Spatial Control of Cell Transfection Using Soluble or Solid-Phase Redox Agents and a Redox-Active Ferrocenyl Lipid

Burcu S. Aytar,[1] John P. E. Muller,[1] Yukishige Kondo,[3] Nicholas L. Abbott,[1] and David M. Lynn[1,2,*]

[1]Department of Chemical and Biological Engineering, 1415 Engineering Drive, and [2]Department of Chemistry, 1101 University Avenue, University of Wisconsin – Madison, Madison, WI 53706; [3]Department of Industrial Chemistry, Tokyo University of Science, Shinjuku, Tokyo 162-8601, Japan

Supporting Information

Experimental Section

Materials. BFDMA was synthesized using methods described previously.[18] Ascorbic acid and lithium sulfate monohydrate were purchased from Sigma Aldrich (St. Louis, MO). Dodecyltrimethylammonium bromide (DTAB) was purchased from Acros Organics (Morris Plains, NJ). Plasmid DNA constructs encoding enhanced green fluorescent protein [pEGFP-N1 (4.7 kb), >95% supercoiled] and firefly luciferase [pCMV-Luc, >95% supercoiled] were purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM cell culture medium, phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) protein assay kits were purchased from Pierce (Rockford, IL). Glo Lysis Buffer and Steady-Glo Luciferase Assay kits were purchased from Promega Corporation (Madison, WI). Mixed cellulose ester membranes (pore size: 25 nm) were purchased from Millipore (Bedford, MA). Immobilized tris(2-carboxyethyl)phosphine (TCEP) disulfide reducing gel and control agarose resins without immobilized TCEP were purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Deionized water (18 MΩ) was used to prepare all buffers and salt solutions. All commercial materials were used as received without further purification, unless otherwise noted.

General Considerations. Electrochemical oxidation of BFDMA was performed as described previously.[21] UV/vis absorbance measurements of BFDMA solutions were made using a Beckman Coulter DU520 UV/vis Spectrophotometer (Fullerton, CA). Fluorescence microscopy images used to characterize expression of enhanced green fluorescent protein (EGFP) in cell transfection experiments were acquired using an Olympus IX71 microscope and processed using Metamorph Advanced V7.7.8.0 (Molecular Devices, LLC, Toronto, Canada). Fluorescence intensity analyses were performed using Image J64 (National Institutes of Health, Washington, DC). Luminescence and absorbance measurements used to characterize luciferase expression and total cell protein were performed using a PerkinElmer EnVision multilabel plate reader (Luciferase: Em, 700 nm cutoff. BCA: Abs 560 nm). Agarose beads with and without immobilized TCEP were washed twice using Opti-MEM cell culture medium before use in cell transfection experiments. Student's *t*-test was used for statistical analyses of data (two different populations, n = 6). Data were considered statistically significant for *P* values less than 0.001.

Preparation of Lipid and Lipoplex Dispersions. Solutions of reduced BFDMA were prepared by dissolving reduced BFDMA in aqueous Li_2SO_4 (1 mM, pH 5.1). Solutions of oxidized BFDMA were prepared by electrochemical oxidation of solutions of reduced BFDMA, as previously reported.[21] To prepare lipoplexes, a solution of plasmid DNA (24 μg/mL in water) was added slowly to an aqueous Li_2SO_4 solution containing an amount of oxidized or reduced BFDMA sufficient to give the final lipid concentrations reported below (oxidized lipid/DNA charge ratios (CRs) were kept constant at 4.2:1; this ratio was selected on the basis of past studies demonstrating that lipoplexes formed at this CR do not transfect cells efficiently, but can be

chemically reduced to yield active lipoplexes that promote high levels of transgene expression).[19,20] Dispersions of lipoplexes were allowed to stand at room temperature for 20 min before use.

