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The influence of titanium surfaces treated by alkalis on macrophage and osteoblast-like cell adhesion and gene expression *in vitro*

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Achieving fast and long-term osseointegration is one of the major goals in dental implant design. Physic-chemical surface modification is a key to osseointegration improvement. Two types of alkaline modifications, 1 M sodium bicarbonate and 0.05 M sodium hydroxide, were applied on the sand-blasting and acid etching (SLA) titanium surface. After the alkaline treatments, superhydrophilic SLA surfaces were produced. The mRNA expression level of IL-1β of Raw264.7 cells cultured on the sodium bicarbonate treated SLA surface was inhibited compared with SLA and sodium hydroxide treated surfaces, while MMP-9 expression on the sodium hydroxide treated SLA surface was increased with comparison to SLA and sodium bicarbonate treated surfaces. Cell adhesion of osteoblast-like MG63 cells on surfaces with alkaline treatments were moderately more pronounced than that on the SLA surface. With comparison to SLA surface, the proliferation of MG63 cells was decreased, while the alkaline phosphatase (ALP) activity was enhanced on the sodium bicarbonate treated SLA surface after 3 days. An increased mRNA expression level of integrin α_v of MG63 cells were observed on the alkali treated surfaces compared with the SLA surface. Integrin β_1, osteocalcin (OCN) and bone morphogenetic protein-2 (BMP-2) were enhanced on the sodium bicarbonate treated SLA surface when compared to the SLA surface. The results imply that the inflammation-related gene expression of macrophage cells is changed on sodium hydroxide and sodium bicarbonate treated SLA surfaces; the early adhesion of osteoblast-like cells is enhanced by both alkaline treatments, while the osteogenic differentiation is improved on sodium bicarbonate treated surface.

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Introduction

Dental implants are surgically inserted into the bone, which is considered to be a direct structural and functional connection between ordered living bone and the surface of the load-carrying implant, referring to the osseointegration by Branemark.[1,2] Osseointegration also takes place due to the balance between the osteoblastic and osteoclastic activity, representing the process of adaptation and formation for repair and function.[3,4] Historically, the mechanical properties and function of the implant are the main concerns when the reconstructive device is being designed. Recent studies focusing on the modification of the titanium surface by mimicking the characteristic of bone help to ensure a more robust and fast bone healing process. It is known that through modification, the surface parameters like roughness, chemistry and wettability would be affected, while consequently, cell and protein adhesion could be changed, especially during the early stage of the osseointegration.[5–7]

Bone tissue–implant interface's formation is a complex process involving many temporal and co-operated factors. Once the implant is placed into the bone, there is a water layer around the surface within 30 seconds to hours, extracellular matrix proteins are observed on the surface of the implant. Neutrophils and later macrophages are involved in clot and necrotic tissue formation and play an important role in the inflammatory response. Followed by undifferentiated mesenchymal cells adhesion, proliferation and differentiation, on the 5th day after implantation, the detective alkaline phosphatase indicates bone matrix formation and mineralization.[8,9] During the interaction of cells and interface, the wettability of the surface could influence the adhesion of the proteins and other macromolecules on the surface, the cluster formation of osteoblast and osteogenic gene expression *in vitro*, as well as the rate of osseointegration *in vivo*.[10]

Chemical modification of the classical sandblasted acid-etched surface improving the surface energy and wettability has been demonstrated to promote the osseointegraion *in vivo*[11] and to increase osteogenic gene expression, such as BMP-6, BMP-2, Runx-2 *etc.* *in vitro*.[12–14] These studies are based on

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sandblasted, acid-etched titanium surface, stored in a saline solution, which is against oxidization protected by N_2 to maintain hydrophilicity. The main outcome of this treatment is that this modified SLA samples have shown increased surface free energy and decreased initial water contact angles from 139° to ~0°.[15] These superhydrophilic titanium implants have shown to result in enlarged bone to implant contact area in the early healing phase.[16]

In previous studies, a rapid and convenient strategy to achieve superhydrophilic titanium surface is reported by application of 0.05 M sodium hydroxide solution at room temperature. Titanium surface chemical conditions were changed by reversible deprotonation and ion exchange processes which induced qualitative and quantitative differences in protein absorption.[17] Mustafa and colleagues investigated that the alkali-treatment could support the early adhesion of fibroblastic cells to titanium alloy surface.[18] Blood clots on the alkali treated surface are thicker and more assembled in an organized, layered architecture.[19] By this alkaline treatment, the potential effect on osseointegration improvement was demonstrated in preliminary studies.[17,20,21] Besides sodium hydroxide, other kinds of alkalis are also candidates for such modification. Sodium bicarbonate, a mild alkaline compound, is currently considered of high priority for food and drug application because of its low hazard potential. To the authors' knowledge, there is limited study on the alkaline treatment with this chemical modification.

Sufficient osteogenic cell attachment to the biomaterial is a necessary condition for further cell proliferation, differentiation, and the extracellular matrix (ECM) mineralization. Cell adhesion is a complex interaction between extracellular ligands and cellular receptors. Integrins are a large family of heterodimeric transmembrane receptors, formed by non-covalent association α and β subunits. There are 16 α subunits and 8 β subunits have been found. Whereas the subunits β_1 and α_v have affinity bond to extracellular matrix proteins like fibronectin.[22,23] Furthermore, the integrin-mediated pathway has been shown to influence cell adhesion and differentiation like osteogenic gene expression, while the adhesion gene expression is specific to different substance surface characteristics.[24–26]

Though the majority of cell culture researches of osseointegration have focused on osteoblastic cell lineage, the other types of cells were also observed to adhere to the surface in this process, like monocytes and macrophages. These cells play an important role in mediating host tissue response to implanted biomaterials; it is known to be influenced by the topography of the surface[4,27] and the chemical characters.[28] After adhesion, activated platelets direct the macrophages migration to the wound site. During this process, different surfaces could influence expression of the inflammatory factors, thus it will potentially influence the osteogenesis and osseointegration[27,29]

The objective of the present study is to investigate the effects of sodium hydroxide and sodium bicarbonate treated titanium surfaces on proliferation and the expression of pro-inflammatory cytokines of the macrophage cells, as well as the adhesion and differentiation of the osteoblast-like cells *in vitro*; ultimately, to understand the influence of alkaline modification of titanium surfaces on the macrophage and osteoblastic cells.

