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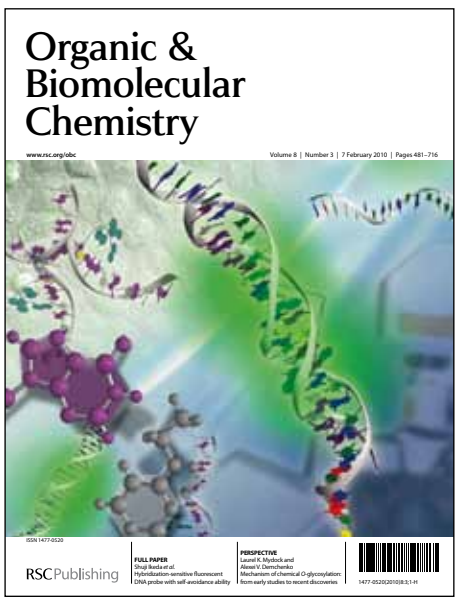

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Disulfide bond reduction-triggered molecular hydrogels of Folic acid-Taxol conjugates

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Molecular hydrogel of therapeutic agents is a novel kind of self-delivery system that can sustainedly release drugs or pro-drugs. We have previously developped a molecular hydrogelator of Folic acid (FA)-Taxol conjugate triggered by phosphatase. In this paper, we reported on a novel molecular hydrogelator system of FA-Taxol conjugates with improved synthetic strategy. The hydrogels are formed by the reduction of disulfide bond by glutathione (GSH). These hydrogels could sustainedly release Taxol through ester bond hydrolysis. Compared with intraveneous (i.v.) injection of clinically used Taxol® with four times of dosage, our hydrogel could inhibit tumor growth more efficiently by a single dose of intra-tumor (i.t.) administration. These observations suggested the big potential of this novel gelation system of Taxol for cancer therapy.

1. Introduction

Molecular hydrogels are formed by small molecules (hydrogelators) with molecular weight usually lower than 2,000.[1-7] Many small molecules of sugar derivatives, amino acid derivatives, and peptides have been demonstrated to be efficient hydrogelators.[8-18] Molecular hydrogels have shown big potential for sensing of bioactive molecules,[19-22] synthesis of organic and inorganic materials,[23-29] cell culture,[30-33] drug delivery,[34-38] etc. Recent research interests in molecular hydrogel focus on the development of novel gelation systems (especially responsive ones[39-41]), novel strategies to improve the mechanical property of molecular hydrogels,[42-46] and novel applications of them. For example, Adams and McNeil groups independently applied polymeric additives to improve the property of short peptide-based molecular hydrogels.[42, 44, 47, 48] Banerjee and co-workers had incorporated carbon materials to endow amino acid-based hydrogels with novel properties.[49, 50]

Most recently, the application of molecular hydrogels in drug delivery attracted extensive research efforts.[51-57] Besides using hydrogels as physically carriers to deliver therapeutic agents, molecular hydrogel of therapeutic agents was a self-delivery system that hold advantages. This recently developped drug delivery system usually had high drug loadings. Importantly, the drug loading could be designable and fixed through conjugation with different compounds such as different peptides.[51] This system could constantly release therapeutic agents without burst release phenomenon.[58] Several examples of molecular hydrogels of therapeutic agents including Taxol,[56, 58-60] Camptothecin,[51, 61] Naprofen,[52] and Olsalazine[53] had been rationally designed and reported up to now.

Our group focuses on the development of molecular hydrogels of hydrophobic therapeutic agents,[57] typically Taxol. We had reported a phosphatase-triggered hydrogel system of a Folic acid (FA)-Taxol conjugate with nanosphere morphology.[60] In the system, FA was connected with Taxol through a phosphorylated tripeptide of GpYK (G: glycine, pY: phosphorylated tyrosine, and K: lysine), which needed five steps of synthesis to obtain it in a relative low yield (total yield in five steps lower than 10%). We recently found that Folic acid (FA) could be a general motif to construct molecular hydrogelators through conjugation with hydrophobic therapeutic agents of triamcinolone acetonide (TA) and rapamycin.[62] The hydrogels of FA-TA and FA-rapamycin conjugates were formed by disulfide bond reduction triggered by glutathione (GSH). Based on these information, we planed to develop a GSH-triggered gelation system based on FA-Taxol conjugates.

