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Supporting Information

P*ara*-sulphonato-calix[n]arene capped silver nanoparticles challenge the catalytic efficiency and the stability of a novel human gut serine protease inhibitor

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Contents:

Experimental details:

Synthesis of para-sulphoanto-calix[n]arenes and nanoparticles

The *para*-sulphoanto-calix[n]arenes were synthesized as per the literature and all physical properties are in agreement with the literature [1]. The *para*-sulphoanto-calix[n]arene nanoparticles were prepared as per the literature and their physical properties correspond to the published values [2].

Bacterial strains, plasmids and media

Eubacterium saburreum DSM 3986 was cultured in chopped meat media as recommended by the supplier (DSMZ). *E. coli* BL21 was grown in Luria Bertani (LB) medium supplemented, when necessary, with kanamycin (50 µg/ml) and IPTG at 1 mM.

Cloning and enzyme purification

To amplify the Serpin gene of *E. saburreum*, two primers were designed based on the *E. saburreum* genomic sequence available in the GeneBank (HMPREF0381_1373, GeneBank accession number AEPW01000054.1). The oligonucleotide sequences were F-Serpin $^{5'}$GGGACATGTGTAAAAAGGGCGGTATACAGT$^{3'}$ and R-Serpin $^{5'}$CCCTTGAATTCCTACTCTATATTCTGAATAAC$^{3'}$. Chromosomal DNA isolated from *E. saburreum* using Wizard® Genomic DNA Purification Kit (Promega) was used as a template. PCR mixture (100 µl) contained *Takara* DNA polymerase amplification buffer, 10 pmol of each primer, 200 ng of genomic DNA, and 2 units of *Takara* polymerase (Appligene). The cycling parameters were 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 120 s, respectively. PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN®) following the manufacturer's instructions. The amplified DNA fragment was sequenced using an automated DNA sequencer (Applied Biosystems) then cloned into pETM-11 expression vector. In this construct the gene encoding for the Serpin was cloned under the control of the T7 promoter.

The recombinant *Escherichia coli* BL21 overexpressing the serpin was grown until OD_{600} of 1.0, the culture was induced overnight using IPTG (1 mM) followed by harvesting the cells by centrifugation (7500 × g, for 10 min at 4 °C). Pellets were resuspended in 20 mM Tris-HCl buffer pH 8.0 and 0.5 M NaCl. Cell disruption was carried out by sonication at 4 °C for 2 min (pulsations of 3 s, amplify 90) using a Vibra-Cell™ 72405 Sonicator and cell debris was

removed by centrifugation (30 000 × g, for 30 min at 4 °C). The protein was purified by 1-ml HiTrap chelating HP column (GE-Healthcare) charged with Ni^{2+} ions using the standard protocol. After imidazole gradient elution, obtained protein was subjected to size-exclusion chromatography using a HiLoad Superdex 200 Increase 10/300 gel filtration column (GE-Healthcare) in 0.02 M Tris-HCl, 0.5 M NaCl buffer pH 8.0.

Protein quantification and electrophoresis

Protein concentration was determined using Bradford's method with bovine serum albumin as the standard [3]. The protein samples were separated in 12% SDS-PAGE according to the Laemmli method [4].

Enzyme assay

Inhibitory activity was evaluated by incubating porcine pancreatic elastase (EPC elastin) with Serpin from *E. saburreum* (Saburopin) and measuring the residual enzyme activity. The reaction was performed in 50 mM phosphate buffer, pH 8 (90 µl) using the methoxysuccinyl-Ala-Ala-Pro-Val-pNA (Bachem) as substrate. Protease at final concentration of 1.7 µM (2µl) was mixed with 17 µM (3 µl) of Serpin in a total volume of 100 µl. After 1 hour of incubation at 25°C, The enzymatic activity was subsequently assayed by adding 5 µl of substrate solution (170 µM) and monitoring the absorbance change for 15 min at 405 nm at 25 °C. Notably nanoparticules (10^{-4} M) were recovered in 50 mM phosphate buffer, pH 8.

