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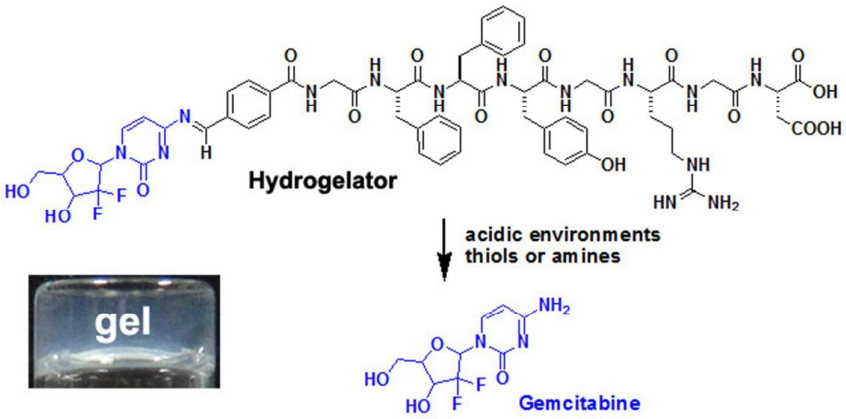

Supramolecular hydrogels can be formed by the addition of gemcitabine to aldehyde-containing peptides.

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ARTICLE TYPE

Gemcitabine induced supramolecular hydrogelations of aldehyde-containing short peptides

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The addition of gemcitabine to aldehyde-containing short peptides leads to supramolecular hydrogelations and the resulting hydrogels can be applied for sustained release of gemcitabine.

Supramolecular nanofibers and hydrogels[1] hold great promise for controllable delivery of therapeutic agents due to their biocompatibility and responsiveness.[2] They can serve as physical carriers to improve the aqueous solubility of hydrophobic drugs and deliver them more efficiently into cancer cells and to tumor tissues.[3] Another alternative approach of using them to deliver drug molecules is to develop drug derivatives that can self-assemble into nanostructures or hydrogels.[4, 5, 6] Materials formed by such drug derivatives possess very high and adjustable drug loadings and original drug molecules can be sustainedly released *via* chemical or enzymatic reactions. For instance, Xu and co-workers have reported on a supramolecular hydrogel that can sustainedly release anti-inflammatory olsalazine through the reduction of azo group.[5] Cui and co-workers have reported on supramolecular nanofibers that can release 10-hydroxy camptothecin through the glutathione triggered reduction of disulfide bond.[6] These nano-materials have shown excellent properties to inhibit cancer cells, bacteria, and inflammation. In this study, we report on another example of supramolecular hydrogel system that can sustainedly release the anti-cancer drug of gemcitabine.

Gemcitabine is a nucleoside analog used to treat various cancers including non-small cell lung cancer, pancreatic cancer, and breast cancer.[7] It has an amine group that will form Schiff bases with aldehydes. We therefore planed to design a hydrogel system based on Schiff base formation.[8] The design principles were described as following: 1) We firstly designed aldehyde-containing peptides that would not form nanofibers and gels by themselves; 2) The addition of gemcitabine would lead to the formation of Schiff bases, increased the hydrophobicity of the conjugates, and might lead to nanofiber and hydrogel formations. Since the Schiff base was not stable in acidic conditions,[9] the original gemcitabine could be liberated when the supramolecular nanofibers encountering acidic environments such as in tumor tissues and within cells.

We then designed 4-Formylbenzoic acid (FBA) capped short peptides of GFFYGRGD and GFFYGRGE, FBA-GFFYGRGD and FBA-GFFYGRGE (Fig. 1). They were synthesized by standard Fmoc- solid phase peptide synthesis directly and purified by reverse phase high performance liquid chromatography. The obtained peptides could form solutions in phosphate buffer saline (PBS, pH = 7.4) at concentrations up to 5 wt% (50 mg/mL). The addition of 1 equiv. of gemcitabine to the peptide solution could indeed lead to hydrogel formations. For example, adding 1 equiv. of gemcitabine to a PBS solution of FBA-GFFYGRGD (final peptide concentration = 0.5 wt%, Fig. 1) led to a hydrogel formation within 30 minutes (Fig. 1). The disappearance of NMR signal of the proton on aldehyde after the addition of gemcitabine also suggested the formation of Schiff base (Fig. S-6). This is the first example of hydrogelator of gemcitabine and the first hydrogel system formed from a hydrogelator containing a Schiff base and the observations clearly demonstrated the success of our design.

