Supporting Information

Heparin-immobilized gold-assisted controlled release of growth factors *via* electrochemical modulation

Boyoung Kim,[‡,a] Kihak Gwon,[‡,a] Seyeong Lee,[a] Young Ha Kim,[a] Myung-Han Yoon[a] and Giyoong Tae[*,a,b]

[a]School of Materials Science and Engineering, Gwangju Institute of Science and Technology (GIST), 261 Cheomdan-gwagiro, Buk-gu, Gwangju 61005, Korea

[b]Center for Theragnosis, Biomedical Research Institute, KIST, Seoul 02792, Korea

E-mail: gytae@gist.ac.kr

Tel: +82-62-715-2305

Fax: +82-62-715-2304

[*] Corresponding authors: Prof. G. Tae

[‡] Equally contributing author

Experimental Section

1. Preparation of thiolated heparin

Thiolated heparin (Hep-SH) was synthesized by converting the carboxyl groups of heparin into thiol groups, as described in a previous report.[1] Hep-SH with 40% degree of thiolation was prepared by reacting the carboxyl groups of heparin with cysteamine. Briefly, heparin was dissolved in DIW at 10 mg/mL. EDC (1.75 molar ratio), HOBT (1.1 molar ratio), and cysteamine (2.2 molar ratio) were sequentially added, and the pH was adjusted to 6.8. After reaction under stirring for 12 h at room temperature, the solution was thoroughly dialyzed by dialysis (3.5 kDa Mw cut-off) to remove unreacted reagents. Then, the excess amount of DTT (10 molar ratio) was added to reduce the oxidized disulfide groups and to form free thiol groups of modified heparin. After 12 h of reaction, this solution was dialyzed against a 0.1 M NaCl solution at pH 3.5, pH 5.0, and in pure DIW, sequentially, and lyophilized. The degree of thiolation was measured using Ellman's reagent at 412 nm. 40% thiolated heparin was used for all experiments.

2. Sample preparation

All experiments (for characterization, electrochemical release, cytotoxicity, and bioactivity) were performed on gold-coated glass or on gold-coated quartz crystals (for QCM/EQCM experiments). Gold-coated glass with evaporated gold at a thickness of 1000 Å and a Ti adhesion layer at a thickness of 50 Å were purchased from EMF (Ithacha, NY, USA). Gold-coated quartz crystals (QCM quartz crystals-QSX 301) were purchased from Biolin Scientific (Stockholm, Sweden). Gold-coated glass was cut into 1.25 cm × 1.25 cm and cleaned by piranha solution (H_2SO_4:H_2O_2 = 3:1) for 15 min, followed by sequential rinsing with DIW. Then, the electrodes were dried under nitrogen. For the chemisorption of Hep-SH

on gold, the substrate was immersed into a Hep-SH solution (1 mg/mL) in PBS for 90 min at 37°C, followed by rinsing with PBS to remove unreacted Hep-SH. Then, Hep-SH-immobilized gold substrates were subsequently immersed in a bFGF solution in PBS (2 μg/200 μL) for 3 h at 37°C. After incubation, unbound bFGF was removed and collected in an Eppendorf tube, and the amount was measured by ELISA (Peprotech, Rocky Hill, NJ, USA) to calculate the amount of bFGF loaded on gold surfaces. QCM measurements were carried out with a QCM-D system from Q-Sense (Västra Frölunda, Sweden), as described in detail.[2] An electrochemistry flow cell was used for the electrochemical QCM-D (EQCM-D) measurements.[3] Gold-coated quartz crystals were cleaned by a solution of DIW:NH_4OH:H_2O_2 with a 5:1:1 volume ratio at 75°C for 5 min, followed by rinsing with DIW. After drying with nitrogen, quartz crystals were treated with UV/ozone for 10 min, and installed in the instrument chamber at 37°C. PBS was injected into the chamber and stabilization was reached. A 1 mg/mL Hep-SH solution was injected into the chamber and incubated for 90 min to induce the chemisorption of heparin on gold, followed by PBS washing to remove unbound Hep-SH. Then, bFGF (2 μg/200 μL) was injected into the chamber to induce the loading of bFGF on heparin and incubated for 3 h, followed by rinsing with PBS. To confirm the specific binding of bFGF on heparin, 1% BSA was injected into the chamber between Hep-SH injection and bFGF injection. Frequency shift is linearly proportional to mass change on gold-coated quartz crystal. All samples were measured with n = 3.

