# Nano-hydroxyapatite–thermally denatured small intestine sub-mucosa composites for entheses applications

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**Abstract:** The objective of the present in vitro study was to estimate the adhesion strength of nanometer crystalline hydroxyapatite (HA)–small intestine sub-mucosa (SIS) composites on model implant surfaces. Techniques of thermal denaturation (60ºC, 20 min) of SIS were used to enhance the adhesion strength of entheses materials to underlying implants. Specifically, results indicated that the adhesion strength of thermally denatured SIS was 2–3 times higher than that for normal unheated SIS. In addition, aqua-sonicated, hydrothermally treated nano-HA dispersions enhanced the adhesion strength of SIS on implant surfaces. Importantly, results of the present study demonstrated that human skeletal muscle cell (hSkMC) numbers were not affected by thermally denaturing SIS in nano-HA composite coatings; however, they increased on aqua-sonicated nano-HA/SIS composites compared with SIS alone. Interestingly, thermally denatured SIS that contained aqua-sonicated, hydrothermally treated nano-HA decreased human osteoblasts (hOBs) numbers compared with respective unheated composites; all other composites when thermally denatured did not influence hOB numbers. Results also showed that the number of hOBs increased on nano-HA/SIS composites compared with SIS composites alone. Human mesenchymal stem cell (hMSC) numbers were not affected by the presence of nano-HA in SIS composites. For these reasons, the collective results of this in vitro study demonstrated a technique to increase the coating strength of entheses coatings on implant surfaces (using thermally denatured SIS and aqua-sonicated, hydrothermally prepared nano-HA) while, at the same time, supporting cell functions important for entheses regeneration.

**Keywords:** bone, entheses, orthopedic implant, tendon, hydroxyapatite, small intestine sub-mucosa composites, adhesion, nanotechnology, tissue engineering

## Introduction

In cases involving tumor resection, complicated end-stage revision, congenital deformities, massive trauma, and joint replacement for advanced arthritis, large amounts of bone are usually replaced with a metallic implant. Also removed with bone are soft tissue attachment sites for tendons, ligaments, and muscles. Failure to re-attach these soft tissues to the newly inserted metallic implants causes an inability to re-establish function of an orthopedic joint. Re-establishment of orthopedic soft tissue to metallic implants is a significant problem, as a total of 491 000 orthopedic joint replacements were performed in 2001 in the US, a number projected to rise by more than 60 000 over the next 10 years (Crompton 2004).

Recreating the entheses (the attachment or insertion site of tendons, ligaments, or joint capsules to bone (Benjamin et al 2002)) on a metallic orthopedic implant has proved challenging due to the complex structure of the natural orthopedic soft and hard tissue interface. The structure of the entheses relates to the need to dissipate stress from the hard–soft tissue into soft tissue and/or bone itself. Based on the character of the tissue at the bone–tendon interface, entheses can be classified as either fibrous or

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fibrocartilaginous. In the limbs, fibrous entheses are regions of tendons that attach to the diaphyses of bones, whereas fibrocartilaginous entheses regions attach to the epiphyses or apophyses parts of bones. A typical fibrocartilaginous entheses is composed of four zones: pure dense fibrous connective tissue, uncalcified fibrocartilage, calcified fibrocartilage, and bone (Benjamin et al 2002).

One approach that seems promising for entheses regeneration is to concentrate on recreating the nanometer constituent sizes of the tissue. For example, mineralized tendons are composed of type I collagen and apatite crystals; both have dimensions in the nanometer scale (Johnson 1960; Likens et al 1960; Nylen et al 1960; Hodge and Petruska 1963; Anderson 1969; Moradian-Oldak et al 1991; Landis et al 1993, 1996; Bonucci 1970; Landis and Silver 2002).

Based on these structural properties, entheses materials were recently created by calcifying collagenous materials (specifically, collagen type I, collagen rich porcine small intestine sub-mucosa [SIS], and gelatin A from porcine skin) with highly dispersed nanometer-sized hydroxyapatite (nano-HA) particles (Perla and Webster 2005). It was found that the best uniform coating of current titanium (Ti) implants was possible with aqua-sonicated, hydrothermally prepared HA nanoparticles (Perla and Webster 2005). Moreover, human skeletal muscle cell (hSkMC) and human osteoblast (hOB) numbers were the highest on entheses composites created with collagen type I and aqua-sonicated, hydrothermally prepared HA nanoparticles (Perla and Webster 2005). Similarly, hOB numbers also increased on gelatin A mixed with aqua-sonicated, hydrothermally prepared HA nanoparticles compared with gelatin A without HA (Perla et al 2005).