Materials and methods

Preparation and scanning electron microscopy (SEM) examing of titanium specimen

1 mm thick, 15 mm diameters commercially pure titanium discs were used in this experiment. The sand-blasting and acid etching (SLA) surface modification was obtained by blasting with the grits of 0.25–0.50 mm Al_2O_3 and acid etched followed by etching with hot HCl (10–16%)/H_2SO_4 (68–75%) at a temperature of 80–90 °C (Wego Jericom Biomaterials Co., Weihai, China). The alkaline treatment was performed by immersing the discs in 1 M sodium bicarbonate for 3 min at the room temperature and rinsing in distilled water for 3 times. The 0.05 M sodium hydroxide treatment was performed following a published research.[17] Specimens were immediately used after the mild alkaline treatment. Both alkali-treated and untreated SLA surface were observed by SEM (S-4800, Hitachi, Japan) at 15.0 kV.

Determination of water contact angle

A standard contact angle goniometer (OCA20, Dataphysics, Germany) with drop image software was used to measure contact angles on sodium bicarbonate treated SLA, sodium hydroxide treated SLA and SLA surface. A number of 4 independent measurements were made on each surface by using deionized water. Titanium discs were dried in vacuum drying chamber directly before the measurements.

Cell culture

RAW264.7, a murine leukaemic monocyte cell line (ATCC, USA) and MG63 osteoblast-like cells (Dr Shaoqing Liu, School of stomatology, Peking University) originally isolated from a human osteosarcoma were cultured in Dulbecco's Modified Eagle Minimum essential Medium (DMEM, Gibco) supplemented with 10% FBS and 1% penicillin penicillin/streptomycin (Gibco, USA) with an atmosphere of 5% CO_2 at 37 °C. The medium was changed at 48 h intervals.

Cell adhesion measurements

Titanium alloy discs were transferred into 24 well culture plates. MG63 cells were plated into the different surface groups at the same density of 6×10^3cells per ml. After 1, 3 and 24 h, cells were washed by phosphate buffered saline (PBS) for three times in order to remove non-adhesive cells. Cells were fixed in 4% paraformaldehyde for 5 min, followed by permeabilization by 0.1% Triton X-100/PBS solution for 5 min. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Cell Signalling Technology, USA). 3 randomly selected microscopic fields (× 10 objective) of each sample (Olympus, Tokyo, Japan) were used and the cell number was counted by Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, USA).

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Scanning electron microscopy (SEM) for adherent cells

MG63 cells were seeded onto Ti disks placed in 24-well plate at a density of 2×10^3 per ml and cultured for 1, 3, 6 and 24 h. The cells on the disks were fixed in 2% glutaraldenhyde for 30 minutes at room temperature. Then samples were serially dehydrated with an increasing ethanol gradient, air-dried and sputtered with gold before to image by SEM (S–3000N, Hitachi, Japan) at 15.0 kV.

Cell adhesion by confocal laser scanning

MG63 cells were seeded onto Ti disks at a density of 2×10^3 per ml for 3 h and 24 h incubation and were rinsed three times with PBS. Cells were fixed by 4% paraformaldehyde for 5 min, then permeabilized by 0.1% Triton X-100/PBS solution for 5 min. Thereafter, 200 μl, 1 μg ml^{-1} phalloidin-FTIC (Sigma, P5282, USA)/PBS solution was added to each sample and stained for 1 h at room temperature. Then disks were washed by PBS for three times. Nuclei were labeled with DAPI. Samples were examined at an excitation wavelength of 488 nm or under UV light by using confocal laser scanning microscopy (Carl Zeiss, Jena, Germany).

Cell proliferation by CCK-8

RAW264.7 cells were plated in the 24-well plate on different types of titanium surface at a density of 1×10^4 per ml for 1 day. MG63 cells were plated at a density of 1×10^4 per ml for 1, 3, 7 days. Cell proliferation was determined by Cell Counting Kit (CCK-8, Dojindo, Japan). The CCK-8 was added and then incubated at 37 °C until the media turned yellow. The optical density (OD) was recorded on an ELX-808 Absorbance Microplate Recorder (BioTek, Winooski, VT) at 450 nm.

The alkaline phosphate activity

MG63 cells were seeded onto the different Ti samples in 24-well culture plate at a density of 1×10^4 cells per well. After 3 days of culture, the alkaline phosphatase (ALP) activity was measured by the ALP kit (JianCheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Results were normalized to levels of total protein.

Quantitative real-time PCR (qrt-PCR)

Total mRNA was extracted from the cells plated on different titanium surfaces by Trizol reagent (Invitrogen, USA). The cDNA was synthesized by using the Reverse Transcription kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Qrt-PCR using the FastStart Universal SYBR Green Master (ROX; Roche, Indianapolis, IN, USA) was performed by applying an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Relative quantification was carried out by the ΔΔCt method. The sequences of gene primers used for qrt-PCR for RAW264.7 were listed in Table 1, while primers for MG63 cells were listed in Table 2.

