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First preparation of a triterpenoid-based supramolecular hydrogel in physiological phosphate buffered saline

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A glycyrrhetinic acid-based (GA, a natural pentacyclic triterpenoid) supramolecular hydrogel was attained in physiological phosphate buffered saline for the first time. Its assembly process and potential applications in drug release were preliminarily explored.

Triterpenoids, one class of natural products consisting of six isoprene units, are found in many plants in the form of free acids or aglycones.[1-3] They have attracted significant attention over the past decades due to their relative low-toxicity and prominent biological activities in anti-virus, anti-tumor, and anti-inflammatory.[4-8] Recently, increasing efforts have been made to fabricate supramolecular assemblies on account of their multiple functional groups, rigid skeleton, and unique stacking manners.[9-15] These features render triterpenoids as one of the ideal building blocks for constructing supramolecular structures by the self-assembly. Currently, many triterpenoid-based supramolecular assemblies have been developed, especially the supramolecular organogels.[16-25] For example, Weiss and co-workers reported nine excellent gelators of the ester of arjunolic acid (**AA**, a natural triterpenoid) in a wide range of organic solvents, independent of the ester groups;[21] our group found that a π-chromophore-contained glycyrrhetinic acid (**GA**, a natural pentacyclic triterpenoid) amphiphile could form supramolecular gels in chloroform/aromatic solvents,[25] and one of the major driving forces was hydrophobic interactions between glycyrrhetate skeletons. Moreover, its molecular chirality could be transferred and magnified through the self-assembly, consequently resulting in the formation of helical nanofibers. However, due to the participation of toxic organic solvents, their biological applications are limited. Thus, it is necessary and beneficial to explore the triterpenoids-based supramolecular hydrogels.

To date, only two works on the triterpenoid-based hydrogel have been reported[26,27] on account of their bulky hydrophobic skeletons. Bag and co-workers found that **AA** could form the supramolecular hydrogel in water/DMF, water/DMSO, and water/ethanol, respectively, and this hydrogel had been utilized in the entrapment and the controlled release of anticancer drugs.[26] Our previous work showed that **GA** afforded a stable hydrogel under the condition of pH > 9.[27] Obviously, these two hydrogels either need organic solvents or high pH values, which

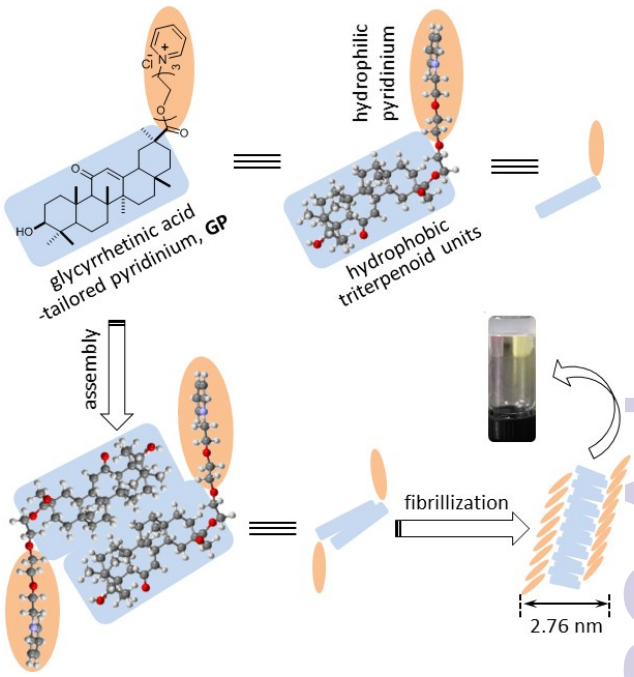

Fig. 1. Schematic representation of the assembly process of the supramolecular hydrog... **GP** in physiological PBS.

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Electronic Supplementary Information (ESI) available: experimental section, synthetic details of **GP** and **AGP**, Table S1-S2, and Fig. S1-S15. See DOI:10.1039/x0xx00000x

prohibited their biological applications. Therefore, the development of supramolecular hydrogel under physiological conditions is particularly called. Herein, we first reported a supramolecular hydrogel assembled from a glycyrrhetinic acid-tailored pyridinium amphiphile (**GP**, Fig. 1) in physiological phosphate buffered saline (PBS), and its potential applications in drug release.

