Understanding the Role of Poly(ethylene oxide) in the Electrospinning of Whey Protein Isolate Fibers

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UNDERSTANDING THE ROLE OF POLY(ETHYLENE OXIDE) IN THE ELECTROSPINNING OF WHEY PROTEIN ISOLATE FIBERS

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Poly(ethylene oxide) (PEO) is known for facilitating the electrospinning of biopolymer solutions, that are otherwise not electrospinnable. The objective of this study was to investigate the mechanism by which PEO enables the formation of whey protein isolate (WPI) electrospun fibers under different pH conditions. This investigation revealed that the addition of PEO increased the viscosity of WPI/PEO (10% w/w WPI; 0.4% w/w PEO) solutions. Difference in pH levels of the polymer solutions affected electrospinnability and fiber morphology. Acidic solutions resulted in smooth fibers (700 ± 105 nm) while neutral solutions produced spheres (2.0 ± 1.0 μm) linked with ultrafine fibers (138 ± 32 nm). In comparison, alkaline solutions produced fibers (191 ± 38 nm) that were embedded with spindle-like beads (1.0 ± 0.5 μm). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analyses revealed that the native globular configuration of WPI was not altered under neutral conditions. By contrast, the electrophoresis and
spectrometry data indicated that WPI was denatured and hydrolyzed under acidic conditions, which facilitated the formation of smooth fibers. C\textsuperscript{13} nuclear magnetic resonance (NMR) and attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopies showed that the increase in random coil and \(\alpha\)-helix secondary structures in WPI contributed to the formation of bead-less electrospun fibers. Also, C\textsuperscript{13} NMR analysis showed no evidence of chemical interaction between WPI and PEO. Scanning transmission electron microscopy coupled with energy dispersive X-rays (STEM-EDAX) revealed that WPI was uniformly distributed within WPI/PEO electrospun fibers. Observations by scanning electron microscopy (SEM) and field emission scanning electron microscopy (FESEM) indicated that fibers possessed a solid core. All these findings suggested that PEO enables the formation of WPI/PEO electrospun fibers by entanglement/entrapment/deposition.

Preliminary studies were conducted on hydroxypropyl methyl cellulose (HPMC). In the absence of PEO, HPMC enabled the formation of WPI electrospun fibers under acidic conditions (124 ± 46 nm). FTIR analyses indicated that there was no interaction between HPMC and WPI, suggesting that HPMC aided in the electrospinning of WPI fibers, also by entanglement/entrapment/deposition. Hence, HPMC and PEO aid in the electrospinning of WPI fibers by entanglement/entrapment/deposition, which can be manipulated by alterations in the protein configuration and solution properties.
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Table of contents

Acknowledgments ........................................................................................................... iv

List of Tables .................................................................................................................... xii

List of Figures .................................................................................................................. xiii

Chapter 1. Introduction ...................................................................................................... 1

Chapter 2. Literature Review .............................................................................................. 5

2.1 Ultrafine fibers ........................................................................................................... 5

2.1.1 Mechanical drawing ............................................................................................... 5

2.1.2 Phase separation .................................................................................................... 6

2.1.3 Template synthesis ................................................................................................. 6

2.1.4 Self-assembly ......................................................................................................... 7

2.2 Electrospinning ........................................................................................................... 8

2.2.1 Intrinsic factors ..................................................................................................... 10

2.2.1.1 Surface tension .................................................................................................. 10

2.2.1.2 Viscosity .......................................................................................................... 11

2.2.1.3 Conductivity ..................................................................................................... 12

2.2.2 Extrinsic parameters ............................................................................................. 14

2.2.2.1 Relative humidity ............................................................................................ 14
2.2.2.2 Spinneret-collector distance .............................................. 15
2.2.2.3 Applied voltage ...................................................................... 15
2.2.2.4 Flow rate .............................................................................. 17
2.2.3 Fiber formation in the electrospinning process ......................... 18
  2.2.3.1 Jet initiation ........................................................................... 18
  2.2.3.2 Jet stretching and whipping .................................................. 20
    2.2.3.2.1 Modes of unsteady bending ........................................... 26
2.3 Poly(ethylene oxide) (PEO) ...................................................... 31
2.4 Whey protein isolate (WPI) ....................................................... 36

Chapter 3. Problem Statement and Objectives ................................... 39
  3.1 Problem statement ..................................................................... 39
  3.2 Overall objectives ...................................................................... 40

Chapter 4. Electrospinning of WPI Fibers ........................................ 41
  4.1 Introduction ................................................................................. 41
  4.2 Materials .................................................................................. 44
  4.3 Methods .................................................................................... 44
    4.3.1 Solution preparation .............................................................. 44
    4.3.2 Solution viscosity ................................................................. 45
4.3.3 Solution pH, conductivity and surface tension ............................................. 45
4.3.4 Electrospinning ................................................................................................. 46
4.3.5 Scanning Electron Microscopy (SEM) Analysis ............................................. 46
4.3.6 Data analysis ..................................................................................................... 47
4.4 Results and discussion ......................................................................................... 47
  4.4.1 Fiber morphology ............................................................................................ 47
  4.4.2 Viscosity ........................................................................................................ 49
  4.4.3 Surface tension ............................................................................................... 53
  4.4.4 Conductivity ................................................................................................... 58
  4.4.5 Spinneret-collector distance effects ............................................................... 60
4.5 Conclusion ........................................................................................................... 66

Chapter 5. WPI Characterization and WPI-PEO Interaction in Electrospun Fibers
........................................................................................................................................ 68
  5.1 Introduction ......................................................................................................... 68
  5.2 Materials ........................................................................................................... 71
  5.3 Methods ............................................................................................................ 71
    5.3.1 Solution preparation ..................................................................................... 71
    5.3.2 Electrospinning and casting ........................................................................ 72
6.3 Methods.................................................................................................................. 99

6.3.1 Solution preparation ............................................................................................ 99

6.3.2 Scanning Electron Microscopy (SEM) Analysis................................................. 100

6.3.3 Scanning Transmission Electron Microscopy (STEM) Analysis and Energy Disperse Analysis of X-rays (EDAX)................................................................. 100

6.3.4 Field Emission Scanning Electron Microscopy (FESEM) Analysis and Ion Beam Milling.............................................................................................................. 101

6.4 Results and discussion............................................................................................ 102

6.4.1 Effect of various WPI and PEO concentrations on the morphology of electrospun WPI/PEO fibers. ................................................................. 102

6.4.3 Transmission Electron Microscopy Analysis-EDAX on different substrates to determine the location of PEO in the electrospun fibers ...... 109

6.4.4 Cross section of electrospun fibers ................................................................. 117

6.5 Conclusions............................................................................................................ 124

Chapter 7. Electrospinning of Hydroxypropylmethyl cellulose (HPMC)/WPI

Electrospun Fibers........................................................................................................ 126

7.1 Introduction.............................................................................................................. 126

7.2 Materials................................................................................................................. 128

7.3 Methods.................................................................................................................. 128
10.1.3. Statistics analysis of the conductivity data ...................................... 177

10.2 Effects of different speeds of the rotational drum as a collector for neutral
electrospun fibers ......................................................................................... 178

10.4 Tensile strength of acidic electrospun fibers............................................ 180
List of Tables

Table 1. Electrospun biopolymer systems with the aid of PEO.......................... 35
Table 2. Physical properties of whey proteins\textsuperscript{64}................................................. 37
Table 3. Conductivity of various polymer solutions................................................. 59
Table 4. Paired comparisons of the viscosity data of polymer solutions in the absence and presence of PEO................................................................. 170
Table 5. ANOVA for the viscosity data of acidic polymer solutions in the presence of PEO................................................................. 171
Table 6. Multiple comparisons by Tukey test for the viscosity data of acidic solutions in the presence of PEO at different cross head speed ...................... 171
Table 7. ANOVA for the viscosity data of neutral polymer solutions in the absence of PEO ................................................................. 172
Table 8. Multiple Comparisons by Tukey test for the viscosity data of neutral polymer solutions in the absence of PEO ................................................................. 172
Table 9. Paired comparisons of the surface tension data of polymer solutions in the presence and absence of PEO................................................................. 173
Table 10. ANOVA for the surface tension data of acidic polymer solutions in the absence of PEO ................................................................. 173
Table 11. Multiple comparisons by Tukey Test for surface tension data of acidic polymer solutions in the absence of PEO ................................................................. 174
Table 12. Paired comparison of the conductivity data of the different polymer solutions ......................................................................................... 177
List of Figures

Figure 1. Diagram of the sequence of the different areas of investigation for the present study ................................................................. 4

Figure 2. Effect of fiber diameter on surface area. Adapted from Ko 12 ............ 5

Figure 3. Typical electrospinning setup ................................................................................................................................. 9

Figure 4. Forces exerted on segment E of the polymer jet. Segment E moves to E’ because of jet perturbation, and the Coulomb forces from segments D and F. Adapted from Ramakrishna et al. and Reneker et al. 14, 40 ................................................. 22

Figure 5. Viscoelastic Dumbbell-Maxwell model to represent the bending instability of the polymer jet during electrospinning: (a) initial bead at the pendant droplet; (b) springs and dashpots to represent the viscoelastic resistance to the jet elongation; (c) final bead on the collector. Adapted from Reneker et al. 40 ..... 23

Figure 6. Operating diagram to predict the mode of instability: (a) PEO/water solution of low viscosity value (1.67 Poise); (b) PEO/water solution of high viscosity value (167 Poise). Adapted from Hohman et al. 38.......................... 25

Figure 7. Illustrations of the rotating bending mode. Adapted from Si et al. 37 ..... 27

Figure 8. Illustrations of the swinging rotating mode. Adapted from Si et al 37 ..... 28

Figure 9. Illustration of the blurry bending mode. Adapted from Si et al. 37 ........ 29

Figure 10. Illustration of three branches of polymer jets during the bending instability at different voltages: (a) two branches at 3.5 kV; (b) three branches at 4 kV; (c) four branches at 4.1 kV. Adapted from Si et al 37 ............................................. 30

Figure 11. Schematic representation of PEO clustering in water due to end chain effect. Adapted from Hammouda et al. 45 ............................................................... 33

Figure 12. Scanning electron micrographs of electrospun fibers from 0.4% PEO solutions at different pH: (a) pH 1; (b) pH 7; (c) pH 12. Top row: low magnification; bottom row: high magnification ......................................................... 48

Figure 13. Scanning electron micrographs of WPI-PEO electrospun fibers from polymer solutions at: (a) pH 1; (b) pH 7; (c) pH 12. Top row: low magnification; bottom row: high magnification ................................................................. 49

Figure 14. Viscosity values for various WPI solutions in the presence and absence of PEO as a function of shear rate ................................................................. 50
Figure 15. Surface tension values as affected by bubble lifetimes of polymer solutions and their respective solvent at different pH conditions. The concentrations of PEO, WPI and WPI:PEO were 0.4, 10 and 0.4:10% (w/w), respectively .......................................................... 54

Figure 16. Scanning electron micrographs of electrospun fibers from the electrospinning of 10% WPI and 0.4% blend at pH 7 under various spinneret-collector distances: (a) 10 cm; (b) 15 cm; (c) 20 cm; (d) 25 cm. Top row: low magnification; bottom row: high magnification ........................................... 61

Figure 17. Scanning electron micrographs of electrospun fibers from the electrospinning of 10% WPI and 0.4% blend at pH 1 under various spinneret-collector distances: (a) 10 cm; (b) 15 cm; (c) 20 cm; (d) 25 cm. Top row: low magnification; bottom magnification: high magnification .................................. 62

Figure 18. Scanning electron micrographs of electrospun fibers from the electrospinning of 10% WPI and 0.4% PEO blend at pH 12 under various spinneret-collector distances: (a) 10 cm; (b) 15 cm; (c) 20 cm; (d) 25 cm. Top row: high magnification; bottom row: high magnification ........................................ 64

Figure 19. ATR-FTIR spectra for: (a) WPI film; (b) WPI-PEO; and (c) WPI-PEO electrospun fibers, prepared from polymer solutions at different pH's. For comparison, the spectrum for PEO fibers electrospun from neutral PEO aqueous solution (4% w/w) is also shown (d)........................................................................ 78

Figure 20. Deconvolution and curve fitting of the FTIR spectrum for WPI fibers electrospun from polymer solutions at different pH's ......................................................... 79

Figure 21. 1H NMR Spectra of: (a) WPI neutral solution; (b) WPI/PEO neutral solution ........................................................................................................ 80

Figure 22. 1H NMR Spectra of: (a) WPI alkaline solution; (b) WPI/PEO alkaline solution ........................................................................................................ 81

Figure 23. 13C NMR spectra for: (a) WPI film; (b) WPI-PEO film; and (c) WPI-PEO electrospun fibers prepared from neutral polymer solutions. For comparison, the spectrum for PEO powder is also shown (d)......................................................... 83

Figure 24. Comparison of 13C NMR spectra of: (a) neutral WPI film; (b) acidic WPI film; (c) acidic WPI-PEO film; and (d) acidic WPI-PEO electrospun fiber. .... 84

Figure 25. Comparison of 13C NMR spectra of: (a) neutral WPI film; (b) alkaline WPI film; (c) alkaline WPI-PEO film; and (d) alkaline WPI-PEO electrospun fibers. ........................................................................................................ 86

Figure 26. SDS-PAGE electrophoretogram for WPI solutions at different pH: (a) WPI/PEO acidic electrospun fibers (pH 1); (b) WPI/PEO alkaline electrospun fibers (pH 12); (c) WPI/PEO neutral electrospun fibers (pH 7) ......................... 90
Figure 27. MALDI-TOF mass spectra of: (a) WPI powder; (b) WPI aqueous neutral solution; (c) WPI/PEO aqueous neutral solution; (d) WPI/PEO neutral electrospun fibers ................................................................. 93

Figure 28. MALDI-TOF mass spectra of: (a) WPI acidic solution; (b) WPI/PEO acidic solution; (c) WPI/PEO acidic electrospun fibers ................................................................. 94

Figure 29. SEM micrographs of WPI/PEO fibers resulted from polymer solutions using glacial acetic acid as solvent with a concentration of 10% WPI and various PEO concentrations: (a) 0.3%; (b) 0.2%; (c) 0.1%; (d) 0.05%. Top row: low magnification; bottom row: high magnification ................................................................. 103

Figure 30. SEM micrographs of WPI/PEO ultrafine fibers resulted from polymers solutions using glacial acetic acid as solvent with a concentration of 0.4% PEO and various WPI concentrations: (a) 0%; (b) 2.5%; (c) 5%; (d) 7.5%. Top row: low magnification; bottom row: high magnification ................................................................. 105

Figure 31. SEM micrographs of WPI/PEO electrospun fibers resulted from polymer solutions using glacial acetic acid as solvent of various WPI and PEO concentrations: (a) 10% WPI, 0.4% PEO; (b) 7.5% WPI, 0.45% PEO; (c) 5% WPI, 0.5% PEO; (d) 2.5% WPI, 0.55% PEO. Top row: low magnification; bottom row: high magnification ................................................................. 108

Figure 32. STEM micrographs and EDAX maps of carbon and sulfur distribution along electrospayed WPI beads and PEO electrospun fibers. Top row: 100% WPI; bottom row: 100% PEO. The area delimited by the black square was examined by EDAX ................................................................. 111

Figure 33. STEM micrographs and EDAX maps of carbon and sulfur distribution along WPI/PEO acidic electrospun fibers of different composition (pH 1). Upper row: the WPI to PEO ratio is 10:90. Lower row: the WPI to PEO ratio is 96:4 . 113

Figure 34. STEM micrographs, and EDAX maps of the distribution of carbon and sulfur along WPI/PEO neutral electrospun fibers of different composition (pH 7). Upper row: the WPI to PEO ratio is 10:90 in the fiber. Lower row: the WPI to PEO ratio is 96:4 in the fiber. The area delimited by the black square was examined by EDAX ................................................................. 114

Figure 35. STEM micrographs and EDAX maps of the distribution of carbon and sulfur along WPI/PEO alkaline fibers of different composition (pH 12). Upper row: The WPI to PEO ratio is 96:4 in the fibers. Lower row: The WPI to PEO ratio is 10:90 in the fiber. The area delimited by the black square was examined by EDAX ................................................................. 116

Figure 36. Cross section of fiber electrospun from 10% WPI and 0.4% acidic solution (pH 1). Left column: SEM micrographs. Right column: FESEM micrographs ................................................................. 120
Figure 37. Cross section of fiber electrospun from 10% WPI and 0.4% PEO neutral solutions (pH 7). Left column: SEM micrographs. Right column: FESEM micrographs ................................................................. 122

Figure 38. Scanning electron micrographs of fiber electrospun from HPMC acidic solutions at various concentrations: (a) 0.5% HPMC; (b) 1% HPMC. Top row: low magnification; bottom row: high magnification .......................... 132

Figure 39. Scanning electron micrographs of electrospun fiber from HPMC water ethanol (50:50) solutions at various concentrations: (a) 0.5% HPMC; (b) 1% HPMC. Top row: low magnification; bottom row: high magnification ............. 133

Figure 40. Scanning electron micrographs of electrospun 10% WPI from different polymer solutions: (a) WPI dissolved in water ethanol (50:50); (b) WPI dissolved in acetic acid. Top row: low magnification; bottom row: high magnification .... 134

Figure 41. Scanning electron micrograph of fibers resulted from the electrospinning of WPI and HPMC in water-ethanol (50:50) at various polymer concentrations. Top row: low magnification; bottom row: high magnification .... 136

Figure 42. Scanning electron micrographs of fibers resulted from the electrospinning of HPMC and WPI in glacial acetic acid at various polymer concentrations: (a) 5% WPI, 0.5% HPMC; (b) 5% WPI, 1% HPMC; (c) 10% WPI, 0.5% HPMC; (d) 10% WPI, 1% HPMC. Top row: low magnification; bottom row: high magnification ................................................................. 137

Figure 43. ATR-FTIR spectra for: (a) WPI powder; (b) electrospun beads from 10% WPI in water-ethanol (50:50); (c) electrospun beads from 10% WPI in glacial acetic acid ........................................................................................................ 141

Figure 44. ATR-FTIR spectra for: (a) HPMC powder; (b) electrospun bead and fibers from 1% HPMC in water-ethanol (50:50); (c) electrospun beads and fibers from 1% HPMC in glacial acetic acid ........................................................................................................ 142

Figure 45. ATR-FTIR spectra for fibers from the electrospinning of HPMC-WPI in water-ethanol (50:50) at various concentrations: (a) 5% WPI, 0.5% HPMC; (b) 10% WPI, 0.5% HPMC; (c) 5% WPI, 1% HPMC; (d) 10% WPI, 1% HPMC ...... 144

Figure 46. ATR-FTIR spectra for fibers from the electrospinning of HPMC-WPI in glacial acetic acid at various concentrations: (a) 5% WPI, 0.5% HPMC; (b) 10% WPI, 0.5% HPMC; (c) 5% WPI, 1% HPMC; (d) 10% WPI, 1% HPMC ......... 145

Figure 47. Scanning electron micrographs of fibers electrospun from 10% WPI and 0.4% aqueous neutral solution collected with a rotational drum at different speeds: (a) 200 rpm; (b) 400 rpm; (c) 1000 rpm and (d) 1000 rpm at different magnifications. Top row: low magnification; bottom row: high magnification .... 179
Figure 48. Illustration of the cardboard frame to fix a single electrospun fiber for tensile testing

Figure 49. Plot of Stress vs Strain for fibers with a diameter of 1400 nm from electrospinning 12.5% WPI, 0.4% PEO in glacial acetic acid. Replicas of test are represented by (a), (b), and (c).
Chapter 1. Introduction

Electrospinning is a versatile fiber-forming technology for producing fibers with diameters ranging from nano- to micrometers using electrostatic force. More than 50 synthetic polymers have been electrospun for a variety of applications such as cosmetics¹,², tissue engineering³,⁴, food packaging⁵,⁶, and protective clothing⁷.

Biopolymers have been electrospun into fibers for food and biomedical purposes due to their biodegradability and biocompatibility. However, in aqueous systems, electrospinning of low-molecular biopolymers, such as proteins and carbohydrates, dissolved in aqueous has been unsuccessful in most cases. To enable the electrospinning of aqueous biopolymer systems, a spinning aid polymer such as poly(ethylene oxide) is commonly added. For example, chitosan, keratin, alginate, eggshell membrane, and soy protein fibers have been successfully electrospun from aqueous solutions when PEO is added in the polymer solution.

PEO is a commonly chosen synthetic polymer as a spinning aid agent because it is non toxic and water soluble. Several studies have speculated the PEO’s role in the electrospinning of biopolymers. For example, Nie et al. reported that the high molecular weight of PEO (1000 kDa) stabilized the polymer jet during the electrospinning of the alginate/PEO blend ⁸. Also, in the electrospinning of soy protein and alginate/pectin fibers, it was reported that the
addition of PEO decreased the electrical conductivity of the polymer solution. This effect has been speculated to be caused by the charge counteracting effect of PEO upon the biopolymers which are polyelectrolytic. Although these are the explanations often cited for explaining the positive effect of PEO, the fundamental reasons remain obscure. Understanding the mechanism by which PEO enables the formation of biopolymer electrospun fibers is crucial for the selection of the replacement of PEO with a food grade biopolymer. The production of electrospun fibers made of food grade materials may expand their applications in food.

To elucidate the mechanism by which PEO enables the formation of fibers, whey protein isolate (WPI) was used in this research as biopolymer model that requires PEO for electrospinnability. The investigation was conducted in four stages as shown in Figure 1. Considering that solution properties are well known for their influence on facilitating or impeding electrospinnability, in the first stage (Chapter 4) of this study, the effects of PEO on the solution properties, and consequently on the resulting fiber morphology of the different WPI/PEO polymer systems were investigated. For this study, viscosity, surface tension and conductivity of the polymer solutions in the absence and presence of PEO were evaluated. Also, the correlation between solution properties and fiber morphology was investigated.

