

SUPPLEMENTARY INFORMATION

**Chemical Exchange Saturation Transfer (CEST): an efficient tool for detecting molecular information on proteins' behaviour**

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**Materials and Methods**

**1) Chemicals**

1,2-Dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG); 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC); 1,2-Dioleoyl-3-trimethyl ammonium-propane (DOTAP); 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Bovin Serum Albumin (BSA - A0281), Human Serum Albumin (HSA - A3782), Sodium Dodecyl Sulfate (SDS - 436143), Sodium glycochenodeoxycholate (GCDA – G0759), Protease (from *Streptomyces griseus* type XIV - P5147) and all the other chemicals were purchased from Sigma Aldrich. Chicken liver Bile Acid Binding Protein (BABP) was recombinantly expressed in *E. coli* cells and purified by anion-exchange and gel-filtration chromatography, followed by delipidation using chromatographic separation. Doubly distilled water was used for all the experiments.

BSA, HSA and BABP were dissolved in phosphate saline buffer (PBS: 30 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 0.1 M NaCl) to maintain the pH at 7. Every solution was freshly prepared and filtered just before the measurements and the protein concentration was spectrophotometrically determined.

**2) Thermal aggregation and enzymatic cleavage of BSA**

The aggregation of BSA was obtained by dissolving 5 mg/ml of BSA in PBS and heating at 80°C for 2, 5, 10, 30 or 60 min.

A 10 mg/ml BSA solution cross-linked after heating at 80°C for 60 min was treated with 150 µg/ml of Protease at 37°C to induce the cleavage of the BSA aggregated for several time points (5-30-60-240 min).

### **3) BSA and HSA interaction with SDS and spectroscopic measurements**

Several concentrations of SDS stocks were prepared in PBS buffer at pH 7 ( $\pm 0.1$ ). SDS titration of the protein was carried out by preparing aliquots of varying concentration of the SDS using different stock solutions and PBS at pH 7.0.

Fluorescence emission spectra of BSA/HSA (0.008 mM) and SDS (at different stoichiometric ratio with the protein, varying from 1:1 to 1:200) have been acquired by using a Fluoromax-4 Spectrofluorimeter (Horiba Jobin Yvon, NJ, USA). Samples were positioned in a cuvette of 1 cm and all emission spectra were recorded at 1 nm wavelength intervals, with emission and excitation bandwidth of 3 nm. Emission spectra of intrinsic tryptophan in proteins were collected from 300 to 400 nm with an excitation wavelength at 270 nm.<sup>1</sup>

### **4) Preparation of Liposomes and DLS characterization**

Large unilamellar vesicles (LUV) were prepared by following the lipidic thin film hydration method. Briefly, the lipidic mixture was dissolved in chloroform and the organic solution was put into a glass balloon and slowly evaporated under rotation to remove the solvent until a thin film was formed. Then film was hydrated with aqueous solution containing PBS (pH 7) and the temperature was maintained higher than the reported gel-liquid transition temperature, *i.e.* 25°C for DMPG and DMPC, and 55°C for DOTAP, DPPG and DPPC.

Four different membrane formulations were prepared:

- A) 100% DMPC, 20 mg/ml of phospholipids;
- B) 80% DPPC, 20% DOTAP, 20 mg/ml of phospholipids;
- C) 100% DMPG, 20 mg/ml of phospholipids;
- D) 100% DPPG, 20 mg/ml of phospholipids.

In order to obtain small unilamellar vesicles (SUV) the resulting liposome suspension was extruded (Lipex extruder, Northern Lipids Inc., Canada) four times through polycarbonate filters of 400 nm and four times through 200 nm filters under Argon pressure (ca. 5 atm).

Upon preparation the vesicles were characterized using Dynamic Light Scattering, DLS, (Zetasizer Nano ZS, Malvern, UK) in order to determine the mean hydrodynamic diameter and the polydispersity of the system, that was maintained lower than 0.1.

The liposomal preparation (D) was divided into four aliquots, varying in the size. The different size has been obtained by changing the extrusion protocol as follows:

- 1) 4 times at 400 nm (to obtain 300 nm liposomes);
- 2) 4 times at 400 nm, 4 times 200 nm (to obtain 134 nm liposomes);
- 3) 4 times at 400 nm, 4 times 200 nm, 2 times 100 nm (to obtain 80 nm liposomes);
- 4) 4 times at 400 nm, 4 times 200 nm, 5 times 100 nm (to obtain 50 nm liposomes).

## 5) MRI CEST protocols

MRI CEST images were recorded with a Bruker Avance 300 7.0 T micro-imaging system with a 35 mm quadrature coil as both excitation and receiver device. All the CEST measurements were conducted with a controlled temperature of 37 °C, except for those of HSA and BSA with SDS where the temperature was about 20 °C to keep the same temperature as for spectrophotometer measurements. A Fast spin-echo (FSE) sequence with centric encoding was applied at different saturation frequency offsets, varying from -10 ppm to 10 ppm with a step of 0.2 ppm, using a continuous wave pre-saturation pulse of 6  $\mu$ T for 5 s. Acquisition parameters were: TR = 10s, TE = 3ms, NEX = 8, RF = 96, FOV = 30mm, MTX=128x128, slice thickness = 4 mm.

## 6) CEST image analysis

CEST images were analyzed using home-made scripts using the Matlab platform (version, Mathworks, Natick, MA) by following the published procedures.<sup>2</sup> Shortly, a pixel-by-pixel analysis was employed, correcting for  $B_0$  inhomogeneity for each pixel, employing the minimum of the cubic spline interpolated Z-spectrum. Saturation Transfer (ST) curves were calculated at different frequency offsets as:

$$ST = 1 - SI_{ON}/SI_{OFF}$$

Where  $SI_{ON}$  is the signal intensity of the label scan and  $SI_{OFF}$  is the signal intensity of the reference scan at the opposite frequency.

**Table S1.** Main properties of the different liposome formulations in PBS (pH = 7)

Name	Membrane formulation	Diameter (nm)	Net charge
A	100% DMPC, 20 mg/ml of phospholipids	80	neutral
B	100% DOTAP, 20 mg/ml of phospholipids	140	positive
C	100% DMPG, 20 mg/mL phospholipids	90	negative
D1	100% DPPG, 20 mg/mL phospholipids	300	negative
D2	100% DPPG, 20 mg/mL phospholipids	134	negative
D3	100% DPPG, 20 mg/mL phospholipids	80	negative
D4	100% DPPG, 20 mg/mL phospholipids	50	negative

1. V. Vetri and V. Militello, *Biophys Chem*, 2005, **113**, 83-91.
2. E. Terreno, J. Stancanello, D. Longo, D. D. Castelli, L. Milone, H. M. Sanders, M. B. Kok, F. Uggeri and S. Aime, *Contrast Media Mol Imaging*, 2009, **4**, 237-247.