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Binding of collagen gene products with titanium oxide

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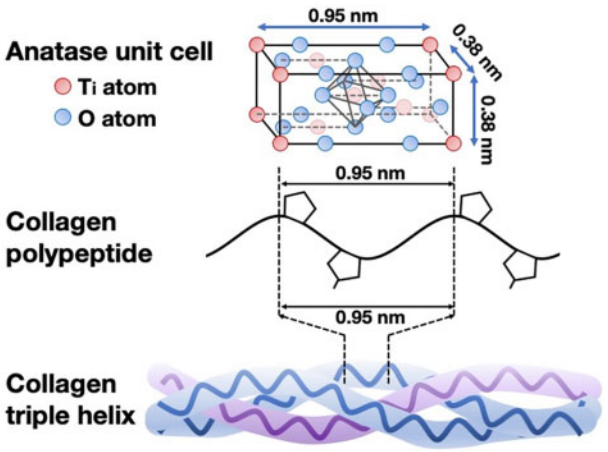
Titanium is the only metal to which osteoblasts can adhere and on which they can grow and form bone tissue *in vivo*, resulting in a strong bond between the implant and living bone. This discovery provides the basis for the universal medical application of Ti. However, the biochemical mechanism of bond formation is still unknown. We aimed to elucidate the mechanism of bond formation between collagen, which constitutes the main organic component of bone, and TiO_2, of which the entire surface of pure Ti is composed. We analysed the binding between the soluble collagen and TiO_2 by chromatography with a column packed with Ti beads of 45 μm, and we explored the association between collagen fibrils and TiO_2 (anatase) powders of 0.2 μm. We ran the column of chromatography under various elution conditions. We demonstrated that there is a unique binding affinity between Ti and collagen. This binding capacity was not changed even in the presence of the dissociative solvent 2M urea, but it decreased after heat denaturation of collagen, suggesting the contribution of the triple-helical structure. We propose a possible role of periodically occurring polar amino acids and the collagen molecules in the binding with TiO_2.

Keywords: anatase; chromatography; collagen; titanium beads; 2M urea.

Abbreviations: I-AC, type I acid-soluble collagen; I-PC, type I pepsin solubilized collagen; PBS, phosphate-buffered saline; SIBLING, small the integrin-binding ligand, N-linked glycoprotein.

Graphical Abstract



An increasing number of older people worldwide have benefitted from artificial joints and dental implants, primarily composed of Ti. However, besides the cost-benefit advantages, it takes a considerable time, up to several months, to achieve fixation between a device and living bone. The ability of living bone to bind to Ti was discovered by Brånemark *et al.* (*1*) about 60 years ago. However, despite numerous efforts to accelerate fixation, few investigations have investigated the underlying biochemical mechanism, although the specific affinity of phosphorylated peptides for TiO_2 is well established and widely applied (*2*). Therefore, our study aimed to accelerate Ti implant fixation by clarifying the binding mechanism. In previous works, we reported for the first time that binding occurs between TiO_2 and phosphoproteins such as caseins, phosvitin and bone phosphoproteins (*2*). Next, we found that some phosphorylated and RGD-containing proteins of the small the integrin-binding ligand, N-linked glycoprotein (SIBLING) family in bone tissue possessed the ability to bind to TiO_2. The Ti implant coated with the SIBLING proteins strongly promoted the formation of bone tissue (*3–6*). These observations provided evidence for their possible role in the fundamental mechanism of preferential bone formation on the Ti-device's surface. However, it is important to consider the initial formation of bone. The maintenance of the interface between Ti implant and bone requires mechanical support, provided by the combination of type I collagen and hydroxyapatite in bone tissue. However, there has been no systematic study of TiO_2 and collagen's interaction as far as we know.

With the ultimate aim of improving fixation of Ti implants, here we report an investigation of the behaviour of soluble collagen using column

chromatography with 45 mm TiO_2 beads. We have also studied the interaction between TiO_2 (anatase) powders and aggregating collagen and observed the attachment of anatase powders and Ti-beads to collagen fibres. The rationale for using TiO_2 (anatase) is that all the surface of titanium, once exposed to open air or aqueous solutions, is known to be covered by a thin layer of TiO_2, which consists of anatase type crystals (*7–9*). Since other polymorphs of TiO_2, such as rutile and brookite, require a heat treatment above 800°C to produce, the newly formed TiO_2 on the pure titanium surfaces under room temperature is usually assumed to be covered by anatase. However, a comparison of the interaction of anatase and rutile with collagen may be worth studying, and this is now being undertaken in this laboratory.

Since collagen behaves differently depending on its solvent environment, we developed a Ti-chromatography regime to test collagen-binding with three different solvents. We observed the binding ability of native type I collagen with Ti in all three solutions. We demonstrated that it decreased upon heat denaturation, indicating that the triple helical structure significantly contributes to the binding. In addition to the importance of the triple helix, we ascribe the binding to the periodical presence of numerous polar amino acids, mainly those of acidic nature, distributed along with the collagen molecules.

Based on our results, we propose a 'periodicity hypothesis of collagen-Ti interaction', in which collagen molecules provide a regular pattern of charged groups along with the triple-helical structure. This periodicity corresponds well to the anatase crystal lattice dimension (0.949 nm), facilitating multiple hydrogens and electrostatic bonds between the two substances.

