Proteomic analysis of bone proteins adsorbed onto the surface of titanium dioxide

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ABSTRACT

Osseointegration is the structural and functional connection between bone tissues and implants such as titanium dioxide (TiO_2). The bone-TiO_2 interface is thought to contain proteoglycans. However, exhaustive analysis of the proteins in this layer has not been performed. In this study, we evaluated the bone protein adhered on the surface of TiO_2 comprehensively. Pig bone protein was extracted by sequential elutions with guanidine, 0.1 M EDTA, and again with guanidine. The proteins obtained from these extractions were allowed to adhere to an HPLC column packed with TiO_2 and were eluted with 0.2 M NaOH. The eluted proteins were identified by LC/MS/MS and included not only proteoglycans but also other proteins such as extracellular matrix proteins, enzymes, and growth factors. Calcium depositions were observed on TiO_2 particles incubated with bone proteins, guanidine-extracted proteins adhered to TiO_2 displayed significantly high amounts of calcium depositions.

1. Introduction

Osseointegration refers to a direct bone-to-metal surface connection without interposition of fibrous tissue [1]. Based on this concept, implant treatment has become one of the prosthetic treatment for replacing missing teeth. Almost all dental implants are made of titanium dioxide (TiO_2), because of its superior biocompatibility and mechanical resistance to force [2]. Osseointegration is mainly evaluated by observing the bone interface using light microscopy or by comparing the bone-to-implant surface contact rate in vivo [1]. However, a layer at the bone-TiO_2 interface has also been reported based on transmission electron microscopy [3,4]. This layer was suggested to be essential for osseointegration. Previous reports speculated that this zone was filled with glycosaminoglycans (GAGs) as observed by immunohistochemical analysis and ruthenium red staining [4,5].

A previous biochemical study attempted to identify the biomolecules adsorbed on TiO_2 [5]. GAG chondroitin-4-sulfate (C4S) was adsorbed onto the surface of TiO_2 powder via calcium ions [6]. However, the proteoglycans adsorbed on TiO_2 were not released upon EDTA-mediated calcium ion chelation, invalidating the electrostatic bridge hypothesis. These histological and biochemical data are several decades old and have previously been summarized elsewhere [7].

Recently, TiO_2 has been used in proteomics for chromatography-based protein purification because of its high affinity for phosphopeptides [8]. TiO_2 has the capacity to bind not only to phosphorylated peptides but also to non-phosphorylated or acidic peptides, excluding hydroxycarboxylic acids, 2,5 dihydroxybenzoic acid, and glycolic acid [9]. The mechanism that binds TiO_2 and the adherent proteins has not been fully elucidated. Gertler and colleagues investigated the binding mechanism and relative binding strength between peptides and a TiO_2 surface using chromatography [10]. This biochemical analysis was performed with peptides or trypsin-digested proteins that were no longer in their three-dimensional conformation, and therefore did not represent the in vivo behavior of proteins. Recently, Tsuchiya and colleagues reported that secreted proteins derived from cultured bone marrow stromal cells, including extracellular matrix (ECM) proteins, cytokines, growth factors, and cellular components, were capable of adhering to TiO_2 particles [11]. Another study revealed that the proteins attached to TiO_2 were related to cell adhesion, transport, coagulation, immune response, and cytokines [12]. Taken together, these previous reports demonstrate that various proteins can be immobilized on TiO_2 surfaces, and the TiO_2 chromatography system is a useful proteomics tool. Research is needed to identify the specific bone-related proteins that are immobilized on the TiO_2 surface.

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There were only a few report of biochemical analysis with *in vivo* proteins on the surface of TiO_2. To clarify the phenomenon of osseointegration, this study was a new trial to use *in vivo* proteins for proteome analysis of proteins adhered on TiO_2.

To achieve this, chromatography was performed with commercially available TiO_2 particles as a carrier. Immobilized proteins were analyzed *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). The mineralization capacity of these proteins was also evaluated by Field emission scanning electron microscopy with X-ray spectroscopy (SEM).

2. Materials and methods

2.1. Preparation of bone powder

Pig calvaria were surgically extracted with a hammer and chisel from the skulls of 24 pigs (6-month-old) with in minutes after sacrifice at the Nambu Market (Nagoya City Central Wholesale Market, Nagoya, Japan). The calvaria were cut into small pieces (approximately 20×10 mm) and the tissue was powdered using a grinder (ASONE, Osaka, Japan).

2.2. Dissociative extraction procedure

The procedure for sequential extraction of bone proteins was performed at 4 °C as described previously [13]. A 50 g bone powder sample was taken from each density fraction. Two 48-h extractions, each at a volume:weight ratio of 50:1, were performed at 4 °C with a solution of 4 M guanidine hydrochloride (GuHCl), 50 mM Tris-HCl (pH 7.4) containing a protease inhibitor cocktail (1 mM AEBSF, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, and 10 μM pepstatin (Calbiochem EMD Chemicals Inc., Gibbstown, NJ, USA)), and 1 mM 1,10-phenanthroline (Sigma-Aldrich, Tokyo, Japan). Insoluble material was pelleted by centrifugation and the supernatant was designated the first guanidine extract (G1S). The remaining insoluble bone powder was washed twice for 30 min in phosphate-buffered saline (PBS) at a volume:weight ratio of 200:1. The samples were then extracted twice with 0.5 M EDTA/50 mM Tris-HCl (pH 7.4) containing the same protease inhibitor cocktail as previously described, each at 100X volume, for 48 h. Insoluble material was pelleted by centrifugation and the supernatant was designated the EDTA extract (E-Sup). The PBS wash procedure was repeated and the tissue residues were again extracted twice at 4 °C with the guanidine extraction solution for 48 h at a volume: weight ratio of 50:1. Residual insoluble material was pelleted by centrifugation. Following centrifugation, the supernatant was designated the second guanidine extract (G2S). All extractions were then dialyzed exhaustively in distilled water at 4 °C.

