Supplementary Information

Continuous Flow Fabrication of Fmoc-Cysteine Based Nanobowl Infused Core-Shell Like Microstructures for pH Switchable on Demand Anti-Cancer Drug Delivery

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1. **Preparation of microfluidic device:** To fabricate the microfluidic device, commercially available SU-8 2100 negative photoresist and 1-Methoxy-2-Propanol (Propylene glycol methyl ether; PGME) developer was purchased from MicroChem Corp., USA. Silicon wafer (n type, Si (100), 4" dia. x 0.525 mm thickness) with 300 nm SiO_2 coated layer was used as a substrate material. Before the start of the lithography process, substrates were thoroughly cleaned with isopropanol alcohol (IPA) and dump rinsed in hot boiling deionized (DI) water. Stream of dry nitrogen gas was used to remove the excess water droplets. The final step involved in wafer cleaning was the use of oxygen plasma to remove contaminants from the surface of Si wafer. Spin coating (spinNXG-P1, Apex Instruments, India) was employed to uniformly coat the resist onto the silicon wafer. The pattern to be

exposed was designed using CleWin software (WieWeb). Durham Magneto Optics Ltd, Microwriter (ML3) mask less lithography setup with 385 nm long-life semiconductor light source was used for exposure. PDMS and curing agent (SYLGARD™ 184 Silicone Elastomer Kit) were procured from Kevin Electrochem, Mumbai, India.

2. Lithography used for the fabrication of SU-8 master template

SU-8 was spin coated on the clean SiO_2 coated silicon wafer side. A soft-baking was employed at 65℃ and 95℃ for 5 min and 35 min respectively. An input exposure dose value of 6975 mJ/cm^2 was employed to crosslink and subsequently harden the photopolymer. Post-exposure bake was carried out at 65℃ for 1 min and 95℃ for 15 min. After the development process, the master mold was dump rinsed in IPA and blown with the stream of dry nitrogen gas to remove any stray material. The micro-patterns were optically examined under microscope before transferring to PDMS.

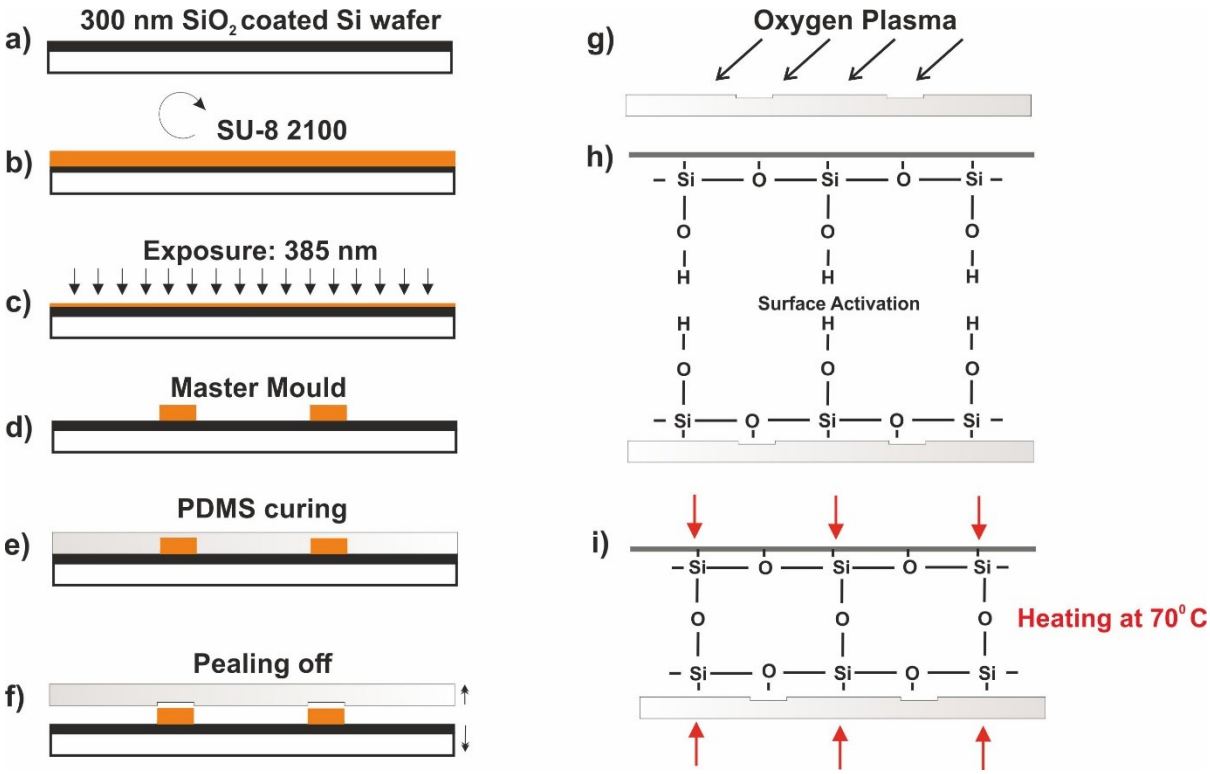


Fig. S1. Overall scheme showing the formation of lithography-based microfluidics chip. a) Clean Si-Wafer, b) SU-8 2100 photoresist coated via spin coating, c) exposure so as to cross

link the photoresist, d) Dissolution of unexposed photoresist in the developer, e) PDMS pouring and curing, f) Pealing of cured PDMS, g) surface activation by oxygen plasma, h) surface activation process, i) glass-PDMS bonding.

