Materials Today Communications 16 (2018) 169–173

Contents lists available at ScienceDirect

Materials Today Communications

journal homepage: www.elsevier.com/locate/mtcomm

Surface mediated non-viral gene transfection on titanium substrates using polymer electrolyte and nanostructured silicate substituted calcium phosphate pDNA (NanoSiCaPs) composites

Sudhanshu Shekhar[a,e], Boeun Lee[a], Abhijit Roy[a], Joe Candiello[a], Prashant N. Kumta[a,b,c,d,e,*]

[a] Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, 15261, USA
[b] Department of Chemical Engineering, University of Pittsburgh, Pittsburgh, PA, 15261, USA
[c] Department of Mechanical Engineering and Materials Science, University of Pittsburgh, Pittsburgh, PA, 15260, USA
[d] Center for Complex Engineered Multifunctional Materials, University of Pittsburgh, Pittsburgh, PA, 15261, USA
[e] McGowan Institute of Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, 15261, USA

ARTICLE INFO

Keywords:
Calcium phosphate (CaP)
Nanoparticles
Gene delivery
Plasmid DNA
Hydroxyapatite
Bioceramics

ABSTRACT

Exploiting gene delivery from the surfaces of bio-functionalized implants is a unique strategy to facilitate tissue regeneration and integration. However, it has been challenging, due to the difficulty for incorporation and delivey of sufficient payloads of plasmid DNA (pDNA) to cells from the implant surface allowing efficient gene expression. Herein, we describe a novel and simple coating approach using nanostructured silicate substituted calcium phosphate pDNA complexes termed as NanoSiCaPs, that preferentially adsorb onto titanium (Ti) substrates coated with poly (diallyldimethylammonium chloride) (PDADMAC). The Ti-polyelectrolyte NanoSiCaPs assemblies aptly called PNA, deliver pDNA to MC3T3-E1 cells (pre-osteoblast cell line), induce protein production, without eliciting any cytotoxicity, while simultaneously encouraging cell attachment on the substrate.

1. Introduction

Surface properties of biomaterials play a crucial role in determining the initial host tissue response, which in turn plays a significant role in determining the long-term success of the implants [1,2]. Surface mediated gene delivery (SMGD) from implants is an attractive approach to stimulate specific cellular response at the molecular level [3]. Currently, layer by layer (LbL) assembly [4] and biomineralization [5] are two common approaches for surface modification of orthopedic implants to incorporate substantial amounts of plasmid DNA (pDNA) on the implants. However, these methods involve prolonged incubation (> 48 h) of substrates in supersaturated calcium phosphate solution [6,7], or multistep procedures comprising alternate dipping cycles in polycationic and polyanionic solutions [8]. Therefore, herein we focused on the development of a novel surface modification technique that: 1) involves a quick and easily adaptable methodology applicable to materials used for dental and bone repair, with different geometries and composition; 2) provides an easily accessible medium promoting cell attachment and proliferation; and 3) acts as a gene-activating material [3].

Commercially available pure Ti is by far one of the most ubiquitously used material for dental and bone repair due to its superior mechanical properties and biocompatibility [9,10]. However, despite its beneficial properties, numerous physical and chemical methods are employed for surface modification of Ti surfaces to promote osseointegration [9,11]. Therefore, gene delivery *via* Ti surface makes it an ideal choice as a substrate for the current study. As outlined in Scheme 1, our goal is to incorporate NanoSiCaPs (complexed with pDNA) on Ti substrates, wherein NanoSiCaPs mediate the delivery of plasmid DNA (pDNA) to MC3T3-E1, a murine pre-osteoblasts cell line, seeded on top of the Ti surface. Our group has recently reported the development of NanoSiCaPs, a unique modification of our previously reported work on nanostructured calcium phosphate particles (NanoCaPs), containing 8.3 mol% or 1.4 wt% of silicate substituted in lieu of the phosphate [12]. This substitution as we have already reported [12] resulted in two-fold increase in the gene transfection capability of NanoSiCaPs, due to its superior solubility enabling endosomal escape, and ensuing materials properties.

