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Supplementary Material

Lipidic mesophase with tunable release properties for local delivery of macromolecules: Apoferritin nanocage, a case study

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Small Angle X-ray Scattering measurements and analysis

SAXS measurements were used to determine the symmetry and unit cell dimensions of the lipidic cubic phases.

Measurements were performed at 37 °C, and the samples were equilibrated for 10 min prior to measurements, while the scattered intensity was collected over 30 min.

A triply periodic minimal surfaces arguments [1] was used to calculate the structural parameter of the phases. Briefly, following determination of the lattice parameter (a) by SAXS, the length of the lipid chain (L_{lip}) was calculated according with the following equation [2]:

$$\phi = 2A_0 (L_{lip}/a) + 4/3\ \pi\ \chi\ (L_{lip}/a)^3$$

Where A_0 and χ are respectively the ratio of the area of the minimal surface in a unit cell to (unit cell volume) $^{2/3}$ and the Euler–Poincaire characteristic whereas ϕ is the lipid volume fraction, which was calculated from the water intake (water content at maximum hydration level) measured by gravimetric method for each formulation (see Table S2) and the density of lipids.

A_0 and χ have the following values, depending on the specific cubic phase:

$A_0 = 3.091$ and $\chi = -8$ for Ia3d

$A_0 = 1.019$ and $\chi = -2$ for Pn3m.

$A_0 = 2.345$ and $\chi = -4$ for Im3m3.

Therefore, one can derive the diameter of the water channels (d_W) by:

$d_{W\ pn3m} = (0.391\ a - L_{lip}) \times 2$

$d_{W\ Ia3d} = (0.248\ a - L_{lip}) \times 2$

$d_{W\ Im3m} = (0.305\ a - L_{lip}) \times 2$

All the obtained information from SAXS experiments is summarized in Table S1.

Table S1. Obtained structural parameters from SAXS experiments. All the formulation were measured in excess of water (70% w/w), while the ratio between the lipids are reported as molar percentage. The protein loaded samples (+ ApoF) contain 1% *w/w* of ApoF (1 mg/100 mg gel)

Formulations	**Phase**	**a (Å)**	**d_w (nm)**
MO	Pn3m	87	3.2
MO + ApoF	Pn3m	100	4.2
MO/ 5% Chol	Pn3m	99	4.1
MO/ 5% Chol + ApoF	Pn3m	120	5.7
MO/ 10% Chol	Pn3m	110	5.0
MO/ 10% Chol/ + ApoF	Pn3m	125	6.1
MO/ 2% DOPS	Im3m	145	5.2
MO/ 2% DOPS + ApoF	Im3m	170	6.7
MO/ 5% DOPS	Im3m	300	14.7
MO/ 5% DOPS + ApoF	Im3m	320	15.9
MO/ 5% DOPS/ 5% Chol	Im3m	365	18.6
MO/ 5% DOPS/ 5% Chol + ApoF	Im3m	385	19.8
MO/ 5% DOPS/ 10% Chol	Im3m	410	21.4
MO/ 5% DOPS/ 10% Chol + ApoF	Im3m	417	21.8
MO/ 2% DOPG	Im3m	142	5.0
MO/ 2% DOPG+ ApoF	Im3m	160	6.1
MO/ 5% DOPG	Im3m	300	14.7
MO/ 5% DOPG+ ApoF	Im3m	314	15.5
MO/ 5% DOPG/ 5% n Chol	Im3m	345	17.4
MO/ 5% DOPG/ 5% Chol + ApoF	Im3m	355	18.0
MO/ 5% DOPG/ 10% Chol	Im3m	370	18.9
MO/ 5% DOPG/ 10% Chol+ ApoF	Im3m	385	19.8
MO/ 2% DOTAP	Ia3d	140	3.3
MO/ 2% DOTAP + ApoF	Ia3d	165	4.5
MO/ 5% DOTAP	Ia3d	211	6.8
MO/ 5% DOTAP + ApoF	Ia3d	230	7.8
MO/ 5% DOTAP/ 5% Chol	Ia3d	301	11.3
MO/ 5% DOTAP/ 5% Chol + ApoF	Ia3d	320	12.2
MO/ 5% DOTAP/ 10% Chol	Ia3d	350	13.7
MO/ 5% DOTAP/ 10% Chol + ApoF	Ia3d	370	14.7

Hydration study.