2.3. Protein adherence on TiO_2 and elution using chromatography

Before chromatographic analysis, it was confirmed that G1S, E-Sup, and G2S proteins were adhered on the TiO_2 powder (particle size, 45 μm) (WAKO, Osaka, Japan) and were subsequently eluted with 0.2 M NaOH (Supplementary Fig. 1). A Bio-Scale MT10 Column (12×88 mm) (Bio-Rad, Tokyo, Japan) packed with TiO_2 powder was used for chromatography. The packed columns were washed consecutively with water and 0.2 M NaOH to desorb surface contaminants. The columns were then pre-equilibrated with 0.01 M PBS. Chromatography was performed on an Agilent 1220 Infinity HPLC system (Agilent Technologies, Tokyo, Japan). Lyophilized protein samples were dissolved in running buffer (10 mg/mL), centrifuged at $5000 \times g$ for 5 min, and filtered through a 0.45-μm syringe filter (Thermo Fisher Scientific K.K., Nagoya, Japan) to remove aggregates prior to injection into the column. Five milliliters of supernatant was injected into the TiO_2 column at a flow rate of 0.02 mL/min to attain a 40 min retention time for adsorption and flow-through of non-adhesive proteins [14]. After 40 min, the flow rate was increased to 3.0 mL/min. PBS (10 mM) lacking Mg^{2+} and Ca^{2+} (pH 7.4), was used as the adsorption (running) medium. Elution was performed using gradients of 0.2 M NaOH and monitored at 254 nm.

2.4. SDS-PAGE

A 100 mg sample of each of the G1S, E-Sup, and G2S extracts was resuspended in 50 μL SDS sample buffer (0.125 mM Tris-HCl, 4% SDS, 10% sucrose, 0.01% bromophenol blue, and 10% 2-mercaptoethanol) and heated to 95 °C for 1 min. Proteins were separated in a NIHON EIDO system (NIHON EIDO, Tokyo, Japan) using 12% Tris-Tricine gels, alongside a protein ladder (Spectra Multicolor Broad Range Protein Ladder, Thermo Fisher Scientific K.K.) to estimate molecular weights. Proteins were stained with Coomassie Brilliant Blue (CBB R-250) and 0.01% Stains-All (Sigma Chemical Co., St Louis, MO).

2.5. Protein identification with liquid chromatography-tandem mass spectrometry (LC/MS/MS)

Proteins in the elution fractions were digested with trypsin (Promega KK, Tokyo, Japan) for 16 h at 37 °C after reduction, alkylation, demineralization, and concentration. Nanoelectrospray tandem mass analysis was performed using an LTQ Orbitrap XL mass spectrometry system (Thermo Fisher Scientific Inc., Waltham, MA) combined with a Paradigm MS4 HPLC System (Michrom BioResources Inc., Auburn, CA). Samples were injected onto the Paradigm MS4 HPLC System equipped with an L-column2 ODS 0.1 mm in diameter and 150 mm in length (Chemicals Evaluation and Research Institute, Japan). Reversed-phase chromatography was performed with a linear gradient (5% B at 0 min; 50% B after 100 min) of solvent A (2% acetonitrile with 0.1% formic acid) and solvent B (90% acetonitrile with 0.1% formic acid) at an estimated flow rate of 500 nL/min. Ionization was performed by an ADVANCE Spray Source (Michrom BioResources Inc., Auburn, CA) at 150 °C with a capillary voltage of 1.7 kV. A precursor ion scan was carried out using a mass to charge ratio (m/z) of 400–2000 prior to MS/MS analysis. Multiple MS/MS spectra were submitted to the Mascot program, version 2.4.1 (Matrix Science Inc., Boston, MA) for the MS/MS ion search.

2.6. Mineralization capacity of proteins adhered on TiO_2

About 100 μg each of G1S, E-Sup, and G2S proteins were dissolved in separate 100-μL aliquots of PBS, added to TiO_2 particles, then incubated overnight at 37 °C. As a negative control, a sample of TiO_2 particles was immersed in PBS alone. After incubation, TiO_2 particles were rinsed three times with PBS, and 100 μL of 1 mM $CaCl_2$ was added for another overnight incubation at 37 °C. Field emission scanning electron microscopy with x-ray spectroscopy (JEOL-JSM7610F SEM) was performed to confirm the presence of calcium deposits on the TiO_2 particle surfaces for the G1S, E-Sup, G2S, and control groups. The amount of calcium deposition was measured with an Alizarin Red S (ARS) Staining Quantification assay (ScienCell Research Laboratories, Carlsbad, USA). Briefly, 40 mM ARS was added to the TiO_2 particles for 30 min at 37 °C. After incubation, semiquantification of ARS in TiO_2 particles was measured by acetic acid extraction and neutralization with ammonium hydroxide, followed by colorimetric detection at 405 nm.

3. Results

3.1. TiO₂ chromatography

The results of TiO₂ chromatography of the G1S, E-Sup, and G2S proteins are shown in Fig. 1A–F. Most of the proteins injected into the column eluted in the flow-through fraction (Fig. 1A, C and E). However, several smaller peaks were detected after elution with 0.2 M NaOH (Fig. 1B, D and F). For the G1S proteins, only one peak was detected in the flow-through fraction (Fig. 1A), while 6 peaks were detected in the eluted fraction (Fig. 1B). For E-Sup proteins, 2 peaks were detected in the flow-through fraction (Fig. 1C) and 5 peaks were detected in the eluted fraction (Fig. 1D). For the G2S proteins, one peak was detected in the flow-through fraction (Fig. 1E) and 4 peaks were detected in the fraction (Fig. 1F).

