Protein Expression of Bone Marrow Mesenchymal Stem Cells Transfected on Green Fluorescent Protein mRNA and Collagen-coated Titanium Disk

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Abstract

New strategies for surface modification have been explored to promote osseointegration and osteogenesis, which are crucial factors for the success of titanium dental implants. Coating the titanium surface with mRNA encoding therapeutic growth factors may achieve these objectives. In our pilot study, we employed green fluorescent protein (GFP) mRNA as a model. Our investigation focused on determining whether titanium disks coated with GFP mRNA encapsulated with lipid nanoparticles and collagen Type I (GFP mRNA-Col) could enhance transfection in human bone marrow mesenchymal stem cells (hBMSCs) in comparison to titanium disks coated with GFP mRNA alone, without collagen. We tested various concentrations of collagen, ranging from 0.8 to 8.1 µg, to evaluate the transfection efficiency. GFP expression was evaluated using a fluorescence microscope, and mean fluorescence intensity was measured through flow cytometry. At 24 hr post-transfection, GFP-expressing cells were clearly observed on the titanium disks coated with GFP mRNA-Col, whereas negligible GFP-expressing cells were detected on the titanium disks coated with GFP mRNA alone. Notably, collagen at 2.7 µg and 8.1 µg significantly increased translation efficiency, as evidenced by enhanced GFP expression in hBMSCs when compared to no collagen. The kinetic study revealed that the peak GFP expression could be observed at 48 hr and then declined at 72 hr. In conclusion, coating titanium with GFP mRNA-Col proved to be a successful method for transfecting hBMSCs, leading to sustained protein expression. These results suggest the potential application of mRNA in lipid nanoparticles and collagen as a novel coating for titanium dental implants.

Keywords**:** *Protein Expression, mRNA, Collagen, Human Bone Marrow Mesenchymal Stem Cells, Green Fluorescent Protein, Titanium Dental Implant*

1. Introduction

Dental implant therapy is crucial for restoring oral function and aesthetics in individuals with missing teeth (Buser et al., 1997). However, the world's demographic changes, marked by a rapidly increasing life expectancy, are leading to an aging society (Nations, 2017). This shift may increase the number of patients with tooth loss and those suffering from comorbidities that affect wound healing and bone metabolism. Consequently, there is a need for continuous improvement in the performance of titanium dental implants.

The intricate process of bone healing and integration with titanium implants (osseointegration) is vital for implant success (Albrektsson et al., 1986; Branemark et al., 2001). A variety of surface modifications, including biological, physical, and chemical techniques, have been applied to titanium implants to achieve these goals. For instance, coating the titanium surface with bioactive molecules such as an osteogenic growth factor protein—recombinant human bone morphogenetic protein-2 (rhBMP-2)—has been explored (Behrens

et al., 2022). However, issues such as high costs, the need for high protein doses, and a short protein half-life present challenges that must be addressed (Deyo et al., 2012; Ren et al., 2020).

Gene therapy, as an alternative to protein-based therapy, offers improved bioavailability of therapeutic protein in target tissues. In 2021, mRNA-based technology, highlighted by the FDA-approved mRNA vaccine against SARS-CoV-2, demonstrated safety and efficacy. This vaccine uses nucleoside-modified mRNA encoding a spike protein antigen, encapsulated with lipid nanoparticles (LNPs) (Fang et al., 2022). Nucleoside-modified mRNA translates more protein than unmodified mRNA by suppressing the inflammatory response. Encapsulation with LNPs protects mRNA from degradation and enhances its stability (Wang, Zhang, & Dong, 2021). Hence, mRNA-LNPs technology is recognized as a leading platform in medicine.

Thus, employing mRNA technology to create therapeutic proteins by coating titanium implant surfaces with mRNA encoding growth factors could enhance bone healing and osseointegration. In this study, we used green fluorescent protein (GFP) mRNA as a model. Our mRNA, N1-methylpseudouridine m1Ψ-modified mRNA, encapsulated in LNPs, shares a similar formulation with the COVID-19 mRNA vaccine, except for the GFP coding sequence. We investigated whether titanium disks coated with m1Ψ-modified mRNA encoding GFP encapsulated in LNPs and collagen Type I (GFP mRNA-Col) could enhance transfection in human bone marrow mesenchymal stem cells (hBMSCs), compared to disks coated only with GFP mRNA-LNPs. We also tested various collagen concentrations, from 0.8 to 8.1 µg, to evaluate transfection efficiency.

