method [20,
138 26] described in detail by Hadorn et al. [23]. All solutions were prepared using Milli-Q water.
139 Sucrose (99.5%) and glucose (99.0%) were purchased from Sigma-Aldrich (Poole, UK). The
140 sucrose solution as well as the aqueous phase (glucose solution) was adjusted to 1000 mM
141 (equiosmolar conditions) to avoid any osmotic pressure that would reduce vesicular stability.
142 Consequently, the sucrose solution as well as the aqueous phase only differed in their densities.

143 The W/O emulsion was prepared in microtubes by adding 50 µL of the sucrose solution to
144 400 µL of the lipid solution prepared above and vigorously grated against a microtube rack for 3
145 min with force to aid emulsification. The intermediate phase was prepared in microtubes by
146 adding 150 µL of the lipid solution to 300 µL of the aqueous phase and incubation at RT for 10
147 min. To generate the nanovesicles, the emulsion was then placed on top of the intermediate
148 phase and centrifuged for 3 min at 1500g at RT. The oil was removed by aspiration and the pellet
149 was resuspended in the osmotically-adjusted glucose (aqueous phase) and kept at 4°C.

150 To prevent the nanovesicles from adhering to surfaces, microscope slides and coverslips
151 were treated with PlusOne Repel-Silane ES (GE Healthcare, Amersham, UK) in accordance with
152 manufacturer's recommendation. Nanovesicle suspension (10 µL) was applied to a microscope
153 slide and covered with a coverslip and then visualized (20x magnification) using an Olympus
154 IX70 fluorescent microscope (Olympus Corporation, Tokyo, Japan).

155

156 **2.3. Nanocomplex formation**

157 Cationic receptor-targeted nanocomplex (RTN) formulations (at a weight ratio of 1:4:1,
158 liposome or nanovesicle: peptide: DNA or siRNA) were made by first adding the peptide to the
159 liposome or nanovesicle DOTMA/DOPE (DOTMA/DOPE and DOTMA/DOPE_{ves}, respectively),
160 followed by addition of the DNA or siRNA with rapid mixing and incubation for 30 min at RT to
161 allow for complex formation. The composition and terminology of the nanocomplexes
162 (lipopolyplexes or nanovesicle complexes) are summarized in Table 1.

163

164 **2.4. Size and zeta potential determinations**

165 Nanocomplex preparations were diluted with distilled water to a final volume of 1 mL at a
166 concentration of 5 µg/mL with respect to DNA or siRNA. They were then analyzed for size and
167 electrophoretic mobility measurements using a Malvern Nano ZS (Malvern, UK). The following
168 specifications were used: automatic sampling time of 10 measurements/sample, refractive index
169 of 1.330 (water) and 1.340 (5% w/v glucose), dielectric constant 78.5 (water) and 77.37 (5% w/v
170 glucose), viscosity 0.8872 cP (water) and 1.1450 cP (5% w/v glucose), and temperature of 25°C.
171 DTS version 5.03, which was provided by the manufacturer, was used for data processing.

172

173 **2.5. Heparin dissociation assay**

174 DNA (0.2 µg) was mixed with PicoGreen reagent (1:150) (Invitrogen, Paisley, UK) at RT
175 in Tris-EDTA buffer and the DNA/PicoGreen mixture was then formulated into nanocomplexes
176 at a 1:4:1 weight ratio (liposome or nanovesicle: peptide: DNA) as described above. Heparin
177 sulfate (Sigma-Aldrich, Poole, UK) was added to the PicoGreen-labelled nanocomplexes in a
178 range of concentrations (0-2 U/mL). In each experiment, naked DNA stained with PicoGreen
179 was used to normalize the PicoGreen signal detected from the nanocomplexes. Fluorescence was
180 analyzed using a fluorescence plate reader, FLUOstar Optima (BMG Labtech, Aylesbury, UK).

181

182 **2.6. Transmission electron microscopy (TEM)**

183 For the electron microscopy investigations, the nanocomplexes were prepared as described
184 above and were placed on a glow-discharged 300-mesh copper grid coated with a
185 Formvar/carbon support film (Agar Scientific). After a few seconds, the grid was blotted with a
186 filter paper. The sample was then negatively stained with 1% (w/v) uranyl acetate or 1% (w/v)

187 phosphotungstic acid, before blotting and then air-dried. Imaging was carried out under a Philips
188 CM120 BioTwin Transmission Electron Microscope and operated at an accelerating voltage of
189 120 KV.

190

191 ***2.7 Cell culture***

192 Murine Neuro-2A and human Kelly neuroblastoma cell lines were obtained from the
193 American Type Culture Collection (Teddington, UK). Neuro-2A cells were maintained in
194 Dulbecco's Minimal Essential Medium (DMEM; Invitrogen, Paisley, UK) supplemented with
195 10% (v/v) FBS, 1% (v/v) non-essential amino acids, and 1% (v/v) sodium pyruvate. Kelly cells
196 were cultured in RPMI1640+GlutaMAX (Invitrogen, Paisley, UK) with 10% (v/v) FBS, 25 mM
197 HEPES and 100 U/mL Penicillin/Streptomycin. The human bronchial epithelial cells
198 16HBE14o- (shortened to HBE) were provided by D. Gruenert, (San Francisco, CA, USA) and
199 were cultured in Eagle's Minimal Essential Medium with HEPES modification (Sigma, Poole,
200 UK), 10% (v/v) FCS and 2 mM L-glutamine. All cells were maintained in a humidified
201 atmosphere of 95% air and 5% CO₂ at 37°C.

202

203 ***2.8 DNA transfection***

204 Neuro-2A and HBE cells were seeded in 96-well plates at 2×10^4 cells per well 24 h prior to
205 transfection. Following the removal of growth medium, 200 μ L of complexes in OptiMEM
206 containing 0.25 μ g of plasmid DNA were added to the cells in replicates of six. Plates were
207 centrifuged at 400g for 5 min and incubated for 4 h at 37°C, then transfection medium was
208 replaced by the complete growth medium and incubated for a further 24 h. Luciferase expression
209 was measured in cell lysates with a luciferase assay (Promega, Southampton, UK) in a FLUOstar
210 OPTIMA luminometer (BMG Labtech, Aylesbury, UK). The amount of protein present in each
211 sample was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel
212 Hempstead, UK) in a FLUOstar OPTIMA luminometer. Luciferase activity was expressed as
213 relative light units per milligram of protein (RLU/mg). Each measurement was performed in
214 groups of six.

215 The same protocol was used for transfections with eGFP plasmid DNA with the only
216 difference being that the cells following transfection were incubated for 48 h at 37°C. They were
217 firstly imaged (20 \times magnification) using an Olympus IX70 fluorescent microscope (Olympus,

218 Southend-on-Sea, UK) and then prepared for flow cytometry by detaching cells from the wells
219 with 50 μ L Trypsin-EDTA (Sigma-Aldrich, Poole, UK) and re-suspending them with 150 μ L
220 Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, Poole, UK). Cells were acquired
221 with a BD FACSAarray flow cytometer (BD Biosciences, Oxford, UK) and analyzed with FlowJo
222 software v. 8.8.3 (Tree Star Inc., Ashland, Oregon, USA).

223

224 ***2.9 Flow cytometry analysis***

225 After 4 h or 24 h of transfection with different nanocomplexes, the Neuro-2A cells were
226 washed with PBS twice and then trypsinized and re-suspended in culture medium in a 96-well
227 plate. The uptake of the siRNA-Dy677 or DNA-Cy3 by cells in each well was analyzed using
228 BD FACSCalibur™. Non-transfected cells were used to set the negative control gate. Acquired
229 data were analyzed using FlowJo software v. 8.8.3 (Tree Star Inc., Ashland, Oregon, USA) to
230 determine the percentage of the Dy677-positive or Cy3-positive cells in each treatment group.

