Delivery of Plasmid DNA Encoding Bone Morphogenetic Protein-2 with a Biodegradable Branched Polycationic Polymer in a Critical-Size Rat Cranial Defect Model

Sue Anne Chew, Ph.D.,¹ James D. Kretlow, Ph.D.,¹ Patrick P. Spicer, B.S.,¹
Austin W. Edwards,¹ L. Scott Baggett, Ph.D.,² Yasuhiko Tabata, Ph.D., D.Med.Sci., D.Pharm.,³
F. Kurtis Kasper, Ph.D.,¹ and Antonios G. Mikos, Ph.D.¹

This study investigated the delivery of plasmid DNA (pDNA) encoding bone morphogenetic protein-2 in the form of polyplexes with a biodegradable branched triacrylate/amine polycationic polymer (TAPP) that were complexed with gelatin microparticles (GMPs) loaded within a porous tissue engineering scaffold. More specifically, the study investigated the interplay between TAPP degradation, gelatin degradation, pDNA release, and bone formation in a critical-size rat cranial defect model. The pDNA release kinetics *in vitro* were not affected by the crosslinking density of the GMPs but depended, rather, on the degradation rates of the TAPPs. Besides the initial release of polyplexes not bound to the GMPs and the minimal release of polyplexes through diffusion or dissociation from the GMPs, the pDNA was likely released as naked pDNA or as part of an incomplete polyplex, after the degradation of fragments of the polycationic polymer. After 30 days, significantly higher amounts of pDNA were released (93%–98%) from composite scaffolds containing naked pDNA or pDNA complexed with P-AEPZ (synthesized with 1-[2-aminoethyl]piperazine, a faster degrading TAPP) compared with those containing pDNA complexed with P-DED (synthesized with N,N-dimethylethylenediamine, a slower degrading TAPP) (74%–82%). Composite scaffolds containing GMPs complexed with TAPP/pDNA polyplexes did not result in enhanced bone formation, as analyzed by microcomputed tomography and histology, in a critical-size rat cranial defect at 12 weeks postimplantation compared with those loaded with naked pDNA. The results demonstrate that polycationic polymers with a slow degradation rate can prolong the release of pDNA from the composite scaffolds and suggest that a gene delivery system comprising biodegradable polycationic polymers should be designed to release the pDNA in an intact polyplex form.

Introduction

BONE TISSUE ENGINEERING systems delivering bioactive factors that have the capability of inducing bone formation, such as bone morphogenetic proteins (BMPs), have been investigated by many groups as an approach to repair bone defects. BMPs can stimulate angiogenesis,¹ promote proliferation of mesenchymal stem cells,^{2–4} initiate the recruitment of osteoprogenitors and mesenchymal stem cells toward the defect site, and stimulate the differentiation of the cells along an osteoblastic lineage.⁵ Delivering the gene encoding a BMP is an alternative approach to delivering the BMP in the protein form. Gene delivery allows for a longer bioavailability of the protein,⁶ and the resulting protein will have a more precise post-translational modification and tertiary structure formation as it is produced *in vivo*.^{7,8} Fur-

ther, the production of the gene is lower in cost and easier compared with the production of the protein. However, these advantages compared with protein delivery are only valid if the gene is successfully delivered into the cell nucleus and results in the production of the protein of interest.

When developing a successful gene delivery system, the gene delivery vector is not the only important factor that has to be taken into consideration. The carrier that delivers the vector/DNA complexes (i.e., scaffold or composite scaffold) also plays a critical part in the gene delivery approach. In an ideal nonviral gene delivery system, the gene delivery vector has to be capable of condensing and protecting the DNA, facilitating delivery of the DNA into the cells and assisting in endosomal escape in order that the DNA can be transcribed and translated in the nucleus. Further, the scaffold or composite scaffold has to be able to deliver the vector/DNA

Departments of ¹Bioengineering and ²Statistics, Rice University, Houston, Texas.

³Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan.

TABLE 1. POLYMER COMPOSITION AND STRUCTURES OF THE TRIACRYLATE AND AMINE MONOMER BUILDING BLOCKS

TAPP	Triacrylate monomer	Amine monomer
P-AEPZ	TMPTA	AEPZ
P-DED	TMPTA	DED

TMPTA, trimethylolpropane triacrylate; AEPZ, 1-(2-aminoethyl)piperazine; DED, N,N-dimethylethylenediamine; TAPP, triacrylate/amine polycationic polymer.

complexes to or near the defect site, control the release kinetics of the complexes, and preferably protect the vector from degrading before the complexes are delivered into the cells.

Previous work in our laboratory has investigated the use of composite scaffolds comprising gelatin microparticles (GMPs) and porous poly(propylene fumarate) (PPF) to deliver BMP-2 for bone tissue engineering.^{9,10} In this work, we investigated the delivery of plasmid DNA (pDNA) encoding BMP-2 complexed with hydrolytically degradable branched triacrylate/amine polycationic polymers (TAPPs), which have been extensively characterized previously in our laboratory,¹¹ using a similar composite scaffold. Acidic GMPs, which are negatively charged at physiological pH, were loaded electrostatically with net positively charged TAPP/pDNA polyplexes. These enzymatically degradable GMPs have been used previously to deliver both growth factors^{9,10,12} and pDNA^{13–15} in a sustained manner.