2. Objectives

1) To evaluate the transfection efficiency of hBMSCs on titanium disks coated with a combination of GFP mRNA-LNPs and collagen at concentrations of 0, 0.8, 2.7, and 8.1 µg.

2) To investigate the cell viability of hBMSCs transfected on GFP mRNA-LNPs and collagen-coated titanium disks.

3) To study the kinetics of protein expression in hBMSCs transfected on GFP mRNA-LNPs and collagen-coated titanium disks.

3. Materials and Methods

3.1 Medium and reagents

Minimum Essential Medium with Alpha modification (Alpha MEM, Sigma-Aldrich®, Germany) supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific, USA), 2 mM GlutaMax-I (Thermo Fisher Scientific, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B (Life Technologies, Thermo Fisher Scientific, USA) were used throughout the study. Collagen I from rat tail was purchased from Thermo Fisher Scientific, USA.

3.2 Preparation of mRNA

m1Ψ-modified GFP mRNA encapsulated LNPs was synthesized by Dr. Norbert Pardi of the University of Pennsylvania, USA (Pardi et al., 2017).

3.3 Preparation of titanium disks

Titanium disks (Ti grade 23, Ti-6Al-4V ELI) with an as-built surface (a roughness of 6.97 ± 0.34 µm) were provided by Associate Professor Dr. B. Lohwongwatana, from Meticuly Co., Ltd. The disks were produced using selective laser melting, a 3D printing technology (Decha-umphai et al., 2021). The dimensions of each of the circular titanium disks are 6 mm in diameter and a thickness of 0.5 mm, which fit inside the flat wells of a 96-well plate. Following production, the disks underwent a degreasing process in acetone (Merck) for 10 min at room temperature (RT) in an ultrasonic bath. Subsequently, the disks were subjected to two washes in ddH_2O, followed by an additional wash in 80% ethanol using an ultrasonic bath (10 min at RT). After the excess ethanol was removed, the disks were left to air-dry inside a laminar flow cabinet. Sterilized disks were then stored in a container until further use (Fayed et al., 2021).

3.4 Coating titanium disks with GFP mRNA and collagen

The titanium disks were coated using physical methods in a petri dish. A total volume of 30 µl was prepared by mixing 3 µg of GFP mRNA-LNP in 3 µl with 27 µl of collagen type I in 20 mM acetic acid at various concentrations (0, 30, 100, and 300 µg/ml). This mixture was immediately deposited onto sterilized titanium disks. The coated disks were left to air-dry in a laminar flow cabinet for 4 hours and then rinsed three times with DPBS. Under these conditions, four types of coated titanium disks were obtained: 1) disks coated with 3 µg of GFP mRNA-LNP alone, 2) disks coated with 3 µg of GFP mRNA-LNP plus 0.8 µg of collagen, 3) disks coated with 3 µg of GFP mRNA-LNP plus 2.7 µg of collagen, and 4) disks coated with 3 µg of GFP mRNA-LNP plus 8.1 µg of collagen.

3.5 Preparation of hBMSCs

Bone marrow cells from the iliac crest were obtained by needle puncture aspiration from three volunteers at the Division of Hematology, Department of Medicine, Chulabhorn Hospital. The experiment procedure was approved by The Human Research Ethics Committee of Princess Srisavangavadhana College of Medicine, Chulabhorn Royal Academy (EC052/2565), and informed consent was obtained from all participants. The hBMSCs were isolated from the bone marrow samples by density gradient centrifugation. The cells were cultured in an alpha-MEM culture medium, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After 48 hr, non-adherent cells were removed, and adequate fresh medium was added. The medium was replaced twice a week. Until confluence was reached at 80–90%, cells were harvested. Over 95% of the cells were positive for CD44, CD73, CD90, and CD105 (cell surface markers of bone marrow mesenchymal stem cells) and negative for CD45 (a common hematopoietic marker) (Drela et al., 2020). The cells from the 3rd to the 8th passages were used in this study.

