ADVANCED MATERIALS

Supporting Information

for *Adv. Mater.*, DOI: 10.1002/adma.201205088

Non-Viral Gene Delivery via Membrane-Penetrating, Mannose-Targeting Supramolecular Self-Assembled Nanocomplexes

*Lichen Yin, Ziyuan Song, Kyung Hoon Kim, Nan Zheng, Nathan P. Gabrielson, and Jianjun Cheng**

Supporting Information

Non-Viral Gene Delivery via Membrane-Penetrating, Mannose-Targeting Supramolecular Self-Assembled Nanocomplexes

Lichen Yin, Ziyuan Song, Kyung Hoon Kim, Nan Zheng, Nathan P. Gabrielson, Jianjun Cheng*

Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, 1304 W Green St, Urbana, IL 61801, USA

*Corresponding author. Tel.: (+1) 217-244-3924; Fax: (+1) 217-333-2736

Email address: jianjunc@illinois.edu

1. Materials and methods

1.1. Materials, cells, and animals

Chitosan (Mw=200 kDa, deacetylation degree of 95%) was purchased from Golden-shell Biochemical Co., Ltd. (Zhejiang, China). tBoc-NH-PEG-succinimidyl valerate (tBoc-PEG-SVA, MW = 3400 Da) was purchased from Laysan Bio (Arab, AL). *N*-hydroxysuccinimide (NHS), oleyol chloride, D-mannosamine hydrochloride, and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasmid DNA encoding luciferase (pCMV-Luc) was purchased from Elim Biopharmaceutics (Hayward, CA, USA). Bright-Glo Luciferase assay kit was purchased from Promega (Madison, WI, USA). Lipofectamine^{TM} 2000 (LFP2000) and YOYO-1 were purchased from Invitrogen (Carlsbad, CA, USA). Poly(ethyleneimine) (PEI, branched, 25 kDa) and jetPEI^{TM}-Macrophage (jetPEI, mannose-conjugated linear PEI) were purchased from Sigma (St. Louis, MO, USA) and Polyplus-Transfection Inc. (New York, NY, USA), respectively, and they were used according to the manufacturer's protocols Spectra/Por RC dialysis tubing with a molecular weight cut-off (MWCO) of 1 kDa was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA).

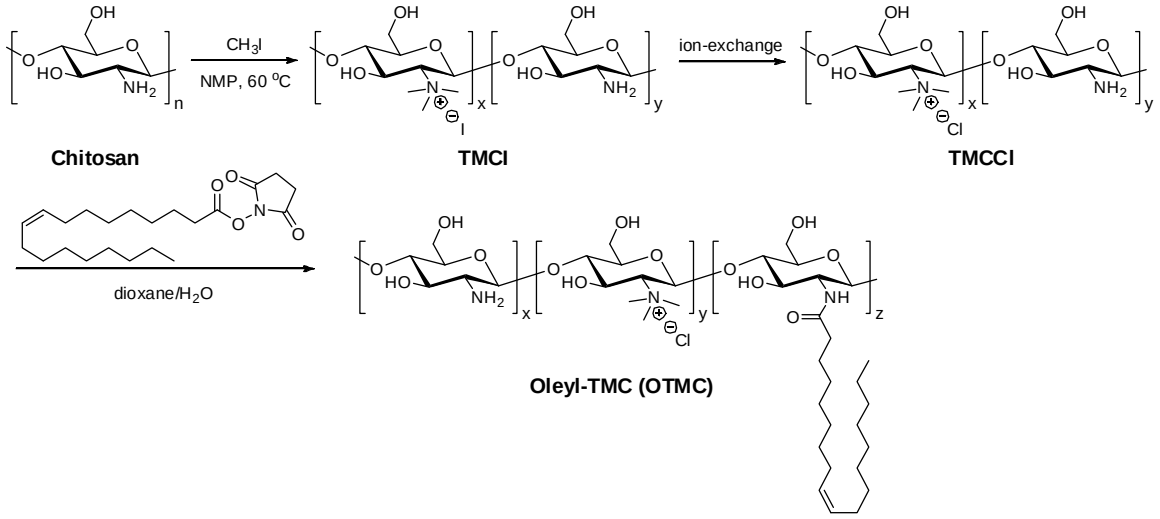
HepG-2 cells (human hepatocellular carcinoma) and Raw 264.7 cells (mouse monocyte macrophage) were purchased from the American Type Culture Collection (Rockville, MD, USA), and cultured in DMEM supplemented with 10% fetal bovine serum (FBS).

Male C57BL/6 mice (8-10 wk) were obtained from Charles River Laboratory (Wilmington, MA, USA) and were housed in a clean room four to a cage with access to water ad libitum, a 12:12 h light–dark cycle (7:00 am–7:00 pm), a temperature of 25±1 °C. The animal experimental protocols were approved by the Institutional Animal Care and Use Committees, University of Illinois at Urbana-Champaign, and followed federal and state laws.

1.2. Synthesis of oleic acid N-hydroxysuccinimide ester (oleyl-NHS)

NHS (2.67 g, 23.2 mmol) and DIEA (2.72 mL, 15.6 mmol) were dissolved in THF (50 mL). The mixture was cooled in an ice bath into which oleoyl chloride (85%, 6.1 mL, 15.6 mmol) was added dropwise over the course of 1 h. The mixture was allowed to stir at 0 ^{o}C for 2 h and then overnight at room temperature. The grey precipitate was removed by centrifugation. After the solvent was removed under vacuum, the resulting yellowish oil was dissolved in DCM (20 mL) and washed with 5% HCl (10 mL × 2). DCM was dried over $MgSO_4$ and then evaporated under vacuum to give the crude product. Oleyl-NHS was recrystallized from ethanol (yield 62%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.34 (m, 2H, C*H*=C*H*), 2.84 (s, 4H, COCH_2CH_2CO), 2.60 (t, 2H, CH_2CH_2COON), 2.01 (m, 4H, CH_2CH=CHCH_2), 1.74 (m, 2H, C$H_2$$CH_2$COON), 1.44-1.24 (m, 20H, other CH_2), 0.87 (t, 3H, CH_3). ESI-MS (*m*/*z*): $C_{22}H_{37}NO_4Na$ (M+Na), calcd., 402.2; found, 402.2.

