Short communication: Electrospinning of casein/pullulan blends for food-grade applications¹

P. M. Tomasula,^{*2} A. M. M. Sousa,^{*} S.-C. Liou,^{*†} R. Li,^{*‡} L. M. Bonnaillie,^{*} and L. S. Liu^{*}

^{*}Dairy and Functional Foods Research Unit, USDA, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19038

[†]Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., South District, Taichung City, Taiwan 402

[‡]State Key Laboratory of Hollow Fiber Materials and Processes, School of Materials Science and Engineering, Tianjin Polytechnic University, No. 399 Binshuixi Road, Xiqing District, Tianjin 300387, China

ABSTRACT

Electrospinning is a complex process that produces fibers with diameters on the micrometer or nano-scale from an electrified jet of a polymer solution. The objective of this study was to create electrospun fibers for food use from aqueous solutions of calcium (CaCAS) or sodium caseinate (NaCAS). Fibers were not formed from electrospinning of solutions of either caseinate (CAS) at 50°C, but were formed from blends of either CAS solution with aqueous solutions of the food-grade polysaccharide, pullulan (PUL), when using mass ratios from 2:1 to 1:4 of PUL/CAS. The CAS in the spinning solutions ranged from 3 to 15% (wt/wt) and the PUL ranged from 5 to 15% (wt/wt). The PUL/CaCAS 1:2 fibers showed the lowest fiber diameter sizes (FDS) of 172 ± 43 nm, as determined by scanning electron microscopy, and were smaller in size than fibers electrospun from 15% (wt/wt) PUL solution. The PUL/NaCAS solutions were more viscous and formed fibers with occasional branching and less uniform FDS at higher NaCAS contents. Reductions in NaCAS in these solutions reduced viscosity and improved jet stabilities with consequent improvement in fiber morphology leading to more uniform FDS. Fibers with less defects and more homogeneous FDS were formed from PUL/CaCAS blends with more CaCAS, showing that each CAS interacted differently with PUL and formed the best fibers at different solution conditions. Calcium bridging may also underlie the anomalous behavior of the PUL/CaCAS blends by forming crosslinks with the phosphoserine residues, further enabling chain entanglements for fiber formation. The PUL/NaCAS fibers tended to be larger than the PUL/CaCAS fibers, which may also be due to other factors such as solution

surface tension and conductivity, which also affect fiber quality and size. The shear viscosities at 100 s^{-1} of the solutions producing fibers were within the range of 0.07 to 0.16 Pa/s, with the smallest standard deviations in FDS noted for solutions with viscosities within about 25% that of PUL. This is the first example of caseinate fibers prepared using a food-grade carrier rendering a product with potential use in food and packaging applications.

Key words: nanotechnology, fiber, protein, polysaccharide

Short Communication

Edible films and coatings have been proposed as a means to reduce petrochemical-based food packaging, to ensure food security by providing better protection for foods and extending shelf-life, and as a means to use proteins, carbohydrates, or by-products for value-added food or consumer products. Films made from calcium or sodium caseinates (**CAS**) may be consumed with food and used to improve its appearance, prevent migration of or bind its components, add antimicrobials, or add and protect nutrients such as vitamins and probiotics (McHugh and Krochta, 1994; Tomasula, 2009). Caseinates have a very low amount of cysteine, no disulfide linkages, and a large number of proline residues that prevent formation of secondary structures and allow bending of the protein chains. Thus, they have a random coil structure (Gennadios et al., 1994) responsible for the ability of CAS to form films. Edible films are usually made using a casting process while coatings are sprayed onto a food substrate (Kozempel and Tomasula, 2004).

Electrospinning is a technology for making nonwoven mats from continuous fibers with diameters on the nano or microscale to build tissue engineering scaffolds or filter media (Greiner and Wendorff, 2007). The fibers have a surface area about $1,000\times$ greater than volume, giving electrospun mats increased surface efficiency compared with films or coatings. Electrospinning may

Received September 9, 2015.

Accepted November 21, 2015.

¹Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

²Corresponding author: peggy.tomasula@ars.usda.gov

be a useful technique for improving the mechanical and barrier properties (Tomasula, 2009) and extending the range of applications of edible films and coatings to include active packaging for targeted nutrition needs through embedding of biologically active compounds or to improve sensory aspects of foods (Kriegel et al., 2008).

Electrospinning involves applying a high voltage to a viscous polymer solution. The positively charged solution is pumped dropwise through a syringe; the electric field causes the drop to form into a Taylor cone which then, depending on the combination of solution properties and processing conditions (Fong et al., 1999), forms a continuous, stable jet due to the presence of chain entanglements and its elongational viscosity, necessary for successful fiber formation (Fong et al., 1999; Shenoy et al., 2005). The jet becomes narrower and forms an open coil as it approaches a counter electrode; the solvent simultaneously evaporates, precipitating the fibers on the counter electrode. The fibers are of no particular length unless a break in the process occurs. The drop is balanced at the nozzle by its surface tension, a measure of the tendency of a liquid to minimize its surface area due to interactions among the molecules, and is ejected when the electric field is opposed by the solution electrostatic forces that become larger than the surface tension (Greiner and Wendorff, 2007).