3.6 In vitro cell transfection of GFP-mRNA and collagen on coated titanium disks

Figure 1 shows an overview of the experimental design for the cell transfection of GFP mRNA-Col on coated titanium disks**.** hBMSCs (20,000 cells) were seeded onto the titanium disks previously coated with GFP mRNA-Col at various concentrations. After 24 hr incubation, transfection efficiency was evaluated. GFP expression was assessed under a fluorescence microscope (Olympus DP27, Microscopy Technologies, Japan). Cells were then trypsinized and mean fluorescence intensity (MFI) was measured through flow cytometry (FACSCalibur instrument, Thermo Fisher Scientific, USA). In addition, cell viability was assessed by alamarBlue (AlamarBlue®, BIO-RAD, UK).

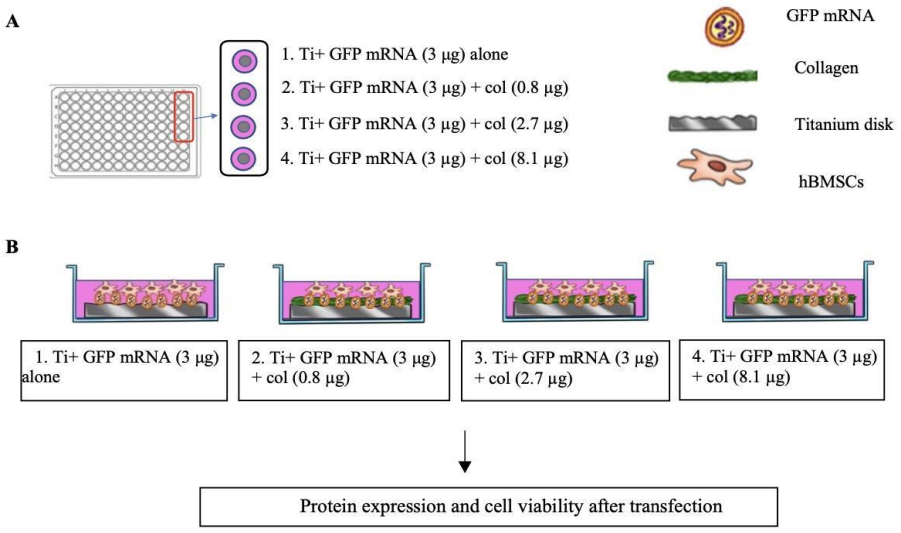

Figure 1 Overview of the experimental design for cell transfection of GFP mRNA coated on titanium disks**.** (A) Cell culture was performed using 96-flat well plates. A magnified image shows four individual wells with titanium disk placement. (B) Titanium disks with four experimental conditions of coating include 1) GFP mRNA (3 µg) alone in

DPBS (control); 2) GFP mRNA (3 µg) with 0.8 µg collagen; 3) GFP mRNA (3 µg) with 2.7 µg collagen; and 4) GFP mRNA (3 µg) with 8.1 µg collagen. hBMSCs were seeded onto the coated disks and incubated for 24 hr. The cell transfection efficiency and cell viability were evaluated.

3.7 Kinetic study of GFP expression in hBMSCs

Collagen at 2.7 µg was selected for the kinetic study. hBMSCs (20,000 cells) were seeded onto titanium disks coated with GFP mRNA and 2.7 µg of collagen. Transfection efficiency was evaluated at 24 hr, 48 hr, and 72 hr using flow cytometry.

3.8 Statistical analysis

The statistical software SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. The normal distribution of data was tested via the Shapiro-Wilk test. The data was normally distributed, the parametric one-way ANOVA test with Dunn's correction (followed by pairwise comparisons) was performed with multiple group comparisons, and p-value less than 0.05 was considered statistically significant.

4. Results and Discussion

4.1 Results

4.1.1 The transfection efficiency of hBMSCs on titanium disks coated with GFP mRNA and collagen

The hBMSCs transfection efficiency of GFP mRNA in different concentrations of collagen (0.8, 2.7, 8.1 µg), which was coated on the titanium disks, was investigated after 24 hr incubation. GFP mRNA alone in DPBS was used as a control. Under the microscope, the GFP expressing cells were clearly observed in all tested concentrations, except for the control (Figure 2A). Flow cytometric analysis showed that the mean MFI in the collagen groups at 0.8 µg, 2.7 µg, and 8.1 µg were 1,699 ± 566, 4,181 ± 428, and 5,607 ± 2609 respectively, whereas in the control group, it was negligible (321 ± 223) (Figure 2B). The transfected hBMSCs in the 2.7 µg and 8.1 µg collagen groups generated a significantly higher expression of GFP than that of the control group ($p < 0.05$). There were no significant differences in GFP expression between the 2.7 µg and 8.1 µg collagen groups. Therefore, the 2.7 µg collagen was selected for the kinetic study.

