Fibrillar assembly and stability of collagen coating on titanium for improved osteoblast responses

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Abstract: Collagen, as a major constituent of human connective tissues, has been regarded as one of the most important biomaterials. As a coating moiety on Ti hard-tissue implants, the collagen has recently attracted a great deal of attention. This article reports the effects of fibrillar assembly and crosslinking of collagen on its chemical stability and the subsequent osteoblastic responses. The fibrillar self-assembly of collagen was carried out by incubating acid-dissolved collagen in an ionic-buffered medium at 37°C. The degree of assembly was varied with the incubation time and monitored by the turbidity change. The differently assembled collagen was coated on the Ti and crosslinked with a carbodiimide derivative. The partially assembled collagen contained fibrils with varying diameters as well as nonfibrillar aggregates. On the other hand, the fully assembled collagen showed the complete formation of fibrils with uniform diameters of ~100–200 nm with periodic stain patterns within the fibrils, which are typical of native collagen fibers. Through this fibrillar assembly, the collagen coating had significantly improved chemical stability in both the saline and collagenase media. The subsequent crosslinking step also improved the stability of the collagen coating, particularly in the un assembled collagen. The fibrillar assembly and the crosslinking of collagen significantly influenced the osteoblastic cell responses. Without the assembly, the collagen layer on Ti adversely affected the cell attachment and proliferation. However, those cellular responses were improved significantly when the collagen was assembled to fibrils and the assembly degree was increased. After crosslinking the collagen coating, these cellular responses were significantly enhanced in the case of the unassembled collagen but were not altered much in the assembled collagen. Based on these observations, it is suggested that the fibrillar assembly and the crosslinking of collagen require careful considerations in the collagen administration as a coating moiety. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 629 – 638, 2005

Key words: collagen coating on Ti; fibril assembly; crosslinking; chemical stability; cell responses

INTRODUCTION

Collagen is the main component of extracellular matrix proteins in the hard and soft tissues, such as bone, dentin, cartilage, tendon, cornea, and skin.1 Therefore, a variety of clinical applications have been proposed and developed over the last decades. A significant stimulus to the demands on biomaterials and tissue engineering has highlighted the potential on native organic matrix, including collagen.2–4 In bone biology, collagen (hereafter means type I collagen) plays an indispensable role in the bone formation process, such as initial cellular responses and subsequent matrix syntheses, as well as in the maintenance of the mechanical functions.5,6 The most intriguing characteristic of collagen is the ability to self-assemble into fibrils, resulting in a hierarchical tissue structure. The structural hierarchy generated by the collagen assembly provides the mechanical resilience and toughness needed for the tissues to function efficiently.6

Fibrillar collagens are known to play a significant role in the initial cell attachment through the mediation of integrin receptor and the following cellular responses, such as movement, growth, and matrix synthesis.7–9 At the very center, the structural characteristics of collagen, such as the amino acid sequence, the helical structure, the fibrillar level, and the size and shape of the large-scale fibers, have a significant impact on its biological and mechanical functions.

Thus far, various types of collagen have been extracted from animals, and some are commercially available for use as biomaterials. Almost identically, the extracted collagen displays a fibrillar assembly process under both in vitro and in vivo conditions.10–17 In utilizing this in vitro assembly character of collagen,
its diverse formulations, such as porous scaffolds, membranes, and coatings on substrate, have been devised. For the most part, their applications have been limited to soft tissues because the mechanical properties of collagen do not fit the level that the bones and teeth require. To improve the mechanical properties, inorganic minerals were introduced to the collagen matrix to produce composites. However, they showed still limited success in view of the mechanical properties, which are satisfactory for real applications.  