Chemical Reduction of Lipoplexes of Oxidized BFDMA. Reduction of lipoplexes of oxidized BFDMA using solutions of AA was performed as reported previously.[20] For experiments designed to characterize the reduction of oxidized BFDMA upon treatment with beads containing immobilized TCEP, samples of oxidized BFDMA were diluted in Opti-MEM to a final lipid concentration of 250 μM and an ~10-fold molar excess of immobilized TCEP beads (in Opti-MEM) was added. The extent of reduction of oxidized BFDMA upon treatment with immobilized TCEP was monitored by measuring UV/vis absorption spectra at wavelengths ranging from 400-800 nm, as described previously. To eliminate clouding, DTAB was added to all lipid solutions prior to performing measurements of absorbance (DTAB does not absorb light in the range of wavelengths used in these experiments). Solutions were incubated for 1-2 min to allow resin beads to settle prior to measurement.

Spatial Control of the Activation of Lipoplexes of Oxidized BFDMA. COS-7 cells used in pEGFP-based cell transfection experiments were grown in clear polystyrene 6-well culture plates at an initial seeding density of 9×10^4 cells/well in 2 mL of growth medium (90% DMEM, 10% fetal bovine serum; 100 units/mL penicillin and 100 μg/mL streptomycin). After plating, cells were incubated at 37 °C until the cell populations were ~80% confluent. Serum-containing cell culture medium was then aspirated and replaced by serum-free medium, Opti-MEM (2 mL), followed by the addition of solution of lipoplexes of oxidized BFDMA (0.5 mL) to yield a final

lipid concentration of 30 μM. A homogenous mixture of the lipoplexes and the cell culture medium was provided by gentle mixing via pipetting inside the well. Spatial control over the introduction of reducing agent to the lipoplexes was achieved using three different methods. In the first method, a hollow cylinder (radius: 0.5 cm) was placed into the middle of the cell culture well (radius: 1.74 cm), followed by the slow addition of AA solution (10-fold molar excess with respect to total lipid concentration) to the lipoplex-containing medium and cells confined within the cylinder. In the second method, a circular, porous hydrophilic membrane (radius: 1.5 cm; average pore size = 25 nm) was fixed in place on the surface of lipoplex-containing cell culture medium (approximately 0.1 cm above cells; the membrane was not in contact with cells in these experiments). This average pore size was selected to allow diffusion of AA but hinder the passage of larger lipoplexes (see text). A droplet of AA solution (volume of 10 μL, radius of 2 mm, 10-fold molar excess with respect to total lipid concentration) was then carefully placed in the center of the membrane and AA was allowed to pass through the membrane to the lipoplex-containing medium. In the third method, an ~10-fold molar excess of a suspension containing TCEP immobilized on agarose microspheres (15 μL; ~50-170 μm in size) was added to a predetermined location in the middle of a cell culture well containing cells (the beads used in these assays were more dense than water and sedimented onto the cells in these experiments). For all cases, at the end of 1 h of incubation, the lipoplex-containing medium was replaced with fresh serum-containing cell culture medium. Cells were incubated for an additional 48 h and relative levels of EGFP expression in cells were characterized using fluorescence microscopy.

Temporal Control of the Activation of Lipoplexes of Oxidized BFDMA. For experiments designed to control the timing of initiation of cell transfection, COS-7 cells were grown in clear

or opaque 96-well culture plates at an initial seeding density of 15×10^3 cells/well in 200 μL of growth medium for EGFP- and pCMV-Luc-based experiments, respectively. After plating, cells were incubated at 37 °C until the cell populations were ~80% confluent. Serum-containing cell culture medium was then aspirated and replaced by Opti-MEM (200 μL), followed by the addition of a solution of lipoplexes of oxidized BFDMA (50 μL) to yield a final lipid concentration of 10 μM. After incubation of the lipoplexes with cells for the time periods reported in the text, AA solution (10-fold molar excess) was added to activate the lipoplexes and initiate cell transfection. After 1 h of incubation, lipoplex-containing medium was replaced with fresh serum-containing cell culture medium, and cells were incubated for an additional 48 h. Relative levels of EGFP expression in cells were characterized using fluorescence microscopy. For experiments in which pCMV-Luc was used, luciferase protein expression measurements were conducted using luminescence-based luciferase assay kits, according to the manufacturer's specified protocol. Luciferase expression data were normalized against total cell protein in each respective well using a commercially available BCA protein assay kit. All cell transfection experiments were conducted in replicates of six.

