

Alginate-peptide amphiphile core-shell microparticles as a targeted drug delivery system

Job Boekhoven,^{a,b} R. Helen Zha,^{a,c} Faifan Tantakitti,^{a,b} Ellen Zhuang,^b Roya Zandi,^b Christina J. Newcomb,^{a,f} Samuel I. Stupp^{a,b,c,d *}

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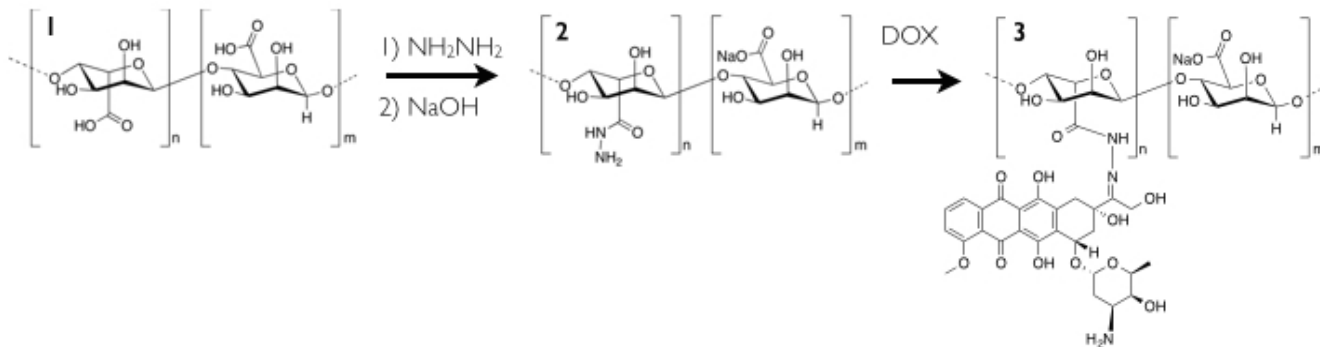
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1. Materials

All chemicals and solvents were ordered from Sigma-Aldrich and used without further purification unless otherwise indicated. Sodium alginate (MW 300–400 kDa) was purchased from FMC Biopolymers (HF120RBS), 4',6-diamidino-2-phenylindole HCl (DAPI), Rhodamine phalloidin and penicillin/streptomycin (P/S) were ordered from Invitrogen (Life Technologies). Dulbecco's Modified Eagle's Media (DMEM), Roswell Park Memorial Institute (RPMI) 1640 media, phosphate buffered saline (PBS) and foetal bovine serum (FBS) were ordered from Gibco (Life Technologies).

1.1 Synthesis of alginate derivatives



Scheme S1. Synthesis of alginate derivatives.

Compound 2

Hydrazide bearing alginate was synthesized according to a published protocol.¹ Briefly, sodium alginate (5.0 gr., 25 mmol in monomer) was dissolved in 500 mL water. This solution was heated and the pH was adjusted to pH 2 by addition of 1M HCl. The gelled solution was cooled down and centrifuged to remove as much water as possible. Subsequently the collected gel was refluxed in 40 ml hydrazine hydrate (41 gr., 0.82 mol) for 14 hours. A solution of 5 M NaOH was titrated to bring the pH back to neutral after which ethanol was added to precipitate

the product. The crude product was filtered and washed with ethanol to remove any hydrazine, water and NaOH. The filtrate was dissolved in water and precipitated by addition of 1 M HCl. The precipitate was collected by filtration and redissolved in water by addition of NaOH. The product was again precipitated with ethanol, filtered and washed thoroughly with ethanol after which it was dried under high vacuum.

