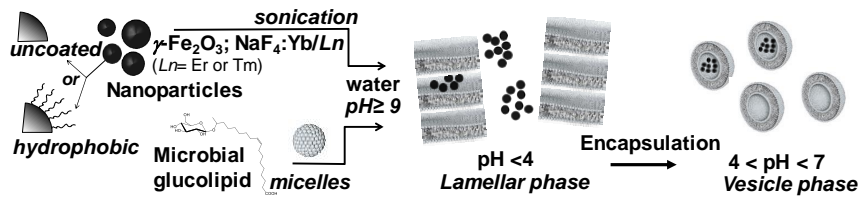


1 **TOC**



2
3 Bulk encapsulation of (coated or hydrophobic) nanoparticle aggregates into vesicles is possible
4 by a simple lamellar-to-vesicle phase transition above pH 4 and using only a pH-responsive
5 microbial glucolipid. Nanoparticle dispersion is assisted by sonication and the encapsulation
6 spontaneously occurs in bulk water, without solvents. The approach does not require the use of
7 phospholipids. The process is developed with magnetic iron oxide nanoparticles to form
8 magnetoliposomes but it is extended to the encapsulation of upconverting nanoparticles with
9 an average encapsulation efficiency of 60%.

10

11 **Easy formation of functional liposomes in water using a**
12 **pH-responsive microbial glycolipid: encapsulation of**
13 **magnetic and up-converting nanoparticles**

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29

30 **Abstract**

31 The compartmentalization of colloids into topologically closed, vesicular, microphases offers
32 an attractive mean to concentrate a functional cargo in aqueous solutions for a range of
33 biomedical, cosmetic, and biotechnological applications. In this paper, we develop a simple,
34 phospholipid-free, phase change method employing a pH-responsive glycolipid. The method is
35 applied to the encapsulation of a sonicated, metastable, aqueous dispersion of functional
36 colloids in the lumen of lipid vesicles: uncoated magnetic maghemite $\gamma\text{-Fe}_2\text{O}_3$ and oleic-acid
37 coated upconverting $\text{NaYF}_4\text{:Yb/Ln}$ (Ln= Er or Tm) nanoparticles (NPs). We find a stable
38 liposomal dispersion containing a sub-population of crowded liposomes with high
39 concentrations of NPs. The encapsulated NPs, formed at nearly neutral pH and room
40 temperature, are stable over time and towards extrusion. The vesicular microphase is entirely
41 composed of pH-responsive glycolipids, which undergo a pH-mediated mesoscopic structural
42 transition from an open lamellar ($2 < \text{pH} < 4$) to topologically closed vesicular state ($\text{pH} > 4$).

43 We also show that encapsulation successfully works with a stable colloidal aqueous dispersion
44 of iron clusters stabilized in ferritin cages. This compartmentalization approach combining self-
45 assembly with an orthogonal nonequilibrium dispersion of nanoparticles shows untapped
46 potential for synthesizing unusual classes of mixed matter.

47

48 **Introduction**

49 Encapsulation of nanoparticles into topologically-closed liposomes represents one of
50 the most valuable advances for therapeutics purposes, because it enables an integration of the
51 properties (e.g., optical, magnetic, and luminescent) of nanoparticles (NPs) with those of
52 liposomes (protection, stability, biocompatibility).¹ Since the early 80s, nanoparticle-loaded
53 liposomes have been explored to probe cell-liposome interactions on gold² and magnetite.^{3,4}
54 More recently, there has been more focus on the the diversity of not only the nature of the
55 encapsulated NPs, like silica and quantum dots,⁵ but also to achieve better encapsulation
56 methods.⁶ The latter allows, for instance, high NPs load uptakes by the liposomes⁷ or the
57 development of stimuli-responsive vectors based on lipids^{8,9} or polymers.^{10,11,12} In the last
58 decade, liposomal encapsulation of iron-loaded ferritin cages has even been associated with the
59 fundamental questions underlying the origins of life.¹³

60 The case of magnetoliposomes, an idea being at least three decades old,^{3,4,14} is
61 particularly interesting. Magnetoliposomes refer to liposomes that contain magnetic NPs
62 (mainly Fe₃O₄ and γ -Fe₂O₃) in their lumen, lipid bilayer or at the vesicle surface,^{1,9,15} and which
63 are particularly interesting for the well-known applications in hyperthermia or as contrast agent
64 in magnetic resonance imaging.¹⁶ Either alone or in combination with drug loading, liposomal
65 protection increases the blood circulating time, bioavailability and delivery.^{1,8,9} The first
66 reported preparation approach to insert NPs into the liposome lumen employed a classical
67 precipitation method:⁴ simple mixing of pre-formed DMPG¹⁷ and DPPG¹⁸ phospholipid
68 liposomes with lauric acid coated Fe₃O₄ particles was shown to precipitate NP-loaded vesicles.
69 Other methods have been employed, such as reverse phase evaporation,^{14,19,20,21} or *in-situ*
70 liposomal precipitation of the NPs.⁶ Thin film hydration, double emulsion methods, or ethanolic
71 injection can also be used.^{13,21,22,23} Recent review papers address the synthesis of
72 magnetoliposomes more extensively.^{1,9,16} Similar encapsulation strategies are employed for
73 other materials such as metal,²⁴ oxides⁵ or luminescent nanoparticles including quantum dots⁵
74 and upconverting lanthanide-doped fluorides.²⁵

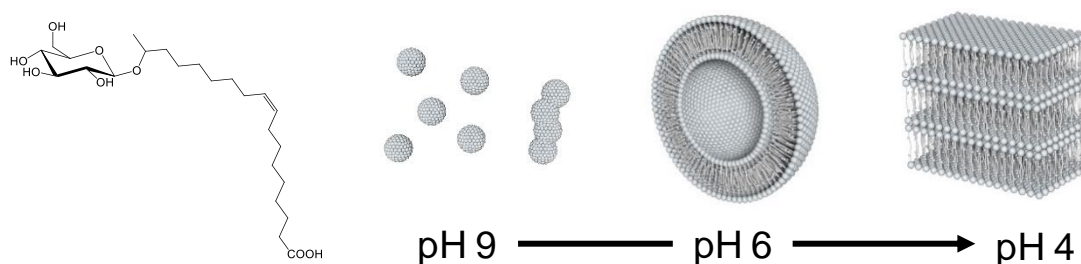
75 Although these methods are valuable, some of them use toxic halogenated solvents (e.g.,
76 chloroform), require elaborate methodologies (e.g., evaporation under vacuum) and in general

77 the use of stable colloids. The latter is an important point. Most, if not all, of these methods
78 require the use of stable colloids in general. In most cases, many nanoparticle systems are
79 passivated by a hydrophobic layer,²⁵ which easily guarantees encapsulation in the vesicle
80 bilayers.^{9,15,16} However, ligand exchange, commonly regarded as a tedious unavoidable step,
81 use of surfactants or hydrophilic coatings, become then necessary for encapsulation in the
82 lumen in water-based media.^{16,21} Moreover, the interaction between the (coated or uncoated)
83 NPs with the phosphate groups of phospholipids may destabilize the bilayer membrane, thereby
84 affecting the vesicle formation mechanism and reducing their stability.^{1,9} It is then interesting
85 to develop an encapsulation water-based method applicable to a broad range of colloids out of
86 thermal equilibrium. Non-equilibrium encapsulation is generally obtained in food and material
87 science through dynamic processes such as spray drying,^{26,27} while recent theoretical work
88 could explore its possibilities in the compartmentalization within elastic geometries like
89 carboxysomes.²⁸

90 Lipid switches^{29,30} were recently combined with classical phospholipid liposomes to
91 deliver a cargo upon application of an external stimulus (pH, ion). In this paper, we take
92 inspiration of this concept to develop a fully water-based (at nearly neutral pH) bulk phase-
93 change method to encapsulate, rather than release, a cargo composed of metastable colloids into
94 vesicles. The latter are compartments entirely free of phospholipids and only composed of pH-
95 responsive lipid switches. Vesicle formation is dynamic, reversible, rapid and entirely
96 controlled through pH. We apply this method to the formation of magnetoliposomes, chosen as
97 model system abundantly described in the literature,^{4,9} from an unstable dispersion of bare
98 (uncoated) maghemite $\gamma\text{-Fe}_2\text{O}_3$ nanoparticle aggregates. We then extend it to the encapsulation
99 of hydrophobic, oleic acid coated, $\text{NaYF}_4\text{:Yb/Ln}$ (Ln= Er or Tm) upconverting nanocrystals.³¹
100 Both systems, otherwise sedimenting/segregating in/from water, are driven out of equilibrium
101 by applying strong sonication before encapsulation through a pH-jump process.

102 The method proposed here involves a lamellar-to-vesicle phase transition, conceptually
103 analogous to the mechanism occurring in thin film hydration or in the preparation of
104 vesosomes,³² but driven by a simple pH jump (generally ~4 to ~6) in bulk water. Instead of
105 common complex formulations combining phospholipids with pH-responsive lipids^{8,33,34} or
106 block-copolymers,³⁵ we use a single pH-sensitive microbial glycolipid (GL) (Figure 1).³⁶
107 Microbial glycolipids are biobased compounds known for their stimuli-responsive
108 properties,^{37,38,39} their biodegradability and low toxicity,⁴⁰⁻⁴⁵ and for these reasons particularly
109 interesting for biomedical applications. GL are known to form a set of closed vesicular
110 microphase via a simple pH-jump below ≤ 6.2 and a lamellar precipitate below pH ~4.⁴⁶

111 Using a combination of cryo-TEM, light and X-ray scattering, we find that high loads
112 of nanoparticles tend to occupy a limited number of vesicular compartments. This feature is
113 also observed upon loading of ferritin nanocages,⁴⁷ a result which agrees with the finding of
114 Luisi et al.,¹³ who proposed that the massive occupation of a small fraction of vesicles,
115 coexisting with a large majority of empty vesicles, could explain the compartmentalization of
116 heregenous genomic material in the early stages of life formation.



118 **Figure 1 - Acidic form of the microbial glycolipid GC18:1 (GL) and its corresponding pH-dependent phase**
119 **behaviour at room temperature: micelles, vesicle, lamellar.**
120

121 **Material and Methods**

122 **Synthesis of glycolipid GC18:1 (GL).** GLs were produced in a scaled-up bioreactor
123 experiment (100 L) by the *S. bombicola* strain Δ *ugtBI* described by Saerens et al.³⁶ The detailed
124 production, purification and hydrolysis process to obtain the compound in Figure 1 has been
125 reported elsewhere.⁴⁶ GLs contain about 90 % and 7 % of the subterminal (glycosidic bond
126 between glucose and the C17 of the fatty acid chain, Figure 1) and terminal (glycosidic bond
127 between glucose and the C18 of the fatty acid chain) congener, respectively. The remaining 3
128 % is constituted by di-unsaturated C18:2 and saturated C18:0 impurities.

