Poster

Structural and functional characterization of hyper-stable human serum albumin variants

Sofia De Felice[1], Stefan T. Huber[2], Christian Buratto[1], Alessia Savio[1], Maria Morbidelli[3], Emanuele Papini[3], Sarel J. Fleishman[4], Olga Khersonsky[4], Arjen J. Jakobi[2], Alessandro Angelini[5], Laura Cendron[1]

1 Department of Biology, University of Padova, Via U. Bassi, 58/B - 35121 Padova, Italy.2 Department of Bionanoscience, Kavli Institute of Nanoscience, Delft University of Technology, Delft, the Netherlands. 3 Department of Biomedical Science, University of Padova, Via U. Bassi, 58/B - 35121 Padova, Italy4 Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot 7610001, Israel 5 Department of Molecular Sciences and Nanosystems, Ca' Foscari University of Venice, Via Torino 155, 30172 Venice, Italy; European Centre for Living Technology (ECLT), Ca' Bottacin, Dorsoduro 3911, Calle Crosera, 30123 Venice, Italy.

laura.cendron@unipd.it

Human serum albumin (hSA) is the most abundant protein in blood plasma, serving as the natural transporter of several endogenous ligands and being well reported to influence drugs bioavailability and pharmacokinetics [1]. Indeed, the interaction with the neonatal Fc receptor (FcRn), rescues albumin from lysosomal degradation, leading to its persistence in the serum for up to three weeks [1]. The prolonged half-life sparked an increasing interest in utilizing albumin- conjugated compounds in the drug discovery field. Such unique feature offers significant benefits, including reduced drug administration frequency for patients, enhancing their well-being and contributing to a more patient-friendly healthcare system.

In this poster we present the *structural and functional characterization* of a panel of *hSA variants,* from now on named hSA1, hSA2, hSA3 (hSA*), engineered *in silico* by the group of Prof. Sarel Fleishman at the Weizmann Institute of Science (Rehovot, Israel), to be hyper soluble, stable and expressible in *Escherichia coli* with a high yield (up to 100 mg/L), [2]. Specifically, hSA* were designed using the PROSS algorithm based on sequence and structure similarity to
other albumins and incorporating mutations spread across its structure, 18, 25 and 73 respectively [2].

On the structural side, hSA *wt* resists crystallization in its apo-form and requires supplementation with myristic acid (Myr) and concentration up to 100 mg/mL to precipitate in an order manner. Due to their high solubility, with a melting temperature higher than 90°C, the crystallization of hSA variants was even a harder task and proved to be successful just for hSA1 [2]. To overcome this problem, we decided to exploit Cryo-Electron Microscopy (Cryo-EM), emerged as a valuable complementary technique for proteins like hSA, difficult to crystallize due to their flexibility and extreme stability [3]. Here I present for the first time, the Cryo-EM structure of one of hSA3, in complex with a megabody (MbAlb1) selected to be cross-reactive for both mouse and human albumin [4], yielding a consensus map at a 3.9 Å

overall resolution (structure under deposition). 0,3 mg/mL solution of hSA6/MbAlb1gel filtrated complex was applied to a glow discharged Quantifoil R 1.2/1.3 Cu300 holey carbon grid. The excess sample was blotted and plunge-freezed into liquid ethane using a Leica plunger (5.0 s blot time, at 25°C under 95% humidity) at the Kavli Institute of Nanoscience (TU Delft). The grids were imaged on the 300 kV Titan Krios microscope (Thermo Fisher Scientific) of the Netherlands Centre for Electron Nanoscopy (NeCEN) facility with a K3 direct electron camera in the super resolution mode at 0.418 Å per pixel. A total of 3732 movies was collected with 60 frames each, a dose of 1 e/Å−2 per frame and a defocus range from -2.4 to -1.6 um. Overall, hSA3 atomic structure is highly conserved with the hSA *wt* one, maintaining the global 3D arrangement and the position of the respective domain. To explore all the hSA* possible applications, we functionally characterize hSA2 and hSA3 performing a panel of affinity measurements by isothermal titration calorimetry (ITC) with the two-gold standard albumin-binding drugs (i.e., warfarin and ibuprofen). Besides, a preliminary size exclusion chromatography (SEC) showed that hSA1 retains the capability of binding its receptor, hFcRn, an appealing feature in the context of its possible drug delivery application. The hFcRn binding will be tested also for hSA2 and hSA3 and surface plasmon resonance (SPR) measurements will be carried out to measure the binding affinity of all hSA variants for the hFcRn. To test whether the hSA* are not toxic to human cells and can be used in cell culture medium, we are setting up a viability test in collaboration with a group in the Department of Biomedical Science (University of Padova), adding hSA* variants to the growth medium of primary cells.

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