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Supplementary Information

Fabrication and Modification of Composite Silica Nano Test Tubes for Targeted Drug Delivery

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Preparation of AAO Membrane and SNTs

Al foil was first mechanically polished with 600-grit sand paper and ultrasonicated in acetone. This was followed by an electrochemical polishing step at 70 °C under 15 V where Al was the anode and a Pb electrode was used as the cathode. A Sorensen DCS 60-20 E power supply was used as the voltage source. The electropolishing solution contained 95 wt% H_3PO_4, 5 wt% H_2SO_4 and 20 g/ L of CrO_3. After electropolishing, the anodization steps were carried out in 5 wt% aqueous oxalic acid solution at 5 °C under 50 V against a cylindrical stainless-steel cathode. A precursor alumina film forms as a result of the first anodization (16 h) which was dissolved in acidic CrO_3 solution (0.2 M CrO_3 and 0.4 M H_3PO_4) at 80 °C after being removed from the oxalic acid electrolyte and rinsed with water. The duration of the second anodization determines the AAO depth[1] and in our case it was set at 6 min to obtain ~ 800 nm deep pores. The second anodization was conducted using the same oxalic acid solution of the first anodization. Finally, AAO templates were extensively washed with purified water and the nanopore diameter was tuned by a pore widening step through immersion into 5 % (v/v) H_3PO_4 solution. It should be noted that the pore width, and hence the resultant tube diameter, can also be controlled by the anodization voltage and electrolyte type. The AAO surface was characterized by Scanning Electron Microscopy (SEM) by using a FEI Quanta 200FE-SEM microscope after the surface was coated with 10 nm-thick Au-Pd conductive layer.

In order to prepare SNTs, the surface sol–gel method[2] which involves layer-by-layer deposition of silica onto a substrate material was employed. An AAO template was first immersed into $SiCl_4$ for 2 min, and then quickly soaked into a hexane-filled beaker for 2 min. In order to remove the non-bound $SiCl_4$, the template was rinsed with hexane and soaked into a second beaker of hexane for 10 min. It

was necessary to limit the hydrolysis of $SiCl_4$ with atmospheric water during these steps,[3] hence, they were conducted under constant nitrogen flow within a polyacrylic box. The template was then placed in a mixture of MeOH and hexane (1:1 v/v) for 2 min, EtOH for 5 min and dried under nitrogen stream. Finally, it was immersed into purified water for 5 min and into MeOH for 2 min. From $SiCl_4$ to the final MeOH treatment, one adsorption/hydrolysis cycle was completed. Here, the templates were treated with 5 such deposition cycles to obtain ~ 5 nm tube thickness and then they were cured under 100 °C for 1 h. To obtain free SNTs, the top surface silica layer which normally connects the tubular structures had to be removed[3] by a brief Ar^+ plasma treatment (1 min). A SAMCO RIE-1C reactive ion etcher system was used where the plasma conditions were 13.56 MHz, 140 W, 20 Pa Ar pressure and 20 sccm Ar flow rate.

FA Modification of SNTs

FA conjugation was done by first dispersing the rinsed SNTs into an APTES solution containing EtOH. Prior to SNT addition, this solution (EtOH:APTES:Acetate Buffer (50 mM, pH= 5.0), 18:1:1, v/v) was hydrolyzed for 20 min with magnetic stirring. For amine coupling, the tubes were kept in this hydrolyzed solution for 80 min, filtered, and then rinsed several times with EtOH and water. While placed on the filter membrane, the tubes were cured overnight in a vacuum oven at 40 °C to obtain a stable amine coating. Similar silanization procedures reported the formation of ~ 3 amines/nm^2 surface density values and such surfaces can be successively modified with EDC-based coupling procedures.[4] The amine-modified tubes (NH_2-SNTs) were then re-suspended in 1 ml PBS (10 mM, pH = 7.4) and mixed with an activated FA solution containing 3.025 mg FA, 2.25 mg NHS, 7.5 mg EDC in 58 ml PBS.[5, 6] Under

magnetic stirring, the NH_2-SNTs were kept in this solution for 3 h for effective FA coupling. Finally, FA-NH_2-SNTs were filtered and washed several times with water.