Materials and Methods

Column chromatography packed with Ti-beads of collagens

Column chromatography was carried out as previously reported (*3–5*), using a column packed with Ti-beads (ø 45 μm, Osaka Ti Technologies, Co., Japan). According to company documentation, the beads were produced by the gas atomizer method and are spherical with a smooth, shiny surface entirely covered with TiO_2. Finer particles were removed by repeated decantation from a suspension in distilled water. The beads were packed into a commercial glass chromatography column equipped with a water jacket (XK16/20, GE Health Care, Tokyo, Japan) to obtain a bed volume of ø 16 × 50 mm. The column was washed with a large amount of dilute HCl (pH 4.0) and subsequently equilibrated with one of three different solvents: dilute HCl at pH 4.0, 2M urea in phosphate-buffered saline at pH 7.4 [phosphate-buffered saline (PBS)] or 2M urea in dilute HCl at a pH of 4.0. These solvents were used for the following reasons: In standard acidic solutions such as dilute HCl (pH 3–4), type I collagen is primarily in a monomeric state but often contains trace amounts of cross-linked polymers, which tend to aggregate under a slight change in the local environment, which may change their affinity with the Ti-column. 2M urea was used because Mizuno and Hayashi (*10*) reported systematically that in a low concentration of urea, such as 2M, collagen maintains its triple helical structure. Secondly, the 2M urea effectively prevents the tendency for collagen aggregation which, if it occurs, could lead to difficulty in discriminating between this and binding to the column.

Type I acid-soluble collagen (I-AC), and type I pepsin solubilized collagen (I-PC) were obtained as 0.3% acidic solutions from Koken Co., Japan. I-AC is essentially a purified acid extract from bovine skin collagen, while I-PC is the product of a limited pepsin-digestion of insoluble bovine skin collagen. This treatment removes the telopeptide parts of the collagen molecules with a high yield of soluble-form collagen, called atelocollagen. Solutions of I-AC and I-PC were heated at 95°C for 15 min in a hot water bath, and circular dichroism was used to confirm that this process destroyed the triple helical structure.

A fixed amount of collagen (9 mg/3 ml) was applied to the top of the column, which was then run at a flow rate of 120 ml/h at 15°C with each buffer, using a ceramic pump (VSP-3200W, Eyela, Tokyo, Japan). The adsorbed fraction was eluted by stepwise addition of 25 mM NaOH solution after the first groups of non-absorption peaks of no-adsorption had been washed out. We confirmed that 25 mM NaOH recovered all the collagen bound to the Ti column. The elusion at 254 nm was monitored with an ATTO Bio-Mini-UV monitor (Atto Co., Tokyo, Japan). We checked that collagen follows Lambert-Beer's law at this wavelength, even though it does not significantly peak at 254 nm. Peak areas in the chromatogram were measured using Image-J software. The volume of collagen was adjusted with a four-way quantitative injector with a precise volumetric syringe.

Observation of the effect of anatase powders on collagen aggregation

A suspension of 0.1% (w/v) anatase powder (Fujifilm-Wako, Co., Japan) of 0.2 μm average particle size in PBS and a solution of 0.01% (w/v) pepsin solubilized collagen in PBS were prepared separately. Then anatase powder was added to 0.1% (w/v) to a 0.01% pepsin-solubilized collagen solution and after gentle mixing, all three preparations were incubated at 37°C for 30 min, without shaking.

Microscopic observation of a mixture of anatase powders and insoluble collagens

Insoluble bovine collagen fibres were obtained from bovine skin, as previously reported (*11*, *12*). Suspensions of 0.1% (w/v) insoluble bovine skin collagen and 0.1% (w/v) anatase powders in PBS were mixed at room temperature with gentle shaking. After washing the mixtures by decantation with PBS, precipitates were observed with bright-field light microscopy and by crossed polarized light microscopy using a Zeiss Primotech D/A microscope (Supplementary Data). The distribution of the anatase powders on the collagen surface was observed by scanning electron microscopy (SEM MA10; Zeiss, Cambridge, UK). Samples for SEM were rinsed with PBS to remove loose powders. The specimens were then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 at 20°C for 10 min, dehydrated in a series of ethanol solutions from 20 to 100%. 100% ethanol was then replaced with hexamethyldisilazane (Sigma, Aldrich, UK), which was evaporated to dryness. Samples were imaged following gold sputter coating.

Results

The interaction between collagen and Ti was studied in three ways. First, by chromatography, secondly by observation the aggregation of collagen with anatase powder by eye, and finally by demonstrating the positive association of anatase powders and Ti-beads with insoluble collagen fibres using regular and polarized light microscopy (Supplementary Data) and SEM.

Chromatographic demonstration of binding between collagen solution and Ti

In the liquid chromatography system, the stationary phase consisted of Ti-beads and the three different solutions described above were used for the liquid phase. According to the principle of chromatography, if a substance is applied which has no binding capacity for the stationary phase and is run through dissolved in the liquid phase, it will pass through the column and be eluted out in the pass-through fraction. Contrastingly, if a substance has any binding capacity for the stationary phase, in this case, Ti-beads, it will

remain in the top of the column and wash out only when a solution is run through that cleaves the bonds between the substance and TiO_2. Based on the above proposition, I-AC, I-PC and their heat-denatured products were chosen as the test substances. 25 mM NaOH was used as a wash-out solution since we verified that this concentration washed out all of the collagen bound to the TiO_2. Thus, collagen in the pass-through peak(s) is the fraction with no binding affinity for Ti whilst that in the wash-out peak, is the fraction with some affinity to Ti. Because 25 mM NaOH removes all of the collagen bound with Ti-column, the sum of the collagen in both peaks is equivalent to the applied amount of the collagen. The percentage of the wash-out peak to the total indicates the strength of binding ability (the bound ratio) (Table I).

