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Supramolecular hydrogelators of N-terminated dipeptides selectively inhibit cancer cells†

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Consisting of N-terminated diphenylalanine, a new type of supramolecular hydrogelators forms hydrogels within a narrow pH window (pH 5.0 to 6.0) and selectively inhibits growth of HeLa cells, which provides important and useful insights for designing molecular nanofibers as potential nanomedicines.

This communication describes supramolecular hydrogelators consisting of N-terminated dipeptides that exhibit selective inhibitory effects against cancer cells. Over the last decade, peptides, as one class of essential biological molecules, have emerged as a favourite and useful choice of building blocks for making nanostructured biomaterials, especially in the form of nanofibers that serve as the network of hydrogels.¹ For example, amphiphilic oligopeptides have become a versatile platform for developing functional, self-assembled nanofibers that promise applications in tissue engineering, regenerative medicine, drug delivery, and anticancer therapeutics.² A simple dipeptide of phenylalanine also self-assembles to form stiff nanotubes that allow the casting of metal nanowires.³ These seminal works have largely stimulated the use of other small peptides as the basic motifs for designing small molecule gelators that not only self-assemble into nanofibers, but also result in molecular hydrogels, which promise applications ranging from biomedicine to energy.⁴ Among the reported molecular hydrogelators containing dipeptide motifs, some have already shown useful properties and promising applications, including separation of protein,⁵ response to the changes of pH or temperatures⁶ or ligand–receptor interactions,⁷ as matrices for three-dimensional cell-cultures,⁸ and the formation of vesicles for the delivery of oligonucleotides.⁹ These promising results suggest that it is worthwhile to further explore the potentials of molecular hydrogelators containing dipeptide motifs because of their versatility, low cost, and well-established chemistry.

Most of the dipeptidic molecular hydrogelators, explored so far, are C-terminated hydrogelators.¹⁰ Though there is one report of a cationic dipeptide that forms vesicles for the possible delivery of oligonucleotides,⁹ the properties of the N-terminated dipeptides are largely unknown, including their

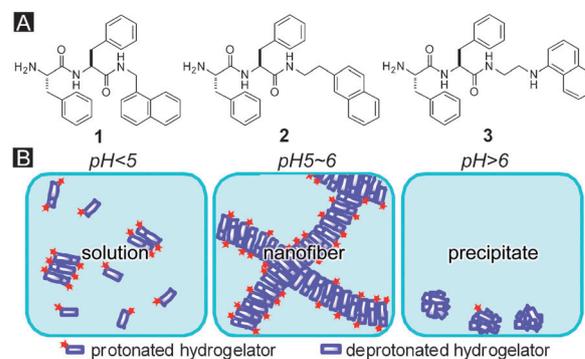


Fig. 1 (A) Molecular structures of the N-terminated dipeptide hydrogelators **1** to **3**. (B) Illustrated pH response of the hydrogelators.

ability to serve as hydrogelators and their cytotoxicity. To address this less explored direction of the development of small peptide hydrogelators, we conjugate naphthalene to the carboxylate end of a diphenylalanine to generate three N-terminated dipeptides (**1–3**, Fig. 1A), which successfully self-assemble into supramolecular nanofibers in water to afford stable hydrogels with the concentration of less than 0.8 wt% and within a relatively narrow pH range (pH = 5.0–6.0). The formation of the molecular nanofibers and hydrogels of **1**, **2** and **3** likely arises from both aromatic–aromatic interaction and the proper protonation of the N-terminal amine group. Besides that, we find that these hydrogelators exhibit significant higher cytotoxicity to HeLa cells than to Ect1/E6E7 cells, a result that represents the first example of molecular hydrogelators selectively inhibiting cancer cells. These results are significant because they may provide important insights for understanding cell specific cytotoxicity that are critical for the applications of hydrogelators and supramolecular hydrogels.

