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SPECIAL ISSUE ARTICLE

# Characterization of Fish Gelatin at Nanoscale Using Atomic Force Microscopy

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**Abstract** Atomic force microscopy (AFM) was used as a meaningful tool to characterize the nanostructure of gelatin from catfish (*Ictalurus punctatus*) skin. The gelatins extracted with pretreatments including acid pretreatment, alkaline pretreatment, and alkaline followed by acid pretreatment (optimized extraction conditions). The resulting gelatins were imaged using AFM and their nanostructure was studied. The AFM images showed that gelatin extracted with acid pretreatment had a coacervate structure while with alkaline pretreatment there were separate aggregates. Spherical aggregates and annular pores were observed in AFM images of gelatin with the optimized extraction conditions. AFM imaging of gelatin with a relative high concentration (0.5%) was successfully done and the results help researchers to understand gelatin structures at the nanoscale.

**Keywords** Nanotechnology · Atomic force microscopy · Gelatin · Fish skin · Nanostructure · Catfish

## Introduction

Gelatin is a polypeptide obtained by thermal hydrolysis of collagen. Pretreatment with alkaline and/or acid and a subsequent hot water extraction are a common technology used for gelatin manufacture. Gelatin's unique functional properties including gelling and melting below 35 °C and its reasonable market prices make it an important ingredient in the food and pharmaceutical industries. The gelatin industry primarily uses cattle hides, beef bones, and pork skin as raw materials to produce gelatin. Recently, fish gelatin has provided an alternative to cattle and pork gelatin for religious, safety, and economic reasons.[1,2] Similar to other biomaterials, gelatin's properties are mainly determined by its structures. Study of gelatin at different size scales can provide different useful information. Therefore, the structures at different scales from the macroscale to the nanoscale should be studied. At the nanoscale level, the physical, chemical, and biological properties of materials fundamentally differ from those at bulk scale. Atomic force microscopy, a nanotechnology tool, has been applied successfully to many fields of food science and technology including imaging of pectin,[3,4] high-purity laboratory-prepared mammalian gelatins,[5–11] and recently gelatin from fish skin.[12–14]

The objective of this work was to characterize the nanostructure of food-grade fish gelatin obtained using different preparation conditions and different concentrations. The atomic force microscopy (AFM) experimental conditions and AFM imaging of the gelatins from fish gelatin were studied and optimized to obtain the most meaningful results.

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## Materials and Methods

### Gelatin Preparation

The extraction of catfish skin gelatin was carried out according to previously developed procedures.[2,12,13] All reagents used in this study were of analytical grade. Frozen catfish skins (Harvest Select Inc., Uniontown, AL, USA) were thawed at 4 °C for about 20 h, then cut into 2–3-cm

squares and washed with tap water (1:6 *w/v*) at 4 °C for 10 min. The washing was repeated three times. Then, the skins were drained using four layers of cheesecloth for 5 min, and the cheesecloth containing the skins was squeezed by hand as much as possible. The squeezed skins were divided into three groups. The first group was pretreated with 0.1 M acetic acid for 60 min and the second group was pretreated with 1.0 M NaOH for 50 min. These two groups were treated at 50 °C water bath for 3 h. In the third group (optimized extraction conditions), the skins were put into a flask and 0.20 M NaOH (1:6 *w/v*) was added for 84 min. After that, the samples were drained including hand squeezing using the cheesecloth and rinsed with tap water (1:6 *w/v*). The washing was repeated two times, and then 0.115 M acetic acid (1:6 *w/v*) was added for 60 min, drained with cheesecloth and rinsed with tap water (1:6 *w/v*) for three times. All the solutions used were at 4 °C. After the above pretreatment, deionized water (1:4 *w/v*) was added to the flasks. The flasks and samples were covered with parafilm (Structure Probe Inc/SPT Supplies, West Chester, PA, USA) and aluminum foil and were put in a 55 °C water bath (Model 86; Precision Scientific Co., Chicago, IL, USA) for 180 min. Then, the solutions were filtered through four layers of cheesecloth and the filtered solution was lyophilized (Labconco Corporation, Kansas City, MO, USA) or frozen until used.

### AFM Imaging

The fish gelatin solutions were thawed or diluted from the prepared gelatin. The solutions were melted in hot water and disrupted for about 3 min with a vortex mixer (Fisher Scientific, Pittsburgh, PA, USA). The solution was then diluted to a designated concentration and a small volume (about 20 μL) was pipetted onto a piece of freshly cleaved mica sheets (Electron Microscopy Sciences, Hatfield, PA, USA). The mica surface was then naturally air-dried for about 1 h at room temperature before AFM imaging. An AFM (Nano-R2™, Pacific Nanotechnology Inc, Santa Clara, CA, USA) was used to characterize the nanostructure of gelatin in air using the noncontact mode. The noncontact mode in this AFM is comparable to the commonly called tapping mode (registered trademark by another company) in many of the previous references. Therefore, "tapping mode" was used to describe this format in the text. The NSC 11/no Al (MikroMasch, Wilsonville, OR, USA) tip with a resonance frequency of 330 kHz and a force constant of 48 N/m was used. The scan speed was about 0.5–2 Hz.[12] For imaging gelatin with high concentration (0.5%), a rubber suction bulb was used to generate strong-forced air to extend the gelatin solutions when they were dropped on the mica.

