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Effect of bacterial cellulose nanofibers incorporation on acid-induced casein gels: microstructures and rheological properties

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Abstract: In this study, the effect of bacterial cellulose nanofibers (BCNF) incorporation on the structural and rheological properties of casein gels was investigated, where the mixed BCNF and casein gels were prepared by adding gluconic acid δ -lactone (GDL) to acidify the mixed polymer solutions at 3.0% casein concentration (w/v) and varying BCNF concentrations (0–0.5%, w/v). By changing the addition amount of GDL, the mechanical and structural properties of the mixed gels were studied at above, near and below the electric point (pI) of the casein. At pH above the pI of the casein, the introduction of BCNF initially increased the gel strength, but further addition of BCNF weakened the mixed gels. At near and below the pI of the casein, the incorporation of BCNF continuously increased the gel strength. Besides, all gels showed good structural homogeneity, without macroscopic phase separation occurring, which indicated good compatibility of BCNF with the casein gels.

Keywords: bacterial cellulose; casein; mixed gels; rheological properties; structure.

1 Introduction

Bacterial cellulose (BC) is produced by some bacteria such as *Acetobacter*, *Agrobacterium*, *Rhizobium* and *Sarcina* in either solid-phase cultivation or submerged culture [1, 2]. Compared with plant-derived celluloses, BC possesses

tremendous mechanical strength, good water-holding capacity, moldability and high purity [1, 2]. These properties allow BC to find an array of applications in packing materials [3], biomedical application [4, 5], food formulations [1, 6] and so on. Especially, in the food industry, BC has been successfully used to improve the textures and stability of some food products such as ice creams, meat balls, bean curds and emulsions [7–9]. In recent years, BC is also reported to have the ability to lower the blood lipid level, increase satiety and modulate the intestinal microbiota [10, 11]. Due to the significant health-promoting effect, BC is generally considered as a good dietary fiber and is recommended to be used in the food industry [12].

Casein is the main protein component in bovine milk and constitutes 78–80% of milk proteins [13]. It is generally accepted that casein consists of α s1-, α s2-, β -, and κ -casein in the approximate ratio of 4:1:4:1 [14]. In milk, casein tends to self-associate into spherically shaped colloidal particles with a wide diameter range (50–500 nm), which is known as “casein micelles”. So far, several models have been proposed to describe the structures of casein micelles, including submicelle model, dual bonding model and interlocked lattice model [14]. In spite of the ongoing debate on the fine structures of casein micelles, their physicochemical properties have been well investigated. It is considered that casein micelles are highly solvated and are soluble at pH >5.5 and <3.5, but insoluble at pH 4.5–5.0 [15]. When slowly acidifying a casein dispersion to its electric point, *i. e.* adding glucono- δ -lactone (GDL), an acid-induced casein gel will be produced [16].

Due to the good gelling capacity, casein has been used as an important building material in designing food gels, including single casein gels, mixed casein and polysaccharide gels. Previously, it was shown that the incorporations of alginate [17], gellan gum [18] and methylcellulose [19] are effective to improve the strength of casein gels. The underlying mechanism is that these polysaccharides can either electrostatically bind with casein to increase the crosslink density in the gels, or electrostatically repulse with casein to increase the casein's self-aggregation. In the latter case, phase separation is often observed. It was reported that with the increasing amount of polysaccharide addition, casein

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gels often showed a structural transition from single casein phase to “water in water” emulsion structure to bicontinuous phase, leading to a nonhomogeneous gel matrix [17–19]. In order to give homogeneous gel structure, naturally occurring nanofibers such as cellulose nanocrystal [20], cellulose microfibril [21], nanofiber cellulose [22] and okara dietary fiber [23] have been suggested to strengthen the protein-based gels without leading to macroscopic phase separation. In this regard, it is assumed that bacterial cellulose nanofibers (BCNF) could play a similar role in casein gels. However, there is no report available concerning the mixed BCNF and casein gels. In the present study, we prepared mixed casein and BCNF gels at a fixed casein concentration (3.0%, w/v) and different BCNF concentrations (0–0.5%, w/v), where GDL was added to slowly acidify the mixed casein and BC solutions to induce gel formation. The effect of GDL addition amounts and BCNF concentrations on the structural characteristics and gelation behaviors of the mixed gels were further studied. It is anticipated that our study can offer a feasible approach to improve the physical properties of casein gels.

