ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

BIOACTIVE PEPTIDE ENCAPSULATION BY ELECTROSPINNING TECHNIQUE: CHARACTERIZATION OF ELECTROSPUN FIBERS AND MATHEMATICAL MODELLING OF RELEASE KINETICS

Ph.D. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

BİYOAKTİF PEPTİTLERİN ELEKTROEĞİRME TEKNİĞİ İLE ENKAPSÜLASYONU: ELEKTROEĞRİLMİŞ LİFLERİN KARAKTERİZASYONU VE SALIM KİNETİĞİNİN MATEMATİKSEL MODELLENMESİ

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Zahide KIRBAŞ, a Ph.D. student of İTU Graduate School student ID 506152509, successfully defended the thesis/dissertation entitled "BIOACTIVE PEPTIDE ENCAPSULATION BY ELECTROSPINNING TECHNIQUE: CHARACTERIZATION OF ELECTROSPUN FIBERS AND MATHEMATICAL MODELLING OF RELEASE KINETICS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my daughter,

FOREWORD

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Zahide KIRBAŞ
(Food Engineer, M.Sc.)

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ABBREVIATIONS

ABTS : 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)
ACE : Angiotensin-Converting Enzyme
ACN : Acetonitrile
ANOVA : Analysis of Variance
ATR : Attenuated Total Reflection
BHT : Butylated Hydroxytoluene
Ca : Calcium
$CaCl_2$: Calcium Chloride
Car : Carnosine
CE : Coaxial Electrospinning
CENFs : Coaxial Electrospun Nanofibers
CUPRAC : Cupric İon Reducing Antioxidant Capacity
DCM : Dicloro Metane
DMF : Dimethylformamide
DPPH : 2,2-diphenyl-1-picrylhydrazyl
EC : Electrical Conductivity
EE : Emulsion Electrospinning
EENFs : Emulsion Electrospun Nanofibers
FTIR : Fourier-Transform Infrared Spectroscopy
GAE : Gallic Acid Equivalents
HCl : Hydrogen Chloride
HHP : High Hydrostatic Pressure Processing
HLB : Hydrophilic-Lipophilic Balance
HPLC : High Performance Liquid Chromatography
IC_{50} : The Half Maximal Inhibitory Concentration
KCl : Potassium Chloride
KH_2PO_4 : Monopotassium Phosphate
MCT : Medium-Chain Triglycerides
$MgCl_2(H_2O)_6$:Magnesium Chloride Heksa Hydrates
NaAlg : Sodium Alginate
$NaHCO_3$: Sodium Bicarbonate
NaOH : Sodium Hydroxide
NFs : Nanofibers
NH_4Ac :Ammonium Acetate
$(NH_4)_2CO_3$: Ammonium Carbonate
O/W : Oil-in-Water
$O_1/W/O_2$: Oil-in-Water-in-Oil
PCL : Polycaprolactone
PELA : Poly(ethylene glycol)-Poly(L-lactide)
PEO : Poly(ethylene oxide)
PGPR : Polyglycerol Polyricinoleate
PLGA : Poly [(D,L-lactic acid)-co-(glycolic acid)]

Pul : Pullulan
SEM : Scanning Electron Microscopy
SGF : Simulated Gastric Fluid
SGI : Simulated Gastrointestinal Tract
SIF : Simulated Intestinal Fluid
SO : Sunflower Oil
SPM : Standard Peptide Mixture
TE : Trolox® Equivalent
TFA : Trifluoroacetic Acid
Triton X-100 : 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol
Trolox® : 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Tween® 80 : Polyoxyethylene (80) Sorbitan Monooleate
UE : Unixial Electrospinning
UENFs : Unixial Electrospun Nanofibers
W/O : Water-in-Oil
$W_1/O/W_2$: Water-in-Oil-in-Water

SYMBOLS

a, b	**:** Scale parameter and shape parameter in Weibull model
A, B	**:** Diffusion and erosion rate constants in Kopcha model
G	**:** Gauge
GHz	**:** Giga Hertz
k	**:** Korsmeyer-Peppas rate constant
K	**:** Consistency index
kV	**:** kilovolt
K_1, K_2	**:** Fickian-diffusion rate constant and erosion rate constant in Peppas-Sahlin model
m	**:** milli (10^{-3})
M	**:** Molar
MHz	**:** Mega Hertz
M_t	**:** Released amount at time t
M_∞	**:** Total released amount
n	**:** nano (10^{-9})
n	**:** Flow behavior index
n	**:** release exponent in Korsmeyer-Peppas model
N	**:** Newton
Pa	**:** Pascal
ppm	**:** parts per million
Q	**:** Volumetric flow rate
r	**:** Correlation coefficients
R	**:** Radius
rpm	**:** revolutions per minute
S	**:** Simens
t	**:** time
T	**:** Lag time of the release process in Weibull model
w	**:** weight
$X(t)$	**:** Percent of dissolved material at time t in Gompertz model
X_{max}	**:** Maximum dissolution in Gompertz model
μ	**:** micro (10^{-6})
ε'	**:** Dielectric constant
τ	**:** Shear stress
$\dot{\gamma}$	**: S**hear rate
η	**:** Apparent viscosity
$\dot{\gamma}_{max}$	**:** Maximum shear rate
ξ	**:** Zeta potential

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BIOACTIVE PEPTIDE ENCAPSULATION BY ELECTROSPINNING TECHNIQUE: CHARACTERIZATION OF ELECTROSPUN FIBERS AND MATHEMATICAL MODELLING OF RELEASE KINETICS

SUMMARY

Bioactive peptides, which are biologically active amino acid groups in the sequence of proteins, exhibit a variety of beneficial effects including antioxidant, anti-inflammatory, antihypertensive, anticancer, antidiabetic, antimicrobial, antithrombotic, hypocholesterolemic, antiaging and opioid activities as well as prevention of cancer, osteoporosis, hypertension, cardiovascular disorders and neurodegenerative diseases such as Parkinson and Alzheimer's diseases. However, the bioactive peptides isolated from plants and animals may be lost during processing and storage. Furthermore, bioactive peptides have short *in vivo* half-lives, low bioavailability and poor stability against gastrointestinal conditions. Therefore, to use of encapsulation technologies such as coacervation, ionic gelation, electrospraying, microfluidic, emulsification, liposomal encapsulation, spray drying and electrospinning have been started to become widespread.