PEO might also interact with WPI for electrospinnability purposes. To gain a deeper understanding of this possibility, WPI was characterized and interactions between WPI and PEO were investigated in the second stage (Chapter 5). For this study, attenuated total reflection-Fourier transform infrared
(ATR-FTIR) spectrometry and nuclear magnetic resonance (NMR) analyses were used to characterize WPI and to investigate whether or not WPI and PEO interact for the formation of electrospun fibers. Also, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and matrix-assisted laser desorption-ionization time-of flight mass spectrometry (MALDI-TOF MS), were used to characterize WPI.

To further elucidate the role of PEO as an aid agent in electrospinning, the ability of WPI and PEO to form electrospun fibers was studied by characterizing the microstructure of these fibers in the third stage (Chapter 6). Scanning electron microscopy (SEM), scanning transmission electron microscopy (STEM) in conjunction with energy dispersive x-rays analysis (EDAX), and field emission electron microscopy were used for the purposes of this chapter.

In the fourth stage (Chapter 7) of this study, the investigation of a food grade polymer as a replacement of PEO for the electrospinning of WPI fibers is reported. Although PEO is a non toxic polymer, it does not form part of the food additive list from Health Canada, and Food and Drug Administration. The selection of the food grade polymer that will potentially replace PEO was based on the proposed mechanism found in previous chapters.
Figure 1. Diagram of the sequence of the different areas of investigation for the present study
Chapter 2. Literature Review

2.1 Ultrafine fibers

Ultrafine fibers have been involved in numerous investigations for different purposes due to their large surface area (Figure 2). Nano-micro fibers can be produced using many methodologies, including mechanical drawing, template synthesis, phase separation, self assembly, and electrospinning.

![Surface Area vs Fiber Diameter](image)

*Figure 2. Effect of fiber diameter on surface area. Adapted from Ko*¹²

2.1.1 Mechanical drawing

Mechanical drawing is one of the simplest techniques to produce a single fiber at the time. In this technique, an ultrafine fiber is pulled from a microdroplet of a polymer solution at a certain temperature and relative humidity to evaporate the solvent. To form the fiber, the viscoelasticity of the polymer is crucial to have enough cohesiveness to support the deformation. After deformation, the solvent
evaporates completely, and the fiber solidifies. Parameters such as drawing rate, evaporation rate, and molecular weight of the polymer determine the size of the fiber\textsuperscript{13, 14}.

### 2.1.2 Phase separation

The phase separation technique has been studied and investigated for the production of microporous membranes for different applications such as tissue engineering and electronics. In this technique, polymer gelation takes places and the solvent is extracted from the gel. Typically, water is used to replace the solvent in the gel. To remove the water, the gel is freeze-dried under vacuum. The porous morphology of the membrane can be controlled during the gelation process by adjusting the polymer concentration, type of polymer, type of solvent and temperature\textsuperscript{15-17}. Also, the incorporation of porogens, such as, paraffin spheres, salt and sugar, has been investigated to control the macroporosity of the membrane\textsuperscript{18, 19}.

### 2.1.3 Template synthesis

Template synthesis is a relatively simple inexpensive technique to produce fibers. In this technique, a monomer solution passes through a microporous membrane with a pore size ranging from 30 nm to 10 \( \mu \)m; the polymer is synthesized along the walls of the membrane since there is a polymerization reagent on the other side of the membrane. When the polymer solidifies, fibers are produced\textsuperscript{20}. The production of hollow or solid fibers can be controlled by the chemistry of the pore. For the formation of hollow fibers, agents called “molecular
anchors" are attached to the walls of the pores in the membrane to bind monomers through non-covalent interactions. The attraction between the molecular anchors and the monomer during polymerization results in hollow fibers\textsuperscript{20, 21}.

### 2.1.4 Self-assembly

The molecular self-assembly techniques are inspired by the natural driving force of biomacromolecules to form functional structures in living beings. Biomacromolecules such as proteins can arrange themselves into different configurations through non covalent bonding such as hydrogen bonding, van der Waals and hydrophobic interactions. Control of the chemistry behind the natural arrangement of biomacromolecular has resulted in the formation of nanofibers\textsuperscript{22}. For example, residues of peptides have been chemically modified to produce nanofibers with a hydrophobic interior and hydrophilic exterior having a diameter of 5 to 8 nm\textsuperscript{15}.

Advantages and disadvantages of the different techniques to produce ultrafine fibers are taken into account for selection purposes. Although drawing is the simplest approach for forming long fibers, it has a low throughput since fibers are produced one at a time. Template synthesis, which needs a nanoporous membrane to form fibrils, cannot produce single continuous long nanofibers. The phase separation and self assembly techniques could be applied to create nanofibers, but the preparation time is longer than the other methods\textsuperscript{23}. The
technology that overcomes the drawbacks of these methods is electrospinning, which will be described in the following section.

2.2 Electrospinning

The pioneering works of electrostatic spinning or electrospinning can be found in the invention of Bose (1745), who created an aerosol spray by exposing a liquid in a capillary tube to a high electrical potential. In 1934, Formhals was granted a patent related to the production of monofilament fibers by the application of an electric field. In this investigation, an electrode was inserted into the polymer solution, and the opposite charged electrode was attached to the electrode. When the electric field was applied, polymer filaments were produced\textsuperscript{12}. In 1952, Vonnegut and Neubauer invented an apparatus to produce filaments. It was a glass tube filled with a polymer solution that received high voltage (5-10 kV). In 1955, Drozin studied the dispersion of aerosols using high voltage. In 1966, Simmons developed and patented a device to produce light fabrics of different patterns made of ultrafine fibers by using electrical spinning. For this invention, the positive electrode was drawn in the polymer solution while the negative electrode was attached to a textile belt. In 1977, Baumgarten improved the electrical spinning apparatus by pumping the polymer solution to a stainless capillary at a constant rate. The development and improvement in the electrospinning techniques have been accelerating since the last three decades. Studies in this area have increased exponentially in the last few years. This technique had been known as electrostatic spinning until 1993\textsuperscript{10}. To date more than 50 synthetic polymers have been electrospun\textsuperscript{14}. 
The electrospinning process requires four main components: (1) direct current power supply; (2) capillary tube spinneret with needle; (3) the polymer solution delivered to the spinneret by a pump and (4) a collector (Figure 3). In a typical setup, the positive electrode is attached to the spinneret filled with the polymer solution to be electrospun, while the collector plate is grounded. As electrical voltage is applied to the spinneret, the pendant droplet of the polymer solution at the tip of the capillary tube becomes charged, resulting in an electrostatic repulsion on the surface of the droplet. When the voltage exceeds the critical level, the electrostatic repulsion force overcomes the surface tension of the polymer solution, causing the polymer to eject towards the collector plate. As the polymer jet takes flight in the air, the solvent vaporizes rapidly, producing a continuous fiber that is laid on the collector as a nonwoven membrane\textsuperscript{14,24}.

![Figure 3. Typical electrospinning setup](image-url)
The electrospinning process is governed by extrinsic and intrinsic parameters. The main intrinsic factors of the polymer solution are: surface tension, electrical conductivity, and viscosity. Some of the key extrinsic parameters are: voltage, spinneret-collector distance, and flow rate. The effects of these selected factors on electrospinning are discussed below.

2.2.1 Intrinsic factors

2.2.1.1 Surface tension

Surface tension is a property of a liquid related to the cohesive force between the molecules, which shows resistance to external forces. As observed in many natural phenomena, the cohesiveness of molecules in solution results in the formation of spherical free-falling structures or suspended droplets. In the electrospinning process, when the polymer solution is pumped to the spinneret, a pendant droplet is formed at the tip of the spinneret. When the droplet is subjected to high voltage, the surface of the droplet is electrically charged either positively or negatively depending on the polarity of the electrode. An excess number of molecules with the same charge results in repulsive charges between them. The repulsion force overcomes the surface tension of the solvent giving rise to a stream of polymer solution jet. Polymer solutions with a relatively lower surface tension value tend to facilitate the formation of polymer jets, while those of higher surface tension value have greater propensity of forming electrospun/electrosprayed bead or bead-fiber materials. Several studies have reported the effects of surface tension on electrospinnability. For example, It was
reported that the addition of Tween40® surfactant reduced the surface tension of a blend solution of cellulose acetate and egg albumen dissolved in acetic acid from 30 to 28 mN/m, which facilitated the electrospinning by reducing the voltage required to initiate the polymer jet formation. Lee et al. reported that surface tension of poly(ε-caprolactone) dissolved in methylene chloride and dimethylformamide (DMF) could be lowered by increasing the amount of DMF. This had an effect of decreasing the formation of beads along the electrospun fiber.

2.2.1.2 Viscosity

Viscosity indicates the resistance of a solution to flow when under shear stress. In general, when the viscosity of a solution is relatively low, the formation of a polymer jet is not stable, and it has a tendency to break up into beads. For instance, PEO solution could be electrospun from bead-fiber morphology into bead-free fibers when its viscosity was raised from 13 to 1835 centi Poise (cPs) by increasing the polymer concentration. This transformation has been attributed to the higher molecular chain entanglement in the more viscous solution that stabilized the polymer jet. Similar phenomena were reported by Doshi and Reneker on the effects of viscosity for aqueous PEO solutions. They observed that the solution electrospun into fiber when the viscosity ranged from 80 to 4000 cPs, while polymer solution with viscosity values lower than 80 cPs could not be spun into fibers. In contrast, solutions with viscosity higher than 4000 had a tendency to dry at the spinneret tip, disrupting the formation of a stable polymer jet. Other studies reported that increasing polymer concentration could result in
the formation of larger fibers. For example, Fong et al. observed that as PEO concentration increased from 2 to 4.5% w/w, the viscosity and fiber diameter increased from 74 to 1835 cPs, and 150 to 250 nm respectively.27

### 2.2.1.3 Conductivity

Electrical conductivity measures the ability of a solution to transmit electrical current. A polymer solution must be conductive enough to allow the movement of charge to the surface of the pendant droplet to establish the required repulsion force to overcome the surface tension during electrospinning. In general, the addition of ions to the polymer solution tends to facilitate electrospinning by reducing the voltage required to overcome the surface tension. Moreover, the fiber diameter tends to be smaller due to increased stretching of the fiber during chaotic whipping.

The size and charge of the added ions are also important factors that affect the electrospinnability of a polymer solution. For instance, Zong et al. demonstrated that the addition of 1% w/w NaCl into poly(D-L lactic acid) solution dissolved in DMF produced electrospun fibers of smallest fiber (210 nm) as compared to fibers prepared from the same polymer solution added with 1% w/w KH₂PO₄ (1000 nm). The reason for this observation was that the addition of ions increased the surface charge density at the pendant droplet causing a reduction of size in beads and fiber diameter; and smaller ions provided a higher surface charge density.29 Su and coworkers reported that the addition of calcium chloride (0.8% w/w) into a chitosan/PEO solution prepared in acetic acid resulted in electrospun fibers that were bead-free. They reported that the added salt...
increased the surface excess charge of the pendant droplet favoring the formation of the polymer jet\textsuperscript{30}.
2.2.2 Extrinsic parameters

2.2.2.1 Relative humidity

Elevated relative humidity has been known to reduce the evaporation rate of water in aqueous polymer solution during electrospinning, thereby delaying the solidification of the fiber, and resulting in thinner fiber than solutions electrospun under lower relative humidity conditions. Porosity in fibers in some polymer solutions increases as relative humidity increases which has been attributed to the water condensates developed on the fibers during the whipping process.\textsuperscript{14} For example, when studying the electrospinning of a polystyrene solution dissolved in tetrahydrofuran, Casper et al. reported that increasing relative humidity of the air from 31-38% to 66-72% increased the fiber pore size from 85 to 135 nm, respectively.\textsuperscript{31} Similarly, Tripatanasuwan et al, reported that the diameter of PEO electrospun fibers decreased from 253 to 144 nm as the relative humidity increased from 4 to 48%.\textsuperscript{32} Due to the importance of relative humidity during the electrospinning, relative humidity should be controlled and monitored during electrospinning investigation. This parameter may be exploited for manipulating the pore size of electrospun fibers.
2.2.2.2 Spinneret-collector distance

The distance from the spinneret to the collector dictates the flight path length of the polymer jet and therefore affects the drying process of the polymer jet. Short distances will limit the stretching and drying of the polymer jet, potentially resulting in wet and/or thick fibers. On the other hand, longer distances decrease the electric field strength between the spinneret and the collector impeding the formation of jet at the spinneret tip. To overcome this difficulty, the voltage applied will have to be increased. In general, the spinneret-collector distance should be optimized for a specific polymer solution to allow the solidification and stretching of the polymer jet, which is necessary to enable the formation of thin and dried fibers\textsuperscript{23}. Doshi and Reneker demonstrated that the diameter of PEO electrospun fibers decreased from 90 $\mu$m to 10 $\mu$m as the distance from the spinneret to the polymer jet increased from 0 to 25 mm\textsuperscript{28}. Ryu et al. reported that distances shorter than 5 cm do not allow the complete evaporation of the solvent formic acid in the polymer system of Nylon 6 in formic acid resulting in wet fibers. On the other hand, distances longer than 5 cm did not facilitate the collection of fibers. As such, the authors reported 5 cm as the optimum spinneret-collector distance for the polymer in study\textsuperscript{33}.

2.2.2.3 Applied voltage

The applied voltage will directly affect the electric field strength. At higher voltage, the electrostatic repulsion force builds faster on the droplet surface of the
polymer solution, thereby ejecting the polymer jet faster, and accelerating the movement of the jet from the spinneret to the collector, impeding solidification and stretching. As such, higher voltage led to the formation of larger fibers with beads. At lower voltage, the electric field strength is weaker causing deacceleration of the polymer jet which increases the time for stretching and solidification. As a consequence, electrospun fibers thin beadless fibers are produced when lower voltages are applied\textsuperscript{14}. Zong et al., investigated the differences in fiber morphology from the electrospinning of poly(D,L-lactic acid) in DMF at different voltages (20, 25, 30 kV). They reported that smaller beadless fibers were produced at 20 kV. At 25 kV, the resulting fibers were larger and the presence of beads was notable. These beads changed their shape from spindle-like to spherical like when the applied voltage was increased to 30 kV\textsuperscript{29}. Doshi and Reneker investigated the optimum voltage to electrospin aqueous PEO solution of different concentrations by studying the formation of the Taylor cone at the spinneret. They concluded that 12 kV were required to enable the formation of fibers from a 4.5% w/w PEO solution\textsuperscript{28}. Deizel et al. found that the amount of beads along PEO electrospun fibers increased when the voltage was raised from 5 to 7 kV, and they observed that the Taylor cone structure at the spinneret tip disappeared when the voltage increased to 7 kV\textsuperscript{34}. These studies show that optimization of the electric force for a given polymer solution is crucial not only to enable the formation of the polymer jet, but also to control fiber morphology.
2.2.2.4 Flow rate

During electrospinning, the control and optimization of the feeding rate are crucial to provide a stable and uninterrupted process. Polymer solution pumped through the spinneret should possess a flow rate that matches the electrospinning throughput that is dependent on the applied voltage. When an excess of polymer solution is fed into the spinneret, the pendant droplet at the spinneret tip will grow faster than the spinning process, and eventually the droplet will drip and/or spatter toward the target. This imbalanced flow will interrupt the spinning process. Conversely, when the flow rate is lower than the spinning process throughput, the Taylor cone will recess within the tip of the spinneret as electrospinning progresses, and eventually the process will stop. The electrospinning process will later resume when sufficient polymer solution has accumulated at the spinneret tip to form the pendant droplet. Zong et al., observed the formation of beads along poly (D,L-lactic acid fibers) when the feeding rate increased from 20 to 75 µl/min.29
2.2.3 Fiber formation in the electrospinning process

Understanding the complex process of electrospinning has inspired the development of mathematical and empirical models. Many of these models explained the formation of fibers during the electrospinning process by two main stages: (i) jet initiation, and (ii) jet stretching and whipping. The following section reviews some of the existing theories on electrospinning.

2.2.3.1 Jet initiation

Gordon Taylor was one of the first investigators who studied the electrospinning phenomena of polymer solutions. Taylor reported that in the presence of an electric field, the electrostatic force causes the pendant droplet of a polymer solution that is hanging at the spinneret tip to deform. When the droplet adapts to the shape of a cone with an angle of $49.3^\circ$, the polymer solution ejects as a jet. At this point, the viscous solution is in equilibrium with the applied electric force. The cone feature is commonly known as the Taylor's cone$^{35}$. 
Decades later, Yarin et al. also calculated the critical angle of the cone for the polymer jet formation. They predicted a theoretical value of 33.5° for viscoelastic fluids, which was comparable to the observed experimental values 30.5 and 37.5 for 6% w/w PEO aqueous solutions.\textsuperscript{36}

Recently Si et al. reported that the angle of the Taylor cone at the pendant droplet depends on the applied voltage for 5% w/w PEO aqueous solutions. At 6 kV, the Taylor cone is formed inside the spinneret while at 4.3 kV the Taylor cone is clearly observed at the tip of the spinneret. They pointed out that in order to form a continuous polymer jet, the Taylor cone structure must be stable.\textsuperscript{37} For clarification, the flow rate was kept constant for this investigation since formation of the Taylor cone at the tip of the spinneret can also be controlled by altering the flow rate while the applied voltage is constant.
2.2.3.2 Jet stretching and whipping

Stretching of the polymer jet during electrospinning is very complex and highly dynamic. Several authors have attempted to model the chaotic whipping process. On the basis of electrohydrodynamics, Hohman et al. modeled the stretching and whipping of polymer jet during electrospinning. They concluded that the stretching and whipping of the jet can be investigated under three types of instabilities: i) axisymmetric Rayleigh instability, which has the lowest field strength; ii) second axisymmetric instability; iii) nonaxisymmetric whipping instability, which has the highest field strength\textsuperscript{38,39}.

To mathematically model the Rayleigh and whipping instabilities, investigators considered the main forces acting on the polymer jet: (i) Coulombic, (ii) electric field, (iii) air drag, (iv) electric field, (v) viscoelastic and (vi) gravitational forces.

Hohman et al. and Reneker et al. developed mathematical and empirical models to predict the axisymmetric and nonaxisymmetric instabilities during the electrospinning process. The model developed by Reneker et al., considered electrical and viscoelastic forces during electrospinning of 6% w/w PEO solution dissolved in water and ethanol (1:1). They observed three distinctive features of the polymer jet: (i) Taylor cone; (ii) the linear portion of polymer; and (iii) the complex whipping instability. The authors pointed out that Rayleigh axisymmetric instability occurs in the absence of the perturbation of the polymer jet mainly because of the relatively larger radius and higher bending stiffness. When the
radius of the polymer jet was smaller, the bending stiffness decreased, thereby forming loops.°

During the nonaxisymmetric whipping instability, the polymer undergoes several important events leading to a decrease in the fiber diameter in this sequence: (i) elongation, (ii) looping, (iii) instability, and (iv) bending. As this cycle repeats, the loops become larger, and the polymer jet becomes thinner. The whipping cycles stops when the polymer jet solidifies as the majority of the solvent is evaporated.°

This phenomenon has been modeled mathematically by Reneker et al, who divided the polymer jet in segments (Figure 4). As shown in Figure 4, segment E moves to E' due to the perturbation during the whipping instability. In addition to the perturbation movement, Coulomb’s forces of segment D and F caused segment E to move even further away from the neighbor segments. When segment E bends, the surface area of this segment increases causing the surface tension to counteract the bending force caused by Coulombic and electric forces. Hence, the bending instability increases and the viscoelastic forces become smaller.°
Figure 4. Forces exerted on segment E of the polymer jet. Segment E moves to E’ because of jet perturbation, and the Coulomb forces from segments D and F. Adapted from Ramakrisha et al. and Reneker et al.\textsuperscript{14,40}
Reneker et al. modeled the trajectory of polymer jet during electrospinning using a viscoelastic Dumbbell-Maxwell model. In their model, beads were used to represent the different segments of the polymer jet (Figure 5). Every bead has mass and energy, and these charged beads are assumed to interact with each other following the Coulomb’s law. The electric field that exists between the spinneret and the collector also exerts a force on the beads. The springs and dashpots represent the viscoelastic resistance of the polymer jet to elongate. These researchers assumed that gravitational and air drag forces were negligible. Their mathematical model described the trajectories of polymer jet, which were determined experimentally using high speed camera.40

![Figure 5. Viscoelastic Dumbbell-Maxwell model to represent the bending instability of the polymer jet during electrospinning: (a) initial bead at the pendant droplet; (b) springs and dashpots to represent the viscoelastic resistance to the jet elongation; (c) final bead on the collector. Adapted from Reneker et al.40.](image-url)
Holman et al. derived complex equations to describe the two modes of instability in the electrospinning for PEO aqueous solution (viscosity 16.7 cm$^2$/s). Their model considered electrical forces assuming negligible air drag and gravitational forces. They also compared theoretical prediction with experimental results. Holman et al. concluded that the model can be applied to relatively low conductivity solutions (<1200 $\mu$S/cm), and the whipping frequency increases linearly with increasing electric field increases but diminishes with increasing square root of viscosity. They also developed operating diagrams to elucidate the effects of flow rate (mL/min) and electric field strength (kV/cm) on the mode of instability of polymer solutions during electrospinning (Figure 6)\textsuperscript{38, 39}. 
Figure 6. Operating diagram to predict the mode of instability: (a) PEO/water solution of low viscosity value (1.67 Poise); (b) PEO/water solution of high viscosity value (167 Poise). Adapted from Hohman et al.\textsuperscript{38}. 
Yarin et al., derived quasi-one dimension partial differential equations to describe the whipping behavior of the polymer jet during electrospinning. They assumed that the radius of the loop or envelope cone during the whipping cone increases as the distance between the spinneret and the collector increases. Using 6% w/w PEO aqueous solution as a model system, they compared their mathematical model with experimental data. The researchers concluded that the model can predict the size of the envelope cone as long as the evaporation and solidification of the polymer system in study are known\textsuperscript{41}.