129 **Preparation of iron oxide nanoparticles (NPs).** Iron oxide NPs were synthesized
130 using the co-precipitation method by adapting the standard protocol^{48,49} to obtain the inverse
131 spinel structure typically observed in magnetite, where $[\text{Fe}^{2+}]/[\text{Fe}^{3+}] = 0.5$. Specifically, 0.177 g
132 of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was mixed with 0.108 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ in a round-bottom flask containing 20
133 mL of Milli-Q water. To this solution, 2.7 mL of a 28 % ammonium hydroxide solution was
134 gradually added under continuous mechanical stirring. The system was maintained in an argon-
135 rich environment to limit oxidation. The resulting black precipitate was extracted by
136 magnetically-assisted sedimentation and washed with MilliQ water. This operation was
137 repeated three times to remove any residual salts.

138 **Preparation of upconverting nanoparticles (UCNP).** Upconverting oleic acid capped
139 $\text{NaYF}_4:\text{Yb}/\text{Er}$ and $\text{NaYF}_4:\text{Yb}/\text{Tm}$ nanoparticles (20%-Yb and 0.5%-Ln, Ln= Er, Tm) molar

140 ratio replacing yttrium ions in the lattice, labelled as UCNPs) of size between 20 nm and 40 nm
141 were prepared according to ref. 50.

142 **Iron-loaded ferritin cages preparations and characterization.** The iron-loaded
143 ferritin cages (AfFtn) are prepared as previously described.^{47,51} Briefly, the ferritin is derived
144 from the archaeon *Archaeoglobus fulgidus* and was produced recombinantly in *E. coli* strain
145 BL21(DE3)CodonPlus-RIL (Stratagene) with IPTG induction. The harvested cells were
146 sonicated and heat-treated at 85°C for 10 minutes. The insoluble fraction and denatured proteins
147 were removed using ultracentrifugation and the supernatant was passed through 0.22 µm filter.
148 No further purification was performed for this report. Iron was loaded by adding Fe₂SO₄
149 dropwise until 4800 Fe/cage had been loaded. To remove the unloaded iron, the sample was
150 desalted, concentrated using 100 kDa MWCO Amicon filter, and sterile filtered using 0.22 µm
151 syringe filter. The iron-loaded ferritin (Fe4800)AfFtn preparation was characterized by
152 dynamic light scattering (DLS) technique to confirm that the hydrodynamic size was ~13 nm
153 and the protein concentration was estimated using Bradford assay.^{47,51}

154 **NPs-containing vesicle preparation.** Ten mL of the NPs solution were set to pH 9 and
155 bath-sonicated during 30 min to one hour. After sonication, the NPs solution was mixed at room
156 temperature to 10 mL of a micellar GL solution (0.29 g GL in 10 mL) at pH 9. The NPs GL
157 mixture at pH 9 (referred to NPs GL, pH9, sample S2) was kept under stirring for few minutes
158 before use. *Note: the NPs solution is not sonicated in the presence of the GL solution.* According
159 to the data in Baccile et al.,⁴⁶ GL self-assembles into vesicles at pH values below 6.2. Thus, to
160 prepare vesicles, two aliquots of the NPs GL solution at pH 9 were acidified using 1 M HCl
161 solution: in aliquot 1, the pH is lowered to 6, and this sample is referred to as NPs GL, pH6
162 (sample S3) and in aliquot 2, the pH is lowered below 4 and then increased again to pH 6 and
163 named NPs GL, pH2→pH6 (sample S4). The latter will also be referred to as the *manual pH-*
164 *jump approach 2*, where *approach 1* corresponds to sample S1, that is a control experiment
165 only containing NPs and free of GL. In a second control experiment (sample S5), the pH of the
166 NPs (GL-free) solution was lowered to 6 with 1 M NaOH, and 0.45 g of non-acetylated acidic
167 sophorolipids were subsequently added, similarly to the two-step procedure described in
168 Baccile et al.⁵⁵ Sophorolipids are microbial glycolipids having a disaccharide headgroup and
169 known to self-assemble into micelles, instead of vesicles, under the acidic pH conditions.⁴⁶ The
170 detailed sample compositions are given in Table 1.

171 **Table 1 – Composition table of the samples studied in this work.** GL: acidic glucolipid GC18:1 (Figure 1),
172 NPs: γ-Fe₂O₃ nanoparticles,⁵⁵ SL: sophorolipids.⁵⁵ The concentration of NPs mother solution is estimated by
173 gravimetric analysis. Samples S2 through S5 are obtained by mixing 1:3 vol:vol of mother GL (40 mg/mL)

174 and NPs (4.2 mg/mL) solutions, both at pH 9. pH_i , pH_m and pH_f respectively indicate the initial, middle and
 175 final pH. If no pH change has occurred, $\text{pH}_i = \text{pH}_f$. All pH variations are performed by hand using 1 M HCl
 176 and 1 M NaOH solutions.

Sample name	Sample code	$C_{\text{GL}} / \text{mg/mL}$	$C_{\text{SL}} / \text{mg/mL}$	$C_{\text{NPs}} / \text{mg/mL}$	pH_i	pH_m	pH_f
GL	-	10	-	-	9	-	6
NPs	S1	-	-	4.2	9	-	9
NPs GL pH 9	S2	10	-	3.2	9	-	9
NPs GL pH 6	S3	10	-	3.2	9	-	6
NPs GL pH 2 \rightarrow pH 6	S4	10	-	3.2	9	2	6
NPs SL pH 6	S5	-	10	3.2	9	-	6

177
 178 The *manual pH-jump (approach 2)* described above has been performed manually and
 179 for this reason we have tested a series of additional methods to evaluate the robustness of the
 180 encapsulation process and to control the vesicle size distribution. These experiments have been
 181 performed on both a NPs-free solution constituted of vesicles only (GL in Table 1) and a mixed
 182 NP and GL (sample S4 in Table 1). *Extrusion (approach 3)*: after the *manual pH-jump*
 183 (*approach 2*) the solution is extruded (10 cycles) at 1 mL/min through a 0.45 μm filter.
 184 Extrusion is commonly used to control vesicle size in the hundred nanometer range.^{52,53,54}
 185 *Sonication (approach 4)*: after the *manual pH-jump (approach 2)*, the solution is sonicated for
 186 20 s using an immersive ultrasound probe. Sonication is commonly used to prepare vesicle of
 187 diameter below 100 nm.⁵² *Controlled pH-jump (approach 5)*: after the pH is lowered to 2, pH
 188 is increased to 6 using a 1 M NaOH solution injected at 0.5 $\mu\text{L}/\text{min}$ under stirring. *Dialysis pH-*
 189 *jump (approach 6)*: after the pH is lowered to 2, pH is increased to 6 by dialyzing the solution
 190 against a water reservoir at pH 6 using a 3500 MWCO (Spectra/Por[®]) membrane. Finally, to
 191 guarantee homogeneous dispersion of the vesicles and NPs, solution are kept under stirring
 192 during the *manual pH-jump* and *controlled pH-jump* processes.

193 **Preparation of iron-loaded ferritin-containing vesicles.** The procedure was adapted
 194 from the NPs-containing vesicle process. The stock (Fe4800)AfFtn solution (0.88 mg/mL, pH
 195 7.4) was mixed with the a micellar GL solution (40 mg/mL, pH 7.4) to achieve respective final
 196 concentrations of 0.66 mg/mL and 10 mg/mL. The pH was then lowered to about 3.9 (using ~
 197 6 μL HCl 5 M) to avoid (Fe4800)AfFtn aggregation. The pH is then increased to 6 using three
 198 selected approaches described above. *Manual pH jump (approach 2)*: pH is manually increased
 199 by manual addition of 1 M NaOH solution. *Controlled pH-jump (approach 5)*: a 1 M NaOH
 200 solution is injected at 0.5 $\mu\text{L}/\text{min}$ under stirring until pH 6 was achieved. *Extrusion (approach*
 201 *3)*: the (manual) pH jump solution is extruded (10 cycles) at 1 mL/min through a 0.45 μm filter.

202 *Note: (Fe4800)AfFtm is water-dispersible, therefore it is never sonicated.*

203 **Preparation of UCNP-containing vesicles.** To a 10 mg/mL micellar GL solution in
204 water at pH 9, we add the dried oleic-acid-capped NaYF₄:Yb/Er and NaYF₄:Yb/Tm UCNP
205 powder at concentration of 5 mg/mL. The UCNP sample is water-insoluble and for this reason
206 sonication is necessary to help dispersion. For the encapsulation, we employ *the manual pH*
207 *jump (approach 2)*: pH is reduced to 4 (manual dropwise addition of μ L amounts of 0.5 M HCl)
208 and then increased to \sim 6 (manual dropwise addition of μ L amounts of 1 M NaOH). The same
209 protocol applies to a control solution only containing the UCNP sample, without adding GL.
210 Despite the hydrophobic character of the oleic acid-coated UCNP, sonication is enough to
211 disperse them in water, although sedimentation is fast for the control, GL-free, experiment.

212 **Sedimentation experiments using photoluminescence spectroscopy.** *Free*
213 *sedimentation*: the UCNP dispersion is placed in a quartz cuvette and irradiated with a CW laser
214 source ($\lambda= 980$ nm) at a power density of 105 W/cm². Emission spectra were recorded after 0,
215 10, 20, 30, 60, 120, 300, 600, 1200, 1800, 3600, 5400 s. *Forced sedimentation*: The setup and
216 conditions for photoluminescence measurements were identical to the free sedimentation.
217 However, the encapsulated UCNP dispersion was centrifuged at 2000 rpm in a time scale
218 comprised between 0 and 300 seconds, and emission spectra were taken after 10, 20, 30, 60,
219 120 and 300 seconds of centrifugation.

220 **Light scattering (LS) experiments.** Light scattering experiments were performed on a
221 Malvern Zetasizer Nano ZS instrument ($\lambda= 633$ nm) at constant shutter opening and same
222 sample-to-detector distance. The diffused light was expressed in terms of the derived count rate
223 in kilocounts-per-seconds (kcps). All solutions were diluted 100 times prior to analysis. Two
224 measurements were performed, the first one on the solution itself and the second one on the
225 supernatant of the same solution, after magnetically-assisted sedimentation of the suspended
226 NPs using a neodymium magnet.

227 **Transmission Electron Microscopy (TEM).** TEM was performed on a FEI Tecnai 120
228 Twin microscope operating at 120 kV and equipped with a high resolution Gatan Orius CCD
229 4k x 4k numeric camera. DigitalMicrograph™ software was used for image acquisition. TEM
230 images acquired in cryogenic mode were obtained from the same instrument. The sample holder
231 was a Gatan Cryoholder (Gatan 626DH, Gatan). DigitalMicrograph™ software was used for
232 image acquisition. Cryo-fixation was done on a homemade device. The solutions were
233 deposited on glow discharged holey carbon coated TEM copper grids (Quantifoil R2/2,
234 Germany). Excess solution was removed and the grid was immediately plunged into liquid
235 ethane at -180°C. All grids were kept at liquid nitrogen temperature throughout all

236 experimentation.