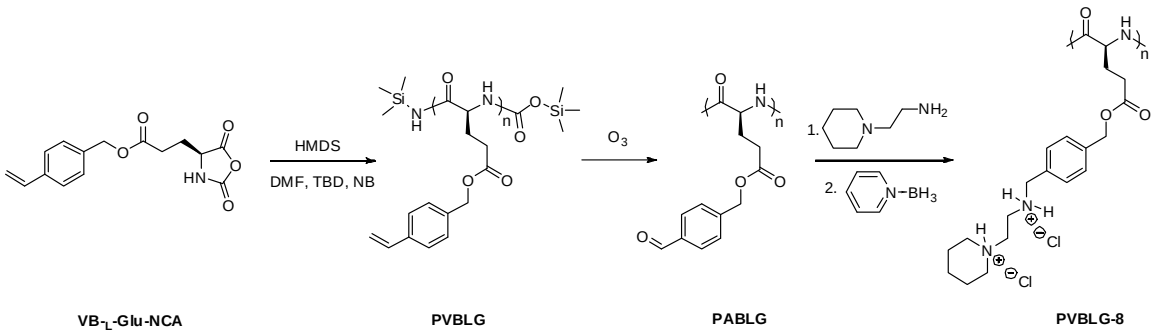
1.3. Synthesis and characterization of OTMC



Trimethyl chitosan chloride (TMC, MW = 200 kDa, quarternization degree of 25.1%) was synthesized as described previously.[1] TMC (50 mg, 0.28 mmol of NH_2) and oleyl-NHS (10.6 mg, 0.028 mmol) were dissolved in dioxane/water (3 mL, 1:1, v/v), into which DMAP (34.1 mg, 0.28 mmol) and triethylamine (TEA, 80 μL, 0.56 mmol) were added. The solution was

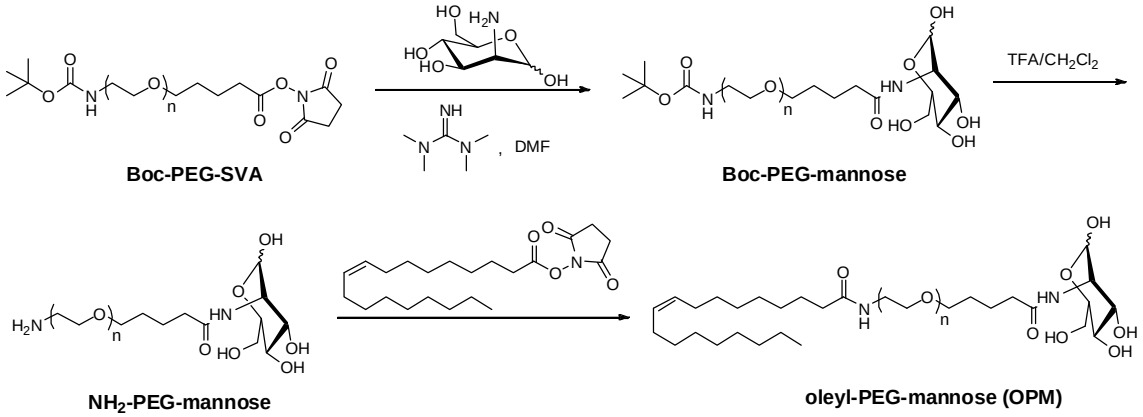
stirred overnight at room temperature. The resulting polymer was then precipitated by ethanol/ether/hexane (30 mL, 1:1:1, v/v/v). After being dissolved in water and precipitated by ethanol/ether/hexane (30 mL × 3, 1:1:1, v/v/v), the product was dissolved in water and further purified by ultrafiltration (MWCO = 10 kDa). ^{1}H NMR was used to characterize the polymers (Supplementary Fig. S1). The quarternization degree was calculated by the integral ratio of trimethyl protons (c) to chitosan backbone C2-C6 protons (b). The oleyl conjugation degree was calculated by the integral ratio of oleyl protons (e) to trimethyl protons (c).

1.4. Synthesis and characterization of PVBLG-8



PVBLG-8 was synthesized via ring-opening polymerization of VB-$_{L}$-Glu-NCA as initiated by hexamethyldisilazane (HMDS) followed by multi-step side-chain derivatization.[2] The degree of polymerization (DP) was 195 with a narrow PDI value of 1.1 as determined by gel permeation chromatography (GPC) as previously described.[2]

1.5. Synthesis and characterization of oleyl-PEG-mannose (OPM)



Boc-PEG-mannose was obtained by reacting Boc-PEG-SVA with D-mannosamine in DMF. D-mannosamine hydrochloride (108 mg, 0.50 mmol) and 1,1,3,3-tetramethylguanidine (120 μL, 0.96 mmol) were dissolved in DMF (10 mL) and the mixture was added to a vial containing Boc-PEG-SVA (MW = 3.4 kDa, 340 mg, 0.10 mmol). The mixture was then stirred for 48 h at 50 ^{o}C and dialyzed against DI water for 24 h (MWCO = 1 kDa). The product, Boc-PEG-mannose, was lyophilized (yield 74%) and characterized by MALDI-TOF-MS. Individual peak shifts (relative to Boc-PEG-SVA) corresponding to the deduction of one NHS group and the addition of one mannosamine per PEG were observed (m/z = +64).

Boc-PEG-mannose (250 mg, 0.07 mmol) was deprotected by TFA/DCM (5.0 mL, 1:1, v/v) to yield NH_2-PEG-mannose. The reaction was allowed to proceed for 1 h at room temperature after which the polymer was precipitated and washed with cold diethyl ether (20 mL × 2, yield 86%). The product (NH_2-PEG-mannose) was characterized by MALDI-TOF-MS. Individual peak shifts (relative to Boc-PEG-mannose) corresponding to the deduction of one Boc group per PEG were observed (m/z = -100).