Undoubtedly, titanium (Ti) is currently the most widely used hard-tissue implant. Because its surface properties are of utmost importance in determining the biocompatibility, a significant attempt has been directed on the surface modification of Ti, such as sandblasting, acid-etching, oxidation, and calcium phosphate coating. As a surface modification moiety, the native proteins are believed to be a strong candidate. Recent studies on a series of protein coatings on Ti have demonstrated their effective roles in the stimulation of cellular responses, such as attachment, proliferation, phenotype expression and protein synthesis. Collagen, being the main constituting protein of the hard tissues, has the potential as a coating moiety on a Ti implant. Therefore, a level of studies on the collagen coating on Ti have been carried out, focusing on the development of a coating methodology and the effects on the biological properties in vitro and in vivo. However, there has been little focus on the characteristics of the collagen coatings. As a result, there is some controversy as to their biological efficacy, eliciting either positive or negative or even null effect on cell attachment, proliferation, and protein synthesis, thereby making it difficult to draw any general conclusions as to the effectiveness of collagen as a coating layer.  

The diversity observed in vitro was believed to be attributed to the differences in the collagen characteristics, mainly to the fibrillar assembly level and the chemical stability. Practically, in the collagen coatings engineered thus far, little consideration has been made on the levels of the fibrillar assembly and its chemical stability. Therefore, in this study we addressed these points as follows: (1) the degree of the fibrillar assembly was controlled by varying the in vitro conditions, (2) the novel crosslinking process was used to improve the chemical stability. The effects of the fibrillar assembly and crosslinking of collagen on its chemical stability as well as on the subsequent osteoblastic cell responses were assessed.

MATERIALS AND METHODS

Collagen fibrillar assembly and coating on Ti

As a substrate for the collagen coating, commercially pure Ti (cp Ti, grade II) was prepared in a disc type (10 × 10 × 1 mm) after polishing with SiC paper #1500 and cleaning in acetone and ethanol/water ultrasonically.  

The acid soluble type I collagen (MW 300,000, RegenMed Inc., South Korea) was dissolved in 50 mM acetic acid at a concentration of 2.5% w/v. The solution was diluted with an equivalent volume of a phosphate-buffered solution (PBS) and then stored at 4°C for fibrillar assembly and coating processes later. To produce the fibrillar form of the collagen, the collagen solution was diluted to 1/10 in PBS (with pH being adjusted at ~4.2) and self-assembled with incubation at 37°C for different periods (from 10 min to 24 h). The degree of the fibrillar assembly with the incubation time was quantified by measuring the absorbance at 313 nm using a spectrophotometer (UV1700, Pharmaspec, Shimadzu, Japan), as the turbidity changed. Based on the turbidity data, two different periods (1 and 8 h) were determined for a further coating process because their turbidities showed a big difference. For the purpose of comparison, the collagen solution without the self-assembly was also prepared after dilution with PBS to 1/10.  

The collagen solutions of 400 μL with and without the fibrillar assembly at the two time points (1 and 8 h) were homogenized slightly and coated on the Ti discs. After immobilization for 60 min at 4°C the remnant was removed gently, followed by washing twice with distilled water and drying. Parts of the collagen-coated Ti were subsequently crosslinked using EDC/NHS solution in ethanol (EDC/NHS at 1:1 each 10 mg/mL) and then stored at 4°C for further tests.  

Quantification of collagen  

The amount of collagen coated on the Ti was quantified using a Sirius Red dye assay, with a slight modification of the method described by Walsh et al. First, 1 mL of the Sirius Red F3BA (Poole, UK) solution, which was diluted to 0.1% in saturated picric acid, was added to the culture plates containing the coated samples and then left at room temperature for 1 h in dark. After rinsing with 1 mL of 0.01 N HCl (three times) until the solution was colorless, the samples were treated with 200 μL of 0.1 N NaOH to elute the red color dye. Aliquots of the eluent were transferred to 96-well plates and the absorbance was read at 540 nm using a microplate reader (Model 550, Hercules, CA). The absorbance was converted to a collagen level when referenced to a standard curve, which was obtained as follows: the 2.5% collagen solution in acid/PBS was diluted with PBS to a concentration of 0.1% (1 mg/mL). The solution was again diluted with PBS to a series of concentrations (from 1000 to 15,625 μg/mL). Aliquots (50 μL) of the diluted solution were transferred to microtubes, and incubated at 37°C for 24 h with sealing and then at 37°C for 24 h without sealing. The dried remnants were assessed using the Sirius Red dye method, as described above, to obtain a standard plot (absorbance vs. collagen content).
Characterization and chemical stability test

The morphology of the collagen coated on the Ti was observed using field emission scanning electron spectroscopy (FE-SEM; JSM 6330F, JEOL) at accelerating voltages of 5–10 kV after gold coating the samples. The assembled collagen was characterized by transmission electron microscopy (TEM; CM20, Philips) after placing the assembled collagen solution on a carbon coated copper grid at an accelerating voltage of 200 kV.