In addition to the most common solvent, dilute HCl (pH 4), we chose two other solutions: 2M urea/PBS (pH 7.4) and 2M urea/HCl (pH 3), to avoid aggregation of collagen solution, if any, in the column, as explained above.

Figure 1A shows the four chromatograms obtained using dilute HCl (pH 4) as a solvent. The combined chromatogram (a) compares the patterns of the native (upper part) and the denatured (lower part) acid-soluble type I collagen (I-AC). Similarly, (b) shows the results of the native (upper part) and denatured (lower part) of pepsin-soluble type I collagens (I-PC). Black arrows in all chromatograms indicate the points of change from diluted HCl (pH 4) to 25 mM NaOH.

When native collagen (upper chromatogram) and denatured collagen (lower chromatogram) in Fig. 1A are compared, it is clear that the (first) pass-through peak increases and the wash-out peak (the second peak) decreases after denaturation. These changes are expressed more clearly as percentages of the wash-out peak to the total amount of collagen applied (Table I). The areas of each peak in the chromatogram were determined using Image-J, and the average calculated from more than three chromatography runs with the same collagen samples. $30.4 \pm 2.4\%$ of the total amount of native I-AC bound to the Ti-column (Table I and Fig. 1Aa upper chromatogram). After denaturation, this percentage reduced to $19.3 \pm 3.6\%$ (Table I and Fig. 1Aa lower chromatogram). Likewise, the percentage of bound collagen (bound ratio) of I-PC to the total charged I-PC was $32.2 \pm 6.1\%$ (Table I and Fig. 1Ab upper) in the native state, which decreased to $20.6 \pm 4.4\%$ after denaturation (Table I and Fig. 1Ab lower). These differences between the peak areas obtained with the native and denatured collagen were significant ($P < 0.05$ according to Student's t-test).

Figure 1B (a and b) shows the results using 2M urea/PBS pH 7.4. Under these solvent conditions, $26.8 \pm 3.2\%$ and $25.9 \pm 4.1\%$ of I-AC and I-PC respectively bound to the column in the native state (Table I). The results indicate that 26–27% of collagen molecules bound to TiO_2, irrespective of telopeptide. But after heat denaturation, the bound amounts of I-AC and I-PC decreased to $17.2 \pm 3.2\%$ and $11.6 \pm 2.5\%$, respectively.

Figure 1C (a and b) shows the results with 2M urea/HCl, pH 3.0. Even with this more potent dissociative solvent, $27.1 \pm 3.5\%$, and $17.0 \pm 2.3\%$, of the native I-AC and denatured I-AC, respectively, bound to the column (Table I). With I-PC, the percentages of bound collagen were markedly higher at $40.8 \pm 3.5\%$ and $24.1 \pm 2.3\%$ for native and denatured forms, respectively. With all the native collagens, some tailing-peaks accompanied the pass-through peaks, but with the denatured collagens, these were not observed (Fig. 1A–C).

Table I summarizes the percentages of the various collagens bound to Ti with the three different solvents, dilute HCl, 2M urea/PBS and 2M urea/HCl (pH 3.0). The figures indicate the ratio (%) of the bound fraction (wash-out peaks with 0.025 M NaOH) to the total areas of the applied sample (sum of pass-through fractions and bound fraction), which corresponds to the Ti-binding ability of each collagen. There appeared to be no essential difference in the chromatographic profiles with the three different solvents used as eluents. However, we found marked differences between the ratios of the pass-through fraction and wash-out peaks (the binding ratios) before and after heat denaturation. In the case of the native pepsin solubilized collagen (I-PC), we observed that the binding ratio showed the highest value of $40.8 \pm 3.5\%$ adsorption in 2M urea (pH 3.0), while the Ti-binding ratio was reduced to $24.1 \pm 2.3\%$ after heat denaturation.

Most interestingly, the Ti-binding ratios decreased after denaturation. These changes occurred with all collagens analysed, with all three solvents, regardless of the presence of telopeptides, as summarized in Table I. This is the first evidence that type I collagen can bind to TiO_2 in dilute HCl (pH 4.0), 2M urea (pH 7.4) and 2M urea/HCl (pH 3.0). Moreover, the

Table I. The ratios (percent) of bound/applied collagen to Ti-column of the acid-soluble collagen (I-AC) and pepsin solubilized collagen (I-PC)

Collagen	Elution buffer		
	Diluted HCl pH 4.0	2M urea/PBS pH 7.4	2M urea/HCl pH 3.0
Acid-soluble collagen (I-AC)			
Native	30.4 ± 2.4	26.8 ± 3.2	27.1 ± 3.5
Denatured	19.3 ± 3.6	17.2 ± 3.2	17.0 ± 2.3
Pepsin-solubilized collagen (I-PC)			
Native	32.2 ± 6.1	25.9 ± 4.1	40.8 ± 3.5
Denatured	20.6 ± 4.4	11.6 ± 2.5	24.1 ± 2.3

In all the Ti-column chromatography, we applied 9 mg of collagen in a 3 ml buffer.

