SUPPORTING INFORMATION

COSAN-stabilised omega-3 oil-in-water nanoemulsions to prolong lung residence time for poorly water soluble drugs.

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**General reagents and methods**

**Reagents**

Cesium salt of bis(1,2-dicarbollide)cobaltate (COSAN) was purchased from Katchem Ltd. (Prague, Czech Republic). Docosahexaenoic acid oil (DHA) (80%) was kindly provided by SendaBio. 3-methoxymethyl-16β,17β-epiestriol-O-cyclic sulfone (MMSE) was purchased from ABX (Advance Biomedical Compounds). Illustra NAP TM-5 columns were purchased from GE Healthcare Life Sciences. Ultrapure water was obtained from a Milli-Q A10 Gradient equipment (Millipore). Iodine, 17β-estradiol and 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo(8.8.8)hexacosane (Kryptofix, K2.2.2) were purchased from Sigma Aldrich Chemical. Phosphate-buffered saline (PBS) and HPLC grade ethanol, dichloromethane and acetonitrile were purchased from Scharlab (Sentmenat, Barcelona, Spain).

**Dynamic Light Scattering (DLS)**

DLS analyses were conducted using a Zetasizer Nano ZS, ZEN3600 Model (Malvern Instruments Ltd). All measurements were performed in disposable sizing cuvettes at a laser wavelength of 633 nm and a scattering angle of 173°. Zeta-potential measurements were performed in disposable zeta potential cells (pH 7.2, 25°C). Samples were dispersed at a concentration of 2 mg of oil droplets/mL in ultrapure water for size and in 1 mM phosphate buffer saline for zeta-potential. Measurements were performed in triplicate.

**Laser diffraction (LD)**

LD analyses were conducted using a Mastersizer 3000 laser diffraction system (Malvern Instruments Ltd). All measurements were performed at a laser wavelength of 430 nm using 600 mL of ultrapure water. Each measurement was performed 5 times (5 seconds per run) in ultrapure water at 1500 rpm and 25°C following Mie dispersion model.

**Cryo-TEM Analysis**

Cryo-TEM analysis of (o/w)-emulsions were performed on a JEM-2200FS/CR (JEOL Europe, Croissy-sur-Seine, France) transmission electron microscope. This microscope is equipped with a field emission gun (FEG) operated at 200 kV and an in-column Ω energy filter.
For sample preparation, freshly glow-discharged 200-mesh grid (R2.2 200 Mesh; QUANTIFOIL) was placed inside the chamber of a Vitrobot Mark III (FEI Company, USA), which is maintained at 8˚C temperature and relative humidity close to saturation (85% rH). Four microliters of non-diluted sample were dropped onto the grid for 15 seconds. After incubation, most of the liquid on the grid was removed by blotting (blot time was 3 seconds, number of blots was set to 1, drain time was zero and blot offset was 1 mm) with absorbent standard Vitrobot filter paper (Ø55/20mm, Grade 595, Thermo Fisher Scientific - FEI). After the blotting step, the grid was abruptly plunged into a liquid ethane bath, previously cooled with liquid nitrogen at approximately -170 ºC. Once the specimen was frozen, the vitrified grid was removed from the plunger and stored under liquid nitrogen inside a cryo-grid storage box.

During imaging, no-tilted zero-loss two-dimensional (2D) images were recorded under low-dose conditions, utilizing the ‘Minimum Dose System (MDS)’ of Jeol software, with a total dose on the order of 10-20 electrons/Å² per exposure, at defocus values ranging from 1.5 to 4.0 µm. The in-column Omega energy filter of the microscope helped us to record images with improved signal-to-noise ratio (SNR) by zero-loss filtering, using an energy selecting slit width of 30 eV centred at the zero-loss peak of the energy spectra. Digital images were recorded on a 4K × 4K (15 µm pixels) Ultrascan4000™ charge-coupled device (CCD) camera (Gatan Inc.) using DigitalMicrograph™ (Gatan Inc.) software, at different nominal magnifications from 4000× to 60000×. Number size distribution was achieved from several micrographs using an automatic image analyser (ImageJ). 500 droplets were selected for the analysis.

**Absorbance analysis**

Absorbance analyses were conducted using a Synergy™ HT Multi-Detection Microplate Reader (Bio-tek Instruments). All measurements were performed in disposable 96 well-plates at λ = 405 nm.

