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Cell-Controlled and Spatially Arrayed Gene Delivery from Fibrin Hydrogels

Pedro Lei¹, Roshan Padmashali¹, and Stelios T. Andreadis^{1,2,*}

¹ Bioengineering Laboratory, Department of Chemical and Biological Engineering, University at Buffalo, State University of New York, Amherst, NY 14260

² Center of Excellence in Bioinformatics and Life Sciences, Buffalo, NY 14203

Abstract

We investigated fibrin-mediated gene transfer by embedding pDNA within the hydrogel during polymerization and using two modes of gene transfection with cells placed either on the surface (2D transfection) or within the hydrogel (3D transfection). Using this model, we found that cell transfection depended strongly on the local cell-pDNA microenvironment as defined by the 2D vs. 3D context, target cell type and density, as well as fibrinogen and pDNA concentrations. When cells were embedded within the fibrin matrix lipofectamine-induced cell death decreased significantly, especially at low target cell density. Addition of fibrinolytic inhibitors decreased gene transfer in a dose-dependent manner, suggesting that fibrin degradation maybe necessary for efficient gene transfer. We also provided proof-of-concept that fibrin-mediated gene transfer can be used for spatially localized gene delivery such as required in cell transfection microarrays. When lipoplex-containing hydrogels were spotted in an array format gene transfer was strictly confined to pDNA-containing fibrin spots with no cross-contamination between neighboring sites. Collectively, our data suggest that fibrin may be used as a biomaterial to deliver genes in an efficient, cell-controlled and spatially-localized manner for potential applications in vitro or in vivo.

Keywords

Fibrin hydrogels; fibrinolysis; matrix-mediated transfection; cell-controlled gene delivery; transfection microarrays

INTRODUCTION

Biomaterial-mediated gene transfer is the use of biomaterials to serve as gene carriers to facilitate gene delivery to target cells. This approach has several advantages including close proximity of vectors and target cells [1,2], localized gene delivery [3], controlled and sustained vector release [4] and increased bioactivity that promotes gene transfer to epidermal stem cells [5]. In general, gene delivery using biomaterials can take place in either of two settings: (1) substrate-mediated and (2) polymeric-release [6].

*Address for all Correspondence: Stelios T. Andreadis, Bioengineering Laboratory, 908 Furnas Hall, Department of Chemical and Biological Engineering, University at Buffalo, State University of New York, Amherst, NY 14260, tel: (716) 645-2911 ext. 2204, fax: (716) 645-3822, email: E-mail: sandread@eng.buffalo.edu.

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Substrate-mediated gene transfer is realized by immobilization of a gene transfer vector on a solid surface, followed by addition of target cells to initiate gene transfer. It is also known as reverse transfection or solid-phase gene transfer. This system offers elevated concentration of gene delivery vehicle in the microenvironment of the cells, and hence significantly enhances gene transfer over traditional bolus delivery system [1,7,8]. The same strategy has been employed to capture different genes or RNAs on microfabricated surface features for engineering high-throughput cell transfection microarrays [9–11].

Polymeric-release gene delivery - also termed gene-activated matrix delivery, is based on plasmid incorporation into a biomaterial and subsequently release in a controllable manner, e.g. pH, temperature or matrix degradation dependent manner, to transfect target cells [12, 13]. Depending on the polymer formulation, the release kinetic profile can range from days to months [4,14], providing sustained vector release to facilitate tissue regeneration and angiogenesis [15,16].

Fibrin hydrogel is a biodegradable polymer that is formed during blood coagulation. It is readily formed under physiological conditions and hence it is amenable to *in situ* delivery by simple injection at the site of interest, where it quickly polymerizes within seconds. Effective isolation of autologous fibrinogen from the patient's own blood can eliminate the risk of immune rejection and viral transmission from allogeneic fibrinogen. Fibrin is known to support cellular infiltration and proliferation while fibrin degradation products have no adverse effects on cell function or viability. These advantages led to widespread use of fibrin as scaffold for tissue engineering of skin [17–19], ocular [20] and neuronal [21] tissues, tendons and ligaments [22], liver [23], bone [24] and blood vessels [25–28].

In addition to serving as scaffold for cell growth and differentiation, fibrin was also employed for drug and protein delivery. For example, fibrin was used to encapsulate liposome-in-chitosan matrix for sustained release of small molecules [29]. In another setting, fibrin was modified to incorporate multi-domain peptides for covalent binding of growth factors or other proteins. Some domains served as sites for enzymatic conjugation to the matrix, while others were heparin-binding domains for incorporation of heparin-binding growth factors or protease recognition sites for cell-controlled cleavage and release of bioactive molecules in the local microenvironment [30]. Growth factors such as VEGF [31,32], NGF [33,34] and KGF [19] have been incorporated into this system resulting in cell-controlled release with localized therapeutic effects. Fibrin hydrogels were also employed for non-viral gene delivery to promote wound healing [35] or vascularization of ischemic myocardium [36] and more recently fibrin was used to deliver KGF- or eNOS-encoding adenovirus to enhance the wound healing response [37,38].