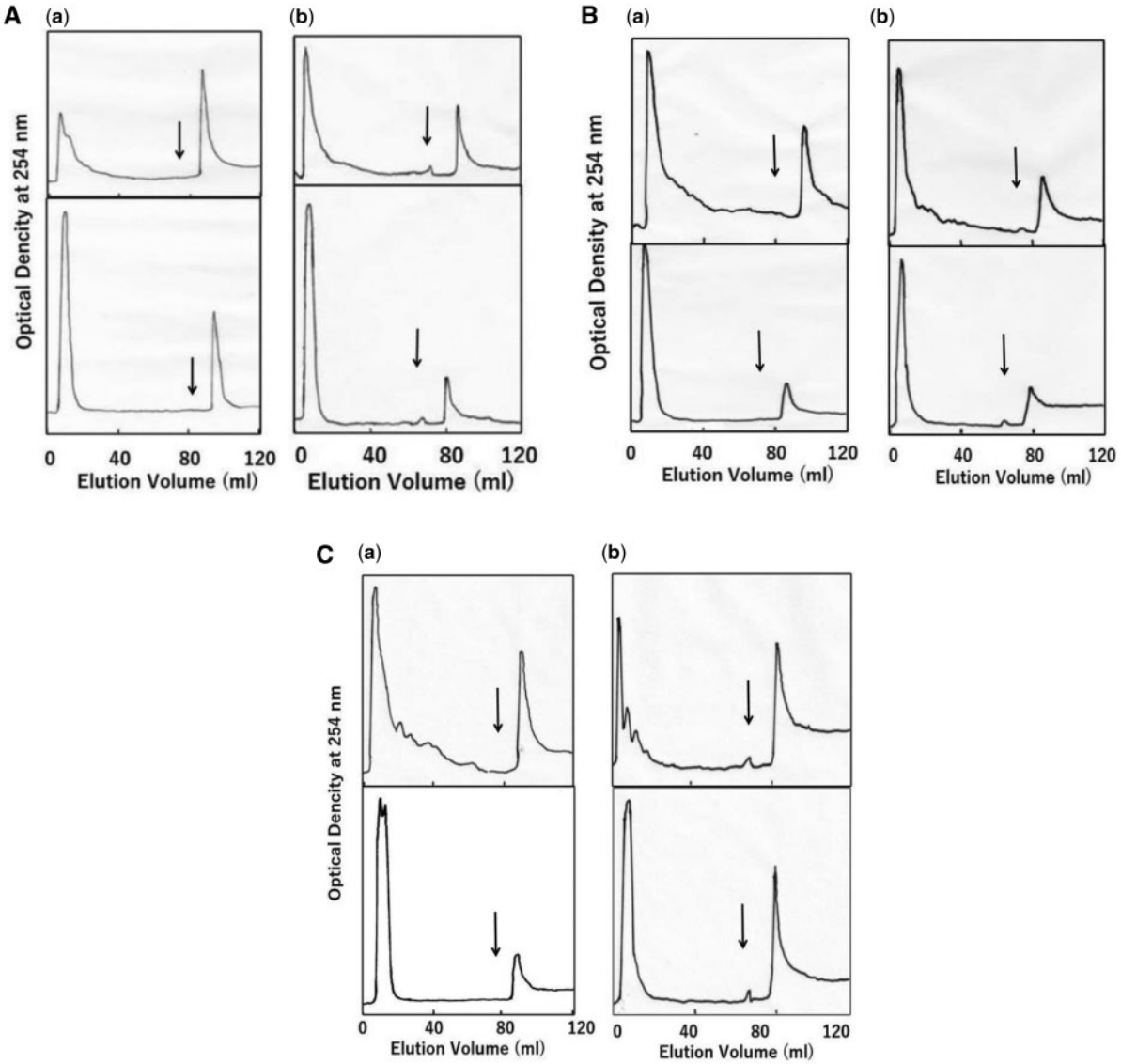


Fig. 1. (**A**) Ti-chromatograms of collagen eluted with dilute HCl at pH 4.0. (**a**) Acid-soluble collagen (9 mg) was applied to a Ti bead packed column (bed volume ø16 × 50 mm), eluted with dilute HCl, pH 4.0 and washed out with 25 mM NaOH after the first fraction had passed through, as indicated by the arrow. Eluted collagen was monitored at 254 nm. The upper and lower chromatograms show the result before and after denaturation at 95°C for 15 min, respectively. (**b**) Pepsin solubilized collagen (9 mg) in the same elution system before (upper) and after heat denaturation (lower), as in (a). (**B**) Ti-chromatograms of collagen eluted with 2M urea at pH 7.4. (**a**) Acid-soluble collagen (9 mg) was applied to a Ti-column eluted with 2 M urea at pH 7.4 and washed out with 25 mM NaOH as described for Fig. 1A. (**b**) Pepsin solubilized collagen (9 mg) eluted with 2 M urea/PBS at pH 7.4. The upper and lower chromatograms show the results before and after denaturation at 95°C for 15 min, respectively. (**C**) Ti-chromatograms of collagen eluted with 2M urea/HCl, pH 3.0. (**a**) I-AC (9 mg) applied to a Ti-bead packed column eluted with 2 M urea/HCl at pH 3.0 and washed out with 25 mM NaOH/2 M urea as described for Fig 1A. (**b**) Pepsin solubilized collagen (9 mg) was analysed in the same elution system. The upper and lower chromatograms show the results before and after denaturation at 95°C for 15 min, respectively.