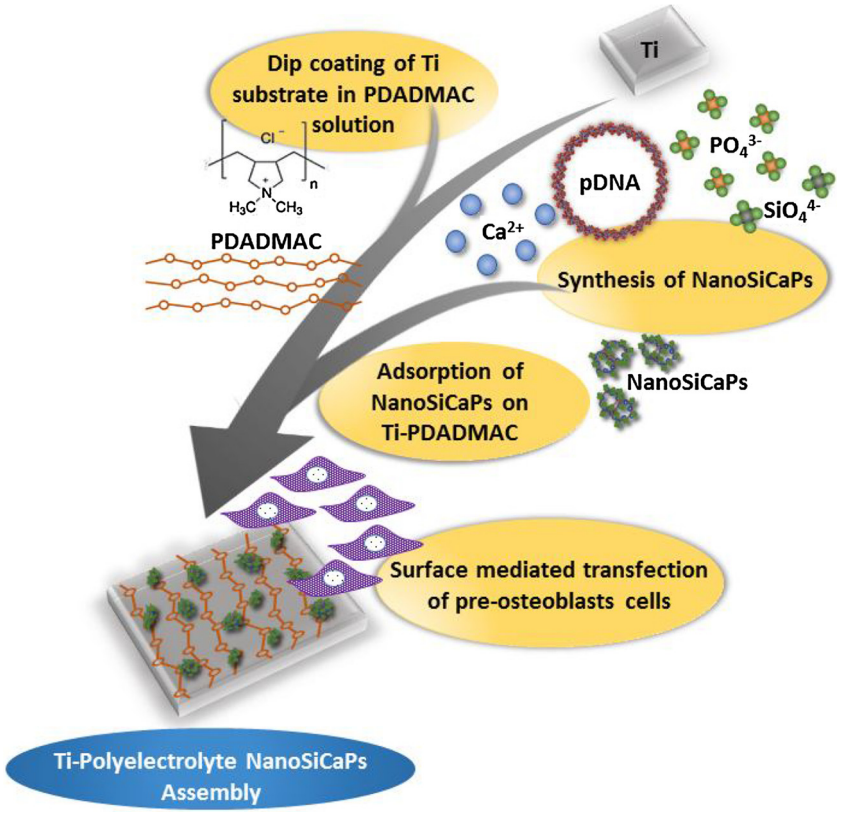
2. Experimental details

2.1. Materials

Calcium chloride ($CaCl_2 \cdot 2H_2O$, 99% purity), trisodium phosphate

* Corresponding author at: 815C Benedum Hall, 3700 O'Hara Street, University of Pittsburgh, Pittsburgh, PA, 15261, USA.
E-mail address: pkumta@pitt.edu (P.N. Kumta).

https://doi.org/10.1016/j.mtcomm.2018.05.008
Received 4 March 2018; Received in revised form 14 May 2018; Accepted 25 May 2018
Available online 26 May 2018



Scheme 1. Steps involved in fabrication of Ti-polyelectrolyte NanoSiCaPs assembly (PNA).

($Na_3PO_4{\cdot}12H_2O$), sodium chloride (NaCl), dextrose, AlamarBlue Cell Viability Assay were purchased from Fisher Scientific (Pittsburgh, PA). Poly-(diallyl-dimethyl-ammonium chloride) medium molecular weight (Mw 100–200 kDa, 20 wt% in water), Potassium chloride (KCl, 99 + %), Paraformaldehyde, and Phalloidin-Tetramethylrhodamine B isothiocyanate (phalloidin-TRITC) was purchased from Sigma Aldrich (St. Louis, MO). 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from PacReacAppliChem (Maryland Heights, MO). Sodium hydroxide (NaOH) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Sodium metasilicate ($Na_2SiO_3{\cdot}5H_2O$) and Titanium (Ti) (99.7%, metal basis), 0.89 mm thick, was purchased from Alfa Aesar (Ward Hill, MA). HEPES (99%) was obtained from EMD Chemicals (Gibbstown, NJ). Reporter plasmid, gWizTM GFP (Green Fluorescent Protein) was purchased from Aldevron LLC (Fargo, ND). All the reagents were used as received without further modification or purification.