The chemical stability of the collagen coated on the Ti was assessed using two different media, the Dulbecco’s Modified Eagle Medium (DMEM, Gibco) for a hydrolytic degradation and collagenase (for type I collagen, the original >125 CDU/mg, Sigma) diluted to 1/100 in distilled water for enzymatic degradation. The coated samples were incubated in 1 mL of the two media for different time periods at 37°C. After incubation, the samples were removed and washed twice with PBS, and the amount of collagen remaining on the Ti was then quantified using the Sirius Red dye method, as described above, and normalized to the initial collagen content prior to the degradation tests.

Cellular responses

Osteoblastic MG63 cells were used to observe the level of cell attachment and proliferation on the collagen-coated Ti, because this cell line exhibits the traits of osteoblast primary cells relatively well at such initial stages. The culture medium was comprised of 10 mL DMEM, 2 mM l-glutamine, 50 IU/mL penicillin and 50 mg/mL streptomycin. After subculturing, the MG63 cells were seeded at a density of 3 × 10⁴ cells/mL on each sample and incubated at 37°C in an incubator humidified with 5% CO₂/95% air for 3 h and 4 days to assess the level of attachment and proliferation, respectively. The cell attachment and proliferation level was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method. At each culturing period, MTT was added to each well and incubated at 37°C for 4 h. The blue formazan product was dissolved in 1 mL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader. The cell morphology at each culturing period was observed using FESEM after fixing the cells with 2.5% glutaraldehyde, dehydrating them with a graded series of ethanols (70, 90, and 100%), and gold coating the samples.

Statistics

The degradation and cellular response tests were performed on six replicate samples for each condition, and data were represented as mean ± 1 SD for n = 6. The statistical analysis was carried out using a Student’s t-test, and statistical significance was considered at p < 0.05 and p < 0.01.

RESULTS

Collagen assembly and morphology

For the quantification of collagen, a standard curve was obtained using a Sirius Red dye method as shown in Figure 1, which is represented as the optical density versus the collagen content in a microtube. Below a concentration of ~35 μg/tube, the optical density showed reasonable linearity with respect to the collagen concentration. A linear regression made within the range (~35 μg/tube) resulted in the relationship: absorbance = 0.00413 X + 0.00033 (R² = 0.99). In the following coating and degradation tests, this curve was used to assess the quantity of collagen on Ti.

Prior to the collagen coating, the collagen solution was assembled into fibrils by incubation at 37°C. The degree of the fibrillar assembly was measured by the change in turbidity of the solution. Figure 2 shows the optical density of the collagen solution (turbidity) at 313 nm with respect to the assembly time of up to 24 h at various collagen concentrations. The collagen assembly was more pronounced at the higher collagen concentration and all time points. At all concentrations, the absorbance was measured at 570 nm using a microplate reader. The cell morphology at each culturing period was observed using FESEM after fixing the cells with 2.5% glutaraldehyde, dehydrating them with a graded series of ethanols (70, 90, and 100%), and gold coating the samples.
bly, the collagen coating did not show any morphological difference from the pure Ti substrate [Fig. 3(a, b)]. However, with assembly for 1 h, the collagen coating had a fibrillar morphology, and the fibril diameters were approximately 100–200 nm [Fig. 3(c, d)]. When the assembly time was extended to 8 h, the fibrillar formation was more noticeable, but the fibril diameter appeared to change little [Fig. 3(e, f)]. When the collagen coatings were crosslinked, the morphologies were similar to those without the crosslinking (data not shown here).

