ENHANCING DENTAL IMPLANT OSSEOINTEGRATION VIA PROTEIN OR NON-VIRAL GENE DELIVERY

by

Tarek Hisham Sharkas

D.D.S. Ajman University (AUST), 2004

Submitted to the Graduate Faculty of the School of Dental Medicine of the requirements for the degree of Master of Dental Science

University of Pittsburgh

2009

UNIVERSITY OF PITTSBURGH

SCHOOL OF DENTAL MEDICINE

This Thesis was presented

by

Tarek Sharkas

It was defended on

November 2, 2009

and approved by

Dr. Steve Kukunas, Assistant Professor, Department of Prosthodontics,

Dr. Prashant Kumta, Professor, Departments of Bioengineering, Mechanical Engineering and Materials Science and Chemical Engineering

Mr. John Close Assistant Pofessor Dental Public Health & Information Management

Thesis Advisor: Dr Charles Sfeir, Associate Professor, Department of Oral Biology

ENHANCING DENTAL IMPLANT OSSEOINTEGRATION VIA PROTEIN OR NON-VIRAL GENE DELIVERY

Tarek Sharkas, D.D.S

University of Pittsburgh, 2009

This project proposes to enhance technology aimed at improving osseointegration following dental implant. Specifically, we focus on the delivery of plasmid DNA (pDNA) using nanostructured hydroxyapatite particles coated on titanium surfaces. Our hypothesis is that localized expression of osteoinductive proteins will improve healing time and facilitate osseointegration, as well as enhance the efficacy of placing implants in porous type 4 bone.

To evaluate the feasibility of intercellular gene delivery off titanium surfaces, we employed a reporter system of green fluorescent protein (GFP) pDNA-NanoCaP particles coated on a titanium surface. MG63 osteoblast cells were seeded on this surface, and later assayed for GFP expression. Twenty-four substrates were assessed: experimental group 2 (Exp2, 400 µl NanoCaP/ 8 µl pDNA) substrates group, demonstrated significantly higher GFP expression values compared to the control (Ctl, 200 µl of ddH_2O and 4 µl of pDNA) and Exp (200 µl of NanoCaP and 4 µl of pDNA) groups.

Our data show that the coated NanoCaP/pDNA complex can transfect plated cells and that the applied amount of NanoCaPs is critical. In conclusion, we have successfully shown the feasibility of a non-viral approach to deliver plasmid DNA from titanium surfaces and suggest that further optimization is needed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

- **To my family for their unlimited support and assistance along my life, and to whom impossible is not an obstacle**

- **To Dr. Charles Sfeir, who opened my eyes to the beautiful world of research! Thank you for such a nice experience!**

- **To Dr. Steven Kukunas, who taught me what real clinician skills should look like!**
- **To Dr. Prashant Kumta, for his extreme support and guide in the dental material world!**

- **To Mr. John Close for his dedicated teaching and support in statistics during this project and in my residency education!**

- **My thanks and deep appreciation to all those who generously contributed their experience and hints by making this project possible: Dr. Elia Beniash, Dr. Hussein Zaki and Dr. Mohsen Azarbal!**

1.0 INTRODUCTION

1.1 DEFINITION

Osseointegration is the process by which clinically asymptomatic rigid fixation of alloplastic materials such as titanium is achieved and maintained in bone during functional loading (T. Albrektsson, Johansson, C., Sennerby, L,, 2000). This definition does not clarify the biological processes controlling bone formation and bone maintenance at the bone-to-implant interface, nor the cellular and molecular cascades triggered by site preparation and placement of the implant (Table-I). The result is primary bone healing, and bone deposition around the implant. This process is both time-dependent and dynamic, with maximum bone deposition achieved by 3-4 months. After this time, the bone-to-implant interface is maintained by lifelong remodeling cycles of bone resorption and apposition.

Table 1: Timeline for Bone Healing Process

TIME LINE	EVENTS
Up to 72 hours	Blood clot formation and platelet activation
Up to 4 weeks	Formation of granulation tissue; angiogenesis and fibroplasia
3 weeks to 2 months	Woven bone formation
2 months to 4 months	Replacement with lamellar bone
4 months onwards	On-going bone remodeling

Osseointegration is temporally controlled. Johansson et al. demonstrated that there is a sparse bone-to-implant contact in the first weeks after implant insertion (Johansson, 1987). Direct contact and increased resistance to torque removal takes place three months post-implant insertion. Moreover, there is a gradually increasing required removal torque for up to three years after placement (Yamanaka, 1992).

Currently, the success rates for endosseous dental implants are 70-85%, depending on the site of placement. Thus, improving the success rate is not the major focus for this study. Rather, the goals of the current project are twofold: 1) to accelerate the time to achieve osseointegration, thus expediting the healing process prior to final restoration placement 2) to provide a viable means of implant placement in porous bone (type 3 and 4). This can be achieved through the application of tissue engineering technology, thereby facilitating an osteoinductive (osteogenic) effect on the bone-implant interface. (T. Albrektsson & Johansson, 2001).

2.0 LITERATURE REVIEW

2.1 DENTAL IMPLANT BIOMATERIALS

A variety of dental implant materials are currently used *in vivo,* including titanium and its alloys, cobalt chromium alloys, austenitic Fe-Cr-Ni-Mo steels, tantalum, niobium and zirconium alloys, precious metals, ceramics, and polymeric materials.

Among the aforementioned dental implant materials, the ceramics (aluminum oxide) bear similar interfacial events of tissue integration, corrosion resistance and biocompatibility compared to metallic surface oxides of titanium and chromium.

2.1.1 Implant Coatings

- Hydroxyapatite (HA) is frequently used as a coating, either through spin coating or plasma spraying, for implant or cobalt alloy substrates. HA is a naturally occurring inorganic component of bone, comprising calcium apatite. HA coating conveys several potential advantages, such as enhancing tissue integration and biocompatibility.

- Plasma spraying, which involves spraying molten powder droplets onto the substrate under high temperatures (15,000° C), is considered an attractive means of achieving a porous/rough coating surface (Hermann H, 1988; Steinemann SG, 1985).
- Sandblasting techniques can provide irregular surfaces with <10 μm scale, nitric and hydrofluoric acids which can etch the titanium implant surface and change the surface chemistry, besides eliminating surface contaminations.

HA coating provides an interconnected surface that has irregular surface pores which enhance bone anchorage on the surface for better stability (Schroeder A, 1981). Additionally, increased porosity usually increases the surface area, thus enhancing the bone cells' attachment and supporting ionic exchange on the interface. The result is a better bone-to-implant interface and a stronger load-bearing capability (25%-30%) (W. P. Deporter DA, Pillar RM et al, 1986, 1988, 1990; W. P. Deporter DA, Pillar RM et al,, 1996; Hench LL, 1982; Kirsch A, 1983; Pilliar RM, 1991; Young FA, 1979).

Moreover, the HA thickness is usually in the range of 50 μm, which is the ideal range for manufacturing. It was shown that bone cells that form close to HA surface display more organized distribution and produce more mineralized bone architecture (J. J. Thomas KA, Cook SD et al,, 1987). Nevertheless, bone formation increased in coated implant substrates as compared to other non-coated implant systems. This increased bone formation in turn improves the biomechanics and the initial load bearing of the implant structure. Also, studies showed that HA coated implants have the ability to possess stronger contact between HA-bone than between HA and the implant (Cook SD, 1987; deGroot K, 1987; K. J. Thomas KA, Cook SD et al,, 1987).

Since HA-coated materials exhibit relatively better integration with bone, it is imperative to ensure the strength of HA surface attachment. A promising method of HA application such as ion-beam sputtering that produced denser and thinner coating (thickness of a few micrometers) may reduce the problem of insufficient shear strength, or fatigue at the HA-implant interface (Lacefield, 1986).

These coating and surface treatment techniques all additionally serve to enhance osteoblast differentiation and proliferation at the bone-to-implant interface, thus enhancing the osseointegration process.

2.2 OSTEOBLAST

Bone deposition is carried out by the osteoblast. When osteoblasts mature they are highly polarized, and secrete a specialized extracellular matrix comprising type I collagen, and non collagenous proteins such as osteopontin, osteocalcin, and bone sialoprotien (Marks, 2002).

Osteoblastic proliferation and differentiation can be enhanced by the presence of specific growth factor such as bone morphogenetic protein (BMPs) that can produce a bio-mimetic surface material. It is needless to say that BMP is normally produced by osteoprogenitor and mature osteoblast cells. Experimentally, a relatively high dose of BMP is needed to produce effective results on bone cells, bringing into question the high cost and the safety associated with it (Lieerman, 2002; Schmitt, 1990).