231

232 ***2.10 In-cell Western analysis***

233 After 4, 24 and 48 h post transfection of Neuro-2A cells with different siRNA-Dy677
234 nanocomplexes, the 96-well plate was washed twice with PBS and scanned by the Odyssey Clx
235 infrared imaging system (LI-COR Biosciences, Cambridge, UK) and the intensity of the 700 nm
236 fluorescent channel for each well was determined using image studio software 3.1.4.

237

238 ***2.11 Viable cell assay***

239 Viable cell assay was assessed in 96-well plates using the CellTiter 96 Aqueous One Solution
240 Cell Proliferation Assay (Promega, Southampton, UK). Neuro-2A cells were seeded and
241 transfected as above. After 24 h the medium was substituted for a growth medium containing 20
242 μ L of the CellTiter 96 Aqueous One Solution reagent. Finally, after incubation for 2 h, the
243 absorbance at 490 nm was measured on a FLUOstar Optima spectrophotometer (BMG Labtech,
244 Aylesbury, UK). Viable cells for each formulation treatment were expressed as a percentage of
245 the viable control cells.

246

247 ***2.12 Complement Activation assays***

248 Details for human serum preparation, characterization and functional assessment of

249 complement pathways were described previously [39-41]. To measure complement activation *in*
250 *vitro*, we determined nanocomplex-induced rise of serum complement activation products C5a
251 and sC5b-9 using respective ELISA kits (Quidel, San Diego, USA) according to the
252 manufacturer's protocol as described earlier [39-41]. Complement activation was initiated by
253 adding the appropriate quantities of nanocomplexes (in 10 μ L) to undiluted human serum (40
254 μ L) in Eppendorf tubes in a shaking water bath at 37°C for 30 min. Reactions were terminated
255 by addition of ice-cold sample-diluent provided in the assay kit containing 25mM EDTA.
256 Nanocomplexes were removed by centrifugation, and complement activation products were
257 measured in ELISA kits. Control serum incubations contained buffers that were used for
258 liposome suspension. Zymosan was prepared as described before [41] and was used as a positive
259 control for generating C5a and sC5b-9 at a concentration of 0.2 mg/mL.

260

261

262 **2.13 *In vivo* experiments**

263 Female C57Bl6 mice were purchased from Charles River (Margate, UK). All procedures
264 were approved by UCL animal care policies and were carried out under Home Office Licenses
265 issued in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (UK).
266 DOTMA/DOPE lipopolyplexes and DOTMA/DOPE_{ves} nanovesicle complexes were prepared at
267 a weight ratio of 1:4:1 (lipid: peptide: DNA) as described previously [18] at a final plasmid DNA
268 concentration of 0.29 mg/mL. 6-week old female C57Bl6 mice were instilled oropharyngeally
269 with nanocomplexes in 55 μ L (made in 5% glucose, v/v) containing 16 μ g pCI-Luc, with
270 untreated mice used as controls. 24 h following instillation, the mice were culled and their lungs
271 extracted and snap frozen. Lungs were defrosted on ice, submerged in reporter gene assay lysis
272 buffer (Roche, Basel, Switzerland), homogenized with a Precellys24 tissue homogenizer
273 (Stretton Scientific, Stretton, Derbyshire, UK) and then centrifuged at 14,170g for 10 min at 4°C.
274 The supernatant was removed and centrifuged for a further 10 min at 4°C and then used in
275 luciferase assays. Results were expressed as relative luminescence units per milligram of protein
276 (RLU/mg).

277 Female NOD-SCID gamma (NSG) mice (Charles River, Margate, UK), 6 to 8 week old,
278 were injected subcutaneously in the right posterior flank with 3×10^6 human neuroblastoma
279 Kelly cells. After approximately 2 weeks, when tumours had reached 8–10 mm in size, 100 μ L

280 of RTN complexes made in 5% (v/v) glucose and containing 16 µg of siRNA-Dy677 were
281 injected into the lateral tail vein. Experiments were performed with replicates of 3 mice. 24 hours
282 after injection, the mice were killed and tumours and organs (lung, liver, heart, kidneys and
283 spleen) were resected and imaged using an IVIS Lumina Series III imaging system
284 (PerkinElmer, Seer Green, UK). The images were processed using the Living Image software
285 (PerkinElmer, Seer Green, UK). The tumours were then placed in 4% (w/v) paraformaldehyde
286 (PFA) for 3 h followed by overnight incubation in 15% (w/v) sucrose/PBS and then a brief wash
287 in 50% (v/v) ethanol and stored briefly in 70% (v/v) ethanol till ready for dissection.

288

289 ***2.14 Preparation of frozen tissue sections***

290 Freshly dissected tissue was placed onto a pre-labelled tissue base mold. Tissue block was
291 covered with cryo-embedding media OCT (Leica microsystems, Milton Keynes, UK). Base mold
292 containing tissue block was snap frozen in isopentane (VWR International, Lutterworth, UK),
293 pre-chilled in liquid nitrogen and then transferred to a cryotome cryostat, which was pre cooled
294 to -20⁰C. 10 µm tissue sections were prepared using the cryotome and mounted on the Superfrost
295 Plus glass slides (Fisher Scientific UK, Loughborough, UK). The sections were dried at RT and
296 then stored at -80⁰C until utilized.

297

298 ***2.15 Staining of frozen sections***

299 Tissue sections were rinsed in PBS briefly to remove any media components and fixed in
300 pre-cooled (-20⁰C) acetone for 10-15 min. Next, tissue sections were rinsed three times in PBS
301 and stained with DAPI for 15 min at RT in dark. Finally, tissue sections were washed three times
302 in PBS and sections were mounted using ProLong® Gold antifade mountant (Thermo
303 Fisher Scientific, Hemel Hempstead, UK). Micrographs were taken using Leica upright
304 fluorescence microscope (Leica DFC310 FX) at 200x magnification.

305

306 ***2.16 Statistical analysis***

307 The data presented in this study are expressed as the mean ± standard deviation (SD) and
308 were analyzed using a two-tailed, unpaired Student's *t*-test or one-way analysis of variance and
309 Bonferroni's post hoc analysis, where applicable.

310

311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341

3. Results

3.1. Biophysical characterization. The sizes and zeta potentials (Fig. 1A and Fig. S1) of nanovesicles, cationic liposomes, nanovesicle complexes and lipopolyplexes were determined first. Nanovesicles were considerably larger than their liposomal counterparts (557.0 ± 82.5 nm vs 76.1 ± 0.9 nm, respectively) consisting of two particle populations (Fig. S1) and had comparable average zeta potential values (66.9 ± 1.7 mV for nanovesicles vs 70.9 ± 2.4 mV for liposomes, respectively). These nanovesicles were capable of forming complexes with peptide and nucleic acid (DNA or siRNA) of 89.6 ± 1.3 nm, when mixed with DNA, or under 140 nm, when mixed with siRNA, respectively (Fig. 1A and Fig. S1). There was no statistical difference in the size of the nanocomplexes, however, LYD_{ves} had the least cationic surface charge among all nanocomplexes (zeta potential of 26.2 ± 0.4 mV for LYD_{ves} vs 47.7 ± 3.0 mV for LYD).

Negative staining transmission electron microscopy (TEM) was used to further characterize nanovesicles (Fig. 1B) and nanovesicle complexes (LYD_{ves}; Fig. 1C). Nanovesicles and nanovesicle complexes were predominantly spherical, however, some rod-shaped objects were also present (in LYD_{ves}; Fig. 1C). The majority of the spherical entities observed by TEM for each formulation were in similar size ranges determined by dynamic light scattering (DLS). DOTMA/DOPE liposomes and LYD lipopolyplexes were also visualized (Fig. S2) and they formed some discrete spherical particles with most being aggregated in clusters. These clusters may have been generated during sample preparation and dehydration processes for TEM.