Within this amphiphilic molecule **GP**, the oligo ethylene glycol (OEG) is used to connect the hydropboic **GA** skeleton and the hydrophilic pyridinium cation, which are utilized to afford possible hydrophobic interactions, van der Waals forces, π-π stacking, and electrostatic interactions during the gel formation process. Its synthetic route is shown in Scheme S1, where the intermediate was obtained by the esterification of **GA**, and subsequently reacted with pyridine at 80 °C to afford the final amphiphile **GP**.

As a general procedure, **GP** in deionized water, Kphos buffer, or PBS was heated until a clear solution formed. Then it was cooled to room temperature and the "inverted test tube method"[28] was used to determine whether a hydrogel formed or not. As shown in Table S1 and Fig. S1, **GP** can form hydrogel both in Kphos buffer (10 mM, pH 7.4 and 8.0; Entries 3 and 6, Table S1) and PBS (1x, pH 7.4; Entry 7, Table S1), but only precipatates in deionized water (Entry 1, Table S1). Either change in pH or concentration will lead to the collapse of hydrogel (Entries 2, 4, 5, and 8, Table S1). In addtion, sodium chloride saline (10 mM) with different pH values was used to investigate the role of phosphate anions during the gelation process. The results show that no gel is attained (Entries 9-11, Table S1), indicating that phosphate anions participate in electrostatic interactions with pyridinium cations during the assembly process. In order to mimic the biological system, PBS was chosen as the solvent in the following study. As a crucial parameter of the hydrogel stability, T_{gel} of **GP** under various concentrations was summarized (Fig. 2a and b), where T_{gel} increases with the concentration of **GP**. Based on the above relationship, the thermodynamic parameters (ΔH, ΔS, ΔG) at 298 K were calculated (Fig. S2), revealing the excellent stability of this hydrogel.

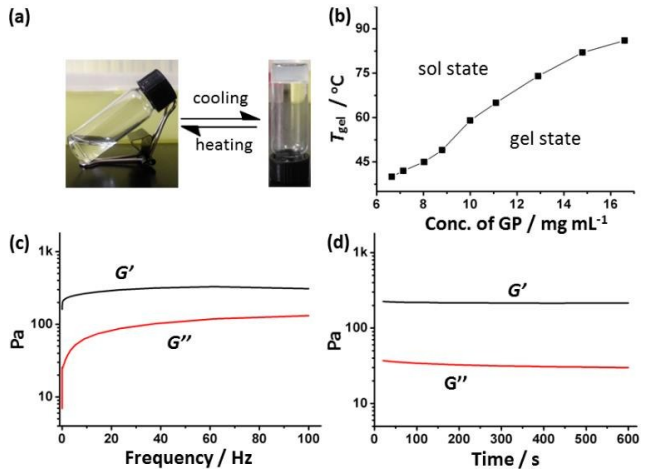

Fig. 2. (a) Photograph of **GP** in gel and sol states (PBS, pH 7.4); (b) Plot of T_{gel} *vs*. the concentration of **GP**; Dynamic frequency (c) and time (d) sweep of the storage modulus *G′* and the loss modulus *G″* of the hydrogel **GP** in PBS (10 mg/mL, pH 7.4). 2% strain is used for (c) and (d), and 1 Hz frequency is used for (d), respectively.

Its viscoelastic behaviour was characterized by the rheological measurements, in which the storage modulus *G′* and the loss modulus *G″* were measured as functions of frequency and time sweep. As shown in Fig. 2c, *G′* is greater than *G″*, which indicates the elastic character of the hydrogel.[29,30] The fact that *G′*/*G″* decreases with the increase in frequency suggests that it is a medium-elastic gel.[31] The dynamic time sweep data show that *G′* and *G″* values remain constant within 10 min gelling time, further suggesting it is a medium-strength physical gel (Fig. 2d).[32-34]