Characterization of the Zeta Potentials of Lipoplexes. The ζ-potentials of lipoplexes were characterized using a Zetasizer Nano-ZS instrument (Malvern Instruments, Worcestershire, UK). Prior to measurement, dispersions of lipoplexes containing TCEP-immobilized agarose beads were incubated for 1-2 min to allow the beads to settle, and only the solution containing the lipoplexes was transferred to the instrument. Characterization of 1 mL samples containing lipoplexes was performed at ambient temperatures using an electrical potential of 150 V. Six measurements were performed for each sample of lipoplexes. The Henry equation was used to

calculate the ζ-potentials from measurements of electrophoretic mobility. For these calculations, the viscosity of the lipoplex solutions was assumed to be same as that of water.

Additional Supporting Results

Demonstrations of Temporal and Spatiotemporal Control over Activation of Lipoplexes.

Because lipoplexes of oxidized BFDMA can be incubated in the presence of cells for prolonged periods without promoting internalization and transgene expression (see main text), delays in the time at which AA is added can be used to exert control over the timing with which cell transfection is initiated. To demonstrate proof-of-concept and establish redox-mediated principles for temporal control of DNA delivery, we conducted quantitative and qualitative transfection assays using lipoplexes prepared using oxidized BFDMA and plasmid DNA constructs encoding either firefly luciferase or EGFP (lipoplexes were prepared at a CR of 4.2:1 and a total lipid concentration of 10 μM). For these experiments, cells were incubated in the presence of quiescent lipoplexes for 2 hrs and then an aliquot of AA was added via pipette to activate the lipoplexes and initiate/trigger transfection (e.g., see schematic illustration in Figure S1A). After 1 h of incubation in the presence of AA, culture medium was replaced and cells were incubated for an additional 48 h prior to characterization.

Figure S1B shows levels of luciferase expression (expressed as relative light units normalized to the total concentration of cell protein) in cells mediated by AA-triggered lipoplexes relative to otherwise identical cells that were cultured in the presence of lipoplexes, but to which no AA was added. The images in Figure S1C-D show qualitative differences in levels of transgene expression resulting from an otherwise identical experiment using lipoplexes

formed using the pEGFP plasmid. The results of these two experiments demonstrate that (i) lipoplexes of oxidized BFDMA do not themselves promote high levels of transgene expression upon prolonged incubation in direct contact with cells, and (ii) the addition of an aliquot of activating agent can be used to initiate transfection at a time defined by the user. The results of additional experiments shown in Figure S1E-F demonstrate that this small-molecule activator approach can also be used to exert combinations of both spatial *and* temporal control over cell transfection. These experiments were performed using the membrane-based setup described and shown in Figure 1E of the main text; localized transfection in this case was initiated by the placement of a small droplet of AA on the membrane 2 hrs after the addition of a uniform dispersion of oxidized BFDMA lipoplexes to cells. Control experiments using aqueous droplets that did not contain AA did not result in significant levels of transfection (data not shown).