The presence of hydrazides was confirmed using IR-spectroscopy. By means of a modified TNBSA assay the reaction yield was quantified. A solution of 2,4,6-trinitrobenzenesulfonic acid (TNBSA, 34 mM in 100 mM sodium carbonate buffer at pH 8) was freshly prepared and 0.5 mL of this solution was added to 1 mL of hydrazide in water and incubated at 37 °C for two h after which the absorption at 500 nm was read in a 96 well plate (n=3). The molar extinction coefficient of TNBSA reacted with a hydrazide was determined using the assay on known concentrations of adipic acid dihydrazide and found to be constant between 6 to 150 μM at $1.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. Using this extinction coefficient the degree of substitution was found to be $16 \pm 1\%$. By means of gel permeation chromatography (GPC, LC-9210NEXT Japan Analytical Industry, W-253 column, using water as eluent), the average molecular weight of the polymer was found to be 315 kDa \pm 98 kDa.

Compound 3

The doxorubicin bearing alginate **3** was synthesised by dissolving 100 mg of the alginate-hydrazide (0.5 mmol in monomer alginate, 0.08 mmol in hydrazide) in dimethylformamide (DMF). To this solution 24.6 mg Doxorubicin (0.045 mmol, 0.56 eq) was added and the red solution was stirred for 48 h at room temperature after which the product was precipitated by addition of ethanol. The precipitate was collected on a glass filter and washed thoroughly with ethanol, after which the product was dried under high vacuum yielding 66 mg of a deep red powder. The degree of substitution (2.6%) was determined by measuring the absorbance of solutions of **3** and dividing this by the molar extinction coefficient of doxorubicin (determined to be $7.9 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 485 nm). By means of GPC, the average molecular weight of the polymer was found to be 335 kDa \pm 135 kDa corresponding to a degree of substitution of 3%.

1.2 Synthesis of Peptide Amphiphiles

All peptide amphiphiles (PAs) were synthesised at the Peptide Synthesis Core at the Simpson Querrey Institute for BioNanotechnology using standard fluoren-9-ylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis on rink amide 4-methylbenzhydrylamine (MBHA) resin (100-200 mesh, 0.55 mmol/gram). Synthesis was performed on a CEM Liberty microwave-assisted peptide synthesizer. For each coupling, 5 equivalents of Fmoc-protected amino acid in DMF was added with 5 equivalents of N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) in DMF and 10 equivalents of N,N-diisopropylethylamine (DIPEA) in N-methyl-2-pyrrolidone. Fmoc removal was accomplished using a solution of 20% piperidine in DMF and 0.1 M 1-hydroxybenzotriazole (HoBt). The palmitic acid tail was added using the same coupling conditions.

For the synthesis of **PA2**, first $\text{C}_{16}\text{V}_3\text{A}_3\text{K}(\text{fmoc})_3\text{-K}(\text{mtt})$ was synthesised. The 4-methyltrityl (mtt) protecting group was removed by shaking in 1% trifluoro-acetic acid (TFA) and 4% triisopropylsilane (TIS) in DCM. Next, folate (0.88 gr, 2 mmol, 10 eq) was dissolved in DMSO and activated with EDC (0.38 gr., 2 mmol, 10 eq.) and sulpho-NHS (0.31 gr., 2 mmol, 10 eq.) by shaking the solution for 2 h. The solution of activated folate was added to the PA on resin and this solution was shaken for 24 h.

Upon completion of the reaction, the PAs were cleaved of the resin by shaking in a solution of 95% TFA, 2.5% TIS and 2.5% water. After cleavage of the PA, the solvent was evaporated under reduced pressure. The remaining solid was taken up in \sim 1 mL of water and the crude product was precipitated out by the addition of cold diethyl ether (\sim 25 mL). This precipitate was filtered and washed with ether before taking it up in 20 mL water with 0.1% TFA. This solution was purified using reversed-phase high-performance liquid chromatography (HPLC, Varian Prostar 363) in a water/acetonitrile gradient containing 0.1% TFA with a starting condition of 2% acetonitrile. The fraction of acetonitrile was increased linearly in 30 min to 40% acetonitrile in water. Purified PAs were lyophilised and stored at -20 °C until further use. The purity of the PAs was confirmed by electrospray ionisation mass spectrometry in positive mode (ESI-MS, Agilent 6510 Q-TOF) and analytical HPLC (Agilent 1260 Infinity, eluted with a gradient of 0.1% NH_4OH in water: acetonitrile from 95:5 to 5:95 in 30 min). The synthesis of rhodamine-PA is described in ref ².