Considering the above, the objectives of this Ph.D. thesis were (i) to produce a nanofibrous delivery vehicles for bioactive peptides without using any synthetic polymers or any hazardous solvents by using electrospinning, to characterize electrospun fibers to evaluate the effect of formulation and properties of feed solutions on electrospinnability and to examine the encapsulation efficiencies of produced nanofibrous delivery vehicles by using a model peptide; (ii) to produce carnosine (Car) loaded water-in-oil-in-water ($W_1/O/W_2$) double emulsions with different formulations using as feed emulsions in emulsion electrospinning study; (iii) to produce carnosine (Car), an antioxidative peptide, loaded pullulan (Pul)-sodium alginate (NaAlg) based composite nanofibers by uniaxial (blending), coaxial and emulsion electrospinning techniques and to characterize electrospun fibers to evaluate the effect of solution /emulsion properties and the role of emulsion parameters; (iv) examining the encapsulation efficiencies of electrospun fibers and to investigate the effect of encapsulation on antioxidant activity of Car; to determine the effects of electrospinning encapsulation and crosslinking on release behavior of carnosine from electrospun nanofibers during *in vitro* digestion and to analyse the release kinetics by establishing corresponding mathematic models.

To achieve these goals, two different experiments (Chapters 3-4) were conducted. In the first part of the thesis (Chapter 1), research framework and objectives of this Ph.D thesis, are introduced. Following that, in the second part of the thesis (Chapter 2), a comprehensive review on biological activities of food-derived peptides and the encapsulation methodologies used to protection and delivery of them. Initially, the sources and biological activities of food-derived peptides were presented with a specific focus on the antioxidative peptides and Car. Then, the studies about encapsulation methods for bioactive peptides have been rewieved. Lastly, electrospinning was scrutinized as an encapsulation method and the studies about

encapsulation of bioactive peptides by single/uniaxial, coaxial-triaxial and emulsion electrospinning techniques regarding principles, advantages, drawbacks and applications have been reviewed.

In the third part of the thesis (Chapter 3); Pul, Pul-NaAlg and Pul-NaAlg-$CaCl_2$ based composite nanofibers were produced by uniaxial electrospinning, without using any synthetic polymers or any hazardous solvents. Then, scanning electron microscopy (SEM) and image analysis were performed to assess the morphology of the fibers and to evaluate the effects of electrical, rheological and surface properties of feed solutions on electrospinnability. Finally, standard peptide mixture (SPM), as model peptide, was encapsulated to realize encapsulation of peptides and to evaluate the encapsulation efficiencies of electrospun fibers. High Performance Liquid Chromatography (HPLC) analysis were carried out to determine the encapsulation efficiencies. According to the results, Pul-NaAlg based uniaxial electrospun fibers was successfully fabrricated and SPM was encapsulated with 77.97 % encapsulation efficiency . Thus, biodegradable and non-toxic nanofibers made only of food-derived polysaccharides could be produced for encapsulation of peptides without using any synthetic polymers and /or harmful solvents. On the other hand, NaAlg and $CaCl_2$ improved electrospinnability of pullulan with enhanced the solution properties such as viscosity, surface tention and electrical conductivity; thus SPM-loaded Pul-NaAlg-$CaCl_2$ composite nanofibers were successfully produced under pre-determined proper conditions. Additionally, incorporation of $CaCl_2$ decreased mean diameter and improved stability of electrospun fibers possibly due to intermolecular interactions between NaAlg and $CaCl_2$.

In the fourth part of the thesis (Chapter 4); Car-loaded $W_1/O/W_2$ double emulsions were produced by using different surfectants, oily phases and Car levels to use in emulsion electrospinning study, nanofibrous delivery vehicles for Car antioxidative peptide were produced without using any synthetic polymers or any hazardous solvents and Pul-NaAlg-Car composite fibers were obtained by uniaxial, coaxial and emulsion electrospinning techniques. Ionic crosslinking was applied by $CaCl_2$ as crosslinking agent to improved stabilities of nanofibers. Then, SEM and image analysis were performed to assess the morphology of the electrospun nanofibers and to evaluate the effects of electrical, rheological and surface properties of feed solutions/emulsions on electrospinnability. Additionally, zeta potential, mean droplet size measurements and optical microscopy were performed to assess the role of emulsion parameters on electrospun fibers. Later, contact angle measurements were performed to characterize the hydrophobicity and stability of the resulting nanofiber mats and the mechanism of crosslinking and possible other interactions between components involved in the emulsion structure were investigated by Fourier-transform infrared spectroscopy (FTIR). Afterwards, the encapsulation efficiencies were determined by HPLC analysis and the effect of electrospinning encapsulation on antioxidant activity of Car was evaluated according to antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and antioxidant activity by cupric ion reducing antioxidant capacity (CUPRAC) assays. Latly, the release behavior of Car was investigated by *in vitro* digestion study and its release kinetics were analyzed by establishing a corresponding mathematical models. According to the results, Car-loaded Pul-NaAlg based composite fibers were produced successfully by uniaxial (blending), coaxial and emulsion electrospinning techniques with 74.11, 81.69 and 68.63% encapsulation efficiencies, respectively. The zeta potential results of double emulsions demonstrated that all emulsions can be considered as stable and incorporation of soy lecithin increased the absolute value of zeta potential, thus