TEM observation revealed the morphology of the assembled collagen more clearly, as shown in Figure 4. The collagen assembled for 1 h [Fig. 4(a–c)] showed a mixed state, that is large-sized fibrils (area “1,” with diameters of ~100–200 nm, as clearly observed also in SEM image), small-sized fibrils (area “2,” with diameters of ~10–20 nm) and left aggregates (area “3”). This feature can be explained by the fact that at 1 h, the assembly of collagen was in the dynamic process of the fibrillar formation.11,12 The collagen assembled for 8 h was almost in the form of large-sized fibrils with ~100–200 nm diameters [Fig. 4(d–f)]. On closer examination, the fibrils typically showed the presence of bands with ~67 nm periodicity.11

**Amount and degradation of collagen coating**

The amount of collagen coated at different states was quantified using the Sirius Red dye method, as shown in Figure 5. The assembled collagen was coated to a higher level than the unassembled one. After crosslinking, the coated-collagen content was slightly lower. In the case of the unassembled collagen coating, a higher concentration of collagen solution (1%) was needed to reach a level similar to the fully-assembled collagen coating (0.4% for 8 h assembly).

The chemical stability of the collagen coatings on Ti were assessed using degradation tests in both DMEM and collagenase media. After the degradation tests, the coating residue was quantified using the Sirius Red dye method, as shown in Figure 6. After testing in DMEM for 3 days, the fibrillar-assembled collagen coating showed a significantly higher stability than the unassembled one [Fig. 6(a)]. The crosslinking of the collagen further increased the stability. In a collagenase medium for 24 h, the degradation of the collagen coating was more conspicuous for all cases [Fig. 6(b)]. The unassembled collagen (without crosslinking) degraded almost completely. However, with the fibrillar assembly, the collagen coating was sustained to a significantly higher level. The crosslinking process improved the enzymatic stability of the collagen, particularly of the unassembled one.

**Cellular responses to collagen coating**

The SEM morphology of the MG63 cells attached on the collagen-coated Ti after culturing for 3 h is presented in Figure 7. Pictures of the two conditions (0.4% collagen unassembled and 8 h-assembled, both without crosslinking) are shown as representative examples. The cells attached and spread well on both types of collagen coatings. However, the cells attached more on the assembled-collagen coating [Fig. 7(b)] than on the unassembled one [Fig. 7(a)]. The cell attachment level on the collagen coatings obtained at different conditions was quantified using an MTT assay, as shown in Figure 8. The MG63 cells were cultured for 3 h and subjected to an MTT assay. On the unassembled-collagen coatings, the level of cell attachment was reduced with respect to the pure Ti substrate, and this downregulation was significant at the higher collagen concentrations (0.4 and 1%). When the assembled collagen was coated, the level of cell attachment was increased with respect to the pure Ti. This improvement became more significant with increasing degree of assembly, and was more conspicuous at the higher collagen concentration. With regard to the crosslinking effect, the cell attachment was significantly improved in the unassembled-collagen coatings at the high concentrations (0.4 and 1%). On the assembled-collagen coatings, the crosslinking step did not significantly affect the level of cell attachment.

Figure 9 shows the proliferation of the MG63 cells on the collagen-coated Ti after culturing for 4 days. The proliferation trends were quite similar to but more prominent than those observed in the cell attachment.
Without assembly, the collagen coating reduced the level of cell proliferation significantly with respect to Ti. However, after crosslinking, the unassembled coatings showed similar levels of proliferation to the pure Ti. When the collagen was assembled, the level of cell proliferation on the coatings was significantly upregulated with respect to the pure Ti or to the unassembled-collagen coatings, particularly at the high collagen concentrations. The crosslinking of the assembled-collagen coatings did not appear to make any significant difference on the cell proliferation level.

**DISCUSSION**

In this study, collagen was administered as a surface modification moiety on a Ti implant for hard tissue applications. Compared with the significant level of research regarding its use as a soft tissue implant, the hard tissue applications of collagen have had limited success in its bulk form such as scaffolds or membranes. On the strong Ti substrate, which is currently regarded as a standard implant for load-bearing parts, collagen immobilization is considered to be a promising strategy. In this study, particular emphasis was placed on the collagen characteristics, mainly on the degree of the fibril assembly of the collagen molecules and the crosslinking because these are believed to affect the chemical stability and the cellular responses.