Ti-binding ratio diminished to approximately 2/3 after the denaturation of the collagen. We obtained a similar reduction of the Ti-binding ratio after denaturation in I-AC and I-PC, as shown in Table I. These reductions were all statistically significant with Student's test, with *P*-values of 0.003, 0.0015 and 0.028, with the three different solvents, respectively.

The importance of telopeptides in the binding is indicated by comparing the chromatograms of I-AC and PC in the different solvents. When dilute HCl (pH 4.0) was used as a solvent, the Ti-binding ratios between I-AC (30.4%) and PC (32.2%) were not significantly different (*P*: 0.56). This difference was much smaller in the denatured collagen, 19.3% in I-AC, and 20.6% in I-PC (*P*: 0.72). However, in the dissociative solvent of 2M urea/HCl (pH 3.0), the percentage of the Ti-binding of I-PC was the highest value, at 40.8%, considerably higher than of I-AC, at 27.1%. This indicates that native I-PC, but not native I-AC, could most efficiently bind to TiO_2 under these conditions.

Effect of anatase powders on collagen aggregation

Figure 2A shows the suspension of anatase powder (0.1% in PBS) the addition of collagen. It was pale grey in appearance with slight precipitations. When I-PC was added to PBS at a final concentration of

0.01% and incubated at 37°C for 30 min, grey cloudy amorphous precipitates appeared (Fig. 2B). But when the same amount of collagen was mixed with the anatase suspension and shaken gently at 37°C, white flocculating fibres appeared (Fig. 2C). This mixing experiment clearly showed an association between the aggregating collagen and the anatase powders.

Positive interaction of insoluble collagen with anatase powders and Ti- beads

Light microscopic observation of the insoluble skin collagen fibres (Fig. 3A) revealed a positive interaction of collagen with anatase powders (Fig. 3B) and titanium beads (Fig. 3D). We observed dense aggregates of attached anatase powder particles (Fig. 3C). In Fig. 3E and F, even the larger Ti-beads with an average diameter of 45 µm (Fig. 4D), appeared to be attached to the collagen fibres. These interactions were also observed using polarized light microscopy. Due to its birefringence, in crossed polarized light, the collagen fibres appeared coloured (*13*), and the attached black anatase powder particles were more clearly discernable. Black particles of anatase covered the lengths of both individual fibrils and bundles of fibres **(**Supplementary Data**)**.

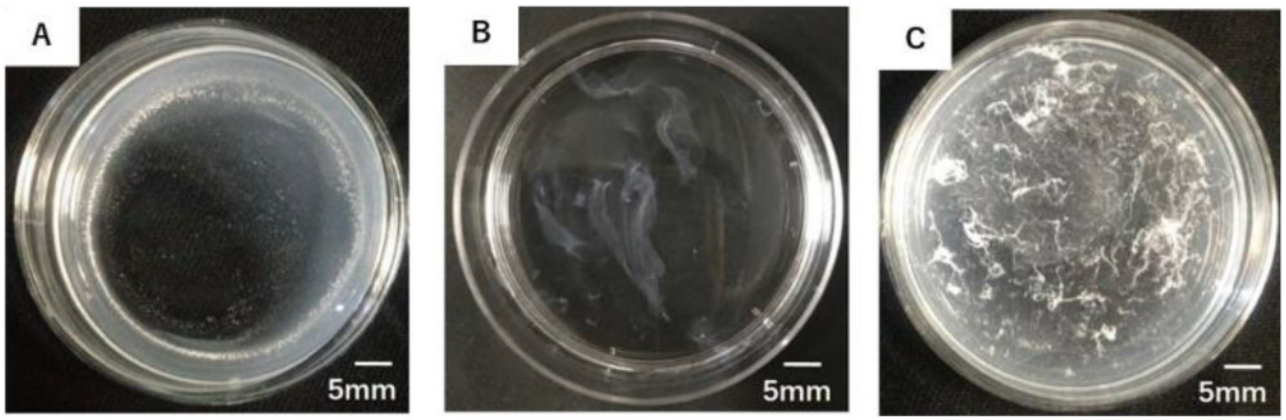


Fig. 2. Effect of anatase powders on the collagen aggregation. Photographs showing (**A**) Suspension of 0.1% (w/v) anatase powders (0.2 µm average size) in PBS, (**B**) 0.01% (w/v) pepsin solubilized collagen incubated at 37°C for 30 min and (**C**) Collagen and anatase mixture is shaken gently at 37°C for 30 min (C).