2 Materials and methods

2.1 Materials

Acid caseins (92% purity, micellar caseins, extracted by acid precipitation technique from bovine milk) and D-(+)-Gluconic acid δ -lactone (GDL, BR, 99% purity) were purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China), and used without further treatment. The ionic species in the casein sample were determined by flame atomic absorption spectrometry, with 0.015% Na⁺ (w/w), 0.009% K⁺ (w/w) and 0.082% Ca²⁺ (w/w) identified. Bacterial cellulose nanofibers (BCNF) were purchased from Guilin Qihong Technology Co., Ltd. (Guangxi, China), and the BCNF is in the form of an aqueous dispersion (0.75%, w/v), featuring 50–100 nm of the diameter and 10–20 μ m of the length. The pH of the BCNF dispersion was determined to be ~6.1. For labelling the caseins, Nile blue A dye was purchased from Yuanye biological technology Co., Ltd. Other chemical reagents, such as sodium hydroxide and hydrochloric acid (AR), were purchased from Jingbo biological technology Ltd (Xi'an, Shaanxi province, China). Unless stated, deionized water was used throughout.

2.2 Characterization of BCNF

Scanning electron microscope (SEM) observations. First, 50 mL of the BCNF dispersion was poured into a petri dish and was quickly frozen by liquid nitrogen, after that the frozen dispersion was immediately transferred to a freeze-dryer for lyophilization at –50 °C for 48 h. The freeze-dried sample was fractured manually and the fracture section was sprayed with gold power. Then, a SEM (TM3030, Hitachi, Japan)

was adopted to observe the morphology of the fracture section at a magnification of 3000 $\times$ and 15.0 kV of high voltage.

Atomic force microscope (AFM) observations. The BCNF dispersion was diluted using deionized water to achieve 0.05% (w/v) concentration. The pH of the diluted BCNF dispersion was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 with 0.01 M HCl and 0.01 M NaOH. A drop of the BCNF dispersion was loaded onto the surface of a freshly cleaved mica sheet and left for 48 h for drying. Subsequently, the morphology of the BCNF was imaged by an atomic force microscope (Dimension ICON, Bruker, Germany) [24].

Fourier transform infrared spectroscopy (FTIR) analysis. The freeze-dried BCNF was mixed with KBr at 1:100 ratio and then ground into powder. Afterwards, the powder was pressed into a semi-transparent pellet and scanned by a FTIR spectrometer (Tensor27, Bruker, Germany) in 4000–400 cm^{–1} of wavenumber range, with 32 scans and 4 cm^{–1} resolution [25].

X-ray diffraction (XRD) measurements. The lyophilized BCNF was ground to powder form, followed by varying the diffraction angle (2θ) from 5° to 60° at a step size of 0.02°/s by using a Powder X-ray Diffractometer (D8 Advance, Germany) equipped with a CuK α lamp and a nickel filter.

Thermogravimetry (TG) analysis. Thermal properties of the BCNF were analyzed by using a thermal analysis equipment (Q600SDT, TA Instrument, USA). About 10 mg of the dried BCNF was placed onto an aluminum pan, which was then heated from room temperature to 600 °C at a linear rate of 10 °C/min, where the pure nitrogen was used as purge gas and an empty aluminum pan was regarded as the reference.