Transmission electron microscopy (TEM) was employed to investigate the hydrogel morphology. Entangled nanofibers are formed with the length at several micrometers and the width around 4.0 nm (Fig. 3a and Fig. S3). In order to study the driving forces during the gelation process, UV-Vis, NMR, and FTIR spectra were performed. As shown in the absorption spectra, a redshift (5 nm) of **GP** in PBS is observed in comparison with the one in water (Fig. 3b), indicating the π-π stacking between pyridinium rings on **GP**.[35,36] Moreover, temperature-dependent ^{1}H NMR spectra show the upfield shifts for pyridinium protons when the temperature decreased from 65 to 35 °C (Fig. 3c), which also points to the π-π stacking of pyridinium rings. In addition, the phosphate peak of PBS shifts downfield in ^{31}P NMR spectra after mixing with **GP** (Fig. 3d), likely caused by the electrostatic interactions between pyridinium cations and phosphates that decreases the electron density.[37,38] Furthermore, FTIR spectra of a powder sample and xerogel of **GP** were compared, where the saturated C-H stretching vibrations of methyl and methylene groups located on **GP** move from 2962, 2925 cm^{-1} to 2956, 2920 cm^{-1} (Fig. S4), respectively upon the gelation. It suggests an increase in van der Waals forces between the neighbouring **GP** skeletons in xerogel.[39] To verify the importance of the hydroxyl group during the gelation process, an acetylated derivative of **GP** was synthesized, in which the hydroxyl group was protected by an acetyl group (Scheme S1). The gelation test shows that the acetylated **GP** could form hydrogel under the similar conditions as **GP** (Fig. S5), indicating that the hydroxyl group is not crucial for the gelation. Given that above, π-π stacking, electrostatic interactions, van der Waals forces, and hydrophobic interactions primarily modulated the hydrogel formation in PBS.

To gain deeper insight into the supramolecular hydrogel, theoretical computation and X-ray diffraction (XRD) were performed. The optimized structure of **GP** is shown in Fig. 3, where the molecular length is 2.18 nm. Meanwhile, XRD pattern of **GP** xerogel shows one diffraction peak corresponding to *d*-spacing of 2.76 nm (Fig. 3f). Considering *d*-spacing of 2.76 nm between the single and the double molecular length of **GP** (2.18 and 4.36 nm) based on the assumption that the OEG spacer takes a fully extended conformation, it very likely corresponds to the length of **GP** dimer with a partial overlap of hydrophobic triterpenoid unit as shown in Fig. 1. This dimer will further arrange orderly to form nanofibers with sequestering the hydrophobic triterpenoid units within the interior and

projecting the hydrophilic pyridinium groups on the outside surface. Consequently, these fibers bind with each other driven by the side-side association[40] and entangle together to form the supramolecular hydrogel as shown in Fig.2a.