Characterization of FA-Modified SNTs

The FA modification of SNTs were characterized by zeta potential, FTIR and XPS studies. The zeta potentials for SNTs, NH_2-SNTs and FA-NH_2-SNTs were obtained by using a Malvern Nanosizer ZS instrument. In each case ~ 5 mg/ml sample was diluted with 10 mM NaCl (1:9) and three measurements were conducted for each sample. In order to follow the modification steps, SNTs were also characterized by FT-IR measurements. Each setup involves the preparation of KBr pellets including ~ 5 mg SNT sample. The IR absorption bands were collected via Mattson 1000 FT-IR instrument at 25 °C and the data analysis were carried out by using Omnic software and library.

FA conjugation onto the test tube surface was also characterized by XPS (K-Alpha, Thermo Scientific) using an Al K-alpha target with 400 μm spot size. The amine peaks at ~ 400 eV for NH_2-SNTs, and FA-NH_2-SNTs were then analyzed by a Mathcad program. Fig. S1A shows the XPS spectra for naked, amine-modified (NH_2-SNTs) and FA-modified (FA-NH_2-SNTs) nanostructures. The peak for NH_2-SNTs at 399 eV demonstrated a single Gaussian distribution and corresponds to the presence of primary amine groups (Fig. S1B). On the other hand, the peak for FA-NH_2-SNTs can be deconvoluted into two different peaks (Fig. S1C), one appearing at 397 eV and corresponds to amide functional groups and N=C bonds while the second one again at 399 eV and stems from primary amines.[7] Similar results were observed in the previous reports[7] and indicate the successful conjugation of the FA moieties onto the SNT surface.