The overall objective of this study was to evaluate the ability of composite scaffolds comprising pDNA complexed

with a TAPP, GMPs, and a porous PPF scaffold to control the release of pDNA and induce bone formation in a critical-size rat cranial defect. We hypothesized that the release of pDNA from these composite scaffolds can be controlled by the degradation rates of the TAPPs and GMPs. To test this hypothesis, composite scaffolds were fabricated with two different types of TAPPs and GMPs and the effects of the degradation rates of the TAPPs and GMPs on the release kinetics of the pDNA *in vitro* were evaluated. Further, we also hypothesized that the delivery of pDNA complexed with a TAPP from the composite scaffolds will result in higher BMP-2 protein expression as reflected by greater bone formation compared with the delivery of naked pDNA from the composite scaffolds. To test this hypothesis, composite scaffolds were implanted into a critical-size rat cranial defect and the capability of these composite scaffolds to induce bone formation was evaluated at 12 weeks postimplantation.

Materials and Methods

Experimental design

All the groups containing pDNA in this study were loaded with 160 μ g of pDNA, which was derived from the dose of pDNA used for the critical-size rat cranial defect study performed by Huang *et al.*¹⁶ Based on previous studies in our laboratory,¹¹ we chose to investigate two different types of TAPPs [“P-AEPZ” and “P-DED,” synthesized with amine monomer 1-(2-aminoethyl)piperazine (AEPZ) or N,N-dimethylethylenediamine (DED), respectively as shown in Table 1] that have different degradation rates and transfection efficiencies. At pH 7.4, ~80% of the esters in P-AEPZ and P-DED degrade after 3 and 14 days, respectively. Polyplexes formed with P-AEPZ and P-DED (polymer/pDNA weight ratio of 300:1) result in 11.8% $\pm$ 2.6% and 30.6% $\pm$ 6.6% transfection in rat fibroblasts with an enhanced green fluorescent protein reporter gene, respectively. Two different types of acidic GMPs (“10mM” and “40mM,” crosslinked with 10 or 40 mM glutaraldehyde, respectively) that have different degradation rates were investigated. About 10 mM acidic GMPs loaded with BMP-2 are completely degraded after 9 days in phosphate-buffered saline (PBS) containing collagenase, whereas 40 mM acidic GMPs do not show visible degradation after 28 days.¹⁷ The difference in crosslinking density and enzymatic degradation rates of the GMPs has been shown to result in different drug re-

TABLE 2. COMPOSITION OF PLASMID DNA, TRIACRYLATE/AMINE POLYCATIONIC POLYMER, AND GELATIN MICROPARTICLES IN EACH GROUP AND NUMBER OF SAMPLES THAT WERE IMPLANTED *IN VIVO* AND RETRIEVED FOR MICROCOMPUTED TOMOGRAPHY AND HISTOLOGICAL EVALUATION

Group	Amount pDNA (μ g)	Type of TAPP	Type of GMP (mM)	Samples implanted	Samples retrieved
Blank_10 ^a	—	—	10	8	8
Blank_40 ^a	—	—	40	8	8
Free_10	160	—	10	8	8
Free_40	160	—	40	8	8
P-AEPZ_10	160	P-AEPZ	10	8	8
P-AEPZ_40	160	P-AEPZ	40	8	7
P-DED_10	160	P-DED	10	9	9
P-DED_40	160	P-DED	40	9	9

^aGroups included in the *in vivo* bone formation study but not in the *in vitro* release kinetics study.

GMP, gelatin microparticle; pDNA, plasmid DNA.

lease kinetics.^{12,17} The resulting groups for the studies are summarized in Table 2.

PPF scaffold preparation and characterization

PPF was synthesized as previously described,¹⁸ and the resulting polymer had a number average molecular weight of 2270 and a polydispersity index of 1.53. Porous PPF scaffolds were fabricated as previously reported,^{9,10} and disc-shaped scaffolds (8 mm diameter × 1 mm thickness) were obtained. Mercury porosimetry (Autoscan 500; Quantachrome Instruments, Boynton Beach, FL) was used to evaluate the porosity of the scaffolds, as performed previously.¹⁹ Microcomputed tomography (microCT) was also used to analyze the porosity and interconnectivity of the scaffolds as previously described.^{10,19} The porosity and interconnectivity analyses were conducted in triplicate.

Composite scaffold fabrication

pDNA with a cytomegalovirus promoter and human BMP-2 gene (pBMP-2, 5.5 kb; Invitrogen, Carlsbad, CA) was amplified in *Escherichia coli* bacteria as done previously.^{11,20} P-AEPZ and P-DED were synthesized by reacting trimethylolpropane triacrylate (TMPTA) with AEPZ and DED, respectively, as previously described.¹¹ Gelatin with an isoelectric point of 5.0 was used to prepare acidic GMPs using a previously established method,²¹ and particles ranging from 50 to 100 μm in diameter were obtained. The composite scaffolds were prepared using an adaptation of previous methods for loading with growth factors.^{9,10} First, a 300 μL solution of polymer/pDNA polyplexes containing 160 μg of pDNA was prepared according to established methods.^{11,20} The solutions were lyophilized to remove the liquid. Dry GMPs were then loaded with the lyophilized polyplexes or naked pDNA that were resuspended in 25 μL of PBS. Each solution was added drop-wise to 5 mg of GMPs, and the mixture was vortexed and left overnight for 20 h at 4°C. The loaded GMPs were then mixed with 30 μL of 24% (w/v) aqueous solution of Pluronic F-127 (Sigma-Aldrich, St. Louis, MO) and injected into the pores of a PPF scaffold. Preliminary studies in our laboratory have shown that pDNA released from the composite scaffold can still be expressed by cells.