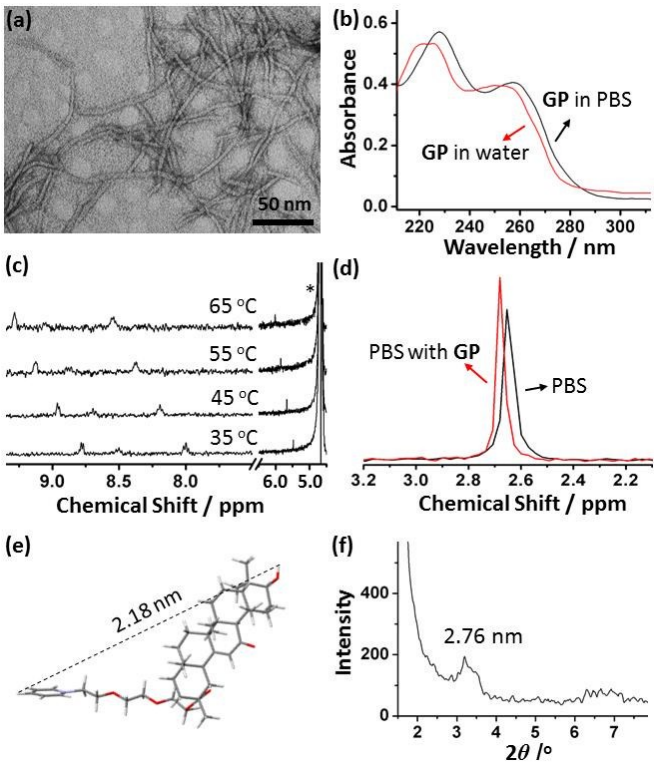

Fig. 3. (a) TEM image of hydrogel **GP** (10 mM) in PBS; (b) UV-Vis spectra of **GP** (3.8×10^{-5} M) in water and PBS, respectively; (c) Temperature-dependent partial ^{1}H NMR spectra of **GP** in PBS (10 mM, * represents D_2O); (d) ^{31}P NMR spectra of PBS and PBS with **GP** (10 mM); (e) Theoretical optimized structure of **GP** by Gaussian 09. Carbon atoms, hydrogen atoms, oxygen atoms, and nitrogen atoms are presented in gray, white, red, and blue, respectively, chlorines are neglected; (f) X-ray diffraction patterns of **GP** xerogel (10 mM).

Since gel networks can be used to entrap guest molecules for the cancer treatment, its biocompatibility was evaluated by Cell-Titer Blue viability assay. The results show that the gelator **GP** has no obvious cytotoxicity to 3T3-L1 fibroblast cells (Fig. S6). Meanwhile, doxorubicin (an anti-cancer drug) was used to investigate the encapsulation and release properties of hydrogel **GP** in PBS. **GP** and doxorubicin were dissolved in PBS by heating, and then the solution was cooled down to form a co-hydrogel with the maximum loading capacity of 8 mg/mL as shown in Table S2 and Fig. S7. After that, PBS was put on top of the co-hydrogel, and the release was monitored by UV-Vis spectra as shown in Fig. 4a. It is obvious that doxorubicin can be released from the hydrogel structures, and reaches the equilibrium within 28 h. Moreover, the assembled structures of co-hydrogel after 72 h was investigated by TEM. The fibrous nanostructures are still observed clearly (Fig. 4b), which suggests that the drug does not change the overall packing patterns in the hydrogel, and are simply trapped inside the 3D fiber networks of **GP** in PBS.

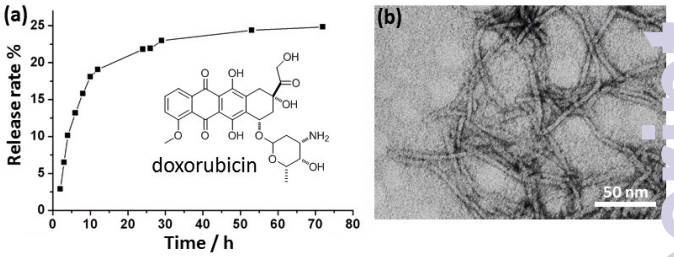

Fig. 4 (a) Time-dependent release rate of doxorubicin from co-hydrogel *via* diffusion; (b TEM image of co-hydrogel after 72 h diffusion. (Conc. **GP** = 10 mM; Conc. Doxorubicin = 0.41 mg/mL).

In conclusion, we firstly obtained a triterpenoid-based supramolecular hydrogel in physiological phosphate buffered saline, primarily modulated by π-π stacking, electrostatic interactions, van der Waals forces, and hydrophobic interactions. Moreover, this hydrogel can encapsulate and release the drug under physiological conditions without changing the overall packing patterns. This result provides a facial approach for constructing the supramolecular hydrogel based on natural triterpenoids under physiological conditions.

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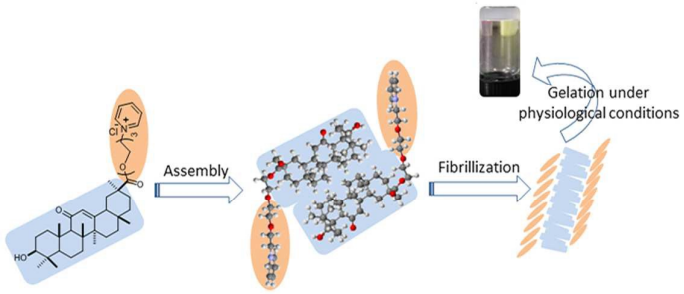
Graphical abstract:



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