Fig. 1A shows the structures of the N-terminated hydrogelators. **1**, **2** and **3** all consist of diphenylalanine and naphthalene motifs. Their structures differ at the linker between the two motifs and the position of substitution on the naphthalene group. **2** has a 2-substituted naphthalene; **1** and **3** both have 1-substituted naphthalene, but the linker of **3** has an extra secondary amine. These slight structural variations result in difference in the properties of the hydrogelation of these three compounds (Table 1). Each of three compounds is able to immobilize a large quantity of water and form a stable supramolecular hydrogel (with more than 99 wt% of water) at a proper pH.

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Table 1 Gelation properties and 48 h IC_{50} of the N-terminated hydrogelators

Compound	MGC ^a		IC_{50} ($\mu\text{M}/\mu\text{g/ml}$)	
	(mM/wt%)	pH	HeLa	Ect1/E6E7
1	4.4/0.20	5.0–6.0	142.0 \pm 3.6/64.1 \pm 1.7	>200/>90.2
2	6.5/0.30	5.0–6.0	>200/>93.1	>200/>93.1
3	16.7/0.80	5.0–6.0	150.2 \pm 3.1/72.1 \pm 1.5	>200/>96.1

^a MGC: minimum gelation concentration.

However, under the same pH conditions (Fig. 2A–C), hydrogelators **1**, **2** and **3**, at their optimal gelation concentrations, all give hydrogels of similar optical appearances despite the different concentrations. Due to the presence of 1-aminonaphthalene in its structure,¹¹ the hydrogel of **3** is fluorescent (Fig. 2D).

Fig. 2E–H demonstrate the phase transition of **3** induced by the change of pH. At the concentration of 1.2 wt%, **3** forms the hydrogel at pH 5 to 6, with the pellucidity gradually changing from transparent to opaque when the pH increases from 5 to 6. When the pH reaches above 6, **3** becomes insoluble in water and mainly exists as white precipitates; when the pH is below 5, **3** dissolves well in water and gives a clear solution. The formation of the hydrogel within a narrow pH range (Fig. 1B) likely arises from a balance of protonation/deprotonation of the N-terminal amine. The N-terminal primary amine, at relatively low pH (*e.g.*, pH < 5.0 in this work), exists as ammonium groups due to the protonation of the amine group. The positive charges on the molecules cause the hydrogelators to repulse each other thus disfavoring the formation of nanofibers (or the networks of the nanofibers), which results in solutions of the hydrogelators. At relatively high pH (*e.g.*, pH > 6.0 in this work), the N-terminal amines exist as neutral amine groups due to deprotonation, which significantly increases the hydrophobicity of the hydrogelators, thus promoting the aggregation and further precipitation of the hydrogelators. Only within a narrow pH window of weak acidic conditions, the N-terminal amine group establishes the subtle balance of protonation/deprotonation that allows a certain degree of aggregation (self-assembly and crosslinking of nanofibers) of the hydrogelators while preserving their strong interactions with water molecules, thus achieving hydrogelation. Despite the extra secondary amine in **3**, which gives it higher solubility than the other two hydrogelators, the three hydrogelators share the same pH window for gelation (Table 1). The higher pK_a (9.7) of secondary amine than primary amine¹² makes it to be protonated within the pH window of hydrogelation, thus,

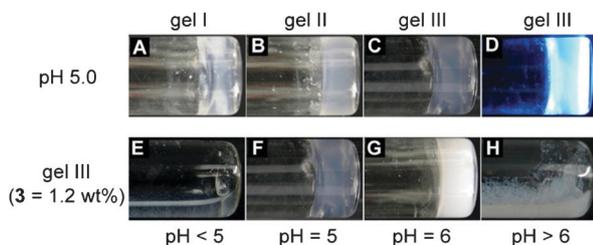


Fig. 2 Optical images of (A) gel I ([**1**] = 0.4 wt%), (B) gel II ([**2**] = 0.4 wt%), (C) gel III ([**3**] = 1.2 wt%). (D) A fluorescent image of gel III ($\lambda_{\text{ex}} = 345 \text{ nm}$). Compound **3** (1.2 wt%) exists (E) as a clear solution at pH < 5.0, (F) a transparent gel at pH = 5.0, (G) an opaque gel at 5.0 < pH < 6.0, and (H) precipitates at pH > 6.0.

