

## Bioinspired Approach for Dental Implant Functionalization: An Experimental Study Evaluating the Effect of Hyaluronate as Bioactive Implant Coating

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**Abstract:** Limited osseointegration of dental implants in areas of poor quantity and quality of bone underscore the need for novel approaches that modulate host cell-implant responses to enhance osseointegration. Bioinspired strategies have emerged and included functionalizing implants with extracellular matrix proteins to augment the biological performance of dental implant. The purpose of this study was to investigate whether coating implant surface with hyaluronate will improve osseointegration compared to uncoated implant surface. Twelve mature New Zealand white rabbits weighing 2.5 - 3.5 kg were implanted with a hyaluronate -coated implant in one tibia and uncoated implant in the other one. Six animals were evaluated by scanning electron microscope for a period of 4 or 8 weeks. Scanning electron microscopy analysis demonstrated that the implants with hyaluronate coating had significantly the least percentage of gap distance at 8 weeks ( $P=0.0079$ ) compared with the uncoated implants. Biofunctionalization of the implant surface with hyaluronate significantly improve bone to implant contact and osseointegration.

[Ghada Bassiouny Ahmed. **Bioinspired Approach for Dental Implant Functionalization: An Experimental Study Evaluating the Effect of Hyaluronate as Bioactive Implant Coating.** *J Am Sci* 2013;9(11):187-192]. (ISSN: 1545-1003). <http://www.jofamericanscience.org>. 25

**Keywords:** Dental implant, osseointegration, surface modifications, biofunctionalization, extracellular matrix, hyaluronate.

### 1. Introduction

Pure titanium and titanium alloys are well established standard materials in dental implants because of their favorable combination of mechanical strength, chemical stability, and biocompatibility. Integration of titanium implants with the surrounding bone is critical for successful bone regeneration and healing. (1)

The first generation of successfully used clinical titanium implants, which were machined with a smooth surface texture, now approach 50 years in clinical use. Since then, implant surfaces have long been recognized to play an important role in molecular interactions, cellular response and osseointegration. (2) Scientists all over the world have developed the second generation implants with surfaces modifications to promote osseointegration, with faster and stronger bone formation. This will likely confer better stability during the healing process, which, preferentially, will improve the clinical performance in the area of poor bone quality and quantity. Furthermore, such promotion may, in turn, accelerate the bone healing and thereby allowing immediate or early loading protocols. (3) The second generation of implants underwent various surface modifications from mechanical blasting coupled or not, with acid etch, (3) anodized, and laser modified (4) to more recently, biofunctionalized surfaces. (5)

The concept of functionalizing the implant surfaces with native or synthetic molecules based on extracellular matrix ECM peptides, proteins and growth factors emerged from the hypothesis that the ability of imitating the environment of bone, which is composed of an organic and inorganic phase, could enhance the implant surface performance, and encouraging the initial biological response. (5)

In bone, the ECM consists mainly of an organic phase known as osteoid, which constitutes approximately 20% of bone mass, and a mineral phase which is composed of hydroxyapatite, and calcium phosphate compound. The organic fraction of bone consists of over 90% type I collagen, other minor collagens such as types III and V, and 5% non collagenous proteins. Those proteins include osteocalcin, osteonectin, osteopontin, adhesion proteins such as fibronectin and vitronectin and proteoglycans such as versican, decorin and hyaluronan. The bone matrix also sequesters growth factors, acting as a reservoir for soluble inductive signals such as bone morphogenic protein (BMP). (6)

Hyaluronate is a widely distributed polysaccharide component of the extracellular matrix of connective tissues and bone. (7,8) It has been reported to play an important role in tissue repair and regeneration. (9,10) Hyaluronate structure consists of polyanionic disaccharide units of glucouronic acid and N-acetylglucosamine connected by alternating  $\beta$  1-3 and  $\beta$  1-4

bonds. (11) There is no antigenic specificity for species or tissues; and thus, these agents have a low potential for allergic or immunogenic reaction. (12). Hyaluronate plays a vital role in the functioning of extracellular matrices, including those of mineralized and non-mineralized tissues. It has been reported that Hyaluronic acid accelerates the regeneration by means of chemotaxis, proliferation and successive differentiation of mesenchymal cells. (13) It shares bone induction characteristics with osteogenic substances such as bone morphogenetic protein-2 and osteopontin. (14) The application of exogenous hyaluronate showed good results in manipulating and accelerating the wound healing process in a large number of medical disciplines. (15,16)

The scientific rationale underlying the development of the current approach stems from the need for an osteoinductive biomaterial with improved biologic properties, facilitating cell migration and attachment to be used as bioactive coating for dental implant for accelerated wound healing and osseointegration. In light of the roles described to hyaluronate during embryonic development and tissue repair, (17) this polysaccharide, may be the promising candidate molecule for this purpose.