2.3 Zeta-potential (ξ -potential) determination of BCNF

The BCNF dispersion was diluted to 0.05% (w/v) concentration with deionized water. Then, the pH of the diluted dispersion was adjusted to 3.0–8.0 using 0.01 M HCl and 0.01 M NaOH. For Zeta-potential determination, ~2 mL solution was transferred into a universal folded capillary cell and measured at 25 °C by using a zeta-potential analyzer (Malvern Zetasizer Nano 3690, Malvern Panalytical Instruments Co. Ltd, Britain) [26].

2.4 Effect of GDL addition on BCNF dispersion

Before preparing mixed BCNF and casein gels, it is considered necessary to investigate the effect of GDL addition on the rheological properties of the BCNF dispersion. Approximately 67 mL of the BCNF dispersion was poured into a 100 mL beaker, followed by adding deionized water to 95 mL. Different amounts of GDL powder (0.3, 0.5 and 1.0 g) were dissolved into 5 mL deionized water, which was immediately added to the BCNF dispersion for homogeneous mixing. The final solutions had a volume of 100 mL, and the BCNF concentration was ~0.5% (w/v). After 16 h of equilibrium at room temperature, the BCNF formed weak gels, and the pH of the gels was determined to be 3.4, 2.9 and 2.7 at 0.3 g, 0.5 and 1.0 g GDL addition, respectively.

The rheological properties of the BCNF gels at different pHs were measured by a stress-controlled rheometer (AR-G2, TA Instruments, USA) equipped with a rough-surfaced parallel plate with 20 mm diameter. Approximately 2 mL of the BCNF gel was transferred to the rheometer sample stage (25 °C), followed by setting the parallel plate

gap at 1 mm. After the sample was held at 25 °C for 5 min for equilibrium, a frequency sweep procedure (0.1–100 rad/s) was performed at 25 °C and 1.0% strain range (within the linear viscoelastic region).

2.5 Preparation of mixed casein and BCNF gels

Casein stock solution (15.0%, w/v) was prepared by dispersing 75.0 g casein powder in 490 mL deionized water. In order to completely hydrate the casein, 5 M NaOH solution (~10 mL) was used to adjust the pH of the dispersion to ~7.0, followed by continuously stirring and holding the dispersion at room temperature for 6 h. On the other hand, the BCNF dispersion (0.75%, w/v) was used as received. For preparing mixed gels, 20 mL of casein stock solution was poured in a 100 mL beaker, followed by adding different volumes of the BCNF dispersion. Then, deionized water was added into the beaker until achieved 95 mL of volume and was then homogeneously mixed. Afterwards, GDL (0.3, 0.5, 1.0 g) was dissolved in 5 mL deionized water, and then immediately added in the mixed BCNF and casein solutions. After sufficient mixing, the solutions were left at room temperature for 16 h for gel formation. The detailed components in mixed gels are listed in Table 1.

2.6 Gel strength determination

The strength of the gels prepared in Section 2.5 was determined using a texture analyzer (TA. XT. Plus, stable micro system, UK) equipped with a 5 mm flat-faced cylindrical probe (P 0.5). All gels (in beakers) were compressed by the probe at 2.0 mm/s of compression rate and 2.0 g of trigger force. The gel strength was defined as the force at the gel rupture point [27]. Each sample was determined at least in triplicate.

2.7 SEM observations

The gels were cut into small cubes with ~2 cm of slide length and were immersed in liquid nitrogen to be quickly frozen [19]. Afterwards, the cubes were immediately transferred to a vacuum freeze-dryer to be lyophilized at -50 °C for 48 h. The lyophilized gel cubes were manually fractured and the fracture surface was sprayed with gold powder before SEM observations. The magnification was 2000× and the high voltage was 15.0 kV.

2.8 Confocal laser scanning microscope (CLSM) observations

Nile blue A staining solution (0.2%, w/v) was prepared by dissolving 20 mg Nile blue A powder in 10 mL deionized water at 50 °C. Then, 0.4 mL Nile blue A solution was homogeneously mixed with 20 mL casein stock solution for 3 h for labelling the casein. Gels were prepared according to the method described in Section 2.5. After adding GDL, a drop of the solution was loaded onto a glass slide, followed by

covering with a cover glass. After 16 h of equilibrium, the sample was observed by CLSM (FV1200, Olympus Corporation, Japan) under both fluorescence model (the excitation wavelength was 488 nm) and the bright field model [18]. The magnification was 60×.