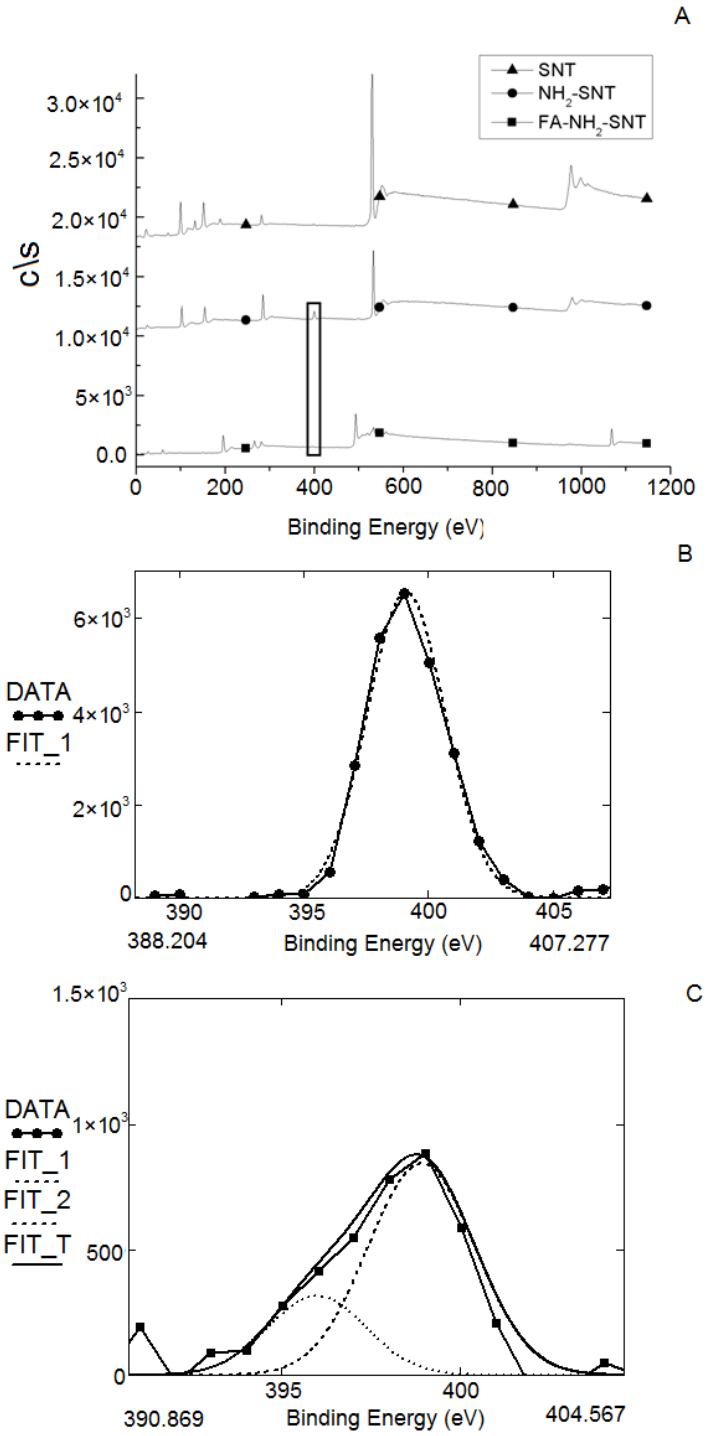

Fig. S1. The XPS spectra for naked, amine and FA-modified SNTs (A), the distribution anaylzes of the spectrum for NH_2-SNTs (B), and for FA-NH_2-SNTs (C). The dashed lines represent the fitted curves and the solid line is their sum.

Preparation and Investigation of pH-responsive Gels

The interior of the SNTs were filled with a gel matrix that involved HEMA, PEG-EEM, AEM, trimethyloylpropane ethoxylate triacrylate (crosslinker), doxorubicin (DOX) hydrochloride, 2,2-diethoxyacetophenone (photoinitiator), IPA and water (see Table S1 for relative ratios. Here, HEMA provided the swelling and thus the drug release mechanism, PEG-EEM allowed the control over mesh size and thus the release rate as well as mildly hydrophobic environment for enhanced DOX solubility, and AEM enhanced the swelling rates at acidic medium that is typically observed in tumor milieu.[8]). Prior to the fabrication of composite SNTs and related cell studies, the pH responsive DOX release from bulk gels were investigated using bulk gels.

Table S1. The relative amounts of the components for gel formulation.

Gel Constituents	**Formulation** (% vol/vol, for * % wt/vol)
HEMA	82.96
PEG-EEM	1.15
Trimetthyloylpropaneethoxylate triacrylate	0.58
2,2-diethoxyacetophenone	1.91
H_2O	9.57
Isopropanol	3.83
AEM*	1.0
DOX*	0.13