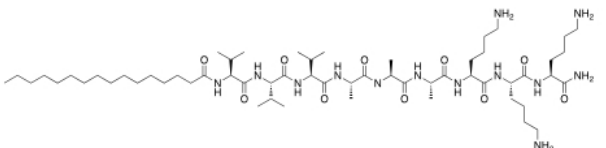
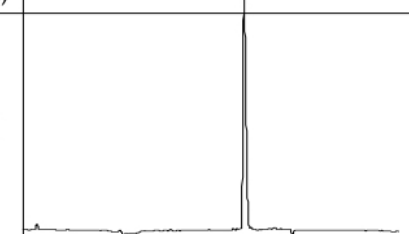
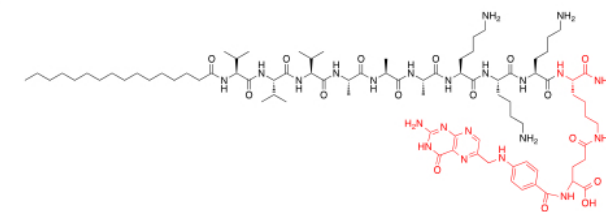
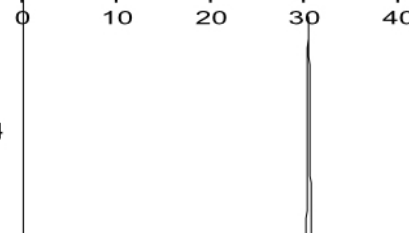
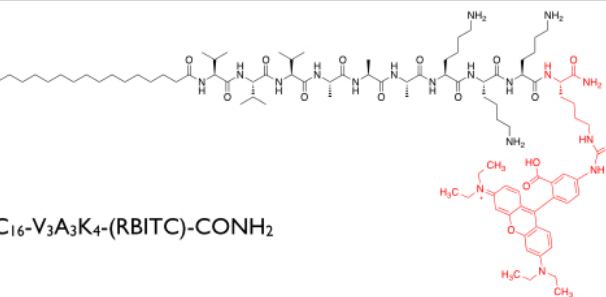
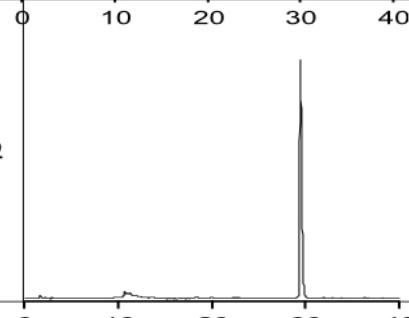
Structure and name	Abbreviation	Exact mass	Obs. mass (deconv.)	HPLC
 $C_{16}\text{-V}_3\text{A}_3\text{K}_3\text{-CONH}_2$	PA1	1149.86	1149.61	
 $C_{16}\text{-V}_3\text{A}_3\text{K}_4\text{-(folate)-CONH}_2$	PA2	1701.08	1701.44	
 $C_{16}\text{-V}_3\text{A}_3\text{K}_4\text{-(RBITC)-CONH}_2$	Rhodamine PA	1778.15	1777.72	

Table S1. Structures, names, abbreviations and masses of all PAs used.

1.3 Cell Media

Three different cell media were prepared:

Growth media: Dulbecco's Modified Eagle's Media (DMEM) supplemented with penicillin (100 units/mL), streptomycin (100 mg/mL), foetal bovine serum (FBS, 10% v/v).

Folate free media: Roswell Park Memorial Institute (RPMI) 1640 media supplemented with penicillin (100 units/mL), streptomycin (100 mg/mL), foetal bovine serum (FBS, 10% v/v).

Starvation media: Roswell Park Memorial Institute (RPMI) 1640 media supplemented with penicillin (100 units/mL), streptomycin (100 mg/mL).