Table 1 The primers for the quantitative real-time PCR for RAW264.7

Genes	Sequence (5′-3′)
GAPDH	5′-AACTTTGGCATTGTGGAAGG-3′
	5′-ACACATTGGGGGTAGGAACA-3′
TNF-α	5′-AGCCCCCAGTCTGTATCCTT-3′
	5′-CTCCCTTTGCAGAACTCAGG-3′
IL-1β	5′-GCCCATCCTCTGTGACTCAT-3′
	5′-AGGCCACAGGTATTTTGTCG-3′
MMP-9	5′-TCCAGTACCAAGACAAAG-3′
	5′-TTGCACTGCACGGTTGAA-3′

Table 2 The primers for the quantitative real-time PCR for MG63 cells

Genes	Sequence (5′-3′)
GAPDH	5′-GGCAAGTTCAACGGCACAGT-3′
	5′-GCCAGTAGACTCCACGACAT-3′
Col1a1	5′-CCAACGAGATCGAGCTCAGG-3′
	5′- GACTGTCTTGCCCCAAGTTCC-3′
RUNX2	5′-TCTTCCCAAAGCCAGAGCG-3′
	5′-TGCCATTCGAGGTGGTCG-3′
OCN	5′-CAGTAAGGTGGTGAATAGACTCCG-3′
	5′-GGTGCCATAGATGCGCTTG-3′
BMP-2	5′-TCAAGCCAAACACAAACAGC-3′
	5′-AGCCACAATCCAGTCATTCC-3′
Integrin α_v	5′-TTGTTTCAGGAGTTCCAAGA-3′
	5′-TGAAGAGAGGTGCTCCAATA-3′
Integrin β_1	5′-GCAGGCGTGGTTGCCGGAAT-3′
	5′-TTTTCACCCGTGTCCCACTTGGC-3′

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Data are subjected to an independent samples *t*-test or one-way ANOVA by using SPSS17.0 software for Microsoft Windows. For all analyses, differences were considered to be significant when $p < 0.05$.

Results

Surface characterization

The SEM micrographs (Fig. 1) showed that after being treated by 1 M sodium bicarbonate and 0.05 M sodium hydroxide, the surfaces showed no morphological differences between these groups (Fig. 1). As the complex microstructure of the SLA surface was produced by acid and sandblasting, the cavities have with an average diameter of 10–50 μm, as well as that of the micropits were approximately 1–2 μm.[15] SEM analysis of treated SLA and SLA surfaces shows no morphological differences after the sodium bicarbonate and sodium hydroxide treatments. We measured the water contact angle to analyze the change in wetting of the surface. SLA surface exhibited water contact angles of 128.45 ± 5.67° (Fig. 2a). The sodium bicarbonate and sodium hydroxide treatments greatly decreased the contact angle to near 0° (Fig. 2b and c) indicating that the alkali-treated SLA become surperhydrophilic surface.

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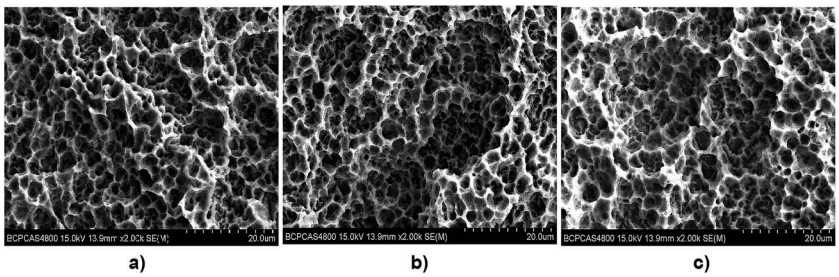

Fig. 1 SEM images of the SLA (a), sodium hydroxide treated SLA (b) and sodium bicarbonate treated SLA (c) surfaces. All the surfaces showed the similarity in morphology and no distinguishable differences on SEM imaging.

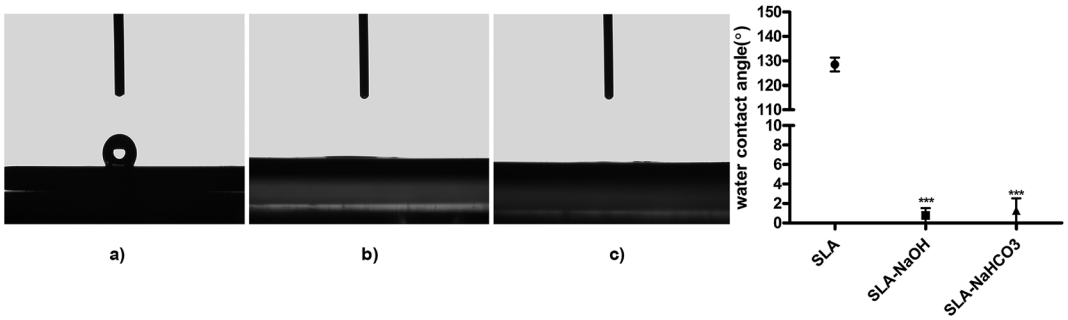

Fig. 2 Water contact angle measurements of SLA surface (a), sodium hydroxide (b) and sodium bicarbonate treated SLA (c) surfaces. After the alkaline treatments, both of the sodium hydroxide treated SLA (b) and sodium bicarbonate treated SLA (c) became superhydrophilic (~0°) (***p < 0.001 when compared with SLA, $n = 4$).

RAW264.7 cells proliferation by CCK-8

We compared RAW264.7 cells on different surfaces of sodium bicarbonate treated SLA, sodium hydroxide treated SLA, SLA and tissue culture polystyrene (TCP) at 24 h post-seeding by CCK-8 assay (Fig. 3). After 24 h cell culture, there was no significant difference in the level of cell proliferation on the titanium groups, while cell proliferation of the sodium bicarbonate treated SLA, sodium hydroxide SLA and SLA groups were lower than that of the TCP group ($p < 0.05$).

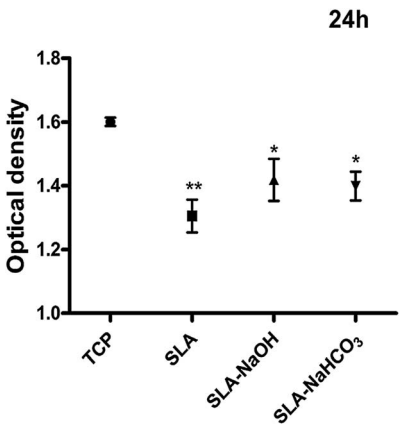

Fig. 3 CCK-8 assay showed the proliferation of RAW264.7 cultured on SLA, sodium hydroxide and sodium bicarbonate treated SLA surfaces were decreased than that on the TCP (tissue culture plate) after 24 hours incubation. There were no significant differences between the alkaline treated SLA surfaces and the SLA surface ($n = 3$, *$p < 0.05$, **$p < 0.01$ compared with the TCP group).

