

Supramolecular Assemblies of a Conjugate of Nucleobase, Amino Acids, and Saccharide Act as Agonists for Proliferation of Embryonic Stem Cells and Development of Zygotes

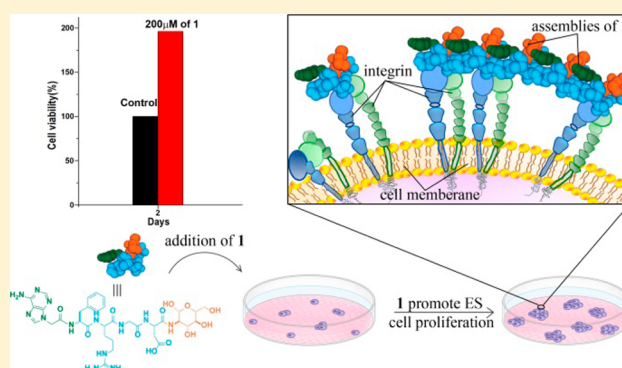
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S Supporting Information

ABSTRACT: The synthetic challenges in glycobiology and glycochemistry hamper the development of glycomaterials for biomedicine. Here we report the use of molecular self-assembly to sidestep the laborious synthesis of complex glycans for promoting the proliferation of murine embryonic stem (mES) cells. Our study shows that the supramolecular assemblies of a small molecule conjugate of nucleobase, amino acids, and saccharide, as a *de novo* glycoconjugate, promote the proliferation of mES cells and the development of zygotes into blastocysts of mouse. Molecular engineering confirms that each motif (i.e., adenine, Arg-Gly-Asp (RGD) domain, and glucosamine) is indispensable for the observed activity of the conjugate. As the first example of using assemblies of the molecular conjugates of multiple fundamental biological building blocks to control cell behaviors, this work illustrates an unprecedented approach to use supramolecular assemblies as multifunctional mimics of glycoconjugates.



Because tissue regeneration requires the integration of new cells with their surroundings, the intricate relationship between stem cells¹ and their microenvironment (i.e., stem cell niches) has to be regulated properly and effectively. Now, we know that stem cell niches interact with stem cells via a number of ways, including cell-to-cell contact, cell-extracellular matrix (ECM) adhesion, and presentation of soluble growth factors.² A common feature of these modes of action is that they all associate with glycoproteins and proteoglycans. For example, every living cell is covered with a complex array of glycans (i.e., glycocalyx);³ almost half of ECM proteins are either glycoproteins or proteoglycans;⁴ most secreted proteins of eukaryotes carry large amounts of covalently attached glycans;⁵ and actions of growth factors usually are mediated by proteoglycans.⁶ These facts, clearly, underscore the pivotal roles of glycans in stem cell biology. Despite the prevalence of glycans in the cellular environment,⁷ the development of glycomaterials for biomedical applications is rather limited because of the challenges in glycobiology and glycochemistry. Barriers to progress include limited *a priori* structural information on glycans and no “codons” for a particular saccharide structure,⁸ along with the daunting nature of glycan chemical synthesis.^{9,10} Unlike nucleic acids and proteins, glycans are more difficult to synthesize due to (i) their typically branched structures, (ii) their anomeric linkages (i.e., α or β glycosidic bond), and (iii) the need for multiple selective protection and deprotection steps. Therefore, no general

methods are available for the preparation of complex glycans.¹¹ These factors greatly impede the development and application of glycomaterials for biomedicine.