Gravimetric analysis were carried out to evaluate the exact amount of water in the fully hydrated and swollen bicontinuous cubic phases. After 72 h of equilibration the gels (70 % *w/w* lipids and 30 % *w/w* 1/3 PBS) were transferred in a tared metal basket, which was immersed in excess of 1/3 PBS. The basket was kept at 37 °C for 1 week to allow total hydration and equilibration; afterward, the basket was extracted from the media, the excess water removed, and the mesophase weighed to assess the final water intake. The percentage of water intake was calculated by the following equation:

Total % of water intake = $[((W_1 - W_0) + V_h)/ W_0] \times 100$

Where V_h is the hydration volume (30 mg), W_0 is the initial gel weight in to metal basket before submersion in the media and W_1 is the weight of the gel after 1 week of immersion in the media. The results are summarized in table S2.

Moreover, the maximum hydration level of the formulation containing 5% of DOPS and 10% of Chol (highest water uptake and biggest water channel) was also carried out by SAXS experiments. The prepared mesophase were hydrated with 45, 50, 55, 60, 62, 65 and 70% w/w of ApoF solution in PBS 1/3 and then was measured by SAXS as described in the method section. The maximum hydration level for this formulation was obtained with 60 % *w/w* aqueous phase, in agreement with the gravimetric measurement.

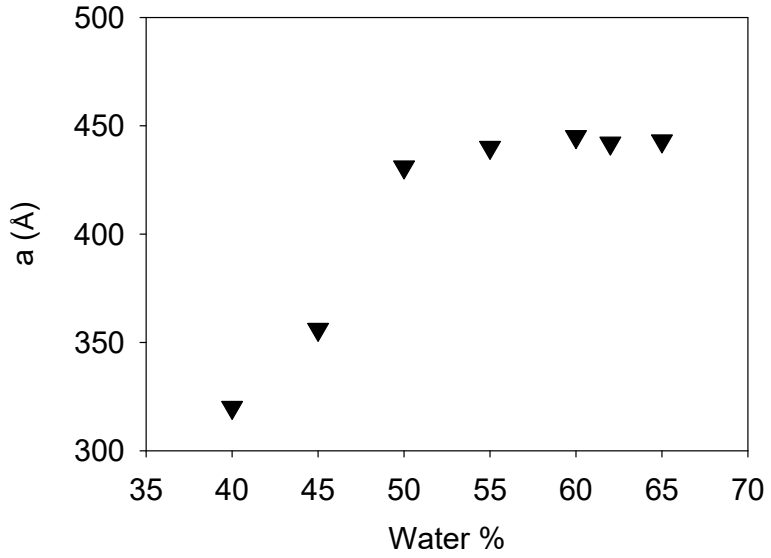

Figure S1. Lattice parameters obtained from SAXS spectra for LMPs composed by MO with 5% DOPS and 10 % Chol hydrated with increasing percentage (w/w) of ApoF solution in PBS 1/3.

Table S2. Maximum Water Intake obtained from gravimetric experiments. Mean ± standard deviation (SD) (n = 3).