The purpose of this study was to investigate whether biofunctionalized implant surface containing hyaluronate influences the bone-implant contact and osseointegration around implants compared to standard uncoated implant surface.

## 2. Materials and Methods

A total of twelve mature New Zealand white rabbits weighing 2.5 - 3.5 kg were used in this study, each rabbit was implanted with a hyaluronate-coated implant in one tibia (test) and uncoated implant in the other **tibia (control)**.

### **Implant material:**

Twenty four 8×4.2 mm: length × diameter Sand-blasted, acid-etched dental implants were used in this study (Dentium, Seoul, Korea)

### **Implants Coating:**

Twelve implants were incubated for two hours in 300 µl of hyaluronate solution (Hyadent, BioScience GmbH, Germany). The treated implants were removed from the coating solutions and allowed to dry under sterile conditions for 12 hours at room temperature. Thereafter, the coated implants were ready for implantation. (18,19)

### **Anaesthetic protocol:**

Under aseptic conditions the surgical procedure was carried out under general anaesthesia produced by an intramuscular injection of Xylazine (Chanazine, Chanelle Pharmaceutical, Ireland) 5mg/kg body weight and ketamine Hydrochloride (Ketamine, Pharmazeutische Präparate, Germany).

30 mg/kg body weight. Local anesthesia with 1ml of 5% Xylocaine (Astra, Sweden) was administered to the tibial metaphysis where the implants were to be inserted.

### **Surgical protocol:**

Once general anaesthesia was established, the medial aspects in the region of the proximal tibia were shaved; the skin was carefully swabbed with mixture of iodine and 70% ethanol. A 30 mm incision was made along the medial aspect of the proximal tibia and the wound advanced down to and through the periosteum. A subperiosteal dissection was then advanced up to the inferior attachment of the knee joint capsule and laterally to the full extent of the flat medial bone surface.

Under continuous irrigation with sterile saline, the twenty four implants were installed in tibiae bone according to the manufacturer's instructions. The prophylactic administration of procaine penicillin (Wyeth Pharmaceuticals, Parramatta, New South Wales.) 60 000 units/kg intramuscularly was commenced during the surgery and continued for three postoperative days to reduce the potential for wound infection.

### **Animal sacrifice:**

To assess bone attachment to the implant surfaces, Six rabbits were sacrificed at 4 and 8 weeks using an intramuscular injection of 60 mg/ml/kg body weight sodium phenobarbitone (Phenobarbitone, Fawns & McAllan Pty Ltd, Melbourne, Victoria).

### **Electron microscopic analysis:**

Block sections of the tibial bone, containing the implants were obtained using a stryker bone saw (Stryker; Kalamazoo, Mich, United States of America). The samples were immersed into 10% buffered formic for 48 hours for decalcification. These specimens were dehydrated in ascending ethyl alcohol concentration 70%, 80% and 90% for 6 hours each and 100% for 10 hours. Then, to displace the alcohol the specimen were immersed in acetone for 12 hours. These specimen were embedded in polymethylmethacrylate resin under vacuum and after polymerization for 24 hours, sections were cut at 150µm by a diamond wafering blade. The specimens were coated with layer of gold with the aid of magnetron-sputtering device. Analysis was performed using scanning electron microscopy (SEM, JXA-840A, JEOL, Japan). The mean gap distance (µm) between the bone and implant in areas among the five threads was calculated.

### **Statistical analysis:**

The data obtained from computer image analysis were presented as mean and standard deviation (SD), tabulated and statistically analyzed. Student's t-test was used for comparisons between the two different observation periods and for

comparison between each group. P value  $\leq 0.05$  was considered statistically significant.

**3. Results**

No difficulties were experienced during the use of the tested implants. All animals survived the surgical procedure and each of the postoperative examinations. During the healing period, no signs of inflammation or negative side effects with regard to the local tissue compatibility were detected.