Production of fibers from aqueous solutions of food proteins, such as casein (Xie and Hsieh, 2003) and whey protein isolates (Sullivan et al., 2014), required use of a carrier polymer, such as polyethylene oxide, that enabled electrospinning through solution viscosity enhancement and possibly lowering of surface tension or electrical conductivity. Proteins electrospun with polyethylene oxide, however, are not for food use. Only production of fibers from zein (Miyoshi et al., 2005; Kanjanapongkul et al., 2010) and gelatin proteins (Zhang et al., 2009) dissolved in aqueous solutions have been demonstrated without a carrier. Electrospinning of protein fibers for food use has been demonstrated using gelatin as a carrier with proteins, such as whey protein isolate, ovalbumin, BSA, soy protein isolate, and NaCAS (Nieuwland et al., 2013). The ability to electrospin aN NaCAS-gelatin solution required an ultrasonic treatment to disrupt aggregated protein prior to electrospinning.

Our objective was to determine the feasibility of creating fibers from the food proteins calcium (**CaCAS**) and sodium (**NaCAS**) caseinate from aqueous solution with and without a food-grade carrier polysaccharide, such as pullulan (**PUL**), and to determine the effect of some of the solution parameters and the operating conditions on fiber morphology. Pullulan is a linear, uncharged polysaccharide with maltotriose units con-

nected by α -(1 $\rightarrow$ 6) linkages associated with randomly ordered chains (Kristo and Biliaderis, 2006) responsible for its amorphous character which has potential for molecular entanglement with CaCAS or NaCAS proteins.

Sodium CAS and CaCAS were obtained from the American Casein Co. (Burlington, NJ). Pullulan was obtained from TCI America (Portland, OR); all materials were used as received. Deionized water used in all solutions was produced using a Barnstead E-pure water system (Dubuque, IA). Aqueous solutions containing 5, 8, 15, and 20% (wt/wt) of PUL and 5, 10, 15, and 20% (wt/wt) of CaCAS or NaCAS were prepared for solution characterization and electrospinning. Stock solutions containing 20% (wt/wt) of either CAS were also diluted with stock 15 or 30% (wt/wt) PUL to prepare spinning solutions with mass ratios from 2:1 to 1:4 of PUL:CAS. The CAS in the solutions ranged from 3 to 15% (wt/wt) and the PUL ranged from 5 to 15% (wt/wt; Table 1). After addition of the CAS or PUL or both to water, the solutions with pH $\sim$ 6.7, were mechanically stirred (Cole-Parmer, Vernon Hills, IL) for 2 h at 1,000 rpm and refrigerated overnight at 4°C to remove air bubbles.

The electrospinning apparatus (NaBond Technologies, Hong Kong, China) consisted of a cabinet housing the equipment, an external syringe pump leading to a tubeless spinneret with a metallic needle, a controllable high-voltage source connected to the needle with range of 0 to 50 kV, and a rotating cylinder wrapped in aluminum foil for collection of the fibrous mats (Sousa et al., 2015). The PUL and CAS solutions were added to the tubeless spinneret before experiments. Preliminary experiments showed that fibers were produced using the following conditions: 12 cm distance from the tip of the needle to the surface of the collector; 0.4 to 3.0 mL/h flow rate; and 50°C temperature. Voltage was varied from about 11 to 23 kV depending on fiber quality. After experiments, the foil wrap holding the dry fibrous mat was removed from the cylinder and placed in a desiccator to protect from moisture. The mats were typically 10 cm wide by 4 cm long or oval in shape with the largest diameter at 3 cm. The experiments were performed in triplicate.

Shear viscosity as a function of shear rate 1.0 to 100 s⁻¹ at 50°C was determined using a rheometer (AR 2000, TA Instruments, New Castle, DE) equipped with a 30-mm inside diameter cup with a 28-mm outside diameter bob. Shear viscosity at 100 s⁻¹ was used to indicate the potential to produce fibers even though the shear encountered in electrospinning is much greater than that obtained from shear viscosity measurements (Kong and Ziegler, 2014). The surface tension of all solutions at 50°C was determined (n = 18) using a Surface Tension Apparatus model 20 (Catalog No. 14-812,

Fisher Scientific, Suwanee, GA) with ring diameter of 1.905 cm. A conductivity cell with built-in temperature sensor and automatic temperature correction (reference temperature = 25°C) was attached to a Model IQ270G conductivity meter (Scientific Instruments, Loveland, CO) to measure electrical conductivity of the solutions at 50°C ($n = 18$). For PUL solutions, the measurements were done close to the detection limit of the device and only a range of values could be indicated. One-way ANOVA and Tukey test were performed (Statistica 8.0, StatSoft Inc., Tulsa, OK) to determine the significant differences between each mean value of surface tension and conductivity, defined as $P < 0.05$.