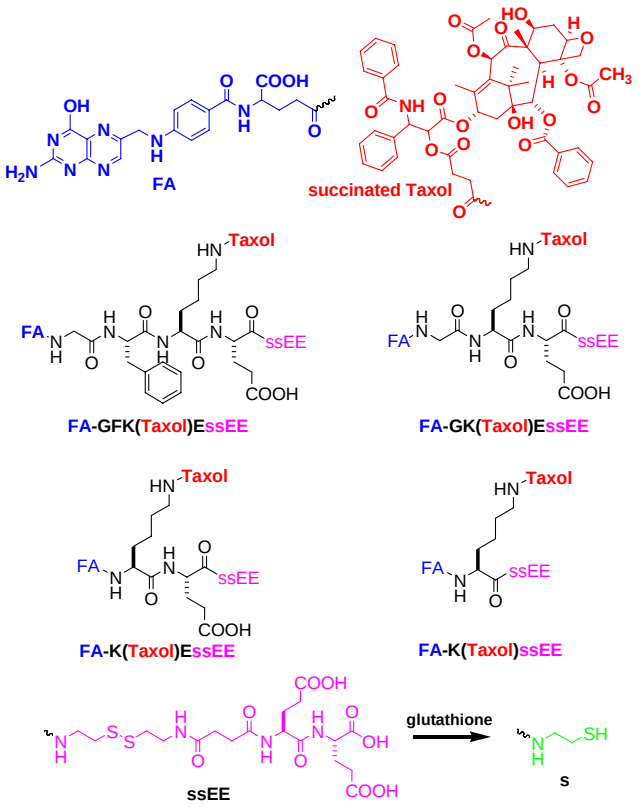
2. Results and discussions

2.1 Molecular design and synthesis

In our previous study, we had shown that the FA-GpYK(Taxol) could be converted to FA-GYK(Taxol) by the enzyme of phosphatase, resulting in a molecular hydrogel with a three dimensional nanosphere network.[60] The relative low yield to obtain FA-GpYK(Taxol) would hinder the futher development and application of this system. We therefore planed to design a novel hydrogel system of FA-Taxol conjugates by disulfide bond reduction. We had demonstrated that disulfide bond was a useful cleavable linker to construct precursors of gelators.[63] The reduction of disulfide bond by Dithiothreitol (DTT) or GSH could release gelators and result in the formation of hydrogels of peptides and conjugates of FA and hydrophobic therapeutic agents.[60, 63]

We firstly designed FA-GFK(Taxol)EssEE (Scheme 1) as a precursor of gelator. The peptide part of GFKE was used to connect FA and Taxol and disulfide bond reduction could generate FA-GFK(Taxol)E-s with a similar chemical structure of FA-GYK(Taxol)-COOH produced by phosphatase in our previous study.[60] The FA-GFKEssEE was obtained by standard Fmoc- solid phase peptide synthesis (SPPS) and then

used to couple with N-hydroxylsuccinimide (NHS)-activated succinated Taxol. The resulting compound was purified by reverse phase high performance liquid chromatography (HPLC). The FA-Taxol conjugates could be obtained in moderate yields of 40% approximately over two steps. We then planed to make other FA-Taxol conjugates with shorter peptide sequences. The use of shorter peptide linkers would make the synthesis of FA-peptides easier and improve the yield of synthesis. Different peptide sequences might also lead to different release profile of resulting gels. What's more, application of shorter peptide linkers would improve the loading of Taxol in the resulting self-assembled nanostructures. In this study, peptides of GFKE, GKE, KE, and K were designed and used (Scheme 1).