2. Methods

2.1 Doxorubicin release profiles

On the bottom of a 1.5 mL Eppendorf tube 50 μ L of 1% alginate and 0.2% **3** in water was gelled by the addition of 50 μ L 50 mM CaCl_2 . The formed gel was washed 3 times with the appropriate buffer (100 mM phosphate buffer pH 5, 6 or 7.4). Next, 300 μ L of the buffer was added and the sample was incubated at 37 °C. At each time point, 300 μ L of the buffer was replaced with fresh buffer and, the concentration of doxorubicin released was determined by measuring the absorbance at 485 nm.

2.2 Preparation of microparticles

A water-in-water emulsion was created by thoroughly mixing an aqueous two phase system containing 10 w% sodium dextran sulphate and 0.01 to 1 w% sodium alginate or **3** (doxorubicin functionalised alginate). Mixing was performed by horn sonication using a Branson digital sonifier at 10% capacity for 3 times 10 seconds. After mixing the alginate micro-droplets in the dextran sulphate continuous phase they were rapidly crosslinked by injecting the solution in a 50 mM solution of CaCl_2 . Typically, per mL of emulsion, 10 mL of CaCl_2 solution was used. The crosslinked microparticles were collected by centrifugation for 20 min at 30 krcf. The supernatant was removed and the particles were washed with water. This process was repeated 3 times to remove all CaCl_2 and dextran sulphate.

Subsequently, the alginate particles were coated with PA by mixing a solution of particles with a 500 μ M solution of PA. The PA solution was horn sonicated for 5 seconds at 10 % capacity to decrease the fibre length. The coated microparticles were isolated by centrifugation 20 min at 30 krcf and resuspended in water by vortexing. The particles were resuspended in water and collected again by centrifugation to wash out all PA. Next, the particles were resuspended in PBS.

2.3 Attenuated Total Reflectance-Fourier Transform Infra-Red spectroscopy

ATR-FTIR spectroscopy was performed using a Bruker Tensor 37 spectrometer fitted with a germanium crystal pike cell accessory. To avoid overlap of the carboxylates of **2**, the product was protonated to its carboxylic acid form. To do so, **2** was dissolved in water at 1 w/w%. This solution was acidified to pH 1 by dropwise addition of 1 M HCl. The formed gel was lyophilised and the resulting powder was examined using ATR-FTIR spectroscopy. Two absorption bands (1537 cm^{-1} and 1625 cm^{-1}) were observed that were not present in alginic acid and assigned to the hydrazides.

2.4 Dynamic Light Scattering

Dynamic light scattering was used to determine the hydrodynamic radii of the particles using a Malvern Zetasizer Nano ZSP. The samples were loaded in a low volume cuvette and measured using a standard operation procedure. The number average diameters was used and listed in table S2.

2.4 Nile Red Assay

The self-assembly of the PAs was assessed by incorporation of the hydrophobic solvatochromic probe Nile Red (9-diethylamino-5-benzo[*a*]phenoxazinone), which exhibits an emission blueshift upon inclusion in hydrophobic environments. Solutions of both PAs, ranging from 1 μ M to 1000 μ M in 10% PBS, were prepared from stock solutions, and Nile Red in ethanol was diluted 200-fold into solutions to a final concentration of 250 nM. Using a NanoLogHJ spectrofluorometer, the samples were excited at 550 nm and the spectra were collected from 580 nm-750 nm. Blueshifts were calculated by subtracting the emission wavelength of the sample by the emission wavelength of Nile Red in 10% PBS and were plotted against concentration to determine the critical aggregation concentration.

2.5 Circular Dichroism

The secondary structure of the PAs was probed using circular dichroism (CD) at 25 °C. PAs were diluted to 500 μM in 10% PBS and stabilized for 24 h before measurement with a JASCO J-715 CD spectrophotometer in a 0.1 mm path length quartz cuvette.