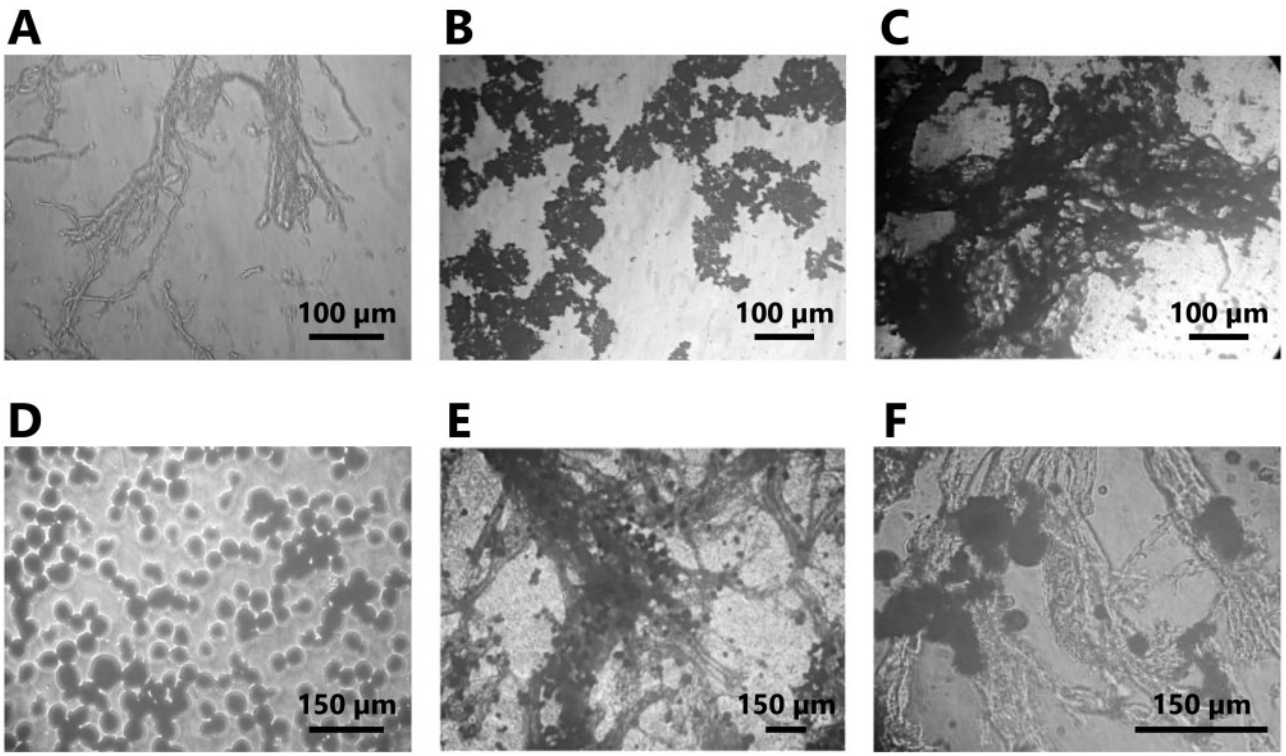


Fig. 3. Associations of anatase powders (**A**–**C**) and Ti-beads (**D**–**F**) with insoluble skin collagen. Light microscope images of insoluble skin collagen fibres (A), anatase powders (B), Ti-beads, average diameter 45 µm (D), attached anatase powders on collagen fibres (C), Ti-beads (D), Ti-beads on collagen fibres (E), enlarged view of D showing Ti-beads attached to the collagen fibres. All materials were suspended in PBS. The scale bars in A, B and C indicates 100 µm, and D, E and F indicate 150 µm.

Scanning electron microscopy

Figure 4 shows scanning microscopic images indicating the attachment of anatase powders to insoluble collagen fibres (Fig. 4A). Anatase powders consist of primary particles (0.1–0.2 μm) and clusters of 2–10 μm (Fig. 4B). Both individual particles and clusters adhered to the surface of collagen fibres (Fig. 4C). The appearance of the collagen surface without particles is visible in the background.

Determination of maximum bound collagen on Ti column

In most of the chromatographic analyses in this study, 9.0 mg of soluble collagen was applied to the column. However, when the amount of collagen is altered, the ratio of bound collagen will change, enabling us to estimate the maximum binding capacity. A series of different amounts of I-AC and denatured I-AC were applied to the column to determine this.

Figure 5A indicates that the highest binding ratios were obtained with the smaller amounts of bound collagen, with a ratio of 96% being achieved with 0.3 mg applied collagen. On the other hand, the Ti-binding ratio decreased more gradually above 9 mg. Observing the changes in the quantity of collagen retained in Fig. 5B, the maximum amount of collagen, which can bind, was estimated to be less than 3 mg. From these experiments, the average amounts of bound collagen per column were estimated to be 2.7 mg for native I-AC and 2.0 mg for denatured I-AC. Since the weight of Ti-beads was 11.3 g, this means that 1 g of Ti-beads can bind a maximum of 0.24 ± 0.005 mg and 0.18 ± 0.002 mg of native I-AC and denatured I-AC, respectively. The 25% decreased binding after

