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Self-assembled nanospheres as a novel delivery system for taxol: a molecular hydrogel with nanosphere morphology†

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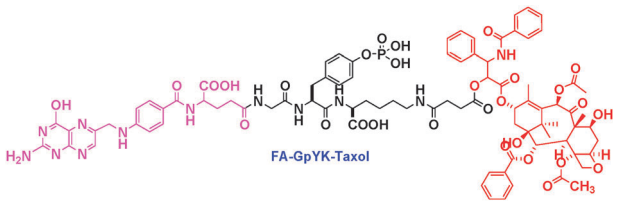
Here we reported on the first example of a Folic acid-based molecular hydrogel with nanosphere morphology as a delivery system for Taxol.

Nanospheres,[1] especially those formed by the self-assembly of amphiphilic polymers, dendrimers, and biomacromolecules such as DNA molecules and peptides, have been extensively investigated in the fields of drug delivery,[2] patterning,[3] lithography,[4] molecular sensing,[5] *etc*. What's more, the morphology and the size of their self-assembled structures can be well controlled. However, the nanospheres formed by small molecules (molecular weight less than 2000) in aqueous solutions are relatively less explored and their morphology is hard to be controlled due to the more dynamic property of small molecules. Up to now, only several examples of nanospheres formed by small molecules have been reported.[6] Among them, the di-phenylalanine and its derivatives are probably the most investigated system.[7] The nanospheres formed by di-phenylalanine derivatives can be used for the delivery of ss-DNA and quantum dots.[8,9] Most recently, the nanospheres formed by the derivative of di-phenylalanine had been demonstrated to be as rigid as metal nanoparticles.[10] Besides the di-phenylalanine system, Hamachi's group demonstrated that the nanospheres formed by amphiphilic small molecules could be used for the detection of biomacromolecules by turning on signals of F-NMR or fluorescence.[11]

Taxol, as an effective anti-cancer chemotherapy drug, is widely used in the clinic.[12] Due to its limited water solubility, it is usually administrated in the forms of liposome, conjugates with proteins and hydrophilic polymers, *etc*. These administration forms, however, frequently suffer from the low loading amounts of Taxol, difficult synthetic pathways, *etc*. We believed that the strategy capable of making Taxol derivatives self-assemble into stable nanospheres would be an alternative approach for the delivery of Taxol because of the well-controlled synthetic pathways for small molecular Taxol derivatives and the much higher loading amount of the Taxol within the self-assembled nanospheres than that in other delivery systems. Stimulated by the first example of molecular hydrogel bearing Taxol,[13] we designed and synthesized a FA–Taxol conjugate of FA-GpYK-Taxol (Scheme 1). It has several features: (1) it is a Taxol derivative which can be considered as a prodrug releasing the Taxol upon ester cleavage; (2) it has the FA moiety, which has a strong tendency to form a stable tetramer[14] and offers the FA–Taxol conjugate the targeting effect to cancer cells;[15] (3) phosphorylated and succinated tripeptide of Glycine-Tyrosine-Lysine (GYK) as a linker to connect the Taxol and FA not only provides the sufficient water solubility to the FA-GpYK-Taxol but also acts as the substrate of the phosphatase.[16] Upon the treatment of the phosphatase, the FA-GpYK-Taxol was converted to the FA-GYK-Taxol with less solubility in aqueous solutions. Due to the self-assembled ability of the FA, the FA-GYK-Taxol might be able to self-assemble into nanospheres.

After the successful synthesis of the FA-GpYK-Taxol, its self-assembly was evaluated by the treatment with the phosphatase. The FA-GpYK-Taxol was highly soluble in aqueous solutions with a solubility of higher than 4 wt% in the phosphate buffer saline (PBS) solutions (pH = 7.4). The phosphatase was then added to the clear and slightly yellow solution containing 0.2 wt% of the FA-GpYK-Taxol (final concentration of the phosphatase = 90 U mL^{-1}). We observed the formation of a transparent hydrogel (Fig. 1A, insert) after incubation of the above solution at room temperature



Scheme 1 Chemical structure of the FA-GpYK-Taxol (purple: Folic acid (FA), black: phosphorylated and succinated tripeptide of Glycine-Tyrosine-Lysine (GYK), and red: Taxol).