2.9 Rheological properties

According to Section 2.5, after GDL was added, the solution was poured into 10 mL beakers for gel formation. After 16 h of equilibrium, the gels were taken out from the beakers and had a column shape with the diameter of ~20 mm. Thus, it is considered that the diameter of the gels is very close to the diameter of the parallel plate. After the gels were cut into slices with 1 mm thickness, a frequency sweep procedure was performed according to the same program in Section 2.4.

2.10 Statistical analysis

All measurements were performed in triplicate and the results were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was performed by SPSS statistics 19 (SPSS, Inc., Chicago, IL, USA). Duncan's multiple range test was adopted to indicate the significant differences between groups ($p < 0.05$ indicates significant difference).

3 Results and discussion

3.1 Structural characterization of BCNF

The purchased BCNF was produced by static cultivation, where the BC existed as a gelatinous membrane on the surface of the cultivation medium [28]. The BCNF was obtained by mechanically shearing the membrane and sold as an aqueous dispersion. Therefore, the physico-chemical and structural features of the BCNF were characterized before the following experiments. As shown in Figure 1a, the SEM result suggested that the BCNF self-aggregated into a sheet-like structure, but the fiber-like structure could be still observed. The length of the BCNF was at micrometer scale, which is consistent with the information provided by the producer. According to Figure 1b, the FTIR curve of the BCNF exhibited significant absorption peaks at ~3300, 2880 and 1630 cm^{-1} , respectively. The absorption peak at 3300 cm^{-1} was attributed to O–H stretching vibrations, which reflected the existence of intermolecular hydrogen bonds in the BC polymer [29]. The peak at 2880 cm^{-1} was caused by C–H stretching vibration in $-\text{CH}_2$ groups. The peak at 1630 cm^{-1} was ascribed to the symmetrical stretching vibration of $-\text{COOH}$ groups, indicating that the BCNF contained a larger number of $-\text{COOH}$ groups. In addition, the peak at 1425.65 cm^{-1} was one of the main principal peaks of cellulose Ia type, which was associated with symmetric ($-\text{CH}_2$) bending vibrations (δ)

Table 1: Components of mixed BCNF and casein gels.

BCNF concentration (% w/v)	0	0.1	0.2	0.3	0.4	0.5
BCNF dispersion (mL)	0	13.33	26.67	40	53.33	66.67
Deionized water (mL)	75	61.67	48.33	35	21.67	8.33

[30]. The peak at 1376.98 cm^{-1} represented C–H bending vibration (δ). The peaks at $1200\text{--}1000\text{ cm}^{-1}$ were the characteristic fingerprint region for 1, 4- β glycoside pyranose ring [30]. These absorption peaks were similar to the results reported by others [25], which demonstrated that the BCNF used in our study was cellulose with high purity.

Moreover, the XRD measurement was also conducted. As shown in Figure 1c, it could be observed that the BCNF exhibited three major diffraction peaks and respectively centered at $2\theta = 14.7^\circ$, 17.1° and 22.8° , corresponding to the crystallographic plane $\langle 101 \rangle$, $\langle 101 \rangle$ and $\langle 002 \rangle$. This result was similar to the reports by others, indicating that the BCNF was featured by typical I type cellulose structure [31, 32]. Subsequently, we also calculated the crystallinity of the BCNF, which was expressed as the ratio of diffraction peak area to total diffractogram area. It was found that the BCNF had a high crystallinity ($78.00 \pm 1.22\%$), which was consistent with the reported value by Zhai et al. [32]. In addition, TG analysis result indicated that the BCNF showed two major weight loss steps, which were below 100°C and $250\text{--}350^\circ\text{C}$, respectively. The first step was due to water evaporation, and the pronounced weight loss centered at $\sim 325^\circ\text{C}$ was caused by the thermal degradation of the BCNF. This result was similar to the TG curve of the BC produced by *Acetobacter pasteurianus* RSV-4 on tomato juice, which had a maximum weight loss centered at 343°C [29]. The slight difference was ascribed to the BC types produced by different fermentation media.

