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A Temporal Gene Delivery System Based on Fibrin Microspheres

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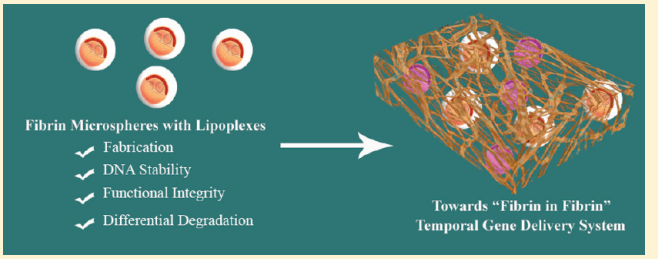
ABSTRACT: Combining complementary nonviral gene delivery vehicles such as tissue engineering scaffolds and liposomes not only is a promising avenue for development of safe and effective gene delivery system but also provides an opportunity to design dynamic extended release systems with spatiotemporal control. However, the DNA loading capacity of scaffolds such as fibrin is limited. Fibrin microspheres carrying DNA complexes can be utilized to extend the capacity of fibrin scaffold. Here, in a proof of concept study, the feasibility of fibrin microspheres for extending gene delivery capacity is described. Toward this goal, fibrin microspheres encapsulating lipoplexes were fabricated. The structural and functional integrity of DNA was assessed respectively by gel electrophoresis and an *in vivo* pilot study, using endothelial nitric oxide synthase (eNOS) as a model therapeutic gene in a rabbit ear ulcer model of compromised wound healing. The results confirmed structural integrity and successful delivery and functional integrity, assessed qualitatively by angiogenic effect of eNOS. Finally, as a step toward development of a "fibrin in fibrin" temporal release system, fibrin microspheres were shown to degrade and release DNA differentially compared to fibrin scaffold. It can thus be concluded that fibrin microspheres can be utilized for gene delivery to extend the capacity of a fibrin scaffold and can form a component of a "fibrin in fibrin" temporal release system.



KEYWORDS: fibrin microspheres, gene delivery, fibrin scaffold, lipoplexes

INTRODUCTION

In recent years, gene therapy, involving manipulation of genetic makeup either to replace a defective gene or to up- or down-regulate the disregulated genes/molecular pathways, has become a common and promising research approach. This surge can be attributed to a number of factors such as better diagnostic tools and advances in molecular biology to identify genetic diseases or diseases with a plausible genetic element. However, the journey of gene therapy from laboratory to clinics is stalled due to lack of an ideal gene delivery system with high transfection efficiency, extended but easily controllable gene expression and optimal safety profile. One of the research avenues toward solving this issue, being investigated recently, is to employ complementary delivery systems, such as nonviral carriers and tissue engineering scaffolds in a combinatorial approach.[1]

Fibrin is a natural polymer formed in the body on need basis and degraded in a highly controlled manner. Thus, the host has full machinery to degrade fibrin scaffolds in a very systematic manner, highly desirable in tissue engineering applications. Also, fibrin scaffolds have not been reported to elicit any untoward inflammatory response, abnormal foreign body reaction, tissue necrosis, or fibrosis.[2] Fibrin is highly versatile and can be fabricated in various forms such as simple or composite gel,[3−5] sphere-templated scaffolds,[6] as gels with extracellular protein coated spheres[7] or as fibrin microspheres.[8−11] Although liposomal systems have been shown to be safer than viral carriers, the factors hindering the success of a liposomal approach appear to be instability of the liposome−DNA complex (lipoplex), toxicity of the cationic lipid, and short half-life of the complexed DNA.[12] The use of scaffolds not only provides opportunity, by virtue of their macro- and microstructure, to manipulate dynamics of liposomal gene delivery, creating controlled extended[13−15] or spatiotemporal release systems[16−19] and/or reservoir systems, but also enhances stability of liposomes[20,21] while reducing their toxicity.[17,22] In order to exploit this complementary benefit, we decided to fabricate a scaffold that has the complementary benefits of fibrin and lipoplexes.

We have previously described a fibrin−lipoplex system to deliver two reporter genes simultaneously for an extended period of seven days postsurgery.[14] Toward improvement of this system for therapeutic use, it was hypothesized that a "fibrin in fibrin" system, devised by incorporation of another fibrin system in the form of microspheres within the fibrin gel, not only would enhance the DNA carrying capacity of the system but at the same time may open newer avenues for development of concentration gradients or a temporal release system as against simultaneous delivery. A number of studies have exploited geometry of microspheres fabricated from either natural or synthetic materials for gene delivery. This spherical/spheroid geometry along with suitable size provides certain advantages such as high surface area and easy fabrication of composite scaffolds.