2.2.3.2.1 Modes of unsteady bending

The bending mode can be steady and unsteady. Steady bending is the most commonly reported phenomenon, where a steady straight jet is ejected from the tip of the Taylor cone. Further away from the spinneret, the polymer jet starts to bend. This model is characterized by a constant linear jet portion and an angle of jet cone envelop. On the other hand, non axisymmetric instability exhibits more complex trajectory as the polymer jet travels towards the collector. The bending instability modes are affected by electrospinning process parameters such as voltage, flow rate, spinneret-collector distance. Four unsteady bending modes have been identified by Si et al.\textsuperscript{37}.
(1) Rotating bending mode:

When electric field increases, the axis that the polymer jet follows rotates away from the linear portion of the polymer jet (Figure 7). In this mode, the position of the electrode attached to the spinneret will also impact the direction of the bending jet since the polymer jet moves away from the electrode.

Figure 7. Illustrations of the rotating bending mode. Adapted from Si et al.\textsuperscript{37}
(2) Swinging bending mode

The polymer jet becomes unsteady and swings horizontally as the electric field decreases while other parameters remain constant. When the electric field is reduced, the electrostatic force of attraction diminishes resulting in the swinging that rotates around the axis of the linear portion of the jet. The formation of a thin uniform polymer jet results from this unsteady bending mode known as “swinging rotating mode” (Figure 8).

Figure 8. Illustrations of the swinging rotating mode. Adapted from Si et al.\textsuperscript{37}
(3) Blurry bending mode

Under certain process conditions, the bending stability is irregular without specific trends, producing a polymer jet that is thin and non-uniform. This is known as 'blurry bending mode' (Figure 9).

Figure 9. Illustration of the blurry bending mode. Adapted from Si et al.37
(3) Branching bending mode

Si et al. reported that the branching mode could occur when the pendant droplet at the spinneret possessed a larger volume. The deformation of the cone caused the ejection of several jets, and the resulting branches separated from each other due to repulsive forces between them. In this mode, jet branches go through the bending instability individually from the spinneret to the collector (Figure 10).

Figure 10. Illustration of three branches of polymer jets during the bending instability at different voltages: (a) two branches at 3.5 kV; (b) three branches at 4 kV; (c) four branches at 4.1 kV. Adapted from Si et al. 37
2.3 Poly(ethylene oxide) (PEO)

Among the polymers that have been investigated in electrospinning research, PEO is the one that has been most well characterized due to its unique properties that endow its ease for electrospinning. Chemically, PEO is similar to poly(ethylene glycol) (PEG) except that PEO has a higher molecular weight. When the molecular weight is lower than 20 kDa, the polymer is commonly known as PEG. PEO is a linear polymer that consists of ethylene and ether segments [-CH$_2$CH$_2$O-]$_n$. The ether oxygen allows this polymer to interact with other hydrophilic species, while the ethylene part participates in hydrophobic interactions. Due to its amphiphilic nature, PEO is soluble in water by forming hydrogen bonding between the oxygen of the PEO ether group and the hydrogen of water molecules$^{42, 43}$. Also, the oxygen-oxygen interdistance on the PEO backbone matches the distance of the oxygen atoms (2.8 Å) in the water molecules, which is believed to be important in making the polymer soluble in water. This theory supports the fact that homologues of PEO such as poly(methyl ethylene) and poly(propyl ethylene) are not water soluble due to the mismatch oxygen-oxygen interdistance with that of water$^{44, 45}$.

Several studies have investigated the unique properties of PEO in water. Most authors agree that hydration of PEO forms a cage that shields the hydrophobic ethylene segments from the hydrophilic environment. Kjellander et al., and Hammouda et al. agreed that two to three molecules of water are required to hydrate a PEO monomer segment$^{42, 45}$. Polverary et al, found that
thermodynamic forces are important in driving the formation of PEO clusters in dilute aqueous solutions but not in chloroform according to dynamic light scattering, static light scattering, and gel permeation chromatography. They concluded that molecular weight and concentration of the polymer are important in the formation of clusters. The formation of clusters was driven by hydrophobic forces between the methyl groups of the PEO chain, and higher levels of polymer concentration had a higher propensity to form clusters. However, Faraone et al. observed that PEO did not aggregate in water for polymers with molecular weights ranging from 8 kDa to 4 000 kDa according to their NMR data, and speculated that the hydrophilic forces of PEO decreased as the molecular weight decreased. Temperature has also been reported to impact the formation of PEO clusters in aqueous solutions. As temperature increases, the entropy of a PEO aqueous solution is also higher, promoting hydrophobic forces between PEO molecules, but reducing interaction between PEO and water. Claeson et al. theorized that the entropic attractive interaction in aqueous PEO solution increases as the temperature increases while the enthalpic repulsive interaction increases as the temperature decreases. Brown et al. stated that short-chain PEO is truly water soluble in the temperature range from 25 to 75°C.

Bekiranov et al. reported that hydrogen bonds between PEO and water break at temperatures close to the boiling point of water, resulting in phase separation. Their data confirmed that at ambient temperatures or increasing the temperature away from the boiling point of the water will result in true PEO solution in water.
Hammouda et al. investigated the clustering behaviours of PEO in water and other organic solvents, specifically studying the effect of polymer chain ends. When both ends of the PEO chain are methyl groups (-CH\textsubscript{3}), more clusters are formed in water solutions. Methyl groups at the end of the chain repel water molecules, and they are attracted to other hydrophobic groups in the system forming a cluster. On the contrary, when both ends of the PEO chain are hydroxyl groups (-OH), they formed hydrogen bonds with water resulting in solubilization. As Figure 11 shows, Branched clusters can be formed when one of the chain end is a methyl group (-CH\textsubscript{3}), and the other is a hydroxyl group (-OH)\textsuperscript{45}.

![Figure 11. Schematic representation of PEO clustering in water due to end chain effect. Adapted from Hammouda et al.\textsuperscript{45}](image-url)
Hammouda et al. also described the effects of chain end on PEO clustering in organic solvents. When both ends of the PEO chain were methyl groups (-OCH₃), PEO can be dissolved completely in benzene. On the other hand, the formation of PEO clusters in benzene resulted when both ends of the PEO chain were hydroxyl groups (-OH) since they repelled benzene, and they were attracted to oxygen in the polymer chain. In methanol, they reported that the effect was negligible since methanol (CH₃-OH) possesses a hydrophobic and hydrophilic group moieties resulting in solubilization of PEO₄⁵.

As discussed in previous sections, many studies have reported successful electrospinning of aqueous solution of PEO, and the polymer has been thoroughly characterized by researchers. Thus, it is ideal as a model polymer to understand the complex electrospinning process for aqueous polymer solutions, especially those prepared from biopolymers. Due to its water solubility, non-toxicity and electrospinnability, PEO has been used as an additive in biopolymer solutions to enable the formation of electrospun fibers. Examples of biopolymer fibers that have been successfully electrospun with the aid of PEO are summarized in Table 1.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Solvent</th>
<th>Intended application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate/PEO</td>
<td>Aqueous</td>
<td>Tissue engineering, and drug delivery</td>
<td>Lu et al.(^{51})</td>
</tr>
<tr>
<td>Keratin/PEO</td>
<td>Aqueous</td>
<td>Filtration: Water purification, and air cleaning</td>
<td>Aluigi et al.(^{52})</td>
</tr>
<tr>
<td>Chitosan/PEO</td>
<td>Acetic Acid</td>
<td>Tissue engineering and drug delivery.</td>
<td>Bhattarai et al.(^{53}) Su et al.(^{30}) Duan et al.(^{54})</td>
</tr>
<tr>
<td>Chitosan/PEO</td>
<td>Aqueous/Acetic Acid</td>
<td>Tissue engineering and drug delivery.</td>
<td>Bhattarai et al.(^{53}) Su et al.(^{30}) Duan et al.(^{54})</td>
</tr>
<tr>
<td>Chitosan/PEO</td>
<td>Methanol/Acetic Acid</td>
<td>Proliferation of cells.</td>
<td>Lou et al.(^{55})</td>
</tr>
<tr>
<td>Alginate/Pectin/PEO</td>
<td>Aqueous</td>
<td>Encapsulation of folic acid for food applications.</td>
<td>Alborzi and Lim.(^{9})</td>
</tr>
<tr>
<td>Silk/PEO</td>
<td>Aqueous</td>
<td>Proliferation of human marrow stroma cells.</td>
<td>Jin et al.(^{56})</td>
</tr>
<tr>
<td>Eggshell protein/PEO</td>
<td>Aqueous</td>
<td>Wound dressing and cosmetic applications.</td>
<td>Kang et al.(^{57})</td>
</tr>
<tr>
<td>Microalga spirulina/PEO</td>
<td>Aqueous</td>
<td>Tissue engineering to treat spinal cord injury</td>
<td>Morais et al.(^{58})</td>
</tr>
<tr>
<td>Soy protein/PEO</td>
<td>Aqueous</td>
<td>Encapsulation of ally isothiocyanate for food packaging applications.</td>
<td>Vega-Lugo and Lim.(^{59})</td>
</tr>
</tbody>
</table>
2.4 Whey protein isolate (WPI)

During cheese making, caseins in milk are precipitated by using acid or rennin enzyme. The resulting liquid byproduct, known as cheese whey, contains lactose, whey proteins, minerals and organic acids. The production of 1 kg of cheese results in 9 kg of whey (6-7% dry matter); thus, large quantities of protein byproduct are created, the discharge of which can cause environmental problems due to its high levels of chemical and biochemical demands. Since whey proteins are highly functional, many studies have been attempted to utilize this industrial by products for various applications.

Whey protein can be isolated by membrane filtration methods, and an ionic exchange tower can be used to separate components by ionic charge. WPI is rich in \( \beta \)-lactoglobulin (3.2 g/L, MW 18 kDa/mol), \( \alpha \)-lactalbumin (1.2 g/L, MW 14 kDa/mol), immunoglobulin (0.7 g/L MW 155 kDa/mol) and serum albumin (0.4 g/L, MW 66 kDa/mol) which are globular proteins.

Whey proteins can be separated according to their physical properties by selective precipitation, membrane filtration, selective adsorption and selective elution. Selective precipitation consists of separating proteins according to their isoelectric point (Table 2), a pH at which the proteins are insoluble and tend to aggregate. Membrane filtration separates proteins by molecular mass, requiring that the proteins should have significantly different molecular mass (at least by a factor of 10). It is important to note that differences in ionic strength can be used to separate proteins with similar molecular mass. For selection adsorption, an
immobilized phase retains specific proteins. The final outcome consists in a pure protein and a solution depleted of that protein. In the selective elution method, whey proteins are retained simultaneously onto the immobilized phase and eluted at different times for their separation\textsuperscript{64}.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (kg/mol)</th>
<th>Concentration (g/L)</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactoglobulin</td>
<td>18</td>
<td>3.2</td>
<td>5.4</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>14</td>
<td>1.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>66</td>
<td>0.4</td>
<td>5.1</td>
</tr>
<tr>
<td>Immunoglobulin G</td>
<td>150</td>
<td>0.7</td>
<td>5-8</td>
</tr>
</tbody>
</table>

When WPI proteins are denatured, non-covalent interactions and disulfide bonds are disrupted, thereby their tertiary structure\textsuperscript{65}. Compared to other dairy proteins, whey proteins are richer in lysine and sulfur aminoacids\textsuperscript{60}. Specifically, β-lactoglobulin is made of 162 aminoacids (pI 5.4) with two disulfide bonds and a free thiol. By contrast, α-lactalbumin consists of 123 aminoacids (pI 4.4), and it has four disulfide bonds\textsuperscript{66}.

Casting of whey protein films is one of the most extensively studied edible film materials. Through thermal denaturation in water followed by casting and drying, standalone films of robust mechanical and oxygen barrier properties can be produced\textsuperscript{67, 68}. To enhance the material properties of the WPI films, various active components have been incorporated into the film-forming solution including lysozyme, lactoferrin and the lactoperoxidase system to produce antimicrobial films against the growth of \textit{Salmonella enterica} and \textit{Escherichia coli} O157:H7 in smoked salmon\textsuperscript{69}. In another study, the antibacterial activities of whey films incorporated with nisin, malic acid, EDTA and grape seed oil were
evaluated on a turkey frankfurter showing that the films could inhibit the growth of
Listeria monocytogenes, Salmonella typhimurium and E. coli 0157:H7\textsuperscript{70}. Besides
antimicrobial agents, antioxidants such as \( \alpha \)-tocopherol, ascorbyl palmitate, and
ascorbic acid have also been tested in WPI coating to inhibit rancidity in food \textsuperscript{71, 72}. One study showed that coating plums with whey protein film incorporated with
flaxseed oil improved the organoleptic characteristics of the fruit during storage\textsuperscript{73}.

From the above literature review, it is clear that whey protein can be
converted into coherent film structures through various denaturation treatments.
Conceivably, similar film-forming methodologies can be applied to prepare whey
proteins fiber-making solutions suitable for electrospinning, an area that has not
been reported before in the literature.
Chapter 3. Problem Statement and Objectives

3.1 Problem statement

Electrospinning of biopolymers has been motivated not only by the ultrafine diameter of the fibers, but also by their biodegradability and biocompatibility. These properties create some potential novel applications as bioactive carriers in nutraceutical, biomedical and food-related areas.

Unfortunately many studies have shown that biopolymers of low molecular weight and high water solubility are not able to form electrospun fibers in the absence of PEO. Moreover, proteins that adopted compact and globular configurations cannot be electrospun into fibers due to the lack of chain entanglement that is important for stabilization during electrospinning. Similar to other proteins, preliminary studies showed that WPI solution prepared in an aqueous medium could not be electrospun (data not shown). However, the addition of PEO in the polymer solution could effectively overcome this hurdle.

While PEO is instrumental for enabling the electrospinning of biopolymers, it is desirable to develop methodology to spin these polymers without using PEO, since the presence of PEO may not be ideal for certain applications. Understanding the mechanism by which PEO enables the electrospinning of biopolymers could contribute to achieve the long-term goal of producing electrospun biopolymer fibers without PEO by eliminating or replacing this synthetic polymer with a food grade additive. Although the positive effect of PEO
on electrospinning has been widely reported, the mechanism by which PEO enables the formation of electrospun biopolymer fibers remains unclear.

### 3.2 Overall objectives

In order to understand the role of PEO during the electrospinning of aqueous biopolymer solution, systematic investigations are needed and currently lacking in the literature. In this study, whey protein was chosen as a model polymer for the investigation to elucidate the positive effect of PEO on electrospinning by using advanced analytical techniques such as Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared Spectroscopy (FTIR), Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF), Scanning Electron Microscopy (SEM), and Scanning Transmission Electron Spectroscopy (STEM)- Energy Disperse X-Rays Analysis (EDAX) to probe the effects of PEO during the formation of WPI/PEO electrospun fibers. The main objective of this study was to develop the fundamental understanding on the mechanism by which PEO promotes the formation of fibers during the electrospinning of WPI/PEO fibers.
Chapter 4. Electrospinning of WPI Fibers

4.1 Introduction

WPI is composed of mainly β-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa), which are globular proteins of relatively low molecular weight. Accordingly, these proteins, in their native state, are expected not to be optimal to electrospin into fiber. Denaturation of the proteins and manipulation of extrinsic and intrinsic electrospinning parameters are expected to be necessary in order to spin the proteins.

In preliminary investigations, attempts were made on electrospinning WPI alone by manipulating solution properties and altering protein configuration through using different solvents (water, acetic acid, formic acid, and ethanol), changing WPI concentrations (0.5 to 15%), modifying pH (1-13), and experimenting with different solution preparation methods. Unfortunately, none of the experimental conditions tested were conducive for fiber formation.
Several studies have successfully electrospun polymers dissolved in organic solvents instead of an aqueous medium. The electrospinning of aqueous biopolymer systems has been reported in literature to be unsuccessful. Many biopolymers can be electrospun readily when dissolved in organic solvents such as 1,1,1,3,3,3-hexafluoro-2-propanol and 2,2,2-trifluoroethanol. Unfortunately, these solvents are toxic, and therefore their use in food applications is restricted.

To overcome this hurdle, many studies have adopted non-toxic aqueous based solvents, in conjunction with the addition of a spinning aid polymer, such as poly(ethylene oxide) (PEO) to enable the electrospinning process. For example, chitosan, keratin, alginate, chitosan/alginate, eggshell membrane, and soy protein fibers have been successfully electrospun from aqueous solutions when PEO is added in the polymer solution. In the electrospinning of soy protein and alginate/pectin fibers, it was reported that the addition of PEO decreased the electrical conductivity of the polymer solution. This effect has been speculated to be caused by the charge counteracting effect of PEO upon the biopolymers that are polyelectrolytic. The positive effect of PEO has been also attributed to its chain entanglement with the biopolymer molecules. Nie et al., reported that molecular weight of PEO should be high enough (~900 kDa) in order to promote the required polymer chain entanglement for stabilizing the polymer jet during the electrospinning of the alginate/PEO blend. Although these are the explanations often cited for explaining the positive effect of PEO, the fundamental reasons remain obscure.
The investigation of the electrospinning of WPI and PEO may lead further understanding of the role PEO has on the electrospinning of biopolymer. In this study, it is hypothesized that the addition of PEO will favour the electrospinning of WPI. The objectives are: (i) to determine viscosity, surface tension and conductivity of polymer solutions of different pH in the presence and absence of PEO; (ii) to electrospin WPI fibers from these polymer solutions; and (iii) to correlate the solution properties with fiber morphology.
4.2 Materials

WPI (protein content >90%) was donated by Glanbia Nutritionals, Inc. (Evanston, IL), PEO (Mw 900 kDa), sodium hydroxide, and glacial acetic acid were purchased from Sigma Aldrich Company Canada (Oakville, ON).

4.3 Methods

4.3.1 Solution preparation

Material concentrations of all the components in the polymer solutions are given in w/w basis. PEO aqueous solutions (4%) were prepared, and added to distilled water, sodium hydroxide aqueous solution (1%) and glacial acetic acid were used as solvents to give 0.4% PEO solution of pH of 7, 12, and 1 respectively. WPI (10%) was then added slowly to the PEO solutions and stirred with the aid of a magnetic stirrer. The neutral and alkaline polymer solutions were stirred for 15 min, while the acidic polymer solution was stirred for 12 h to achieve complete dissolution of WPI. The final concentrations of WPI and PEO in the resulting final polymer solutions were 10 and 0.4%, respectively. The ratio between the WPI and PEO content in the resulting electrospun fibers was 96:4. Polymer solutions without PEO were prepared similarly except that the addition of PEO was omitted. Polymer solutions were prepared at room temperature (22 ± 2°C).
4.3.2 Solution viscosity

A shear capillary rheometer mounted on an Instron Universal Testing Machine (Model 1122, Instron, Norwood, MA) was used to determine viscosity of the polymer solutions. The area of the plunger was 5.3 cm$^2$. The length of the capillary was 15 cm and the inner diameter was 1.5 mm. Protein solutions (50 ml) were introduced into the barrel and shear at four crosshead velocities (200, 300, 400, 500 mm/min) were applied to determine solution viscosity at different shear rates. All tests were run at room temperature (22 ± 2°C). Viscosity values were calculated from pressure drop (related to shear stress) and flow rate (related to shear rate), considering the polymer solution as non-Newtonian fluids$^{11, 80, 81}$.

4.3.3 Solution pH, conductivity and surface tension

Electrical conductivity and pH of the polymer solution were determined by using a pH/conductivity meter (Accumet XL20, Fisher Scientific, ON). Surface tension of polymer solution was measured by using a bubble tensiometer (SITA pro line f10, SITA Messtechnik, Dresden, OH), which was based on the maximum bubble pressure method. All tests were conducted at room temperature (22 ± 2°C).
4.3.4 Electrospinning

WPI solutions were electrospun using a vertical electrospinning setup. The polymer solution was delivered to the spinneret at 0.4 ml/h using an infusion pump (Model KD200, KD Scientific, Holliston, MA). The spinneret was connected to the positive electrode, while the stainless steel collector plate was grounded. A constant voltage ranging from +15 to +25 kV was applied to the positive electrode using a DC supply (Model ES30R-5W/DM, Gamma High Voltage Research, Ormand, Beach, FL). The distance between the spinneret and the collector was set at 26 cm. For the investigation of the spinneret-collector distance effects, the distance was set from 10 to 25 cm, and the applied voltage was set at 25 kV. Electrospinning was conducted under ambient conditions (22 ± 2°C; 15 ± 5% RH).

4.3.5 Scanning Electron Microscopy (SEM) Analysis

Morphology of the electrospun fiber was examined using a scanning electron microscope (SEM S-570, Hitachi High Technologies Corporation, Tokyo, JP) at an accelerating voltage of 10 kV. Samples were coated with gold/palladium (15 nm) using a sputter coater (Model K550, Emitech, Ashford, Kent, England). The average fiber diameter was determined by taking at least 50 measurements for each image using Image analysis software (Pro-Plus 5.1, Media Cybernetics Inc., Bethesda, MA)
4.3.6 Data analysis

Quantitative analysis of solution properties (viscosity, conductivity and surface tension) was conducted in triplicate. Statistical analyses of the solution properties at different pH were performed using one way ANOVA, Tukey’s test, and paired comparison t-test ($\alpha = 0.05$), using SPSS 17 statistical software (SPSS Inc., Chicago, IL).