enhancing the stability of emulsions. The microscopy results indicated that the type B $W_1/O/W_2$ double emulsion was successfully obtained and several small inner water droplets were encapsulated in the relatively larger oil globules. Addition to these, DPPH and CUPRAC assays results revealed that all electrospinning methods demonstrated the protective effect on the antioxidant activity of Car and thus helped in enhancing its antioxidant potential significantly. Results showed that, ionic crosslinking improved stability of nanofiber mats. In fact, the SEM analysis provides evidence that, among all emulsion electrospun nanofibers (EENFs), the EE-VIII NFs were characterized by fine, beadless and uniform nanofibrous structure, and were thus selected to apply crosslinking and some further characterizations. According to the results, crosslinked nanofibers, except for the coaxial electrospun nanofibers (CENFs) were able to largely preserve their well-defined nanofibrous structure after crosslinking, while the morphologies are quite different from the uncurving shape of the non-threated ones. Furthermore, fiber diameters of nanofibers were increased after crosslinking treatment, except for the EE-VIII NFs which retained their original size. According to *in vitro* digestion results, it can be stated that the $CaCl_2$ crosslinking treatment enhanced sustained release of Car in the gastrointestinal tract. The initial burst release of Car from EE-VIII NFs was significantly lower than uniaxial electrospun nanofibers (UENFs) and CENFs in both gastric phases, besides the release from EE-VIII NFs in intestinal phase was followed by sustained release, with/without crosslinking treatment. The results provided by mathematical modelling of release kinetics revealed that, Fickian diffusion mechanism was predominant in releasing Car from Pul-NaAlg NFs in the gastrointestinal tract.

Finally, in the fifth part of the thesis (Chapter 5), based on the outcomes of the previous chapters, all results obtained within this work were evaluated together, conclusions and recommendations for future research are provided. The main conclusions derived in each section were summarized including Pul-NaAlg-$CaCl_2$ based composite nanofibers can be considered as a promising material to encapsulate various bioactive peptides with a variety of health effects for food purposes and simultaneously encapsulation of bioactive peptides in $W_1/O/W_2$ double emulsions and in Pul-NaAlg nanofibers provided sustained release during *in vitro* gastrointestinal conditions.

BİYOAKTİF PEPTİTLERİN ELEKTROEĞİRME TEKNİĞİ İLE ENKAPSÜLASYONU: ELEKTROEĞRİLMİŞ LİFLERİN KARAKTERİZASYONU VE SALIM KİNETİĞİNİN MATEMATİKSEL MODELLENMESİ

ÖZET

Biyoaktif peptitler, protein diziliminde yer alan biyolojik olarak aktif amino asit grupları olup, antioksidan, antiinflamatuar, antihipertansif, antikanser, antidiyabetik, antimikrobiyal, antitrombotik, hipoglisemik, yaşlanma karşıtı ve opioid aktiviteler ile kanser, osteoporoz, hipertansiyon, kardiyovasküler hastalıklar ile Parkinson ve Alzheimer gibi nörodejeneratif hastalıkların önlenmesi gibi pek çok faydalı etkiye sahiptir. Bununla birlikte, bitkiler ve hayvanlardan isole edilen biyoaktif peptitler işleme ve depolama sırasında kayba uğramaktadır. Ayrıca, biyoaktif peptitler gastrointestinal sindirim koşullarına karşı kısa *in vivo* yarılanma ömrü, düşük biyoyararlık ve zayıf stabiliteye sahiptirler. Bu nedenle, koaservasyon, iyonik jelleşme, elektro püskürtme, mikroakışkan, emülsifikasyon, lipozomal enkapsülasyon, püskürtmeli kurutma ve elektroeğirme gibi enkapsülasyon teknolojilerinin kullanımı yaygınlaşmaya başlamıştır.

Yukarıdakiler göz önüne alındığında, bu doktora tezinin hedehleri; (i) elektroeğirme yöntemi kullanılarak herhangi bir sentetik polimer veya zararlı çözgen kullanmaksızın biyoaktif peptitler için nanolif yapıda salım araçları üretmek, besleme çözeltisi formülasyonları ve özelliklerinin elektroeğrilebiliklik üzerine etkilerini değerlendirebilmek için elde edilen nanolifleri karakterize etmek ve model bir peptit kullanarak üretilen nanolif yapıdaki salım araçlarının enkapsülasyon etkinliklerini incelemek; (ii) emülsiyon elektroeğirme çalışmasında besleme emülsiyonu olarak kullanılmak üzere farklı formülasyonlara sahip karnosin yüklenmiş su-içinde-yağ-içinde-su ($W_1/O/W_2$) çift katlı/çoklu emülsiyonları üretmek; (iii) tek eksenli (karışım), çift eksenli ve emülsiyon elektroeğirme yöntemleri kullanılarak antioksidatif bir peptit olan karnosin (Car) yüklenmiş pullulan (Pul)-sodyum aljinat (NaAlg) bazlı kompozit nanolifler üretmek ve çözelti/emülsiyon özellikleri ve emülsiyon parametrelerinin etkisini incelemek amacıyle elde edilen nanolifleri karakterize etmek; (iv) nanoliflerin enkapsülasyon etkinliklerini incelemek ve enkapsülasyon işleminin karnosinin antioksidan aktivitesi üzerine etkilerini incelemek; elektroeğirme yöntemi ile enkapsülasyon ve çapraz bağlama işlemlerinin karnosinin *in vitro* simdirim sırasında salım davranışı üzerine etkilerini tespit etmek ve uygun ilgili matematiksel modeller vasıtasıyla salım kinetiğini analiz etmektir.