Samples of the fibrous mats were coated with a thin film of gold and then examined using scanning electron microscopy (FEI, Hillsboro, OR). The high-vacuum secondary electron imaging modes at an accelerating voltage of 10 kV and working distance of 12.5 mm were used. Fiber diameter sizes (**FDS**) for 100 fibers of fibrous mat samples were measured using the image analysis software XT Document (FEI) and used to construct a diameter histogram. The Shapiro-Wilk W test adapted to large sample sizes by Royston (1982) was used for testing normality ($P < 0.05$) with Statistica 8.0 software. Means of FDS following the normality and homogeneity of variance requirements were compared

using Tukey test ($P < 0.05$), whereas Mann-Whitney test ($P < 0.05$) was used for comparing means of FDS with non-normal distributions.

Scanning electron microscopy images and histograms of FDS of the fibrous mats electrospun from aqueous PUL solutions at 50°C, 3 mL/h, and 20 kV are shown in Figure 1. Electrospinning of the 5% (wt/wt) PUL solution (Figure 1A) generated only droplets. The 8% (wt/wt) PUL solution (Figure 1B) showed evidence of electrospinning with beaded and elongated shapes interspersed among the fibers, an indication that minimal entanglement of the chains was established to stabilize the jet, whereas the 15% (wt/wt) PUL solution (Figure 1C) resulted in fiber formation. These results are consistent with previous reports showing the transition from no fibers to beaded fibers to fibers alone as PUL concentration, and consequently shear viscosity, were increased (Shenoy et al., 2005; Stijnman et al., 2011; Kong and Ziegler, 2014).

Fibers produced from 8% (wt/wt) PUL solution (Figure 1B) showed a region of small FDS at 36 ± 11 nm, excluding the beads and other nonfibrous shapes (301 ± 90 nm). Fibers with beads leading to bimodal FDS distributions may occur from solutions or polymers with inadequate properties for spinning (Fong et al., 1999) or from inadequate spinning conditions (De-

Table 1. Properties of the pullulan (PUL), calcium caseinate (CaCAS), and sodium caseinate (NaCAS) stock solutions, and respective PUL/CAS blends (mean $\pm$ SD, $n = 18$)

Solution	PUL:CAS	PUL wet (%)	CAS wet (%)	Surface tension $\times 10^3$ (N/m)	Shear viscosity at 100 s ⁻¹ (Pa/s)	Conductivity (μ S/cm)	Morphology
PUL	1:0	5.0	0.0	51.2 ± 0.9^a	0.00991	20.6–29.0	No fiber
	1:0	8.0	0.0	59.2 ± 0.8^b	0.0710	30.0–39.4	Fibers and beads
	1:0	15.0	0.0	61.8 ± 1.0^c	0.0820	49.9–61.1	Fibers
	1:0	20.0	0.0	ND ¹	ND	ND	Not spinnable
	1:0	30.0	0.0	ND	ND	ND	Not spinnable
CaCAS	0:1	0.0	5.0	44.9 ± 0.3^d	0.00139	$1,049 \pm 5^a$	No fiber
	0:1	0.0	10.0	44.9 ± 0.6^d	0.00227	$1,755 \pm 13^b$	No fiber
	0:1	0.0	15.0	46.1 ± 0.7^e	0.0141	$2,289 \pm 9^c$	No fiber
	0:1	0.0	20.0	50.2 ± 0.8^{af}	0.104	$2,332 \pm 35^d$	No fiber
	0:1	0.0	5.0	42.8 ± 0.4^g	0.00229	$1,655 \pm 7^e$	No fiber
NaCAS	0:1	0.0	10.0	42.4 ± 0.3^g	0.00801	$2,862 \pm 12^f$	No fiber
	0:1	0.0	15.0	45.9 ± 0.5^{eh}	0.0977	$3,719 \pm 29^g$	No fiber
	0:1	0.0	20.0	49.9 ± 0.4^f	4.39	$4,148 \pm 26^h$	No fiber
	1:2 ²	5.0	13.3	45.7 ± 0.5^{deh}	0.0687	$1,692 \pm 8^e$	Fibers and defects
	1:4 ³	6.0	16.0	47.0 ± 0.3^i	— ⁴	$1,809 \pm 52^i$	Fibers and ribbons
PUL/CaCAS	1:1 ²	7.5	10.0	46.1 ± 0.9^{eh}	0.115	$1,324 \pm 20^j$	Fibers and a few beads
	1:2 ³	10.0	13.3	47.8 ± 0.8^i	— ⁴	$1,495 \pm 19^k$	Fibers and some ribbons
	1:4 ³	3.0	16.0	45.0 ± 1.0^d	0.385	$3,412 \pm 39^l$	Fibers and defects
	1:2 ⁵	5.0	13.3	45.2 ± 1.2^{dh}	0.158	$2,699 \pm 93^m$	Fibers and defects
	1:1 ⁵	7.5	10.0	44.9 ± 0.6^d	0.0961	$2,079 \pm 25^n$	Fibers
PUL/NaCAS	2:1 ⁵	10.0	6.7	44.8 ± 1.4^d	0.120	$1,420 \pm 55^o$	Fibers

^{a–o}Means within a column with different superscripts differ ($P < 0.05$).