Despite promising results from the use of fibrin for gene delivery, understanding the fundamental parameters that govern fibrin-mediated transfection remains incomplete. Previous studies focused on ways to immobilize polyplexes on the surface or within fibrin scaffolds [39–42] but essential parameters that may control the transfection process such as fibrinogen concentration, pDNA concentration, as well as the importance of cell-mediated fibrin degradation for productive gene transfer have not been addressed. The current study addressed these issues in the context of two- and three-dimensional microenvironment. We also examined the potential of fibrin hydrogels as a means to achieve spatially localized gene delivery that maybe useful in engineering cell-transfection microarrays or gene delivery *in vivo*.

MATERIALS & METHODS

Vector construction

The *Zoanthus* sp. green-encoding vector (pCSCZ) was derived from the lentiviral vector pCSCG (Miyoshi, 1998) by replacing the GFP in the original vector with ZsGreen between the *NheI* and *XhoI* restriction sites. The DsRed2-encoding vector (pCSCD) was constructed by substituting ZsGreen in pCSCG with DsRed2 between the same sites. The sequence of the cloned genes was confirmed by sequencing with ABI PRISM 3130XL Genetic Analyzers (Applied Biosystems, Foster City, CA).

Cell culture

Human embryonic kidney cells (293T/17, ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 1% (v/v) Antibiotic-Antimycotic (Gibco) and 10% (v/v) fetal bovine serum (FBS; Gibco), at 37°C with 10% CO₂. NIH-3T3 mouse fibroblasts (ATCC) were cultured in DMEM supplemented with 1% (v/v) Antibiotic-Antimycotic and 10% (v/v) bovine serum (Gibco) under the same conditions as 293T/17 cells.

Transfection methods

Three different protocols were used to transfect cells in 24-well tissue culture treated plates (Greiner Bio-One, Monroe, NC). Lipofectamine 2000 (4 µL, Invitrogen, Grand Island, NY) was diluted in 50 µL Opti-MEM-I Reduced Serum Medium (Invitrogen). At the same time 2 µg pDNA was added to 50 µL Opti-MEM-I in a separate conical. In some experiments the amount of pDNA varied between 1–10 µg but the ratio of pDNA/lipofectamine remained the same, namely 1 µg pDNA/2 µl lipofectamine. After 5 min of incubation, the lipofectamine and pDNA were mixed and incubated at RT for at least 25 min. The pDNA/lipofectamine mixture was then added to bovine thrombin (12.5U/mL in PBS; Sigma-Aldrich, St. Louis, MO), which was mixed with plasminogen-depleted human fibrinogen (Enzyme Research Laboratories, South Bend, IN) at 1:4 ratio to form fibrin gels (300 µl per well) with final concentration of 2.5 U/mL thrombin and 4 mg/mL fibrinogen. In some experiments, the final concentration of fibrinogen varied from 1 to 16 mg/mL as indicated. To study the effect of fibrinolytic inhibitors on gene transfer, aprotinin (0 to 100 µg/mL Sigma) or ε-aminocaproic acid (εACA, 0 to 20 mg/mL, Calbiochem, La Jolla, CA), was added to the thrombin-containing mixture before mixing with fibrinogen. For the “in-gel” protocol, cells were also added in the thrombin fraction and 15 min after gelation, the cell-containing hydrogels were overlaid with fresh culture medium (500 µL/well). For the “on-gel” protocol, 15 min after gelation target cells were added on the surface of lipoplex-containing fibrin gels at the indicated densities in 500 µL fresh medium. Finally, cells at the indicated densities were seeded in tissue culture treated 24-well plates in 500 µL fresh medium and transfected with lipoplexes the next day (“no-gel” protocol). One day after transfection, the culture medium was replenished in all samples and thereafter, the medium was changed every 2 days until all gels were degraded and the cells were processed for flow cytometry.

Measurement of transfection efficiency by flow cytometry

Cells were washed once with PBS and detached from the surface with 0.25% Trypsin-EDTA (Gibco). The trypsin was inactivated with DMEM containing 10% serum, cells were centrifuged (5 min; 1500 g) and resuspended in PBS containing 1.0 µg/mL of propidium iodide (PI; Molecular Probes, Eugene, OR) for 15 min at 4°C. Only viable cells (PI-) were used to determine the fraction and fluorescence intensity of transfected (ZsGreen+) cells.

