ADVANCED MATERIALS

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Triple-Helix Scaffolds of Grafted Collagen Reinforced by Al_2O_3–ZrO_2 Nanoparticles**

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Recently, the design and fabrication of bioscaffolds exhibiting biocompatibility and structural stability has received much attention for engineering functional tissues[1,2] and for clinical applications.[3] Collagen, a bioscaffold polymer whose stalks consist of right-handed supercoils made from three left-handed polyproline II-type helices, with the major amino acid sequence being $(Gly–Pro–Hyp)_n$,[4] has attracted considerable interest because of its appealing characteristics, such as availability in appreciable quantities, a variety of processable forms, a large number of dipoles and molecule-bound charges, and so forth.[5] However, unprocessed collagen exhibits low stability and weak mechanical strength, which limit its application in many areas. Therefore, collagen needs to be modified to improve its mechanical and thermal properties. Up until now, extensive efforts have been made to mimic[6] or stabilize[6–9] the triple-helix conformation of collagen. Although these approaches have been successful in improving the thermal stability of the triple-helix form, not much attention has been devoted to improving the mechanical properties of collagen. Recently, Castano and co-workers[10] and Wang et al.[11] have used nanotubes to reinforce the various polymer chains. These significant research efforts have stimulated us to attempt to incorporate metal oxide nanoparticles to stabilize the collagen structure. This approach is inspired by the abundance of oxygen on the surface of the metal oxide nanoparticles, which can potentially interact with collagen molecules containing abundant $–NH_2$ and –OH groups by forming covalent or hydrogen bonds. In this work, Al_2O_3–ZrO_2 composite nanoparticles have been synthesized in a single step by a simple sol–gel method and then anchored onto the grafted collagen matrix to form hybrid nanocomposites. The incorporation of the composite nanoparticles serves to strengthen the mechanical properties of the matrix, as well as to improve the thermal stability of the triple helices by interactions between the Al_2O_3–ZrO_2 nanoparticles and the collagen peptide chains. Collagen is first grafted with methyl methacrylate to improve its biocompatibility for potential medical applications.[12]

Figure 1 shows the tensile stress–strain curves of the Al_2O_3–ZrO_2-nanoparticle/grafted-collagen hybrid nanocomposites. As seen in the representative plots, no noticeable yield point is observed for collagen and the grafted collagen, whereas the hybrid nanocomposites with different amounts of composite nanoparticles show a distinctive yield point, indicating a change from an elastomer to a tough plastic. After grafting the collagen triple helix, the tensile strength increases by about 8.9 % relative to bulk collagen (from 359 to 391 kPa), and the elongation at break is increased to 132 %. When the collagen triple helices are doped with Al_2O_3–ZrO_2 composite nanoparticles, the tensile properties are greatly improved. As is evident from Figure 1, the stress strength increases gradually and the elongation at break decreases gradually as increasing amounts of Al_2O_3–ZrO_2 composite nanoparticles are doped into the matrix. Samples containing 7.3 wt % composite nanoparticles show an increase in the tensile stress from 359 to 911 kPa and a decrease in the elongation at break from 132 to 93 %. The 154 % improvement in the tensile stress is much greater than the 75.4 % enhance-

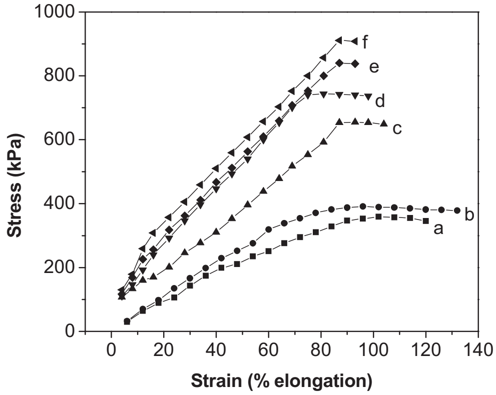

Figure 1. Tensile stress–strain curves of a) collagen, b) grafted collagen, c) 1.2 %, d) 2.6 %, e) 4.7 %, and f) 7.3 % Al_2O_3–ZrO_2-nanoparticle/grafted-collagen hybrid nanocomposites. The nanoparticle content is denoted in terms of weight percent.

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ment observed by incorporating chemically functionalized multiwalled carbon nanotubes into poly(methyl methacrylate) (PMMA) by in situ polymerization.[10] These observations indicate that the hybrid nanocomposites gradually become brittle, and that the nanoparticles reduce the plasticity of the hybrid nanocomposites. When more composite nanoparticles (> 7.3 wt %) are incorporated into the matrix, the change in the tensile stress is very slight, indicating that only a limited amount of composite nanoparticles can be immobilized in the matrix, and that the binding sites on the triple helices of grafted collagen are saturated.

