D-Glucosamine-based supramolecular hydrogels to improve wound healing

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A simple supramolecular hydrogel based on D-glucosamine, a naturally occurring aminosaccharide, promises new biomaterials for applications such as wound healing.

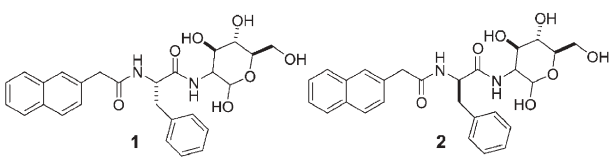
Glucosamine, a naturally occurring compound found in healthy cartilage, serves as a normal constituent of glycoaminoglycans in cartilage matrix and synovial fluid in the form of glucosamine sulfate, which strengthens cartilage and aids the synthesis of glycosaminoglycan. Glucosamine acts as one of the components in a widely used pain management for osteoarthritis (OA) patients and achieves moderate effectiveness.[1] Glucosamine also plays a role in the process of wound healing,[2] which has led to the successful demonstration that the dendrimer of glucosamine prevents the formation of scar tissue in a clinically relevant rabbit model.[3] Apparently, the dendrimer of glucosamine inhibits Toll-like receptor 4 (TLR4) to achieve defined immuno-modulatory and antiangiogenic effects for synergistically preventing the formation of scar tissue.[3] The biological importance of glucosamine, the success of the design and application of polyvalent glucosamine,[3] and the successful generation and applications of low molecular weight gelators based on carbohydrates[4,5] encourage us to incorporate glucosamine into supramolecular hydrogelators as the starting point towards self-assembled polyvalency of D-glucosamine for wound healing and other biomedical applications.

Despite intense research interest in glucosamine[6] and the increased efforts on supramolecular gelators or self-assembled nanofibers,[4,7–9] the use of glucosamine as a building block to generate supramolecular hydrogels remains unexplored, except that Hamilton *et al.* suggested that the conformational rigidity of sugars plays an important role in hydrogelation *via* directing the intermolecular hydrogen-bond networks.[7] Moreover, the polymeric hydrogels that incorporate glucosamine or aminosugars exhibit increased adhesion with neural tissue of the host, improved vascularization, and enhanced infiltration of non-neuronal cells of the host.[10] This observation suggests that glucosamine may exert similar beneficial effects on the supramolecular hydrogels and render them a new type of biomaterials for applications in biomedicine.

We found that the attachment of appropriate hydrophobic groups to the glucosamine affords supramolecular hydrogelators with good biocompatibility. More importantly, the resulting hydrogel assists wound healing and prevents the formation of scars on a mouse model. The result of this work also supports the notion that the self-assembly of bioactive molecules[8,11] to form networks of nanofibers in the hydrogel may offer a useful and effective way to generate biomaterials.

Scheme 1 illustrates the structures of the two hydrogelators, which consist of D-glucosamine, L- or D-phenylalanine, and a naphthalene group. L-phenylalanine or D-phenylalanine reacts with *N*-hydroxy succinimide (NHS) activated ester of 2-(naphthalen-2-yloxy)acetic acid to afford (*S*)-2-(2-(naphthalen-2-yl)acetamido)-3-phenylpropanoic acid (**3**) and (*R*)-2-(2-(naphthalen-2-yl)acetamido)-3-phenylpropanoic acid (**4**), respectively. Then, NHS assisted coupling between **3** and D-glucosamine gives pure compound of **1** in 66% yield after HPLC, and the coupling between **4** and D-glucosamine affords pure compound of **2** in 63% yield after HPLC. Both **1** and **2** are effective hydrogelators. Typically, after 2 mg of **1** are suspended in 1.0 mL of water, the increase of the temperature to ~80 °C gives a clear solution. Cooling the solution to room temperature leads to a slightly opaque hydrogel (Gel **I**, Fig. 1A). A similar procedure affords the hydrogel of **2** (Gel **II**, Fig. 1B). The pH values of Gels **I** and **II** are around 7, and the hydrogels are stable at room temperature for several months. We also synthesized Nap-D-glucosamine and Nap-L-Phe-L-Phe-D-glucosamine, which fail to form supramolecular hydrogels. This result indicates that the balance between hydrophobicity and hydrophilicity is important for a molecular hydrogelator.

Fig. 1C and 1D show the rheological data of Gels **I** and **II**. Using the mode of dynamic strain sweep at the frequency of 10 rad s^{-1}, we determined the optimal conditions for the measurements of dynamic frequency sweep. As shown in Fig. 1C, the values of G′ and G″ remain constant from 0.1 to about 0.7% strain for Gel **I** and from 0.1 to 1.0% strain for Gel **II**. Both samples' values of G′ are larger than values of G″, indicating that both samples are viscoelastic. Although the value of G′ of Gel **I** is larger than that of Gel **II**, the range of plateau of Gel **II** is wider than that of Gel **I**, suggesting that Gel **I** is slightly more viscoelastic, but Gel **II** is more tolerant of external force. Based on



Scheme 1 The chemical structures of the hydrogelators, Nap-L-Phe-D-glucosamine (**1**) and Nap-D-Phe-D-glucosamine (**2**).