2.2. Synthesis and fabrication of Ti-PNA

The general steps involved in the fabrication of Ti-PNA substrates are shown in Scheme 1. Briefly, Ti substrates were cut into squares of 1 cm × 1 cm × 0.89 mm, and cleaned using acid etching and repeatedly washing with acetone. The substrates were then polished with 320, 600 and 1200 grit SiC paper and cleaned under ultrasonication using acetone. The substrates were subsequently coated with a single layer of, PDADMAC-5 mg/ml in phosphate buffer (pH-7.5), medium molecular weight (M_w100–200 kDa), using a dip coater (Desktop Dip Coater, MTI Corporation) for 15 min followed by air drying and subsequent washing in deionized water for 1 min to generate the Ti-PDADMAC. NanoSiCaPs incorporating pDNA encoded for green fluorescent protein (GFP) were freshly synthesized according to a protocol previously described [12]. Ti-PDADMAC substrates were then incubated in 1 mL of freshly synthesized NanoSiCaPs solution for 10 min to generate the Ti-PNA substrates. For some studies, where specified, Ti-PDADMAC-pDNA substrates were also made, by directly immersing Ti-PDADMAC substrates in equal volume of pDNA solution (25.6 µg in 1 ml DI water). It should be noted that the entire coating and drying process is performed under sterile conditions.

2.3. Surface characterization techniques

PDADMAC polymer solution, bare Ti and Ti-PDADMAC were analyzed using attenuated total reflectance Fourier transform infrared spectroscopy (FTIR; Nicolet 6700 Thermo Scientific). The spectra were collected in the range of 4000–500 cm^{-1}. The surface morphology of Ti-PNA was studied using scanning electron microscopy (SEM) with a Philips-XL30 FEG operating at 10.0 kV. All the samples used for SEM analysis were coated with Pd using a sputter coater system. The surface topography and roughness of the bare Ti, Ti-PDADMAC and Ti-PNA were characterized by non-contact atomic force microscopy (AFM) using MFP-3D AFM (Asylum Research) and Igor Pro (Wavemetrics) analysis software as previously described [13]. Topography images of 80 by 80 microns were taken with a silicon nitride conical tip (k = 40Nm^{-1}, Mikromasch, Ltd) at a scan rate of 1 Hz and 512 by 512-pixel resolution. Root mean square (RMS) surface roughness quantification was performed at (n = 3) random 20 by 20 µm regions within the images. Water contact angle was determined using a Theta Lite Optical Tensiometer (Attension, Biolin Scientific) equipped with image analysis software (Theta Lite). By applying the sessile drop method, a drop of 200 pixels was created at the tip of the syringe and carefully placed on the top surface of the substrate. Measurements were conducted immediately after placing the drop at RT. The contact angle was calculated using the Young-Laplace fit equation.

2.4. In vitro studies

Murine osteoblast cell line MC3T3-E1 was obtained from ATCC (Manassas, VA). Cells were cultured in humidified incubator at 37 °C and 5% CO_2, in minimum essential medium alpha (MEM α; Gibco,

Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells at third to seventh passage were used in all these experiments. For *in-vitro* transfection, the cells were plated at a density of 4.0×10^4 cells on tissue culture polystyrene (TCPS) used as the control, Ti-PDADMAC-pDNA and Ti-PNA substrates placed in a 24-well plate, respectively. The level of GFP-expression was qualitatively assessed using a fluorescence microscope (Olympus, CKX41). For quantitate analysis of GFP expression, flow cytometry was performed on day 3 using the Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI), as per previously described protocol [12]. *In vitro* viability of MC3T3-E1 cells at different time points (n = 3) was also performed using the AlamarBlue Cell Viability Assay according to the manufacturers protocol. Media containing 1/10 vol of Alamarblue was freshly prepared and incubated at 37 °C for 3 h to measure cell activity. After completion, the media was transferred to 96 well plated then followed by measuring the fluorescence at 560 nm/585 nm (excitation/emission) using the microplate reader (Biotek, Synerge2). Qualitative assessment of pDNA coverage on coated substrates was performed by DAPI staining. DAPI staining solution was diluted in PBS at 300 nM and then added to the bare Ti, Ti-PDADMAC-pDNA and Ti-PNA substrates. The substrates were incubated for 15 min, washed in PBS and then imaged using fluorescence microscope (Olympus, CKX41). Cytoskeletal staining of filamentous actin (F-actin) was performed as per previously described and published protocol [14].