4.4 Results and discussion

4.4.1 Fiber morphology

In the absence of PEO, WPI polymer solutions could not be electrospun under all three pH conditions investigated. At the spinneret, the pendant droplet for these solutions tended to elongate and wiggle as the voltage increased, and eventually detached from the spinneret and spattered towards the collector as large droplets when the voltage was increased to $\sim$30 kV. Although pure 10% WPI solutions could not be electrospun, pure 0.4% PEO solutions could be electrospun at different pH levels (Figure 12). On the other hand, the blend of 0.4% PEO and 10% WPI solution could form a polymer jet at approximately 15, 20, and 25 kV for the acidic, alkaline, and neutral solutions, respectively. Scanning electron micrographs showed that WPI/PEO fibers electrospun from the acidic solutions were bead-free with an average diameter of $705 \pm 105$ nm (Figure 13a), while those produced from alkaline solutions had smaller diameter ($191 \pm 38$ nm) with spindle-like beads (Figure 13c). On the other hand, the
neutral WPI/PEO solutions produced mainly spherical particles linked to ultrafine fibers (138 ± 32 nm) (Figure 13b). The observed differences in morphology might be correlated to changes in solution viscosity, surface tension and conductivity. The effects of these solution parameters on fiber morphology are discussed in the following sections.

Figure 12. Scanning electron micrographs of electrospun fibers from 0.4% PEO solutions at different pH: (a) pH 1; (b) pH 7; (c) pH 12. Top row: low magnification; bottom row: high magnification.
4.4.2 Viscosity

Viscosity values of the polymer solutions, together with the solvents used for preparing these solutions are summarized in Figure 14. The addition of trace amounts of PEO (0.4% w/w) to the pure solvents resulted in a small increase in viscosity for the acidic polymer solution, but did not induce significant change in viscosity for the alkaline and neutral solutions. However, when WPI was added, considerable increases in viscosity were observed for all polymer systems, especially for the acidic and alkaline polymer solutions. This observation could be caused by the changes in net charge on the WPI molecules when exposed to
different pH conditions. Since whey proteins have an average isoelectric point of ~ pH 5.2\textsuperscript{82}, WPI will carry a net positive charge at pH 1, but negative charge at pH 7 and 12. The lone pair of electrons on the ether oxygen of PEO might have interacted with the net positively charged protein under acidic conditions, resulting in the greater viscosity in comparison to the neutral and alkaline solutions when PEO was added.

![Figure 14. Viscosity values for various WPI solutions in the presence and absence of PEO as a function of shear rate](image)

Another factor that contributed to the different material morphologies observed in Figure 13 is protein configuration. In neutral solution, WPI adopted globular conformation, limiting its interaction with the PEO macromolecules. However, in acidic and alkaline environments, the proteins are more unfolded, allowing the entanglement/entrapment/deposition of WPI with PEO. WPI might have had interacted with PEO by entrapment/deposition of WPI in PEO or by
chain entanglement between the polymers. As shown in Figure 14, when PEO was added to the WPI acidic and alkaline solutions, the magnitude of increase in viscosity was significantly larger as compared to the neutral solution (p<0.05). The changes in viscosity for PEO solutions (in the absence of WPI) were minimal under the different pH conditions tested. The smooth WPI/PEO fibers produced under acidic conditions can be correlated with the high viscosity for the fiber-forming solution, which favored polymer chain entanglement/entrapment/deposition by stabilizing the polymer jet during electrospinning. Similar results have been reported for polyamide 6 dissolved in formic acid-dichloromethane blend\(^83\).

The neutral WPI/PEO polymer solutions had the lowest viscosity value in comparison to the other treatments. The low viscosity of the neutral solution favored the capillary breakup of the polymer jet to form small droplets (i.e., electrospraying; Figure 13b) due to the dominant surface tension effect of the solvent. The ultrafine fibers observed in Figure 13b could represent the PEO-rich domain while the spherical beads are likely the protein rich fractions that lack polymer chain-chain interaction. Following on a similar reasoning, the transformation of spherical beads to elongated spindles (Figure 13) as the pH increased from 7 to 12 solutions could be due to the increased WPI-PEO polymer chain entanglement that stabilized the polymer jet, counteracting the surface tension effect that tends to promote capillary breakup.

A number of theories have been put forward to explain the electrospinning behavior of biopolymers. Many have correlated the jet stabilization during electrospinning with complex rheological properties of polymer solutions\(^84,85\). The
elasticity of an aqueous solution of high molecular weight PEO has been attributed to its linear and flexible chain structure that is conducive for the formation of uniform fibers during electrospinning. On the other hand, the conformation of globular proteins (e.g., WPI, bovine serum albumen (BSA)) can change depending on the solvent environment in which they are dispersed (e.g., pH, temperature, presence of denaturant). Conditions that induce the unfolding of the globular protein will favor the formation of fibers due to the enhanced elasticity of the polymer jet during the electrospinning process, as a result of increased polypeptide chain entanglement and chain-chain interaction. For instance, Dror et al. observed that a low viscosity solution of globular BSA protein, prepared in 9:1 trifluoroethanol (TFE):water solution, can be modified into a spinnable polymer solution using β-mercaptoethanol and pH conditions away from the isoelectric point (pH 5.4) of the protein. Both the extreme pH’s and denaturant are advantageous for the unfolding of the native globular protein. This is consistent with the findings of this investigation, which show that electrospun fibers could be produced when WPI solutions were far away from the isoelectric point. However, unlike the findings of this investigation, Dror et al. were able to spin the protein solution without the addition of the second polymer. Besides disrupting the hydrophobic core of the globular protein and causing the exposure of the hydrophobic segments of the polypeptides to the solvent, the TFE solvent employed by Dror et al. and Regev et al. was also less conductive than the aqueous solvent used in the present study. Thus, although unfolding of globular protein is beneficial in promoting of the formation of fiber, adequate build
up of excess surface charge on the polymer solution is also critical to initiate the jetting phenomenon, and to generate adequate repulsion force along the fiber to stretch the polymer jet into thin fiber.

**4.4.3 Surface tension**

Surface tension is another important factor that affects the electrospinning behavior of a polymer solution. The higher the surface tension, the greater the required excess electrostatic charge to initiate jetting of the polymer solution from the spinneret. Considering that electrospinning is a dynamic process, in this study, surface tension was determined using the dynamic bubble pressure method instead of the typical static methods (e.g., Du Nouy ring, Whilhelmy plate). In bubble pressure tensiometry, air is pumped through a capillary tube of which the tip is submerged in the test liquid. The size of the bubble increases as the air is forced into the solution. Eventually the bubble will dislodge from the capillary tip and rise to the surface of the solution. During the bubble formation process, the air pressure increases as the bubble is inflated in the test liquid. When the bubble reaches a hemispherical shape with a diameter equals to the diameter of the capillary tip, the maximum pressure is reached. The maximum pressure can be converted to surface tension value according to Laplace equation for spherical bubble in a liquid\(^{88, 89}\). With this method, bubbles can be produced at different frequencies, which can also be expressed as bubble lifetime, defined as the time interval from the formation of the meniscus to the detachment of the bubble from the capillary tube, i.e., time taken for completing one bubbling cycle\(^{90, 91}\).
Figure 15. Surface tension values as affected by bubble lifetimes of polymer solutions and their respective solvent at different pH conditions. The concentrations of PEO, WPI and WPI:PEO were 0.4, 10 and 0.4:10% (w/w), respectively.

Surface tension values of WPI, PEO, and WPI-PEO solutions prepared using acidic, neutral and alkaline solvents are summarized in Figure 15. At long bubble lifetimes (>800 ms), the surface tension values for the neutral and alkaline polymer solutions were lower than their respective solvents, showing that WPI behaved as an surface-active agent in these solutions. In comparison, the surface tension values for the acidic polymer solution were higher than the pure acidic solvent. The higher cohesive force in the acidic solution could be related to the adsorption of unfolded and entangled macromolecules at the surface that strengthen the newly formed bubble surface. Overall, the surface tension values for all polymer solutions increased as the bubble lifetime decreased, i.e., when the rate of dilatational deformation on the bubble surface increased. Depending on the adsorption kinetics of the polymer molecules and how fast the
macromolecules rearrange themselves onto the newly formed bubble surface, the solution exhibit different surface tension properties.

At the trace amount added (0.4% w/w), PEO alone exhibited a surfactant-like behavior in the aqueous neutral and alkaline solution. Here, minimal changes in the surface tension values were observed as a function of bubble lifetime, indicating the PEO chains were able to adsorb and rearrange themselves readily at the air-liquid interface as the bubbles were formed. On the other hand, neutral and alkaline WPI solutions (10% w/w) displayed strong time-dependent behavior; although their surface tension values were significantly lower than the solvent when the bubble lifetime was long, as the bubbling rate increased, the surface tension increased and converged to that of the pure solvent. This suggested that the adsorption/rearrangement of WPI molecules to the newly formed bubble surface was slower than that of PEO chains, possibly due to the more flexible backbone of the latter.

The addition of PEO resulted in an increase in surface tension for all WPI solutions tested, indicating that PEO had an effect on increasing the cohesive energy of the polymer layer adsorbed at the air-liquid bubble interface. This effect could be attributed to the introduction of high molecular weight and flexible PEO macromolecules to the air-liquid interface that strengthened the adsorbed layer through polypeptide-PEO chain interaction. The information presented in Figure 15 can be used to elucidate the dynamic changes in surface tension during an electrospinning process. Before the formation of a polymer jet, the surface tension of the pendant droplet is in equilibrium, which can be approximated from
long bubble lifetime asymptote of dynamic tensiometry measurement. As the polymer jet started to form, the rapid deformation of the Taylor cone will result in continuous creation of new surface, of which the surface tension can be determined from the plot when the bubble lifetime is short. These phenomena can be used to explain the different WPI morphologies observed in Figure 13 considering the surface tension profile for the neutral WPI/PEO solution. Here, reducing the bubble lifetime resulted in the convergence of surface tension values for the WPI/PEO solution towards those of the pure solvent. This observation suggests that the diffusivity of polymer molecules to the interface and/or conformational change of the polymer chains at the surface were not fast enough to reduce the surface cohesive force of the solvent. Consequently, although the equilibrium surface tension at long bubble lifetime was significantly lower than the pure solvent (which should favor the formation of the jet), the rapid increase of surface tension as the solution started to stretch, coupled with the low viscosity of the solution Figure 14, will cause the solution to break into droplets due to the development of Rayleigh instability. This may explain why spheres were predominantly observed when the neutral WPI solution was electrospun (Figure 13b).

From Figure 15, it can be seen that the surface tension profiles for the acidic WPI solution were markedly different from the neutral and alkaline WPI solutions. The addition of polymers to the acidic solution resulted in higher surface cohesive force than the pure solvent throughout the range of bubble lifetimes investigated. On the basis of classical Stokes-Einstein theory on the
diffusion of solute in solution, the diffusion of WPI macromolecules at pH 7 should be faster than those at pH 1, considering that the protein was more compact and the solution was less viscous in the former. This may explain why surface tension values of the neutral solution were less sensitive to changes in bubbling rate than those of the acidic solution, since the protein molecules can migrate and adhere to the surface quicker in the former. The increasing surface tension values under short bubble lifetime for the acidic solution could be related to the viscous resistance provided by the polymer chains acting against the growing bubble due to the high viscosity values for the acidic solution (Figure 14). Manglik et al. reported similar observations on a viscous polyacrylic acid solution, of which the surface tension values determined from dynamic measurements were strongly affected by the viscosity of the polymer solution.\(^{92}\)

In general, low surface tension is desirable for electrospinning as this reduces the required electric field strength to initiate the formation of a polymer jet.\(^{14, 25, 93}\) In the present study, although the addition of PEO has resulted in increased surface tension of the WPI solutions, the fact that the resulting WPI/PEO solutions could still be electrospun suggested that surface tension was not the only determining factor that dictated the electrospinnability of the WPI solutions. Rather, both surface tension and viscosity were important in affecting the electrospinning behavior of WPI solutions. For instance, the combined low surface tension and high viscosity of the acidic polymer solution can be correlated to the smooth fibers (Figure 13a). Although the alkaline solution had a higher surface tension value than the neutral solution, the fact that the alkaline solution produced fibers while the neutral counterpart resulted in spheres linked
to ultrafine fibers implied that surface tension was not the limiting factor in determining the morphology of the electrospun/electrosprayed materials. The observation suggested that chain entanglement, which favored the formation of fibers, was likely the dominating factor. The formation of viscoelastic air-liquid interface during the electrospinning process is likely important for stabilization of polymer jet, as proposed by Regev et al. 87.

4.4.4 Conductivity

Electrical conductivity determines the ability of charge to move to the surface of the pendant droplet, directly affecting the buildup of electrostatic repulsion force that is critical to initiate jetting. As shown in Table 3, the addition of PEO to WPI solution did not alter significantly the conductivity value (p>0.05) for all the pH conditions tested. Overall, polymer solutions prepared in acetic acid had the lowest conductivity values, while the alkaline solutions had the highest due to the presence of large amount of Na\(^+\) and OH\(^-\) ions. The high conductivity is desirable since it increases the repulsion charges on the pendant droplet at the tip of the spinneret. The higher conductivity also caused greater transfer of surface charge to the spinning polymer jet, resulting in greater electrostatic repulsion force responsible for inducing bending instability and stretching critical for forming submicron fibers. This is consistent with the observation that fibers spun from the alkaline solution had a smaller diameter when compared to the acidic polymer solution. Other studies also reported positive effects of added ions to fiber-forming solutions. For instance, Marsano and co-workers reported that the addition of LiCl to polyamide 6 dissolved in TEF increased the conductivity of
the polymer solution, producing bead-free electrospun fibers. In this study, it is noteworthy that at pH 1, the conductivity value was substantially lower in comparison to the neutral and the alkaline solutions, but yet, continuous fibers could be electrospun. This observation implies that conductivity was not the main contributor of jet formation for the acidic polymer solution. Here, the reduced surface tension and high viscosity probably dominated the low conductivity effect, as discussed in the previous section.

Table 3. Conductivity of various polymer solutions

<table>
<thead>
<tr>
<th>pH</th>
<th>Polymer</th>
<th>Conductivity (mS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>None</td>
<td>47.180 ± 0.190</td>
</tr>
<tr>
<td>12</td>
<td>PEO</td>
<td>43.170 ± 0.710</td>
</tr>
<tr>
<td>12</td>
<td>WPI</td>
<td>10.920 ± 0.150</td>
</tr>
<tr>
<td>12</td>
<td>WPI/PEO</td>
<td>9.640 ± 0.090</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>7</td>
<td>PEO</td>
<td>0.021 ± 0.001</td>
</tr>
<tr>
<td>7</td>
<td>WPI</td>
<td>1.440 ± 0.020</td>
</tr>
<tr>
<td>7</td>
<td>WPI/PEO</td>
<td>1.300 ± 0.010</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>1</td>
<td>PEO</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>1</td>
<td>WPI</td>
<td>0.170 ± 0.010</td>
</tr>
<tr>
<td>1</td>
<td>WPI/PEO</td>
<td>0.490 ± 0.008</td>
</tr>
</tbody>
</table>
4.4.5 Spinneret-collector distance effects

As the polymer jet travels from the spinneret to collector it undergoes axisymmetric and non axisymmetric instabilities. As the fiber stretches, the fiber diameter decreases, and finally solidifies when the solvent is evaporated. Accordingly, when the spinneret-collector distance is too short, non axisymmetric bending instability is impeded resulting in the deposition of wet fibers on the collector. On the other hand, when the distance increases, the “window” for non axisymmetric bending instability increases, which has an effect of promoting stretching and evaporation of the solvent to produce thinner fibers. However, while keeping the applied voltage constant, increasing the distance will also weaken the electric field between the spinneret and the collector, thereby decreasing the electrostatic force acting on the pendant droplet. Thus, the voltage will need to be increased proportionally to maintain the same electric field strength. In this section, the effect of spinneret-collector distances on the fiber morphology while the rest of the electrospinning parameters remained constant was investigated.

At pH 7, the polymer solution tended to electrospray, forming spherical beads, rather than fibers, especially when the spinneret-collector distance was short (Figure 16). At large distance, the presence of fibers was more evident than at short distance. Moreover, the area of deposition of the electrospun/electrosprayed materials varied according to the spinneret-collector distances. At 10 cm, the area of coverage deposited by the polymer deposition was much smaller (2 cm diameter) while the area of deposition for a distance of
25 cm was greater than 20 cm (data not shown). Figure 16 shows the trend from high fiber deposition concentration (10 cm) to low fiber deposition concentration (25 cm). At short distance (10 cm), the small flight length limited the whipping motion of the polymer jet, preventing the formation of fiber. The polymer jet broke into beads due likely to poor solution properties of the polymer system, i.e. relatively low viscosity and high surface tension.

Figure 16. Scanning electron micrographs of electrospun fibers from the electrospinning of 10% WPI and 0.4% blend at pH 7 under various spinneret-collector distances: (a) 10 cm; (b) 15 cm; (c) 20 cm; (d) 25 cm. Top row: low magnification; bottom row: high magnification
Figure 17. Scanning electron micrographs of electrospun fibers from the electrospinning of 10% WPI and 0.4% blend at pH 1 under various spinneret-collector distances: (a) 10 cm; (b) 15 cm; (c) 20 cm; (d) 25 cm. Top row: low magnification; bottom magnification: high magnification.
For the acidic polymer solution, at the shortest distance (10 cm), wet fibers were deposited on the collector causing the fibers to fuse together (Figure 17). A drastic transformation in morphology occurred when the spinneret-collector distance increased from 10 to 15 cm, where individual fibers overlaid on each other were observed. This shows that a minimum of approximately 15 cm is needed for the present experimental condition, to provide long enough flight for adequate evaporation of solvent from the polymer jet. At 15 cm distance, irregular clusters embedded between the fibers were detected. At 20 and 25 cm, electrospun materials obtained had similar morphology and appeared to be smooth and dried (Figure 17 c and d) meaning that the distance between the spinneret and the collector is optimum to allow the axisymmetric and non axisymmetric instabilities stretching and solidification of the polymer jet. It is speculated that the observed clusters were rich in WPI, and changes in the spinneret-collector distance affected the entrapment/deposition of WPI along the PEO jet during the bending instability causing changes in fiber morphology. When the spinneret-collector distance was optimal (20-25 cm) resulting in the formation of smooth fibers.
In the case of the alkaline solution, at 10 cm, a heterogeneous mixture of irregular cluster, beads and spheres linked with short fibers was observed (Figure 18). It is speculated that the irregular structures, beads and spheres are composed of WPI. At 15 cm, the irregular clusters disappeared and transformed into beads attached to fibers, with occasional presence of spheres. Fibers electrospun at 20 and 25 cm have similar morphology. Both samples showed individual fibers carrying elongated beads along the fibers, and there was no evidence of fused junctions, indicating that 20 and 25 cm allowed the necessary
bending instability for stretching, and evaporation of solvent to produce solid fibers. Changes in the spinneret-collector distances could have affected the incorporation of WPI in PEO, if the protein incorporation occurred during the bending instability resulting in fiber morphology differences. Although the distance of 20-25 cm was optimal for achieving the required bending instability, the formation of smooth fibers was not attainable due to the influence of solution properties and protein configuration for WPI/PEO alkaline solutions, which will be the subject of investigation in the next chapter.
4.5 Conclusion

This study showed that PEO is essential for the electrospinning of WPI fibers, and its presence affected the solution properties of WPI solutions. The addition of PEO increased the viscosity value implying that probably chain entanglement has occurred between WPI and PEO. Also, entrapment/deposition of WPI along the PEO fiber jet could have occurred. WPI/PEO Acidic polymer solutions possessed the relatively highest value of viscosity while WPI/PEO neutral polymer solutions possessed the relatively lowest value of viscosity. Increases in viscosity indicated that WPI and PEO entangled closer under acidic conditions in comparison to alkaline and neutral system. Chain entanglement between WPI and PEO probably enabled the elongation of the polymer jet during electrospinning for the formation of smooth fibers. Although the acidic polymer solution possessed the lowest value of conductivity, viscosity and surface tension overode the low conductivity properties and the formation of smooth fibers was evident. Differences in the surface tension values of the polymers confirmed that lower values of surface tension favored the electrospinning of WPI. The advantageous low surface tension of the acidic polymer solutions facilitated the formation of beadless fibers. By contrast, WPI/PEO neutral polymer solution possessed the highest values of surface tension, and the lowest values of viscosity resulting in the formation of spheres linked to smaller ultrafine fibers was evident. In addition to the solution properties and protein configuration, the effects of bending instability, which can be controlled by manipulating the spinneret-
collector distance, was also related to chain entanglement/entrapment/deposition. Overall, bead-free fibers were correlated to relatively higher values of viscosity, lower values of surface tension, and optimum spinneret-collector distance. These factors had a positive influence on the chain entanglement between WPI and PEO. It is assumed that differences in protein configuration due to changes in pH, also contributed to differences in solution properties and interfered with the chain entanglement between WPI and PEO. To confirm this assumption, the investigation of WPI configuration, and interactions between WPI and PEO under different polymer system is discussed in Chapter 5.
Chapter 5. WPI Characterization and WPI-PEO Interaction in Electrospun Fibers

5.1 Introduction

Results from Chapter 4 showed that PEO could facilitate the formation of electrospun WPI/PEO fibers under the pH and experimental conditions investigated. The addition of trace amount of PEO (0.4%) not only allowed the formation of fibers but also caused changes in solution properties. Notably, the addition of PEO to acidic WPI solutions resulted in considerable increases in viscosity, which could be the main contributor to the formation of smooth fibers. It is speculated that the acidic conditions facilitated the interaction between WPI and PEO, possibly through chain entanglement between the polymers. Another possibility is that WPI was entrapped along the PEO fiber jet. These effects might have endowed the polymer solution with an optimal viscoelasticity important for stabilizing the polymer jet as it takes flight in the air, preventing the jet from breaking up into droplets. Similar conclusions were reported by Fong et al. and Wei et al. in their investigation of the electrospinning of aqueous PEO solutions and polyamide 6 formic acid/dichloromethane solutions respectively. In both cases, increases in viscosity of the polymer solution facilitated the formation of beadless fibers\textsuperscript{27, 83}.