In vitro polymer cytotoxicity

The cytotoxicity of the two different types of TAPPs were evaluated with methyl tetrazolium (MTT) assay (Sigma-Aldrich) using a previously established method.^{11,20} Briefly, polymer and polyplex (formed at 300:1 polymer/pDNA weight ratio) solutions at different polymer concentrations (mass of polymer/volume of void space in the scaffold) were prepared with fetal bovine serum (FBS)-free Dulbecco's modified Eagle's medium (DMEM) and exposed to CRL 1764 rat fibroblasts. Branched polyethylenimine (PEI) (molecular weight ~25 kDa) (Sigma-Aldrich) solutions were used as negative controls. The MTT assay was performed 24 h after exposure. This study was conducted at $n = 4$.

In vitro pBMP-2 transfection

Fifty microliters of solutions of polymer/pDNA polyplexes containing 1 μg of pDNA was placed on an orbital

shaker (70 rpm) in a 37°C warm room throughout the duration of the study to allow the TAPPs to degrade. At each time point (0, 1, 2, 3, 7, and 14 days), the solutions were lyophilized to remove the liquid. CRL 1764 rat fibroblasts were seeded in 24-well plates at a seeding density of 50,000 cells/well. After 24 h of cell attachment, the lyophilized polyplexes were resuspended in 100 μL of FBS-free DMEM (1 μg of pDNA/100 μL solution) and added drop-wise to the cells. The following solutions served as controls: naked pDNA (i.e., Free) and complexes formed with Lipofectamine 2000 (a commercial transfection reagent; Invitrogen). After 4 h of incubation, 400 μL of DMEM/FBS (10% [v/v]) was added to the cells. At 48 h post-transfection, the supernatants were collected and the concentration of BMP-2 was measured using a human BMP-2 enzyme-linked immunosorbent assay development kit (Peprotech, Rocky Hill, NJ) according to the manufacturer's guidelines. This study was conducted at $n = 4$.

In vitro pDNA release kinetics

Composite scaffolds were placed in 1 mL of PBS containing bacterial collagenase type 1A (Sigma-Aldrich) at a physiologically relevant concentration (400 ng/mL) to allow for the enzymatic degradation of the GMPs.²² The samples were placed on an orbital shaker (70 rpm) in a 37°C warm room throughout the duration of the study. At each time point (0.5, 1, 2, 3, 7, 10, 17, 24, and 30 days), the release solutions were collected and completely replaced with 1 mL of fresh PBS containing collagenase. Preliminary studies in our laboratory have shown that the PicoGreen reagent (Molecular Probes, Eugene, OR) cannot fully bind to pDNA that is in a polyplex, as also reported by Kim *et al.*²³ The assay does not allow us to accurately differentiate between and measure the amount of pDNA with different extents of complexation (i.e., fully complexed, partially complexed, and uncomplexed pDNA) at each time point. Thus, after each time point, the collected release solutions were further incubated at 37°C for an additional 14 days to allow the TAPPs to degrade and release the pDNA from the polyplexes. The PicoGreen Quantification assay was then performed on the solutions to quantify the total amount of pDNA released at each time point, which would include uncomplexed or previously complexed pDNA. The cumulative release at each time point was calculated using the equation below. This study was conducted at $n = 4$.

$$\% \text{ Cumulative release at time } X = \frac{\sum \text{pDNA released up to time } X}{\text{pDNA loaded}}$$

In vivo bone formation

Animal surgery, euthanasia, and implant retrieval. This study was conducted in accordance with an animal protocol approved by the Rice University Institutional Animal Care and Use Committee. The composite scaffolds were compiled using aseptic techniques and implanted into 11–12-week-old male syngeneic Fischer-344 rats (Harlan, Indianapolis, IN) weighing ~250–274 g. The general inhalational anesthesia, creation of an 8-mm-diameter critical-size cranial defect with

a trephine drill, placement of composite scaffolds in the defect site, and postoperative animal care were performed as previously described.⁹

The samples from the 4 control groups (i.e., Blank_10, Blank_40, Free_10, and Free_40, with $n = 8$ per group) were implanted into 32 animals without any complications or abnormal behavior observed in the animals. Three out of eight of the animals receiving composite scaffolds from Group P-AEPZ_10 experienced seizures in the immediate postoperative period. Two out of three of these animals were euthanized upon the recommendation of the consulting veterinarian. To continue the study, the animal protocol was modified and approved to include a preoperative intraperitoneal injection of fosphenytoin (60 mg/kg)²⁴ 30 min before surgery for the remaining animals receiving composite scaffolds from Groups P-AEPZ_10 (to replace the euthanized animals), P-AEPZ_40, P-DED_10, and P-DED_40. Each animal also received intraperitoneal injections of fosphenytoin at 12 (20 mg/kg), 24 (20 mg/kg), and 36 (15 mg/kg) h after surgery for maintenance. Some of the animals receiving a composite scaffold containing P-AEPZ (i.e., from Groups P-AEPZ_10 and P-AEPZ_40) and one animal receiving a composite scaffold containing P-DED (i.e., from Group P-DED_10), which were given fosphenytoin before surgery, still experienced seizures. However, euthanasia was not recommended for these animals. Fosphenytoin was found to be capable of reducing the occurrence, severity, and length of the seizures in this study and previous studies in both rats²⁴ and humans.²⁵ Long-term treatment with an antiepileptic drug (such as fosphenytoin) can decrease calcium absorption and bone density.²⁶ However, the short-term administration (up to 36 h postoperatively) of fosphenytoin in this study would not be expected to affect bone formation. Animals receiving samples from groups without a TAPP did not experience seizures. Given the general complexity of seizures, neither the specific factor(s) contributing to the occurrence of seizures in this study nor the mechanism by which the seizures occurred could be readily identified.