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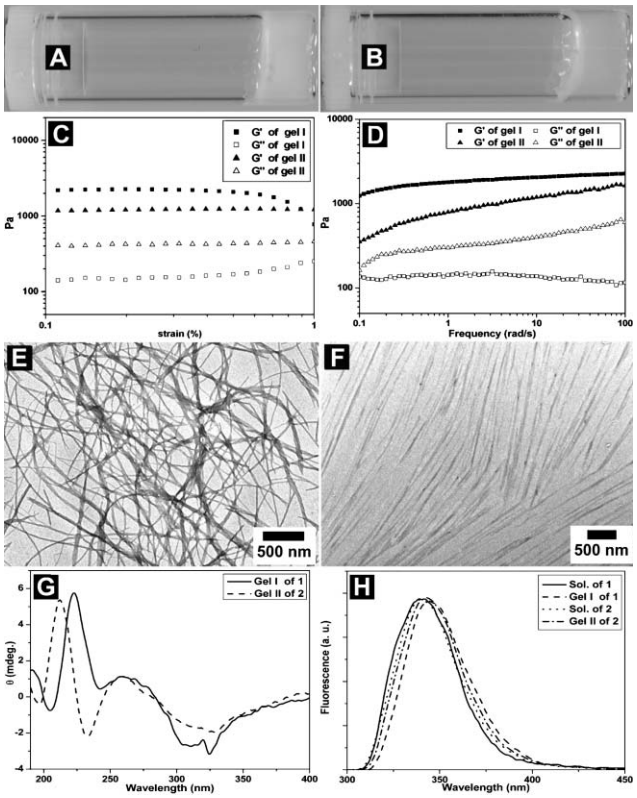

Fig. 1 Optical images of (A) Gel **I** and (B) Gel **II**, (C) strain and (D) frequency dependence of dynamic storage moduli (G′) and loss moduli (G″) of the hydrogels, TEM images of (E) Gel **I** and (F) Gel **II**, (G) the circular dichroism (CD) of the hydrogels, and (H) the emission spectra of **1** and **2** in solution and in the hydrogels.

the above results, we measured the dynamic frequency sweep of both hydrogels at the strain of 0.4%. The values of their storage moduli (G′) exceed those of their loss moduli (G″) by a factor of 10 (for Gel **I**) and 1.5 (for Gel **II**),[10] indicating that these two samples are viscoelastic and behave like a typical hydrogel. For Gel **I**, the value of G′ exhibits weak dependence on frequency (from 0.1 to 100 rad s^{-1}) at the stress above 1000 Pa; for Gel **II**, its value of G′ changes from about 200 Pa at low frequency (0.1 rad s^{-1}) to more than 1000 Pa at high frequency (100 rad s^{-1}). This observation indicates that the matrices of Gel **I** have a good tolerance to the change of external force.

To study the microstructure of Gels **I** and **II**, we obtained the transmission electron micrograph (TEM) images of the hydrogels. As shown in Fig. 1E, the irregular small ribbons form large bundles and tangle with each other in Gel **I**. We also observed a small amount of helical fibers with a width ranging from 27 to 55 nm in Gel **I**. For Gel **II** (Fig. 1F), small rigid ribbons with a width of 35–50 nm form well-distributed matrices. The sizes of the ribbons in Gel **II** are more uniform than those in Gel **I**. The density of nanostructures in Gel **I** is higher than that in Gel **II**, which accounts for a slightly larger value of G′ of Gel **I** than that of Gel **II**. The different morphologies in the two gels are likely a result of their different structures because the concentration of **1** or **2** is the same in their corresponding hydrogels and the only difference is the configuration of phenylalanine (L- for **1** and D- for **2**). According to TEM images, compound **1** with an L-phenylalanine has a stronger tendency to aggregate to form larger bundles and a more crosslinked network.

The circular dichroism (CD) and fluorescence spectra of the hydrogels also help further understanding of the molecular arrangements in Gels **I** and **II**. As shown in Fig. 1G, the peak at 191 nm and the trough at 205 nm in Gel **I** result from exciton splitting of the peptide π–π* transition, while the peak at 222 nm is due to the peptide n–π* transition. In Gel **II**, the peptide π–π* and n–π* transition bands appear at 195 nm and 212 nm, respectively. The peaks at around 218 nm indicate unordered conformations of peptide bonds of both compounds (**1** and **2**) in their gel phases. These CD signals (below 240 nm) share common features with the CD of the β-sheet of a polypeptide,[12] suggesting that the self-assembly of the hydrogelators leads to a β-sheet like super-structure. We also observed a broad positive peak centered at about 272 nm (nπ* of aromatic parts) and a broad negative peak centered at about 315 nm (ππ* of aromatic parts). The CD signals of Gels **I** and **II** exhibit similar shapes, suggesting that they were mainly induced by the D-glucosamine.