3. Pattern transfer to PDMS and bonding

For this, initially PDMS and curing agent were mixed in the ratio of 10:1 and vigorously stirred. The well-blended mixture of PDMS and curing agent was poured into petri plate containing the mold and kept inside the vacuum desiccators to remove the trapped air. Once all the air bubbles were removed the mold was kept in the oven at 70 ℃ for 4 h for enhanced crosslinking of the polymer. After the system cools to room temperature, the PDMS was cut and pulled out carefully avoiding damage to the micro-patterns. Punch was made with 1.5 cm outer diameter puncher for all inlets and outlet. After this, the patterned PDMS and a glass slide were activated under oxygen plasma in vacuum condition of 1×10^{-2} mbar, oxygen gas flow rate of 0.47 sccm and RF power of 80 W. To initiate the PDMS-glass bonding, the activated PDMS and glass slide were placed on top of each other with the pattern side facing glass slide. This arrangement was heated (70 ℃) using a programmable hot plate to strengthen the bonding with mild pressure applied from the top. Syringes and tubes for both inlet and outlet were connected in the punched holes of the mold. Flow rates were controlled using a software-driven syringe pump (Cetoni GmbH, NEMESYS-120). The output was attached to eppendorf for collecting the synthesized product.

4. Microreactor fabricated via lithography process

Image of microreactor was captured through digital camera. Coin was taken as reference for the microreactor dimensions.

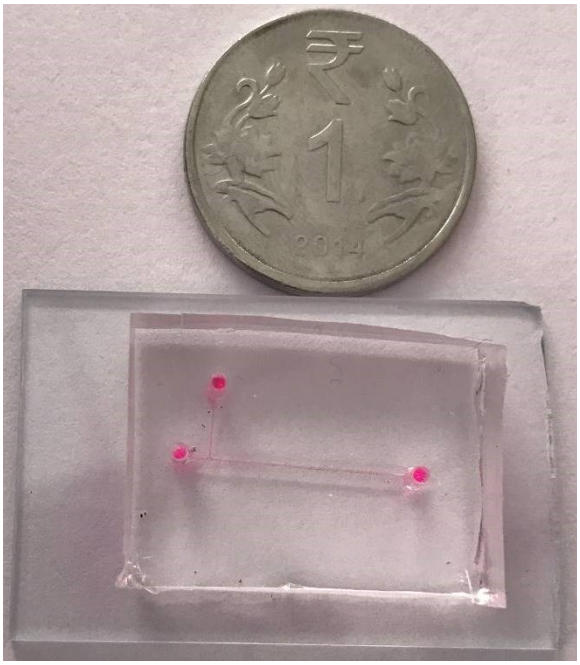


Fig. S2: The microreactor fabricated using lithography process

5. NBs used as containers:

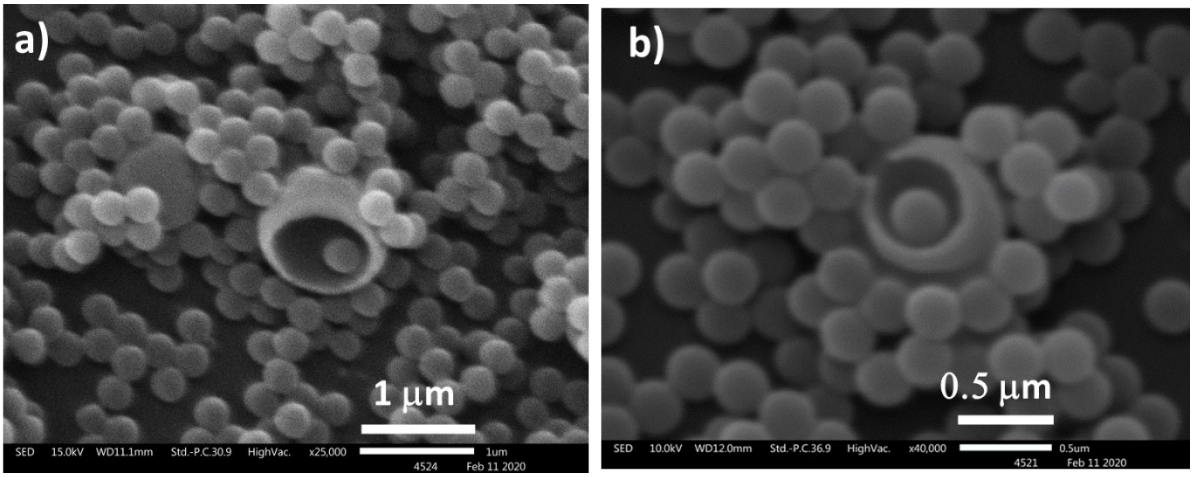


Fig. S3: SEM images (a, b) demonstrating the NBs as hollow containers with polystyrene beads of 300 nm diameter entrapped inside.

Above figure shows a typical example where the bowl-shaped nanoparticles were co-dispersed with 300 nm polystyrene solid beads and briefly sonicated for 15 min to load the polystyrene beads inside their cavities.

6. Stability studies of NB-shells using DLS

Stability studies of NB-shells was done in PBS up to 48 h. Results demonstrated the particles to be stable with not much variation in their mean particle size during this period of observation (as per figure shown below).