The animals were euthanized and implants were harvested at 12 weeks postimplantation using a previously established method.⁹ The samples were placed into 10% neutral buffered formalin for 5 days and then placed into 70% ethanol. One of the animals receiving a composite scaffold from Group P-AEPZ_40 had a large abdominal tumor that was discovered around 7 weeks postimplantation. The animal was euthanized upon the recommendation of the consulting veterinarian before the end of the study and thus was not included in the microCT and histology evaluations. The occurrence of a tumor was only observed in one animal that received a composite sample; thus, it could not be concluded that the occurrence of the tumor was associated with the implanted material. Laboratory rodents may develop tumors as a result of their genetic predisposition.

Microcomputed tomography. MicroCT was used to quantify the amount of bone formed within the defect as previously described.^{9,10} Maximum intensity projections (MIPs) for each sample were generated. An 8-mm-diameter circular region of interest (ROI) was created in the MIP, and a binary image was obtained with a global binarization threshold of 70–255. The percentage of area within the circular ROI of the two-dimensional MIP projection that

was covered with bone was determined with the equation below.

$$\% \text{ Bone area} = \frac{\text{Binarized object area}}{\text{ROI area}} \times 100\%$$

The extent of bony bridging and union within the defect was also determined from the MIPs by two blinded observers (J.D.K and P.P.S.) separately according to the grading scale in Table 3 as done previously.^{9,10} A consensus score was obtained for each sample.

Histological processing and scoring. After microCT scanning, the samples were dehydrated in a graded series of ethanol solutions (70%–100%), followed by methylmethacrylate embedding as done previously.²⁷ After polymerization, 5- μ m-thick coronal sections were prepared from the middle of the sample using a microtome (Leica RM2165, Wetzlar, Germany). Three sections per sample were stained with hematoxylin and eosin (H&E). One section per sample was stained with von Kossa/Van Gieson and one section per sample was stained with Goldner's Trichrome. The sections stained with H&E were quantitatively evaluated separately by three blinded observers (F.K.K., J.D.K., and P.P.S.) using light microscopy. The hard tissue response (1) at the scaffold–bone interface and (2) within the pores of the scaffold were evaluated using the quantitative grading scale in Table 4 as done previously.^{9,10} A consensus score was obtained for each sample.

Statistical methods

For the results of the release kinetics of pDNA *in vitro* in the different phases, repeated measures analysis of variance was used to identify if there were significant differences among groups ($\alpha = 0.05$). Tukey–Kramer multiple comparison test was then conducted to identify the specific groups that differed statistically significantly. For the BMP-2 concentration produced *in vitro*, cumulative release after 30 days *in vitro* and the microCT bone volume and area results obtained from the *in vivo* study, multifactor analysis of variance was used to identify if there were significant differences among groups ($\alpha = 0.05$). Tukey's Honestly Significantly Different test was then conducted to identify the specific

TABLE 3. SCORING GUIDE FOR EXTENT OF BONY BRIDGING AND UNION OBTAINED USING MAXIMUM INTENSITY PROJECTIONS OF MICROCOMPUTED TOMOGRAPHY DATASETS

Description	Score
Bony bridging over entire span of defect at longest point (8 mm)	4
Bony bridging over partial length of defect	3
Bony bridging only at defect borders	2
Few bony spicules dispersed throughout defect	1
No bone formation within defect	0

TABLE 4. SCORING GUIDE FOR QUANTITATIVE HISTOLOGICAL ANALYSIS

Description	Score
Hard tissue response at scaffold–bone interface	
Direct bone to implant contact without soft interlayer	4
Remodeling lacuna with osteoblasts and/or osteoclasts at surface	3
Majority of implant is surrounded by fibrous tissue capsule	2
Unorganized fibrous tissue (majority of tissue is not arranged as capsule)	1
Inflammation marked by an abundance of inflammatory cells and poorly organized tissue	0
Hard tissue response within the pores of the scaffold	
Tissue in pores is mostly bone	4
Tissue in pores consists of some bone within mature, dense fibrous tissue, and/or a few inflammatory response elements	3
Tissue in pores is mostly immature fibrous tissue (with or without bone) with blood vessels and young fibroblasts invading the space with few macrophages present	2
Tissue in pores consists mostly of inflammatory cells and connective tissue components in between (with or without bone) OR the majority of the pores are empty or filled with fluid	1
Tissue in pores is dense and exclusively of inflammatory type (no bone present)	0

groups that differed statistically significantly. For the microCT bone union and histology hard tissue response scoring results obtained from the *in vivo* study (discrete ordinal values), ordered logistic regression was used to identify if there were significant differences among groups ($\alpha = 0.05$). An analysis of effects was then performed to identify the specific groups that differed statistically significantly.

Results

PPF scaffolds porosity and interconnectivity

The porosity of the PPF scaffolds was determined by microCT and mercury porosimetry and was found to be $64.3\% \pm 1.0\%$ and $67.1\% \pm 0.8\%$, respectively, which is comparable to what was reported by Patel *et al.* for the same porous PPF scaffold formulation ($\sim 70\%$).⁹ More than $98.8\% \pm 0.3\%$ of the pores in the scaffolds were connected to the outside environment through an opening of $32\mu\text{m}$ or larger (Fig. 1).

In vitro polymer cytotoxicity

The cell viability of rat fibroblasts exposed to different concentrations of TAPPs by themselves or in a polyplex form was evaluated with an MTT assay (Fig. 2). The cells remain viable (above 100% cell viability) after exposure to the TAPPs and polyplexes formed with the TAPPs at concentrations corresponding to a release of 1% or lower ($\leq 14,500\mu\text{g/mL}$) of the amount of loaded polymer or polyplexes in the composite scaffold. In contrast, PEI caused cell death at all the concentrations tested ($100\text{--}14,500\mu\text{g/mL}$, 6%–8% cell viability). At concentrations corresponding to a release of 3.5%–10% ($50,000\text{--}145,000\mu\text{g/mL}$) and 12.5%–100% ($181,000\text{--}1,450,000\mu\text{g/mL}$), 20%–26% and 5%–13% cell viability was observed, respectively, for cells exposed to P-DED (polymer and polyplexes). For cells exposed to P-AEPZ (polymer and polyplexes) at concentrations corresponding to a release of 3.5%–100% ($50,000\text{--}1,450,000\mu\text{g/mL}$), 2%–5% cell viability was observed.