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So, to realize our hypothesis, the primary objective of this "proof of concept" study was to fabricate fibrin microspheres capable of encapsulating lipoplexes for gene delivery. To the best of our knowledge, this is the first study describing the use of fibrin microspheres for gene delivery. Toward this goal, we fabricated fibrin microspheres encapsulating lipoplexes, by a modified "preheated oil emulsion" method developed in our laboratory. This study investigates the structural and functional integrity of the DNA to establish that the fabrication procedure does not affect the integrity of the DNA. As a step toward development of the proposed "fibrin in fibrin" system, the degradation of fibrin microspheres and the release of DNA were studied in comparison with the fibrin scaffold to fathom the plausibility of spatiotemporal control over the release kinetics from the system.

MATERIALS AND METHODS

Fabrication of Fibrin Microspheres with Lipoplexes. Fibrinogen and thrombin were sourced from Baxter Healthcare, Vienna. Fibrin microspheres (FM) were fabricated by modification of a "preheated oil emulsion" method.[8] Briefly, fibrinogen solution (20 mg/mL) and thrombin solution (4 IU), containing lipoplexes (with 25 μg of pDNA), were mixed. Prior to formation of gel, while it is still in liquid phase, the solution was poured dropwise into a preheated mineral oil at 75 °C and stirred overnight at 250 rpm to evaporate the water phase. Two surfactants were used. Tween20 was used in water phase and Spam80 was used in oil phase. After overnight stirring, the oil was decanted. This was done in two phases, first at low speed (1000 rpm) centrifugation and then at high speed (4500 rpm). The FM were washed by ethanol, hexane and then air-dried.

Transmission and Scanning Electron Microscopy and Size Analysis. Formation of microspheres was confirmed by transmission electron microscopy (TEM). The FM were resuspended in water, and a drop was placed directly on a Formvar carbon coated grid (Agar Scientific) and viewed under TEM (Hitachi H7500 transmission electron microscope). In addition, scanning electron microscopy (SEM) was used for surface morphological analysis. The samples were first gold coated and visualized under vacuum using SEM (Hitachi S-4700 scanning electron microscope). The mean size of FM was measured by laser light diffraction using a Zetasizer (Nano ZS, Malvern). The air-dried FM were suspended indirectly in water and vortexed gently. Three experimental replicates were used for these measurements. The particle size was expressed as mean diameter (± standard deviation).

Plasmid DNA Labeling and Confocal Microscopy. Plasmid DNA was labeled by Cy 5 using *Label* IT Cy5 Labeling Kit (Mirus Bio, Madison, WI) using the manufacturer's instructions. Briefly, plasmid DNA and Cy 5 marker were mixed together and incubated at 37 °C for 1 h, with a quick spin after 30 min to reduce the effect of evaporation. The labeled plasmid DNA was then extracted by using the spin column provided in the kit and stored at −20 °C until use. Fibrin microspheres fabricated with the labeled DNA complexes were then studied under a confocal microscope (Zeiss LSM 510 Axiovert inverted confocal microscope) to confirm encapsulation of DNA. Fibrin microspheres with labeled DNA complexes were further labeled by FITC and were also studied under confocal microscope (Zeiss LSM 510 Axiovert inverted confocal microscope).

Stability of DNA. The integrity of plasmid DNA within the FM was assessed by agarose gel electrophoresis. The fibrin microspheres were dissolved in 100 nM human plasmin for 45 min, and the released lipoplexes were treated with Triton-X to get free DNA. Agarose gel electrophoresis was then performed in 0.7% (w/v) agarose gels containing 0.01% SYBR safe for visualization for 30 min at 100 V. A 1kb DNA ladder was also run. The resultant gels were visualized under UV transilluminator at wavelength of 365 nm.

***In Vivo* Model.** The ability of FM to deliver the gene of interest *in vivo* was investigated qualitatively. An alloxan induced hyperglycemic model of rabbit ear ulcer was utilized for this purpose. Two New Zealand white rabbits (3−3.5 kg) were used in the study. The protocol was approved by the ethics committee of the National University of Ireland, Galway, and the study was conducted under a license granted by the Department of Health and Children, Dublin, Ireland. Rabbits were housed in individual cages with a 12 h light/dark cycle and controlled temperature and humidity. Rabbits were fed a standard chow diet and water ad libitum.

