Supplementary Information

A novel pH-responsive polysaccharidic ionic complex for proapoptotic D-(KLAKLAK)$_2$ peptide delivery

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Materials and experimental design

Materials

Glycol chitosan (GCS, Mw = 250 kDa; degree of deacetylation = 82.7%), 2,3-dimethylmaleic anhydride (DMA), fluorescein isothiocyanate (FITC), dimethylsulfoxide (DMSO), pyridine, N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodimide (DCC), triethylamine (TEA), and 1% formaldehyde were purchased from Sigma-Aldrich (USA). D-$(KLAKLAK)_2$ proapoptotic peptide (>95% purity) and tetramethylrhodamine (TAMRA)-labeled D-$(KLAKLAK)_2$ [D-$(KLAKLAK)_2$-TAMRA, >95% purity] were obtained from Peptron Inc. (Republic of Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Welgene Inc. (Korea). Chlorin e6 (Ce6) was purchased from Frontier Scientific Inc. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies Inc. (Japan).

Synthesis of GCS-g-DMA

DMA was grafted to GCS in DMSO using TEA and pyridine at room temperature for 7 days, as described in detail in our previous reports.[S1] The degree of substitution (DS, defined as the number of DMA blocks per 1 primary amine of GCS) was estimated from the ^{1}H-NMR (DMSO-d_6 with TMS) peaks using the integration ratio of the peaks from δ 1.88 (-C**H**$_3$, DMA block) and δ2.73 (-C**H**-, repeating sugar unite of GCS) (Supplementary Figure 1). As a consequence, the degree of substitution (DS, defined as the number of DMA blocks per 1 primary amine of GCS) was 0.82, according to the results of ^{1}H-NMR.

Preparation and characterization of GCS-g-DMA/D-$(KLAKLAK)_2$ ionic nanocomplex

GCS-*g*-DMA (0.5 mg/ml) and D-$(KLAKLAK)_2$ (0.1 mg/ml) were dissolved in a HCl (or NaOH)–$Na_2B_4O_7$ buffer solution (pH 8.0, 1 mM) at room temperature. The resultant solution was vigorously mixed at 14,000 rpm for 30 sec and was then mixed with phosphate buffer saline (PBS) solution (ionic strength= 0.15, pH 7.4-6.4). After dialyzing the ionic nanocomplex (GCS-*g*-DMA 0.5 mg/ml, D-$(KLAKLAK)_2$-TAMRA 0.1 mg/ml) solution against pH 7.4 (ionic strength= 0.15) for 1 day (in order to remove noncomplexed peptide), purified GCS-*g*-DMA/D-$(KLAKLAK)_2$ ionic nanocomplex was obtained. This nanocomplex was used for

the following experiments. In addition, for measuring peptide drug encapsulation efficiency, the pH of the solution was adjusted to pH 1.0 using 1 M HCl (in order to disintegrate ionic complexes). The concentration of D-$(KLAKLAK)_2$-TAMRA in the resultant solution was analyzed at λ_{ex} 544 nm and λ_{em} 580 nm with a Spectrofluorometer (Shimadzu RF-5301PC, Japan). The complexation efficiency of the peptide was approximately 75±2%.[S1]

The particle size distribution of the GCS-*g*-DMA/D-$(KLAKLAK)_2$ ionic nanocomplex was measured using a Zetasizer 3000 (Malvern Instruments, USA) equipped with a He-Ne Laser beam at a wavelength of 633 nm and a fixed scattering angle of 90°.[S1] The morphology of GCS-*g*-DMA/D-$(KLAKLAK)_2$ ionic nanocomplex was confirmed with a Field Emission Scanning Electron Microscope (FE-SEM, Hitachi s-4800, Japan).[S1]

Fluorescence resonance energy transfer (FRET) study

FITC-labeled GCS-*g*-DMA [GCS-*g*-DMA (FITC)] (1 mg/ml) was complexed with D-$(KLAKLAK)_2$-TAMRA (0.2 mg/ml) at room temperature in PBS (pH 7.4, ionic strength= 0.15). Herein, GCS-*g*-DMA (FITC) was synthesized with GCS-*g*-DMA (500 mg) and FITC (2.5 mg) in DMSO (5 ml) containing TEA (0.5 ml), finally producing chemical conjugation between residual primary amine of GCS-*g*-DMA and FITC. The reaction was conducted for 2 days at room temperature. The solution obtained was then dialyzed against pH 9.0 borate buffer (1 mM) for 2 days, followed by the lyophilization.