3. Sample characterization

To evaluate the surface properties of the modified substrate at each step, the water contact angles were measured by a Phoenix apparatus (Surface Electro Optics Co., Korea). All samples were measured with n = 3. Additionally, cyclic voltammetry (CV) was carried

out to analyze the electrochemical properties of modified gold surfaces in PBS buffer (scan rate: 100 mV/s). Although bFGF was bound on heparinized gold, the CV curve was similar to that before bFGF loading, indicating that the adsorption of bFGF on heparinized gold did not cause changes in the electrochemical properties of heparin-chemisorbed gold. The reduction peak of gold-thiol was under 0.8 V.

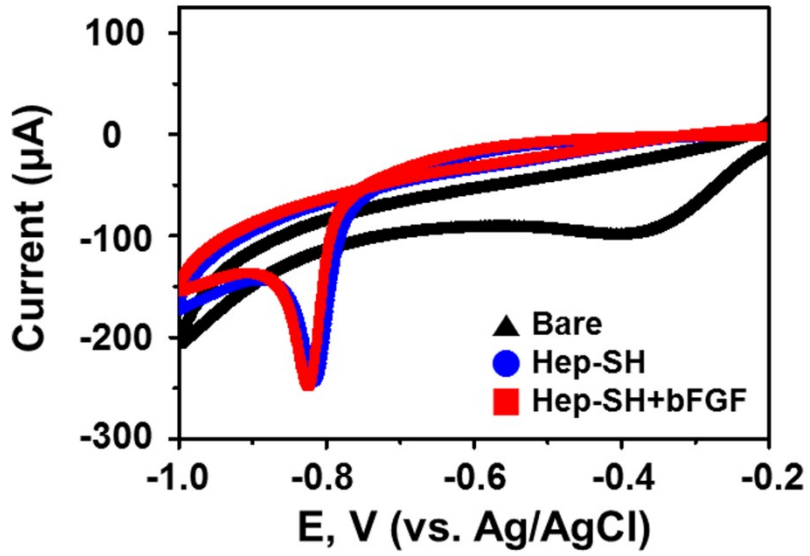

Fig. S1 Cyclic voltammogram of bare gold (▲), heparinized gold (●), and heparinized gold after bFGF loading (■). Scan rate = 100 mV/s

4. Electrochemical analysis

All electrochemical stimulations (ES) were applied using CH Instrument (Austin, TX, USA) based on a three-electrode system: a gold-coated glass or gold-coated quartz crystal sensor as a working electrode, Ag/AgCl as a reference electrode, and a platinum wire as a counter electrode. Two types of electrochemical controlling methods (voltage control and current control) were employed. In the case of voltage control, a given voltage source was applied between the working and counter electrodes. In the case of current control, a given

current source was applied between the working and counter electrodes and the desired currents were passed. Various types of ES pulse forms are available: monophasic, charge balanced biphasic, and charge unbalanced biphasic.[4] In monophasic pulsing, a constant pulse is applied for a defined time. In biphasic pulsing (both charge balanced and unbalanced), a constant pulse is applied in one direction, followed by the reverse pulse direction. The charge balanced form applies the same charge during the stimulation phase and the reversal phase, whereas charge unbalanced form has different charges between the stimulation phase and the reverse phase.[4] Various types of ES were applied to the gold electrode for the electrochemical release of bFGF: monophasic potential (-0.8 V), biphasic balanced potential (±0.2 V), biphasic unbalanced potential (0.2/-0.8 V, 0.2/-1.2 V), biphasic balanced current (±20 μA), and biphasic unbalanced current (20/-50 μA, 20/-100 μA, 20/-160 μA, 20/-300 μA). In the case of biphasic ES, each phase of ES was applied for 1 s (Fig. S2). The magnitudes of potential and current in various ES were set to be in the safe range of cytotoxicity and electrode corrosion.[4] After stimulation, electrochemically released bFGF in the buffer was collected in an Eppendorf tube and the amount released was measured by ELISA.