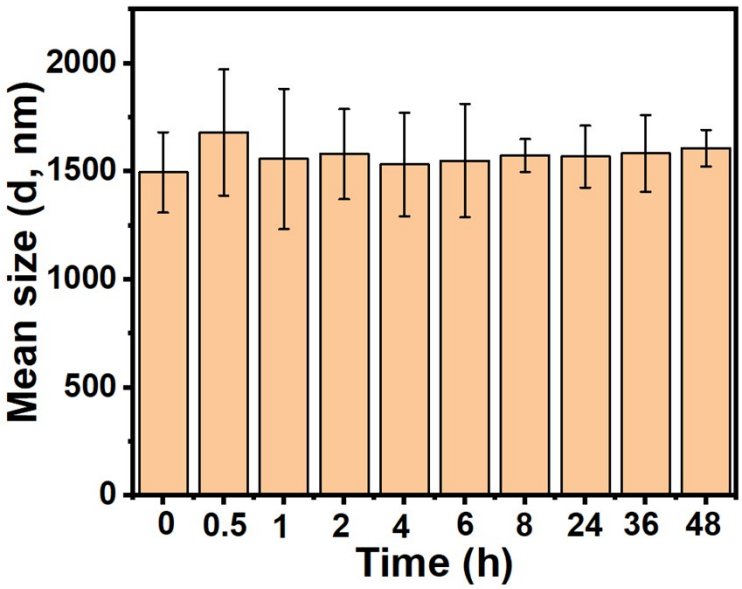


Fig. S4: DLS graph representing the mean size of *Nanobowl Infused Core-Shell Like Microstructures* (NB-shells) dispersed in PBS.

7. Covering onto NBs using Fmoc-Cys(Trt)-OH

We also tried coating the NBs with Fmoc-Cys (Trt)-OH, but could not get uniform coating, rather many uncovered NBs with altered size and shapes were observed (**Fig. S5**).

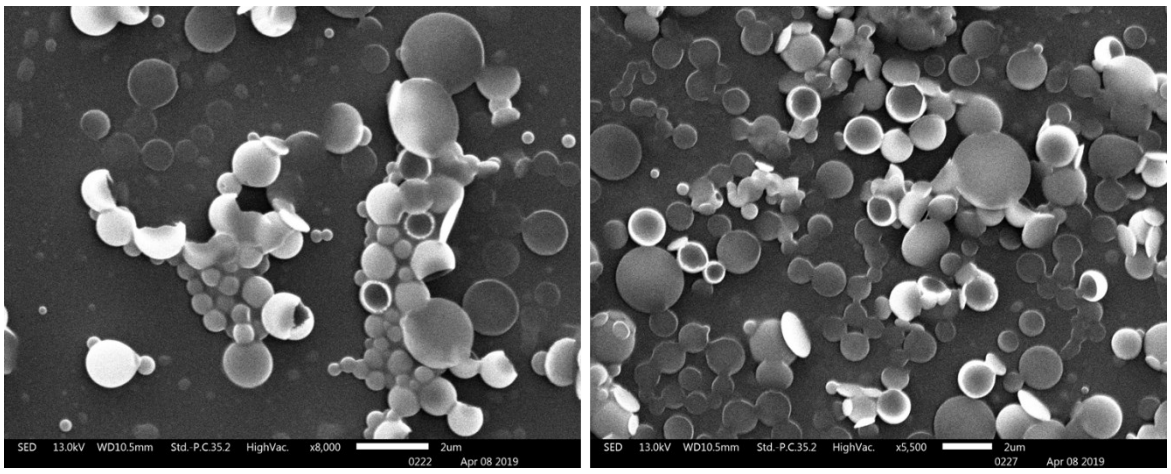


Fig. S5: Covering of the NBs using Fmoc-Cys(Trt)-OH shell.

8. Confirmation of acid triggered amino acid degradation determined using HPLC analysis

The acid triggered deprotection/dissociation of acid labile moieties (Trt/Boc) from the amino acid Boc-Cys(Trt)-OH was monitored using HPLC. For this, the amino acid was first dissolved in a mixture of acetone-water and then pH of the solution was set to 7, 5 and 2. The amino acid was incubated under these pH conditions for a period of 24 h. After the incubation period was over, samples were analysed using HPLC connected with ESI-MS. Briefly, samples were dissolved in minimum amount of acetonitrile (ACN) and H_2O containing 0.1% trifluoroacetic acid and passed through reverse phase C18 column in a Waters HPLC setup at an ACN-H_2O linear gradient (total flow rate of 1 mL min^{-1} over 36 min). Then the absorption intensities were observed at a wavelength of 205 nm and further molecular weight corresponding to the major peaks were determined by using the attached ESI-MS setup.

We hypothesized that the opening of pores at acidic pH was due to the cleavage or deprotection of the acid labile groups, Trt and -Boc, from the amino acid Boc-Cys(Trt)-OH. This was confirmed by carrying out combined analytical HPLC and mass spectrometric analysis. HPLC chromatograms and their corresponding mass peaks are presented in **Fig. S6: a, b, c**. Boc-Cys(Trt)-OH initially demonstrated a single peak with a mass of 462.36 at pH 7, whereas at pH 5 and pH 2 multiples peaks were observed having different masses. At pH 5, two peaks were observed having masses of 462.38 (corresponding to the mass of Boc-Cys(Trt)-OH) and 243.29 (c*orresponding to the mass of only trityl group*). However, at pH 2 a higher degree of deprotection was observed resulting in the emergence of multiple peaks in the HPLC chromatogram. Peaks corresponding to the masses of Boc-Cys(Trt)-OH) (equivalent to 462); trityl group (equivalent to 243) and to NH_2-Cys-OH after cleavage of both Trt and Boc groups

(equivalent to 122) were observed in this case (**Fig. S6).** These results were very encouraging, as they clearly supported our hypothesis of the acid induced deprotection of the assembling amino acids leading to pore formation of the NB-shells. The acid induced cleavage of –Trt group from Fmoc-Cys(Trt)-OH also cannot be overruled in this case.

