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Effect of extraction methods on the preparation of electrospun/electrosprayed microstructures of *tilapia* skin collagen

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Collagen plays a pivotal role in human physiological functions and extracted collagen has multiple potential applications. *Tilapia* skin can be applied to extract collagen for maximizing the profit of *tilapia* processing. Electrospinning/electrospraying is novel micro- and nano-techniques to fabricate microfibers and microspheres in a simple and easy way. In this work, we extract collagens from *tilapia* skin by three types of extraction methods: acetic acid method, hot water method, and sodium hydroxide method. Then, these extracted collagens are characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Fourier transform infrared spectrometry. These extracted collagens have different molecular weights and different protein secondary structures. Finally, these extracted collagens are applied to fabricate electrosprayed microspheres, electrospun microfibers, and mixed microspheres/microfibers with multiple potential applications by adjusting the collagen concentrations. Higher polymer molecular weight only needs lower concentration to produce microfibers. The microfiber diameter increases with the increase of collagen concentration. This work proves that extraction methods have obvious effect on the preparation of electrospun/electrosprayed microstructures of *tilapia* skin collagen and provide a way to maximize resource utilization of *tilapia* processing waste.

[**Key words:** Fish skin collagen; Electrospinning; Extraction; Microfiber; Microsphere]

Collagen is the most common insoluble fibrous protein in animal connective tissue and plays a pivotal role in the distinctive physiological functions of tissues in bones, skin, tendons, and cartilage (1). Due to its excellent biocompatibility and biodegradability, collagen has been widely applied in food, pharmaceutical, biomedical, leather, cosmetics, cell culture (2) and tissue engineering industries (3). Collagen is thought to be expensive and some cost-effective alternatives such as silk fibroin have been developed in these fields (4,5). Hence, it is necessary to produce cost-effective collagen.

Conventionally, collagen is extracted from the skins and bones of land-based animals such as cows and pigs. However, the outbreaks of bovine sponge encephalopathy, transmissible spongiform encephalopathy and foot-and-mouth disease have led to anxiety that collagen from the land-based animals may introduce cross-infection between human and land-based animals. In addition, pig-derived collagen cannot be used in some foods due to religious reasons (6). Therefore, these issues have caused limitations on the use of collagen from land-based animals. It is necessary to find safe and cost-effective collagen sources. Recently, scientists started to explore collagen extraction from by-products of aquatic products such as skin, bone, fins, and scale (7,8). These collagens are cost-effective and can be used as substitutes for collagens from land-based animals in the future due to their excellent characteristics.

Tilapia has become the shining star of aquaculture with farms and the most important food fish in the world. *Tilapia* is sometimes described as aquatic chicken due to their fast growth speed, adaptability to a wide range of environmental conditions, ability to grow and reproduce in captivity, easy feed on low trophic level, and easy processing to fish fillets (9). *Tilapia* culture has been expanding rapidly and is now practiced in more than one hundred countries in the world. Approximately 60–70% of the fish body is processing waste, which includes meat remains, head, bones, skin, scale, and viscera (10). *Tilapia* skin is a good resource for the production of collagen. It also can solve the problem of waste disposal and, in addition, create a value-added product. Collagen has been successfully extracted from *tilapia* skin by several methods such as acetic acid method (11) and pepsin hydrolysis (12).

Electrospinning/electrospraying is emerging micro- and nano-techniques using the same instrument, namely electrostatic spinning machine, to simply and easily fabricate microfibers and

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microspheres. Due to huge advantages of microfibers such as easy fabrication, easy functionalization, high surface-to-volume ratio, and surface bionics to the extracellular matrix for application in tissue engineering and regenerative medicine, electrospun nanofibrous scaffolds have been extensively explored in tissue engineering (13–18), drug delivery (19–21), energy science (22–24), sensors (25–27), catalyst (28,29), filtration (30,31), food science (32–34), and environmental science (35). Electrospinning has been applied to fabricate micro- and nano-fibers from acid soluble calf-skin collagen (36), acid soluble bovine dermis collagen (37), alkali soluble *tilapia* skin collagen (38), and acid soluble *tilapia* skin collagen/polycaprolactone (39). Due to huge advantages of microspheres such as high entrapment efficiency of bioactive substances, ideal mask of unwanted odor and taste, and ideal bioactivity protection (40), electrosprayed microspheres have been widely explored for controlled release bioactive drugs and food substances (41,42). Electrospraying was applied to fabricated microspheres from pig skin gelatin (42). Electrospinning/electrospraying could play important roles in the maximization of resource utilization of *tilapia* skin collagens for tissue engineering, drug delivery, and food science. Hence, it is necessary to analyze the effect of extraction methods on the preparation of electrospun/electrosprayed microstructures of *tilapia* skin collagen.