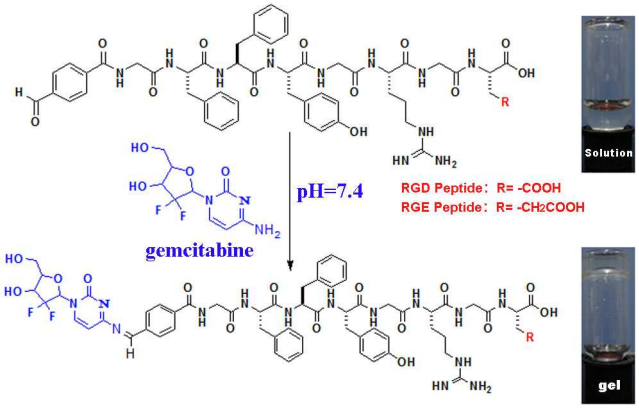

Fig. 1. Chemical structures of aldehyde-containing short peptides (4-Formylbenzoic acid (FBA)-GFFYGRGD and FBA-GFFYGRGE), the illustration of gemcitabine induced supramolecular hydrogelations, and the optical images of the solution and the resulting gel

Rheology was performed to characterize the mechanical properties of the resulting hydrogels. After peptide mixing with gemcitabine at pH 7.4, dynamic time sweep was conducted at a strain of 0.5%. Results in Fig. S-8 showed that both the storage moduli (G') and the loss storage (G'') values increased with time extension and achieved balances about 30 minutes later. The dynamic frequency sweep was then performed. As shown in Fig. 2A, the G' value of the gemcitabine-RGD hydrogel (gel I) was about an order of magnitude greater than its G'' value, indicating a true hydrogel formation.[10] Furthermore, both values of G' and G''

exhibited weak frequency dependences at the range from 0.1 to 100 rad/s and weak strain dependences at a range from 0.1 to 10% (Fig. 2A and S-8C). Similar results were observed for gemcitabine-RGE hydrogel (gel II, Fig. 2B and Fig. S-8D). These observations demonstrated the presence of high elastic networks in both gels.

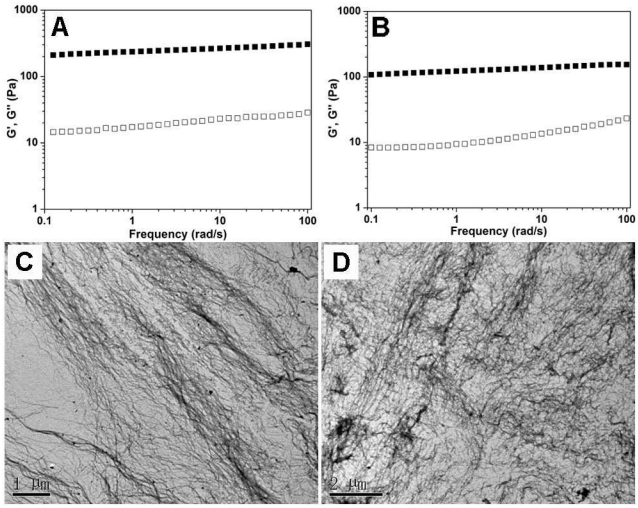

Fig. 2. Dynamic frequency sweep at the strain of 0.5% of A) gel I formed by adding 1 equiv. of gemcitabine to PBS solution of FBA-GFFYGRGD and B) gel II formed by adding 1 equiv. of gemcitabine to PBS solution of FBA-GFFYGRGE and TEM images of C) gel I and D) gel II (the peptide concentration in gels is 0.5 wt% (5 mg/mL))

Transmission electron microscopy (TEM) was then used to characterize the morphology of nanostructures in the peptide solutions and the hydrogels. Both solutions of peptides exhibited no regular nanostructures (Fig. S-9A and S-9B), suggesting that the peptides themselves were lack of good self-assembly abilities. As shown in Fig. 2C and Fig. 2D, nanofibers were observed in both hydrogels. The width of nanofibers was about 50-100 nm and the nanofibers were longer than several microns. They entangled with each other to form dense networks for the hydrogel formations.

Table 1. IC_{50} value of gemcitabine and gemcitabine-peptide conjugates to cancer cells (SD was used for the statistical analysis)

IC_{50} (nM)	PAC-2	CFPAC-1	BxPC-3
Gemcitabine-RGD peptide	157±23	390±12	487±18
Gemcitabine-RGE peptide	173±20	547±40	570±21
Gemcitabine	223±12	473±11	480±6