Fluorescence spectra of the ionic complex, free GCS-*g*-DMA (FITC) (1 mg/ml), and free D-$(KLAKLAK)_2$-TAMRA (0.2 mg/ml) was measured using a spectrofluorometer. The excitation wavelength was fixed at 495 nm. The slit width was 1.5 nm for both excitation and emission.[S2]

D-$(KLAKLAK)_2$ peptide release test

Peptide release behavior from the ionic nanocomplex (GCS-*g*-DMA 1 mg/ml, D-$(KLAKLAK)_2$-TAMRA 0.2 mg/ml) was conducted in PBS (ionic strength= 0.15, 0.01 wt.% sodium azide) with different pH values. In brief, the dialysis membrane bag (Spectra/Por MWCO 10K) containing an ionic nanocomplex solution (1 ml) was sealed and subsequently immersed in a vial containing 20 ml of PBS solution adjusted to different

pH values (pH 7.4, 6.8, 6.4).[S2] The peptide release test was conducted under mechanical shaking (120 rev./min) at 37 °C. A small volume of the release medium was removed at each time point and analyzed using a spectrofluorometer at λ_{ex} 544 nm and λ_{em} 580 nm.

Cell culture

The mouse melanoma B16(F10) cells (from Korean Cell Line Bank) were maintained in DMEM medium with 1% penicillin–streptomycin and 10% FBS in a humidified standard incubator at 37°C and a 5% CO_2 atmosphere. Prior to testing, cells (1×10^5 cells/ml) grown as a monolayer were harvested via trypsinization using a 0.25 % (w/v) trypsin/0.03 % (w/v) EDTA solution. B16(F10) cells suspended in a DMEM medium (200 μl) were seeded into 6-well plates and cultured for 24 hours prior to *in vitro* cell testing.

Cell cytotoxicity

B16(F10) cells (1×10^5 cells/ml) were treated with PBS solution (means "non-treated") or GCS-*g*-DMA/D-$(KLAKLAK)_2$ [GCS-*g*-DMA 50 μg/ml, D-$(KLAKLAK)_2$ 10 μg/ml] or free D-$(KLAKLAK)_2$ (10 μg/ml) at each pH (pH 7.4~6.4) for 2 hours. The surviving cell numbers were analyzed via a CCK-8 assay.[S3] Additionally, the photographic images of B16(F10) cells were obtained after 2 hours of incubation with GCS-*g*-DMA/D-$(KLAKLAK)_2$-TAMRA ionic nanocomplexes.

Flow cytometry

The cellular uptake of D-$(KLAKLAK)_2$ peptide was confirmed with a FACSCalibur™ Flow Cytometer (Becton Dickinson, USA). The B16(F10) melanoma cells (1×10^6 cells/ml) in DMEM medium were seeded onto 6-well plates. After 1 hour of incubation of B16(F10) cells with GCS-*g*-DMA/D-$(KLAKLAK)_2$-TAMRA [equivalent D-$(KLAKLAK)_2$-TAMRA 0.2 mg/ml] at pH 7.4, 6.8, 6.4, the cells were washed three times with PBS and demounted via trypsinization using a 0.25% (w/v) trypsin/0.03% (w/v) EDTA solution. The resultant cell solution was fixed with 1% formaldehyde and subsequently analyzed with a Flow Cytometer.

Fluorescence microscope

D-$(KLAKLAK)_2$ peptide accumulation in B16(F10) cells (1×10^5 cells/ml) by GCS-*g*-DMA/D-$(KLAKLAK)_2$-TAMRA ionic nanocomplexes [equivalent D-$(KLAKLAK)_2$-TAMRA 10 μg/ml] were monitored according to different pH signals (pH 7.4, 6.8, 6.4), using a fluorescence microscope (at λ_{ex} 544 nm and λ_{em} 580 nm, E-SCOPE 1500F). The cells incubated for 2 hours with the ionic complexes were washed three times in PBS and fixed with 1% formaldehyde.[S4] In order to ameliorate any fluorescence photobleaching, a drop of anti-fade mounting solution (5% N-propyl galate, 47.5% glycerol and 47.5% Tris–HCl, pH 8.4) was added to the cells.

