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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Scheme S1. The synthetic route of the hydrogelators 2 and 3.

i) L-Asp(OctBu) or L-Glu(OctBu), DIEA; ii) 20% piperidine; iii) L-Gly, HBTU, DIEA; iv) L-Arg(Pbf), HBTU, DIEA; v) L-Phe, HBTU, DIEA; vi) bis-Boc-adenylacetic acid, HBTU, DIEA; vii) 20% TFE in DCM; viii) D-glucosamine, HBTU, DIEA; ix) TFA:TIS:water (95:2.5:2.5)
The synthesis of 1 has been reported.\textsuperscript{[1]} \textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}_6): 7.86 (d, 2H, \( J = 4 \) Hz), 8.31 (m, 3H), 7.54 (m, 1H), 7.13–7.26 (m, 4H), 7.00 (s, 2H), 6.41 (s, 1H), 4.81–4.92 (m, 4H), 4.55–4.63 (q, 1H, \( J = 2 \) Hz), 3.45–3.70 (m, 6H), 3.29 (m, 1H), 2.99–3.10 (m, 5H), 2.72–2.75 (m, 4H), 1.49 (s, 1H), 1.45 (m, 4H); \textsuperscript{13}C NMR (100MHz, DMSO-\textit{d}_6) \( \delta \) 176.07, 175.02, 174.76, 173.86, 171.32, 168.72, 162.01, 161.72, 161.68, 159.63, 152.10, 140.55, 132.37, 132.37, 131.15, 131.15, 129.38, 120.83, 93.58, 75.23, 75.17, 73.99, 73.66, 73.52, 64.13, 57.67, 57.13, 55.40, 52.45, 48.31, 32.10, 29.43, 28.03; ESI MS(m/z) [M]\textsuperscript{+} calcd. for C\textsubscript{34}H\textsubscript{43}N\textsubscript{13}O\textsubscript{12} 829.35, found [M-H]\textsuperscript{−} 828.45. 

The synthesis of 2 is rather straightforward. Following the procedures of making nucleobase acetic acids reported by Porcheddu,\textsuperscript{[2]} we synthesized (\( \text{N}^6\text{-bis}-\text{Boc-adenine-9-y1})\text{-acetic acid for covalently coupling with the protected peptide fragments, Phe-Arg(Pbf)-Gly-Asp(tBu), which was synthesized by solid phase method. The removal of the protecting groups of the intermediate, adenine-Phe-Arg(Pbf)-Gly-Glu(tBu) results in 2.\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}_6): 8.57–8.67 (m, 2H), 8.14–8.31 (m, 5H), 7.59 (m, 1H), 7.14–7.26 (m, 6H), 4.84 (m, 2H), 4.51–4.54 (m, 2H), 4.24–4.25 (q, 1H, \( J = 2 \) Hz), 4.05 (m, 2H), 3.70–3.72 (d, 2H, \( J = 4 \) Hz), 2.99–3.05 (m, 3H), 2.45–2.64 (m, 2H), 1.49 (s, 1H), 1.45 (m, 4H); \textsuperscript{13}C NMR (100MHz, DMSO-\textit{d}_6) \( \delta \) 175.32, 174.98, 174.89, 171.90, 171.52, 168.97, 169.04, 161.90, 161.51, 159.85, 148.19, 140.45, 132.35, 132.35, 131.85, 131.14, 131.14, 109.95, 59.74, 57.12, 55.29, 54.74, 51.66, 51.62, 32.18, 28.06, 27.89, 27.14; ESI MS(m/z) [M+H]\textsuperscript{+} calcd. for C\textsubscript{28}H\textsubscript{36}N\textsubscript{12}O\textsubscript{8} 668.28, found [M+H]\textsuperscript{+} 669.57. 