Scheme 1. Chemical structures of all Folic acid (FA)-Taxol conjugates used for GSH-triggered hydrogelations

2.2 Gelation test and characterization of hydrogels

The gelation ability of obtained compounds were tested. As presented in Fig. 1A, we found that all of them could form hydrogels in the phosphate buffer saline (PBS, pH = 7.4) upon the treatment of 4 equiv. of GSH. The gels formed by the compounds were named as the GFKEgel, GKEgel, KEgel, and Kgel for compound of FA-GFK(Taxol)EssEE, FA-GK(Taxol)EssEE, FA-K(Taxol)EssEE and FA-K(Taxol)ssEE, respectively. The minimum gelation concentration (MGC) was about 0.25 wt%, 0.175 wt%, 0.3 wt%, and 0.4 wt% for GFKEgel, GKEgel, KEgel, and Kgel, respectively. The different colors of these gels might be due to the presence of different amounts of free thiol compounds. The observations, in combine with our previous results of hydrogels of conjugates of FA and TA/rapamycin, suggested that FA was a useful motif to construct hydrogelators through conjugating with other drug molecules.

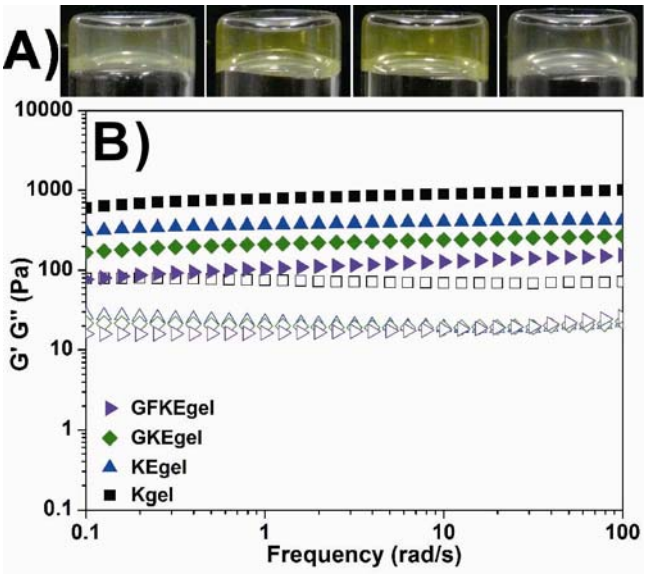


Fig. 1. A) Optical images of the hydrogels from PBS solution containing 0.5 wt% of the precursors with 4 equiv. of GSH (from left to right: GFKEgel, GKEgel KEgel, Kgel, respectively) and B) Dynamic frequency sweep of four gels formed at 2 hour time point at the strain of 1% and at 25 ℃ (the solid symbols represent elasticity (G') and the hollow ones represent viscocity (G'')).

We then characterized the mechanical property of the hydrogels by a rheometer. As shown in Fig. 1B, a PBS solution containing 0.5 wt% of the compound with 4 equiv. of GSH was transferred to the rheometer. After two hours' incubation, a dynamic frequency sweep was then performed at the strain of 1% and at 25 ℃. The results showed that G' value of the gel was about 130, 230, 360, and 800 Pa for GFKEgel, GKEgel, KEgel, and Kgel, respectively. The G' value was at least one magnitude bigger than G'' value for

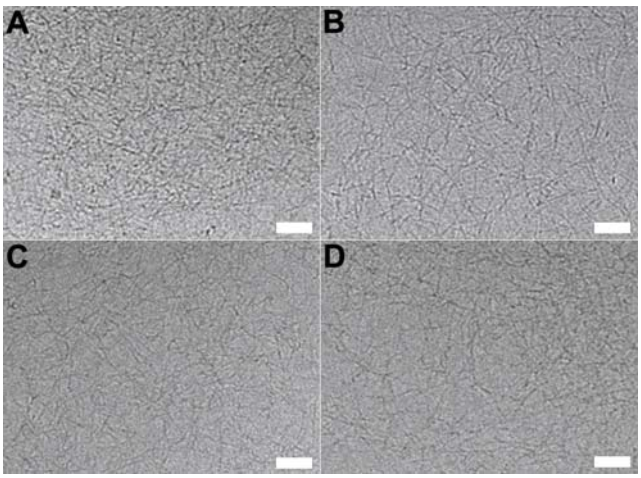


Fig. 2. Cryo-TEM images of A) GFKEgel, B) GKEgel, C) KEgel, and D) Kgel (the scale bars represent 50 nm)