MTT assay for cell viability

Cells were transfected with 1–10 μg pDNA/lipofectamine (lipoplexes) in the presence or absence of fibrin hydrogels as described in “transfection methods”. One day after transfection 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Research Organics, Cleveland, OH) was added to each well and incubated for 3 hr at 37°C, 10% CO_2 . Afterwards, the MTT-containing medium was removed and each sample was incubated with 250 μL human plasmin (0.2 U/mL in PBS; Calbiochem, La Jolla, CA) for 5 hr at 37°C, 10% CO_2 and followed by overnight treatment with 250 μL of 20% sodium dodecyl sulfate (SDS; Fisher Scientific, Fair Lawn, NJ) at 37°C, 10% CO_2 . The optical density of cell lysates was measured at 570 nm with an absorbance microplate reader (SpectraMax 340, Molecular Devices, Menlo Park, CA) and the values were corrected for non-specific background by subtracting the optical density at 650 nm. Gels without cells were treated identically and served as negative controls.

pDNA release from fibrin hydrogels

The amount of pDNA was measured using the dye Hoechst 33342 (Molecular Probes), which fluoresces only when it is bound to double-stranded DNA. A standard curve was generated with known amounts of pDNA (0–2 μg per 0.5 mL buffer) in the absence or presence of lipofectamine (pDNA: lipofectamine = 1 μg : 2 μL) to examine whether lipofectamine affected Hoechst binding to pDNA.

To measure pDNA release from the matrix, lipoplexes (2 μg of pDNA per gel at pDNA:lipofectamine ratio = 1 μg :2 μL) were embedded in fibrin hydrogels with varying fibrinogen concentrations between 1–20 mg/mL and 2.5 U/mL thrombin in 24-well plates (300 μL per well) as described above. Fifteen minutes after gel formation, gels were overlaid with PBS (250 μL per well), which was harvested and replenished with fresh PBS at the indicated times. After 24 hr, human plasmin (0.2 U/mL in PBS; 250 μL per well) was added to degrade fibrin in order to measure the amount of pDNA that remained in each gel. All samples were stored at –20°C until use. To quantify the amount of pDNA, Hoechst 33342 was added to each sample (1:400 dilution; 5 min) and the fluorescence intensity was measured using a fluorescence microplate reader (SpectraMax Gemini, Molecular Devices) with excitation and emission at 360 nm and 460 nm, respectively. The amount of pDNA in each sample was back-calculated from the standard curve.

pDNA-containing fibrin spots and arrays

A solution of thrombin/lipoplex was mixed with fibrinogen to final concentrations of 2.5 U/mL thrombin, 4 mg/mL fibrinogen, 2 μg pDNA and 4 μL lipofectamine. Immediately after mixing, this solution (0.2–2 μL) was rapidly spotted onto 35 mm tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) in order to examine the effect of gel volume on the spot size. The image of each spot was photographed with an ORCA-ER CCD camera (Hamamatsu, Bridgewater, NJ) and the area (A) of the spot was determined using the Image J software. Finally, the spot diameter was calculated as $D = \sqrt{4A/\pi}$.

To create pDNA-containing fibrin arrays, a solution (300 μL) containing fibrinogen (4 mg/mL), thrombin (2.5 U/mL), and lipoplexes (2 μg pDNA and 4 μL lipofectamine) was prepared as mentioned above and 0.3 μL of the mixture containing 2 ng of pDNA was rapidly spotted onto 35 mm tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). After gelation for 15 min at 37°C, 293/T17 cells (3×10^5 cells/cm², which corresponds to confluent density) were added to initiate gene transfer. Culture medium was replenished every 3 days. Five to six days later when all gels were dissolved, transfected cells were visualized at 5X magnification using a Zeiss Axio Observer.Z1 (Carl Zeiss MicroImaging, Thornwood, NY) fluorescence microscope.

and photographed with an ORCA-ER CCD camera (Hamamatsu). Images of all spots were merged using the AxioVision software (Carl Zeiss MicroImaging).

Statistical analysis

The results were analyzed using ANOVA for multiple comparisons followed by the Fisher's t-test for pairwise comparisons (significance $p < 0.05$). The sample size was $n = 3-4$ and each experiment was repeated three times unless indicated otherwise.