Most importantly, SIS was found to be a very interesting entheses material when combined with nano-HA particles. For example, compared with SIS alone, hSkMC numbers increased 8% on SIS mixed with hydrothemally prepared HA (Perla and Webster 2005). In addition, the numbers of hOBs doubled on SIS mixed with either conventional (or micron grain size) HA or aqua-sonicated, hydrothermally prepared nano-HA particles. Functions of hSkMCs and hOBs are important for entheses regeneration.

In spite of having identified several positive properties of aqua-sonicated, hydrothermally prepared nano-HA dispersions in SIS (specifically, uniform coatings on implant materials and positive cell responses), it is not clear how strongly these potential entheses materials were adherent on the underlying implants. Better adhesion properties of entheses materials on implants prevent delamination and damage during handling and/or implantation. For this reason, the objective of this in vitro study was to estimate the adhesion strength of entheses materials created by mixing SIS and different types of HA (including aqua-sonicated, hydrothermally prepared nano-HA particles). While doing so, the present investigation also incorporated a technique of thermal denaturing to enhance the adhesion strength of nano-HA–SIS composite materials on implant surfaces. Due to their importance in entheses regeneration, the last objective of the present in vitro study was to determine human skeletal muscle cell (hSkMC), human osteoblast (hOB), and human mesenchymal stem cell (hMSC) numbers on such composites.

## Materials and methods

### Synthesis of nano-HA materials for better coating adhesion

#### Synthesis of conventional (or micron grain size) HA

HA was precipitated through an established wet chemical process (Ioku and Yoshimura 1991; Narasaraju and Phebe 1996). Concentrated ammonium hydroxide (Fisher Scientific, Pittsburgh, PA, USA) was added to 300 mL of deionized water to stabilize the pH at 10. Calcium nitrate solutions (1.0 M) and diammonium hydrogen phosphate solutions (0.6 M) were separately prepared by dissolving respective chemicals (Sigma-Aldrich, St Louis, MO, USA) in deionized water. The diammonium hydrogen phosphate solution was added to deionized water at a pH of 10 and the calcium nitrate solution was dripped into the mixture at a rate of 3 mL/min. The diammonium hydrogen phosphate solution and the calcium nitrate solution were added to yield a stoichiometric ratio of Ca/P = 1.67. With stirring, HA precipitation occurred as indicated by the reaction below (Sato et al 2005).

$$10Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 8NH_4OH = Ca_{10}(PO_4)_6(OH)_2 + 6H_2O + 20NH_4NO_3 \quad (1)$$

The solution was stirred for 10 min at room temperature. The precipitated solution was centrifuged (1500xg) and rinsed with deionized water two times at room temperature. The precipitates were dried in an oven at 80ºC overnight.

#### Synthesis of hydrothermally prepared nano-HA

The HA synthesis method using hydrothermal treatment was previously described by Ioku and Yoshimura (1991). High crystallization was achieved at relatively low temperatures but under a higher pressure than that sintered at the same temperature in air. The precipitated HA solution after stirring

for 10 min (as described in the earlier section) was then centrifuged (1500xg) one time. Fifty mL of the concentrated HA precipitated solution was added into a 125-mL Teflon liner. The Teflon liner was sealed tightly in an autoclave (Parr Acid Digestion Bombs 4748; Parr Instrument, Moline, IL, USA) and processed hydrothermally at 200°C for 20 hours. As a result, agglomerated nano-sized HA crystallites were obtained. After hydrothermal treatment, the HA particles were rinsed with $dH_2O$ two times and dried at 80°C overnight.

### Production of nano-HA dispersions for synthesis of entheses materials

Agglomerated nano-HA particles were transformed into nanoparticulate HA dispersions by aqua-sonication as described in earlier reports (Perla and Webster 2005). Specifically, conventional (or micron grain size) HA and hydrothermally prepared HA powders in deionized water (1 mL of 10% HA) were sonicated in an aqua-sonicator (Aquasonic 50T; VWR Scientific Products, West Chester, PA, USA) for 6 and 2 hours, respectively. After aqua-sonication, contents were vortexed thoroughly and used in the synthesis of entheses materials with SIS (as described in the next section).

## In vitro synthesis of the calcified zone of tendon entheses

In order to synthesize the calcified zone of tendon entheses, porcine small intestine submucosa (SIS) in powder form was obtained from DePuy Orthopaedics, Inc. (Warsaw, IN, USA). Without further characterization, this matrix material was directly used in the creation of the calcified zone of tendon entheses. The SIS solution (1% powder in 1x phosphate buffered saline) was mixed (vortexed) with different types of hydroxyapatite dispersions described above (0.25 weight % of SIS) and the bottom surface of 96-well polystyrene cell culture plates were coated with such composites using a pipette with 20 µl of solution per well. Coated plates were allowed to dry. Composites were sterilized under UV light exposure for about 6 hours with continuous air flow. These plates were used directly for cell culture as described below.