237 **Small Angle X-ray Scattering (SAXS).** SAXS experiments were recorded at the high
238 brilliance ID02 beamline at the ESRF synchrotron (Grenoble, France) using a 1 m detector-to-
239 sample distance and a flow-through polycarbonate capillary. Acquisition time was
240 1 s per pH value. pH was changed in-situ in the experimental hutch and followed live. More
241 details on the experimental setup have been provided in Baccile et al.⁴⁶ Data were acquired
242 using a CCD camera and integrated azimuthally to obtain a typical $I(q)$ spectrum. Contribution
243 of the solvent (water at pH 11.6) and capillary were measured prior to the experiment and
244 accordingly subtracted during the data treatment. All data were corrected for the transmission
245 of the direct beam.

246 Additional SAXS experiments were performed on a laboratory three-pinhole type S-
247 MAX 3000 RIGAKU Nanoviewer instrument using a monochromatic Cu-K α radiation
248 produced by a microfocus (20 microns x 20 microns) sealed tube X-ray source (MicroMax 002+
249 RIGAKU working at 40 W) and equipped with a two-dimensional multi-wire proportional gas
250 detector. The sample-to-detector distance was set to 1469 mm. The applied voltage and filament
251 current were 40 kV and 50 mA respectively. The q -range calibration was made using a silver
252 behenate standard sample ($d_{\text{ref}} = 58.38 \text{ \AA}$). The measured intensity was always divided by the
253 sample transmission and appropriate masking was done to eliminate the beam-stop shadow
254 contribution and imperfection of the 2D detector. Quartz sample holders of 1 mm diameter were
255 used in a flow-through mode using a home-made device, thus assuring that the background
256 signal was constant for all samples. The subtracted background was constituted by the water-
257 containing quartz capillary. The acquisition time per sample is 1 h.

258 **X-ray Diffraction (XRD).** XRD data were recorded on the dried powders using a
259 Bragg-Brentano θ -2 θ ($\lambda = 1.54 \text{ \AA}$) goniometer Bruker D8 Discover instrument.

260 **Differential thermal analysis (DTA).** DTA was performed in a Mettler-Toledo
261 TGA/SDTA851e/LF/1600 instrument. The analysis of the powders was carried out in Pt
262 crucibles at a heating rate of 5 °C/min under air atmosphere over the range of 20-900 °C and
263 using alumina as reference.

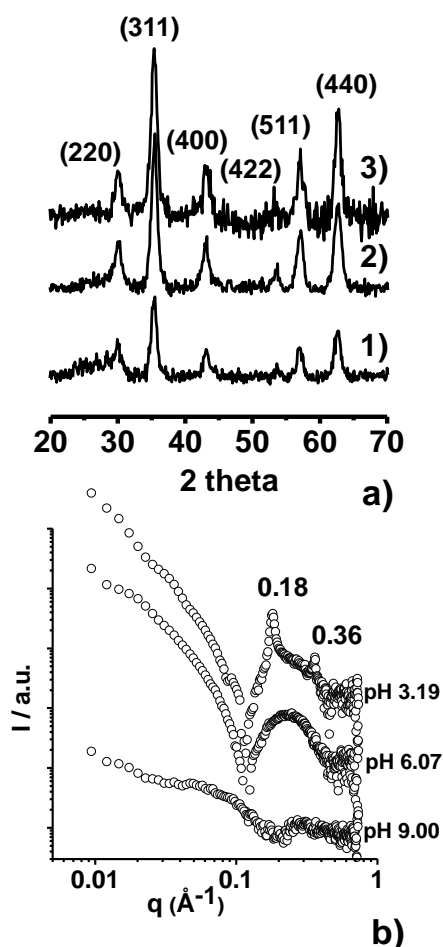
264 **Photoluminescence spectroscopy.** Upconversion photoluminescence was recorded
265 using an infrared laser diode RLMDL-980-2W module (980 nm \pm 5 nm, 2 W cw) from
266 Roithner LaserTechnik, and a Black Comet CXR (StellarNet) optical fiber as detector. The
267 emission was measured using dispersions of the UCNPs (10 mg in 1mL of ethanol for bare
268 nanoparticles, or in 1 mL of water for the samples encapsulated in glycolipids) and focusing
269 the laser on the samples at a pump density of 105 W/cm².

270

271 **Results and discussion**

272 **Encapsulation of sonicated iron oxide nanoparticles.** The XRD of the bare iron oxide
273 NPs is represented by the diffractogram N°1 on Figure 2, which shows the d-values: 2.967,
274 2.527, 2.092, 1.714, 1.614, 1.478 Å, corresponding, to the (220), (311), (400), (422), (511),
275 (440) Bragg diffraction planes of the iron oxide spinel cubic structure (JCPDS file, N° 19-
276 0629), respectively. This is attributed to a maghemite structure, previously identified using
277 Mössbauer spectroscopy.⁵⁵ The typical size (below 10 nm) and aggregation state of this sample
278 are shown by TEM in Figure S 1 (sample referred to as NPs).⁵⁵ The magnetic nature of the NPs
279 (Figure 3) shows the attraction of the NP sample to the magnetic field of a neodymium magnet
280 leaving the supernatant solution completely clear. The observation is also confirmed by the
281 light scattering (LS) data in Figure 3 (sample S1) before (white bars, strong scattering) and after
282 (grey bars, no scattering) exposure to the magnet. This is explained by the strong aggregation
283 of the uncoated NPs (Figure S 1, NPs sample) in water. On the other hand, GL mainly assembles
284 into micelles in water at pH 9, as shown by the characteristic SAXS scattering signal, shown in
285 Figure 2b. The same compound is also known to form vesicles when the pH is lowered from 9
286 to 6, as previously demonstrated by us^{37,46} and shown here by the typical SAXS scattering signal
287 recorded at pH 6.07 in Figure 2b. The SAXS pattern is characterized by a broad oscillation at
288 q above 0.1 \AA^{-1} , attributed to the form factor of a lipid bilayer, and an intense scattered intensity
289 at q below 0.05 \AA^{-1} , the slope of which in log-log scale is close to -2, a typical value found for
290 planar surfaces. A detailed analysis of the form factor through model-dependent fitting of SAXS
291 data presented in Figure 2b can be found in ref. 37.

292 Addition of GLs to the bare nanoparticle solution at pH 9 does not modify the NPs
293 crystal structure (XRD pattern N°2, Figure 2a), nor the overall aggregation state (sample NPs
294 GL, pH9 in Figure S 1). However, upon exposure to the magnet most NPs are removed from
295 the solution, but the latter still has a light brown colour, indicating a small dispersion of NPs in
296 solution. This is confirmed by the slightly higher scattering signal in LS data (sample S2) in
297 Figure 3. Given the strong molecular similarity between GLs and sophorolipids (SLs), GLs can
298 certainly stabilize a small fraction of the iron oxide NPs by a simple adsorption at their surface
299 via the carboxylate group, a phenomenon observed in a previous study.⁵⁵

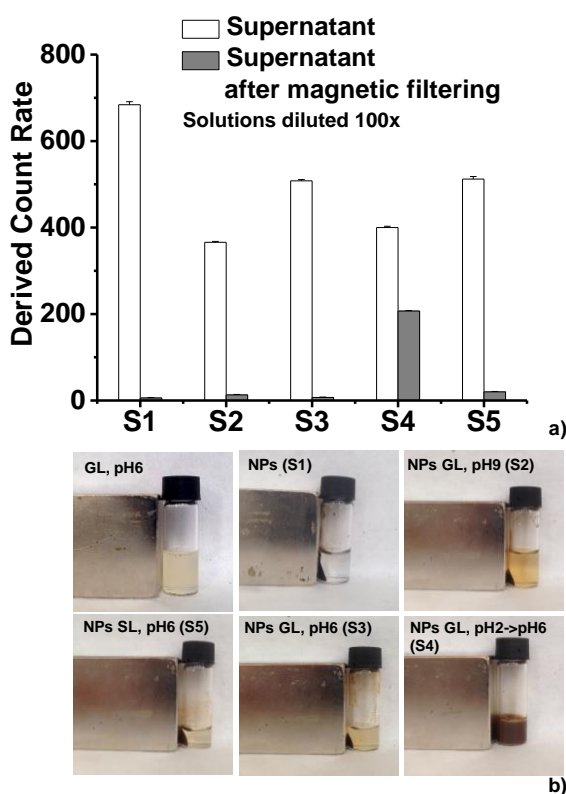


300

301 **Figure 2 – a) X-ray diffraction patterns of 1) iron oxide NPs, 2) NPs GL at pH 9 and 3) NPs GL at pH 6.**
 302 **Reported values relate to the Miller (hkl) indices of the Bragg diffraction planes. b) SAXS curves were**
 303 **recorded on the ID02 line of ESRF synchrotron for a pure GL solution at pH values between 3 and 9. The**
 304 **solutions at pH 6.07 and pH 3.19 have been directly obtained from the solution at pH 9.00 by adding 1 M**
 305 **HCl solution.**
 306

307 When the pH of the NPs GL solution is lowered from 9 to 6, one can confirm that the
 308 nanoparticle crystal structure is intact (XRD pattern N° 3, Figure 2a). Upon approaching an
 309 external magnet to the vial, most NPs are attracted, thus leaving the solution slightly yellowish
 310 (Figure 3b, sample S3) due to the spurious presence of GL-coated NPs in solution. Light
 311 scattering of sample S3 (diluted, Figure 3a) qualitatively confirms the loss in the scattered
 312 intensity of the supernatant upon magnetically-assisted sedimentation. Nevertheless, Figure 3b
 313 shows that sample S3 (undiluted) supernatant is slightly turbid, if compared to samples S1 or
 314 S2; this is explained, and actually expected,⁴⁶ by the presence of vesicles, confirmed by SAXS

315 (Figure 2b) and the image in Figure 3b (GL, pH 6), both corresponding to a NP-free GL solution
 316 at pH 6. The TEM image of sample 3 (NPs GL pH6, Figure S 2a) confirms that nanoparticle
 317 size, morphology, structure and aggregation are not affected by the presence of the GL.
 318 However, GL can stabilize the nanoparticle surface, as commented above and as expected from
 319 the previous work performed on sophorolipids-stabilized iron oxide nanoparticles.⁵⁵ Light
 320 scattering experiments performed on sample S3 (Figure 3b), as well as its dim yellowish colour
 321 and aggregation state (Figure S 2a) are comparable to a control system composed of
 322 sophorolipid-stabilized iron oxide nanoparticles (sample S5, Figure 3a, Figure S 1, Figure 3b).