In vitro pBMP-2 transfection

Rat fibroblasts were exposed to TAPP/pBMP-2 polyplexes, which were allowed to degrade over a 14 day period,

and the concentration of BMP-2 secreted by the cells was quantified using an enzyme-linked immunosorbent assay (Fig. 3). Cells transfected with polyplexes formed with the positive control, Lipofectimine 2000, produced a significantly higher concentration of BMP-2 ($581.6 \pm 82.7\text{pg/mL}$) compared with the other groups. Polyplexes formed with P-AEPZ did not result in a significantly higher concentration of BMP-2 production compared with naked DNA (i.e., Free, $55.1 \pm 17.0\text{pg/mL}$) for all the different time points tested ($83\text{--}123\text{pg/mL}$). Cells transfected with polyplexes formed with P-DED that had not been allowed to degrade (i.e., still in an intact polyplex form, time point day 0) produced a significantly higher concentration of BMP-2 ($154.5 \pm 10.2\text{pg/mL}$) compared with naked DNA (i.e., Free).

In vitro pDNA release kinetics

The *in vitro* release of pDNA from the composite scaffolds was evaluated in PBS containing a physiological amount of collagenase over 30 days (Fig. 4). The amount of pDNA released was divided into four time segments as done in previous studies^{10,12,17,22} to facilitate the comparison of the

FIG. 1. Scaffold pore interconnectivity obtained by micro-computed tomography (microCT) analysis at different minimum connection sizes. The results represent means $\pm$ standard deviations of $n = 3$.

FIG. 2. Cytotoxicity of the polycationic polymers and polyplexes formed with the polycationic polymers on CRL 1764 rat fibroblasts after 24 h as evaluated by an MTT assay with polyethylenimine (25 kDa) as negative control. The results are expressed as means $\pm$ standard deviation for $n = 4$.

release rates of the different groups (Table 5). The type of GMP (10 or 40 mM) did not have an overall significant effect on the pDNA release kinetics. However, the type of TAPP (no polymer [i.e., delivered as naked pDNA], P-AEPZ or P-DED) did have an overall significant effect on the pDNA release kinetics ($p < 0.0001$). Groups Free_10 and Free_40 had a large burst release of pDNA ($92.6\% \pm 1.1\%$ and $88.9\% \pm 2.5\%$ pDNA per day, respectively) in the first 24 h (Phase 1) and significantly lower release rates in the subsequent phases (0.1% – 2.6% per day). All other groups had significantly lower release rates in Phase 1 (10.7% – 16.6% per day) compared with Groups Free_10 and Free_40. Groups P-AEPZ_10 and P-AEPZ_40 had significantly higher release rates in Phase 2 ($31.7\% \pm 10.2\%$ and $32.1\% \pm 9.2\%$ per day, respectively) compared with the other groups (1.7% – 11.0% per day).

Although no difference in release kinetics was observed for 10 and 40 mM GMPs, visual observation of the samples at the end of the release study demonstrated that there was more degradation of 10 mM GMPs compared with 40 mM

GMPs, as expected. After 30 days, almost all of the 10 mM GMPs in samples from Groups Free_10 and P-AEPZ_10 were degraded. However, samples from Group P-DED_10 still had some 10 mM GMPs remaining in the sample tubes. For samples with 40 mM GMPs, there were still some GMPs remaining in all the sample tubes. However, samples from Groups P-AEPZ_40 and P-DED_40 had a higher amount of 40 mM GMPs remaining in the sample tubes compared with samples from Group Free_40.

In vivo bone formation

The ability of the composite scaffolds to induce bone formation was evaluated in a critical-size rat cranial defect over 12 weeks. The percent of bone volume that formed in the defect was obtained by microCT (Fig. 5A). Group Free_10 had significantly higher bone formation ($5.0\% \pm 3.4\%$) compared with Groups P-AEPZ_10 and P-AEPZ_40 ($0.7\% \pm 0.9\%$ and $0.5\% \pm 0.7\%$, respectively). The amount of bone healing was also analyzed by comparing the percent of bone area in the MIPs that were obtained by microCT (Fig. 5B). As observed for the bone volume results, Group Free_10 had a significantly higher amount of bone area ($21.9\% \pm 15.7\%$) compared with Groups P-AEPZ_10 and P-AEPZ_40 ($5.0\% \pm 5.9\%$ and $2.6\% \pm 3.0\%$, respectively).

FIG. 3. Bone morphogenetic protein 2 (BMP-2) produced by CRL 1764 rat fibroblasts exposed to polyplexes formed with $1 \mu\text{g}$ pBMP-2 DNA, which were allowed to degrade for different durations, as evaluated by an enzyme-linked immunosorbent assay. Naked plasmid DNA (Free) and polyplexes formed with Lipofectimine 2000 served as controls. The results are expressed as means $\pm$ standard deviation for $n = 4$. #A statistically significant difference between one group from all other groups ($p < 0.05$). *A statistically significant difference between groups ($p < 0.05$).

FIG. 4. Profiles of percent cumulative plasmid DNA released *in vitro* in phosphate-buffered saline containing collagenase. The results represent means $\pm$ standard deviations of $n = 4$.