¹ND = not determined.

²Prepared from 15% wt/wt PUL and 20% wt/wt CaCAS stock solutions.

³Prepared from 30% wt/wt PUL and 20% wt/wt CaCAS stock solutions.

⁴Could not be measured due to the formation of a protein-polysaccharide film during testing.

⁵Prepared from 15% wt/wt PUL and 20% wt/wt NaCAS stock solutions.

itzel et al., 2001; Greiner and Wendorff, 2007). Mean FDS from 15% (wt/wt) PUL solution was 217 ± 26 nm, showing an increase in fiber size with increasing solution concentration and shear viscosity (Table 1).

In addition to shear viscosity, solution properties such as surface tension and electrical conductivity may be important for fiber formation. The value of surface tension increased with PUL concentration ($P < 0.0001$;

Table 1), as did the very low electrical conductivities ($<100 \mu\text{S}/\text{cm}$; Table 1). Impurities have shown to affect significantly the conductivity of PUL and other non-ionic polymer solutions (Kishikawa et al., 2013; Rošić et al., 2013) and may have contributed to the observed increase in conductivity. However, the shear viscosity of the 15% (wt/wt) PUL solution was about 88% greater than that of the 5% (wt/wt) solution (Table 1). The

Figure 1. Scanning electron microscopy images and fiber diameter size (FDS) analysis of electrospun fibers obtained from 5, 8, and 15% (wt/wt) pullulan (PUL) solutions at 50°C. Flow rate = 3 mL/h, Voltage = 20 kV. (A) 5% (wt/wt) PUL solution (1,000× magnification); (B) 8% (wt/wt) PUL solution (25,000× magnification); (C) 15% (wt/wt) PUL solution (25,000× magnification).

elimination of beads, with consequent improvement noted in morphology leading to a normal distribution of FDS ($P = 0.23$; Figure 1C) when electrospinning the 15% (wt/wt) PUL solution, was most likely due to their increased shear viscosities, which tends to indicate that the viscoelastic forces in the jet were more important for producing bead-free fibers (Fong et al., 1999). Additional experiments are needed to confirm this finding.

It was not possible to electrospin the neat CaCAS or NaCAS solutions at 20°C because they were too viscous. Electrospinning at 50°C, which lowered solution viscosity (Konstance and Strange, 1991), resulted only in droplet formation or beads, indicating the inability of the proteins to entangle and to stabilize a jet for fiber formation. Calcium CAS and NaCAS solutions up to 15% (wt/wt) showed lower shear viscosities compared with the 10 or 15% (wt/wt) PUL solutions (Table 1). Solutions of 20% (wt/wt) CaCAS were gel-like and had shear viscosity over 20% greater than that of 15% (wt/wt) PUL solution. These solutions did not form fibers, nor did solutions of 20% (wt/wt) NaCAS, because their high viscosities prevented formation of a drop at the needle opening to initiate electrospinning. In addition, the CaCAS and NaCAS solutions showed high electrical conductivities in the ranges of 1,044 to 2,367 and 1,648 to 4,174 $\mu\text{S}/\text{cm}$, respectively (Table 1); further research is needed to examine if the high conductivities are an impediment to fiber formation.

Kozempel et al. (2003) showed that films formed if cast from aqueous dispersions of CaCAS $\leq 10\%$ (wt/wt) and did not form as readily if CaCAS dispersions were $\geq 10\%$ (wt/wt), which is approximately the point at which solution viscosity and intermolecular interactions increase (Manski et al., 2007) as CaCAS or NaCAS solutions became more concentrated. Also, CaCAS becomes less soluble as concentration increases. Khwaldia et al. (2004) suggested that 10% (wt/wt) most likely represents an upper limit for the concentration of NaCAS in aqueous solutions with volume fraction of the caseinate low enough to enable chain entanglements.

Other factors peculiar to either NaCAS or CaCAS in aqueous solution (McMahon and Oommen, 2013) may have contributed to the inability to electrospin the pure caseinates. Dissolution of NaCAS in water results in short protein strands and small agglomerates of protein which will not entangle and form fibers. Numerous Ca bridges form between the phosphoserine groups in CaCAS as it is converted from acid casein to CaCAS, resulting in an accumulation of protein chains, but the chains are probably too dense for fiber formation. Methods to enable fiber formation need to be explored.