RESULTS

Transfection of 293T/17 and NIH-3T3 cells using fibrin

At confluent density (5×10^5 cells per well in 24-well plates, area = 1.9 cm^2), transfection of 293T/17 cells with the in-gel protocol yielded the highest transfection efficiency ($97.8 \pm 1.1\%$ GFP+ cells, Fig. 1A), compared to the no gel ($94.4 \pm 1.5\%$) and on-gel protocol ($86.3 \pm 1.6\%$). In addition, the mean fluorescence intensity of transfected cells – a measure of transgene expression – was the highest for the in-gel protocol (335.8 ± 39.8 , Fig. 1B). Interestingly, more dramatic differences were observed at lower target cell density (3×10^4 cells per well). Although the in-gel protocol consistently yielded the highest level of gene transfer ($96.5 \pm 1.8\%$ GFP+ cells, Fig. 1A), the no gel and on-gel protocol yielded only $77.1 \pm 8.2\%$ and $50.9 \pm 2.1\%$ GFP+ cells, respectively. In agreement to transfection efficiency, the level of transgene expression was the highest using the in-gel protocol (Fig. 1B). In contrast, transfection was not observed by any of the aforementioned protocols in the absence of lipofectamine (data not shown).

Next, we tested whether fibrin-mediated gene transfer could be applied with other cell types such as mouse fibroblasts (NIH-3T3 cells). Similar to 293T/17 cells, the in-gel protocol yielded the highest fraction of transfected cells and transfection at higher cell densities generally resulted in higher transfection efficiency. At high cell density (1.5×10^5 cells per well), the in-gel and on-gel protocols yielded comparable transfection efficiency, which was significantly higher than the control, no gel transfection (Fig. 2A). At low cell density (2×10^4 cells per well), we observed the highest difference between fibrin-mediated and liquid-phase gene transfer. Specifically, the transfection efficiency remained highest for the in-gel followed by the on-gel protocol, but the control, liquid-phase transfection decreased dramatically (Fig. 2A). Similar to transfection efficiency, fibrin-mediated transfection yielded higher transgene expression than control, at high and low cell densities (Fig. 2B).

Cell viability during fibrin-mediated transfection

Next we examined whether transfection in the presence of fibrin hydrogels decreased the cytotoxic insult induced by pDNA lipoplexes. To this end, we transfected NIH-3T3 cells with the aforementioned protocols and measured cell viability using the MTT assay. The results were normalized to viability of control cells that were never exposed to lipoplexes. This experiment was conducted with NIH-3T3 cells that were highly sensitive to lipofectamine 2000 at low cell density.

We found that cell viability depended on the amount of pDNA lipoplexes, cell density, and the transfection protocol. At high cell density (1.5×10^5 cells; Fig. 3A), the on-gel protocol did not show significant decrease in cell number for pDNA lipoplexes containing between 1–10 μg pDNA (pDNA:lipofectamine = $1 \mu\text{g}/2 \mu\text{l}$ for all pDNA amounts tested). For in-gel transfection, viability remained high at 1 and 2 μg pDNA but decreased to about 65% at 10 μg pDNA per gel. In contrast, viability decreased significantly for the liquid-phase transfection ranging from less than 60% even at 1 μg pDNA to less than 40% at 10 μg pDNA per gel.

At low target cell density (2×10^4 cells; Fig. 3B) viability was further compromised in liquid-phase transfection exceeding 90% reduction when lipoplexes contained more than 2 μ g pDNA per gel. Viability also decreased with increasing pDNA concentration for the in-gel protocol albeit to a much lesser extent. Notably, cell viability remained high for the on-gel protocol at all pDNA concentrations tested.

pDNA dose and transfection efficiency

Subsequently, we determined the amount of pDNA that yielded the highest level of fibrin-mediated gene transfer by embedding various amounts of pDNA (1–10 μ g; complexed with lipofectamine at 1 μ g:2 μ l ratio) in fibrin hydrogels during polymerization. Both the in-gel and on-gel protocols showed increased gene transfer efficiency with increasing pDNA from 1 to 2 μ g per gel but higher amounts of pDNA decreased the fraction of transfected cells and the level of transgene expression as measured by fluorescence intensity (Fig. 4).

Effect of fibrinogen concentration on gene transfer

To determine whether fibrinogen concentration affects transfection efficiency, pDNA lipoplexes were embedded in fibrin gels containing various concentrations of fibrinogen (1–16 mg/mL) and target cells were transfected with the on-gel or in-gel protocols. Interestingly, the effect of fibrinogen on gene transfer depended on cell type. For 293T/17 cells gene transfer decreased with increasing fibrinogen concentration from 1 to 4 mg/mL. Higher concentrations of fibrinogen did not reduce gene transfer further (Fig. 5A). The in-gel protocol showed a similar trend but the decrease was more gradual. Transgene expression was higher with the in-gel protocol but decreased significantly with increasing fibrinogen concentration. On the other hand, the on-gel protocol yielded lower levels of transgene expression at all fibrinogen concentrations tested (Fig. 5B).