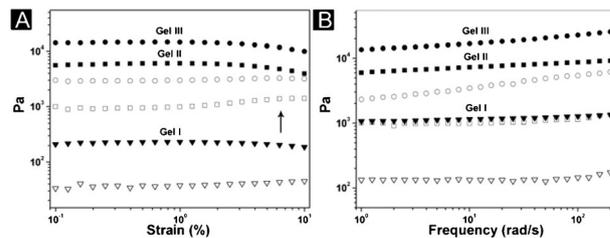


Fig. 3 Rheological properties of hydrogels formed by **1**, **2** and **3**. (A) Strain sweep and (B) frequency sweep of gel I ([**1**] = 0.4 wt%: ∇), gel II ([**2**] = 0.4 wt%: \blacksquare) and gel III ([**3**] = 1.2 wt%: \bullet). Storage moduli (G' : filled symbols); loss moduli (G'' : open symbols). Arrow points to the weak strain overshoot of gel II.

like **1** and **2**, partial protonation derived from the primary amine of **3** at pH 5.0–6.0 leads to hydrogelation.

We use rheology to characterize the viscoelastic properties of the hydrogels formed by **1**, **2** and **3**. Fig. 3A shows the dependence of G' (storage moduli) and G'' (loss moduli) of the hydrogels over a wide range of strain amplitudes. Unlike gel I and gel III, whose moduli exhibit a trend of decrease at high strain amplitude (*i.e.*, strain thinning¹³), gel II displays a weak strain overshoot in amplitude response: while G' decreases with increasing strain amplitude, G'' , with the increase of strain amplitude, rises first before it drops rapidly. This weak strain overshoot indicates that the molecules of **2** align with each other and associate into superstructures (in this case, bundle of fibers) that can resist the deformation up to a certain strain value (G'' increase) before a large deformation disrupts the structure (*i.e.*, G'' starts to decrease). The strain resistant structure of gel II explains that G' of gel II is larger than that of gel I while the concentration of hydrogelators is the same (Fig. 3B). In frequency sweep, all three hydrogels have G' and G'' essentially independent of frequency, in addition to G' dominating G'' at all frequencies, thus indicating the elastic nature of the three hydrogels. The mechanical properties of hydrogels formed by N-terminal hydrogelators classify these hydrogels as extensively cross-linked networks.¹⁴

Transmission electron micrographs (TEM) of uranyl acetate stained¹⁵ samples of the hydrogels reveal the nanostructures of the matrices of the hydrogels formed by these N-terminal hydrogelators. As the dominant structures of all the three hydrogels are nanofibers, each hydrogelator forms the nanofibers bearing a slightly different feature. Long and extended helical nanofibers with the pitch of about 40 nm (Fig. 4A and D) are the main morphology of the network of gel I; gel II consists of straight nanofibers that tend to align parallelly (Fig. 4B); the major morphology of the network of gel III is randomly tangled nanofibers (Fig. 4C). Unlike the nanofibers in gel I and gel II, nanofibers in gel III have irregular widths.

We use the MTT cell viability assay to examine the cytotoxicity of the N-terminal hydrogelators against HeLa cells and its counterpart cell line, Ect1/E6E7 cells, after treatment for 48 hours. HeLa, an epithelial cell line from cervical tumor, is the most widely used cell line in biology; Ect1/E6E7 is an immortalized normal epithelial cell line from cervix. These two cell lines serve as each other's reference, making a cancer-normal cell pair that is largely used as model cell pair in cancer studies.¹⁶ Though hydrogelators **1**, **2** and **3** exhibit similar

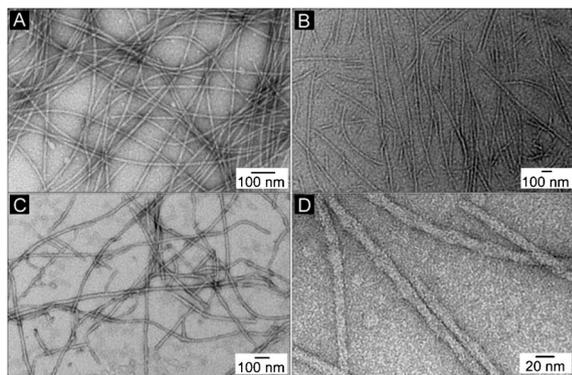


Fig. 4 The TEM images of negative stained (A) gel I, (B) gel II, (C) gel III, and (D) the magnified image of the helical nanofibers in gel I.