The ability of nanocomplexes to package DNA efficiently and to dissociate following heparin challenge was assessed (Fig. 1D). PicoGreen-labelled DNA was formulated into cationic LYD and LYD_{ves}. Packaging was inferred from fluorescence quenching compared with free DNA as 100%. The packaging efficiency refers to the extent of nucleic acid protection. Therefore, the higher the packaging efficiency, the better the protection of the nucleic acid cargo. Both formulations resulted in high packaging efficiency: 81% for LYD lipopolyplexes compared with 94% for LYD_{ves} nanovesicle complexes. In addition, they had a different heparin release profile. LYD achieved 50% dissociation at 0.41 U/mL heparin, whereas LYD_{ves} achieved 50% dissociation at 0.84 U/mL heparin, thus making the latter less responsive to polyanions.

3.2. Cellular uptake and targeting specificity of siRNA-containing nanocomplexes. Next, we

342 determined cellular uptake of Dy677-labelled siRNA nanocomplexes following transfection of
343 Neuro-2A cells in comparison with liposomes. The following complexes were used: nanovesicle
344 complexes made from fresh nanovesicles and incorporating targeting peptides (LYR_{ves-new} and
345 LMER_{ves-new}) or a non-targeting peptide (LK16R_{ves-new}), nanovesicle complexes made with
346 nanovesicles previously stored for 1 year at 4°C and incorporating targeting peptides (LYR_{ves-old1}
347 and LMER_{ves-old1}), lipopolyplexes with targeting peptides (LYR and LMER) or non-targeting
348 peptide (LK16R), peptide/siRNA complexes (Y/ siRNA-Dy677 and ME27/ siRNA-Dy677),
349 liposome/siRNA (LR) or nanovesicle/siRNA (LR_{ves-new}). Two complementary methods were
350 used for analysis: in-cell Western analysis of the siRNA-Dy677 uptake (Fig. 2A-B) and
351 measurement of the fluorescent intensity of the Dy677 (which reflects the uptake level of the
352 siRNA; Fig. 2C). The results show a time-dependent uptake of nanocomplexes. In particular,
353 lipopolyplexes had a higher and statistically significant uptake than their respective nanovesicle
354 complex counterparts at 4 h (LYR_{ves} uptake was 35.4% vs 53.8% for LYR, $p < 0.001$), but this
355 was not significant at later time points. Similar observations were made with formulations
356 incorporating the targeting peptide ME27. The nanocomplexes that were made with nanovesicles
357 stored for one year in the fridge had approximately a 2.5-fold increased size (215-240 nm)
358 compared with their fresh counterparts and this might explain why they showed less uptake than
359 freshly made counterparts. Other comparisons that were significant for all time points were those
360 of LR (liposome/siRNA) or LR_{ves} (nanovesicle/siRNA) formulations, which displayed inferior
361 uptake to lipopolyplexes ($p < 0.001$). Peptide/siRNA (PR) formulations also displayed
362 significantly less cell uptake compared with lipopolyplexes ($p < 0.001$). Particularly, the
363 formulations with the targeted peptides resulted in a much higher uptake than those with non-
364 targeted peptides ($p < 0.001$ for all comparisons), however, this effect was more pronounced with
365 the nanovesicle complexes. For example at 24 h, LYR_{ves} resulted in approximately 10-fold higher
366 uptake than LK16R_{ves}, which was considerably higher than the difference in uptake of LYR,
367 which in turn was 2.6-fold higher than that of LK16R.

368 Flow cytometry analysis (Fig. 2C), showed a similar uptake pattern to that of in-cell
369 Western analysis at 4 h post transfection. The comparisons described above as being significant
370 (Fig. 2A-B) were also significant in Fig. 2C. For example, the targeted formulations resulted in a
371 much higher uptake than their non-targeted counterparts ($p < 0.001$ for all comparisons), however
372 again this effect was more pronounced with the nanovesicle complexes. FACS analysis was also

373 performed to investigate the uptake of Cy3-labelled DNA nanocomplexes in Neuro-2A cells.
374 The trend was the same as the one found for siRNA uptake; again there was no statistical
375 difference between nanovesicle complexes and lipopolyplexes at 24 h and the use of targeting
376 peptides resulted in higher uptake than non-targeting formulations (Fig. S3). However, PR and
377 peptide/DNA (PD) complexes showed a significantly different nucleic acid uptake profile. For
378 example at 4 h post-transfection, PD complexes (Fig. S3) achieved 22.5% uptake, which was
379 significantly more than the 4.1% seen with the PR formulations (Fig. 2C; $p < 0.05$).

380

381 **3.3. *In vitro* transfection efficiencies.** Nanocomplexes were formulated and used for
382 transfection of Neuro-2A and HBE cells (Fig. 3). LMED formulations were significantly better
383 in transfection than the LMED_{ves} ($p < 0.05$) in Neuro-2A cells (Fig. 3A), however, this difference
384 was not statistically significant for LYD and LYD_{ves} nanocomplexes in HBE cells (Fig. 3B).
385 Importantly, the receptor-targeted formulations showed considerable differences in transfection
386 efficiency compared with the non-targeted formulations. Targeted lipopolyplexes LMED
387 (Neuro-2A cells) and LYD (HBE cells) resulted in a 3.6-fold and a 4.3-fold enhancement of
388 transfection compared with non-targeting LK16D ($p < 0.001$ for both Neuro-2A and HBE cells),
389 whereas the vesicular targeted formulations LMED_{ves} (Neuro-2A cells) and LYD_{ves} (HBE cells)
390 resulted in a 10.5-fold and 8.9-fold ($p < 0.001$ for both Neuro-2A and HBE cells) increase in
391 transfection efficiency compared with non-targeting LK16D_{ves}, respectively.

392 The transfection efficiency was further evaluated with the plasmid expressing enhanced
393 green fluorescent protein (GFP) in Neuro-2A cells 48 h after transfection. Fluorescent
394 microscopy images of LMED_{ves} (Fig. 3C) and LMED (Fig. 3D) nanocomplexes provided
395 evidence of the high transfection efficiency of both formulations. Flow cytometry analysis of
396 GFP transfections was then performed (Fig. 4) and showed that $28.6 \pm 1.9\%$ and $33.5 \pm 2.2\%$ of
397 cells expressed GFP following transfection with LMED_{ves} and LMED, respectively ($p < 0.05$).

398

399 **3.4. Complement activation assay and cell viability.** We challenged undiluted human
400 serum with LYD and LYD_{ves} formulations and measured the two pathway-independent soluble
401 end-point complement activation products C5a and sC5b-9, respectively [39, 42]. The
402 complement system is a key effector of both innate and cognate immunity recognizing danger
403 signals through pattern recognition [43]. C5a is an anaphylatoxin and chemoattractive agent,

404 whereas soluble sC5b-9 is a measure of whole complement activation. The results in Fig. 5A
405 show that neither formulations elevated sC5b-9 levels above the background compared on the
406 basis of equivalent surface area. On the other hand, both formulations caused very small
407 increases of serum C5a levels (Fig. 5B). For comparison zymosan treatment induced massive
408 rises of C5a and sC5b-9 levels above background (201.3 ± 10.1 ng/mL C5a and 31.7 ± 1.6
409 $\mu\text{g/mL}$ sC5b-9, respectively). On the basis of our findings, our formulations were poor activators
410 of the complement system and could be used for intravenous applications. Indeed, the extent of
411 complement activation by these preparations was considerably lower than PEGylated regulatory
412 approved liposomes (Doxil®) on equivalent surface area (46 cm^2) basis (8560 ± 108.1 ng/mL
413 sC5b-9) [44].

414 The cell viability assessment showed no particular differences between nanocomplexes,
415 which included lipopolyplexes and nanovesicle complexes (Fig. 5C). However, the cationic
416 liposomes DOTMA/DOPE (from either a fresh or an older batch) were significantly more
417 cytotoxic (65% viable cells) compared with untreated controls ($p < 0.001$ for both batches). On
418 the contrary, all the batches of the nanovesicles DOTMA/DOPE_{ves} did not induce any apparent
419 cytotoxicity.

