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Multifunctional, Biocompatible Supramolecular Hydrogelators Consist Only of Nucleobase, Amino Acid, and Glycoside

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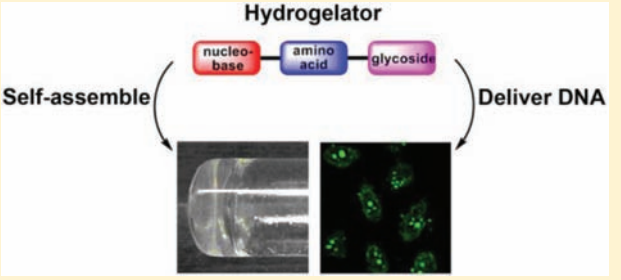
Supporting Information

ABSTRACT: The integration of nucleobase, amino acid, and glycoside into a single molecule results in a novel class of supramolecular hydrogelators, which not only exhibit biocompatibility and biostability but also facilitate the entry of nucleic acids into cytosol and nuclei of cells. This work illustrates a simple way to generate an unprecedented molecular architecture from the basic biological building blocks for the development of sophisticated soft nanomaterials, including supramolecular hydrogels.



INTRODUCTION

This article reports a novel class of hydrogelators, which consist only of life's three fundamental building blocks (nucleobase, amino acid, and glycoside), to self-assemble in water for generating multifunctional, biocompatible, and biostable supramolecular nanofibers/hydrogels. Due to their morphological similarity to extracellular matrices (ECMs) in tissues, hydrogels,[1−3] which consist of cross-linked matrices and large amounts of water, have emerged as an important class of biomaterials under intensive development. Although both natural (e.g., collagen, gelatin, hyaluronic acid, and alginate) and synthetic polymers (e.g., poly(DL-lactide-*co*-glycolide), poly(*N*-isopropyl acrylic amide), and poly(ethylene oxide)) have been serving as the matrices of hydrogels for biomedical applications (e.g., tissue engineering and drug delivery),[1] each of them still has its own limitation: the separation and purification of natural polymers are a nontrivial matter; the synthetic polymers are largely passive (despite the functionalization).[4] Therefore, it is necessary and beneficial to explore alternative matrices for developing hydrogels that mimic the ECM both morphologically and functionally.

Among various alternative approaches, nanofibers of self-assembled peptides as the matrices of supramolecular hydrogels have exhibited considerable promises by serving as scaffolds to guide the differentiation of neuron progenitor cells,[5] as media for cell culture,[6] and as carriers for drug releases.[7] Like the modified peptides, derivatives of glycosides are able to self-assemble to form nanofibers to result in supramolecular organogels[8] or hydrogels,[9] which has led to the development of semiwet peptide/protein arrays as biosensors and intelligent soft materials. Recently, nanostructures of deoxynucleic acid (DNA)[10] have been demonstrated as the matrices of hydrogels, albeit the hydrogels of DNA alone fail to promote cell growth.[11] These results not only attest that molecular self-assembly is an ubiquitous process but also imply that it is possible to combine the basic building blocks (i.e., nucleobase, amino acid, and glycoside) of the three major biomacromolecules (i.e., nucleic acid, proteins, and glycans) for exploring new molecular architectures to construct nanostructures that serve as the matrices of supramolecular hydrogels. Moreover, the existence of glycoproteins[12] and nucleopeptides[13] for a variety of biological functions in nature and the recent demonstration of hydrogelators of nucleopetides[14] support the notion that the integration of nucleobase, amino acid, and glycoside into a molecule to form the nanostructured matrices of supramolecular hydrogels will be an effective approach to impart hydrogels with both supramolecular orders and multiple functions.