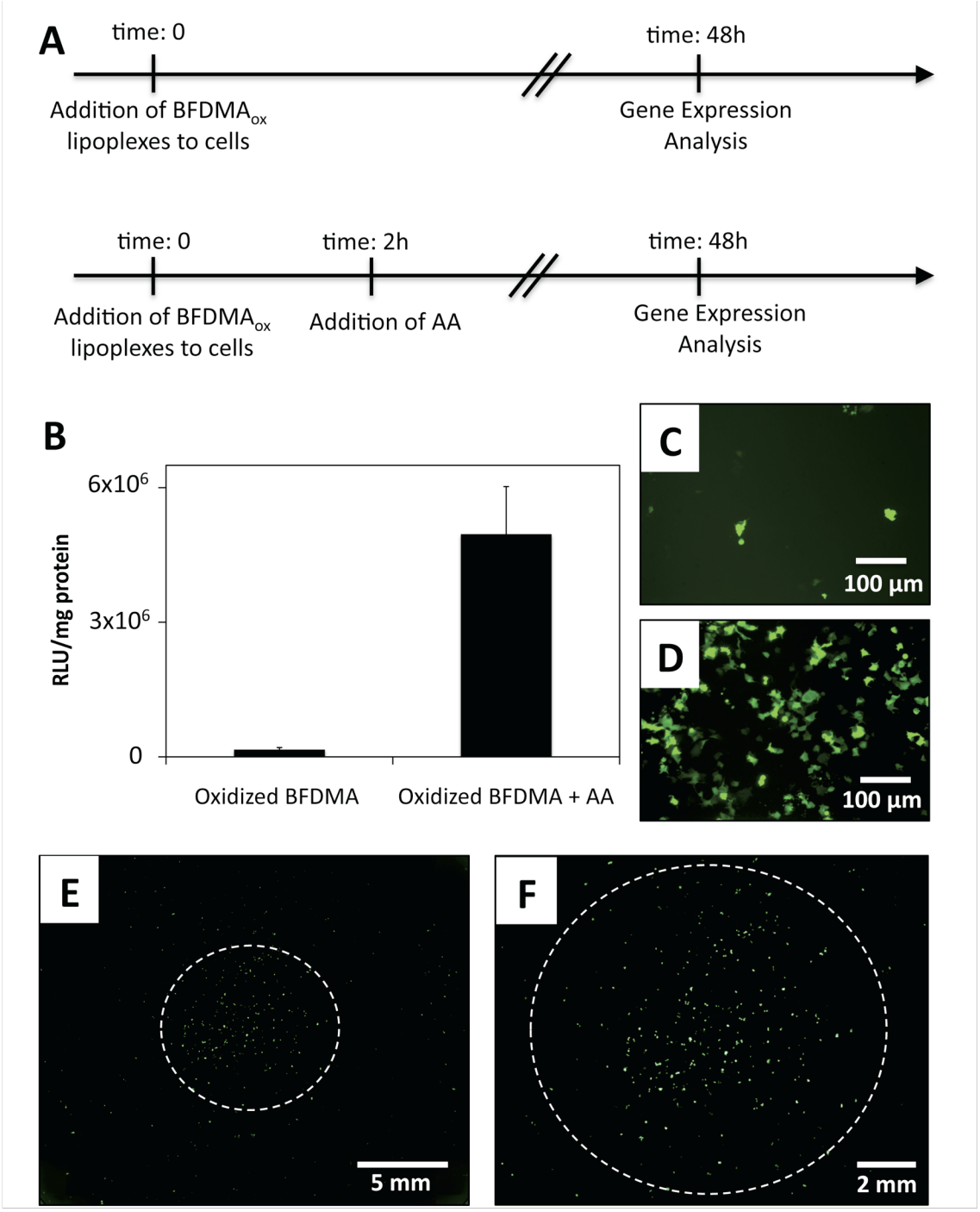

Figure S1: Demonstrations of temporal control over the activation of lipoplexes of oxidized BFDMA (see text on previous pages for additional discussion and context). (A) Schematic showing the experimental procedure. Lipoplexes of oxidized BFDMA were added to cell culture wells containing COS-7 cells. After two hours of incubation, AA was introduced (bottom schematic). Some wells remained untreated (top schematic) to permit comparison of the levels of EGFP expression mediated by AA-treated and AA-untreated lipoplexes. (B) Normalized luciferase expression mediated by untreated lipoplexes and lipoplexes treated with AA after two hours. (C,D) Representative fluorescence micrographs of cells showing EGFP expression mediated by (C) untreated lipoplexes and (D) lipoplexes treated with AA after two hours. (E,F) Spatiotemporal control over the activation of lipoplexes of oxidized BFDMA. (E) Composite fluorescence micrographs of a confluent monolayer of COS-7 cells. Localized EGFP-expression was obtained by the placement of a small droplet of a solution of AA on top of a membrane positioned above cells two hour after the administration of lipoplexes of oxidized BFDMA to cells. The dotted circle represents the approximate location of the culture well immediately below the added droplet. (F) A magnified view of the circled area in (E).

