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Scalable Nanomanufacturing of Cyclic Peptide-based Nanorobots for *In Vivo* Sensing

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Objective

This grant provides funding for the development of a scalable nanomanufacturing platform for fabricating cyclic peptide-based nanorobots for biomedical applications, such as *in vivo* sensing, disease diagnosis, and targeted drug delivery. The nanomanufacturing platform will be used to assemble various types of cyclic peptide-based nanotubes conjugated with DNA-based aptamers. The core body of the nanorobots will be formed by self-assembling individual cyclic peptide subunits under controlled reaction conditions. After the core body has been formed, aptamers will be conjugated to the open ends of the nanotubes, and serve as the sensing as well as actuating components. Upon binding of a target biomarker to the aptamers, a conformational change takes place allowing the nanorobots to release their payload. In an effort to optimize the design, a library of cyclic peptides with varying diameters, controlled by the number of peptide subunits will be fabricated. To further demonstrate the modularity of the approach, aptamers for a variety of biomarkers related to specific diseases will be conjugated to the nanorobots and tested. To scale-up the fabrication process, phase equilibrium method, self-assembly in bulk solution, and layer-by-layer assembly method will be examined. After prototype fabrication, the nanomanufacturing process will be further optimized in terms of reliability, yield and manufacturing efficiency.

If successful, the results of this research will lead to manufacturing of cyclic peptide-based nanorobots for *in vivo* sensing, disease diagnosis and targeted drug delivery. The primary goal of this research is to determine the fundamental engineering principles related to scalable nanomanufacturing of self-assembled bio-molecules. The principles learned through this research will be applicable to various nanomanufacturing processes with self-assembly as a key step for bottom-up manufacturing. Upon development and optimization of the fabrication process, the medical community will benefit from the nanorobots.

A cyclic peptide nanotube (CPNT) used for antitumor drug delivery *in vitro* has been fabricated by our group. Based on the study, we currently are fabricating a stimulus-responsive hybrid nanorobot with controllable sizes using DNA-based adaptors and single CPNTs for *in vivo* sensing and disease diagnosis. Here we report the results from *in vitro* antitumor drug delivery using cyclic peptide nanotube (CPNT) bundles.

Methods and Results

1. Cyclic Peptide Design

1/2PCP6: PEG conjugated *cyclo*-(-L-Glu-D-Ala-)3
1/4PCP8: PEG conjugated *cyclo*-(-L-Glu-D-Ala-L-Gln-D-Ala-L-Glu-D-Ala-L-Gln-D-Ala-)
1/2PCP8: PEG conjugated *cyclo*-(-L-Glu-D-Ala-)4
1/2PCP10: PEG conjugated *cyclo*-(-L-Glu-D-Ala-)5
1/6PCP12: PEG conjugated *cyclo*-(-L-Glu-D-Ala-L-Gln-D-Ala-L-Glu-D-Ala-L-Gln-D-Ala-L-Gln-D-Ala-)
1/4PCP12: PEG conjugated *cyclo*-(-L-Glu-D-Ala-L-Gln-D-Ala-L-Glu-D-Ala-L-Gln-D-Ala-L-Glu-D-Ala-L-Gln-D-Ala-)
1/2PCP12: PEG conjugated *cyclo*-(-L-Glu-D-Ala-)6

D=Np/Na

D: Density.
Np: Number of PEG chains.
Na: Number of amino acids.

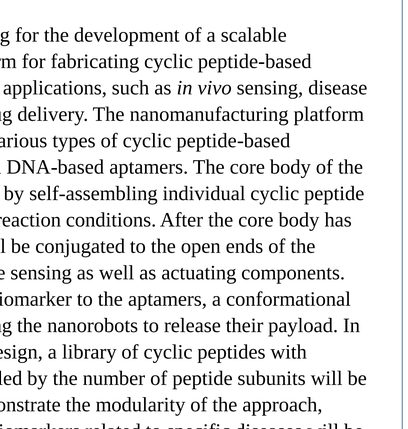

Fig. 1 Cyclic peptide design based on density (**D**) of the PEG chains and diameter of the cyclic peptide (number of the amino acids).

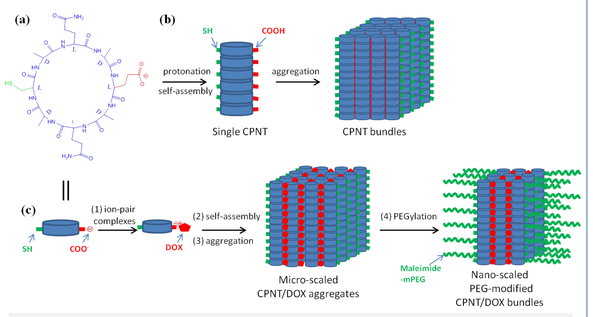

Fig. 2 Structure of the cyclic octapeptide (a) used in this study, pH-driven self-assembly of the blank cyclic peptide nanotube (CPNT) bundles (b), and fabrication of PEG-modified DOX-loaded CPNT bundles (c).

