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Noncovalent interactions between linear-dendritic copolymers and carbon nanotubes lead to liposome-like nanocapsules

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Experimental section

Materials

MWCNT with 20 nm diameter were prepared by chemical vapor deposition procedure in the presence of Co/Mo/MgO as catalyst at 900 ºC. Polyethyleneglycol (MW=1000), $AgNO_3$, sodium methoxid, nitric acid, sulfuric acid, methanol, DOX were purchased from Merck. Glycidol was purchased from Aldrich. The murine colon adenocarcinoma tumor C26 and the mouse tissue connective fibroblast adhesive L929 cancer cells were obtained from the National Cell Bank of Iran (NCBI) Pasteur institute, Tehran, Iran. MTT powder, Annexin-V FLUOS Staining Kit, was obtained from Sigma.

Characterization

Nuclear magnetic resonance (^{1}H NMR) spectra were recorded in D_2O solution on a Bruker DRX 400 (400 MHz) apparatus with the solvent proton signal for reference. Infrared spectroscopy (IR) measurements were performed using a Nicolet 320 FT-IR. Ultraviolet (UV) spectra were recorded on a shimadzu (1650 PC) scanning spectrophotometer. Ultrasonic bath (Model: 5RS, 22 KHZ, Made in Italy) was used to disperse materials in solvents. The particle size and polydispersity of materials were determined using Dynamic Light Scattering (DLS) (zetasizer ZS, Malvern Instruments).

Surface imaging studies were performed using atomic force microscopy (AFM) to estimate surface morphology and particle size distribution. The samples were imaged with the aid of Dualscope/Rasterscope C26, DME, Denmark, using DS 95-50-E scanner with vertical z-axis resolution of 0.1 nm. Thermogravimetric analysis (TGA) were carried out in a thermal analyzer (model: DSC 60, shimadzu, Japan) under dynamic atmosphere of an inert gas (i.e. N_2) at 30

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ml/min (room temperature). The Transmission electron microscopic (TEM) analyses were performed by a LEO 912AB electron microscope with accelerating voltage of 200 kV.

Opening of MWCNTs

MWCNTs were purified and opened according to reported procedures in literature [1]. Briefly, MWCNTs (2 g) were added to 40 mL of sulfuric and nitric acid mixture (3:1) in a reaction flask and refluxed for 24 h at 120ºC. The mixture was cooled and diluted by distillated water and then it was filtrated. The product (MWCNT-COOH) was washed by distillated water and dried at 60ºC for 3 h by vacuum oven.

Preparation of PG-PEG-PG/MWCNT$_S$ LLNs

In this work, two molecular weights of PG-PEG-PG linear-dendritic copolymers were synthesized using different ratios of polyethyleneglycol (PEG) to glycidol (G) (1:10 and 1:20) and they were used to interact with MWCNTs non-covalently. PG-PEG-PG linear-dendritic copolymers (0.001 g) were dissolved in 5 ml distilled water and solution was added to MWCNTs (0.002 g).

Determination of the concentration of MWCNTs in the PG-PEG-PG/MWCNT$_S$

6 mg of acid treated-MWCNT was dispersed in 10 ml of water. PG-PEG-PG linear-dendritic copolymer (3 mg, 6mg and 12 mg) was added to the above solution by sonication for 30 min using ultrasonic bath. PG-PEG-PG/MWCNT$_S$ after passing through 0.45μ filter represented concentration of 0.47, 0.35 and 0.25 mg/ml nanotube with respect to the mass of PG-PEG-PG linear-dendritic copolymer respectively.

UV/vis spectra of PG-PEG-PG/MWCNTs were recorded and concentrations of MWCNTs were calculated using a calibration curve and Y=0.4094x + 0.001 equation (Fig.1 and Fig.2).

Loading capacity

DOX hydrochloride (11.75 mg) was stirred with the PG-PEG-PG/MWCNT LLNs (2.35 mg) in a pH 7.0 of purified water (5 mL) sonicated for 30 minutes and then stirred for overnight at room temperature.

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Unbound doxorubicin was removed by filtering and washing using Amicon Ultra 30 kDa centrifugal filter devices (Millipore).

Ultracentrifugation was continued with purified water until the filtrate became color free. The products LLNS containing the loaded DOX molecules were then collected by re-suspending in 5 mL of water.

Cell culture

Murine colon adenocarcinoma tumor C26 was cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1%peniciline-streptomycin at 37ºC in a humidified incubator with 5% CO2. The cells were maintained in an exponential growth phase by periodic subcultivation.