**Liquid-Liquid Interfacial Tension (IT) analysis by reverse pendant drop method**

Interfacial Tension (IT) analyses were conducted by reverse pendant drop method using the Attension Theta Flex optical tensiometer from Biolin Scientific. The liquid with a lower density (i.e. DHA oil) was dispensed using a hooked needle (C205/C205A/C201, Biolin
Scientific) for oil drop generation. The liquid with a higher density (i.e. aqueous phase) was placed in a GC10 glass cell (Dataphysics instrument GmbH). The drop was captured using a CCD camera at 3.5 fps. Data treatment was performed in One-Attension commercial software using Surface tension (Young-Laplace) analysis mode.

**Effect of the amount of COSAN on the size distribution of DHA emulsion droplets**

In order to determine the optimal amount of COSAN for the formation of the NEs, experiments using different amounts of this compound (1-8 mg) were carried out. In brief, the corresponding amount of COSAN (1-8 mg) was introduced in an 8 mL glass vial containing ultrapure water (3.6 g). DHA (oil phase; 400 mg, 425 µL) was added, and the emulsion was then formed by sonication (0°C, under stirring) using an UP400S (Hielscher) system at 100% of amplitude and pulse during 4 minutes (400 W) with a H3 sonotrode tip (3 mm diameter, 100 mm length).

A fraction of the final emulsion (500 µL) was purified by size exclusion chromatography (SEC) using an Illustra NAP TM-5 column (**Fig. S1**), and the collected fraction was diluted with ultrapure water to a final volume of 3 mL. A different fraction of the non-purified emulsion (500 µL) was directly diluted with ultrapure water to the same final volume (3 mL). The samples were freeze dried for water removal, diluted with dioxane/MeCN (80/20, 350 µL) and submitted to LD analysis (purified NEs; **Fig. S2**) and absorbance analysis (all samples; **Fig. S3** and Table S1).

**Figure S1.** Example of COSAN emulsion purification by SEC (red arrow indicates the presence of free COSAN as a yellowish solution inside the column). From left to right; initial collection of emulsion droplets, last collection of emulsion droplets and collection of traces without free COSAN.
**Figure S2.** Size distribution of NEs generated using different amounts of COSAN (1-8 mg) as determined by LD.

**Quantification of the amount of COSAN in the NEs.**

The amount of COSAN present in the NEs before and after purification was determined using absorbance analysis ($\lambda = 405$ nm). With that aim, a calibration curve was first generated by dissolving increasing amounts of COSAN (0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 mg/mL) in DHA/dioxane/MeCN (12.5/70/17.5) (Fig. S3).

**Figure S3.** Calibration curve obtained using increasing amounts of COSAN (0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 mg/mL) dissolved in DHA/dioxane/MeCN (12.5/70/17.5).
Quantification of the samples (Table S1) showed that the whole amount of COSAN remained inside the emulsion after SEC purification when 1-2 mg of COSAN were used for the preparation of the NEs, suggesting that all the COSAN is used to stabilize the oil droplet and located at the oil/water interface. As the solubility of COSAN in water is 0.5 mg/mL, all the emulsions produced with > 2 mg in the water phase represent an excess of COSAN in the aqueous phase. Still, quantification of the amount of COSAN in these NEs suggests that the majority of COSAN is incorporated in the NE.

Table S1. Amount of COSAN in the NEs before (non-purified) and after (purified) purification, as determined by absorbance analysis ($\lambda = 405$ nm). Values are expressed as mean ± standard deviation, n=3 per experimental scenario. Excess of COSAN in the aqueous phase is calculated by the difference between purified and non-purified emulsions.

| COSAN used for NE preparation / mg | 1     | 2     | 3     | 4     | 5     | 6     | 8     |
|-----------------------------------|-------|-------|-------|-------|-------|-------|-------|
| COSAN found in non-purified NE / mg| 0.84 ± | 1.98 ± | 2.92 ± | 3.34 ± | 3.95 ± | 5.90 ± | 7.75 ± |
| COSAN found in purified NE / mg    | 1.09 ± | 2.10 ± | 2.62 ± | 2.93 ± | 2.98 ± | 4.34 ± | 6.21 ± |
| COSAN excess in aqueous phase      | -0.25 | -0.12 | 0.31  | 0.40  | 0.97  | 1.56  | 1.53  |

**Determination of COSAN solubility in DHA oil**

COSAN (2 mg) was added to DHA (1 mL) and the mixture was heated at 50°C under continuous stirring. After 15 minutes, the presence of precipitate was confirmed by visual inspection, and the mixture was allowed to cool down to room temperature. After filtration, a small sample (50 µL) was diluted with Dioxane/MeCN (80/20, 350 µL) and submitted to absorbance analysis ($\lambda = 405$ nm) using the calibration curve shown in Figure S3. COSAN solubility was determined as 0.77 ± 0.05 mg/mL.