3.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Fig. 2A–F shows the 12% gel electrophoretic profiles of peaks

from the TiO₂ chromatography performed on the G1S, E-Sup, and G2S proteins. Several bands were detected with CBB and Stains-All staining. Proteins with various molecular weights were detected in each experimental group. The expression pattern of each sample derived from the peaks was not significantly different between the three experimental groups.

3.3. Protein identification with liquid chromatography-tandem mass spectrometry (LC/MS/MS)

Using LC/MS/MS, the proteins from each fraction that adhered to TiO₂ were identified. From the 0.2 M NaOH elution peaks, 151, 116, and 43 proteins were detected from the G1S sample, E-Sup sample, and G2S sample, respectively. Table 1 shows representative proteins detected in the three experimental groups based on their Exponentially Modified Protein Abundance Index (emPAI) value.
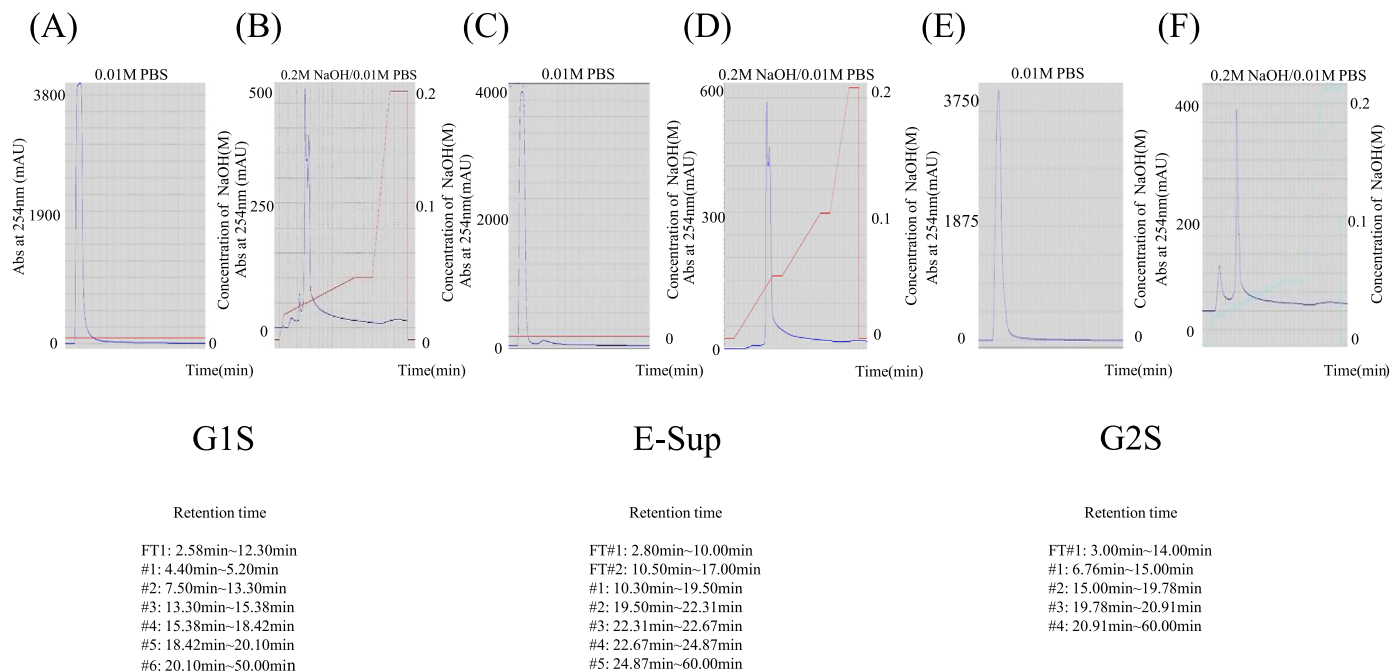

G1S

Retention time

FT1: 2.58min~12.30min
#1: 4.40min~5.20min
#2: 7.50min~13.30min
#3: 13.30min~15.38min
#4: 15.38min~18.42min
#5: 18.42min~20.10min
#6: 20.10min~50.00min

E-Sup

Retention time

FT#1: 2.80min~10.00min
FT#2: 10.50min~17.00min
#1: 10.30min~19.50min
#2: 19.50min~22.31min
#3: 22.31min~22.67min
#4: 22.67min~24.87min
#5: 24.87min~60.00min

G2S

Retention time

FT#1: 3.00min~14.00min
#1: 6.76min~15.00min
#2: 15.00min~19.78min
#3: 19.78min~20.91min
#4: 20.91min~60.00min