The atelocollagen used in this study is normally extracted from bovines (skin or tendon) and treated enzymatically to minimize the various antigenic and immunogenic problems. Therefore, it has been applied as tissue scaffolds and wound dressings as well.

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**Figure 3.** FESEM image of the collagen coated-Ti: collagen solution used for coating (a,b) without assembly and with assembly for (c,d) 1 h and (e,f) 8 h.
as in the studies of fibrillar formation. Normally, this collagen is dissolved in an acidic solution (such as acetic acid), and is then reconstituted into fibrils by means of increasing the temperature and/or neutralizing the pH. The ultrastructure of the fibrils depends on the medium conditions, such as pH, temperature, and ionic strength, as well as the sequence of the assembly steps. Among the choices in changing the degree of fibril formation, only the incubation period was varied in this study with all the other conditions fixed (pH at \(4.2\), temperature at \(37°C\), ionic strength of \(123 \text{ mM NaCl}\) and the assembly procedure as described in the Method section). The lateral interaction between the triple-helical domains basically accounts for the self-assembly process of the collagen. During this process, the fibrils exhibited a typical banded pattern, as a result of the specific parallel and mutually staggered alignment of the triple-helical domains of the collagen fibrils.

The degree of fibrils was measured using a turbidity test. Because the turbidity (OD at 313 nm) was created by the collagen assembly and is proportional to the degree of collagen fibril formation, the method has been used extensively to interpret the kinetics of the collagen assembly. As observed in Figure 2, the turbidity was increased within a short time (a few hours), and stabilized for a prolonged period. For convenience, two time points (1 and 8 h) were chosen for the coating solutions, where the turbidities were quite different, that is, the collagen was considered to be

Figure 4. TEM image of the collagen assembled for (a–c) 1 h and (d–f) 8 h. With assembly for 1 h, the collagen status was mixed, with large fibrils (area “1”), small fibrils (area “2”) and unrevealed aggregates (area “3”). With assembly for 8 h, the collagen was mainly in the large fibrils (diameter \(\sim 100–200 \text{ nm}\)), and the fibrils retained the typical periodic strands (arrowed in (e), \(-67 \text{ nm periodicity}\)).

Figure 5. Quantification of collagen on Ti after coating with the different collagen solutions. The coating with higher collagen concentration (1%) was also compared. Data were quantified using the Sirius Red dye method and normalized to the standard curve in Figure 1. Significantly higher values were observed \((\bullet p < 0.05 \text{ and } \bullet \bullet p < 0.01)\) with respect to those without the assembly at 0.4% collagen. (mean \(\pm 1 \text{ SD, } n = 6\)).
partially assembled at 1 h and almost completely assembled at 8 h. The morphology of the collagen coatings at these two conditions showed a marked contrast in the fibrillar assembly (Fig. 4). With the 1-h assembly, the collagen fibrils consisted of various fibril sizes, that is, large-sized fibrils with 100–200 nm diameters, which have been normally observed in the mature stage of fibrillar formation, and the small-sized fibrils with diameters of 10–20 nm, which have been found in the early or intermediate stage, and are more widespread than the larger ones in this case. In addition, there was a significant amount of seemingly unassembled collagen, a state similar to the aggregates found in a collagen-dissolved solution, which is known to consist of ~1.5-nm diameter collagen monomers (not clearly revealed in the electron micro-

![Figure 6](image)

**Figure 6.** Percentage of collagen remaining on Ti after the degradation test in (a) DMEM (1 mL) for 3 days and (b) collagenase solution (1 mL, approximately over 1.25 U) for 24 h, both at 37°C. Significant difference was observed with respect to that without assembly (∗∗p < 0.05 and ∗∗∗p < 0.01) and that without crosslinking (∗p < 0.05 and ∗∗p < 0.01) (mean ± 1 SD, n = 6).