3.2 Effect of pH on the BCNF aggregation

To understand the electrostatic properties of the BCNF, we determined the zeta-potential of the BCNF dispersion at different pHs. As shown in Figure 2a, the BCNF carried negative charges in the range of pH 3.0–8.0. At pH 8.0, the zeta potential was $\sim -45\text{ mV}$; with decreasing pH, the charge density of the BCNF was decreased. At pH 6.0, the zeta potential of BCNF was $\sim 37\text{ mV}$, suggesting a good stability of the BCNF dispersion. Moreover, the molecular morphology of the BCNF at different pHs was also visualized by AFM. As shown in Figure 2b, the fiber-like structure was clearly observed; with decreasing pH, the fibers tended to self-aggregate and gradually formed a dense network. This was understood by the fact that decreased pH facilitated the self-aggregation of the BCNF by lowering the intermolecular electrostatic repulsion.

The rheological properties of the BCNF dispersion at different pHs were also investigated. As shown in Figure 3a, without GDL addition, the BCNF dispersion exhibited a higher G' than G'' within $0.1\text{--}1\text{ rad/s}$ of frequency range, during which the G' and G'' were almost plateaued. With increasing sweep frequency, the G' dramatically dropped and the G'' significantly increased, indicating that the gel structure was destroyed at higher shearing frequency. With increasing amount of GDL addition, the G' in the plateau region became higher, indicating that the BCNF gels showed increased structural strength. Previously, it was reported

Figure 1: Structural characterizations of bacterial cellulose nanofibers (BCNF).

a) Scanning electron microscope (SEM) images; b) Fourier transform infrared spectroscopy (FTIR) spectrum; c) X-ray diffraction (XRD) pattern; d) TG/DTG curve. In right hand set axis of d), Deriv. Weight (%) means the first derivative of the weight change of the sample, which reflected the rate of the weight decrease during heating.

Figure 2: Zeta potential (a) and molecular morphology (b) of bacterial cellulose nanofibers (BCNF) at different pH values. In (b), different lowercase letters indicate the significant difference between different pH values ($p < 0.05$).

that aqueous suspensions of colloid cellulose were mainly stabilized by electrostatic repulsion arose from deprotonated carboxyl groups on the fiber surfaces. And it was more effective in reducing the surface charge by decreasing the pH than by increasing salt concentration [33]. In our study, the pHs of the BCNF dispersions were determined to be ~ 6.1 (without GDL addition), 3.4 (0.3 g GDL), 2.9 (0.5 g GDL) and 2.7 (1.0 g GDL), respectively. Thus, the structural strengthening of the BCNF gels was believed to arise from the weakened intermolecular electrostatic repulsion, which led to a larger extent of BCNF aggregation and thereby increased the crosslink density in the gels.

3.3 Gel strength of mixed BCNF and casein gels

In this section, the mixed BCNF and casein gels were prepared at different BCNF concentrations and different

GDL addition levels. As shown in Figure 4, without BCNF addition, the casein only formed a weak gel at 0.3 g GDL addition level. At a higher level of GDL addition (0.5 g), the gel became stronger, but followed by a decrease in gel strength when the addition amount of GDL continuously increased to 1.0 g. The effect of GDL on casein gels should be attributed to different pH values, which were determined to be 5.1, 4.7 and 4.3 at 0.3, 0.5 and 1.0 g GDL addition, respectively. It was obvious that at 0.5 g GDL addition level, the pH of the casein gel was close to the electric point of the casein ($pI \sim 4.6$) [14], at which the casein could have the biggest extent of aggregation and thus exhibit the highest gel strength.