TABLE 5. RELEASE KINETICS OF PLASMID DNA FROM COMPOSITE SCAFFOLDS IN FOUR DIFFERENT PHASES

Group	Phase 1 (%/day) First 24 h	Phase 2 (%/day) Days 1–3	Phase 3 (%/day) Days 3–17	Phase 4 (%/day) Days 17–30
Free_10	92.6 ± 1.1 ^{a,b}	1.7 ± 0.4	0.1 ± 0.0	0.1 ± 0.0
Free_40	88.9 ± 2.5 ^{a,b}	2.6 ± 0.9	0.2 ± 0.2	0.1 ± 0.0
P-AEPZ_10	13.4 ± 7.7 ^b	31.8 ± 10.2 ^{a,b}	1.0 ± 1.1	0.3 ± 0.1
P-AEPZ_40	14.1 ± 16.6 ^b	33.0 ± 6.2 ^{a,b}	0.6 ± 0.1	0.4 ± 0.3
P-DED_10	16.0 ± 1.6 ^c	9.4 ± 0.8 ^c	1.6 ± 0.2	1.3 ± 0.2
P-DED_40	19.0 ± 2.1 ^b	11.1 ± 1.9 ^b	2.0 ± 0.2	1.6 ± 0.2

The results are expressed as means ± standard deviation (% release per day) for $n = 4$.

^aStatistically significant difference ($p < 0.05$) between one group from all other groups excluding the group with the same TAPP treatment (i.e., Free_10 and Free_40, P-AEPZ_10 and P-AEPZ_40, P-DED_10, and P-DED_40 are not significantly different from each other) within the same phase.

^bStatistically significant difference ($p < 0.05$) between one phase from all other phases within the same group.

^cStatistically significant difference ($p < 0.05$) between one phase from Phase 3 and 4 within the same group.

The MIPs were also used to evaluate the extent of bony bridging and union based on the guide shown in Table 3, and the average scores are shown in Figure 6A. Groups P-AEPZ_10 and P-AEPZ_40 had average scores of around 1 (1.3 ± 0.9 and 0.9 ± 0.9 , respectively), whereas the other groups had scores closer to 2 (1.9 – 2.3). The groups containing only GMPs, Pluronic F-127, and a PPF scaffold (without any drug loaded) in this study (i.e., Groups Blank_10 and Blank_40) had comparable bone scores (2.0 ± 1.3 and 1.9 ± 1.0 , respectively) to that observed by Patel *et al.* (Group BLANK, 2.0 ± 1.1).⁹ The bone that formed in the defects was mostly on the dural side of the cranium. The bone usually formed from the defect margins, and, in some of the animals, bone formed further toward the middle of the defect (Fig. 7A, C).

Histological analysis was performed on the samples to further investigate the tissue response to the implanted composite scaffolds. The scoring of the hard tissue response at the scaffold–bone interface and within the pores of the scaffolds was performed on the H&E sections based on the guide shown in Table 4, and the average scores are shown in Figure 8. All the groups had a majority of implants that were surrounded by a fibrous tissue capsule, which corresponds to a score of 2, except for samples from Groups P-AEPZ_10 and P-AEPZ_40 (Fig. 9A). The implants from these groups were mostly surrounded by unorganized fibrous tissue that was not arranged in a capsule, which corresponds to a score of 1. The pores of the implants were mostly filled with immature fibrous tissue with some blood vessel formation, which corresponds to a score of 2 (Fig. 9B). Groups P-AEPZ_10 and P-AEPZ_40, however, had more samples where the majority of the implant pores were empty or filled with fluid which corresponds to a score of 1.

Bone formation was mostly observed on the defect margin and on the outer surface of the implant (Fig. 10A, C) as confirmed with von Kossa/Van Gieson staining, which stains mineralized tissue black. However, several samples also had bone formation in the pores of the scaffold (Fig. 10H) and toward the middle of the defect. As observed by microCT, histological analysis also showed that the newly formed bone was mostly on the dural side of the defect (Fig. 10A, C). Minimal osteoid formation was observed in some samples as indicated by the dark red staining of the matrix by Goldner's Trichrome staining (shown in Supplementary Figs. S1 and S2;

Supplementary Data are available online at www.liebertonline.com/ten). The osteoid matrix was observed mostly at the edge of the newly formed bone or the defect margin (i.e., host bone) and usually next to a line of osteoblasts arranged linearly along the developing bone. Qualitative observation of the slides stained with von Kossa/Van Gieson and Goldner's Trichrome did not reveal observable differences in mineralized tissue and osteoid formation. Thus, further analysis of the slides stained with von Kossa/Van Gieson and Goldner's Trichrome was not performed.

Discussion

Porous PPF scaffolds have been used previously in our laboratory with GMPs to deliver BMP-2 in a controlled and sustained manner in a critical-size rat cranial defect.^{9,10} In this work, we investigated the application of these composite scaffolds to deliver pDNA encoding BMP-2 complexed with biodegradable branched polycationic polymers and evaluated their ability to enhance bone formation *in vivo*.