Animal care

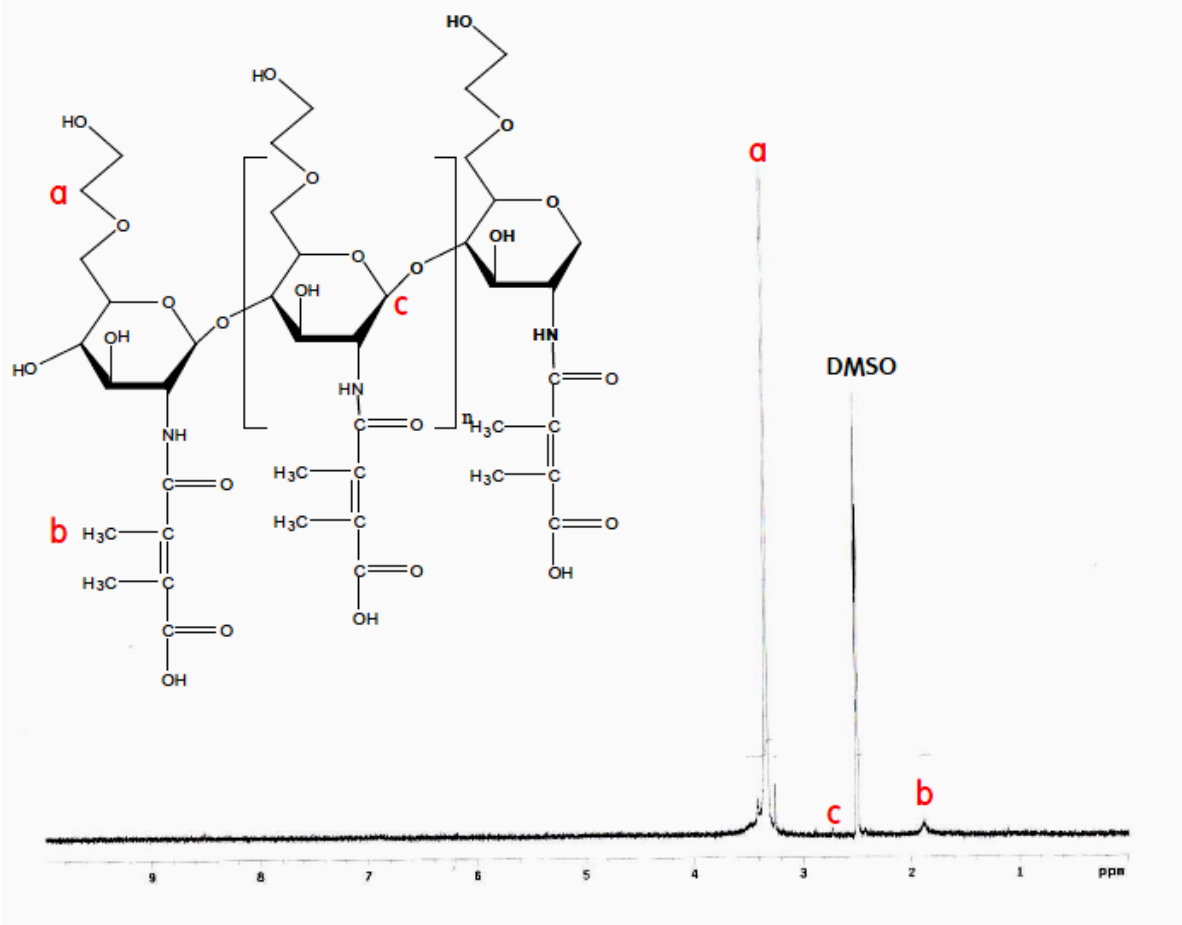
In vivo studies were conducted in 4–6 week old female nude mice (BALB/c nu/nu mice, Institute of Medical Science, Tokyo). Nude mice were maintained under the guidelines of an approved protocol from the Institutional Animal Care and Use Committee (IACUC) of the University of Yonsei (Republic of Korea). All experiments were performed in compliance with the relevant laws and institutional guidelines