Results from Chapter 4 also led us to hypothesize that changes in pH could alter the protein configuration which in turn affects the chain entanglement
with PEO. This assumption was based on the fact that smooth fibers were obtained when WPI was exposed to extreme pH conditions while the presence of spheres linked with ultrafine fibers was observed when neutral WPI solutions were used. It is well known, as it has been shown that pH far away from the isoelectric point can promote the denaturation of WPI, causing the protein to unfold, which in theory should favor its chain entanglement with PEO. Unfolding of the protein has been correlated with the promotion of intermolecular interactions in films and emulsions. For example, Quinn et al. stated that exposure of whey protein to extreme alkaline conditions favored intermolecular interaction for the formation of whey protein film. Demetriades et al. also reported that pH values away from the isoelectric point favored the whey protein stabilization of emulsions. They explained that whey protein is pH sensitive which could have an impact on intermolecular interactions, and consequently emulsion stability. As such, the effects of protein denaturation on electrospinnability and fiber morphology will be investigated.

Although it is clear from the results of the previous chapter that the addition of PEO enabled the electrospinning of WPI, the underlying mechanisms for this effect still remains obscure. A study of the interactions between WPI and PEO will contribute to the understanding on how PEO enhances the electrospinning of WPI fibers. In this chapter, it is hypothesized that the positive effect of PEO on electrospinning of WPI can be attributed to polymer-polymer interactions, which are favoured by unfolding of WPI.

In this chapter the objectives are: (i) to study the secondary structure of WPI and the interaction between the protein and PEO using attenuated total
reflection – Fourier transform infrared (ATR-FTIR) and nuclear magnetic resonance. Analyses were performed for WPI solutions, WPI films and electrospun fibers of different pH; (ii) to investigate the unfolding and hydrolysis of WPI under the different pH conditions using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF) analysis.
5.2 Materials

WPI (protein content >90%) was donated by Glanbia Nutritionals, Inc. (Evanston, IL), PEO (MW 900 kDa), sodium hydroxide, and glacial acetic acid were purchased from Sigma Aldrich Company Canada (Oakville, ON).

5.3 Methods

5.3.1 Solution preparation

Material concentrations of all the components in the polymer solution are given in w/w basis. PEO aqueous solutions were prepared, and added to distilled water, sodium hydroxide aqueous solution (1%) and glacial acetic acid were used as solvents to give 0.4% PEO solutions of pH 7, 12, and 1 respectively. WPI (10%) was then added slowly to the PEO solutions and stirred with the aid of a magnetic stirrer. The neutral and alkaline polymer solutions were stirred for 15 min, while the acidic polymer solution was stirred for 12 h to achieve complete dissolution of WPI. The final concentrations of WPI and PEO in the resulting final polymer solutions were 10 and 0.4%, respectively. The ratio between the WPI and PEO content in the resulting electrospun fibers was 96:4. Polymer solutions without PEO were prepared similarly except that the addition of PEO was omitted. Polymer solutions were prepared at room temperature (22 ± 2°C).
5.3.2 Electrospinning and casting

WPI solutions were electrospun using a vertical electrospinning setup. The polymer solution was delivered to the spinneret at 0.4 ml/h using an infusion pump (Model KD200, KD Scientific, Holliston, MA). The spinneret was connected to the positive electrode, while the stainless steel collector plate was grounded. A constant voltage ranging from +15 to +25 kV was applied to the positive electrode using a DC supply (Model ES30R-5W/DM, Gamma High Voltage Research, Ormand, Beach, FL). The distance between the spinneret and the collector was set at 26 cm. Electrospinning was conducted under ambient conditions (22 ± 2°C; 15 ± 5% RH). To prepare cast film specimens, polymer solutions (5 ml) were poured into a 47 mm diameter polystyrene petri dish (Fisher Scientific, Waltham, MA) and the solutions were allowed to dry overnight (12 h) under ambient conditions (22 ± 2°C; 15 ± 5% RH). The reason why WPI cast films were investigated is that WPI cannot be electrospun alone into a membrane. In order to form a thin material, a casting method was used instead.

5.3.3 Attenuated Total Reflection –Fourier Transform Infrared (ATR-FTIR) Spectrometry

WPI films in the presence and absence of PEO as well as electrospun fibers were characterized by using a FTIR spectrometer (IRPrestige21, Shimadzu Corp. Japan) equipped with an attenuated total reflection accessory (Pike Tech, Madison, WI.). The test was set at 4 cm⁻¹ resolution and an average of 40 scans was taken for each sample. Solid electrospun fibers were placed on the ATR
crystal for analysis at room temperature (22 ± 2°C). The FTIR spectrum in the region of 1700 cm⁻¹ to 1600 cm⁻¹ was curve fitted by using the Gaussian function with the Grams/32 spectral analysis software (Galactic Industries Corp., Salem, NH). The 2nd derivative function was obtained to determine the wavenumber of the bands of interest.

5.3.4 Nuclear Magnetic Resonance (NMR) Analysis

In the presence and absence of PEO, neutral and alkaline WPI solutions were prepared by replacing 8% w/w of the aqueous medium with deuterium dioxide (D₂O). The acidic solutions were not suitable for 1H-NMR due to the excessive noise caused by the presence of H₃O⁺ ions in the polymer solution. The ¹H-spectra of the alkaline and neutral WPI solutions was recorded on a Bruker 600 MHz Avance II WB operating at 600 MHz equipped with a 5 mm ¹H-probe. ¹³C NMR cross polarization/magic angle spinning (CP/MAS) analysis of the electrospun fibers and films was performed on a Bruker 500 MHz Avance II WB operating at 125.77 MHz. The spectrometer was equipped with a 4 mm HXY MAS probe using a ZrO₂ rotor. Electrospun fibers and films were ground into powder, tightly packed into the rotor and spun at 10 KHz at room temperature. Spectra were acquired with a proton 90° pulse length of 2.7 μs and ¹³C contact time of 2000 μs. Each sample spectrum was obtained with 2000 scans with a repetition delay time of 5 s and processed with 10 Hz line broadening.
5.3.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE electrophoresis was carried out to characterize the molecular weight distribution of WPI according to Laemmli\textsuperscript{97}. WPI/PEO dried electrospun fibers (5 mg) were dissolved in 820 µl buffer, which had 10\%15 mM Tris (pH 6.8), 1.2\% SDS, 30\% glycerol, 15\% β-mercaptoethanol, and 0.0018\% bromophenol blue. WPI mixtures were hold for an hour, and then heated for 5 min (95°C). When WPI solutions were cooled at room temperature, they were loaded (2 µl) onto Biorad Ready Gel 12\%Tris-HCl Gel (4\% stacking gel 15-well 15 µl) in a Biorad Mini Protean III Cell SDS PAGE system (Biorad, Hercules, CA). A running buffer (0.01\% SDS, 0.0303\% Tris and 0.144\% glycin) was poured into the chamber, and the system was set at 200 VDC for 1 hour. When the electrophoresis run was completed, the gel was stained with a Commassie blue aqueous solution (0.0025\% Coomassie brilliant blue R-250, 45\% methanol, and 10\% acetic acid. The destaining solution contained 45\% methanol, 10\% acetic acid and 45\% water. Gels were observed by using Chemi Genius2 Bioimaging System (Syngene, Cambridge, U.K).
5.3.6 Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF spectrometry analysis was conducted for neutral and acidic polymer systems in the liquid and solid state. Preliminary studies showed that the alkaline polymer system was not suitable for the chemical conditions of this analytical tool due to the excessive presence of NA\(^+\) and OH\(^-\) ions in this polymer system. For the mass spectrometry analysis of the neutral and acidic polymer systems, dried fibers (\(~ 2\) mg) were dissolved in 100 \(\mu\)l of water. Acidic and neutral polymer solutions were prepared as described previously (section 5.5.1). After dissolution, the samples were mixed directly with matrix solution (2 mg sinapinic acid in 100 \(\mu\)L of 50:50 acetonitrile:water, with 0.1\% trifluoroacetic acid) in an analyte to matrix ratio of 1:1 (v/v). An aliquot of 1 \(\mu\)L of the solution was spotted on a MALDI plate and allowed to dry at room temperature (22 \(\pm\) 2°C). Analysis of the liquid and solid samples was performed by using a matrix-assisted laser desorption ionization-time of flight mass spectrophotomer (MALDI-TOF MS, Reflex II, Bruker, Germany) equipped with a 337 nm nitrogen laser (Biological Mass Spectrometry Facility, University of Guelph, ON, Canada). Positive ions were detected in linear mode scanning from 1,000 to 20,000 m/z and using ion suppression up to 500 m/z. For all experiments, the ion sources 1 and 2 were held at 20 kV and 16.35 kV respectively.
5.4 Results and discussion

5.4.1 Attenuated Total Reflection–Fourier Transform Infrared (ATR-FTIR) Analysis

FTIR spectral analysis provides vibrational information of the functional groups present in the sample useful for probing the interaction between the components in the electrospun fibers, as well as for characterizing the secondary structure of protein. In the present study, the main peak of interest is the Amide I band (1700-1600 cm\(^{-1}\)) mainly due to the stretching vibrations of carbonyl groups of the peptide bond of the protein. Another band of interest is due to the stretching vibrations of the ether peak located at around 1100 cm\(^{-1}\) wavenumber. Unfortunately, due to the small amount of PEO present in the specimens, the ether peak of PEO was masked by the stretching vibrations of the carboxylic acid peaks (1300-1100 cm\(^{-1}\)).

As shown in Figure 19, there is no appreciable difference between the spectra of electrospun fibers and cast films at the same pH, implying that electrospinning did not cause a detectable effect on the molecular properties of the protein. It is worth highlighting here that the molecular characterization of polymer produced from casting and spinning methods are likely to be different, due to differences in stress imposed on the material during the drying process. In film casting, the polymer solution was allowed to dry slowly under ambient condition in the absence of strain. By contrast, electrospinning process will induce a certain degree of orientation along the direction of stretch. Nevertheless, comparing cast and electrospun specimens will allow one to study
the effects of electrospinning on the polymers such as changes in the secondary structure of the protein, and interactions with PEO.

The effect of pH was investigated by studying the Amide I band, which can be used to characterize the secondary structures (random coil, α-helix, and β-sheet) of the protein. For neutral WPI electrospun fibers, the spectral deconvolution analysis of the Amide I region revealed that the fraction of β-sheet was higher than α-helix for the neutral electrospun sample. For fibers electrospun from glacial acetic acid solution, the proportion of α-helix peak at 1658 cm\(^{-1}\) and random coil at 1645 cm\(^{-1}\) were greater than those detected in the neutral fibers (Figure 20). In the case of alkaline fibers, the ATR-FTIR results showed increases in the α-helix at 1650 cm\(^{-1}\) and the random coil at 1640 cm\(^{-1}\) while a drastic decrease in the proportion of β-sheets is evident when compared to the neutral fibers. These results show that the protein conformation between the fiber samples was different depending on the pH of the polymer solution.
Figure 19. ATR-FTIR spectra for: (a) WPI film; (b) WPI-PEO; and (c) WPI-PEO electrospun fibers, prepared from polymer solutions at different pH's. For comparison, the spectrum for PEO fibers electrospun from neutral PEO aqueous solution (4% w/w) is also shown (d).
Figure 20. Deconvolution and curve fitting of the FTIR spectrum for WPI fibers electrospun from polymer solutions at different pH's

5.4.2 Nuclear Magnetic Resonance (NMR) Analysis

To further elucidate the interaction between WPI and PEO, NMR analyses were conducted on the fiber-forming solutions and the electrospun fibers. As shown in Figure 21 and Figure 22, the spectra of neutral and alkaline solutions in the presence of PEO display a sharp peak at 3.5 ppm that is absent in the spectrum for protein solutions. This peak is originated from PEO due to the spinning of the \(^1\)H at CH\(_2\)O position of the polymer chain. From Figure 21 and Figure 22, it is clear that the addition of PEO into WPI solution did not affect the other resonance peaks of the protein. This observation revealed that the ether segment of PEO did not affect the molecular properties of WPI in the solution.
Figure 21. 1H NMR Spectra of: (a) WPI neutral solution; (b) WPI/PEO neutral solution
Figure 22. $^1$H NMR Spectra of: (a) WPI alkaline solution; (b) WPI/PEO alkaline solution

In the solid $^{13}$C NMR spectrum, the carbonyl peak (-C=O) resonance was located at 170-180 ppm, which was well separated from that ether group (-C-O-) at 70 ppm. The effect of the ether oxygen segment of PEO on the molecular properties of protein can be investigated under different pH conditions. Under neutral pH conditions, no detectable changes were observed for the chemical shifts of carbonyl carbon for electrospun WPI-PEO fibers, WPI-PEO film, and WPI film (Figure 23). This result suggests that the exposure of high voltage during electrospinning did not result in detectable changes on the protein structure. Moreover, the chemical shift for the ether oxygen did not alter when the
PEO was blended with the protein (Figure 23) indicating that the magnetic shielding properties of carbon next to the ether oxygen were not affected by the presence of WPI under neutral conditions.

For fibers prepared from acidic polymer solution, a chemical shift on the carbonyl peak was evident when compared to the neutral pH condition (Figure 24). The chemical shift for carbonyl $^{13}$C peak between 172 and 176 ppm reflects the secondary structure of the protein. The peak at 176 ppm is indicative that the main-chain carbonyl group is in the $\alpha$-helix configuration while the peak at 172 ppm is related to $\beta$-sheet configuration$^{99,101}$. In acidic pH, the carbonyl $^{13}$C at 176 ppm is evident showing the $\alpha$-helix configuration has developed after electrospinning (Figure 24). This data is consistent with the ATR- FTIR analysis.
Figure 23. $^{13}$C NMR spectra for: (a) WPI film; (b) WPI-PEO film; and (c) WPI-PEO electrospun fibers prepared from neutral polymer solutions. For comparison, the spectrum for PEO powder is also shown (d).
Figure 24. Comparison of $^{13}$C NMR spectra of: (a) neutral WPI film; (b) acidic WPI film; (c) acidic WPI-PEO film; and (d) acidic WPI-PEO electrospun fiber.
For the alkaline WPI/PEO electrospun fibers, the resonance for carbonyl $^{13}\text{C}$ appeared at 172 ppm indicating that the proportion of $\beta$-sheet configuration in the protein secondary structure was substantial (Figure 25). The presence of a left shoulder close to the main-chain carbonyl group at $\sim$180 ppm has been attributed to the side-chain carbonyl carbons of aspartic acid and glutamic acid$^{99}$. The increased signal from these amino acid residues suggested that the protein might have been unfolded at pH 12 which might have led to a higher chain entanglement with PEO macromolecules. PEO showed a peak at 70 ppm due to $^{13}\text{C}$ attached to the ether oxygen on the polymer chain. The position of this peak was not affected by the presence of WPI, suggesting that the shielding environments of the ether carbon were similar between WPI-PEO and PEO fibers. Studies conducted by Claesson et al. and Jeon et al. concluded that the interaction of PEO with protein was mainly attributed to electrostatic and hydrophobic forces$^{102,103}$. Topchieva et al. also reported that the interactions between poly(ethylene glycol) and proteins ($\alpha$-chymotrypsin, lysosome, BSA) were non covalent, and that the presence of PEO did not induce any changes on the secondary structure of the protein$^{104}$. Kelarakis et al. workers reported similar findings concluding that PEO did not alter the molecular properties of BSA in aqueous solutions$^{105}$. 
Figure 25. Comparison of $^{13}$C NMR spectra of: (a) neutral WPI film; (b) alkaline WPI film; (c) alkaline WPI-PEO film; and (d) alkaline WPI-PEO electrospun fibers.
Based on the NMR results from the present studies, and the available information from the literature, the electrospinning-enabling effect of PEO on aqueous WPI solutions was mainly due to chain entanglement and/or entrapment/deposition. There was no evidence to suggest interaction at specific sites between the two polymers, nor alteration of secondary structure of the protein due to the presence of PEO. FTIR and NMR analyses showed that WPI was unfolded under acidic and alkaline conditions promoting chain entanglement/entrapmen/deposition with PEO.

However, acidic extreme pH and alkaline pH might have also partially hydrolyzed WPI, and the resulting peptides that might have interacted with PEO, through deposition on PEO chain under shear as the polymer jet ejects from the Taylor cone. To verify whether WPI was denatured and hydrolyzed under the extreme pH conditions, SDS-PAGE and MALDI–TOF mass spectrometry analyses were carried out.
5.4.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

From SDS-PAGE analysis (Figure 26), the appearance of the β-lactoglobulin and α-lactalbumin bands in whey protein isolate is evident for alkaline and neutral polymer system. The presence of the β-lactoglobulin and α-lactalbumin at the corresponding molecular weight implied that the polypeptide was not hydrolyzed under these pH conditions. Nevertheless, the polypeptide could still undergo substantial unfolding, especially for the alkaline condition when the protein is far away from the isoelectric point, as evidenced from the FTIR and NMR data. By contrast, for the acidic polymer system, there are no defined bands along the gel. This observation can be partially attributed to the hydrolysis of the protein, resulting in the formation of smaller peptides. It is assumed that the low abundance of the peptides was not detected by SDS-PAGE analysis due to the poor sensitivity of the technique\textsuperscript{106, 107}. The second possible reason is that the initial charge of the protein due to the exposure to acidic conditions may have interfered with the SDS-PAGE analysis. Although, it is assumed that the addition of the detergent SDS results in negative charged proteins to allow their separation by molecular weight, several anomalous behaviors have been reported in electrophoresis. For example, Garcia-Ortega et al. reported that a very acidic protein Ribonuclease U2 does not bind with SDS under typical SDS-PAGE conditions affecting the analysis. They explained that the binding of the protein with SDS-PAGE is through electrostatics and hydrophobic interactions, which are affected by the net charge of the protein\textsuperscript{108}. 
Armstrong and Roman investigated the effect of replacing two basic residues with two acidic residues of the protein papillomavirus 16E7 on the gel mobility during SDS-PAGE analysis. They concluded that the net negative charge affected the binding with SDS through electrostatic interactions affecting mobility during the test, and impeding accuracy for the determination of molecular weight\textsuperscript{109}. Also Alves et al. revealed that the acidic protein Gir2 from \textit{Saccharomyces cerevisae} showed a slow migration during the SDS-PAGE analysis, and they also attributed this phenomenon to the net charge of the protein as responsible for impeding the binding between protein and SDS\textsuperscript{110}. To clarify whether WPI has been denatured or hydrolyzed, mass spectrometry-MALDI analysis was conducted, and the results are presented in the next section.
Figure 26. SDS-PAGE electrophoretogram for WPI solutions at different pH: (a) WPI/PEO acidic electrospun fibers (pH 1); (b) WPI/PEO alkaline electrospun fibers (pH 12); (c) WPI/PEO neutral electrospun fibers (pH 7)
5.4.4 Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

From the MALDI-TOF mass spectra for WPI aqueous neutral system, it is evident that whey protein isolate was not hydrolyzed during the solution preparation nor the electrospinning process (Figure 27). The peaks that correspond to $\alpha$-lactalbumin and $\beta$-lactoglobulin were evident in the whey protein powder (Figure 27a) at approximate 14 and 18 kDa respectively, which agreed with the literature\textsuperscript{111, 112}. For the WPI solutions in the presence and absence of PEO (Figure 27b and Figure 27c) the $\alpha$-lactalbumin peak was present but there is no evidence of the $\beta$-lactoglobulin peak. The absence of $\beta$-lactoglobulin peak could be attributed to the sample preparation. Nevertheless, it is clear that MALDI-TOF mass spectra for WPI aqueous neutral solution in the absence and presence of PEO are similar. Consequently, the addition of PEO did not have an effect on the molecular weight distribution of WPI under neutral conditions. Due to molecular weight limitations of the MALDI-TOF mass spectroscopy technique, observation of a PEO peak is beyond the limitations for this analytical tool\textsuperscript{113, 114}. For WPI/PEO neutral fibers (Figure 27d), the presence of the $\beta$-lactoglobulin and $\alpha$-lactalbumin peaks suggests that the electrospinning process did not have an effect on the molecular weight distribution of WPI.

For clarification, MALDI-TOF MS is a qualitative but not quantitative technique. MALDI-TOF is a relatively novel technique that allows the characterization of biomolecules, which otherwise would be fragmented by typical
mass spectrometry methods. In MALDI-TOF MS, analyte and matrix co-crystallize simultaneously, but the analyte does not crystallize nor distribute uniformly in the matrix resulting in areas of high and low concentration which impedes reproducibility and quantification\textsuperscript{113-116}. When the laser beam targets the matrix/analyte mixture, the matrix absorbs most of the energy protecting the analyte from fragmentation, and provides protons to the sample for ionization. A phenomenon called ion suppression may occur at this stage, in which an ion suppresses the signal of another one. In proteins, hydrophobic residues suppress the signal of other residues while basic residues favor the ionization process. As such, the ion suppression effect is another obstacle for analyte quantification by MALDI-TOF MS\textsuperscript{113-116}. Hence, the peak intensity in the MALDI-TOF spectra does not represent the quantity of the different peptides in the sample.

MALDI-TOF spectra agreed with SDS-PAGE analysis confirming the presence of $\beta$-lactoglobulin and $\alpha$-lactalbumin, which is consistent with the FTIR and NMR analyses in that the addition of PEO and electrospinning process did not affect the chemical structure of WPI.
For the acidic polymer system, MALDI-TOF mass spectra shows that not just β-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa) are evident in all cases but additional peaks were also present at approximately at 7, 10, 12 and 16 kDa (Figure 28). The presence of additional peaks implied that β-lactoglobulin and α-lactalbumin were hydrolyzed under the acidic conditions (pH 1) producing shorter peptides. This is consistent with the SDS-PAGE data. Comparing the spectra for samples with and without PEO (Figure 28b and Figure 28c), it can be seen that they are highly similar. MALDI-TOF spectrum of WPI/PEO acidic fibers is not
different from the WPI acidic solutions indicating that the electrospinning process did not affect the molecular weight distribution of WPI under acidic conditions.