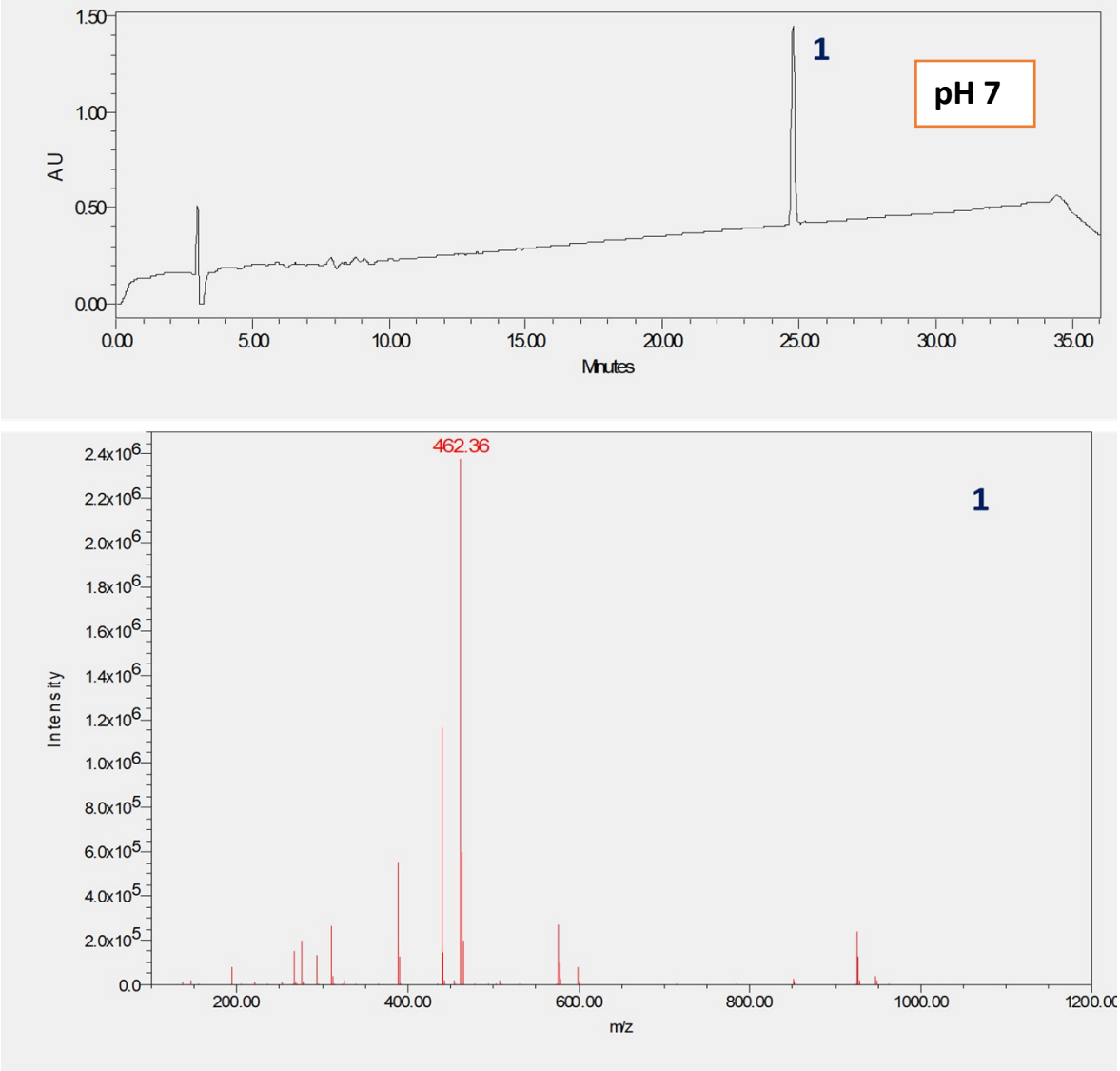
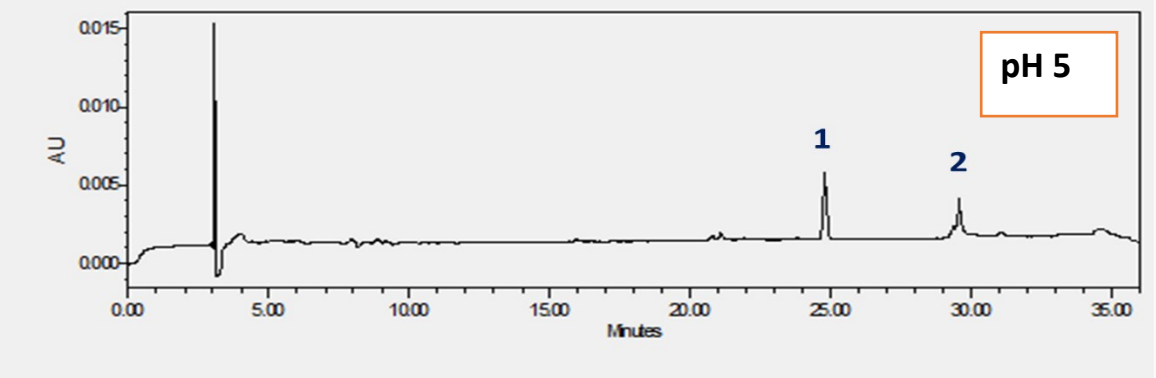