2.5. Statistical analysis

Statistical calculations were performed using the GraphPad Prism software. The data was analyzed to test for significant ($p < 0.05$) mean differences on contact angle, RMS values, *in vitro* cell viability results for different samples using one-way analyses of variance (ANOVA). Each ANOVA on these response variables was significant at $p < 0.05$. *Post hoc* tests for pair-wise differences and identification of homogeneous subgroups were performed using the Tukey HSD procedures for contact angle and RMS measurements. For *in-vitro* transfection analysis using flow cytometry, paired *t*-test was performed with a $p < 0.05$.

3. Results and discussion

FT-IR was used to confirm the presence of PDADMAC on Ti substrates. The FT-IR spectrum of Ti substrate coated with PDADMAC matched the spectrum of pure PDADMAC with IR absorption peaks at 1470cm^{-1} (C—H_2 stretching) and 1409cm^{-1} (symmetric bending mode of the $^{+}$N—CH_3 group) [15,16] indicating the presence of PDADMAC on the Ti substrates (Fig. 1a). The surface morphology of the coated substrates was then investigated using scanning electron microscopy (SEM) and atomic force microscoy (AFM). SEM images of the Ti-PNA are shown in Fig. 1b and c, which suggests that NanoSiCaPs are uniformly coated on the entire Ti substrate (Fig. 1b). Moreover, the primary particle sizes of the NanoSiCaPs are in the range of 50–100 nm as reported in our earlier work [12] (Fig. 1c). AFM images of theTi-PNA films are generated using the non-contact mode shown in Fig. 1d and e. Root mean square (RMS) surface roughness quantification was performed at (n = 3) random 20 by 20-micron regions within the images. The surface morphology of the coated substrates changes from a smooth surface for bare Ti, RMS ~ 80 nm and Ti-PDADMAC films, RMS ~ 45 nm (see Supp. information, Fig. S1), to a much rougher surface covered by the complexed particles after NanoSiCaPs adsorption, RMS ~ 145 nm. For further illustrating these differences, 3D-image reconstructions for the Ti-PNA films is presented in Fig. 1e. Additionally, surface wetting properties was also investigated by measuring the water contact angle at three separate locations on the coated substrates. It was determined that the contact angle of bare Ti (61.5 ± 2.1°) was similar to Ti-PDADMAC (63 ± 3.5°), as shown in Fig. 1f. However, the contact angle decreased to 29 ± 1.4° (statistically significant), with accompanying adsorption of NanoSiCaPs complexes on the substrates, which suggests that the adsorption of NanoSiCaps renders the surface more hydrophilic. Presence of pDNA on the Ti-PNA films was detected by DAPI staining of the substrates (see Supp. information, Fig. S2). Overall, the surface characterization results demonstrate that PNA coating strategy is successful in obtaining a nanoceramic coating on Ti substrates which has a direct influence on the surface properties of the substrates.

In-vitro transfection potential of Ti-PNA was evaluated using pre-osteoblast (MC3T3-E1) cells. Subject to the same conditions, another group received equal amounts of pDNA on Ti-PDADMAC substrates, but without NanoSiCaPs. The substrates were prepared on the day of cell seeding. GFP expression of the transfected cells was monitored using a fluorescent microscope. The transfected cells were observed as early as day 1 and the GFP expression peaked at day 3 (Fig. 2a) for Ti-PNA, while the substrates lacking NanoSiCaPs (only pDNA) showed much reduced GFP expression, Fig. 2b. GFP expression of MC3T3-E1 cells was also quantified using flow cytometry at day 3 as shown in Fig. 2c. The Ti-PNA coated substrates exhibited transfection in ~13% of cells, whereas the substrates lacking NanoSiCaPs showed negligible transfection in agreement with fluorescence microscopy results (statistically significant). These results thus indicate that Ti-PNA serves as a gene-activating material, and also further highlights the importance of incorporating a transfecting agent in the coating to achieve successful delivery of pDNA to the cells.