2.6 Cryogenic-Transmission Electron Microscopy

Cryogenic transmission electron microscopy was performed using a JEOL 1230 TEM fitted with a LaB6 filament working at an accelerating voltage of 100 kV. Samples were plunge frozen using a Vitrobot Mark IV (FEI) vitrification robot operating at 25 °C with 100% humidity. The sample (5 μL) was deposited on a 300 mesh copper grid with lacey carbon support, blotted, and plunged into a liquid ethane reservoir cooled by liquid nitrogen. Following vitrification, the sample was transferred to a Gatan 626 cryo-holder under liquid nitrogen with the aid of a transfer stage. Images were acquired using a Gatan 831 bottom-mounted CCD camera. PAs were imaged at a concentration of 500 μM in 10% PBS.

2.7 Scanning electron microscopy (SEM)

SEM samples were prepared by spinning down the microparticles in an eppendorf tubes and the samples were then dehydrated by exchanging the water with ethanol. This exchange was done exchange the solvent with solutions with increasing amount of ethanol (10, 20, 30,..., 100%). By means of critical point drying the ethanol was removed in a critical point dryer using supercritical CO₂. The dried powders were then attached to SEM stubs using conductive tape. Finally, the samples were coated with 4 nm Osmium using an osmium plasma coater to reduce charging while imaging. The SEM samples were imaged on a Hitachi SU8030 electron microscope, operating at 2.0 kV and a typical current of 5 μA . In case of charging on the specimen the current was decreased.

2.8 Cell culture

MDA-MB-231 human breast cancer cells were cultured in growth media at 37 °C, 5% CO₂, and 95% relative humidity. Confluent cultures were rinsed with phosphate buffered saline (PBS) and split (1:3) every 3 days using trypsin/ ethylenediaminetetraacetic acid (EDTA).

2.9 Cytotoxicity assays

The *in vitro* cytotoxicity of doxorubicin was measured using the methyl thiazolyl tetrazolium (MTT) viability assay in MDA-231 cells. Cells were seeded into 96-well cell-culture plate at 3×10^3 /well and then incubated for 24 h at 37 °C, 5% CO₂ and 95% relative humidity in folate free media. To determine the IC₅₀ of doxorubicin the solutions of doxorubicin (100 μL /well in folate free media) were added to the wells after which the cells were incubated at 37 °C for 72 h.

To determine the toxicity of the microparticles, solutions of microparticles (100 μL /well in folate free media) were added to the wells of the treatment group. The cells were then incubated at 37 °C and 5% CO₂ for 2 h after which the microparticle solution was removed, the cells were washed 3 times with PBS and incubated for 72 h in folate free media.

After 72 h of incubation the media was removed and replaced with MTT solution (prepared according to protocol) and incubated for an additional 1 h at 37 °C under 5% CO₂. After the addition of 10% sodium dodecyl sulfate (SDS, 100 μL /well), the assay plate was allowed to stand at room temperature for 12 h. Next, the absorbance was measured at 490 nm using a microplate reader. The background signal was subtracted and the following formula was used to determined the cell viability.

Cell viability = mean of absorbance value of treatment group / mean of absorbance value of control * 100%

The viability was measured in triplicate and the IC₅₀ values were determined by fitting a log[inhibitor] vs. normalised response using Graphpad Prism software.

2.10 Cellular uptake of folate PA and microparticles.

For flow cytometry experiments and microscopy experiments, the cells were seeded in a 12-well culture plate (1×10^5 cells per well) and incubated for at 37 °C at 5% CO₂ for 24 h in folate free media. After incubation, a solution of fluorescent particles or fluorescent PA in folate free media was added and incubated at 37 °C at 5% CO₂ for 1 h.

After incubation the cells were washed three times with with PBS (3x) and imaged using a Nikon Eclipse TE2000U inverted microscope with a 10x objective.

For confocal microscopy the cells were plated on 12 mm L-PDL coated coverslips.

from their surface with 0.05% trypsin/EDTA and centrifuged down. Next, the cells were either resuspended in PBS (1mL) and stored on ice before analysis with a flow cytometer or replated on fibronectin coverslips for confocal microscopy. Fluorescence signal of each cell was quantified using a LSRFortessa cell analyser (BD Biosciences) and per condition 1.0×10^4 cells were counted. Forward and side scatter data were used to fix a gate around live cell population. The confocal micrographs were obtained with a Zeiss LSM 510 UV confocal microscope.