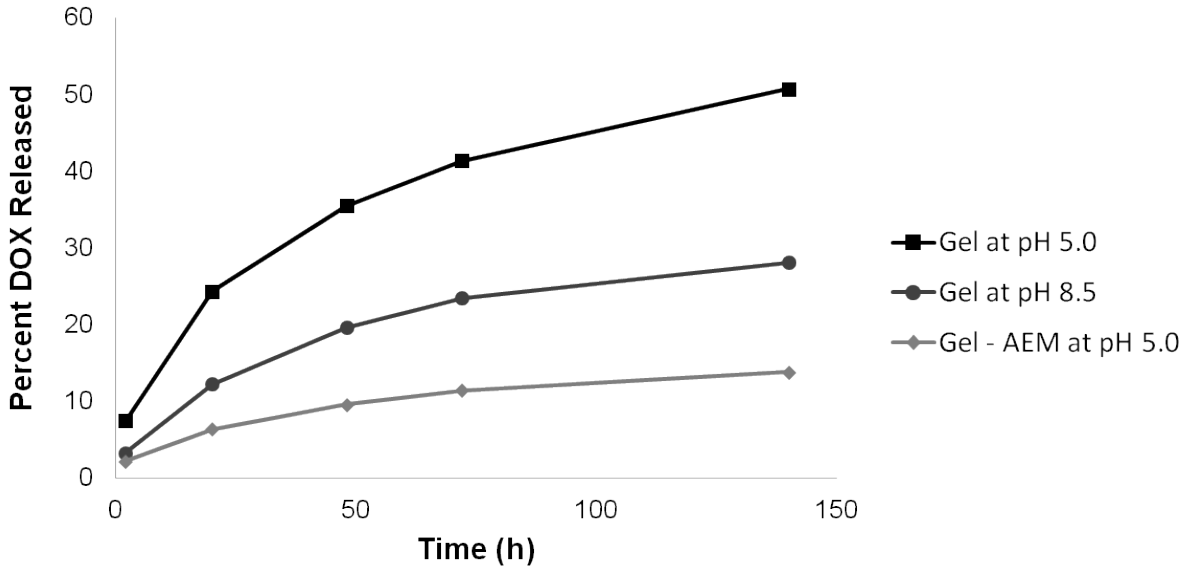

Fig. S2. DOX release from bulk gels to acetate (pH=5) vs. phospate buffer (pH=8.5), and also DOX release trend from bulk gel without AEM content at pH=5.

Here, disk-shaped 0.5 cm^2 gels were prepared according to the relative ratios indicated in Table S1. DOX release was quantified at different time points for gels in acidic or basic media as well as for a gel formulation that does not involve AEM. More than 2.5 folds DOX release was obtained for AEM-containing gels in acidic release media (pH=5.0, acetate buffer) compared to a basic one (phosphate buffer at pH=8.5) (Fig. S2). This result originates from the extensive protonation of the primary amine groups (pKa ~ 7.6,[9]) of the AEM at the acidic pH. More extensive gel swelling was observed and hence higher drug release patterns were attained. Moreover, DOX release was greatly suppressed even in an acidic buffer if AEM was not involved in the prepolymer mixture. These studies revealed that AEM is necessary for making a responsive gel as well as obtaining increased drug release.

Cells and Culture

SK-BR3 breast cancer cells (ATCC) were maintained in Dulbecco's modification of Eagle's medium with 10% fetal bovine serum and 1% penicillin in T-25 flasks at 37 °C in 5% CO_2/air. MCF-12A normal human mammary gland epithelial cells (ATCC) were maintained in Dulbecco's modification of Eagle's medium with 5% horse serum, 20 ng/ml human epidermal growth factor, 0.01 mg/ml bovine insulin and 500 ng/ml hydrocortisone in T-25 flasks at 37 °C in 5 % CO_2/air. For both cell types, cell media was changed with new one every other day. When the growth rate reached ~ 80 %, the cells that were fully spread out in flasks were removed from surface by using Trypsin/EDTA. After reaching sufficient maturity, the cells were transferred to 96-well plates, each well including 1×10^4 cells. After 48 h, SNTs of different compositions were applied to the cells for the viability tests.

Cell viabilities were examined via WST-1 Cell Proliferation Kit Assay.[10] Cells exposed to various SNT types were kept in 96-well plates for 48 h at 37 °C in 5% CO_2/air. WST-1 solution was then prepared by using 10 µl kit which was added to each well and the plate was kept for 1 minute on an orbital shaker. After this process, the plate was incubated for 2 more hours at 37 °C, and finally, the absorbance value at 450 nm was measured. The percentages of live cells for each SNT sample were presented as mean ± SD values and reported relative to the control cells which were not treated with SNTs. Here, 3-5 parallel wells were used for each SNT sample. In order to compare two groups, unpaired Student's *t* test was performed and a value of $P < 0.05$ was considered significant.

Free Drug Cytotoxicity

DOX formulations with different final concentrations were prepared and applied to SK-BR3 cells and cytotoxicity values were assessed by applying the protocol mentioned above via the WST-1 kit (Fig. S3).

