Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

Intracellular Protein Delivery by Glucose-Coated Polymeric Beads

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Supporting Results and Discussion

Analysis by transmission electron microscopy (TEM) of translocation experiments

Transmission electron micrographs also provided direct evidence that the glucose-coated polymeric beads were internalized. The TEM image for R1 mES cells untreated (a in Fig. S1) and treated with non-glucose-coated beads (b in Fig. S1) did not show any particles with diameters of about 150 nm inside a cell. In contrast, the polymeric beads were detected inside a cell in the TEM images for a cell treated with 5%- glucose-coated beads (c and d in Fig. S1). The size of the particles is same to that of the polymeric beads.

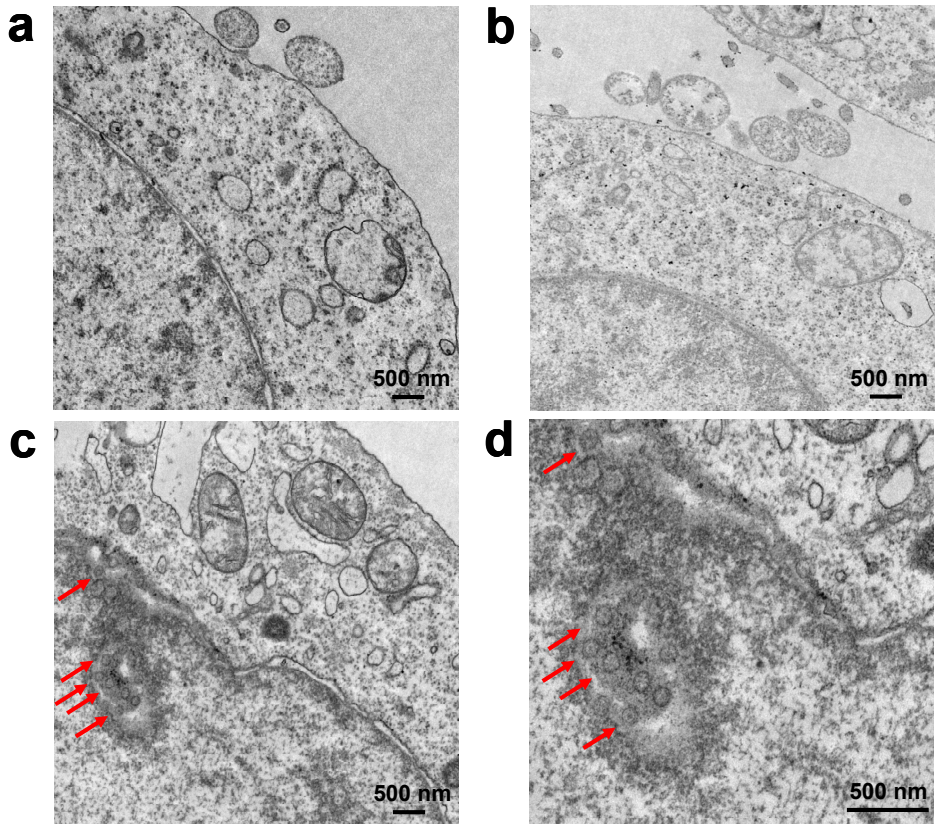

Fig. S1 Analysis by transmission electron microscopy (TEM) of translocation experiments of the beads into R1 mES cells. a) a cell untreated. b) a cell treated with non-glucose-coated beads. c) a cell treated with 5%-glucose-coated beads d) the amplified image of c. The arrows indicate the polymeric beads.

Transduction of the beads into Hela cells at 37 ºC and mouse embryonic stem cells at 4 ºC

Hella cells were tested to evaluate further applicability of our delivery system. Similar results were observed in the transduction experiment with Hela cells (Fig. S2). The confocal images for Hela cells treated with EGFP alone or non-glucose-coated beads did not show fluorescence in the cells (1-a, 1-b, 2-a, and 2-b in Fig. S2). However, the cells treated with 5%-glucose-coated beads clearly showed fluorescence (3-a in Fig.S2) and the

overlap image indicates that fluorescence is present in the cells (3-b in Fig. S2). It can therefore be concluded that glucose plays the key role in enabling protein-conjugated beads to cross membranes.