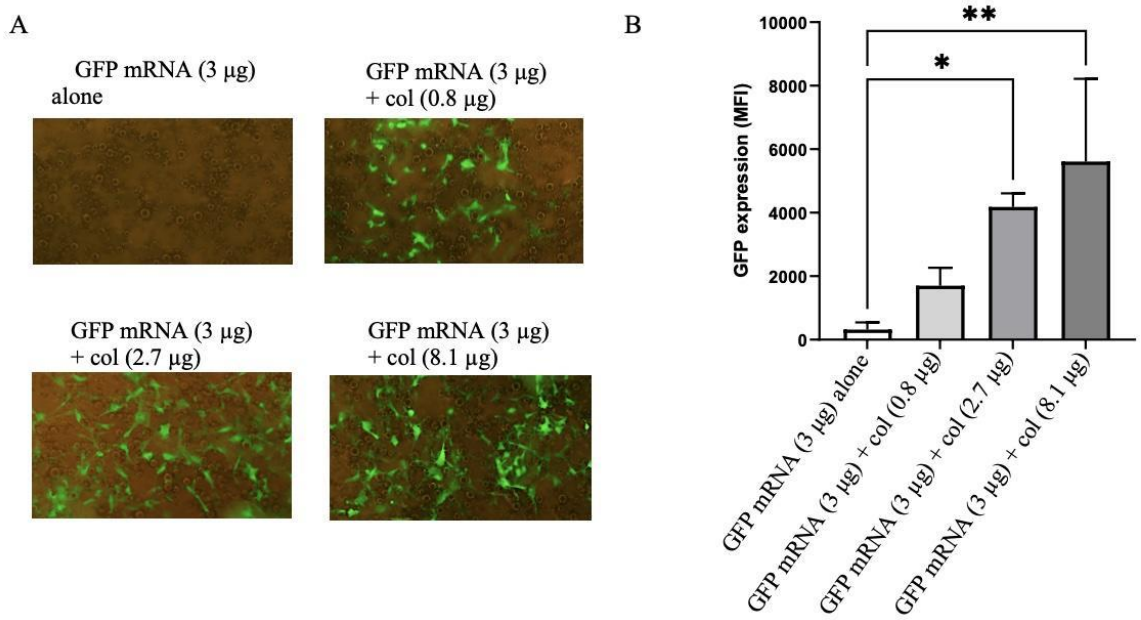

Figure 2 GFP expression in transfected hBMSCs on coated titanium disks. hBMSCs were transfected with GFP mRNA in varying concentrations of collagen (0, 0.8, 2.7, 8.1 µg), which was coated onto the titanium disks. After 24 hr of

transfection, GFP expression was assessed under a fluorescence microscope and by flow cytometry. (A) Representative fluorescence image of transfected cells (B) The data shown represent the mean fluorescence intensity (MFI) ± SE (n = 3). *p < 0.05, **p < 0.05 indicates a significant difference between the collagen groups and the control.

4.1.2 Cell *viability of hBMSCs transfected on GFP mRNA and collagen-coated titanium disks*

After hBMSCs transfection with GFP mRNA and collagen (0.8, 2.7, 8.1 µg) on the titanium disks for 24 h, the alamarBlue solution was added for 4 hr, and cell viability was assessed by a microplate reader. Cell transfection with GFP mRNA alone in DPBS was used as a control. As shown in Figure 3, mRNA-transfected cells were viable in all mRNA + collagen groups. The percentages of cell viability ranged from 112% to 123% compared to the control, with the highest viability observed at 8.1 µg of collagen and the lowest at 0.8 µg. There were no significant differences between collagen groups and the control ($p > 0.05$). Our results indicate that the transfection with mRNA and collagen was non-toxic to hBMSCs.