Temperature, pH and thermostability profiles

The effect of temperature on the activity was determined by incubating the purified serpin at temperatures ranging from 4 to 70 °C. The Saburopin pH profile was obtained by measuring the activity at various pH values ranging from 2 to 10 using 50 mM phosphate buffer. Concentrations of PPE, Saburopin, SC and SC-Ag-Np were the same as described in the enzyme assay section. The enzyme stability as a function of temperature and pH was investigated by incubating the enzyme at different temperature and pH, with drawing the samples at defined intervals, placing them on ice and measuring residual activity at 25 °C and pH 8.0.

Stoichiometry of inhibition

Stoichiometry of inhibition was determined by mixing varying amount of Saburopin with 845 nM of PPE. Proteases and Saburopin were gently mixed yielding to molar ratios of inhibitor/protease ranging from 0 to 8 during 5 min at 37 °C. Therefore, substrate was then added and activity was assessed for 30 min by measuring absorbance at 405 nm. Fractional activity (velocity of the inhibited enzyme reaction/velocity of the non-inhibited enzyme reaction) was subsequently calculated and plotted to the concentration ratio of the inhibitor to enzyme ([I0/E0]). The stoichiometry of inhibition was determined by linear regression as the x-intercept.

Kinetic analysis

Kinetic of PPE inhibition by Saburopin was determined using the progress curve methodology [5, 6] under pseudo-first-order conditions, where the initial concentration of Saburopin was varied from 0 to 5-fold greater than that of proteases. Initial reaction mixture contains 170 µM of substrate and different amounts of Saburopin incubated at 37 °C during 10 min. Then, 5µl PPE (845 nM) was added to the reaction and the substrate hydrolysis rate was monitored at 405 nm for 30 min. The progress curves were fitted by non-linear regression and the pseudo first order association rate constant, *k obs*, was calculated using the Eq. 1 [5, 6].

$$P = (V_0/ K_{obs})/ 1 - e^{-t.kobs} \qquad (1)$$

where P is the product concentration, v_0 is the substrate hydrolysis velocity and t is the reaction time. Calculated K_{obs} were plotted as a function of Saburopin concentration. The apparent second order associate constant k'_{app} was considered to be the slope of the fitted linear curve. Seeing the competitive nature of inhibition by serpin, the second order association rates were then corrected using the Eq. 2 [5, 6].

$$Ka = k'_{app} (1+[S]/Km) \qquad (2)$$

where [S] is the substrate concentration and Km is the Michaelis–Menten constant determined from the Lineweaver–Burk plot. Km value was 275µM.

Dynamic light scattering studies

Samples were centrifuged at 20,000 g during 30 min before the determination of the apparent dynamic radius using the Zetasizer NanoS apparatus (Malvern). The mean size distribution was generated as an intensity distribution then transformed to a volume distribution using the DTS software v5.0.

Statistical analysis

All data are shown as means ± SEM. For statistical analysis, R package 'Stats' (Version 3.5.3) was used. Differences in PPE activity were assessed using Mann Whitney test. Statistical significance was accepted at $p < 0.05$.

Supporting Table :

Table S1 Determination of the inhibition constant of saburopin alone and in presence of SC and SC-Ag-Np.

Saburopin	**Alone**	**SC4**	**SC4-Ag-NP**	**SC6**	**SC6-Ag-NP**	**SC8**	**SC8-Ag-NP**
***Ki* (nM)**	1.1 ± 0.05	0.98 ± 0.03	0.99 ± 0.02	0.98 ± 0.02	0.97 ± 0.05	0.99 ± 0.01	0.047 ± 0.02

Supporting Figures:

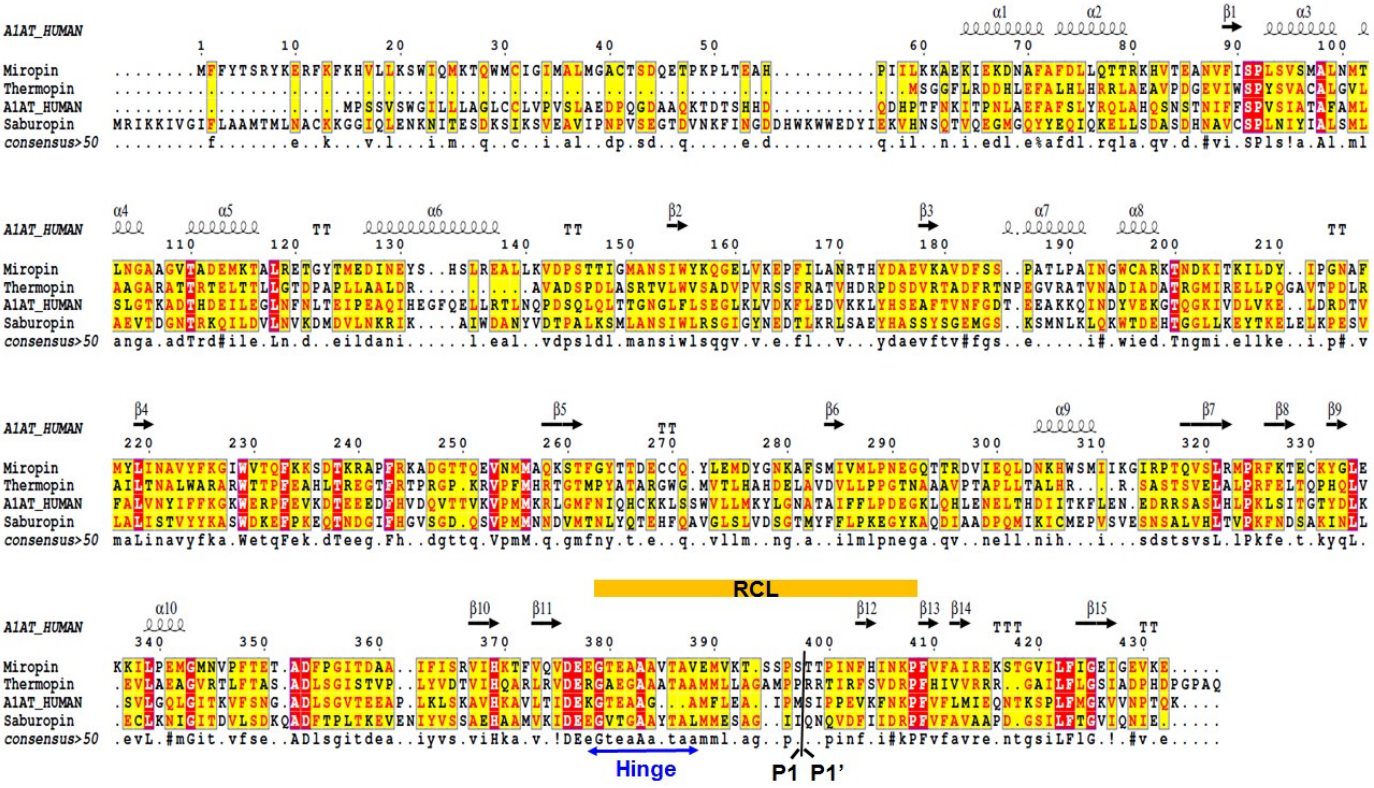

Fig. S1 Structure-based multiple sequence alignment of Saburopin with human α-1-antitrypsin (UniProt accession number P01009), Miropin (Uniprot accession number G8UQY8), and Thermopin (UniProt accession number Q47NK3). Structural elements shown above the alignment were generated using the native α-1-antitrypsin structure (PDB ID: 1QLP) sequence. Residues invariable between sequences are typed red on a yellow background and residues conserved within each group are displayed as white letters on a red background. Underlined sequences represent the predicted hinge region (blue) and reactive center loop (yellow). The predicted cleavage site is marked with a vertical line. P1 and P1' residues are labeled. Sequence inspection of saburopin with other serpins reveals that it display significant homology of 35% with the human α-1-antitrypsin. Comparison with previously studied bacterial serpins show that saburopin shares 30 and 34% of sequence homolgies with serpin from *Thermobifida fusca* (Thermopin) and *Tannerella forsythia* (Miropin).