Studies demonstrated that surface roughness not only can affect cell adhesion properties but also can affect cell behavior. Current literature shows that increasing surface roughness can improve osteoblast cell attachment, alkaline phosphatase, osteocalcin, TGF-β, prostaglandin production and decrease cells proliferation (Boyan, 2001; Lohmann, 2002).

The reaction to surface roughness also depends on the quality of bone architecture arrays (osteoblast cell arrangement) that possess a direct effect on the osseointegration process in the healing phase.

2.3 DENSITY OF BONE

Lekholm et al. defined four bone qualities in the anterior region of jawbones: Quality 1: homogeneous compact bone; Quality 2: a thick layer of compact bone surrounding a core of dense trabecular bone; Quality 3: a thin layer of cortical bone surrounding dense trabecular bone of favorable strength; and Quality 4, a thin layer of cortical bone surrounding a core of low-density trabecular bone (Lekholm U Zarb GA, 1985).

Each bone quality type has its advantages and disadvantages when it comes to primary stability, healing and long term prognosis. Quality 1 bone is highly mineralized and thus able to withstand high loads. Cortical lamellar bone heals with little interim woven bone formation, providing superior bone strength during the healing stage (Roberts EW, 1987; Roberts WEL, 1993).

In Quality 3 bone, the use of coating technology is needed to compensate for the poor initial bone-to-implant contact and stability. Examples of these coatings are titanium plasma spray (TPS), or hydroxyapatite (HA). Each have been shown to improve both surface area, and initial bone-to-implant contact. Blood supply and circulation in this bone quality usually lead to faster healing and regeneration, because migration of the regenerative cells is easier through collateral blood vessels.

In addition, initial bone-to-implant contact is 50%, therefore, coating is important in implant design in this bone quality, and that is to provide support for the implant healing through the coating which provides 20-30% more surface area and more initial trabecular bone at the bone-to-implant interface (Block MS at al, April 1988; Block MS, 1987).

The healing time frame for this bone quality can take up to 6 months in order to increase the lamellar bone formation and mineralization.

Quality 4 bone represents porous bone architecture, and thus poor initial stability because of the poor bone-to-implant contact at the time of implant placement. Healing on this bone quality needs more time compared to the above mentioned bone qualities; eight months or more of healing time may be needed to improve the trabeculation and mineralization of bone structure around implant, and undoubtedly, coating is needed for implant surface to improve the surface area (30%) and to enhance the initial healing mechanism.

2.4 BONE-TO-IMPLANT HEALING (OSSEOINTEGRATION)

The process of cutting the bone to provide space for an implant creates an injury, and subsequent healing cascade.

Following implant placement, the gap between the bone and the implant will fill with blood. This blood brings factors which will initiate the healing process, culminating eventually in intramembranous bone formation, (Schenk R, 1994) and *de novo* bone formation (Davies JE 1998).

In large or small defects, bone healing takes the same pattern; it all starts with blood clot formation, angiogenesis, osteoprogenitor cell migration, woven bone formation, compaction of woven bone by deposition of parallel-fibered and lamellar bone, and secondary remodeling of the woven bone.

Roberts et al. found that it takes three months for the remodeling process to reach the balancing stage (1.5 mo for woven bone formation + 1.5 mo for turnover) at the interfacial gap (Roberts EW, 1987). If complete bone healing is required, then six months or more are necessitated, depending on the initial bone density.

Furthermore, the cement line that normally exists between secondary osteon and pre-existing bone has been found to be similar at a healing bone-to-implant interface. Additionally, the presence of extracellular matrix proteins such as osteopontin and bone sialoprotein indicates that the healing that occurs at this post-injury interface takes a similar pattern to the natural osteogenic process (McKee MD, 1993).

2.5 TISSUE ENGINEERING

Tissue engineering is new field of research that relies on a combination of materials, growth factors and cells to regenerate diseased tissues. According to Langer and Vacanti, “tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or produce a whole organ” (Langer).

Scientific advances in biomaterials and stem cell technologies, as well as the knowledge of growth and differentiation factors and biomimetic environments, have been rapid. These advances have led to the development of powerful new methods of producing tissue *in vitro* from the combined set of engineered extracellular matrices ("scaffolds"), biologically active molecules, and cells. Among these treatment methods exists the use of gene therapy—namely the ability to deliver genes encoding proteins like growth factors, transcription factors, or extracellular matrix molecules, locally to somatic cells. These genes assist in regenerating the tissue by recapitulating the normal biological process through the expression of bioactive molecules (Bonadio J, 1999).

The most prominent advantages of gene therapy over the local delivery of growth factors in a protein form, are increased half--life, low dose requirement, low cost, wide distribution, and no need for repeated applications (Wozney JM, 1998).

Gene therapy offers different approaches for the introduction of DNA to host cells. Of the many approaches available, they are roughly classified into: viral and non-viral delivery mechanisms.

2.5.1 Viral Approach (Transduction)

Viral delivery (transduction), refers to the use of a virus to transfer DNA into host cells. Transduction is similar to the process of “infection”, however, transduction does not end in disease of the cell.

Viruses can be divided into integrating and non-integrating subtypes based on their influence on the cell’s genome.

Non-integrating viruses maintain the genetic material into the nucleus as a non-integrated (episomal) form, instead of transferring it into the cell’s DNA.

Adenovirus is an example of a small, non-integrating DNA virus that has the advantage of precisely infecting non-dividing host cell DNA at chromosome 19 (Samulski, 1991).

2.5.1.1 Advantages

The most important advantage of the viral approach is high transduction efficiency conveyed through the virus’ natural ability to infect host cells.

2.5.1.2 Disadvantages

The virus as a gene delivery agent has its complications, such as: endogenous recombination, oncogenic potential, and, most significantly, potential for toxic immunological reactivity (Ferber

2001; Somia N, 2000). This immunologic response is particularly critical, as it can lead to destruction of the viral vector or the host cells (Ferber 2001). The significant drawbacks of viral gene delivery have necessitated the study and development of non-viral methods.

2.5.2 Non-Viral Approach (Transfection)

The successful transfer of DNA into the cell nucleus using non-viral vectors is defined as "transfection" (Graham FL, 1973; Vaheri, 1965). The great early achievements of Vaheri, Pagano, Graham, and van der Eb, through DEAE-dextran and calcium phosphate-mediated transfection techniques, opened the door for further experiments involving DNA transfer into cultured eukaryotic cells.

Prior to the development of molecular biological techniques for cloning plasmid DNA, advancement in transfection technology were relatively slow. However, cloning techniques facilitated the preparation of the DNA sequence and the production of unlimited amounts of pure DNA for transfection experiments (Melton, 1984).

Great progress in developing reporter gene technology for monitoring of the efficiency of DNA transfer opened new horizons for gene therapy applications. Gorman et al. established the reporter gene concept by using a bacterial chloramphenicol acetyltransferase (CAT) gene and an associated assay system. By using a reporter gene that is not endogenous to the cell, along with an assay system specifically sensitive for that gene product, the scientist is able to study the reporter gene under different conditions (Gorman, 1982; Groskreutz, 1997).

This approach, combined with transfection reagents such as calcium phosphate, founded the basics for studying promoter-enhancer sequences, *trans*-acting proteins such as transcription factors, protein/protein interactions, mRNA processing, translation, and recombination.
Since the introduction of the CAT assay system, other reporter systems have been developed including luciferase, β-galactosidase, alkaline phosphatase and green fluorescent protein (GFP) (Groskreutz, 1997).

2.5.2.1 Advantages

Non-viral gene delivery methods have advantages such as tissue specific targeting (Li Y, 2004), ease of large scale production (Li Y, 2004; Wilson SP, 1995a; Wu D, 2003), the capacity to carry large DNA inserts (Corsi K, 2003), and low immunogenicity (Huard J, 2003; Li Y, 2004; Wu D, 2003).

2.5.2.2 Disadvantages

The relative disadvantage of non-viral approaches is a decreased transfection efficiency (Huard J, 2003; Wu D, 2003), as compared to viral approaches.

2.5.2.3 Transfection Efficiency Factors

In general, the factors that determine the efficiency of gene transfection are: the physical and chemical stability of the DNA in the extracellular space, cellular uptake, DNA escape from the

endosomal network, cytosolic transport, and nuclear localization of the DNA for transcription (Chowdhury EH, 2003; Jordan M, 2004).