### Synthesis of entheses materials by thermally denaturing SIS

In an effort to increase coating strength on metals, thermally denatured SIS was created by heating the SIS solution (1% powder in 1x phosphate buffered saline) in a water bath at 60ºC for 20 min. Calcification of thermally denatured SIS with different types of HA was performed before heating.

### Characterization of entheses materials

Various entheses composites were coated on Ti (Biomedical Grade 2; Osteonics) by soaking Ti pieces in the respective entheses solutions for 5 min at room temperature. Characterization of the surfaces of wrought Ti coated with entheses materials were performed by SEM (JEOL-JSM-840) at an accelerating voltage of 5kV using digital Scan Generator Plus Software. For this purpose, samples were sputter-coated with gold-palladium in a vacuum of 100 millitorr for 3 min at 10 mA current. The conventional and nanometer crystalline HA were characterized in a similar manner using SEM with an additional TEM characterization (using a JEOL 2000FX TEM) completed.

## In vitro estimation of the adhesion strength of entheses materials

Adhesion strengths of entheses materials created by SIS, thermally denatured SIS, and the different types of HA was estimated by a penetrometer (Fruit Texture Analyzer; FTA) and associated FTA software. After a brief vortex for 1 min, 5 µl of the test coating solution was added to the center of a glass slide with 7.62 x 2.54 x 0.12 cm dimensions (M6134; Baxter). A clean second glass side was mounted parallel and lengthwise and was pressed on the surface of first glass side in such a way that the labeled portion (painted white) on one of the glass sides was exposed. This glass slide pair with test coating was left at room temperature for about 12 hours. After that time period, the exposed portion of second slide was clamped and the exposed portion of first slide was tested against a penetrometer probe. Utmost care was taken to prevent exposure of the glass slide pair to unwanted forces while handling during clamping and testing. Probe trigger threshold (0.20), forward speed (10 mm/s), reverse speed (40 mm/s), measured speed (5 mm/s), measured distance (20 mm), calibration weight (2501 g), maximum distance (90 mm), maximum load (14 000 g), firmness ratio (840), step ratio (6221), frequency (1 536 000), and gradient (1) were set with FTA software. After testing against the penetrometer probe, the strength value at the failure of entheses material coatings was recorded with aid of the FTA software.

## Cell adhesion on entheses materials

### Cells

Without further characterization, human skeletal muscle cells (hSkMCs; Cell Applications, Inc., San Diego, CA, USA; population numbers 3–7) in 100 µl of hSkMC media (Cell Applications, Inc.) were seeded at 10 000 cells/well (96-well

format) and were placed in an incubator under standard cell culture conditions (37°C temperature, 5% $CO_2$ and 95% humidified air) for 48 hours. At that time, cell proliferation was assessed via MTT cell proliferation assay kits.

Human osteoblasts (bone forming cells; hOBs; CRL-11372 American Type Culture Collection, population numbers 3–8) in 100 µl of Dulbecco's Modified Eagle Media (HyQ® DME/High glucose, phenol free; Hyclone, Logan, UT, USA) supplemented with 5% fetal bovine serum (Hyclone) and 1% penicillin–streptomycin (Hyclone) were seeded at 10 000 cells/well and cultured for 48 hours under standard cell culture conditions. Cell proliferation was assessed via MTT assay kits.

Similarly, human mesenchymal stem cells (hMSC; PT2501 Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA; population number 1) in 135 µl of mesenchymal stem cell growth media (PT-3001, MSCGM bulletkit, Cambrex Bio Science Walkersville, Inc.) were seeded at 10 000 cells/well and cultured for 48 hours under standard cell culture conditions. Cell proliferation was assessed via MTT assay kits.

### MTT cell proliferation assay

MTT assays were performed as per the protocol provided by the vendor (ATTC; catalog number 30-1010K). After 48 hours of cell culture, 10 µl of the MTT reagent was added to each well containing 100 µl of media (1:10 ratio of MTT and media). Plates were returned to the incubator. After 4 hours of dark incubation, 100 µl of the detergent reagent was added to all the wells (1:1 ratio of detergent and media). Plates with covers were left in the dark for 4 hours at room temperature. Contents were mixed and transferred to microtubes. These microtubes were centrifuged at 5585xg for 5 min to sediment HA particles that may have been removed from the coatings during the MTT assay. From the top aqueous phase, 100 µl of supernatant was transferred to another micro titer plate and the color absorbance was measured at 570 nm in a microtiter plate reader (Spectramax-190; Molecular Devices, Sunnyvale, CA. USA). After deducting blank values, the total number of cells per well was estimated from standard curve obtained for each cell type under similar experimental conditions.

## Statistical analysis

Data were collected as per standard statistical procedures and the significant differences were assessed with the probability associated with a two-tailed Student's t-test. Unless otherwise mentioned, mean values reported in this study were estimated from three experiments with three repeats each.