each gel, indicating the formation of true gels.[64] The morphology of self-assembled structures in the hydrogels was characterized by Cryo-transmission electron microscopy (Cryo-TEM). As shown in Fig. 2, all hydrogels exhibited filamentous structures. The diameter of the fibril was about 4-6 nm for all hydrogels (about 5.1, 4.9, 4.8, and 4.6 nm for GFKEgel, GKEgel, KEgel, and Kgel, respectively). This observation suggested that shorter peptide linkers would lead to smaller sized fibrils in the gels. The small fibrils entangled with each other and formed three dimensional network structure that served as the matrix of the hydrogels.

2.3 Release profile

The releasing profile of Taxol or Taxol conjugates from gels at physiological temperature condition (37 ℃) *in vitro* was then studied. We added a 0.25 mL of PBS buffer solution on top of 0.2 mL of gels (original concentration of all compounds was 0.5 wt%). The 0.2 mL of upper solutions were taken out at desired time points for measurement of accumulating release amount of Taxol from gels, and a fresh PBS buffer solution (0.2 mL) was then added. As shown in Fig. 3, the four gels exhibited different release profiles. They released Taxol at a mean rate of 0.16, 0.25, 0.34, and 0.11 μg/mL per hour for GFKEgel, GKEgel, KEgel, Kgel respectively for 24h, and at a mean rate of 40.00, 54.41, 52.79, and 35.24 μg/mL per hour for GFKEgel, GKEgel, KEgel, Kgel respectively for 14 days (Fig. S-12). The results indicated that gels formed by gelators with hydrophilic amino acid of E could obviously release Taxol at higher rates, and the presence of hydrophobic F could result in the release of Taxol at lower rates. We only observed Taxol itself got released from gels, indicating that the peptide derivatives were trapped in the matrix of gels and Taxol was released through ester bond hydrolysis. The accumulating released percentage of Taxol were about 0.13%, 0.22%, 0.30%, 0.40% correspond to Kgel, GFKEgel, GKEgel, KEgel, respectively during the 24h experimental period. These observations implied that our Taxol hydrogels could be used for sustainedly release of Taxol for cancer therapy.

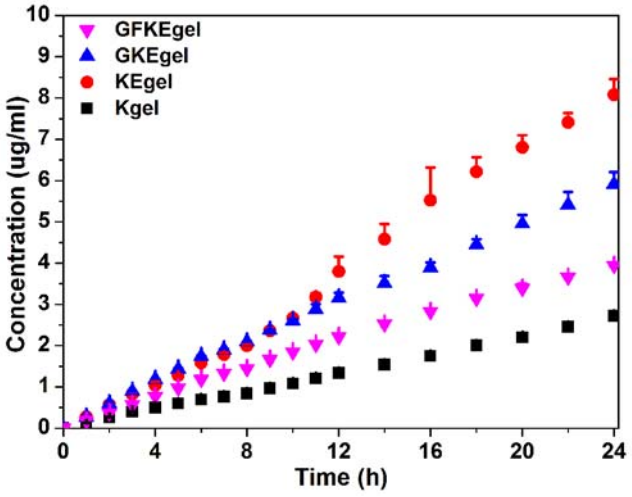


Fig. 3. Release profile of Taxol from hydrogels to PBS buffer solution at 37 ^{0}C

