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Original Paper

Type I Collagen Grafting on Titanium Surfaces Using Low-temperature Glow Discharge

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To improve the bioactivity of titanium surfaces, glow discharge was used to facilitate collagen grafting on titanium disks. Titanium test specimens were pre-treated by glow discharge fed with a mixture of argon and allylamine (AA) gases. Treated titanium disks were then grafted with type I collagen using glutaraldehyde (GA) as a crosslinking agent. The surfaces of collagen-grafted titanium disks were evaluated using scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS) and X-ray photoelectron spectroscopy (XPS). MG-63 osteoblast-like cells were cultured on the grafted titanium surfaces to examine the effect of collagen grafting in terms of cell morphology. Our results demonstrated that collagen component elements could be detected on the titanium surfaces. Morphology of the cells on the surfaces of collagen-grafted titanium disks indicated differentiation. These findings showed that type I collagen could be successfully grafted onto titanium surfaces using glow discharge technology, with enhanced biofunctionality demonstrated on osteoblastic cells.

Key words: Glow discharge, Titanium, Collagen

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INTRODUCTION

Titanium dental implants have been used for oral rehabilitation since 1965. According to Brånemark *et al.*, osseointegration is defined as "direct contact between an artificial implant and the living bone"[1,2]. However, if based on this definition, the level of osseointegration between implant and surrounding bone has remained unsatisfactorily lower than 70%. This is because at a microscopic level, a thin intervening layer is always found between the implant and bone. This thin layer, which is a gligoamino-glucose substrate of *circa* 20–100 nm thickness, is an imperfect structure. Therefore, it is a barrier to successful completion of osseointegration and which may subsequently cause implantation failure.

In modern implant dentistry, osseointegration is defined as direct functional and structural connection between implant and bone, with no intervening layers of fibrous tissue. Accompanying osseointegration is satisfactory long-term clinical performance without rejection[1]. It is noteworthy that early interaction at the bone and implant interface allows early or immediate implant loading. On this note, various surface treatments have been investigated with a view to improving bone cell adhesion on and bone integration of titanium implants[3,4].

The gas plasma process can strip a material surface of chemical bonds and establish new ones. In general, electrons and ions gain energy by heating. Energy is then transferred by collisions between atoms, which increase excitation and cause decomposition and ionization. Therefore, for years, this technique has been used for surface cleaning and sterilization of titanium and titanium alloys[5,6]. Recently, low-temperature gas plasma discharge, which operates far below atmospheric pressure and room temperature, has been introduced for surface treatment[7,8]. Due to this low operative pressure and temperature, it is also termed as cold plasma or glow discharge plasma (GDP). The main advantage of GDP is that it provides a non-destructive reaction, hence avoiding subsequent reaction failure during surface chemical modification of biodegradable polymers[9].

Glow discharge plasma can be used to treat material surfaces by etching, deposition, copolymerization, and chemical modification[7-11]. GDP-treated surfaces can be used to immobilize biofunctional groups and increase surface wettability[10,11], thereby enhancing the biocompatibility of the target materials[12,13].

On improving bone remodeling on titanium surfaces, Yamamoto *et al.*[14] incubated GDP-treated titanium disks in a typical culture medium containing

extracellular matrix proteins (ECM). It was found that adsorption of ECM proteins to the titanium surface was achieved. In 2007, Alves *et al.* facilitated the adsorption of albumin and fibronectin on titanium plates using oxygen-based glow discharge plasma treatment[15]. Although these studies demonstrated that plasma modification clearly promoted the proliferation of osteoblastic cells, there was no direct evidence that the proteins chemically bound to the titanium surface. Further, non-specific bioadhesion is a problem that limits and hampers the clinical utilization of biomaterials[16].

