Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Highly Efficient Anti-Cancer Therapy using Scorpion 'NanoVenin'

*Santosh K. Misra, † Mao Ye, † Sumin Kim and Dipanjan Pan**

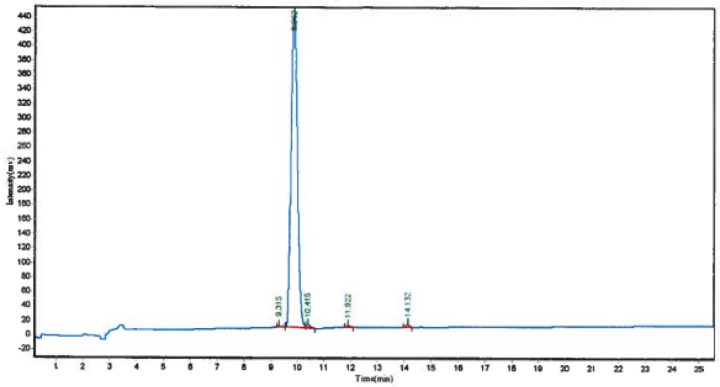
Dept. of Bioengineering, University of Illinois, Urbana-Champaign, IL, and Beckman Institute of Advanced Science and Technology, Urbana, IL, USA, 61801

[†] These authors contributed equally to this work.

*Corresponding author:

Prof. Dipanjan Pan
Dept. of Bioengineering,
University of Illinois at Urbana-Champaign
and Beckman Institute of Advanced Science and Technology,
Urbana, IL, USA, 61801
Phone: 217-244-2938
E-mail: dipanjan@illinois.edu

Column : 4.6×250mm, Venusil MP C18-5
Solvent A : 0.1% trifluoroacetic in 100% acetonitrile
Solvent B : 0.1% trifluoroacetic in 100% water



Peak No.	Ret Time	Height	Area	Conc.
1	9.315	765.473	10869.534	0.1493
2	9.862	432588.063	7167724.500	98.4244
3	10.415	4771.325	54604.293	0.7498
4	11.922	1469.437	13748.378	0.1888
5	14.132	4258.548	35520.512	0.4878
Total				100.0000

Figure S1. Raw HPLC traces of ε-Ahx-TsAP-1 showing 98.42% from C18 linear gradient (200 nm).

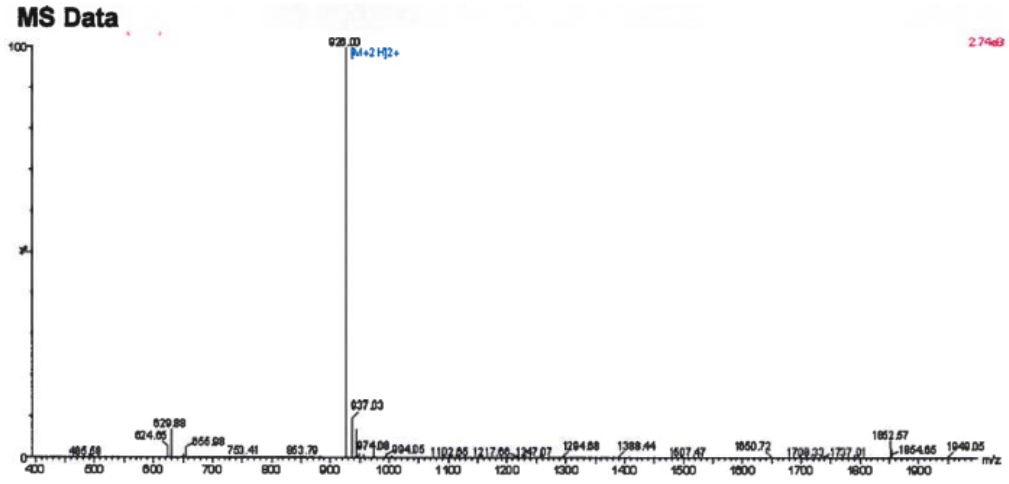


Figure S2. HRMS spectroscopic data $(M+2H^{+})^{2+}$.