The final product OPM was synthesized by reacting oleyl-NHS (50.0 mg, 0.13 mmol), DIEA (30 μL, 0.17 mmol), and NH_2-PEG-mannose (200 mg, 0.06 mmol) in 1,4-dioxane/DMF/H_2O (6.0 mL, 4:1:1, v/v/v). The solution was allowed to stir overnight at room temperature, after which it was dialyzed against DI water for 24 h (MWCO = 1 kDa).

OPM, the final product, was lyophilized (yield 68%) and characterized by MALDI-TOF-MS. Individual peak shifts (relative to NH_2-PEG-mannose) corresponding to the addition of one oleyl group per PEG were observed (m/z = +264).

1.6. Preparation and characterization of SSANs

OTMC, PVBLG-8, OPM, and DNA were separately dissolved in deionized water at 1 mg/mL, 1 mg/mL, 10 mg/mL, and 1 mg/mL, respectively. OTMC, PVBLG-8, and OPM were mixed at various weight ratios, and the mixture was added to the DNA solution at determined OTMC/DNA or PVBLG-8/DNA weight ratios. It was subsequently vortex for 30 s and incubated at 37 °C for 30 min. The obtained SSANs were characterized for size and zeta potential using dynamic laser scanning (DLS, Zetasizer Nano-ZS, Malvern). The condensation of DNA by the cationic polymers was evaluated using the gel retardation assay. SSANs or naked DNA were loaded in a 1% agarose gel at 100 ng DNA/well, and gel electrophoresis was run at 100 V for 30 min. Migration of DNA was visualized by gel documentation following staining with ethidium bromide (EB). Morphology of the SSANs was observed by scanning electronic microscopy (SEM, Hitachi-4800, Japan).

The stability of DNA against DNase I was quantitatively assayed by utilizing the hyperchromic effect.[3] Briefly, SSANs containing 20 μg DNA were incubated with an equal volume of DNase I (20 IU) in 10 mM Tris–HCl, 150 mM NaCl, 1 mM $MgCl_2$ (pH 7.4) at 37 °C. Naked DNA treated with DNase I served as the positive control. Absorbance at 260 nm was continuously monitored at 37°C up to 1 h.

1.7. In vitro gene transfection

Cells were seeded on 96-well plates at 1×10^4 cells/well and incubated for 24 h prior to transfection studies. The medium was replaced by serum-free DMEM, into which complexes were added at 0.1 μg DNA/well. After incubation for 4 h, the medium was replaced by

serum-containing DMEM and cells were further cultured for 20 h. Luciferase expression was assayed using a Bright-Glo Luciferase assay kit while cellular protein level was determined using a BCA kit. Results were expressed as relative luminescence unit (RLU) associated with 1 mg of protein. Commercial transfection reagents, including LPF2000, jetPEI, and PEI served as positive controls and were used according to the manufacturers' protocols.

To monitor the transfection efficiencies of SSANs in the presence serum, SSANs were incubated with cells in DMEM supplemented with 0%, 10%, 20%, 30%, and 50% FBS for 24 h (0.3 μg DNA/well) followed by luminescence measurement.

1.8. Cell uptake of SSANs

DNA (1 mg/mL) was labeled with YOYO-1 (20 μM) at one dye molecule per 50 bp DNA.[4] The resultant YOYO-1-DNA was then allowed to form SSANs with OTMC, PVBLG-8, and OPM as described above.

Cells were seeded on 96-well plates at 1×10^4 cells/well and cultured for 24 h. The medium was replaced by 100 μL of serum-free DMEM, and SSANs containing YOYO-1-DNA were added (0.1 μg DNA/well). Following incubation at 37 °C for 4 h, cells were washed three times with cold PBS containing heparin sulfate (20 U/mL) to completely remove surface-bound cationic proteins from cells. Cells were then lysed with 100 μL of the RIPA lysis buffer, and YOYO-1-DNA content in the lysate was quantified by spectrofluorimetry (λ_{ex}=488 nm, λ_{em}=530 nm). Protein content was quantified using the BCA kit. Uptake level was expressed as the amount of YOYO-1-DNA (ng) associated with 1 mg of cellular protein. In order to elucidate the effect of mannose-receptor recognition during the internalization of SSANs, the cell uptake study was performed in serum-free DMEM supplemented with 600 μmol/L of mannose. In order to visualize the internalization and intracellular distribution of the SSANs, rhodamine B-labeled PVBLG-8 (RhB-PVBLG-8) and YOYO-1-DNA were used for the complex formation. Raw 264.7 cells were seeded on

coverslips in a 6-well plate at 2×10^4 cells/well and cultured for 24 h. SSANs were added at 1 μg DNA/well and the cells were cultured for a determined time. Following three PBS washes and nuclei-staining with DAPI, cells were observed by confocal laser scanning microscopy (CLSM, Zeiss-700, Germany).

1.9. Mechanistic probing into the intracellular kinetics

To elucidate the mechanisms underlying the cellular internalization of SSANs, we performed uptake studies at 4 °C or in the presence of various endocytic inhibitors. Cells were pre-incubated with endocytic inhibitors including chlorpromazine (10 μg/mL), genistein (200 μg/mL), methyl-β-cyclodextrin (mβCD, 50 μM), dynasore (80 μM), and wortmannin (50 nM) for 30 min prior to SSANs application and throughout the 2 h uptake experiment at 37 °C. Results were expressed as percentage uptake level of the control where cells were incubated with SSANs at 37 °C for 2 h.

To explore the involvement of endosomal entrapment and escape, we performed the transfection experiment in the presence of chloroquine or Bafilomycin A1. Briefly, SSANs were incubated with cells in DMEM supplemented with 100 μM chloroquine or 50 nM Bafilomycin A1 for 4 h. The media was then replaced by serum-containing DMEM and cells were further cultured for 20 h before assessment of luciferase expression.

The endosomal entrapment and escape of SSANs were also evaluated by CLSM observation. Briefly, Raw 264.7 cells were seeded on coverslips in a 6-well plate at 2×10^4 cells/well and cultured for 24 h. SSANs containing YOYO-1-DNA were added at 1 μg DNA/well and the cells were cultured for 4 h at 37 °C. Following wash by PBS three times, the nuclei were stained with DAPI and the endosomes/lysosomes were stained with Lysotracker® Red (Invitrogen). Cells were then observed by CLSM (Zeiss-700, Germany).