We have also examined the variation of the yield strength and tensile modulus of the hybrid nanocomposites as a function of the amount of anchored Al_2O_3–ZrO_2 composite nanoparticles (wt %). The incorporation of 7.3 wt % Al_2O_3–ZrO_2 increases the yield strength of grafted collagen from 14 to 31 kPa, and improves the elastic modulus by about 143 % (from 328 to 796 kPa). These results are similar to previous studies of clay-enhanced polymer nanocomposites,[13,14] where a dramatic improvement in the tensile strength and modulus has been observed. The improvement in the tensile modulus is much more significant here than previously reported by Wang et al.,[11] wherein the formation of chitosan/multi-walled-carbon-nanotube nanocomposites was seen to produce a 93 % improvement in the tensile modulus of the polymer chains.

Figure 2 shows the variation of the storage modulus of the hybrid nanocomposites at 1 Hz as a function of temperature. The mechanical behavior of collagen and grafted collagen are typical of amorphous polymers. However, when the Al_2O_3–ZrO_2 composite nanoparticles are incorporated into the grafted collagen matrix, the storage modulus level gradually increases over the entire temperature range studied. In comparison to grafted collagen, hybrid nanocomposites with 7.3 wt % nanoparticles show a 504 % improvement in the storage modulus under glassy conditions (0 °C) and a 149 % improvement under rubbery conditions (100 °C). At 40 °C, the improvement in the storage modulus upon incorporating the nanoparticles is 390 %, which is much higher than the 88 % enhancement observed upon incorporating 1.5 wt % chemically functionalized multiwalled carbon nanotubes into PMMA.[10] The remarkable improvement obtained here can be attributed to the interactions between the composite nanoparticles and the triple helices in grafted collagen.

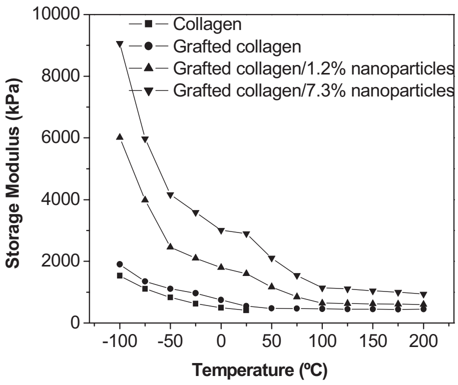


Figure 2. Plot of storage modulus versus temperature.

The hybridization of the composite nanoparticles in the grafted collagen matrix should also increase the hardness and modulus versus displacement. The hardness and modulus of Al_2O_3–ZrO_2-nanoparticles (7.3 wt %)/grafted-collagen composites versus displacement is reinforced by a factor of 3–4.5, as compared to the grafted collagen matrix, which is similar to the values obtained for mesoporous silica-reinforced polymer nanocomposites.[15] The improvements in the mechanical parameters discussed above are summarized in Table 1.

As a control experiment, 7.3 wt % of the Al_2O_3–ZrO_2 composite nanoparticles were added to an ungrafted collagen matrix. The resulting hybrid composites show relatively poorer mechanical properties compared to grafted collagen. In comparison to collagen, the tensile stress and storage modulus under glassy conditions (at 0 °C) increases by only about 96.7 and 254 %, respectively, and the elongation at break decreases by 85 %. This can possibly be attributed to the smaller diameter of the ungrafted collagen chains, which are thus not able to bind all the composite nanoparticles.

To evaluate the influence of the Al_2O_3–ZrO_2 composite nanoparticles in enhancing the mechanical properties of the hybrid nanocomposites, the surface morphology and microstructure of these nanocomposites have been studied. Figure 3A shows a transmission electron microscopy (TEM) image of the Al_2O_3–ZrO_2 composite nanoparticles, which are seen to have a fine spherical shape with diameters ranging from 10 to 18 nm. The materials connecting the particles include some salts and organic impurities such as citric acid

Table 1. Mechanical properties of collagen and different hybrid nanocomposites.