**Scanning electron microscope examination**

**I-Comparison between control and experimental groups**

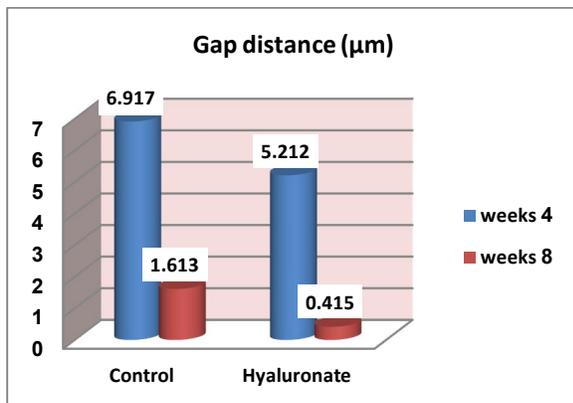
Measurement of gap distance by the aid of the scanning electron microscope four weeks postoperatively revealed greater measurement in the control group ( $6.917 \pm 3.268$ ), while the least value was recorded in the hyaluronate group ( $5.212 \pm 0.802$ ). The same pattern was observed eight weeks post-operatively, where the gap distance was greater in the control group ( $1.613 \pm 1.195$ ) compared to hyaluronate group that revealed the least value ( $0.415 \pm 0.422$ ).

Scanning electron microscopic measurements revealed that the gap distance was greater in the control group compared to hyaluronate group at both observation periods. Student's t test revealed that the difference between these two groups was not statistically significant at 4weeks ( $p=0.1265$ ), but was very statistically significant at 8 weeks ( $p= 0.0079$ ), (Table 1, Fig.1).

Table (1) Mean ( $\pm$ standard deviation) of gap distance ( $\mu$ m) of control and Hyaluronate group and statistical significance of the difference (Student's t test)

	Control	Hyaluronate	tvalue	p value
4 weeks	$6.917 \pm 3.268$	$5.212 \pm 0.802$	1.6023	0.1265
8 weeks	$1.613 \pm 1.195$	$0.415 \pm 0.422$	2.9893	0.0079**

\*\* very statistically significant



**Fig. (1) Mean of gap distance ( $\mu$ m) in control and hyaluronate group**

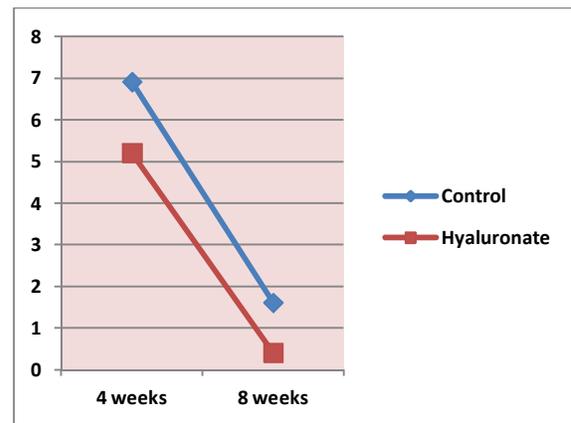
**II- Change by time in gap distance in control and experimental groups**

Both groups exhibited a decrease in gap distance throughout time. Student's t test revealed that the difference in gap distance throughout the experiment (four and eight weeks post-operatively) was extremely statistically significant ( $p < 0.0001$ ) in the control and experimental groups (Table 2, Fig.2-6).

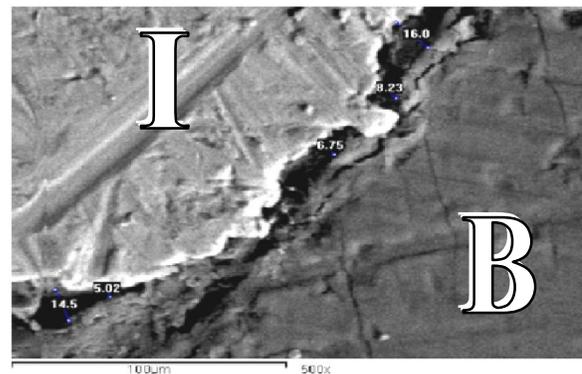
**Table (2) Change by time in gap distance of each group and statistical significance of the difference (Paired Student's t test)**