The membrane disruption capacity of the polypeptides was evaluated in terms of the cell uptake of a membrane-impermeable dye (FITC).[5] Cells were seeded on 24-well plate at 5 ×

10^4 cells/well and incubated for 24 h. The medium was changed to serum-free DMEM, into which polypeptides were added at 2 μg/well and FITC was added at 1 μg/well. After incubation for 2 h, cells were washed with cold PBS for three times and lysed with the RIPA lysis buffer. FITC content in the cell lysate was quantified by spectrofluorimetry (λ_{ex} = 488 nm, λ_{em}=530 nm), and the protein content was determined using the BCA kit. Cells incubated with free FITC in the absence of polypeptide served as the control. The uptake level was expressed as ng FITC associated with 1 mg of cellular protein.

1.10. Cytotoxicity of SSANs

Cells were seeded on a 96-well plate at 1×10^4 cells/well and cultured for 24 h before replacement of the media with serum-free DMEM (100 μL/well). Individual components (OTMC, PVBLG-8, and OPM) were added to each well at various final concentrations. SSANs (OTMC/PVBLG-8/DNA weight ratio of 15/15/1, 10 mol% OPM) were also added to each well at various DNA concentrations. Following incubation for 4 h, the medium was replaced with serum-containing DMEM. Cells were further cultured for 20 h before viability assessment using the MTT assay.

1.11. In vivo transfection in macrophages

SSANs containing YOYO-1-DNA were i.p. injected to male C56/BL6 mice at 5 μg DNA/mouse (four mice per group). Murine peritoneal exudate cell macrophages (PECs) were harvested 4 h following administration and washed with PBS. They were subjected to flow cytometry analysis, and the percentage uptake level of SSANs in PECs were quantified. PECs from non-treated mice served as blank, and treated with LPF2000, PEI, jetPEI, and naked DNA served as controls.

The transfection efficiencies of SSANs in PECs were evaluated as follows. SSANs were i.p. injected to male C56/BL6 mice at 5 μg DNA/mouse (four mice per group). PECs

were harvested 4 h following administration, washed with PBS, and seeded onto 96-well plate at 5×10^4 cells/well. After incubation for 20 h, the luciferase expression level was assayed using a Bright-Glo Luciferase assay kit while cellular protein level was determined using a BCA kit. Results were expressed as RLU associated with 1 mg of protein.

1.12. Statistical analysis

Statistical analysis was performed using Student's t-test and differences were judged to be significant at $*p<0.05$ and highly significant at $**p<0.01$.

Supplementary Figure

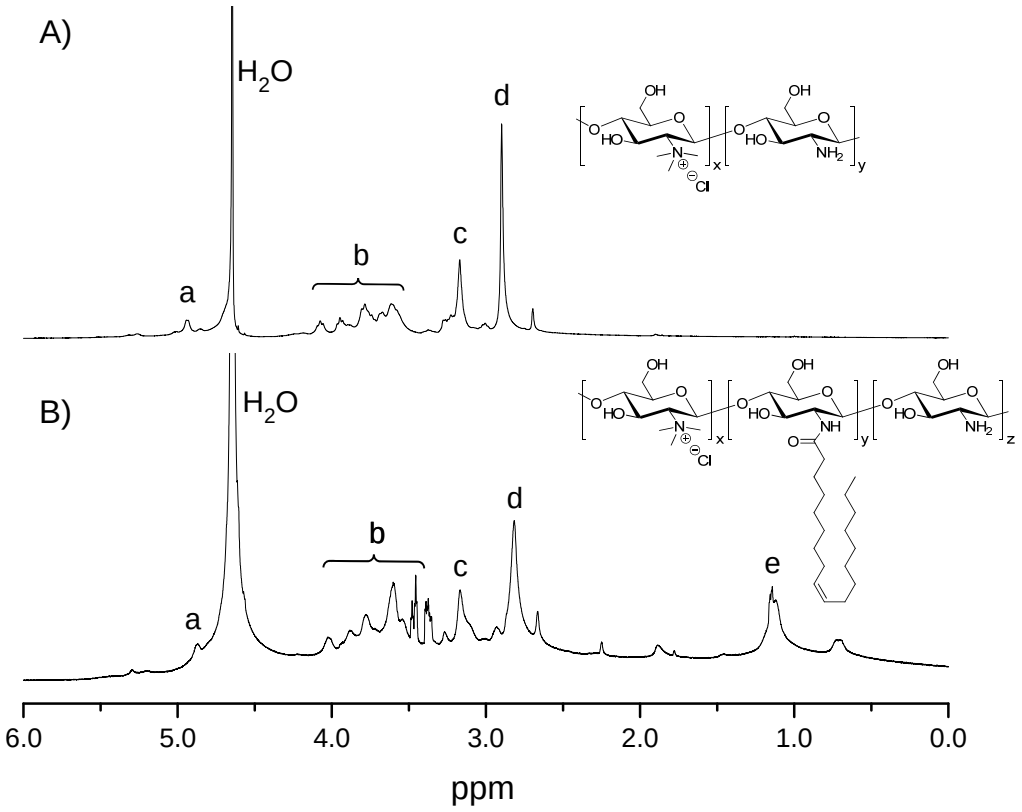


Fig. S1. ^{1}H NMR spectra of TMC (A) and OTMC (B). (a) chitosan backbone C1 proton; (b) chitosan backbone C2-C6 protons; (c) trimethyl protons; (d) dimethyl protons; (e) oleyl protons. The quarternization degree was calculated by the integral ratio of (c) to (b); the oleyl conjugation degree was calculated by the integral ratio of (e) to (c).