Surprisingly, gene transfer to NIH-3T3 cells depended on fibrinogen concentration in a different way according to the protocol used. For the on-gel protocol, the fraction of transfected cells and transgene expression decreased with increasing fibrinogen concentration beyond 1 mg/mL. However, when cells were transfected within the fibrin gel, both the fraction of transfected cells and transgene expression increased with fibrinogen concentration reaching a maximum at 8 mg/mL. Higher fibrinogen concentration (16 mg/mL) decreased the fraction and fluorescence intensity of transfected cells (Fig. 5C, D).

Effect of fibrinolytic inhibitors on gene transfer

To examine whether fibrin degradation was necessary for successful gene transfer, we prepared fibrin gels (fibrinogen: 4 mg/mL) in the presence of various concentrations of two fibrinolytic inhibitors, aprotinin or ϵ -aminocaproic acid. We found that addition of aprotinin decreased gene transfer for both 293T/17 and NIH-3T3 cells in a dose dependent manner. Interestingly, the on-gel protocol was influenced to a larger extent by aprotinin compared to the in-gel protocol. At 100 μ g/mL aprotinin, the fraction of ZsGreen+ 293T/17 cells decreased from 85% to 10% (90% reduction) for the on-gel protocol and from 94% to 44% (55% reduction) for the in-gel protocol (Fig. 6A). Similarly results were observed for NIH-3T3 cells. While the percent of transfected cells decreased from 43 to 12% (72% reduction) using the on-gel protocol, the reduction was less dramatic - from 74 to 55% (25% decrease) - for the in-gel protocol (Fig. 6C).

Surprisingly, aprotinin decreased transgene expression of 293T/17 cells to a greater extent for the in-gel than the on-gel protocol (64% vs. 43% reduction at 100 μ g/mL aprotinin; Fig. 6B). On the other hand, aprotinin did not affect NIH-3T3 transgene expression significantly for either protocol (Fig. 6D). As expected, aprotinin had no effect on liquid-phase (no gel) gene

transfer, indicating that this fibrinolytic inhibitor did not affect the intrinsic steps of transfection (Fig. 6).

These findings were verified by use of a second fibrinolytic inhibitor, ϵ -amino-caproic acid (eACA). We found that the effect of eACA on gene transfer also depended on the mode of gene transfer (2D vs. 3D). Regardless of cell type, the on-gel protocol showed a sharp decrease in transfection efficiency and transgene expression as eACA increased from 0 to 1 mg/mL, with no further decrease up to 20 mg/mL eACA. On the other hand, transfection with the in-gel protocol decreased more gradually with increasing eACA concentration, reaching the lowest transfection efficiency and transgene expression level at 5 mg/mL eACA (Fig. 7). As expected, eACA had no effect on gene transfer in the absence of fibrin hydrogels. Taking together, these data showed that fibrin degradation by target cells is necessary for successful gene transfer.

Release kinetics of pDNA lipoplexes from fibrin hydrogels

Since fibrin hydrogels are highly porous, it was not clear whether the pDNA lipoplexes remained in the fibrin matrix or diffused out of the gels over time thereby transfecting target cells as in the traditional matrix-free transfection protocol. To address this question pDNA lipoplexes were embedded in fibrin hydrogels prepared with 1, 4 or 20mg/mL fibrinogen and the amount of pDNA in the medium was measured at the indicated times using the nucleic acid binding dye, Hoechst. The fluorescence intensity was similar for naked or lipofectamine-complexed pDNA at the same concentrations (data not shown) suggesting that Hoechst binds to pDNA with the same efficiency and fluoresces with the same quantum yield whether pDNA is associated with lipofectamine or not.

For 1 mg/mL fibrinogen, a significant amount of pDNA (40–50%) diffused out of the gel at early times (< 0.5 hr; burst release) with little if any more pDNA diffusing out of the gel up to 24 hr thereafter. In contrast, when the fibrinogen concentration increased to 4 mg/mL, no pDNA diffused out of the gel at any time. Indeed, plasmin degradation of fibrin gels 24 hr later recovered all pDNA that was initially embedded (Fig. 8), indicating that pDNA lipoplexes remained in the fibrin matrix for the time period tested.

Transfection arrays using fibrin-embedded pDNA lipoplexes

Since pDNA is efficiently trapped within fibrin gels, we examined whether fibrin can be used to achieve spatially localized gene delivery. To this end, we first determined the effect of hydrogel volume on spot size by manually pipetting different volumes of fibrin on glass slides. As expected the size of each spot depended on the volume of the deposited solution, with the smallest volume of 0.2 μ L resulting in $\sim$ 1 mm diameter spots (Fig. 9A). To show that gene transfer was localized to each spot we arrayed ZsGreen or DsRed encoding plasmids in alternate columns and seeded 293T/17 cells at high density (2.5×10^5 per cm^2) to form an almost confluent monolayer. Although cells covered the whole surface, ZsGreen- or DsRed-expressing cells were observed only on the spots containing the corresponding pDNA. There were very few transfected cells between the spots and cross-contamination was not observed i.e. ZsGreen+ cells in DsRed containing spots or vice-versa (Fig. 9B). Taken together, these data demonstrate that transfection was spatially confined to the cells on pDNA containing spots, suggesting that fibrin hydrogels can be used for engineering transfection arrays for high-throughput experiments.