# Results

## Ti implant coating characterization

### Effective coatings with thermally denatured SIS and nano-HA

When coating model Ti surfaces (Figure 1A) with solutions of normal, unheated, and unmodified SIS powders alone, results

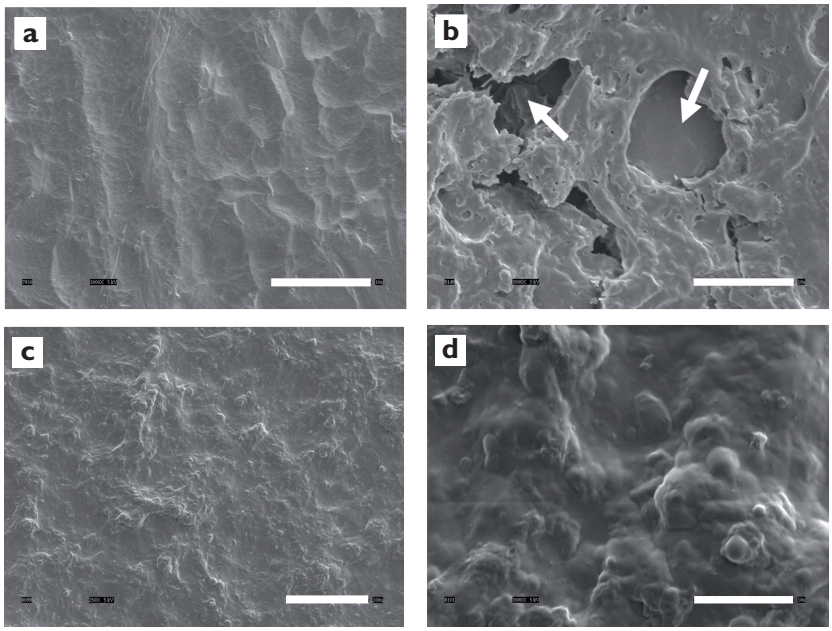


**Figure 1** SEM images of Ti surfaces uncoated (a) and coated with unheated SIS (b) and thermally denatured SIS (c and d). Uncovered regions on Ti using unheated SIS (arrows in image b) were covered by using thermally denatured SIS (c and d). Bars = 10 µm in a, b, and d (magnification = 3000x); Bar = 100 µm in c (magnification = 250x).
**Abbreviations:** SIS, small intestine sub-mucosa

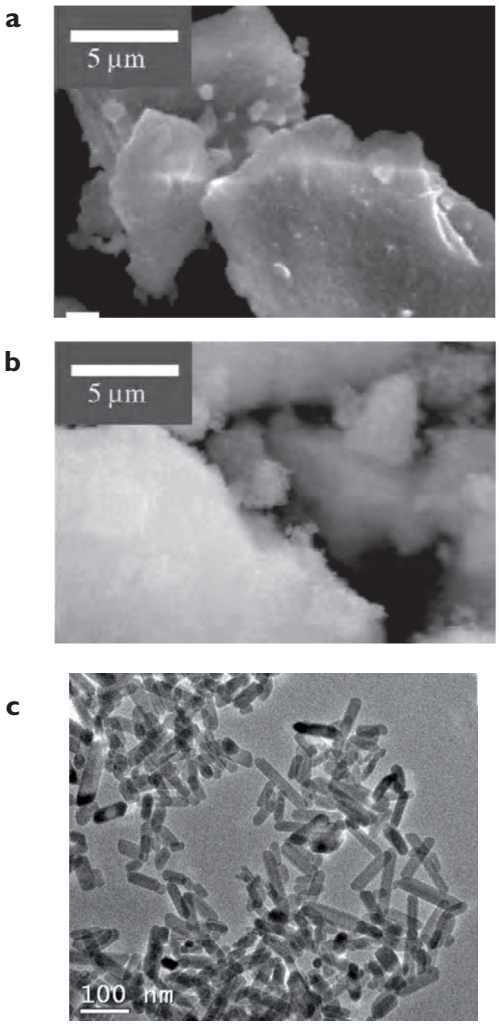


**Figure 2** SEM images of (a) conventional (or micron grain size) and (b) nanometer grain size HA as well as a (c) TEM image of nanometer grain size HA. These HA particles were used in composite formulations.
**Abbreviations:** HA, hydroxyapatite.