420
421 **3.5. *In vivo* lung delivery and tumour distribution.** We further determined whether the *in vitro*
422 results translate to *in vivo* performance. Firstly, LED (size: 95.3 ± 1.4 nm; ζ potential 54.5 ± 1.3
423 mV) and LED_{ves} (size: 96.3 ± 0.5 nm; ζ potential 43.5 ± 0.6 mV) nanocomplexes were delivered
424 to the airways of murine lungs (Fig. 6). 24 h after administration, luciferase assay was performed
425 on lung extracts. The mean luciferase expression from LED_{ves} was higher than that of LED (6160
426 RLU/mg protein for LED_{ves} vs 4596 RLU/mg protein for LED), but this was not statistically
427 significant.

428 Finally, we investigated whether LMER_{ves} nanocomplexes can be delivered to tumours
429 following systemic administration in xenograft mouse models of neuroblastoma. 24 h after
430 intravenous administration, the organs and the tumours were removed and imaged using the IVIS
431 III system for siRNA-Dy677 distribution. The LMER_{ves} nanocomplexes showed high retention in
432 tumours (17.5% of the initial injected dose; the radiant efficiency of the initial dose was
433 measured at 1.9×10^{10} photons $\text{s}^{-1} \text{ cm}^{-2}$ steradian $^{-1}$ per $\mu\text{W cm}^{-2}$), while leaving other normal
434 tissues with extremely low (heart, liver, kidneys and spleen) or moderate uptake (lung; 5.2% of

435 the initial injected dose) (Fig. 7A-B). The fluorescent radiant efficiency was 3.39-fold higher in
436 tumours than in the lungs (Fig. S4, $p < 0.01$). Immunostaining of the tumours (Fig. 7C-H)
437 revealed that the siRNA-Dy677 was strongly present throughout the tumour mass and the
438 staining was very intense in mice injected with targeted LMER_{ves}, whereas the intracellular
439 fluorescence signals were not detected in the tumour tissues collected from control untreated
440 mice.

441

442

443 **4. Discussion**

444 In this study, we replaced the liposomal component of lipopolyplexes, which was derived
445 through sonication of multi-lamellar vesicles [8, 15-18, 45], with GUVs as initial templates for
446 vector assembly (nanovesicles and nanovesicle complexes). Following further processing, these
447 preparations were characterized and compared with sonicated liposomes. These modifications
448 resulted in formation of large unilamellar vesicles, with the majority being less than 1 μm . The
449 engineered vesicles comprise of a bilayer that isolates the aqueous lumen of the intermediate
450 vesicles loaded with sucrose from the external hosting glucose solution [19, 46, 47]. When
451 complexed with peptide ligands and nucleic acids, we were able to produce nanovesicle
452 complexes that were less than 140 nm. The electrostatic forces involved between nanovesicles,
453 peptides and nucleic acids most likely play a role in compaction processes and, hence, the
454 observed reduction in size of the nanovesicle complexes compared with native nanovesicles.
455 Indeed, the size of the nanocomplexes should preferentially be less than 200 nm to allow for
456 efficient internalization through different endocytic processes as well as for tumour targeting
457 [48].

458 While extracellular stability is an essential requirement for formulation of an efficient
459 nucleic acid delivery system, effective transfection is also influenced by the extent of cargo
460 release intracellularly. For instance, cationic lipopolyplexes may interact favourably with actin
461 during internalization causing destabilization and partial release of nucleic acids directly into the
462 cytoplasm [49, 50]. To compare particle stability and nucleic acid dissociation, nanocomplexes
463 were incubated with heparin, which mimics actin [9, 49]. Both types of nanocomplexes achieved
464 approximately 65% maximum release at a heparin concentration of 2 U/mL, thus suggesting
465 their suitability for nucleic acid delivery and release.

466 Next we compared cell uptake of the engineered formulations. The higher positive charge of
467 the lipopolyplexes compared with nanovesicles could explain the differences in cell uptake,
468 either through better plasma membrane destabilization (causing direct nucleic acid release into
469 the cytoplasm) and/or improved interaction with anionic components of cell surface
470 proteoglycans [51]. Furthermore, it has been shown that shape is an important factor in cellular
471 uptake and that rods may enter cells more readily than spheres under static conditions and
472 particularly from a side-on mode of contact with plasma membrane [52, 53]. Indeed, nanovesicle
473 particles were mostly spherical in shape, whereas lipopolyplexes contained a high number of
474 rods and torroids [9, 16, 45, 54] as well as particle clusters, which could explain their higher
475 uptake. Our results further showed that the nanocomplexes that were made with nanovesicles
476 after prolonged storage had larger size and reduced cell uptake. It is likely that fusion processes
477 may have caused vesicular destabilization and partial release of complexed nucleic acids prior to
478 cell incubation.

479 The initial barriers to transfection are cell binding and uptake [54, 55]. Differences in
480 efficiencies of these two processes could potentially explain the different transfection
481 efficiencies of the nanocomplexes. LPD complexes had similar biophysical characteristics with
482 LPD_{ves}, but displayed improved cellular uptake and this may explain the differences in their
483 transfection efficiencies, providing that cell viability is not compromised. Our results show that
484 plasmid DNA in LPD_{ves} nanovesicle complexes is more tightly packaged (94%) than in LPD
485 lipopolyplexes (81%; $p < 0.001$), however, DNA was more easily released from LPD
486 lipopolyplexes than from LPD_{ves} nanovesicle complexes. Thus LPD having a greater cell uptake,
487 while resulting in better DNA release within the cell, achieves the greater transfection
488 efficiencies observed. The targeted formulations were more efficient than their non-targeting
489 counterparts due to the presence of the targeting peptide, which resulted in a higher uptake in the
490 cells as corroborated in Fig. 2.

491 Although nanovesicle complexes did not show advantages in transfection and cell uptake
492 compared with lipopolyplexes, they were considerably less cytotoxic than their liposomal
493 counterparts. This is most likely due to their lesser cationic charge. In addition, cells may use the
494 glucose that was present in the nanovesicle complexes (e.g., DOTMA/DOPE_{ves}) to maintain
495 oxidative phosphorylation and ATP synthesis. It is well documented [56, 57] that cationic
496 liposomes are cytotoxic and this could explain the reduced cell viability observed in our assay

497 for DOTMA/DOPE. The LPD lipopolyplexes were also found to be significantly less cytotoxic
498 than the cationic DOTMA/DOPE liposome, which implies that DNA or siRNA may have
499 sequestered the cationic lipid reducing its damaging effect on cells.

500 Our *in vivo* studies further showed the suitability of LED_{ves} in nucleic acid delivery. For
501 instance, following oropharyngeal administration nanovesicle complexes were more effective in
502 nucleic acid delivery and transfection than lipopolyplexes (LED). This observation contrasted the
503 *in vitro* findings where the lipopolyplexes showed superiority. We have previously shown that
504 nanocomplexes target mainly the airway epithelia [18], thus a plausible explanation for these
505 differences may arise from a relatively higher destabilization of LED at the apical surface of the
506 lung cells compared with the sugar-containing LED_{ves}. As for tumour targeting, we used a near-
507 infrared fluorescent probe, Dy677, which results in low autofluorescence and scattering of light
508 and enables good tissue penetration of light, which is ideal for *in vivo* imaging [58]. Our
509 nanocomplexes, following intravenous administration, were mainly localized to tumours and
510 showed less deposition to the lungs. Others have also shown that at 24 h following intravenous
511 administration, cationic nanoparticles coupled to $\alpha_v\beta_3$ ligands were mainly expressing luciferase
512 in tumours with minimal activity detected in the lung and none in other organs [59]. This
513 distribution pattern is very important as the nanocomplexes were able to largely avoid the
514 reticuloendothelial system (RES), which is the major clearance mechanism of nanoparticles from
515 the circulation [60]. This might be attributed to the targeting peptide utilized here and to the
516 enhanced permeation and retention effect (EPR) and the leakiness of the tumour neovasculature
517 in our *in vivo* model [61]. Another contributing factor may be poor complement opsonization of
518 the engineered nanocomplexes and hence their poor recognition by macrophages of the RES,
519 thereby allowing more nanoparticles to reach tumours. These results collectively indicate that the
520 targeted nanovesicle complexes could efficiently deliver siRNA to the tumour tissue, and thus
521 might have potential applications in therapeutic oncology. Finally, considering the high level of
522 nanovesicle complex accumulation in tumour interstitium, their poor complement activating
523 nature is clinically advantageous. Indeed, intratumoural complement activation has been
524 suggested to accelerate tumour growth [62, 63].