DISCUSSION

Fibrin hydrogels have been used extensively in tissue engineering and regenerative medicine e.g. to accelerate reepithelialization and wound healing [17,18,43], engineer vascular grafts

[26–28,44], repair articular cartilage defects [45] or promote vascularization [32]. Fibrin is particularly attractive because it is a natural biomaterial that acts as a scaffold for tissue regeneration during wound healing and has been widely used as an adhesive in plastic and reconstructive surgery. In combination with novel methods that have been developed to incorporate peptides and growth factors [19,32–34,46,47] fibrin formulations may be ideal for cell, growth factor and gene delivery to accelerate tissue regeneration. Here we demonstrate the use of fibrin hydrogels to achieve highly efficient, cell-controlled and spatially localized gene delivery to target cells.

Our results showed that co-localization of target cells and lipoplexes within fibrin hydrogels (in-gel) yielded higher gene transfer efficiency compared to the case where cells were seeded on top of the matrix (on-gel). In this mode of delivery the lipoplexes are immobilized in a 3D microenvironment surrounding the target cells, which degrade the gel locally and uptake the pDNA. The presence of lipoplexes in the immediate vicinity of target cells may be one reason for the increased gene transfer efficiency within the 3D matrix.

In addition, fibrin hydrogels may improve gene transfer by decreasing the cytotoxic effects of the transfection agents such as lipofectamine. Indeed, our results showed that cytotoxicity was significantly reduced when lipoplexes were immobilized within the hydrogels compared to traditional transfection protocol, especially at low cell densities when target cells were more susceptible to apoptosis. Although the mechanism through which fibrin increased cell viability is not understood, interaction of cells with fibrin – possibly through integrin $\alpha\beta3$ – may suppress pathways that are activated by lipofectamine such as caspase activation and reactive oxygen species generation [48], ultimately promoting cell survival.

Alternatively, increased survival might be attributed to the localized nature of fibrin-mediated gene delivery. In a 3D matrix target cells – whether on top or inside the gel – encounter only lipoplexes positioned within their local microenvironment following fibrin degradation. While in traditional gene transfer target cells are exposed to high concentrations of lipoplexes at once, in fibrin-mediated delivery cells are exposed to lipoplexes gradually as they degrade the gel. This gradual exposure to cytotoxic agents may attenuate activation of pro-apoptotic pathways thereby minimizing cell death. This argument implies that there may be a critical level of lipofectamine that is required to induce cytotoxicity. The critical lipofectamine concentration may vary depending on cell type, as NIH-3T3 cells displayed much higher cell death upon exposure to lipoplexes than 293T/17 cells. With lipoplexes embedded in fibrin matrix, the critical lipofectamine concentration may be reached at a rate that may depend on the rate of fibrin degradation, which in turn varies according to cell type and fibrinogen concentration. Accordingly, our results showed that gene transfer to NIH3T3 depended on fibrinogen concentration in a different way than 293T/17 cells. Therefore, fibrinogen and pDNA concentration may need to be optimized for each cell type in order to yield maximum transfection efficiency and minimum cytotoxicity.

Surprisingly, increasing pDNA above 2 μg per gel did not yield higher gene transfer efficiency for either cell type. To the contrary, we observed a significant reduction in the fraction of transfected cells and transgene expression. This decrease maybe – at least in part – due to the cytotoxic effects of higher lipofectamine concentrations since the pDNA:lipofectamine ratio remained constant. However, high pDNA concentrations decreased gene transfer in both protocols but compromised viability only when target cells were incorporated within the matrix. The viability of target cells on the surface of fibrin gels remained unaffected, suggesting that other factors such as cell-matrix interactions in 2D vs. 3D microenvironment may also affect gene transfer possibly by modulating cellular physiology.

Experiments with fibrinolytic inhibitors showed that gene transfer decreased with increasing eACA or aprotinin concentration suggesting that fibrin degradation by target cells is necessary for gene delivery. Since matrix degradation is a major limiting step that depends on target cells, fibrin-mediated transfection may be a cell-controlled process. Neither eACA nor aprotinin affected traditional, liquid transfection and no cytotoxic effects were observed even at the highest concentrations tested, thereby excluding the possibility that these inhibitors may have acted by affecting cell viability. Our data also showed that the on-gel protocol was more sensitive to the presence of fibrinolytic inhibitors than the in-gel protocol. This may be because the probability of gene encounter by target cells may be different in 2D vs. 3D microenvironment. When embedded in the hydrogel, target cells are surrounded by lipoplexes and therefore, minimal fibrin degradation may be required for the cells to encounter and uptake the lipoplexes.