**Determination of interfacial tension**

Interfacial tension analysis was carried out using the pendant drop method (see Figure S4 for experimental set up). Experiments were carried out in three different scenarios: (i) DHA / water; (ii) DHA (COSAN-saturated) / water; and (iii) DHA (COSAN-saturated) / water (COSAN-saturated).
Determination of the amount of COSAN at the oil/water interface

Taking into account the hydrodynamic diameter of the NEs obtained via DLS (due to the high polydispersity of the oil droplets, this diameter was preferred compared to the number average diameter obtained by cryo-TEM), the amount of COSAN ($m_{\text{COSAN}}$) required to cover the total surface area of the droplet was calculated, assuming that the coverage of COSAN was around 2 nm$^2$ (ref 24 in the manuscript).

It is known that the total surface area of colloids in a dispersion can be calculated from the following equation:

\[ A_s = \frac{6m}{\rho \cdot D} \]

Where:
- \( m \) is the weight of particles or droplets. In our case it is the weight of oil emulsified (400 mg).
- \( \rho \) is the density of the particles. In our case the density of the oil, i.e. 0.93 g cm\(^{-3}\)
- \( D \) is the diameter of the oil droplet, here 170 nm

Knowing the area occupied by a single molecule of COSAN, i.e. \( A_c = 2 \text{ nm}^2 \), the number of COSAN \( (N) \) molecules required to cover all the total surface area of the emulsion droplet would be:

\[
N = \frac{A_s}{A_c}
\]

Finally, the weight of Cosan \( (M_c) \) required to cover the droplet of the emulsions can be calculated with the Avogadro number \( (N_a) \) and the molecular weight of the COSAN \( (M_w) \):

\[
M_c = \frac{N \ M_w}{N_a}
\]

Thus it was found that 6.5 mg of COSAN was required to cover the entire droplets, which is pretty similar to the amount of COSAN found after purification. So, these results suggest that most of the COSAN is located at the interface and also that the COSAN is adsorbed as monolayer at the oil/water interface.

**Stability of the NEs**

**Long term stability in water at different storing conditions (accelerated test conditions)**

COSAN-stabilized DHA-in-water emulsion was freshly prepared as described above (using 8 mg of COSAN) and 1 mL fractions were stored at 4 different conditions (A: light at r.t., B: dark at r.t., C: dark at 5 °C and D: dark at 40 °C) for 3 months. Emulsion stability at 1, 2 and 3 months was subsequently evaluated by visual inspection; the hydrodynamic diameter was determined by DLS and volume average diameter by LD (Fig. S5).
**Figure S5.** NE long-term stability at 3 different time points (1, 2 and 3 months) after storing at 4 different conditions (A: light at r.t., B: dark at r.t., C: dark at 5 °C and D: dark at 40 °C) as determined by LD.

**Stability in phosphate buffer saline (PBS)**

COSAN-stabilized DHA-in-water emulsion (330 µL) was diluted in PBS 1X (660 µL). The mixture was kept at rest at room temperature and lightness during 20 hours. Destabilization phenomena were evaluated by visual inspection at regular time points (Fig S6).

**Figure S6.** Visual inspection of the overtime stability of COSAN stabilised DHA-in-water emulsion in phosphate buffer saline (5 mM of phosphate)

**Radiochemistry**

**Synthesis of 16α-[\(^{18}\)F]Fluoroestradiol**

\(^{18}\)F]fluorine was produced by irradiation of \([^{18}\text{O}]\text{H}_2\text{O}\) with 18MeV protons using an IBA Cyclone 18/9 cyclotron. \(^{18}\)F]F\(^-\) was trapped in an Accell™ Plus QMA anion exchange resin (Waters) and eluted into a V-shaped vial using sequentially a solution of Kryptofix K\(_{2,2,2}\) in MeCN (15 mg/mL; 1 mL) and an aqueous K\(_2\text{CO}_3\) solution (6 mg/mL; 0.5 mL).
The solvent was evaporated to dryness via azeotropic distillation in two steps (80°C, 10 min; 100°C, 5 min) under helium flow. Subsequently, a solution containing 1 mg of 3-methoxymethyl-16β,17β-epiestriol-O-cyclic sulfone (MMSE) in anhydrous MeCN (1 mL) was added and the mixture was heated at 100°C for 10 min. After fluorination, a 0.1M HCl solution in MeCN (prepared by mixing 9 parts of MeCN and 1 part of 1 M HCl) was added and the solvent was evaporated to dryness at 100°C under helium flow. After repeating this operation and cooling to 40°C, the reaction crude was diluted with Water/MeCN (40/60, 2.5 mL) and the resulting mixture was eluted through a Sep-Pak C18 plus cartridge (Waters) and purified by high performance liquid chromatography (HPLC) using radioactive detection. A Nucleosil™ 100-7 C18 column (10 x 250 mm, 5 μm) was used as stationary phase and Water/MeCN (40/60) as the mobile phase at a flow rate of 5 mL/min. The desired fraction (retention time ~ 11.8 min, Fig. S7) was collected, diluted with water (40 mL) and reformulated by trapping in a Sep-Pak C18 Plus Short Cartridge (waters) and further eluting with dichloromethane (2 mL).