On the basis of the above rationales, we simply connect a nucleobase (e.g., thymine), an amino acid (e.g., phenylalanine), and a glycoside (e.g., D-glucosamine) via covalent bonds and obtain **1T**. Compound **1T** forms molecular nanofibers to result in a supramolecular hydrogel at pH 7.0 and concentration 3.0 wt %. The replacement of thymine with other nucleobases (e.g., adenine, cytosine, or guanine) and/or the introduction of diphenylalanine in **1T** also results a series of novel hydrogelators (**1A**, **1G**, **2T**, **2C**, **2A**, and **2G**) that self-assemble in water to form molecular nanofiber/hydrogels at 3.0 wt % and proper pH. Besides that these hydrogelators hardly inhibit the growth of mammalian cells, the inclusion of glycoside in the hydrogelators significantly enhances their resistance to proteases. Moreover, after the self-assembly, the nanofibers exhibit significant interbase interaction with nucleic acids. The hydrogelators are able to facilitate oligonucleic acids entering cells and the nuclei of cells. Thus, this work illustrates a simple way to generate unprecedented molecular architecture from the basic biological building

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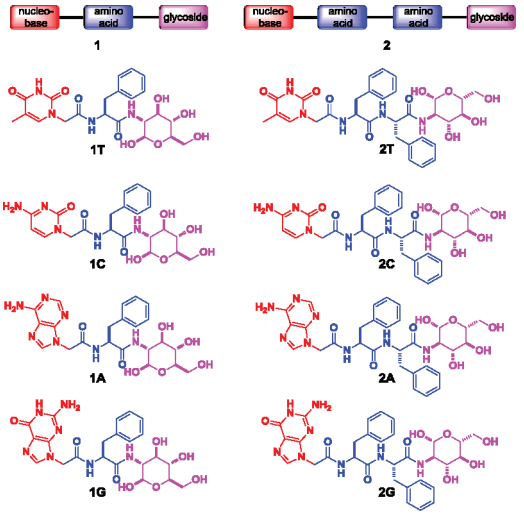
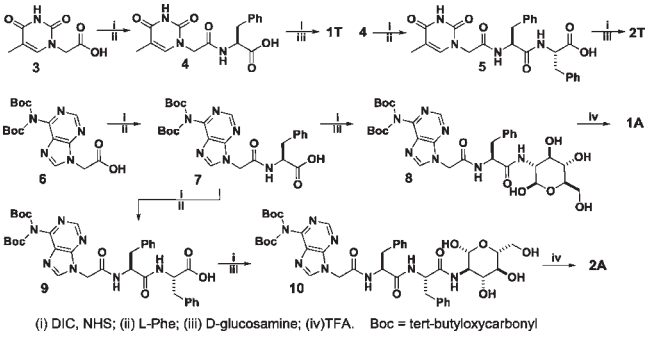
Scheme 1. Structures of the Hydrogelators (Except 1C) Consist Only of Nucleobase, Amino Acid, and Glycoside



Scheme 2. Typical Synthetic Route of Hydrogelators of 1 and 2



blocks for developing sophisticated soft nanomaterials that promise a wide range of applications.

RESULTS AND DISCUSSION

Scheme 1 shows the molecular design of two types of hydrogelators (**1** and **2**). **1** consists of a nucleobase (e.g., thymine, cytosine, adenine, or guanine), a phenylalanine, and a D-glucosamine; **2** consists of a nucleobase, a diphenylalanine,[15] and a D-glucosamine. In both **1** and **2**, the nucleobase and the D-glucosamine connect to the N-terminal and C-terminal, respectively, of the amino acid(s). Scheme 2 outlines typical synthetic routes for making these hydrogelators, exemplified by the cases of **1T**, **2T**, **1A**, and **2A**. The thymine acetic acid (**3**), being activated by *N*-hydroxysuccinimide (NHS), reacts with L-Phe to afford **4**. After undergoing the same NHS activation, **4** couples with D-glucosamine to give the hydrogelator **1T**. The addition of a second phenylalanine to **4** affords **5**, which couples with D-glucosamine to yield the hydrogelator **2T**. The synthesis of other hydrogelators (i.e., **2C**, **1A**, **2A**, **1G**, and **2G**) and compound **1C** starts from the protected nucleobases (i.e., (N^4-bis-Boc-cytosine-1-yl)-acetic acid, (N^6-bis-Boc-adenine-9-yl)-acetic acid, and (N^2-bis-Boc-guanine-9-yl)-acetic acid). As exemplified by the process for making the hydrogelators consisting of adenine, following the procedures of making the nucleobase acetic acid reported by