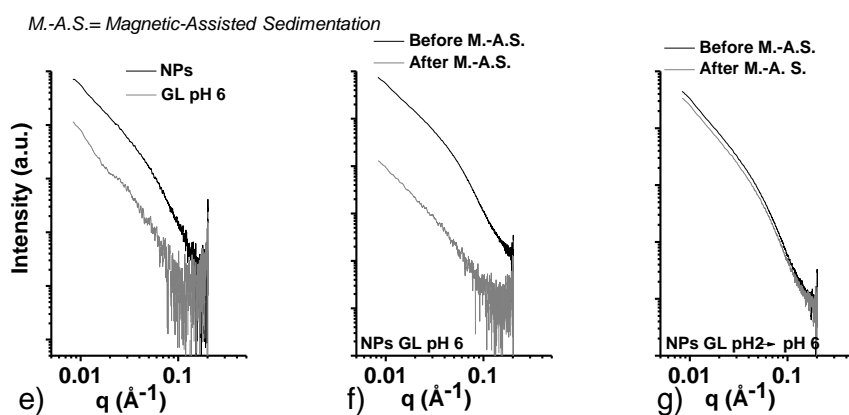
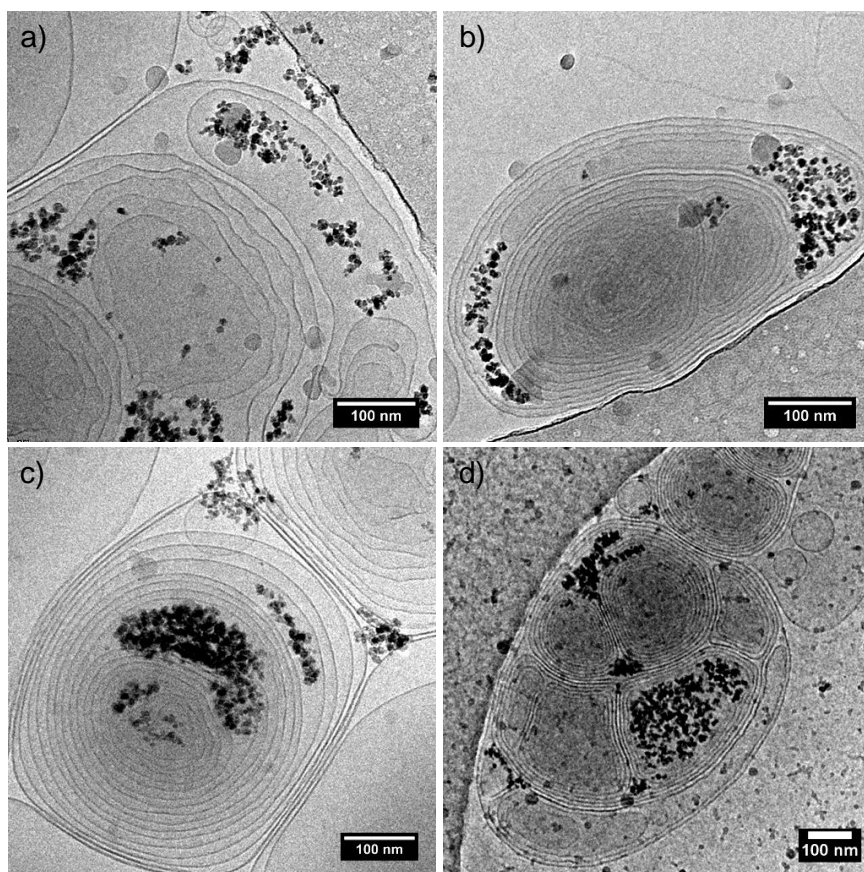


323
 324 **Figure 3 – Light scattering data (a) and magnetically-assisted sedimentation images (b) for bare NPs**
 325 **(sample S1), GL-containing NPs at pH 9 (NPs GL, pH9, sample S2), GL-containing NPs at pH 6 (NPs GL,**
 326 **pH6, sample S3), GL-containing NPs at pH 6 after a transition at pH 2 (NPs GL, pH 2 → pH 6, sample S4),**
 327 **NPs-free GL solution at pH 6 (GL, pH6) and SLs-containing nanoparticle solution at pH 6 (NPs SL pH6,**
 328 **sample S5. Sample S5: control sample composed of sophorolipids-stabilized iron oxide NPs.⁵⁵**
 329

330 GLs are known to precipitate into a lamellar phase when the pH is lowered below 4,⁴⁶
 331 also illustrated by the SAXS pattern recorded on a nanoparticle-free GL solution at pH 3.19 and
 332 shown in Figure 2b. In the last figure, two sharp diffraction peaks at $q = 0.18 \text{ \AA}^{-1}$ and 0.36 \AA^{-1}
 333 overlaps the typical vesicle form factor profile; they respectively correspond to the first and
 334 second order interplanar distances lamellar stacking ($d = 34.8 \text{ \AA}$ and $d = 17.4 \text{ \AA}$). The cryo-TEM

335 analysis of a GL nanoparticle solution at pH below 4 displays the coexistence of nanoparticle
336 clusters and flat lamellae (Figure S 3). Under these conditions, NPs can be easily removed by
337 magnetically-assisted sedimentation, leaving a solid white precipitate in the vial. However,
338 when pH is increased again to 6, the nanoparticle solution becomes extremely stable towards
339 magnetically-assisted sedimentation: light scattering of sample S4 (Figure 3) indicates strong
340 scattering before and after sedimentation. This is confirmed by the images showing the
341 magnetically-sedimented undiluted sample (Figure 3b, sample S4): the dark solution indicates
342 that most NPs are now stabilized. We anticipate that stability is observed over several hours of
343 continuous exposure to the neodymium magnet.

344 Figure S 2b-d show that particle size (~10 nm) is unchanged compared to the GL-free
345 NP solution and high magnification (Figure S 2b) and Fourier transform (Figure S 2c) indicate
346 the crystallinity (Figure S 2d) of the particle with a typical distance of 2.9 Å, attributed to the
347 (220) plane of maghemite.

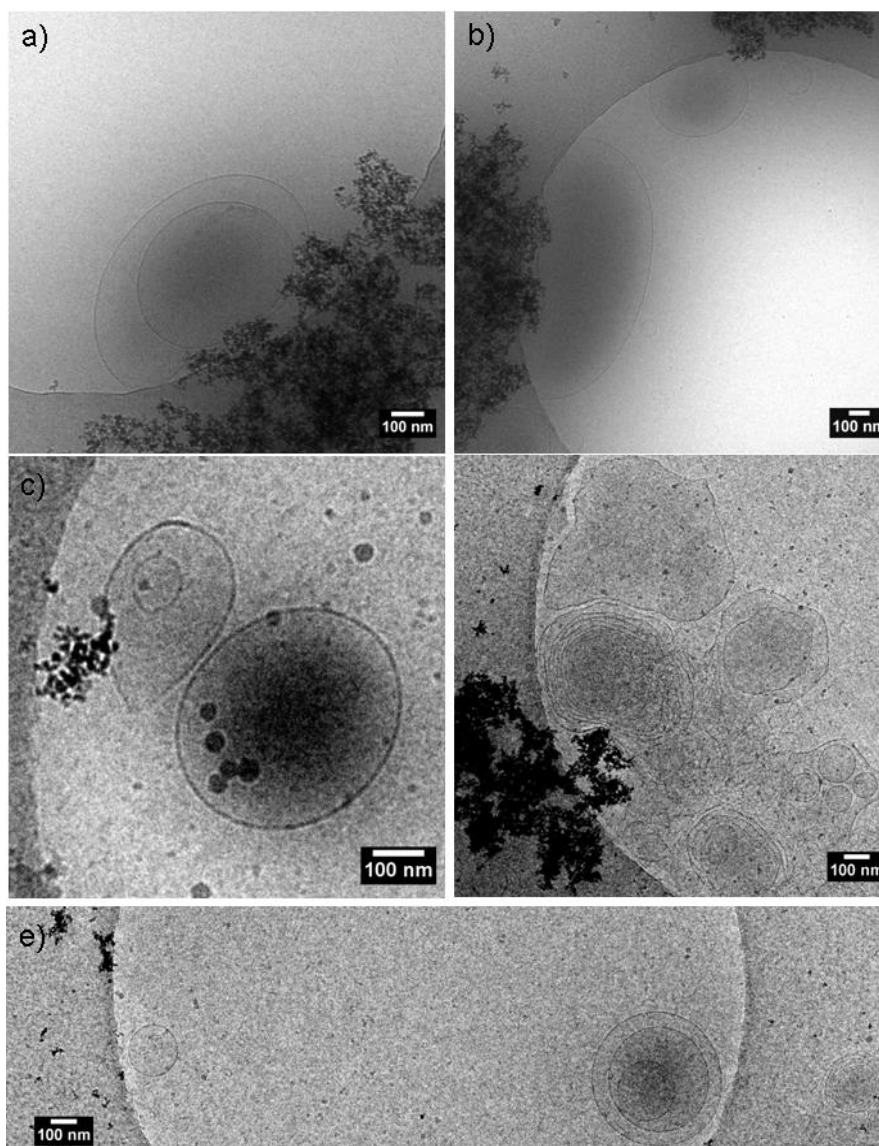


348
 349 **Figure 4 – a-d) Cryo-TEM images of NPs GL samples obtained after a pH jump (pH 9 →) pH 2 → pH 6**
 350 **(NPs GL pH2→pH6, sample S4). e-g) Laboratory SAXS data recorded on e) bare NPs and NPs-free GLs at**
 351 **pH 6 (GL pH6), f) NPs GL samples at pH 6 directly adjusted from pH 9 (NPs GL pH6, sample S3) before**
 352 **(black curve) and after (grey curve) magnetically-assisted sedimentation, g) NPs GL samples at pH 6**
 353 **adjusted from pH 9 after passing through pH 2 (NPs GL pH2→pH6, sample S4) before (black curve) and**
 354 **after (grey curve) magnetically-assisted sedimentation**
 355

356 Although GL adsorption is not excluded, the only surface passivating effect of GL
 357 cannot explain the colloidal stability at pH 6, because only a fraction of the NPs are in fact
 358 concerned, as already shown for sample S3 and control sample S5.⁵⁵ The remarkable stability

359 of the nanoparticle suspension is rather explained by the encapsulation of nanoparticle clusters
360 within (mainly) multilamellar vesicles, as shown by cryo TEM experiments in Figure 4a-d.
361 These data are statistically-confirmed by complementary SAXS experiments presented in
362 Figure 4e-f: panel a) shows the typical signatures of bare NPs (black curve) and GL in solution
363 at pH 6 (grey curve). The difference in the scattering intensity (although not in absolute scale)
364 reveals the strong difference between NPs and GL in terms of contrast with the solvent (water).
365 Additionally, the high-q region above 0.1 \AA^{-1} in the GL sample, although very noisy, shows the
366 beginning of the form factor oscillation, which is very clear in the synchrotron-collected data
367 on a larger q-scale (Figure 2b, pH 6.07). The highly stable pH 2 \rightarrow pH 6 system (Sample S4,
368 Figure 4g) shows that the signal is dominated by the NPs scattering before and after
369 magnetically-assisted sedimentation, thus confirming that vesicles prevent their removal.