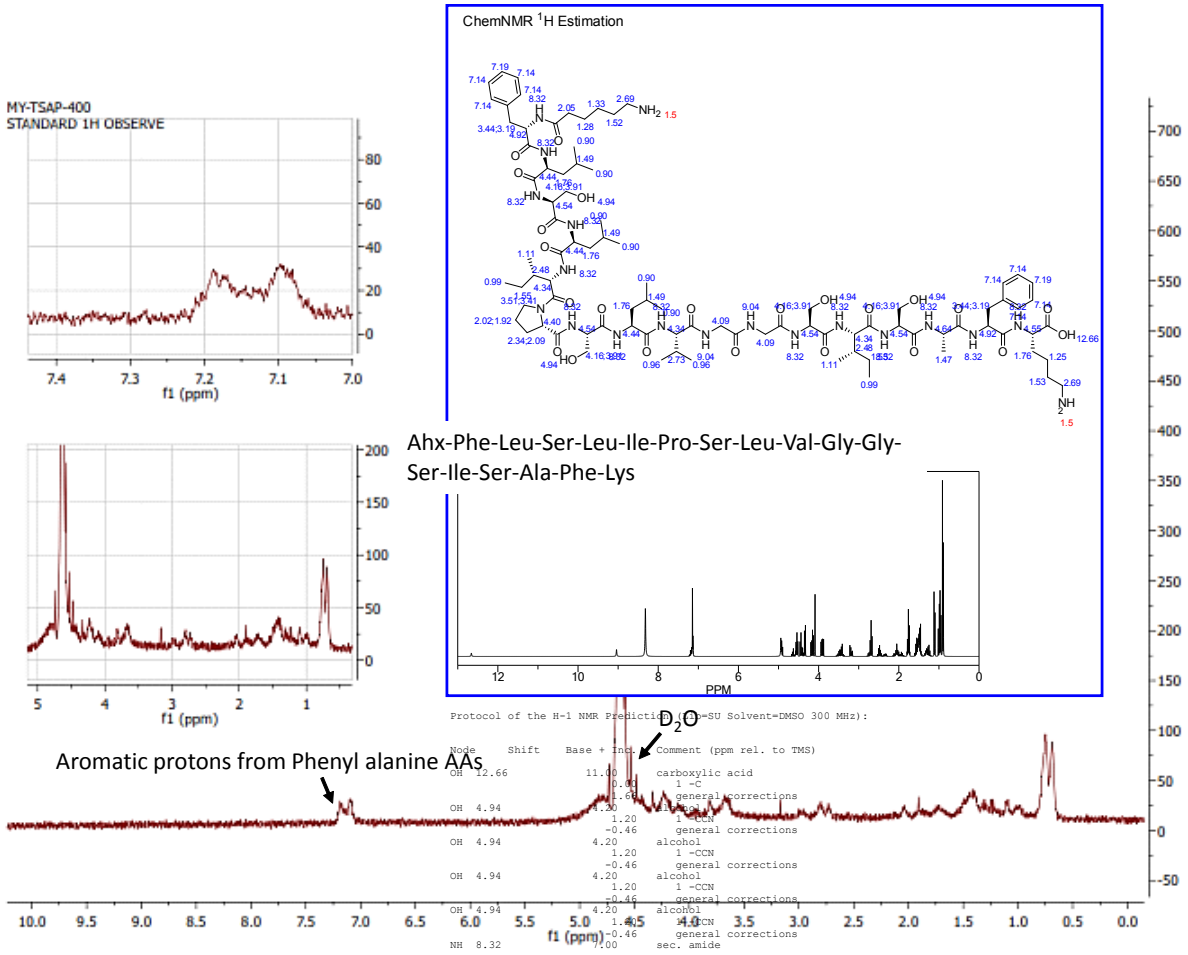


Figure S3. 1H-NMR spectroscopic data of Ahx-TsAP-1 (brown) with ChemNMR 1H estimation values shown in blue box.