Induction of Hyperglycemia. Rabbits were sedated with subcutaneous injection of 1 mL/kg acepromazine. Hair was shaved off at the back of the ears, and an anesthetic cream (EMLA, AstraZeneca, USA) was rubbed into the back of the ear and left in situ for 20 min. Alloxan (150 mg/kg) (Sigma−Aldrich, Ireland) in 30 mL of saline was prepared and administered via an ear vein using a butterfly syringe, at a rate of 1.5 mL/min. Alloxan is known to cause necrosis of pancreatic islets, thereby inducing hyperglycemia.[23] After alloxan treatment, water containing one tablespoon of glucose per liter was given to the rabbits for a 48 h period, to avoid possible hypoglycemia. Blood glucose readings were taken daily, using blood glucose test strips and meter (Accu-chek test advantage meter, Accu-chek advantage II strips, Roche Diagnostics, United Kingdom). Food and water intakes were monitored daily. Hyperglycemia was confirmed if blood glucose readings were in the range of 20−28 mmol/L. Insulin treatment was not required to control hyperglycemia in any animals.

Surgical Procedure. Four weeks post-alloxan treatment, rabbits were anesthesized using intramuscular injection of 0.1 mL/kg xylazine and 0.12 mL/kg ketamine. This is half the dose of anesthesia, as a full dose will likely prove fatal under hyperglycemic conditions. Two 6 mm punch biopsy wounds were created in each ear, using sterile disposable 6 mm diameter punch biopsies (Panvet, Ireland), exposing bare cartilage. Each wound was treated with one of two randomized treatment groups: no treatment or fibrin gel containing fibrin microspheres encapsulating lipoplexes with eNOS plasmid DNA. Wounds were covered with a polyurethane dressing (Opsite, Smith and Nephew Ltd.) until day 7. At day 7, rabbits were euthanized. At necropsy, ears were surgically removed and cut across the center line. One half of the ear was fixed in formalin for histological and immunohistochemical analysis, in order that a cross section of the wound could be analyzed. The other half was stored in RNA*later* at −80 °C for further analysis.

Histology and Immunohistochemistry. Formalin fixed paraffin embedded sections were cut at 5 mm thickness. Six consecutive sections were saved from the block, as soon as the tissue was reached in the block. This ensured that all sections were saved at the cross section of the wound. Identification of blood vessels was confirmed by immunohistochemistry with endothelial cell marker CD31 using standard protocol.

Briefly, enzymatic antigen retrieval was carried out at 37 °C using 1X proteinase K (20 mg/mL, Sigma-Aldrich) solution in TE buffer (50 mM Tris Base, 1 mM EDTA, pH 8.0, Sigma-Aldrich).

Primary antibodies used were monoclonal mouse anti-CD31 (DakoCytomation, Dublin, Ireland) (1:30 in 0.01 M PBS containing 1% BSA, 0.1% cold fish skin gelatin), with an incubation time of 90 min at room temperature. Blocking buffer was added to three slides as negative control. Endogenous peroxidase was blocked with 3% hydrogen peroxide (Sigma-Aldrich). Secondary goat anti-mouse IgG (1:100 in 0.01 M PBS, Dako Cytomation, Ireland) was applied for 45 min at room temperature, followed by streptavidin−avidin−biotin complex HRP (DakoCytomation, Ireland), and developed using DAB chromagen (Sigma-Aldrich, Ireland).

RNA Isolation and Real Time PCR. For isolating RNA, the wound tissue from treated and control groups was first crushed in pestle and mortar under liquid nitrogen and then homogenized in 1 mL of Tri-Reagant per sample. RNA was separated by chloroform and precipitated by ethanol. The samples were then applied to Qiagen RNAeasy column and RNA was extracted according to the manufacturer's instructions (Qiagen RNAeasy kit). RNA was collected in RNase free water, and the purity and concentration were checked using a spectrophotometer (ND-1000 UV−vis, NanoDrop Technologies, USA). The extracted RNA was reverse-transcribed using ImProm-II reverse transcription system (Promega) to obtain cDNA. Real time PCR was then carried out using a StepOnePlus real-time PCR system, software version 2.1 (Applied Biosystems). The relative expression of eNOS was normalized to 18S rRNA as endogeneous control and calculated by comparative CT method. Table 1 details the specific primer sequences used.