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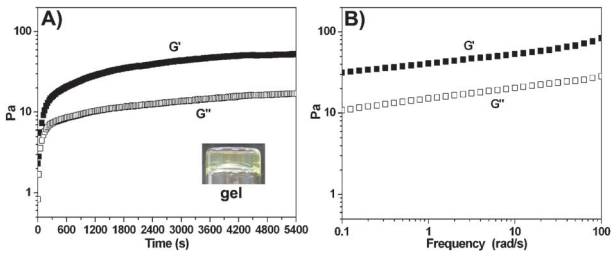

Fig. 1 The rheological measurements with the mode of (A) dynamic time sweep at the frequency of 2 rad s^{-1} and the strain of 2% for the PBS solution containing 0.2 wt% of FA-GpYK-Taxol and 90 U mL^{-1} of phosphatase (insert: optical image of the gel) and (B) dynamic frequency sweep at the strain of 2% for the gel formed 2 h after the addition of the phosphatase.

(22–25 °C) within 3 min (66% mol of FA-GpYK-Taxol was converted to the FA-GYK-Taxol at the gelling point, determined by the HPLC). The hydrogel was stable at room temperature for at least 6 months without disturbing. The minimum concentration of FA-GpYK-Taxol needed for gelation was about 0.08 wt% in the PBS buffer solutions. These results indicated that FA-GYK-Taxol was an effective gelator and it had an excellent self-assembly ability.

A rheological measurement with the mode of dynamic time sweep was used to characterize the kinetics of enzymatic hydrogelation. As shown in Fig. 1A, the gel formed immediately after the addition of the enzyme, as indicating by the value of elasticity (G′) dominating than that of viscosity (G″). The enzyme of phosphatase kept converting the FA-GpYK-Taxol to the FA-GYK-Taxol post the gelling point, thus leading to the increase of the values of both G′ and G″ and the formation of a more rigid hydrogel. The conversion was recorded at a low rate 1 h post the incubation, as indicating by the observation in the rheological measurement that there was only a slightly increase of the values of both G′ and G″ from one to one and a half hour time points (the molar percentage of FA-GpYK-Taxol converted was similar at these two time points, both were about 86%). And then the rheological measurement with a mode of dynamic frequency sweep was used to characterize the resulting hydrogel. As shown in Fig. 1B, the gel showed a slight dependence to the frequency—the value of G′ changed from about 30 Pa to about 80 Pa at the frequency of 0.1 and 100 rad s^{-1}, respectively, which implied the existence of a weak matrix in the gel.

The atomic force microscopy (AFM) was then used to characterize the nanostructures in the gel. Unlike most of small molecular hydrogels with nanofibers and tubular structures, highly uniform nanospheres with the size of about 50 nm were observed in the gel (Fig. 2A). This result clearly proved the success of our design. Though the reason for why FA-GYK-Taxol could self-assemble into nanospheres remained unknown, we proposed that both the strong tendency of the FA to form the tetramer structure (Fig. 2C and D) and the hydrophobic interaction between Taxol molecules might help to extend the supramolecular chain and form a dendrimer-like structure (Fig. 2B), thus resulting in the formation of the nanospheres.

To evaluate the stability of the nanospheres in different kinds of aqueous solutions, the gels were dispersed by vortex in both the PBS buffer solution and the DMEM solution with 10% of FBS (final concentration of FA-GYK-Taxol was about 0.003%, 30 μL of gels were diluted to 1.8 mL by these two solutions). As shown in Fig. 3A and B, the as formed nanospheres exhibited small aggregates in both solutions—their average sizes were 183 nm and 252 nm in the PBS buffer solution and the DMEM solution with 10% of FBS, respectively. The relative larger size of the small aggregates in DMEM solution than that in PBS buffer solution was probably due to the existence of Taxol binding proteins and FA binding proteins in FBS. The size of the small aggregates in both solutions decreased slightly after being incubated at room temperature for 24 h (about 136 nm and 120 nm in both solutions, respectively). These results clearly indicated that the self-assembled nanospheres possessed good stability in both solutions with an anti-dilution property. We then studied the chemical stability of the FA-GYK-Taxol in gels. The gels were firstly incubated at room temperature overnight for the enzymatic conversion to reach the balance, gels were then incubated in a water bath at 37 °C. The molar percentage of the FA-GYK-Taxol remained in the gels was determined by the LC-MS—there was 92, 78, and 68% molar of FA-GYK-Taxol remained in the gels at 12, 24, and 48 h time points, respectively. The degradation of FA-GYK-Taxol conjugate underwent complex pathways—we observed a small peak from Taxol formed by the ester bond hydrolysis[18] and several other peaks from unknown compounds probably due to the decomposition of FA.[17] When calculating the weight percentage of the Taxol in the nanospheres, the value was 49.4%, which was much higher than that in liposome systems or in Taxol–polymer/dendrimer conjugates. The high stability of the self-assembled nanospheres and the high weight percentage of Taxol in the nanospheres suggested that they could be developed into a novel delivery system for Taxol and for cancer therapy.