Pro-inflammation cytokine gene expression of RAW264.7 cells

Pro-inflammation cytokines as IL-1β, TNF-α and MMP9 are shown in Fig. 4 that the expression of IL-1β of sodium bicarbonate treated SLA was significantly down-regulated compared with sodium hydroxide treated SLA, SLA and TCP surfaces after 24 h culture ($p < 0.001$). The IL-1β expression of sodium hydroxide group and SLA group was down-regulated compared with the TCP group ($p < 0.001$). There was no difference between the sodium bicarbonate treated SLA and SLA surface of MMP9 and TNF-α expression ($p > 0.05$), while the TNF-α and MMP9 level of SLA group was inhibited compared with the TCP group ($p < 0.05$). Besides, the MMP9 expression level of Raw264.7 on the sodium hydroxide treated SLA was increased compared with the other three groups. TNF-α production by Raw264.7 cultured on the titanium groups were down-regulated compared with the TCP group ($p < 0.05$).

Cell adhesion and spread morphology of MG63 cells

The alkaline treatment enhanced the MG63 cells adhesion at the time of 1 h ($p < 0.05$). No significant changes between these two alkaline treatment groups were observed at 1, 3 and 24 h (Fig. 5). This result indicated that early cell adhesion on the SLA surfaces might be enhanced by these alkaline treatments. By SEM analysis, on both of the sodium bicarbonate and sodium

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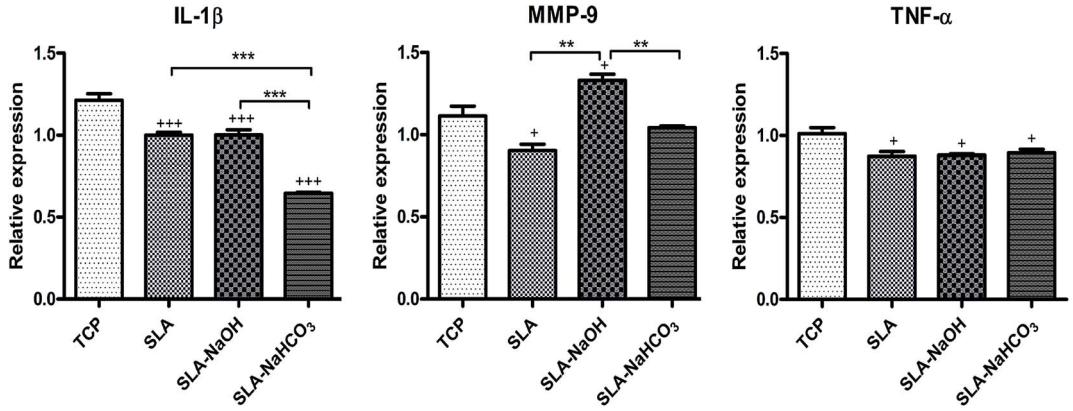

Fig. 4 The mRNA expression levels of IL-1β, MMP-9 and TNF-α expression in Raw264.7 cells after 24 hours culture on TCP, SLA, sodium hydroxide SLA and sodium bicarbonate treated SLA surfaces. GAPDH was used as a control for equal loading ($^{+}p < 0.05$, $^{+++}p < 0.001$ when compared with TCP, $^{**}p < 0.01$, $^{***}p < 0.001$).

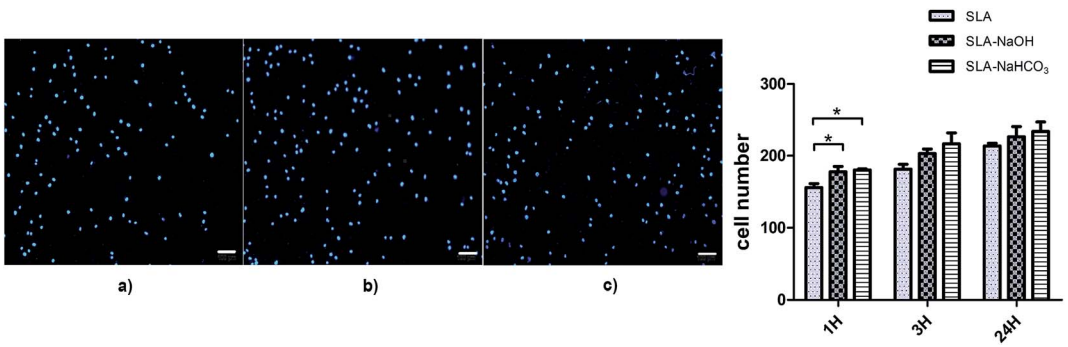

Fig. 5 Assessment of MG63 cells adhesion on different surfaces by DAPI staining after 1 hour, 3 hours and 24 hours incubation. Representative photos of cell adhesion on SLA (a), sodium hydroxide treated SLA (b) and sodium bicarbonate treated SLA (c) surfaces after 1 hour incubation. Cellular attachments of MG63 cells to sodium hydroxide treated SLA (b) and sodium bicarbonate treated SLA (c) surfaces after 1 hour incubation showed more pronounced compared with the untreated SLA surface ($n = 3$, $^{*}p < 0.05$). Scale bar = 100 μm.

hydroxide treated SLA surfaces, MG63 cells exhibited a flatter and more elongated morphology than that on the untreated SLA surface at the time point of 1, 3 and 6 h, no more morphological differences were observed at 24 h. Cells on the treated SLA surfaces extended more lamellipodia and filopodia after 3 h compared with the SLA group (Fig. 6). By the stain of cytoskeleton, the results were consisting with the SEM analysis, after 3 h incubation, the cells on the treated surfaces were flatter and bigger, with more lamellipodia and filopodia than that on the SLA surface. Longitude stress fibers in cells cultured on the sodium bicarbonate treated SLA surface seemed to be clearer than that on the other groups after 3 h incubation. There were no obvious differences between the groups after 24 h (Fig. 7).