![Figure 7](image)

**Figure 7.** FESEM morphologies of the MG63 cells attached for 3 h on the collagen-coated Ti at a concentration of 0.4% (a) without and (b) with assembly for 8 h (both were not crosslinked).
This monomer collagen state constitutes most of the collagen coating without the assembly process. However, with the prolonged assembly for 8 h, almost all the collagen fibrils were in the state of the large-sized mature form. When the fibrillar assembly continues and approaches this mature stage, the fibrils are known to have a unique diameter of approximately hundreds of nanometers with a narrow distribution. Although the assembly time was different, the mature fibril diameter was similar, with the only difference being observed in the fibrillar state. This was attributed to the fact that the assembly conditions (pH, temperature, ionic strength, and the assembly process), which are known to affect the final fibrillar structure and dimension, were fixed in this study. However, the different collagen state, that is, whether it is in monomer or fibril form or in the early/intermediate/mature stage of the fibril, is of utmost importance in the chemical and biological properties of the collagen.

The initial content of the collagen immobilized on Ti, whether it exists in the fibrillar or nonfibrillar form, was already different depending on the degree of assembly, as measured by the Sirius Red dye method (Fig. 5). This was attributed to the fact that the nonfibrillar collagen could be removed more readily during the washing step, which was carried out after the collagen coating. With crosslinking, the amounts of
the collagen coating appeared to be slightly lower (but not significant) than those without the step, and more so in the unassembled collagen. It is possible to some extent that the unassembled collagen would also be dissolved by the crosslinking solution. Although initially different in the coating amount depending on the preparation conditions, the degradation result, which was normalized to the initial coating content, showed a representative trend reflecting the effect of the assembly and the crosslinking of the collagen (Fig. 6). The assembled-collagen coating remained to a significantly higher degree than the unassembled one at both the degradation media (DMEM and collagenase).

Moreover, the crosslinking further improved the collagen stability, and its effect was more significant in the unassembled-collagen coating. The crosslinking of collagen has been reported to improve the chemical stability including the thermal and mechanical properties of collagen.\(^{35,36}\)

This different chemical stability of the collagen coating afforded by its fibrillar assembly and crosslinking influenced the cellular responses. The unassembled-collagen coating was found to be unfavorable for the cell attachment and proliferation, which was more significant at the higher collagen concentration (Figs. 8 and 9). However, when the collagen was assembled, its coating on Ti proved to be effective in improving the osteoblastic responses. This enhancement was more significant as the degree of assembly was increased. Regarding the crosslinking effect, the cellular responses appeared to be better in the unassembled-collagen coating. However, in the assembled-collagen coatings, the crosslinking did not significantly alter the cellular responses. Although the chemical stability of the assembled collagen was improved by the following crosslinking, its cellular responses were not influenced significantly by the treatment. Based on this, it is possible that the chemical stability of collagen would not directly correlate with the cellular responses. However, it should be noted that the crosslinking step was highly effective in the unassembled-collagen coating. The assembled collagen is believed to have sufficient chemical stability to stimulate the favorable cellular responses, so the synergetic outcomes by both fibril assembly and crosslinking were not manifested at the cellular level. Taken together, the crosslinking process is believed to be effective on the unassembled collagen coating but not on the fully assembled case. Moreover, the fibrillar assembly of collagen is ultimately required to improve the chemical stability and the cellular responses on its coating on Ti.

**CONCLUSIONS**

In the collagen coating on Ti, the fibrillar assembly and crosslinking of the collagen were observed to have a significant effect on the chemical stability and osteoblastic cellular responses. To address this phenomenon, the degree of the collagen assembly was precisely controlled and the amount of collagen was quantified. The fibrillar assembly of collagen significantly improved the chemical stability as well as the level of cell attachment and proliferation. The subsequent crosslinking also improved the chemical stability of all the collagen coatings without or with self-assembly. However, in terms of improving those cellular responses, the crosslinking was only effective in the unassembled-collagen coating. These observations are considered to provide important information and strategies in the collagen administration as a coating moiety on Ti implant.

**References**