Finally, we determined the cell viability of MC3T3-E1 cells seeded on the coated substrates using Alamar blue assay™. Tissue culture polystyrene (TCPS) and bare Ti were used as the control groups. Cell viability of the coated substrates was no different than the control groups (TCPS or bare Ti) with no statistically significant difference noted at each time point (Fig. 3a). The cell morphology was also observed to evaluate cell attachment and spreading on the substrates as cytoplasmic extensions are crucial for cell adhesion, migration and formation of cell-cell junctions [17,18], which are also key factors for dictating tissue integration of the implants [19]. MC3T3-E1 cells attached on the substrates were stained for F-actin (filamentous-actin), a cytoskeletal element (in red) and nucleus (in blue), on day 3, Fig. 3b–d. Fig. 3b–d clearly demonstrates that bare Ti, Ti-PDADMAC, and Ti-PNA favorably supported attachment and spreading of the pre-osteoblasts cells since the increase in the cell viability at different time points in all groups (bare and coated Ti), including the control (TCPS) is due to the increase in the number of cells. These results thus collectively indicate that Ti-PNA enables attachment, spreading and proliferation of pre-osteoblast cells.

4. Conclusions

We have developed a novel and a user-friendly coating strategy on Ti surfaces using electrostatic interaction between a polycation, PDADMAC and negatively charged NanoSiCaPs complexes. Interestingly, the PNA coating is compatible with pre-osteoblast cells and encourages cell attachment and spreading. Most notably, the entire PNA fabrication process requires only 30 min to obtain a nanoceramic complexed coating, and this methodology can be easily used to coat various substrates of different composition and geometries due to its simplicity. The *in-vitro* transfection results indicate that Ti-PNA is an excellent platform for successful realization of surface mediated gene delivery (SMGD) and transfection (SMGT), offering the potential to generate and herald a new class of surface functionalized gene-stimulating biomaterials for myriad clinically relevant applications. These include ‘smart’ surface functionalized gene activated scaffolds promoting healing and bone regeneration as well as biomaterials that could enable implants to modulate immune response and/or surrounding tissue response contributing to faster healing and implant fixation within the native bone tissue. Future studies will involve testing these biomaterials in animal experiments to further validate the