Compared with PEI, a higher concentration of the biodegradable polycationic polymers (i.e., P-AEPZ and P-DED) can be used for transfection, without resulting in high cell death. For *in vitro* transfection studies in this work and our previous work with the plasmid enhanced green fluorescent protein,^{11,20} the cells were exposed to only 1 and 5 μg of pDNA, respectively. This results in a polymer concentration of 3000 $\mu\text{g}/\text{mL}$, which was not toxic to the cells. However, for the *in vivo* study in this work, a large amount of pDNA (160 μg) was loaded in the composite scaffolds, which resulted in a high concentration of polymer (1,450,000 $\mu\text{g}/\text{mL}$). When tested *in vitro*, a release of larger than 1% of the amount of loaded polymer resulted in low cell viability. However, the same observation may not be observed *in vivo*, where circulation of fluid at the defect site would reduce the concentration of the polyplexes. In the present study, the cytotoxicity was assessed for the nondegraded polymers to provide an indication of the immediate worst-case cytotoxicity of the polymers upon implantation, with the assumption that the degraded polymers would be less toxic compared with the nondegraded polymers, as the degradation of the polymers by ester hydrolysis, which results in lower molecular weight polymer fragments, was expected to reduce the cytotoxicity of the polymers as seen by Kloeckner

FIG. 5. Percent bone (A) volume formed within an 8-mm-diameter and 1.5-mm-thick cylindrical volume of interest and (B) area formed within an 8-mm-diameter circular region of interest of the maximum intensity projections as measured by microCT at 12 weeks. The results are expressed as means $\pm$ standard deviation for $n = 7-9$. *A statistically significant difference between groups ($p < 0.05$).

FIG. 6. (A) Average bone union score within the defect as measured from maximum intensity projections obtained by microCT at 12 weeks. The results are expressed as means $\pm$ standard deviation for $n = 7-9$. *A statistically significant difference between one group from all other groups ($p < 0.05$) excluding the group with the same triacrylate/amine polycationic polymer treatment [i.e., P-AEPZ_10 and P_AEPZ_40 are not significantly different from each other]. *A statistically significant difference between groups ($p < 0.05$). (B) Bone union score distribution.

FIG. 7. Representative images of maximum intensity projections of rat cranial defects obtained by microCT at 12 weeks. (A) Group Blank_10: 6.0% bone volume, 25.6% bone area, bone score = 3. (B) Group Blank_40: 3.0% bone volume, 17.3% bone area, bone score = 2. (C) Group Free_10: 10.2% bone volume, 47.0% bone area, bone score = 3. (D) Group Free_40: 2.9% bone volume, 15.6% bone area, bone score = 2. (E) Group P-AEPZ_10: 1.0% bone volume, 6.2% bone area, bone score = 1. (F) Group P-AEPZ_40: 0.1% bone volume, 0.1% bone area, bone score = 0. (G) Group P-DED_10: 6.6% bone volume, 26.1% bone area, bone score = 2. (H) Group P-DED_40: 5.8% bone volume, 19.3% bone area, bone score = 2. Bar represents 2 mm.

*et al.*²⁸ The accumulation of acidic degradation products in hydrolytically degradable microspheres can be detrimental to the bioactivity of the protein or DNA that are encapsulated within them.^{29,30} However, for polycationic polymers, this may not be such a concern as the acidic degradation products can be more easily released to avoid accumulation in the polyplexes.

When tested *in vitro*, intact polyplexes formed with P-DED produced a significantly higher concentration of BMP-2 compared with naked pDNA (i.e., Free). However, these polyplexes began losing their transfection ability over time as part of the polymer degrades, which can decrease the zeta potential of the polyplexes and the ability of the polymer to condense the pDNA. Polyplexes formed with the TAPPs were not very efficient and were unable to produce a high concentration of BMP-2 *in vitro* compared with complexes formed with the commercial transfection reagent, Lipofectimine 2000. In the pBMP-2 transfection study in this work, rat fibroblasts were used as a model cell line. However, *in vivo*, other cell types are present at the defect site that could result in different transfection efficiencies and BMP-2 production rates.

Naked pDNA, which is negatively charged due to the phosphate backbone, is not able to interact electrostatically with the negatively charged acidic GMPs used in this work. This results in inefficient loading of the GMPs with pDNA and thus, the large burst release of the loaded pDNA from the composite scaffolds. In contrast, groups containing a TAPP only had a slight burst release in Phase 1, which could

have resulted from the initial release of polyplexes not bound to the GMPs, as seen when growth factors were delivered from a similar composite scaffold.^{10,12} The TAPP/pDNA polyplexes have a net positive charge¹¹ and thus are able to interact electrostatically with the GMPs and result in a potentially stronger binding and more efficient loading of the pDNA to the GMPs.

Although release of the drug from the GMPs by diffusion or dissociation through a change in environment (such as a change in ionic concentration) can occur after the initial burst release in Phase 1, previous studies indicated that the release is usually dominated by the enzymatic degradation of the GMPs.^{10,12} Unlike the previous studies with BMP-2, in this study, we found that the GMP crosslinking density (which influences the degradation kinetics of the GMPs) did not affect the release profiles of the pDNA and that the pDNA release depended on the known degradation rates of the TAPPs instead. The TAPPs were degrading and releasing the pDNA faster than the GMPs were degrading and releasing the whole polyplexes. The slower degradation rate of 10 mM GMPs in this study compared with the previous study where they were completely degraded within 9 days¹⁷ suggests that the polyplexes may be shielding the GMPs from enzymatic degradation. The polyplexes formed with P-DED (i.e., TAPP with a lower degradation rate), which can stay attached to the GMPs for a longer time, were able to better shield the GMPs from degradation. Thus, beyond the initial release of polyplexes not bound to the GMPs in Phase 1 and the

FIG. 8. Average histological scores for hard tissue response (A) at the scaffold–bone interface and (B) within the scaffold pores of the defect as assessed from hematoxylin and eosin staining of histological sections of rat cranial defects at 12 weeks. The results are expressed as means $\pm$ standard deviation for $n = 7$ –9. Groups not connected with the same letter are significantly different from each other ($p < 0.05$).

minimal release of intact polyplexes through diffusion or dissociation, the remaining pDNA in polyplexes that were bound to the GMPs were likely released from the composite scaffolds as naked pDNA or in polyplexes with a lower amount of polymer (i.e., lower polymer/pDNA weight ratio), as fragments of the polymer had to degrade to dislodge and release the pDNA from the GMPs.