3. Supporting Figures

[alginate]	diameter	PDI
0.01 w%	0.66 μm	0.33
0.05 w%	0.87 μm	0.39
0.1 w%	1.1 μm	0.38
0.5 w%	1.2 μm	0.21
1.0 w%	2.3 μm	0.70

Table S2: Hydrodynamic diameters of particles against concentration alginate in the all aqueous emulsion. Diameters and polydispersity indices are obtained from DLS.

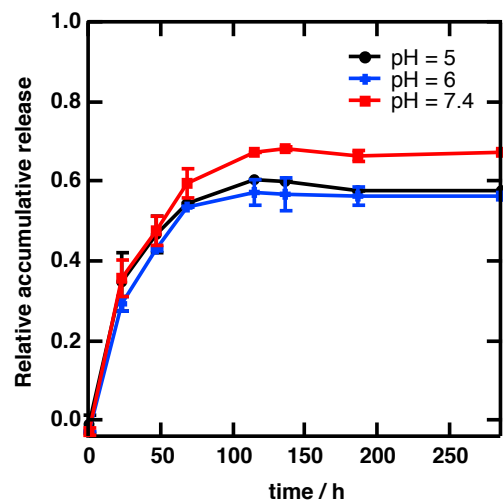


Fig. S1: Accumulative release profiles of doxorubicin from gels of 3 at pH 5, 6 and 7.4.

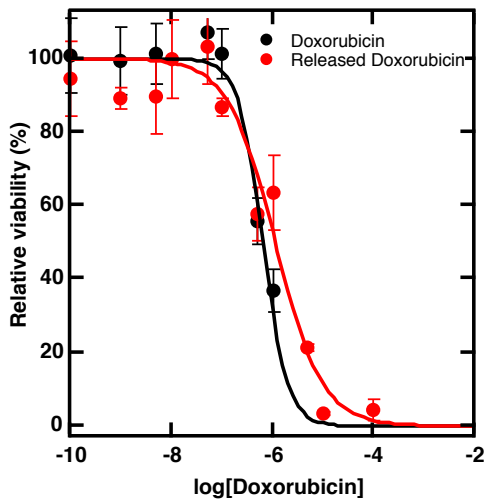


Fig. S2: Toxicity of doxorubicin. Viability assay of commercial doxorubicin and doxorubicin released from a gel of 3. The IC_{50} values were found to be 0.64 and 1.12 μM , respectively.

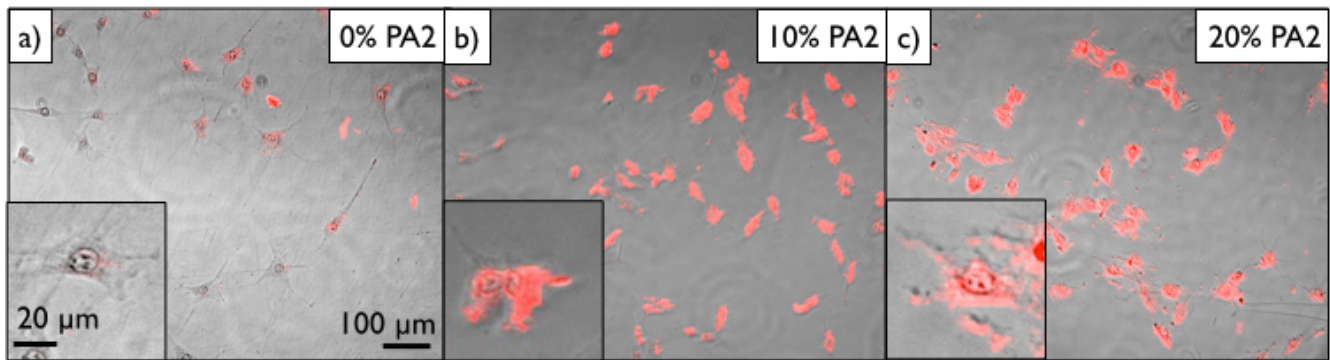


Fig. S3: Uptake of PA fibres by MDA-MB-231 cells by microscopy. Fluorescence microscopy images laid over brightfield microscopy images of MDA-MB-231 cells incubated with nanofibres with varying ratio PA2 and 5% Rhodamine-PA.