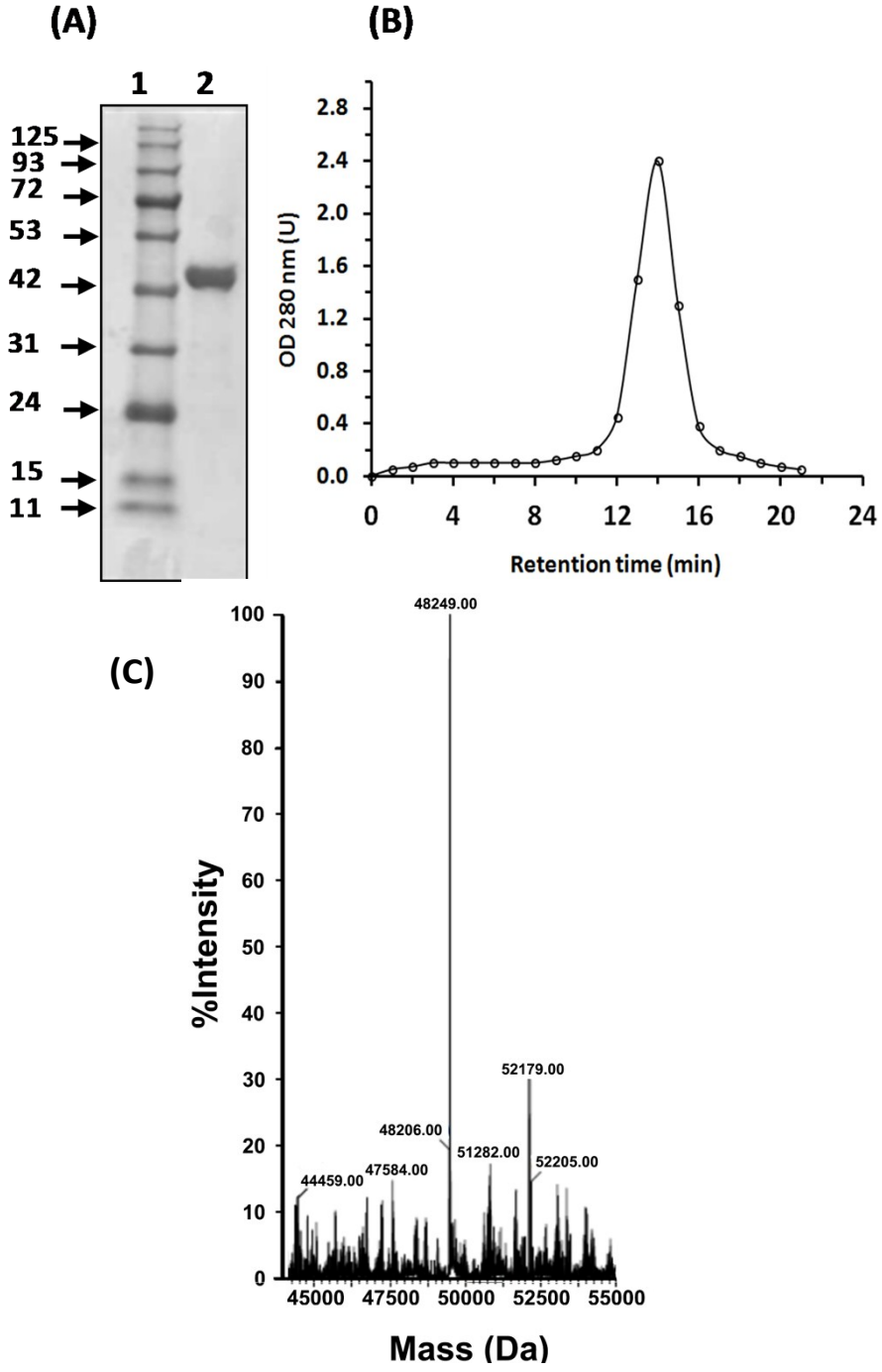

Fig.S2 A. Coomassie brilliant blue-stained gel, under reducing conditions. Lane 1 protein marker (molecular masses in kilodaltons); lane 2, purified Saburopin. B. Size exclusion chromatography profile of the purified Saburopin with a retention time (RT) of 14.8 min. Used protein markers of 669 kDa (RT = 8.9 min), 440 kDa (RT = 11.3 min), 158 kDa (RT = 13.2 min),

75 kDa (RT = 14.1 min), 44 kDa (RT = 15.3 min) and 29 kDa (RT = 16.9 min). C. Mass spectrometry analysis of Saburopin.

