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Angaben zur Veröffentlichung / Publication details:

Kolk, Andreas, Cornelia Haczek, Christian Koch, Stephan Vogt, Martin Kullmer, Christoph Pautke, Herbert Deppe, and Christian Plank. 2011. "A strategy to establish a gene-activated matrix on titanium using gene vectors protected in a polylactide coating." *Biomaterials* 32 (28): 6850–59. <https://doi.org/10.1016/j.biomaterials.2011.05.071>.

A strategy to establish a gene-activated matrix on titanium using gene vectors protected in a polylactide coating

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1. Introduction

The repair of bony defects or bone detachment from implant interfaces poses a major challenge in cranio-maxillofacial and orthopedic reconstructive surgery [1]. Recombinant growth factors have been used with great success to promote and improve bone healing, and the first FDA-approved products have appeared on the market [2–4]. However, the routine clinical applicability of recombinant growth factors is limited by high product costs, their limited half-lives *in vivo* [5,6] implying high and repeated doses, and the difficulty of restricting the activity of the growth factor to the area of application in order to avoid undesired systemic side effects [6,7]. For these reasons, the development of sustained and controlled release technologies involving implantable biomaterials

and biodegradable surface coatings has been a major focus of biomedical research during recent years [8]. Poly(lactide) and poly(lactide-co-glycolide) surface coatings for medical implants have been used clinically for several years, and have been demonstrated to deliver pharmaceutical agents in a sustained manner to influence bone healing [9,10]. More recently, our research group, among others, has shown that poly(lactide) surface coatings can be formulated to integrate growth factors for sustained release to promote bone regeneration in animal models [11,12]. Transfecting or transducing growth factor genes into target tissues is a viable alternative to the use of their gene products (i.e. recombinant growth factors) [5]. According to the gene-activated matrix (GAM) concept [13,14], vectors for nucleic acid delivery are immobilized on implantable materials, and cells colonizing such matrices express the gene(s) encoded by the vector upon becoming transfected/transduced on the matrix. The GAM concept has shown promising results in combination with a broad range of different matrices with numerous nonviral and viral gene vectors and in multiple envisaged applications for tissue regeneration, notably bone healing [14,15]. The matrix materials used so far have been mostly gels, sponges or foams prepared from biopolymers

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such as collagen and fibrin [13–19] or from synthetic polymers like poly(lactides) or poly(lactides-co-glycolides) [20–22]. Most advanced in clinical development is a collagen matrix comprising an adenoviral vector encoding platelet-derived growth factor (PDGF) applied in clinical trials as a treatment for diabetic ulcers [23].

The most frequent implants in bone reconstructive surgery are metallic ones. Numerous complications are associated with their use such as loosening due to inadequate osseous integration with the host tissue [6]. Surprisingly little is known about the suitability of metallic implants to serve as gene-activated matrices. Our goal is devising such materials for improving bone regeneration and implant integration with the host tissue. Here we report on a strategy for preparing such materials and show their functionality as gene-activated matrix in cell culture. This strategy is based on embedding copolymer-protected gene vectors (COPROGs), which we have described previously [24,25], in a poly(lactide) surface coating on titanium implants. COPROGs are polycation-DNA nanoparticles shielded with an electrostatically bound layer of an anionic PEG-peptide derivative copolymer [24] that we have previously shown to be highly suitable in the context of the GAM concept [16,17]. Our aim is combining the transfection efficiency of COPROGs with the stability of PDLLA on titanium surfaces.

The questions examined in this study are (I) whether a suspension of lyophilized COPROGs in an organic solution of PDLLA is suitable for preparing stable coatings on planar model surfaces, (II) whether plasmid DNA remains intact during the homogenizing and coating procedure, (III) what the vector release profile is, (IV) whether the coating is compatible with cell growth and whether cells become transfected when colonizing the coating and (V) whether there is any correlation between coating composition, vector release, toxicity and transfection efficiency. The overall aim of this study is to establish an efficient and safe *in situ* gene transduction system on titanium implants for improved bone healing *in vivo*.

2. Materials and methods

2.1. Materials

If not stated otherwise, reagents were purchased from Sigma–Aldrich (Deisenhofen, Germany). Branched 25 kD polyethylenimine used in this study was prepared as an aqueous stock solution neutralized with hydrochloric acid. Poly(D,L-lactide) – Resomer 203 – was purchased from Boehringer Ingelheim, Ingelheim, Germany. All components (plasmid DNA, PEI, protective copolymer) were kept as stock solutions in deionized water.

2.2. Plasmids

The plasmid p55pCMV-IVS-luc+, coding for the firefly luciferase as a reporter gene under the control of the CMV promoter, was kindly provided by Andrew Baker, Bayer Corp., USA. The plasmid pB-BMP-2 was derived from p55pCMV-IVS-luc+ by removing the luciferase-encoding sequence using the Qiagen (Hilden, Germany) gel extraction kit after Hind III/Fse I digestion and by inserting the BMP-2 coding sequence which was PCR-amplified in order to introduce Hind III/Fse I restriction sites. Plasmids were expanded and purified using chromatographic techniques by Plasmid Factory GmbH & Co. KG, Bielefeld, Germany. The coding sequence of the β -galactosidase gene was cloned into the same plasmid backbone in a similar manner. This expression plasmid pBLacZ was purified by cesium chloride gradient.

2.3. Copolymer-protected gene vectors (COPROGs)

Copolymer-protected gene vectors used in this study were prepared from 25 kD branched polyethylenimine (PEI) and plasmid DNA at an N/P ratio of 8 coated with a layer of the negatively charged protective copolymer P6YE5C. P6YE5C was synthesized according to Finsinger et al. [24] with slight modifications as described recently [17,25]. Briefly, diamino-PEG-6000 was oligomerized with a thiol-reactive linker molecule and a negatively charged peptide (YE5)₂KahxC was coupled via disulfide bond to the polymer backbone. PEI–DNA–P6YE5C complexes (COPROGs) were prepared by mixing equal volumes of PEI, DNA and P6YE5C stock solutions in water, first adding the DNA solution to the PEI solution using a micropipette and

after 15 min adding the resulting complex to the P6YE5C solution. The concentrations of the stock solutions were 125.1 $\mu\text{g/ml}$ for PEI, 120 $\mu\text{g/ml}$ for DNA and 1.09 mM negative charge for P6YE5C. This results in a polyplex with a DNA concentration of 40 $\mu\text{g/ml}$ coated with 3 charge equivalents of protective copolymer (charge equivalent refers to the ratio of negative charges of the copolymer to the negative charges of DNA). Aliquots of DNA complexes were either transferred into cylindrical tubes of a 10 ml glass homogenizer (Schütt Labortechnik, Göttingen, Germany) or into 15 ml conical polypropylene tubes (Techno Plastic Products, Trasadingen, Switzerland) and were frozen either in ethanol/dry ice (glass tubes) or in liquid nitrogen (plastic tubes). The preparations were lyophilized overnight and the dried DNA complexes were dispersed in PDLLA solutions in ethyl acetate as described below.