After BCNF were incorporated, the casein gels were significantly strengthened, where all gels showed self-supporting properties. As shown in Table 2, at 0.3 g of GDL addition, with increasing BCNF concentration, the gel strength showed an initial increase, and peaked at 0.3% of BCNF, then followed by a decrease. However, at 0.5 and 1.0 g GDL addition level, with increasing BCNF addition, the strength of the gels was monotonically increased. At 0.5% of BCNF concentration, the gel strength increased by almost 15 $\times$ and 40 $\times$ fold when compared with the pure casein gels at 0.5 and 1.0 g of GDL addition, respectively. Besides, it was observed that all gels exhibited homogeneous structure, without the macroscopic phase separation occurring. As for the high viscosity of the BCNF dispersion, we did not further increase the BCNF concentration in the mixed gels. Despite this, the usage level of the BCNF in our study was similar to the case of mixed whey protein isolate and cellulose microfibril gels, where 0–0.4 wt% of the microfibrils were used [21].

3.4 Microscopic structures of mixed BCNF and casein gels

SEM and CLSM were used to explore the structural characteristics of mixed BCNF and casein gels. As shown in Figure 5, at 0.3 g GDL addition, the pure casein gel showed a network with sheet-like structures, indicating the self-aggregation of the casein. With increasing BCNF addition, the gel network became denser and featured by a dominant sheet-like network with big pores and numerous fibers entrapped. At 0.5 g GDL addition level, the change of gel network showed a similar trend, but the network was denser at identical BCNF concentrations, reflecting a larger extent of casein aggregation. At 1.0 g GDL addition, the gel network

Figure 3: Frequency sweep curves of bacterial cellulose nanofibers (BCNF) dispersions at different gluconic acid δ -lactone (GDL) additions. a) Without GDL addition (pH ~ 6.1); b) 0.3 g GDL (pH ~ 3.4); c) 0.5 g GDL (pH ~ 2.9); d) 1.0 g GDL (pH ~ 2.7).

Figure 4: Appearance of mixed bacterial cellulose nanofibers (BCNF) and casein gels.

was similar to that of 0.5 g GDL, however, at higher BCNF concentration, the gel network was more significant and the BCNF was entrapped into the pores of sheet-like network.

CLSM was further adopted to visualize the gel morphology. Under fluorescent mode, the casein labelled by Nile blue A should be presented as bright regions and the unlabelled BCNF should be presented as dark areas [17].

As shown in Figure 6, at 0.3 g GDL addition, the casein was uniformly distributed, and the BCNF was filled in the casein matrix at lower concentration. However, at higher BCNF concentration, the casein matrix was divided into several regions, indicating that the homogeneity of gel matrix was disrupted by BCNF addition. Besides, at higher levels of GDL addition, the casein was found to exist around the

Table 2: Gel strength (g) of the mixed BCNF and casein gels.

BCNF concentration (% <i>, w/v</i>)	0.3 g GDL	0.5 g GDL	1.0 g GDL
0	9.43 ± 0.12 ^{Be}	19.90 ± 1.04 ^{Ae}	10.02 ± 0.89 ^{Bf}
0.1	48.58 ± 3.90 ^{Bbc}	92.17 ± 6.74 ^{Ad}	86.77 ± 7.85 ^{Ae}
0.2	53.61 ± 2.57 ^{Cb}	124.93 ± 8.21 ^{Bc}	151.34 ± 14.21 ^{Ad}
0.3	63.39 ± 4.91 ^{Ca}	135.80 ± 21.38 ^{Bc}	235.24 ± 22.64 ^{Ac}
0.4	45.28 ± 3.56 ^{Cc}	183.87 ± 1.16 ^{Bb}	323.93 ± 21.64 ^{Ab}
0.5	37.27 ± 3.12 ^{Cd}	333.29 ± 14.27 ^{Ba}	382.14 ± 31.46 ^{Aa}

Different lowercase letters in the same column indicate the significant difference between different BCNF concentrations ($p < 0.05$). Different capital letters in the same row indicate the significant difference between different GDL amounts ($p < 0.05$). All data are the means ± standard deviations of three replicates.

