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In-vivo study of adhesion and bone growth around implanted laser groove/RGD-functionalized Ti-6Al-4V pins in rabbit femurs

J. Chen [a,*], R.A. Bly [a], M.M. Saad [b], M.A. AlKhodary [b], R.M. El-Backly [b], D.J. Cohen [a], N. Kattamis [a], M.M. Fatta [b], W.A. Moore [b], C.B. Arnold [a], M.K. Marei [b], W.O. Soboyejo [a]

[a] Princeton Institute of Science and Technology of Materials and Department of Mechanical and Aerospace Engineering, Princeton University, Princeton, NJ 08544, USA
[b] Tissue Engineering laboratories, Faculty of Dentistry, Alexandria University, Alexandria, Egypt

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ABSTRACT

Titanium surfaces were designed, produced, and evaluated for levels of osseointegration into the femurs of rabbits. A total of 36 Ti-6Al-4V pins (15 mm length, 1.64 mm diameter) were prepared into three experimental groups. These were designed to test the effects of osseointegration on laser grooved, RGD coated, and polished control surfaces, as well as combined effects. Circumferential laser grooves were introduced onto pin surfaces (40 μm spacing) using a UV laser (λ = 355 nm). The tripeptide sequence, Arginine-Glycine-Aspartic acid (RGD), was functionalized onto laser grooved surfaces. Of the prepared samples, surface morphology and chemistry were analyzed using scanning electron microscopy (SEM) and Immunoflourescence (IF) spectroscopy, respectively. The experimental pin surfaces were surgically implanted into rabbit femurs. The samples were then harvested and evaluated histologically. Sections of the sample were preserved in a methylmethacralate mold, sliced via a hard microtome, and polished systematically. In the case of the RGD coated and laser grooved surfaces, histological results showed accelerated bone growth into the implant, pull-out tests were also used to compare the adhesion between bone and the titanium pins with/without laser textures and/or RGD coatings.

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1. Introduction

The success of an endosseous implant in achieving adequate bone regeneration, stability, and optimal required function is greatly dependent on the nature of the biomaterial and its surface characteristics [1]. One of the most frequently used biocompatible materials is the Ti-6Al-4V [2]. This is due to its excellent combination of biocompatibility, mechanical properties and its inert reactions with tissue once implanted [2,3].

The ability of a biomaterial to support cellular adhesion and spreading is an essential factor reflecting its level of biocompatibility and subsequent osseointegration [1,2]. The key elements for tissue regeneration and integration in the peri-implant area are the recruitment of sufficient osteoprogenitor cells and their differentiation [4].

Surface properties of the implant have proven to play a major role in cellular response and the subsequent tissue healing at the implant interface [1]. This has stimulated prior studies on the influence of surface roughness on cell differentiation, extra cellular matrix synthesis and osseointegration [5]. Furthermore, there are several methods for modifying implant surfaces to promote cellular adhesion [6,7]. These include methods that result in random and uniform surface textures.

Alumina blasting is a commonly used process that creates randomized surface textures [2,3,7]. It generally increases the surface area. However, this method can also give rise to the development of random bone cell orientations that may contribute to scar tissue formation [8,9]. In addition, blasting techniques can lead to increased concentrations of cytotoxic elements at the implant surface.

Another promising technique for producing controlled surface topography is lasergrooving [3,8,10–12]. Laser grooved surfaces with groove widths of 20 μm have been shown to exhibit increased cell attachment and cell spreading in *in vitro* studies [3,10,12]. Furthermore, similar grooving technology has been shown to be effective in humans, and is currently in use clinically, fabricated by BioHorizons Corporation (Birmingham, AL) for dental screws [13].

Additionally, the surface of an implantable material may be modified so that the biology of the surface is better served to interact with the surrounding environment. Endogenous adhesive proteins found in blood and interstitial fluids can be adsorbed on implant surface and are becoming an essential clue in mediating cell behavior, and tissue regeneration around the implant [4,14,15].

Arginine-Glycine-Aspartic acid (RGD) is a tripeptide sequence and is one of the known cell adhesion motifs found in a variety of extracellular proteins, such as fibronectin as the α5β1 integrin, laminin and collagens [11,16]. These integrins act as anchor points for

* Corresponding author.
E-mail address: jianboc@gmail.com (J. Chen).

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which the actin cytoskeleton to bind. They also form structural components of the cell [17]. Several studies have shown the role of extracellular adhesive proteins in cell adhesion and migration. RGD has also been shown to play a particularly important role in the osseointegration behavior of osteoblasts [4].

The improved osseointegration observed on RGD-coated surfaces has stimulated recent efforts to attach RGD to oxide coated metals and their alloys such as Ti and Ti-6Al-4V. This involves the attachment of the RGD sequence to alkyl phosphonate and cystiene linker molecules that are physiologically stable [18]. An *in vitro* study has shown increased cellular adhesion [19], while *in vivo* studies have shown increased bone formation in animal models [20–22].