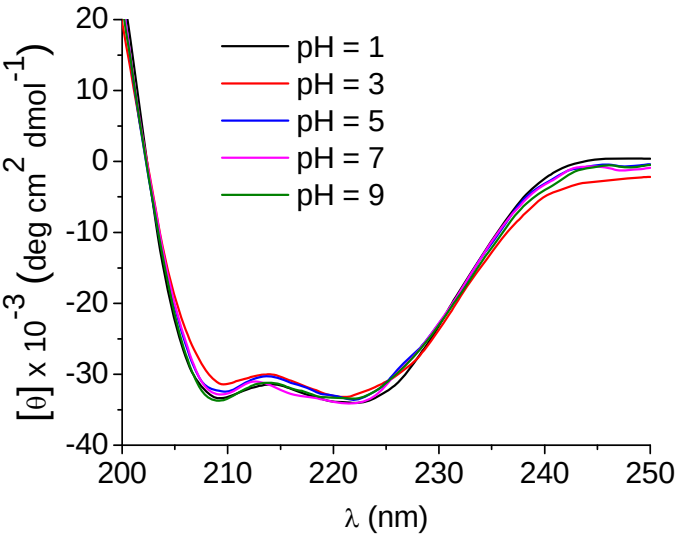


Fig. S2. CD spectra of PVBLG-8 at different pH.

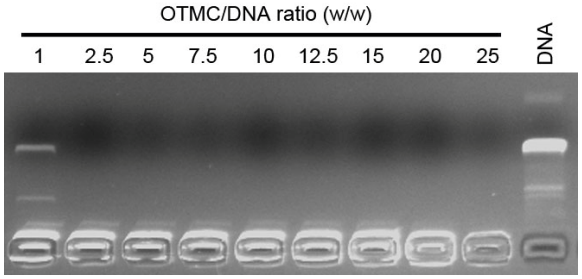


Fig. S3. Gel retardation assay showing the condensation of DNA by OTMC at different weight ratios. Naked DNA served as the control.

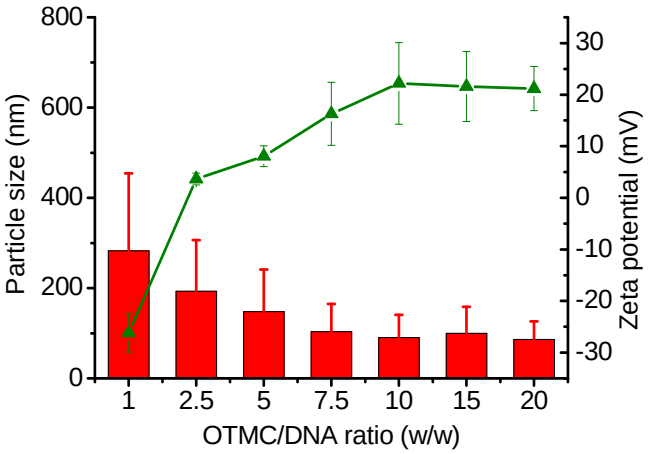


Fig. S4. Size (column) and zeta potential (scattered line) of OTMC/DNA complexes at different weight ratios.

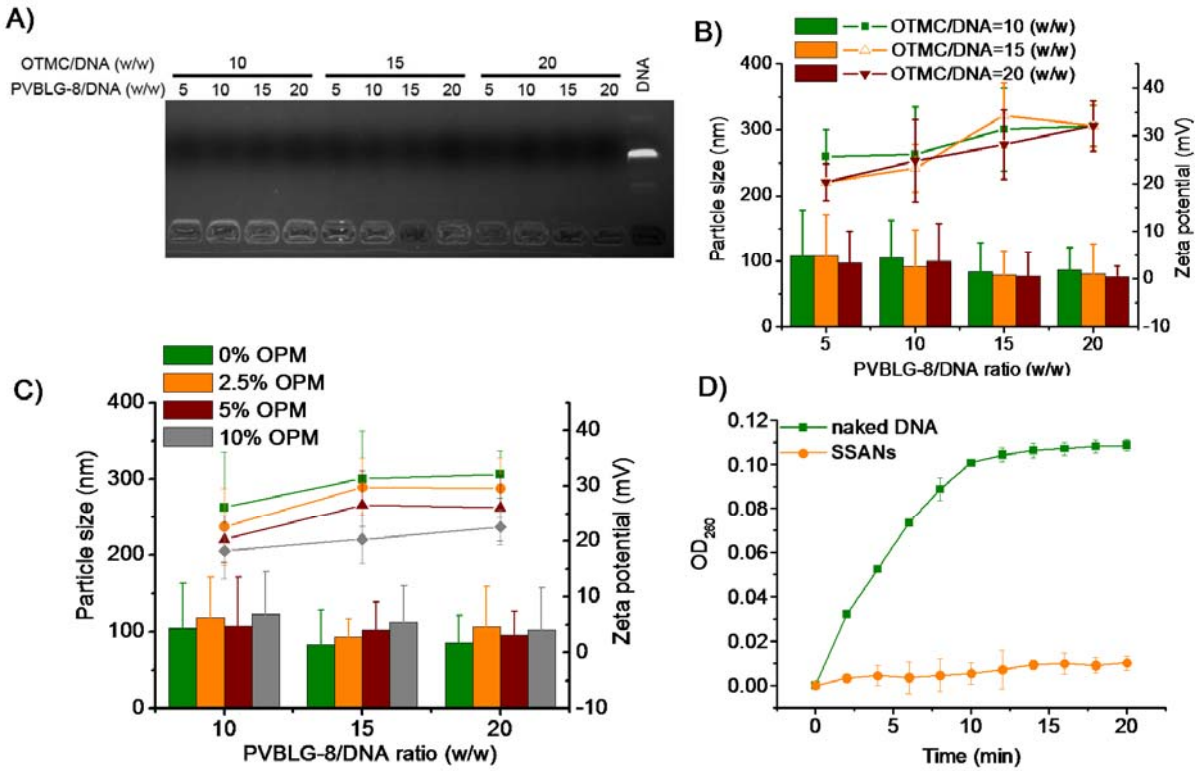


Fig. S5. Characterization of SSANs. (A) Gel retardation assay showing the condensation of DNA at different OTMC/DNA or PVBLG-8/DNA weight ratios. (B) Size (column) and zeta potential (scattered line) of OTMC/PVBLG-8/DNA complexes at different OTMC/DNA or PVBLG-8/DNA weight ratios. (C) Size (column) and zeta potential (scattered line) of SSANs at various OPM contents. OTMC/DNA and PVBLG-8/DNA weight ratios were kept constant at 15 and 15, respectively. (D) SSANs improved DNA stability against enzymatic hydrolysis as shown by the shifts of OD_{260} values upon incubation with DNase I at 37 °C. Results were indicated as mean ± SD (n=3).