Next, PUL/CaCAS solutions (Table 1) were electrospun at 23 kV with increasing amounts of PUL. The PUL/CaCAS 1:2 containing 5% (wt/wt) PUL and

13.3% (wt/wt) CaCAS was spun at 1 mL/h and formed submicron fibers with FDS of 172 ± 43 nm, but with some flat fibers, branching, and other defects (Figure 2A). The FDS were smaller ($P < 0.0001$) than the 15% (wt/wt) PUL fibers alone (Figure 1C), but with some overlap in fiber sizes even though the percent (wt/wt) CaCAS was greater than the percent (wt/wt) PUL. This suggested that the combined viscoelastic properties of CaCAS and the PUL carrier contributed to an elongational viscosity sufficient for maintaining a jet during electrospinning. Even though PUL was at only 5% (wt/wt), the smaller FDS may be indicative of strong interactions between CaCAS and PUL, which increased the shear viscosity of the solution to 0.0687 Pa/s (Table 1) below that of the 15% (wt/wt) PUL solution (0.0820 Pa/s) and about 5 times that of shear viscosity of the 15% (wt/wt) neat CaCAS dispersion (0.0141 Pa/s). Interaction of Ca salts with the hydroxyl groups of PUL has been shown to increase the viscosity of 20% (wt/wt) PUL by almost 20% (Tsujioka and Mitsuhashi, 1993) and may indicate interaction of CaCAS and PUL by a similar mechanism. The decrease in the electrical conductivity of the solution (1,692 $\mu\text{S}/\text{cm}$) was 20% lower than the interpolated value of 2,107 $\mu\text{S}/\text{cm}$ for a neat 13.3% CaCAS solution. Given its high electrical conductivity, CaCAS may have also increased the charge density of the jet, which elongates the jet resulting in thinner fibers, relative to PUL alone, as observed when salts such as NaCl were added to polymer solutions (Zong et al., 2002; Tan et al., 2005).

For PUL/CaCAS of 1:4, with 16% (wt/wt) CaCAS and 6% (wt/wt) PUL, FDS increased to 225 ± 57 nm (Figure 2B) and became non-normally distributed ($P = 0.034$) with some overlap of the FDS, seen in Figure 2A, but with less uniform fiber morphology. A few wide fibers were evident and appeared similar to ribbons from electrospinning of polymers (Ramakrishna et al., 2005; Reneker and Yarin, 2008). These ribbons can result from a skin that forms around the jet, first forming a hollow tube as the solvent evaporated and then collapsing into a flat ribbon shape because the still wet fibers flattened upon impact with the drum collector. The thinner, flat fibers noted in Figure 2A may have also resulted from this effect. For the other solutions of PUL/CaCAS in Table 1, with PUL increasing from 6 to 7.5 to 10% (wt/wt), the FDS increase shear viscosity. A decrease in electrical conductivity relative to the neat CaCAS solutions reflects interaction with PUL, as noted. Aceituno-Medina et al. (2013) also suggested possible interactions between protein and PUL when reporting a similar increase in viscosity and a decrease in conductivity upon PUL addition to amaranth protein isolate solutions. In their study, the protein and pullulan were dissolved in formic acid and spun with the aid

of a surfactant, whereas, in our study, only water was used. The addition of a salt, such as NaCl, was thought to aid fiber development; for example, by lowering the concentration of polymer required for fiber formation by increasing electrical energy (Shenoy et al., 2005) but not the number of chain entanglements. Similar to salt addition, the blending of positively charged proteins with PUL may have lowered the polymer concentration

required for fiber formation from 15% to as low as 5% (wt/wt) in CaCAS/PUL spinning solutions.

Shear viscosity was not reported for samples PUL/CaCAS 1:4 and 1:2 prepared from the 30% (wt/wt) PUL stock solution (Table 1) because a film of PUL/CaCAS was formed during rotational viscometry testing. The film was off-white and flexible and formed after about 20 min on the surface of the solution. The

Figure 2. Scanning electron microscopy images and fiber diameter size (FDS) analysis of electrospun fibers obtained from either 15 or 30% pullulan (PUL) solutions and 20% calcium caseinate (CaCAS) solutions in various mass ratios at 50°C. Flow rate = 1 to 2.5 mL/h, Voltage = 23 kV. (A) 15% PUL, 20% CaCAS solution (1:2); (B) 30% PUL, 20% CaCAS (1:4); (C) 15% PUL, 20% CaCAS solutions (1:1); and (D) 30% PUL, 20% CaCAS solution (1:2). Magnification is 5,000×.

film did not form after testing the neat CaCAS or PUL solutions, PUL/CaCAS solutions with TS content <20% (wt/wt), or the PUL/NaCAS solutions described below. This type of concentration and time-dependent thickening flow has been described for polysaccharides, such as acacia gum (Sanchez et al., 2002), and for proteins, such as BSA (Sharma et al., 2011), but apparently not for protein-polysaccharide systems. Sanchez et al. (2002) ruled out dehydration of the solution, but this may be a possibility because the viscosity testing was conducted at an elevated temperature of 50°C and evaporation would be expected from the uncovered sample, which could serve to cool the surface facilitating aggregation due to reduced viscosity. However, the film still formed even when the sample was covered during testing. Additional experiments are needed to elucidate the conditions for the observed rheology of these samples.