525

526

527 **5. Conclusion**

528 Giant liposomes have been used for biophysical investigations, namely the interaction of
529 cytoskeleton proteins with membranes, the dynamic structures of biomembranes and the change
530 of liposomal shapes [64-66]. These vesicles have the advantage over traditional smaller
531 liposomal preparations of being easier to prepare in small quantities and by high throughput
532 procedures [19, 23, 26, 47, 67, 68]. Here, we initially modified the procedure of making GUVs
533 that resulted in nanovesicles of less than 1 μm in size and then for the first time, we reported the
534 development of nanovesicle complexes using these nanovesicles (derived from GUV
535 precursors). These engineered vesicles exhibited good transfection efficiency, however, unlike
536 conventional cationic lipoplexes, nanovesicles and nanovesicle complexes neither exhibited
537 considerable cytotoxicity nor activated the complement system. These observations are of
538 importance, since nanovesicle complexes were able to deliver nucleic acids to both lung and
539 tumour tissues *in vivo*. Nanovesicle complexes therefore represent a promising tool for
540 improving our arsenal of safer non-viral vectors for site-specific delivery of therapeutic nucleic
541 acids.

542

543 **Acknowledgement**

544 We would like to thank the Maeshima Medical Office, Action Medical Research and
545 Cystic Fibrosis Trust for funding this work. This work was further supported by the National
546 Institute for Health Research Biomedical Research Centre at Great Ormond Street Hospital for
547 Children NHS Foundation Trust and University College London. We would like to thank Dr
548 Boenzli for helpful discussions and for proofreading the manuscript.

549

550 **References**

551 [1] N. Pardi, S. Tuyishime, H. Muramatsu, K. Kariko, B. L. Mui, Y. K. Tam, T. D. Madden, M.
552 J. Hope, D. Weissman. Expression kinetics of nucleoside-modified mRNA delivered in lipid
553 nanoparticles to mice by various routes, *J. Control. Release* 217 (2015) 345-351.

554 [2] S. C. Semple, A. Akinc, J. X. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Y. Sah, D.
555 Stebbing, E. J. Crosley, E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K.
556 F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M.
557 Srinivasulu, M. J. Weinstein, Q. M. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T.
558 Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A.

559 Tracy, A. de Fougerolles, I. MacLachlan, P. R. Cullis, T. D. Madden, M. J. Hope. Rational
560 design of cationic lipids for siRNA delivery, *Nat. Biotechnol.* 28 (2010) 172-176.

561 [3] A. D. Tagalakis, I. A. Diakonov, I. R. Graham, K. A. Heald, J. D. Harris, J. V. Mulcahy, G.
562 Dickson, J. S. Owen. Apolipoprotein E delivery by peritoneal implantation of encapsulated
563 recombinant cells improves the hyperlipidaemic profile in apoE-deficient mice, *BBA-Mol. Cell.*
564 *Biol. L.* 1686 (2005) 190-199.

565 [4] H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin, D. G. Anderson. Non-viral
566 vectors for gene-based therapy, *Nat. Rev. Genet.* 15 (2014) 541-555.

567 [5] C. Sheridan. Gene therapy finds its niche, *Nat. Biotechnol.* 29 (2011) 121-128.

568 [6] Y. Zhang, A. Satterlee, L. Huang. In vivo gene delivery by nonviral vectors: overcoming
569 hurdles?, *Mol. Ther.* 20 (2012) 1298-1304.

570 [7] A. Kwok, D. McCarthy, S. L. Hart, A. D. Tagalakis. Systematic Comparisons of
571 Formulations of Linear Oligolysine Peptides with siRNA and Plasmid DNA, *Chem. Biol. Drug*
572 *Des.* 87 (2016) 747-763.

573 [8] A. D. Tagalakis, L. He, L. Saraiva, K. T. Gustafsson, S. L. Hart. Receptor-targeted liposome-
574 peptide nanocomplexes for siRNA delivery, *Biomaterials* 32 (2011) 6302-6315.

575 [9] A. D. Tagalakis, L. Saraiva, D. McCarthy, K. T. Gustafsson, S. L. Hart. Comparison of
576 Nanocomplexes with Branched and Linear Peptides for SiRNA Delivery, *Biomacromolecules* 14
577 (2013) 761-770.

578 [10] A. Weng, M. D. I. Manunta, M. Thakur, R. Gilibert-Oriol, A. D. Tagalakis, A. Eddaoudi,
579 M. M. Munye, C. A. Vink, B. Wiesner, J. Eichhorst, M. F. Melzig, S. L. Hart. Improved
580 intracellular delivery of peptide- and lipid-nanoplexes by natural glycosides, *J. Control. Release*
581 206 (2015) 75-90.

582 [11] C. Yu-Wai-Man, A. D. Tagalakis, M. D. Manunta, S. L. Hart, P. T. Khaw. Receptor-
583 targeted liposome-peptide-siRNA nanoparticles represent an efficient delivery system for MRTF
584 silencing in conjunctival fibrosis, *Sci. Rep.* 6 (2016) 21881.

585 [12] G. D. Kenny, C. Villegas-Llerena, A. D. Tagalakis, F. Campbell, K. Welsler, M. Botta, A. B.
586 Tabor, H. C. Hailes, M. F. Lythgoe, S. L. Hart. Multifunctional receptor-targeted nanocomplexes
587 for magnetic resonance imaging and transfection of tumours, *Biomaterials* 33 (2012) 7241-7250.

588 [13] Q. H. Meng, S. Irvine, A. D. Tagalakis, R. J. McNulty, J. R. McEwan, S. L. Hart.
589 Inhibition of neointimal hyperplasia in a rabbit vein graft model following non-viral transfection

590 with human iNOS cDNA, *Gene Ther.* 20 (2013) 979-986.

591 [14] M. M. Munye, A. D. Tagalakis, J. L. Barnes, R. E. Brown, R. J. McAnulty, S. J. Howe, S.
592 L. Hart. Minicircle DNA Provides Enhanced and Prolonged Transgene Expression Following
593 Airway Gene Transfer, *Sci. Rep.* 6 (2016) 23125.

594 [15] A. D. Tagalakis, S. M. Grosse, Q. H. Meng, M. F. M. Mustapa, A. Kwok, S. E. Salehi, A. B.
595 Tabor, H. C. Hailes, S. L. Hart. Integrin-targeted nanocomplexes for tumour specific delivery
596 and therapy by systemic administration, *Biomaterials* 32 (2011) 1370-1376.

597 [16] A. D. Tagalakis, G. D. Kenny, A. S. Bienemann, D. McCarthy, M. M. Munye, H. Taylor,
598 M. J. Wyatt, M. F. Lythgoe, E. A. White, S. L. Hart. PEGylation improves the receptor-mediated
599 transfection efficiency of peptide-targeted, self-assembling, anionic nanocomplexes, *J. Control.*
600 *Release* 174 (2014) 177-187.

601 [17] A. D. Tagalakis, D. H. D. Lee, A. S. Bienemann, H. Y. Zhou, M. M. Munye, L. Saraiva, D.
602 McCarthy, Z. X. Du, C. A. Vink, R. Maeshima, E. A. White, K. Gustafsson, S. L. Hart.
603 Multifunctional, self-assembling anionic peptide-lipid nanocomplexes for targeted siRNA
604 delivery, *Biomaterials* 35 (2014) 8406-8415.

605 [18] A. D. Tagalakis, R. J. McAnulty, J. Devaney, S. E. Bottoms, J. B. Wong, M. Elbs, M. J.
606 Writer, H. C. Hailes, A. B. Tabor, C. O'Callaghan, A. Jaffe, S. L. Hart. A receptor-targeted
607 nanocomplex vector system optimized for respiratory gene transfer, *Mol. Ther.* 16 (2008) 907-
608 915.