Fig. 1. Chromatogram of the flow-through fraction of (A) G1S, (C) E-Sup, and (E) G2S protein from a column packed with titanium powder. Chromatogram of the 0.2 M NaOH elution fraction of (B) G1S, (D) E-Sup, and (F) G2S protein from a column packed with titanium powder. All peaks were detected with a UV monitor at 254 nm.
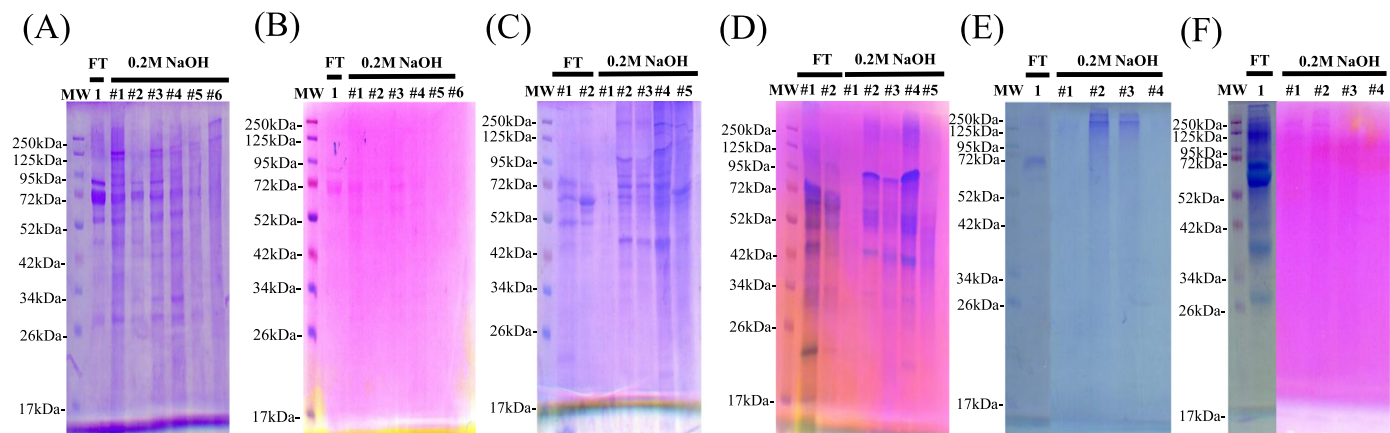

Fig. 2. SDS electrophoretic pattern of peaks on a 12% gel detected in the flow-through and elution fraction of experimental groups stained with Coomassie Blue (A, C, E), and Stains-All (B, D, F).

Table 1
List of Proteins Identified on TiO$_2$ Particles.

G1S proteins immobilized on TiO$_2$, as detected by LC/MS/MS

#	Accesion no	Protein	emPAI
1	P02067	Hemoglobin subunit beta	30.17
2	P08835	Serum albumin	21.95
3	P089571	Serotransferrin	21.67
4	P01846	Ig lambda chain	14.46
5	O97788	Fatty acid-binding protein, adipocyte	5.55
6	P50828	Hemopexin	4.26
7	Q8SPS7	Haptoglobin	4.21
8	P01965	Hemoglobin subunit alpha	4
9	Q2KIS7	Tetranectin	2.5
10	P18648	Apolipoprotein A-I	2.24
11	P50447	Alpha-1-antitrypsin	2.12
12	P19620	Annexin A2	1.76
13	Q49I35	Galectin-1	1.53
14	P23284	Peptidyl-prolyl cis-trans isomerase	1.44
15	P02080	Hemoglobin subunit beta-C(NA)	1.43
16	P18650	Apolipoprotein E	1.19
17	Q5E9F7	Cofilin-1	1.11
18	P52552	Peroxiredoxin-2	1.07
19	P01972	Hemoglobin subunit alpha-1/2	0.99
20	Q5E956	Triosephosphate isomerase	0.94
21	P35700	Peroxiredoxin-1	0.88
22	P08758	Annexin A5	0.82
23	P00795	Cathepsin D	0.77
24	Q61398	Procollagen C-endopeptidase enhancer 1	0.76
25	Q29549	Clusterin	0.32

E-Sup proteins immobilized on TiO$_2$, as detected by LC/MS/MS

#	Accesion no	Protein	emPAI
1	P20112	SPARC	9.43
2	P08493	Matrix Gla protein	4.17
3	O46390	Biglycan	4.08
4	Q19AZ8	Prothrombin	3.8
5	P0DMA9	Apolipoprotein A-I	3.1
6	P14460	Fibrinogen alpha chain (Fragment)	2.89
7	Q9GLP2	Vitamin K-dependent protein C	2.17
8	P48819	Vitronectin	2.15
9	P79281	Pleiotrophin	1.96
10	Q27972	Chondroadherin	1.83
11	Q3ZBN5	Asporin	1.76
12	P16293	Coagulation factor IX	1.72
13	P62935	Peptidyl-prolyl cis-trans isomerase	1.63
14	P45845	Protein-lysine 6-oxidase	1.62
15	Q2KJH6	Serpin H1	1.52
16	Q05717	Insulin-like growth factor-binding protein 5	1.21
17	P36955	Pigment epithelium-derived factor	1.01
18	Q62009	Periostin	0.93
19	Q863H1	Secreted frizzled-related protein 2	0.87
20	F1N152	Serine protease HTRA1	0.85
21	P32262	Antithrombin-III	0.85
22	P0C225	Osteocalcin	0.79
23	P78539	Sushi repeat-containing protein SRPX	0.51
24	P19879	Mimecan	0.51
25	O01305	Calcium vector protein	0.46

G2S proteins immobilized on TiO$_2$, as detected by LC/MS/MS

#	Accesion no	Protein	emPAI
1	Q9XSD9	Decorin	7.52
2	Q711S8	Secreted phosphoprotein 24	4.26
3	P29700	Alpha-2-HS-glycoprotein	3.32
4	P50609	Fibromodulin	1.69
5	P14287	Osteopontin	1.33
6	Q05443	Lumican	1.1
7	P12273	Prolactin-inducible protein	0.86
8	Q9XSJ7	Collagen alpha-1(I) chain	0.82
9	P02465	Collagen alpha-2(I) chain	0.75
10	Q28178	Thrombospondin-1	0.59
11	Q9TTY1	Metalloproteinase inhibitor 2	0.53
12	Q29116	Tenascin	0.5
13	P20908	Collagen alpha-1(V)	0.46
14	Q28944	Cathepsin L1	0.46