Samples	Maximum tensile strength [kPa]	Yield strength [kPa]	Tensile modulus [kPa]	Modulus [kPa]	Hardness [kPa]
Collagen	359	14±0.9	328±33	470±9	830±30
Grafted collagen (GC)	391	16±1.1	401±29	501±5	950±29
GC/1.2% nanoparticles	655	20±0.6	505±31	1376±35	1189±9
GC/2.6% nanoparticles	744	22±1.2	637±64		
GC/4.7% nanoparticles	840	25±2.1	698±58		
GC/7.3% nanoparticles	911	31±1.2	796±66	2100±28	2476±17

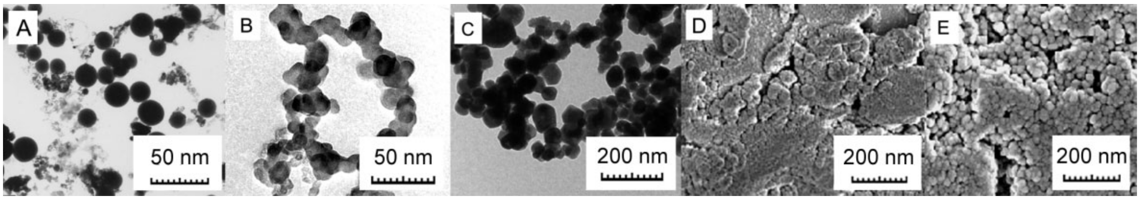


Figure 3. TEM images of A) Al_2O_3–ZrO_2 composite nanoparticles, B) collagen, and C) grafted collagen. SEM images of hybrid nanocomposites with a composite nanoparticle content of D) 1.2 wt% and E) 7.3 wt%.

wrapped in a gel. TEM observations of collagen indicate a catenarian conformation with the chain diameters ranging from 10 to 20 nm (Fig. 3B). After the grafting process, the grafted collagen chains show a grain-like morphology with the particle size ranging from 30 to 60 nm (Fig. 3C), which is larger than the individual size of the Al_2O_3–ZrO_2 nanoparticles and collagen chains. After doping 1.2 wt % Al_2O_3–ZrO_2 nanoparticles into the grafted collagen matrix, scanning electron microscopy (SEM) images show a compact and disordered stacking arrangement with some agglomeration of the grafted collagen (Fig. 3D). In contrast, a higher weight ratio of 7.3 wt % Al_2O_3–ZrO_2 nanoparticles leads to the formation of a homogeneous and well-distributed nanocomposite structure (Fig. 3E). The isolated particles occasionally observed are due to small cracks or the uneven surface of the composite. As is well known, the key factors pertaining to the performance of the hybrid nanocomposite are its microstructure and the dispersion of the hybridized nanoparticles. Hybrid nanocomposites with 7.3 wt % Al_2O_3–ZrO_2 composite nanoparticles show optimally enhanced mechanical properties. This is because at lower particle concentrations, the Al_2O_3–ZrO_2 composite nanoparticles occupy relatively fewer binding sites on the grafted collagen helices, which leads to the disordered agglomeration of grafted collagen. In contrast, at higher particle concentrations, more binding sites on the peptide chains are anchored by hydrogen bonding to the nanoparticles, and thus no agglomeration of grafted collagen is observed. For even higher particle loadings, the excess nanoparticles cannot be immobilized or anchored,[16] and thus are not able to further enhance the mechanical properties of the hybrid material.

The thermal behavior of the hybrid nanocomposites has been investigated by thermogravimetric analysis (TGA) and differential thermal analysis (DTA). From the TGA curve of the hybrid nanocomposite (Fig. 4a), a large improvement in the thermal stability can be observed. An obvious weight loss starts to occur at 264 °C, associated with the elimination of the collagen polymer component. Commercial collagen can only endure temperatures up to 39 °C.[17] When 7.3 wt % Al_2O_3–ZrO_2 composite nanoparticles are added to ungrafted collagen, an obvious weight loss is observed starting at 89 °C. The improved thermal stability is due to the synergistic effect between the two components in the nanocomposite. The DTA curve for the nanocomposite shows an exothermic peak at a temperature of 350 °C and a single endothermic peak at about 432 °C (Fig. 4b). The former peak can be attributed to the destruction of the collagen chain, while the latter peak originates from the fact that entire composite is a single-component structure. Therefore, the DTA curve provides indirect evidence of the strong interactions between the grafted collagen matrix and Al_2O_3–ZrO_2 composite nanoparticles.[16]