### Analysis of AFM Images

The AFM images were analyzed offline using a NanoRule+™ AFM software provided by the company. The software can use a flattening correction to improve the contrast of the images. Different scales were used with the vertical and horizontal axes. Both the height mode (including 3D and 2D versions) and the error signal mode images were obtained. The height mode images recorded the height information and the error signal mode removed some of the variations in the topography, but strengthened the feature edges. Section analysis of the software can be applied to quantitatively determine the dimensions of the objects.

## Results and Discussion

### Effects of Alkaline and Acid Pretreatment on the Nanostructure of Gelatin

Figure 1 shows the nanostructure of gelatins obtained with acid and alkaline pretreatments. Gelatins with acid pretreatment (Figure 1a) show a coacervate structure without definite geometric shape, which is similar to that of acid-treated pig gelatin reported.[8] Gelatins with alkaline pretreatment (Figure 1b) show separate aggregates with relatively clear border. The results show that aggregates with alkaline pretreatment have nonregular shapes (Figure 1b).[1] reported that alkaline extrac-

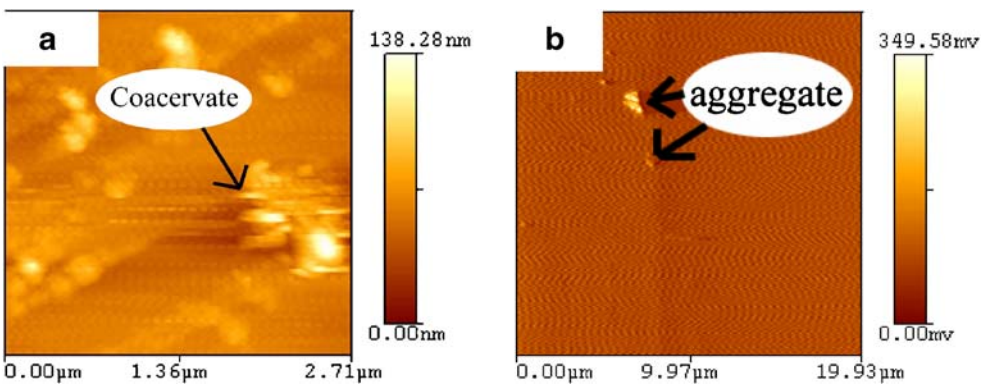


**Fig. 1** AFM images of gelatin pretreated with acid and alkaline. **a** Pretreatment with 0.1 M acetic acid for 60 min and **b** pretreatment with 1.0 M NaOH for 50 min. Note: the gelatins were extracted at 50 °C water bath for 3 h