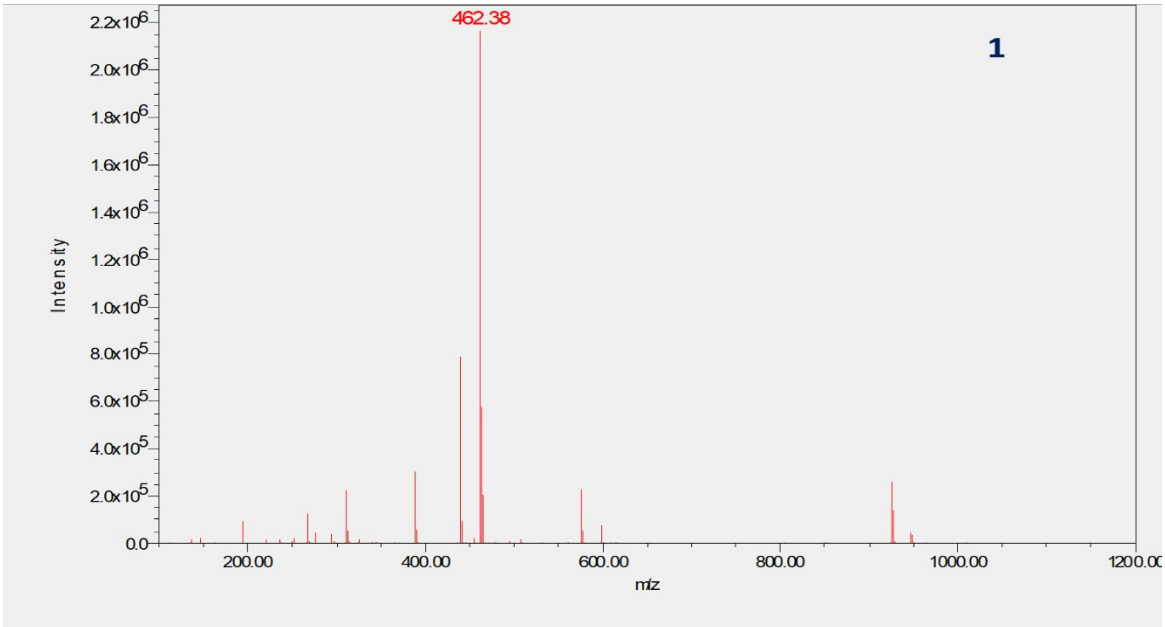
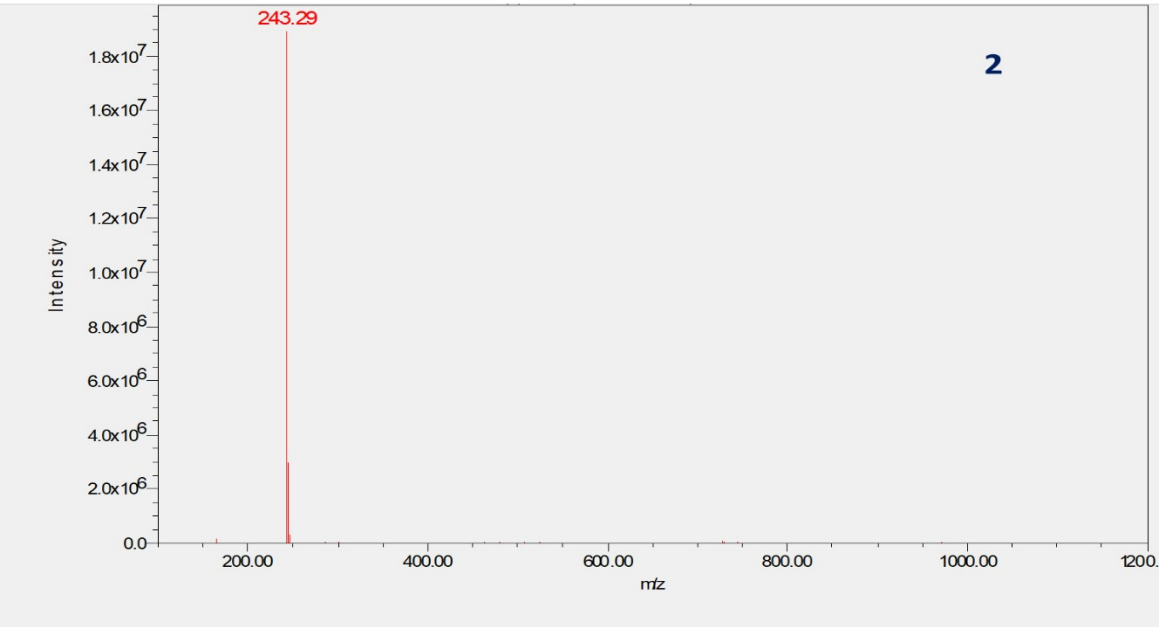