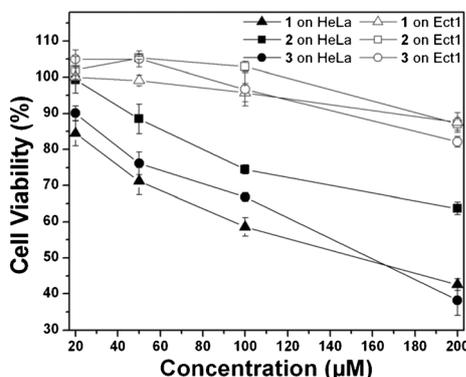


Fig. 5 48 h cytotoxicity vs. concentration curves of 1–3 on cancerous cells (HeLa) and counterpart normal cells (Ect1/E6E7).

cytotoxicity towards same cell lines, it is clear that **1**, **2** and **3** all have lower cytotoxicity against Ect1/E6E7 cells than against HeLa cells (Fig. 5). Same cytotoxicity tests were performed on another cancer cell line T98G to verify if the cytotoxicity of the N-terminated hydrogelators only occurs on HeLa cells or is a general behavior on cancer cells. IC₅₀ values of N-terminated hydrogelators on T98G (Fig. S1, ESI[†]) are in the same order of magnitude with HeLa cells and **2** is also being slightly less toxic than **1** and **3**, which demonstrate that N-terminated hydrogelators are generally toxic towards cancer cells. **2** differs from the other two compounds as it has 2-substituted naphthyl instead of the 1-substituted naphthyl group. The cytotoxicity of **2** on HeLa cells is also lower than that of **1** or **3**, thus implying a possible correlation between substitution patterns with the cytotoxicity. The difference of the cytotoxicity against the two cell lines increases with the concentration, and the difference is as large as 40% (in the case of **3**) at the highest concentration tested.

The cause of difference in cytotoxicity of the N-terminated hydrogelators toward cancer and normal cell lines, we speculate, unlikely originates from the positive charges provided by the hydrogelators since the excess of positive charge should also lead to the death of Ect1/E6E7 cells, but might lie in one or the combination of the following reasons: first, normal cells might have a poor permeability to the N-terminated hydrogelators; second, as acidification is a significant feature of cancer cells,¹⁷ normal cells have a pH of 7.4–7.6, so the N-terminated hydrogelators are less likely to self-assemble in normal cells than in cancer cells; third, while the growth of Ect1/E6E7 cells

is serum-free, the growth of HeLa cells requires serum, so the N-terminated hydrogelators may interact with some essential proteins through ionic bonds and consequently inhibit growth of HeLa cells. This ability to distinguish cancer and normal cells has not been discovered on any of the C-terminal hydrogelators (though it is possible for the precursors of hydrogelators¹⁸), which makes it a unique property of the N-terminated hydrogelators that warrants further investigation.

In conclusion, we design and synthesize a series of dipeptides based hydrogelators with exposed N-terminal amine that, unlike their C-terminal analogues, forms hydrogels only within a narrow pH range. These N-terminated hydrogelators, as a new group in the family of dipeptide based hydrogelators, not only provide an alternative option for designing functional hydrogelators that allow bioactive groups to be conjugated through their C-terminals, but also have a unique property of selective inhibition of the growth of HeLa cells, which is of significance to the understanding of cellular response to hydrogels and hydrogelators and may eventually lead to a route for the applications of supramolecular hydrogels and hydrogelators in cancer therapies.

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