We also performed the transduction experiment at 4 ºC because the experiment could provide a clue for the uptake mechanism. In general, inhibition of the uptake at 4 °C indicates that the internalization process presumably involves an endocytic pathway (*S1*). The mES cells were cultured for 8 hr at 4 °C before being incubated with the beads. Incubation at 4 °C for 8 hr did not cause internalization of EGFP or any glucose-coated beads in the cells (4-a, 5, 6, and 7 in Fig. S2). Again a repeated experiment of incubation at 37 °C resulted in internalization of the glucose-coated beads (8 in Fig. S2). Although the detailed mechanism of the cellular uptake of the protein-conjugated glucose-coated beads is unclear at this time, the result indicates that the glucose-coated beads are presumably internalized by endocytic machinery.

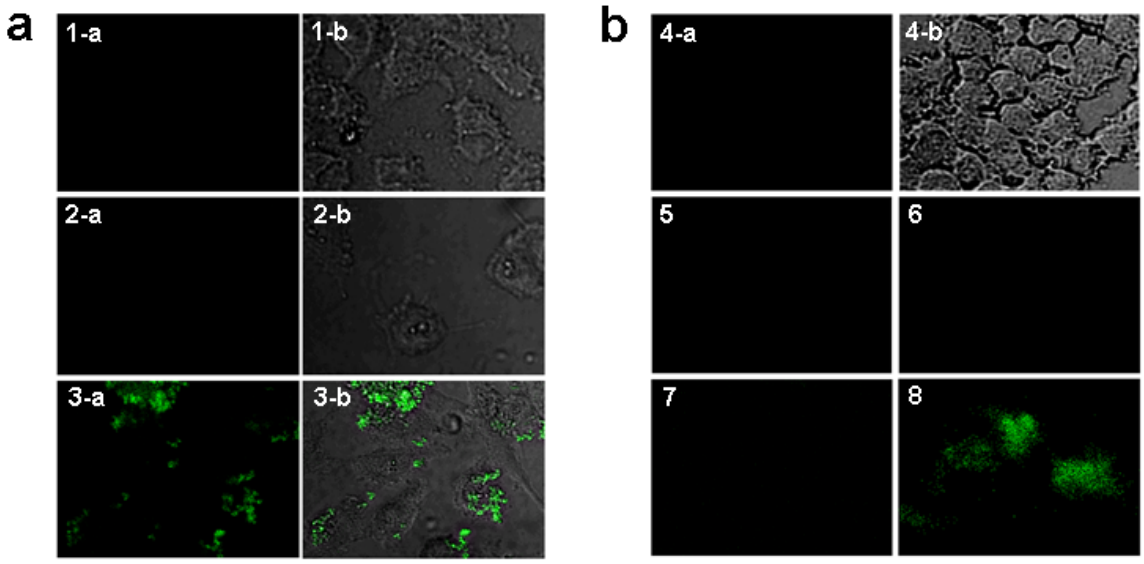

Fig. S2 Translocation experiments of the EGFP-ligated beads into HeLa cells at 37 °C, and into R1 mouse embryonic stem cells at 4 °C (4, 5, 6, and 7) and at 37 °C (8). (a) The confocal microscopic images of Hela cells. 1-a and 1-b, cells treated with EGFP alone; 2-a and 2-b, cells treated with non-glucose-coated beads; 3-a and 3-b, cells treated with 5%-glucose-coated beads. 1-a, 2-a, and 3-a are dark field images. 1-b, 2-b, and 3-b are merged images (dark-bright fields). (b) The confocal microscopic images of R1 mouse embryonic stem cells. 4-a and 4-b, cells treated with EGFP alone (4-a is a dark field image and 4-b is a merged image.) at 4 °C; 5, cells treated with non-glucose-coated beads at 4 °C; 6, cells were treated with 5%-glucose-coated beads at 4 °C; 7, cells were treated with 10%-glucose-coated beads at 4 °C; 8, cells treated with 5%-glucose-coated beads at 37 °C. 5, 6, 7, and 8 are dark field images.