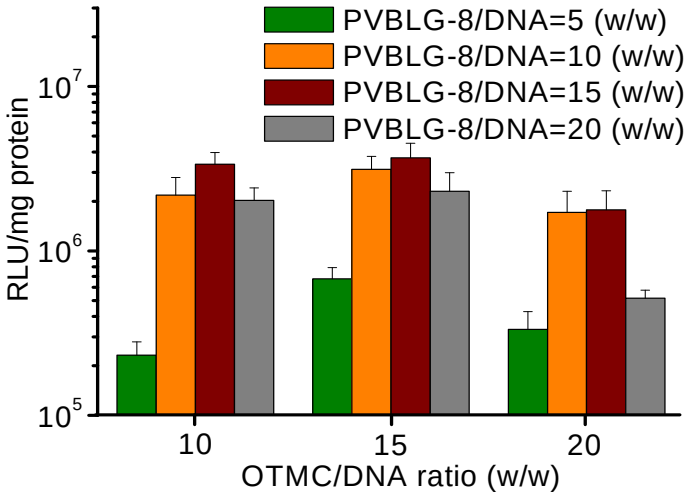


Fig. S6. Transfection efficiencies of OTMC/PVBLG-8/DNA complexes in Raw 264.7 cells at various OTMC/DNA or PVBLG-8/DNA weight ratios. Results were indicated as mean ± SD (n=3).

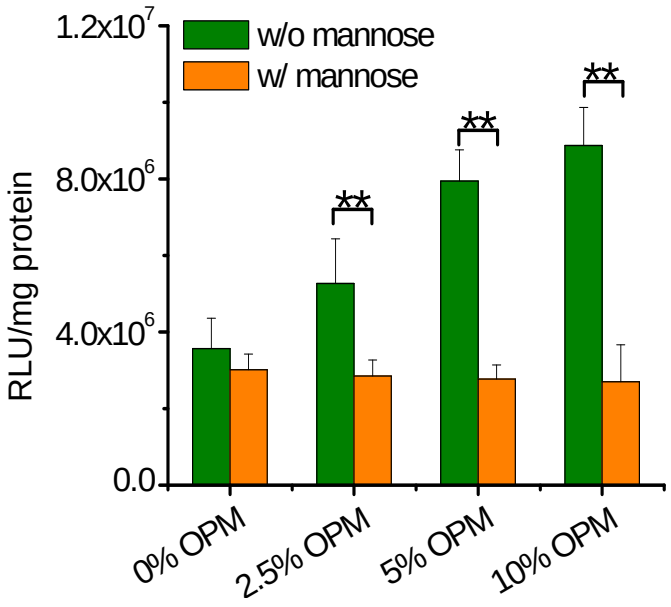


Fig. S7. Transfection efficiencies of OTMC/PVBLG-8/DNA/OPM complexes with various OPM contents in Raw 264.7 cells in the presence or absence of free mannose. Results were indicated as mean ± SD (n = 3).

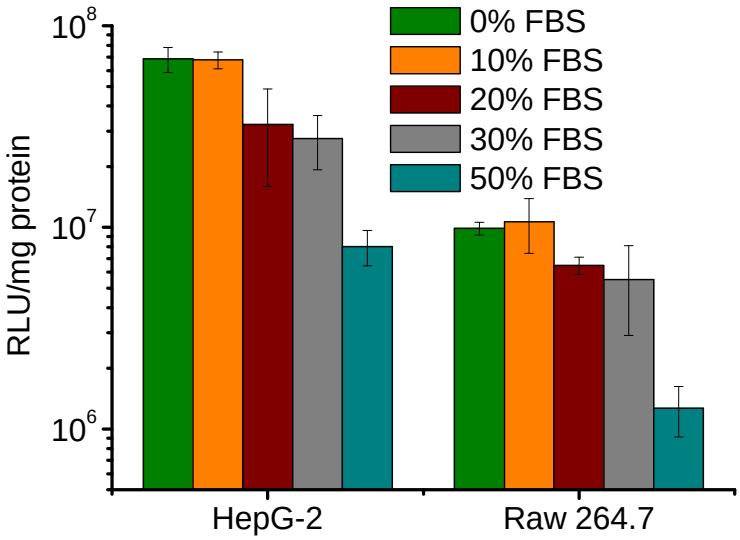


Fig. S8. Transfection efficiencies of SSANs (OTMC/PVBLG-8/DNA weight ratio of 15/15/1, 10 mol% OPM) in HepG-2 and Raw 264.7 cells in the presence of serum. Results were indicated as mean ± SD (n = 3).

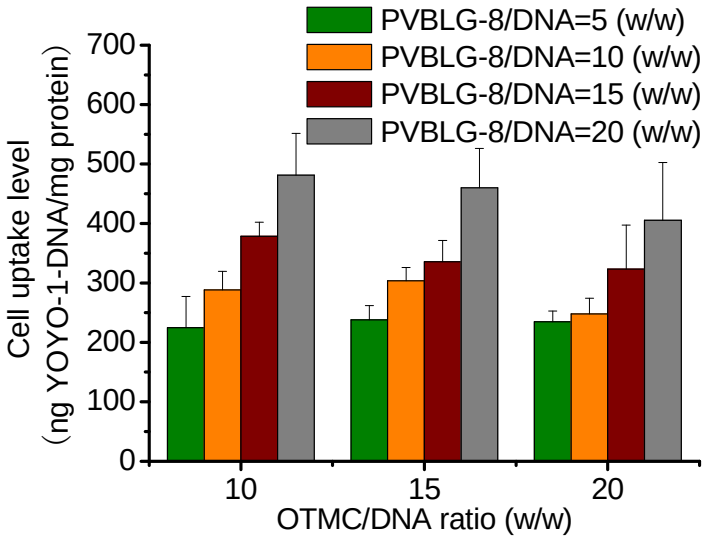


Fig. S9. Uptake levels of YOYO-1 labeled DNA in Raw 264.7 cells when complexed with OMTC and PVBLG-8 at different weight ratios. Results were indicated as mean ± SD (n = 3).