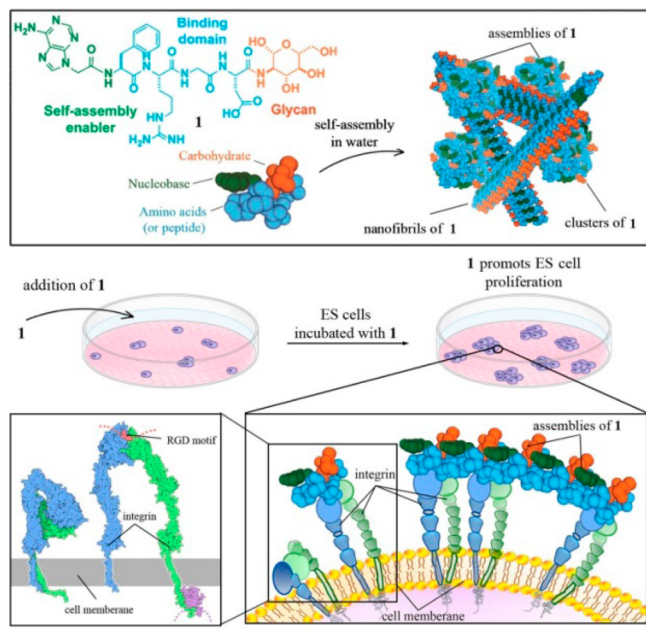
Thus, to sidestep the laborious synthesis of complex glycans, we choose self-assembly of small glycoconjugates, as a new approach, for mimicking the functions of glycoproteins/proteoglycans. We make this choice based on three facts. First, the tremendous advance of and the understanding of supramolecular chemistry^{13,14} have established the foundation for exploring more complex features of molecules such as the emergent properties of the assemblies of small molecules.^{13,15} In fact, our recent observation that the nanofibers of small molecules selectively inhibit cancer cells¹⁶ validates that supramolecular assemblies of small molecules can exhibit emergent properties drastically different from the constituent small molecules. Second, and most importantly, it is known that the clustered saccharide patches can mimic the binding of a disparate glycan,¹⁷ which implies that the assemblies of small saccharides are able to function as glycans. Third, the dendrimers¹⁸ or the nanofibers of glucosamine improve wound healing in animal models,^{19,20} which further supports that assemblies of small glycoconjugates can provide a facile way to mimic certain aspects of glycoproteins or proteoglycans.

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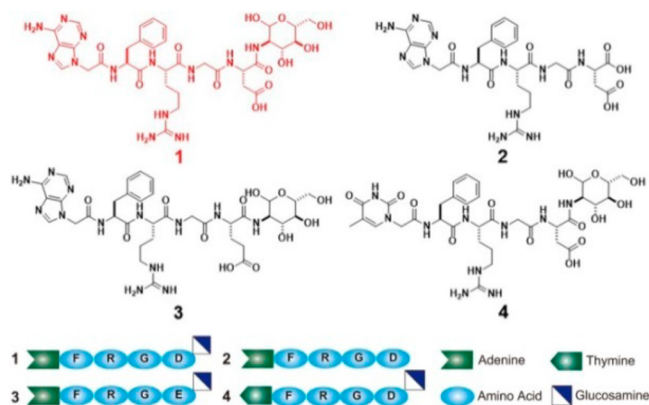
Based on the above-described rationales and encouraged by progress in glycochemistry and glycobiology,^{9,21} particularly the generation of self-assemblies of saccharide derivatives,^{20,22} we examine the functions of assemblies of a new type of small glycoconjugates,^{23,24} which consist of nucleobases, amino acids, and saccharides, for promoting the proliferation of mES cells and the development of zygotes of mouse. Our results indicate that the simple conjugate of adenine, Phe-Arg-Gly-Asp, and glucosamine (**1** in Scheme 1) self-assembles to form supra-

Scheme 1. Promoting the Proliferation of mES Cells via Plausible Interactions of Supramolecular Assemblies with Integrins¹²



molecular assemblies that contain nanoparticles and nanofibers. When they interact with cells, the assemblies of **1** nearly double the proliferation of mES cells without compromising their pluripotency. This stimulating effect requires the conjugation of the saccharide, the binding between the RGD epitope on **1** and integrins²⁵ on the cell surface (Scheme 1), and the presence of adenine because the removal of any one of the three motifs results in an ineffective analogue of **1** (Scheme 2). Moreover, the assemblies of **1** promote the development of zygotes of

Scheme 2. Molecular Structures and Representations of the Multifunctional Small Glycoconjugate (**1**) and Its Analogues



mice into blastocysts, implying the multiple functions of **1** and its assemblies. These results, together, establish the feasibility of using nanoscale assemblies of small glycoconjugates to emulate natural multifunctional glycans (e.g., heparan sulfate⁶), which may lead to a general approach that bypasses the laborious synthesis of glycans and focuses on the functional mimics (not the structural mimics) of glycoconjugates for regulating the behaviors of cells *in vitro* and *in vivo*.

Toward the goal of developing supramolecular assemblies of small molecules to mimic functional glycoconjugates, we examine the cell response to the nanoscale assemblies of **1** and its analogues. We specifically include the RGD sequence in **1** because RGD, as the well-established functional tripeptide motif²⁶ binding to integrins,²⁷ can carry out multiple functions. Meanwhile, we synthesize three analogues of **1** (Scheme 2) for evaluating the contribution of each module to the activity of the conjugate. For example, the removal of glucosamine from **1** gives **2**, which should help determine the necessity of the glucosamine, the replacement of aspartic acid in **1** to glutamic acid results in **3** that has a RGE sequence instead of RGD sequence, which would verify the interactions between the conjugate and integrin,²⁸ the substitution of adenine by thymine affords **4**, which could further help determine the role of the nucleobase for the observed biological activities of the conjugates.