2.5.2.4 Methods

The methods of using non-viral gene delivery are: naked DNA injection (Hickman MA, 1994; Sikes ML, 1994), electroporation (Somiari S, 2000) , gene gun administration (Cheng L, 1993; Jiao S, 1993), cationic (Li B, 2000; Maurer N, 1999), and anionic (Fillion P, 2001; Patil SD, 2005), lipids, cationic polymers (Kunath K, 2003; Li Y, 2004; Oster CG, 2005), peptides (McKenzie DL, 2000; Trentin D, 2005), and ceramic particles of calcium phosphate (CaP).(Batard P, 2001; Bisht S, 2005; Graham FL, 1973; Olton D, 2007b; Orrantia E, 1990; Roy I, 2003).

The most important methods aforementioned are:

- Naked DNA injection into embryonic cells, is commonly used in the production of transgenic organisms. However, this approach is not appropriate for studies requiring a high number of transfected cells (Cappechi, 1980; Hickman MA, 1994).
- Electroporation was first reported in 1982. This approach is often used in plant protoplast cells that are particularly defiant to normal gene transfer methods (Shigekawa, 1988; Wong, 1982). Based on the electrical stimulus of the cell membrane and consequent opening of pores which allow the passage of nucleic acid, this technique requires fine-tuning and great optimization of the electrical pulse for each cell type.

- Calcium phosphate: Extensive studies and investigations led by Graham and van der Eb, published in a paper in 1971, showed that calcium phosphate co-precipitation is an effective technique for non-viral transfection. In their study, they evaluated cationic and anionic and phosphate concentration along with the pH of the solution and its effect on transfection efficiency (Graham FL, 1973).This precipitate is taken up by the cells via endocytosis or phagocytosis. In addition, the calcium phosphate provides protection against intracellular nucleases.

2.5.3 Nano-scale Calcium Phosphate/pDNA complex

One of the most attractive methods for calcium phosphate mediated transfection is the incorporation of pDNA with nanostructured calcium phosphate (CaP). CaP's many clear advantages include its biocompatibility, biodegradability, ease of handling and adsorptive capacity for pDNA (Olton D, 2007a; Wilson SP, 1995b).

Nano-scale calcium phosphate particles are typically 20 - 40 nanometers (nm) and are usually synthesized through mixing of $CaCl_2$ and Na_3PO_4 to form a colloidal suspension of HA. The Ca/P ratio and pH is maintained to control the rate of nucleation and growth of the HA precipitates which will ultimately form a nanocrystalline calcium phosphate (Olton D, 2007a).

3.0 HYPOTHESIS

Coating titanium implants with a functional system for delivery of a gene or growth factor, would improve and expedite osseointegration.

This project will propose to focus on gene delivery of plasmid DNA (pDNA) using nanostructured hydroxyapatite. This HA-pDNA will be coated on titanium surfaces in an attempt to achieve transfection of eukaryotic cells in the milieu. The therapeutic goal is the enhancement of osseointegration through modifying cells which migrate during the healing process, to the interface of titanium/bone.

The hypothesis is that local transfer of pDNA, such as that which encodes bone morphogenetic protein (BMP), would be likely to improve bone healing at the implant site, and potentially accelerate the rate of osseointegration. Similarly, the transfer of bioactive genes at a site of type four (porous) bones may increase the efficiency of osseointegration and bone union at the site.

4.0 SPECIFIC AIM

To assess the efficacy of precoating titanium implants with therapeutic plasmid DNA (pDNA)--delivered using non-viral vehicles--as a means to improve and accelerate the process of osseointegration. Our assay systems employs a green fluorescent protein (GFP) pDNA complexed with NanoCaPs particles coated on a titanium surface, over which MG63 osteoblast cells will be grown.

5.0 MATERIALS AND METHODS

5.1 IMPLANT SUBSTRATE (TI6AL-4V)

Titanium foil, 6AL-4V [Aluminum 5.50-6.75%, Vanadium 3.50-4.50%], 0.127mm (0.005in) thick, 99.99+% (metals basis) (Titanium Metal Supply, Inc.) was used as a substrate for this study; each substrate (total of 24) has been sectioned in 1cm X 1cm dimension.

5.2 APPLICATION OF CAP COATING OVER TITANIUM IMPLANT SUBSTRATE

A non-aqueous sol-gel process was used for depositing a porous CaP phase on the titanium implant substrate by a spin coating technique:

Step One: The deposition of the coating fluid onto the titanium implant substrate.

Step Two: The substrate is accelerated up to its final, desired, rotation speed (2000 rpm).

Step Three: The substrate is spinning at a constant rate and fluid **viscous forces dominate** fluid thinning behavior.

Step Four: The substrate is spinning at a constant rate and **solvent evaporation dominates**, enabling the formation of a thin coating.

Finally, the titanium implant substrate is heat-treated in air at 900˚ C for 1 hour forming a porous layer of hydroxyapatite.

5.3 EVALUATION OF COATING TOPOGRAPHY USING SCANNING ELECTRON MICROSCOPY (SEM)

Calcium phosphate (CaP) coating of the titanium surface was assessed by Philips XL-30FEG equipped with an EDS detector system, comprising an ultrathin beryllium window and Si (Li) detector operating at 25 kV.

Two groups were assessed:

1. Titanium substrate coated with porous HA *without* NanoCaPs/pDNA complex.

2. Titanium substrate coated with porous HA *with* NanoCaPs/pDNA complex.

5.4 SYNTHESIS AND CHARACTERIZATION OF NANO-HYDROXYAPATITE (NANOCAPS)

A novel aqueous solution chemistry-based technique used to synthesize HA: specifically, $CaCl_2$ and Na_3PO_4 will be reacted in deionized water leading to the immediate formation of a colloidal suspension of HA. The Ca/P ratio in the reacting solutions will be maintained at 167 to control the supersaturated state, thereby preventing the rapid nucleation and growth of the HA precipitates.

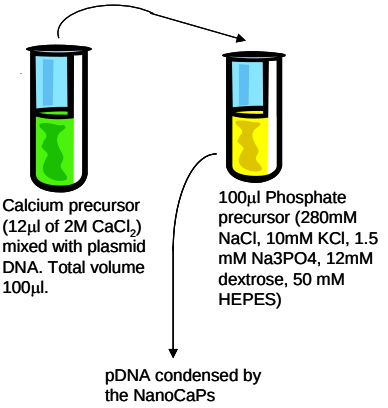

Figure 1: The DNA was first added to the calcium precursor and then the phosphate precursor was added to the mix [NanoCaP/pDNA]

This, combined with the addition of suitable pH buffering agents (50mM Hepes), will ensure an invariant reaction pH of 7.5 during the entire reaction. These conditions are suitable for synthesizing the CaP structures in a nanocrystalline form while also maintaining their chemical composition. This is critical for biocompatibility and attaining efficient transfection of DNA.

A nanostructured form of hydroxyapatite (HA) was synthesized by mixing stoichiometric amounts of the above Ca salts and phosphate precursors [Fig-1]. 4 µg of p-DNA was added to 12µl of 2M $CaCl_2$ solution in 100µl of water. The mixture was added to 100µl of phosphate precursor (280mM NaCl, 10mM KCl, 1.5 mM Na_3PO_4, 12mM dextrose, 50 mM HEPES). The

reaction conditions, namely temperature, pH and the chemical concentration, were adjusted to achieve a particle size in the nanometer range.

In the case of HA, we have determined the Ca/P ratio in the reacting solution to be 167 to control the supersaturated state, thereby preventing rapid nucleation and growth of the HA precipitates. A dilution factor of 100 was used to increase the Ca concentration in the solution such that the Ca/P ratio is 167 instead of 1.67 for stoichiometric HA to prevent rapid growth and coalescence.

5.5 APPLICATION OF NANOCAP/PDNA COMPLEX OVER THE HA-COATED TITANIUM IMPLANT SUBSTRATE

The NanoCap/pDNA or pDNA then added to each well/substrate. A Wallac 1450-606S Black Visiplate (Perkin-Elmer) plate was used, due to its special characteristics including low background fluorescence, minimal light scatter and reduced crosstalk.

A total of 24 wells are divided into three main groups. Each group (n = 8) received different composition of NanoCaP/pDNA or pDNA complex as shown below:

Exp - Group: including in each well/substrate: 200 µl of NanoCaP and 4 µl of pDNA

Exp2 - Group: including in each well/substrate: 400 µl of NanoCaP and 8 µl of pDNA

Ctl - Group: including in each well/substrate: 200 µl of ddH_2O and 4 µl of pDNA

5.6 DETERMINATION OF *IN VITRO* TRANSFECTION EFFICIENCY USING REPORTER GENE (GFP)

Osteoblast (MG63) cells were cultured in 75 cm^2 flasks in a humidified incubator at 37 °C and 5%CO_2; Cells were maintained at sub-confluency and passaged every 2–3 days. Each well/substrate received 8 $x10^4$ cells that were grown in α-MEM-F12 containing 10% FBS and 1% penicillin/streptomycin. The cells were allowed to grow for 6 days and then tested for GFP expression.

