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Dr. Khershed P. Cooper

Bio-inspire Fluorescent Dipeptide Nanoparticles for Targeted Cancer Cell Imaging and Real-time Monitoring of Drug Release

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Objective

This grant provides funding for the development of a scalable nanomanufacturing platform to fabricate 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. Upon binding of a target biomarker to the aptamers, a conformational change takes place allowing the nanorobots to release their payload (dyes or drugs). To demonstrate modularity of the approach, aptamers for a variety of biomarkers related to 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 research goal of this project is to determine 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 manufacturing of nanorobots in nanomedicine.

Peptide nanostructures are biodegradable and are suitable for many biomedical applications. However, peptides have limited intrinsic optical properties to become useful imaging probes. As an interim progress report for this grant. Here, we show the formation of tryptophan-phenylalanine dipeptide nanoparticles (DNPs) that can shift the peptide's intrinsic fluorescent signal from ultra-violet to visible range. The visible emission signal allows the DNPs to act as imaging and sensing probes. The peptide design is inspired by the red-shift seen in the yellow fluorescent protein resulting from π-π stacking, and enhanced fluorescence intensity seen in the green fluorescent protein mutant, BFPms1, that results from structure rigidification by Zn(II). We show that DNPs are photostable, biocompatible, have narrow emission bandwidth and visible fluorescence properties. DNPs functionalized with MUC1 aptamer and doxorubicin can target cancer cells and be used to image and monitor drug release in real time.

Methods and Results

1. Bio-inspired Self-assembly of the DNPs.

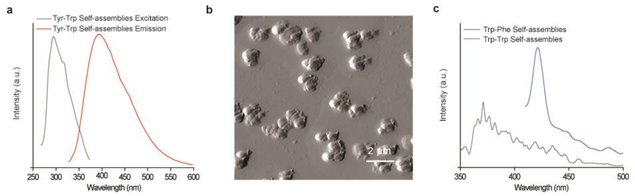

Fig. 1 Optical and nanomorphological properties of Tyr-Trp and Trp-Trp dipeptide self-assemblies. a, Fluorescence excitation and emission spectra of Tyr-Trp self-assemblies. The fluorescence excitation peak of the Tyr-Trp self-assemblies is around 295 nm. The fluorescence emission peak of the Tyr-Trp self-assemblies is around 395 nm. **b,** Atomic force microscopy image of the Trp-Trp self-assemblies. The Trp-Trp dipeptide did not self-assemble into organized nanoparticles. **c,** Fluorescence emission spectra of the Trp-Phe and Trp-Trp self-assemblies. The fluorescence emission peak of Trp-Trp self-assemblies is approximately 370 nm, compared to 423 nm for Trp-Phe dipeptide self-assemblies.

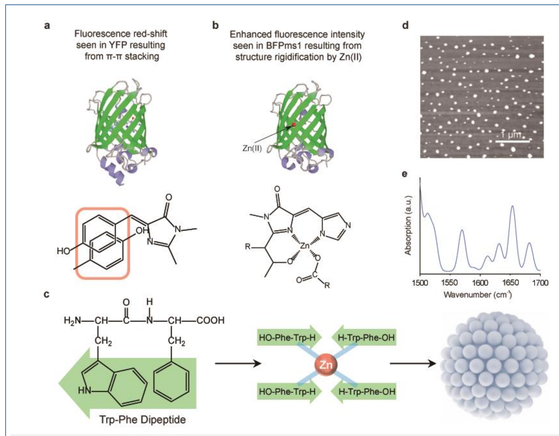

Fig. 2 Design of the self-assembled fluorescent DNPs through bio-inspiration of molecular principles underlying the GFP family. a, Yellow fluorescence protein and the chemical structures of its chromophore. **b,** Blue fluorescent protein and the chemical structure of its chromophore. **c**, Bio-inspired synthesis of fluorescent DNPs with tryptophan-phenylalanine (Trp-Phe) dipeptides as the basic unit (left). Through hydrogen bonding, π-π stacking interactions and Zn(II) co-ordinations (middle), the dipeptide self-assembles to form spherical nanoparticles (right). **d,** Atomic force microscopy image of the DNPs. **e,** FTIR absorption spectra in the amide I region of 1 mg/mL DNPs in water showing the presence of well-ordered aggregates for the Trp-Phe dipeptides.