Fig. 1H shows the emission spectra of both **1** and **2** in solution and gel phases. Both compounds exhibit broad peaks centered at 340 nm in their corresponding solution phase. In their gel phases, the peaks show slightly red shifts (to 347 nm for Gel **I** and to 343 nm for Gel **II**). These small red shifts indicate the lack of efficient π–π stacking of naphthalene groups of both compounds in their gel phases. The observation of a slightly bigger red shift and a higher shoulder peak at 375 nm in Gel **I** than in Gel **II** correlates well with the results obtained from rheological measurements (higher elasticity or bigger G′ value for Gel **I**) and TEM images (more entangled fibers in Gel **I**).

After characterizing the physiochemical properties of the hydrogels, we evaluated their biocompatibility, one of the major requirements for the application of the hydrogels. The cytotoxicity assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) indicates that 73.8% and 79.0% of HeLa cells survive in 100 μM of **1** and **2** at 24 h, respectively. Based on this result, we chose Gel **II** to test its ability to reduce formation of scar tissue at the wound site on the mice wound model[13] since **2** is more biocompatible than **1**. Subsequently, we tested whether the hydrogel would improve the wound healing using the following protocol:† Six Balb/C mice, aged 6 weeks, were randomly divided into two groups *viz*: treatment and control. A cut (7 mm in length, 2 mm in width, and 2 mm in depth) was made on the middorsal skin of each mouse. After 30 seconds, 1 mL (2 mg of **2** in PBS) of Gel **II** was applied on the cut (a liquid bandage was also used to fix the hydrogel). For the negative control, only a liquid bandage was applied on the wounds. No clinical, hematological or biochemical (including blood glucose) toxicity was observed, and there were no local or systemic bacterial, viral, or fungal infections in either of the two groups treated over 18 days. On inspection, the mice treated with Gel **II** exhibited a much faster wound healing process and smaller scars than those of the control group at day 6 (Fig. 2 A & B). Histological examination of the skins at the wound site also shows that higher density of fibroblasts (scar tissues) is presented in the skin of the mice in the control group. A large amount of keratinocytes migrate to the extracellular matrix (ECM) near the scar tissue (Fig. 2C), which indicates that on day 6, the wound on the untreated mice is at the re-epithelialization phase, one of the five typical phases of wound healing.[14] In contrast, there is

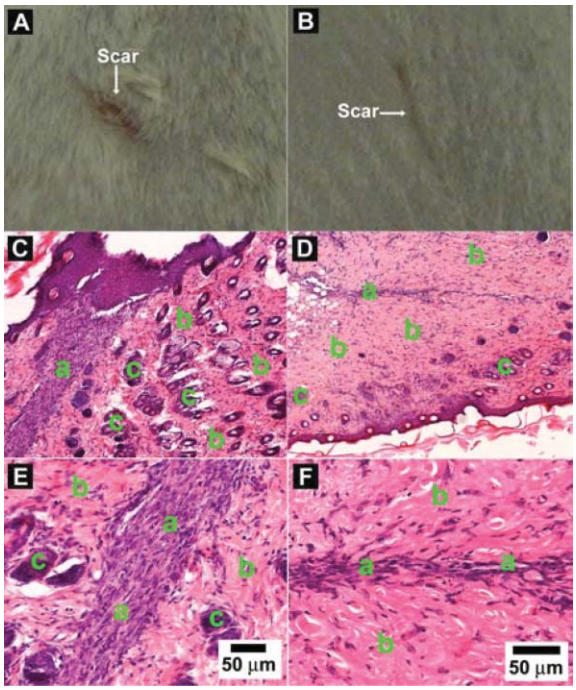

Fig. 2 Gross appearance (A, B), histological cross-section images (C, D) and enlarged images (E, F) of the dorsal skins of Balb/C mice on day 6 after wounding. (A, C, and E) negative control and (B, D, and F) Gel **II**-treated immediately after the incision was made. Histological specimens were embedded in paraffin wax and stained with hematoxylin and eosin. a, scar tissue; b, extracellular matrix (ECM); c, keratinocytes.

minimal formation of scar tissue at the wound site of the Gel **II**-treated mice on day 6. A large amount of ECM forms between the fibroblasts and the keratinocytes, which indicates the Gel **II** treated mice are at the later matrix deposition phase of wound healing (Fig. 2D). These results are consistent with the appearance of the skin of the wound site after healing.

In summary, based on a biologically important aminosaccharide derivative, we successfully synthesized two novel small molecule hydrogelators, which form biocompatible and stable hydrogels. The mice with wounds on their back recovered more rapidly when one of the hydrogels was applied than those without the treatment. This result indicates that the strategy reported in this paper promises a new way to develop candidates for wound healing. Further work will focus on studying the detailed relationship between self-assembly of the glucosamine-based hydrogelators and the beneficial effects of the hydrogels for other biomedical applications.

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Notes and references

† All animal procedures were approved by a local animal ethics committee.

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