2. Characterization of the CPNT, CPNT/DOX, and PEG-modified CPNT/DOX Bundles

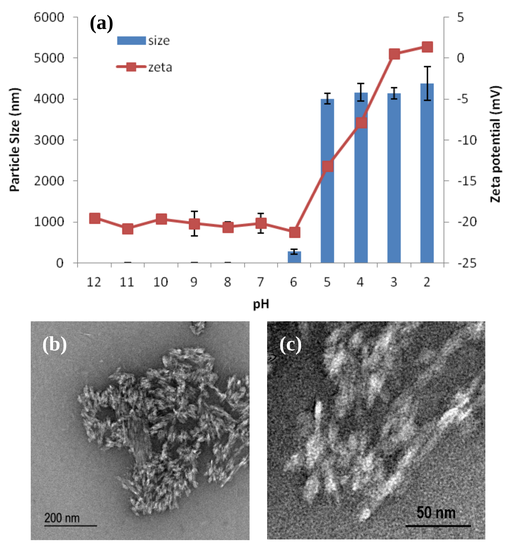

Fig. 3 pH-driven self-assembly of cyclic peptide nanotube (CPNT) bundles. (a) Self-assembly profile of the cyclic octapeptide, *cyclo*-(-L-Gln-D-Ala-L-Glu-D-Ala-L-Gln-D-Ala-L-Cys-D-Ala-), over a pH range of 2.0-12.0; (b-c) Representative TEM images of the CPNT bundles prepared at pH 2.0 by trifluoroacetic acid/acetonitrile method (see Materials and Methods).

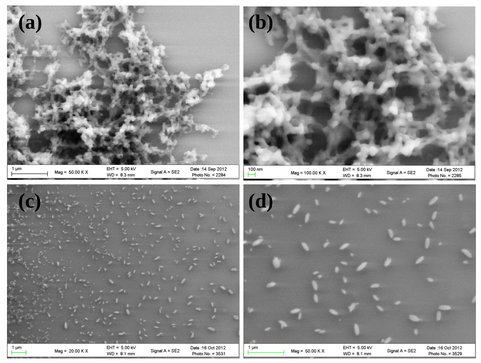

Fig. 4 Doxorubicin was loaded into the CPNT bundles and PEG-modified CPNT/DOX bundles. (a-b) SEM images of CPNT/DOX bundles in aggregates at pH=7.0; (c-d) SEM images of PEG-modified CPNT/DOX bundles.

3. DOX-Loaded CPNT Bundles Sensitized Resistant MCF-7/ADR Tumor Cells In Vitro

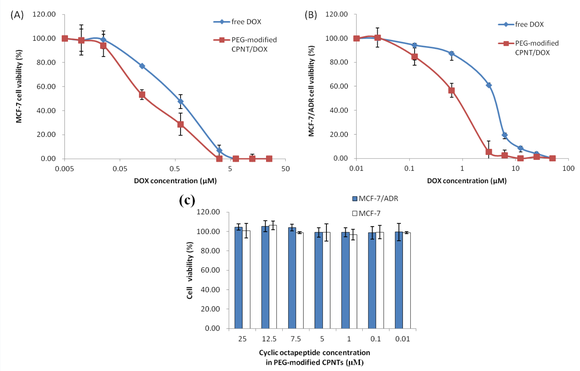

Fig. 5 Cytotoxicity of PEG-modified CPNT/DOX bundles against MCF-7 (a) and MCF-7/ADR cell lines (b). Blank PEG-modified CPNT bundles (c) were used as a control.

Table 1. IC_{50} (mean±S.D.) and resistance reversion index (RRI) of PEG-modified CPNT/DOX bundles against human breast MCF-7 cells and multidrug resistant MCF-7/ADR cells

Formulation	MCF-7	MCF-7/ADR	
	IC_{50}(μM)	IC_{50}(μM)	RRI*
Free DOX	0.53±0.05	4.21±0.65	--
PEG-modified CPNT/DOX bundles	0.16±0.03 **	0.84±0.04 **	5.01

*Resistance reversion index (RRI), i.e. ratio of IC_{50} of free DOX solution to PEG-modified CPNT/DOX bundles.
**P<0.01, vs free DOX.