A Wallac 1420 Victor³V fluorescence plate reader (Perkin Elmer) was used to assess the fluorescence intensity of each substrate/well.

5.7 STATISTICAL ANALYSIS

The data were analyzed for significant ($p \leq 0.05$) mean differences of fluorescent readings between the groups: Ctl (control), Exp (experimental) and Exp2 (experimental-2) using one-way analysis of variance (ANOVA). Post-hoc analysis for pairwise differences and identification of homogeneous subgroups were performed using Fisher's Protected Least Significant Difference (LSD).

Auto-fluorescence of calcium phosphate is well documented in the literature (Muddana HS, 2009). Therefore, titanium implant substrates with HA-coating only (Blank, n = 8) were used to determine the statistical means of fluorescence which might be contributed by background noise for Ctl, Exp and Exp2. These controls were also evaluated for pure determination of the cells' native fluorescence values.

Blank substrates (Titanium substrate coated with porous HA *without* NanoCaPs/pDNA complex) as 4th group were also evaluated to determine if there is a significant difference among Ctl, Exp and Exp2 groups.

6.0 RESULTS

6.1 MORPHOLOGY OF COATED SUBSTRATE UNDER ELECTRON SCANNING MICROSCOPY (SEM)

The HA coating on the implant surface contains pores which play an important role in retaining the NanoCaP/pDNA complex. Also noticeable by SEM analysis, is the presence of grooves between HA islands, conveying an overall roughness. This roughness, in turn, facilitates the processes of cell adhesion, growth, and transfection by the NanoCaPs/pDNA.

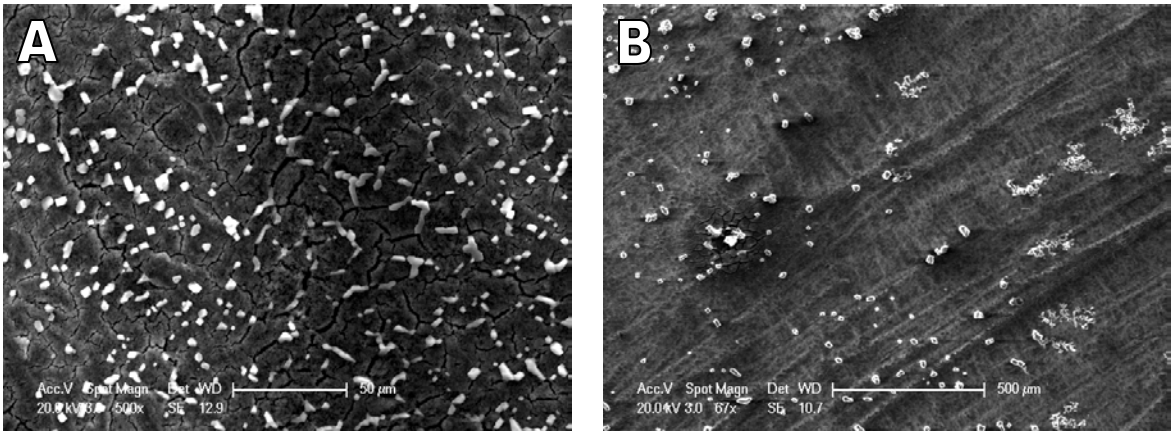

Figure 2: SEM images of HA-coated titanium substrate under different magnifications (67x – 500x).

A, B: note the agglomeration of NanoCaP/pDNA complex over the surface of HA-coated implant.

A: Note the agglomeration of NanoCaP/pDNA complex into the cracks between HA islands

Low magnification SEM shows that these grooves between HA islands seem to also serve as a good attachment site for the Nano-CaP/pDNA complex. [Figure 2A].

Higher magnification SEM analysis shows a thin film coating of NanoCaPs on the CaP film and also shows clusters of NanoCaP/pDNA complex [Fig3/A]. These NanoCaP/pDNA clusters remained in clusters on the substrate even after cell culture, as shown below in the fluorescence images analysis section.NanoCaP/pDNA was found to be incorporated into the grooves between the HA islands. In addition, NanoCaP/pDNA complex was also found on the pores of the HA-coating itself.

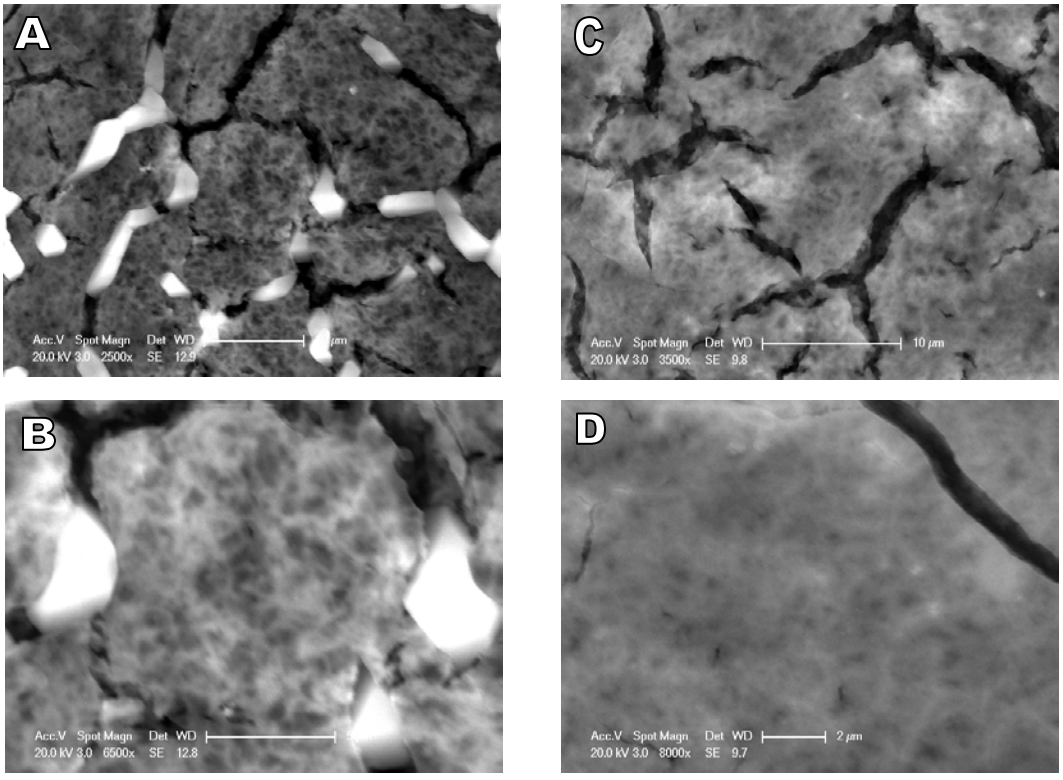

Figure 3: SEM images of HA-coated titanium substrate under different magnifications (2500x – 8000x). A, B: with NanoCaP/pDNA complex sinking into cracks or fissures of HA-coating. C, D: without NanoCaP/pDNA complex.

6.1.1 NanoCaPs and Transfection Efficiency:

Evaluation of MG63 cell transfection can be easily conducted through measurement of green fluorescent protein (GFP) levels, once the pDNA encoding GFP is taken up by the cell and expressed.

Twenty-four substrates were assessed for GFP expression using the Wallac 1420 Victor^{3}V fluorescence plate reader. Green fluorescence is indicative of the transfection efficiency of the various NanoCaP/pDNA treatment groups.

Exp - Group: 200 µl of NanoCaP and 4 µl of pDNA

Exp2 - Group: 400 µl of NanoCaP and 8 µl of pDNA

Ctl - Group: 200 µl of ddH_2O and 4 µl of pDNA

Exp2 (400 µl NanoCaP/ 8 µl pDNA) substrates group, demonstrated significantly higher GFP expression values compared to the Ctl *($p < .003$)* and Exp ($p > .006$) groups.

Moreover, Exp group (200 µl of NanoCaP and 4 µl of pDNA) showed no significant difference to Ctl (200 µl of plain water and 4 µl of pDNA) groups in GFP expression values.

A negative control group was also evaluated for GFP expression to determine and thereby subtract the background noise, however no significant differences were found following noise subtraction from the groups Exp2, Exp, and Ctl.