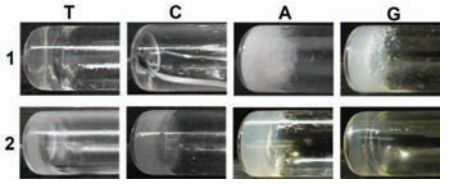


Figure 1. Optical images of the hydrogels of **1T** (pH 7.0), **2T** (pH 8.5), **2C** (pH 7.5), **1A** (pH 5.0), **2A** (pH 5.0), **1G** (pH 4.0), and **2G** (pH 4.0) and the solution of **1C** (pH 7.0). All are at 3.0 wt %.

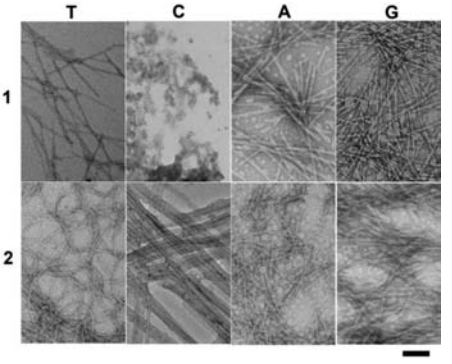


Figure 2. Transmission electron micrographs of the negatively stained[18] hydrogels of **1T**, **2T**, **2C**, **1A**, **2A**, **1G**, and **2G** and solution **1C**. Scale bar = 100 nm, and the concentration and pH values for each of them are the same as in Figure 1.

Nieddu,[16] we first synthesize bis(*tert*-butyloxycarbonyl) (bis-Boc) protected adenine, (N^6-bis-Boc-adenine-9-yl)acetic acid (**6**). After being activated by NHS, **6** reacts with L-Phe to afford **7**, which undergoes the same NHS activation and D-glucosamine coupling to give the product **8**. Subsequent removal of the Boc-protecting groups by the addition of trifluoroacetic acid (TFA) gives the hydrogelator **1A** in 42% total yield. The addition of the second phenylalanine to compound **7** gives **9**, which reacts with D-glucosamine to afford intermediate **10**. After the Boc groups are removed, **10** turns into hydrogelator **2A**. This five-step synthesis affords **2A** in 37% total yield. On the basis of the same strategy, we obtain **1C**, **2C**, **1G**, and **2G** in 45%, 39%, 41%, and 43% total yields, respectively.

We find that all compounds, except **1C**, behave as hydrogelators and self-assemble in water to form hydrogels (Figure 1). This result indicates that the covalent connection of nucleobase, amino acid, and glycoside presents a valid, simple approach to construct supramolecular hydrogelators. Since **1C**, **2C**, **1A**, **2A**, **1G**, and **2G** have amine groups on the nucleobases, we dissolve these compounds at low pH (via the protonation of their amine group(s)) and trigger hydrogelation by increasing the pH. Without any amine group for protonation, **1T** and **2T** dissolve completely in water at 3.0 wt % and pH 10.0 upon gentle heating. The change of the pH values of the solutions of **1T** and **2T** from 10.0 to 7.0 and 8.5, respectively, results in transparent hydrogels. **1C**, however, remains as a solution at the same condition. **1A** forms an opaque hydrogel at pH 5.0; **1G** produces a semitransparent hydrogel at pH 4.0. Hydrogelators **2T**, **2C**, **2A** and **2G** all self-assemble in water to form semitransparent hydrogels at the concentration 3.0 wt % and pH around 8.5, 7.5, 5.0, and 4.0, respectively. The different optical appearances of the hydrogels