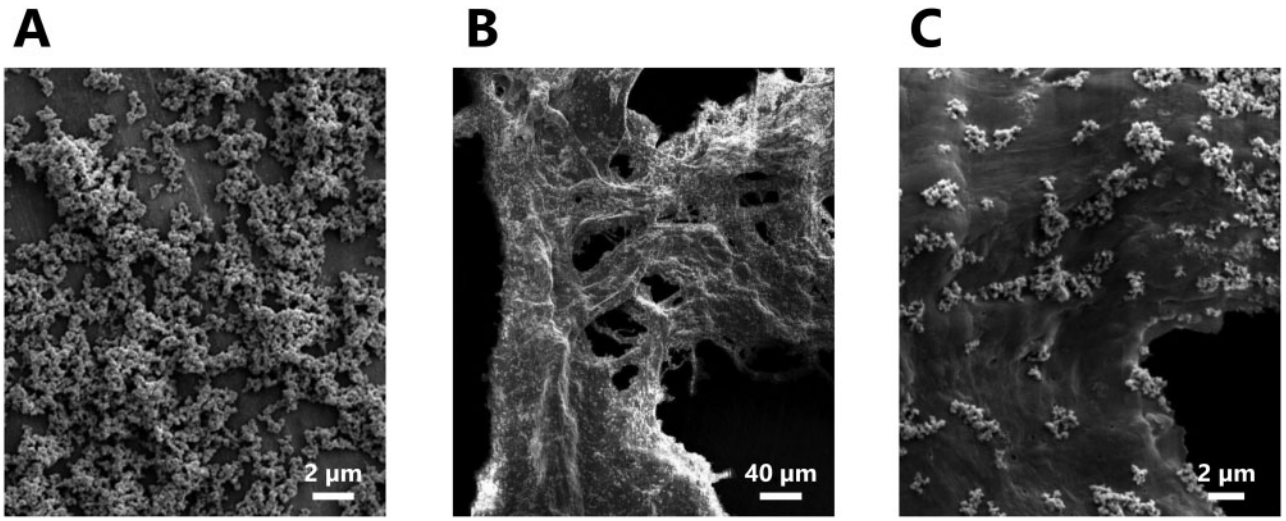


Fig. 4. Scanning microscopic images of the positive association between the anatase powders and insoluble collagen fibres. Anatase powders (0.2 μm) from the suspension used in the experiment, showing clustering, scale bar = 2 μm (**A**); insoluble skin collagen with attached anatase powders at low magnification, scale bar = 20 μm (**B**); higher magnification of B showing clusters of attached anatase powders, scale bar = 2 μm (**C**).

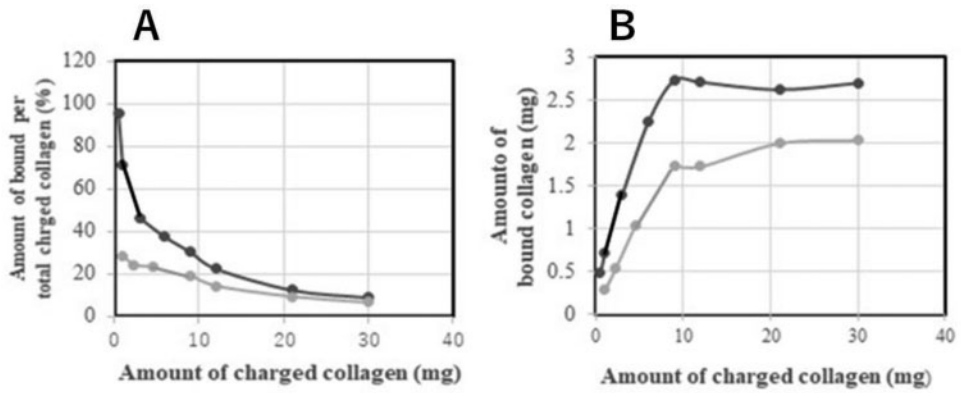


Fig. 5. Dose-dependent binding of collagen to titanium. Different amounts of I-AC and denatured I-AC collagen (9.0–30 mg) were applied to the same Ti-bead packed column (1.2 × 5.0 cm) and run under the same conditions with an eluent of dilute HCl, pH 4. The flow rate was 120 ml/h. The solid lines indicate native I-AC and, the gray lines denatured I-AC. (**A**) The influence of the amount of collagen charged to the column on the bound collagen as a percentage of the total applied (the binding ratios). As the applied amount increased, the ratio of binding decreased down to 9% with 30 mg collagen. (**B**) The amount of bound collagen versus the quantity of collagen charged to the column. An asymptote appeared at quantities above 9 mg with I-AC, and 20 mg with denatured I-AC. The average amounts of bound collagen at 21 and 30 mg were 2.7 mg for native I-AC, and 2.0 mg for denatured I-AC.

denaturation indicates the importance of the helical structure of collagen for binding to TiO_2.

Discussion

From the above results, we concluded that the destruction of the helical structure significantly reduces the binding ability of collagen with TiO_2 but does not entirely abolish it. We assume that the triple helicity in collagen is important for the Ti-binding ability and that three-dimensional alignment of the reactive chemical groups, probably periodic charged amino acid sequences, may be a significant factor for Ti-binding, which we will discuss in more detail later.

We also concluded that in 2M urea/HCl (pH 3.0), the presence of the telopeptide in I-AC may have some inhibitory function on the binding with collagen and TiO_2. We assume that in this solution, acid pepsin solubilized molecules (I-PC) can interact more freely with TiO_2 than in I-AC, which lacks the telopeptide.

All the surface of pure titanium, once exposed to open air or aqueous solutions, is covered by a thin layer of TiO_2, which consists of anatase type crystals, and exhibits a characteristic colour, depending upon the thickness (*7–9*). Therefore, our topic more precisely concerns the interaction of TiO_2 with collagen gene products. Thus, we used anatase powder to study its interaction with collagen and observed enrichment of fibrous collagen aggregates when we added the powder particles to the collagen fibre-forming system, as shown in Fig. 2A–C. We also observed the association of anatase powders to the preformed-formed collagen fibres (insoluble collagen), as in Fig. 3A–C.