Table 1. Primer Sequences Used in the qPCR

eNOS_forward	5′-CTG AGA GAC CAG CAG AGA TAC CAC-3′
eNOS_reverse	5′-CTG AAG CTC TGG GTC CTG AT-3′
18S_forward	5′-GTA ACC CGT TGA ACC CCA TT-3′
18S_reverse	5′-CCA TCC AAT CGG TAG TAG CG-3′

Degradation of Fibrin Microspheres vs Fibrin Gel. To compare the degradation of FM with fibrin gel, 1 mg of FM was incubated with 2 mL of 0.1 N NaOH for 24 h at room temperature. The supernatant was collected and subjected to reduced 4−12% gradient SDS−PAGE. Supernatant from fibrin gel under same conditions was used as control. Next, the supernatant from FM in 0.1 N NaOH was discarded and FM were visualized under scanning electron microscope to qualitatively assess the degradation.

***In Vitro* Release Study.** Cumulative DNA release from fibrin gel and microspheres was assayed to compare their DNA release profile. For this study, fibrin gel and microspheres containing lipoplexes were incubated with 0.1 N NaOH at 37 °C with continuous shaking. Samples from supernatant collected at 0, 1/2, 1, 6, 12, 24, and 48 h and stored at −20 °C. Equal amounts of fresh 0.1 N NaOH were replaced each time the samples were collected. The quantity of DNA in each collected sample was assessed using PicoGreen assay using manufacturer's instructions (Quant-iT PicoGreen dsDNA Reagent, Invitrogen). All the experiments were done in triplicate.

RESULTS AND DISCUSSION

Over a decade ago, fibrin microspheres, termed then as fibrin derived microbeads, were first developed as cell carriers. These fibrin microbeads were shown to be haptotactic to cells such as endothelial cells, smooth muscle cells and fibroblasts, and their

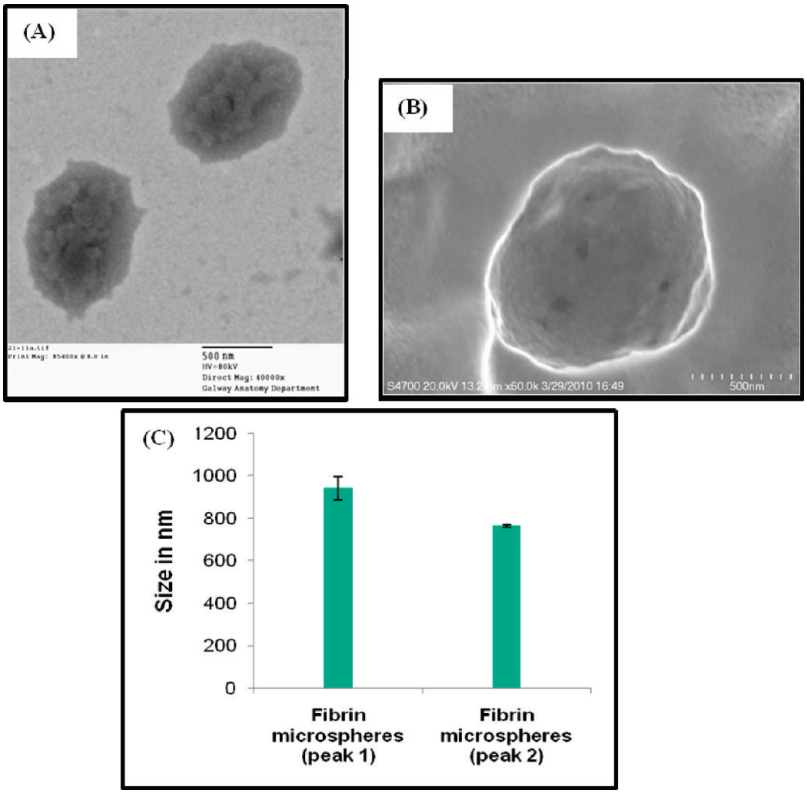

Figure 1. Formation of fibrin microspheres. (A) TEM and (B) SEM micrographs showing fibrin microspheres. (C) Graph showing the results of size analysis (n = 3, error bars represent standard deviation). Two peak sizes, ∼700 nm and ∼1000 nm, were observed.