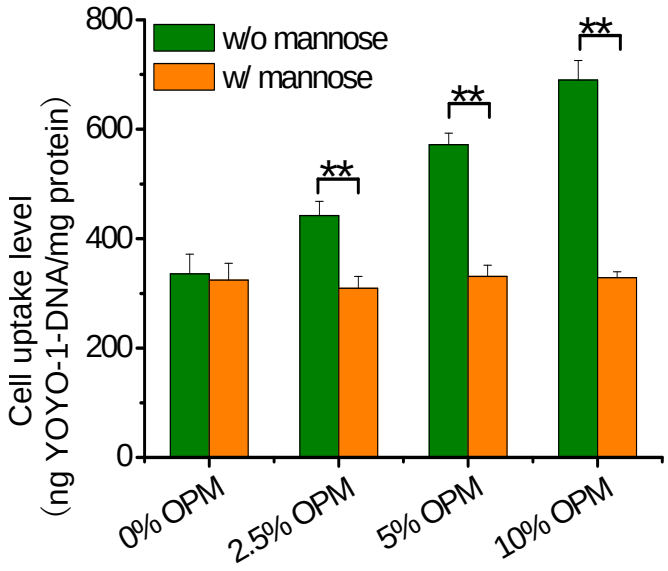


Fig. S10. Uptake levels of SSANs in Raw 264.7 cells in the presence or absence of free mannose. Results were indicated as mean ± SD (n = 3).

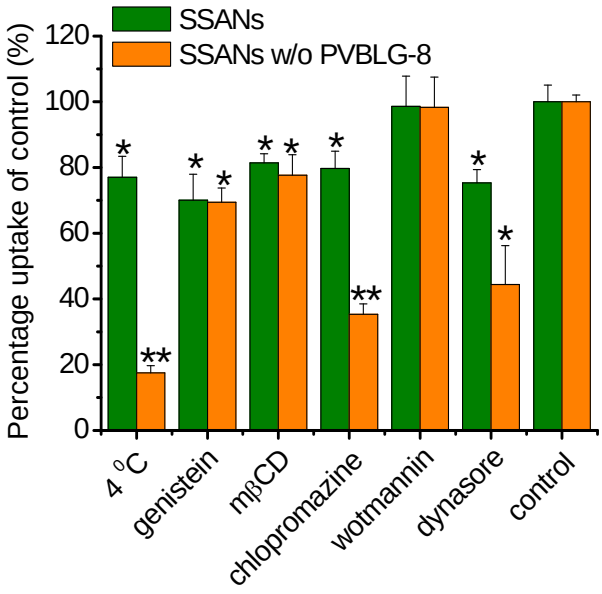


Fig. S11. Cell uptake level of SSANs in Raw 264.7 cells in the presence of various endocytic inhibitors.

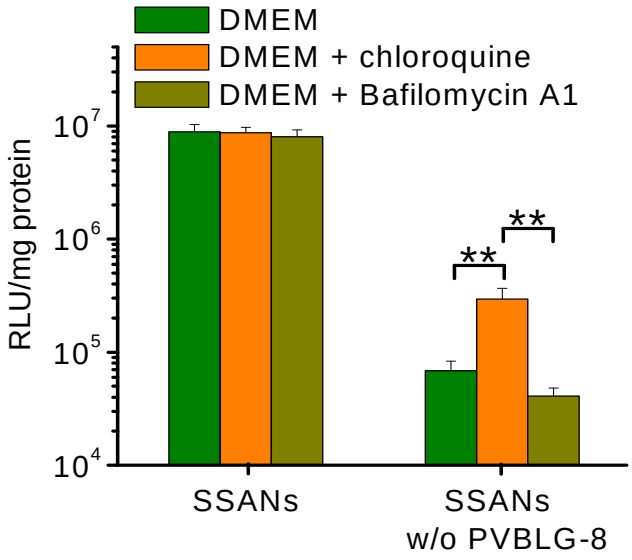


Fig. S12. Transfection efficiencies of SSANs w/ and w/o PVBLG-8 in Raw 264.7 cells in the presence and absence of chloroquine or Bafilomycin A1. Results were indicated as mean ± SD (n = 3).

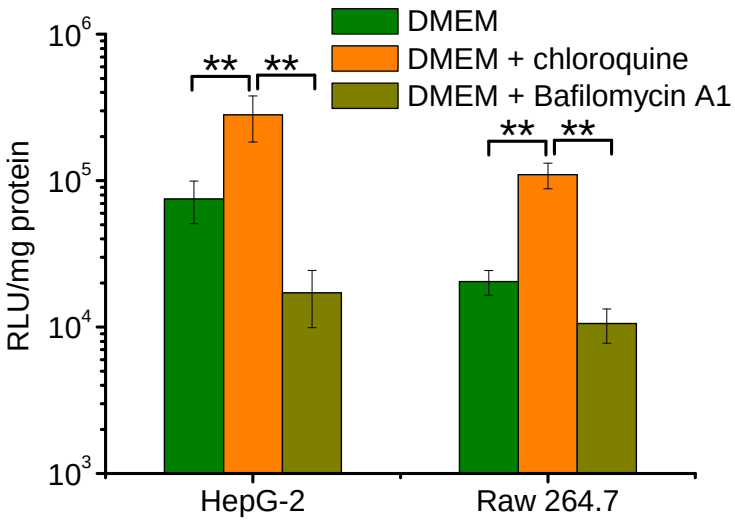


Fig. S13. Transfection efficiencies of OTMC/DNA complexes (w/w = 15) in HepG-2 and Raw 264.7 cells in the presence and absence of chloroquine or Bafilomycin A1. Results were indicated as mean ± SD (n = 3).