Formulation	Total % of water intake (wt %)
MO	38 ± 3
MO/ 5% Chol	42 ± 4
MO/ 10% Chol	43 ± 2
MO/ 2% DOPS	46 ± 3
MO/ 5% DOPS	50 ± 4
MO/ 5% DOPS/ 5% Chol	55 ± 3
MO/ 5% DOPS/ 10% Chol	61 ± 5
MO/ 2% DOPG	45 ± 3
MO/ 5% DOPG	51 ± 3
MO/ 5% DOPG/ 5% Chol	54 ± 3
MO/ 5% DOPG/ 10% Chol	59 ± 4
MO/ 2% DOTAP	42 ± 3
MO/ 5% DOTAP	45 ± 2
MO/ 5% DOTAP 5% Chol	51 ± 1
MO/ 5% DOTAP / 10% Chol	55 ± 4

ApoF calibration curve.

The calibration curve of the protein was carried out by fluorescence spectroscopy exploiting the fluorescence of tryptophan (λ_{em} 320–430 nm and λ_{ex} at 295 nm) using an Infinite 200 Pro F-Plex plate reader (Tecan, Männedorf, Switzerland).

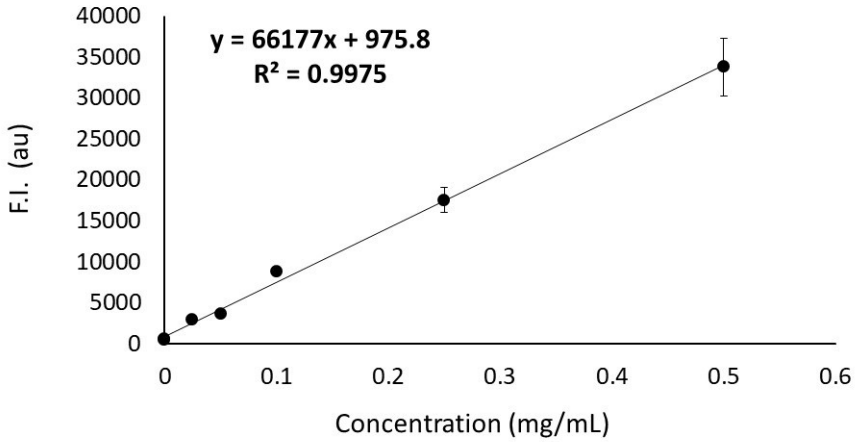

Figure S2. ApoF calibration curve obtained at 37 °C.

Dynamic Light Scattering (DLS)

Particle size and ζ potential determinations of the ApoF were performed immediately after the preparation of protein solution and after the release experiments. Litesizer 500 (Anton Paar, Graz, Austria) equipped with a 175° backscatter angle detector and a semiconductor laser with λ = 658 nm was used. Briefly, the protein solutions were transferred into disposable semi-micro cuvettes and after equilibrating the sample at 37 °C, the measurement was performed (10 runs x 30 seconds for each sample). The zeta potential was determined by means of continuously monitored phase-analysis light scattering (cmPALS) in an Omega cuvette (Anton Paar, Graz, Austria) where a refractive index of 1.33 and a viscosity of 0.89 mPa/s was set for the solvent. The intensity size distribution of the ApoF was unimodal in the case of freshly prepared solution therefore the autocorrelation function was analyzed according to the cumulant method and the width of the DLS hydrodynamic diameter distribution is indicated by the PDI (polydispersion index). Whereas in the case of samples measured after the release experiments a bimodal distribution was observed and thus a CONTIN method was used by the Kalliope™-software (Anton Paar).