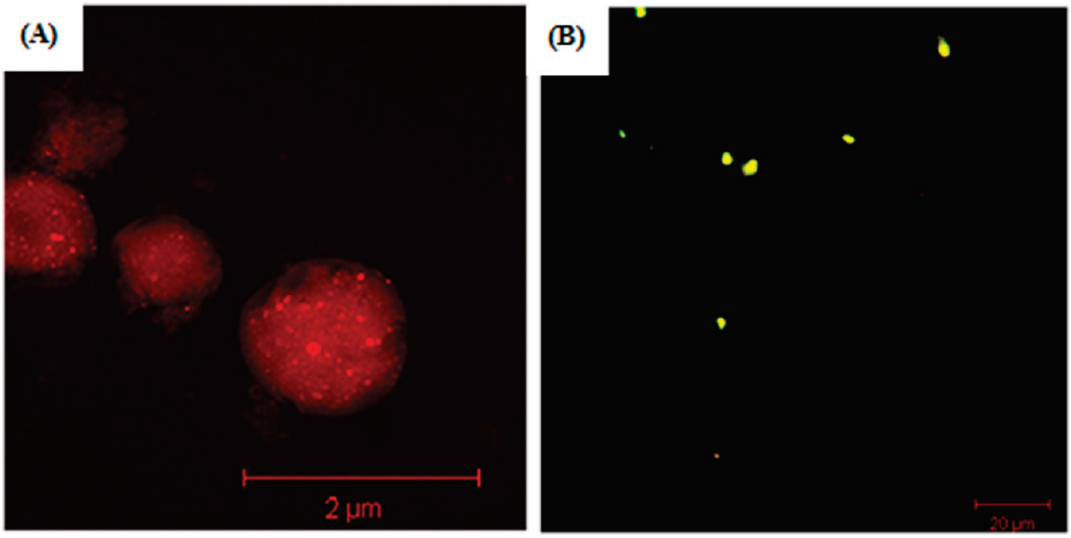

Figure 2. Confocal microscopy of (A) unlabeled and (B) FITC labeled fibrin microspheres encapsulating CY5 labeled DNA complexes. Fluorescence from the fibrin microspheres confirms encapsulation of DNA complexes.

use in wound healing was investigated.[8] Fibrin microbeads have also been used as cell carriers for a variety of cells such as chondrocytes, periosteal-derived cells, and nucleus pulposus cells.[11] Recently, these microbeads have been extensively utilized for isolation, expansion and differentiation of mesenchymal stem cells[10,24−27] and investigated in a variety of applications such as bone tissue engineering[28] and renal diseases.[29] However, as described earlier, in the present study, for the first time, fibrin microspheres have been fabricated for gene delivery, mainly to investigate their ability to encapsulate and deliver DNA complexes without damage to the structural and functional integrity.

Fabrication of Fibrin Microspheres. For fabricating FM suitable for gene delivery, the preheated oil emulsion method was modified. Previously, in FM fabrication, studies have used a variety of oil phase such as a mixture of corn oil and isooctane,[8] vegetable oil,[9] medium chain triglyceride oil[29] or recently mineral oil.[28] It is preferable to use mineral oil or vegetable oil as continuous phase to encapsulate a water-soluble drug[30] and in this study DNA complexes. Mineral oil is commonly used as a preferred oil of choice in fabrication of a variety of microspheres,[31−34] hence in this study, mineral oil was chosen as oil phase. To achieve a more stable emulsion, two surfactants, one for each phase, were utilized. The dynamics of surfactants at the water−oil interface directly influence the stability of emulsion, since they affect the fundamental processes occurring during emulsification, namely, droplet rupture and droplet recoalescence.[35] And since the fibrin microspheres were being fabricated with plasmid DNA complexes, the stability of emulsion was of utmost importance in order to protect the DNA. So, we used two surfactants simultaneously, namely, Tween20 in water phase and Spam80 in oil phase. Formation of microspheres was confirmed by TEM (Figure 1A) and SEM (Figure 1B). The size analysis revealed two peaks, ∼750 nm and ∼1000 nm (Figure 1C). The fibrin microspheres, used as cell carriers, are generally 50−200 μm in size. It was notable that the microspheres obtained by the modified method were much smaller. This smaller size provides a number of advantages such as higher surface area and ease of uniform or gradient distribution as required within the scaffold.

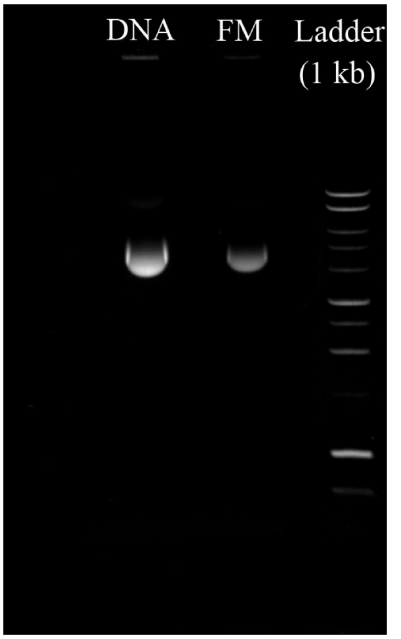

Figure 3. Gel electrophoresis of DNA eluted from the fibrin microspheres. The presence of a distinct band similar to that of control DNA confirms the integrity of the plasmid DNA within the fibrin microspheres.