Experimental

Materials and Methods

Polyoxyethylene (20) cetyl ether and poly(styrene)-block-poly(acrylic acid) (PS-*b*-PAA) were obtained from Sigma Life Sciences (St. Louis, MO, U.S.A). Tetrahydrofuran (THF) was obtained from Avantor Performance Materials (Center Valley, PA, U.S.A.). Scorpion venom peptide ε–Ahx-TsAP-1 (TsAP-1) was synthesized by solid phase peptide synthesis and obtained from Sigma Biosciences Corp. (Rockville, MD, U.S.A). The hydrodynamic

diameter was measured on Malvern Zetasizer machine equipped with 633 nm laser. Zetapotential measurement was performed on Malvern Zetasizer instrument. Atomic force microscopy was performed on MFP-3D AFM from Asylum Research using Igor Pro software. The TEM images were acquired on JEOL 2100 Cryo TEM machine and imaged by Gatan UltraScan 2kx2k CCD. The cast film XRD data was collected on instrument Siemens-Bruker D5000 diffractometer and analyzed using software Jade X-ray analysis. Flow Assisted Cell Sorting was performed oniCyt Reflection machine from iCyt Mission Technology equipped with Software Win List 3D.

Preparation of RCMs

Polyethylene glycol cetyl ether (2 mg) was melted at 65 °C for 5 min followed by the dropwise addition of 2 ml of water (approximately 1 drop/sec). The solution was allowed to stir for 20 min at 1150 rpm. Simultaneously, a solution of poly(styrene)$_{67}$-block-poly(acrylic acid)$_{27}$ (PS_{67}-*b*-PAA_{27}, M_n 1,600-1,950 (poly(acrylic acid)), M_n 6,500-7,000 (polystyrene), M_n 8,100-9,100, average M_n 8,700, mp: 192-197 °C; M_w/M_n=1.2) was prepared by adding 2 mg of the amphiphilic polymer and 1 ml of tetrahydrofuran (THF) to a glass vial. After the polyethylene glycol cetyl ether miceller suspension was stirred for 20 min; 250µl of PS_{67}-*b*-PAA_{27}/THF solution was added drop-wise (approximately 1 drop/10 sec) to the solution. The solution was left for stirring overnight to allow THF evaporation. At the end of the procedure, volume was made up to 2 ml with autoclaved nanopure water (0.2 µM). The suspension was further allowed to stir for 10 min at room temperature. The hydrodynamic diameter of prepared nanoparticles was measured using a nano series Zetasizer. Finally, the suspension was stored at 4°C overnight for curing the core of the particle and the particle size measurement was repeated. The nano particles were purified by dialysis against nanopure (0.2µM) water using a 20,000 Da MWCO cellulose membrane for prolonged period of time

and then passed through a 0.45 µm Acrodisc Syringe filter. The nanoparticles were stored under argon atmosphere typically at 4°C in order to prevent any bacterial growth.

DLS (D_{av})/nm =19 ± 02 nm; TEM (D_{ah})/nm = 25 ± 10 nm AFM (H_{av})/nm =25 ± 11 nm; Zeta (ζ)/mV = -38 ± 04 mV; PDI: 0.14±0.05; XRD (2Θ) 28.5, 31.6, 43.6 and 45.5 associated with different d spacing.

Preparation of S-NanoVenin

25 µM of TsAP-1 (MW: 1849.26 g/mol) solution was made from dilution of 200 µM of TsAP-1 aqueous solution with nanopure water. 40 µl of 25 µM TsAP-1 and 960 µl of RCMs (as synthesized above) were mixed to make the TsAP-1 1 µM (5 wt%) in the mixed suspension and subsequently vortexed. This suspension was then incubated at room temperature for 30 min and stored at 4 °C.