Since collagen molecules bind to TiO_2, it is reasonable to assume that the anatase particles' attachment to the collagen molecules accelerates the aggregation process (Fig. 3C). We believe that individual particles of anatase attract soluble collagen molecules. The local collagen density increases, thereby enabling neighbouring collagen molecules to bind to each other to form the visible fibrous aggregates. We did not characterize the aggregates in detail, and they may not be the standard physiological collagen fibres with the typical 'D' (67 nm) periodicity. Interestingly, the increase in fibrous collagen on the addition of TiO_2 presented a different appearance when we used rutile instead of anatase (Kuboki *et al.*, unpublished data). Anatase and rutile are of the same chemical composition but the size of the unit cell of anatase is $0.949 \times 0.378 \times 0.378$ nm, while that of rutile is $0.295 \times 0.458 \times 0.458$ nm (*14*). This fundamental difference will influence their interaction with collagen molecules.

The effects of the anatase powders on collagen fibre formation have relevance for medical implantation of Ti-devices into living tissues. Hayashi and Nagai (*15*) demonstrated that various factors affected collagen fibre formation but did not mention anatase. Our results further strengthen the evidence of binding between TiO_2 and collagen gene products.

Positive interactions between insoluble collagen and anatase powders were supported by polarized microscopy imaging (Supplementary Data). The colours observed are due to collagen's birefringence (*13*) and interference patterns produced as the light rays recombine. This colour contrast allowed us to visualize the fibres suspended in water more clearly.

By using scanning electron microscopy, as shown in Fig. 4A–F, we confirmed the size of Ti beads and showed their clustering. Although the clusters could be partly due to the sample preparation, the images suggest that the powders of anatase are distributed relatively regularly on the collagen fibre surface. The above three imaging experiments with insoluble collagen fibres and anatase powders or Ti-beads provided morphological evidence for the interaction between insoluble collagen and TiO_2, supporting our previous conclusion.

Regarding the chromatographic difference between natural and denatured collagen (gelatin), we have already highlighted the importance of the three-dimensional alignment of the reactive chemical groups, which are probably periodic charged amino acid sequences. We propose that the Ti binding sites with collagen and gelatin are the same two-dimensionally, but differ three-dimensionally and that regularly placed charged amino acids may line up with repetitive features of the anatase lattice. Gelatin, without the triple helix structure, also has the periodic sequences of charged amino acids, but the charged amino acids do not line up, which is essential for the Ti-binding ability.

McLachlan (*16*) showed that there are many periodic patterns in the amino acid sequences in collagen. The most eminent and most extended periodicity is the 'D' length, which extends 67 nm along the length of typical collagen fibrils. There are many recurrences in the collagen molecule ranging from D/5 to D/39, which contain charged amino acid sequences (*16*). The anatase lattice is a tetragonal crystal (*14*). If there is periodic alignment between active sites of the two substances, we can explain the mechanism of binding more clearly, as follows.

Based on the results in this study, the interaction between native collagen and TiO_2 can at least be partially attributed to the formation of hydrogen and electrostatic bonds between the two substances, which are enabled by the alignment of periodically occurring charged amino acids on the surface of the triple-helical collagen molecules with the regularly positioned oxygen atoms in the TiO_2 (mainly anatase) crystal lattice. We propose the above explanation as a 'periodicity hypothesis'. In collagen type, I, the numerous longitudinally arranged, periodically occurring polar amino acids give rise to the characteristic periodic striations that can be observed by electron microscopy after staining with phosphotungstic acid (*17–19*). On the other hands, the lattice structure of anatase crystals exhibits a regular arrangement of oxygen and Ti atoms due to the unit cell of anatase (*14*). If alignment occurs, numerous hydrogen and electrostatic bonds will form between the polar amino acids on the surface of collagen molecules and the oxygen atoms in the crystal lattice of TiO_2.

The Ti-beads used in this study are spherical, with an average diameter of 45 μm, whilst the anatase powders, whose component particles, with an average size

of 0.2 μm, should provide sufficient binding sites to enable binding to the triple helices of collagen (the length of which is about 0.3 μm).

All interactions have so far been tested in aqueous solutions, but the role of water in the binding is still unknown. We hypothesize that the water molecule may form a bridge between the oxygen atoms in TiO_2 lattice, as well as the hydroxyl, carboxyl, and phosphate groups of the collagen molecule.

Concerning the specificity of Ti-binding proteins, we previously confirmed that albumin and lysozyme did not bind with the Ti-column (less than 5%), but that protein which is rich in phosphoserine or phosphothreonine such as phosvitin, caseins, phosphophoryn and bone phosphorylated proteins [small the integrin-binding ligand, N-linked glycoprotein (SIBLING) family] bound well with TiO_2 in the column (50–70%), in addition to fibronectin (about 50%) and collagen (this study). Recently, we found that phosphorylated chitin also binds with TiO_2 (*20*, *21*). Moreover, it was recently reported that collagen itself could be enzymatically phosphorylated (*22*). The above results led us to a tentative conclusion that the high-molecular substances with the periodic alignment of multiple acidic residues may bind with Ti. However, the binding mechanism of fibronectin with TiO_2 is not understood at present.

Titanium is the only metal to which osteoblasts can adhere and grow forming bone tissue. Therefore, it is the most useful metal for mechanical supports in various medical applications, especially the reconstruction of bone and teeth. The biochemical interactions of this metal and biomolecules are increasingly attracting attention (*23*–*25*). Chromatographic and visual confirmations of the interactions between collagen and Ti contribute to our understanding of the importance of collagen in the support and maintenance of clinical Ti devices and may be useful in designing and developing new artificial organs.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

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