In agreement with the requirement for fibrin degradation, transfection of 293T/17 cells decreased with increasing fibrinogen concentration for both the in-gel and on-gel methods. Similar reduction in transfection efficiency was also observed for NIH-3T3 cells that were transfected on the surface of fibrin gels. In 2D transfection gel degradation may be accompanied by cell migration into the matrix where the lipoplexes reside. Cell migration may increase the probability of pDNA-target cell encounter or may affect pDNA uptake through activation of intracellular signaling pathways or cytoskeletal reorganization. At high fibrinogen concentrations gel degradation and migration of cells into the matrix may be impaired thereby decreasing the transfection efficiency.

Notably, the opposite result was observed with NIH-3T3 cells that were embedded within the fibrin matrix. In this case, transfection efficiency and transgene expression increased significantly as the concentration of fibrinogen increased from 1 to 8 mg/mL. In 3D matrices mesenchymal cells such as fibroblasts, myofibroblasts and smooth muscle cells tend to generate mechanical forces and contract fibrin hydrogels, thereby bringing lipoplexes closer to the cell surface. Contractility may be enhanced in denser matrices that may provide more points of contact between cells and the matrix. However, when fibrinogen concentration exceeds a certain level (in our experiments 16 mg/mL), the fibrin network may be too dense for cells to contract or degrade, thereby decreasing transfection efficiency. As we showed previously, at high fibrinogen concentrations cell viability may also be compromised possibly due to impaired nutrient diffusion in the matrix [49]. More experiments are required to determine the mechanism(s) at work and the role – if any – of cell migration vs. contractile force generation during transfection in 2D vs. 3D microenvironments.

Although at low fibrinogen concentrations (1 mg/mL) lipoplexes could diffuse out of the gels, higher fibrinogen concentrations (4 or 20 mg/mL) prevented lipoplex diffusion, possibly by reducing the average pore size [50]. Intermediate fibrinogen concentrations may be desirable as they prevent pDNA diffusion and at the same time they can be degraded by target cells. This is important for both in vivo and in vitro applications of this gene delivery system. Implantation of lipoplex-containing fibrin gels in vivo may allow transfection of tissue cells that migrate into the matrix thereby minimizing non-specific gene delivery to surrounding tissues or into the systemic circulation. In vitro, fibrin hydrogels can be used to “print” pDNA onto defined spots to achieve gene delivery only to cells that spatially co-localized with the lipoplexes.

Specifically, fibrin-based transfection may provide an effective means of generating cell transfection arrays to achieve dynamic measurements of gene expression in real-time using promoter elements to drive expression of marker genes e.g. EGFP or DsRed. Cell transfection arrays may be useful for high throughput overexpression or knockdown of hundreds of genes to evaluate their effects on a variety of biological problems ranging from cancer signaling to embryonic stem cell differentiation. In contrast to surface immobilization strategies that

employ covalent modification, fibrin gels entrap pDNA non-covalently thereby facilitating its uptake by target cells. In addition, 3D hydrogels can encapsulate higher amount of pDNA compared to 2D spots of the same surface area thereby increasing the likelihood of gene transfer. Taken together, our results suggest that lipoplex-containing fibrin-based microarrays that yield high efficiency of gene transfer in a localized and cell-controlled manner may provide an effective platform for high-throughput transfection experiments.

CONCLUSIONS

Embedding lipoplexes within fibrin hydrogels leads to efficient gene delivery while minimizing cytotoxicity. Fibrin-mediated transfection depends on the position of target cells on the surface or within the hydrogel, suggesting that gene transfer in this setting may be a function of cell-matrix interactions and the local microenvironment. Appropriate fibrinogen concentration ensures confinement of pDNA within the hydrogel thereby necessitating fibrin degradation for gene transfer to occur. This mode of cell-controlled gene delivery enables transfection in a spatially-controlled manner with potential applications in engineering transfection microarrays to facilitate high-throughput studies.

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Figure 1. Co-localizing targets cells and transfection complexes inside fibrin hydrogels yields high transfection efficiency

293T/17 target cells at 5×10^5 or 3×10^4 cells per gel were transfected using the indicated protocol. **(A)** % transfected (ZsGreen+) cells and **(B)** mean fluorescence intensity of transfected cells were determined by flow cytometry. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment ($n=3$). The asterisks (*) denote $p < 0.05$ between the indicated samples and the in-gel protocol at the same cell density.