609 [19] M. Hadorn, E. Boenzli, K. T. Sorensen, D. De Lucrezia, M. M. Hanczyc, T. Yomo. Defined
610 DNA-mediated assemblies of gene-expressing giant unilamellar vesicles, *Langmuir* 29 (2013)
611 15309-15319.

612 [20] S. Pautot, B. J. Frisken, D. A. Weitz. Engineering asymmetric vesicles, *Proc. Natl. Acad.*
613 *Sci. U S A* 100 (2003) 10718-10721.

614 [21] P. A. Beales, J. Nam, T. K. Vanderlick. Specific adhesion between DNA-functionalized
615 "Janus" vesicles: size-limited clusters, *Soft Matter* 7 (2011) 1747-1755.

616 [22] M. Hadorn, E. Boenzli, M. M. Hanczyc. Specific and Reversible DNA-Directed Self-
617 Assembly of Modular Vesicle-Droplet Hybrid Materials, *Langmuir* 32 (2016) 3561-3566.

618 [23] M. Hadorn, P. Eggenberger Hotz. DNA-mediated self-assembly of artificial vesicles, *PLoS*
619 *One* 5 (2010) e9886.

620 [24] L. Parolini, B. M. Moggetti, J. Kotar, E. Eiser, P. Cicuta, L. Di Michele. Volume and

621 porosity thermal regulation in lipid mesophases by coupling mobile ligands to soft membranes,
622 Nat. Commun. 6 (2015) 5948.

623 [25] A. S. Cans, M. Andes-Koback, C. D. Keating. Positioning lipid membrane domains in giant
624 vesicles by micro-organization of aqueous cytoplasm mimic, J. Am. Chem. Soc. 130 (2008)
625 7400-7406.

626 [26] M. Hadorn, E. Boenzli, P. E. Hotz. A quantitative analytical method to test for salt effects
627 on giant unilamellar vesicles, Sci. Rep. 1 (2011) 168.

628 [27] R. Kwok, E. Evans. Thermoelasticity of large lecithin bilayer vesicles, Biophys. J. 35
629 (1981) 637-652.

630 [28] M. Mally, J. Majhenc, S. Svetina, B. Zeks. The response of giant phospholipid vesicles to
631 pore-forming peptide melittin, Biochim. Biophys. Acta 1768 (2007) 1179-1189.

632 [29] C. Mauroy, T. Portet, M. Winterhalder, E. Bellard, M. C. Blache, J. Teissie, A. Zumbusch,
633 M. P. Rols. Giant lipid vesicles under electric field pulses assessed by non invasive imaging,
634 Bioelectrochemistry 87 (2012) 253-259.

635 [30] S. L. Veatch, O. Soubias, S. L. Keller, K. Gawrisch. Critical fluctuations in domain-forming
636 lipid mixtures, Proc. Natl. Acad. Sci. U S A 104 (2007) 17650-17655.

637 [31] Y. Yu, J. A. Vroman, S. C. Bae, S. Granick. Vesicle budding induced by a pore-forming
638 peptide, J. Am. Chem. Soc. 132 (2010) 195-201.

639 [32] J. Zhou, A. L. Loftus, G. Mulley, A. T. Jenkins. A thin film detection/response system for
640 pathogenic bacteria, J. Am. Chem. Soc. 132 (2010) 6566-6570.

641 [33] R. Dimova, S. Aranda, N. Bezlyepkina, V. Nikolov, K. A. Riske, R. Lipowsky. A practical
642 guide to giant vesicles. Probing the membrane nanoregime via optical microscopy, J. Phys.
643 Condens. Matter 18 (2006) S1151-1176.

644 [34] A. Hall, L. Parhamifar, M. K. Lange, K. D. Meyle, M. Sanderhoff, H. Andersen, M.
645 Roursgaard, A. K. Larsen, P. B. Jensen, C. Christensen, J. Bartek, S. M. Moghimi.
646 Polyethylenimine architecture-dependent metabolic imprints and perturbation of cellular redox
647 homeostasis, Biochim. Biophys. Acta 1847 (2015) 328-342.

648 [35] L. R. Montes, A. Alonso, F. M. Goni, L. A. Bagatolli. Giant unilamellar vesicles
649 electroformed from native membranes and organic lipid mixtures under physiological conditions,
650 Biophys. J. 93 (2007) 3548-3554.

651 [36] M. J. Writer, B. Marshall, M. A. Pilkington-Miksa, S. E. Barker, M. Jacobsen, A. Kritz, P.

652 C. Bell, D. H. Lester, A. B. Tabor, H. C. Hailes, N. Klein, S. L. Hart. Targeted gene delivery to
653 human airway epithelial cells with synthetic vectors incorporating novel targeting peptides
654 selected by phage display, *J. Drug Target.* 12 (2004) 185-193.

655 [37] J. Bella, P. R. Kolatkar, C. W. Marlor, J. M. Greve, M. G. Rossmann. The structure of the
656 two amino-terminal domains of human ICAM-1 suggests how it functions as a rhinovirus
657 receptor and as an LFA-1 integrin ligand, *Proc. Natl. Acad. Sci. U S A* 95 (1998) 4140-4145.

658 [38] S. C. Chan, D. K. Shum, G. L. Tipoe, J. C. Mak, E. T. Leung, M. S. Ip. Upregulation of
659 ICAM-1 expression in bronchial epithelial cells by airway secretions in bronchiectasis, *Respir.*
660 *Med.* 102 (2008) 287-298.

661 [39] S. M. Moghimi, I. Hamad, T. L. Andresen, K. Jorgensen, J. Szebeni. Methylation of the
662 phosphate oxygen moiety of phospholipid-methoxy(polyethylene glycol) conjugate prevents
663 PEGylated liposome-mediated complement activation and anaphylatoxin production, *FASEB J.*
664 20 (2006) 2591-2593.

665 [40] A. J. Andersen, J. T. Robinson, H. Dai, A. C. Hunter, T. L. Andresen, S. M. Moghimi.
666 Single-walled carbon nanotube surface control of complement recognition and activation, *ACS*
667 *Nano* 7 (2013) 1108-1119.

668 [41] N. K. Banda, G. Mehta, Y. Chao, G. K. Wang, S. Inturi, L. Fossati-Jimack, M. Botto, L. P.
669 Wu, S. M. Moghimi, D. Simberg. Mechanisms of complement activation by dextran-coated
670 superparamagnetic iron oxide (SPIO) nanoworms in mouse versus human serum, *Part. Fibre*
671 *Toxicol.* 11 (2014)64.

672 [42] I. D. Azmi, L. Wu, P. P. Wibroe, C. Nilsson, J. Ostergaard, S. Sturup, B. Gammelgaard, A.
673 Urtti, S. M. Moghimi, A. Yaghmur. Modulatory effect of human plasma on the internal
674 nanostructure and size characteristics of liquid-crystalline nanocarriers, *Langmuir* 31 (2015)
675 5042-5049.

676 [43] S. M. Moghimi, A. J. Andersen, D. Ahmadvand, P. P. Wibroe, T. L. Andresen, A. C.
677 Hunter. Material properties in complement activation, *Adv. Drug Deliv. Rev.* 63 (2011) 1000-
678 1007.

679 [44] P. P. Wibroe, D. Ahmadvand, M. A. Oghabian, A. Yaghmur, S. M. Moghimi. An integrated
680 assessment of morphology, size, and complement activation of the PEGylated liposomal
681 doxorubicin products Doxil(R), Caelyx(R), DOXOrubicin, and SinaDoxosome, *J. Control.*
682 *Release* 221 (2016) 1-8.

683 [45] A. D. Tagalakis, S. Castellaro, H. Y. Zhou, A. Bienemann, M. M. Munye, D. McCarthy, E.
684 A. White, S. L. Hart. A method for concentrating lipid peptide DNA and siRNA nanocomplexes
685 that retains their structure and transfection efficiency, *Int. J. Nanomed.* 10 (2015) 2673-2683.