Yukarıdaki hedeflere ulaşmak için iki farklı deneysel çalışma (Bölüm 3–4) yapılmıştır. Tezin birinci kısmında (Bölüm 1), bu doktora tezinin araştırma çerçevesi ve hedefleri tanıtılmıştır. Bunu takiben, tezin ikinci kısmında (Bölüm 2), gıdalardan elde edilen peptitlerin biyolojik aktiviteleri ve bunların korunması ve salımında kullanılan enkapsülasyon yöntemlerine ilişkin kapsamlı bir derleme sunulmuştur. Başlangıç olarak, antioksidatif peptitler ve karnosin başta olmak üzere, gıdalardan elde edilen peptitlerin kaynakları ve biyolojik aktiviteleri özellikle antioksidatif peptitlerden bahsedilmiştir. Sonrasında, biyoaktif peptitlerin enkapsülasyonunda kullanılan

yöntemlere ilişkin çalışmalar derlenmiştir. Son olarak, elektroeğirme bir enkapsülasyon yöntemi olarak irdelenmiş ve biyoaktif peptitlerin enkapsülasyonunda kullanılan tek eksenli, çift-üç eksenli ve emülsiyon elektroeğirme tekniklerinin prensipleri, avantajları, dezavantajları ve uygulamaları ile ilgili çalışmalar derlenmiştir.

Tezin üçüncü kısmında (Bölüm 3), Pul, Pul-NaAlg and Pul-NaAlg-$CaCl_2$ kompozit nanolifler tek eksenli, çift eksenli ve emülsiyon elektroeğirme yöntemleri kullanılarak herhangi bir sentetik polimer veya zararlı çözgen kullanılmaksızın üretilmiştir. Sonrasında, nanoliflerin morfolojisini incelemek ve besleme çözeltilerinin elektriksel, reolojik ve yüzey özelliklerinin elektroeğrilebilirlik üzerine etkilerini değerlendirebilmek için taramalı elektron mikroskopisi (SEM) ve görüntü analizi gerçekleştirilmiştir. Son olarak, üretilen nanoliflerle peptit enkapsülasyonunu gerçekleştirmek ve enkapsülasyon etkinliklerini değerlendirmek amacıyla standart peptit karışımı (SPM) model bir peptit olarak enkapsüle edilmiştir. Enkapsülasyon Etkinliğinin belirlenmesi için HPLC analizi gerçekleştirilmiştir. Sonuç olarak, Pul-NaAlg bazlı tek eksenli nanolifler başarılı bir şekilde üretilmiş ve SPM %77,97 enkapsülasyon etkinliği ile enkapsüle edilmiştir. Böylece, peptit enkapsülasyonu için sadece gıda kaynaklı, polisakkaritlerle, herhangi bir sentetik polimer veya zararlı çözgen kullanılmaksızın, biyobozunur ve toksik olmayan nanolifler üretilebilmiştir. Bununla birlikte, NaAlg ve $CaCl_2$, besleme çözeltilerinin viskozite, yüzey gerilimi ve elektriksel iletkenlik gibi bir takım özelliklerini geliştirerek pullulanın elektroeğrilebirliğini iyileştirmiş; böylece SPM-yüklü Pul-NaAlg-$CaCl_2$ kompozit nanolifler önceden belirlenen uygun koşullar altında başarılı bir şekilde imal edilmiştir. Ayrıca, $CaCl_2$ ilavesinin nanoliflerin ortalama çapını azalttığı stabilitesini iyileştirdiği tespit edilmiş olup, bu durum NaAlg and $CaCl_2$ arasında gerçekleşen moleküllerarası etkileşime bağlanabilir.

Tezin dördüncü kısmında (Bölüm 4), emülsiyon elektroeğirme çalışmasıda kullanılmak üzere farklı sürfaktanlar, yağ fazları ve karnosin içerikleriyle karnosin yüklü $W_1/O/W_2$ çift katlı/çoklu emülsiyonları hazırlanmış, karnosin antioksidatif peptidinin salımı için geliştirilen nanolifler herhangi bir sentetik polimer veya zararlı çözgen kullanılmaksızın üretilmiş ve Pul, Pul-NaAlg and Pul-NaAlg-$CaCl_2$ kompozit nanolifler tek eksenli, çift eksenli ve emülsiyon elektroeğirme yöntemleri kullanılarak herhangi bir sentetik polimer veya zararlı çözgen kullanılmaksızın üretilmiş olup, Pul-NaAlg-Car kompozit nanolifler tek eksenli, çift eksenli ve emülsiyon elektroeğirme teknikleri ile elde edilmiştir. Elde edilen nanoliflerin stabilitelerini geliştirmek için $CaCl_2$ ile iyonik çapraz bağlama işlemi uygulanmıştır. Sonrasında, nanoliflerin morfolojisini incelemek ve besleme çözeltilerinin elektriksel, reolojik ve yüzey özelliklerinin elektroeğrilebilirlik üzerine etkilerini değerlendirebilmek için taramalı elektron mikroskopisi (SEM) ve görüntü analizi gerçekleştirilmiştir. Buna ek olarak, emülsiyon parametrelerinin nanolifler üzerindeki etkilerini değerlendirmek amacıyla çift katlı/çoklu emülsiyonların zeta potansiyel ve ortalama damlacık boyutu ölçümleri gerçekleştirilmiş ve optik mikroskopi ile emülsiyonlar incelenmiştir. Ayrıca, elde edilen nanoliflerin hidrofobisite ve stabilitelerinin karakterizasyonu için temas açısı ölçümleri gerçekleştirilmiş ve çapraz bağlamanın mekanizması ve emülsiyon yapısında yer alan bileşikler arasındaki olası diğer etkileşimler Fourier-dönüşümlü kızılötesi spektroskopisi (FTIR) metoduyla incelenmiştir. Daha sonra, enkapsülasyon etkinlikleri HPLC analizi ile belirlenmiş ve elektroeğirme yöntemiyle enkapsülasyonun karnosinin antioksidan aktivitesi üzerine olan etkileri 2,2-diphenyl-1- picrylhydrazyl (DPPH) tarafından radikal yakalama deneyi ve kuprik iyon