Encapsulation and Stability of DNA. Another challenge was to encapsulate the DNA complexes within the microspheres without causing any degradation of the plasmid DNA. To confirm encapsulation, we used labeled DNA complexes as described in Materials and Methods. The confocal microscopy (Figure 2A) showed fluorescent microspheres suggesting that the labeled DNA complexes were encapsulated within. To further confirm that the fluorescence seen was indeed from the microspheres, we labeled the microspheres with FITC. The green signal from microspheres and the red signal from DNA complexes colocalized to give a yellow signal (Figure 2B). This confirmed the encapsulation.

In view of the high temperature at which the fabrication procedure was carried out, the stability and integrity of plasmid DNA was assessed by performing gel electrophoresis of the DNA released from fibrin microspheres. The distinct bands seen in the lane with DNA from FM were similar to those seen in the lane with control plasmid DNA (Figure 3), suggesting that the integrity of the plasmid DNA is maintained. We have previously shown that there exists a biomolecular interaction between fibrinogen and lipofectin.[14] We presume that, on account of this interaction, the plasmid DNA complexes get physically covered by the fibrin (or fibrinogen) even before formation of microspheres and thus remain protected from undue exposure to high temperature and hence from degradation.

Functional Integrity in the *in Vivo* Model. With an aim to investigate the efficacy of fibrin microspheres for gene delivery and establish functional integrity of plasmid DNA, a pilot *in vivo* study was performed. The results of real time PCR (Figure 4) suggest that at seven days postdelivery the level of eNOS mRNA is 28-fold higher than in the control group.

It is known that eNOS knockouts have defects in angiogenesis,[36] and studies have previously shown that eNOS delivery enhances angiogenesis.[36−38] Therefore, in order to assess the functional integrity of eNOS delivered via FM, we decided to investigate the effect on angiogenesis in an alloxan induced compromised wound model of rabbit ear ulcer, which has been shown to have defects in angiogenesis.[39] The FFPE wound tissues from eNOS treated and control groups were analyzed histologically by H & E staining. Wounds treated with eNOS via FM showed a number of blood vessel like structures when compared to control group (Figure 5). To confirm that these structures were indeed blood vessels, we performed immunohistochemistry for CD31, which is a specific endothelial cell marker.

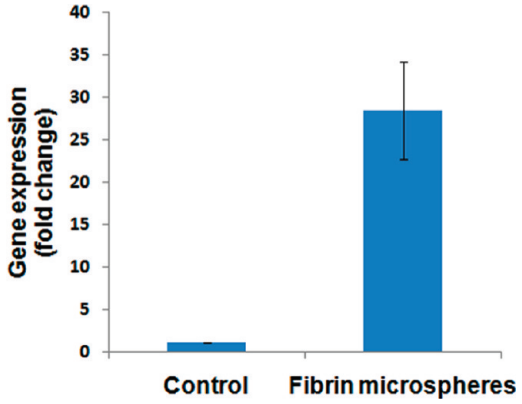

Figure 4. Real time PCR showing ~28-fold increase in eNOS mRNA following treatment with fibrin microspheres carrying eNOS gene compared to control.

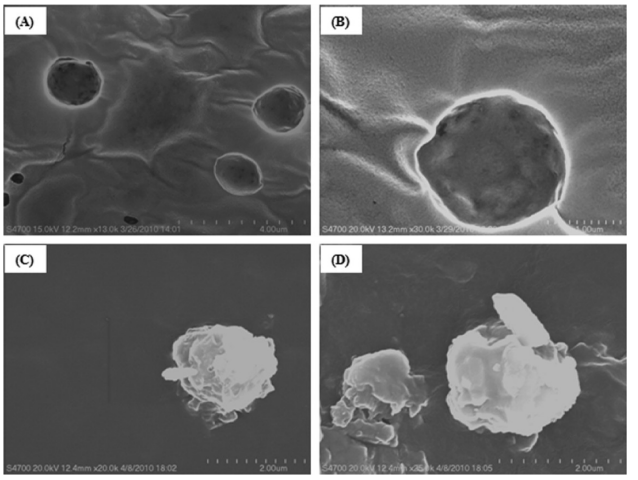

Figure 6. SEM micrographs of fibrin microspheres in water (A, B) and in 0.1 N NaOH for 24 h (C, D). Partially degraded microspheres can be seen (C, D) after incubation with 0.1 N NaOH for 24 h.