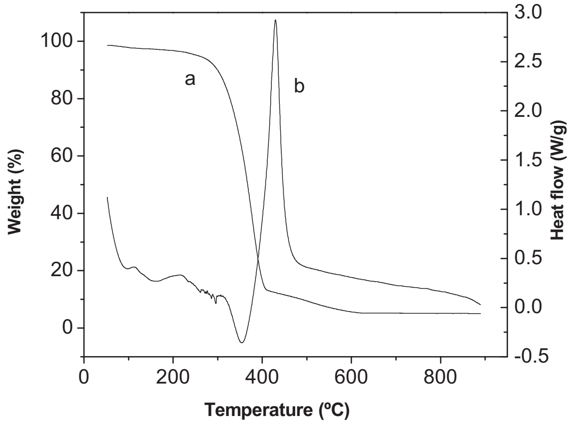
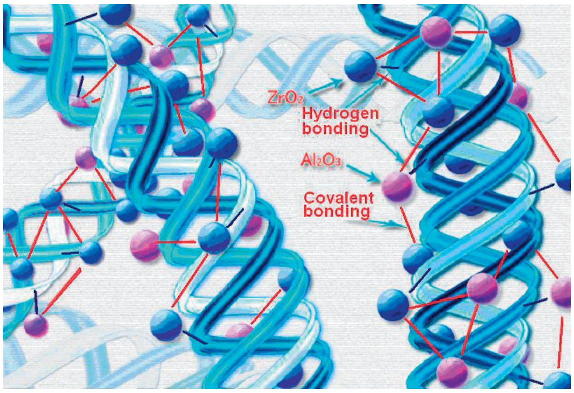


Figure 4. a) TGA and b) DTA curves for Al_2O_3–ZrO_2/grafted-collagen hybrid nanocomposites with 7.3 wt% Al_2O_3–ZrO_2 composite nanoparticles.

A schematic model for spatial interactions existing in this system is shown in Scheme 1. Hydrogen bonds may be formed between the oxygen atom of the bimetallic oxide nanoparticles and the –OH or $–NH_2$ groups on the grafted collagen (blue line); additionally, covalent bonds can link adjacent



Scheme 1. Triple-helix scaffolds of grafted collagen supported by Al_2O_3–ZrO_2 composite nanoparticles. Covalent bonds (red line) link adjacent Al_2O_3 (violet) and ZrO_2 (blue) species, which in turn link triple-helix scaffolds of grafted collagen by hydrogen bonding (blue line).

Al_2O_3 (violet) and ZrO_2 (blue) species (red line),[16] resulting in the formation of the composite nanoparticles shown in Figure 3A. The resulting hybrid nanocomposites reduce the exposure of the collagen to collagenase, and are thus expected to significantly increase the resistance against collagenase degradation.[18,19] At the same time, chemical crosslinking may reduce the immunogenicity of collagen when used in biomedical and bionic fields,[18] and thus it is expected that the enhanced hybrid nanocomposite will act as less of an antigen than collagen alone. The synthesis strategy presented here is facile and can be used as a model system to study the influence of nanoparticles on polymer matrices.

In conclusion, this paper demonstrates the feasibility of using Al_2O_3–ZrO_2 composite nanoparticles to enhance the mechanical properties of triple helices of collagen. The incorporation of an appropriate metal oxide into a surface-modified polymer matrix can provide substantial control over the crystalline phase and morphology of the polymer matrix. The enhanced mechanical and thermal stabilities of the collagen matrix are attributed to the structural and morphological changes induced by the nanoparticles. Our results not only illustrate a potential route to reinforce biological matrices with metal-oxide-functionalized nanoparticles, but also offer some preliminary insight into the interaction mechanisms existing in such hybrid materials. This work provides a new approach for reinforcing the mechanical and thermal properties of biopolymers, and therefore can potentially be used for biomedical and bionic applications.

Experimental

Preparation of Al_2O_3–ZrO_2 Composite Nanoparticles: 1.0 g pure aluminum and 3.4 g zirconium were dissolved in 10 mL nitric acid (6 mol L^{-1}). Then, 0.115 g citric acid (0.01 mol L^{-1}) and 50 mL water were added into this system. The reaction mixture was maintained at 60 °C for 2 h. Subsequently, 0.05 mol L^{-1} ammonia was added dropwise until a system pH of 6–6.5 was reached. After this step, a sol was formed. The sol solution was allowed to stand for 2 h and then filtered to obtain the gel particles. Subsequently, the gel particles were separated by filtration under vacuum, dried, and ground into powder. The powder was heated at 500 °C for 4 h to produce the final Al_2O_3–ZrO_2 composite nanoparticles.

Preparation of Grafted Collagen Matrix: 2.0 g collagen was dissolved in a 160 mL 1:3 (w/w) methanol/water mixture. A calculated amount of methyl methacrylate monomer (0.5 mol L^{-1}) was added into the solution, followed by a 1:1 mixture of 0.01 M ammonium cerium (IV) nitrate (CAN, 99.9 wt %) in 1 mol L^{-1} nitric acid. 0.01 mol L^{-1} 2,2′-azobis(2-methylpropionitrile) in methanol was added as the polymerization initiator. The resulting system was heated to 60 °C and maintained at this temperature for 2 h with vigorous stirring under a nitrogen atmosphere. The obtained products were then separated by filtration, and the recovered solid was washed with distilled water and extracted with acetone to remove the loosely bound homopolymer. This process was continued until no remaining homopolymer was observed.