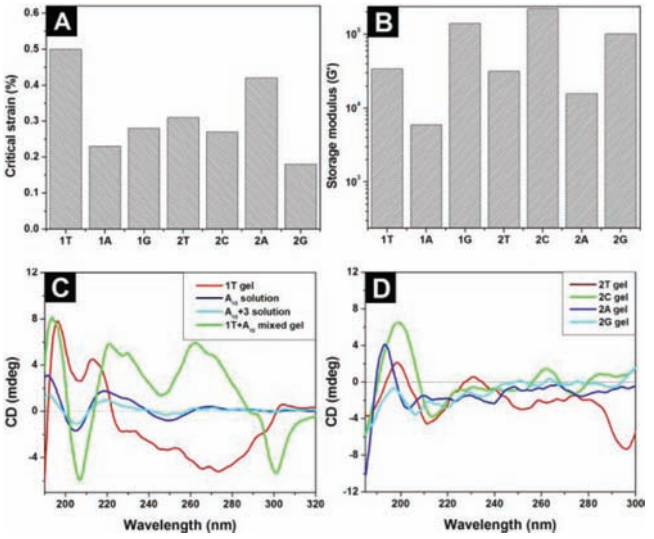


Figure 3. (A) Critical strain and (B) dynamic storage moduli (G') of the hydrogels of **1T**, **2T**, **2C**, **1A**, **2A**, **1G**, and **2G**. (C) CD spectra of the hydrogel of **1T**, the solution of deoxyadenosine (A_{10}), the mixture solution of thymine acetic acid with deoxyadenosine (A_{10}) in a 1:1 molecular ratio, and the hydrogel of **1T** mixed with deoxyadenosine (A_{10}) in a 1:1 molecular ratio; (D) CD spectra of hydrogels of **2T**, **2C**, **2A**, and **2G**. The concentrations and pH values are the same as in Figure 1.

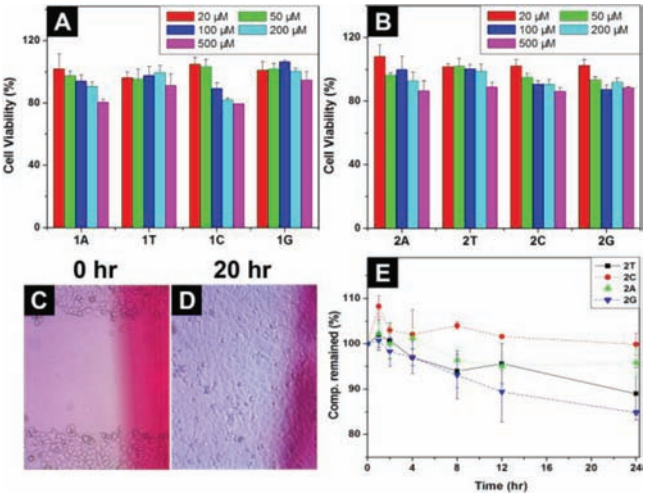


Figure 4. 72 h cell viability test of (A) hydrogelator **1** and (B) hydrogelator **2**. Optical images of the scratch-wound assay to assess the effects of **2T** in the media on wound closure; optical images of HeLa cells on the surface (C) 0 h and (D) 20 h after the creation of a wound in the presence of **2T** (by adding 500 μM **2T** in the media). (E) Time-dependent course of the digestions of hydrogelators of **2T**, **2C**, **2A**, and **2G** by proteinase K.

and the pH values for hydrogelation suggest the subtle difference of the self-assembly of these hydrogelators. Unlike **2T**, **2A**, **2C**, and **2G**, naphthalene−diphenylalanine−glucosamine fails to form a hydrogel properly,[17] suggesting a fundamental difference between the naphthalene unit and the nucleobases. Optical images of the hydrogels are shown in Figure 1.

As revealed by transmission electron microscopy (TEM), each hydrogelator leads to a characteristic morphology of the nanostructures (Figure 2) in the corresponding hydrogels. For example, the nanofibers of **1T** are thin and straight with a diameter of 12 nm; the nanofibers of **2T** (15 nm in diameter) appear to bend easily and to cross-link relatively efficiently, thus forming a fine network. The TEM of the solution of **1C** only shows featureless aggregates. The hydrogel of **2C** consists of nanofibers (25 nm wide) that cross-link into a network. The nanofibers of **2C** also form bundles, which likely contributes to the highest storage modulus among these hydrogels (Figure 3B). Both short nanofibers (14 nm in width and 200 nm in length) and nanoparticles (average diameter of 18 nm) present as the solid phase in the hydrogel of **1A**. The hydrogel of **2A**, similarly, consists of nanofibers (10 nm in width and 120 nm in length) and nanoparticles (average diameter of 21 nm), which tend to physically cross-link to afford the network. The hydrogel of **1G** appears to contain thin nanofibers (9 nm in width) and aggregated nanoparticles whose diameters are about 27 nm. In addition to forming nanoparticles with 20 nm average diameters, hydrogelator **2G** mainly self-assembles in water to form long thin nanofibers with the width 13 nm, and the nanofibers in **2G** entangle with each other to form a dense nanofiber network.

