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Supporting information

Microfluidic approach for highly efficient synthesis of heparin-based bioconjugates for drug delivery †

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Experimental

1. Materials

Unfractionated heparin (UFH, 12000 Da) was obtained from Mediplex Co. (Korea). All-trans-retinoic acid (RA), folic acid (FA), pyridine, ethylenediamine (EDA), formamide, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased form Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). N,N'-dicyclohexylcarbodiimide (DCC) was obtained from Fluka (Buchsm, Switzerland). Tri-n-octylphosphine oxide (TOPO)-capped CdSe/ZnS quantum dots were received from Chungju National University. Penicillin-streptomycin, fetal bovine serum (FBS), 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution, and EMGM medium were purchased from American Type Culture Collection (Rockville, MD, USA). RPMI-1640 medium (without FA) was obtained from Pharmacia Biotech AB (Uppsala, Sweden). All reagents were analytical grade and used as received without further purification.

2. Fabrication of fluoropolymer microfluidic device

Solvent-resistant and thermally stable fluoropolymer microfluidic devices were fabricated using low molecular weight perfluoropolyether.[15]Device fabrication was accomplished according to the procedure illustrated in Fig. S1. In a typical fabrication, fluoropolymer-containing photoinitiator (Duracur 1173) was poured to make a thick layer (roughly 2 mm) of the material into a PDMS barrier mold surrounding the silicon wafer containing the desired SU-8 photoresist pattern. This wafer was irradiated with UV light for 2 min. At the same time, adhesive coating was performed onto a glass slide consisting of inlet outlet holes, using MPTMS and dried in 80^{o}

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C oven for 1 h. A thin layer of fluoropolymer was spin coated to a thickness of 10 μm (2000 RPM) onto glass slide. This was then placed into the UV curing chamber and irradiated for 10 s. The thick layer was then carefully aligned with and placed on top of the thin layer and the entire device was irradiated for 10 min.

3. Synthesis of aminated retinoic acid and aminated folic acid

a. Preparation of aminated RA. Carboxy group of RA (1 mmol) was activated with EDC (2 mmol) and NHS (2 mmol) in N,N-dimethyl formamide (DMF) (30 mL) for 6 h, following by the reaction with EDA (10 mmol) at room temperature overnight. After the reaction, unreacted EDA was removed via evaporation at 25 °C for 4 h under vacuum. The crude product was precipitated in an excess of cold deionized water and washed several times with deionized water. The aminated RA powder was obtained after lyophilization.

b. Preparation of aminated FA. FA (1 mmol) was reacted with DCC (1 mmol) and NHS (2 mmol) in 20 mL of dimethyl sulfoxide (DMSO) at room temperature for 12 h. EDA and pyridine (200 μL) were added into the activated FA solution and allowed to react at room temperature overnight. Then, excess acetonitrile was added to the reactant solution for precipitation and removal of DCC and NHS. The aminated FA was obtained after centrifugation and dried under vacuum.

4. Characterization of HR and HFR bioconjugates

The coupling ratios of FA and RA were quantified using a UV-vis spectrophotometer (S-3100, SCINCO, Korea). Briefly, the lyophilized HR and HFR were dissolved in formamide to final concentration of 0.05 mg/mL. The absorbance of FA at 290 nm and of RA at 373 nm was

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measured using established calibration curves with known concentrations of aminated RA and aminated FA in formamide. The critical aggregation concentrations (CACs) of HR and HFR bioconjugates were analyzed by fluorescence spectrophotometry (FluoroMax-4, Horiba Jobin Yvon), using pyrene as a fluorescence probe. Pyrene stock solution ($3x10^{-4}$M) in acetone was added into the test tubes, and followed evaporation to remove the organic solvent. Then, various concentrations of aqueous bioconjugate solutions (10 mL) were added to each vial (the final concentration of pyrene was 6.0×10^{-7} M in each vial), and sonicated for 3 h at 60°C to equilibrate pyrene with the nanoparticles, and left to cool at room temperature for 3 h. The emission spectra of pyrene were recorded in the range of 350-450 nm at the excitation wavelength of 336 nm. For the measurement of the intensity ratio of the first (374 nm) and the third highest energy bands (386 nm) in the pyrene emission spectra, the slit opening was set at 2.5 nm.