indirgeme antioksidan kapasite (CUPRAC) tayinine göre değerlendirilmiştir. Son olarak, karnosinin salım davranışı *in vitro* sindirim çalışmasıyla incelenmiş ve uygun ilgili matematiksel modeller vasıtasıyla salım kinetiğini analiz edilmiştir. Sonuç olarak, karnosin yüklü Pul-NaAlg nanolifler tek eksenli (karışım), çift eksenli ve emülsiyon elektroeğirme teknikleri kullanılarak, sırasıyla %74,11, 81,69 ve 68,63 enkapsülasyon etkinliği ile başarılı bir şekilde üretilmiştir. Emülsiyonların zeta potansiyel sonuçları, tüm emülsiyonların stabil olarak kabul edilebileceğini ve emülsiyonlara ilave edilen soya lesitini miktarının artmasıyla zeta potansiyelinin mutlak değerinin arttığı ve böylece emülsiyonların stabilitesini geliştiğini göstermektedir. Mikroskopi sonuçları ise, birçok küçük iç su damlacıklarının görece büyük yağ globülleri içinde yer aldığı ve böyle B tipi $W_1/O/W_2$ çift katlı/çoklu emülsiyonların başarılı bir şeklide elde edildiğini göstermektedir. Bunlara ek olarak, DPPH ve CUPRAC analiz sonuçları elektroeğirme töntemi ile enkapsülasyonun karnosinin antioksidan özellikleri üzerine koruyucu etki gösterdiğini ve böylece antioksidan potansiyelin geliştirilmesine katkı sağladığını ortaya koymuştur. SEM sonuçlarından elde edilen bilgiyle, emülsiyon elektroeğirme ile elde edilen nanolifler (EENFs) arasında en uygun nanolif özelliklerine (ince, boncuksuz ve tekdüze) sahip olması nedeniyle EE-VIII NFs, tek eksenli nanolifler (UENFs) ve çift eksenli nanoliflerle (CENFs) birlikte çapraz bağlama işlemine tabi tutulmuş olup, daha ileri karakterizasyon çalışmaları için şeçilmiştir. Sonuçlar iyonik çapraz bağlamanın nanoliflerin stabilitesini iyileştirdiğini göstermektedir. Elde edilen sonuçlara göre, çapraz bağlama işlemi sonrasında, CENFs hariç olmak üzere çapraz bağlanmış nanolifler iyi tanımlanmış nanolif yapısını korumuş olmakla birlikte morfolojik özellikleri işlem uygulanmamış nanoliflerin kıvrımsız yapısına nazaran önemli ölçüde değişiklik göstermektedir. Ayrıca, orjinal çaplarını korumuş olan EE-VIII NFs hariç olmak üzere, çapraz bağlama işlemi uygulanan örneklerin lif çaplarında önemli düzeyde artış saptanmıştır. *In vitro* sindirim çalışması sonuçlarına göre, $CaCl_2$ ile çapraz bağlama işleminin karnosinin gastrointestinal kanal içinde yavaş salımını geliştirdiği tespit edilmiştir. EE-VIII NFs'de karnosinin mide fazında gerçekleşen başlangıç aşamasındaki ani salımı, UENFs ve CENFs'e kıyasla önemli düzeyde düşüktür, ayrıca bağırsak fazında EE-VIII NFs, çapraz bağlama uygulanıp uygulanmamasına bakılmaksızın, yavaş salım davranışı sergilemiştir. Ayrıca, salım kinetiğinin matematiksel mdellenmesiyle elde edilen sonuçlar, karnosinin Pul-NaAlg nanoliflerinden gastrointestinal koşullarda salımında Fickian difüzyon mekanizmasının baskın olduğunu ortaya koymuştur.

Son olarak, tezin beşinci kısmında (Bölüm 5), önceki bölümlerde elde edilen veriler dikkate alınarak, tüm çalışma boyunca elde edilen veriler bir arada incelenmiş ve hepsi birlikte son bir kez yorumlanmış ve gelecekteki araştırmalar için tavsiyeler aktarılmıştır. Her bir bölümden elde edilen başlıca yorumlar, Pul-NaAlg-$CaCl_2$ kompozit nanoliflerin pek çok sağlık etkisi bulunan biyoaktif peptitlerin enkapsülasyonu için gelecek vaadeden bir materyal olarak ortaya konduğu ve biyoaktif peptitlerin emüülsiyon elektroeğirme yöntemi sayesinde çift/çoklu emülsiyon ve Pul-NaAlg nanolifler içerisinde eş zamanlı olarak enkapsulasyonunun *in vitro* gastrointestinal koşullarda yavaş salımını sağladığı şeklinde özetlenmiştir.

1. INTRODUCTION

The current relevance of health-promoting ingredients and food supplements in human nutrition and healthy living has led to an increased interest in developing encapsulation technologies to carry, protection and delivery of bioactive agents. Encapsulation, is a concept by which one bioactive compound or a complex contain bioactive substances is coated with or entrapped within another complex body, which provide physical and chemical stability to bioactive compounds and sustained delivery into gastrointestinal tract (Risch, 1995). In addition to, encapsulated compounds are protected against environmental conditions such as light, temperature, humidity, oxygen and pH changes (Fang & Bhandari, 2010). In recent years, polyphenols, essential oils, probiotic microorganisms, vitamins, flavor substances and bioactive proteins or peptides have been encapsulated by various techniques (Hosseini et al., 2019; Pinilla & Brandelli, 2016; Wang et al., 2015).