Cytotoxicity of glucose-coated beads on the mES cells

We have measured degeneration rate (death rate) of the mES cells after treatment of 5%-glucose-coated beads (Fig. S3). The rate was dependent on the amount of beads used. Addition of below 0.25 μL, that is the experimental condition in this study, did not show considerable death rate compared to the control experiment. However, adding above 0.5 μL

showed significant toxicity on the cells.

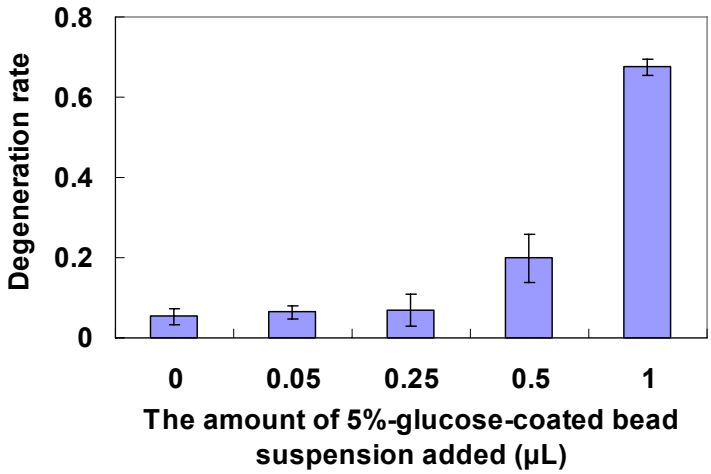

Fig. S3 Cytotoxicity of 5%-glucose-coated beads on the mES cells. Toxicity was measured by degeneration rate (death rate). The number of dead cells was counted using a hemocytometer after being stained with 0.4% trypan blue.

Materials and Method

Chemicals, buffers, lysozyme, and concanavalin A were purchased from Sigma-Aldrich. *Pfu* DNA polymerase and restriction enzymes (*Xho*I and *BamH*I) were purchased from Enzynomics (Daejeon, Korea). DNA oligomers were obtained from Sigma-Proligo (Singapore). The vector (pET-15b) was purchased from Merckbiosciences. DNA sequencing was performed by Solgent Co. (Daejeon, Korea). The Ni- NTA agarose resin was purchased from QIAGEN. ^{1}H-NMR spectrum was recorded from D_2O solutions on a Varian 500 MHz spectrometer. The SEM images were obtained by a Nano-SEM at KIST

(Seoul, Korea) or JSM-7500F at SWU (Seoul, Korea). The TEM images were obtained by H7600 (Hitachi, Japan).

Synthesis of 6-O-methacryl glucose

The monomer (6-*O*-methacryl glucose) was prepared similarly to the literature method (*S2, S3*). Novozyme 435 (3 g) was added to a mixture of D-(+)-glucose (6 g, 33 mmol) and vinyl methacrylate (11 g, 100 mmol) in *t*-butyl alcohol (100 mL), and the reaction mixture was stirred at 50 °C for 12 hours. After Novozyme 435 was removed by filtration, 6-*O*-methacryl glucose (4.7 g, 55%) was obtained by evaporation of the solvent. The product was used for polymerization without further purification. The NMR data were obtained as a mixture of the α- and β-forms of 6-*O*-methacryl glucose.

^{1}H-NMR (500 MHz, D_2O) δ 6.02 (d, 2H), 5.59 (s, 2H), 5.08 (d, 1H), 4.52 (d, 1H), 4.33-4.25 (m, 4H), 3.92 (m, 1H), 3.58 (m, 2H), 3.41-3.37 (m, 4H), 3.12 (t, 1H), 1.80 (s, 6H); ^{13}C-NMR (125 MHz, D_2O): δ 169.6, 135.8, 127.2, 96.1, 92.3, 75.6, 74.2, 73.6, 72.7, 71.6, 69.8, 69.7, 69.4, 63.7, 17.5 27.8; MS (ESI) *m/z* calcd for $[M+Na]^+$: 271.22, found 271.0.