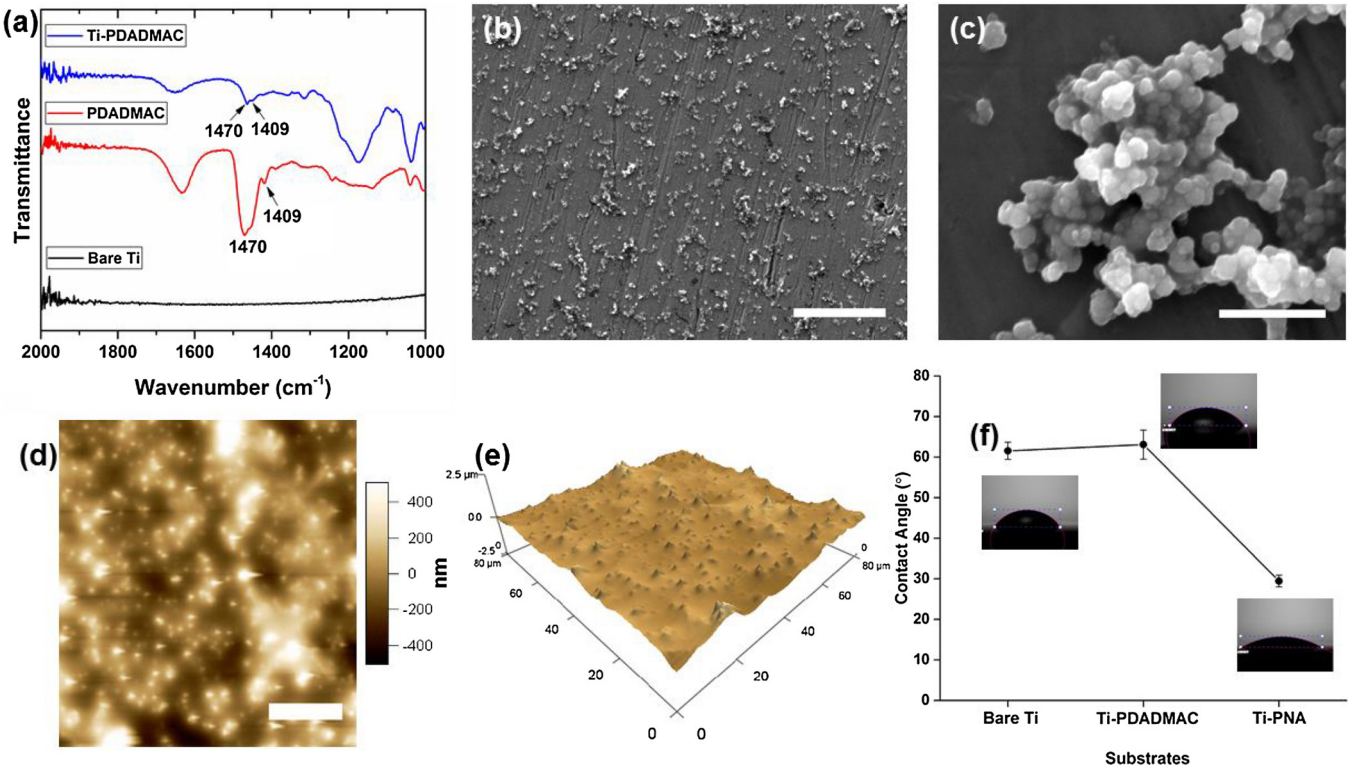


Fig. 1. Surface characterization of coated substrates (a) FT-IR spectrum; SEM micrograph of Ti-PNA (b) *scale 20* µm and (c) *scale 1* µm; (d) AFM height image of Ti-PNA (scale 20 µm); (e) 3-D image of the morphology of Ti-PNA films; and (f) water contact angle measurements on bare Ti, Ti-PDADMAC and Ti-PNA (n = 3). Data reported as mean ± SD ($P < 0.05$).

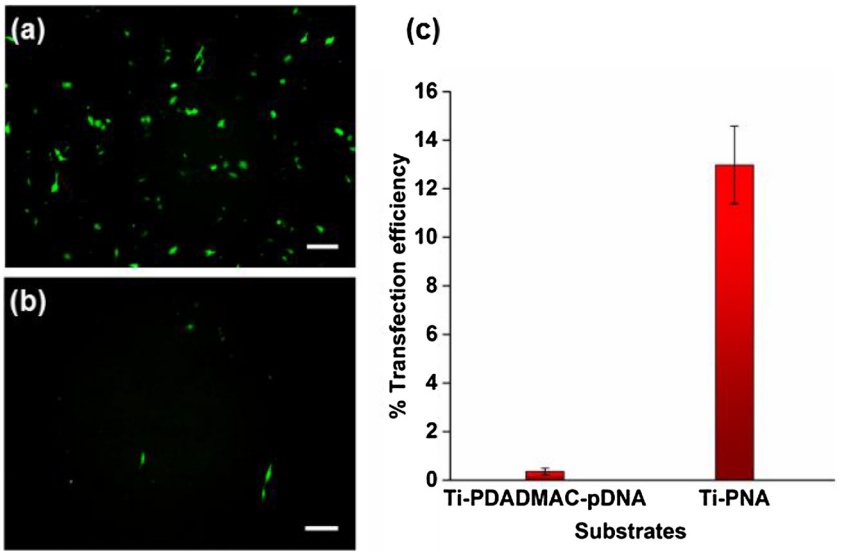


Fig. 2. Fluorescence microscopy images of MC3T3-E1 cells on (a) Ti-PDADMAC-pDNA and (b) Ti-PNA on day 3 (*scale* 200 µm). (c) Flow cytometry analysis measuring GFP expression in MC3T3-E1 cells at day 3. The data were reported ± SD, n = 3 ($P < 0.05$).

bioactivity of the coated PNA architectures.