2.4. Suspension of lyophilized COPROGs in PDLLA solutions in ethyl acetate and coating procedures

2.4.1. Procedure for homogenization in a glass homogenizer and coatings on polypropylene flat bottom 96-well plates

Ethyl acetate was added to lyophilized COPROG powder to result in a concentration in terms of DNA content of 400 $\mu\text{g/ml}$. The preparation was thoroughly dispersed by hand using the Teflon pestle of the homogenizer. From this original dispersion, a 1:1 dilution series was prepared in ethyl acetate, resulting in DNA concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 $\mu\text{g/ml}$. PDLLA was dissolved in ethyl acetate at concentrations of 20, 40, 80, and 160 mg/ml. Aliquots of 25 μl each of the COPROG dispersions were transferred to wells of a flat bottom polypropylene 96-well plate (Greiner Bio-One, Frickenhausen, Germany) and were mixed with 25 μl each of the four different PDLLA concentrations in ethyl acetate. The solvent was evaporated on air, followed by drying under high vacuum. This procedure resulted in an array of PDLLA films (500, 1000, 2000, and 4000 μg PDLLA per well) comprising for each quantity of PDLLA 78, 156, 312.5, 625, 1250, 2500, 5000 and 10,000 ng plasmid DNA, respectively, in COPROG formulation. The combinations ultimately used in the vector release and gene delivery studies are summarized in Table 1.

2.4.2. Procedure in conical polypropylene plastic tubes and coating on titanium foils

COPROGs comprising the firefly luciferase plasmid were prepared in water at a final DNA concentration of 40 $\mu\text{g/ml}$ as described above and were aliquoted into conical 15 ml polypropylene tubes to contain 70, 140, 280, and 560 μg , respectively, in four-fold repeats. The preparations were shock-frozen in liquid nitrogen and were lyophilized overnight. PDLLA stock solutions in ethyl acetate were prepared at concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 mg/ml and were sterile filtered (0.2 μm , Minisart, Sartorius, Germany). Aliquots of 700 μl each of these stock solutions were added to lyophilized COPROG preparations to obtain for each DNA dose DNA/PDLLA weight ratios of 0.2, 0.4, 0.8 and 1.6% or in other words DNA concentrations of 100, 200, 400 and 800 $\mu\text{g/ml}$. The tubes were briefly vortexed, and the COPROGs were thoroughly dispersed using a custom-made Teflon pestle that exactly fits the conical bottom of the 15 ml polypropylene tubes (dimensions of the Teflon pestle cone: $d = 13.5$ mm, $h = 21.5$ mm; the diameter is 250 μm smaller than the inner diameter in the conical bottom of the polypropylene tube). For this, the Teflon pestle was mounted via a stainless steel rod to an electric drill fixed in a stand and spun at 200 rpm for 120 s. Aliquots of 100 μl of each dispersion were applied drop wise to the surfaces of titanium discs (16 mm diameter, foil thickness 20 μm ; Friedent, Mannheim, Germany) using a micropipette. This procedure generated PDLLA films of various “thickness” comprising amounts of COPROGs as specified below. The table shows the weight percentage of DNA relative to PDLLA for each examined composition.

Comment: For economic reasons, methods and coating procedures were first established on plastic surfaces (polypropylene cell culture dishes). Selected coating formulations were subsequently evaluated on titanium foils.

Table 1

COPROG–PDLLA combinations used for coating polypropylene 96-well plates for release and gene delivery studies. The percentages in the table designate the w/w ratio of COPROG to PDLLA.

	PDLLA (mg/well)	6	4	2	1	0.5
	PDLLA ($\mu\text{g/mm}^2$)	180.8	120.5	60.3	30.1	15.1
COPROG ($\mu\text{g/well}$)	COPROG (ng/mm ²)					
5000	150.7	0.083%	0.125%	0.250%	0.500%	1.000%
2500	75.3	0.042%	0.063%	0.125%	0.250%	0.500%
1250	37.7	0.021%	0.031%	0.063%	0.125%	0.250%
625	18.8	0.010%	0.016%	0.031%	0.063%	0.125%
312.5	9.4	0.005%	0.008%	0.016%	0.031%	0.063%
156.25	4.7	0.003%	0.004%	0.008%	0.016%	0.031%
78.125	2.4	0.001%	0.002%	0.004%	0.008%	0.016%

2.4.3. Integrity of plasmid DNA upon dispersion of COPROGs using a glass homogenizer

Lyophilized COPROGs were prepared and lyophilized in a glass homogenizer as described above with 40, 20, and 7.5 μg plasmid DNA. The lyophilized powder was dispersed with a pestle in plain ethyl acetate exactly as described above for PDLLA solutions in ethyl acetate. The solvent was evaporated on air overnight. The dry preparations were rehydrated with 500 μl water. Aliquots thereof were run on a 0.6% basic agarose gel in 0.05 M sodium hydroxide, 1 mM EDTA in comparison with freshly prepared COPROGs (non-lyophilized, non-dispersed) and naked plasmid DNA (60 V, 30 min. Gel prepared with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide).

2.5. Characterization of PDLLA/COPROG films by scanning electron microscopy

Samples for SEM were prepared by coating of titanium discs with 100 μl ethyl acetate suspensions containing 5 μg DNA in COPROG formulation and 50 mg PDLLA. The specimens were gold coated with a Pelco SC-6 sputter coater (Ted Pella, Inc., Redding, CA), and imaged on a Hitachi S3500-N EDX electron microscope (Hitachi[®], High Technologies America, Pleasanton, CA). Four random view fields were taken from each of three samples. The COPROG particle size distribution was determined based on all particle populations from 12 view fields.

2.6. Release studies

Plasmid DNA was labeled with Iodine¹²⁵ using the Commerford method as modified by Terebesi et al. [26] and subsequently by Mykhaylyk et al. [27]. The labeling mixture was separated using a PD-10 gel filtration column (Amersham Biosciences, Freiburg, Germany) with water as the eluent. Iodinated DNA was analyzed by gel electrophoresis and autoradiography. This labeling procedure yields a specific radioactivity of ca. 47,000 Bq/ μg DNA. COPROGs and coatings on polypropylene 24-well plates and titanium discs were prepared as described above using a mixture of labeled and non-labeled DNA at a ratio of 1:4 (w/w) in order to result in a radioactive DNA dose per well of ca. 2000 Bq and ca. 8300 Bq per titanium disc, respectively. For the release study in the polypropylene 96-well plate, the absolute amounts of COPROGs (in terms of DNA dose) applied per well were 0.625, 1.25, 2.5 and 5 μg comprised in 500, 1000, 2000 and 4000 μg PDLLA. For the release study from 16 mm titanium discs, the absolute amounts of COPROGs (in terms of DNA dose) applied per disc were 10, 20, 50, 100, and 200 μg , all comprised in coatings of 2.5 mg PDLLA (0.4%, 0.8%, 2%, 4%, 8% DNA/PDLLA). Experiments were carried out in 6 repeats per composition. Two hundred μl each of Dulbecco's Modified Eagle Medium were added to each well of the polypropylene plate. Titanium discs were incubated in 24-well plates with 1 ml cell culture medium each, and kept in a standard cell culture incubator at 37 °C in an atmosphere of 5% CO₂ and 99% humidity. Supernatants were removed for radioactivity determination at the time points indicated in Fig. 5 and replaced with fresh medium (DMEM, Biochrom, Berlin, Germany). Radioactivity in the supernatants (i.e. released plasmid DNA) was determined using a Wallac 1480 Wizard 3[®] gamma counter (Perkin Elmer Wallac, Freiburg, Germany). The cumulative release was calculated as the percentage of the applied DNA dose.

2.7. Cell culture and transfections

Cells were grown at 37 °C in an atmosphere of 5% CO₂ at 99% relative humidity. Human embryonic kidney cells (HEK 293) (DSMZ ACC305, Germany) and NIH 3T3 mouse fibroblasts (DSMZ ACC 59) were incubated in DMEM containing 10% FCS, 2 mM-glutamine, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Human mesenchymal stem cells (hMSCs) (Cambrex Bio Science Walkersville, Inc. East Rutherford, NJ) were cultured in DMEM supplemented with 4 mM-glutamine, 4.5 g/l glucose, 25 mM HEPES buffer, 10% fetal bovine serum (Gibco), 10 U/ml penicillin G sodium salt, 10 $\mu\text{g}/\text{ml}$ streptomycin, 25 $\mu\text{g}/\text{ml}$ amphotericin B (Gibco), and 100 $\mu\text{g}/\text{ml}$ L-ascorbic acid (Sigma–Aldrich, Oakville, Ontario, Canada).