Furthermore, Blank group (Titanium substrate coated with porous HA *without* NanoCaPs/pDNA complex) demonstrated significantly low fluorescence expression values, and as a result a large significant difference when compared to Exp2 ($p > .0004$), Exp ($p > .009$) and Ctl ($p > .017$) groups.

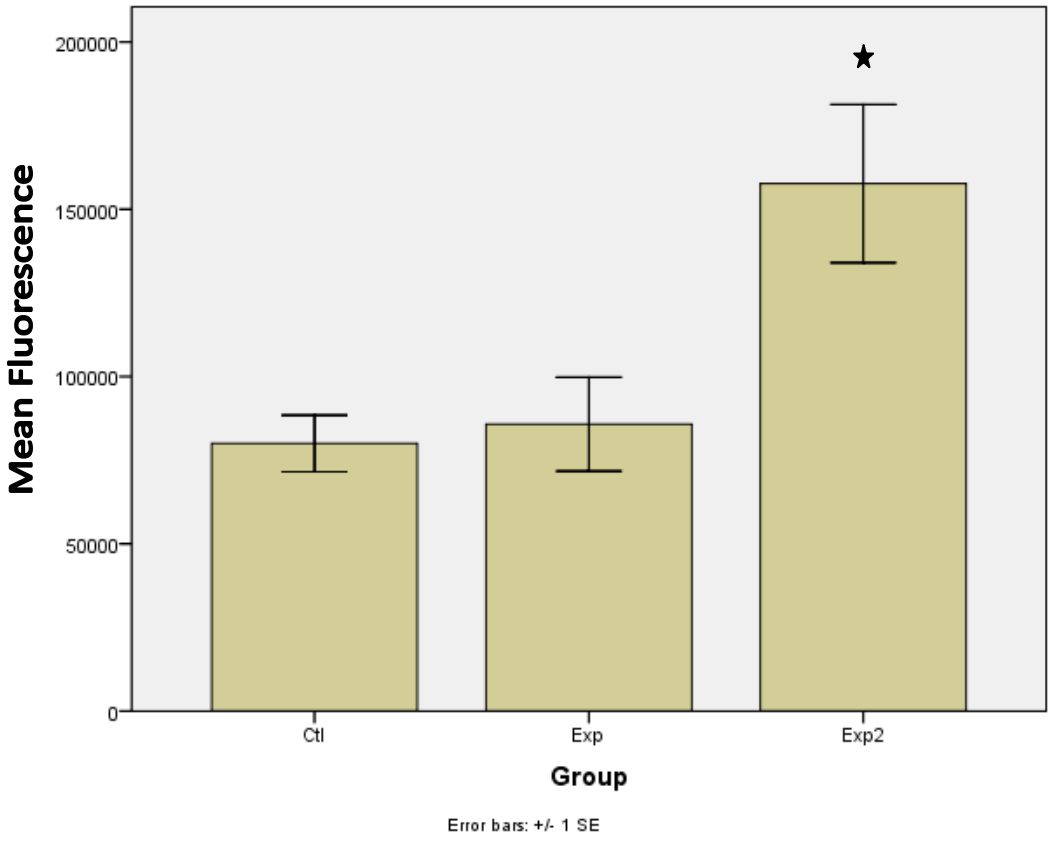

Figure 4: Comparison of GFP expression of transfected MG63 cells: mean values for Ctl, Exp, and Exp2 groups, using Wallac 1420 Victor³V fluorescence plate reader.

($n = 8$ for each group, *difference is significant at $p \leq 0.05$).

6.2 TRANSFECTION EFFICIENCY AND VISUALIZATION OF FLUORESCENCE

Fluorescence microscopy analysis was performed to visualize the transfected MG63 cells expressing GFP. A Nikon inverted microscope (Nikon Instruments, Inc.) was used. All three groups (Ctl, exp, Exp2), in addition to a blank group, were examined for GFP expression. Images were taken at 10 x magnifications to visualize a large surface area of the transfected cells plated on coated titanium.

Figure 5 shows, that Exp2 group displays a significantly higher surface area of green fluorescence expression [Fig-5/D]. This could be due to the higher amount of NanoCaP/pDNA present on the substrate.

Neither the Ctl [Fig-5/B] nor blank [Fig-5/A] groups contained any NanoCaP, however, the Ctl group (pDNA vector control) shows slight green fluorescence expression in MG63 cells compared to the blank group.

Besides, the difference between the Exp [Fig-5/C] and Ctl [Fig-5/B] in green fluorescence expression values was not significant.

Furthermore, Exp [Fig-5/C], Exp_2 [Fig-5/D] groups showed a general distribution of NanoCaP clusters over the substrate and was always related to high green fluorescent expression values as measured with (Nikon) NIS-Element software. It should, however, be noted that the florescence analysis was not quantitative, but rather qualitative.

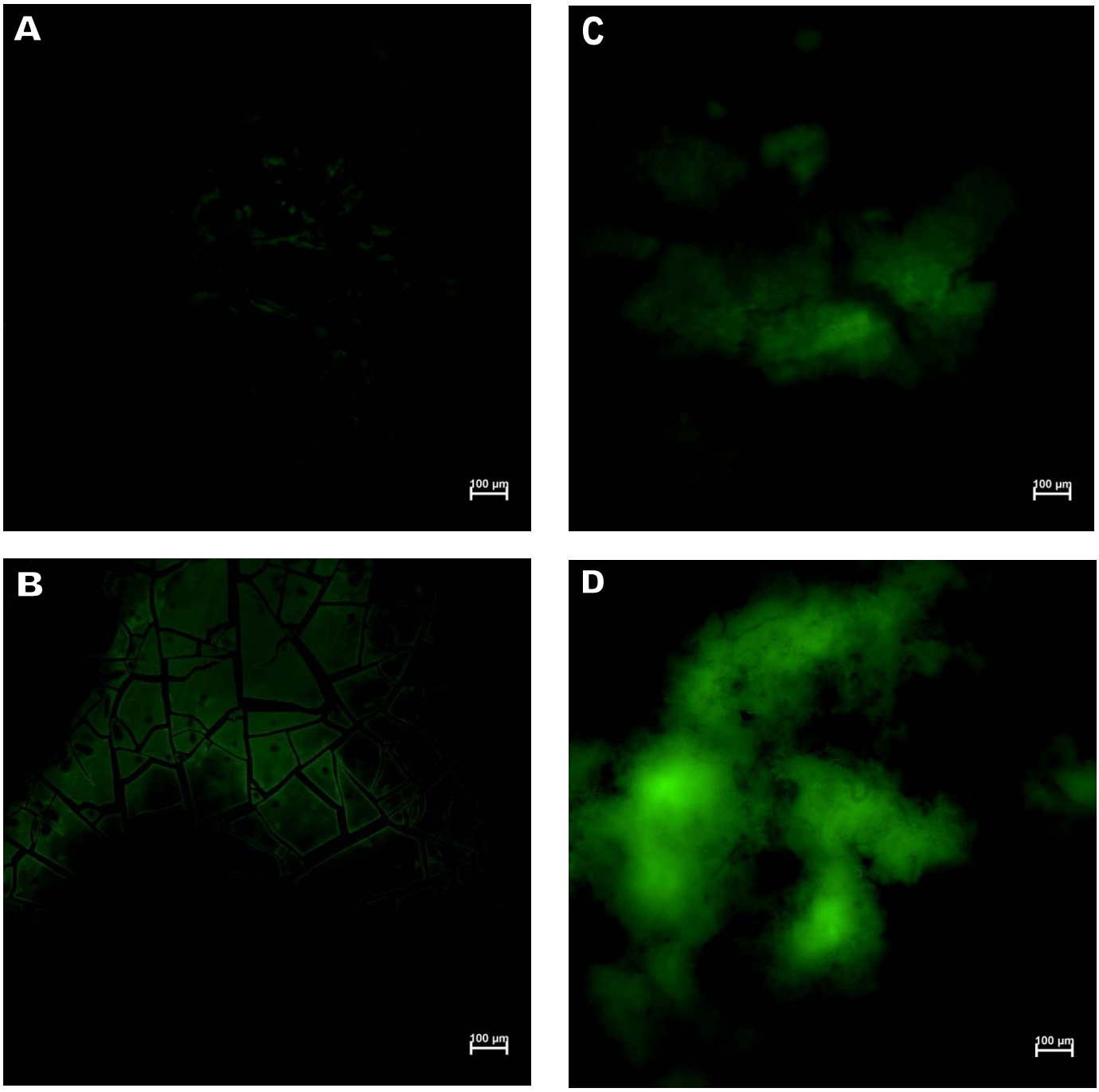

Figure 5: Comparison of four microscopic flourescence images representing the four groups: blank (A), Ctl (B), Exp (C), Exp2 (D), all taken at 10x, note the scattering of pDNA/cells on Ctl group and the clusters of NanoCaP/pDNA/cells on Exp and Exp2.