MTT cell viability test was also performed to measure the IC_{50} values of gemcitabine and gemcitabine-peptide conjugates to three kinds of pancreatic cancer cells: PAC-2, CFPAC-1, and BxPC-3 (Fig. S-10-S-12). Results in Table 1 clearly demonstrated that gemcitabine-peptide conjugates possessed comparable or slightly better inhibition capacities to three kinds of pancreatic cancer cells comparing with the free gemcitabine. The observations indicated that the Shiff base formation in both conjugates would not attenuate the inhibition capacity of gemcitabine to cancer cells. The conjugate of RGD peptide showed better inhibition capacities to three kinds of cancer cells than that of RGE peptide. For example, the IC_{50} value of gemcitabine-RGD peptide to PAC-2 cell was 157 nM (47 μg/mL), while this value for the gemcitabine-RGE peptide was 173 nM (52 μg/mL). The better inhibition capacities of the gemcitabine-RGD peptide conjugate were probably due to the targeting effect of RGD peptide to cancer cells.[11]

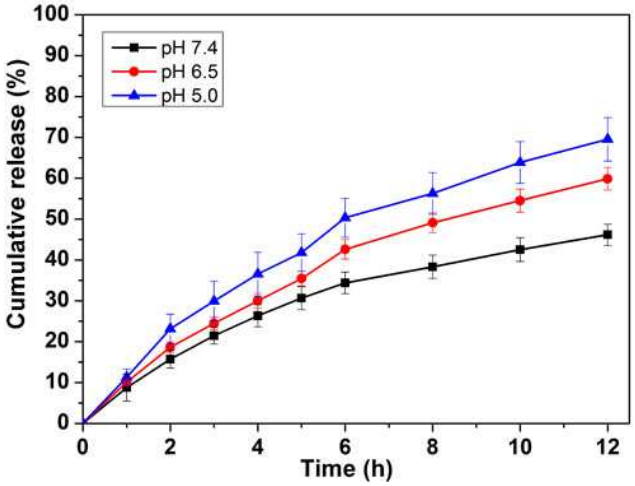

Fig. 3. Release profile of gemcitabine from gel I at different pH values (the peptide concentration in gels is 0.5 wt% (5 mg/mL))

We studied the release behaviour of gemcitabine from the gel I at different pH values and at 37℃. A 0.25 mL of PBS solution with selected pH value was placed on top of 0.2 mL of gel I. The upper solution was totally taken out at desired time points following by another 0.25 fresh PBS solution being added. The accumulation amounts of released gemcitabine from the gel were determined by LC-MS. As shown in Fig. 3, the gel I exhibited a constant release rate of gemcitabine during the 12 h experimental period without any burst release phenomenon at three pH values. The release rate and the accumulative release percentage of gemcitabine were different at different pH values. That is, gel I exhibited bigger release rates and higher accumulative released percentages at lower pH values. For instance, there was about 70 and 46% of gemcitabine being released from gel I during 12h at pH of 5.0 and 7.4, respectively. The pH- dependence release of gemcitabine was in accordance with the property of Schiff base which would deform at acidic conditions. Since there are acidic in tumor micro-environments and within cells, the pH responsive release property of our gel suggests its big potential in cancer therapy. Generally, Schiff bases will rapidly decompose in acidic environments. However, we observed a slow decomposition of Schiff base in our hydrogel system, which is probably due the shielding of imine within the hydrophobic compartment of supramolecular nanofibers.

Our hydrogel was formed through the Schiff base formation. Therefore, the release of gemcitabine might be enhanced in the presence of competitive nucleophiles such as amines and thiols. In order to test this hypothesis, we performed the release experiment in the presence of glycine and cysteine. As shown in Fig. S-13, the results indicate that in the presence of

glycine with an amine group, the release of gemcitabine is faster. In the presence of cysteine with amine and thiol groups, the release of gemcitabine is even faster than that in the presence of glycine. Our hydrogel can therefore be used as a pre-formed hydrogel material for topical administration. Besides, Upon dilution, a supramolecular hydrogel will be converted to a dispersion of nanostructures that can be used for i. v. injection. Upon encountering acidic tumor micro-environments or entering cancer cells with large amounts of glutathione, the nanofibers might rapidly release gemcitabine to kill cancer cells.

In summary, we have developed a novel hydrogel system based on the Schiff base formation. The hydrogels showed excellent anticancer activities to pancreatic cancer cells and they could sustainedly release gemcitabine with a pH controllable manner. Recent studies indicated that the fast release of original anti-cancer drugs after being uptake of nanomaterials of drug-peptide conjugates are crucial to the good inhibition capacities of supramolecular drug amphiphiles.[12] Our system was based on Schiff base formation between aldehyde-containing peptide and amine-bearing anti-cancer drugs, which can liberate original anti-cancer drugs instantly encountering acidic environments. There are many therapeutic agents having amine groups such as the doxorubicine and vancomycin. Therefore, we image the big potential of our hydrogel system in controllable delivery of anti-cancer drugs for cancer therapy. One of shortcoming of our system is the relative low stability of aldehyde on peptides. This might be overcome by using more stable ketone compounds such as the 4-acetylbenzoic acid that can still form Schiff bases with primary amines.

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