Transfection efficiency was increased upon chloroquine treatment while was decreased upon Bafilomycin A1 treatment, indicating that the complexes experienced endosomal entrapment and could partly escape from the endosomes via the proton sponge effect.

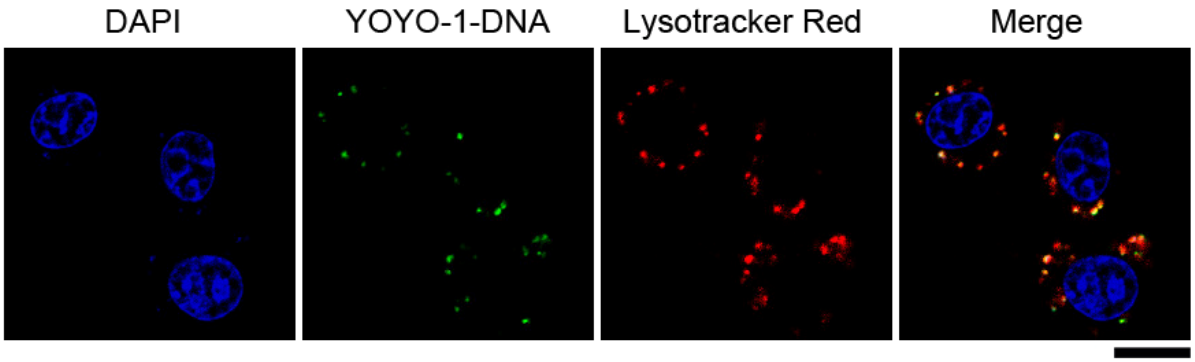


Fig. S14. CLSM images of Raw 264.7 cells treated with OTMC/YOYO-1-DNA complexes (w/w=15) for 4 h and stained with Lysotracker Red. Bar represents 20 μm.

In the CLSM observation, co-localization of complexes with Lysotracker Red-stained endosomes was noted, which suggested endosomal entrapment of the complexes upon endocytosis. Only a small proportion of the complexes did not co-localize with endosomes, indicating that OTMC triggered endosomal escape with a relatively low efficiency.

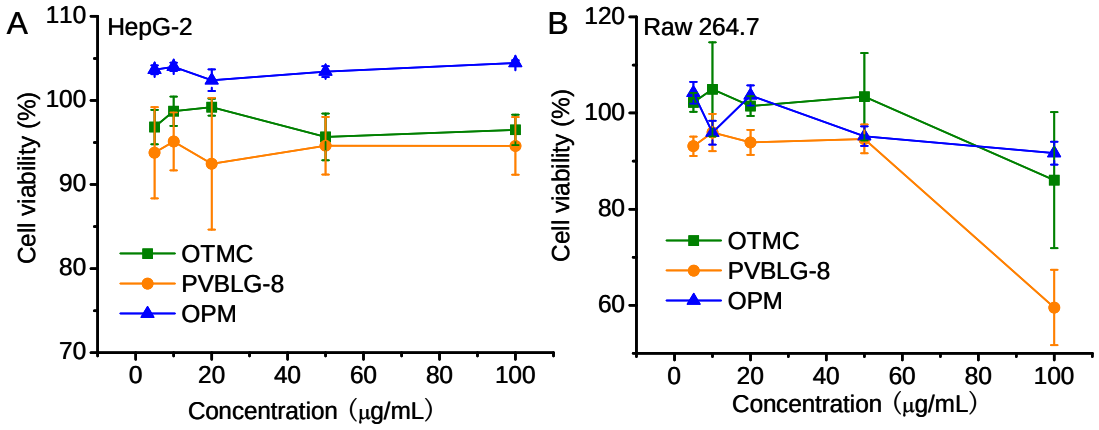


Fig. S15. Cytotoxicity of individual components in SSANs towards (A) HepG-2 and (B) Raw 264.7 cells as determined by the MTT assay. Results were indicated as mean ± SD (n = 3).

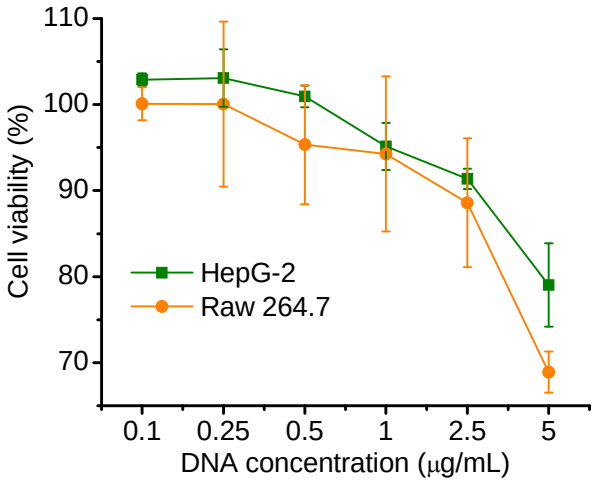


Fig. S16. Cytotoxicity of SSANs (OTMC/PVBLG-8/DNA weight ratio of 15/15/1, 10 mol %OPM) towards HepG-2 and Raw 264.7 cells as determined by the MTT assay. Results were indicated as mean ± SD (n = 3).

Reference

[1] L. C. Yin, J. Y. Ding, C. B. He, L. M. Cui, C. Tang, C. H. Yin, *Biomaterials* **2009**, *30*, 5691.

[2] H. Lu, J. Wang, Y. Bai, J. W. Lang, S. Liu, Y. Lin, J. Cheng, *Nat. Commun.* **2011**, *2*, 206.

[3] C. Moreira, H. Oliveira, L. R. Pires, S. Simoes, M. A. Barbosa, A. P. Pego, *Acta Biomaterialia* **2009**, *5*, 2995.

[4] N. P. Gabrielson, J. J. Cheng, *Biomaterials* **2010**, *31*, 9117.

[5] G. Ter-Avetisyan, G. Tuennemann, D. Nowak, M. Nitschke, A. Herrmann, M. Drab, M. C. Cardoso, *J. Biol. Chem.* **2009**, *284*, 3370.