The synthesis of 3 is similar to 2. First, we synthesized (\( \text{N}^6\text{-bis}-\text{Boc-adenine-9-y1})\text{-acetic acid for covalently coupling with the protected peptide fragments, Phe-Arg(Pbf)-Gly-Glu(tBu). After being activated by N-hydroxysuccinimide(NHS), adenine-Phe-Arg(Pbf)-Gly-Glu(tBu) reacts with D-glucosamine to give the intermediate compound, which turns into 3 after the removal of the protecting groups from it.\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}_6): 7.86 (d, 2H, \( J = 4 \) Hz), 8.20–8.37 (m, 3H), 7.75–8.05 (m, 4H), 7.55 (m, 1H), 7.15–7.21 (m, 6H), 6.41 (s, 1H), 4.84–4.95 (m, 4H), 4.37–4.55 (m, 3H), 4.24–4.25 (q, 1H, \( J = 2 \) Hz), 3.45–3.71 (m, 6H), 3.24 (m, 1H), 2.99–3.10 (m, 5H), 2.69–2.84 (m, 2H), 2.20–2.22 (m, 2H), 1.84 (m, 2H), 1.66 (s, 1H), 1.43 (m, 4H); \textsuperscript{13}C NMR (100MHz, DMSO-\textit{d}_6) \( \delta \) 177.37, 174.92, 174.26, 173.93, 171.31, 168.60, 162.26, 161.96, 161.61, 159.83, 149.24, 140.48, 132.34, 132.34, 131.15, 131.15, 129.27, 120.78, 93.65, 75.57, 75.23, 74.19, 73.76, 73.58, 68.43, 59.34, 57.66, 57.03, 54.54, 51.54, 48.45, 33.24, 32.07, 31.46, 28.11; ESI MS(m/z) [M]\textsuperscript{+} calcd. for C\textsubscript{35}H\textsubscript{49}N\textsubscript{13}O\textsubscript{12} 843.36, found [M-H]\textsuperscript{−} 842.16.
Figure S1. The transmission electron microscope (TEM) images of 1 at the concentration of (A) 100 µM and (B) 200 µM in PBS buffer (pH 7.4). Scale bar for TEM is 100 nm. The sizes of the highlight parts are congruous to those of dynamic light scattering.
Figure S2. (A) The intensity of SLS of 1, 2, 3, and 4 at 500 µM. The TEM images of (B) 2, (C) 3, and (D) 4 at the concentration of 500 µM in PBS buffer (pH 7.4). Scale bar for TEM is 100 nm; all compounds are in PBS buffer, pH 7.4; the PBS buffer is the control in (A).

Figure S3. Relative cell viability (determined by counting the cell numbers; 100% represents the control, i.e., 0 µM of the Assemblies) of the mES cells incubated with 1, 2, 3, or 4. The initial number of mES cells is 2.0×10^4/well, and are cultured for 48 hrs according to the protocol.

Figure S4. Relative cell viability (determined by counting the cell numbers; 100% represents the control, i.e., 0 µM of the aggregates) of mES cells when incubated with 1. The mES cells are cultured on Primary Mouse Embryo Fibroblasts (PMEF) according to the protocol. The initial number of mES cells is (A) 20,000; (B) 2,000.
Figure S5. Cell number counting of mES cells when incubated with 1. The mES cells are cultured on Primary Mouse Embryo Fibroblasts (PMEF) according to the protocol. The initial number of mES cells is (A) 20,000; (B) 2,000.
**Figure S6.** Cell number counting of mES cells when incubated with 1 for four trials. The mES cells are cultured on Primary Mouse Embryo Fibroblasts (PMEF) according to the protocol. The initial number of mES cells is 20,000.

**Figure S7.** (A) The average cell number counting of mES cells when incubated with 1 in the four trials as shown in Fig. S6. *P < 0.05; **P < 0.01 versus control (i.e., 0 μM of the aggregates). (B) The average of relative cell viability (determined by counting the cell numbers; 100% represents the control, i.e., 0 μM of the aggregates) of mES cells when incubated with 1 as shown in Fig. S6.

**Figure S8.** Relative cell viability (determined by counting the cell numbers) of mES cells when incubated with the mixture of adenine, FRGD, and glucosamine. The mES cells are cultured on Primary Mouse Embryo Fibroblasts (PMEF) according to the protocol. The initial number of mES cells is 20,000.
Figure S9. The optical images of the second day mES cells growth (A) incubated with 1 (200 µM) and (B) incubated without 1 and at the initial mES cell number of 20,000 (scale bar = 100 µm).