686 [46] T. Bhatia, P. Husen, J. Brewer, L. A. Bagatolli, P. L. Hansen, J. H. Ipsen, O. G. Mouritsen.
687 Preparing giant unilamellar vesicles (GUVs) of complex lipid mixtures on demand: Mixing small
688 unilamellar vesicles of compositionally heterogeneous mixtures, *Biochim. Biophys. Acta* 1848
689 (2015) 3175-3180.

690 [47] M. Hadorn, E. Boenzli, P. Eggenberger Hotz, M. M. Hanczyc. Hierarchical unilamellar
691 vesicles of controlled compositional heterogeneity, *PLoS One* 7 (2012) e50156.

692 [48] H. Gao, W. Shi, L. B. Freund. Mechanics of receptor-mediated endocytosis, *Proc. Natl.*
693 *Acad. Sci. U S A* 102 (2005) 9469-9474.

694 [49] M. Mannisto, M. Reinisalo, M. Ruponen, P. Honkakoski, M. Tammi, A. Urtti. Polyplex-
695 mediated gene transfer and cell cycle: effect of carrier on cellular uptake and intracellular
696 kinetics, and significance of glycosaminoglycans, *J. Gene Med.* 9 (2007) 479-487.

697 [50] C. Schweiger, R. Hartmann, F. Zhang, W. J. Parak, T. H. Kissel, P. Rivera Gil.
698 Quantification of the internalization patterns of superparamagnetic iron oxide nanoparticles with
699 opposite charge, *J. Nanobiotechnol.* 10 (2012) 28.

700 [51] X. X. Zhang, T. J. McIntosh, M. W. Grinstaff. Functional lipids and lipoplexes for improved
701 gene delivery. *Biochimie* 2012;94:42-58.

702 [52] S. E. Gratton, P. A. Ropp, P. D. Pohlhaus, J. C. Luft, V. J. Madden, M. E. Napier, J. M.
703 DeSimone. The effect of particle design on cellular internalization pathways, *Proc. Natl. Acad.*
704 *Sci. U S A* 105 (2008) 11613-11618.

705 [53] J. M. Williford, J. L. Santos, R. Shyam, H. Q. Mao. Shape Control in Engineering of
706 Polymeric Nanoparticles for Therapeutic Delivery, *Biomater. Sci.* 3 (2015) 894-907.

707 [54] Z. X. Du, M. M. Munye, A. D. Tagalakis, M. D. I. Manunta, S. L. Hart. The Role of the
708 Helper Lipid on the DNA Transfection Efficiency of Lipopolyplex Formulations, *Sci. Rep.* 4
709 (2014) 7107.

710 [55] M. M. Munye, J. Ravi, A. D. Tagalakis, D. McCarthy, M. G. Ryadnov, S. L. Hart. Role of
711 liposome and peptide in the synergistic enhancement of transfection with a lipopolyplex vector,
712 *Sci. Rep.* 5 (2015) 9292.

713 [56] B. Ballarin-Gonzalez, K. A. Howard. Polycation-based nanoparticle delivery of RNAi

714 therapeutics: adverse effects and solutions, *Adv. Drug Deliv. Rev.* 64 (2012) 1717-1729.

715 [57] K. Romoren, B. J. Thu, N. C. Bols, O. Evensen. Transfection efficiency and cytotoxicity of
716 cationic liposomes in salmonid cell lines of hepatocyte and macrophage origin, *Biochim.*
717 *Biophys. Acta* 1663 (2004) 127-134.

718 [58] X. B. Xiong, A. Lavasanifar. Traceable multifunctional micellar nanocarriers for cancer-
719 targeted co-delivery of MDR-1 siRNA and doxorubicin, *ACS Nano* 5 (2011) 5202-5213.

720 [59] J. D. Hood, M. Bednarski, R. Frausto, S. Guccione, R. A. Reisfeld, R. Xiang, D. A.
721 Cheresh. Tumor regression by targeted gene delivery to the neovasculature, *Science* 296 (2002)
722 2404-2407.

723 [60] S. D. Li, L. Huang. Nanoparticles evading the reticuloendothelial system: role of the
724 supported bilayer, *Biochim. Biophys. Acta* 1788 (2009) 2259-2266.

725 [61] W. L. Monsky, D. Fukumura, T. Gohongi, M. Ancukiewicz, H. A. Weich, V. P. Torchilin, F.
726 Yuan, R. K. Jain. Augmentation of transvascular transport of macromolecules and nanoparticles
727 in tumors using vascular endothelial growth factor, *Cancer Res.* 59 (1999) 4129-4135.

728 [62] M. M. Markiewski, R. A. DeAngelis, F. Benencia, S. K. Ricklin-Lichtsteiner, A.
729 Koutoulaki, C. Gerard, G. Coukos, J. D. Lambris. Modulation of the antitumor immune response
730 by complement, *Nat. Immunol.* 9 (2008) 1225-1235.

731 [63] S. M. Moghimi. Cancer nanomedicine and the complement system activation paradigm:
732 Anaphylaxis and tumour growth, *J. Control. Release* 190 (2014) 556-562.

733 [64] M. Honda, K. Takiguchi, S. Ishikawa, H. Hotani. Morphogenesis of liposomes
734 encapsulating actin depends on the type of actin-crosslinking, *J. Mol. Biol.* 287 (1999) 293-300.

735 [65] J. Kas, E. Sackmann. Shape transitions and shape stability of giant phospholipid vesicles in
736 pure water induced by area-to-volume changes, *Biophys. J.* 60 (1991) 825-844.

737 [66] O. Sandre, L. Moreaux, F. Brochard-Wyart. Dynamics of transient pores in stretched
738 vesicles, *Proc. Natl. Acad. Sci. U S A* 96 (1999) 10591-10596.

739 [67] S. H. C. Askes, N. L. Mora, R. Harkes, R. I. Koning, B. Koster, T. Schmidt, A. Kros, S.
740 Bonnet. Imaging the lipid bilayer of giant unilamellar vesicles using red-to-blue light
741 upconversion, *Chem. Commun.* 51 (2015) 9137-9140.

742 [68] S. Pautot, B. J. Frisken, D. A. Weitz. Production of unilamellar vesicles using an inverted
743 emulsion, *Langmuir* 19 (2003) 2870-2879.

744

745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778

| Name | Components |
|--|--|
| LYD (lipopolyplex) | DOTMA/DOPE/Peptide Y/DNA |
| LED (lipopolyplex) | DOTMA/DOPE/Peptide E/DNA |
| LMED (lipopolyplex) | DOTMA/DOPE/Peptide ME27/DNA |
| LK16D (lipopolyplex) | DOTMA/DOPE/Peptide K16/DNA |
| LYD _{ves} (nanovesicle complex) | DOTMA/DOPE nanovesicle/Peptide Y/DNA |
| LED _{ves} (nanovesicle complex) | DOTMA/DOPE nanovesicle/Peptide E/DNA |
| LMED _{ves} (nanovesicle complex) | DOTMA/DOPE nanovesicle/Peptide ME27/DNA |
| LK16D _{ves} (nanovesicle complex) | DOTMA/DOPE nanovesicle/Peptide K16/DNA |
| LYR (lipopolyplex) | DOTMA/DOPE/Peptide Y/siRNA |
| LMER (lipopolyplex) | DOTMA/DOPE/Peptide ME27/siRNA |
| LYR _{ves} (nanovesicle complex) | DOTMA/DOPE nanovesicle/Peptide Y/siRNA |
| LMER _{ves} (nanovesicle complex) | DOTMA/DOPE nanovesicle/Peptide ME27/siRNA |

779

780 **Table 1.** Terminology of nanocomplexes (lipopolyplexes or nanovesicle complexes).