Figure 2. Immobilizing targets cells and transfection complexes inside fibrin hydrogels enhances gene transfer to NIH-3T3 cells

NIH-3T3 target cells at 1.5×10^5 or 2.0×10^4 cells per gel were transfected using the indicated protocol. (A) % transfected (ZsGreen+) cells and (B) mean fluorescence intensity of transfected cells were determined by flow cytometry. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment (n=3). The asterisks (*) denote p<0.05 between the indicated samples and the in-gel protocol at the same cell density.

Figure 3. Fibrin hydrogels prevent pDNA lipoplex induced cytotoxicity

NIH-3T3 cells at (A) 1.5×10^5 cells or (B) 2×10^4 cells per gel were transfected with various amounts of pDNA lipoplexes using the indicated protocols. The next day cell viability was determined using the MTT assay and optical density values were normalized to that of cells that were never exposed to lipoplexes. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment (n=2). The asterisks (*) denote $p < 0.05$ between the indicated samples and the no-gel protocol at the same pDNA level.

Figure 4. Highest gene transfer efficiency is attained at intermediate pDNA concentrations
pDNA at the indicated amounts was mixed with lipofectamne 2000 at 1g:2l ratio. (A, B) 293T/17 (5×10^5 cells per gel) or (C, D) NIH-3T3 (1.5×10^5 cells per gel) target cells were transfected with the indicated protocols. (A, C) % transfected (ZsGreen+) cells and (B, D) mean fluorescence intensity of transfected cells were determined by flow cytometry. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment (n=3). The asterisks (*) denote $p < 0.05$ between the indicated samples and the 2 g pDNA/gel sample transfected with the same protocol.

Figure 5. Fibrinogen concentration strongly affects fibrin-mediated gene transfer
 (A, B) 293T/17 (5×10^5 cells per gel) or (C, D) NIH-3T3 (1.5×10^5 cells per gel) target cells were transfected with the on-gel or in-gel protocol using fibrin gels prepared with the indicated concentrations of fibrinogen. In this experiment, each gel contained 0.5g of pDNA and 1l lipofectamine. (A, C) % transfected (ZsGreen+) cells and (B, D) mean fluorescence intensity of the transfected cells were measured by flow cytometry. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment (n=3). The asterisks (*) denote $p < 0.05$ between the indicated samples and the 1 mg/mL fibrinogen sample transfected with the same protocol.

Figure 6. Aprotinin decreases fibrin-mediated gene transfer in a dose dependent manner (A, B) 293T/17 (5×10^5 cells per gel) or (C, D) NIH-3T3 (1.5×10^5 cells per gel) target cells were transfected with the in-gel or on-gel protocols in the presence of the indicated aprotinin concentrations. (A, C) % transfected (ZsGreen+) cells and (C,D) mean fluorescence intensity of transfected cells were measured by flow cytometry. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment (n=3). The asterisks (*) denote $p < 0.05$ between the indicated samples and the sample without aprotinin within the same transfection protocol.

Figure 7. eACA decreases fibrin-mediated gene transfer in a dose dependent manner (A, B) 293T/17 (5×10^5 cells per gel) or (C, D) NIH-3T3 (1.5×10^5 cells per gel) target cells were transfected with the in-gel or on-gel protocols in the presence of the indicated eACA concentrations. (A, C) % transfected (ZsGreen+) cells and (C,D) mean fluorescence intensity of transfected cells were measured by flow cytometry. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment (n=3). The asterisks (*) denote $p < 0.05$ between the indicated samples and the sample without eACA within the same transfection protocol.

Figure 8. Successful entrapment of pDNA lipoplexes within fibrin hydrogels

pDNA lipoplexes were embedded in fibrin hydrogels containing fibrinogen at the indicated concentrations and overlaid with PBS. At the indicated times after polymerization, the buffer was collected and gels were overlaid with fresh PBS. The amount of pDNA lipoplexes remaining in the matrix was measured 24 hr later, after plasmin (PD) degradation of fibrin. The amount of pDNA in each sample was quantified using Hoechst. All values represent the mean $\pm$ SD of quadruplicate samples in a representative experiment (n=2 for (A); n=3 for (B)).

Figure 9. Cell controlled gene delivery on defined pDNA-containing fibrin hydrogel spots
(A) Fibrin hydrogels at the indicated volumes were spotted on tissue culture treated plastic. The diameter of each spot was calculated from its surface area and plotted as the mean $\pm$ SD of quadruplicate samples ($n=4$). A representative 1 L fibrin gel is shown (**inset**). **(B)** ZsGreen or DsRed encoding-plasmids embedded in fibrin hydrogels were spotted in alternate columns. After gelation, 293T/17 cells were seeded to initiate gene transfer. A representative 5 $\times$ 5 transfection array is shown.