Data availability statement

The raw data used to process the research findings will be available upon communication with the authors of this manuscript.

Acknowledgements

The authors would like to acknowledge the NSF (CBET-0933153) and NSF-ERC (Grant # EEC-0812348) for partial support of this research. PNK would also like to acknowledge the Edward R. Weidlein Chair Professorship Funds and the Center for Complex Engineered Multifunctional Materials (CCEMM), Swanson School of Engineering, and the University of Pittsburgh for providing partial support in the form of support for graduate student, reagents, equipment and other accessories much needed for conducting and completing this research. Specifically, authors would like to thank Dr. Ipsita Banerjee, Department of Chemical Engineering, University of Pittsburgh, for the use of flow cytometry and Dr. Yadong Wang, Department of

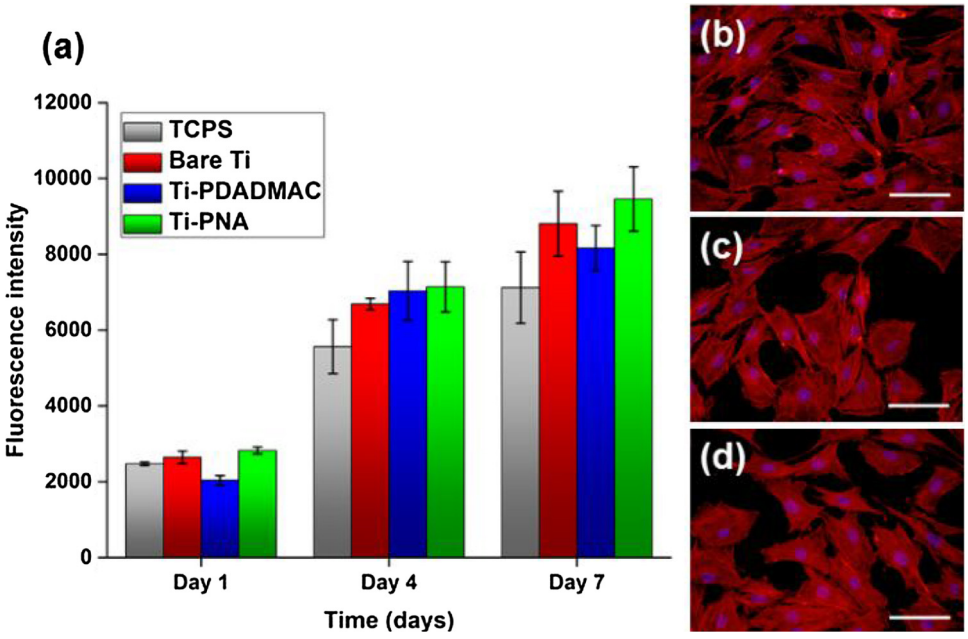


Fig. 3. (a) Cell viability assay at different time points ($P < 0.05$). The data were reported ± SD; cytoskeletal staining showing F-actin (red) and nucleus (blue) for (b) bare Ti, (c) Ti-PDADMAC and (d) Ti-PNA (*scale* 50 μm).

Bioengineering, University of Pittsburgh for the use of the tensiometer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mtcomm.2018.05.008.