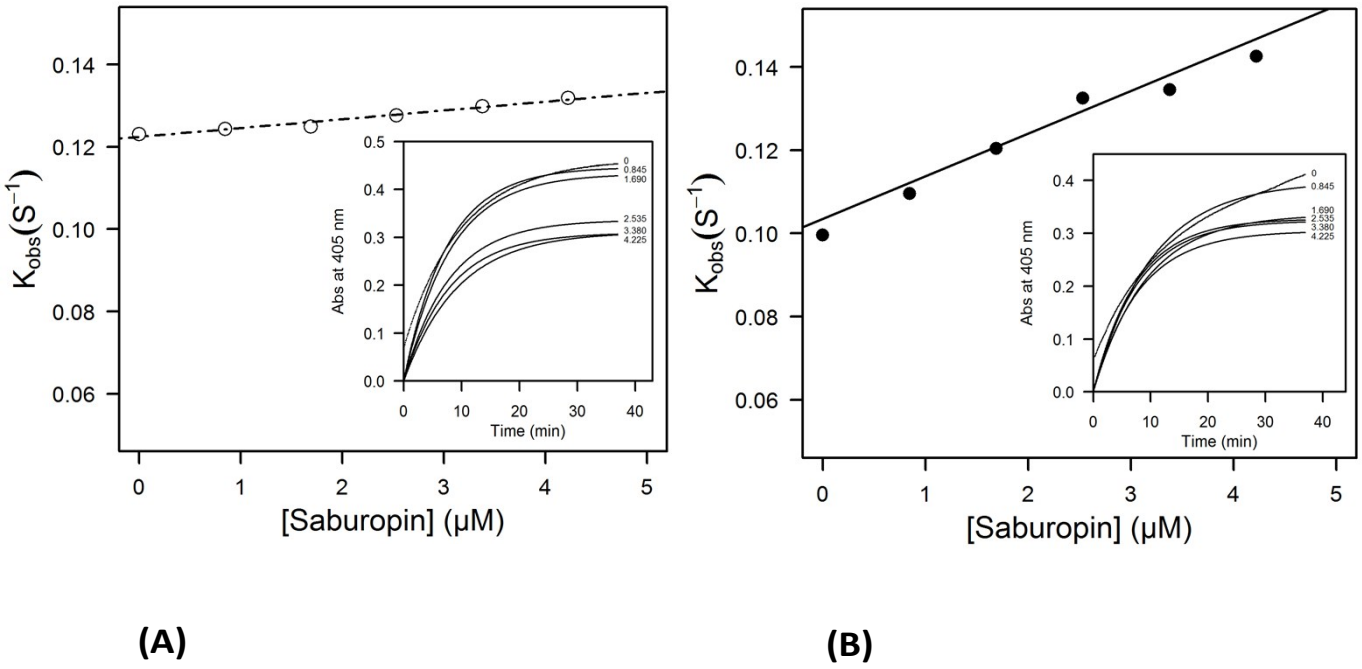

Fig. S3 Progress curves for PPE inhibition by Saburopin. (A) Saburopin alone. (B) Saburopin in presence of SC8-Ag-Np. Constant concentrations of PPE were used with various amount of Saburopin. Used concentration of Saburopin (µM) are shown beside each progress curve. Obtained k_{obs} rate were plotted against the Saburopin concentration. The apparent second-order association constant k'_{app} was calculated as the slope of the fitted linear curve and then corrected to determine the second-order association constant k_a.