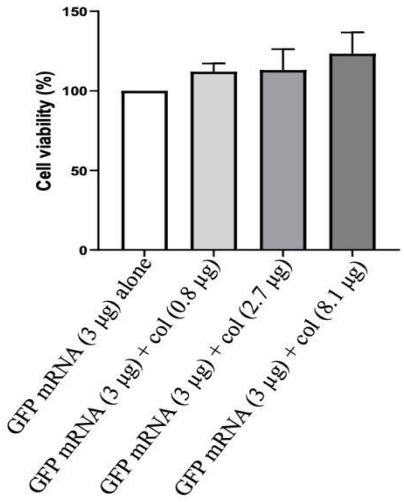

Figure 3 Percent cell viability of transfected hBMSCs on coated titanium. The hBMSCs were transfected with GFP mRNA in varying concentrations of collagen (0.8, 2.7, 8.1 µg), which was coated onto the titanium disks. After 24 hr of transfection, cell viability of the GFP mRNA + collagen groups were assessed using the almarBlue assay and compared with the control group (mRNA alone). The data shown represent the mean ± SE (n = 3).

4.1.3 Kinetics of protein expression in hBMSCs transfected on GFP mRNA and collagen-coated titanium disks

The time response of hBMSCs transfected with GFP mRNA in 2.7 µg collagen was examined. The mean MFI of GFP expression was 5,383 ± 1233 at 24 hr, reached its peak of 8,100 ± 955 at 48 hr, and then declined to 4,942 ± 1392 at 72 hr (Figure 4).

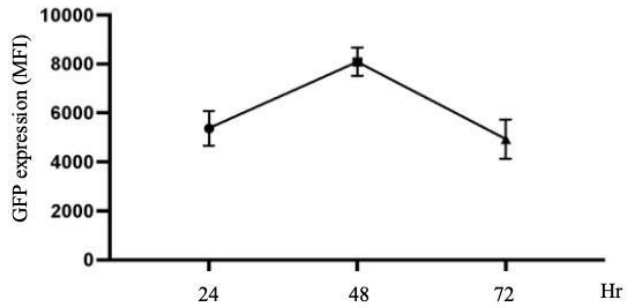

Figure 4 Kinetics of GFP expression in transfected hBMSCs on coated titanium disks. The hBMSCs were transfected with GFP mRNA in the presence of 2.7 µg collagen, which was coated onto the titanium disks for 24 hr, 48 hr, and 72 hr. The data shown represent the mean fluorescence intensity (MFI) ± SE (n = 3).

4.2 Discussion

Surface modification of titanium implants is a desirable and feasible method to improve osseointegration and bone healing. Coating titanium with mRNA could be a promising approach for delivering therapeutic proteins required for these biological processes. Using mRNA encoding GFP as a model, we found that collagen type I could promote binding of mRNA to titanium disks, leading to cell transfection as indicated by the expression of GFP in hBMSCs.

Before the advent of mRNA technology, human recombinant growth factor proteins were applied to implant systems to enhance osseointegration and bone regeneration (Hunziker et al., 2012). The FDA-approved rhBMP-2 and recombinant human platelet-derived growth factor (rhPDGF-BB) have received significant attention. However, the use of proteins presents problems, including a short half-life (7-16 min for rhBMP-2, FDA-approved INFUSE, and 30 min for rhPDGF-BB), the requirement for supraphysiological doses, unpredictable adverse effects, and high costs (Bowen-Pope et al., 1984; FDA, 2002). Gene therapy technology emerged as a means of transferring genetic information to specific cells to direct the synthesis of endogenous therapeutic proteins. Early studies utilized plasmid DNA (pDNA) and viral vector-based approaches for growth factor delivery. The delivery of pDNA-BMP-2 loaded with chitosan nanoparticles was shown to enhance bony defect healing in ligature-induced dog periodontitis (Li et al., 2017). Administration of adenovirus encoding the PDGF-BB gene with a collagen matrix was found to enhance alveolar bone repair in rats (Chang et al., 2009). However, safety concerns are a major challenge for using pDNA and viral vectors because DNA must integrate into the host genome, which might cause tumor formation (Romano et al., 2000). In recent years, mRNA-based therapeutics have emerged as a promising approach due to the negligible risk of mRNA integration into the genome, low immunogenicity, transient mode of expression, natural biodegradation pathways, and efficient translation of therapeutic proteins (Khorsand et al., 2019). Elangovan et al. (2015), pioneer researchers, used modified mRNA encoding BMP-2 complexed with polyethylenimine (PEI) and embedded into collagen scaffolds, which were then implanted into rat calvarial bone defects (Elangovan et al., 2015). After four weeks, the matrices activated by PEI–BMP-2 mRNA significantly improved bone regeneration compared to matrices activated by PEI-complexed BMP-2 pDNA, as analyzed by micro–computed tomography. The osteogenic potential of nucleoside-modified BMP-2 mRNA treatment was also confirmed in a rat femur bone defect model (Balmayor et al., 2016).