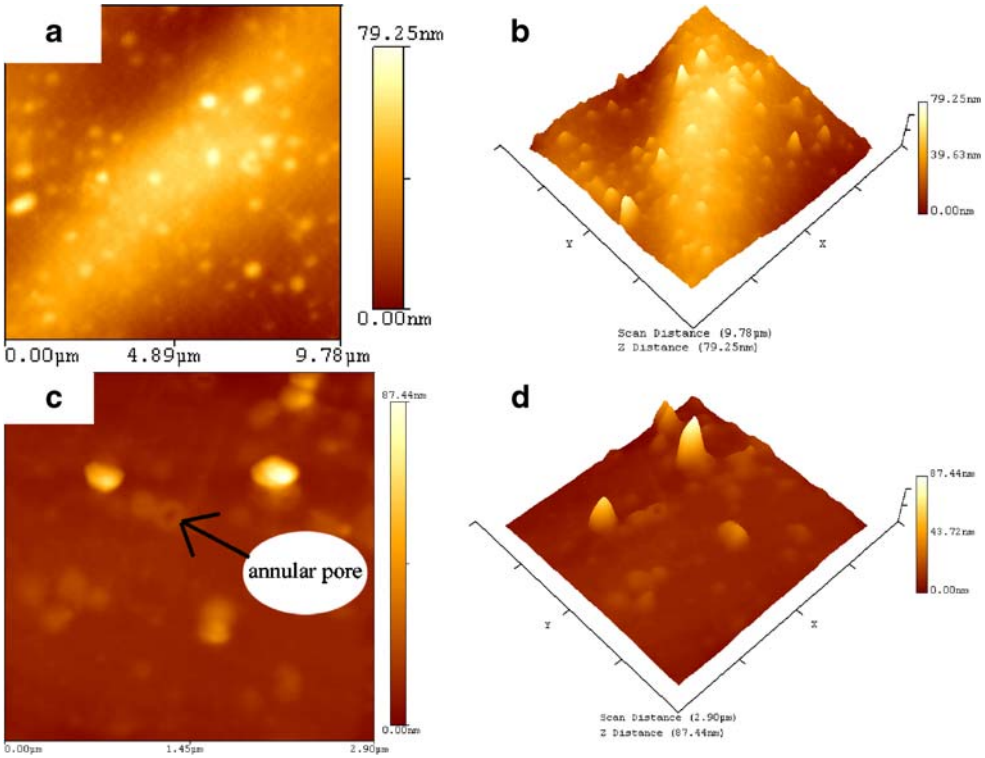


**Fig. 2** AFM images of gelatin extracted using optimized conditions. **a** Height mode image, **b** corresponding 3D image, **c** annular pore image in height mode, and **d** corresponding 3D image

tion caused the breakdown of some polypeptide chains of collagen into small pieces, which probably result in the irregular shape of the aggregates.[1] The different shapes of the nanoscale structures of gelatins between acid and alkaline pretreatment indicates that the pH of the pretreatment influenced the nanostructure of the gelatin. The pretreatments influenced the primary structure of the polypeptides by hydrolysis of collagen at different amino acid locations.

## AFM Images of Gelatin Extracted Using the Optimized Conditions

Figure 2 shows AFM images of gelatin extracted using the optimized conditions previously described.[2] The results indicate that most gelatin aggregates show a spherical structure with different diameters. Only a few samples with annular pores were found. We proposed a mechanism to describe the formation of the aggregates and annular pores observed in AFM images of gelatin in our previous study.[12] Similar arguments can be used to explain the results in Figure 2. Two pathways for the aggregation of fish gelatin during hydrolysis and sample drying were proposed. In pathway 1, a large amount of water with cations and anions from the alkaline and/or acid penetrates into the collagen molecules during hydrolysis and aggregation, then more gelatin molecules conglomerate at the outer layer of the water aggregates during aggregation. Therefore, a large water pool is formed with a spherical shape. The water in this pool is evaporated during the air-drying before AFM imaging and is then observed as a hollow structure surrounded by gelatin (annular pores). In pathway 2, the solution penetrates into the gelatin molecules with minimal

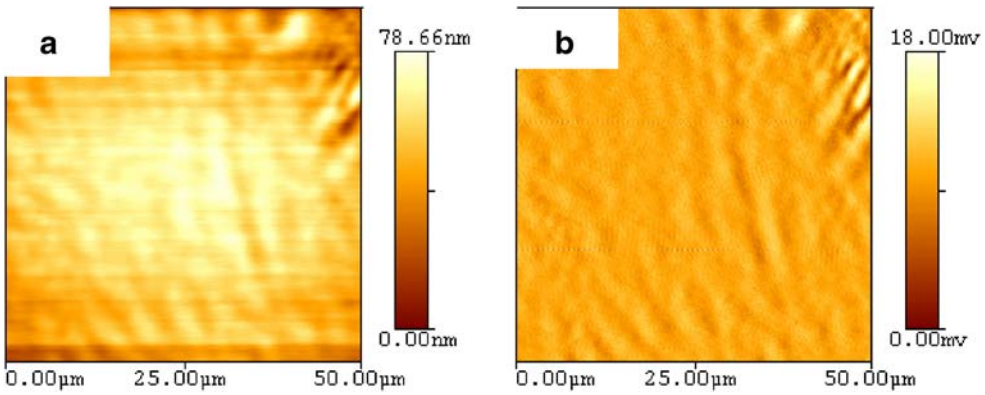


**Fig. 3** AFM images of 0.5% gelatin extracted using optimized condition. **a** Height mode image and **b** corresponding error signal mode image

aggregation. Therefore, although many small water droplets and ions penetrate into the gelatin molecules, they are separated from one another by gelatin molecules. Thus, the result of the hydrolysis is a more even distribution and aggregation of the water and salt ions. In this case only small water pools are formed in the process. The encompassing gelatin molecules are more inclined to join together during the water evaporation before AFM imaging, leading in the end to the formation of compact spheres. Furthermore, the quantitative aspects of the aggregates or annular pores can be determined from the sectional analysis.[5,12,13]

We also tried to image the gelatin at a relatively high concentration, which may be well correlated to the macroscale analysis of physical properties. Images of gelatin with relatively high concentration (0.5%) were obtained (Figure 3). The fibril structure of gelatin that is seen in the images is similar to the electronic microscopy images reported by Chang et al. (2003).[15] Further work will be done to obtain gelatin images at even high concentrations.

## Conclusions

AFM images of the nanostructure of fish skin gelatin were studied. Gelatin with acid pretreatment showed coacervate structure, with alkaline pretreatment gelatin showed a structure with separate aggregates. Spherical aggregates and annular pores were seen for gelatin extracted at the optimized conditions. Imaging of a relatively high concentration of gelatin (0.5%) was successfully developed.

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