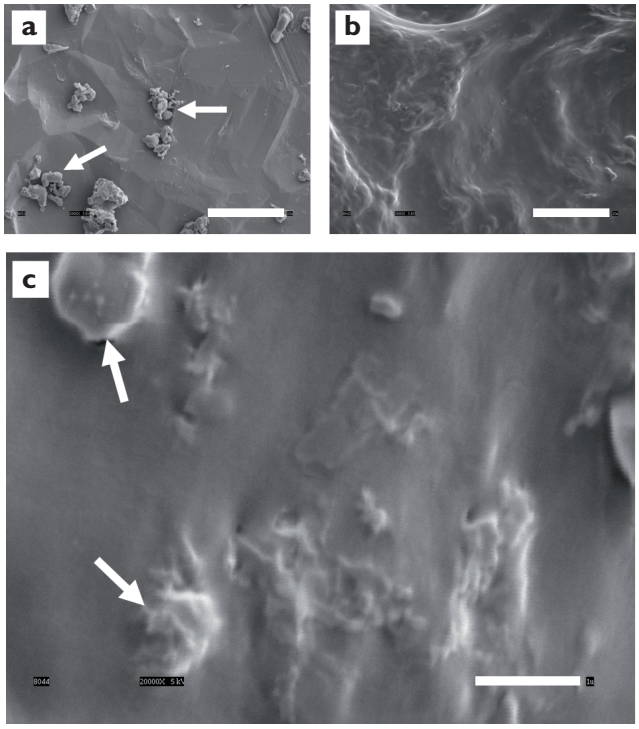


**Figure 3** SEM images of Ti surfaces coated with conventional (or micron grain size) HA (a) and conventional HA with thermally denatured SIS (b and c). Arrows indicate the presence of unevenly distributed micron range particles of HA. Bars = 10 µm in a and b (magnification = 3000x); bar = 1 µm in c (magnification = 20000x).
**Abbreviations:** HA, hydroxyapatite; SIS, small intestine sub-mucosa.

of this study showed that the coatings were not uniform (Figure 1B); uncovered areas on the Ti surface were obvious. Visual observations suggested that these coatings were not uniformly attached on the Ti surface (Figure 1B).

In contrast, SEM analysis demonstrated complete coverage of Ti surfaces (without any uncovered areas) through the application of thermally denatured SIS solutions (Figures 1C and 1D). It appeared that the thermally denatured SIS coatings were much more firmly attached on the Ti surface than normal, unheated, SIS coatings.

Importantly, results from this study provided evidence of the synthesis of conventional (or micron grain size) and nanometer grain size HA (Figure 2). Not surprisingly, results of this study also demonstrated that the creation of entheses calcified zones through the use of conventional (or micron grain size) HA particles alone were not sufficient to cover Ti (Figure 3A). Visual observations suggested that micron range HA agglomerations were not well dispersed and appeared loosely attached on the Ti surface when used alone (Figure 3A).

In contrast, SEM analysis provided evidence that the surfaces of Ti were partially covered when thermally denatured SIS was mixed with conventional HA (Figures 3B and 3C). However, although conventional HA agglomerations were not well dispersed, when combined with thermally denatured SIS, the composite appeared to be uniformly covering the Ti surface (Figure 3C). Importantly, previous studies have demonstrated the best Ti surface coverage occurred through the use of nano-HA and thermally denatured SIS (Perla and Webster 2005).

## Adhesion strength

### Greater adhesion strength for thermally denatured SIS and nano-HA dispersions in thermally denatured SIS

This study demonstrated that the adhesion strength of thermally denatured SIS was 2-3 times stronger than normal (unheated) SIS at all the concentrations (0.5, 1.0, 2.5, and 5.0%) tested (Figure 4). Results also provided evidence that

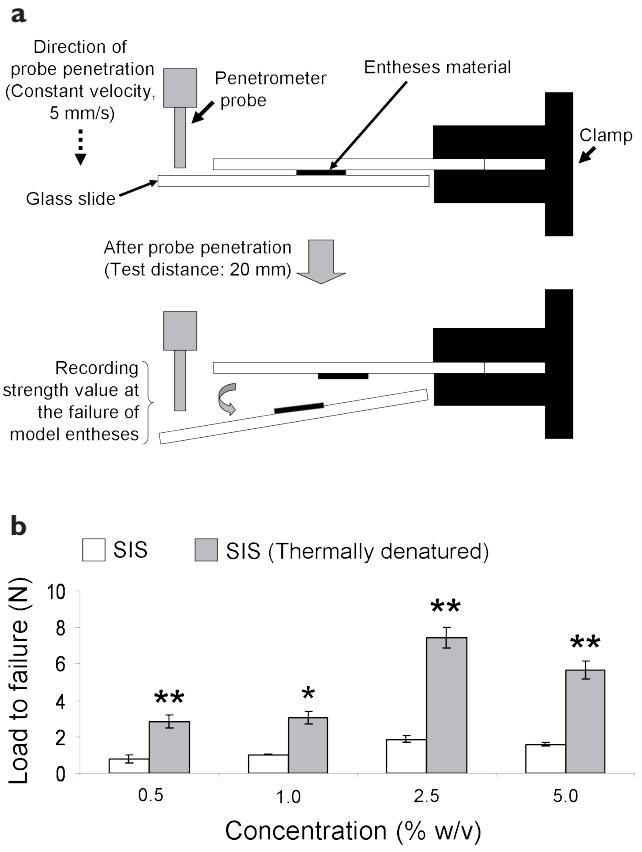


**Figure 4** In vitro estimation of the adhesion strength of entheses materials. (a) Schematic representation of the estimation of coating strength (load to failure) of entheses materials on underlying materials by the penetrometer method. (b) SIS (unheated) and thermally denatured SIS penetrometer results. Data = mean + SE; n = 3; * $p \leq 0.05$ and ** $p \leq 0.01$ compared with SIS (unheated) at respective concentrations.
**Abbreviations:** SIS, small intestine sub-mucosa.