In vivo *fluorescence imaging of GCS-g-DMA/D-(KLAKLAK)$_2$ ionic nanocomplexes*

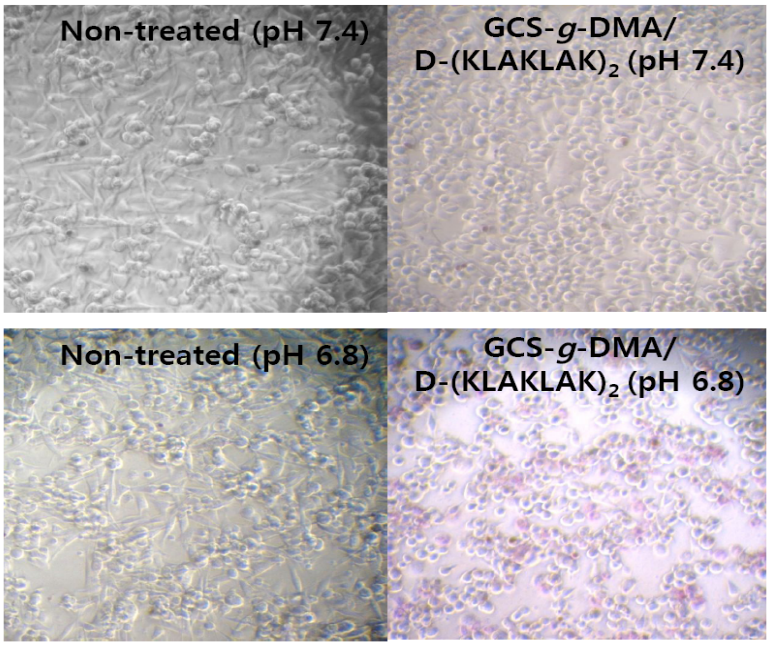
Fluorescent dye (Ce6) (1.1 mM) [S5] was coupled with D-$(KLAKLAK)_2$ peptide (1 mM) in DMSO containing DCC (1 mM), NHS (1 mM), and TEA (50 mM) at room temperature for 3 hours. The solution was filtrated to remove dicyclohexylurea (DCU) and then dialyzed (in order to remove non-reacted Ce6) using a dialysis membrane bag (Spectra/Por MWCO 1K), followed by lyophilization. Ce6-labeled D-$(KLAKLAK)_2$ mixture was used for the following experiment.

For the *in vivo* animal experiments, B16(F10) tumor cells were introduced twice into female nude mice via subcutaneous injection of 1×10^3 cells suspended in PBS 7.4 (ion strength: 0.15) medium. When the tumor volume reached 200 mm^3, Ce6-labeled GCS-*g*-DMA/D-$(KLAKLAK)_2$ ionic nanocomplexes [equivalent D-$(KLAKLAK)_2$ 0.4 mg/kg body)] or free D-$(KLAKLAK)_2$ were injected intravenously into B16(F10) tumor-bearing nude mice through a tail vein. A 12-bit CCD camera (Image Station 4000 MM; Kodak, New Haven, CT) prepared with a special Cmount lens and a long wave emission filter (600-700 nm; Omega Optical,

USA) were used to measure fluorescence images of nude mice[2]. The fluorescence images were monitored for 1-72 hours.



Supplementary Figure 1. ^{1}H-NMR peak of GCS-*g*-DMA



Supplementary Figure 2. Photographs of mouse melanoma cell B16(F10) cells treated with GCS-*g*-DMA/D-$(KLAKLAK)_2$ ionic nanocomplex at pH 7.4 or pH 6.8 (incubation time: 2 hours). This Figure shows photograph images of mouse melanoma B16(F10) tumor cells treated with the nanocomplexes at pH values of 7.4 and 6.8. The selectively delivery of the peptide drug to tumor cells at acidic pH (pH 6.8) was observed as a red color of D-$(KLAKLAK)_2$-TAMRA.

References

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