Groups Values	Control		Hyaluronate	
	4 weeks	8 weeks	4weeks	8 weeks
Mean $\pm$ SD	$6.917 \pm 3.268$	$1.613 \pm 1.195$	$5.212 \pm 0.802$	$0.415 \pm 0.422$
t value	4.8203		16.7387	
P value	$< 0.0001^{***}$		$< 0.0001^{***}$	

\*\*\*extremely statistically significant



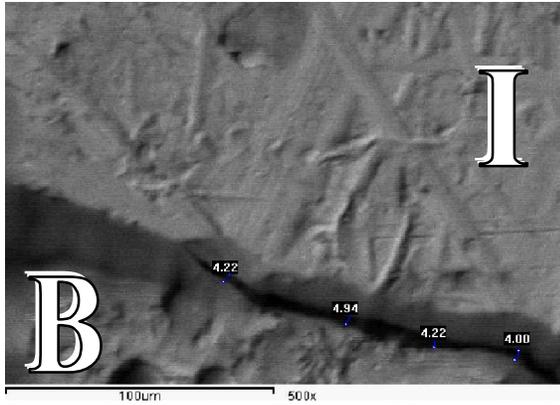
**Fig. (2) Change by time in gap distance in control and experimental groups**



**Figure 3. Scanning Electron Micrograph for control group at the end of 4 weeks showing gap distance of bone along the implant surface (SEM X 500).**

B: Bone

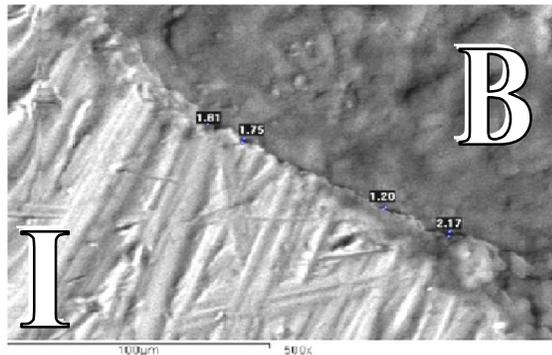
I : Implant



**Figure 4. Scanning Electron Micrograph for hyaluronate group at the end of 4 weeks showing gap distance of bone along the implant surface (SEM X 500).**

**B: Bone**

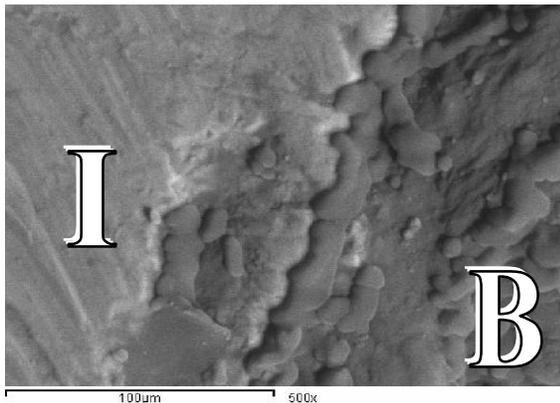
**I : Implant**



**Figure 5. Scanning Electron Micrograph for control group at the end of 8 weeks showing gap distance of bone along the implant surface (SEM X 500).**

**B: Bone**

**I : Implant**



**Figure 6. Scanning Electron Micrograph for hyaluronate group at the end of 8 weeks showing gap distance of bone along the implant surface (SEM X 500).**

**B: Bone**

**I : Implant**

#### 4. Discussion

Implant osseointegration is a prerequisite for clinical success in orthopaedic and dental applications, many of which are restricted by loosening. Current orthopedic implant surface technologies, including porous coatings and calcium phosphate overcoats, seek to promote bone cell ingrowth and mineral formation.(20,21) Although these approaches are successful in many cases, they can be restricted by slow rates of osseointegration and poor mechanical anchorage, especially in challenging clinical cases, such as those associated with large bone loss and poor bone quality. In addition, these Surface modification approaches rely on costly and manufacturing-intensive processes. (22)

As an alternative to these surface technologies, emerging biomimetic strategies, they have focused on the presentation of biological motifs, including extracellular matrix sequences and growth factors.(23,24) The general paradigm of these bio-inspired approaches is the covalent immobilization of the biological entities onto the underlying material support, which often involves multi-step procedures to render the support suitable for biofunctionalization.(25,26) In contrast, a recently described simple, one-step coating procedure that relies on the passive adsorption of ECM biomaterial onto biomedical grade titanium to enhance osseointegration has been introduced. (19)

The present study was conducted to examine the ability of a biomimetic implant coating strategy to promote bone tissue healing and implant osseointegration. This coating relies on the physisorption of hyaluronate onto the surface of sand blasted acid etched implant as a simple, clinically-translatable strategy to functionalize dental implants.