One characteristic property of hydrogels is their viscoelasticity, reflecting the mechanical properties to resist the deformation. We use rheometry to study the viscoelastic properties of the hydrogels. According to the results from the strain sweep (Figure S2 of the Supporting Information), the hydrogel of **1T** shows the critical strain value of 0.5% (Figure 3A). The critical strain values of the hydrogels of **1A**, **1G**, **2T**, **2C**, **2A**, and **2G** are at 0.23, 0.28, 0.31, 0.27, 0.42, and 0.18%, respectively, suggesting that the networks in these hydrogels lose the integrity relatively easily upon the application of an external force. The frequency sweep of the hydrogels shows that the dynamic storage moduli (G') of the hydrogels (**1T**, **2T**, **2C**, **1A**, **2A**, **1G**, and **2G**) dominate their dynamic loss moduli (G'') (Figure S2 of the Supporting Information), indicating that all samples behave as viscoelastic materials. Among these hydrogels, the hydrogel of **2C** exhibits the highest storage modulus (220 kPa). The hydrogels of **1G**, **2G**, **1T**, **2T**, and **2A** possess relatively high storage moduli of 139, 101, 34, 32, and 15 kPa, respectively. The hydrogel of **1A** exhibits the lowest storage modulus (6 kPa). Moreover, the addition of an oligomeric deoxyadenosine (A_{10}) to the viscous solution of **1T** (2.1 wt %, pH 7.0) affords a stable gel (Figure S5 of the Supporting Information), accompanied by the increase of storage modulus (G') from 4.4×10^2 Pa (of the solution of **1T**) to 9.5×10^2 Pa (of the hydrogel of **1T** plus A_{10}), which suggests the interbase interaction between the self-assembly of **1T** and A_{10} to favor molecular aggregation.[14,19]

As a useful tool to study the secondary structures of proteins, circular dichroism (CD) also provides insightful information about the self-assembled superstructures[20] in the gel phase or the liquid crystal phases.[21] We use CD to study the secondary structures of the self-assembled hydrogelators in the gel phase. As shown in Figure 3C, the hydrogel of **1T** exhibits a peak near 195 nm and a trough around 210 nm, suggesting that the backbones of the hydrogelators adopt β-sheet-like configurations in the self-assembled structures. The addition of A_{10} to the hydrogel of **1T** results in distinctive changes in the CD spectra (the increase of the CD intensity at around of 194 and 207 nm (belonging to the β-sheet structure) and two new peaks at around 230 and 262 nm), suggesting the formation a DNA−**1T** complex, which will enhance the base stacking of A_{10} and affect the superstructure of the self-assembly of **1T** through interbase

pairing and phosphate−sugar interactions.[19,22,23] The CDs of hydrogels of **1A** and **1G** display a maximum around 201 nm and a minimum near 210 nm (Figure S3 of the Supporting Information), slightly red-shifted from the CD signals of typical β-sheets, indicating that they share the common feature of a β-sheet structure but have a less ordered conformation or contain a mixture of β-sheet and random coil structures.[24] This result also agrees with the TEM of the hydrogel of **1A** or **1G**. The solution of **1C** exhibits the weakest CD signals (Figure S3 of the Supporting Information), agreeing with that fact that **1C** fails to self-assemble in water to form a hydrogel. As shown in Figure 3D, hydrogels of **2T**, **2C**, **2A**, and **2G** all exhibit a positive peak near 198, 198, 193, and 197 nm and a negative peak around 211, 213, 202, and 206 nm, respectively, suggesting that the backbones of the hydrogelators adopt β-sheet-like configurations in the self-assembled structures. The CD of the hydrogel of **2T** shows a negative broad band around 296 nm, which likely originates from the formation of a mesophase of **2T**[25] because it locates far from the chromophoric absorption region (ca. 268 nm) of **2T** (Figure S2 of the Supporting Information).