We first characterize the supramolecular assemblies of **1**. As shown in Figure 1A, static light scattering (SLS) of the

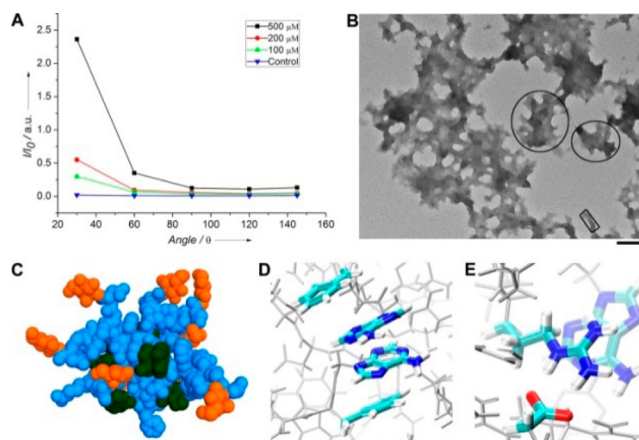


Figure 1. Supramolecular assemblies of **1**. (A) The intensity of SLS of **1** at 100, 200, and 500 μM . (B) TEM image of **1** at 500 μM . The sizes of the circled parts are congruous with those indicated by dynamic light scattering. Scale bar = 100 nm; all compounds are in PBS buffer, pH 7.4; the PBS buffer is the control in (A). (C) Cluster of nine molecules of **1**, with saccharide atoms in orange, nucleobase in dark green, and amino acid in blue; the two unseen saccharides are located on the solvent-exposed back face of the cluster. (D) Adenine and phenylalanine side chain π -stacking in the cluster. (E) Salt bridge between arginine and aspartate side chains, and π -stacking of arginine with adenine.

solutions of 100, 200, and 500 μM of **1** show much higher intensities than that of the PBS buffer, suggesting that **1** starts to form assemblies in the PBS buffer even at 100 μM . Dynamic light scattering (DLS) of the solution of **1** at 100 μM indicates the average hydrodynamic radii of the particles is about 140 nm, further supporting the results from SLS. Moreover, the intensity of SLS of **1** increases nonlinearly with decreasing detection angle, suggesting that the assemblies exhibit

anisotropic shapes (especially at the concentration of 500 μM). Consistent with SLS and DLS, transmission electron microscopy (TEM) of **1** (Figure 1B) at 500 μM shows a significant amount of assemblies that consist of dense clusters (diameter at about 200–400 nm) and short nanofibrils (width at about 20 nm and length at about 100 nm), which agrees with **1** forming a hydrogel at higher concentration.²⁴ At concentration higher than 100 μM , TEM images (Figure S1) show that **1** results in assemblies (around 100 nm), which agrees with DLS. These results confirm that **1** forms nanoscale, heterogeneous assemblies at a concentration below its critical gelation concentration (36 mM).²⁴ The ability of **1** to form assemblies in PBS buffer suggests that **1**, at and above 100 μM , is able to self-assemble to form assemblies, either in culture medium or on the surface of cells (Scheme 1).

Although the precise and complete molecular interactions of **1** within assemblies remain to be elucidated, all-atom explicit-solvent molecular dynamics simulations²⁹ provide insight into the molecular interactions stabilizing small clusters that can seed formation of larger assemblies. In a 100 ns trajectory of a system consisting of nine molecules of **1** with sodium and chloride ion concentrations and titratable residue protonation states consistent with PBS, formation of a single stable cluster is observed. The individual molecules, initially constructed with a linear peptide backbone and evenly spaced from each other, readily associate upon intermolecular collision. The final conformation at 100 ns has several notable features (Figure 1C,D,E): the saccharide moieties are solvent-exposed and poised on the periphery of the cluster, there are numerous intra- and intermolecular π -stacking interactions involving both adenine and the phenylalanine side chain (and occasionally the guanidinium group in arginine), and salt bridges form between the arginine and aspartate side chains. These features point toward π -stacking for the cohesion of assemblies, and toward the importance of the saccharide moiety for solubility and for interfacing with pockets of water in the assembled state.