2.4 Tumor inhibition

The anti-tumor efficacy of our hydrogels in mice tumor model (4T1-luciferase breast tumors in mammary fat pad of female mice) was also evalutaed *in vivo*. We choose Kgel for the evaluation because of the highest drug loading and the lowest release speed in the four gels. When the volume of breast tumors reached about 500 mm^3, different dosages (10, 20, and 40 mg/kg of Taxol) of the hydrogel was injected directly into tumors with the same volume (100 μL) by variation of the concentration of the compound in gels. As shown in Fig. 4, our hydrogels could efficiently hinder tumors growth in the 14 days. The final volume of tumors was about 376%, 353%, and 398% bigger than the original volume of tumors (~500 mm^3) and there were not obvious difference among different dosages (10, 20, and 40 mg/kg of Taxol) of our hydrogels (Fig. S-10). However, the final relative tumor volumes were 756% and 832% in groups administrated with intravenous (i.v.) injection of 40 mg/kg of Taxol® (10 mg/kg * 4 every other day) and i.v. injection of a PBS solution (100 μL), respectively. These observations clearly indicated that our hydrogel of Taxol could efficiently hinder the growth of breast tumors. We also monitored the body weight of the mice. There were no obviously body weight losses in groups of mice administrated with our hydrogels (Fig. S-11), compared to the control group of mice without any treatments. These results suggested low toxicity and high efficiency to inhibit tumor growth of our hydrogels.

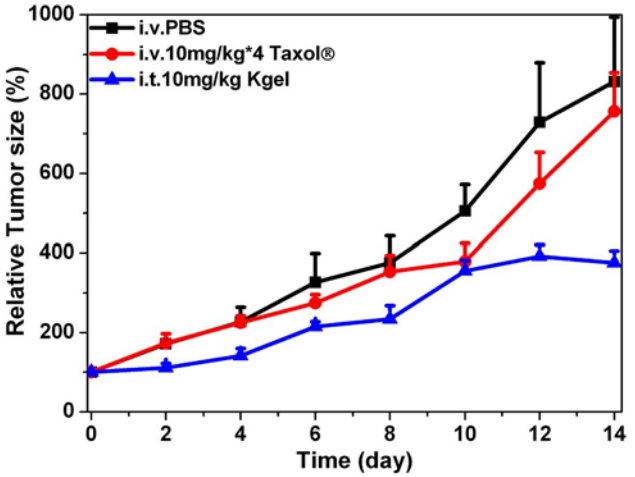


Fig. 4. Kgel inhibits xenografted mouse breast tumor (4T1-luciferase) growth in vivo (gels were administrated into i.t. spaces after tumor sizes reaching ~500 mm^3, n=5, data were represented as Mean±SEM)

3. Experimental

3.1 Peptide derivate synthesis: Peptide derivates of FA-GFKEssEE, FA-GKEssEE, FA-KEssEE, FA-KssEE were prepared by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and the corresponding N-Fmoc protected amino acids with side chains properly protected by a tert-butyl group. The first amino acid (Fmoc-Glu(O^{t}Bu)-OH) was loaded on the resin at the C-terminal with the loading

efficiency about 1.0 mmol/g. 20% piperidine in anhydrous N,N'-dimethylformamide (DMF) was used during the deprotection of Fmoc group. Then the next Fmoc-protected amino acid was coupled to the free amino group using O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU) as the coupling reagent. The growth of the peptide chain was according to the established Fmoc SPPS protocol. At the final step, Folic acid was used to couple with the peptide. After the last coupling step, excessive reagents were removed by a single DMF wash for 5 min (5 ml per gram of resin), followed by five steps of washing using dichloromethane (DCM) for 2 min (5 ml per gram of resin). The peptide derivatives were cleaved from the resin by ice-cold reagent B (95% Trifluoroacetic acid, 2.5% Triisopropylsilane, 2.5% double-distilled water) and the mixture was stirred at room temperature for 30 min, filtered, and poured into ice-cold diethylether. The resulting precipitate was centrifuged for 10 min at 4℃ at 10,000 rpm. Afterward the supernatant was decanted and dissolved in double-distilled (dd) water and lyophilized.