For the PUL/CaCAS 1:1 blend, increasing PUL to 7.5% (wt/wt) with CaCAS reduced to 10% (wt/wt) for a total concentration of 17.5% (wt/wt) led to an increase in FDS ($P < 0.0001$; 283 ± 67 nm; Figure 2C) compared with the PUL/CAS 1:4 fibers (Figure 2B). A few elongated beads along with fibers were spun from the PUL/CaCAS 1:1 blend at 2 mL/h, with electrical conductivity decreasing below that of the 10% (wt/wt) neat CaCAS solutions ($P < 0.0001$; Table 1). Adjustment of the PUL and CaCAS concentrations, further manipulation of the electrospinning parameters, or both would most likely prevent bead formation (Deitzel et al., 2001; Greiner and Wendorff, 2007); however, it is unknown if these changes would affect any bead formation resulting from Ca bridging of the CaCAS. As shown in Figures 2A to 2C, the percentage of CaCAS in the fibers was higher than that of PUL and the critical concentration for electrospinnability of PUL was decreased from 8 or 15% in pure solution to as low as 5% (wt/wt) in the presence of CaCAS. Electrical conductivity for the PUL/CaCAS solutions decreased further (Table 1) corresponding to that of neat 10% (wt/wt) CaCAS likely because of the Ca and PUL interactions.

Increasing PUL to 10% (wt/wt) and CaCAS to 13.3% (wt/wt) for a total solution concentration of 23.3% (wt/wt) caused the FDS distribution to become less uniform and the mean FDS to increase to 451 ± 145 nm (Figure 2D); this was different than FDS means of other PUL/CaCAS blends ($P < 0.0001$). Relatively smooth fibers and a few flat fibers or ribbons that appeared similar to those in Figure 2B were formed, probably resulting from the increase in flow rate to 2.5 mL/h to facilitate electrospinning. As the total solution concentration was increased, a higher flow rate was found necessary to elongate the drop at the needle tip as the solution became more viscous.

Although shear viscosity measurements are not available for 2 of the solutions, these results show that shear viscosity may be important for defining the region in which PUL/CaCAS is electrospinnable; in our study, this was in the narrow range from approximately 0.07 to 0.12 Pa/s at 100 s^{-1} (Table 1). Further adjustments of solution properties and operating parameters may result in a wider range, although examination of the effects of the CaCAS manufacturing process on fiber formation (McMahon and Oommen, 2013) should also be considered.

For comparison, solutions of PUL with NaCAS replacing CaCAS were subjected to electrospinning. Scanning electron microscopy images are shown in Figures 3A to 3D in order of increasing PUL concentration and decreasing NaCAS concentration. For various PUL/NaCAS blends, the higher shear viscosities of the blended solutions (Table 1) appear to be due to the contribution of the higher viscosity NaCAS to the aqueous mixture with PUL. For production of fibers, preliminary trials indicated that voltage should be reduced to 11 kV, half of that used for electrospinning of PUL/CaCAS, with adjustment of flow rate from 0.4 to 2 mL/h, as needed, to elongate the drop to overcome the viscosity at the needle tip and ensure a jet. The PUL/NaCAS solutions at 1:1 and 2:1 formed more uniform fibers with normal distributions of FDS ($P = 0.21$ and $P = 0.34$, respectively), possibly because of the lower solution viscosities (Table 1). The non-normal distributions of FDS ($P < 0.0001$) and occasional branching noted for the PUL/NaCAS blends with a higher percent (wt/wt) of NaCAS (Figures 3A and 3B) could be due to jet instabilities, more commonly observed in solutions with high concentration and viscosity (Deitzel et al., 2001). Only Figure 3A showed the presence of ribbon-like fibers, but large fibers in the range of 764 to 2,622 nm were apparent in Figures 3A and 3B, in which the electrical conductivities of the solutions showed a difference ($P < 0.0001$) with that of the equivalent neat NaCAS solution (Table 1). An increase of PUL concentration in the spinning solution led to more uniform distributions of FDS (Figures 3C and 3D) and to the formation of defect-free fibers (Figure 3D). This suggested improved jet stabilities, which occurred as the shear viscosity decreased (Table 1); likewise, the electrical conductivities of these solutions were lower than those of the corresponding neat NaCAS solutions. Although viscosity is the most important solution variable governing FDS, CaCAS and NaCAS appear to interact differently with PUL and formed fibers best under very different solution conditions; whereas the former showed fibers with less defects when the PUL content in the fiber was lower, the best fibers of the latter were formed from blends with higher PUL concentration.

The solution electrostatic forces overcame the surface tension for ejection of the jet. It is doubtful that the relatively small decreases in surface tension of the PUL/CaCAS or PUL/NaCAS solutions relative to that of PUL solution alone (Table 1) contributed to fiber formation as much as viscosity. The consistency in fiber diameters may be further improved by adjustment in operational parameters.