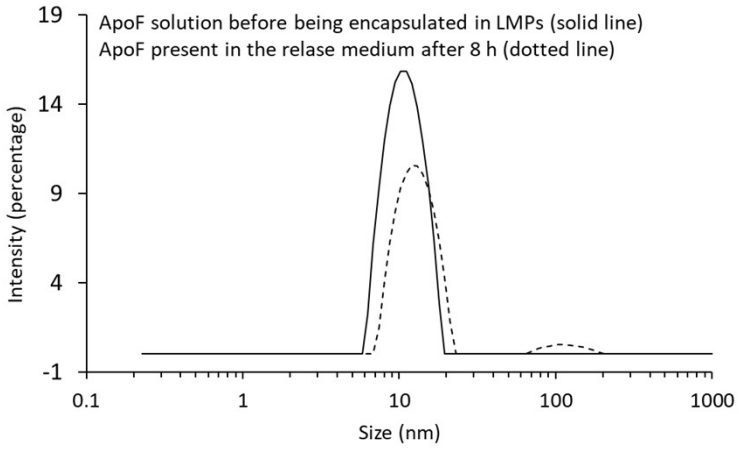

Figure S3. ApoF size distribution profile. ApoF solution measured by DLS before being encapsulated in LMPs (solid line; PDI 0.170) and after the release experiments (Release medium after 8 h, dotted line).

Release rate from MO/ DSPG gel.

A swollen gel with Ia3d symmetry was produced by using a lipid mixture reported in literature [3]. Briefly, a lipidic mixtures of monoacylglycerol monopalmitolein (MP) with 8% *w/w* of distearoyl phosphatidylglycerol (DSPG) (total lipid amount: 25 mg) were prepared by co-dissolving the appropriate volume amounts of lipids stock solutions in chloroform. Solvent was then completely removed and the obtained dried lipids mixture were hydrated by mixing weighed quantities of a protein solution in 1/3 PBS achieving 1% w/w of ApoF loaded-gel (1 mg ApoF/100 mg gel). After 72 h of equilibration, the release experiment was carried out as described in the method section.

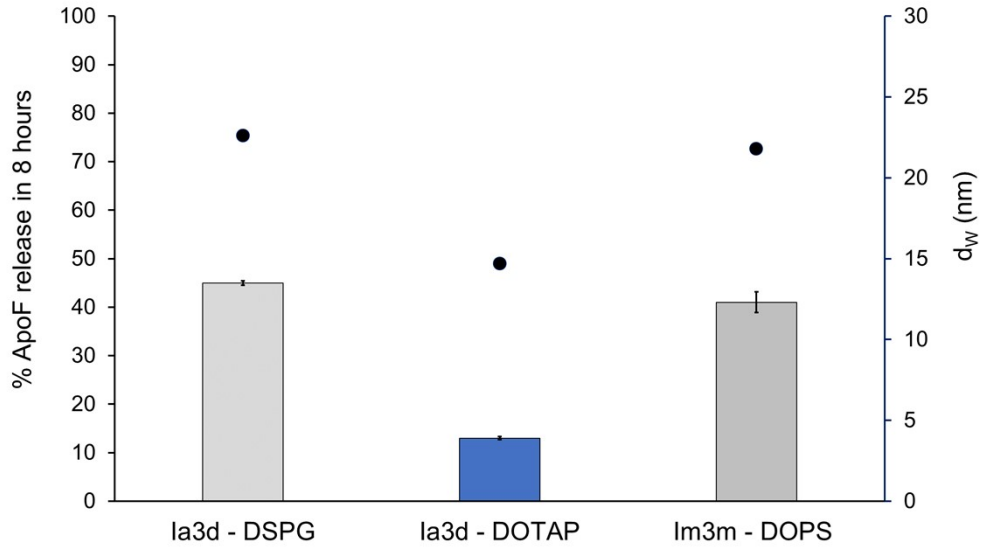

Figure S4. ApoF release rate after 8 h (reported as bar plot; left axis) and d_W (black circles; right axis) from LMP obtained by MP doped with 8% w/w of DSPG (Ia3d), LMPs obtained by MO doped with 5% DOTAP and 10% Chol (Ia3d) and LMPs obtained by MO doped with 5% DOPS and 10% Chol (Im3m). Mean ± standard deviation (SD) (n = 3). The reported d_W value for the MP/ DSPG formulation was taken from literature [3].

Model and fitting parameters.

ApoF release rates from the gels were fitted by using Higuchi and Weibull model.