MATERIALS AND METHODS

Materials All chemicals were of analytical reagent grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and were used without purification. All the solutions were prepared by using ultrapure water.

***Tilapia* skin collagen extraction** *Tilapia* skins were provided by Zhanjiang Shuanghu Food Co. Ltd. (Guangdong Province, China). The *tilapia* skins were immediately frozen by a double drum spiral freezer after the *tilapia* processing in the company, refrigerated transport to the laboratory, and then were stored at −20°C. Before use, the skins were thawed in tap water at room temperature and cut into about 5 × 5 mm pieces by a pair of scissors. Skin pieces (50 g) were washed with ultrapure water at room temperature for 30 min, mixed with 2 volumes (v/w) ethanol for 24 h at room temperature without stirring to remove fat and other residues. After that, the skin pieces were washed with ultrapure water three times and were set aside till water is drained out. Then collagens were extracted by three different methods: acetic acid method (1000 mL 0.5 M acetic acid for 24 h at room temperature), hot water method (1000 mL 80°C ultrapure water for 6 h in an oscillatory water bath shaker incubator at 10 rpm), and sodium hydroxide method (1000 mL 0.12 M NaOH for 24 h at room temperature). Then the samples were filtered with a double layer of gauze. The resultant solution was dialyzed in ultrapure water for 72 h using dialysis cassettes (MW: 2000 KD, Spectra/Por 7 dialysis membranes, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). This step was done at 4°C for acetic acid method and sodium hydroxide method and was done at room temperature for hot water method. Finally, pure collagen solutions were acquired and were vacuum freeze dried to get pure dried collagen. The obtained collagen samples were photoed by a digital camera.

Collagen yield During the *tilapia* skin collagen extraction process, the collagen production yields were calculated as the obtained collagen mass divided by the used fish skin mass. Briefly, after that the fish skins were cut into about 5 × 5 mm pieces, the wet skin pieces were set aside untill water is drained out dripped dry and then weighed (W_0). The prepared freeze-dried collagens were weighed (W_1). Finally, the collagen production yield (based on the wet weight of fish skin) was calculated according to below equation:

$$\text{Production yield (based on the wet weight of fish skin) (\%)} = (W_1\ [\text{g}]/W_0\ [\text{g}]) \times 100\% \quad (1)$$

Tilapia fish skin contains the collagen at the level of 21.89 g/100 g (43). Therefore, the collagen production yield based on the initial collagen presented in the skin or recovery (%) was calculated according to below equation:

$$\text{Production yield (based on the initial collagen in the fish skin)} = \text{Production yield (based on the wet weight of fish skin) (\%)}/21.89\% \quad (2)$$

SDS-PAGE The purified collagens were analyzed on 8% SurePAGE Bis-Tris gels (GenScript, Nanjing, China). The collagen samples were dissolved in chilled ultrapure water (4°C, 1 mg/mL) for 24 h. The solution pH was adjusted o pH 7.0. The solubilized samples were mixed at 1:1 (v/v) ratio with 2X SDS-PAGE sample loading buffer (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China) and were boiled for 5 min. Next, samples (5 μL) were loaded onto in each well. The electrophoresis was carried out for 50 min with a DYCZ-24KS electrophoresis cell (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China) and a DYY-6D electrophoresis power supply (Beijing Liuyi Biotechnology Co., Ltd.). The electrophoresis voltage was set as 120 V. To estimate the molecular weight of proteins, broad multi color pre-stained protein standard (GenScript) was used. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 25% (v/v) isopropanol and 10% (v/v) acetic acid for 3 h. Then it was destained by a destaining solution with 5% (v/v) methanol and 10% (v/v) acetic acid until clear bands could be observed. The destained gel was photoed by a digital camera.

FIG. 1. Digital camera image of *tilapia* skin collagen extracted by acetic acid method (AAM), hot water method (HWM), and sodium hydroxide method (SHM).