the addition of conventional HA (2a in Figure 5) enhanced the coating strength of unheated SIS (1a in Figure 5). Similarly, the addition of nano-HA particles (3a in Figure 5) enhanced the coating strength of unheated SIS (1a in Figure 5). However, the strength obtained by thermally denaturing SIS (1b in Figure 5) was greater than the strength obtained by adding either nano- or conventional-HA particles to unheated SIS (2a and 3a in Figure 5). In addition, the thermal denature process coupled with aqua-sonication (to increased HA dispersion) significantly enhanced the strength of unheated SIS coatings after adding either nano or conventional HA (4b and 5b in Figure 5). For the composites, the greatest adhesion strength measured was for thermally denatured SIS with aqua-sonicated, hydrothermally prepared nano-HA (5b in Figure 5).

Importantly, the strength of the coatings to the underlying substrates was measured in this study as fracture exclusively occurred at that interface. That is, none of the coating materials themselves fractured during the penetrometer tests. However, it is important to note that due to ease of experimentation, glass coverslips were used as the underlying substrate in this study (not Ti).

## Cell culture experiments

### hSkMC cell numbers were not affected by nano-HA–thermally denatured SIS composites

Due to the enhancement of implant coating strengths as just mentioned from: (i) thermally denaturing SIS and (ii)

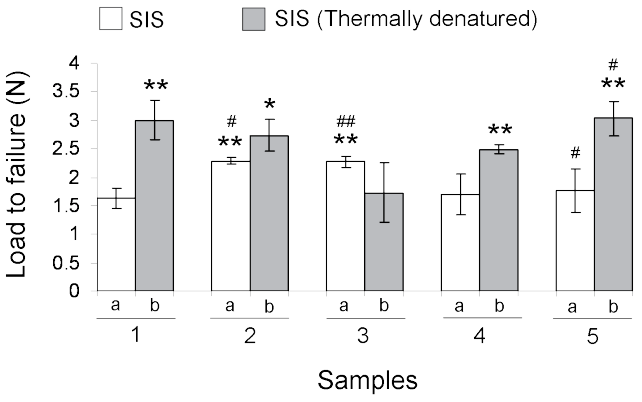


**Figure 5** Effect of thermal denaturation of SIS on the strength of entheses coatings with HA. 1 = SIS; 2 = SIS + conventional HA; 3 = SIS + hydrothermally prepared nano-HA; 4 = SIS + conventional HA with aqua-sonication for 6 hours; and 5 = SIS + hydrothermally prepared nano-HA with aqua-sonication for 2 hours. Data = mean + SE; n = 3; * $p \leq 0.05$ and ** $p \leq 0.01$ compared with SIS (unheated) alone (1a); # $p \leq 0.01$ and ## $p \leq 0.01$ compared with thermally denatured SIS alone (1b).
**Abbreviations:** HA, hydroxyapatite; SIS, small intestine sub-mucosa.

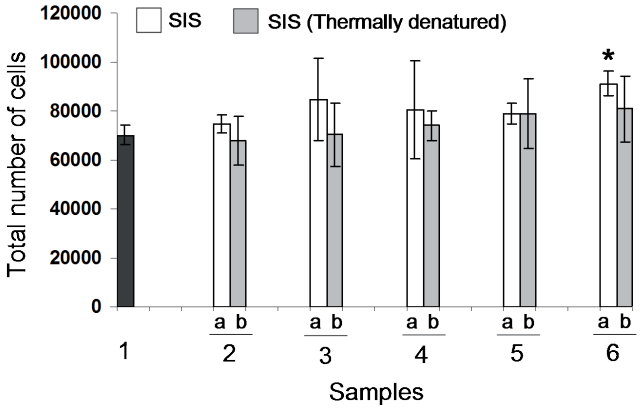


**Figure 6** Increased hSkMC numbers on SIS mixed with aqua-sonicated, hydrothermally prepared nano-HA particles. 1 = polystyrene cell culture plate (control); 2 = SIS; 3 = SIS + conventional HA; 4 = SIS + hydrothermally prepared nano-HA; 5 = SIS + conventional HA with aqua-sonication for 6 hours; and 6 = SIS + hydrothermally prepared nano-HA with aqua-sonication for 2 hours. Data = mean ± SE; n = 3; * $p \leq 0.05$ compared with SIS (unheated) alone (2a).
**Abbreviations:** HA, hydroxyapatite; hSkMC, human skeletal muscle cell; SIS, small intestine sub-mucosa.