It has been proposed to use amine groups to solve the problem of non-specific bioadhesion, as they serve as ideal attachment vehicles between biomaterials and cells[17]. To graft amine groups onto biomaterials, allylamine (AA) radio-frequency GDP was developed to produce thin films with amine groups on biopolymer surfaces[16,18]. Then, in 2007, Stine *et al.* first used glutaraldehyde (GA) as a bifunctional linker to covalently couple proteins on aminated silicon surfaces[19]. Although several techniques have been used to verify the existence of amine-specific molecules on material surfaces, biocompatibility checking using cell culture experiments has not been performed to date.

The aim of this study was to improve the surface activity of titanium. To this end, radio-frequency GDP was used to immobilize biofunctional amine groups on a titanium surface, and then type I collagen was grafted onto the amine group interfacing with GA linker. Biocompatibility of the surface-modified titanium disks was subsequently evaluated through observation of morphological changes in the cultured osteoblastic cells.

MATERIALS AND METHODS

Titanium disk cleaning

Grade II titanium disks (BioTech One Inc., Taipei, Taiwan) with a diameter of 10 mm were prepared. Before treatment with glow discharge plasma, the disks were cleaned as described previously[5]. Briefly, the titanium specimens were ultrasonically cleaned in acetone, detergent solution, and pure distilled water for 15 minutes separately, and then dried at 25℃ for 24 hours. Disk surfaces were subsequently cleaned using glow discharge (PJ, AST Products Inc., North Billerica, MA, USA) at 85 W and 13.56 MHz in argon gas at room temperature and at a low pressure (100 millitorr) for 15 minutes. At this stage, the titanium disks were defined as original specimens.

Glow discharge treatment and protein grafting

Adhesion of NH groups to the titanium surface was achieved by glow discharge fed with AA gas (Fig. 1a). The reaction pathways for allylamine degradation and recombination using glow discharge plasma are depicted in Fig. 1b. To deposit allylamine onto the titanium surface and create NH groups, the GDP reactor was set to expose the specimens to 85 W/13.56 MHz gas plasma at room temperature and at a low pressure (60−100 millitorr) for 30 minutes. Following which, glow discharge-treated titanium disks were immersed in GA solution (Merck, NJ, USA) for 30 minutes. To determine the optimal GA concentration, the specimens were divided into three groups treated with 1%, 2%, or 3% GA solution.