In conclusion, this is the first example of creation of electrospun fibers from the milk proteins CaCAS

and NaCAS using an aqueous solvent and a food-grade polysaccharide, PUL, instead of a spinnable polymer to facilitate molecular entanglement. It was not possible to create fibers from the neat CaCAS or NaCAS solutions due to insufficient chain entanglements, but addition of PUL to CAS allowed fiber production and also lowered the concentration of PUL required for fiber formation. Additional studies are needed to determine the compositions of the fibers and the distribution of CAS within the fiber.

Figure 3. Scanning electron microscopy of electrospun fibers and fiber diameter size (FDS) analysis obtained from 15% pullulan (PUL) and 20% sodium caseinate solutions in various mass ratios: (A) 1:4 (5,000 $\times$ magnification); (B) 1:2 (5,000 $\times$ magnification); (C) 1:1 (10,000 $\times$ magnification); and (D) 2:1 (10,000 $\times$ magnification). Flow rate = 0.4 to 2 mL/h, Voltage = 11 kV.

ACKNOWLEDGMENTS

The authors thank Raymond Kwoczek for the technical assistance in the characterization of the spinning solutions and Joseph Uknalis for the SEM analysis (both of USDA, ARS, ERRC, Wyndmoor, PA). This study was funded under National Program 306, Dairy and Functional Foods Research Unit, USDA, ARS, ERRC Project 8072-41000-096 "Improving the Sustainability and Quality of Food and Dairy Products from Manufacturing to Consumption via Process Modeling and Edible Packaging."