incorporating aqua-sonicated, hydrothermally prepared nano-HA to SIS, it was important in this study to determine if cell responses were also altered under these conditions. Results showed that hSkMC numbers were not significantly affected on composites with thermally denatured SIS compared with unheated SIS (Figure 6). In other words, the SIS thermal denature process at 60°C for 20 min maintained the proliferation capacity of hSkMC. Results of cell culture experiments also revealed that hSkMC numbers significantly increased on composites of SIS with aqua-sonicated, hydrothermally prepared nano-HA compared with unheated SIS alone; the greatest hSkMC numbers were observed on SIS with aqua-sonicated, hydrothermally prepared nano-HA.

### Increased hOB numbers on nano-HA–SIS composites

In contrast to hSkMC numbers, significant decreases in hOB numbers were observed on composites made with thermally denatured SIS with aqua-sonicated, hydrothermally prepared nano-HA compared with respective composites unheated (Figure 7). Results of the present investigation also indicated that the number of hOBs significantly increased on composites of SIS mixed with aqua-sonicated, hydrothermally prepared nano-HA compared with SIS alone (Figure 7). Among all the composites, hOB numbers were the greatest on unheated SIS with either aqua-sonicated conventional HA or hydrothermally prepared nano-HA (both were statistically similar to each other).

### hMSC cell numbers were not affected by nano-HA–SIS composites

The present investigation also revealed that the SIS composites made with either unsonicated or aqua-sonicated nano-HA did not significantly affect hMSC numbers (Figure 8). All entheses formulations had the same hMSC numbers.

It is important to note that since these assays were performed after 48 hours of cell culture, cell adhesion was not specifically determined in this study; rather differences in cell proliferation in conjunction with cell adhesion were determined. Nonetheless, the results of the present study provided significant promise for the use of nano-HA–thermally denatured SIS composites to increase coating strength while maintaining attractive cytocompatibility properties.

## Discussion

The introduction of "nanotechnology" to the biomaterials field has provided unique opportunities to engineer a new class of implants. Bone as well as entheses structures are examples of biological materials whose properties are dependent on their nanoscale structure. Bone is an inorganic-organic nanostructured material consisting of collagen in which tiny HA crystals (10–50 nm in length) reside (Weiner and Wagner 1998; Taton 2001; Webster and Ejiofor 2004). In fact, several studies have positively correlated the adhesion and functions of bone cells on metals (Webster and Ejiofor 2004), ceramics (Webster et al 1999; Webster et al 2000), polymers (Webster

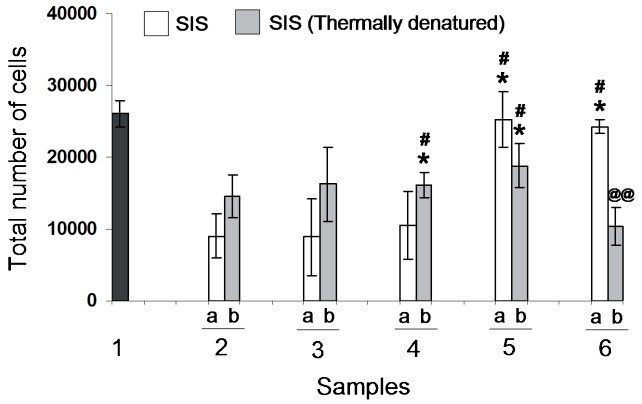


**Figure 7** Increased hOB numbers on SIS mixed with HA. 1 = polystyrene cell culture plate (control); 2 = SIS; 3 = SIS + conventional HA; 4 = SIS + hydrothermally prepared nano-HA; 5 = SIS + conventional HA with aqua-sonication for 6 hours; and 6 = SIS + hydrothermally prepared nano-HA with aqua-sonication for 2 hours. Data = mean ± SE; n = 3; * $p \leq 0.05$ compared with SIS (unheated) alone (2a); # $p \leq 0.05$ compared with SIS (unheated) with non-sonicated, conventional HA (3a); @@ $p \leq 0.001$ compared with SIS (unheated) with aqua-sonicated, hydrothermally prepared nano-HA (6a).
**Abbreviations:** HA, hydroxyapatite; hOB, human osteoblast; SIS, small intestine sub-mucosa.