Synthesis of polymeric nanoparticles

Styrene (5 mL, 44 mmol) washed with a sodium hydroxide solution (0.1 M), acrylic acid (274 μL, 3 mmol), and 6-*O*-methacryl glucose (1.1 or 0.55 g, 4 or 2 mmol, repectively) were added to preheated methanol (50 mL) containing water (10%) and hexane (5%) at 70 °C. The reaction mixture was stirred at 70 °C for 20 hours after potassium persulfate (125 mg, 0.43 mmol) was added. The solvent was removed by decanting after

centrifugation at 10,000 rpm. The prepared beads (1.2 g) were washed three times with distilled water (25 mL) and dried under vacuum. The acrylic acid beads were prepared similarly without addition of 6-*O*-methacryl glucose.

Removing glucose from beads

The beads (10% glucose-coated beads, 1 g) were added to an HCl solution (6 M, 100 mL) and refluxed for 16 hours. After washing three times with distilled water (25 mL), the beads were dried under vacuum.

Expression and purification of the EGFP

The EGFP gene was subcloned from EGFP-C2 (Clontech) into the pET-15b (Merckbiosciences). Gene specific primers used were as follows: EGFP-XhoI-F1(5'-GCAAACTCGAGGTGAGCAAGGGCGAGGAGCTG-3') and EGFP-BamHI-R1(5'-CAGCCGGATCCTTACTTGTACAGCTCGTCCATGCCG-3'). The primers containing restriction sites for *Xho*I and *BamH*I were used to introduce these two sites into the genes and to clone the genes into the pET-15b vector. The plasmid containing the EGFP gene was transformed into BL21(DE3).

An overnight culture of BL21(DE3) (1 mL) containing the EGFP gene was added to LB medium (100 mL; ampicillin, 100 μg/mL) and incubated at 37 °C and 200 rpm to an OD_{600} of 0.5. Protein expression was induced by adding an IPTG solution (1 mL; 2% w/v) and the expression culture was incubated for 6 h at 25 °C and 200 rpm, at which point the OD_{600} was ~1.5. The cells were harvested by centrifugation (10 min, 3,800 × g, 4 °C) and

the supernatant was discarded. The cell pellet (~0.8 g) was resuspended in the lysis buffer (5 mL/g wet weight; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 10 mM; adjusted to pH 8.0 with NaOH), and lysozyme was added to 1 mg/mL. Incubation on ice for 45 min was followed by a freeze-thaw cycle at -20 °C and room temperature. The viscous lysate was passed several times through a sterile 20-gauge syringe needle and centrifuged (10 min, 10,000 × g, 4 °C). The supernatant was separated from the cell debris. Ni-NTA agarose resin (1 mL of 50% slurry) was added to the supernatant (4 mL) and the mixture was shaken at 25 °C for 1 h. The lysate-Ni-NTA mixture was loaded on a Poly-Prep column (Bio-Rad), drained, and then washed three times with the wash buffer (4 mL; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 20 mM; adjusted to pH 8.0 with NaOH). The His_6-EGFP was eluted from the column with four volumes of the elution buffer (0.5 mL; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 250 mM; adjusted to pH 8.0 with NaOH).

Eluate (2 mL) containing the purified EGFP from the Ni-NTA column was exchanged from the elution buffer to PBS buffer (0.1 M, pH 7.3) using a centrifugal device (Amicon Ultra-15, Millipore). The protein amount was estimated from the absorption at 280 nm (molar absorptivity = 22,015 M^{-1} cm^{-1}, calculated with tools at Swiss Prot Expasy, http://ca.expasy.org/tools/protparam.html)

Conjugation of polymeric nanoparticles with the EGFP

The EGFP was conjugated to the polymeric beads similarly to the method in the literature (*S4*). The polymeric beads (100 mg) were resuspended in MES buffer (5 mL, 0.1 M, pH 5.0) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 100 mg)

was added. The mixture was stirred for 20 min and centrifuged to remove the buffer. The activated beads were washed three times with PBS buffer (5 mL, 0.1 M, pH 7.3) and resuspended in PBS buffer (1 mL). The resuspended beads in PBS buffer (100 μL) and EGFP solution (100 μL, 15 mg/mL) were mixed and incubated overnight at 4 °C. The EGFP-bound beads were washed three times with PBS buffer (1 mL) and resuspended in PBS buffer (100 μL). The amount of bound EGFP was determined to be 1.4 mg per g of beads by the difference in absorbance of the supernatant at 280 nm, before and after incubation.