Bioactive peptides are biologically active amino acid groups in the sequence of proteins which have several benefits to human health through antioxidant, anti-inflammatory, antihypertensive, anticancer, antidiabetic, antimicrobial, antithrombosis, hypocholesterolemic, antiaging and opioid activities (Maestri et al., 2016; Mohanty et al., 2016). However, the bioactive peptides isolated from plants and animals may be lost during processing and storage. Furthermore, bioactive peptides have short *in vivo* half-lives, low bioavailability and poor stability against gastrointestinal conditions (Pei et al., 2022; Sun et al., 2020). In this sense, encapsulation technologies such as coacervation, ionic gelation, electrospraying, micro-fluidic, emulsification, liposomal encapsulation, spray drying and electrospinning have been started to become widespread.

Electrospinning is a simple, flexible and promising encapsulation technology because of the controlled release possibilities based on high encapsulation efficiency, enhanced bioavailability, good thermal, light and storage stability, advanced protection of bioactive compounds from chemical degradation and sustained release of loaded compounds (H. Huang et al., 2022; Rostamabadi et al., 2020). Electrospinning is the

stretching and elongation of an electrically conductive feed solution into the electrical field generated by a voltage supplier between the tip of a conductive needle and collector plate (Kulkarni et al., 2010).

Electrospun fibers are ideal vehicles for the delivery of bioactive compounds due to their high surface area to volume ratio, which provide more efficient mass transfer of biomolecules. Core-sheath nanofibers are more suitable vehicles to eliminate the burst release for bioactive molecule delivery applications. In general, coaxial and triaxial spinnerets were used to fabricate core-sheath nanofibers to incorporate the bioactive compouns into the core part of the nanofibers. On the other hand, emulsion electrospinning, is the alternative technique to obtain core – sheath fibers, has attracted growing interests in the last years. This technique is relatively simple and has various other advantageous such as minimize the amount of organic solvents and other hazardous chemicals, thus environmentally friendly and suitable for organic solvent sensitive hydrophilic components including peptides and proteins (Crespy et al., 2012; Pant et al., 2019).

This Ph.D. thesis is structured taking into account the above mentioned conditions, and lack of information on these topics. In this thesis, firstly, to fabricate a nanofibrous delivery vehicles for bioactive peptides, without using any synthetic polymers or any hazardous solvents was aimed. Pullulan-sodium alginate based composite nanofibers were fabricated, characterized and a model peptide mixture was encapsulated to evaluate the encapsulation performance of produced nanofibrous delivery vehicles. Secondly, carnosine, an antioxidative peptide, loaded pullulan-sodium alginate based composite fibers were produced by uniaxial (blending), coaxial and emulsion electrospinning techniques and characterized to evaluate the effect of solution properties and the role of emulsion parameters on electrospun fibers. Carnosine-loaded water-in-oil-in-water double emulsions were prepared with different formulations including three different surfactant combination, surfactant, two different oily phase and two different carnosine level to evaluate the effect of formulation on electrospinnability and used as feed emulsion in emulsion electrospinning study. Additionally, ionic crosslinking was applied by $CaCl_2$ as crosslinking agent to improve the stability of electrospun nanofibers. Third part of the thesis was implemented by examining the encapsulation efficiencies of electrospinning techniques and the effect of encapsulation on antioxidant activity of carnosine was also investigated. Finally,

the effects of electrospinning encapsulation and crosslinking on release behavior of carnosine was investigated by *in vitro* digestion and the release kinetics was analyzed by establishing corresponding mathematic models.

2. LITERATURE REVIEW

2.1 Biological Activities of Food Derived Peptides

Bioactive peptides are unique biologically active fragments, usually comprised of 2−20 amino acid residues, in the sequence of proteins. Most of the physiological functions of proteins are performed by peptide sequences that remain inactive in precursor protein, but when released they may interact with selected receptors and become active (Korhonen & Pihlanto, 2006; Mohanty et al., 2016; Shuhui Zhang et al., 2021). The continuously updated and modified bioinformatics database, BIOPEP-UWM, provide opportunities to addition of new bioactive peptides and reach information about the structural and physiological properties of previously identified ones (Minkiewicz et al., 2019). Bioactive peptides display various bio-functions including antioxidant, anti-inflammatory, antihypertensive, anti-obesity, antimicrobial activities, antidiabetic, antiaging effects, blood-lipid-lowering effect, opioid role, ability to bind minerals and angiotensin-converting enzyme (ACE)-inhibitory, immunomodulatory properties therefore playing important role in the prevention of cancer, osteoporosis, hypertension and some other disorders by the way regulate immunological, gastrointestinal, hormonal and neurological responses (Akbarian et al., 2022; Chakrabarti et al., 2014; Erdmann et al., 2008; Toldrá et al., 2020). Furthermore, bioactive peptides have the advantages of safety, low toxicity, good healing effect due to the selectivity and no accumulation in organisms compared to synthetic drugs (Abd-Talib et al., 2022; Shuhui Zhang et al., 2021).