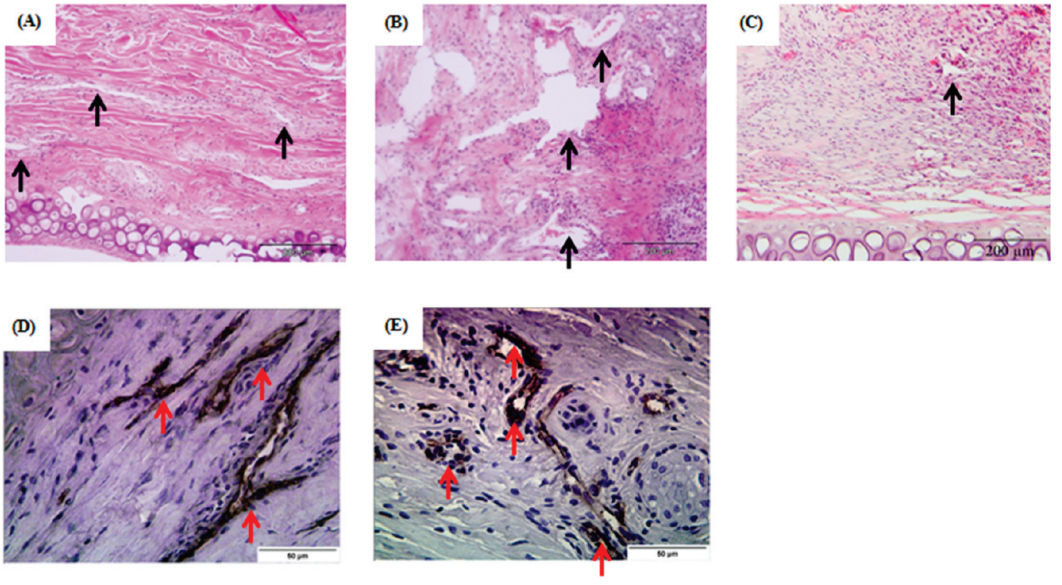

Figure 5. Functional integrity of eNOS, as observed by angiogenesis in response of eNOS delivered via fibrin microspheres. A number of blood vessel like structures are seen in wounds treated with fibrin microspheres carrying eNOS (A, B) while only modest angiogenesis is seen in control wounds (C) as shown by black arrows. CD31 immunohistochemistry (D, E) confirmed that these structures are indeed entholial cells. CD31 positive cells stained brown as indicated by red arrows.