The nano particles were purified by dialysis against nanopure (0.2µM) water using a 20,000 Da MWCO cellulose membrane for prolonged period of time and then passed through a 0.45 µm Acrodisc Syringe filter. The hydrodynamic diameter of synthesized nanoparticles was measured with Malvern zetasizer nanoseries.

DLS (D_{av})/nm =34 ± 03 nm; TEM (D_{ah})/nm = 26 ± 08 nm AFM (H_{av})/nm =25 ± 10 nm; Zeta (ζ)/mV = -30 ± 04 mV; PDI: 0.12±0.07; XRD (2Θ) 19.9, 21.0, 21.7, 22.7, 23.7 and 24.7 28.4, 31.6, 45.4. associated with different d spacing.

Molecule sketch-up and minimization studies.

The polymer and ε-Ahx-TSAP-1 peptide were sketched and subjected to energy minimization using Powell method with Sybyl-X 2.0.[1] Termination: Gradient at 0.05 kcal/mol. Max Iterations: 100. Energy settings are as followings: Force field: Tripos. NB

Cutoff: 8.00. Dielectric FunctionL Distance. Dielectric Constant: 1.00. For the solvation method, silverware algorithm was chosen. All other settings are selected with default.

Docking:

MOE 2013.08[2] was used for the docking. Both target and active site were chosen as the minimized whole polymer molecule. Peptide ligand molecules were docked with rotate bonds setting. Triangle matching was used as the placement method. Duplicated poses were removed and best 30 poses were retained. The best S_Score poses were chosen as the docked poses, which are -12.255 for non-aqueous media docking and -7.058 for water media docking.

In non-aqueous media, following key interactions are identified: The amino groups of the backbone of Leu2, Leu4, Ile5, Ser7, Leu8, Gly11, Ser12, Ile13 and Ser14 and of the 6-aminohexanoic group, and the hydroxyl groups of side chains of Ser7, Ser12 and Ser14 form hydrogen bond interactions with oxygen of carboxylic acid of the polymer. The side chains of Leu2, Leu4, Leu8, Ala15 and Lys17 form hydrophobic interactions with phenyl and carbon atoms of the backbone of the polymer. In water media, following key interactions are identified: The amino groups of the backbone of Ile5, Gly10, Phe16 and of the e-aminohexanoic group, the hydroxyl groups of side chains of Ser14, and the C-end carboxylic acid form hydrogen bond interactions with oxygen of carboxylic acid of the polymer. The side chains of Ile13 and phenyl ring of Phe1 form hydrophobic interactions with phenyl and carbon atoms of the backbone of the polymer. Figure 1 shows the Molcad surface of the two docking poses in non-aqueous media and in water solvent. The former shows the intertwined docking structure and the hinder shows the roughly paralleled docking structure.