Cytotoxicity assay

In vitro cytotoxicity of the nanomaterials was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells (2500 cells/well) were seeded in 96-well plates. Nanomaterials were then added to the wells in triplicates and incubated for 72 hours. After the incubation period, 20 µl of MTT dye (5 mg/ml in PBS) was added to each well, and they were incubated in the dark at 37 ºC for 5 hours. Then media were removed and formazan crystals were dissolved in 200 µL dimethylsulfoxide (DMSO) and 20 µl of glycine buffer. Then the absorbance of each well was measured by an ELISA reader (Statfax–2100 Awareness Technology, USA) at 570 nm.

Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = (\text{Ints}/\text{Intscontrol}) \times 100 \quad (1)$$

where “Ints” is the colorimetric intensity of the cells incubated with the samples, and “Intscontrol” is the colorimetric intensity of the cells incubated with the Media only (positive control).

It is notable that owing to the absorption of vitamins, amino acids and ions at the surfaces of nanoparticles, it is clear that the common *in vitro* examination method can yield erroneous cell viability values.

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Outlier Detection

All MTT experiments were performed in triplicate or more, with the results expressed as mean ± standard deviation; standard deviation values are indicated as error bars in the MTT results plots. The results were statistically processed for outlier detection using a "T procedure" using MINITAB software (Minitab Inc., State College, PA). One-way analysis of variance (ANOVA) with $p<0.05$ was performed for each set of MTT assay test repeats. Outlier samples have then been excluded from the corresponding asset viability calculations.

In this method, a *T*-ratio is calculated as follows

$$T = \frac{X - \bar{X}}{S} \qquad (2)$$

Where X is the suspected outlier point (normally the smallest or the largest value in a set of measurements), $\bar{X}$ is the sample mean, and S is the (estimated) standard deviation. If the calculated value of T is equal to or exceeds a critical value, the outlier point is removed with a significance level of 0.05. In the latter case, assuming that the data were sampled from a normal distribution, there is at least a 95% chance that the suspected point is in fact far from other points.

Result and discussion

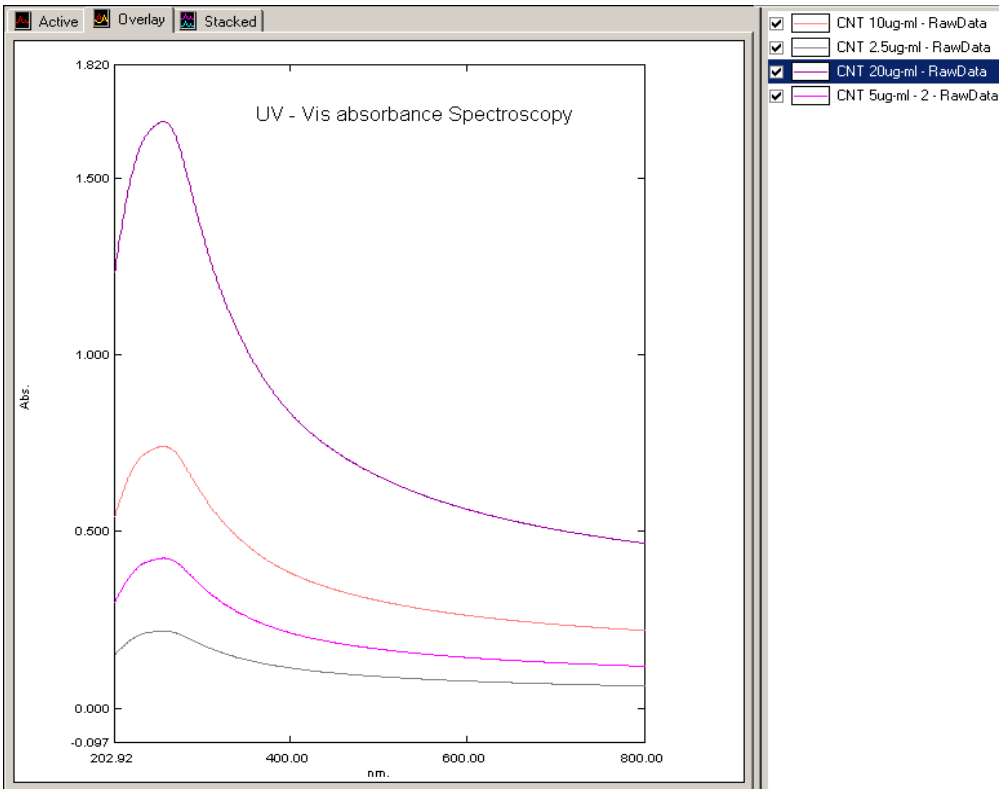

Fig. 1. UV/vis absorbance spectra of PG-PEG-PG/MWCNTs in water.