Figure 5: Scanning electron microscope (SEM) images of mixed bacterial cellulose nanofibers (BCNF) and casein gels.

surfaces of the BCNF, probably due to the electrostatic attraction between the two polymers.

3.5 Rheological properties of mixed BCNF and casein gels

As shown in the frequency sweep curves of the mixed gels (Figure 7), all gels exhibited higher G' than G'' over a wide frequency range, suggesting a relatively strong gel structure. In order to better compare the gel strength, the G' values of the gels at 1 Hz were used for further analysis (Table 3). At 0.3 g GDL addition, the G' was almost tenfold higher than pure casein gels in the presence of 0.1% BCNF. At 0.1–0.3% of BCNF concentration, the G' did not significantly increase, but discernibly decreased when the BCNF

concentration was above 0.3%. At 0.5 and 1.0 g GDL addition, the G' were monotonically increased as the BCNF concentration increased. Because G' was the predominant measure of elasticity and reflected the structural strength of gel, these results were well consistent with the outcomes in Section 3.3.

3.6 Discussion on the gel properties and structural mechanism

Recent years we have seen a trend for nanofibers usage in food industry to control the physical and textural properties of foods [9, 34]. In terms of food gels improvement, a combination of fiber and gel can provide new mechanical properties for the gels. In this respect, the mixed food gels

Figure 6: Confocal laser scanning microscope (CLSM) images of mixed bacterial cellulose nanofibers (BCNF) and casein gels.

containing nanofibers have attracted great attention, such as whey protein isolate-cellulose nanocrystal composite gels [20], whey protein isolate-cellulose microfibril gels [21], gelatin-nanofiber cellulose gels [22] and okara dietary fiber-tofu gels [23]. In our study, BCNF was obtained from mechanically shearing the BC membrane and was accessible as an aqueous dispersion. Since the BCNF dispersion had a relatively high charge density at the natural pH (pH ~ 6.1), the dispersion showed good stability and the aggregation of BCNF was prevented. When preparing mixed gels, the BCNF dispersion and the casein solution were homogeneously mixed at desired ratios, followed by adding GDL to acidify the mixture to form gels. Three GDL addition levels were considered to give the pH of the final gels above, near and below the pI of the casein, respectively. For the mixed gels with 0.3 g GDL addition (pH 5.1, above the pI of the casein), the incorporation of BCNF increased the gel strength at 0–0.3% of BCNF concentration, but the gel strength was slightly decreased with further BCNF addition. This trend is similar to the result reported by Wang et al., who investigated the effect of carboxylated nanofiber cellulose on gelatin gels. It was

reported that the addition of nanofiber cellulose from 0 to 0.5% (w/v) increased the gel strength of gelatin gels, whereas further increased the cellulose concentration decreased the gel strength [22]. Also, Guo et al. [34] investigated the effect of nano BC fibers on the gelation of soy protein isolate, and they found that at low concentration, the BC fibers were homogeneously distributed into the protein matrix, but the protein network was disrupted at high dose of BC addition. The possible reason was that at low concentration, the nanofibers filled into the protein gel matrix and led to a more compact gel structure; at relatively high concentration, however, the addition of nanofibers disrupted the structure of the protein gel matrix, possibly resulting from the aggregation of nanofibers (Figure 6). At 0.5 g GDL addition (pH 4.7, near the pI of the casein), the incorporation of BCNF monotonically increased the gel strength. The underlying reason was that at near the pI of the casein, the intermolecular repulsion between the BCNF and the casein greatly decreased, which reduced the aggregation extent of BCNF in the mixed gels. In this situation, the addition of BCNF was considered to cause a more compact structure of the casein gels and minimize the

Figure 7: Rheological properties of mixed bacterial cellulose nanofibers (BCNF) and casein gels at different gluconic acid δ -lactone (GDL) additions.

structural disruption (Figure 5). At 1.0 g of GDL addition (pH 4.3, below the pI of the casein), it was expected that the intermolecular electrostatic attraction between the BCNF and the casein was pronounced, which created strong

interfaces in the mixed gels and therefore greatly increased the gel strength [9].