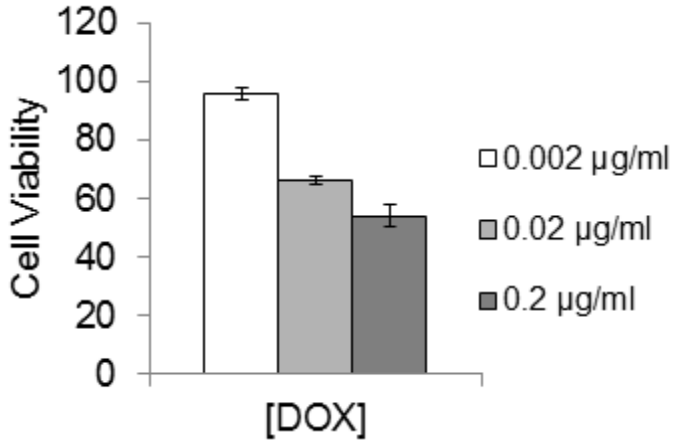

***Fig. S3.** Cell viability data obtained with WST-1 Kit for SK-BR3 cells against free DOX at different concentrations.

Calculation of DOX loading

The drug loading capacity of the SNTs were calculated by analysing the released drug via square wave voltammetry.[11] The characteristic DOX peak at ~ - 0.49 V vs. Ag/AgCl reference electrode (Fig. S4A) was investigated in a voltammetry setup where potential was scanned between 0 V and -0.8 V (frequency=50Hz, amplitude=0.025V) through a CHI 660D potentiometer with electrochemical workstation program. The electrolyte (release media) was 50mM acetate buffer at pH 5, the tube concentration was ~ 2 x 10^{10} SNTs / ml, and the release time was two weeks. The working electrode was BAS controlled growth mercury electrode and a Pt wire was used as the counter electrode. Using standard addition method with a 3.5

μM stock solution, the concentration of released DOX was quantified as 0.15 μM (Fig. S4B), corresponding to ~ 4500 DOX / tube. Note that the calculated DOX concentration is dictated by the initial DOX content which can be increase by using more concentrated starting solutions, and hence, values larger than the reported concentrations[12] are attainable. Moreover, when the release media was changed to PBS buffer (pH=7.4), no faradaic peak could be observed, again indicating the importance of pH for appreciable drug release.

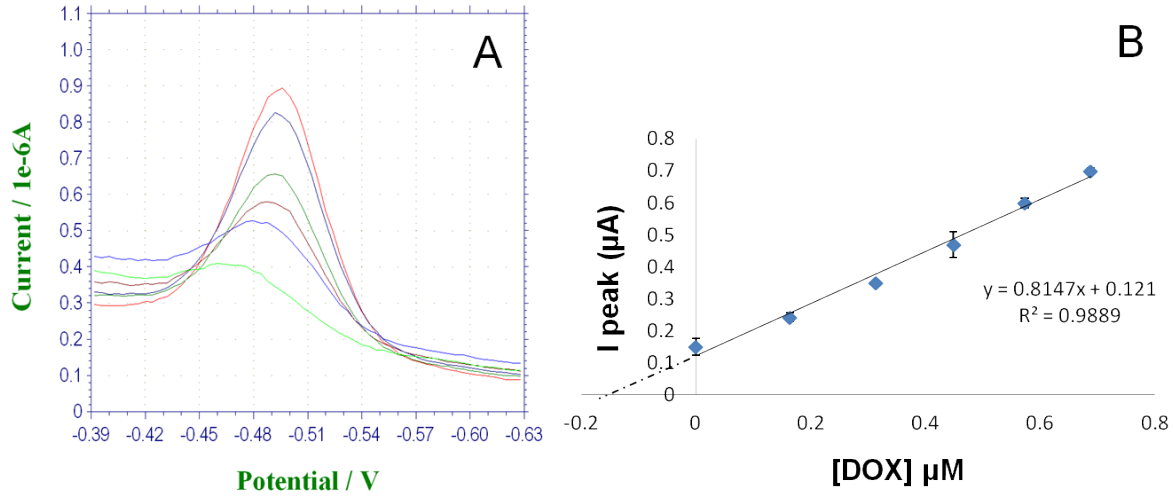

Fig. S4. Electrochemical detection of DOX signal from SNTs. The lowest curve on A stems from the original sample and the larger curves belong to DOX additions from the stock solution to attain the concentration by the standard additon method (B).

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