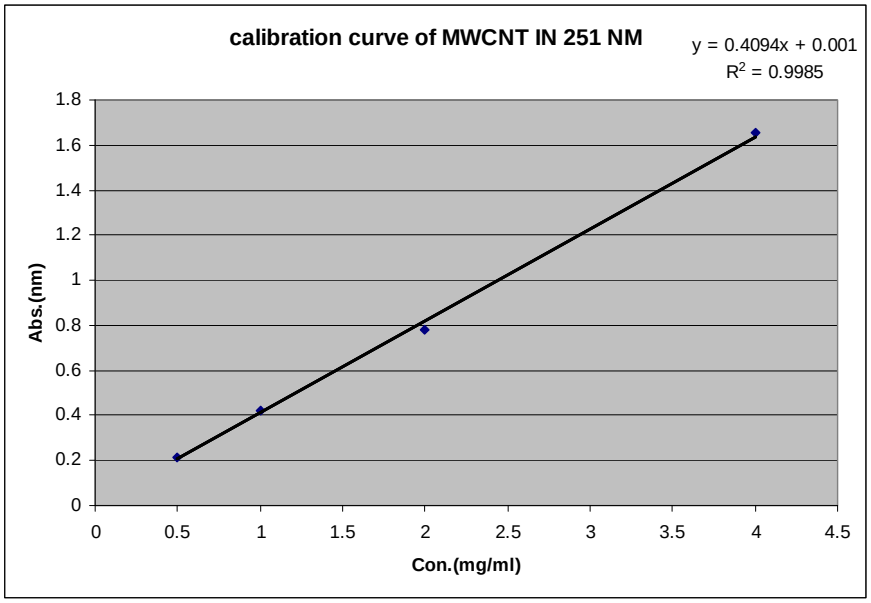

Fig. 2. Calibration curve for PG-PEG-PG/MWCNTs in water.