Thus far, there has been only one study investigating the *in vitro* transfection efficiency of titanium disks with mRNA coating (Fayed et al., 2021). In this study, the titanium disks were coated with luciferase mRNA in combination with different types of biomaterials, including fibrin, fibrinogen, and poly-D,L-lactic acid. It was found that fibrinogen coatings provided the best results in terms of mRNA transfection in mouse embryonic fibroblasts (NIH3T3 cell line). In our study, hBMSCs were used as clinically relevant target cells, which are the source of progenitor cells in close proximity to dental implant sites.

The surface topography of titanium implants plays a crucial role in osseointegration and osteogenesis. Rough titanium surfaces are generally preferred over smooth machined surfaces for dental implants due to their superior performance in promoting bone-implant integration, mechanical stability, and

the long-term success of the implants (Buser, Sennerby, & De Bruyn, 2017; Wennerberg, Hallgren, Johansson, & Danelli, 1998). Unlike the smooth surface of titanium foil in a previous study (Fayed et al., 2021), we used a 3D-printed Ti6Al4V disk with an as-built surface made by the selective laser melting process. As-built surface titanium disks offer a rough surface texture and were previously shown to enhance cell adhesion and proliferation when seeded with human osteoblasts, as well as promote osteogenic differentiation when seeded with both osteoblasts and hBMSCs (Przekora et al., 2022). Treating the as-built titanium surface with growth factor mRNA may add the value of translated therapeutic proteins for promoting the integration of bone to implant and bone regeneration.

Like fibrinogen and fibrin, collagen is a natural polymer that has been researched for use in coating metallic implants (Ao et al., 2014; Fayed et al., 2021; Sartori et al., 2015; van der Stok et al., 2015). Collagen type I is the predominant structural protein in the extracellular matrix and is commonly used in tissue engineering as the primary healing scaffold in the body. It provides support for cell adhesion, migration, and differentiation (Bonnans, Chou, & Werb, 2014; Rico-Llanos et al., 2021). In this study, collagen type I was used to physically entrap bioactive molecules—GFP mRNA-LNPs—and subsequently adsorb onto the titanium disks. This physical coating method was similar to that employed in a previous study (Fayed et al., 2021). The benefit of combining mRNA with collagen could be observed since coating titanium disks with mRNA alone, without collagen, led to poor cell transfection and poor protein translation after 24 hr of transfection. This may be associated with the inability of mRNA to bind to the titanium surface. Significantly greater protein expression (a 2- to 3-fold increase) was observed when the mRNA was incorporated with all of the tested concentrations of collagen. The collagen coating showed a trend of improved transfection efficiency in a dose-dependent manner. In addition, cell toxicity was not observed.

To the best of our knowledge, this study is the first to report the utilization of collagen to promote the binding of GFP mRNA to titanium, which successfully enhanced cell transfection as indicated by the protein expression in hBMSCs. This coating method facilitated sustained protein expression for up to 72 hr. Our future direction involves assessing the effectiveness of mRNA encoding BMP-2, incorporated with collagen on titanium surfaces, in facilitating *in vitro* cell transfection and differentiation. This innovative combination of mRNA and collagen presents a promising avenue for developing advanced coatings on titanium surfaces, aimed at enhancing osseointegration and bone healing.

5. Conclusion

We have investigated mRNA coating on titanium surfaces. We found that as-built titanium coating using collagen significantly improved the transfection efficiency of GFP mRNA as indicated by the protein expression in hBMSCs. Negligible protein expression was observed in the mRNA coating without collagen. Sustained protein expression was observed up to 72 hr. Our results suggest the potential application of mRNA and collagen as a novel coating for titanium dental implants aimed at enhancing osseointegration and bone healing.

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7. References

Albrektsson, T., Zarb, G., Worthington, P., & Eriksson, A. R. (1986). The long-term efficacy of currently used dental implants: a review and proposed criteria of success. *The International Journal of Oral & Maxillofacial Implants*, *1*(1), 11-25.