5. Preparation of quantum dots (QDs) loaded HR and HFR nanoparticles

CdSe/ZnS QDs loaded nanoparticles were prepared by dialysis method. Briefly, the bioconjugates (20 mg) were dissolved in a mixture of 10 mL formamide and 5 mL DMSO, followed by the addition of 100 μL QDs in chloroform (40 μM). The mixture was sonicated for 3 h to evaporate chloroform, followed by dialysis against distilled water for 2 days (MWCO: 2000). The precipitated QDs were removed by centrifugation at 10,000 rpm and filtration through 0.45 μm syringe. The reddish products were obtained after lyophilization.

6. Preparation and characterization of self-assembled HR and HFR nanoparticles

The self-assembled HR and HFR nanoparticles were prepared by dispersing HR and HFR bioconjugates in deionized water at a concentration of 1 mg/mL under gentle shaking, followed

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by sonication for several minutes. After filtration with a 0.45 μm syringe filter, the average particle size, size distribution and zeta-potential of the nanoparticles were measured using a dynamic light scattering (DLS) instrument (ELS-Z series, Otsuka Electronics, Japan). The morphologies of HR and HFR nanoparticles were imaged by scanning electronic microscopy (SEM) (JSM-6700, GELO, Japan) at 10 kV. Specimens were prepared by dropping a solution of HR or HFR nanoparticles to the surface of a silicon wafer, followed by drying under vacuum. The fluorescent spectra of free QDs in chloroform and QDs loaded nanoparticles in water were recorded by fluorescence spectrophotometry (FluoroMax-4, Horiba Jobin Yvon).

7. Cellular uptake

To visualize the cellular uptake of HR and HFR nanoparticles, KB and A549 cells were exposed to QDs loaded nanoparticles and visualized using confocal laser scanning microscope (CLSM) (Leica LSM5, Germany). KB and A549 cells were seeded at 1×10^5 cells/well in 96-well plates and preincubated for 24 h before the assay in a Lab-Tek® II chamber slide (Nalge Nunc, Napevillem, IL). The QDs loaded HR and HFR bioconjugates synthesized by bulk and microfluidic device were dispersed in RPMI-1640 medium at 0.25 mg/mL, and added to each well of the chamber, followed by the incubation for 3 h at 37 °C. The cells were then washed three times with PBS buffer (pH 7.4) to remove the nanoparticles that were not taken up by the cells, fixed by 4% formaldehyde in phosphate buffer saline for 15 min. Finally, the cells were mounted by the mounting medium and observed under CLSM.

8. Cytotoxicity test

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KB cells (human nasopharyngeal carcinoma cell line) and A549 cells (human lung carcinoma cell line) were used for cytotoxicity test. KB and A549 cells were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. KB and A549 cells were seeded at 5×10^5 cells/well in 96-well plates and preincubated for 24 h before the assay. Then, the FA-free RPMI medium was replaced with fresh medium containing free RA dissolved in 1% tween 80 solution, HR, HFR, or QD loaded nanoparticles at different RA concentrations. After 48 h incubation, 200 μL of MTT solution in culture medium (concentration of MTT solution was 0.5 mg/mL) was added to each well. The plate was then incubated for an additional 4 h, followed by the addition of 200 μL of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol, Sigma, Milwaukee, WI, USA) to each well. The solution was gently mixed to dissolve MTT formazan crystals. The absorbance of each well was measured with a microplate reader at 570 nm. The background absorbance of the well plate at 690 nm was measured and subtracted. The cytotoxicity was calculated by dividing the optical density values (OD) of the treated groups (T) by the OD of the controls (C) ([T/C×100%]).

9. Statistics

All data were presented as mean ± SD from three to six independent measurement and analyzed using descriptive statistics and single-factor analysis of variance. A probability level of $p<0.05$ was considered significant.

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Table S1. Characterization of QDs loaded HR and HFR nanoparticles

Samples	CAC* (mg/L)	Average size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
HR –MF	51	141.3 ± 1.2	0.162	-36.51 ± 0.09
HR-bulk	79	155.4 ± 0.9	0.244	-35.5 ± 0.8
HFR –MF	14	130.8 ± 2.0	0.143	-34.51 ± 0.71
HFR-bulk	24	139.1 ± 2.9	0.265	-30.1 ± 0.7
QD-HR-MF		129 ± 3.9	0.178	-24.51 ± 2.4
QD-HR-bulk		145.4 ± 9.2	0.213	-30.68 ± 0.47
QD-HFR-MF		123.1 ± 1.8	0.101	-31.16 ± 0.46
QD-HFR-bulk		131.1 ± 9.8	0.170	-28.37 ± 1.12