Turbidity measurement of aggregation of concanavalin A by the prepared beads

The prepared beads (1 mg) were suspended in PBS buffer (1 mL) by sonication. The suspension of beads (50 μL) was added to PBS buffer (750 μL) in a cuvette (1 mL). The absorbance change at 600 nm was monitored for 3 hours after adding a concanavalin-A solution (200 μL, 2 mg/mL of PBS buffer).

mES cell culture and transduction of beads into mES cells

Undifferentiated R1 mouse embryonic stem (mES) cells were grown on gelatin-coated tissue culture plates in the mES medium (5 mL, Dulbecco's modified eagles medium supplemented with 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 10% fetal calf serum). The mES medium contained the 1000 U/mL leukemia inhibitory factor (LIF, Chemicon). The mES cells were passed every 3 or 4 days onto freshly prepared

gelatin-coated plates, and fed every day with the mES medium (5 mL) at 37°C with 5% CO_2 and > 95% humidity.

The mES cells were plated on gelatin-coated cover glasses for the test of the transduction activity of the prepared beads. The cover glasses were placed in each well of a 24-well culture plate and the mES medium (0.5 mL) was added. After the mES cells were grown for 2 or 3 days to 80% confluency, the mES medium was replaced with the mES medium (0.5 mL) containing the EGFP solution (0.25 μL, 15 mg/mL) or the above EGFP-conjugated bead suspension (0.25 μL, 100 mg/mL). The mES cells were additionally incubated for 8 hr at 37 °C. The confocal-microscopy images were obtained under the unfixed condition after washing three times with PBS buffer.

To evaluate temperature dependency of the transduction of the beads, a transduction experiment at 4 °C was performed. The mES cells were preincubated for 8 hr at 4 °C maintaining 5% CO_2 in air before adding the beads. After addition of the beads, the mES cells were additionally incubated for 8 hr at 4 °C and then analyzed with a confocal microscope.

Fluorescent intensity analysis

The intensity of intracellular EGFP was analyzed using a confocal microscope operated by FluoView (Ver1.26). After XYX scanning, the images were accumulated and analyzed for measuring the intensity from a single mES cell. Experiments were repeated 6 times and the measurements were repeated 10 times at each experiment.

Hela cell culture and transduction of beads into Hela cells

Hela cells (CRL-1658, ATCC) were cultured under the standard condition with Dulbecco's modified Eagle medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; GIBCO), 100 μg/mL penicillin, and 100 U/mL streptomycin. HeLa cells were plated on a culture slide glass (Nalgene, Rochester, NY) and grown for 2 or 3 days until 80% confluency at 37 °C with 5% CO_2 in the culture medium supplemented with 10% fetal bovine serum. The cells were treated with the beads and analyzed in the same manner as the mES cells.

Cytotoxicity of the glucose-coated beads in mES cells

The mES cells were cultured on the 24-well culture plate in the mES medium containing LIF (1000 iu/ml). When the cells reached 80-90% confluency, the mES medium was replaced with the medium containing various amount of 5% glucose bead suspension. After incubation for 8 hr, the cells were treated with trypsin-EDTA (Gibco BRL), and harvested and washed with DMEM. The trypan-blue-viability test was performed in order to analyze cytotoxicity. Briefly, 0.4% trypan blue (Sigma) was added into the cell suspension and the cell suspension was incubated for 15 min. The number of dead cells was counted using a hemocytometer. The test was repeated 3 times.

Supporting References

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