4. DOX-loaded CPNT bundles inhibited P-gp function in MCF-7/ADR cell line

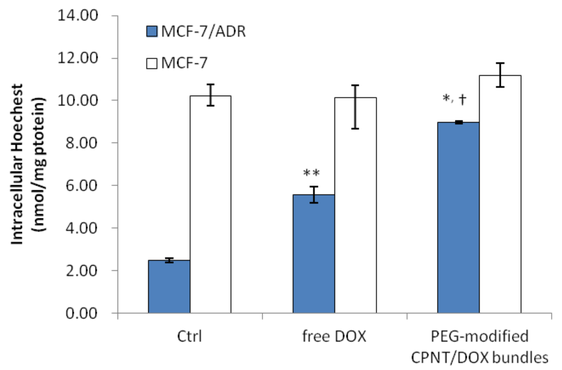

Fig. 6 P-gp activity in the presence of free DOX and PEG-modified CPNT/DOX bundles in the human multidrug resistant breast tumor MCF-7/ADR cell line. The cells were incubated for 4 h in fresh medium (ctrl) or with 10 μM doxorubicin (DOX) or PEG-modified CPNT/DOX bundles, and 4 μM Hoechst 33342 was added to the culture media for another 30-min incubation prior to quantitative analysis. The cultures were then washed, lysed and analyzed fluorimetrically for the intracellular content of the dye. The sensitive breast tumor MCF-7 cells were used as a control. Measurements were performed in triplicate and data are presented as mean±SD (n = 3). **p < 0.01, vs MCF-7/ADR cells ctrl; *P<0.001, vs MCF-7/ADR cells ctrl; †P<0.05, vs MCF-7/ADR cells treated with free DOX.

5. DOX-Loaded CPNT Bundles Enhanced Intracellular DOX Uptake and Altered Intracellular Distribution of DOX in Tumor Cells

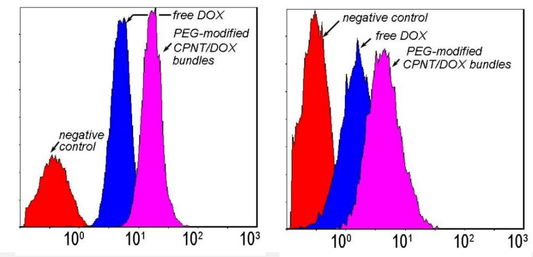

Fig. 7 Flow cytometry analysis for cellular uptake of PEG-modified CPNT/DOX bundles in human breast cancer MCF-7 cells (a) and the resistant counterpart MCF-7/ADR cells (b). Both cells were treated with the PEG-modified CPNT/DOX bundles or free DOX at a DOX concentration of 10 μM for 4 h, and then the mean DOX fluorescence associated with the cell was measured by collecting 20000 events for each sample.

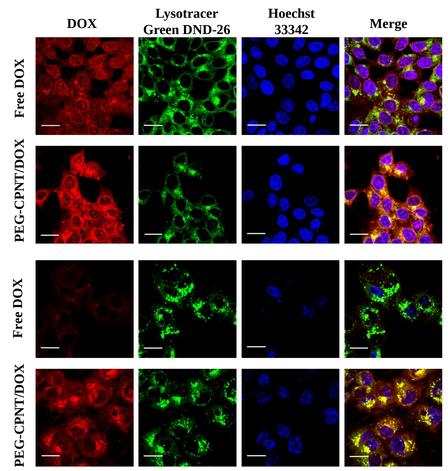

Fig. 8 Intracellular distributions of PEG-modified CPNT/DOX bundles and free DOX at DOX concentration of 10 μM in human breast cancer MCF-7 cells (a) and the multidrug resistant cell line MCF-7/ADR (b). The cells were incubated with both samples at 37 °C, 5% CO_2 for 4 h, and then 100 nM Lysotracker Green DND-26 and 4 μM Hoechst 33342 were added for a 30-min incubation prior to visualization by confocal microscopy. Scale bars represent 20 μm.

Conclusions

In this study, cyclic peptide-based nanotube (CPNT) bundles with a diameter of ~10 nm and a length of ~50-80 nm were prepared . PEG-modified DOX-loaded CPNT bundles showed a high drug encapsulation ratio and good dispersion. More importantly, the PEG-modified CPNT/DOX (PCPNT) bundles demonstrated the activity to overcome the multidrug resistance in a human breast cancer cell line *in vitro*. Further, the PCPNT bundles increased the uptake of DOX, altered the intracellular DOX distribution, and inhibited P-gp activity in MCF-7/ADR cells. The findings of this study indicate that the PEG-modified DOX-loaded CPNT bundles may be a useful nanocarrier for drug delivery to resistant tumor cells.

Reference

Yongzhong Wang, Sijia Yi, Leming Sun, Yujian Huang, Scott C. Lenaghan, Mingjun Zhang. Doxorubicin-loaded Cyclic Peptide Nanotube Bundles Overcome Chemoresistance in Breast Cancer Cells, Journal of Biomedical Nanotechnology, 10, 445-454, 2014.