Fourier transform infrared spectroscopy measurements The functional groups of chemicals were analyzed by a Fourier transform infrared (FTIR) spectrometer (Nicolet iS5, Thermo Scientific, Madison, WI, USA) in a wavenumber range of 650–4000 cm^{-1}. *Tilapia* skin collagen powders were analyzed by a KBr pellet method. Briefly, dried collagen proteins and KBr were mixed and grinded into powders. Then, the mixture was pressed into self-supported discs (13 mm in diameter). Finally, the infrared spectra of these discs were analyzed by the FTIR spectrometer with the subtraction of KBr background. The areas of 1600–1700 cm^{-1} were chosen to analyze the secondary structures of collagens using PeakFit v4.12 (SeaSolve Software Inc., San Jose, CA, USA).

Preparation of electrospun/electrsprayed microstructures Electrospun/electrsprayed microstructures (microfibers and microspheres) were fabricated by a custom-designed electrospinning instrument. Briefly, dried *tilapia* skin collagen (1%, 5%, 11%, 18%, 25%, 34%, and 43%, w/v) were added into 1,1,1,3,3,3-hexafluoro-2-propanol, and then the solution was magnetically stirred (100 rpm) for 24 h. The collagen solution was loaded in a 5 mL syringe that was fitted with a needle (inner diameter of 0.41 mm) and placed on a syringe pump. An applied voltage of about 20 kV was produced using a high voltage power supply (Tianjin Dongwen High Voltage Power Supply Co., Ltd, Tianjin, China). The feeding rate was 1.8 mL/h. A grounded stainless-steel rotating rod (200 mm in length and 10 mm in diameter) attached to a laboratory mixer motor with a rotation speed of 50–120 rpm was applied as a potential collector for electrospun microfibers. The distance between the syringe needle and the collector (round stainless-steel rod) was 15–20 cm.

Microstructures morphology observation Collagen microstructures (microfibers and microspheres) were collected by a square cover glass (22 × 22 mm, extra thin) and then their morphologies were observed by an inverted optical microscope (MS500W, Shanghai Minz Precision Instruments Co. Ltd., Shanghai, China).

RESULTS AND DISCUSSION

Preparation and production yields of collagen samples There are several collagen extraction methods such as acetic acid method, hot water method, sodium hydroxide method, and pepsin method. Acetic acid method has less effect on triple helix structure of collagens. Hot water method commonly denatures collagens. Sodium hydroxide method commonly destroyes

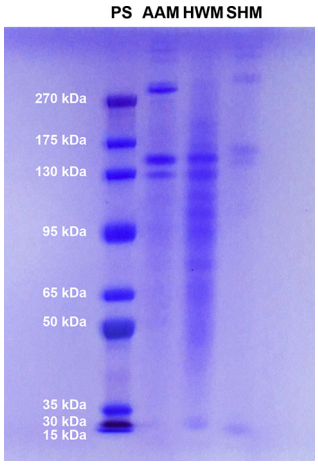

FIG. 2. SDS-PAGE pattern of *tilapia* skin collagen extracted by acetic acid method (AAM), hot water method (HWM), and sodium hydroxide method (SHM). The first lane is the protein standard (PS).

the amino acid structures of collagens. Pepsin method introduces enzymes into the final collagen products, which may affect the application of electrospun collagen microfibers. Therefore, in this work, three types of classical extraction methods with simple preparation steps and different effects on extracted collagens were chosen. Using three different extraction methods (acetic acid method, hot water method, and sodium hydroxide method), *tilapia* collagens were successfully prepared. As shown in Fig. 1, *tilapia* skin collagen samples extracted by acetic acid method and hot water method showed white color, whereas *tilapia* skin collagen sample extracted by sodium hydroxide method showed light yellow color. The production yields (based on the wet weight of fish skin) were: acetic acid method (11.7%) > hot water method (10.7%) > sodium hydroxide method (8.5%). *Tilapia* fish skin contained the collagen at the level of 21.89 g/100 g (43). Therefore, the yield based on the initial collagen presented in the skin or recovery (%) for acetic acid method, hot water method, and sodium hydroxide method were 53.4%, 48.9%, and 38.8%, respectively. These values were significantly higher than that from unicorn letherjacket skin (18.47%) (44), dry *tilapia* fish skin (19.80% for acetic acid method and 20.03% for pepsin method) (43), bigeye snapper skin (1.59%) (45), and deep-sea redfish skin (10.3%) (46). These values were similar to that from grass carp skin (46.6%) (47), Japanese sea-bass bone (40.7%), ayu bone (53.6%), yellow sea bream bone (40.1%), and horse mackerel bone (43.5%) (8,48). It should be noted that the extraction temperature strongly affected extraction efficiency (49). If high extraction efficiency is needed, optimization of the extraction temperatures for these three types of preparation methods will be necessary to be studied.