**Figure S7.** Chromatograms obtained during purification of [¹⁸F]FES. The peak with retention time of ca. 11.8 min in the radioactive chromatogram (top) corresponds to [¹⁸F]FES; bottom, UV profile.
Synthesis of Cs[8-I-3,3’-Co(1,2-C₂B₉H₁₀)(1’,2’-C₂B₉H₁₁)] (I-COSAN)

Iodine (0.83 g, 3.28 mmol) was added to a solution of cobalt bis(dicarbollide) (0.75 g, 1.64 mmol) in EtOH (10 mL). The reaction mixture was left to stand overnight at room temperature and was then heated under reflux for 2.5 h. The excess iodine was decomposed by the addition of a solution of Na₂S₂O₃ (0.42 g, 2.63 mmol) in water (8 mL) and the resulting mixture was boiled for 5 min. The mixture was concentrated until the precipitation of an orange solid was observed. The precipitate was filtered, washed with water and hexane, and dried under vacuum (0.58 g, 36 %). ¹H NMR (500 MHz, acetone-d₆): δ 4.54 [C–H, 2H, s], 4.29 [C–H, 2H, s], 2.83 [B–H, 17 H, m]; ¹¹B {¹H} NMR (500 MHz, acetone-d₆): δ 6.15 [1B, s], 3.19 [1B, s], 0.97 [1B, s], -2.01 [2B, d], -5.01 [5B, s], -5.74 [2B, s], -16.32 [2B, s], -17.77 [2B, s], -21.20 [1B, s], -23.40 [1B, s].

Synthesis of Cs[8-[¹²⁴I]-3,3’-Co(1,2-C₂B₉H₁₀)(1’,2’-C₂B₉H₁₁)] ([¹²⁴I]-COSAN) via Isotopic exchange.

Acetonitrile (200µL) was added to Na[¹²⁴I]I (50 µL, 44.8 MBq) and the resulting solution was introduced in a 2.5 mL conic vial. The solvent was evaporated to dryness (110°C, 30 min) and 1 mg of iodo-COSAN dissolved in acetonitrile (100 µL) was added together with trans-bis(acetate)bis[o-(di-o-tolylphosphino)benzyl] dipalladium (II) (Herrmann’s catalyst, HC, 0.1 mg, 0.101 µmol) dissolved in toluene (100 µL). The reaction mixture was heated at 100°C for 5 min, the solvent was removed under a constant helium flow and the resulting solid was dissolved in 0.5 mL of 0.1M ammonium formate (AMF)/acetonitrile (1/2), further diluted with water (1:100) and purified by and passing through a Sep-Pak C18 plus cartridge (Sep-Pak® Light, Waters). The retained compound was eluted with ethanol (3x500 µL). The solvent was finally evaporated to dryness. Quality control was performed by radio-HPLC (Fig. S8).
**Figure S8.** Chromatograms corresponding to the reaction crude during the production of $[^{124}]$I-COSAN by isotopic exchange reaction. The peak with retention time of ca. 13.5 min in the radioactive chromatogram (bottom) corresponds to $[^{124}]$I-COSAN; top, UV profile.

Analytical conditions were: Stationary phase: Mediterranea Sea18 column (4.6x150 mm, 5 µm particle size, Teknokroma, Spain); mobile phase A: 0.1M ammonium formate (AMF) buffer pH= 3.9; B: acetonitrile; flow rate = 1mL/min; gradient: 0 min: 60% A- 40% B; 2min: 60% A- 40% B; 6min: 20% A- 80% B; 14min: 0% A- 100% B; 16min: 0% A- 100% B; 18min: 60% A- 40% B; 20min: 60% A- 40% B (retention time: 13.5min).