370 Finally, we have specifically verified the orthogonality between vesicle-formation and
371 NPs dispersion: the pH 9 \rightarrow pH 2 \rightarrow pH 6 method is applied to a NPs-free GL solution and the
372 corresponding cryo-TEM images in Figure S 4 confirm the presence of vesicles, and in
373 particular of multilamellar vesicles. One can compare these results to the sample S3 (NPs GL
374 pH 9 \rightarrow pH 6), of which the cryo-TEM (Figure 5) and SAXS (Figure 4f) before (black curve)
375 and after (grey curve) magnetically-assisted sedimentation indicate that the NPs are not
376 preferentially encapsulated. Cryo-TEM shows that the NPs mostly aggregate around vesicles,
377 while SAXS shows the typical signal of the NPs before and a pattern typical of the vesicles
378 after magnetically-assisted sedimentation.



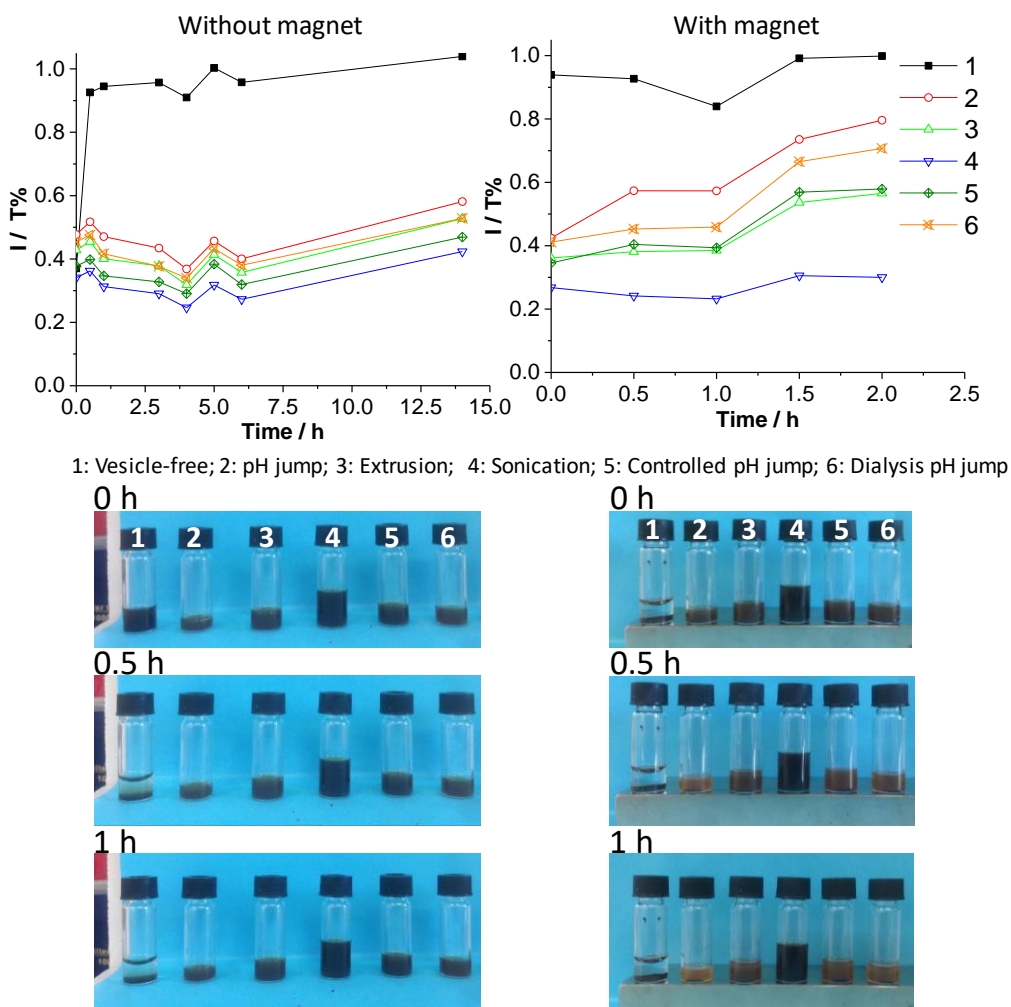
379

380 **Figure 5 – Cryo-TEM images of NPs GL samples prepared at pH 6 directly adjusted from pH 9 (NPs GL**
 381 **pH 6, sample S3)**

382

383 **Stability, control of the size distribution and quantification.** Time-dependent stability and
 384 size control of the encapsulated NPs are two important parameters for potential applications in
 385 nanomedicine and they are tested hereafter. Figure 6 shows the time stability of a GL solution
 386 mixed with NPs in the presence and absence of a magnet employing different approaches of
 387 encapsulation, but all performed using the pH jump (pH 9 → pH 2 → pH 6) process (sample
 388 4). *Approach 1* and *approach 2* are, respectively, the vesicle-free NPs (sample S1) control and
 389 the manually-controlled pH jump (sample S4). Among the others, *approach 3* and *approach 4*
 390 respectively use extrusion and sonication after encapsulation, both being classical methods to
 391 control vesicle size in the 100 nm range (extrusion)^{52,53,54} and below 50 nm (sonication).⁵²
 392 Extrusion is also commonly employed to obtain unilamellar vesicles from multilamellar

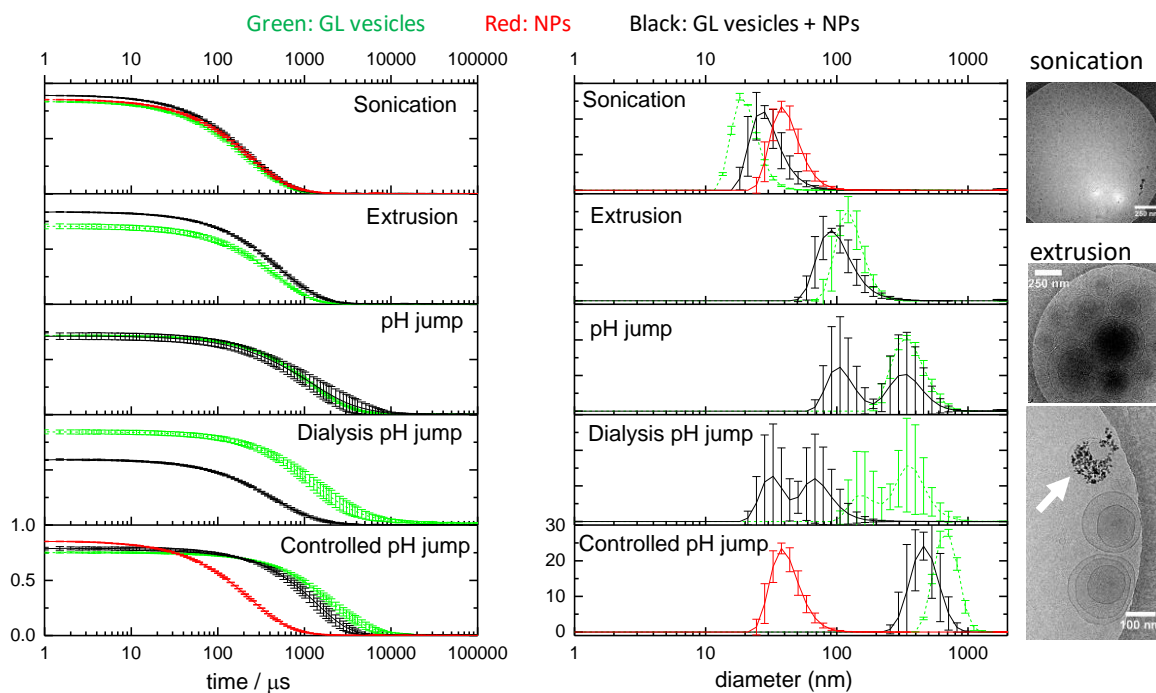
393 vesicles. Finally, *approach 5* and *approach 6* are meant to perform the pH 2 → pH 6 jump
 394 either through a controlled NaOH injection (1 M NaOH at 0.5 μL/min, *approach 5*) or through
 395 a very slow modification in pH (*approach 6*, using dialysis). Without vesicles (filled squares,
 396 Figure 6), the NPs sediment after several minutes in the absence of magnet, as expected from
 397 the Stokes law, while sedimentation is immediate upon use of a magnet. Whichever the
 398 approach of encapsulation, all vesicles-stabilized NPs solutions are similarly stable in time
 399 against sedimentation (Figure 6, left-hand): after 15 h, the transmittance is almost constant,
 400 while the vesicle-free control is clear. In the presence of a magnet (Figure 6, right-hand), none
 401 of the solution shows the same immediate collapse as found in the vesicle-free control (black
 402 squares), thus indicating that encapsulation is always successful. However, after 2 h of exposure
 403 to the magnet, the transmission is not equivalent among the tested solutions, probably indicating
 404 a disparity in terms of the encapsulation efficiency. Extruded, sonicated and controlled pH jump
 405 (respectively, approach 3, 4 and 5) seem to be the most efficient approaches.
 406



407
 408 **Figure 6 – Time-dependent stability of NPs GL pH 2 → pH 6 (sample S4) using approaches 1 through 6**

409 (refer to figure and materials and methods section). Data on the left-hand side refer to simple decantation
410 (no magnet) while data on the right-hand side are acquire using magnetically-assisted sedimentation of the
411 solution (vials sit on a neodymium, as shown on figure)

412
413 Extrusion is known to be a severe process that can destabilize the most stable
414 nanoparticle systems.⁵⁶ Vesicles-stabilized NPs prepared by pH jump provide an ultra-stable
415 dispersion of NPs upon extrusion (*approach 3*). Video 1 shows that a vesicles-stabilized NP
416 solution can be extruded repeatedly (we tested up to ten cycles) without aggregation of the NP
417 onto the filter, while a vesicle-free NP solution cannot be extruded once (Video 2). The
418 advantage of employing various sample preparation approaches is the possibility to control the
419 vesicles size distribution, as formulated theoretically in the development of nonequilibrium
420 compartments.²⁸ Figure 7 shows the autocorrelation function and the corresponding number
421 distribution of the (vesicle-free) iron oxide NPs solution (in red), the (NPs-free) GL vesicle
422 solution obtained through the pH jump (in green) and the vesicles-stabilized NPs solution (in
423 black) obtained with approaches 2 through 6. Both manual and dialysis pH jump approaches
424 provide a heterogenous dispersion of vesicles and NPs with a broad size distribution, in
425 agreement with the cryo-TEM images of sample 4 in Figure 4. On the contrary, extrusion,
426 sonication and controlled pH jump provide a homogeneous set of NPs dispersion, of which the
427 size distribution can be tuned between 50 nm (sonication) and 500 nm (controlled pH jump). In
428 particular, the comparison between the autocorrelation function of the NPs-free vesicles and
429 the vesicles-stabilized NPs systems in the controlled pH jump approach clearly shows that the
430 size distribution of the latter is controlled by the process of vesicle formation. Selected cryo-
431 TEM images for the sonication and extrusion processes are also shown in Figure 7 (right-hand
432 side) to support the DLS data. Cryo-TEM shows that sonication produces vesicles between 20
433 nm and 50 nm, while extrusion produces vesicles of size contained between about 100 nm and
434 300 nm. In both cases the average size and size distribution are in good agreement with the DLs
435 data. White arrow in the cryo-TEM image recorded on the extruded vesicles-stabilized NPs
436 system also shows a typical vesicle highly loaded with iron oxide NPs.