Figure 28. MALDI-TOF mass spectra of: (a) WPI acidic solution; (b) WPI/PEO acidic solution; (c) WPI/PEO acidic electrospun fibers
Although both MALDI-TOF and SDS-PAGE data suggested that hydrolysis of WPI proteins had occurred under the acidic conditions, the fact that the protein solution could be electrospun into smooth and uniform fibers provided an important hint that the shorter peptides could still easily be integrated into the fiber, most probably by depositing along large molecular weight PEO under high shear, as the polymer jet is ejecting from the Taylor cone. Since NMR and FTIR data also revealed the presence of secondary structures in the electrospun fibers, it could be also assumed that the presence of larger polypeptide fractions which are conducive for creating chain entanglement with PEO that is important for stabilizing the polymer jet during electrospinning.
5.5 Conclusion

This chapter showed that molecular characteristics of the protein had an effect on the electrospinning behavior of WPI fibers affecting the fiber. Although there was no evidence of interaction between WPI and PEO, unfolding of WPI had an effect on electrospinnability. According to frequency shifts observed on the Amide I band in FTIR analysis, WPI acidic and alkaline fibers possessed greater proportions of α-helix and random coil than WPI neutral fibers, which correlated with the formation of beadless electrospun fibers. In agreement with this, NMR data also indicated a higher proportion of the α-helix configuration under acidic conditions. Analysis of PEO using FTIR spectroscopy was not possible due to its low concentration in the fiber in comparison to WPI, and overlapping with absorption bands of the protein. However, the higher sensitivity of NMR analysis allowed the detection of the characteristic PEO peak in the NMR spectra. Overall, PEO did not show any chemical shift in the NMR spectra for all of the polymer systems investigated, suggesting that there is no evidence of interaction between PEO and WPI by this analytical tool. SDS-PAGE electrophoresis revealed the presence of α-lactoalbumin and β-lactalbumin in the WPI alkaline and neutral fibers, but substantial hydrolysis was detected for the acidic fiber. MALDI-TOF mass spectra for neutral aqueous solutions and fibers showed that solution properties and the electrospinning process did not have an effect on the molecular weight distribution of WPI. For the acidic electrospun fibers, MALDI-TOF spectrum indicated the presence of shorter peptides (7 -16...
kDa) in addition to the corresponding peaks of \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin. These results are consistent with SDS-PAGE results.

In summary, results from this chapter suggested that the hydrolysis and unfolding of WPI under acidic conditions facilitated the incorporation of WPI along the PEO scaffold polymer chain to produce smooth and uniform fibers. However, there is no evidence of interaction between WPI and PEO by the analytical techniques used in this investigation. The study opens up more questions on the mechanism by which PEO enables the formation of WPI fibers: would entanglement/entrapment/deposition between WPI PEO enable the formation of electrospun fibers? More specifically, how does WPI entangle with PEO, and what is the distribution of PEO within the WPI fiber matrices. In an attempt to answer these questions, the following chapter focuses on studying the distribution of WPI along the fibers using various electron microscopy and sample preparation techniques.
Chapter 6. Microstructural Characterization of WPI/PEO Fibers

6.1 Introduction

Studies conducted in Chapter 5 revealed that specific interactions between PEO and WPI were not detected using NMR and FTIR analysis. Furthermore, it was established that denaturation of WPI is important to facilitate the formation of smooth fibers. On the basis of these findings, it is theorized that the positive effect of PEO on the electrospinning of WPI was mainly due to chain entanglement or mechanical deposition of short polypeptides on PEO scaffolds. The chain entanglement idea has been invoked by the work of Nie et al. who explained why the addition of PEO to aqueous alginate solution could enable the electrospinning of the polysaccharide. Their explanation was supported by the fact that only high molecular weight PEO (1000 kDa) could facilitate the electrospinning of aqueous alginate solution, whereas the low molecular weight counterpart (20 kDa) did not, even when a large amount of the latter was used to considerably increase the viscosity of the polymer solution74.

The quest to seek out the evidence of chain entanglement between WPI and PEO polymers has prompted us to ask several questions: Would WPI and PEO concentration affect the entanglement? How PEO would distribute within the electrospun WPI fiber; would it concentrate near the core or be randomly dispersed throughout the entire cross section of the fiber? Would globular native WPI at pH 7 form beads impeding its incorporation to the PEO ultrafine fibers?
To answer some of these questions, and to gain a deeper understanding on the role of PEO, the studies in this chapter are designed to determine the distribution of WPI and PEO in the fiber. In this investigation, it is hypothesized that the distribution of PEO and WPI in the electrospun fiber is determined by the chain entanglement/entrapment/deposition between the two polymers that relies on the denaturation of WPI. The objectives of this chapter are: (i) to investigate the effects of polymer concentration on the chain entanglement between WPI and PEO; (ii) to determine the distribution of PEO and WPI in the electrospun fiber by using electron microscopy and X-ray analyses.

6.2 Materials

WPI was donated by Ganblia Nutritionals, Inc. (Evanston, IL), PEO (Mw 900 kDa), sodium hydroxide, and glacial acetic acid were purchased from Sigma Aldrich Company (Oakville, ON).

6.3 Methods

6.3.1 Solution preparation

Material concentrations of all the components in the polymer solution are given in w/w basis. PEO aqueous solutions were prepared and added to distilled water, sodium hydroxide aqueous solution (1%) and glacial acetic acid were used as solvents to give PEO solutions of pH 7, 12, and 1 respectively. To prepare the neutral and alkaline solutions, WPI was added to PEO aqueous solutions of pH 7 and pH 12 respectively, and they were dissolved with the aid of a magnetic stirrer
for 15 min. For the acidic solution, WPI was added to the PEO acidic solution and stirred overnight (12 h) to be dissolved. All polymer solutions were prepared at room temperature (22 ± 2°C). Final WPI and PEO concentrations in the polymer solution and in the electrospun fibers were indicated in the results and discussion section according to every study.

6.3.2 Scanning Electron Microscopy (SEM) Analysis

The morphology of electrospun fibers was determined by using a scanning electron microscope (SEM S-570, Hitachi High Technologies Corporation, Tokyo, Japan) at an accelerating voltage of 10 kV. To observe the fiber morphology, fibers were electrospun on aluminum foil, and they were carbon taped on the SEM stub. To observe the cross section of the fibers, samples were previously frozen by using liquid nitrogen, and they were fractured by using a sharp razor blade. All samples were coated with gold/palladium (15 nm) using a sputter coater (Model K550, Emitech, Ashford, Kent, England). The average fiber diameter was determined by taking at least 50 measurements for each image using image analysis software (Pro-Plus 5.1, Media Cybernetics Inc., Bethesda, Ma).

6.3.3 Scanning Transmission Electron Microscopy (STEM) Analysis and Energy Disperse Analysis of X-rays (EDAX)

The distribution of WPI and PEO along the electrospun fibers was examined by using a scanning transmission electron microscope (Titan STEM, FEI Co., Hillsboro, OR) at 80 kV in conjunction with an energy dispersive X-rays
micro-analysis detector (EDAX). Neutral and acidic fibers were electrospun directly on silicon nitride grids (Durasin™, Protochips Inc., Raleigh, NC). Alkaline fibers were electrospun directly on copper grids, which were previously Formvar™ and carbon coated.

6.3.4 Field Emission Scanning Electron Microscopy (FESEM) Analysis and Ion Beam Milling

A cross section of the electrospun fibers was observed by using a field emission electron microscope (FESEM, Hitachi, High Technologies Corporation, Tokyo, JP). Samples were electrospun on aluminum foil, and they were folded to place the electrospun fibers in between two layers of aluminum foil. The electrospun fibers and the aluminum layers were cross sectioned by using ion beam milling (IM4000, Hitachi, High Technologies Corporation, Tokyo, JP).
6.4 Results and discussion

6.4.1 Effect of various WPI and PEO concentrations on the morphology of electrospun WPI/PEO fibers.

Figure 29 shows that small changes in the amount of PEO can cause dramatic changes in fiber morphology. At 0.05% PEO, irregular particles ranging from 3 μm to 100 nm and ultrafine fibers (~100 nm) were observed. As the concentration of PEO increased, the number of particles decreased while the diameter of electrospun fibers increased. At 0.3% PEO, the particles disappeared, and smooth uniform fibers were observed. The PEO polymer used has a relatively high molecular weight (900 kDa) composed of homogeneous ethyl ether groups (-CH₂-CH₂-O-)ₙ. The amphipilic structure of PEO allows this polymer to interact with hydrophobic and hydrophilic functional groups in WPI, while at the same time being water soluble. PEO is soluble in water by forming hydrogen bonds resulting in a flexible α-helix structure. Also the distance between oxygen atoms in PEO matches that of water molecules. On the other hand, homologous of PEO such as poly(methylene oxide) and poly(propylene oxide) are insoluble in water due to the mismatch of oxygen-oxygen distance. The amphipilic nature and water solubility of PEO might have allowed PEO to serve as a scaffold for the protein to assemble into fibers during electrospinning, possibly involving non-covalent PEO and WPI interactions. Sheth and Israelachvili speculated that the formation of protein-poly(ethylene glycol) complex involves electrostatics and hydrophobic interactions. Also, Jeon et
al. reported that when PEO is fixed on a surface, it interacts through weak hydrophobic interactions with finite spherical proteins\textsuperscript{103}. Hence, it is speculated that WPI is carried by PEO through non covalent interactions during the electrospinning process, and consequently the protein became part of the chemical composition of the fibers.

Figure 29. SEM micrographs of WPI/PEO fibers resulted from polymer solutions using glacial acetic acid as solvent with a concentration of 10\% WPI and various PEO concentrations: (a) 0.3\%; (b) 0.2\%; (c) 0.1\%; (d) 0.05\%. Top row: low magnification; bottom row: high magnification

Figure 30 shows that in the absence of WPI, 0.4\% PEO can be electrospun into beads linked with ultrafine fibers. Also, changes in WPI content did not cause large changes in morphology when compared to PEO (Figure 29),
but increases in WPI content resulted in the formation of beadless electrospun fibers that were thicker in diameter. For example at 2.5% WPI, beads linked with thicker fibers (~241 nm) were produced while beadless thicker fibers (~366 nm) were produced from 10% WPI polymer solution. The observation that 0.4% (w/w) PEO alone could not be electrospun into fibers, and that smooth fibers could be produced from the same PEO solution when it was fortified with WPI suggested that the protein could interact synergistically with PEO to enable the electrospinning of the polymer solutions. From this perspective, the results imply that the protein actually acted as an electrospinning aid for PEO solution when it was below its critical concentration at which fiber electrospinning was not possible. These observations along with the viscosity-enhancing effect of WPI upon PEO (Figure 14) provided an important hint that the denatured WPI, even if it is considerably hydrolyzed (as unfolded protein and shorter peptide chains), must have interacted with the long chain PEO macromolecules in response to the shear field during electrospinning. It is theorized that two main modes contributed to the electrospinning of WPI. First, due to random mixing of the two compatible polymer solutions, the two unfolded protein molecules and longer hydrolyzed polypeptides entangled with the flexible PEO polymer chains. As the PEO elongated and was ejected from the Taylor cone under electric field, the entangled protein components were carried along with the PEO backbone. Second, as the entangled PEO-WPI chains eject from the Taylor cone under high shear rate, it is conceivable that the jet also sweeps along the surrounding peptides fragments that are shorter, carrying the shorter peptides with it. In this model, the entangled PEO-WPI chains basically serve as a structural scaffold,
allowing the short peptides to be deposited on them, allowing the buildup of thicker and smoother electrospun fibers.

Figure 30. SEM micrographs of WPI/PEO ultrafine fibers resulted from polymers solutions using glacial acetic acid as solvent with a concentration of 0.4% PEO and various WPI concentrations: (a) 0%; (b) 2.5%; (c) 5%; (d) 7.5%. Top row: low magnification; bottom row: high magnification

The chain entanglement concept has been invoked by a number of researchers to explain electrospinnability of biopolymers. For instance, Shenoy et al. emphasized the importance of chain entanglement by formulating a model to predict the fiber morphology from beads to smooth fibers on the basis of molecular weight and entanglement of the polymer in study. They concluded that
one entanglement per chain on polymers was conducive for the formation of incomplete fibers while more than 2.5 entanglements per chain resulted in the formation of complete smooth fibers. They confirmed their predictions with various polymer systems: polystyrene in tetrahydrofuran, PEO in water, poly(D,L-lactic acid) in dimethylformamide, poly (L-lactic acid) in dichloromethane, poly (L-lactic acid) in chloroform, poly (L-lactic acid) in 1,1,2,2 tetrachloroethane, and poly(vinyl pyrrolidone) in ethanol. Nie et al. demonstrated that chain entanglement is important to enable the electrospinning of aqueous alginate solutions by investigating the effect of glycerol. They concluded that glycerol increased the flexibility of alginate chains and facilitated the electrospinning process. Wang et al. reported that increasing entanglement in a polystyrene solution by increasing the polymer concentration resulted in smooth electrospun fibers. Unfortunately this approach is not ideal for the system as gelation of the solution will occur at concentrations higher than 12.5% under the investigated conditions. In the present study, the use of PEO as a spinning aid was needed. The chain entanglement phenomenon between WPI and PEO is expected to be concentration dependent.

Figure 31 shows the morphology of electrospun fibers that resulted from polymer solutions, where WPI content varied from 2.5% to 10%, while the PEO content varied from 0.4 to 0.55%. In this investigation, small changes in PEO content were paired with large changes in WPI content. Although the fiber composition was different, the resulting fiber morphology and fiber diameter were quite similar confirming the importance of chain entanglement between polymers. Kowalewski et al. attempted to evaluate the fiber jet of PEO aqueous solutions.
during whipping bending stability to model and further understand the electrospinning process. They concluded that evaluation of the dynamic electrospinning process is challenging, and depends on a complex number of factors, and further investigation is needed to simulate the process. They also emphasized that one of the main factors that determines electrospinnability of the polymer is the possibility of the polymer to resist the required deformation into fibers\textsuperscript{120}. In this investigation, it is speculated that PEO must be able to support the deformation into fibers during the electrospinning, and WPI may support this deformation even more by chain entanglement or entrapment/deposition; this enhancement can be manipulated by polymer concentration and level of denaturation/hydrolysis of the protein.
Figure 31. SEM micrographs of WPI/PEO electrospun fibers resulted from polymer solutions using glacial acetic acid as solvent of various WPI and PEO concentrations: (a) 10% WPI, 0.4% PEO; (b) 7.5% WPI, 0.45% PEO; (c) 5% WPI, 0.5% PEO; (d) 2.5% WPI, 0.55% PEO. Top row: low magnification; bottom row: high magnification.
6.4.3 Transmission Electron Microscopy Analysis-EDAX on different substrates to determine the location of PEO in the electrospun fibers

Although it is clear that PEO is essential for the formation of electrospun fibers, and the WPI/PEO ratios determines fiber morphology, the distribution of WPI and PEO along the electrospun fibers of different composition is unknown. In this section, STEM and EDAX techniques were employed to study the distribution of these polymers within the fibers in an attempt to elucidate chain entanglement behaviors between WPI and PEO.

Figures 31-34 show STEM micrographs of electrospun fibers prepared from different WPI/PEO compositions, along with EDAX maps for the distribution of carbon and sulfur. STEM was selected over the conventional TEM and SEM because it offers resolution as high as TEM, and allows mapping and imaging as in SEM. STEM allows a much precise detection of X-rays. STEM can have different detectors to receive the signal from secondary electrons, backscattered electrons, x-rays, electron energy loss, bright field and dark field\textsuperscript{121}. In this investigation, STEM was used in conjunction with EDAX to capture the signal of the X-rays from the elements that constituted WPI/PEO electrospun fibers. During the electron microscopy analysis, electrons from the electron beam will cause excitation of the electrons of elements in the WPI/PEO electrospun fiber. When an electron becomes excited, it will leave the grounded shell in the atom to
cause ionization. Electrons from higher inner shells drop down to occupy the vacancy, releasing energy in the form of X-rays\textsuperscript{122}. The X-ray pattern is specific for every element and supports visual assessment of the amount present at a chosen location on the fiber. In WPI/PEO electrospun fibers, the elements that constitute PEO are carbon, hydrogen and oxygen while the elements in WPI are carbon, hydrogen, oxygen, nitrogen, and sulfur. Hence, sulfur was selected as the indicator element for WPI since it is absent in PEO. Bae et al., also used sulfur to track cysteine-containing proteins by EDAX when investigating the binding ability of proteins to silica nanotubes\textsuperscript{123}. Another example is the study by Mishra and Thomas, in which they characterized sulfur as indication of the location of protein in phospholipid cones through EDAX \textsuperscript{124}.

Control samples of 100% PEO were characterized by the absence of sulfur and the presence of carbon, and oxygen. However, other sources of carbon can emit X-rays during the analysis. A source of carbon comes from the typical TEM/STEM copper grids coated with Formvar\textsuperscript{TM} poly(vinyl formal) and carbon. To overcome this hurdle and to increase the reliability of the study, Durasin\textsuperscript{TM} silicon nitride grids were used to eliminate the carbon source. By using sulfur as the element indicator, the distribution of WPI in fibers was tracked.

Control samples: electrosprayed WPI in the absence of PEO, and PEO electrospun fibers were analysed by STEM-EDAX. Figure 32 shows the dominant content of carbon and a null content of sulfur in PEO electrospun fibers. In comparison, the WPI beads possessed both carbon and sulfur as expected.
Figure 32. STEM micrographs and EDAX maps of carbon and sulfur distribution along electrosprayed WPI beads and PEO electrospun fibers. Top row: 100% WPI; bottom row: 100% PEO. The area delimited by the black square was examined by EDAX.
Figure 33 shows the STEM-EDAX results of acidic electrospun fibers of different WPI/PEO ratios revealing the distribution of carbon and sulfur in an electrospun fiber that contains 96% (upper row) compared to 10% WPI (lower row). The fact that the distribution of WPI along the fiber is uniform when the concentration of WPI is as low as 10% implied that WPI is well mixed in the PEO chain. A uniform distribution of WPI along the acidic fibers may be attributed to the unfolding and hydrolysis of globular WPI exposing more functional groups to interact with the long chains of PEO (900 kDa). In this experiment, STEM-EDAX analysis revealed uniformity in the distribution of WPI along WPI/PEO electrospun fibers under acidic conditions, STEM-EDAX analyses for the neutral and alkaline systems are described in turn.
Figure 33. STEM micrographs and EDAX maps of carbon and sulfur distribution along WPI/PEO acidic electrospun fibers of different composition (pH 1). Upper row: the WPI to PEO ratio is 10:90. Lower row: the WPI to PEO ratio is 96:4.

Figure 34 shows the STEM micrographs, and the EDAX map of the distribution of carbon and sulfur for neutral electrospun fibers/beads. At 10% and 96% WPI, the EDAX analysis showed that WPI was uniformly distributed. Even though WPI is present in ultrafine fibers, it is speculated that the interaction between native WPI and PEO was restricted due to the globular configuration of the protein impeding chain entanglement/entrapment/deposition. The impediment to incorporation of WPI in PEO due to the globular configuration, increased the concentration of free solvent molecules in the polymer solution. During
electrospinning, the solvent molecules tended to aggregate in spherical shape, because of the surface tension causing the formation of beads\textsuperscript{14}.

Figure 34. STEM micrographs, and EDAX maps of the distribution of carbon and sulfur along WPI/PEO neutral electrospun fibers of different composition (pH 7). Upper row: the WPI to PEO ratio is 10:90 in the fiber. Lower row: the WPI to PEO ratio is 96:4 in the fiber. The area delimited by the black square was examined by EDAX.
Figure 35 shows the STEM-EDAX micrographs of electrospun fibers formed from alkaline polymer systems, and the EDAX maps of distribution of carbon and sulfur along the fibers. For this analysis, silicon nitride grids were not used since the alkaline fibers interacted with the silicon nitride coating impeding observation with STEM. Thus, typical copper grids coated with Formvar™ were used to analyse fibers electrospun from alkaline polymer systems for STEM-EDAX analysis. Although the alkaline electrospun fibers also reacted with the Formvar™, the observation and analysis of alkaline electrospun fibers was possible. The effect of alkaline conditions on the Formvar™ was notable in the STEM micrograph, where sodium salts crystallized along the edges of the electrospun fibers. The origin of sodium atoms was from the solution preparation stage, where sodium hydroxide was added to adjust the pH (pH 12), and foreign contaminants on Formvar™ such as chlorine resulted in the formation of sodium salts. However, the effect of sodium on the Formvar™ did not affect the analysis and investigation of the distribution of carbon and sulfur along the fibers. At 96% and 10% WPI, the distribution of carbon and sulfur was uniform along the fibers (Figure 35). The distribution of WPI along neutral, alkaline and acidic fibers is uniform at each WPI concentration, which confirms uniform mixing between WPI and PEO. Also, there is no phase separation of these polymers during electrospinning under the conditions investigated.
Figure 35. STEM micrographs and EDAX maps of the distribution of carbon and sulfur along WPI/PEO alkaline fibers of different composition (pH 12). Upper row: The WPI to PEO ratio is 96:4 in the fibers. Lower row: The WPI to PEO ratio is 10:90 in the fiber. The area delimited by the black square was examined by EDAX.
6.4.4 Cross section of electrospun fibers

The preceding techniques provided two-dimensional results on sulfur and carbon distribution, but do not answer questions such as whether the fiber/beads are hollow, porous or solid. In order to shed additional light on the distribution of material within the fibers, the cross section of the electrospun specimens were examined using two methods: in the first method, electrospun fibers were frozen by liquid nitrogen, fractured, and then examined using SEM; in the second method, electrospun were cross sectioned by using ion beam milling process, followed by examination with FESEM.