Notably, the stimulation at 0.2/-0.8 V and that at 20/-100 μA showed a similar negative maximum potential of ~ -0.8 V (Fig. S3A–B), but the current control provided more efficient electrochemical bFGF release than that of voltage control. In case of voltage control, most of the current was generated at the onset of pulse as a sharp peak (Fig. S3C–D). Moreover, by changing the ES direction, the release of cumulative charge occurred more slowly by current control, indicating more accumulated charges at the working electrode (Fig. S3E-F). Thus, the stimulation efficacy by current control could be higher than that of voltage control.

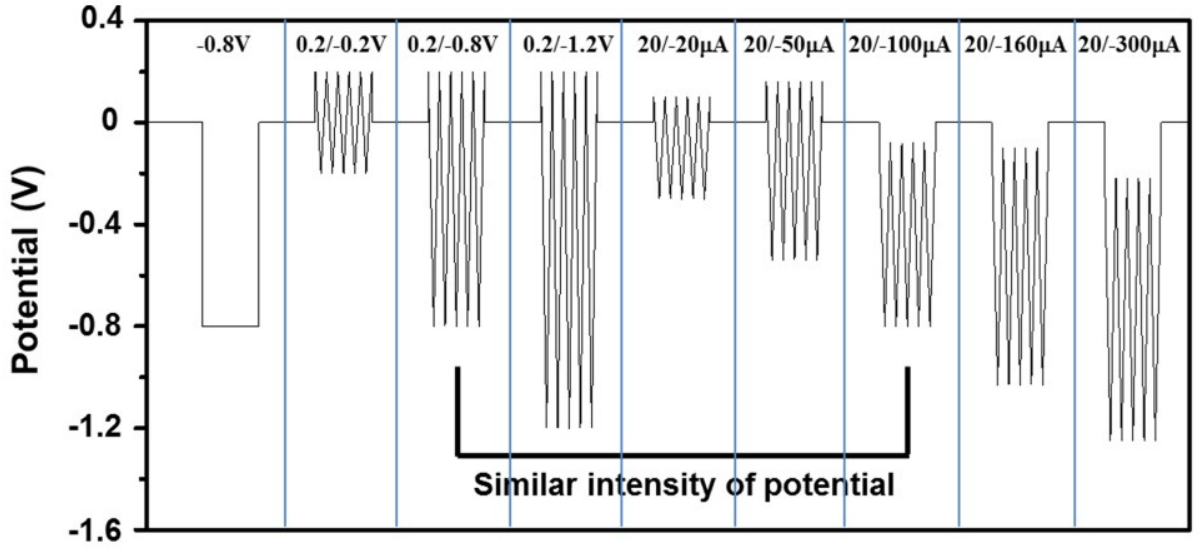

Fig. S2 Various types of electrochemical stimulations.