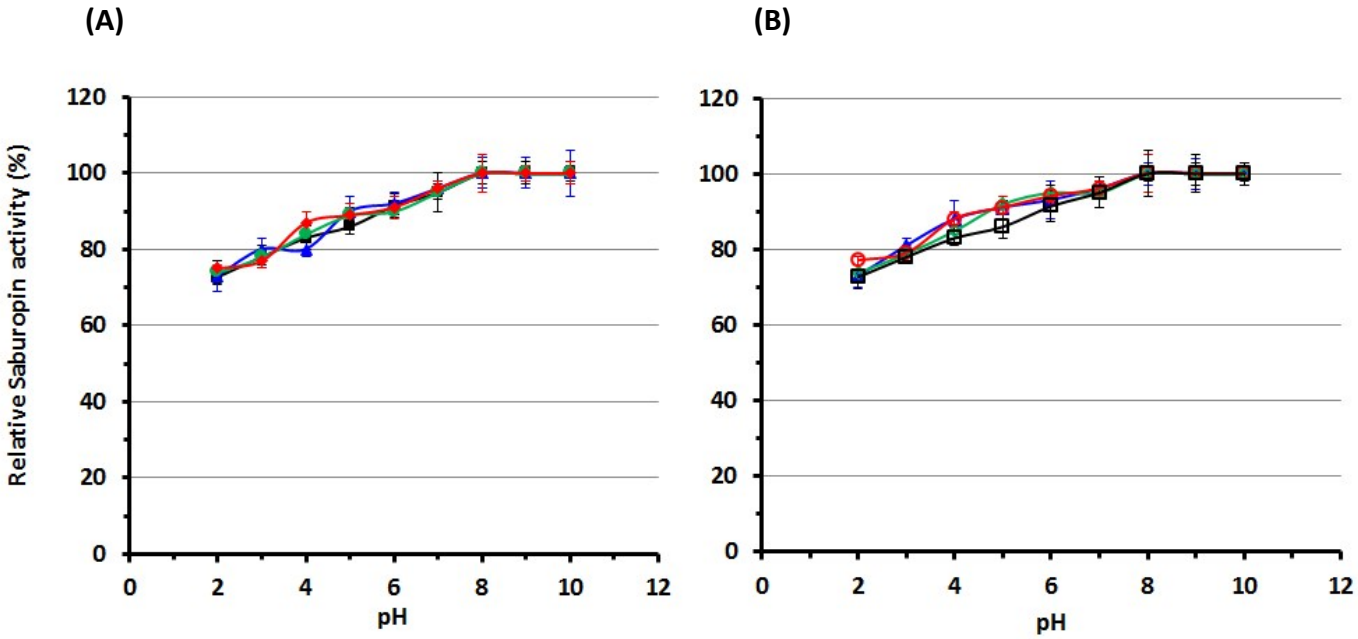

Fig. S4 Measurement of the Saburopin relative activity profile at different pH in presence of (A) calix[n]arenes and (B) calix[n]arene capped silver nanoparticules. Black : Saburopin alone, Blue : SC4/SC4-Ag-Np, Green: SC6/SC6-Ag-Np and Red : SC8/SC8-Ag-Np. Activities at optimal pH were defined as 100%. Error bars represent the SEM from three independent experiments.