For the transfection studies in polypropylene plates, 7000 NIH 3T3 cells/well in 200 μl medium were seeded on the COPROG^{Luc}/PDLLA films. After 48 h cultivation on the films, the cells were washed with PBS and then incubated with 100 μl lysis buffer (0.1% Triton X-100 250 mM Tris–HCl pH 7.8). Fifty μl each of the cell lysates were mixed with 100 μl Luciferase Assay Reagent (Promega, Madison, WI) and luciferase activity was measured using a TopCount instrument (Canberra Packard, Groningen, The Netherlands). From the relative light units, the amount of expressed luciferase was calculated using standard curves obtained with recombinant luciferase protein. Luciferase levels were normalized to the total protein contents of the lysates which were determined with the BioRad protein assay reagent.

Titanium discs coated with COPROG^{BMP-2}/PDLLA were placed in 24-well plates and 200 μl medium with 8×10^4 HEK 293 cells or 16×10^4 hMSCs, respectively, were added drop wise to the foils. After 2 h incubation to allow sedimentation and attachment of the cells, 800 μl medium were added. At indicated time points, the supernatants were collected and replaced with fresh medium. The expression of secreted BMP-2 was determined by ELISA carried out according to the instructions of the manufacturer (human BMP-2 Quantikine, R&D Systems, Wiesbaden, Germany).

The percentage of transfected cells was determined on titanium discs coated with COPROG^{LucZ}/PDLLA. 2×10^5 hMSCs were seeded on the discs and cultivated for 72 h. Subsequently the cells were stained with X-gal for 4 h, and the percentage of blue-stained cells compared to total cell-covered area in randomly selected fields was measured using a KS400 imaging system (Carl Zeiss Vision GmbH, Hallbergmoos, Germany).

2.8. Evaluation of cytotoxicity

For cells grown in COPROG/PDLLA-coated polypropylene plates, cell viability was determined by the WST[®]-1 assay (Roche, Mannheim, Germany) according to the manufacturer instructions. The cell viability on coated titanium discs was assessed by MTT cytotoxicity assay. Titanium foils coated with PDLLA at various concentrations or PDLLA of various concentrations comprising various amounts of COPROGs were seeded with 10^4 HEK 293 or NIH 3T3 cells per foil. The foils were placed in 48-well tissue culture plates, were covered with DMEM containing 10% FBS and were

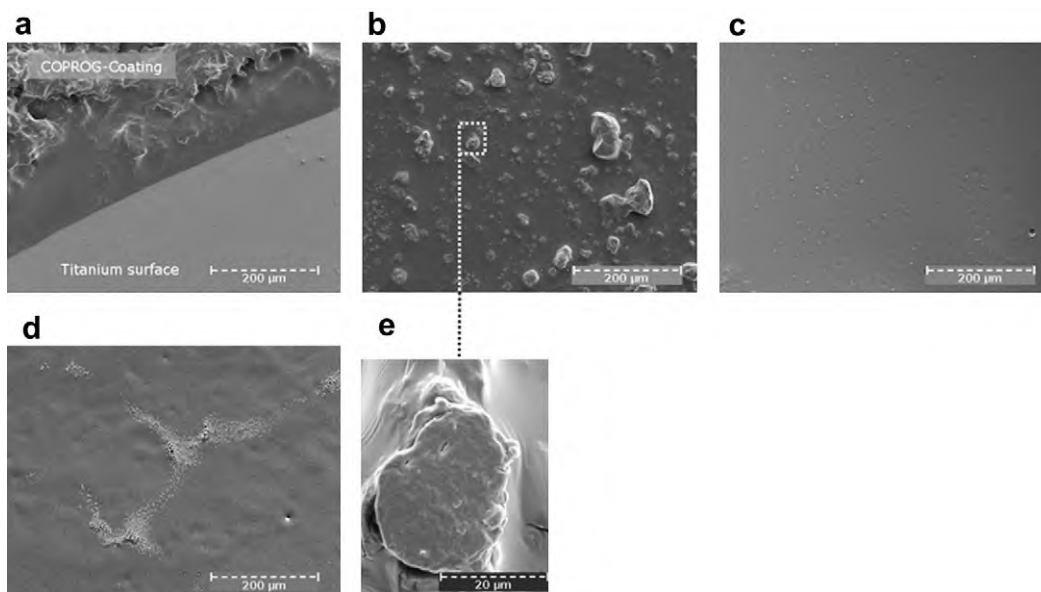


Fig. 1. Scanning electron microscopy (SEM) (a) of the adjacent boundary layer of the titanium/COPROG–PDLLA coating, (b) of the COPROGs after homogenization in PDLLA with the Teflon pestle procedure, (c) after homogenization with the glass potter, (d) of the native PDLLA coating and (e) 10 \times magnification of b (dotted frame). Scale bar represents 200 μm (a–d), respective 20 μm (e).

incubated for 24 h. Then, 25 μ l of MTT stock solution (2 mg/ml in PBS) were added to each well. After 4 h of incubation at 37 °C, the medium was removed and 150 μ l of DMSO was added to each well to dissolve the formazan crystals formed by proliferating cells. Plates were incubated for an additional 4 h at 37 °C in a tissue culture incubator. Cells cultivated in a 48-well plate under standard conditions were treated with MTT/DMSO in the same manner and were taken as a control for 100% viability. Cells grown under standard conditions without MTT addition were used as a blank. The absorbance was measured at 595 nm using a Bio-Tek EL-311 micro plate reader. The cell viability (%) was calculated as percentage relative to untreated control cells according to the following equation:

$$\text{Cell viability} = 100 \times \frac{\text{OD}_{595} \text{ sample} - \text{OD}_{595} \text{ blank}}{\text{OD}_{595} \text{ control} - \text{OD}_{595} \text{ blank}}$$

Similarly, a lactic acid dehydrogenase (LDH) assay was performed using the cytoTox 96™ non radioactive cytotoxicity assay kit (Promega, Madison, WI). In this case, cells were seeded in a 24-well plate at a density of 2.5×10^4 cells/cm² on COPROG/PDLLA-coated foils and were cultivated for 48 h. Supernatants were taken after 24 h and 48 h to determine LDH release to the supernatant. As a measure of cell damage, the percentage of released LDH relative to total LDH determined in 0.9% Triton X-100 cell lysates was calculated.

2.9. Analysis of correlations between COPROG/PDLLA ratio, vector release, cell viability and gene transfer efficiency

The data obtained when plotting the percentage of COPROG release versus the PDLLA film thickness (Fig. 4b) and when plotting vector release versus cell viability (Fig. 4a) where fitted using the “log(inhibitor) vs. response – Variable slope (four parameter)” function of GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The obtained logistic functions were used to calculate a matrix of cell viability in dependence of film thickness and COPROG dose. Contour plots of these data (Fig. 4c) were generated using OriginPro 8.1G, OriginLab Corporation, Northampton, MA, USA.

3. Results

3.1. DNA integrity and scanning electron microscopy of COPROG/PDLLA coating on titanium

The integrity of plasmid DNA was assessed by agarose gel electrophoresis under conditions where COPROGs are disrupted (data not shown). Intact plasmid bands were detected which indicated that neither lyophilization nor the dispersion of the COPROG powder in ethyl acetate affected DNA integrity.