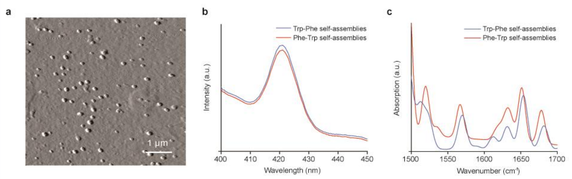

Fig. 3 Characterization of Trp-Phe dipeptide self-assemblies through Zn(II) coordination. a, TEM image of the Trp-Phe dipeptide self-assemblies through Zn(II) coordination.. **b,** Dynamic lighting scattering data for the Trp-Phe dipeptide self-assemblies through Zn(II) coordination.

2. Optical Properties for the DNPs.

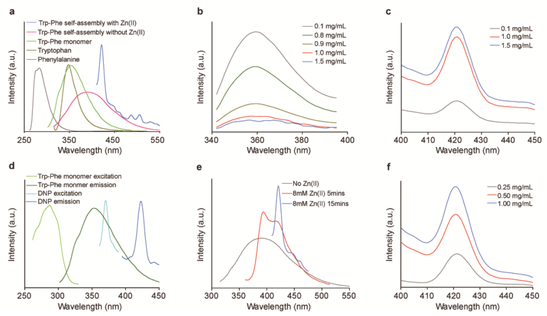

Fig. 4 Optical characterizations of the DNPs. a, Fluorescence emission spectra of Trp-Phe self-assembly with Zn(II), Trp-Phe self-assembly without Zn(II), Trp-Phe monomer, Tryptophan and Phenylalanine, respectively. **b**, Fluorescence emission spectra of Trp-Phe self-assembly at concentrations of 0.1, 0.8, 0.9, 1.0 and 1.5 mg/mL (excitation: 280 nm). **c**, Fluorescence emission spectra of Trp-Phe self-assembly at several concentrations (excitation: 370nm). **d**, Fluorescence excitation and emission spectra of Trp-Phe monomers and DNP. **e-f,** Dynamic fluorescence emission spectra upon **e**) sequential Zn(II) addition, and **f**) sequential dipeptide self-assembly.

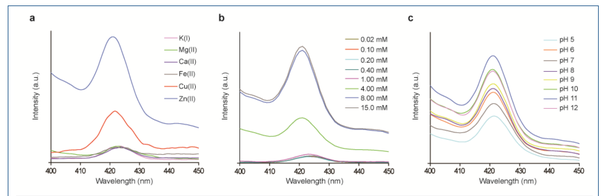

Fig. 5 Effects of metal ions (left), metal ion concentration (middle) and aqueous pH values (right) to fluorescence properties of the DNPs. a, Fluorescence emission spectra of the Trp-Phe dipeptide self-assembly with various metal ions, including K(I), Mg(II), Ca(II), Fe(II), Cu(II) and Zn(II). **b,** Fluorescence emission spectra of Trp-Phe dipeptide self-assembly with Zn(II) at concentrations of 0.02, 0.10, 0.20, 0.40, 1.00, 4.00, 8.00 and 15.0 mM with fixed dipeptide concentration at 2 mM. **c,** Fluorescence emission spectra of Trp-Phe dipeptide self-assembly under various aqueous pH (5, 6, 7, 8, 9, 10, 11, 12).

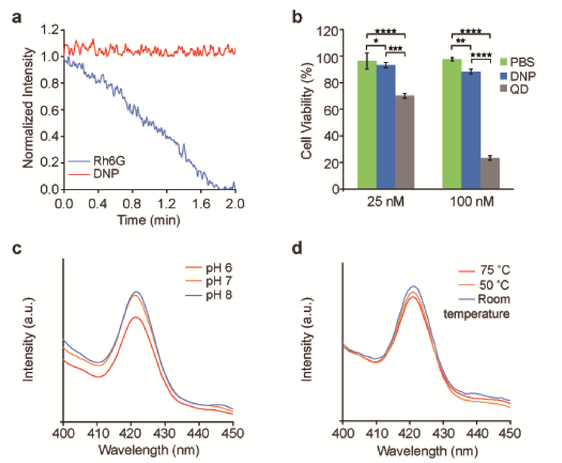

Fig. 6 Comparison of the DNPs with organic dyes rhodamine 6g (Rh6G) and QDs. a, Photostability evaluation of DNP and organic dye rhodamine 6g. The fluorescence intensity of DNPs remained stable after continuous irradiation for 120 seconds, indicating better photostability as compared with organic dye rhodamine 6g. **b**, Toxicity evaluation and comparison for the control (phosphate-buffered saline), DNPs and CdSe QDs against NIH3T3 cells. Error bars indicate standard derivation (s.d.) Statistical significance was determined by using a two-tailed Student's t-test. $P < 0.05$ was considered to be statistically significant. *P>0.05; **P<0.05; ***P<0.01; ****P<0.001. **c**, Fluorescence intensity change after dissolving the DNPs at various pH. **d**, Fluorescence intensity change after dissolving the DNPs at room temperature, 50°C and 75°C.