Table 1 (continued)

G2S proteins immobilized on TiO$_2$, as detected by LC/MS/MS

#	Accesion no	Protein	emPAI
15	A6QLP7	Translocon-associated protein subunit alpha	0.25
16	P04785	Protein disulfide-isomerase	0.21
17	P07589	Fibronectin	0.19
18	P51888	Prolargin	0.18
19	P05009	Interferon alpha-C	0.18
20	Q15198	Platelet-derived growth factor receptor-like protein	0.09
21	Q6AY22	Spermatogenesis-associated protein 1	0.07
22	Q8BMK4	Cytoskeleton-associated protein 4	0.06
23	P08003	Protein disulfide-isomerase A4	0.05
24	P08253	72 kDa type IV collagenase	0.05
25	Q95116	Thrombospondin-2	0.03

3.4. Mineralization assay

Fig. 3 shows the mineralization capacity of the proteins that adhered on TiO$_2$ particles. TiO$_2$ particles incubated with proteins from the G1S and G2S fractions displayed increased calcium deposition compared to TiO$_2$ particles incubated with PBS or the E-Sup fraction (Fig. 3A). As measured by the Alizarin Red S Staining Quantification Assay, more calcium depositions were observed on TiO$_2$ particles treated with G1S and G2S proteins in comparison to the PBS control group. Few calcium depositions were seen in the TiO$_2$ particles treated with E-Sup protein (Fig. 3B).

4. Discussion

The specific bone-related proteins adhered on TiO$_2$ are not completely understood. Previous reports on the bone-TiO$_2$ interface rely on histological observations, but this method is not able to comprehensively identify the adherent biomolecules. In this report, native proteins were extracted from pig bone and subjected to TiO$_2$ particle binding using chromatography. Adhered proteins were analyzed using SDS-PAGE and LC/MS/MS. This analysis revealed that the interface between bone and TiO$_2$ existed a sort of proteins, such as extracellular matrix, enzyme, and growth factor and some of the adhered proteins showed mineralization capacity.

Evaluation of the proteins that adhered to TiO$_2$ particles was performed with chromatography. In contrast to the current findings, a previous report analyzing TiO$_2$ adhesive proteins using chromatography detected only one peak [15]. However, in that study, bovine metatarsal proteins were dissolved in 2 M urea, which denatures proteins and disrupts their conformation [16]. In the current study, extracted proteins were dissolved in PBS before analysis so that their conformation would be maintained closer to their actual *in vivo* state. Thus, it is possible that the buffer caused the differences in results between this previous report and the current study. Moreover, the presence of one peak in their study indicates that this protein fraction was adsorbed on TiO$_2$ with a certain affinity at a neutral pH, and was released at an alkaline pH. The presence of multiple peaks during a linear gradient elution with 0.2 M NaOH buffer indicates that the eluted proteins have different retention times. This suggests that the analyzed bone proteins display differences in affinity for TiO$_2$. At about 15% of 0.2 M NaOH (30 mM NaOH), the column was completely eluted. This is in accordance with results from a previous study that reported complete protein elution using 25 mM NaOH [14]. This suggests that the adherence strength between TiO$_2$ and the bone proteins did not depend on protein conformation.

Furthermore, there was substantial overlap between the peaks found for each experimental group. At the same time, there were significant differences between the chromatograms of all three
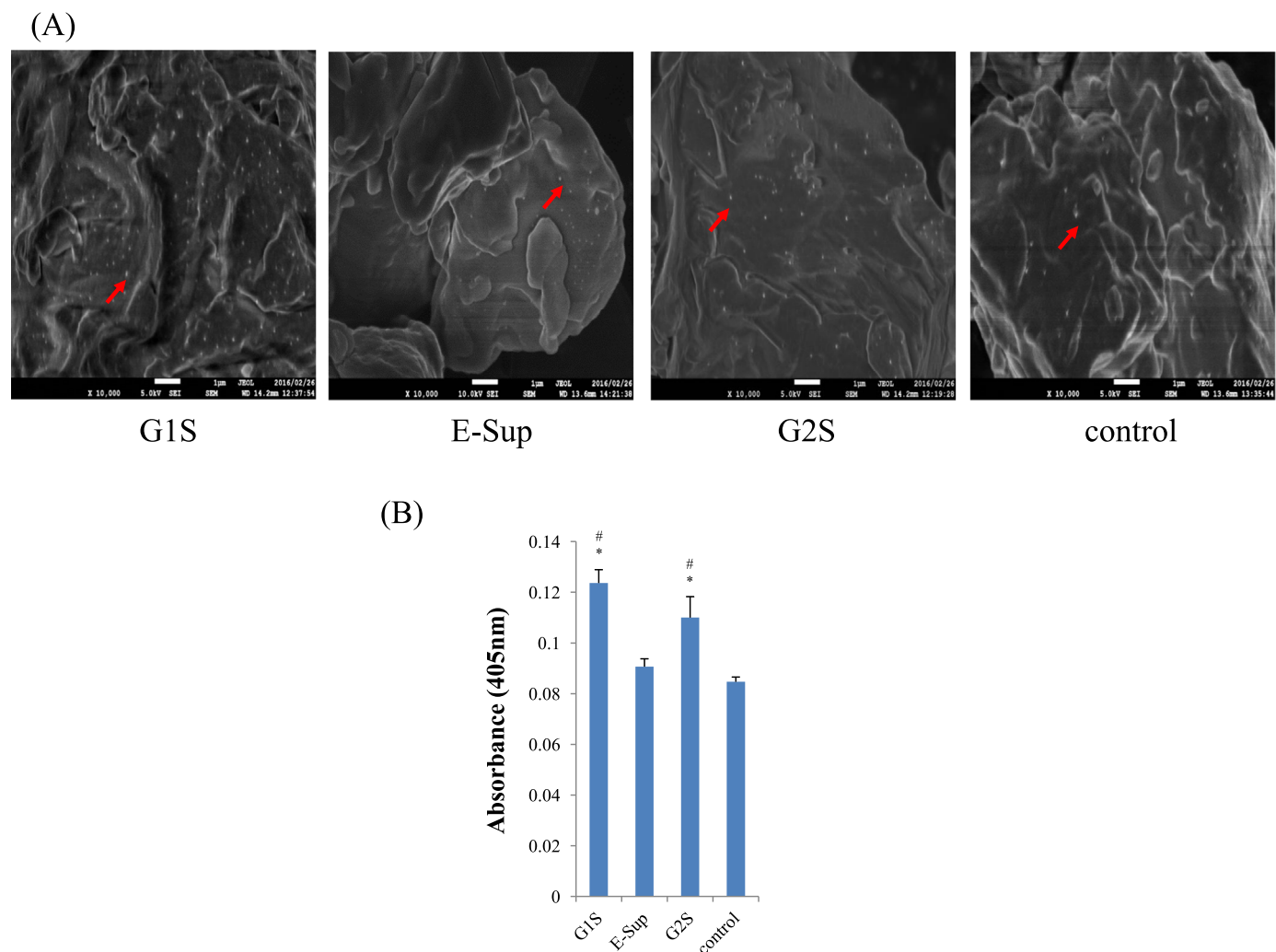
(A)