781

782

783

784

785

786

787

788

789

790

791

792

793

794 **FIGURE LEGENDS**

795 **Fig. 1.** Biophysical characteristics of nanovesicles, liposomes, nanovesicle complexes and
796 lipopolyplexes (A) Size and surface charge measurements of cationic liposomes, nanovesicles,
797 nanovesicle complexes and lipopolyplexes. Particle size was measured by dynamic light
798 scattering. DD=DOTMA/DOPE, LYD=DOTMA/DOPE/Peptide Y/DNA, LYR=
799 DOTMA/DOPE/Peptide Y/siRNA, DD_{ves}= DOTMA/DOPE nanovesicles, LYD_{ves}=
800 DOTMA/DOPE nanovesicle/Peptide Y/DNA and LYR_{ves}= DOTMA/DOPE nanovesicle/Peptide
801 Y/siRNA. (B) Negative staining TEM with 1% (w/v) uranyl acetate was used to visualize
802 DOTMA/DOPE nanovesicles. Scale Bar= 2 μ m. (C) Negative staining TEM with 1% (w/v)
803 uranyl acetate was used to visualize LYD_{ves} nanovesicle complexes. Scale Bar= 500 nm. (D) The
804 dissociation properties of nanocomplexes LYD and LYD_{ves} were investigated. PicoGreen
805 fluorescence of complexes, after incubation with heparin (0-2 U/mL), was expressed as a
806 percentage of relative fluorescence units (RFU) relative to free DNA. All experiments were
807 repeated at least 3 times.

808
809 **Fig. 2.** In-cell Western and flow cytometry analysis of the siRNA-Dy677 uptake of different
810 nanocomplexes. Neuro-2A cells were transfected with different nanocomplexes and 4 h (A) or
811 24 h (B) later the plates were scanned for in-cell Western analysis. The plates are shown in the
812 left panel. The graphs (right panel) show the relative fluorescence units (RFU) of each
813 formulation to that of the naked siRNA, which is set at 1. A1-A6= LYR_{ves-new}, A7-A12= LYR,
814 B1-B6= LMER_{ves-new}, B7-B12= LMER, C1-C6= LYR_{ves-old1}, C7-C12= LMER_{ves-old1}, D1-D6=
815 LK16R, D7-D12= LK16R_{ves-new}, E1-E6= LR, E7-E12= LR_{ves-new}, F1-F6= DOTMA/DOPE_{ves-new},
816 F7-F12= DOTMA/DOPE, G1-G6= peptide Y/siRNA-Dy677, G7-G12= peptide ME27/siRNA-
817 Dy677, H1-H6=siRNA-Dy677, H7-H12= control untransfected cells. Ves-old1 refers to
818 nanovesicles made one year earlier and stored at 4°C and ves-new refers to freshly made
819 nanovesicles. (C) The uptake of siRNA-containing nanocomplexes following transfection of
820 Neuro-2A cells was measured 4 h post-transfection by flow cytometry. In all the graphs each
821 column represents the mean \pm SD from six wells. Asterisks indicate comparison of
822 specific formulations with statistical significance (***, $p < 0.001$).

823
824 **Fig. 3.** *In vitro* transfections of nanovesicle complexes and lipopolyplexes. (A) Nanocomplexes

825 LMED_{ves} and LMED (with targeting peptide ME27) and LK16D_{ves} and LK16D (with non-
826 targeting peptide K16) were used in luciferase transfections in Neuro-2A cells (B)
827 Nanocomplexes LYD_{ves} and LYD (with targeting peptide Y) and LK16D_{ves} and LK16D (with
828 non-targeting peptide K16) were used in luciferase transfections in HBE cells. The cells were
829 assessed for luciferase expression 24 h later. Each column represents the mean \pm SD from six
830 wells, and the experiment was repeated 3 times. Asterisks indicate comparison of
831 specific formulations with statistical significance (*, $p < 0.05$; ***, $p < 0.001$). (B) (C-D) GFP
832 transfection efficiency of the LMED_{ves} and the LMED nanocomplexes. Two formulations,
833 LMED_{ves} nanovesicle complexes (C) and LMED lipopolyplexes (D) were used to transfect
834 Neuro-2A cells. GFP expression was observed by epifluorescence microscopy 48 h later
835 (representative cells are shown in phase-contrast on the left and transfected cells appear green on
836 the right images; Scale Bar = 100 μ m). Peptide ME27 was used for all formulations.

837

838 **Fig. 4.** Flow cytometry analysis of GFP expression in Neuro-2A cells. The intensity of GFP
839 expression was evaluated at 48 h following transfection. (A) Control untransfected cells. (B)
840 Cells transfected with LMED_{ves} nanovesicle complexes. (C) Cells transfected with LMED
841 lipopolyplexes. FL1= fluorescence intensity, SSC= side scatter. Each experiment was performed
842 in triplicate wells.

843

844 **Fig. 5.** Complement activation assays and cell viability show a lack of cytotoxicity. (A)
845 Quantification of complement activation product of sC5b-9 in human serum after incubation
846 with different concentration of nanocomplexes. Blank (PBS) and positive control (200 μ g/mL
847 Zymosan, sC5b-9: 31678 ng/mL serum) were tested during the experiment, as were glucose
848 (used in LYD_{ves}) and water (used in LYD). (B) Quantification of complement activation product
849 of C5a in human serum after incubation with different concentration of nanocomplexes. Blank
850 (PBS) and positive control (200 μ g/mL Zymosan, C5a: 201 ng/mL serum) were tested during the
851 experiment, as were glucose (used in LYD_{ves}) and water (used in LYD). Asterisks indicate
852 comparisons of specific formulations with statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***,
853 $p < 0.001$). (C) Viability of Neuro-2A cells following transfection for 24 h with different
854 nanocomplexes. Viability values were normalized to the untransfected control cells. All
855 transfections were performed in groups of six. Asterisks indicate comparisons of

856 specific formulations to the control untransfected cells with statistical significance (***,
857 $p < 0.001$). DD=DOTMA/DOPE, LYD_{old}=DOTMA/DOPE liposome one year old/peptide
858 Y/DNA, LYD_{new}=DOTMA/DOPE fresh liposome/peptide Y/DNA, LYD_{ves-old1}= DOTMA/DOPE
859 nanovesicles 1 year old/peptide Y/DNA, LYD_{ves-old2}= DOTMA/DOPE nanovesicles 6 months
860 old/peptide Y/DNA, LYD_{ves-new}= DOTMA/DOPE fresh nanovesicle/peptide Y/DNA, LD_{ves-}
861 _{old1}=DOTMA/DOPE nanovesicles 1 year old/DNA, LD_{ves-new}=DOTMA/DOPE fresh
862 nanovesicle/DNA, PD=peptide Y/DNA, LYR_{ves}=DOTMA/DOPE fresh nanovesicle/peptide
863 Y/siRNA.

864

865 **Fig. 6.** Transgene expression following *in vivo* transfections of mice lungs. Luciferase activity in
866 mice lungs was detected 24 h following oropharyngeal instillation of LED_{ves} (nanovesicle
867 complexes) or LED lipopolyplexes. Values are background subtracted and the bar represents
868 mean RLU/mg.

869

870 **Fig. 7.** Tumour uptake of formulations following intravenous administration. 24 h later the mice
871 were culled (n=3 per group) and tumours and organs were extracted and imaged for
872 fluorescence. (A) organs (heart, lung, liver, kidneys, spleen) and tumour of a mouse that received
873 LMER_{ves} (DOTMA/DOPE nanovesicle/peptide ME27/siRNA-Dy677) nanovesicle complexes
874 and (B) mice tumours: control tumour (mouse was not injected) and tumour from a mouse that
875 received LMER_{ves} nanovesicle complexes. The fluorescence signal was also investigated in
876 histological sections of tumours of control mice (C-E) or from mice following tail-vein injections
877 of LMER_{ves} nanovesicle complexes (F-H). The tumours were removed 24 h after the injection
878 and the fluorescence recorded. The cell nuclei were stained with DAPI (blue) and the siRNA-
879 Dy677 in red. (C, F) DAPI staining, (D, G) siRNA-Dy677 and (E, H) merged images. Scale Bar
880 = 50 μ m.

881

882

883

884

885

886

887

888