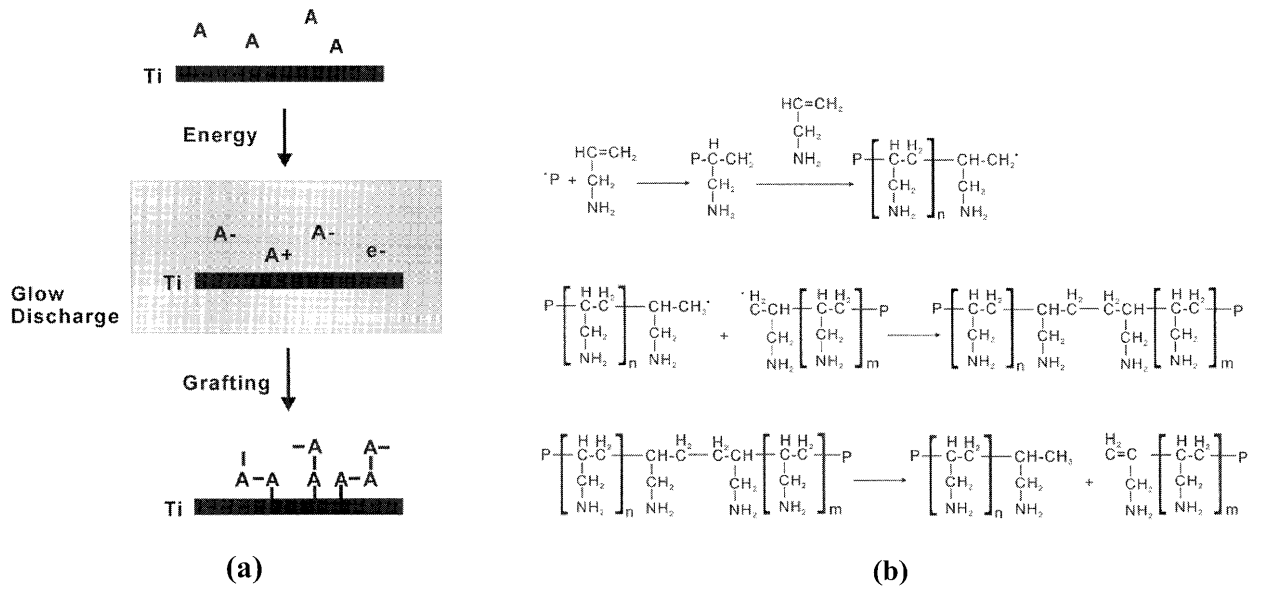


Fig. 1 (a) Schematic diagram of bonding mechanism between Ti and AA; (b) Reaction pathways for degradation and recombination of allylamine fed by glow discharge plasma (A denotes allylamine).

Treated titanium disks were subsequently immersed in 0.05% type I collagen solution (Sigma-Aldrich Co., St. Louis, MO, USA) for 30 minutes. Tris-phosphate buffer (pH 7.4) was used as a stop solution (30-minute immersion). In each experiment, six samples were prepared and tested.

Surface analysis

After crosslinking treatment, the samples were washed with PBS and coated with gold ions in a sputtering apparatus (IB-2, Hitachi Ltd., Tokyo, Japan). The disks were then observed using scanning electron microscopy (SEM) (S-2400, Hitachi Ltd., Tokyo, Japan), with the Ronatec system used for energy dispersive spectroscopy (EDS) microanalysis with a lifetime of 35−90 seconds as described previously[5]. In this study, the [N]/[Cl] ratio was used to determine the collagen content. Differences in [N]/[Cl] ratio between the three GA concentration groups were tested using one-way analysis of variance (ANOVA). Probability values of less than 0.05 were considered significant for all tests.

X-ray photoelectron spectroscopy (ESCA system, VG Scientific, West Sussex, UK) with a monochromated Al X-ray source at 1486.6 eV was used for element chemical analysis of the treated surfaces. Spectra were collected at an electron take-off-angle normal to the sample surfaces. High-resolution [C1s], [N1s], [C1s], and [Ti2p] spectra were obtained from the samples at different surface treatment stages.

MG-63 osteoblast-like cell culture

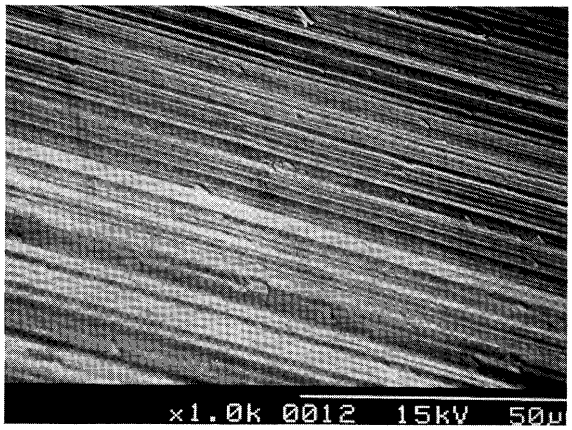
MG-63 (ATCC CRL 1427) is an osteoblast-like cell line established from a rat osteosarcoma. When grown as a monolayer, the cells possess osteoblast-like morphological features. In this study, MG-63 cells were seeded onto the surfaces of type I collagen-grafted titanium disks in 24-well petri dishes (Nunclon, Nunc, Roskilde, Denmark). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with L-glutamine (4 mmol/L), 10% fetal bovine serum (FBS), and 1% penicillin streptomycin. Cultures were incubated in a 5% CO_2 atmosphere at 37℃ and 100% humidity. For all experiments, the cells were first incubated for attachment for 24 hours, with this defined as time point 0 hour for all tests.