Figure 36 shows the cross section of acidic electrospun fibers by SEM and FESEM. FESEM micrographs show that the acidic electrospun fibers possessed a solid interior. Although it is not as clear as in the FESEM micrographs, the SEM micrographs also showed a solid core. The inner morphology of the fiber, hollow or solid, has been related to the rate of evaporation of the solvent. When the electrospun fibers travel from the spinneret to the collector for drying, the wet fiber whips following different electrical forces according to several electrospinning models \(^{40, 125-127}\). The fiber is stretched from right to left forming loops during the whipping/drying process, and the acetic acid is released uniformly from the interior to the exterior of the polymer jet causing the formation of the solid fibers. Several studies have emphasized that the morphology of electrospun fibers depends mainly on the polymer concentration and evaporation.
of the solvent. For example, Tripatanauswan et al., reported that slow evaporation rate affected fiber diameter and morphology in aqueous polyethylene systems. They controlled the evaporation rate of the solvent by adjusting the relative humidity. As the relative humidity increased from 5.1 to 48.7%, the fiber diameter decreased from 253 to 144 nm. Moreover, the presence of beads was evident when the relative humidity was higher than 50%, and the authors attributed this phenomenon to the capillary instability before solidification of the polymer jet. Also, Jarusuwannapoom et al. investigated eighteen solvents on the electrospinnability of polystyrene solutions, and they concluded that just four solvents (dimethylformamide, methyl ethyl acetate, methylethylketone and tetrahydrofuran) enabled the formation of fiber due to their solvent properties emphasizing the importance of the volatility of the solvent. They explained that an extreme rapid solvent evaporation impeded the formation of fibers. Yao et al. reported that the electrospinning of zein (30-50% w/w) in ethanol-water (70:30) resulted in ribbon-like fibers. The authors explained that the high volatility of ethanol is responsible for the formation of the ribbon-like fibers. They indicated that the rapid evaporation of ethanol caused the formation of an outer skin that collapsed during the electrospinning process. With the same reasoning, several papers explained the formation of ribbon-like fibers when electrospinning zein (30-50% w/w) in ethanol-water (80:20). Jiang et al. electrospun zein using a less volatile solvent dimethylformamide instead of the ethanol-water mixture, and the resulted fibers from the electrospinning of zein solutions with a polymer concentration of 500 mg/mL or higher, were smooth and round-shaped confirming that the volatility of the solvent has an impact on the fiber
morphology$^{132}$. Analysis of the fact that acidic fibers possessed a solid core in conjunction with the STEM-EDAX investigation led to the inference that entanglement/entrapment/deposition between WPI and PEO is high enough to prevent the formation of hollow fibers.

In Figure 36, the FESEM images also appeared to show that fibers have an outer and inner shell of different density. This could be the artifact due to deposition of aluminum through the ion beam. This is based on the observation that the thickness of the outer shell increases as the distance from the fiber to the aluminum foil is closer.
Figure 36. Cross section of fiber electrospun from 10% WPI and 0.4% acidic solution (pH 1). Left column: SEM micrographs. Right column: FESEM micrographs
Figure 37 shows SEM and FESEM micrographs of the cross section for neutral ultrafine fibers. The SEM and FESEM micrographs showed that the interior of neutral ultrafine fiber was solid. It is noteworthy that beads tend to show a thick outer shell with a hollow interior with FESEM analysis. This morphological features was not captured in cryofractured specimens from SEM since the fracture tends to take place on the fibers, rather than across the beads.

During the whipping and drying of the electrospun fibers, a moisture content gradient established within the beads might have been created. As the solvent evaporates, a polymer shell was created forming hollow beads. Figure 37 also shows that the cross section of some beads is filled with a material of different density; it is speculated that this filling is aluminum which was deposited by the ion beam.
Figure 37. Cross section of fiber electrospun from 10% WPI and 0.4% PEO neutral solutions (pH 7). Left column: SEM micrographs. Right column: FESEM micrographs

Other researchers have studied the cross section of electrospun fibers by other methods such as SEM, TEM and AFM. For example, Li and Xia reported evidence of having a core-shell electrospun fiber by showing the cross section of titania/poly (vinyl pyrrolidone) composite electrospun fibers, which were fractured with a razor blade and observed by SEM. Megelski et al., reported the ribbon shaped cross section of polystyrene electrospun fibers by TEM by embedding samples in epoxy resin, stained with RuO₄, and microtomed before the
Wang et al. reported the elliptical cross section of silk/PEO fibers by atomic force microscopy analysis; the fibers were also embedded in an epoxy resin, and sectioned with an ultramicrotome using a diamond knife. Also, Wang et al. also cross sectioned silk/PEO ultrafine fibers by freezing them in liquid nitrogen and fracturing them to be observed under SEM. In preliminary attempts to observe the cross section of electrospun fibers, a similar approach was adopted, and the electrospun WPI-PEO fibers were not stable in the resin, causing considerable disruption of original fiber morphology. Unlike these approaches, revealing the cross section features using ion beam milling preserves the original morphology much better with less sample preparation artifacts, allowing a more reliable observation of the original morphology of the cross section of electrospun fibers. To our knowledge, the ion beam milling device as a tool for cross sectioning of ultrafine fibers has not been reported before in the literature.

It was observed that the fracture of electrospun fibers by using ion beam milling is much more precise than any other of the previous techniques allowing a more reliable observation of the original morphology of the cross section of electrospun fibers. The use of aluminum interferes favorably in the interaction between the electrospun fiber and the ion beam protecting the fiber from heat damage. Hence, ion beam milling was successfully used to fracture, with precision, heat-sensitive WPI/PEO electrospun fibers, resulting in high resolution images.

Under acidic and neutral conditions, the WPI/PEO electrospun fibers were solid confirming that there is no sign of phase separation between the two
polymers, further suggesting that entanglement/entrainment/deposition between PEO and WPI impeded the formation of hollow fibers.

6.5 Conclusions

Findings of this chapter further provide evidence that entanglement/entrainment/deposition of WPI in PEO could be the main mechanism for enabling the electrospinning of WPI since one uniform phase of polymer blends was observed. The use of sulfur as an indicator of protein allowed the observation of WPI distribution along the electrospun fibers by STEM-EDAX analysis. The presence of WPI was uniformly distributed along the acidic, neutral, and alkaline electrospun fibers. Small changes in PEO concentration (0.3-0.5%) caused dramatic changes in the fiber morphology of electrospun fibers. Although changes in WPI concentration (2.5-10%) were not as drastic as modification in PEO concentrations, WPI content also affected fiber morphology. Manipulation of WPI and PEO concentrations to control the fiber morphology resulted in the formation of electrospun fibers of similar morphology but different composition.

Imagining the cross-section of the electrospun fibers were obtained by two methods: (i) freezing of the sample with liquid nitrogen to razor snap and cross section the fibers for observations under SEM, and (ii) using ion beam milling to cross section the fiber for observations under FESEM. Solid fibers were observed for acidic and neutral conditions. In the neutral polymer system, there is evidence of hollow beads. It is speculated that the rate of the evaporation of the water
might have induced the formation of a hard shell and empty core resulting in the formation of beads.

Overall, WPI and PEO are distributed uniformly to form solid fibers. This entanglement/entrainment/deposition is determined by the facility of entrapment/deposition of WPI along PEO. Thus, if the mechanism by which PEO enables the formation of WPI/PEO fibers is based on the entanglement/entrainment/deposition between the two polymers, would any other polymers that entangle with WPI be able to form electrospun fibers?
Chapter 7. Electrospinning of Hydroxypropylmethylcellulose (HPMC)/WPI Electrospun Fibers

7.1 Introduction

Studies from previous chapters indicated that PEO and WPI played a synergistic role in enabling the electrospinning of WPI/PEO fibers. Data available led to the speculation that WPI entanglement/entrapment/deposition with PEO is responsible for the formation of WPI/PEO electrospun fibers. As long as the polymer system is optimized for electrospinning of PEO, low-molecular weight biopolymers (e.g. hydrolyzed WPI) should be electrospinnable through chain entanglement and/or deposition. The question arises then: does the same relationship exist with other electrospinnable polymers besides PEO? To test this hypothesis, this study was undertaken to investigate another biopolymer that is inherently electrospinnable in aqueous solution.

PEO was chosen from more than 50 synthetic electrospinnable polymers, as a model polymer for this thesis research due to the fact that it has been well characterized in water. Moreover, PEO has been extensively used in electrospinning of various biopolymers by many researchers. Nevertheless in the food industry is restricted since it is not a food grade additive, despite that the polymer is non-toxic and it is commonly used in pharmaceutical applications. To exploit the uses of electrospun fibers for edible applications, this study focused on studying a food grade polymer, hydroxypropylmethylcellulose (HPMC) to
further validate the proposed chain entanglement/entrapment/deposition mechanism.

Although, most biopolymers dissolved in aqueous medium require the aid of PEO to enable the formation of fibers, Frenot et al. reported that HPMC could be electrospun in the absence of PEO. The authors reported that 2.86% HPMC was dissolved in a mixture of water and ethanol (1:1) to enable the formation of smooth electrospun fibers\(^{136}\).

HPMC is a derivative from cellulose, such as from wood and cotton. The production involves exposing cellulose to a series of reactions involving methyl chloride and propylene oxide to produce the desired derivative. HPMC has a relatively high molecular weight between 85 to 150 kDa, and it is soluble in cold water, but not in hot water, acetone nor chloroform. The unique properties of HPMC make it ideal for thickening cold aqueous mixtures\(^{137}\). Health Canada has approved the use of HPMC in foods as thickening agent for human consumption. HPMC has been used in several applications such as drug release\(^{138, 139}\), food coating\(^{140, 141}\), food antistaling and as thickening agents \(^{142, 143}\). Due to its high molecular weight and water solubility, HPMC is ideal as an aid agent for the electrospinning of WPI-containing fibers.

To further validate the proposed mechanism of PEO in the electrospinning of WPI fibers, HPMC was investigated as a replacement for PEO. It is expected that both polymers aid in the electrospinning of WPI-containing fibers by entanglement/entrapment/deposition with PEO during the electrospinning process. It is hypothesized that HPMC will be able to aid in the electrospinning of WPI by the proposed mechanism found for PEO. The objectives for this study
are: (i) to optimize the formulation of WPI-HPMC polymer system to form electrospun fibers; (ii) to evaluate the fiber morphology of the electrospun fibers at various HPMC and WPI concentrations; (iii) to investigate the interaction between WPI and HPMC using Fourier transform infrared analysis.

7.2 Materials

WPI (protein content >90%) was donated by Glanbia Nutritionals, Inc. (Evanston, IL), HPMC (90 kDa) and glacial acetic acid were purchased from Sigma Aldrich Company Canada (Oakville, ON).

7.3 Methods

7.3.1 Solution preparation

Material concentrations in all formulations were in w/w basis. Preliminary experiments were carried out to investigate the electrospinning of HPMC as an independent polymer and to select HPMC and WPI content for further investigations. As controls, WPI powder was dispersed in two different solvents: ethanol-water (50:50) and glacial acetic acid. WPI solutions possessed a concentration of 5 and 10%. HPMC powder was also dispersed in two different solvents: ethanol-water (50:50) and glacial acetic acid. HPMC solutions possessed a concentration of 0.5 and 1%. WPI and HPMC were also dispersed together in ethanol-water (50:50) and glacial acetic acid to give polymer solutions of different concentrations as indicated in the results sections. All polymer
solutions (10 ml) were stirred with the aid of a magnetic stirred overnight (12 h) at room temperature (22 ± 2°C).

7.3.2 Electrospinning

Polymer solutions were electrospun using a vertical electrospinning setup. The polymer solution was delivered to the spinneret at 0.4 ml/h using an infusion pump (Model KD200, KD Scientific, Holliston, MA). The spinneret was connected to the positive electrode, while the stainless steel collector plate was grounded. A constant voltage ranging from +15 to +20 kV was applied to the positive electrode using a DC supply (Model ES30R-5W/DM, Gamma High Voltage Research, Ormand, Beach, FL). The distance between the spinneret and the collector was set at 26 cm for all experiments. Electrospinning was conducted under ambient conditions (22 ± 2°C; 15 ± 5% RH).

7.3.3 Scanning Electron Microscopy (SEM) Analysis

Morphology of the electrospun fiber was examined using a scanning electron microscope (SEM S-570, Hitachi High Technologies Corporation, Tokyo, JP) at an accelerating voltage of 10 kV. Samples were coated with gold/palladium (15 nm) using a sputter coater (Model K550, Emitech, Ashford, Kent, England). The average fiber diameter was determined by taking at least 50 measurements for each image using Image analysis software (Pro-Plus 5.1, Media Cybernetics Inc., Bethesda, MA).
7.3.4 Attenuated Total Reflection –Fourier Transform Infrared (ATR-FTIR) Spectrometry

Polymer films and electrospun fibers were characterized by using a FTIR spectrometer (IRPrestige21, Shimadzu Corp. Japan) equipped with an attenuated total reflection accessory (Pike Tech, Madison, WI.). The test was set at 4 cm\(^{-1}\) resolution and an average of 40 scans was taken for each sample. Solid films and electrospun fibers were placed on the ATR crystal for analysis at room temperature (22 ± 2 °C). The FTIR spectrum in the region of 1700 cm\(^{-1}\) to 1600 cm\(^{-1}\) was curve fitted by using the Gaussian function with the Grams/32 spectral analysis software (Galactic Industries Corp., Salem, NH). The 2\(^{nd}\) derivative function was obtained to determine the wavenumber of the bands of interest.

7.4 Results and discussion

7.4.1 Electrospinning of WPI/HPMC fibers

Preliminary studies showed that HPMC solutions with concentrations ranging between 1 and 2.5% in water and ethanol (50:50) could be electrospun into fibers. At 5% WPI, the addition of greater than 1% HPMC caused the solution to gel, impeding the formation of electrospun fibers. Considering these preliminary studies and the results from the previous chapters, concentration ranges chosen for investigation were: 0.5-1% and 5-10% for HPMC and WPI respectively. The solvents selected for the study of HPMC and WPI were: glacial
acetic acid and water-ethanol (50:50). Glacial Acetic acid was selected for comparison with data from the previous chapters.

In the absence of WPI, 0.5% acidic HPMC solutions were electrosprayed into irregular particles and ultrafine fibers that were connected to the particles (Figure 38). When HPMC concentration was increased to 10%, ultrafine fibers and irregular beads were abundant. These results can be explained by the fundamentals of electrospinning. Key determining factors for electrospinnability of a polymer solution are: viscosity, molecular weight and concentration. At low polymer concentrations, the majority of the solvent molecules are interacting among themselves while only a small fraction is interacting with the polymer. This results in the dominating surface tension effect of the solvent, causing the polymer jet to break into droplets during electrospinning. This phenomenon is more prevalent for 0.5% HPMC polymer solution, where polymer chain entanglement, which is important for stabilizing the polymer jet, is limited during electrospinning. On the other hand, 1% HPMC polymer solution contained more polymer molecules that not only increased the polymer-solvent interaction, but also had an effect of stabilizing the polymer jet through chain entanglement. As a result, more fibers are observed for the 1% HPMC solution than the 0.5% counterpart.
Figure 38. Scanning electron micrographs of fiber electrospun from HPMC acidic solutions at various concentrations: (a) 0.5% HPMC; (b) 1% HPMC. Top row: low magnification; bottom row: high magnification

In comparison, the morphology of electrospun materials for HPMC solution prepared in 50% aqueous ethanol is notably different (Figure 39). As shown, at 0.5%, the presence of beads linked to ultrafine fibers is evident. When compared to 1% HPMC, the amount of beads decreased. In comparison to the acidic polymer system, the water-ethanol mixture as a solvent might have provided a better dispersion of HPMC.
According to the results analyzed in previous chapters, the electrospinning of WPI in the absence of secondary polymers is not feasible regardless of WPI content in the investigated polymer systems. In this experiment, WPI was dissolved in water-ethanol (50:50) and acetic acid as controls for the investigation of electrospinning HPMC and WPI. As shown in Figure 40, WPI was not able to form electrospun fibers in any of the tested polymer systems. Instead of forming fibers, the electrospinning of pure WPI resulted in the production of irregular clusters. As described in previous chapters, the low molecular weight (14-18kDa) impeded the formation of a polymer jet required for the formation of fibers. When the high voltage is applied, free solvent molecules aggregate independent of
WPI. The solvent evaporates, and the WPI clusters are deposited on the collector. This experiment confirms that WPI under the investigated conditions cannot be electrospun into fibers without the aid of a secondary polymer. The results of electrospinning HPMC-WPI mixtures under different systems are discussed as follows.

Figure 40. Scanning electron micrographs of electrospun 10% WPI from different polymer solutions: (a) WPI dissolved in water ethanol (50:50); (b) WPI dissolved in acetic acid. Top row: low magnification; bottom row: high magnification
Figure 41 shows that WPI and HPMC content have a dramatic impact on electrospun material morphology. Solutions at 5% WPI and 0.5% HPMC concentration, predominantly produced irregular beads (Figure 41a). Increasing WPI content to 10% while holding HPMC content at 0.5% caused an increase in number and size of irregular beads (Figure 41b). At higher HPMC content (1%), similar trends were observed except that more fibrous morphologies were observed (Figure 41c and 40d). When HPMC and WPI contents were increased simultaneously to 1% and 10% respectively, the polymer solution did not electrospin, continuously drying at the tip of the spinneret after 1 min of applying high voltage. Because of this phenomenon, the polymer solution containing 1% HPMC and 10% WPI was considered not suitable for electrospinning. Although, most formulations of HPMC and WPI were able to form electrospun fibers, the excessive amount of irregular clusters led to the speculation that WPI tended to form aggregates in this polymer system impeding the entanglement with HPMC.

As explained in the previous chapters, the globular configuration of the protein might have been an obstacle for the entanglement between HPMC and WPI impeding the formation of smooth fibers. Also WPI might have precipitated in aqueous ethanol solution at concentrations higher than 40% ethanol. It has been reported that casein is precipitated with a 40% ethanol treatment while whey protein remained solubilized under these conditions. Morr and Lin reported that 72% ethanol treatment led to the precipitation of 45 to 60% whey proteins. These results suggested that the water-ethanol system was not suitable for
promoting entanglement/entrapment/deposition between HPMC and WPI for improvement in electrospinnability.

Figure 41. Scanning electron micrograph of fibers resulted from the electrospinning of WPI and HPMC in water-ethanol (50:50) at various polymer concentrations. Top row: low magnification; bottom row: high magnification

Replacement of ethanol with glacial acetic acid resulted in dramatic changes in fiber morphology (Figure 42). As shown, when 5% WPI was added to 0.5% HPMC, ultrafine fibers intermingled with irregular beads were produced (Figure 42a), but as WPI and HPMC contents increased (Figure 42c and 41d) ultrafine fibers were formed. In accordance with results from the previous chapters, these findings suggest that WPI might have been unfolded under extreme acidic pH (pH 1) facilitating the entanglement/entrapment/deposition with HPMC to enable the formation of fibers. Similar to the results for PEO, HPMC
acted synergistically with WPI for the formation of fibers, as the two polymers were not able to individually form continuous electrospun fibers under the conditions investigated. The positive effect of HPMC on the electrospinning of WPI is speculated to be similar to PEO, i.e., mainly due to the chain entanglement between WPI and the polymer aid.

Several studies have reported the synergistic effect of polymers blends favouring electrospinning. For example, Zoccola et al. reported that the electrospinning of keratin in formic acid was improved when fibroin was added to the polymer system. They reported that smooth electrospun fibers resulted from
the electrospinning of blends of keratin and fibroin, while beads linked with fiber resulted were produced from the electrospinning of pure keratin\(^\text{147}\). Similar results were observed in previous studies wherein soy protein and PEO acted synergistically in aqueous system for the formation of smooth electrospun fibers, and fiber morphology can be controlled by manipulating polymers concentrations\(^\text{11}\).

A recent study from Yu et al. demonstrated that elasticity determines electrospinnability and fiber morphology\(^\text{84}\). They blended PEO (MW 672-1030 kDa) with poly(ethylene glycol) (PEG;10 kDa) to prepare aqueous Boger fluids, which are fluids that exhibit constant viscosity but different elasticity values. They adopted capillary break-up extensional rheometry to study the elasticity of the fluids, allowing them to isolate the elasticity effect from the other parameters in the investigation. They reported that inelastic PEG liquid resulted in electrospaying of droplets due to Rayleigh instability driven by the surface tension. By contrast, the addition of PEO to PEG aqueous solutions increased the elasticity of the fluids, which suppressed completely or partially the Rayleigh instability before the whipping of the jet, forming smooth fibers or beads linked to elastic fibers.

From a molecular structure standpoint, the HPMC used a molecular weight of 80 kDa comprised of a relatively bulky and stiff backbone of repeated modified glucose monomer units. By contrast, the PEO used had a molecular weight of 900kDa with relatively much flexible backbones made of ethylene oxide...
monomers. Therefore, the modes of chain entanglement between these polymer aids with WPI were likely different.