7.0 DISCUSSION

Titanium implant surface chemistry has been studied over the years to identify the best surface in terms of biocompatibility and stability. It is well documented that titanium is biocompatible due to its bioinert chemistry (Chiang CY, 2009). In addition, the ion composition of the titanium surface was shown to be important to support cell growth upon coatings of substrates such as calcium phosphate (Park JW, 2009). Furthermore, the CaP coated implants were shown to have a bone-to-implant contact of 77.6% +/- 5.1% after 15 years of loading (Iezzi G, 2009).

Our study has focused on coating titanium foil 6AL-4V, using a spin coating technique; all substrates received the same amount of CaP particles to produce homogeneous layer thickness. The titanium coated substrates were examined via scanning electron microscopy to evaluate the pores/grooves on the surface at the micron-scale.

SEM images showed a homogenous surface with multiple scattered grooves that formed the shape of an island (HA island). This calcium phosphate coating should yield a surface with higher roughness, which would be desirable since previous studies showed that implant surface roughness in three micron range could improve the differentiation of osteoblasts *in vitro* (Marinucci L, 2007).

In this study, surface roughness was not quantified or measured. Future *in vitro* evaluation of the coating will be done by Atomic Force Microscopy (AFM) to evaluate the surface texture and angle of the surface grooves (Catauro, Raucci, de Marco, & Ambrosio, 2006).

A nanostructured form of hydroxyapatite (HA) was successfully synthesized by mixing stoichiometric amounts of the Ca-salts and phosphate precursors.

Three groups were used in this project, each of which showed different transfection results reflecting different NanoCaP concentrations. High transfection efficiency was achieved as shown by fluorescence measurement when 400µl of NanoCaP were used, and low transfection values were achieved in the control group that has 4 µg of pDNA only.

Our study showed the efficacy of using NanoCaP as a carrier method. Previous studies that used NanoCaPs supported the same results, which found that use of NanoCaP particles for transfection proved to enhance odontogenic differentiation (rat dental pulp stem cells), when cultured on 3-dimensional scaffolds. Furthermore, pDNA/NanoCaP complexes have been shown to be very effective as non-viral carriers, and were instrumental in facilitating odontogenic differentiation mediated by BMP-2 growth factor transfection (Yang, et al., 2008).

The next step in this project is to incorporate the proper growth factor gene such as BMP-2 (osteoinductive) with the NanoCaP to form a complex over the osteoconductive HA-coated surface and subsequently improving and expediting bone formation.

In previous studies, soybean trypsin inhibitor was incorporated into a biodegradable polymer that was coated on a titanium implant surface. The release of the protein from the surface improved ingrowths of bone cells. Besides, the biodegradable carrier system degraded completely after 11 weeks. Moreover, it was concluded that after 42 days, 76% and 71% of IGF-

I, TGF-β1, respectively, were found in the elution fluid by ELISA (Agrawal, Pennick, Wang, & Schenck, 1997).

It is well documented from the literature that the use of Gentamicin on Ti6Al4V/coated-calcium alginate and gelatin composite has an antibacterial affect against staphylococcus aureus—an advantage which in turn might improve bone formation (Xiao, Zhu, Liu, Zeng, & Xu, 2009). Drug release of Gentamicin on Ti6Al4V/coated-calcium alginate and gelatin composite was released within 0.5 hour, and it lasted for 10 days furthermore, the drug release can be extended up to 3 weeks period with anodized nano-tubular titanium with calcium phosphate crystals and antibiotic penicillin-based.

Therefore, in addition to growth factor release from the surface of the implant, the incorporation of antibiotic such as Gentamicin on the titanium surface toward the collar of the dental implant might prove to be very beneficial.

Future incorporation of Gentamicin in a specified area such as the collar and the BMP onto the remaining surface seems a feasible approach (Brohede, et al., 2009; Catauro, et al., 2006; Xiao, et al., 2009; Yao & Webster, 2009).

Following this feasibility study, optimizing the concentration of BMP-2 delivery is very important as shown by Liu et al. They incorporated BMP-2 on a metallic titanium surface and on a calcium phosphate coated implant surface. The results showed implant osteoconductivity was the lowest when BMP-2 was adsorbed (burst-release fashion), and was improved when BMP-2 was incorporated into calcium phosphate coating. (Liu, Enggist, Kuffer, Buser, & Hunziker, 2007).We have used a non-viral approach to deliver plasmid DNA from titanium surfaces and our data is promising, showing the feasibility of the method. It would be of interest to compare the efficacy of a non-viral approach to viral delivery as described by Song et al., who delivered

an adeno-associated virus encoding BMP7 around HA-coated titanium implant in rabbit bilateral tibia.

Gaps were created around the dental implant so it could be filled with Bio-Oss coated with adeno-BMP7.

It was concluded that rAAV-BMP7/Bio-Oss can enhance the bone formation and trigger enhanced bone reaction at the bone-to-implant interface (Song, Du, Luo, & Cao, 2008).

Clark et al. assessed the use of TGF-β1 growth factor coated onto titanium implant and then placed into rabbit humerus. They demonstrated that TGF-β1 enhanced bone-to-implant contact at the interface by 96%, and bone ingrowths into the porous surface by 50%. Furthermore, their study supported the physiologic incorporation of drug into the porous surface instead of adsorption fashion (Clark, Moioli, Sumner, & Mao, 2008).

In addition to TGF-β1 growth factor, rhFGF-4 was also assessed as a drug for osseointegration. Franke et al. used rhFGF-4 which was injected into the femur and tibia of rabbits. They showed that local delivery of rhFGF-4 in a collagen sponge (altelocollagen) stimulated bone formation at bone-to-implant interface more than the control implant, which has collagen alone (Franke Stenport, Johansson, Sawase, Yamasaki, & Oida, 2003).

Other growth factors assessed include vascular endothelial growth factor (VEGF). Wolf et al. incorporated the VEGF into 3-dimentional collagenous matrices on Ti6Al4V implant substrates. The release pattern decreased after the first 24 hours, then increased on the third day on heparinized matrices with VEGF, compared to pure collagen and the unmodified surface. Moreover, the proliferation of human dermal micro-vascular endothelial cells was enhanced after the release of VEGF, which in turn proved that VEGF may increase and enhance bone formation

and healing at the bone-to-implant interface (Wolf-Brandstetter, Lode, Hanke, Scharnweber, & Worch, 2006).

8.0 CONCLUSION

We have successfully coated the titanium implant substrate with nano-sized calcium phosphate (NanoCaP)/pDNA complexes, which subsequently transfected MG63 osteoblast-like cells.

Evaluation and assessment of the MG63 cells were performed and showed that the NanoCaP/pDNA did adhere to the surface of the CaP and was successfully taken up by the cells.

Our data show that the amount of NanoCaP/pDNA complex is critical to achieve efficient transfection.

More studies are needed to optimize the amount of NanoCaP/pDNA that needs to be coated on the titanium surface. In conclusion, we have shown that NanoCaPs introduced on a titanium substrate have the ability to transfect cells. We suggest that this technology could be used to improve bone healing at the implant osteotomy site, and potentially expedite the rate of osseointegration.

8.1 FUTURE STUDIES FOR THIS PROJECT MAY INVOLVE: (BY THE AUTHOR)

- The transfer of bioactive genes at the site of type four (porous) bones may increase the efficiency of osseointegration and bone union at the bone-to-implant interface.

- Antibiotic also could be used to provide an antibacterial effect on the implant site, and to improve the healing process.
- Selective osseointegration could be explored as a way to improve healing the most in stress-areas such as in crestal bone areas around dental implants.

Further understanding and evaluation of bone proteins and growth factors is needed to take this project to the next level of *in vitro* and *in vivo* studies. With advanced knowledge, this technology could facilitate the best bone gene delivery on implant surfaces for both faster and better osseointegration.

BIBLIOGRAPHY

Agrawal, C. M., Pennick, A., Wang, X., & Schenck, R. C. (1997). Porous-coated titanium implant impregnated with a biodegradable protein delivery system. *J Biomed Mater Res, 36*(4), 516-521.