*Critical aggregation concentration

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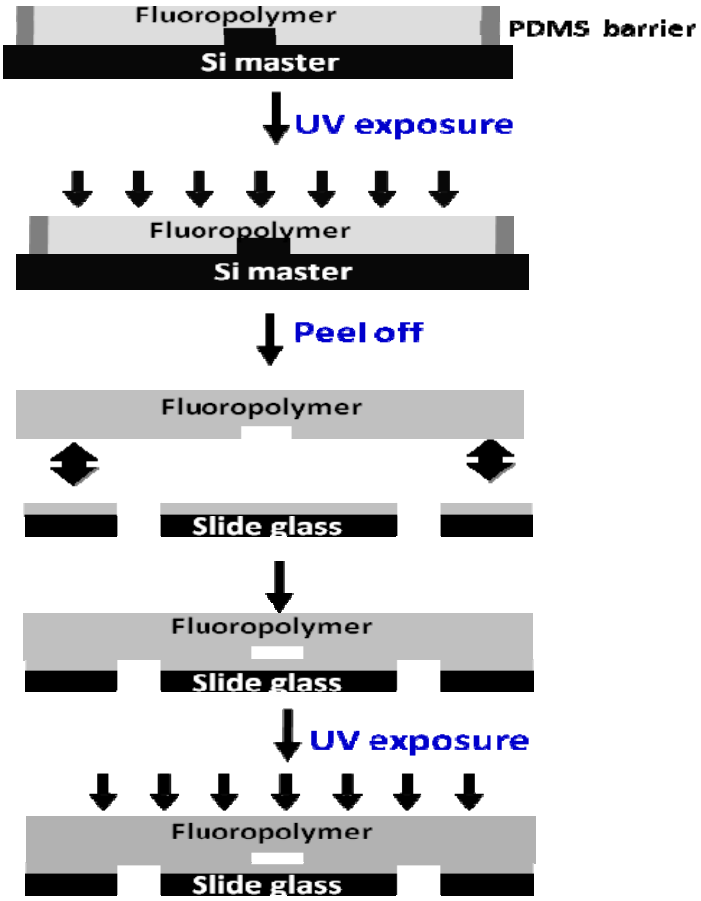
(a)



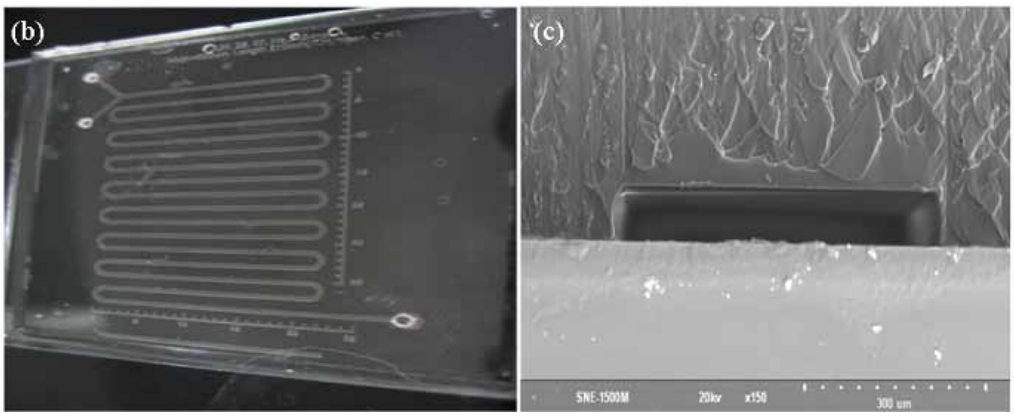


Fig. S1. (a) Schematic fabrication of fluoropolymer microchannel by imprinting process; (b) Optical image and (c) Cross-section view of microfluidic channel.