REFERENCES

- Aceituno-Medina, M., S. Mendoza, J. M. Lagaron, and A. López-Rubio. 2013. Development and characterization of food-grade electrospun fibers from amaranth protein and pullulan blends. *Food Res. Int.* 54:667–674.
- Deitzel, J. M., J. Kleinmeyer, D. Harris, and N. C. B. Tan. 2001. The effect of processing variables on the morphology of electrospun nanofibers and textiles. *Polymer (Guildf.)* 42:261–272.
- Fong, H., I. Chun, and D. H. Reneker. 1999. Beaded nanofibers formed during electrospinning. *Polymer (Guildf.)* 40:4585–4592.
- Gennadios, A., T. H. McHugh, C. L. Weller, and J. M. Krochta. 1994. Edible Coatings and Films Based on Proteins in Edible Coatings and Films to Improve Food Quality. J. M. Krochta, E. A. Baldwin, and M. Nisperos-Carriedo, ed. Technomic Publishing Co. Inc., Lancaster, PA.
- Greiner, A., and J. H. Wendorff. 2007. Electrospinning: A fascinating method for the preparation of ultrathin fibers. *Angew. Chem. Int. Ed. Engl.* 46:5670–5703.
- Kanjanapongkul, K., S. Wongsasulak, and T. Yoovidhya. 2010. Investigation and prevention of clogging during electrospinning of zein solution. *J. Appl. Polym. Sci.* 118:1821–1829.
- Khwalidia, K., S. Banon, C. Perez, and S. Desobry. 2004. Properties of sodium caseinate film-forming dispersions and films. *J. Dairy Sci.* 87:2011–2016.
- Kishikawa, Y., Y. Seki, K. Shingai, R. Kita, N. Shinyashiki, and S. Yagihara. 2013. Dielectric relaxation for studying molecular dynamics of pullulan in water. *J. Phys. Chem. B* 117:9034–9041.
- Kong, L., and G. R. Ziegler. 2014. Rheological aspects in fabricating pullulan fibers by electro-wet-spinning. *Food Hydrocoll.* 38:220–226.
- Konstance, R. P., and E. D. Strange. 1991. Solubility and viscous properties of casein and caseinates. *J. Food Sci.* 56:556–559.
- Kozempel, M., A. J. McAloon, and P. M. Tomasula. 2003. Drying kinetics of calcium caseinate. *J. Agric. Food Chem.* 51:773–776.
- Kozempel, M., and P. M. Tomasula. 2004. Development of a continuous process to make casein films. *J. Agric. Food Chem.* 52:1190–1195.
- Kriegel, C., A. Arrechi, K. Kit, D. J. McClements, and J. Weiss. 2008. Fabrication, functionalization, and application of electrospun biopolymer nanofibers. *Crit. Rev. Food Sci. Nutr.* 48:775–797.
- Kristo, E., and C. G. Biliaderis. 2006. Water sorption and thermomechanical properties of water/sorbitol-plasticized composite biopolymer films: Caseinate-pullulan bilayers and blends. *Food Hydrocoll.* 20:1057–1071.
- Manski, J. M., L. E. van Riemsdijk, A. J. van der Goot, and R. M. Boom. 2007. Importance of intrinsic properties of dense caseinate dispersions for structure formation. *Biomacromolecules* 8:3540–3547.
- McHugh, T. H., and J. M. Krochta. 1994. Milk protein-based edible films and coatings. *Food Technol.* 48:97–103.
- McMahon, D. J., and B. S. Oommen. 2013. Casein micelle structure, functions, and interactions. Pages 185–210 in *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects*, 4th ed. P. L. H. McSweeney, and P. F. Fox, ed. Springer, New York, NY.
- Miyoshi, T., K. Toyohara, and H. Minematsu. 2005. Preparation of ultrafine fibrous zein membranes via electrospinning. *Polym. Int.* 54:1187–1190.
- Nieuwland, M., P. Geerdink, P. Brier, P. van den Eijnden, J. T. M. M. Henket, M. L. P. Langelaan, N. Stroeks, H. C. van Deventer, and A. H. Martin. 2013. Food-grade electrospinning of proteins. *Innov. Food Sci. Emerg. Technol.* 20:269–275.
- Ramakrishna, S., K. Fujihara, W.-E. Teo, T.-C. Lim, and Z. Ma. 2005. *An Introduction to Electrospinning and Nanofibers*. World Scientific Publishing Co. Ltd., Singapore.
- Reneker, D. H., and A. L. Yarin. 2008. Electrospinning jets and polymer nanofibers. *Polymer (Guildf.)* 49:2387–2425.
- Rošić, R., J. Pelipenko, J. Kristl, P. Kocbek, M. Bešter-Rogac, and S. Baumgartner. 2013. Physical characteristics of poly (vinyl alcohol) solutions in relation to electrospun nanofiber formation. *Eur. Polym. J.* 49:290–298.
- Royston, J. P. 1982. An extension of Shapiro and Wilk's W test for normality to large samples. *Appl. Stat.* 31:115–124.
- Sanchez, C., D. Renard, P. Robert, C. Schmitt, and J. Lefebvre. 2002. Structure and rheological properties of acacia gum dispersions. *Food Hydrocoll.* 16:257–267.
- Sharma, V., A. Jaishankar, Y.-C. Wang, and G. H. McKinley. 2011. Rheology of globular proteins: apparent yield stress, high shear rate viscosity and interfacial viscoelasticity of bovine serum albumin solutions. *Soft Matter* 7:5150–5160. <http://dx.doi.org/10.1039/c0sm01312a>.
- Shenoy, S. L., W. D. Bates, H. L. Frisch, and G. E. Wnek. 2005. Role of chain entanglements on fiber formation during electrospinning of polymer solutions: good solvent, non-specific polymer-polymer interaction limit. *Polymer (Guildf.)* 46:3372–3384.
- Sousa, A. M. M., H. K. S. Souza, J. Uknalis, S.-C. Liu, M. P. Gonçalves, and L. Liu. 2015. Electrospinning of agar/PVA aqueous solutions and its relation with rheological properties. *Carbohydr. Polym.* 115:348–355.
- Stijnman, A. C., I. Bodnar, and R. H. Tromp. 2011. Electrospinning of food-grade polysaccharides. *Food Hydrocoll.* 25:1393–1398.
- Sullivan, S. T., C. Tang, A. Kennedy, S. Talwar, and S. A. Khan. 2014. Electrospinning and heat treatment of whey protein nanofibers. *Food Hydrocoll.* 35:36–50.
- Tan, S.-H., R. Inai, M. Kotaki, and S. Ramadrihna. 2005. Systematic parameter study for ultra-fine fiber fabrication via electrospinning process. *Polymer (Guildf.)* 46:6128–6134.
- Tomasula, P. M. 2009. Using dairy ingredients to produce edible films and biodegradable packaging materials. Pages 589–624 in *Dairy-Derived Ingredients—Food and Nutraceutical Uses*. M. Corredig, ed. Woodhead Publishing Ltd. and CRC Press LLC, Boca Raton, FL.
- Tsujisaka, Y., and M. Mitsuhashi. 1993. Pullulan. Pages 447–460 in *Industrial Gums: Polysaccharides and Their Derivatives*. 3rd ed. R. L. Whistler and J. N. BeMiller, ed. Academic Press, San Diego, CA.
- Xie, J., and Y.-L. Hsieh. 2003. Ultra-high surface fibrous membranes from electrospinning of natural proteins: Casein and lipase enzyme. *J. Mater. Sci.* 38:2125–2133.
- Zhang, S., Y. Q. Huang, X. P. Yang, F. Mei, Q. Ma, G. Q. Chen, S. Ryu, and X. Deng. 2009. Gelatin nanofibrous membrane fabricated by electrospinning of aqueous gelatin solution for guided tissue regeneration. *J. Biomed. Mater. Res. A* 90:671–679.
- Zong, X., K. Kim, D. Fang, S. Ran, B. S. Hsiao, and B. Chu. 2002. Structure and process relationship of electrospun bioabsorbable nanofibers membranes. *Polymer (Guildf.)* 43:4403–4412.