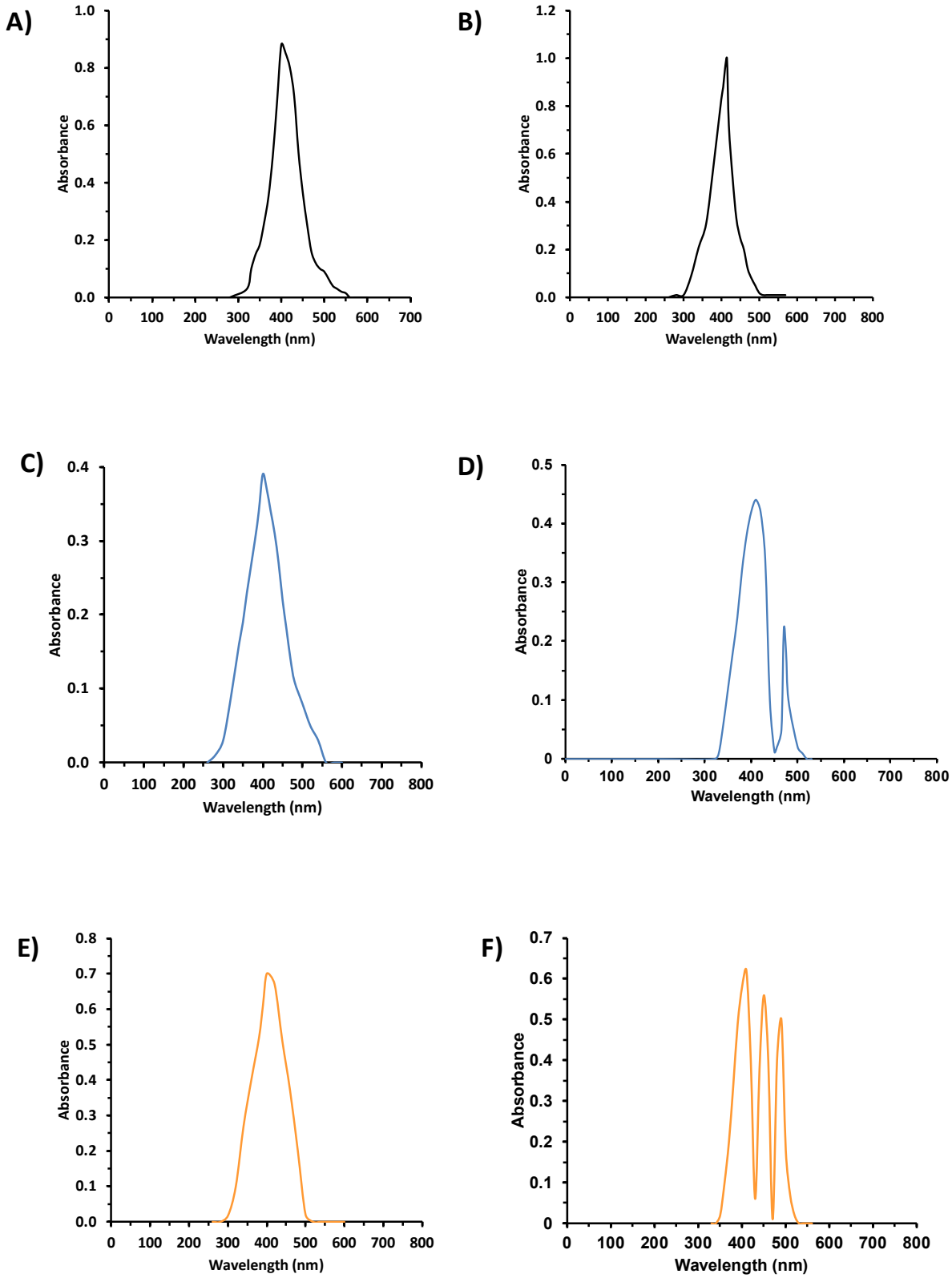

Fig. S5 The spectra of the nanoparticles and the complexes with Saburopin. A : SC4-Ag-Np, B: SC4-Ag-Np + Saburopin, C : SC6-Ag-Np, D: SC6-Ag-Np + Saburopin, E : SC8-Ag-Np, F: SC8-Ag-Np + Saburopin.