SDS-PAGE pattern of collagens Previously work has already demonstrated that collagen in *tilapia* fish skin is type I collagen, a heterotrimer containing two identical α_1 and one α_2 chain in the molecular form (43). SDS-PAGE patterns of collagen samples in this work are shown in Fig. 2. The protein patterns of collagen extracted by acetic acid method showed three clear bands of about 280 kDa, 150 kDa, and 130 kDa. They corresponds to β-chain, α_1 chain, and α_2 chain, respectively. The band intensity of α_1 chain was approximately 2-fold higher than that of α_2 chain. The protein patterns of collagen extracted by hot water method showed two clear bands (about 150 kDa and 130 kDa) and a wide blurry band (about 170 kDa–45 kDa). There was no band of 280 kDa in this collagen sample. Moreover, the band intensities of α_1 and α_2 chains from acetic acid method were higher than those of α chains from hot water method. The protein patterns of collagen extracted by sodium hydroxide method showed four blurry bands of about 280 kDa, 160 kDa, 140 kDa, and 10 kDa. It suggested the collagen molecules were mostly hydrolyzed during the sodium hydroxide method.

FTIR spectra of collagens The representative FTIR absorption spectra of three types of collagen samples in the 4000 to 650 cm^{-1} range are shown in Fig. 3 and Table 1. The shapes of three types of collagen samples were similar. The FTIR spectra of the collagen samples were characterized by a set of absorption regions known as the amide modes. They are resulted from the vibration of the peptide groups and can provide secondary structure information of proteins.

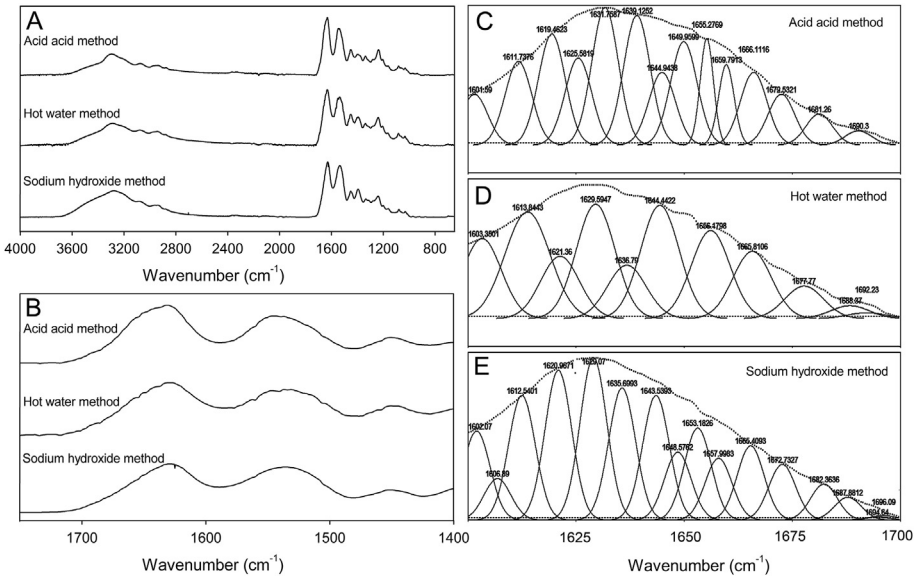

FIG. 3. FTIR spectra of *tilapia* skin collagen extracted by acetic acid method (AAM), hot water method (HWM), and sodium hydroxide method (SHM) with different wavelength range: (A) 4000–650 cm^{-1}; (B) 1750–1400 cm^{-1}; (C–E) 1600–1700 cm^{-1}. The dotted lines are the spectra and the solid lines are the deconvoluted peaks by PeakFit software.

TABLE 1. Attenuated total reflectance (ATR)-FTIR spectra peak locations and assignment for *tilapia* skin collagen samples extracted by three different preparation methods.