G1S   E-Sup   G2S   control

(B)

Fig. 3. (A) SEM of the calcium depositions of proteins adhered on titanium particles, at $10,000 \times$ magnification. Red arrows refer to calcium depositions. (B) Calcium deposition quantification using the ARS assay. Data are expressed as the mean (n=3) with error bars representing standard deviations, #p < 0.05 compared to control, and *p < 0.05 compared to E-Sup.

experimental groups. This suggests that the type of chemical bond responsible for the adhesion of proteins on TiO_2 is not dependent on the protein. The gradient conditions of the 0.2 M NaOH elution buffer were changed in an attempt to improve peak resolution. However, complete separation of the peaks could not be obtained (data not shown). Further optimization of the TiO_2 carrier column is required to improve the separation of the protein peaks upon elution. In this study, the method of chromatography method was available to evaluate soluble proteins adhered to TiO_2. Evaluation of the insoluble proteins derived from bone could not be performed with chromatography, because samples to analysis with this chromatography system were limited to soluble molecules. Further research is required to address the effect of insoluble proteins adsorption.

SDS-PAGE and LC/MS/MS revealed the presence of a wide variety of proteins in each of the three experimental fractions. In the G1S fraction, the most abundant TiO_2 adhesive proteins were albumin and hemoglobin subunit beta, according to the current emPAI results. These serum proteins are known to adhere to TiO_2 [17] and are found in bone tissue upon extraction. However, previous studies reported that fibronectin was the most abundant TiO_2-adhesive protein in serum [12,18]. This discrepancy can be explained by the fact that small proteins such as albumin may be replaced by larger proteins such as fibronectin and fibrinogen during chromatography, due to an exchange process known as the

Vroman effect [19]. Additionally, the protein samples analyzed in the two studies are completely different. While bone tissue and serum both contain fibronectin, the relative protein content is different, possibly affecting the degree of fibronectin adsorption on TiO_2.

Previous studies used TiO_2 disks to study protein adherence [20,21]. It is known that, besides structural selectivity, affinity of large biomolecules can also arise from physical surface properties including hydrophobicity, topology [21], and charge [20]. Our results indicate that these factors were different between TiO_2 particles and TiO_2 disks.

The ECM components detected, especially biglycan, glycosylated decorin, and chondroitin/dermatan sulfate, which contain sulfate groups, were negatively charged [22]. The keratin sulfate proteoglycans fibromodulin, lumican, and mimecan also contain sulfate groups [23]. A previous report showed that these small leucine-rich proteoglycans (SLRPs) can adhere to TiO_2 [24]. However, this report was based on *in vitro* gene expression analysis, which does not provide direct evidence for the adherence of SLRPs onto TiO_2. This is the first report that unequivocally demonstrates SLRPs adhering to TiO_2.

Thrombospondin-1 [25], SPARC [26], osteopontin [27], and tenascin [28] are glycoproteins with O- and N-linked glycosylation, but they have not yet been reported to have sulfate groups. Osteopontin was detected in the interface between TiO_2 and bone in

a previous histological analysis [29]. This indicates that sulfate groups are not necessary for TiO_2 adhesion. Furthermore, osteocalcin, periostin, and matrix gla protein were also detected. These proteins play a role in mineralization and do not have a carbohydrate chain. Osteocalcin was also previously detected in the bone-TiO_2 interface [29]. Our results are in agreement with these previous reports and suggest that these proteins, with the exception of proteoglycan, can be found in the interface between TiO_2 and bone.

It was previously suggested that GAGs bind TiO_2 *via* Ca^{2+} [6]. However, the proteins in this study had the capacity to bind TiO_2 in the absence of Ca^{2+} and Mg^{2+}, as these ions were not present in the buffer. This inconsistency was previously reported by Ozawa and Kasugai (1996) who suggested that proteins from bone marrow stromal cells and osteoblasts could adhere on TiO_2 in the absence of these divalent cations [30]. Further research is required to address the effect of Ca^{2+} and Mg^{2+} on TiO_2 protein adsorption.