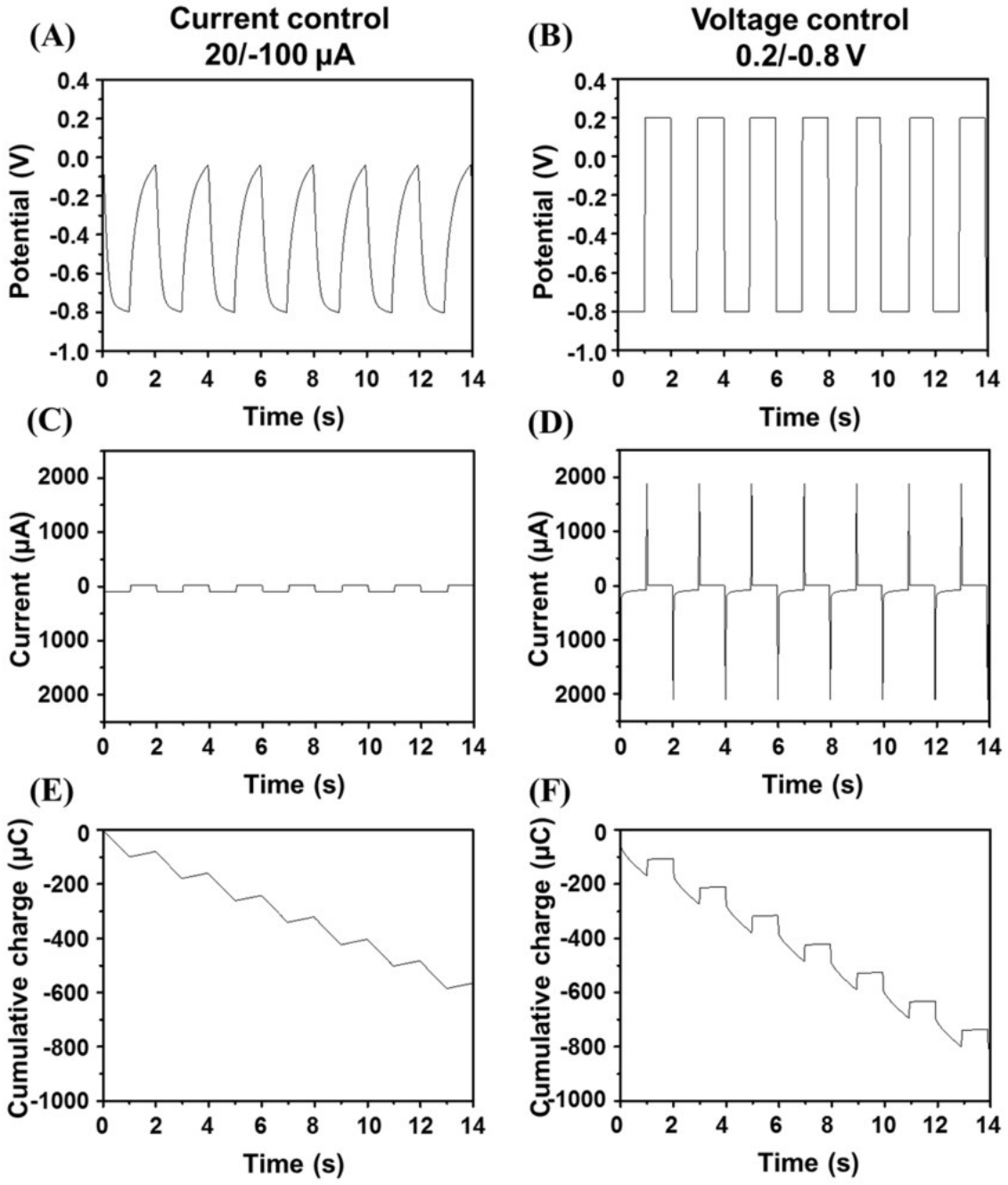

Fig. S3 Comparison of electrochemical stimulations with current stimulation (20/-100 μA) (left) and potential stimulation (0.2/-0.8 V) (right). (A) & (B): applied potential, (C) & (D): applied current, (E) & (F): cumulative charge.

5. Cytotoxicity of electrochemical stimulation

In this study, electrochemical stimulation (20/-100μA) was applied to bFGF and heparin complex immobilized gold to modulate the release of bFGF. Cytotoxicity analysis was performed to analyze whether the electrochemical stimulation itself might cause any toxic effect on cells in any potential applications of the present method. The cytotoxicity of the applied electrochemical stimulation was first analyzed using a double staining live/dead assay. Balb/c 3T3 cells were seeded on glass (3.0×10^4 cells/1.5 cm^2 glass) in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200 U/mL penicillin, and 200 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2. After 12 h of incubation, the cell-seeded glass was transferred to a 3-electrode system installed beaker (immobilized gold glass as a working electrode, Ag/AgCl as a reference electrode, and a platinum wire as a counter electrode) and filled with 6 mL cell culture media. Then, cells were indirectly subjected to electrochemical simulation (20/-100μA) for 1 h. After electrochemical stimulation, cell viability was analyzed by a Live/Dead double staining by adding 2 mL cell culture media containing 0.67 μM AO and 7.5 μM PI for 15 min in the dark. After rinsing with PBS, stained cells were imaged using a fluorescence microscope (TE2000-U, Nikon Co, Tokyo, Japan). Cell viability was quantified by calculating the percentage of live cells. Additional quantitative analysis of cell viability was done using WST-assay, which is similar to MTT assay and measures the metabolic rate of cells. The same density of cells was prepared on glass substrates and the electrochemical simulation was applied to one group whereas without applying ES was used as the control group. Then, the cell proliferation rates in both cases were quantitatively analyzed by a standard WST-8 assay at several time points (before stimulation, day 1, and day 3). At each measurement point, cells on the glass were incubated with 500 μL cell culture media containing WST-8 assay solution for 90 min. After incubation, 100 μL of incubated media was transferred to 96