To verify the biocompatibility of the hydrogelators, we add hydrogelators **1** and **2** into the culture of mammalian cells and measure the proliferation of the cells. According to the results of the MTT assay shown in Figure 4A and B, after being incubated with 500 μM of the hydrogelator (**1T**, **1G**, **2T**, **2C**, **2A**, or **2G**) for 72 h, the cell viability remains at 90%. Although the cell viability decreases slightly when they are incubated with 500 μM **1C** or **1A** for 72 h, the value of IC_{50} is still >500 μM. These results prove that hydrogelators **1** and **2** are biocompatible. In order to further examine the biocompatibility, we conduct a simple *in vitro* wound-healing assay[26] with hydrogelator **2T**. As shown in Figure 4D, the presence of hydrogelator **2T** in cell culture has little inhibitory effect on the migration of cells, further confirming its biocompatibility.

Besides biocompatibility, biostability is also an essential requisite for a biomaterial. Thus, we examine the stability of hydrogelators **2** by incubating them with proteinase K, a powerful protease that catalyzes the hydrolysis of a wide range of peptidic substrates.[27,28] As shown in Figure 4E, more than 85% of **2T** and **2G** and 95% of **2C** and **2A** remain intact after 24 h of the incubation with proteinase K, indicating that hydrogelators **2** have excellent resistance to enzymatic digestion.

To further confirm that the incorporation of glycoside at the C-terminal of the peptides enhances the biostabilities of our hydrogelators to resist the proteinase K digestion, we have synthesized another molecule (thymine-FRGD-glycoside, **1T′**) by conjugating thymine, tetrapeptide (FRGD), and D-glucosamine together, and we examine its biostability by treating with proteinase K. We found that **1T′** self-assembles to form nanofibers (25 nm in diameter) and affords a hydrogel at the concentration 3.0 wt %. After the addition of proteinase K, almost 100%, more than 60%, and almost 50% of **1T′** remains at 4 h, 12 h, and 24 h, respectively (Figure S8 of the Supporting Information). Without the conjugation of glycoside, thymine-FRGD hydrolyzes completely in 4 h upon the same treatment, as does thymine-FF.[14] This result further confirms the advantage of the conjugation of glycosides.

Despite the rapid progress in the design and synthesis of peptidic supramolecular hydrogels from L-version amino acids,[3,29] the inherent susceptibility of L-peptides toward proteolytic digestion *in vivo* has reduced their efficacy and limited their scope of applications when long-term bioavailability is required.[30]

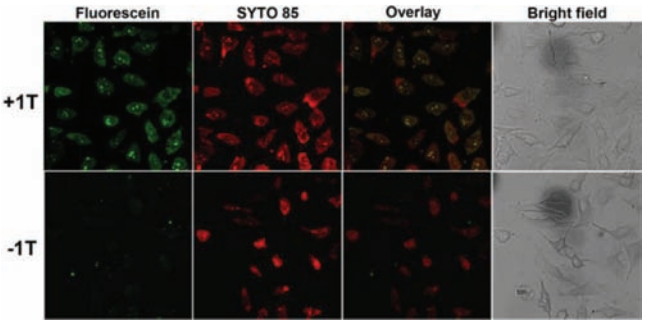


Figure 5. Fluorescence and bright field microscopy images showing the subcellular distribution of A_{10}, which is labeled with fluorescein dye (green). Cell nuclei were stained with SYTO 85 (orange). (Top) 500 μM **1T** and 1 μM FAM-A_{10} incubated with HeLa cells for 24 h. (Bottom) 1 μM FAM-A_{10} incubated with HeLa cells for 24 h.