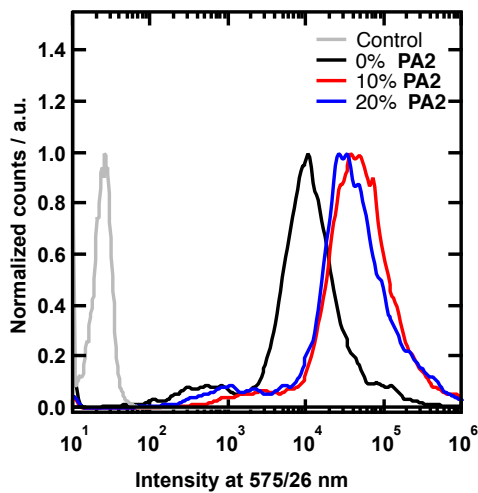


Fig S4. Uptake of PA fibres by MDA-MB-231 cells by flow cytometry. Histogram of flow cytometry data of MDA-MB-231 cells incubated with 50 μM PA consisting of PA1 and 0, 10 or 20% of PA2 and 5% rhodamine PA.

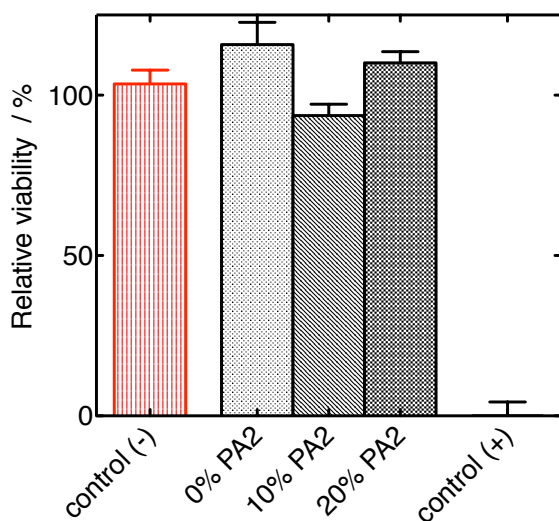


Fig S5. Toxicity of particles. Viability assay of cells treated with soft core-shell particles with 0, 10 or 20% PA2 in their shell. Non of the particles was found to be significantly more toxic as compared to a control with non-treated cells.

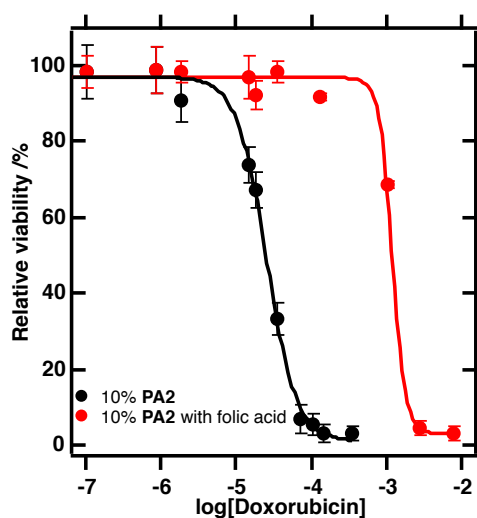


Fig S6. Toxicity of particles with doxorubicin in presence of free folic acid. Viability assay of cells treated with soft core-shell particles with 10% PA2 in their shell in the presence of 1 mM free folic acid. In the presence of folic acid the toxicity these particles the IC50 value shifted from 24 μ M to 1.2 mM.

¹ Anson, I., Novikova, V. & Iozep, A. *Russ. J. App. Chem.* 2008, **81**, 647-650.

² R.H. Zha, S. Sur, S. I. Stupp, *Adv. Healthc. Mater.* 2013, **2**, 126-33.