**Preparation of labelled NEs (NE-$[^{18}]$FES and $[^{124}]$I-NE-FES)**

NE-$[^{18}]$FES and $[^{124}]$I-NE-FES were prepared following the procedure described above for non-labelled NEs, with minor modifications. For NE-$[^{18}]$FES, 50 µL of a trace solution of $[^{18}]$FES in dichloromethane were poured into the DHA phase and mixed before sonication. For $[^{124}]$I-NE-FES, a COSAN solution (containing 7 mg of COSAN) was spiked with $^{124}$I-labelled COSAN (1 mg of iodo-COSAN) in 3.6 mL of ultrapure water. Sonication and purification were carried out as above.

**In vivo experiments**

**General**
Animal handling was conducted in accordance with the European Council Directive 2010/63/UE. All experimental procedures were approved by the Ethical Committee at CIC biomaGUNE and local authorities.

**Aerosol administration**

Endotracheal insufflations were carried out using the Penn-Century MicroSprayer® Aerosolizer (FMJ-250 High Pressure Syringe Model, Penn-Century. Inc. Wyndmoor, USA; hereinafter referred to as “Penn-Century Aerosolizer”). Deep sedation was induced to the animals by inhalation of 5% isoflurane in pure O₂. The tip of the delivery needle was carefully positioned just above the carina and 50 μL (established by using a spacer in the syringe plunger) of injectable emulsion (amount of radioactivity: 1.85-9.32 MBq) was administered. A small animal Laryngoscope (Penn-Century, Model LS-2) was used for correct visualization of the epiglottis. Immediately after administration, rats were submitted to *in vivo* imaging.

**In vivo Imaging experiments**

Without recovering from sedation, animals were positioned in an eXploreVista-CT small animal PET-CT system (GE Healthcare, USA) to perform *in vivo* studies. During imaging, rats were kept normothermic using a heating blanket (Homeothermic Blanket Control Unit; Bruker). Anaesthesia was maintained by inhalation of 1.5-2% isoflurane in pure O₂. Dynamic PET images (energy window: 400-700 KeV) were acquired with the following frames: 4 x 15 s, 4 x 30 s, 3 x 60 s and 3 x 90 seconds. In all cases, four beds were defined to acquire whole body images (total acquisition time = 42 min). After each PET acquisition, a CT scan (X-Ray energy: 40 kV, intensity: 140 μA) was performed for a later attenuation correction in the image reconstruction and for unequivocal localization of the radioactivity. Random and scatter corrections were also applied to the reconstructed image (filtered back projection reconstruction algorithm), generating a 175x175x220 dimension image, with a 2 mm axial FWHM spatial resolution in the centre of the Field Of View (FOV). PET-CT images of the same animal were co-registered and analysed using PMOD image processing tool. First, Volumes of interest (VOIs) were manually delineated on the lungs to assess the amount of radioactivity deposited. Time–activity curves (decay corrected) were obtained as cps/cm³ in each VOI and values were then normalised to the
amount of radioactivity in the lungs in the first frame, and expressed in percentage (see Fig. S9 for time activity curves obtained for the different labelled species).

![Time-activity curves](image)

**Figure S9.** Time-activity curves in the lungs after intratracheal administration of $^{124}$I-COSAN, $^{124}$I-NE-FES, NE-$^{18}$F-FES and $^{18}$F-FES. Results are expressed as percentage of injected dose (%ID). Displayed values correspond to mean ± standard deviation, n=2 per compound.

**Emulsion loading capacity**

Increasing amounts of cold 17β-estradiol (20 mg, 30 mg and 100 mg) were solved overnight in 1 g of DHA oil. After dissolution, 400 mg of each mixture (2, 3 and 10 % wt.) and 50 µL of a trace solution of $^{18}$F-FES in dichloromethane were poured into 3.6 mL of ultrapure water containing 8 mg of COSAN. Emulsions were produced as described above. Once produced, 500 µL of each emulsion were purified by SEC using an Illustra NAP TM-5 column and diluted with ultrapure water to a final volume of 2 mL in plastic tubes. The same dilution was applied for 500 µL of non-purified emulsions. The radioactivity of the samples was measured using a Gamma Counter. The incorporation of $^{18}$F-FES was subsequently calculated by relating the amount of radioactivity obtained for each non-purified emulsion (equivalent to a theoretical 100% $^{18}$F-FES incorporation) with its purified analogous (X% of $^{18}$F-FES incorporation). Size distribution was also evaluated by DLS (Fig. S10).
Figure S10. NE size distribution after encapsulation of different amount of FES (0, 2, 3, and 10% wt.) as determined by DLS.