Cell morphology

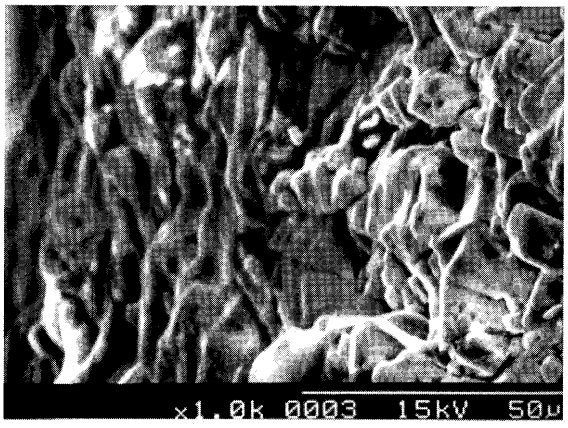
At 4 hours, the culture media were removed and the samples were rinsed three times with PBS. The samples were then fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 20 minutes, and then with 1% osmium tetroxide in 0.1 mol/L PBS for 30 minutes. After initial fixation, the samples were rinsed and postfixed in 1% osmium tetroxide for one hour. The samples were then washed with PBS and dehydrated in an ethanol series at ascending concentrations of 70%, 80%, 90%, 95%, and 100% in a critical point dryer (HCP-2, Hitachi Ltd., Tokyo, Japan). A thin layer of palladium-gold was coated onto the samples using a sputtering apparatus (IB-2, Hitachi Ltd., Tokyo, Japan). The morphological features of the cells were examined using a scanning electron microscope (S-2400, Hitachi Ltd., Tokyo, Japan). For each group, six samples were prepared and with nine fields within each sample being examined.

RESULTS

Analysis of surface topography revealed that the texture of collagen-grafted disks had been altered. For the original disks, the surface showed planar



(a)



(b)

Fig. 2 (a) Control group (titanium disks not treated with allylamine-GDP) showed additional surface structure; (b) Surface of collagen-grafted titanium disks, treated with allylamine-glow discharge and GA collagen crosslinking showed an irregular folding structure on the surface (×1000).

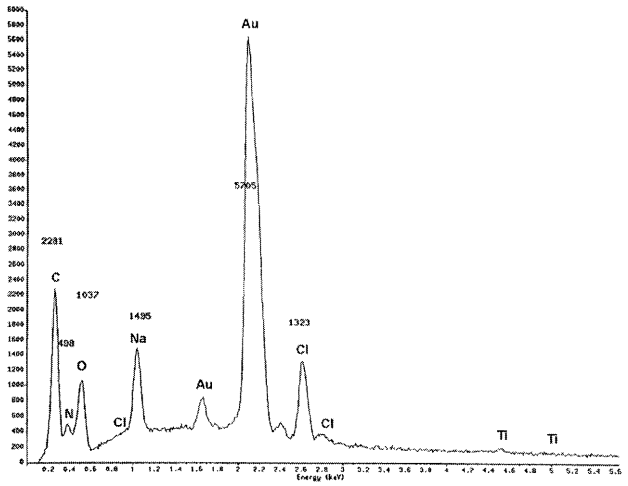


Fig. 3 Typical spectrum of SEM-EDS analysis demonstrating detection of C, N, O, and Cl elements on collagen-grafted titanium disks using 3% GA as the crosslinking agent.

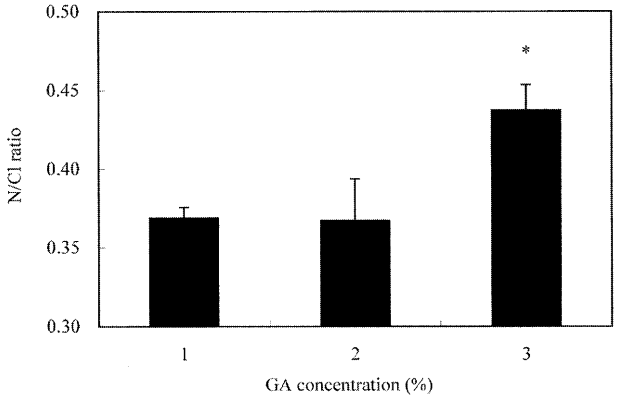


Fig. 4 Plots of [N]/[Cl] ratios for different GA concentration groups calculated from SEM-EDS results, showing relationship between content of type I collagen and concentration of crosslinking agent.