The self-assembly and proteolytic resistance²⁴ of **1** allow us to test **1** in mES cell culture. Our result shows that the assemblies of **1** promote the proliferation of mES cells. As shown in Figures 2A and S3, the growth of mES cells is 162%,

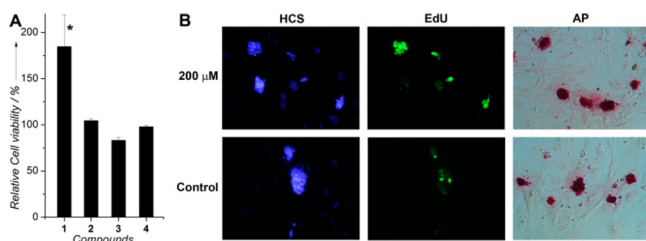


Figure 2. Promotion of the proliferation of mES cells. (A) Relative cell viability (determined by counting the cell numbers; 100% represents the control, i.e., 0 μM of **1**) of the mES cells incubated with **1**, **2**, **3**, or **4** at the concentration of 200 μM . *Averaged from four trials (Figure S6 and S7). The initial number of mES cells is 2.0×10^4 /well, and are cultured for 48 h according to the protocol.³² (B) The treated mES cells (by **1** at 200 μM for 72 h) and the control (by mES growth medium only for 72 h) were incubated with EdU or alkaline phosphatase (AP) prior to imaging. (Left) nuclear staining by HCS NuclearMask™ Blue stain; (middle) EdU staining by Alexa Fluor azide (scale bar = 100 μm); (right) photomicrographs of AP staining of undifferentiated mES cells (scale bar = 50 μm). The initial number of mES cells is 2.0×10^4 /well.

196%, or 135% after being incubated with 100 μM , 200 μM , or 500 μM of **1** for 48 h, respectively. This result clearly indicates that the molecules of **1** stimulate the proliferation of mES cells. The decrease of the agonistic effect for cell proliferation at 500 μM (Figure S3) is consistent with the formation of assemblies of the conjugates as a recent study has shown “bell-shaped” dose–response curves associated with the formation of aggregates of both antagonists and agonists.³⁰ Moreover, it is not uncommon for a growth-factor (e.g., PDGF) to exhibit positive signal at low concentration and negative signal at high concentration.³¹ Thus, the non-monotonic dose-dependence of **1**, in fact, supports that the assemblies of **1** are able to mimic certain features of growth factors, most of which are glycoproteins.⁴ Moreover, at the concentrations tested (100, 200, and 500 μM), **1** likely exists as molecular assemblies to promote cell proliferation. In addition, the effect of **1** appears to depend on the number of mES cells. When the initial number of cells is 2.0×10^3 per well, the respective growth of the mES cells is 105%, 151%, or 118% after 48 h and 214%, 168%, or 178% after 72 h (Figure S4). Only slightly promotion of the proliferation of mES cells incubated with 100 μM or 500 μM of **1** for 48 h fits with “bell-shaped” dose–response of agonists of assemblies.³⁰ That stimulation effects differ at 48 and 72 h reveals that proliferation depends on cell numbers, which implies that the assemblies of **1** likely play a role in cell–cell communication. These results indicate that the stimulating effects of **1** apparently correlate both with the concentrations of **1** and the numbers of mES cells.

According to Click-iT EdU HCS assay,³³ after a 72 h treatment of the mES cells with **1** (at 100, 200, or 500 μM), HCS-positive nuclei are EdU-positive by characteristic bright green fluorescence within the nuclei (Figures 2B and S10), confirming the proliferation of mES cells. However, the Click-iT EdU HCS assay of the control mES cells (treated with the normal mES cell growth medium without adding **1**) shows mainly blue fluorescence in the nuclei and little green fluorescence of the colonies. Moreover, almost all the mES cells treated with **1** are EdU-positive (89%), but less than half of the control mES cells are EdU positive (18%) (Figure S11). These results further confirm that the assemblies of **1** promote the proliferation of the mES cells. In addition, alkaline phosphatase (AP) staining³⁴ shows that after 72 h of incubation, mES cells treated with **1** (at 100, 200, and 500 μM) maintain a relatively high rate of growth of colonies without losing the high activity of AP (i.e., the ratio of AP-positive colonies is nearly 100%, Figures 2B and S12) and are morphologically normal, which is identical to control cells (Figure 2B). This result confirms the pluripotency of the mES cells treated with assemblies of **1**.

Moreover, assemblies of **1** promote the early development of mouse zygote. At the early stage of embryogenesis, the growth from a zygote to a blastocyst is a rather slow process since it starts with a single cell. Since ES cells constitute the inner cell mass of a blastocyst, promoting the proliferation of ES cells helps the zygote reach blastocyst stage. As shown in Figures 3 and S13, after 4 days incubation with KSOM medium containing **1** at 500 μM , 84% of zygotes become blastocysts, which is much higher than the control (where only 31% zygotes reach the blastocyst stage). This result further confirms that assemblies of **1** promote the proliferation of mES cells.