Bioactive peptides are classified into two main groups: endogenous and exogenous peptides. Endogenous peptides are produced into the body of organism by different cells and glands. On the other hand, Exogenous peptides refer to peptides that enter the body from outside through different sources as foods, nutraceuticals and therapeutics (Akbarian et al., 2022). Numerous methods are used to produce exogenous bioactive peptides. Enzymatic hydrolysis, microbial fermentation and chemical hydrolysis are the most commonly used techniques to produce bioactive peptides and can be classified as conventional methods (Ulug et al., 2021;

Wisuthiphaet et al., 2016; J. Wu et al., 2015; Zambrowicz et al., 2013). The microbial fermentation is a safer and healtier technique to produce bioactive peptides, when using food – derived proteins and GRAS microorganisms. The bioactive peptide production by microbial fermantation is advantageous compared to enzymatic hydrolysis due to high level and diversity of protease, low cost, simple purification, microbial safety and being environmentally friendy (Cruz-Casas et al., 2021). Except those, some novel technologies were reported to produce bioactive peptides. Recombinant production of bioactive peptides, performed by *in vitro* or *in vivo* expression of peptide genes, is used for research scale production of specific peptides and in combination with chemical methods for the production of some pharmaceutical peptides (Akbarian et al., 2022). High hydrostatic pressure processing (HHP), subcritical water hydrolysis, pulsed electric fields, ultrasounds, ohmic heating and microwave assisted extractions were other novel technologies reported to improve the degree of hydrolysis to produce bioactive peptides (Ahmed & Chun, 2018; Z. Chen et al., 2014; Costa et al., 2018; Ghosh et al., 2019; Piccolomini et al., 2012; Uluko et al., 2013).

Food derived bioactive peptides are promising nutraceuticals and functional foods and responsible such health claims that antibiosis, a reduction in blood pressure, a reduction in blood cholesterol level, anti-microbial activity, antithrombotic and antioxidant, the enhanced absorption of trace minerals, cytoimmunomodulation and opioid activity (Maestri et al., 2016; Mohanty et al., 2016). Naturally occurring bioactive peptides derived from food sources are advantageous compared to synthetic chemicals as pharmaceuticals. Over the last few years, a wide variety of food-derived bioactive peptide sequences have been identified, with multiple health beneficial activities, from plants (cereals, oil seeds, legumes and other vegetable proteins) and animal sources (egg, dairy, marine and meat sources) (Abd-Talib et al., 2022; Akbarian et al., 2022).

2.1.1 Plant-derived bioactive peptides

Bioactive peptides derived from plant sources especially demonstrated antibacterial, antifungal and antiviral properties, in addition to antidiabetic, immunomodulatory, antimicrobial, hypocholesterolemic, opioid, antihypertensive, and antioxidant activities (Akbarian et al., 2022). Soybeans are one of the most abundant plant protein

sources with the high (36–56%) protein content (B. P. Singh et al., 2014). Various ACE inhibitor peptides were reported to be isolated from enzymatic hydrolysates of soybean protein and fermentation products, and it has been observed that they reduce blood pressure by showing anti-hypertensive activities (Korhonen & Pihlanto, 2003; J. Wu & Ding, 2001). Furthermore, according to previous studies, many of the bioactive peptides isolated and purified from soybean had immunomodulatory, antioxidant, anticancer, anti-obesity and hypocholesterolemic activities (Badger et al., 2005; Kong et al., 2008; Sugano et al., 1990; Takenaka et al., 2000; J.-H. Yang et al., 2000).

By-products from the processing of maize appear as a valuable source for the production of plant-derived bioactive peptides. Numerous studies have shown that several peptides (YALMCH, YFCLT, GLLLPH) identified in maize or its hydrolysates have antioxidant activity (Tang et al., 2010; L. Wang et al., 2016; Yanwei Wang et al., 2015) . In the study of Liang and coworkers (2018), it was proved that , peptides identified from maize with sequences PPYLSP, IIGGAL and FLPPVTSMQ exhibited *in vitro* anti-inflammatory properties and *in vitro* study showed that maize derived peptides succeeded in preventing cardiovascular disease (Liang et al., 2018). Similarly, Liu et al. (2020) proved immunomodullatory activity of zein hydrolysates and maize germ digested with thermolysin by an *in vivo* study. Salouti et al. (2016) reported the antimicrobial effect of maize-derived peptides against Staphylococcus aureus-infected wounds. Flaxseed meal is the de-oiled by-product of the processing of flaxseed and another valuable plant protein source with the high (up to 40%) protein content (S. Wu et al., 2019). Peptides derived from flaxseed meal show various biological activities: ACE inhibition effect, antibacterial and antioxidant activity (Silva et al., 2017; Hwang et al., 2016; Wu et al., 2009). According to Wu et al. (2009) the peptides derived from de-oiled flaxseed meal produced by alcalase and thermolysin hydrolysis showed antihypertensive activity *in vitro*.

2.1.2 Animal-derived bioactive peptides

Animal products and the wastes originating from animal tissues are the most abundant protein sources to produce bioactive peptides. The most important and rich animal sources of bioactive peptides are milk, egg, marine and meat sources and that peptides are responsible such biological activities that antimicrobial, antihypertensive,

antioxidant, anti-inflammatory, anticancer, antidiabetic and hypocholesterolemic activities (Davalos et al., 2004; Hernández-Ledesma et al., 2014; Hung et al., 2014; Lafarga & Hayes, 2017; Y. Ma et al., 2016; Zanutto-Elgui et al., 2019).