References

[1] J.M. Anderson, A. Rodriguez, D.T. Chang, Foreign body reaction to biomaterials, Semin. Immunol. 20 (2008) 86–100.
[2] S. Mitragotri, J. Lahann, Physical approaches to biomaterial design, Nat. Mater. 8 (2009) 15–23.
[3] L.L. Hench, J.M. Polak, Third-generation biomedical materials, Science (New York N.Y.) 295 (2002) 1014–1017.
[4] C.M. Jewell, D.M. Lynn, Multilayered polyelectrolyte assemblies as platforms for the delivery of DNA and other nucleic acid-based therapeutics, Adv. Drug Deliv. Rev. 60 (2008) 979–999.
[5] A. Oyane, X. Wang, Y. Sogo, A. Ito, H. Tsurushima, Calcium phosphate composite layers for surface-mediated gene transfer, Acta Biomater. 8 (2012) 2034–2046.
[6] H. Shen, J. Tan, W.M. Saltzman, Surface-mediated gene transfer from nano-composites of controlled texture, Nat. Mater. 3 (2004) 569–574.
[7] R.R. Rao, J. He, J.K. Leach, Biomineralized composite substrates increase gene expression with nonviral delivery, J. Biomed. Mater. Res. A 94A (2010) 344–354.
[8] Y. Hu, K. Cai, Z. Luo, R. Zhang, L. Yang, L. Deng, K.D. Jandt, Surface mediated in situ differentiation of mesenchymal stem cells on gene-functionalized titanium films fabricated by layer-by-layer technique, Biomaterials 30 (2009) 3626–3635.
[9] A. Civantos, E. Martínez-Campos, V. Ramos, C. Elvira, A. Gallardo, A. Abarrategi, Titanium coatings and surface modifications: toward clinically useful bioactive implants, ACS Biomater. Sci. Eng. 3 (7) (2017) 1245–1261.
[10] Z.Y. Qiu, C. Chen, X.M. Wang, I.S. Lee, Advances in the surface modification techniques of bone-related implants for last 10 years, Regen. Biomater. 1 (2014) 67–79.
[11] R. Junker, A. Dimakis, M. Thoneick, J.A. Jansen, Effects of implant surface coatings and composition on bone integration: a systematic review, Clin. Oral Implants Res. 20 (Suppl. 4) (2009) 185–206.
[12] S. Shekhar, A. Roy, D. Hong, P.N. Kumta, Nanostructured silicate substituted calcium phosphate (NanoSiCaPs) nanoparticles—efficient calcium phosphate based non-viral gene delivery systems, Mater. Sci. Eng. C 69 (2016) 486–495.
[13] S. Kunjukunju, A. Roy, M. Ramanathan, B. Lee, J.E. Candiello, P.N. Kumta, A layer-by-layer approach to natural polymer-derived bioactive coatings on magnesium alloys, Acta Biomater. 9 (2013) 8690–8703.
[14] N. Ostrowski, B. Lee, N. Enick, B. Carlson, S. Kunjukunju, A. Roy, P.N. Kumta, Corrosion protection and improved cytocompatibility of biodegradable polymeric layer-by-layer coatings on AZ31 magnesium alloys, Acta Biomater. 9 (2013) 8704–8713.
[15] H. Li, C.P. Tripp, Infrared study of the interaction of charged silica particles with TiO2 particles containing adsorbed cationic and anionic polyelectrolytes, Langmuir: ACS J. Surfaces Colloids 21 (2005) 2585–2590.
[16] X.Y. Zhao, Y.J. Zhu, F. Chen, B.Q. Lu, C. Qi, J. Zhao, J. Wu, Calcium phosphate hybrid nanoparticles: self-assembly formation, characterization, and application as an anticancer drug nanocarrier, Chem. Asian J. 8 (2013) 1306–1312.
[17] P. Wang, L. Zhao, J. Liu, M.D. Weir, X. Zhou, H.H. Xu, Bone tissue engineering via nanostructured calcium phosphate biomaterials and stem cells, Bone Res. 2 (2014) 14017.
[18] S. Lavenus, J.C. Ricquier, G. Louarn, P. Layrolle, Cell interaction with nano-patterned surface of implants, Nanomedicine (Lond. Engl.) 5 (2010) 937–947.
[19] M.M. Stevens, J.H. George, Exploring and engineering the cell surface interface, Science (New York N.Y.) 310 (2005) 1135–1138.