In this study the implant used was sand blasted acid etched dental implant. It has been reported that the modification on the topographic pattern of surface increases not only the bone-implant contact, but also the biomechanical interaction of that interface at early implantation periods.(5) Furthermore this surface texture allows for ingrowth of bone into the implant surface, thus promoting osseointegration of the implant into the bone. (27,28)

Importantly, in this study the biomimetic surface approach utilized a simple, dip-coating of hyaluronate to pre-sterilized Ti implants, a quick and versatile surface application conducted under physiological conditions that the surgeon can employ seconds before implantation. This single-step procedure, in turn, minimizes the chance of infection, reduces implant surface treatment variability, and minimizes cytotoxicity concerns inherent with

covalent immobilization schemes, while maintaining the surgeon's dexterity. (18,19)

The animal model chosen to demonstrate this provides ideal conditions for the investigation of bone regeneration and implant osseointegration.(5) During the study, the use of standardized surgical procedures and randomized implant placement ensured the greatest possible comparability between the experimental groups.

No difficulties were experienced during the use of the tested implants. All animals survived the surgical procedure and each of the postoperative examinations. During the healing period, no signs of inflammation or negative side effects with regard to the local tissue compatibility were detected.

Concerning the gap distance; the Scanning electron microscopic measurements revealed that the gap distance was greater in the control group compared to hyaluronate group at both observation periods. Student's t test revealed that the difference between these two groups was not statistically significant at 4 weeks ( $p=0.1265$ ), but was very statistically significant at 8 weeks ( $p= 0.0079$ ), (Table 1, Fig. 1).

Based on our findings, hyaluronate coated implants have recorded the least values of gap distance as compared to uncoated implants at both observation periods which was very statistically significant at 8 weeks post operative. Thus biofunctionalization of the implant surface with hyaluronate significantly improve bone to implant contact and osseointegration.

Different mechanisms have been proposed to explain the effect of hyaluronate in accelerating wound healing, osteogenesis, and promoting bone to implant contact.

Firstly, hyaluronate is highly hydrophilic.(29) so by coating the implant with hyaluronate, titanium surface is protected and its surface free energy is maintained thus implant surface coated by this biomaterial will attract blood and bone growth factors more rapidly than a standard uncoated implant (30)

Secondly, It has been proposed that hyaluronate accelerate wound healing in the bone matrix due to stimulation of angiogenesis (31) Thus, hyaluronate coating may encourage new blood vessel growth around the implant, expediting the healing process.

Thirdly, hyaluronate has osteoinductive potential and can induce osteogenesis on the surface of coated implant, as evidenced in a laboratory experiment, that concluded that human osteoblast cells respond more readily to hyaluronate coated surfaces and suggests that, hyaluronate coated surface is better able to retain osteoblast cells compared to standard un coated surface.(30) Other studies have supported the osteogenic properties of hyaluronate

when tested in vitro with bone cells, both through the intramembranous and the endochondral paths of osteogenesis, with the assumption that this biomaterial provide differentiation of stem or progenitor cells before attaching to a surface. (17,32) Moreover it has been found that hyaluronate shares bone induction characteristics with osteogenic substances such as bone morphogenetic protein-2 and osteopontin.(14)

Finally, it has been reported that, hyaluronate plays an important anti-inflammatory role through modulation of inflammatory cells, interaction with the proteoglycans of the extracellular matrix and scavenging of free radicals.(15,16) Thus inhibits tissue destruction and facilitates healing. (13)

Recreating the biological function of ECM using hyaluronate coating may therefore be a powerful biomaterial strategy to enhance bone to implant contact and osseointegration owing to its hydrophilic, angiogenic, osteoinductive, and anti-inflammatory potentials.

## Conclusion

Biofunctionalized dental implant surface containing hyaluronate enhanced bone-to-implant contact and osseointegration around implants compared to standard uncoated implant surface.

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10/7/2013