MG63 cells proliferation by CCK-8

Fig. 8a showed the proliferation of MG63 cells on sodium bicarbonate treated SLA was statistically lower than that on the SLA surface ($p < 0.05$), and there was no significant difference between the sodium hydroxide treated SLA and SLA after 3 days seeding. While, at the 1 day, 3 days and 7 days, the proliferation of titanium groups was inhibited than that of the TCP group, and at 1 d and 7 d, there was no significant difference between the three titanium surfaces.

ALP activity of MG63 cells

As showed in Fig. 8b, the ALP activity of MG63 cells on the three titanium surfaces was statistically enhanced compared with that of the TCP group, especially the sodium bicarbonate treated SLA group ($p < 0.001$). Besides, the ALP activity of the sodium bicarbonate treated SLA was greatly enhanced compared with the SLA and the sodium hydroxide treated group ($p < 0.01$, $p < 0.05$) followed by 3 days culture, and no statistical changes were observed between the sodium hydroxide treated SLA and SLA group at the same time point.

Gene expression of MG63 cells adhesion and differentiation by quantitative real-time PCR

By assessment of mRNA expression level of the integrin β_1 and integrin α_v subunits at 48 h post-seeding, the MG63 cell adhesion was quantified. After the sodium bicarbonate treatment, the integrin β_1 and integrin α_v expression was highly enhanced compared with the other three group ($p < 0.001$). For the sodium

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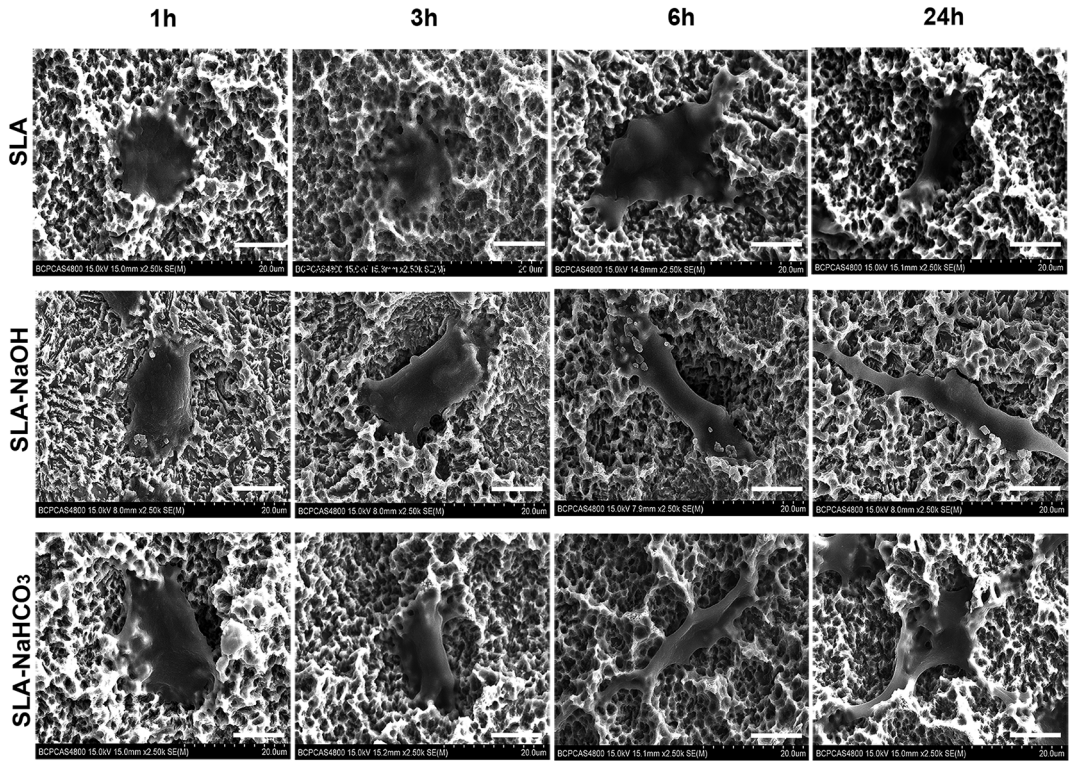

Fig. 6 SEM imaging for representative morphology of specimens with fixed MG63 cells on SLA, sodium hydroxide treated SLA and sodium bicarbonate treated SLA surfaces after 1 hour, 3 hours, 6 hours and 24 hours incubation. MG63 cells extended more lamellipodia and filopodia than that on the untreated SLA surface at the time point of 1 hour, 3 hours and 6 hours, no morphological differences were observed at the later time of 24 hours. Scale bar = 10 μm.

hydroxide group, the gene of integrin α_v expression was higher than that of the SLA group ($p < 0.05$), while the gene integrin β_1 expression was inhibited (Fig. 9). For MG63 cells differentiation, the gene expression of OCN, Runx-2, Col1a1, BMP-2 at 48 h was investigated. On the sodium bicarbonate treated SLA, OCN expression level of MG63 cells was greatly increased than that on the other three groups ($p < 0.001$). After 2 days culturing, the Runx-2 gene expression of MG63 cells on the sodium hydroxide treated SLA was enhanced compare with the TCP group ($p < 0.05$). The gene expression of BMP-2 was enhanced after sodium bicarbonate treatment compared with the SLA and sodium hydroxide groups ($p < 0.01$).

Discussion

Primary stability of implant post-insertion for 3–6 months has been considered for years, in order to reduce the healing time, many modification methods have been developed by variations of surface energy and wettability. A methodology was applied to produce hydroxylated/hydrated surface on the sand-blasted, large grit and acid-etched (SLA) surface, named modified SLA (mod SLA). The main outcomes of mod SLA samples have shown that it greatly decreases the initial water contact angles from the 139.9° of SLA to nearly 0° with increased hydrophilicity. However, there were no topographical and morphological differences between the modSLA and SLA surfaces.[15,30,31] In the present study, the distinguishable differences between the morphology of the alkaline treated SLA surfaces and SLA surface were not observed, while it altered the surface energy and wettability after alkali-treatments. The change of wettability to ~0° of sodium bicarbonate and sodium hydroxide treated titanium surfaces resulted in high surface energy. After these alkaline treatments, the superhydrophilicity of the titanium surface is might ascribe to the enhancement of deprotonation and ion exchange process combines with the rough surface.[32]