Table 1 Elemental composition of Ti samples at different treatment stages. AAGD, GA, and Col represent allylamine glow discharge, glutaraldehyde, and type I collagen respectively. Data are expressed as percentages

Stages	Conditions	%[Cls]	%[Nls]	%[Ols]	%[Ti2p]	N/C	O/C
1	original	57.07	2.25	32.99	4.68	0.039	0.578
2	AAGD	46.75	2.84	40.30	10.10	0.061	0.86
3	AAGD+GA	62.94	3.86	27.49	2.97	0.061	0.427
4	AAGD+GA+Col	58.37	9.94	20.34	2.13	0.170	0.348

morphology with regular surface lines (Fig. 2a). However, grafted disks in the treated groups showed an irregular folding structure (Fig. 2b).

To find the optimal GA concentration for collagen grafting, [N]/[Cl] ratios were calculated from the EDS spectra. A typical result of EDS analysis for the surface of GDP-treated titanium disk grafted with type I collagen (treated with 3% GA) is presented in Fig. 3. The spectrum revealed elements correlated with collagen components (e.g., carbon, oxygen, and nitrogen). However, no significant peaks corresponding to these elements were detected for the control groups (data not shown), indicating that the carbon, oxygen, and nitrogen elements detected in the treated groups might have originated from collagen. Plotted [N]/[Cl] ratios against the different GA concentration groups (Fig. 4) revealed that the grafted collagen volume increased significantly (from 0.368 ± 0.007 to 0.437 ± 0.016, $p<0.05$) when the concentration of crosslinking agent (GA) increased from 1% to 3%. Therefore, 3% GA was used in the subsequent experiments.

The atomic concentrations derived from XPS testing of the titanium specimens at different treatment stages are presented in Table 1. The mean [N1s] value from the sample surfaces at the original stage was 2.25%, with this value increasing to 9.94% after collagen grafting. A similar tendency was also seen for [N1s]/[C1s] value, which increased from a low of 0.039 at the original stage to 0.17 when the disks were collagen-grafted. The highest and lowest [Ti2p] levels were found on the specimen surfaces at treatment stages 2 and 4 respectively.

The recorded [C1s] surface spectra of the test specimens at different treatment stages are presented in Fig. 5a. In the figure, O=C−N peaks could be found on the titanium surfaces treated with GDP and grafted with type I collagen, irrespective of GA addition. However, the major spectral peak for the sample surfaces without AA glow discharge was O−C=O. High-resolution XPS spectra of the [N1s] peaks showed that AA glow discharge samples exhibited C−N structure on the titanium surface (Fig. 5b). In contrast, no N-related peaks could be identified in the samples without GDP treatment.