We also examined the requirement for each motif in the conjugate. According to the SLS data (Figure S2A) and TEM images (Figures S2B, C), the tendency of **2** or **3** to form

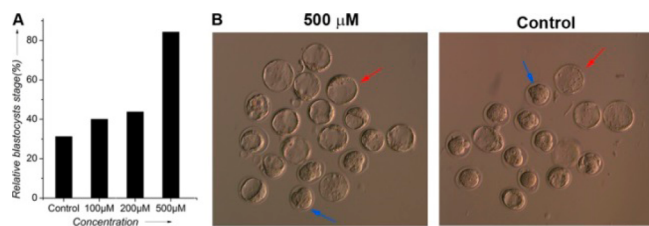


Figure 3. Promotion of the development of zygotes of mouse. (A) Percentage of zygotes reaching the blastocyst stage after incubation without **1** (control) and with **1** at concentrations of 100 μM , 200 μM , and 500 μM for 4 days in KSOM medium. (B) Photomicrographs of embryos incubated with 500 μM of **1** and without **1** (control) for 4 days (scale bar = 50 μm). Red arrows point to blastocysts, and blue arrows to morulas.

assemblies is comparable to that of **1**, suggesting that the removal of glucosamine from **1** or the replacement aspartic acid (D) on **1** by glutamic acid (E) changes little with regard to the tendency for self-assembly. Because thymine has fewer hydrogen bond donors and acceptors than adenine has, it is plausible that **4** is less effective at self-assembly in water. TEM imaging, however, shows assemblies of **4**, at 500 μM (Figure S2D), to have similar morphologies as assemblies of **1**. Despite this similarity, as shown in Figure 2A, **2**, **3**, or **4** hardly promote the proliferation of mES cells. Since **2** and **3** have similar abilities for self-assembly as **1**, the lack of stimulating effect of **2** and **3** on cell proliferation confirms that the glucosamine and/or RGD on **1** is indispensable for this biological activity. The incubation of mES cells with **4** at 500 μM hardly promotes the proliferation (Figure 2A). This result suggests that the replacement of the nucleobase results in the ineffectiveness of **4** for promoting cell proliferation. In addition, the mixture of the three components (adenine, FRGD, and glucosamine) hardly promotes proliferation of mES cells (Figure S8), further supporting that supramolecular assemblies of **1** act as the functional species for the promotion of stem cell proliferation. Combined, our results confirm that assemblies of **1** are critical for the observed stimulative effects and that the glucosamine, RGD domain, and adenine are all indispensable motifs for the activity of **1**.

In conclusion, the study of assemblies of **1** on the proliferation of mES cells confirms that self-assemblies of this conjugate of nucleobase, amino acids, and saccharide promote the proliferation of mES cells. In addition, analogues of **1** underscore the critical importance of each component (i.e., glucosamine, RGD, adenine) and reveal that the action of **1** involves integrin mediated pathways. Cell proliferation depends on optimal clustering of integrins,³⁵ and may explain the correlation of the stimulative effects of **1** with both its concentration and the number of cells. In fact, the “bell-shaped” dose response not only is a feature of assemblies (or aggregates) of small molecules^{30,36} or amyloids,³⁷ but also has been observed in the phenotypes involving glycans.³⁸ As experimental evidence, the non-monotonic dose response, thus, supports the notion that assemblies of **1** are able to mimic the function of glycoproteins (e.g., growth factors) and contributes to the mechanistic understanding of the functions of **1**. Furthermore, the requirement for both glucosamine and adenine suggests that assemblies of **1** may interact with proteins/enzymes other than integrins. Since one unique feature of assemblies of **1** is heterogeneities at nanometer to micrometer scales, this approach may provide a new way to

mimic certain aspects of glycoconjugates since microheterogeneity is a hallmark of glycans in cellular environments.⁵ Moreover, self-assemblies containing different biological building blocks as described here may generate diverse structural variations for interaction with cellular proteins in a fundamentally new way, which ultimately may lead to broader applications of supramolecular chemistry in cellular environment. And finally, the observed promotion of development of the zygotes of mouse to blastocysts points toward the *in vivo* application of such assemblies for directing cell behavior.

■ ASSOCIATED CONTENT

📄 Supporting Information

Synthesis of the hydrogelators, TEM images, relative cell viability, the optical images, cell assays, and molecular simulation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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