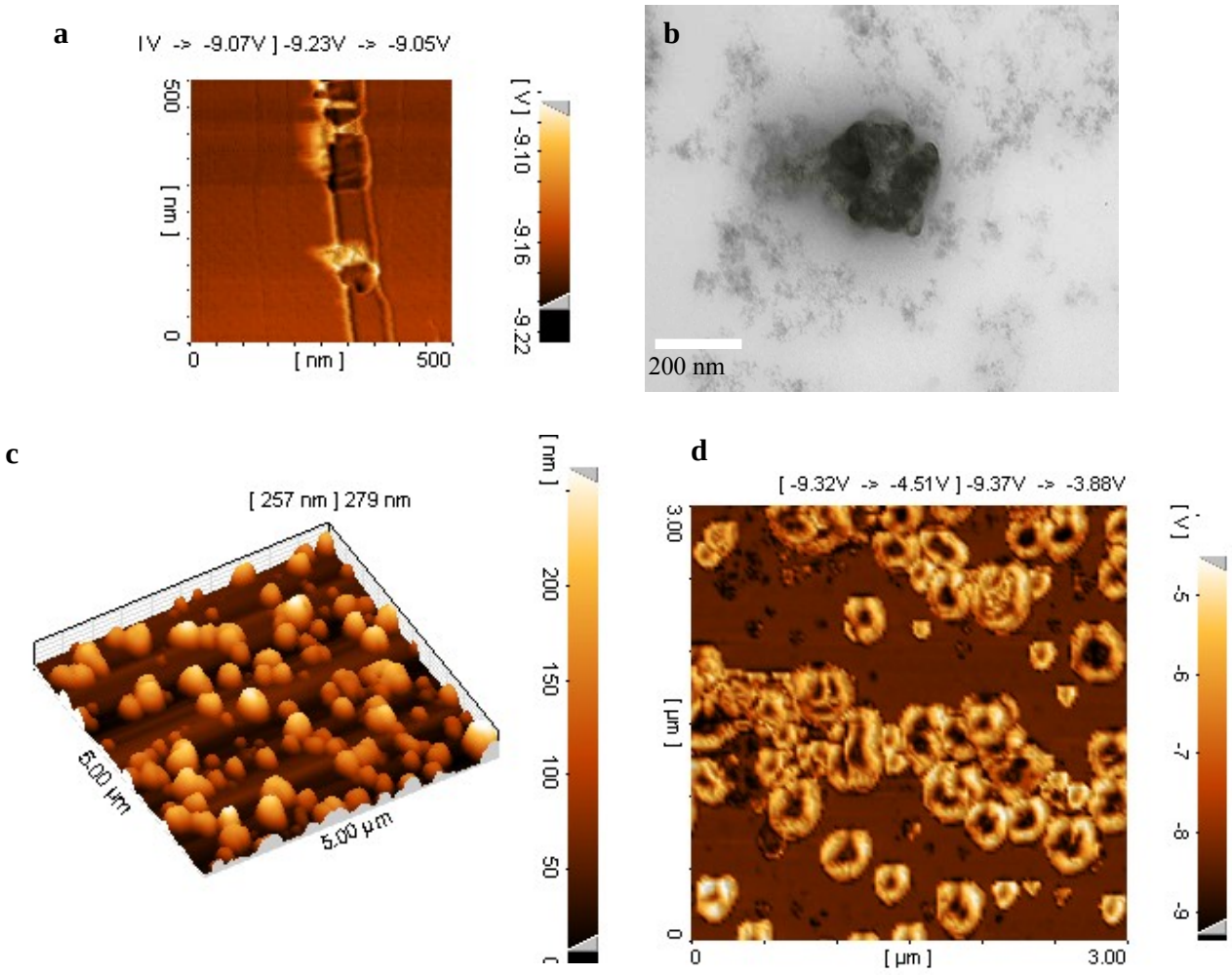

Fig. 3. (a) Topographic AFM image of an acid treatment MWCNT: the created defects on the surface of carbon nanotubes during refluxing in HNO3/H2SO4 mixture can be clearly seen. (b) TEM image of a LLN consisting of MWCNT, polycitric acid grafted onto its surface and paclitaxel molecules conjugated to the carboxyl functional groups of polycitric acid.

Fig. 4. MWCNTs (a) Opened MWCNTs, (b) PG-PEG-PG/MWCNTs and (c) DOX/PG-PEG-PG/MWCNTs in water after several months.

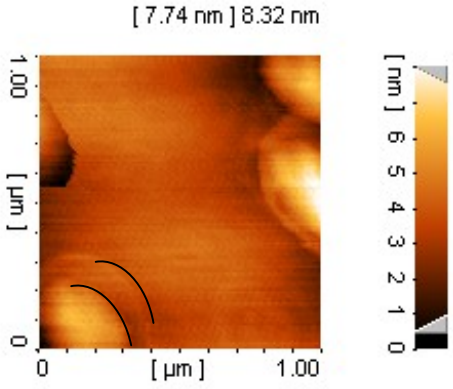

Fig. 5. Topographic AFM image of PG-PEG-PG/MWCNT LLNs. A shell with 100 nm thickness can be seen around a rigid core.

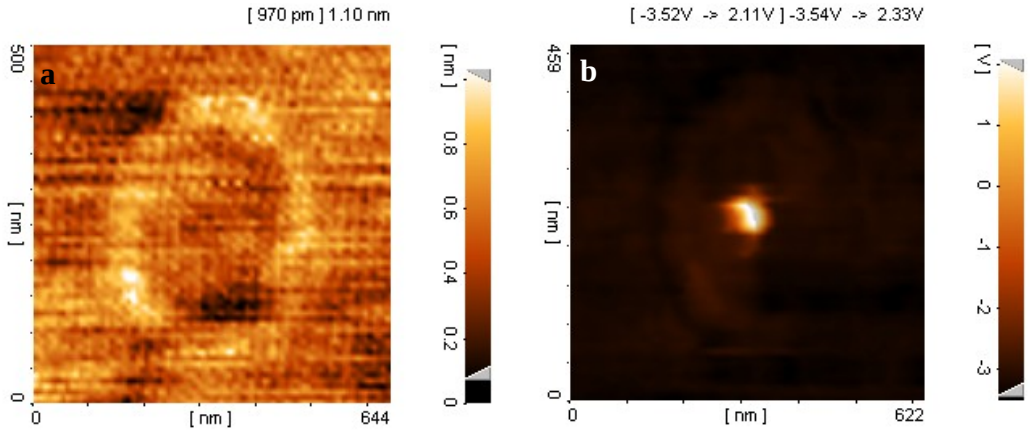

Fig. 6. (a) Topographic and (b) phase contrast AFM images of a PG-PEG-PG/MWCNT LLN containing encapsulated DOX molecules.

References

1. Adeli M, Bahari A, Hekmatara H. NANO: Brief Reports and Reviews 2008;3:37-44.