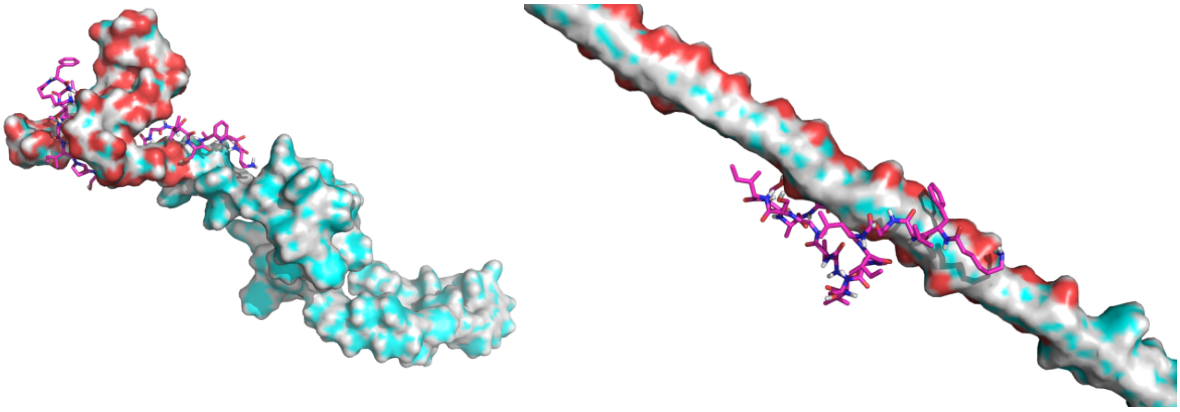


Figure S4. Molcad surfaces of the docking structures. In non- aqueous media (left) and in aqueous media (right).

Plausible mechanism of ε-Ahx-TsAP-1 release from S-NanoVenin *in vitro*

Experimental release of therapeutic agents from nanovehicles has been generally based on pH responsiveness and interaction with anionic lipids and surfactants. These factors are used to mimic endosomal pH and inner compartmental layers, which are supposed to be the major contributing factors in controlling release of various payloads from nano-vehicles. On the basis of chemical characteristics, these nano-vehicles will ideally take one of the preferential pathways to release the active component in cytosolic medium of the cells.

We have used an experimental strategy to mimic and narrow down the preferential release pathways of ε-Ahx-TsAP-1 from S-NanoVenin. S-NanoVenin were prepared as discussed in the experimental section below followed by the incubation at pH 4.6 and either in presence of sodium dodecyl sulphate (SDS, 1 mM) or Tween80 (0.5%) for 0.5, 3 and 6h. At the end of incubation period, hydrodynamic diameter of various suspensions was acquired using dynamic light scattering measurements. Changes in hydrodynamic diameter of S-NanoVenin in water were used as control. (Figure S5)