Titanium discs of 16 mm diameter were used to determine the morphology of a COPROG/PDLLA film by scanning electron micrograph microscopy (SEM) (Fig. 1a–e). Surface coatings were prepared by dispersing COPROGs either with a Teflon pestle or with a glass potter. SEM images indicate a smooth gap free transition between the PDLLA coating and the titanium support (Fig. 1a) at the boundary layer of the coating. Films generated with the Teflon pestle exhibited aggregates of non-uniform size where the smaller aggregates were evenly distributed in the coating (Fig. 1b and e). The diameters of the aggregates varied from 60 to 110 μ m with an inordinate aspect. In contrast, films generated with the glass potter produced a more homogenous film with much smaller aggregates with a spherical, smooth morphology and diameters of 6–10 μ m (Fig. 1c). The different homogenization procedures were analyzed with respect to their influence on gene transfer efficiency in HEK 293 and NIH 3T3 cells grown on COPROG/PDLLA-coated titration foils (data not shown). As the Teflon pestle procedure led to

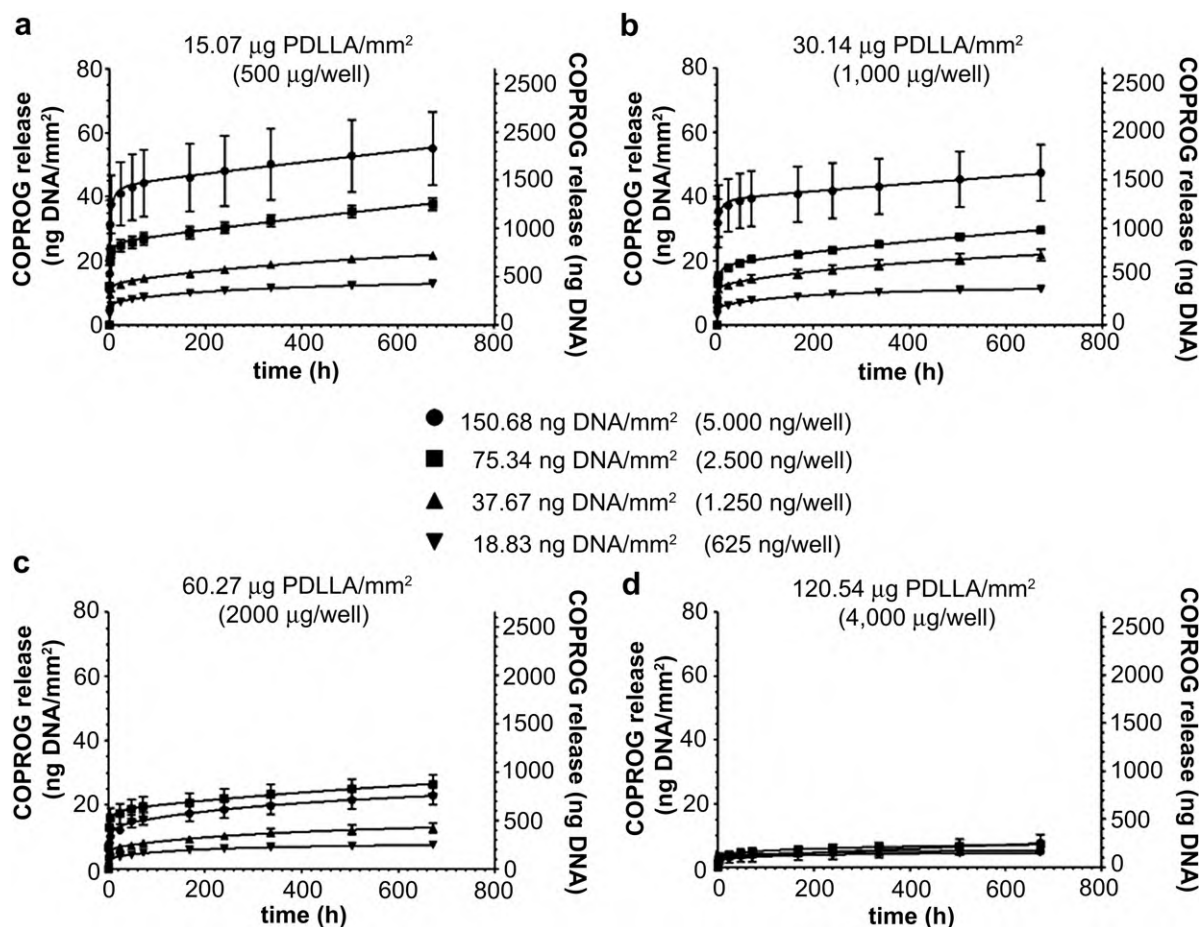


Fig. 2. COPROG release profile over 28 days from COPROG/PDLLA-coated polypropylene wells, from (a) to (d) stepwise doubling the PDLLA film thickness results in a reduction of vector release at all examined vector doses.

a significantly higher luciferase expression compared to the glass potter method, all further experiments were performed using the Teflon pestle system for *COPROG* dispersion in PDLLA solutions in ethyl acetate.

3.2. *COPROG* release, gene delivery and toxicity profile with *COPROG*/PDLLA coatings on polypropylene plates

For cost reasons, *COPROG*/PDLLA coatings were initially established and evaluated on polypropylene dishes. Release profiles were obtained using ^{125}I -labeled DNA. The higher the *COPROG* dose at a given PDLLA concentration, the higher was the amount of released DNA (Fig. 2a–d). The higher the PDLLA concentration at a certain *COPROG* dose, the lower was the amount of released vector such that at a “thickness” of 4000 μg PDLLA (120.5 $\mu\text{g}/\text{mm}^2$) only little release was detectable (Fig. 2d). DNA release in terms of the percentage of applied dose was inversely proportional to the applied dose, i.e. the lower the absolute *COPROG* dose, the higher was the release in percentage of the applied dose. At low dose, *COPROG*s possibly occupied primarily the surfaces of the coatings, whereas at higher doses a fraction occupied areas deeper in the PDLLA layer and thus was not readily released. The release profiles are characterized by an initial burst of release within the first few hours followed by a more steady state of release until the final time point of the study which was after 28 days. The data are shown in Fig. 2. The dose–response profiles of reporter gene expression in dependence of coating thickness obtained with NIH 3T3 cells grown on the *COPROG*/PDLLA coatings were consistent with the release profiles up to a DNA dose of 625 ng per well (18.8 ng/mm^2) or 1.25 μg per well (37.7 ng/mm^2), respectively, in the case of the highest PDLLA concentration (4000 $\mu\text{g}/\text{well}$ or 120.5 $\mu\text{g}/\text{mm}^2$). Up to this dose, reporter gene expression increased with DNA dose and decreased with PDLLA film thickness (Fig. 3a). However, beyond this DNA amount, the reporter gene expression profile is dominated by the toxicity of the released vector (Fig. 3b). Expression levels tend to decrease with increasing DNA dose and to increase with cumulating coating thickness, which reduces DNA release and thus toxicity. The combined effects of coating thickness, responsible for the release profile, and vector toxicity give rise to an optimum composition of the *COPROG*/PDLLA coating around $\sim 20 \text{ ng}/\text{mm}^2$ DNA in 15–30 μg PDLLA/ mm^2 .