437
 438 **Figure 7 – Autocorrelation functions (left-hand panels) and number size distribution (right-hand panels) of**
 439 **NPs GL pH 2 → pH 6 solutions (sample 4) prepared using approaches 2 through 5, respectively referring**
 440 **to (manual) pH jump, extrusion, sonication, controlled pH jump and dialysis pH jump. The experimental**
 441 **detail of each approach is provided in the materials and methods section. To support the DLS data on size**
 442 **control, cryo-TEM images are provided on the right-hand side for the sonication and extrusion methods.**
 443 **White arrow point at a typical vesicle highly loaded with iron oxide NPs.**
 444

445 Vesicles-stabilized NPs can be reproducibly prepared by the pH jump approach, which
 446 controls a lamellar-to-vesicle phase transition when passing from highly acidic pH (it works
 447 between 2 and 4) to pH ~6. Stability and size control of the NP-encapsulated vesicles depends
 448 on the method the lamellar-to-vesicle phase transition line is crossed. This behaviour identifies
 449 a kinetically controlled process, where the non-equilibrium NPs dispersion is randomly
 450 encapsulated during the process of bilayer folding into vesicles. To estimate the repeatability
 451 of the encapsulation process and the loading efficiency, the manual pH jump experiment
 452 (sample S4 in Figure 3, approach 2 in Figure 6) was repeated twenty times. By a simple
 453 gravimetric method, we find an encapsulation efficiency of 59.2 ± 16.0 %. These values are
 454 obtained by weighting the mass of the NPs collected through the magnetically-assisted
 455 sedimentation with respect to the total mass of the initial NPs load. Figure S 5 qualitatively
 456 shows two systems, where the NPs retain rate in solution is in the order of 50 % (Figure S 5b)
 457 and above 50 % (Figure S 5c), and where Figure S 5a shows the control image of a typical NPs-
 458 free GL vesicle solution. Considering the broad variation across experiments in the

459 encapsulation efficiency ($\pm 16.0\%$), we did not estimate useful to perform more precise
460 measurements. However, loading efficiency values above 50% can be compared with the
461 highest values reported in the literature for magnetoliposome systems, with encapsulation
462 occurring both in the lumen and bilayer.^{57,58,59,60}

463
464 **Understanding the uneven encapsulation profile.** At the moment, the only hypothesis
465 supporting the encapsulation of magnetic iron oxide NPs (magnetoliposomes) is the lamellar-
466 to-vesicle transition occurring during the pH 2 \rightarrow pH 6 jump process, a similar process
467 described for vesosomes from cochleate cylinders,³² or in more standard thin film hydration.¹³
468 In this work, such a mechanism is strongly suggested by the fact that both unilamellar and
469 multilamellar vesicles are produced during the micelles-to-vesicle (pH 9 \rightarrow pH 6)⁴⁶ and
470 lamellar-to-vesicle (pH 9 \rightarrow pH 2 \rightarrow pH 6, Figure S 4) transition. The folding process of the
471 bilayer membrane into closed objects during the pH 2 \rightarrow pH 6 jump, and which was already
472 shown to be driven by temperature for GL,⁴⁶ internalizes the NPs in solution. Nanoparticles are
473 locally aggregated but their volumetric distribution is homogeneous at a macroscopic scale due
474 to previous sonication (in the absence of GL vesicles) and stirring (in the presence of GL
475 vesicles). Nonetheless, the variability in loading efficiency is quite high ($\pm 16\%$), a fact which
476 can be explained by several factors. Figure 6 and Figure 7 show the impact of the encapsulation
477 process on the stability and size distribution, where the finest control provides the most stable
478 systems. Manual variation, although quite robust and easy to perform, obviously suffers from
479 an intrinsic variability, especially in a kinetically controlled process. Locally, cryo-TEM shows
480 a coexistence of heavily-loaded vesicles and empty vesicles, rather than a homogeneous
481 encapsulation event. Interestingly, a similar behaviour was reported by Bothun⁵⁹ and by Luisi,¹³
482 respectively on water-dispersible magnetite and iron-loaded ferritin, the latter using a statistical
483 approach employing cryo-TEM. Unfortunately, any attempt to improve the homogeneity of the
484 encapsulation among the vesicles, for instance using different molarities (e.g., 1 M and 0.1 M)
485 of NaOH, was vain, suggesting other mechanisms behind the encapsulation phenomenon.

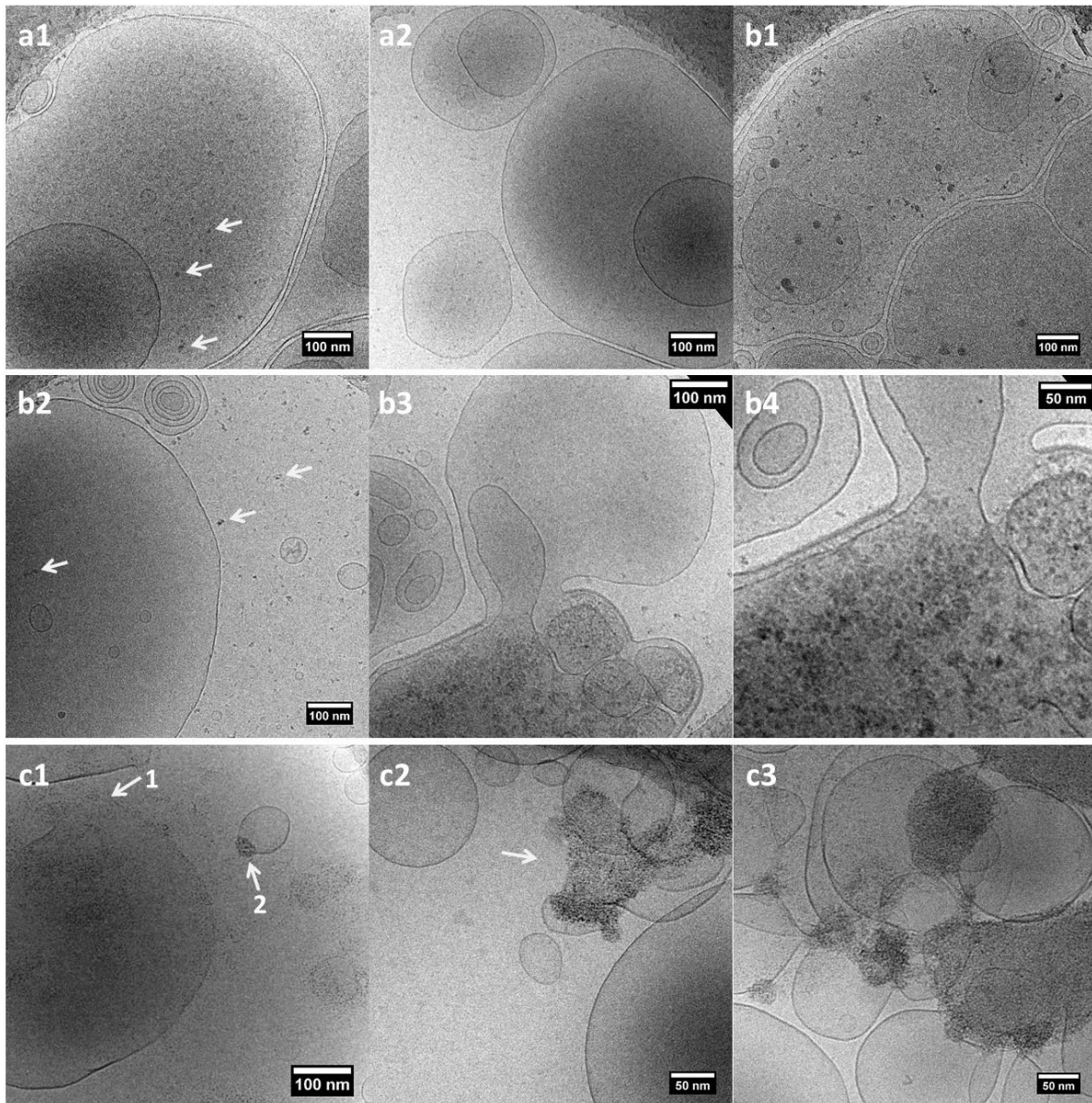
486 To better understand whether or not the uneven encapsulation profile is specific to this
487 system, we have decided to repeat the pH-driven encapsulation experiment using ferritin
488 nanocages containing an iron core. Luisi et al.¹³ have demonstrated that encapsulation of ferritin
489 (performed through thin film hydration and ethanol injection) in the lumen of 1-palmitoyl-2-
490 oleoyl-sn-glycero-3-phosphatidylcholine vesicles is not described by a classical Poisson-
491 Boltzmann distribution function, but rather by a small fraction of heavily-loaded vesicles
492 surrounded by empty ones. (Fe4800)AfFtn, characterized in ref. 51, is a water-dispersible

493 material similar to the one employed by Luisi et al.;¹³ it is characterized by an iron core (4800
494 iron atoms) of 8 nm enclosed in a proteic cage of 13 nm in diameter.

495 (Fe4800)AfFtn is encapsulated in GL vesicles using three main approaches: *manual pH*
496 *jump (approach 2)*, *controlled pH jump (approach 5)* and *extrusion (approach 3)*. These
497 experiments are all analyzed by cryo-TEM presented in Figure 8. Typical (Fe4800)AfFtn is
498 indicated by the white arrows in Figure 8a1,b2. Irrespective of the encapsulation approach, we
499 systematically observe empty, or close to empty, vesicles coexisting with loaded vesicles
500 (Figure 8a1,b1,c1), and in few cases, highly loaded vesicles (a thorough statistical analysis is
501 however out of the scope of this work). In particular, Figure 8b3,b4,c2,c3 show the side-to-side
502 coexistence of extremely crowded vesicles next to empty ones, as concluded by Luisi et al.¹³
503 Whether there may be an influence of the encapsulation approach or not, we do not have enough
504 statistically-meaningful data in our possession to conclude on this point, but this is not excluded,
505 as shown by cryo-TEM images corresponding to the extrusion approach. Figure 8c1, for
506 instance, shows a typical example of a large burst vesicle (arrow 1), from which (Fe4800)AfFtn
507 particles are free of diffusing from inside to outside and vice versa. One can also observe small
508 portions of a smaller vesicle being heavily loaded (arrow 2). We did not observe similar events
509 occurring in the manual and controlled pH jump. This can be explained by the reduction in
510 vesicle size polydispersity (Figure 7) after extrusion, a fact that can involve vesicle bursting
511 and intervesicular fusion.

512 These experiments show that the phase change encapsulation approach developed in
513 this work using the pH-responsive GL provides the same uneven distribution profile of the
514 ferritin nanocages within the lumen of the vesicular compartments, similarly to what was
515 described in ref. 13. To this regard, the data shown in Figure 4 are neither specific to the
516 encapsulation of iron oxide NPs nor to the pH-driven encapsulation method, but they seem to
517 follow an accepted, although not fully understood, distribution profile, which cannot be
518 described by a standard Poisson-Boltzmann distribution function. On another level, the
519 encapsulation of (Fe4800)AfFtn also suggest that the method developed in this work could be
520 extended to the encapsulation of a broader range of water-stable nanocolloids.