Cellular components of immune system play an important role during the host responses to the artificial implant. The macrophage response to biomaterials is of great importance due to the direct regulation of the host inflammatory and immune processes. Subsequently, the pro-inflammatory cytokines, such as interleukins (IL-1β, IL-6), tumor necrosis factor (TNF-α) are secreted.[33] In this study, the RAW264.7 cell attachment on the sodium bicarbonate treated and sodium hydroxide surface was similar to the SLA surface within the first 24 h. Milleret *et al.*[19] found that the number of macrophages/monocytes on the titanium surface treated by sodium hydroxide was decreased compared with that on the untreated surfaces. This variation might be attributed to the different cell type, the CD14-positive cells, and the post-seeding time point, which was only 2 hours after the incubation. In another *in vitro* experiment, the attachment of macrophages after 1, 2 and 24 h incubation, had no significant difference in the level of attached viable cells between chemically modify hydrophilic SLA

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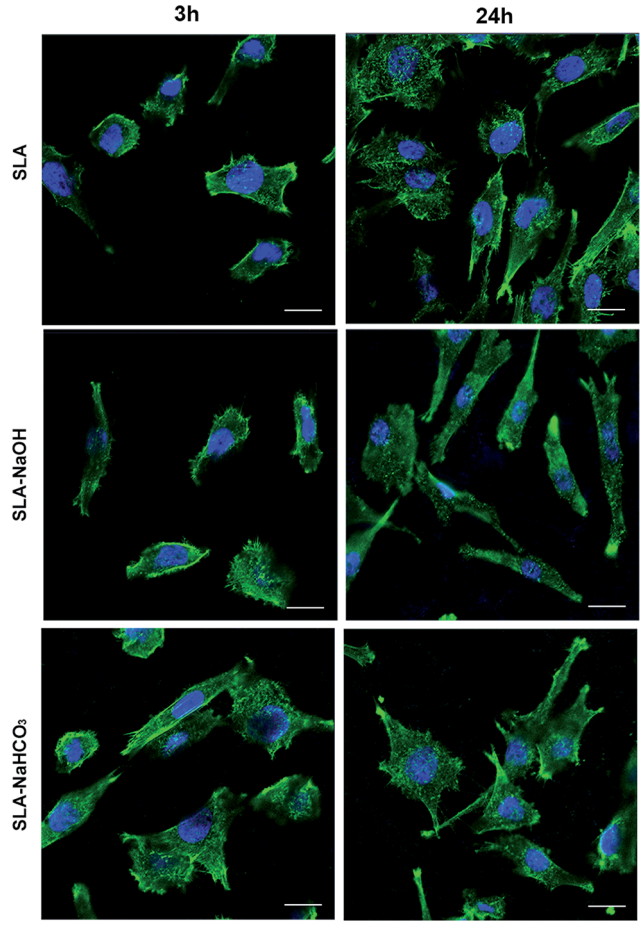

Fig. 7 Assessment of MG63 cells adhesion on SLA, sodium hydroxide treated SLA and sodium bicarbonate treated SLA after 3 hours and 24 hours by FITC-conjugated phalloidin (green signal) and DAPI (blue signal) staining. Cells were with clearer longitude stress fibers after 3 h incubation on the sodium bicarbonate treated SLA surface. Scale bar = 10 μm.

titanium and the SLA group, which is in agreement with the present study.[34]

Previous study elucidated that IL-1β is implicated periodontitis and peri-implantitis pathogen.[35,36] TNF-α in the TNF signaling pathway has an important role in bone homeostasis and inflammatory joint disease, whilst inhibit of TNF-α has led to significant improvement, as TNF-α was known as the inhibitor of osteoblast differentiation and activator of the osteoclastogenesis.[37] IL-1β and TNF-α were expressed in periodontitis along with the loss of the bone and connective tissue, and it could induce bone resorption by activating the osteoclast precursors.[36] In this present study, the IL-1β and TNF-α expression level on the titanium surfaces were down-regulated than that on the TCP group. As the previous research reported, the topography of titanium has been demonstrated the ability of activation of macrophage cytokine expression.[38] Increased roughness without the lipopolysaccharide (LPS) stimulation, the pro-inflammation factor of interleukin 1-beta (IL-1β) was increased during the first 48 h; in contrast, the IL-6 levels were reduced on the rough surface.[39] The variation of the IL-1β and TNF-α between the titanium groups and TCP might be due to the surface roughness. In addition, there were few reports analyzing the effect of chemical modification, especially the alkaline modification of the surface on macrophages' activation and pro-inflammation expression. Hamlet and Ivanovski *et al.* investigated the nanoscale calcium phosphate modified surface markedly down-regulate the pro-inflammatory cytokines and chemokines, and this down-regulation effect may promote the wound healing and osseointegration.[28] In the present study, the IL-1β expression on the sodium bicarbonate treated surface was inhibited than that on the SLA surface and the sodium hydroxide treated SLA surface. In agreement with this study, Hamlet *et al.* found that the alkali modification SLA down-regulated the overall pro-inflammation cytokines in macrophages RAW264.7, which may potentially facilitate the early bone formation.[28] MMP-9 is highly expressed during the early stages of osteoclast development and it is regarded as the type IV collagenase. Activation of MMP-9 has been demonstrated that to be restricted to macrophages in pseudomembrane of the bone-implant interface.[40] Previous studies have demonstrated that the inhibition of MMP-9 resulted in a reduced bone resorption to wear particles.[41] It is interesting to notice that after the sodium hydroxide treatment; the matrix metalloproteinase-9 (MMP-9) expression was activated compared with the other three groups. Though the exact mechanism of MMP-9 in bone remodelling, especially around prosthetic implants is still not clear. Since we merely observed the activation of MMP-9, there were possibly other active proteases influence the inflammatory process on these three different titanium surfaces.