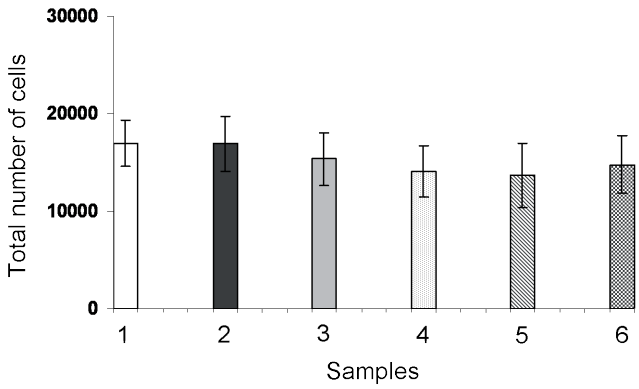


**Figure 8:** hMSC numbers were not affected by SIS mixed with nano-HA particles. 1 = polystyrene cell culture plate (control); 2 = SIS; 3 = SIS + conventional HA; 4 = SIS + hydrothermally prepared nano-HA; 5 = SIS + conventional HA with aqua-sonication for 6 hours; and 6 = SIS + hydrothermally prepared nano-HA with aqua-sonication for 2 hours. Data = mean ± SE; n = 3.
**Abbreviations:** HA, hydroxyapatite; hMSC, human mesenchymal stem cells; SIS, small intestine sub-mucosa.

et al 2001), and composites (Elias et al 2000; Kay et al 2002; Price et al 2003) with nanoscale compared with micron (or conventional) surface features. Increased initial adsorption of proteins important for osteoblast adhesion (specifically, fibronectin, and vitronectin) was shown to be the underlying mechanisms for these events (Webster et al 2001).

For the first time, this in vitro study aimed at understanding the adhesion strength of a so-called new class of biomaterials: nanostructured entheses materials. Results from this study provided the first evidence of the increased adhesion strength of composites made from aqua-sonicated, hydrothermally prepared nano-HA and thermally denatured SIS. Apart from their strength, these composites were also found effective in terms of cytocompatibility pertinent for entheses regeneration. This is evident from the cell number data associated with hSkMCs and hMSCs.

It was reported that the superior uniform coatings achieved with aqua-sonicated, hydrothermally prepared HA might be due to the presence of much smaller particle agglomerations compared with what was present in the non-hydrothermally prepared, conventional HA dispersions; that is, if properly controlled, individual HA nanoparticles can disperse much greater than micron particles to create for more uniform implant coatings and provide increased cytocompatibility properties to cells (Perla and Webster 2005).

In addition to nano-HA, the presence of SIS in these composites is essential. SIS contains 40% collagens (Badylak 1993), 2.1% glycosaminoglycan (GAG) content (Hodde et al 1996), and 1.1% fibronectin (Hynes 1990; McPherson and Badylak 1998). Apart from these, SIS also contains proteoglycans (Roskelley et al 1995), cytokines, and glycoproteins (Roskelley et al 1995; McPherson et al 1998). GAG content may help regulate the activity of certain growth factors and hydration of the extracellular matrix while fibronectin may aid in cell adhesion, migration, proliferation, and angiogenesis (Hynes 1990; Roskelley et al 1995; McPherson et al 1998). In addition, cytokines are a unique family of growth factors that stimulate both the humoral and cellular immune responses, as well as the activation of phagocytic cells. Not only did SIS aid in cell functions in the present study, but as demonstrated, it also increased the adhesion strength of HA to the underlying implant surfaces.

For the present study, however, it is not clear why hOB numbers decreased on aqua-sonicated, hydrothermally treated nano-HA mixed with thermally denatured SIS compared with respective unheated composites. Clearly, it is possible that some of the crucial ingredients that are required for hOB proliferation may have been lost during heating of SIS. Further investigations, though, will have to determine why when not aqua-sonicated, thermally denaturing SIS with HA had no adverse influence on hOB cell numbers. Perhaps the combined thermal denaturing and aqua-sonication influenced the bioactivity of SIS in these composites for hOBs. But, it is important to note that the bioactivity loss of heating SIS for hOBs was gained through the addition of either aqua-sonicated conventional or nano-HA to SIS. Adhesion may also be different on the various coatings; this was not directly measured in this study.

In contrast, bioactivity differences through the use of thermally denatured SIS were not seen for hSkMCs and hMSCs; rather they were maintained compared with unheated counterparts. Moreover, the present study clearly demonstrated the importance of using thermal denaturation of SIS and adding nano-HA in increasing composite adhesion strength to implant surfaces.

## Conclusions

Results of this study provided the first evidence that the adhesion strength of entheses materials can be greatly enhanced through the incorporation of aqua-sonicated, hydrothermally prepared nano-HA and thermal denaturation of SIS (at 60ºC for 20 min). In addition, while more studies are needed, functions of cells were maintained on such composite coatings. Collectively, the results of the present in vitro study thus suggested that entheses composites consisting of thermally denatured SIS and aqua-sonicated, hydrothermally prepared nano-HA should be further investigated for orthopedic applications.

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