521



522

523 **Figure 8 - Cryo-TEM images of GL samples containing Ferritin-4800 (average diameter of 13 nm) obtained**
 524 **after a pH jump (pH 7.4 → pH 3.9 → pH 6). The following approaches (please refer to the method section**
 525 **for more information) are employed: a1-a2) controlled pH jump; b1-b4) manual pH jump; c1-c3) extrusion**
 526 **after manual pH jump.**
 527

528

529 **Extending the pH-jump encapsulation to aqueous dispersions of hydrophobic oleic-**
 530 **stabilized upconverting nanoparticles**

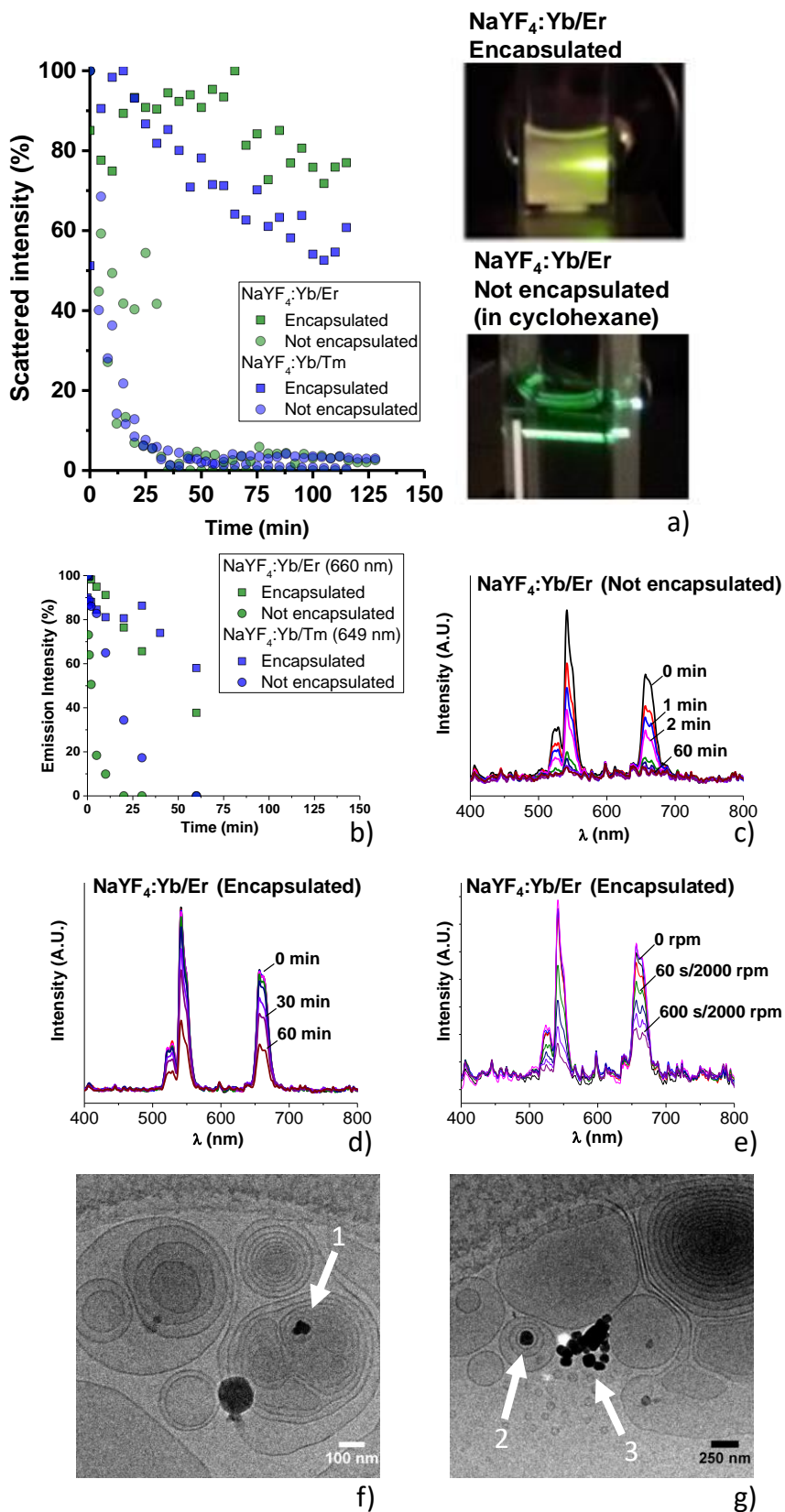
531 UCNP are interesting colloids with a wide variety of applications for their ability to
 532 absorb energy in the near infrared (NIR) and convert it to visible light. The most important
 533 concerns the field of bioimaging because living tissues are transparent in the NIR.³¹ The actual
 534 synthesis procedure occurs in organic media, from which the UCNP result in poor water

535 solubility due to their hydrophobic coating. Strategies to make UCNP hydrosoluble, which
536 generally consists in tedious ligand exchange procedures, are crucial for their application in the
537 biomedical field. In this sense, it could be very interesting to avoid the ligand exchange step
538 and to directly encapsulate the UCNP bearing their original hydrophobic coating. The
539 sonication-assisted phase change method developed in the previous section for bare iron oxide
540 NPs could be an interesting alternative to more classical encapsulation approaches,²⁵ which
541 would require surface engineering steps to make UCNP water-dispersible. Furthermore, as
542 shown in many magnetoliposome systems, stable NPs bearing a hydrophobic coating
543 classically partition in the lipid bilayer of the vesicles,^{1,9,15,16,56,57} and in general only the
544 employment of surfactants, which guarantee oil-water phase transfer, forces them to aggregates
545 in the lumen.²¹ Otherwise, standard hydrophilic stabilizers (e.g., citric acid, polymers) are used
546 to surface stabilizers that help them aggregate in the lumen.^{16,57,59,60} In this section, we make
547 the hypothesis that the sonication-assisted phase-change method employing the pH-responsive
548 GL can also be used to encapsulate hydrophobic colloids under the same conditions employed
549 to form magnetoliposomes. We employ UCNP used with an average diameter between 20 nm
550 and 40 nm; they are composed of NaYF₄:Yb/Er and NaYF₄:Yb/Tm, both coated with oleic acid.
551 Due to their oleic coating, they aggregate in water, either sedimenting or immediately adsorbing
552 on the glass vial.

553 NaYF₄:Yb/Er and NaYF₄:Yb/Tm UCNP are prepared according to a standard
554 procedure,⁵⁰ their typical XRD pattern (Figure S 6a) depicts a classical mixture of hexagonal
555 and cubic crystal structures, while the corresponding TEM micrographs (Figure S 6b) show a
556 polydisperse set of sub-50 nm nanoparticles (size distribution is given in Figure S 6c). The oleic
557 hydrophobic coating is shown by DTA analysis (red profile in Figure S 6e). Given their
558 hydrophobic coating, they are hardly water-dispersible, unless sonication is applied. In this
559 case, the UCNP powder mix with water but it precipitates within the order of minutes, as shown
560 by both light scattering (Figure 9a) and photoluminescence spectroscopy (Figure 9b,c). When
561 the UCNP are added to a GL solution and are submitted to a *manual pH jump (approach 2)*, we
562 find a prompt dispersibility in water and an enhanced colloidal stability. Combination of light
563 scattering (Figure 9a, sensitive to both nanoparticles and vesicles), with photoluminescence
564 spectroscopy (Figure 9b,d, only sensitive to UCNP), show stability of the encapsulated UCNP
565 in solution up to one hour. The stability is also confirmed by photoluminescence spectroscopy
566 data recorded on a series of centrifuged samples: encapsulated UCNP emit significant amount
567 of light up to 2000 rpm for as long as 1 min of centrifugation time (Figure 9e). These results
568 are independent of the nature of doping element (Er or Tm). These data are also completed by

569 XRD and DTA results (Figure S 6d,e) showing that encapsulation does not affect the crystalline
570 structure of the UCNP and, most importantly, that the passivating hydrophobic layer is not
571 exchanged with GL. The typical DTA signature of GL (black curve in Figure S 6e) is different
572 than the signature of the hydrophobic coating in both as-synthesized and encapsulated UNCP
573 samples (Figure S 6e).

574 These experiments show that hydrophobic, aggregated, UCNPs can easily be dispersed
575 in an aqueous environment assisted by sonication followed by exposure to GL and employing
576 the phase change method. Absence of oleic-GL ligand exchange shown by DTA indicates that
577 the stabilization process mainly occurs by their encapsulation in the GL vesicles, as shown by
578 cryo-TEM (arrows 1 and 2 point at encapsulated NaYF₄:Yb/Er particles in Figure 9f,g). The
579 colloidal stability, tested here up to one hour, is impressive if compared to the control system
580 composed of oleic acid coated UCNP in water, although less long-lasting than what we have
581 found for iron oxide NPs. At the moment, we do not have a clear explanation for this, but the
582 hydrophobic coating should certainly favour phase separation more than the bare surface of
583 iron oxide NPs, and cryo-TEM data (arrow 3 in Figure 9g) also shows that it is not uncommon
584 to find aggregated UCNP clusters outside of the vesicles.