Region	Acetic acid method	Hot water method	Sodium hydroxide method	Assignment
Amide A	3296	3281	3271	N–H stretch, coupled with hydrogen bond
Amide B	2938	2939	2936	Asymmetrical stretch of CH_2 and NH_3^+
Amide I	1631	1629	1628	C=O stretch/hydrogen bond coupled with COO^-
Amide II	1544	1536	1536	N–H bend coupled with CN stretch
Amide III	1236	1236	1241	CH_2 group wagging vibration in the glycine backbone proline side chains

The amide A band is mainly associated with N–H stretch and the peak position will shift from a range of 3440–3400 cm^{-1} to a lower peak position (about 3300 cm^{-1}) if the N–H group is coupled with hydrogen bond in the peptide chain (50). According to the amide A peak position of three types of collagens (Table 1), it could be speculated that the amounts of NH group that participate in the hydrogen bond association were SHM > HWM > AAM. The amide B band is related to asymmetrical stretch of CH_2 and NH_3^+ and the shift of amide B to lower wavenumber is resulted from a decrease of free NH_3^+ groups from lysine residues of N-terminal (50). According to the amide B peak position of three types of collagens (Table 1), it could be speculated that the amounts of free NH_3^+ group in the protein chains were: HWM > AAM > SHM. The amide I band is mainly related to the C=O stretching vibrations and the lower wavenumber corresponds to higher hydrogen bonding potential (51). According to the amide I peak position of three types of collagens (Table 1), it could be speculated that the hydrogen bond amounts between α-chains were SHM > HWM > AAM. The amide II band is mainly related N–H bend coupled with CN stretch and the lower wavenumbers indicates the increase of hydrogen bonds between α-chains by NH groups (52), which is attributed to higher structure order in protein chains. According to the amide I peak position of three types of collagens (Table 1), it could be speculated that the NH group-based hydrogen bond amounts between α-chains were SHM = HWM > AAM. The amide III is normally very week in the FTIR spectra and is assigned to the CH_2 group wagging vibration in the glycine backbone proline side chains (52).

The areas of 1600–1700 cm^{-1} (amide I band, Fig. 3C–E) were chosen to analyze the collagen secondary structures (53,54): β-sheet (peak center 1610–1642 cm^{-1}), random coil (peak center 1642–1650 cm^{-1}), α-helix (peak center 1650–1660 cm^{-1}), β-turn (peak center 1660–1680 cm^{-1}), and β-antiparallel (peak center 1680–1700 cm^{-1}). Deconvoluted peak areas were statistically calculated to obtain the contents of secondary structural compositions of collagens, as shown in Table 2.

Preparation and characterization of electrospun/electrosprayed microstructures of collagens In this work, electrospinning technique was applied to prepare electrospun/electrosprayed microstructures of collagens. *Tilapia* skin collagen (1%, 5%, 11%, 18%, 25%, 34%, and 43%, w/v) solution in 1,1,1,3,3,3-hexafluoro-2-propanol were used. The obtained microstructures were collected on cover glass and were observed by optical microscopy, as shown in Fig. 4. For collagen extracted by acetic acid method, electrosprayed microspheres were fabricated using 1% collagen solution (Fig. 4A). A large number of long uniform electrospun microfibers were fabricated using the 5% (Fig. 4B), 11% (Fig. 4C), and 18% collagen solutions (Fig. 4D). Moreover, the microfiber diameter increased with the increase of collagen concentration. The 25% (Fig. 4E), 34% (Fig. 4F), and 43% collagen solutions (data not shown) were too thick to be applied to fabricate ideal uniform microfibers. For collagen extracted by hot water method, electrosprayed microspheres were fabricated using 1% collagen solution (data not shown). Electrosprayed microspheres and electrospun microfibers were fabricated using 5% collagen solution (Fig. 4G). A large number of long uniform electrospun microfibers were fabricated using the 11% (Fig. 4H), 18% (Fig. 4I), 25% (Fig. 4J) and 34% collagen solutions (Fig. 4K). Moreover, the microfiber diameter increased with the increase of collagen concentration. The 43% collagen solution (Fig. 4L) was too thick to be applied to fabricate ideal uniform microfibers. For collagen extracted by sodium hydroxide method, electrosprayed microspheres were fabricated using 1% (data not shown), 5% (Fig. 4M), and 11% collagen solution (Fig. 4N). A large number of long electrospun microfibers were fabricated using the 18% (Fig. 4O) and 25% collagen solutions (Fig. 4P). Moreover, the microfiber diameter increased with the increase of collagen concentration. The 34% (Fig. 4Q) and 43% collagen solutions (Fig. 4R) were too thick to be applied to fabricate ideal uniform microfibers.