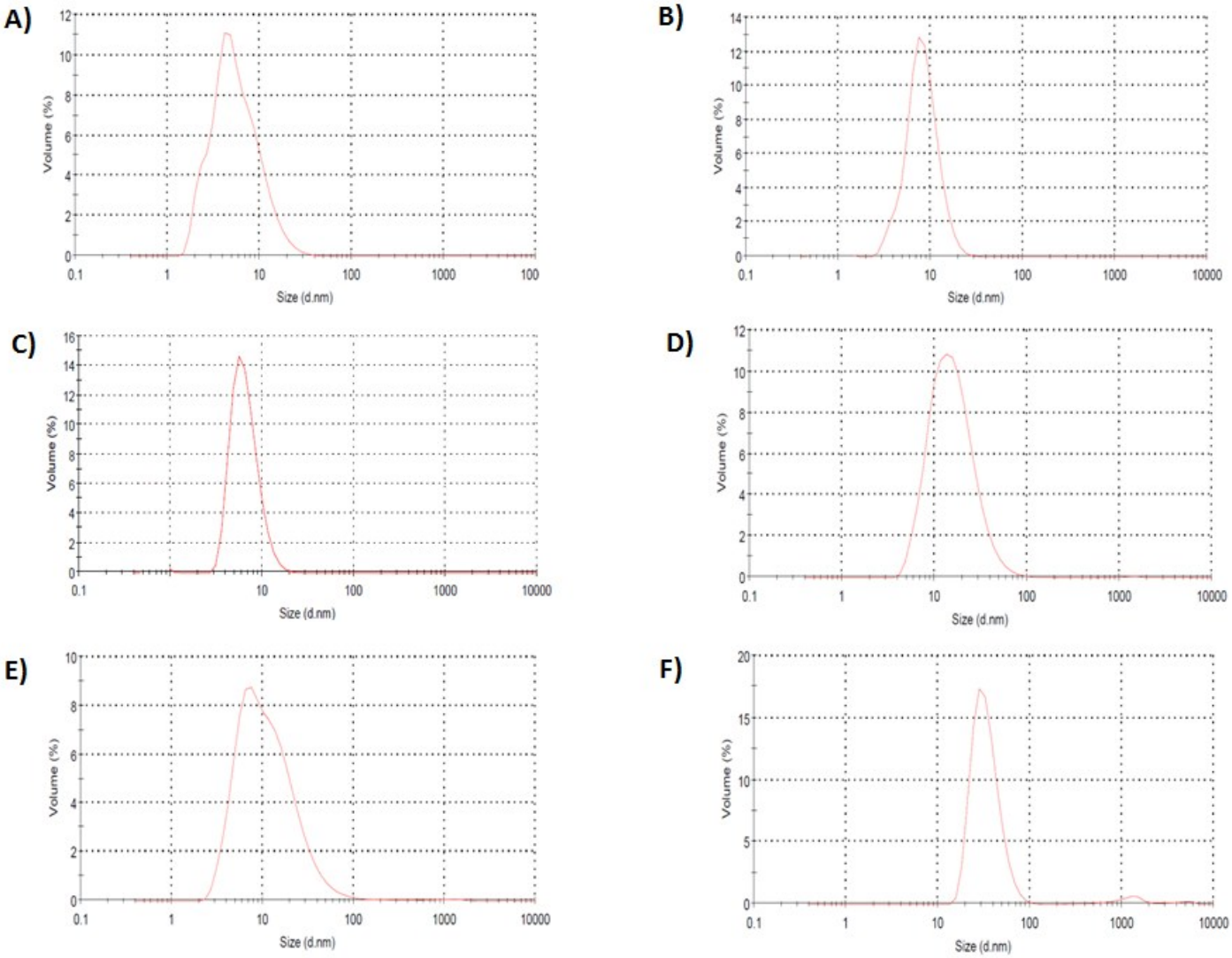

Fig. S6 Dynamic light scattering analysis of the nanoparticles and the complexes with Saburopin. A : SC4-Ag-Np, B: SC4-Ag-Np + Saburopin, C : SC6-Ag-Np, D: SC6-Ag-Np + Saburopin, E : SC8-Ag-Np, F: SC8-Ag-Np + Saburopin.

References and Note

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