Table S3. Obtained R^2 for all the fitting shown in figure 4. The release rate for the DOTAP based-gels were fitted by using either Weibull or Higuchi model

Formulation	Higuchi R^2	Weibull R^2
MO	0.9739	
MO/ 5% Chol	0.9693	
MO/ 10% Chol	0.9948	
MO/ 5% DOPS	0.967	
MO/ 5% DOPS/ 5% Chol	0.9881	
MO/ 5% DOPS/ 10% Chol	0.9925	
MO/ 5% DOPG	0.984	
MO/ 5% DOPG/ 5% Chol	0.9906	
MO/ 5% DOPG/ 10% Chol	0.9885	
MO/ 5% DOTAP	0.8971	0.9970
MO/ 5% DOTAP/ 5% Chol	0.9177	0.9980
MO/ 5% DOTAP/ 10% Chol	0.9101	0.9973

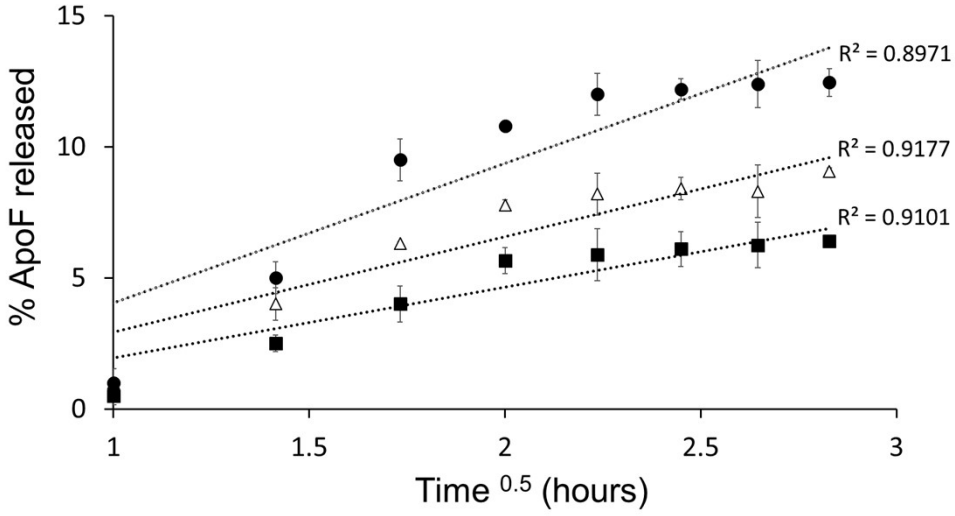

Figure S5. ApoF release rate from gels composed by MO doped with DOTAP plotted against square root of time. 5% DOTAP (black squares), 5% DOTAP and 5% Chol (white triangles), 5% DOTAP and 10% Chol (black circles). Mean ± standard deviation (SD) (n = 3).The R^2 obtained for all the fitting are reported in the table S2.

Release experiment at different water percentage

The entrapment efficacy percentage (EE%) and the release experiments were also performed in case of the gel composed of MO with 5% of DOPS, together with 10% of Chol. The gels were hydrated with 50, 60 and 70% *w/w* PBS 1/3 solution containing ApoF.