The effect of concentration on the preparation of main microstructures for the three types of collagens was summarized. Electrosprayed microspheres could be fabricated by ACM collagen sample at concentrations of <5%, HWM collagen sample at concentrations of <5%, and SHM collagen sample at concentrations of <18%. This provided a way to fabricate collagen microspheres to encapsulate bioactive substances (55,56). Electrospun microfibers could be fabricated by ACM collagen sample at concentrations of 5%–18%, HWM collagen sample at concentrations of 11%–34%, and SHM collagen sample at concentrations of 18%–25%. This provided a way to fabricate collagen microfibers as scaffolds in many scientific fields such as tissue engineering and food science (34,57,58). Furthermore, mixed microspheres/microfibers could be fabricated by HWM collagen at a concentration of 5%. This also provided a way to fabricate microsphere-loaded nanofibrous scaffolds for controlled release of bioactive substances (59). Combined with the collagen molecular weight (Fig. 2), we could conclude that higher polymer molecular weight only needs lower concentration to produce microfibers and the border value between microsphere fabrication and microfiber fabrication was related with the polymer molecular weight. This is consistent with our previous work using synthetic polymer (60). According to the electrospinning results, all the three types of classical extraction methods could be applied for the preparation of electrosprayed microspheres and electrospun microfibers. They will be beneficial for high entrapment and controlled release of drug and nutrient substances (40) and tissue engineering regeneration (18).

To sum up, in this work, *tilapia* skin collagens were extracted by three different methods: acetic acid method, hot water method, and sodium hydroxide method. These extracted collagens had different molecular weights and different protein secondary structures

TABLE 2. Secondary structure percentage (%) analysis of three types of collagens by analyzing the areas of 1600–1700 cm^{-1} in ATR-FTIR spectra.

Preparation methods	β-sheet	Random coil	α-helix	β-turn	β-antiparallel
Acetic acid method	52.27 ± 2.49	16.00 ± 1.45	13.66 ± 3.44	13.18 ± 0.60[a]	4.88 ± 0.53[a]
Hot water method	51.29 ± 1.08	17.66 ± 2.09	13.64 ± 1.39	14.70 ± 0.51[b]	2.72 ± 0.07[b]
Sodium hydroxide method	51.38 ± 1.70	16.70 ± 1.27	12.79 ± 0.25	12.63 ± 0.21[a]	6.50 ± 0.52[c]

For the same column, different letters indicate significant differences ($p < 0.05$).