585

586 **Figure 9 – Sedimentation of static (0 rpm) GL-free and GL-encapsulated NaYF₄:Yb/Ln (Ln= Er or Tm)**

587 **UCNP samples measured by a) light scattering ($\lambda = 633$ nm, $\theta = 90^\circ$, constant shutter opening, two replica**

588 **are presented) and b) photoluminescence spectroscopy ($\lambda_{exc} = 980$ nm), of which the time evolution of the**

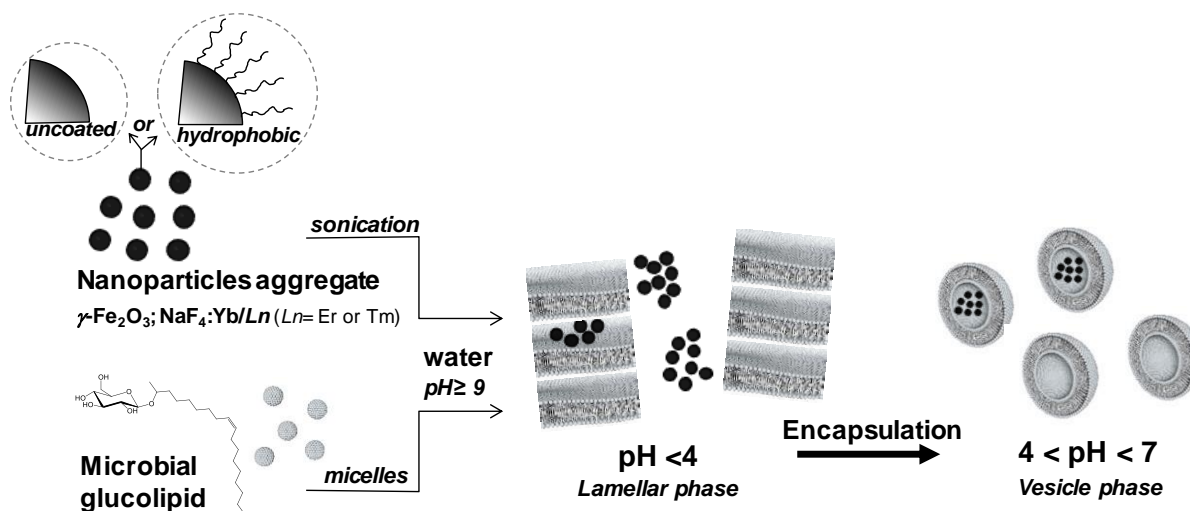
589 emission spectra for free sedimentation of NaYF₄:Yb/Er are given in c) (nanoparticles in water) and d)
590 (encapsulated nanoparticles). Pictures in a) correspond to irradiated ($\lambda_{\text{exc}}= 980 \text{ nm}$) NaYF₄:Yb/Er UCNP
591 dispersed in GL vesicles (top picture) and cyclohexane (bottom image). e) Time evolution of the
592 photoluminescence emission ($\lambda_{\text{exc}}= 980 \text{ nm}$) of encapsulated NaYF₄:Yb/Er UCNP with forced sedimentation
593 (centrifugation at 2000 rpm). f-g) Typical cryo-TEM image of an encapsulated (arrows 1, 2) and non-
594 encapsulated (arrow 3) NaYF₄:Yb/Er sample
595

596 The results presented in this work are summarized in Figure 10. Non-equilibrium
597 entrapment of metastable functional nanoparticles can occur via the same lamellar-to-vesicle
598 phase change process assisted by sonication. In this work, we successfully tested the
599 encapsulation of bare (uncoated) iron oxide NPs and oleic acid coated UCNP. Iron-loaded
600 ferritin nanocages were also encapsulated with the goal of understanding the origin of the
601 uneven distribution of the colloids within the vesicular compartments, in comparison to the
602 Luisi's encapsulation experiments.¹³ However, these preliminary data suggest that stable
603 colloids could also be encapsulated through the same method. As a general remark, it seems
604 that this approach could be potentially extended to a broad class of nanocolloids both stable and
605 unstable in water, including those bearing a hydrophobic coating. This is an interesting point
606 compared to the literature, where efficient encapsulation in the vesicle lumen commonly
607 requires surface stabilization of the nanoparticles via hydrophilic coatings, while hydrophobic
608 coatings tend to partition the nanoparticles in the lipid bilayer.^{16,57} In terms of encapsulation
609 efficiency, the highest standards in the literature settle around 50%, but with the clear distinction
610 between hydrophilic and hydrophobic colloids, respectively encapsulated in the lumen and
611 bilayer.^{16,57} An interesting work by Amstad et al.⁵⁶ shows that iron oxide nanoparticles bearing
612 a stable catechol-based hydrophobic coating partition in the bilayer membrane with an
613 encapsulation efficiency of about 30%, while labile carboxylic hydrophobic induce
614 uncontrolled NPs aggregation outside the vesicles and stable catechol-based hydrophilic
615 coatings induce encapsulation in the lumen. Uncoated NPs aggregates can actually be
616 encapsulated in the lumen, but the efficiency is very low (< 10%) compared to the use of stable
617 colloids bearing a hydrophilic coating and for which efficiency can reach 50%.⁶⁰

618 Our data collected with the phase-change method using GL show that the encapsulation
619 efficiencies in the lumen can reach values as high as 70% on uncoated iron oxide NPs
620 aggregates (estimated through gravimetric methods), or about 50% (after 1 h) using
621 hydrophobic oleic acid coated UCNP (estimated with emission luminescence spectroscopy).
622 The values settle among the highest found in the literature and they also show that the

623 sonication-assisted phase change method of encapsulation in the lumen can be easily applied to
 624 a broad range of as-prepared colloids, for which the classical steps involving the tedious ligand
 625 exchange process could be possibly avoided. Nonetheless, despite the fact that our data do not
 626 suggest that surface chemistry has a major impact on the vesicle-formation process from the
 627 lamellar phase above pH 4, we cannot exclude an impact on the other features, like the
 628 encapsulation efficiency, the NPs distribution within the vesicles or effects on the vesicle size
 629 and stability, as found by others.⁵⁶ Furthermore, the average encapsulation efficiency found
 630 here settles around 60% with very broad variability ($\pm 16\%$). It is also not excluded that the
 631 latter could be influenced by the surface chemistry of the nanocolloids but also by the process
 632 of bringing the NPs out of equilibrium through sonication, a parameter of which the role should
 633 be better explored in future work. Finally, for realistic applications, the stability of the GL
 634 vesicles in a broader pH range, especially above pH 6-7, and in cell culture media must be
 635 studied in more details.

636



637

638 **Figure 10 – NPs encapsulation into vesicles: the pH jump method used in this work start from a glucolipid**
 639 **solution above pH 9 (micellar) and then lowered to pH < 4 (lamellar). Sonication assists dispersion of NPs,**
 640 **which coexist with the micellar and lamellar phase, according to the pH value. The cartoon at pH< 4 shows**
 641 **that clusters of NPs coexist with the lamellar phase, although exact positioning of the clusters (intra or**
 642 **interlamellar) is still unclear. The lamellar-to-vesicle transition occurs above pH 4 and it is responsible for**
 643 **the encapsulation process, resulting a small fraction of heavily-loaded vesicles.**

644

645 Conclusion

646 This paper shows the possibility to form magnetoliposomes from a biobased lipid source
 647 simply using pH as a physico-chemical trigger in water at room temperature. The vesicle-
 648 forming properties of a new family of microbial glycolipids is exploited to encapsulate

649 magnetite (γ -Fe₂O₃) NPs within glucolipid (GLs) vesicles. More precisely, acidic C18:1 GLs,
650 produced by *S. bombicola* strain *AugtB1* are compounds constituted by a β -D-glucose
651 headgroup linked through a glycosidic bond to the subterminal carbon (C17) of hydroxylated
652 oleic acid, thus leaving a free-standing COOH group. As shown by SAXS and cryo-TEM, this
653 compound mainly forms micelles at neutral to basic pH, vesicles between pH 4 and pH 6 and a
654 lamellar phase below pH 4. Direct exploitation of the lamellar-mediated vesicle-forming
655 mechanism when the pH is increased back from below 4 to pH 6 leads to an encapsulation of
656 NPs simultaneously dispersed in the GL solution. Cryo-TEM shows the presence of few but
657 densely-crowded magnetoliposomes. We have repeated the encapsulation experiment using
658 iron-loaded ferritin (Fe4800)AfFtn and we do obtain analogous results: very few heavily loaded
659 vesicles coexisting with empty ones. A similar distribution profile was reported for ferritin
660 nanocages encapsulated within the lumen of liposomes obtained from standard phospholipids
661 using more classical embedding approaches (ethanol injection, thin film hydration). The
662 analogy between our data and the literature for the specific ferritin system suggests that the
663 uneven distribution of NPs within vesicles found in this work does not depend on our conditions
664 of work, but it seems to be a more general, yet misunderstood, phenomenon.

665 In our experiments, we evaluate an encapsulation efficiency in the lumen of about 60%,
666 a value which settles among the highest in the literature, especially if compared to the use of
667 either uncoated or hydrophobic nanoparticles, whereas the former have shown to be
668 encapsulated in the lumen but with efficiencies below 10% and the latter are generally
669 encapsulated in the lipid bilayer. Nonetheless, variability is quite high ($\pm 16\%$), a fact which
670 can depend both on the surface chemistry of the NPs and, above all, on the sonication step
671 required to bring the NPs out of equilibrium. Although at the moment it is not clear how to
672 achieve a precise control of the encapsulation efficiency, we were able to control the average
673 diameter between tens and several hundred of nanometers by varying the encapsulation process,
674 still keeping the same pH change approach. Sonication was found to provide the smallest
675 vesicle size while controlled pH change provides the largest vesicles. The extrusion
676 experiments, besides stabilizing the average vesicle diameter around 100 nm, nicely
677 demonstrate the high stability of the magnetoliposomes towards multiple extrusion cycles.

678 Besides unprotected iron oxide nanoparticles, using the simple phase change
679 encapsulation process in the presence of GL, we were able to encapsulate and stabilize two
680 upconverting sub-50 nm nanoparticles systems composed of oleic-acid coated NaYF₄:Yb/Ln
681 (Ln= Er or Tm). These water-insoluble materials could be dispersed in water and stabilized up
682 to 50% (according to luminescence emission spectroscopy) in the order of one hour under static

683 sedimentation conditions, and in the order of minutes under centrifugation at 2000 rpm. These
684 results suggest that the present method of encapsulation in the lumen may be applied to a broad
685 range of colloids, for which typical, but tedious, ligand exchange steps could be potentially
686 avoided.

687

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704

705 **Keywords**

706 Biosurfactants; Encapsulation; Glycolipids; Magnetoliposomes; Vesicle

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Supporting Information

Figure captions

Figure S 1 - TEM images of bare iron oxide NPs, NPs-SLs at pH 6 (NPs SL pH6, sample S5) and NPs- GLs at pH 9 (NPs GL pH9, sample S2). Solutions have been dried directly on the TEM grid before observation.

Figure S 2 - a) TEM images of NPs GL samples at pH 6 (sample S3). b-d) TEM images of sample S4 (NPs GL, pH 2 → pH 6). Solutions are dried on the TEM grid before observation. The Fourier Transform (FT) performed on the rectangle-highlighted region has been denoised (bottom left image c)) to better put in evidence the crystalline network in the inverse FT image (bottom right d)).

Figure S 3 - Cryo-TEM images of NPs GL samples at pH below 4 adjusted from pH 9

Figure S 4 – Cryo-TEM images of a NP-free GC18 :1 solution at 0.5 w% in water at the final pH 6, after treating the solution at pH 9 → pH 2 → pH 6, by adding few μ L of 1M HCl and 0.1 M NaOH

Figure S 5 – Qualitative illustration of the encapsulation efficiencies for different batches. a) Control: GL vesicles in water at pH 6 next to a neodymium magnet; b) Typical image of a succesful encapsulation event with encapsulation efficiency lower than 50%. c) Typical image of a succesful encapsulation event with encapsulation efficiency higher than 50%.

Figure S 6 – a) XRD, b) TEM and c) size distribution (from TEM) of NaYF₄:Yb/Ln (Ln= Er or Tm) nanoparticle samples used in this work. d) Typical XRD pattern of NaYF₄:Yb/Er encapsulated in GL vesicles after *manual pH jump (approach 2)*. The sample has been freeze-dried before characterization. e) DTA profiles of NaYF₄:Yb/Er, encapsulated (in GL) NaYF₄:Yb/Er and free GL. All NaYF₄:Yb/Ln samples are stabilized by an oleic acid coating.