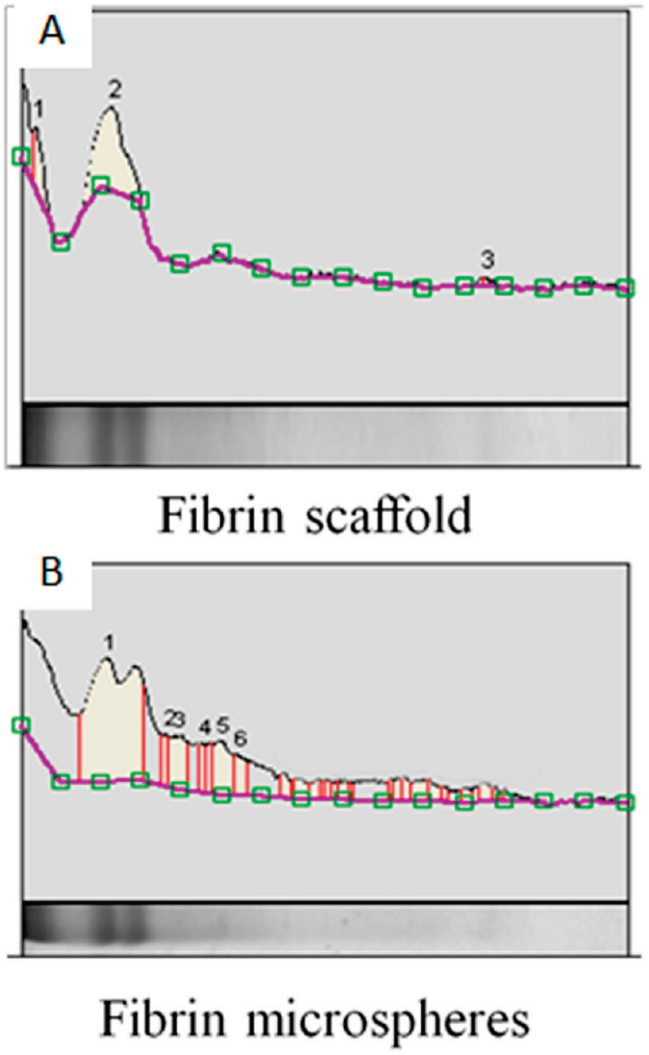

Figure 7. Densitometry curves from the SDS−PAGE performed on the supernatant from (A) fibrin scaffold and (B) fibrin microspheres after 24 h in 0.1 N NaOH solution at room temperature. High degree of cross-linking in fibrin microspheres is evident by the presence of a high number of peaks representing high and low molecular weight fractions.

The brown staining seen in eNOS treated wounds (Figure 5D,E) suggests that these cells are CD31 positive, confirming them to be endothelial cells forming blood vessels after eNOS delivery via fibrin microspheres.

A Step toward "Fibrin in Fibrin" System. Having investigated the feasibility of FM for gene delivery, this study will form the basis of the overall idea of exploiting the potential of FM to extend the capacity of fibrin scaffold to deliver genes. The amount of DNA that can be carried in a fibrin scaffold is limited by the dilution factor added by liquid phase of the lipoplexes. FM carrying lipoplexes can be embedded within fibrin scaffold, allowing a higher load of DNA to be carried in the system without adding a dilution factor. This "fibrin in fibrin" system can provide at least one more very crucial advantage by virtue of the differential degradation of its components. To test this hypothesis, we degraded FM and fibrin scaffold in 0.1 N NaOH for 24 h. Scanning electron microscopy of FM after 24 h incubation with 0.1 N NaOH showed FM were only partially degraded (Figure 6). The densitometry analysis of SDS−PAGE (Figure 7) from the supernatant of partially degraded FM showed a higher number of peaks than fibrin gel. Generally, in the process of normal clotting, fibrin loses α and γ bands and also α−α multimers and only shows γ−γ dimers[8] whereas the presence of multiple peaks of both high and low molecular weight fragments in the FM lane suggests that FM has more cross-links than fibrin scaffold. A previous study has observed similar results.[8] This high cross-linking, we believe, accounts for the slower degradation of FM. An *in vitro* release study comparing the DNA release profiles of fibrin scaffold and FM was performed. The quantification of released DNA (Figure 8) suggested that indeed fibrin scaffold and FM had different release profiles. After 30 min of incubation, fibrin scaffold showed an almost linear release profile of DNA and by 24 h almost all DNA was released from fibrin scaffold, whereas DNA released from FM was significantly lower at all data points from 30 min onward. Moreover, it is thought that the release from FM will be even slower when embedded in a fibrin scaffold. Thus, potentially the "fibrin in fibrin" system can be utilized as a simple temporal release system where DNA complexes from a fibrin scaffold will be released first followed by temporal release of DNA complexes from FM.

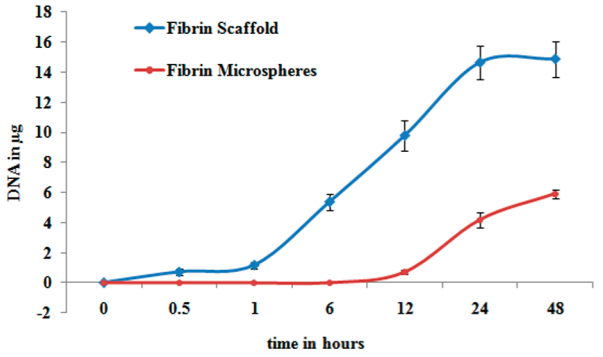

Figure 8. *In vitro* release of DNA from fibrin scaffold and fibrin microspheres. Significantly lower DNA released from fibrin microspheres from 30 min to 48 h suggests possibility of using fibrin microspheres embedded in fibrin scaffold as a temporal release system. Statistical significance was tested using *t* test ($p < 0.05$, $n = 3$).

Thus, in conclusion, fibrin microspheres of around one micrometer size can be fabricated by modified preheated oil emulsion method. This study showed that fibrin microspheres are capable of encapsulating DNA complexes without any degradation of DNA. It can also be concluded that these microspheres are able to deliver genes *in vivo* up to 7 days with the functional integrity of genes maintained. The differential degradation of fibrin microspheres compared to fibrin gel can be exploited to design the "fibrin in fibrin" system which can potentially be used as temporal release system.

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