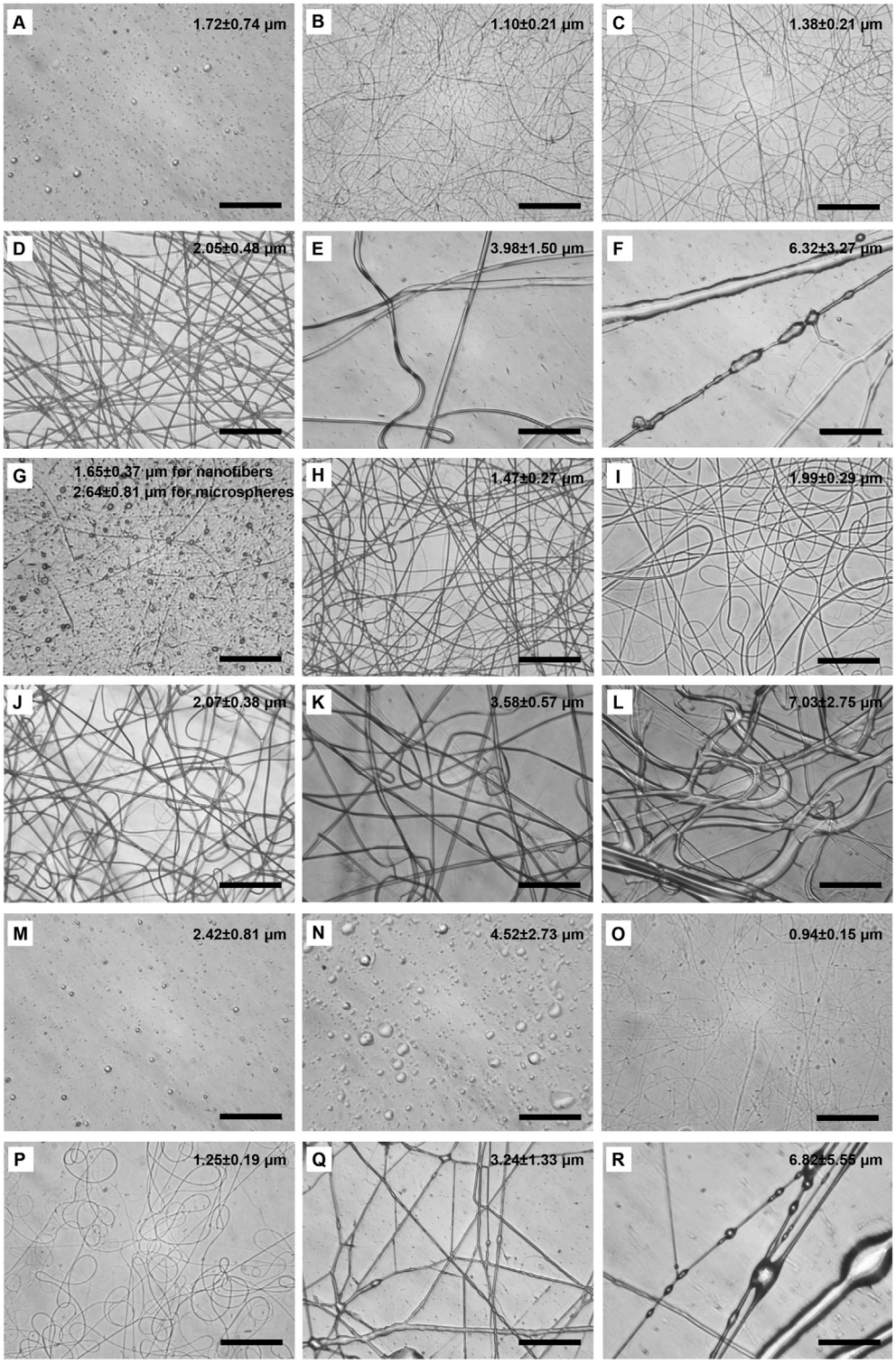

FIG. 4. Optical microscopy images of electrospun microstructures of *tilapia* skin collagen by acetic acid extraction (A–F), hot water extraction (G–L), and sodium hydroxide extraction (M–R). (A) 1%, (B) 5%, (C) 11%, (D) 18%, (E) 25%, (F) 34%, (G) 5%, (H) 11%, (I) 18%, (J) 25%, (K) 34%, (L) 43%, (M) 5%, (N) 11%, (O) 18%, (P) 25%, (Q) 34%, (R) 43%. Scale bar indicates 50 μm. The numbers on the top-right position of the image indicate the size of microspheres or nanofibers (n = 40).

(mainly different in the amounts of β-turn and β-antiparallel structures). Finally, using electrospinning technique, these collagens were applied to fabricate electrosprayed microspheres and electrospun microfibers with multiple potential applications by adjusting the collagen concentrations. Higher polymer molecular weight only needs lower concentration to produce microfibers and the border value between microsphere fabrication and microfiber fabrication was related with the polymer molecular weight. The microfiber diameter increased with the increase of collagen concentration. This work proved that extraction methods had obvious effect on the preparation of electrospun/electrosprayed microstructures of *tilapia* skin collagen and provided a way to convert *tilapia* skin into a value-

added product and to maximize resource utilization of aquatic processing waste such as fish skin. Furthermore, this work will definitely strengthen the economy of the aquatic processing industry. We also expect that future works can be focusing on the detection of molecular interactions such as molecule–surface interaction (61–63) for the understanding of the structure, function, and application of *tilapia* collagen. In addition, previous work demonstrated electrospinning process would induce about 64% reduction in collagen content (64,65), and therefore, it is necessary to explore the effect electrospinning process on the *tilapia* skin collagen prior to their further application.

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