Although the study design did not allow for the differentiation between pDNA of different extents of complexation (i.e., fully complexed, partially complexed, and uncomplexed pDNA), the release data (free DNA detected after the 14 day incubation) coupled with the known TAPP degradation kinetics indicate that the pDNA release was dependent upon the TAPP degradation. In Phase 2, the significantly higher pDNA release rate for Groups P-AEPZ_10 and P-AEPZ_40 compared with the other groups indicates that in 1–3 days, enough ester groups in P-AEPZ had degraded to result in a burst release of pDNA compare to groups with P-DED, which has a significantly lower degradation rate. At the end of the study, almost the entire loaded dose of pDNA (93%–98%) from groups with naked pDNA and polyplexes formed with P-AEPZ had been released, which was significantly higher than the amount of pDNA release from groups with polyplexes formed with P-DED (74%–82%). Thus, there was likely more pDNA (18%–26%, $\sim 1/4$ to $1/5$ of the total loaded pDNA) remaining in the composite scaffolds from these groups that could potentially be released beyond the 30 day endpoint of the present study. The slower degradation rate of P-DED was able to prevent the release of a large amount

of pDNA too early during the delivery process and may also have been able to prolong the condensation and protection of the pDNA in the polyplexes. This is beneficial for the delivery of pDNA encoding BMP-2, as later expression of BMP-2 is usually observed for osteoprogenitor cell differentiation *in vitro*³¹ and bone formation *in vivo*.³² Although the release kinetics *in vivo* may differ from the results obtained *in vitro*, the *in vitro* analysis provides a predictive model and contextual data for the interpretation of the results from the *in vivo* bone formation study.

The results from the *in vivo* study indicated that the addition of the biodegradable TAPP component to the delivery system did not improve the ability of the composite scaffolds to induce bone formation (Figs. 5 and 6). The delivery of pBMP-2 with this composite scaffold system also resulted in less bone formation compared with the delivery of BMP-2 in the protein form with this system in previous studies.^{9,10} The reason for these observations are unclear as several factors are involved during the pDNA delivery as well as wound healing processes. We hypothesized that, even though a polycationic polymer vector component was incorporated into the delivery system, the low amount of bone formation observed could have resulted from the unsuccessful delivery of most of the loaded pDNA in an intact polyplex form. The release of the pDNA in the free or partially complexed form at the defect site results in a decrease in transfection efficiency^{11,16,20} as seen in the *in vitro* pBMP-2 transfection study and increases the possibility of pDNA degradation by enzymes, nucleases^{33–35} and the low pH

FIG. 9. Histological score distribution of hard tissue response (A) at the scaffold–bone interface and (B) within the scaffold pores of the defect as assessed from hematoxylin and eosin staining of histological sections of rat cranial defects at 12 weeks.

FIG. 10. Representative histological sections of samples stained with von Kossa/Van Gieson at 12 weeks (A) Group Blank_10, (B) Group Blank_40, (C) Group Free_10, (D) Group Free_40, (E) Group P-AEPZ_10, (F) Group P-AEPZ_40, (G) Group P-DED_10 and (H) Group P-DED_40. P, poly(propylene fumarate) scaffold; B, new bone. Bar represents 500 μ m.

(~4)³⁶ in the wound healing environment. Even though the pDNA released from the composite scaffolds may have resulted in BMP-2 protein production, the composite scaffolds in this study may have not delivered enough complexed and undegraded pDNA over a prolonged period to allow for a sufficient amount and duration of protein expression to induce bone formation^{37,38} compared with the protein delivered in the previous studies.^{9,10} Although cytotoxicity of the polymers or their degradation products may have contributed to the low bone formation observed *in vivo*, we hypothesized that it may not be the primary reason for this observation as the circulation of fluid at the defect site would reduce the concentration and cytotoxicity of the polymer.

In general, implants from Groups P-DED_10 and P-DED_40 had comparably more tissue ingrowth where almost all the pores were filled with fibrous tissue (Fig. 10G and H, respectively). This suggests that these groups may have enhanced the production of BMP-2, which has the capability of initiating the recruitment of cells to the defect site. As seen in the *in vitro* release study, groups containing P-DED (i.e., Groups P-DED_10 and P-DED_40) were able to prolong the release of pDNA and may also have delivered more of the pDNA in the intact polyplex form due to the slower degradation rate of P-DED; however, the effect of polyplex release kinetics on bone formation remains unknown. Further, the higher amount of tissue ingrowth for these groups could have also resulted from the higher transfection efficiency of polyplexes formed with P-DED compared with naked pDNA and polyplexes formed with P-AEPZ.

Conclusions

In this work, pDNA complexed with biodegradable branched polymers were delivered from a composite containing acidic GMPs and a porous PPF scaffold. The *in vitro* results showed that the degradation rate of the polycationic polymers can control the pDNA release kinetics. The *in vivo* results showed that the premature degradation of polycationic polymers may trigger the release of naked pDNA with minimal effect on bone formation in a critical-size rat cranial defect. The collective results showed that the degradation rate of different polymers comprising a pDNA carrier would affect not only its delivery but most importantly its transfection capability and therapeutic effect *in vivo*.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
 Antonios G. Mikos, Ph.D.
 Department of Bioengineering
 Rice University
 MS-142, P.O. Box 1892
 Houston, TX 77251-1892
 E-mail: mikos@rice.edu

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