We examined the growth of the mES cells daily to maintain healthy colonies, and changed the corresponding medium every day. We measured the cell proliferation by counting the mES cell number using the hemocytometer after incubating the cells with 0.05% trypsin-0.53 mM EDTA at 37 °C for 10 minutes. As shown in Figure S9, the size of the colonies of mES cells incubated with 1 are significantly larger than that of the mES cell without the presence of 1.
Figure S10. Control mES cells and the treated mES cells (by 1 at 100 µM, 500 µM for 72 h) were incubated with EdU in mES cell growth medium for 2 hrs prior to fluorescence microscope. Figures on the left show nuclear staining by HCS NuclearMask™ blue stain; figures on the right show EdU staining by alexa fluor azide (scale bar = 100µm).
**Figure S11.** Control mES cells and the treated mES cells (by 1 at 200 µM for 72 hrs) were incubated with EdU in mES cell growth medium for 2 hrs prior to fluorescence microscope. Figure shows the quantitative result of the number of mES cells using EdU out of all the cells.

**Figure S12.** Photomicrographs of the alkaline phosphatase stained undifferentiated mES cells cultured in the presence of (A) 100µM 1; (B) 500µM 1 in growth medium; the initial number of mES cells is 20,000. It shows the alkaline phosphatase staining of undifferentiated mES cells at 72 hrs (scale bar = 50µm).
Figure S13. Photomicrographs of the embryos incubated with 1 at the concentration of 100 µM, 200 µM, and 500 µM for 4 days (scale bar = 50 µm). Red arrows show the blastocysts, blue arrows for the morulas, and black arrows for the dead cells.

The method for mES cell culturing and cell number counting: Prior to thawing EmbryoMax primary mouse embryo fibroblasts (PMEF) feeder cells, the 12 well plate was coated with 1.0 mL EmbryoMax mES cell qualified 0.1% gelatin solution per well for 1h. After removing the gelatin solution from the plate, 1.0 mL PMEF feeder cell suspension per well containing 1.0×10⁵ cells was incubated at 37 °C and 7% CO₂ for 24h. After that, we removed the PMEF feeder cell medium and seeded 2.0×10⁴ or 2.0×10³ mES cells per well onto the PMEF coated plate with mES cell medium or mES cell medium containing different concentrations of compound 1, 100 µM, 200 µM, or 500 µM. The mES cells needed to be examined daily since they divided rapidly and the cell cultures would become crowded with large colonies. The corresponding medium was changed every day. We counted the mES cell numbers by using the hemocytometer after incubating the cells with 0.05% trypsin-0.53 mM EDTA at 37 °C for 10 min.

The growth medium used for mES cell culture is Dulbecco's modified Eagle's medium (DMEM) (high glucose, Invitrogen Life Technologies 10829-018) with the following supplements: Glutamine to 2mM (Invitrogen Life Technologies 35050-06); MEM nonessential amino acids to 0.1 mM (Invitrogen Life Technologies 11140-050); 0.1 mM β-mercaptoethanol (βME) (Sigma M7522); Sodium pyruvate to 1mM (Invitrogen Life Technologies 11360-070); Penicillin to 50
U/ml and streptomycin to 50 μg/ml (Invitrogen Life Technologies 15070-063); 15% Fetal bovine serum (FBS) (Invitrogen Life Technologies 10082-147); Leukemia inhibitory factor (LIF), 500 units/ml (Invitrogen Life Technologies PMC4051).