Albrektsson, T., & Johansson, C. (2001). Osteoinduction, osteoconduction and osseointegration. *Eur Spine J, 10 Suppl 2*, S96-101.

Albrektsson, T., Johansson, C., Sennerby, L, (2000). Biological aspects of implant dentistry : osseointegration. *Periodontology 2000, 2, 58-73*.

Batard P, J., Wurm F (2001). Transfer of high copy number plasmid into mammalian cells by calcium phosphate transfection. *Gene, 270(1&2):61-8*.

Bisht S, B. G., Mitra S, Maitra A (2005). pDNA loaded calcium phosphate nanoparticles: highly efficient non-viral vector for gene delivery. *Int J Pharm 288(1):157-68*.

Block MS at al (April 1988). HA-coated and grit- blasted titanium Implants in dogs. *proceeding of the world biomaterials Congress.*

Block MS, K. J. K. J. (1987). Evaluation of hydroxylapatite-coated titanium dental implants in dogs. *J Oral maxillofac Surg 45:601*.

Bonadio J, S. E., Patil P, et al (1999). Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. *Nat Med, 5, 753*.

Boyan, B. D., Lohmann, C.H., Sisk, M., Liu, Y., Sylvia, V.L., Cochran, D.L., Dean, D.D. and Schwartz, Z. (2001). Both cyclooxygenese-1 and cyclooxygenese-2 mediate osteoblast response to titanium surface roughness. *J. Biomed. Mater. Res. , 55, 350-359.*

Brohede, U., Forsgren, J., Roos, S., Mihranyan, A., Engqvist, H., & Stromme, M. (2009). Multifunctional implant coatings providing possibilities for fast antibiotics loading with subsequent slow release. *J Mater Sci Mater Med, 20*(9), 1859-1867.

Cappechi, M. R. (1980). *Cell 22, 479*.

Catauro, M., Raucci, M. G., de Marco, D., & Ambrosio, L. (2006). Release kinetics of ampicillin, characterization and bioactivity of TiO2/PCL hybrid materials synthesized by sol-gel processing. *J Biomed Mater Res A, 77*(2), 340-350.

Cheng L, Z. P., Yang NS, (1993). In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proc Natl Acad Sci USA, 90(10):4455-9*.

Chiang CY, C. S., Yang WE, Hsu ML, Yung MC, Tsai ML, Chen LK, Huang HH (2009). Formation of TiO2 nano-network on titanium surface increases the human cell growth. *Dent Mater, Aug;25(8):1022-9. Epub 2009 Mar 28*.

Chowdhury EH, S. T., Nagaoka M, Kundu AK, Akaike T (2003). Transfecting mammalian cells by DNA/calcium phosphate precipitates: effect of temperature and pH on precipitation. *Anal Biochem, 314(2):316-8*.

Clark, P. A., Moioli, E. K., Sumner, D. R., & Mao, J. J. (2008). Porous implants as drug delivery vehicles to augment host tissue integration. *FASEB J, 22*(6), 1684-1693.

Cook SD, K. J., Thomas KA et al, (1987). Interface mechanics and histology of titanium and HA coated titanium for dental implant applications. *Int J Oral Maxillofac Impl 2(1):15-22*.

Corsi K, C. F., Yahia L, Fernandes JC, (2003). Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. *Biomaterials 24(7):1255-64*.

Davies JE (1998). Mechanisms of endosseous integration. *Int J Prosthodont, 11:391-401*.

deGroot K, G. R., Klien C et al, (1987). Plasma sprayed coatings of hydroxyapatite. *J Biomed Mater Res, 21:1375-1381*.

Deporter DA, W. P., Pillar RM et al (1986). A histological assessment of the initial healing response adjacent to porous surfaced titanium alloy dental implants in dogs. *J Dent Res 5(8):1064-1070*.

Deporter DA, W. P., Pillar RM et al (1988). A histological evaluation of a functional endosseous, porous-surfaced, titanium alloy dental in the dog. *J Dent Res 67:1990-1995*.

Deporter DA, W. P., Pillar RM et al (1990). A histological comparison in the dog of porous-coated versus threaded dental implants. *J Dent Res 69:1138-1145*.

Deporter DA, W. P., Pillar RM et al, (1996). A prospective clinical study in humans of an endosseous dental implant partially covered with a powder-sintered porous coating: 3 to 4 years results. *Int J Oral Maxillofac Impl 11:87-95*.

Ferber , D. (2001). Gene therapy. Safer and virus-free? . *Science, 294, 1638*.

Fillion P, D. A., Sayasith K, Lagace J, (2001). Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. *Biochim Biophys Acta 1515(1):44-54*.

Franke Stenport, V., Johansson, C. B., Sawase, T., Yamasaki, Y., & Oida, S. (2003). FGF-4 and titanium implants: a pilot study in rabbit bone. *Clin Oral Implants Res, 14*(3), 363-368.

Gorman, C. M., Moffat, L.F . and Howard, B.H (1982). *Mol. Cell. Biol, 2, 1044*.

Graham FL, v. d. E. A. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology 52(2):456-67*.

Groskreutz, D. a. S., E.T (1997). Methods in Molecular Biology. *Humana Press, NJ, 63, 11 ed. R. Tuan*.

Hench LL, C. A. (1982). Adhesion to bone I Williams DF. *editor: Biocompatibility of orthopaedic implants, , vol 2 Boca Raton, Fla , CRC Press*.

Hermann H (1988). Plasma spray deposition processes. *MRS Bull 60-67*.

Hickman MA, M. R., Lehmann-Bruinsma K, Sih TR, Knoell D, Szoka FC, et al, (1994). Gene expression following direct injection of DNA into liver. *Hum Gene Ther, 5(12):1477-83*.

Huard J, L. Y., Peng H, Fu FH, (2003). Gene therapy and tissue engineering for sports medicine. *J Gene Med, 5(2):93-108*.

Iezzi G, O. S., Pecora G, Piattelli A (2009). Histologic and histomorphometric evaluation of the bone response around a hydroxyapatite-coated implant retrieved after 15 years *Int J Periodontics Restorative Dent, Feb;29(1):99-105*.

Jiao S, C. L., Wolff JA, Yang NS, (1993). Particle bombardmentmediated gene transfer and expression in rat brain tissues. *Biotechnology (NY), 11(4):497-502*.

Johansson, C. A., T, (1987). Integration of screw implants in the rabbit. A 1 - Year follow - up of removal of titanium implants. *International Journal of Oral and Maxillofacial Implants, 2, 69-75*.

Jordan M, W. F. (2004). Transfection of adherent and suspended cells by calcium phosphate. *Methods 33(2):136-43*.

Kirsch A (1983). the two phase implementation method using IMZ intramobile Cylinder. *J oral Implanton, 11(2); 197-210, .*

Kunath K, v. H. A., Fischer D, Petersen H, Bickel U, Voigt K, et al, (2003). Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecularweight polyethylenimine. *J Control Release, 89(1):113-25*.

Lacefield, W. (1986). The coating of hydroxylapatite onto metallic and ceramic implants. *Proceedings Trans 12th Ann Mtg Soc Biomaterials, St Paul, Minn, June*.

Langer, R. V. J. *Tissue engineering. Science, 260, 920-6;*.

Lekholm U Zarb GA (1985). patient selection and preparation. *In Branemark P .I, Zarb GA, alberktsson T, editors: tissue- integrated prostheses,Chicago, quintessence,* p 201.

Li B, L. S., Tan Y, Stolz DB, Watkins SC, Block LH, et al, (2000). Lyophilization of cationic lipid-protamine-DNA (LPD) complexes. *J Pharm Sci, 89(3):355-64*.

Li Y, O. M., Pelisek J, Roedl W, (2004). Stability and release characteristics of poly (D, L-lactide-co-glycolide) encapsulated CaPi- DNA coprecipitation. *Int J Pharm 269(1):61-70*.

Lieerman, J. R., Daluiski, A. and Einhorn, T.A. (2002). The role of growth factors in the repair of bone. Biology and clinical applications. *J Bone and Miner. Res. , 17, 997-978*.

Liu, Y., Enggist, L., Kuffer, A. F., Buser, D., & Hunziker, E. B. (2007). The influence of BMP-2 and its mode of delivery on the osteoconductivity of implant surfaces during the early phase of osseointegration. *Biomaterials, 28*(16), 2677-2686.

Lohmann, C. H., Tandy, E.M., Sylvia, V. L., Hell-Vocke, A. K., Cochran, D. L., Dean, D. D., Boyan, B. D. and Schwartz, Z. (2002). Response of normal female human osteoblasts (NHOst) to 17beta-estradiol is modulated by implant surface morphology. *J. Biomed. Mater. Res., 62, 204-213*.