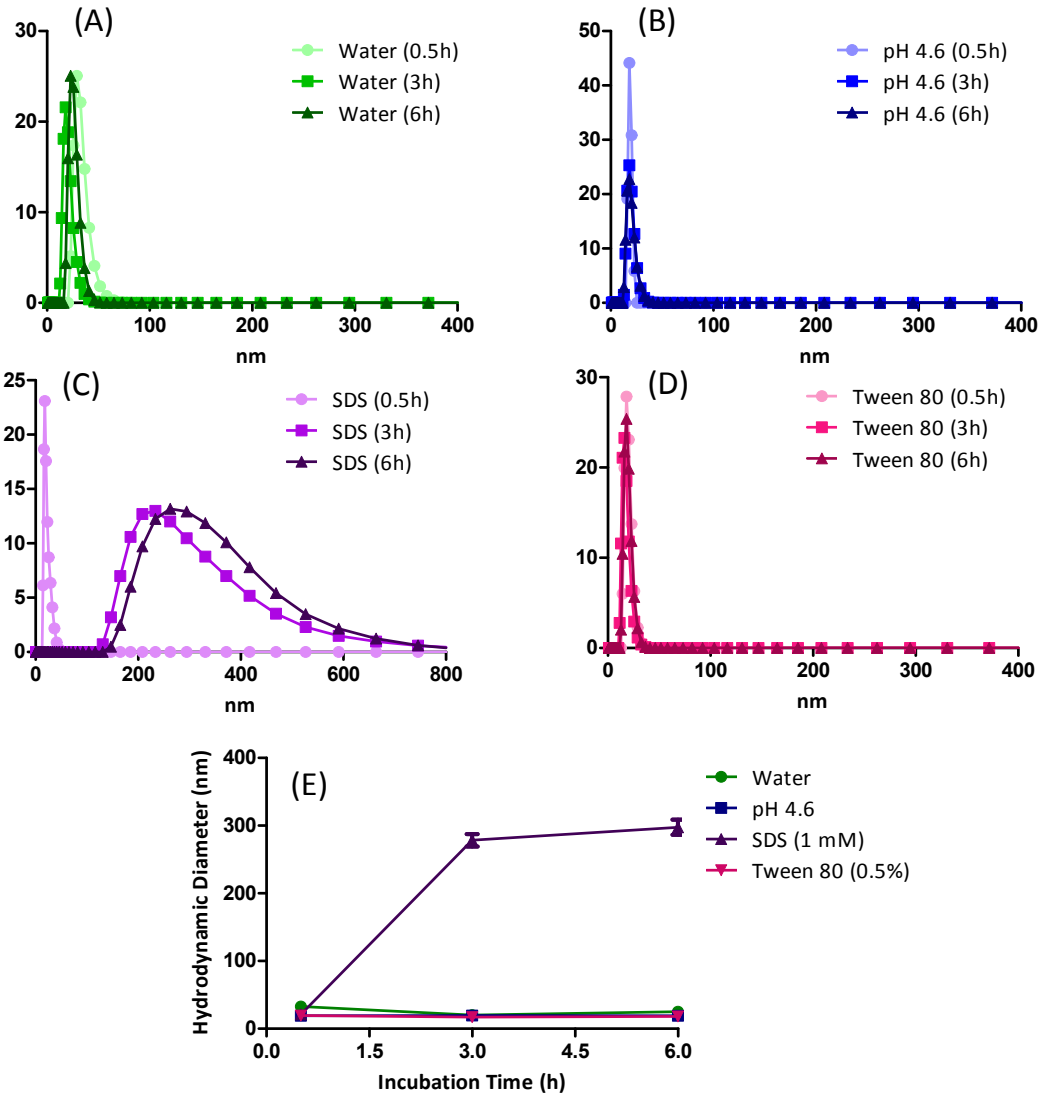


Figure S5. Hydrodynamic size distribution of S-NanoVenin suspension in water (A); at pH 4.6 (B); in presence of SDS (1 mM) (C) and Tween80 (0.5%); (D) after 0.5, 3 and 6 h of incubation (E).

DLS results clearly show the variation in size of S-NanoVenin only in presence of SDS (1 mM) leaving the effect of lower pH and mild surfactant Tween80 nominal. It supports the plausible occurrence of polymer-SDS interactions, which would lead to release of ε-Ahx-TsAP-1 from S-NanoVenin. This indicates that the anionic components of endosomal compartment would be interacting with S-NanoVenin to rearrange the assembly and ε-Ahx-

TsAP-1 would be getting released in the process. Lowering in pH did not affect the association of ε-Ahx-TsAP-1 in S-NanoVenin presumably because of the non-pH-responsive nature of outer corona of RCM particles used in preparation of S-NanoVenin. Lack of enough extraction efficiency of Tween80, which is a mild surfactant, bars its association with S-NanoVenin and subsequently the release of ε-Ahx-TsAP-1.

Dynamic light scattering measurements

Hydrodynamic diameter distribution and distribution averages for S-NanoVenin and RCMs in aqueous solutions were determined using a Malvern Zetasizer nano series–Nano ZS90. Scattered light was collected at a fixed angle of 90°. A photomultiplier aperture of 400 mm was used, and the incident laser power was adjusted to obtain a photon counting rate between 200 and 300 kcps. Only measurements for which the measured and calculated baselines of the intensity autocorrelation function agreed to within +0.1% were used to calculate nanoparticle hydrodynamic diameter values. The measurements for S-NanoVenin and RCMs were made at 0 h, 24 h, 48 h, 72 h, 96 h, and 120 hr after synthesis to evaluate the stability. All determinations were made in multiples of five consecutive measurements.