The proliferation assay: mES cells proliferation was assessed using the Click-iT® EdU HCS assay according to the instructions of the manufacturer (Invitrogen). Briefly, cells plated on the confocal plates were treated with the mES cell growth medium or 1 of different concentrations in medium for 72 hrs. The mES cells were then incubated with 5-ethynyl-2′-deoxyuridine (EdU; 5 μM) in medium for 2 hrs. After the EdU was removed, cells were then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and washed twice with PBS buffer. The cells were permeabilized with 0.1% TritonX-100 in PBS for 15 min at room temperature. The cells were then washed twice with PBS prior to addition of the Click-iT® reaction cocktail for 30 min at room temperature in the dark. The cells were then washed with Click-iT® reaction rinse buffer prior to nuclear staining with HCS NuclearMask™ blue stain solution for 30 min at room temperature in the dark. After all the steps above, the samples were ready for imaging by fluorescence microscope.

The pluripotency assay: mES cells pluripotency assay was assessed using the alkaline phosphatase (AP) detection kit according to the instructions of the manufacturer (Millipore, SCR004). Cells plated on the confocal plates were treated with the mES cell growth medium or 1 of different concentrations in medium for 72 hrs prior to analyzing AP activity. After aspirating medium, cells were fixed with 4% formaldehyde in PBS for 2 min at room temperature and then washed with TBST rinse buffer. AP staining solution was added into the dish and mES cells were incubated for 15 min at room temperature in the dark. After aspirating the staining solution and rinsing the plate with TBST rinse buffer, the cells were ready for imaging.

The method for embryos collection and culture: Ten 3-week-old (B6×SJL)F1 females are injected with PMSG (Pregnant Mare's Serum Gonadotropin) at 3 p.m. and with hCG (human Chorionic Gonadotropin) 44 hrs later. 4 hrs after the administration of hCG, 1 female is placed in a cage with 1 stud (B6×SJL)F1 male and is checked the next morning for a copulation plug. After quick and humane sacrifice of a female mouse by cervical dislocation and dissection of reproductive organs of a female mouse, we can locate the two horns of the uterus, the oviducts, and the ovaries, cut the oviducts, and transfer the oviduct and attached segment of uterus to M2 medium at room temperature. After isolation of zygotes from dissected oviduct, the zygotes are incubated in the hyaluronidase solution for several minutes until the cumulus cells fall off, and then the zygotes are picked up by using pipettes and transferred to fresh M2 medium to rinse off the hyaluronidase solution, cumulus cells, and debris. Finally, the zygotes can be transferred to 50 μL KSOM medium with an oil overlay and incubated at 37 °C and 5% CO2. After the collection of zygotes, they are incubated with KSOM medium or KSOM medium containing different concentrations of compound 1, 100 μM, 200 μM, or 500 μM for 5 days, and then the growth of embryos will be examined daily.
Details of molecular modeling: Compound 1 was modeled using the CHARMM all-atom additive force fields (version C36) for proteins, nucleic acids, and carbohydrates, and a modified version of the TIP3P model was used to represent water. Nine copies of Compound 1 were constructed using force field internal geometries, yielding nine linear molecules with an extended polypeptide backbone. Consistent with literature values for the α:β anomeric ratio for GlcNAc, six of the nine were built with α-GlcNAc and the other three with β-GlcNAc. The individual molecules were evenly spaced on a 3x3 grid, with their long axes perpendicular to the grid and centered in a box of solvent with an edge length of ~7 nm and having sodium and chloride ions consistent with the NaCl concentration in PBS buffer. Water molecules overlapping with Compound 1 atoms were deleted, and the final concentration of Compound 1 was ~50 mM.

After energy minimization and a brief heating stage with restraints on Compound 1 non-hydrogen atoms, a 100-ns unrestrained, unbiased molecular dynamics simulation was performed. Simulations were done using cubic periodic boundary conditions, a 10 Å cutoff for nonbonded interactions, particle mesh Ewald for electrostatic interactions beyond the cutoff, a switching function between 8 and 10 Å for Lennard-Jones interactions, and an isotropic correction to the pressure for Lennard-Jones interactions beyond the cutoff. Covalent bonds involving hydrogen were constrained to their equilibrium lengths and water molecules were constrained to their equilibrium geometries. A 2 fs timestep was used with the BBK integrator, and temperature was maintained at 310 K and pressure at 1 atm with Langevin thermostating and Langevin piston barostating.

System construction and trajectory analysis were done with the CHARMM program; MD simulations were done with the NAMD program.

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