Although RGD functionalized surfaces and laser microgrooved surfaces have been shown independently to exhibit increased and accelerated cellular attachment, prior studies have not explored the combined effects of RGD-coating and laser-grooving on osseointegration. This study examines the combined effects of RGD coating and laser-grooving (of Ti-6Al-4V) on *in vivo* bone formation and osseointegration.

2. Materials and methods

The Ti-6Al-4V material that was used in this study was obtained from Titanium Industries, Rockaway, NJ, in the form of 1.64 mm diameter and 18 mm long rods. To test the combined effects of RGD and Laser grooved surfaces, 36 pins were produced with the various surface modifications and were implanted into rabbit femurs. The following three types of titanium pins were produced for animal studies of wound healing and osseointegration: (i) 12 polished Ti-6Al-4V pins, (ii) 12 laser microgrooved Ti6-Al-4V pins, and (iii) 12 laser microgrooved and RGD-coated Ti6-Al-4V pins. The pins were sterilized with ethylene oxide prior to implantation.

2.1. Sample preparation — Pin production

2.1.1. Laser processing

Prior to laser grooving, the surfaces of the Ti-6Al-4V specimens were polished to a uniform roughness using 320 grit polishing paper. Grooves (25 μm wide, 40 μm spacing) were machined on to the surface of the pins with a frequency tripled Nd:YVO_4 laser operating at 355 nm (Spectra-Physics THP-40, Mountain View, CA). A spot size of 20 μm was obtained using a microscope objective. The pin was rotated at 2800 rpm (RPM) on an X–Y stage (Aerotech A3200, Pittsburgh, PA).

The laser was fired at 1000 Hz for 1.25 s (75 rotations of the pin) to produce one circumferential groove. The pin was then translated laterally in increments of 40 μm to produce multiple grooves. The resulting grooves were 25 ± 2 μm wide, with a spacing of 40 ± 1 μm. The average groove depth was 11 ± 2 μm, as determined by profilometry. A total of 375 grooves were placed in a 15-mm section of each pin (Fig. 1).

The laser-grooved pins were characterized in a scanning electron microscope (SEM). This revealed uniform machined grooves, as well as the nano-scale and sub-micro-scale roughness left behind by the laser ablation process (Fig. 2).

2.1.2. RGD functionalization

The laser grooved samples were functionalized with the RGD amino acid sequence via an alkyl phosphonate (AP) and cystiene tether. This was done using a technique described previously by Schwartz and co-workers [18]. To confirm the successful functionalization of the AP molecule onto the laser grooved surfaces, immunoflourescence (IF) was used. Alexafluor was tagged to the AP molecule, and when visualized under IF microscopy, a well adhered coating was observed on the laser grooved topography (Fig. 3). The concentration of RGD coated on the Ti-6Al-4V pins via this technique is on the order of 2 nmol/cm^2 [27].

2.2. Surgical implantations

A total of 6 adult New Zealand rabbits, weighing an average of 1.5 kg (±10%), were anaesthetized and draped with a sterile technique at Alexandria University. An incision was made, first through the skin and fascia, and then through the muscle, revealing the mid-portion of the femur (Fig. 4). The periosteum was cut with a single pass, and then reflected using a dissector. A 1.6-mm diameter drill bit (RPM 500) was used to create three holes transversely through the femur. The middle hole was placed at the midpoint of the femur, and the two others were positioned 1 cm distal and proximal from the center hole.

Three pins were inserted into the femur of each rabbit. The middle pin was the control surface (polished), while the distal pin was the laser grooved + RGD surface, and the proximal pin was the laser

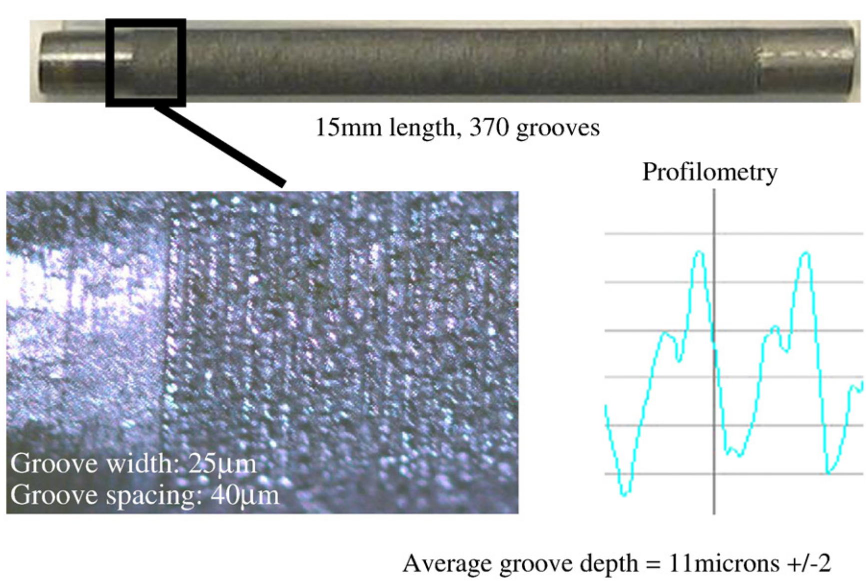

Fig. 1. Laser grooved pin profilometry.