In comparison to the group treated using glow

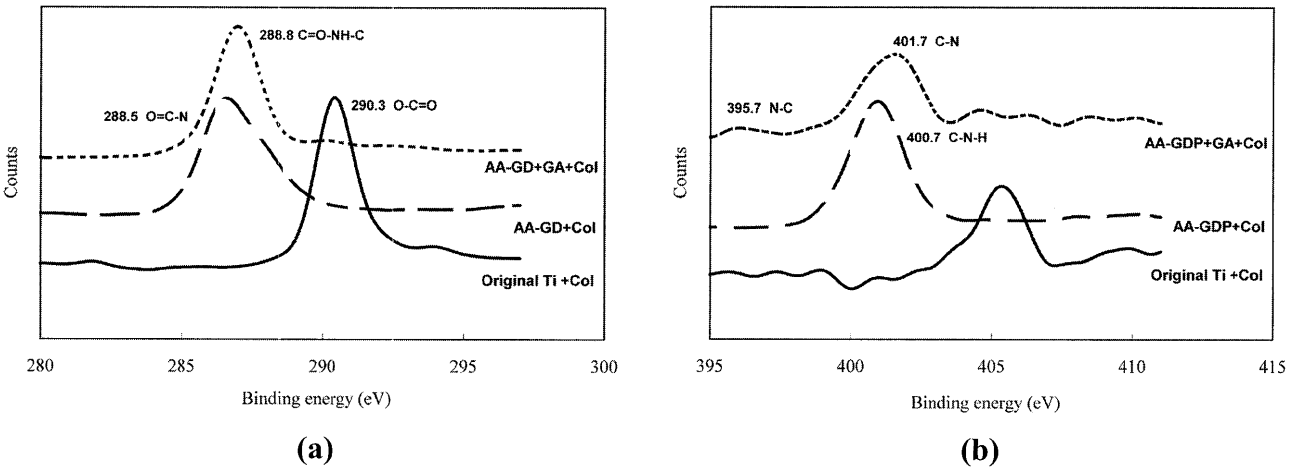


Fig. 5 XPS peaks of [C1s] (a) and [N1s] (b) for titanium surfaces at different treatment stages.

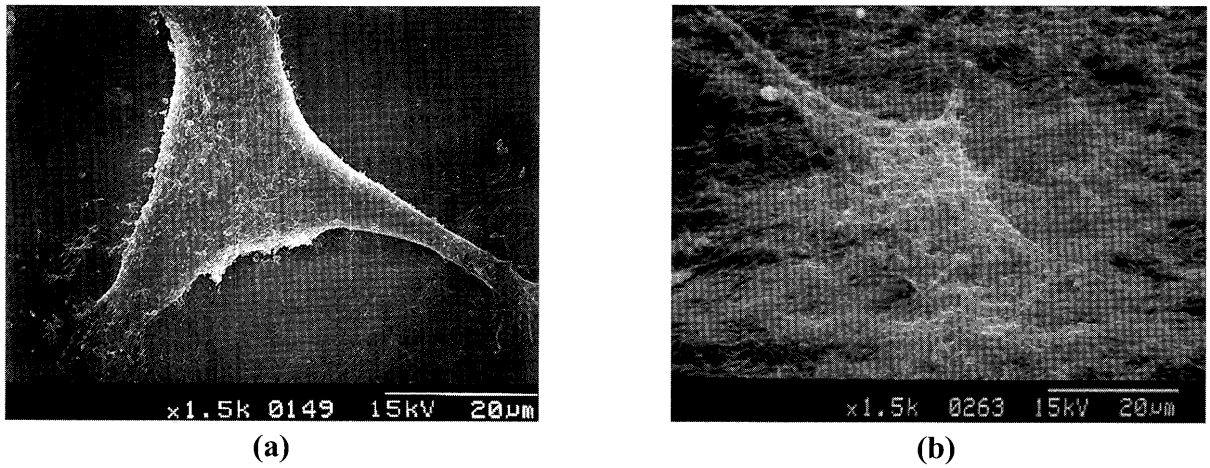


Fig. 6 Scanning electron microscopy images of MG-63 cells cultured on (a) original and (b) type I collagen-grafted titanium surfaces. In the original group, morphology was characterized by a relatively thin monolayer (a). After seeding on collagen-grafted titanium, the cells formed a more shape covered with large quantities of fiber (b) (original magnification ×1500).

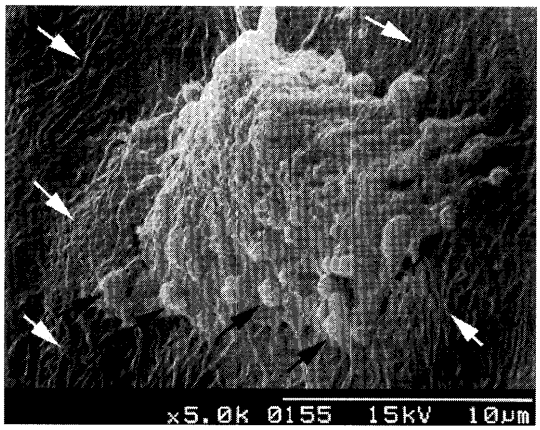


Fig. 7 SEM at higher magnification showing the fiber (white arrows) and matrix vesicles (black arrows) released by MG-63 cells cultured on type I collage-grafted titanium surfaces at four hours after attachment (original magnification ×5000).