Preparation of Al_2O_3–ZrO_2-Nanoparticles/Grafted-Collagen Hybrid Nanocomposite: The obtained grafted collagen matrix and Al_2O_3–ZrO_2 composite nanoparticles were completely dispersed in ethanol and stirred together for 8 h. After ultrasonication for 4–6 h, the resulting product was refrigerated for 2 h to remove any impurities and to obtain the nanocomposites.

Characterization: Stress–strain curves were recorded using a model 5567 Instron tester. Tensile tests were carried out for at least five specimens of each sample at room temperature at a cross-head speed of 50 mm min^{-1}. Storage modulus tests were performed with a DMA Q800 spectrometer (TA Instruments, USA) working in the tensile mode. The hardness and modulus tests were performed on a MTS Nano Indenter XP instrument (MTS Corp., TN). TEM images were obtained using a Hitachi H-600 microscope operated at 80 kV with a 35 µm objective. Specimens for sectioning were embedded in a resin and cured prior to sectioning 70 nm thick samples. Sectioned samples were placed on a carbon-coated copper grid. SEM images were obtained using a Hitachi X650 SEM operating at an accelerating voltage of 20 kV. The thermal analysis data was obtained on a TA Q-600 TGA/DTA apparatus (TA Instruments, USA) operated at a heating rate of 20 °C min^{-1}, to simultaneously determine the correlation between the temperature and weight loss and reaction enthalpies of the materials.

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[1] C. A. Vacanti, J. P. Vacanti, R. Langer, *Tissue Engineering Using Synthetic Biodegradable Polymers*, ACS Symposium Series, Vol. 540, ACS, Washington, DC **1994**, p. 16.

[2] R. P. Lanza, R. Langer, W. L. Chick, *Principles of Tissue Engineering*, Academic, New York **1996**, p. 273.

[3] H. O. Ho, T. Tsai, C. M. Liu, M. T. Sheu, *J. Biomed. Mater. Res.* **2002**, *62*, 22.

[4] T. Koide, M. Yuguchi, M. Kawakita, H. Konno, *J. Am. Chem. Soc.* **2002**, *124*, 9388.

[5] G. M. Sessler, *Electrets*, Springer, Berlin **1980**, p. 330.

[6] G. A. Kinberger, W. Cai, M. Goodman, *J. Am. Chem. Soc.* **2002**, *124*, 15 162.

[7] L. E. Bretscher, C. L. Jenkins, K. M. Taylor, M. L. DeRider, R. T. Raines, *J. Am. Chem. Soc.* **2001**, *123*, 777.

[8] I. R. Babu, K. N. Ganesh, *J. Am. Chem. Soc.* **2001**, *123*, 2079.

[9] R. Berisio, V. Granata, L. Vitagliano, A. Zagari, *J. Am. Chem. Soc.* **2004**, *126*, 11 402.

[10] C. V. Santos, A. L. M. Hernandez, F. T. Fisher, R. Ruoff, V. M. Castano, *Chem. Mater.* **2003**, *15*, 4470.

[11] S. F. Wang, L. Shen, W. D. Zhang, Y. J. Tong, *Biomacromolecules* **2005**, *6*, 3067.

[12] K. Se, K. Aoyama, *Polymer* **2004**, *45*, 79.

[13] T. Lan, T. J. Pinnavaia, *Chem. Mater.* **1994**, *6*, 2216.

[14] H. Shi, T. Lan, T. J. Pinnavaia, *Chem. Mater.* **1996**, *8*, 1584.

[15] X. Ji, J. E. Hampsey, Q. Hu, J. He, Z. Yang, Y. Lu, *Chem. Mater.* **2003**, *15*, 3656.

[16] Y. Cao, Y. M. Zhou, Y. Shan, H. X. Ju, X. J. Xue Z. H. Wu, *Adv. Mater.* **2004**, *16*, 1189.

[17] Y. Nomura, S. Toki, Y. Ishii, K. Shiral, *J. Agric. Food Chem.* **2000**, *48*, 2028.

[18] S. Basu, L. P. Cunningham, G. D. Pins, K. A. Bush, R. Taboada, A. R. Howell, J. Wang, P. J. Campagnola, *Biomacromolecules* **2005**, *6*, 1465.

[19] S. P. Zhong, W. E. Teo, X. Zhu, R. Beuerman, S. Ramakrishna, L. Y. L. Yung, *Biomacromolecules* **2005**, *6*, 2998.