Zeta Potential Measurements

Zeta potential (ζ) values for the RCMs and S-NanoVenin were determined with a nano-series Malvern Zetasizer zeta potential analyzer. Measurements were made following dialysis (MWCO 20 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of nanoparticle suspensions into water. Data were acquired in the phase analysis light scattering (PALS) mode following solution equilibration at 25 °C. Calculation of ζ from the measured nanoparticle electrophoretic mobility (μ) employed the Smoluchowski equation: $\mu = \varepsilon\zeta/\eta$,

where ε and η are the dielectric constant and the absolute viscosity of the medium, respectively. Measurements of ζ were reproducible to within ±5 mV of the mean value given by 20 determinations of 10 data accumulations.

Cast-film X-Ray Diffraction Measurement

The crystalline behavior of RCMs and S-NanoVenin were determined by X-ray diffraction measurements. The aqueous aggregates of each formulation were placed on a pre-cleaned glass plate which, upon air drying, afforded a thin film of the formulations on the glass plate. X-ray diffraction (XRD) of an individual cast film was performed using the reflection method with a Siemens-Bruker D5000 diffractometer. The X-ray beam was generated with a Cu anode and the Cu-Kα beam of wavelength 1.5418 Å was used for the experiments. Scans were performed for 2Θ range of 20 to 60.[3]

Gel electrophoresis

The DNA fragmentation efficiency of S-NanoVenin was determined by gel electrophoresis on extracted genomic DNA. The efficiency was compared with TsAP-1 and RCM formulations. In order to find out the comparative DNA fragmentation efficiency of these formulations, we performed the assay on 2% agarose gel and TAE buffer. The extracted genomic DNA (1.0 µg/well) was used for running the gel. Gel was finally stained in ethidium bromide (20 mg/100 ml) for 10 min and washed for 5 min to remove excess of EB staining. Gel was imaged under UV light.

Atomic Force Microscopy Measurements (AFM) and Transmission Electron Microscopy Measurements (TEM)

Atomic force microscopy (AFM) was performed to observe the morphological topography in structures of RCMs. The samples were drop cast onto freshly cleaved mica sheets and air-

dried for 24h. Topographic imaging of all the formulations was obtained by operating the AFM in a tapping mode with an Asylum Cypher AFM instrument. The average particle height (H_{av}) values and standard deviations were generated from the analyses of a minimum of 50 particles from three micrographs. Analysis of the AFM images was processed using ImageJ.

The Transmission electron microscopy (TEM) was performed on RCMs and S-NanoVenin to evaluate their morphologies. Imaging was performed on samples prepared on copper grids that are coated with a formvar plastic and then coated with carbon for stability followed by negative staining with 7% Uranyl acetate.

Blood-smear experiment

A single smear was made per slide by putting a drop of fresh pig blood on the slide (near the end). The drop was spread by using another slide ("spreader"), placing the spreader at a 45° angle and backing into the drop of blood. The spreader catches the drop and it spreads by capillary action along its edge. Smear is allowed air dry for 1min and cover-slip before placed directly on the microscope and observed under 40 x magnifications. A ratio of 1:9 NanoVenin (300 µl) and pig blood was used for preparation of the smear.

Human transformed cancer cell culture

MD-MB231 cells (ER (-) breast cancer cells) and MCF-7 cells (ER (+) breast cancer cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS) in T25 culture flasks (Cellstar®; Germany) and were incubated at 37 °C in a 99% humidified atmosphere containing 5% CO_2. Cells were regularly passaged by trypsinization with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in DPBS (pH 7.4). Non-synchronized cells were used for all the experiments.

MTT Asssay

The cell viability of RCMs, S-NanoVenin and TsAP-1 formulations in used cells was investigated by using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay in presence of 10% FBS in antibiotic free media.[4] Experiment was performed in 96 well plates (Cellstar®; Germany) growing 8,000 cells per well 24 h before treatments. Experiments were performed for various concentrations of TsAP-1 ranging from 6.25 to 100 μM present in free or S-NanoVenin form while same amount of RCM was used as negative control. Cells were incubated for 72h before performing the MTT assay. After incubation period, cells were treated with MTT as 20 ml (5 mg/mL) per well and further incubated for 5 h. At the end of the incubation entire medium was removed from wells and 200 μL DMSO was added to dissolve blue colored formazan crystals. The percentage cell viability was obtained from plate reader and was calculated using the formula % Viability = {[A_{630}(treated cells)- (background)]/[A_{630}(untreated cells)-background]}x100.