Summarizing, the release characteristics, cell viabilities and transfection efficiencies were strongly dependent on the ratios of *COPROG*-to-poly lactide. To further analyze the interdependencies and assuming that the initial burst of vector release governs the observed gene transfer efficiency and toxicity, we plot in a first step the initial release (cumulative release at 2 h in terms of ng or ng/mm^2 DNA) versus the *COPROG* input dose (in terms of ng or ng/mm^2 DNA) for each film thickness (μg PDLLA/ mm^2) and obtain linear relationships. Plotting the percent DNA release per 2 h (or in other words the slopes of the obtained linear regression curves) versus the film thickness, we obtain a strong inverse relationship (e.g. decreasing release with increasing film thickness) which can be fitted with a logistic function (Fig. 4a). From the equation one can calculate predicted release quantities (at 2 h) for every combination of *COPROG* dose and film thickness. If one plots the measured cell viability versus the logarithm of the measured vector release quantity at 2 h one obtains another relationship which also can be fitted with a logistic function (Fig. 4b). For NIH 3T3 cells grown on *COPROG*–PDLLA coated on polypropylene one finds an IC_{50} of 278 ng (8.4 ng/mm^2) released vector (in terms of DNA content) within 2 h.

Having the two logistic functions, one can now calculate for any combination of film thickness and *COPROG* dose a matrix of predicted cell viabilities which matches very well the observed cell

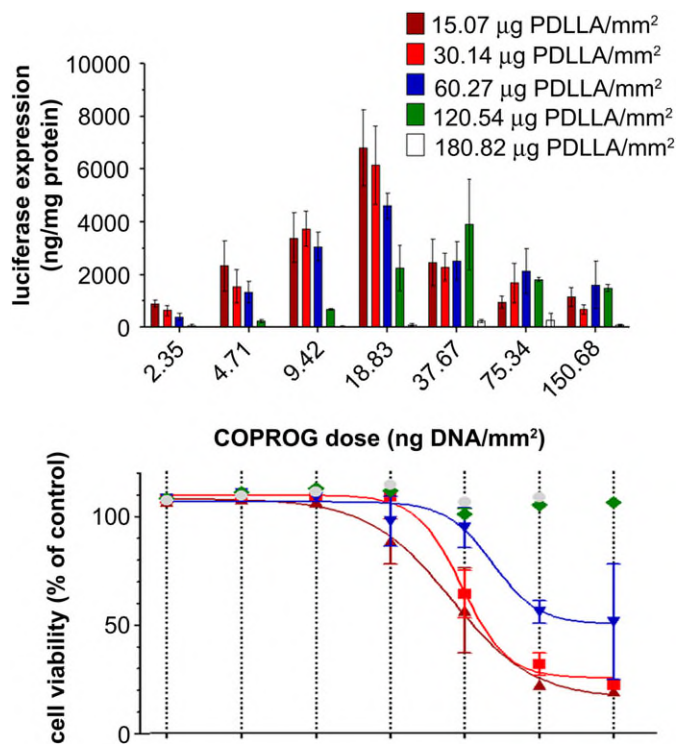


Fig. 3. Reporter gene expression (a: upper figure) and cell viability (b: lower figure) in *COPROG*^{Luc}/PDLLA-coated polypropylene wells in dependence of film thickness and DNA dose. Gene transfer efficiency is dependent on vector dose, film thickness and cell viability. Reporter gene expression decreases parallel to compromised cell viability or to high film thickness.

viabilities (Fig. 4c and d). Hence one can derive predictions on useful combinations of *COPROG* and PDLLA (Fig. 4d). Finally, one can overlay a contour plot of the reporter gene expression data (shown in Fig. 4) on the calculated cell viability plot (Fig. 4d). The result shows that there is only a narrow useful range of combinations of *COPROG* and PDLLA covering 2.5–160 ng/mm^2 in terms of *COPROG* dose and 10–200 $\mu\text{g}/\text{mm}^2$ in terms of film thickness.

3.3. *COPROG* release, gene delivery and toxicity profile with *COPROG*/PDLLA coatings on titanium discs

Release studies were carried out over a period of 11 weeks with 16 mm titanium discs coated with one constant amount of PDLLA (2.5 mg; 12.4 $\mu\text{g}/\text{mm}^2$) comprising various amounts of *COPROG*. This particular amount of PDLLA corresponds approximately to the coating with 500 μg PDLLA per well in the polypropylene plates (15.1 $\mu\text{g}/\text{mm}^2$) which yielded optimal results in gene delivery (Fig. 3). The observed release characteristics were similar to those with coatings on polypropylene. There was a strong linear relationship between applied and released *COPROG* dose. There was an initial release burst of 14–23% of the applied doses within the first 5 h followed by a phase of slow continuous release at the later time points (Fig. 5a). Among the examined coating compositions, the one with 10 μg DNA (49.7 ng/mm^2) in *COPROG* formulation embedded in 2.5 mg PDLLA (12.4 $\mu\text{g}/\text{mm}^2$) on titanium is comparable to the coating with 1.25 μg DNA (37.7 ng/mm^2) in *COPROG* formulation embedded in 500 μg (15.1 $\mu\text{g}/\text{mm}^2$) PDLLA on polypropylene. The release curves for these two compositions on the two different support materials expressed in ng DNA released per mm^2 of coated material were almost identical during four weeks