Proteins can have several binding sites, depending on their conformation and amino acid sequence. Some growth factors that were detected in this study have the ability to bind proteoglycans [22], and SLRPs are known to interact with collagen during collagen fibrillogenesis [31]. The protein samples used in this study were dissolved in 0.01 M PBS (pH 7.4) to mimic an *in vivo* environment, and it is therefore possible that the proteins detected in this study were bound to each other in a complex before adhering to TiO_2. Therefore, future investigations should analyze purified proteins of interest to evaluate whether they adhere to TiO_2 directly.

The mineralization capacity of the proteins experimentally found to adhere on TiO_2 was evaluated by SEM. In the G1S fraction, the most abundant protein was albumin. However, several studies have reported that the presence of albumin decreases the amount of calcium phosphide precipitate on a Ti surface [32]. The G1S fraction included not only albumin but also other proteins, and the findings suggest that one or several of these other proteins are involved in the formation of calcium deposits. However, the specific protein(s) in G1S that are associated with mineralization were not identified and further study is required to determine the players contributing to increased mineralization. The E-Sup fraction contained calcium-binding proteins such as osteocalcin, periostin, and matrix gla protein, however, we only found limited calcium deposition in this fraction. It is possible that the calcium-binding proteins directly adhere onto TiO_2, inhibiting the binding of calcium. The role of the interaction between calcium-binding proteins and TiO_2 in the mineralization process is unclear and requires further research. The most abundant protein in the G2S fraction was the SLRP decorin. SLRPs control the formation and orientation of collagen fibrils and contribute to mineralization [33]. A previous study showed that the GAG chain of decorin can bind calcium [33]; it is possible that decorin contributed to an increased formation of calcium deposits in comparison to the control.

Besides using histological analysis, osseointegration has also been studied by comparing the removal torque or the force necessary for pushing out implants placed in animals [1,3]. For obvious ethical concerns, these experimental methods are not appropriate to study osseointegration in clinical applications. In contrast to these invasive methods, which require the removal of the operated implants from patients, biochemical methods that detect a given protein profile related to osseointegration cause no harm to the bone tissue or implant. The current study represents an important step forward in the development of a biomarker of osseointegration for clinical application.

To conclude, native proteins were extracted from pig bone in this study. These proteins were subjected to TiO_2 chromatography and were identified with LC/MS/MS. We identified that the interface between bone and TiO_2 existed a sort of proteins, such as extracellular matrix, enzyme, and growth factor. Interestingly, except for previously detected proteins, the detected ECM proteins were not glycosylated. TiO_2 incubated with proteins from guanidine-extracted proteins displayed increased calcium depositions. Proteome analysis using TiO_2 chromatography is a useful tool for investigating which bone proteins adhere to TiO_2.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.007.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.007.

References

[1] R. Brånemark, L.O. Öhrnell, P. Nilsson, P. Thomsen, Biomechanical characterization of osseointegration during healing: an experimental *in vivo* study in the rat, Biomaterials 18 (1997) 969–978.

[2] F.J. Gil, N. Manzanares, A. Badet, C. Aparicio, M.P. Ginebra, Biomimetic treatment on dental implants for short-term bone regeneration, Clin. Oral. Investig. 18 (2014) 59–66.

[3] L. Linder, T. Albrektsson, P.I. Brånemark, et al., Electron microscopic analysis of the bone-titanium interface, Acta Orthop. Scand. 54 (1983) 45–52.

[4] Y. Ayukawa, F. Takeshita, T. Inoue, An ultrastructural study of the bone-titanium interface using pure titanium coated plastic and pure titanium rod implants, Acta Histochem. Cytochem. 29 (1996) 243–254.

[5] D.E. Steflik, A.L. Sisk, G.R. Parr, P.J. Hanes, F. Lake, M.J. Song, P. Brewer, R.V. McKinney, High-voltage electron microscopy and conventional transmission electron microscopy of the interface zone between bone and endosteal dental implants, Biomed. Mater. Res. 29 (1992) 529–545.

[6] I.J. Collis, G. Embery, Adsorption of glycosaminoglycans to commercially pure titanium, Biomaterials 13 (1992) 548–552.

[7] M.M.1 Klinger, F. Rahemtulla, C.W. Prince, L.C. Lucas, J.E. Lemons, Proteoglycans at the bone-implant interface, Crit. Rev. Oral. Biol. Med. 9 (1998) 449–463.

[8] Z. Wang, M. Gucek, G.W. Hart, Cross-talk between GlcNAcylation and phosphorylation: site-specific phosphorylation dynamics in response to globally elevated O-GlcNAc, Proc. Natl. Acad. Sci. USA 16 (2008) 13793–13798.

[9] P.A. Connor, A.J. McQuillan, Phosphate adsorption onto TiO_2 from aqueous solutions: an *in situ* internal reflection infrared spectroscopic study, Langmuir 15 (1999) 2916–2921.

[10] G. Gertler, G. Fleminger, H. Rapaport, Characterizing the adsorption of peptides to TiO_2 in aqueous solutions by liquid chromatography, Langmuir 26 (2010) 6457–6463.

[11] S. Tsuchiya, K. Hara, M. Ikeno, Y. Okamoto, H. Hibi, M. Ueda, Rat bone marrow stromal cell-conditioned medium promotes early osseointegration of titanium implants, Int. J. Oral. Maxillofac. Implants 28 (2013) 1360–1369.