Cell Imaging and DNA Fragmentation Assay

To establish the cell morphological variation in MCF-7 and MD-MB231 cells and post-apoptotic DNA fragmentation, microscopic imaging and gel electrophoresis assay was performed. MCF-7 and MD-MB231 cells (60,000/ well) were plated in 24 well plates and grown till it achieved ~80 % confluence. At the end of ~24h incubation at ambient condition, cells were washed with plain DMEM and treated with 50 μM of TsAP-1 in either free or S-NanoVenin form. Cells were incubated for 72h before imaging them for cell morphology variations. Cells were trypsinized and collected in 400 μL of lysis buffer. DNA extraction was performed using manufacturer's protocol using Thermo Scientific DNA extraction kit. Extracted genomic DNA were washed with 70% EtOH and dissolved in water after air drying.

General procedure of Preparation of S-NanoVenin

Polyethylene glycol cetyl ether (2 mg) was melted at 65 °C for 5 min followed by the dropwise addition of 2 ml of water (approximately 1 drop/sec). The solution was allowed to stir for 20 min at 1150 rpm.[5] Simultaneously, a solution of poly(styrene)$_{67}$-block-poly(acrylic acid)$_{27}$ (PS67-b-PAA27, Mn 1,600-1,950 (poly(acrylic acid)), Mn 6,500-7,000 (polystyrene), Mn 8,100-9,100, average Mn 8,700, mp: 192-197 °C; Mw/Mn=1.2) was prepared by adding 2 mg of the amphiphilic polymer and 1 ml of tetrahydrofuran (THF) to a glass vial. After the polyethylene glycol cetyl ether miceller suspension was stirred for 20 min; 250µl of PS_{67}-*b*-PAA_{27}/THF solution was added drop-wise (approximately 1 drop/10 sec) to the solution. The solution was left for stirring overnight to allow THF evaporation. At the end of the procedure, volume was made up to 2 ml with autoclaved nanopure water (0.2 µM). The suspension was further allowed to stir for 10 min at room temperature. Finally, the suspension was stored at 4°C overnight for curing the core of the particle and the particle size measurement was repeated.

The nano particles were purified by dialysis against nanopure (0.2µM) water using a 20,000 Da MWCO cellulose membrane for prolonged period of time and then passed through a 0.45 µm Acrodisc Syringe filter. The nanoparticles were stored under argon atmosphere typically at 4°C in order to prevent any bacterial growth. 25 µM of TsAP-1 (MW: 1849.26 g/mol) solution was made from dilution of 200 µM of TsAP-1 aqueous solution with nanopure water. 40 µl of 25 µM TsAP-1 and 960 µl of RCMs (as synthesized above) were mixed to make the TsAP-1 1 µM in the mixed suspension and subsequently vortexed.[6] This suspension was then incubated at room temperature for 30 min and stored at 4 °C.

The nano particles were purified by dialysis against nanopure (0.2µM) water using a 20,000 Da MWCO cellulose membrane for prolonged period of time and then passed through a 0.45

μm Acrodisc Syringe filter. The hydrodynamic diameter of synthesized nanoparticles was measured with Malvern zetasizer nanoseries.

General procedure of computational studies

The diblock-copolymer and ε-Ahx-TsAP-1 peptide was sketched and was subjected to energy minimization using Powell method with default setting using Sybyl-X 2.0. Docking studies were performed using MOE 2013.08. Active site was chosen as the whole polymer molecule. Triangle matching was used as the placement method, and the docked poses were scored using default settings. The docked poses were exported and visualized with PyMOL.[7]

References

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