3.2 Synthesis of FA-Taxol conjugates: 0.15 mmol of compounds and 95.4 mg of Taxol-succinic acid N-Hydroxysuccinimide (NHS) active ester (0.1 mmol) were dissolved in 5 mL of DMF, 41.25 μL of diisopropylethylamine (DIPEA, 0.25 mmol) was then added. The resulting reaction mixture was stirred at room temperature overnight. FA-Taxol conjugates were obtained by HPLC (yields of 40-50%).

3.3 Hydrogel formation: 1 mg of compond was dissolved in 0.15 mL of PBS buffer solution containing 6 equiv. of Na_2CO_3 (2 equiv. of Na_2CO_3 were used to neutralize the compounds and the additional 4 equiv. of Na_2CO_3 were used to neutralize GSH to make the final pH value to 7.4). 0.05 mL of PBS buffer solution containing 4 equiv. of GSH was then added. Gels would form after being kept at room temperature 25℃ for less than 10 minutes.

3.4 Characterization of hydrogels:

Rheology: Rheology test was done on an AR 2000ex (TA instrument) system, 40 mm parallel plates was used during the experiment at the gap of 500μm. For the dynamic time sweep, the solution of compounds upon adding 4 equiv. of GSH was directly transferred to the rheometer and it was conducted at the frequency of 1 rad/s and the strain of 1% after 1 hour. Before dynamic sweep, dynamic strain sweep was performed to find out the linear region. The gels were characterized by the mode of dynamic frequency sweep in the region of 0.1-100 rad/s at the strain of 1%.

Cryo-Transmission Electron Microscopy (Cryo-TEM): Cryo-TEM samples (hydrogels containing 0.5%wt compounds) were prepared at 25℃ and stored at 4℃. Sample was loaded onto a lacey support TEM grid by using a micropipette which was held by tweezers. The samples were quickly plunged into a reservoir of liquid ethane at its melting temperature (-183 ℃) upon waiting for about 10 s to relax any stresses induced during the blotting. The vitrified samples were then stored in liquid nitrogen. The samples were then transferred to a cryogenic sample holder (Gatan 626) were examined with a JEM 2200FS TEM (200 keV) at about -174 ℃. The phase contrast was enhanced by under focus. The images were recorded on a Gatan multiscan CCD and preceded with Digital Micrographs. Transmission electron microscopy (TEM) was performed on a MODEL H-800 Electron microscope operating at an acceleration voltage of 100 kV.

3.5 Release profile: A hydrogel in PBS (PH=7.4) solution containing 0.5 wt% of compound was formed in Eppendorf tube at 25℃. After 24h, we added 0.25 mL of PBS on the surface of the hydrogels, 0.2 mL solution was taken out at the desired time point and 0.2 mL PBS was added back. For the following time points, 0.2 mL of PBS was taken out and 0.2 mL of PBS was added back at each point. We then monitored and calculated the release profile from the gel formed by a LCMS-20AD (Shimadzu) system. The experiment was performed at 37 ^{0}C.

3.4 Tumor inhibition assay: Female Balb/c mice were inoculated with 2×10^5 4T1-luciferase cells in the mammary fat pad for building breast tumor model. The tumor growth was monitored every other day. Tumor volume was recieved through calculating by the formula: length × width × (Length + Width)/2. When tumors size reached ~500 mm^3, mice were randomly divided into different treatment groups. The day of giving drugs was designated as day 0. Mice weight was also monitored after receiving treatment.

4. Conclusions

In summary, we have developed a series of molecular hydrogels of FA-Taxol conjugates formed by disulfide bond reduction by GSH. All designed FA-Taxol conjugates could form hydrogels by GSH, demonstrating in a further step that FA could be a versatile molecular to construct molecular gelators through conjugation with other drug molecules. The hydrogels of FA-Taxol conjugates in this study could sustainedly release Taxol through ester bond hydrolysis. Compared with i.v. injection of Taxol with four times of dosage, our hydrogel could inhibit tumor growth more efficiently by a single dose of i.t. administration. The prolong and sustainedly release profile, good biocompatibility, and enhanced anti-cancer efficacy of our hydrogels suggested their big potential for cancer therapy.

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Notes and references

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