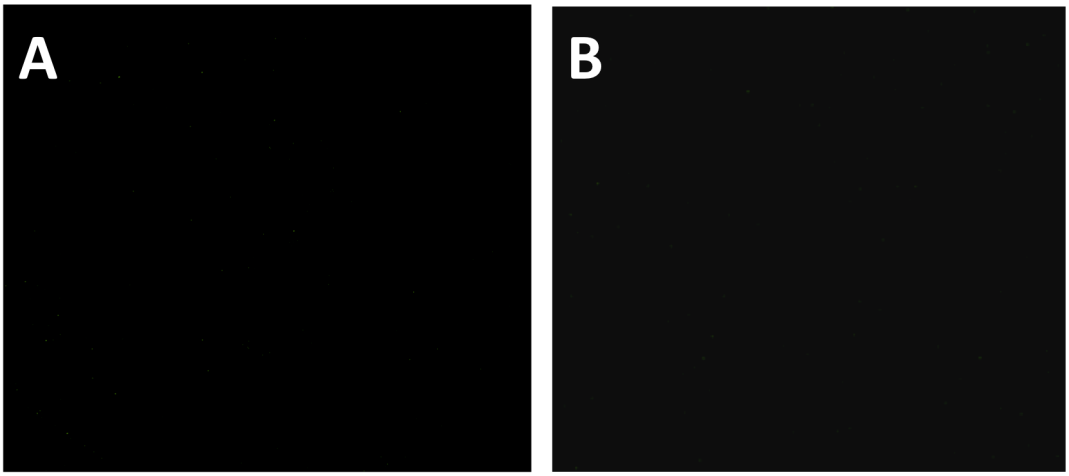

Figure S2: Composite fluorescence micrographs of confluent monolayers of COS-7 cells arising from control experiments in which aqueous droplets containing lipoplexes formed using (A) reduced BFDMA or (B) Lipofectamine were placed on top of nanoporous membranes positioned above cells (see main text and experimental section for additional details).

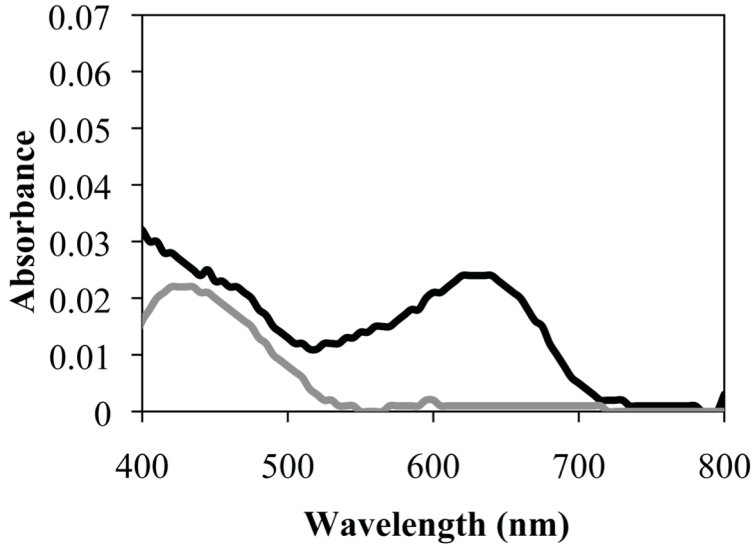

Figure S3: UV/visible absorbance spectra of solutions of lipoplexes of oxidized BFDMA (black line), and lipoplexes of oxidized BFDMA treated with 10-fold molar excess of polymer beads containing immobilized TCEP (grey line) in OptiMEM cell culture medium (see text for additional details). The absorbance peak characteristic of oxidized BFMDA at 630 nm disappears after treatment with immobilized TCEP, and a new absorbance peak characteristic of reduced BFDMA at 430 nm. For additional reference and details related to interpretation of the absorbance spectra of oxidized and reduced BFDMA, see: (1) C. M. Jewell, M. E. Hays, Y. Kondo, N. L. Abbott, and D. M. Lynn, *Bioconjugate Chemistry* **2008**, *19*, 2120-2128, and (2) B. S. Aytar, J. P. E. Muller, S. Golan, S. Hata, H. Takahashi, Y. Kondo, Y. Talmon, N. L. Abbott, and D. M. Lynn, *Journal of Controlled Release* **2012**. *157*. 249-259.