Ao, H., Xie, Y., Tan, H., Wu, X., Liu, G., Qin, A., Zheng, X., & Tang, T. (2014). Improved hMSC functions on titanium coatings by type I collagen immobilization. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, *102*(1), 204-214.

Balmayor, E. R., Geiger, J. P., Aneja, M. K., Berezhanskyy, T., Utzinger, M., Mykhaylyk, O., Rudolph, C., & Plank, C. (2016). Chemically modified RNA induces osteogenesis of stem cells and human tissue explants as well as accelerates bone healing in rats. *Biomaterials*, *87*, 131-146.

Behrens, C., Kauffmann, P., von Hahn, N., Schirmer, U., Liefeith, K., & Schliephake, H. (2022). Collagen-Based Osteogenic Nanocoating of Microrough Titanium Surfaces. *International Journal of Molecular Sciences*, *23*(14), 7803.

Bonnans, C., Chou, J., & Werb, Z. (2014). Remodelling the extracellular matrix in development and disease. *Nature Reviews Molecular Cell Biology*, *15*(12), 786-801.

Bowen-Pope, D. F., Malpass, T. W., Foster, D. M., & Ross, R. (1984). Platelet-derived growth factor in vivo: levels, activity, and rate of clearance. *Blood*, *64*(2), 458–469.

Branemark, R., Branemark, P., Rydevik, B., & Myers, R. R. (2001). Osseointegration in skeletal reconstruction and rehabilitation: a review. *Journal of Rehabilitation Research and Development*, *38*(2), 175-182.

Buser, D., Mericske-stern, R., Pierre Bernard, J. P., Behneke, A., Behneke, N., Hirt, H. P., Belser, U. C., & Lang, N. P. (1997). Long-term evaluation of non-submerged ITI implants. Part 1: 8-year life table analysis of a prospective multi-center study with 2359 implants. *Clinical Oral Implants Research*, *8*(3), 161-172.

Buser, D., Sennerby, L., & De Bruyn, H. (2017). Modern implant dentistry based on osseointegration: 50 years of progress, current trends and open questions. *Periodontology 2000*, *73*(1), 7-21.

Chang, P.-C., Cirelli, J. A., Jin, Q., Seol, Y.-J., Sugai, J. V., D'Silva, N. J., Danciu, T. E., Chandler, L. A., Sosnowski, B. A., & Giannobile, W. V. (2009). Adenovirus encoding human platelet-derived growth factor-B delivered to alveolar bone defects exhibits safety and biodistribution profiles favorable for clinical use. *Human Gene Therapy*, *20*(5), 486-496.

Decha-umphai, D., Chunate, H.-t., Phetrattanarangsi, T., Boonchuduang, T., Choosri, M., Puncreobutr, C., Lohwongwatana, B., & Khamwannah, J. (2021). Effects of post-processing on microstructure and adhesion strength of TiO2 nanotubes on 3D-printed Ti-6Al-4V alloy. *Surface and Coatings Technology*, *421*, 127431.

Deyo, R. A., Ching, A., Matsen, L., Martin, B. I., Kreuter, W., Jarvik, J. G., Angier, H., & Mirza, S. K. (2012). Use of bone morphogenetic proteins in spinal fusion surgery for older adults with lumbar stenosis: trends, complications, repeat surgery, and charges. *Spine*, *37*(3), 222.

Drela, K., Stanaszek, L., Snioch, K., Kuczynska, Z., Wrobel, M., Sarzynska, S., Legosz, P., Maldyk, P., & Lukomska, B. (2020). Bone marrow-derived from the human femoral shaft as a new source of mesenchymal stem/stromal cells: an alternative cell material for banking and clinical transplantation. *Stem Cell Research & Therapy*, *11*, 1-13.

Elangovan, S., Khorsand, B., Do, A.-V., Hong, L., Dewerth, A., Kormann, M., Ross, R. D., Sumner, D. R., Allamargot, C., & Salem, A. K. (2015). Chemically modified RNA activated matrices enhance bone regeneration. *Journal of Controlled Release*, *218*, 22-28.

Fang, E., Liu, X., Li, M., Zhang, Z., Song, L., Zhu, B., Wu, X., Liu, J., Zhao, D., & Li, Y. (2022). Advances in COVID-19 mRNA vaccine development. *Signal Transduction and Targeted Therapy*, *7*(1), 1-31.