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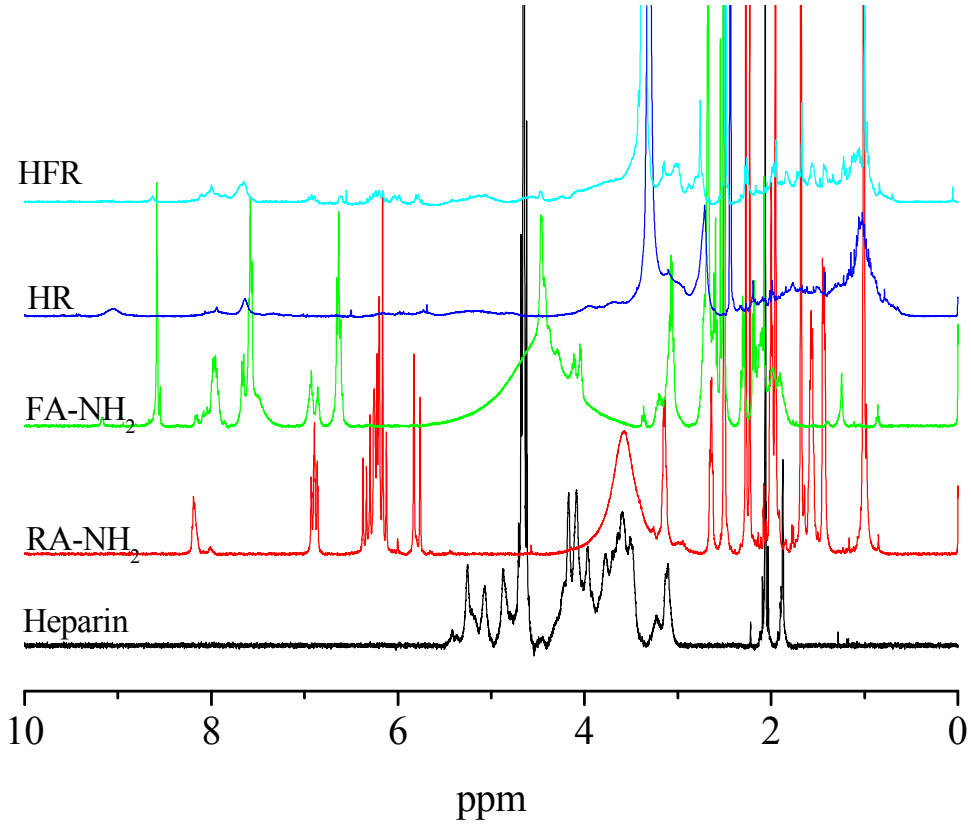

Fig. S2. ^{1}H-NMR of heparin in D_2O, RA-NH_2, FA-NH_2, HR and HFR in DMSO.

The characteristic chemical shifts of UFH were observed at 1.8, 3.1, 3.6, and 4.0 ppm. The proton assignments of aminated RA were given at 1.0, 1.2, 1.4, 2.3-2.8, and 6.2-6.4 ppm. Besides the characteristic peaks of UFH and RA, the typical peaks of aminated FA were observed at 4.5, 6.7, and 8.6 ppm in the ^{1}H NMR spectrum of HFR.

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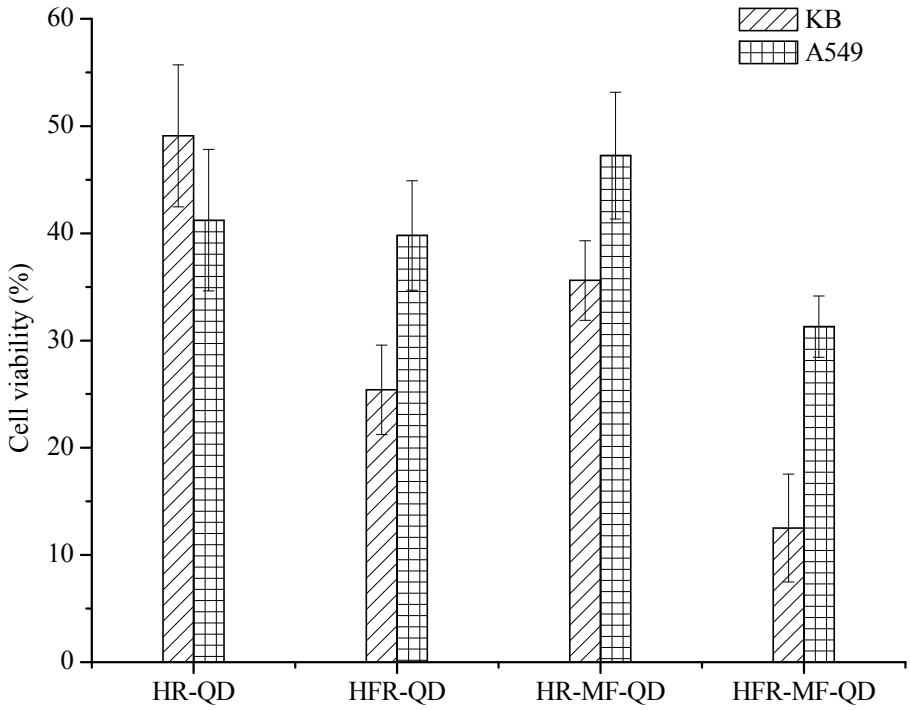

Fig. S3. Cytotoxicity in KB and A549 cells of QDs loaded nanoparticles at 50 μg/mL equivalent RA.