Regev et al. studied the electrospinning of bovine serum albumin (BSA) of different degrees of denaturation induced by the addition of $\beta$-mercaptoethanol when dissolved in 2,2,2-trifluoroethanol, and they concluded that the fully denatured BSA possessed interfacial viscoelastic properties that were required for the formation of smooth electrospun fibers\textsuperscript{87}. They theorized that denaturation was critical to unfold the protein, allowing it to migrate to the surface of the fluid jet and organize as protein skin layer along the fluid jet when the solvent evaporates. This provides the interfacial viscoelasticity needed for the stabilization of the polymer jet. As a result smooth fibers were produced. These observations from Regev et al are consistent with the current investigation; the smooth fibers obtained from the acid-induced WPI might be attributable, at least partially to the interfacial viscoelasticity properties of the unfolded protein. To gain a deeper understanding of the electrospinning of WPI and HPMC, the interaction between the two polymers was investigated by FTIR analysis and the results are discussed as follows.
7.4.2 Attenuated Total Reflection–Fourier Transform Infrared (ATR-FTIR) Analysis

Figure 43 shows that the IR spectrum of WPI powder is not different from that of electrosprayed particles from 10% WPI prepared in water-ethanol solution suggesting that the exposure of WPI to water-ethanol solution, and the electrospinning process did not have an effect on the chemical structure of WPI. The results confirmed that WPI dissolved in water-ethanol maintained the globular configuration impeding the formation of electrospun fibers. Comparing the frequency of Amide I band (1700-1600 cm\(^{-1}\)) from electrosprayed materials for 10% WPI polymer solutions prepared in water ethanol with 10% WPI polymer solutions prepared in glacial acetic acid, it can be seen that there was a wavenumber shift for the Amide I peak. The wavenumber shift at the absorption band of the amide I band reveals the secondary structure of the protein. In this case, the wavenumber shift was the same as the one reported in chapter 5 (section 5.4.1), since the protein was exposed to the same acidic conditions (pH 1). This frequency shift revealed that the extreme acidic conditions has promoted the unfolding of the protein altering the secondary structure of the protein from β-sheet to α-helix and random coil (section 4.4.1), which might have promoted the entanglement/entrapment/deposition between WPI and HPMC facilitating the formation of electrospun fibers.
Figure 43. ATR-FTIR spectra for: (a) WPI powder; (b) electrospun beads from 10% WPI in water-ethanol (50:50); (c) electrospun beads from 10% WPI in glacial acetic acid

ATR-FTIR spectra of HPMC were also determined to understand the effect of solvent on the polysaccharide. HPMC powder (Figure 44a) shows a broad peak between 1300 and 1000 cm\(^{-1}\) which corresponds to the stretching vibrations of C=O-C and C=O groups, and small peaks between 1200 and 1400 cm\(^{-1}\) correspond to the stretching vibrations of CH, CH\(_2\), and CH\(_3\). Comparing this with the spectrum of electrospun beads/fibers 1% HPMC in aqueous ethanol (Figure 44b) and glacial acetic acid (Figure 44c), there is no evidence of wavenumber shift for the characteristic bands between the spectra. The appearance of additional peaks 1600 cm\(^{-1}\) for samples electrospun from acetic acid likely belongs to the absorption band of the carbonyl group C=O of the residual acetic acid. Based on these observations, one can conclude that the
dispersion of HPMC in aqueous ethanol and glacial acetic acid, as well as the exposure to the high voltage during the electrospinning process, did not have an effect on the molecular properties of the polymer.

Figure 44. ATR-FTIR spectra for: (a) HPMC powder; (b) electrospun bead and fibers from 1% HPMC in water-ethanol (50:50); (c) electrospun beads and fibers from 1% HPMC in glacial acetic acid
Figure 45 and 46 show the FTIR spectra for WPI/HPMC fibers electrospun from aqueous ethanol mixture and glacial acetic acid, respectively. In both polymer systems, there is no evidence of wavenumber shift for the Amide I band (1700-1600 cm\(^{-1}\)) nor the HPMC absorption bands at 1300-1000 cm\(^{-1}\) (C-O-C) caused by changes in polymer concentrations. Hence, ATR-FTIR analysis proved that there is no evidence of chemical interaction evidence between WPI and HPMC under any of the tested conditions that could be detected by this analytical tool. These results are consistent with the previous findings (Chapter 5) regarding the interaction between WPI and PEO. It is speculated that HPMC plays a similar role as PEO in the electrospinning of WPI fibers. HPMC and PEO entrapped/entangled WPI along the polymer jet for the formation of electrospun fibers. When WPI is unfolded by exposure to extreme acidic conditions (pH 1), the entrapment/deposition of WPI is facilitated in favour of the formation of smooth fibers.

Smother fibers were observed from the WPI-PEO acidic system (Figure 13, Chapter 5) when compared to the WPI-HPMC system (Figure 42). The explanation for this difference in morphology can be found in the chemical structure of HPMC and PEO. Monomers in HPMC are bulkier and more rigid when compared to ethyl ether monomers in PEO, which might have reduced the ability of the polysaccharide to interpenetrate and entangle with the protein molecules and/or hydrolyzed peptides. Further investigation is required for the optimization of the electrospinning of WPI and HPMC, but the results obtained
suggested that PEO can be replaced by HPMC for the electrospinning of WPI in acidic polymer systems.

![ATR-FTIR spectra](image)

**Figure 45.** ATR-FTIR spectra for fibers from the electrospinning of HPMC-WPI in water-ethanol (50:50) at various concentrations: (a) 5% WPI, 0.5% HPMC; (b) 10% WPI, 0.5% HPMC; (c) 5% WPI, 1% HPMC; (d) 10% WPI, 1% HPMC
Figure 46. ATR-FTIR spectra for fibers from the electrospinning of HPMC-WPI in glacial acetic acid at various concentrations: (a) 5% WPI, 0.5% HPMC; (b) 10% WPI, 0.5% HPMC; (c) 5% WPI, 1% HPMC; (d) 10% WPI, 1% HPMC
7.5 Conclusions

Results from this chapter showed that HPMC could enable the electrospinning of WPI fibers when the polymers are dissolved in glacial acetic acid. Similar to PEO, synergistic behaviors were observed between HPMC and WPI in fiber formation, but on the basis of FTIR analysis, no specific interactions were detected. The electrospinning-enabling properties of HPMC upon WPI is likely due to entanglement or deposition of WPI in HPMC, similar to that of PEO. It is theorized that the entanglement of PEO and HPMC with WPI enabled the formation of WPI by increasing the elasticity of the polymers solutions. Also, it is speculated that denaturation of WPI might have enhanced the interfacial viscoelastic properties of the fluid in favour of electrospinnability.

For the water-ethanol system, the addition of WPI to the HPMC solutions did not promote the formation of electrospun fibers due to globular configuration of WPI in the water-ethanol system.

For the glacial acetic acid system, the entanglement/entrainment/deposition was likely facilitated by the unfolding of WPI resulting in smoother fibers. As HPMC and WPI contents increased, electrospun fibers became smoother and larger. The extreme acidic conditions caused denaturation and hydrolysis of WPI facilitating the entanglement/entrainment/deposition with HPMC.

It is confirmed that acidic conditions caused a change in the secondary structure of WPI as described in Chapter 5, which is important for entanglement
between WPI and HPMC. The polysaccharide was likely a scaffold to entrap WPI during the electrospinning process forming WPI/HPMC electrospun fibers. In summary, HPMC played the same role as PEO for the electrospinning of low molecular weight molecules. This analysis suggests that HPMC, a food grade additive, can replace PEO.
Chapter 8. Conclusions and Future Work

8.1 Conclusions

Electrospinning of WPI in the absence of a secondary electrospinnable polymer is not feasible under the wide range of conditions used here. PEO was added to WPI polymer systems to enable the formation of electrospun fibers. The addition of PEO to WPI solutions increased the viscosity value in all polymer systems, which it is speculated to be due to a higher chain entanglement between WPI and PEO. The addition of PEO in acidic solution resulted in a dramatic increase in the viscosity value. By contrast, neutral polymer solutions possessed the lowest value of viscosity. It was speculated that a high values in viscosity reflected favorable chain entanglement for the formation of smooth electrospun fibers. Although the addition of PEO caused drastic changes in the viscosity, it does not cause a change in the other solution properties that affected drastically the electrospinning. For example, the acidic polymer solution possessed the lowest value of conductivity, and it was speculated that viscosity and surface tension override the low conductivity properties in the formation of beadless fibers. From the electrospinning fundamentals, low surface tension values are beneficial for electrospinning. In this study, it was found that the low surface tension of the acidic solutions was correlated to the formation of smooth fibers. However, the low values of surface tension were related to the solvent used, and not to the addition of PEO.
Analysis of the results from investigating the effects of the spinneret-collector distance on fiber morphology in the acidic polymer system, led to the speculation that WPI is incorporated along the PEO polymer jet during the bending instability stage in the electrospinning process, the effects of bending instability, which can be controlled by manipulating the spinneret-collector distance, was also related to chain entanglement/entrapment/deposition.

To understand the interaction between PEO and WPI for the formation of the chain entanglement, ATR-FTIR and NMR analyses were conducted. It was found that exposure to extreme acidic and alkaline pH unfolded the protein and caused changes in the secondary configuration of WPI. Wavenumber shifts of Amide I absorption peak in the FTIR analysis indicated that WPI possessed greater proportions of $\alpha$-helix and random coil in comparison to neutral WPI. In accordance, NMR analysis indicated that the $\alpha$-helix protein configuration was dominant under acidic conditions. Evidence of WPI denaturation was correlated to the formation of beadless fibers. When WPI was unfolded, the exposure of functional groups increased facilitating chain entanglement/entrapment/deposition with PEO, and consequently affecting fiber morphology. For the investigation of interactions between WPI and PEO, low content of PEO in the polymer systems, and overlapping of the ether peak of PEO with WPI absorption bands impeded the detection of PEO by FTIR. However, NMR analysis allowed the detection of the PEO peak in the NMR spectra for the different polymer systems. Overall, NMR analysis indicated that
there is no interaction between WPI and PEO in the investigated polymer systems.

For the investigation of whether or not there is also hydrolysis of WPI due to extreme pH, SDS-PAGE and MALDI-TOF mass spectroscopy analyses were conducted. SDS-PAGE electrophoresis confirmed the presence of α-lactalbumin and β-lactoglobulin in the WPI alkaline and neutral fibers. For the acidic conditions, no evidence of a specific band along the SDS-PAGE gel was observed. MALDI-TOF mass spectra for neutral aqueous solutions and fibers proved that the molecular weight distribution of WPI was not affected by the polymer system nor the electrospinning process since the presence of α-lactalbumin and β-lactoglobulin was evident. For the acidic electrospun fiber, MALDI-TOF spectrum suggested that WPI was hydrolyzed since not just the peak of α-lactalbumin and β-lactoglobulin were present but also peaks corresponding to shorter peptides (7, 10, 12 and 16 kDa) were evident. Denaturation and hydrolysis of WPI under acidic conditions might have facilitated the entanglement with PEO.

Experiments to investigate the chain entanglement between WPI and PEO indicated that WPI and PEO were entangled/entrapped for the formation of electrospun fibers. Although, the effect of changes in PEO content had a drastic impact on fiber morphology, WPI content also affected the formation of electrospun experiments. The synergetic effect of WPI and PEO for the formation of smooth electrospun fibers suggested that there is chain entanglement between
the two polymers, which it is likely the mechanism by which PEO enables the formation of fibers.

Studies to characterize the distribution of WPI along PEO were conducted by using STEM-EDAX analysis. In all cases, STEM-EDAX analysis revealed uniform distribution of WPI along the electrospun fibers and beads.

To further understand the formation of electrospun fibers, the cross-section of the electrospun fibers was examined by two methods: (i) fibers were frozen and cross sectioned by a razor blade to be observed by SEM, and (ii) fibers were cross sectioned by using ion beam milling, and they were observed with FESEM. Electrospun fibers from all polymer systems revealed a solid interior. However, hollow beads in the neutral polymer system were observed. The hollow interior in the beads was probably caused by the formation of a shell around the beads due to fast rate of evaporation of the solvent from the surface.

HPMC, a water-soluble food grade polymer, which is able to form electrospun fibers in aqueous solutions was selected to replace PEO for the electrospinning of WPI fibers. In the absence of PEO, HPMC enabled the formation of WPI/HPMC fibers when dissolved in glacial acetic acid. Positive effects of the chain entanglement between WPI and HPMC were evident since pure WPI and HPMC did not form fibers under the conditions investigated. Similar results were not found when a water-ethanol system was used instead of glacial acetic acid. It was attributed to the globular configuration of WPI in the water-ethanol system acting as obstacle for chain entanglement/entrapmen/deposition affecting electrospinnability. Although PEO and HPMC possessed different chemical structure and molecular weights, it is
also speculated that they both entangled/entrapped WPI to increase the elasticity of the fluid for the formation of electrospun fibers.

ATR-FTIR analysis revealed that there is no chemical interaction between WPI and HPMC suggesting that the polysaccharide played similar role as PEO in the electrospinning of WPI fibers. WPI was likely entangled/entrapped with HPMC for the formation of electrospun fibers. HPMC being a food grade polymer, can potentially replace PEO in the electrospinning of WPI.
8.2 Future work

According to the findings of this study, the following experiments are proposed to further investigate the mechanism by which PEO enable the electrospinning of aqueous biopolymer systems:

1. **Depth studies of elasticity of the polymer solutions**

   Elasticity of the polymer solutions can be studied by complex rheological techniques such as the use of the capillary extensional rheometer. These studies can be designed to understand the viscoelasticity of polymers in relation to electrospinnability, which may contribute to better elucidation of the mechanism by which PEO enables the formation of the polymer jet in company of low molecular weight biopolymers.

2. **Further investigation of the effects of high voltage on the chain entanglement between polymers**

   To our knowledge, the exploration of the influence of the electric field on the entanglement of polymers is still unclear. Designing of experiments to investigate the effects on polymers chain entanglement when the high voltage is applied is challenging due to very dynamics of the process. However, the strategic use of stains for polymers as well as high speed cameras might be helpful for this investigation. Differences in chain entanglement before and after the application of high voltage will contribute to understand in depth the role of PEO as a carrier of biopolymers during electrospinning.
3. **Further investigation of the incorporation of WPI during the bending instability**

Previous findings suggested that WPI also entangles with the PEO polymer jet during the whipping/bending instability, but further experiments are needed to confirm this statement. The use of markers on the WPI proteins might help to follow the process of integration of WPI along the PEO polymer jet. These experiments will contribute to increase understanding on the entanglement/deposition of WPI with/on PEO jet during the bending instability.

4. **Optimization of the formulation for the electrospinning of WPI/HPMC electrospun fibers**

HPMC enables the formation of electrospun fibers but optimization in the formulation of the polymer system will allow the manipulation of the fiber morphology. For this investigation, other solvents that are able to disperse both polymers can be tested as well as different concentration of both polymers. According to the intended application, fiber morphology can be manipulated by electrospinning parameters. The investigation of the use of food grade electrospun fibers opens the application fields for these relatively novel products.
5. **Investigation of the electrospinning of other biopolymers to be electrospun with the aid of HPMC**

Results of this investigation suggest that any other low molecular biopolymer can be electrospun with the aid of an electrospinnable secondary polymer. The design of experiments including other biopolymers that cannot be electrospun without the aid of PEO, and the replacement of PEO by HPMC should be conducted.

6. **Investigation of other replacements for PEO**

HPMC was chosen as a replacement, but other food grade polymers must also be able to play the same role. The investigation of the electrospinning of low molecular weight aqueous biopolymer with the aid of others food grade electrospinnable food grade biopolymers should be conducted.
Chapter 9. References


44. Lusse, S., K. A. 1996. The interaction of poly(ethylene glycol) with water studied by \(^1\)H and \(^2\)H NMR relaxation time measurements. Macromolecules 29: 4251.


Chapter 10. Appendixes

10.1 Statistics analysis of solution properties data

In this section, examples of the statistics analysis are given for the different polymer systems. Due to the numerous tables resulting from the analysis, representative tables were selected.

10.1.1 Statistics analysis of the viscosity data

Table 4. Paired comparisons of the viscosity data of polymer solutions in the absence and presence of PEO

<table>
<thead>
<tr>
<th>Pair</th>
<th>Type</th>
<th>Paired Differences</th>
<th>95% Confidence Interval of the Difference</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Mean</td>
<td>Std. Deviation</td>
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<td>.02397</td>
</tr>
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<td>Pair 2</td>
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<td>.02675</td>
</tr>
<tr>
<td>Pair 3</td>
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<td>.03874</td>
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Table 5. ANOVA for the viscosity data of acidic polymer solutions in the presence of PEO

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<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
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<td>.116</td>
<td>46.714</td>
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<tr>
<td>Within Groups</td>
<td>.020</td>
<td>8</td>
<td>.002</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>.368</td>
<td>11</td>
<td></td>
<td></td>
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Table 6. Multiple comparisons by Tukey test for the viscosity data of acidic solutions in the presence of PEO at different cross head speed

<table>
<thead>
<tr>
<th>(I) speed</th>
<th>(J) speed</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
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<td>.17924</td>
<td>.04070</td>
<td>.010</td>
<td>.0489 - .3096</td>
</tr>
<tr>
<td></td>
<td>400.00</td>
<td>.36448</td>
<td>.04070</td>
<td>.000</td>
<td>.2341 - .4948</td>
</tr>
<tr>
<td></td>
<td>500.00</td>
<td>.43850</td>
<td>.04070</td>
<td>.000</td>
<td>.3082 - .5688</td>
</tr>
<tr>
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<td>200.00</td>
<td>-.17924</td>
<td>.04070</td>
<td>.010</td>
<td>-.3096 - -.0489</td>
</tr>
<tr>
<td></td>
<td>400.00</td>
<td>.18524</td>
<td>.04070</td>
<td>.008</td>
<td>.0549 - .3156</td>
</tr>
<tr>
<td></td>
<td>500.00</td>
<td>.25926</td>
<td>.04070</td>
<td>.001</td>
<td>.1289 - .3896</td>
</tr>
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<td>.04070</td>
<td>.000</td>
<td>-.4948 - -.2341</td>
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<tr>
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<td>.04070</td>
<td>.008</td>
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<td>.04070</td>
<td>.332</td>
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<td>.04070</td>
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<td>.04070</td>
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Table 7. ANOVA for the viscosity data of neutral polymer solutions in the absence of PEO

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<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
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</thead>
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<td>Total</td>
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Table 8. Multiple Comparisons by Tukey test for the viscosity data of neutral polymer solutions in the absence of PEO

<table>
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<th>(I) speed</th>
<th>(J) speed</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
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</thead>
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<td>.01486</td>
<td>.992</td>
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### 10.1.2 Statistics analysis of the surface tension data

Table 9. Paired comparisons of the surface tension data of polymer solutions in the presence and absence of PEO

<table>
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<tr>
<th>Pair</th>
<th>Condition</th>
<th>Paired Differences</th>
<th>95% Confidence Interval of the Difference</th>
<th>Sig. (2-tailed)</th>
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<td></td>
<td>Mean</td>
<td>Std. Error</td>
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<td>Lower</td>
</tr>
<tr>
<td>Pair 1</td>
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<td>.11500</td>
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<tr>
<td>Pair 2</td>
<td>Alkaline 1.28200</td>
<td>.12911 .07454</td>
<td>.96128</td>
<td>1.60272</td>
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<tr>
<td>Pair 3</td>
<td>Acidic -.31600</td>
<td>.01442 .00833</td>
<td>-.35183</td>
<td>-.28017</td>
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</tbody>
</table>

Table 10. ANOVA for the surface tension data of acidic polymer solutions in the absence of PEO

<table>
<thead>
<tr>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
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</thead>
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Table 11. Multiple comparisons by Tukey Test for surface tension data of acidic polymer solutions in the absence of PEO.

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* The mean difference is significant at the 0.05 level.
### 10.1.3. Statistics analysis of the conductivity data

**Table 12. Paired comparison of the conductivity data of the different polymer solutions**

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<th>Std. Error Mean</th>
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<th>T</th>
<th>df</th>
<th>Sig.</th>
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<td>-9.90943 to -9.05590</td>
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*where nn: neutral in the absence of PEO; np: neutral in the presence of PEO; ak: alkaline in the absence of PEO; akp: alkaline in the presence of PEO; ac: acidic in the absence of PEO; acp: acidic in the presence of PEO.
10.2 Effects of different speeds of the rotational drum as a collector for neutral electrospun fibers

To investigate the effects of different speeds of the rotational drum on the fiber morphology, neutral polymer solution were electrospun at the previously established conditions (section 4.2.2) using a dynamic drum instead of a static metal collector. As shown in Figure 47, there is no difference in morphology when the fibers were collected at different speeds (200-1000 rpm). At 1000 rpm, SEM micrographs at low magnification (Figure 47d), indicated that a considerable proportion of fibers not were randomly collection but aligned. Although beads linked with ultrafine fibers were aligned, the fiber morphology was not affected. These results are coherent with the study of the spinneret-collector distance effects (section 4.6.4), in which it was observed that neutral fibers adopted and dried the final morphology at a very short spinneret-collector distance (10 cm). When the fiber is complete dried, the collection of the fibers at high speed (200-1000 rpm) did not have any other effect on it. It is speculated that if electrospun fibers were wet, collection of the fibers with a drum at different speeds would have an effect on fiber morphology.
Figure 47. Scanning electron micrographs of fibers electrospun from 10% WPI and 0.4% aqueous neutral solution collected with a rotational drum at different speeds: (a) 200 rpm; (b) 400 rpm; (c) 1000 rpm and (d) 1000 rpm at different magnifications. Top row: low magnification; bottom row: high magnification
10.4 Tensile strength of acidic electrospun fibers

To further characterize electrospun fibers, experiments to evaluate the mechanical properties of the fibers were carried out. Tensile strength of acidic electrospun fibers was feasible. However, neutral and alkaline fibers were smaller (section 4.6.1) and their fragility impeded mechanical characterizations due to limitations of the nano-tensile equipment.

To determine the tensile strength of acidic fibers, the following methodology was applied.

- Alignment of the acidic fibers during the electrospinning process. To align the fibers, two rectangular metal plates with a length of 5 cm and a width of 2 cm were used as a fibers collector. The metal places were placed at a distance of 26 from the spinneret, and with a 2 cm gap between the plates. Electrospun fibers were forced to be deposited in between the plates promoting the alignment of the fibers.

- Aligned fibers were collected using a cardboard frame (Figure 48). Cyanoacrylate adhesive (Krazy Glue ®) was applied on the cardboard frame to fix the electrospun fibers.

- A single fiber or several fibers were selected, removing the undesirable fibers.
- The cardboard frame with the single was placed in the nano tensile tester instrument (Nano Bionix tensile tester, MTS Systems Corp, Oak Ridge, TN).

- Tensile tester was carried out applying a tension trigger of 1 uN, and a strain rate of 0.0005 1/s.

Results of the tensile strength for acidic electrospun fibers are shown in Figure 49. Although, it was not feasible to compare the tensile strength of the different polymer system, the methodology could be used for future studies.

Figure 48. Illustration of the cardboard frame to fix a single electrospun fiber for tensile testing
Figure 49. Plot of Stress vs Strain for fibers with a diameter of 1400 nm from electrospinning 12.5% WPI, 0.4% PEO in glacial acetic acid. Replicas of test are represented by (a), (b), and (c).