Fayed, O., van Griensven, M., Tahmasebi Birgani, Z., Plank, C., & Balmayor, E. R. (2021). Transcript-activated coatings on titanium mediate cellular osteogenesis for enhanced osteointegration. *Molecular Pharmaceutics*, *18*(3), 1121-1137.

FDA. (2002). *INFUSE® Bone-accessdata.fda.gov. Summary Of Effectiveness 2002.* Retrieved November 14, 2023, from https://www.accessdata.fda.gov/cdrh_docs/pdf/p000058b.pdf

Hunziker, E. B., Enggist, L., Küffer, A., Buser, D., & Liu, Y. (2012). Osseointegration: the slow delivery of BMP-2 enhances osteoinductivity. *Bone*, *51*(1), 98-106. https://doi.org/10.1016/j.bone.2012.04.004

Khorsand, B., Elangovan, S., Hong, L., Kormann, M. S., & Salem, A. K. (2019). A bioactive collagen membrane that enhances bone regeneration. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *107*(6), 1824-1832.

Li, H., Ji, Q., Chen, X., Sun, Y., Xu, Q., Deng, P., Hu, F., & Yang, J. (2017). Accelerated bony defect healing based on chitosan thermosensitive hydrogel scaffolds embedded with chitosan nanoparticles for the

delivery of BMP2 plasmid DNA. *Journal of Biomedical Materials Research Part A*, *105*(1), 265-273.

Nations, U. (2017). World population prospects: the 2017 revision, key findings and advance tables. *Department of Economics and Social Affairs PD, editor. New York: United Nations*, 46.

Pardi, N., Secreto, A. J., Shan, X., Debonera, F., Glover, J., Yi, Y., Muramatsu, H., Ni, H., Mui, B. L., Tam, Y. K., Shaheen, F., Collman, R. G., Karikó, K., Danet-Desnoyers, G. A., Madden, T. D., Hope, M. J., & Weissman, D. (2017). Administration of nucleoside-modified mRNA encoding broadly neutralizing antibody protects humanized mice from HIV-1 challenge. *Nature Communications*, *8*(1), 14630. https://doi.org/10.1038/ncomms14630

Przekora, A., Kazimierczak, P., Wojcik, M., Chodorski, E., & Kropiwnicki, J. (2022). Mesh Ti6Al4V material manufactured by selective laser melting (SLM) as a promising intervertebral fusion cage. *International Journal of Molecular Sciences*, *23*(7), 3985.

Ren, X., Zhao, M., Lash, B., Martino, M. M., & Julier, Z. (2020). Growth factor engineering strategies for regenerative medicine applications. *Frontiers in Bioengineering and Biotechnology*, *7*, 469.

Rico-Llanos, G. A., Borrego-González, S., Moncayo-Donoso, M., Becerra, J., & Visser, R. (2021). Collagen type I biomaterials as scaffolds for bone tissue engineering. *Polymers*, *13*(4), 599.

Romano, G., Micheli, P., Pacilio, C., & Giordano, A. (2000). Latest developments in gene transfer technology: achievements, perspectives, and controversies over therapeutic applications. *Stem Cells*, *18*(1), 19-39.

Sartori, M., Giavaresi, G., Parrilli, A., Ferrari, A., Aldini, N. N., Morra, M., Cassinelli, C., Bollati, D., & Fini, M. (2015). Collagen type I coating stimulates bone regeneration and osteointegration of titanium implants in the osteopenic rat. *International Orthopaedics*, *39*, 2041-2052.

van der Stok, J., Koolen, M., de Maat, M., Amin Yavari, S., Alblas, J., Patka, P., Verhaar, J., van Lieshout, E., Zadpoor, A. A., & Weinans, H. (2015). Full regeneration of segmental bone defects using porous titanium implants loaded with BMP-2 containing fibrin gels. *European Cells & Materials*, *2015*(29), 141-154.

Wang, C., Zhang, Y., & Dong, Y. (2021). Lipid Nanoparticle-mRNA Formulations for Therapeutic Applications. *Accounts of Chemical Research*, *54*(23), 4283-4293. https://doi.org/10.1021/acs.accounts.1c00550

Wennerberg, A., Hallgren, C., Johansson, C., & Danelli, S. (1998). A histomorphometric evaluation of screw-shaped implants each prepared with two surface roughnesses. *Clinical oral implants research*, *9*(1), 11-19.