In the present study, the early adhesion of MG63 cells was enhanced after 1 h incubation and cells were flatter and more elongated on the superhydrophilic alkaline treated surfaces by morphology analysis. Cells extended more lamellipodia and filopodia on the superhydrophilic surfaces rather than roundish and small on after 3 h culturing. Consisting with our finding, L929 and MG63 cells adhesion counting were enhanced on the alkali treated surface for 1 h.[18] It is well known that protein adsorption and cell adhesion are highly influenced by the surface energy and wettability, while the process of cell adhesion on the superhydrophilic surfaces is complex.[32,42] For cell adhesion, the process is mediated by a complex network of cell transmembrane receptors known as the integrins and the cytoplasmic proteins linking the extracellular matrix (ECM) to cytoskeleton. When spreading, cell membrane extensions at footpads or the protrusion of a cytoplasmic sheet *i.e.* a lamella play an important role during this process.[4] Integrin-mediated cell adhesion triggers the substance cellular attributes, and the integrin β_1 is the major expressed one in osteoblasts when binding to the ECM. It has been previously reported that overexpression of a dominant negative integrin β_1 in transgenic mice inhibited bone formation. In our study, the sodium bicarbonate treated SLA surface resulted in increase of integrin β_1 and α_v gene level compared with the SLA surface after 2 days incubation. While, for the sodium hydroxide treated SLA, the integrin α_v expression was enhanced compared with the SLA group. Though there were two kinds of integrins tested in the

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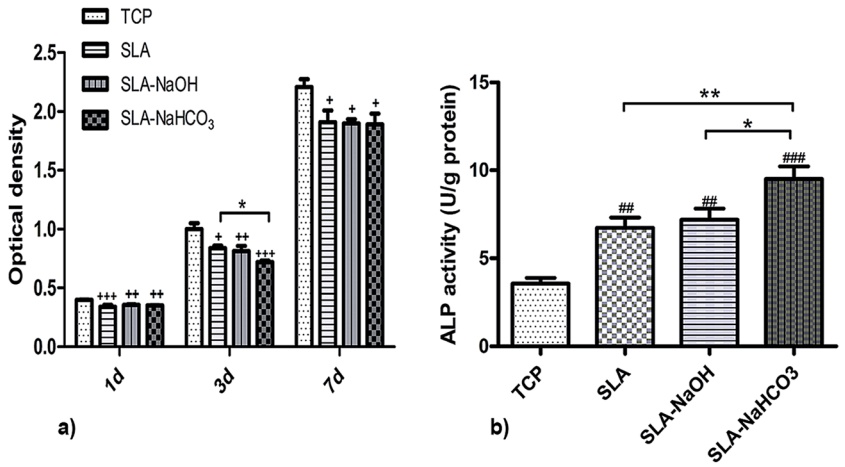

Fig. 8 (a) CCK-8 assay showed the proliferation of MG63 cells cultured on the TCP, SLA, sodium hydroxide treated SLA and sodium bicarbonate treated SLA after 1 day, 3 days and 7 days of incubation. The OD values indicated the proliferation of MG63 cells on sodium bicarbonate treated SLA was statistically lower than that on the SLA surface ($p < 0.05$) after 3 days incubation (*$p < 0.05$ compared with SLA, $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ when compared with the TCP). (b) Comparison of ALP activity of MG63 cells seeded on different titanium surfaces after 3 days incubation. The ALP activity of MG63 cells on the sodium bicarbonate treated SLA was enhanced compared with that on the SLA and sodium hydroxide treated surface ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ when compared with the TCP surface; *$p < 0.05$, **$p < 0.01$, compared with SLA or sodium hydroxide treated SLA surface).