We then evaluated the activity of the nanospheres by treating HepG2 cells at serials of concentrations. After 72 h of incubation with the cells, the FA-GYK-Taxol in the nanosphere exhibited an IC50 value of 27.3 nM (the gel dispersed in the DMEM medium with 10% of the FBS was used directly for the test), which is comparable to that of Taxol (24.8 nM). We proposed that the cellular uptake of the nanospheres occurred by the endocytosis, which was a well-known phenomenon for many delivery systems.[8,19] The phosphorylated peptide without the Taxol showed no inhibitions on HepG2 cells at 250 μM. FA-GpYK-Taxol exhibited an IC50 of 19.6 nM, which was also comparable to those of Taxol and FA-GYK-Taxol. In addition, the shapes of the regression curves of the three compounds were similar. These results indicated that the activity of Taxol was conserved successfully in the precursor and the hydrogelator, which conferred the great potentials for the system here to be used as a novel delivery system for Taxol.

In summary, we have successfully obtained nanospheres of a Taxol derivative without comprising its activity and reported a novel gelation system based on the FA. We are unable to predict performance of the self-assembled system in this study because of the existence of Taxol binding proteins and FA binding proteins in blood stream. And it needs to be studied by *in vivo* experiments future. Aside from Taxol which was chosen

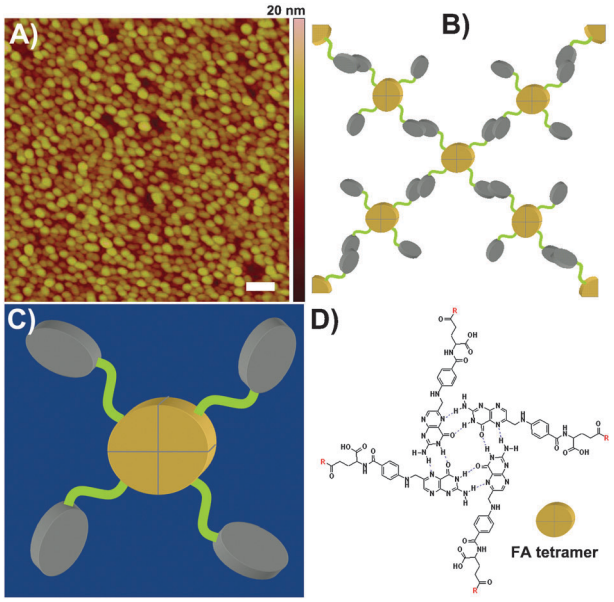

Fig. 2 (A) The atomic force microscopy (AFM) image of the nanospheres obtained by treating the PBS solution containing 0.2 wt% of FA-GpYK-Taxol with 90 U mL^{-1} of phosphatase (the scale bar represents 150 nm) and the proposed molecular arrangement of FA-GYK-Taxol in the nanospheres: (B) a dendrimer like structure (size of the proposed structure does not match the actual size of nanospheres in Fig. 2A), (C) a cartoon diagram of FA-GYK-Taxol tetramer, and (D) schematic illustration for FA tetramer.

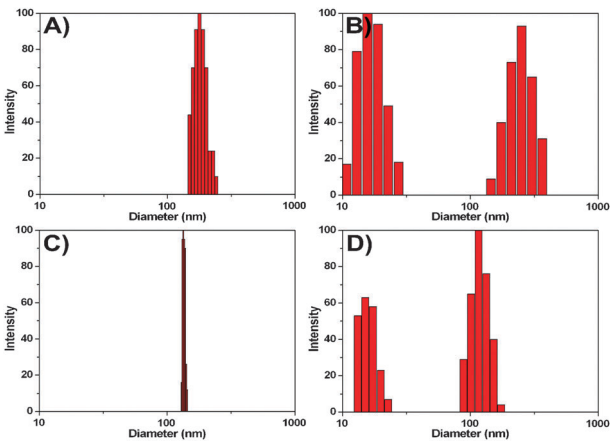

Fig. 3 Light scattering data of the nanospheres in the PBS buffer solutions (A and C) and in the DMEM solutions with 10% of FBS (B and D), as prepared samples (A and B) and samples being incubated at room temperature for 24 h (C and D), final concentrations of FA-GYK-Taxol are ~0.003 wt% in both solutions, the nanospheres smaller than 50 nm in DMEM solutions with 10% of FBS come from the medium itself.[17]

to connect with the FA–peptide, other hydrophobic drug molecules could also be used for the construction of functional hydrogels for biomedical applications. We believed that the hydrogels with nanospheres would have big potentials to be developed into a novel kind of delivery system for hydrophobic drugs to treat different diseases.

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