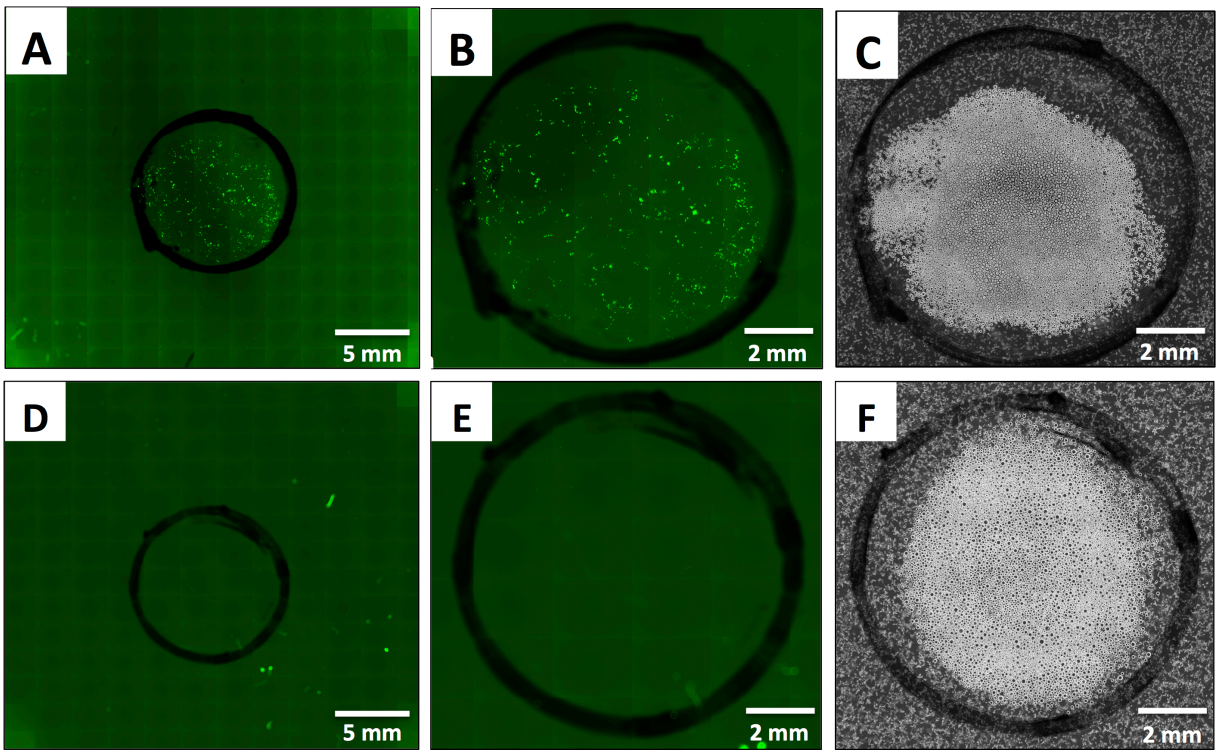

Figure S4: (A-C) Additional results demonstrating spatial control over the activation of lipoplexes of oxidized BFDMA using polymer beads containing immobilized TCEP. (A) A composite of fluorescence micrographs of COS-7 cells. The black circle in these images marks the approximate location to which TCEP-immobilized beads were added. (B) Magnified view of the circled area shown in (A). (C) Phase contrast micrograph showing the locations of the TCEP-immobilized beads in this experiment. (D-F) Results of an otherwise identical control experiment using agarose beads that did not contain immobilized TCEP; no significant levels of cell transfection were observed.