well plates and the absorbance of the produced formazan was measured at 450 nm (Thermo max microplate reader, Molecular Devices, Sunnyvale, CA, USA). The number of proliferated cells was quantified by a standard curve of cells. Data showed that at all time points, the cell proliferation was same for both cases (w/ and w/o electrochemical stimulation group), confirming non-cytotoxicity of the electrochemical stimulation used for bFGF release.

6. Bioactivity of bFGF released by electrochemical stimulation

In order to characterize the bioactivity of electrochemically released bFGF upon the ES of 20/-100 μA from heparinized gold substrates, released bFGF was collected and assayed for bFGF-dependent cell proliferation. Balb/c3T3 cells (1.0×10^4 cells/well) were cultured in DMEM with 10% FBS, 200 U/mL penicillin, and 200 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2. After 8 h of incubation, cells were washed with PBS and then ultra-MEM (GIBCO Grand Island, NY) (with 200 U/mL penicillin and 200 μg/mL streptomycin, no FBS) containing either the released bFGF or pristine bFGF (10 ng/mL) was added to each well. Cells were incubated for 2 days, and then cell proliferation was assayed using a standard WST-8 assay. WST-8 reagent was added and incubated for 90 min, and the absorbance at 450 nm was measured using a microplate reader.

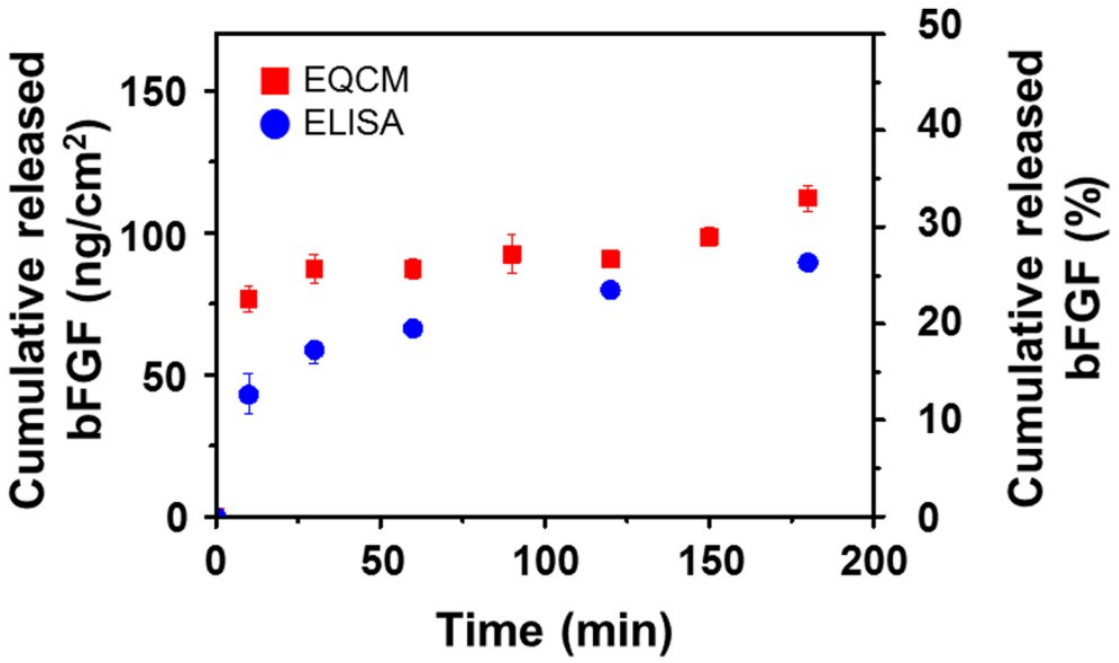

Fig. S4 Comparison of release profiles between EQCM (■) and ELISA (●) measurements. Error bars: standard deviation with n = 3.

References

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