Fig. S6: (a) LCMS data of Boc-Cys(Trt)-OH at pH 7


pH 5
1
2
AU
0.015
0.010
0.005
0.000
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Minutes


462.38
1
Intensity
2.2x10^6
2.0x10^6
1.8x10^6
1.6x10^6
1.4x10^6
1.2x10^6
1.0x10^6
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
0.0
200.00
400.00
600.00
800.00
1000.00
1200.00
m/z


243.29
2
Intensity
1.8x10^7
1.6x10^7
1.4x10^7
1.2x10^7
1.0x10^7
8.0x10^6
6.0x10^6
4.0x10^6
2.0x10^6
0.0
200.00
400.00
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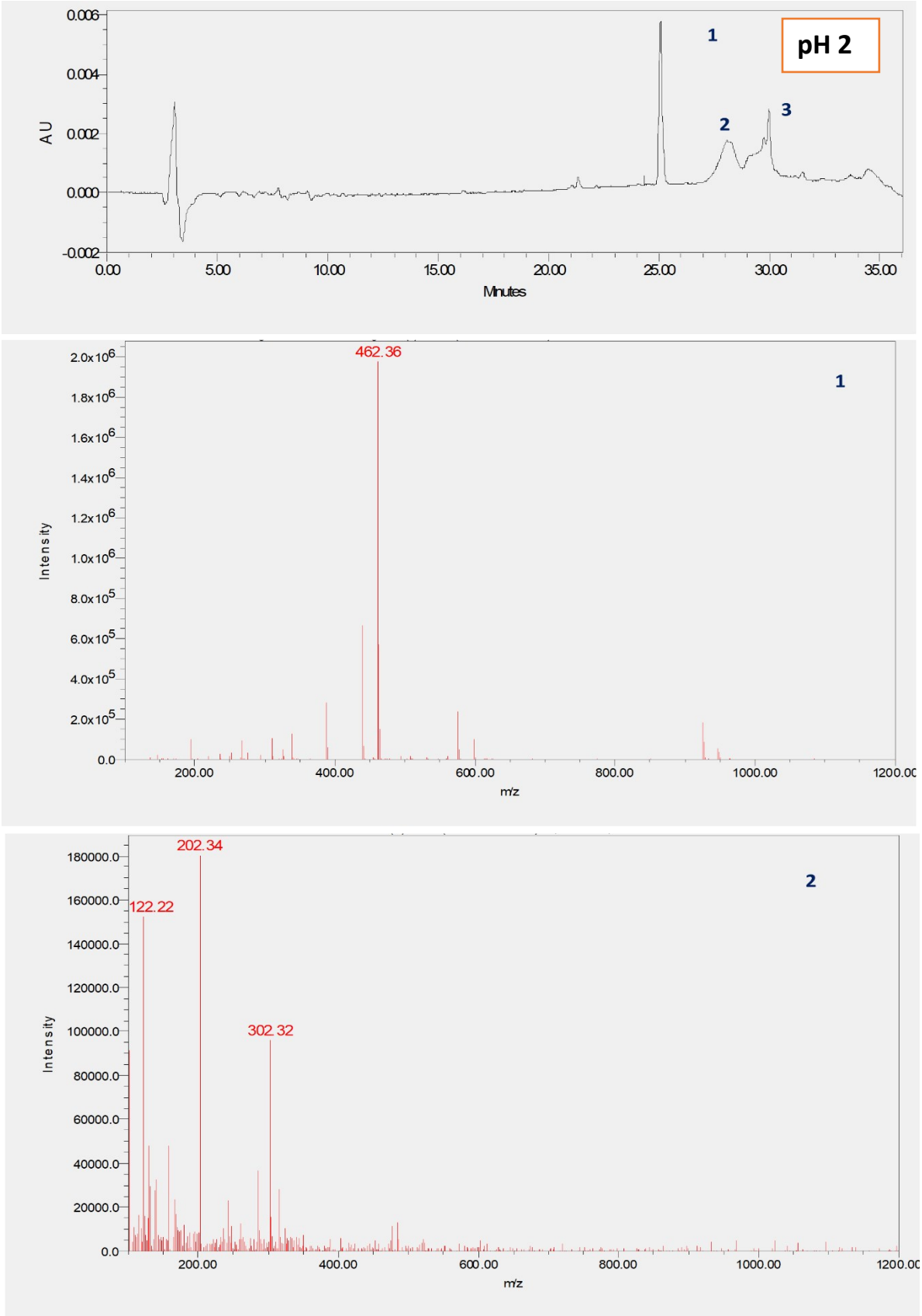
m/z