Moreover, it should be noted that the BCNF-strengthened casein gels showed homogeneous structure, without macroscopic phase separation being observed. This characteristic is different from the cases of mixed casein and other polysaccharide gels, such as mixed methylcellulose/casein gels [19], gellan gum/casein gels [18] and so on. For these gels, the phase separation occurred during the acidification process by GDL addition, which was attributed to the intermolecular repulsion between the casein and polysaccharides. In our study, however, the BCNF existed as insoluble solid particles rather than soluble polysaccharide molecules. Under this circumstance, although the BCNF showed self-aggregation by GDL addition in mixed gels, it was filled into the casein-based gel matrix and formed “filled gels” [9]. For this type of gels, the occurrence of macroscopic phase separation is less than the mixed polysaccharide-protein gels due to a good compatibility between filled fibers and gelling matrix. Therefore, the BCNF showed a greater potential to strengthen the casein gels than other soluble polysaccharides.

4 Conclusions

Mixed BCNF and casein gels were developed at different GDL addition levels, which was corresponding to above, near and below the pI of the casein. At 0.3 g GDL addition, the introduction of BCNF greatly improved the gel strength at 0–0.3% of concentration, and further increased BCNF concentration slightly reduced the gel strength. This was because lower concentration of BCNF promoted the self-aggregation of casein by electrostatic repulsion, but higher concentration of BCNF disrupted the structure of casein gels. In comparison, at 0.5 g GDL addition, the incorporation of BCNF monotonically increased the gel strength, because the lower intermolecular repulsion retarded the self-aggregation of BCNF and thus minimized the disruption of casein gel structure. At 1.0 g GDL addition, the strong intermolecular electrostatic attraction created an intense interface between the BCNF and the casein gel, which greatly increased the gel strength. Besides, at all pHs, the incorporation of BCNF not only greatly strengthened the casein gels, but also created a homogeneous gel structure. Therefore, it is concluded that the incorporation of BCNF provides a feasible way to physically strengthen the casein gels, showing a greater potential than other soluble polysaccharides.

Table 3: Storage modulus (G' , Pa) of the mixed BCNF and casein gels.

GDL addition amounts (g) BCNF concentration (% w/v)	0.3 G'	0.5 G'	1.0 G'
0	212.1 ± 6.5362 ^{Cf}	684.5 ± 15.6785 ^{Af}	427.5 ± 11.3657 ^{Bf}
0.1	2229 ± 43.6547 ^{Ac}	3110 ± 56.3334 ^{Ae}	2470 ± 30.2547 ^{Ae}
0.2	2354 ± 42.3578 ^{Cb}	3608 ± 39.5475 ^{Bd}	4845 ± 96.3789 ^{Ad}
0.3	2607 ± 57.6324 ^{Ca}	5623 ± 63.9785 ^{Bc}	10,500 ± 87.6354 ^{Ac}
0.4	2025 ± 30.2664 ^{Cd}	10,450 ± 108.2365 ^{Bb}	12,150 ± 120.3873 ^{Ab}
0.5	1824 ± 12.6327 ^{Ce}	15,830 ± 120.9887 ^{Ba}	17,640 ± 113.3254 ^{Aa}

Different lowercase letters in the same column indicate the significant difference between different BCNF concentrations ($p < 0.05$). Different capital letters in the same row indicate the significant difference between different GDL addition levels ($p < 0.05$). All data are the means ± standard deviations of three replicates.

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