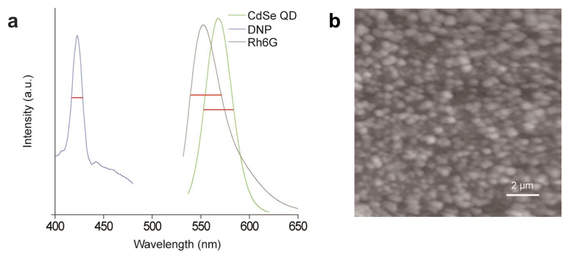

Fig. 7 a, Fluorescence emission spectra of DNPs, rhodamine 6g (Rh6G) and CdSe QDs. The full width at half maximum (red line) measurement indicates that the DNPs have narrowest fluorescence emission bands compared to rhodamine 6g and CdSe QDs. **b, Atomic force microscopy image of the Trp-Phe self-assemblies without Zn(II) coordination.** Without Zn(II) coordination, the Trp-Phe self-assemblies retain the spherical shape with a relatively larger size (approximately 600 nm).

3. Fluorescence Bioimaging using the DNPs.

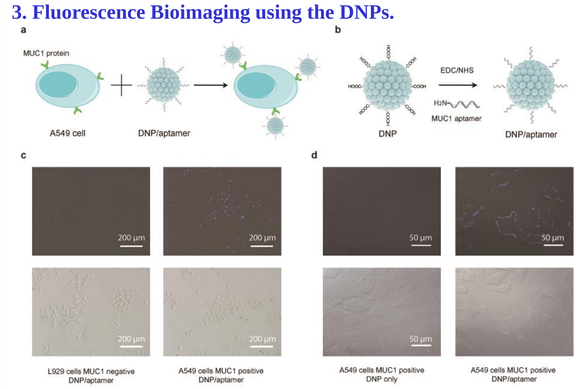

Fig. 8 DNPs modified with MUC1 aptamer target human lung cancer A549 cells. a, DNPs modified with MUC1 aptamer (curvy line) binds to MUC1 protein found on the cell membrane of A549 cells. **b,** Schematic representation of MUC1 aptamer functionalized DNP (DNP/aptamer). **c,** *In vitro* fluorescence imaging of L929 cells (MUC1 negative) and A549 cells (MUC1 positive) treated with DNP/aptamer. The blue color represents the fluorescence signal of the DNPs. **d**, *In vitro* confocal fluorescence cellular imaging of A549 cells (MUC1 positive) treated with DNP and DNP/aptamer.

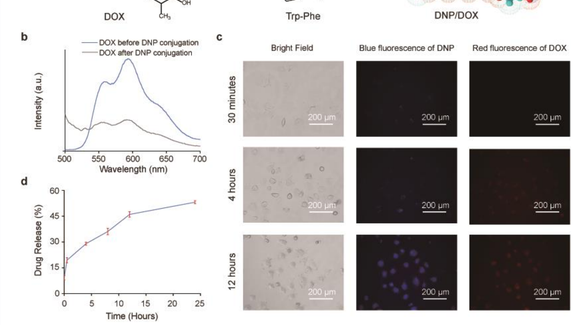

Fig. 9 Fluorescence monitoring of DOX loading and releasing from the DNPs. a, DOX is conjugated to DNPs through π-π stacking. **b**, Fluorescence emission spectra of DOX before and after conjugation with DNPs. **c**, Representative fluorescence images of A549 cells incubated with 10 μg/mL DNP/DOX conjugates after 30 mins, 4 hours and 12 hours. **d**, Characterization of the release of DOX from the DNP/DOX conjugates.

Conclusions

The tryptophan-phenylalanine dipeptide nanoparticles that can shift the peptides' intrinsic fluorescence signal from ultra-violet to visible range have been developed in this study. Through the optimization of the synthesis parameters, a tunable synthesis approach was obtained to produce the DNPs demonstrating visible fluorescence property. Compared with organic fluorophores, QDs and GFPs, the DNPs are photostable, biocompatible, have narrow emission bandwidth and visible fluorescence properties. In addition, the DNPs functionalized with aptamer and doxorubicin can target cancer cells and be used to image and monitor drug release in real time.

References

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2. Tunable Synthesis of Self-assembled Cyclic Peptide Nanotubes and Nanoparticles, **Soft Matter**, 11, 3822-3832, 2015.
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