The replacement of L-amino acids to D-amino acids or β-amino acids easily reduces the proteolytic digestion but leads to the loss of the bioactivity of peptides. Many efforts have been focused on designing and synthesizing different peptide molecules from D-amino acids or β-amino acids to mimic the structures and functions of peptides or proteins for prolonged or controlled bioavailability,[28,31] but the utilization of such unnatural amino acids raises certain safety concerns and limits their *in vivo* applications.[32] Since glycosylation is a strategy, used by cells, for enhancing the stability of proteins without comprising functions,[33] the incorporation of glycoside to the C-terminal of amino acid/peptide would be an advantageous approach for developing biostable and multifunctional hydrogels for applications that require long-term biostability.

To further explore the interbase interaction between the hydrogelators and nucleic acids, an attractive feature of these hydrogelators, we investigate whether these hydrogelators facilitate the delivery of nucleic acids into live cells and examine the subcellular distribution of the delivered nucleic acids. Using a fluorescein-labeled single strand oligonucleotide (FAM-A_{10}), which contains the same sequence (A_{10}) as that used in both circular dichroism (CD) and rheology studies, we incubate HeLa cells with **1T** and FAM-A_{10}. After 24 h of the incubation, we remove the culture medium, wash the cells with PBS buffer, and take fluorescent images. As shown in Figure 5, with the assistance of **1T**, the green fluorescence is in both the cytosols and the nuclei of the HeLa cells, indicating the presence of FAM-A_{10} in HeLa cells. The bright green spots overlay with the fluorescence of SYTO 85, a nuclear staining dye, further confirming the FAM-A_{10} enters the nuclei. In the control experiment (i.e., without using **1T**), green fluorescence is absent from the cytosols and nuclei of the HeLa cells, indicating that it is **1T** to interact with and to deliver the oligonucleotide into live cells. Moreover, the replacement of **1T** with **1G** or **1A** fails to deliver the FAM-A_{10} into the cells (Figure S9 of the Supporting Information), confirming that the matched interbase interactions are critical for the delivery. This result is the first example of the use of a nucleobase−amino acid−glycoside conjugate as a new neutral, biocompatible, and biostable molecule that allows the delivery of oligonucleotides into human cells. Although recent years have also witnessed intensive research activities with the development of various nonviral vectors for gene delivery, including cationic lipids and polymers, these synthetic vectors have suffered from low gene-transfer efficiency, toxicity, and *in vivo* instability.[34] These limitations necessitate the development of new biocompatible

carriers. Thus, these neutral and nontoxic small molecular hydrogelators that act as a successful scaffold for the delivery of oligonucleotide may lead to the development of new nonviral vectors for both *in vitro* and *in vivo* applications.

CONCLUSION

In conclusion, we have demonstrated that the integration of nucleobase, amino acid, and glycoside, life's three fundamental building blocks, generates a new type of hydrogelators that self-assemble in water to afford ordered nanostructures and supramolecular hydrogels with multifunctional properties, such as biocompatibilities and biostabilities. Besides exhibiting excellent cell compatibility, as do hydrogelators of nucleopeptides,[14] these hydrogelators are able to bind and deliver nucleic acids. This feature is particularly useful and warrants further exploration by incorporating different biofunctional peptides or molecular recognition motifs to achieve nucleic acids condensation, blocking metabolism, endosomal escape, nuclear localization, and receptor targeting.[35] Compared to the hydrogelators of glycosyl−nucleoside−lipid,[19,23] the inclusion of a peptide imparts more diverse functions to the hydrogelators than a lipid does. The recent work on sugar−amino acid−nucleoside[36] as potential glycosyltransferase inhibitors, in fact, supports the notion that the integration of sugar, amino acid, and nucleobase into hydrogelators will lead to multifunctional and bioactive soft materials. So this work not only introduces a facile way to expand the current repertoire of building blocks for generating supramolecular assemblies from biomolecules but also promises more functional supramolecular hydrogels[37] for a variety of potential applications, including tissue engineering, drug delivery, and gene delivery.

ASSOCIATED CONTENT

Supporting Information. Synthesis of **1A**, **1T**, **1G**, **2A**, **2T**, **2G**, **2C**, and **1C**, CD spectra, rheological measurements, cell viability tests, and other control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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