Fig. S6: (b) LCMS data of Boc-Cys(Trt)-OH at pH 5



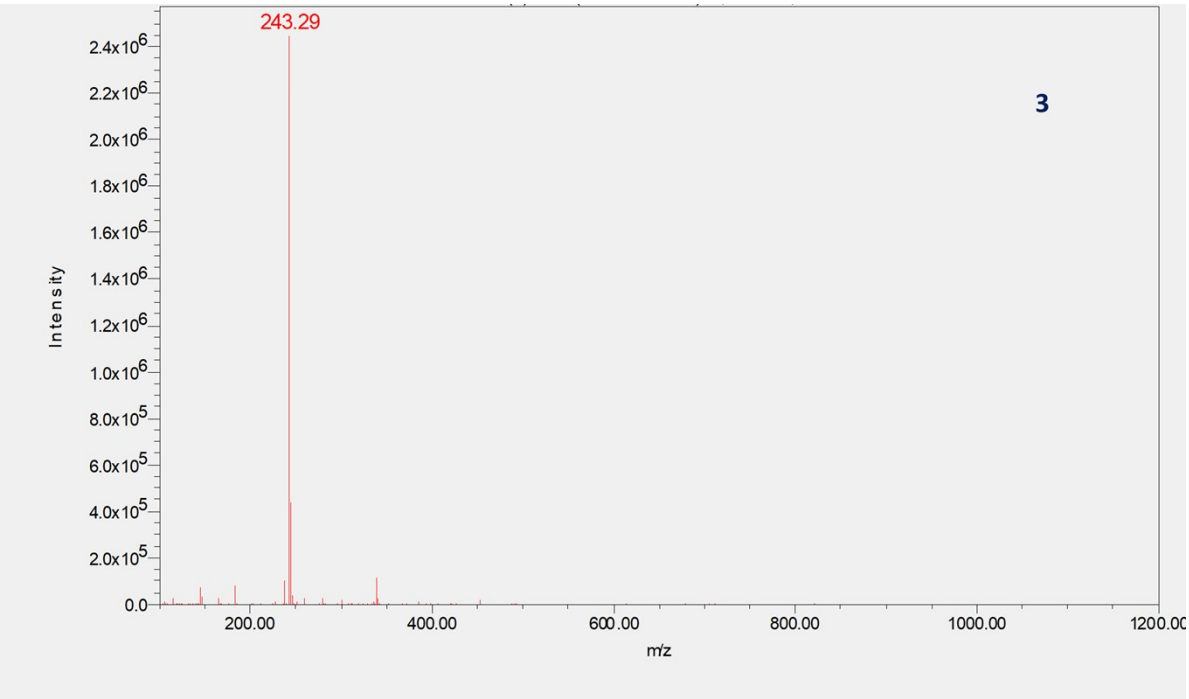


Fig. S6: (c) LCMS data of Boc-Cys(Trt)-OH at pH 2

9. Images taken at different depths of the NBs using confocal microscopy

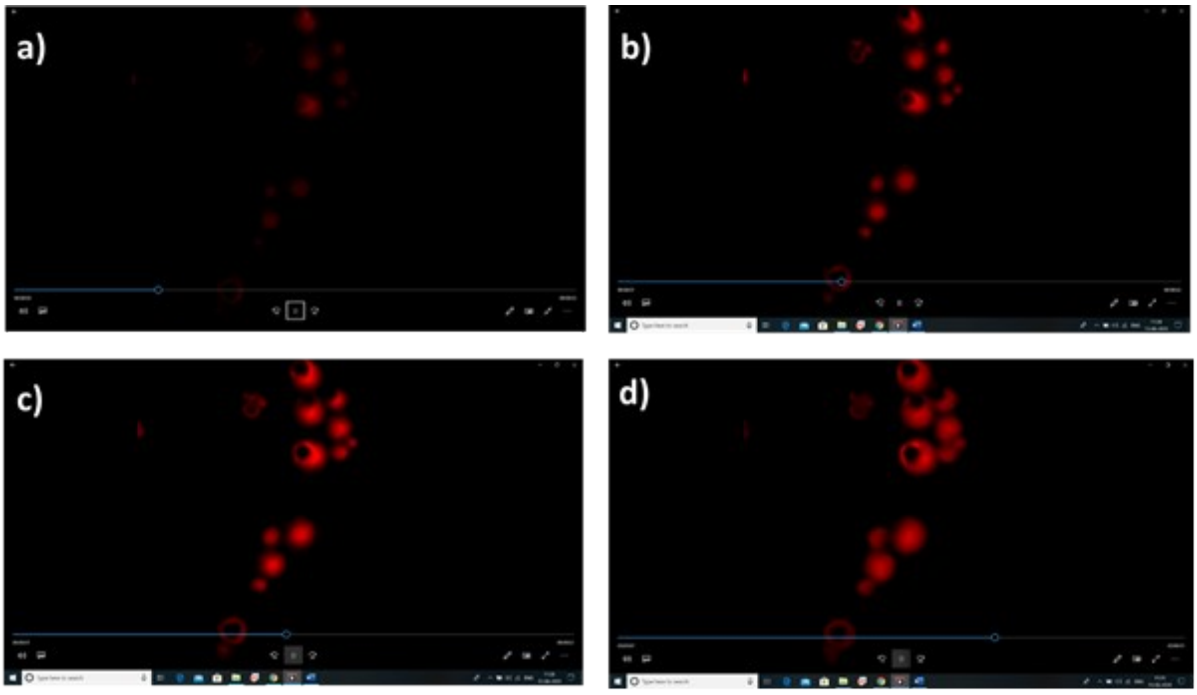


Fig. S7: Confocal images taken at different depths of the Dox loaded NBs

10. Encapsulation of Dox in the NB-shells and study their release behaviour under different pH conditions

After confirmation of drug loading and coating on to the drug loaded NBs at pH 7, pH of the sample was changed to pH 5 (**Fig. S8, a**) and pH 2 (**Fig. S8, b**). Confocal microscopic studies at pH 5 and pH 2 further indicated pH triggered opening of pores of the drug loaded NB-shells

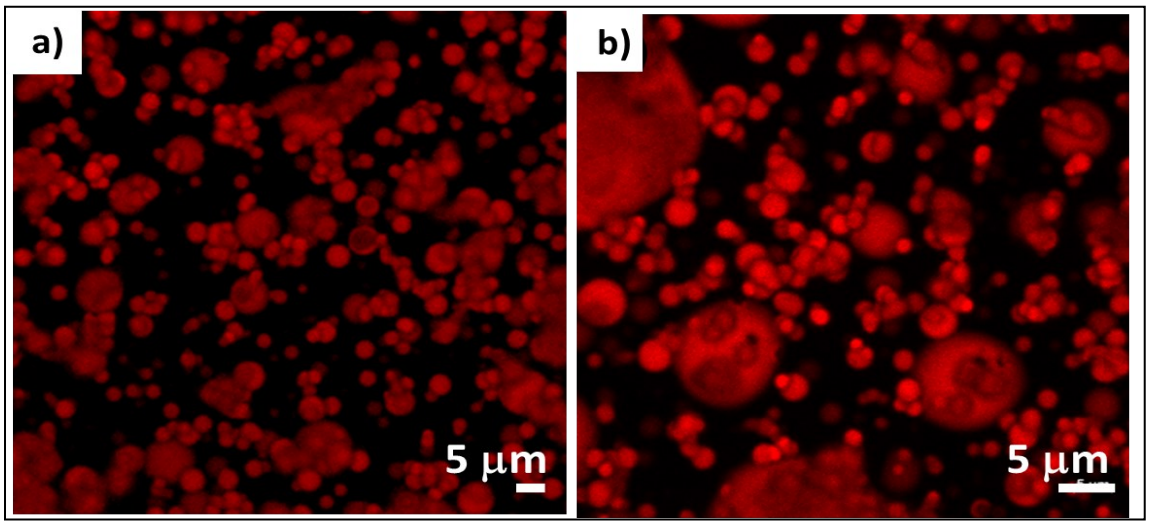


Fig. S8: Confocal microscopic images of Dox loaded NB-shells at pH 5 (a) and at pH 2 (b).