discharge only, the collagen-grafted titanium disks revealed greater attachment of MG-63 cells and activation in the early stage of culture. At 4 hours, cells grown on the surfaces of original specimens were well distributed, appearing to form a relatively thin monolayer (Fig. 6a). As a result of cell growth on specimens with type I collagen grafting, the MG-63 cells underwent morphological alteration from polygonal to a more stellar shape. In addition, these MG-63 cells were covered with a large quantity of extracellular matrix (Fig. 6b). Simultaneous release of clusters of matrix vesicles from the plasma membrane were observed around the MG-63 cells (Fig. 7). These released vesicles and accumulated extracellular fibers become the interface between the cells and the surrounding substrate.

DISCUSSION

Glow discharge or cold plasma provides a stable, low energy source of ions, which possess sufficient energy to rupture molecular bonds at ambient temperature. The distinguishing characteristic of glow discharge is that the chemical reactions induced by partially ionized gas and/or gases are limited only to the surface[7], with significantly reduced penetration depth (a few hundred angstroms) compared to high-energy radiation. This characteristic of glow discharge makes it applicable for surface modification.

In osteoblastic differentiation, type I collagen (15−20% of total cell protein) is required for extracellular matrix mineralization[20]. This collagen type possesses unique physiochemical, mechanical, and biological properties which can be utilized in medical applications. It can enhance the healing process in extraction sockets and alveolar bone defects[21]. In light of these beneficial effects of type I collagen, it has inevitably become a primary focus in the investigation of implant materials.

In this study, allylamine gas was used to modify titanium surfaces to allow collagen bonding. The amine group was used for this purpose because it exhibits a positive charge which may attract negatively charged biomolecules[17]. A significant increase in [N]/[Cl] ratio was determined for AA glow discharge specimens grafted with type I collagen crosslinked with 3% GA (Fig. 4). As [N] could be detected only from allylamine and collagen, and since the detected [Cl] was due to the buffer solution, the [N]/[Cl] ratio was used to determine collagen content. SEM-EDS data revealed that GA was indeed an effective crosslinking agent for type I collagen.

No C−N-related peak was revealed for original titanium disks without AA-GDP pretreatment — despite being immersed in collagen solution, thereby demonstrating a lack of collagen grafting capability (Fig. 5). As the treatment process continued, the [N1s] and [N]/[C] ratios increased (Table 1). These results were similar to the findings of Feng *et al.*, who also used XPS to demonstrate that the [N1s] peak from adsorbed collagen corresponded to NH^+ protein group[22].

It has been suggested that osteoblast differentiation is promoted in glow discharge-treated titanium plates[13]. Shibata *et al.* also demonstrated that stress fiber formation of osteoblast-like cells on titanium plates was enhanced by GDP pretreatment. Further, it was found that ECM protein released by osteoblastic cells on the titanium surface increased, even after just one hour of incubation[12]. Our SEM images also showed that MG-63 cells released matrix vesicles (Fig. 7) and accumulated extracellular fibers on collagen-grafted titanium disks at four hours after attachment (Fig. 6b). These released matrix vesicles can promote mineral deposition, and it has been suggested that extracellular fibers are the primary mineralization sites[23-25]. Therefore, the cells progress into the matrix maturation stage. This result suggested that collagen-grafted surfaces were more bioactive.

Within the limitations of this study, our results indicated that allylamine glow discharge combined with collagen grafting treatment might enhance ECM protein expression by osteoblast-like cells, especially through glutaraldehyde crosslinking. Based on these results, and despite the lack of animal and clinical evidence for application of this technique in this study, it appeared reasonable to suggest that collagen grafting of titanium using glow discharge plasma could potentially reduce the duration of the osseointegration process and improve the stability of dental implants in the early stages.

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