Marinucci L, B. S., Becchetti E, Belcastro S, Guerra M, Calvitti M, Lilli C, Calvi EM, Locci P (2007). Effect of titanium surface roughness on human osteoblast proliferation and gene expression in vitro. *Int J Oral Maxillofac Implants, Jan-Feb;22(1):19*.

Marks, S. C. a. O., P.R., Bilezikian, J P., Raisz, L. G. and Rodan, G. A., Eds., (2002). Structure and development of the skeleton, In Principiles of bone Biology. *Academic Press, New York*, pp. 3-15.

Maurer N, M. A., Palmer L, Monck MA, Mok KW, Mui B, et al, (1999). Lipid-based systems for the intracellular delivery of genetic drugs. *Mol Membr Biol 16(1):129-40*.

McKee MD, N. A. (1993). Ultrastructural, cytochemical and immunocytochemical studies on bone and its interfaces. *Cells and Materials 3:219-243*.

McKenzie DL, K. K., Rice KG (2000). A potent new class of reductively activated peptide gene delivery agents. *J Biol Chem, 275(14):9970-7*.

Melton, D. e. a. (1984). *Nucl. Acids. Res, 12, 7035*.

Muddana HS, M. T., Adair JH, Butler PJ (2009). Photophysics of Cy3-Encapsulated Calcium Phosphate Nanoparticles. *Nano Lett, Apr;9(4):1559-66*.

Nikon, I. I. NIS-Elements.

Olton D, L. J., Wilson ME, Rogers T, Close J, Huang L, Kumta PN, Sfeir C (2007a). Nanostructured calcium phosphates (NanoCaPs) for non-viral gene delivery: influence of the synthesis parameters on transfection efficiency. *Biomaterials Feb ,28(6):1267-79*.

Olton D, L. J., Wilson ME, Rogers T, Close J, Huang L, Kumta PN, Sfeir C (2007b). Nanostructured calcium phosphates (NanoCaPs) for non-viral gene delivery: influence of the synthesis parameters on transfection efficiency. *Biomaterials, Feb ,28(6):1267-79*.

Orrantia E, C. P. (1990). Intracellular distribution of DNA internalized through calcium phosphate precipitation. *Exp Cell Res, 190(2)170-4*.

Oster CG, K. N., Grode L, Barbu-Tudoran L, Schaper AK, Kaufmann SH, et al, (2005). Cationic microparticles consisting of poly(lactide- co-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination. *J Control Release, 104(2):359-77*.

Park JW, J. J., Lee CS, Hanawa T (2009). Osteoconductivity of hydrophilic microstructured titanium implants with phosphate ion chemistry. *Acta Biomater, Jul;5(6):2311-21. Epub 2009 Feb 27*.

Patil SD, R. D., Burgess DJ, (2005). Biophysical characterization of anionic lipoplexes. *Biochim Biophys Acta 1711(1):1-11*.

Pilliar RM, D. D., Watson PA et al (1991). Dental implant design-effect on bone remodeling. *J Biomed Mater Res, 25:467-483*.

Roberts EW, T. P., Brezniak, N et al (1987). bone physiology and metabolism. *J calig Dent Assoc 15:54-61, 1987*.

Roberts WEL (1993). fundamental principles of bone physiology, metabolism and loading *In Naert I, van Steenberghe D, worhengton P editors: Osseointegration in oral rehabitation,, Carol Stream, IL., Quintessence.*, pp157-170.

Roy I, M. S., Maitra A, Mozumdar S (2003). Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. *Int J Pharm, 250(1):25-33*.

Samulski, R. J. e. a. (1991). Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J, 10,6*.

Schenk R, H. E., Brighton CT, Friedlander G, Lane JM, editors. Rosemont,IL: (1994). Histologic and ultrastructural features of fracture healing. In: Bone formation and repair. *American Academy of Orthopaedic Surgeons.*, pp. 117-146.

Schmitt, J. M., Hwang, K., Winn, S.R. and Hollinger, J.O. (1990). Bone morphogenetic proteins: an update on basic biology and clinical relevance. *J Orthop.Res, 17,269-278*.

Schroeder A, V. d. Z. E., Stich H et al, (1981). the reactions of bone, connective tissue and epithelium to endosteal implants with titanium sprayed surfaces. *J Maxillofac Surg, 9:15-25*.

Shigekawa, K. a. D., W.J (1988). *BioTechniques, 6, 742*.

Sikes ML, O. M. J. B., Finegold MJ, Ledley FD, (1994). In vivo gene transfer into rabbit thyroid follicular cells by direct DNA injection. *Hum Gene Ther, 5(7):837-44*.

Somia N, V. I. (2000). Gene therapy: trials and tribulations. *Nat Rev Genet, 1, 91*.

Somiari S, G.-M. J., Drabick JJ, Gilbert RA, Heller R, Jaroszeski MJ, et al, (2000). Theory and in vivo application of electroporative gene delivery. *Mol Ther 2(3):178-87*.

Song, K., Du, J. M., Luo, R. H., & Cao, Y. G. (2008). [Effect of Bio-Oss loading with rAAV-BMP7 on regeneration of bone defects around dental implant]. *Hua Xi Kou Qiang Yi Xue Za Zhi, 26*(4), 421-424, 429.

Steinemann SG, P. S., Muller ME (1985). Titanium alloys as metallic biomaterials. In Lutjering G, Zwicker U, Bunk Weditors. *Proceeding of the 5th international Conference on titanium, . Dutsch gref MaterialKundle eV, Vol 2 1327-1334*.

Thomas KA, J. J., Cook SD et al, (1987). The effect of surface macrostructure and hydroxylapatite coating on the mechanical strengths and histologic profiles of titanium implant materials. *J Biomed Mater Res, 21:1395-1414*.

Thomas KA, K. J., Cook SD et al, (1987). Effect of surface microtexture and hydroxylapatite coating on the mechanical strengths and histologic profiles of titanium implant materials. *J Biomed Mater Res, 21 (12): 1395-1414*.

Trentin D, H. J., Hall H, (2005). Non-viral gene delivery for local and controlled DNA release. *J Control Release, 102(1):263-75*.

Vaheri, A. a. P., J.S (1965). *Virology 27, 434*.

Wilson SP, L. F., Wilson RE, Housley PR, (1995a). Optimization of calcium phosphate transfection for bovine chromaffin cells: relationship to calcium phosphate precipitate formation. *Anal Biochem, 226(2): 212-20*.

Wilson SP, L. F., Wilson RE, Housley PR, (1995b). Optimization of calcium phosphate transfection for bovine chromaffin cells: relationship to calcium phosphate precipitate formation. *Anal Biochem 226(2): 212-20*.

Wolf-Brandstetter, C., Lode, A., Hanke, T., Scharnweber, D., & Worch, H. (2006). Influence of modified extracellular matrices on TI6AL4V implants on binding and release of VEGF. *J Biomed Mater Res A, 79*(4), 882-894.

Wong, T. K. a. N., E (1982). Biochem. *Biophys. Res. Commun, 107, 584*.

Wozney JM, R. V. (1998). Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. *Clin Orthop Relat Res, 26*.

Wu D, R. P., Grande DA, (2003). Gene therapy and tissue engineering in repair of the musculoskeletal system. *J Cell Biochem, 88(3):467-81*.

Xiao, J., Zhu, Y., Liu, Y., Zeng, Y., & Xu, F. (2009). A composite coating of calcium alginate and gelatin particles on Ti6Al4V implant for the delivery of water soluble drug. *J Biomed Mater Res B Appl Biomater, 89B*(2), 543-550.

Yamanaka, E., Tjellstrom, A., Jacobsson, M. & Albrektsson, T, (1992). Long - term observations on removal torque of directly bone-anchored implants. *Transplants and Implants in Otology, II.Ingelheim: Kugle*.

Yang, X., Walboomers, X. F., van den Dolder, J., Yang, F., Bian, Z., Fan, M., et al. (2008). Non-viral bone morphogenetic protein 2 transfection of rat dental pulp stem cells using calcium phosphate nanoparticles as carriers. *Tissue Eng Part A, 14*(1), 71-81.

Yao, C., & Webster, T. J. (2009). Prolonged antibiotic delivery from anodized nanotubular titanium using a co-precipitation drug loading method. *J Biomed Mater Res B Appl Biomater*.

Young FA, S. M., Kresch CH, (1979). Porous titanium endosseous dental implants in Rhesus monkeys: microradiography and histological evaluation. *J Biomed Mater Res 13:843-856*