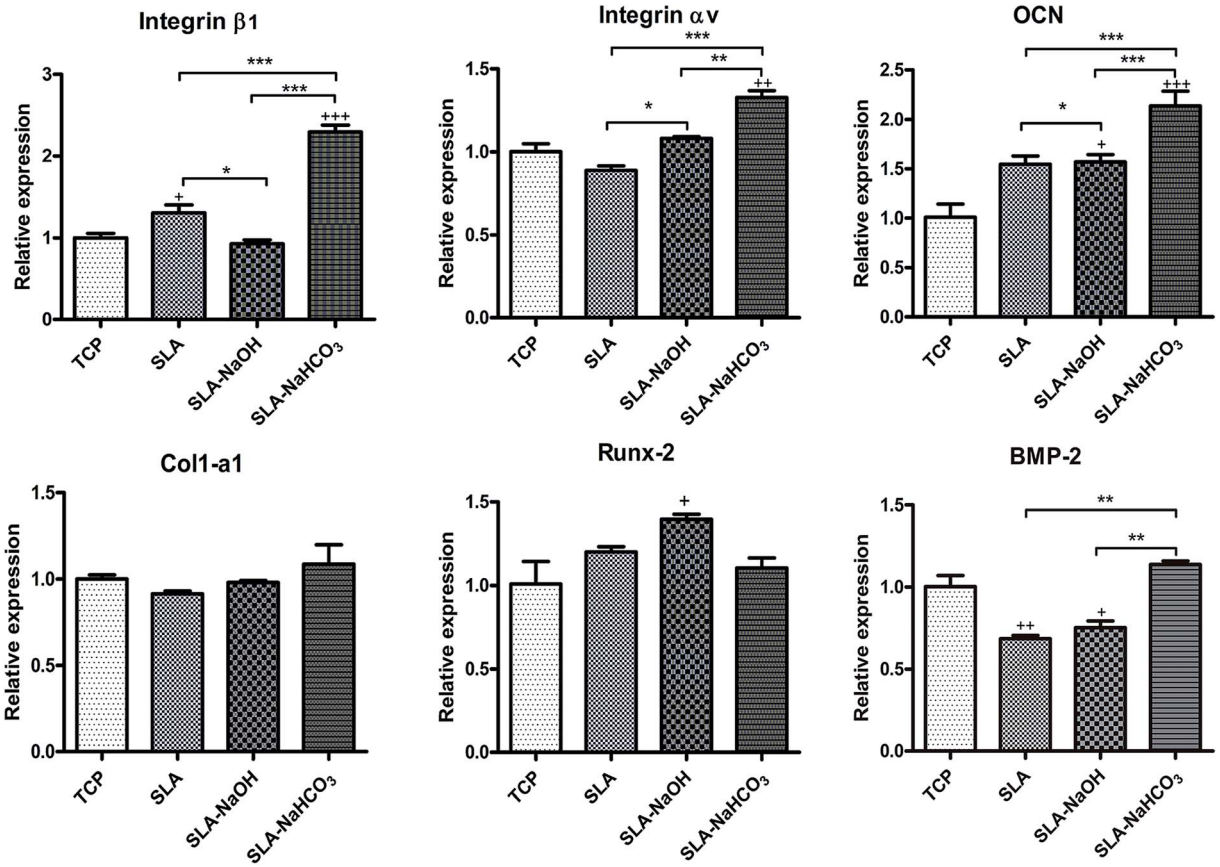

Fig. 9 The mRNA expression levels of integrin β1, integrin α_v, OCN, type 1 collagen (Col1a1), runt-related transcription factor-2 (Runx-2) and bone morphology protein 2 (BMP-2) in MG63 cells cultured on TCP, SLA, sodium hydroxide treated SLA and sodium bicarbonate treated SLA surfaces after 2 days incubation. Increased mRNA expression level of integrin α_v of MG63 cells were observed on the sodium bicarbonate treated SLA and sodium hydroxide treated SLA surfaces, while, integrin β1, OCN and BMP-2 were enhanced on the sodium bicarbonate treated SLA compared with the SLA surface ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ when compared with TCP control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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present experiment, two different alkaline treatments resulted in different integrins expression. Several studies demonstrated that hydrophilic modSLA surface enhances the integrin β_1 and α_v gene expression.[43] In another animal study, the early gene expression of the peri-implant bone tissue by the PCR found that the high levels of integrin β_1 and α_v on the anodic oxidized titanium surface.[44] Previous studies demonstrated that osteoblasts adhesion to osteoactivin, one ECM protein through integrins β_1 and α_v, resulting in focal adhesion complex formation with vinculin which is responsible for the cytoskeleton reorganization as well as the osteoblasts adhesion through β_1 integrin stimulates phophorylation of focal adhesion kinase (FAK) and EKR signaling.[22] These results indicated that by enhancing α_v and β_1 integrin gene expression level, the osteoblast adhesion was improved, which resulted in enhanced differentiation.

The process of new bone formation around the implant on the interface of the titanium implant during the wound healing is similar to the natural process. During the natural bone remodeling process, the osteoclasts dissolved the inorganic matrix to create a complex three-dimensional structured surface for new bone formation. Osteoblasts secret collagen fibers and then become organized and mineralized. While the micro-topographical titanium surface could mimic the three-dimension contour of a bone surface after the osteoclasts resorption facilitates the new bone formation.[9,45,46] Osteoblast-like MG63 cells used in this study are to monitor the chemical modification of the titanium surface which will influence the proliferation and differentiation, respectively, during a comparatively short time. In the present experiment, cellular alkaline phosphatase activity of MG63 cells cultured on the sodium bicarbonate treated SLA was enhanced than that on SLA after the early 3 days post-seeding. The results indicate that alkaline modification of SLA enhances the MG63 osteoblast-like cell differentiation and the cellular production of matrix vesicles which are associated with initial calcification. ALP is activated when the osteoblast precursor committed to differentiate to a bone-forming cell and the activity of ALP is elevated in matured osteoblasts.[47] From the mRNA level, OCN was increased on the alkaline modified surfaces compared with the SLA and TCP surface. OCN is the most abundant non-collagenous proteins of mineralized tissues, which is predominantly synthesized by osteoblasts and plays an important role in both bone resorption and formation.[48] Runx-2 gene expression was enhanced on the sodium hydroxide surface, which has been identified as an essential transcription factor for osteogenic cell differentiation with sequential expression of the Runx-2 regulated phenotypic genes.[43] BMP-2, most osteogenic bone morphogenetic protein, has been identified to stimulate bone formation around endosseous implants.[49] The BMP-2 expression of sodium bicarbonate treated SLA group was up-regulated which in consist with the previous study14. Cell proliferation showed that the lowest cells proliferation was on the sodium bicarbonate treated SLA surface compared with SLA and TCP group after 3 days incubation. The results are consistent with other studies, which indicated there was a reverse relationship between the early cell proliferation and the surface hydrophilicity.[13,43] Taken all together, it suggests that cells on the sodium bicarbonate treated SLA expressed a more differentiated phenotype of osteoblast. For the sodium hydroxide treated SLA, the cell proliferation and ALP activity showed no changes compared with the SLA group. Similarly, as Al Mustafa *et al.* found that after 24 h incubation on the sodium hydroxide treated surface, it showed similar behaviour to the untreated surface including cell viability, protein synthesis, and cell proliferation.[18]

Though sodium bicarbonate and sodium hydroxide applied in the present study are similar from the chemical characteristics, cellular responses to titanium surfaces with these two kinds of modifications are moderately different. Whether the differences are due to the chemical surface characteristics after these two kinds of alkaline treatment influenced some important factors such as the surface charge and ionic strength *et al.* during the cellular process which is largely unknown. Because before the integrin-receptor mediated cell adhesion, the chemical bonding could also influence this process, *i.e.* ionic, covalent, hydrogen or charge-transfer bond during the initial attachment.[50] For example, the interaction between the electron acceptor on the titanium surface and electron donor site on the osteoblast cell could result in the cell adhesion. So, more studies are needed to be done to get a general understanding of the different chemical characteristics of the titanium surfaces after these two kinds of alkaline treatments.

Conclusions

In conclusion, by both of the sodium bicarbonate and sodium hydroxide modifications, the inflamation-related gene expression of macrophage cells on the titanium surfaces is moderately different. Besides, the early adhesion of osteoblast-like cells is enhanced by both alkaline treatments, while the osteogenic differentiation is improved on sodium bicarbonate treated surface. Although there was still space to optimize the alkaline treatment such as the selection of the alkalis and the treatment conditions; the alkaline modifications can be a potential candidate for promotion of peri-implant bone formation.

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