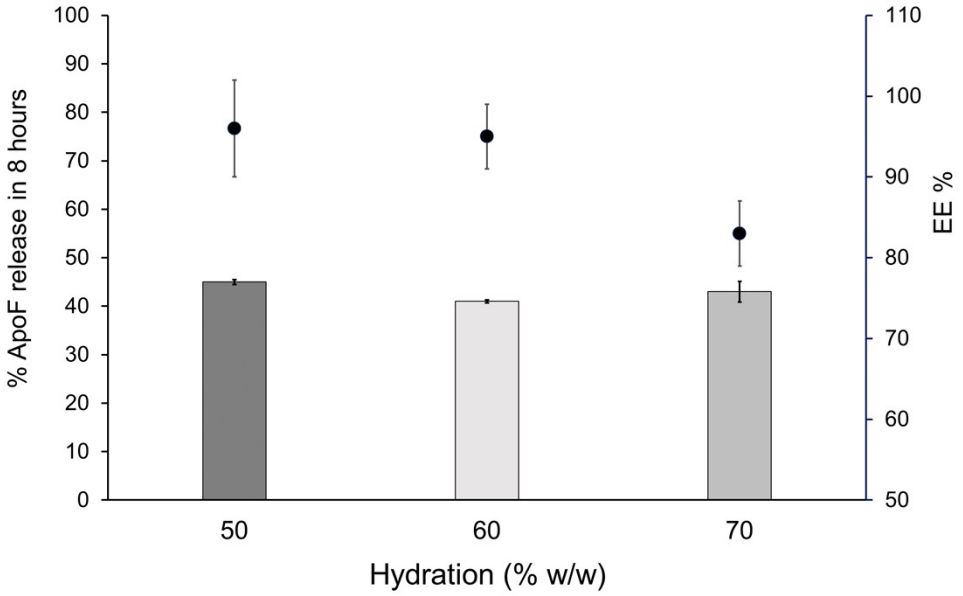

Figure S6. Protein released in 8 hours (reported as bar plot; left axis) and ApoF EE % (black circles; right axis) from LMPs hydrated with 50, 60 and 70% *w/w* of ApoF solution in PBS 1/3. Mean ± standard deviation (SD) (n = 3).

Release experiment using a basket

At the day of experiment, the LMPs gel (40*% w/w* lipids, 60 % *w/w* aqueous phase containing ApoF) was weighed and then transferred in a homemade metal basket (*ca.* 200 mg/basket), which was then immersed in 40 mL of release media (PBS at pH 7.4). The release media was removed periodically after 1, 2, 3, 4, 5, 6, 7 and 8 h, concentrated 10'000 times for spectroscopic determination of the ApoF and replaced with the 40 mL of fresh solution. The concentration of the protein was evaluated by fluorescence spectroscopy exploiting the fluorescence of tryptophan (λ_{em} 310 nm and λ_{ex} at 295 nm) using an Infinite 200 Pro F-Plex plate reader (Tecan, Männedorf, Switzerland) and a calibration curve (see SI; Figure S2).

Rheological experiments

A stress-controlled rheometer (Modular Compact Rheometer MCR 72 from Anton Paar, Graz, Austria) was used in cone-plate geometry, 0.993° angle, and 49.942 mm diameter. The temperature control was set at 25 °C. First, a strain sweep was performed at 1 Hz between 0.008 and 200% strain to determine the linear range. Then, oscillatory frequency sweeps were performed at 0.1% strain between 0.013 and 628 rad/s. Measurement of the storage G′ and loss G″ moduli as a function of the shear frequency, ω, can reveal important information about the characteristic behaviour of viscoelastic materials. Frequency sweep measurements were performed at a constant strain in the linear viscoelastic regime (LVR), as determined by the oscillation strain sweep (amplitude sweep) measurement performed for each sample. Within the linear viscoelastic region, in fact, the material response is independent of the magnitude of the deformation and the material structure is maintained intact; this is a necessary condition to accurately determine the mechanical properties of the material.

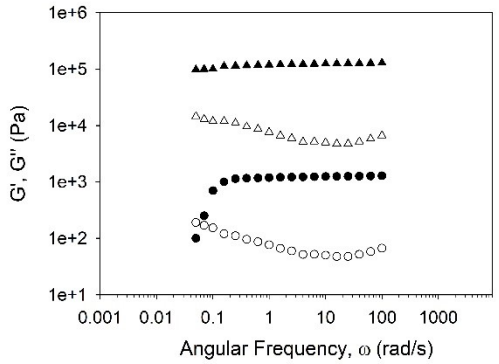

Figure S7. Storage, G′ (black symbols), and loss, G″ (white symbols), moduli as a function of shear frequency of cubic phases composed of MO/W (triangles) and 5% DOPS/10% Chol (circles)

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