[12] C.G. Dodo, P.M. Senna, W. Custodio, A.F. Paes Leme, A.A. Del Bel Cury, Proteome analysis of the plasma protein layer adsorbed to a rough titanium surface, Biofouling 29 (2013) 549–557.

[13] C. Domenicucci, H. Goldberg, T. Hofmann, D. Isenman, S. Wasi, J. Sodek, Characterization of porcine osteonectin extracted from foetal calvariae, Biochem. J. 253 (1988) 139–151.

[14] A.D. Roddick-Lanzilotta, A.J. McQuillan, An *in situ* infrared spectroscopic investigation of lysine peptide and polylysine adsorption to TiO(2) from aqueous solutions, J. Colloid Interface Sci. 217 (1999) 194–202.

[15] Yoshinori Kuboki, Toshitake Furusawa, Masaaki Sato, Yongkun Sun, Hidero Unuma, Shigeaki Abe, Ryuichi Fujisawa, Tsukasa Akasaka, Fumio Watari, Hiroko Takita, Rachel Sammons, Bone enhancing effect of titanium-binding proteins isolated from bovine bone and implanted into rat

calvaria with titanium scaffold, Bio-Med. Mater. Eng. 24 (2014) 1539–1548.

[16] A.K. Bhuyan, Protein stabilization by urea and guanidine hydrochloride, Biochemistry 41 (2002) 13386–13394.

[17] M.N. Sela, L. Badihi, G. Rosen, D. Steinberg, D. Kohavi, Adsorption of human plasma proteins to modified titanium surfaces, Clin. Oral. Implants Res. 18 (2007) 630–638.

[18] H. Noh, E.A. Vogler, Volumetric interpretation of protein adsorption: competition from mixtures and the Vroman effect, Biomaterials 28 (2007) 405–422.

[19] H.G. Xie, X.X. Li, G.J. Lv, W.Y. Xie, J. Zhu, T. Luxbacher, R. Ma, X.J. Ma, Effect of surface wettability and charge on protein adsorption onto implantable alginate-chitosan-alginate microcapsule surfaces, J. Biomed. Mater. Res. 92A (2010) 1357–1365.

[20] K. Tsougeni, P.S. Petrou, D.P. Papageorgiou, S.E. Kakabakos, A. Tserepi, E. Gogolides, Controlled protein adsorption on microfluidic channels with engineered roughness and wettability, Sens. Actuators B 161 (2012) 216–222.

[21] Y. Ayukawa, F. Takeshita, M. Yoshinari, T. Inoue, Y. Ohtsuka, M. Shimono, T. Suetsugu, T. Tanaka, An immunocytochemical study for lysosomal cathepsins B and D related to the intracellular degradation of titanium at the bone-titanium interface, J. Periodontol. 69 (1998) 62–68.

[22] G. Stöcker, H.E. Meyer, C. Wagener, H. Greiling, Purification and N-terminal amino acid sequence of a chondroitin sulphate/dermatan sulphate proteoglycan isolated from intima/media preparations of human aorta, Biochem. J. 274 (1991) 415–420.

[23] Y.- Sommarin, M. Wendel, Z. Shen, U. Hellman, D. Heinegârd, Osteoadherin, a cell-binding keratan sulfate proteoglycan in bone, belongs to the family of leucine-rich repeat proteins of the extracellular matrix, J. Biol. Chem. 273 (1998) 16723–16729.

[24] H.K. Nakamura, F. Butz, L. Saruwatari, T. Ogawa, A role for proteoglycans in

[25] J. Hofsteenge, K.G. Huwiler, B. Macek, D. Hess, J. Lawler, D.F. Mosher, J. Peter-Katalinic, C-mannosylation and O-fucosylation of the thrombospondin type 1 module, J. Biol. Chem. 276 (2001) 6485–6498.

[26] B. Kaufmann, S. Müller, F.G. Hanisch, U. Hartmann, M. Paulsson, P. Maurer, F. Zaucke, Structural variability of BM-40/SPARC/osteonectin glycosylation: implications for collagen affinity, Glycobiology 14 (2004) 609–619.

[27] B. Christensen, M.S. Nielsen, K.F. Haselmann, T.E. Petersen, E.S. Sørensen, Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications, Biochem. J. 390 (2005) 285–292.

[28] S.P. Giblin, K.S. Midwood, Tenascin-C: form versus function, Cell Adhes. Migr. 9 (2015) 48–82.

[29] Y. Ayukawa, F. Takeshita, T. Inoue, M. Yoshinari, M. Shimono, T. Suetsugu, T. Tanaka, An immunoelectron microscopic localization of noncollagenous bone proteins (osteocalcin and osteopontin) at the bone-titanium interface of rat tibiae, J. Biomed. Mater. Res. 41 (1998) 111–119.

[30] S. Ozawa, S. Kasugai, Evaluation of implant materials (hydroxyapatite, glass-ceramics, titanium) in rat bone marrow stromal cell culture, Biomaterials 17 (1996) 23–29.

[31] S. Kalamajski, A. Oldberg, The role of small leucine-rich proteoglycans in collagen fibrillogenesis, Matrix Biol. 29 (2010) 248–253.

[32] C. Combes, C. Rey, M. Freche, In vitro crystallization of octacalcium phosphate on type I collagen: influence of serum albumin, J. Mater. Sci. Mater. Med. 10 (1999) 153–160.

[33] G. Embery, R. Hall, R. Waddington, D. Septier, M. Goldberg, Proteoglycans in dentinogenesis, Crit. Rev. Oral Biol. Med. 12 (2001) 331–349.

mineralized tissue-titanium adhesion, J. Dent. Res. 86 (2007) 147–152.