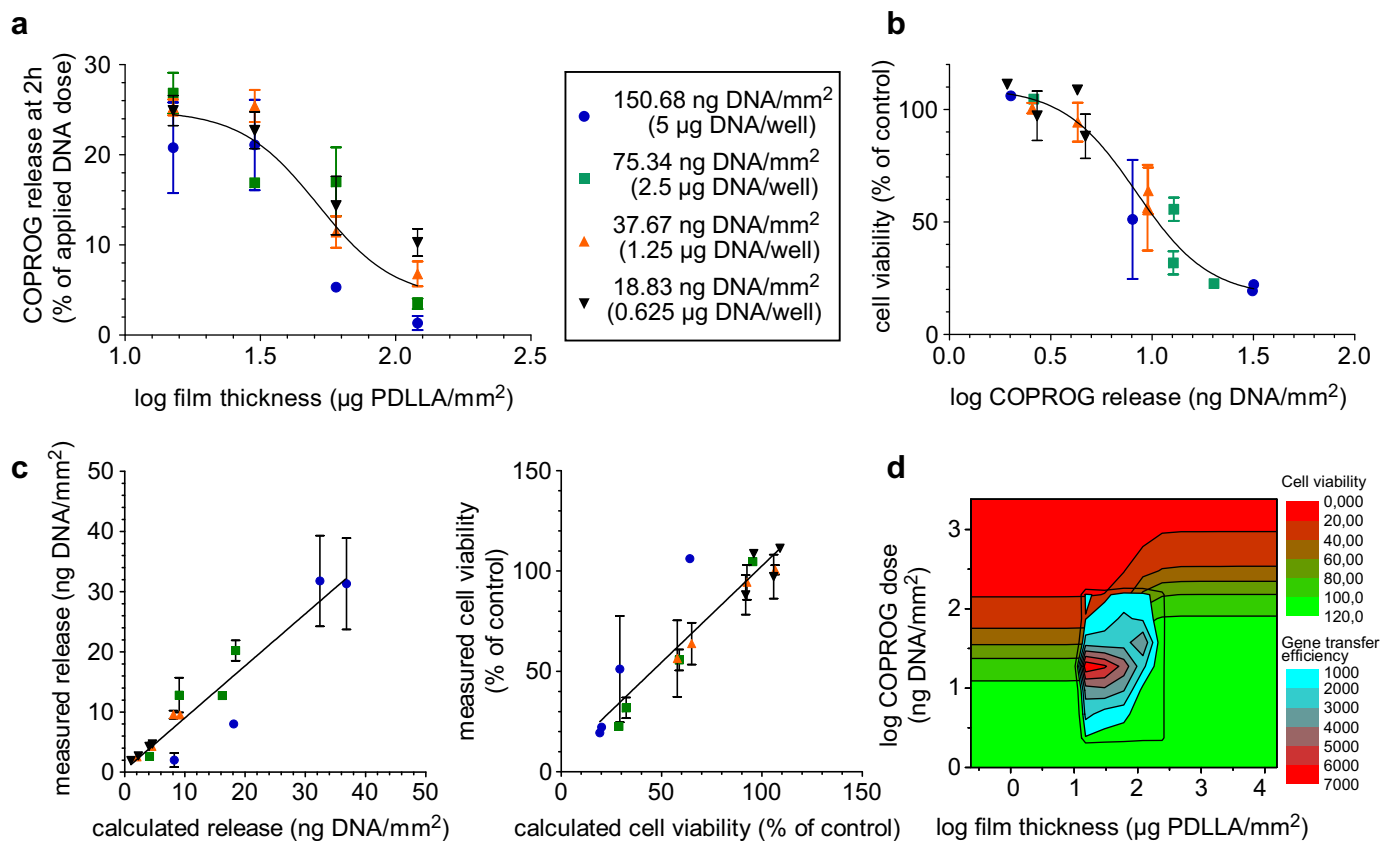


Fig. 4. Correlations between *COPROG* dose, *COPROG* release, cell viability and gene transfer efficiency. (a) *COPROG* release is dependent on film thickness. The release data can be used to predict *COPROG* release for any given combination of *COPROG* and PDLLA, according to the fitted logistic function $Y = 4.006 + (24.92 - 4.006) / \{1 + 10^{[(1.713 - X)^{-3.053}]}\}$. (b) Cell viability decreases with increasing *COPROG* release. The data can be fitted with the logistic function $Y = 16.79 + (109.6 - 16.79) / \{1 + 10^{[(0.923 - X)^{-2.42}]\}$. (c) Calculated release versus measured release and calculated cell viability versus measured cell viability match very well for NIH 3T3 cells grown on *COPROG*-PDLLA, except for the highest examined *COPROG* dose. (d) From the derived logistic functions, the cell viability can be predicted for any combination of *COPROG* dose and PDLLA film thickness shown in a contour plot. The plot is overlaid with another contour plot derived from the gene delivery data in Fig. 3. The data in (a), (b) and (c) are means \pm SEM ($n = 3$).

(Fig. 5b). Concluding, the essential factors governing *COPROG* release are on the one hand the ratio of *COPROG* to PDLLA and at a given ratio the *COPROG* dose per unit area of coated material.

Gene delivery studies were carried out with HEK 293 seeded on titanium foils coated with the *COPROG*^{BMP-2}/PDLLA compositions

specified in Table 2. An initial burst of BMP-2 expression was observed on days 1 and 2 and cumulative quantities of BMP-2 were evaluated over a period of 14 days. As can be seen in Fig. 6, the major factors driving the extent of BMP-2 expression are the “thickness” of the PDLLA coating and the concentrations of

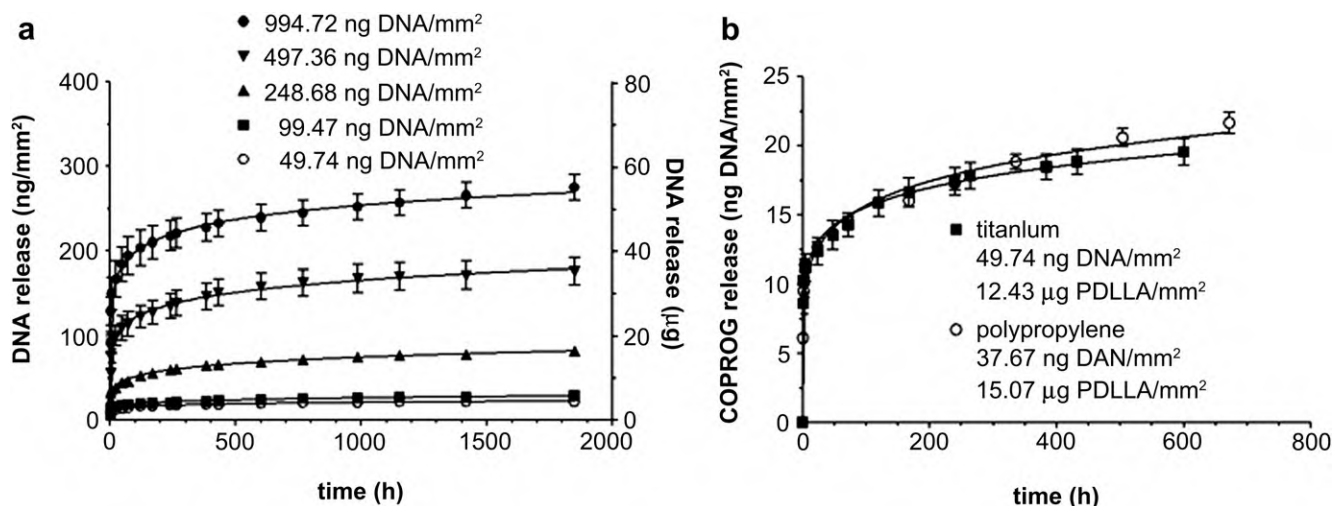


Fig. 5. (a) *COPROG* release profile over 77 days from *COPROG*/PDLLA-coated titanium foils. (b) comparison of release from comparable coatings on polypropylene and on titanium.

Table 2

COPROG–PDLLA combinations used for coating titanium for release and gene delivery studies. The combinations shaded in gray were used for gene delivery studies in HEK 293 cells, the combinations shaded in gray and italicized were used for gene delivery to MSCs. The percentages in the table designate the w/w ratio of *COPROG* to PDLLA.

		PDLLA	40	20	10	5	2.5	1.25	0.625
		(mg/disc)							
		PDLLA	198.9	99.5	49.7	24.9	12.4	6.2	3.1
		($\mu\text{g}/\text{mm}^2$)							
<i>COPROG</i>	<i>COPROG</i>	Release studies							
($\mu\text{g}/\text{disc}$)	(ng/mm^2)								
200	994.7							8.00%	
100	497.4							4.00%	
80	397.9		0.20%	0.40%	0.80%	1.60%			
50	248.7							2.00%	
40	198.9		0.20%	0.40%	0.80%	1.60%			
20	99.5		0.10%	0.20%	0.40%	0.80%		1.60%	
10	49.7				0.20%	0.40%		0.80%	1.60%

*COPROG*s in the coatings. We observed an inverse dose–response profile with respect to the *COPROG* concentration at a PDLLA “thickness” of 2.5 mg ($12.4 \mu\text{g}/\text{mm}^2$) and 5 mg ($24.9 \mu\text{g}/\text{mm}^2$) PDLLA per foil (i.e. increasing amounts of *COPROG* per foil yielding decreasing BMP-2 expression). In contrast, at 10 mg ($49.7 \mu\text{g}/\text{mm}^2$) PDLLA “thickness” per foil this relationship changes to a positive

dose–response profile, with growing amounts of vector yielding increasing BMP-2 expression. The highest expression levels were found with the lowest examined *COPROG* dose which was 10 μg ($49.7 \text{ ng}/\text{mm}^2$) DNA per foil at a PDLLA “thickness” of 2.5 mg ($12.4 \mu\text{g}/\text{mm}^2$) and 5 mg ($24.9 \mu\text{g}/\text{mm}^2$) per foil. Due to the fact that BMP-2 expression decreased sharply on day 3 (Fig. 6c), the cumulative expression did not increase significantly after this time point (Fig. 6a). As is the case with *COPROG*/PDLLA coatings on polypropylene, the observed dose–response relationships on titanium are probably the result of vector toxicity beyond a certain amount of released vector during the initial burst. On polypropylene, toxicity for NIH 3T3 cells is observed roughly beyond 7 ng DNA/ mm^2 (in *COPROG* formulation) released during the initial burst. For the 2.5 mg ($12.4 \mu\text{g}/\text{mm}^2$) PDLLA coating on titanium which as mentioned is comparable with the 0.5 mg ($15.1 \mu\text{g}/\text{mm}^2$) PDLLA coating on polypropylene, all examined vector doses release *COPROG* above this threshold level. Hence, decreasing reporter gene expression with increasing *COPROG* dose can be explained. In fact, substantial cytotoxicity in HEK 293 and NIH 3T3 cells as measured with an MTT assay was observed beyond a DNA dose (in *COPROG* formulation) of 12.5 μg ($62 \text{ ng}/\text{mm}^2$) in the PDLLA coating of 5 mg per foil ($24.9 \mu\text{g}/\text{mm}^2$) (Fig. 6d). The IC_{50} in NIH 3T3 cells was 71.5 ng/mm^2 (in terms of coated DNA dose in *COPROG* formulation). This corresponds to 9.6 $\text{ng DNA}/\text{mm}^2$ *COPROG* released within 2 h according to the release data. This is in excellent agreement with the IC_{50} measured for NIH 3T3 grown on *COPROG*^{Luc}–PDLLA coated