11. Cellular uptake studies using Confocal Microscopy

Quantitative analysis performed using Zen Blue software also depicted higher fluorescence intensity and hence higher cellular uptake in case of Dox loaded NB-shells. Dox loaded NB-shells demonstrated approximately 4-fold higher fluorescence intensity as compared to free Dox (**Fig. S9, a**). Cytotoxicity experiments were carried out using bare NB-shells in order to address biocompatibility of the particles. As shown in **Fig. S9, b**, approximately 94-95% of cells were found to be viable following the treatment with bare NB-shells.

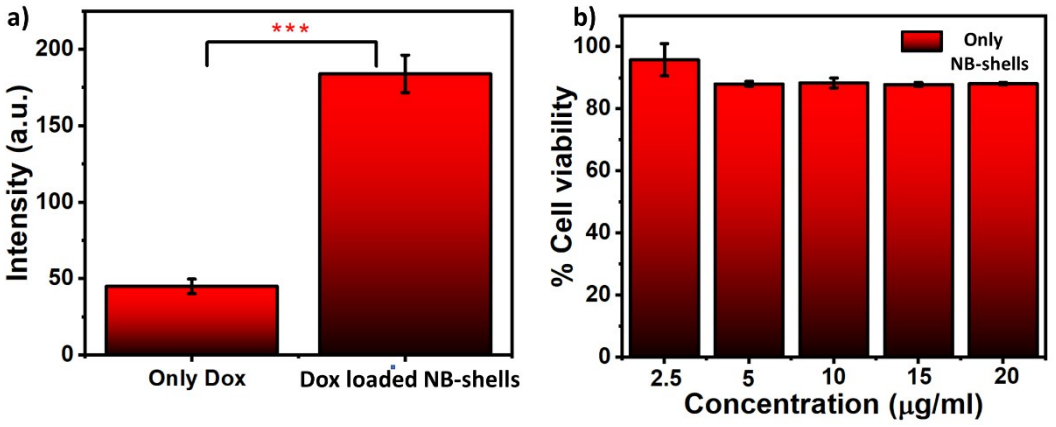


Fig. S9: (a) Graph showing comparison between cellular intensity of only Dox and Dox loaded NB-shells determined through Zen-blue software. (b) Cytotoxicity of bare NB-shells in C6 cells. *** represents levels of significance ($P < 0.001$).

12. Removal of acetone from the formulation using dialysis method and assessment of retention of particle structure after acetone removal and dialysis

To prepare samples for *in-vitro* and *in-vivo studies*, acetone was removed from the formulations using dialysis bag method for 24 h. This was followed by SEM imaging which confirmed the

retention of covering on to the NBs as well as their spherical shape even after acetone removal and dialysis for a period of 24 h **Fig. S10**.

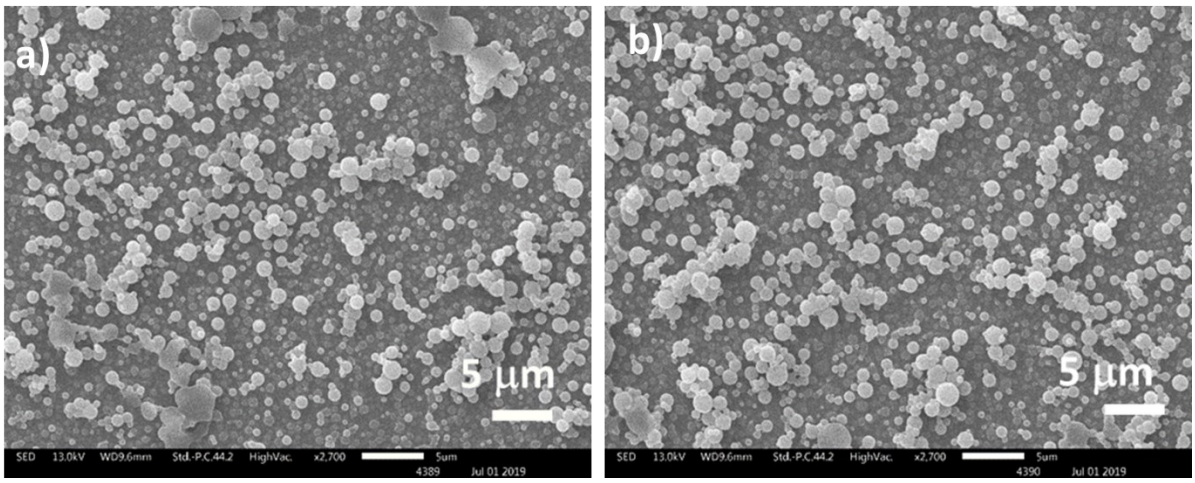


Fig. S10: SEM images of the dialysed NB-shells taken after 24 h of dialysis.