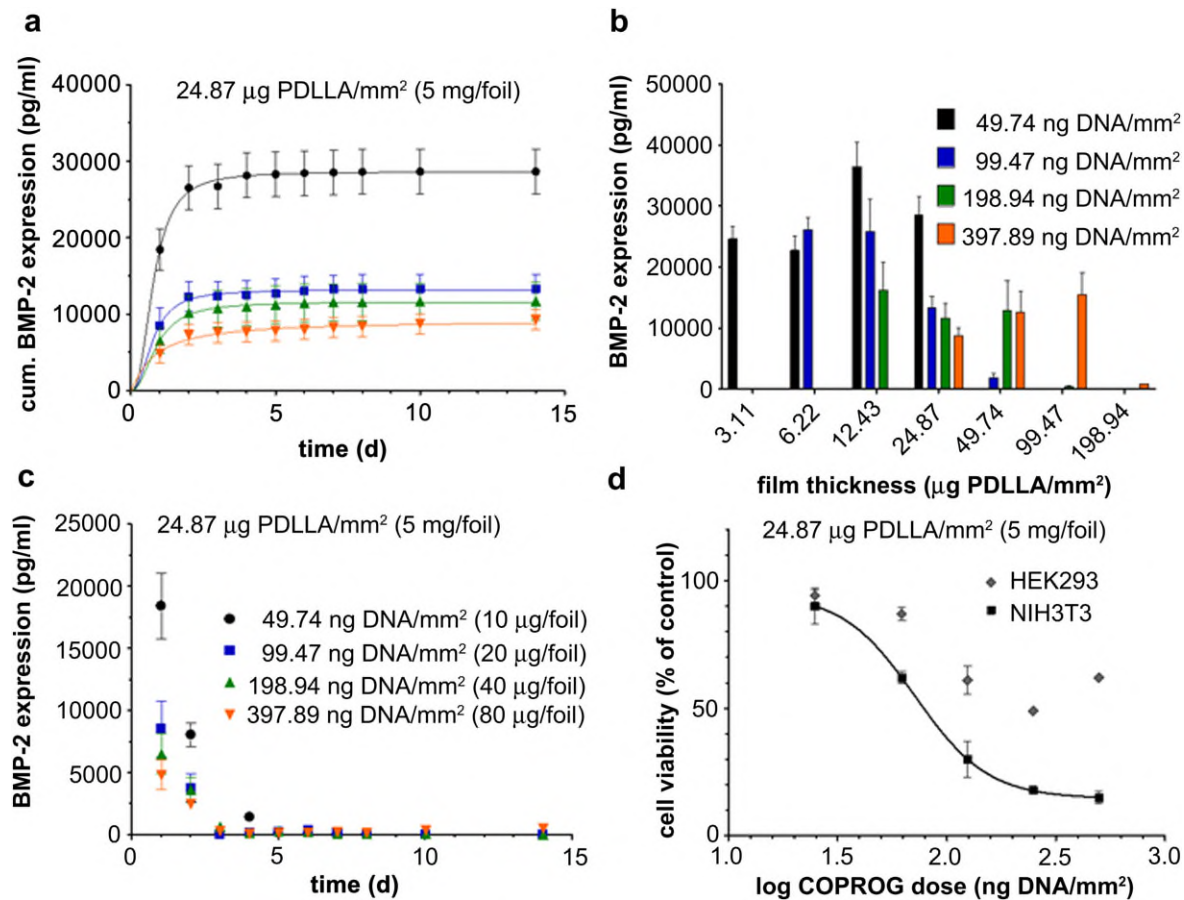


Fig. 6. BMP-2 expression in HEK 293 cells grown on *COPROG*/PDLLA-coated titanium foils. (a) Cumulative expression over 14 days at intermediate film thickness ($24.9 \mu\text{g}$ PDLLA/ mm^2 or 5 mg/foil). (b) Cumulative BMP-2 expression by day 10 at various combinations of *COPROG*s and PDLLA. (c) Expression kinetics at a film thickness of $24.9 \mu\text{g}$ PDLLA/ mm^2 at various *COPROG* doses. (d) Cell viability as determined by MTT test in HEK 293 and NIH 3T3 cells grown on *COPROG*/PDLLA titanium foils of intermediate film thickness ($24.9 \mu\text{g}$ PDLLA/ mm^2 or 5 mg/foil).

polypropylene plates which was 8.4 ng/mm^2 released DNA in *COPROG* formulation (Fig. 4b).

In a final experiment we examined whether human mesenchymal stem cells can be transfected on *COPROG*^{BMP-2}/PDLLA titanium foils. While the transgene expression of HEK 293 cells on *COPROG*^{BMP-2}/PDLLA on titanium peaked at day 1, the expression kinetics of hMSCs was significantly different. With these cells, transgene expression started late and reached a peak only after 5–6 days of cultivation on the vector-coated foils (Fig. 7). The expression was about 40-fold lower than the one observed with HEK 293 cells with the same coating composition ($10 \mu\text{g DNA}/5 \text{ mg PDLLA}$ per foil or 49.7 ng DNA/mm^2 and $24.9 \mu\text{g PDLLA/mm}^2$). BMP-2 expression decreased with decreasing film thickness (Fig. 7a). When increasing the starting film thickness in the titration by a factor of 4 and doubling the starting *COPROG* dose, strongly increasing BMP-2 expression was observed (Fig. 7b). The sigmoidal nature of the relationship between the film thicknesses, *COPROG* dose and cell viability may explain the observed expression profile (Fig. 4d shows such a relationship for NIH 3T3 cells). Quantitative transfection efficiency in terms of the percentage of transfected cells was assessed by cultivation of hMSCs on titanium foils coated with *COPROG*^{LacZ}/PDLLA. LacZ staining indicated that 11% of cells became transfected (data not shown).

4. Discussion

The ultimate aim of this work is generating a gene-activated matrix on metallic surfaces for use in bone regeneration, bone substitution and osseous integration of metallic implants. The matrix should be abrasion-resistant during implantation. Therefore we have focussed our work on a PDLLA coating on titanium as an already established implant coating process [11]. The general motivation for this work is that insufficient osseous integration of implants and implant loosening are frequent problems in clinical practice [6,28]. Due to the demographic changes in industrialized countries and the rising incidence of degenerative musculo-skeletal diseases there is an increasingly urgent need for bioactive implants.

The patients' own regenerative potential can be activated by growth factors which consequently have been used successfully in tissue regeneration [5,19,29,30]. On the other hand, suitable surface and material properties of implants alone can be supportive of regenerative processes [6,31]. For example, the osteoconductive properties of polylactide coatings on metallic implants are well

described [32]. Furthermore, their mechanical stability and high abrasion resistance [9] are highly favorable when implants are screwed or punched into bone. Also, polylactide coatings have been used successfully to embed recombinant growth factors which mediated complete bone reconstitution in critical bone defect models [33]. Therefore, we are interested in whether similar or even better bioactivity can be achieved when a gene vector encoding an appropriate growth factor is embedded in a polylactide coating on metallic surfaces like titanium. This material is routinely used in orthopaedic surgery or implant dentistry. In different context, gene vectors like plasmid DNA have been combined advantageously with polylactides. For example, Huang et al. have described poly(lactide-co-glycolide; PLGA) scaffolds obtained by a gas foaming process and comprising a PEI-condensed plasmid DNA encoding BMP-4 [20]. This gene-activated matrix was suitable to mediate bone regeneration in a rat cranial defect model [21]. In yet another context, microparticles prepared from PLGA [34,35] or poly(beta-amino ester)s [36] and plasmid DNA or gene vectors are known for their high potential as genetic vaccines.

However, so far there have been only few reports on direct polylactide coatings on metallic surfaces which comprise a gene vector or a plasmid. As gene vectors are insoluble in organic solvent, emulsion procedures have been applied previously for incorporation into polylactide microparticles [34,36,37] or for embedding in polyurethane or PLGA coatings on stents [38,39]. Simple dispersion in organic polylactide solutions has been applied for lyophilized recombinant growth factors in order to get them incorporated into polylactide films upon evaporation of the solvent [11,40,41]. For this simple procedure, the properties of copolymer-protected gene vectors (*COPROGs*) which we have developed previously [16] turned out advantageous. *COPROGs* are obtained upon shielding polyethylenimine-DNA nanoparticles with a copolymer of PEG and an anionic peptide derivative by electrostatic interaction [24]. As *COPROGs* can be lyophilized with little loss of activity without addition of further components such as cryoprotectants [16] we reasoned that it should be possible to disperse lyophilized *COPROGs* in organic polylactide solutions. A first concern was potential plasmid damage during the dispersion process. It is known for example that shear stress during emulsification procedures for the preparation DNA-containing microspheres can nick supercoiled plasmid DNA [42]. Therefore, we initially tested different potters for the homogenization of *COPROG* lyophilizates in PDLLA solutions. Even when a glass potter was used for dispersion which generates

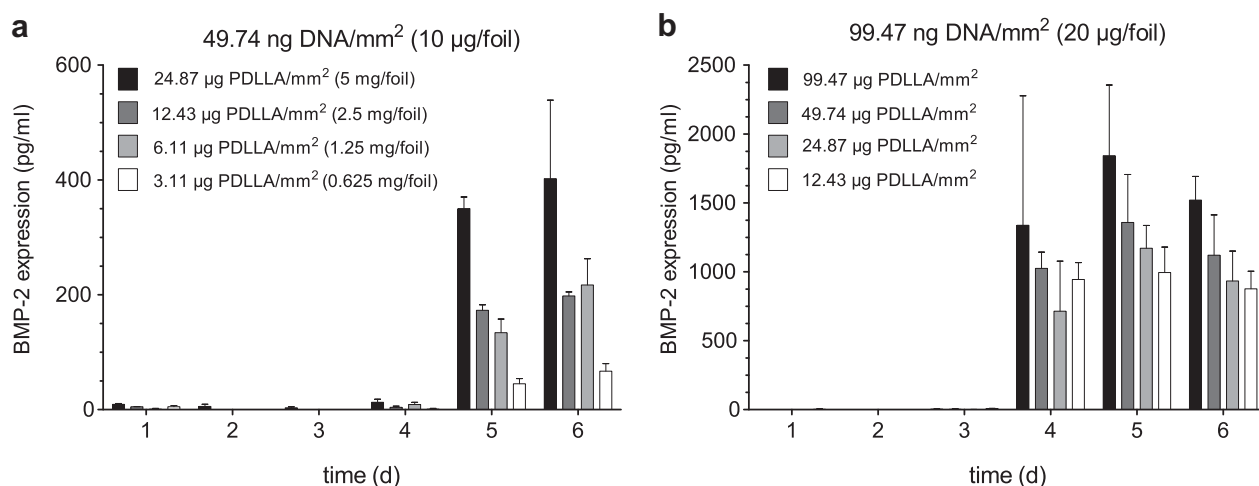


Fig. 7. BMP-2 expression in mesenchymal stem cells grown on *COPROG*/PDLLA-coated titanium foils at (a) $10 \mu\text{g DNA}/\text{foil}$ and (b) $20 \mu\text{g DNA}/\text{foil}$. BMP-2 expression was determined by ELISA in the supernatants at the days indicated in the figure.

strong shear forces and resulted in a fine dispersion of relatively small particles (Fig. 1c), the DNA stayed intact.

For establishing the system, we systematically varied the ratio of COPROG-to-poly(lactide) and their concentrations in ethyl acetate and prepared coatings on polypropylene 96-well plates. These films released COPROGs with kinetics that was dependent on the COPROG-to-poly(lactide) ratio and the film thickness. Based on the assumption that gene transfer efficiency and toxicity to cells grown on COPROG-PDLLA films is governed by the initial burst of release, we founded the further analysis of putative correlations upon the 2 h release data. Correlating, in a first step, film thickness with COPROG release and in a second step the release with cell viability allowed us to establish a formula with which for any combination of COPROG and PDLLA the cell viability could be predicted. As shown in the results part, the predicted values were quite accurate (Fig. 4c). Although such predictions are valid only for one cell type under consideration (in our case NIH 3T3 cells), the found relationships are quite useful in identifying suitable COPROG/PDLLA combinations for coating the expensive titanium foils. This is highlighted for example by the viability of NIH 3T3 cells grown on COPROG^{BMP-2}/PDLLA foils (Fig. 6d) which was consistent with the predicted values. Furthermore, the BMP-2 expression data shown in Figs. 6 and 7 were obtained with COPROG doses which are above the predicted useful dose range, except for the lowest applied dose. Consistent with this, the expression of the transfected BMP-2 gene and the cell viability declined with increasing COPROG dose.

A conclusion of this study is that there is a delicate balance between vector toxicity and gene transfer efficiency, with the former being governed by the vector composition and its dose. The COPROGs used here are polyplexes based on 25 kD poly(ethyleneimine) which is known for its toxicity *in vitro* and *in vivo* [43–45]. The acute toxicity for cells is likely due to free PEI in the complexes [43,44,46]. The protective copolymers used to prepare COPROGs can “neutralize” this free PEI by forming “ghost particles”, i.e. particles consisting of PEI and protective copolymer only [25]. Thus we speculate that protective copolymers eliminate the acute toxicity mediated by free PEI in PEI-DNA polyplexes while fully maintaining or even improving transfection competence of PEI polyplexes [24]. This may be the reason why COPROGs turned out superior to unshielded PEI polyplexes also on gene-activated collagen sponges and fibrin clots [16,17]. A variety of known nonviral vectors, shielded or non-shielded have advantageous toxicity profiles and may be a good alternative to using COPROGs for preparing gene-activated matrices. The strategy presented here will be useful to identify suitable combinations of a given gene vector with a given coating material in a rapid screening process and to forecast ideal combinations.

5. Conclusion

We demonstrate a versatile method to embed nonviral gene vectors in a PDLLA surface coating on titanium. The ratio vector to coating material, the vector dose per surface unit and the coating film thickness appear to govern the observed and inter-dependent cell viability and transfection efficiency. The described screening and analysis procedure allows identifying and predicting useful combinations of vectors and coating materials.

Acknowledgments

This work was supported by the German Federal Ministry of Education and Research (grant 0312019A) and by the Nanosystems Initiative Munich (NIM).

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