The most studied group of peptides from animal sources are milk-derived bioactive peptides that have a range of biological activities. Enzymatic hydrolysis and microbial fermentation are the most common methods used to produce milk-derived bioactive peptides at the commercial scale. Casecidins are the first antimicrobial peptides isolated from casein by cymosin digestion method (Hill et al., 1974). So far, many studies have examined the antimicrobial activity of milk-derived bioactive peptides (Abdel-Hamid et al., 2020; Mohanty et al., 2016; Nielsen et al., 2017; Zanutto-Elgui et al., 2019). In the study of Nielsen and colleagues (2017), it was showed that, 207 antimicrobial peptides identified from milk hydrolysates exhibited inhibitory activity with action on gram-positive and gram-negative bacteria (Nielsen et al., 2017). In another study, Abdel-Hamid et al. (2020) proved antimicrobial activity of camel milk-derived peptides against antibiotic-resistant microorganisms such as Methicillin-resistant Staphylococcus aureus and Pseudomonas aeruginosa. antihypertensive peptides have been identified in milk or various other daily products (Sultan et al., 2018). Numerous ACE inhibitor peptides were reported to be isolated from milk or various other daily products, and it has been observed that they reduce blood pressure by showing anti-hypertensive activities (Aluko, 2019; Mizuno et al., 2004; Zhuo et al., 2013). Sah and co-workers (2018) observed that whey protein-derived peptides contains an amino acid such as Leu, Ile, Tyr, Ala, and Trp and casein-derived peptides contains an amino acid such as Tyr, Leu, Pro, His, and Gln mainly responsible for the antioxidant activity of peptides originating bovine milk (Sah et al., 2018). Furthermore, many bioactive peptides isolated and purified from milk and dairy products was reported to have antidiabetic (Lacroix & Li-Chan, 2013), anti-inflammatory (Chakrabarti & Wu, 2015), anticancer (Perego et al., 2012), opioid activities (Nguyen et al., 2015).

Egg proteins are another valuable source to produce bioactive peptides due to its rich source of amino acids. Several peptides identified in egg white and egg yolk hydrolysates have antimicrobial, antihypertensive, antioxidant, anti-inflammatory, anticancer and immunomodulating activities (Bhat et al., 2015; Ibrahim et al., 2001) . Marine sources including fish, sponges, seaweeds, mollusk, and crustacean as well as

wastes of marine industry are one of the most abundant sources to produce bioactive peptides (C. Jo et al., 2017; Phadke et al., 2021; Xing et al., 2022). In previous studies, its reported that, peptides derived from marine sources demonstrated various biological activities: antimicrobial, antihypertensive, anticancer, anticoagulant, antidiabetic, anti-inflammatory and antioxidant activity (Gong et al., 2020; Jemil et al., 2017; Karanam et al., 2020; Qiao et al., 2018; R. Yang et al., 2009). In the study of Jemil and colleagues (2017), it was proved that , peptides identified from sardinelle protein hydrolysate produced by hydrolysis with Bacillus subtilis A26 proteases and exhibited antibacterial, antioxidant and ACE-inhibitory activities (Jemil et al., 2017).

The meat industry generates large volumes of by-products, such as blood, bones, meat scraps, skin, and fatty tissues, among others. Commonly, red meat and poultry industry wastes are used for animal feed and to produce organic fertilizers. However, they are valuable bioactive peptide sources with the high protein contents (Lafarga & Hayes, 2014; Mora et al., 2014; Romero-Garay et al., 2022). In this regard, many studies have focused on the isolation and purification of bioactive peptides from meat-derived protein sources. In previous studies, its stated that, bioactive peptides derived from meat and poultry sources demonstrated antimicrobial (Jang et al., 2008), antihypertensive (Katayama et al., 2008), antioxidant (Escudero et al., 2014; Katayama et al., 2008) , antithrombotic (Shimizu et al., 2009), opioid (Piot et al., 1992)and lipid regulatory activities (Khiari et al., 2014). According to Lafarga and co-workers (2015), a number of peptides derived from bovine fibrinogen fraction showed antihypertensive activity with angiotensin-I-converting enzyme (ACE-I) and renin half maximal inhibitory concentration (IC_{50}) values of 0.17 and 7.2 mM, respectively (Lafarga et al., 2015). Ryder et al. (2016) evaluated five different commercial microbial protease to hydrolyse meat myofibrils and connective tissue collagen protein extracted from bovine tissues and successfully produced novel antioxidant and antihypertensive peptides by a bacterial-derived protease (Ryder et al., 2016).

2.2 Antioxidative Peptides

Antioxidants are bioactive compounds that delay or inhibit oxidation of a substance and compounds and protective against diseases related to oxidative stress such as atherosclerosis and cancer, thus they play an important role in human health (Samarakoon & Jeon, 2012). Food derived peptides are potential natural antioxidants

and there are many antioxidative peptides which isolated from animal tissues (S. K. Kim et al., 2001; J. Liu et al., 2013), soy bean (H.-M. Chen et al., 1995), milk casein (Suetsuna et al., 2000), egg albumen (Nimalaratne et al., 2015) and egg yolk proteins (P.-J. Park et al., 2001). Antioxidative peptides derived from foods are considered to be natural, safe and healthy agents in order to use as nutraceuticals and to produce functional foods.

In the study of Panchal and colleagues (2020), it was proved that the antioxidant capacity of the peptides derived from fermented goat milk produced by Lactobacillus fermentum found maximum antioxidant activities i.e. 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS) assay (52.27%), hydroxyl free radical scavenging activity (55.73%) and superoxide free radical scavenging activity (43.03%) (Panchal et al., 2020). Wang and co-workers (2014) produced that two novel oysters peptides exhibited high antioxidative actions based on their hydroxyl and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities with the amino acid sequences proline-valine-methionine-glycine-aspartic acid and glutamine-histidine-glycine-valine by suptisilin hydrolysis (Qiukuan Wang et al., 2014). Similarly, Zhu et al. (2022) investigated three novel antioxidative peptides from Auxenochlorella pyrenoidosa protein hydrolysates and found the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidative capacity of 6.20 ± 0.23 mmol TE/g (Z. Zhu et al., 2022).

Numerous studies have shown that several peptides identified in poultry skin and meat have antioxidant activity (Fukada et al., 2016; Nadalian et al., 2019; Sarbon et al., 2018) . In the study of Sarbon and colleagues (2018), it was proved that , peptides identified and purified from chicken skin gelatin hydrolysate with enzymatic hydrolysis combined ultrafiltration exhibited greater antioxidative activity compared with the equal amounts of commercial antioxidants butylated hydroxytoluene (BHT), trolox and ascorbic acid (10 mg/ml) (Sarbon et al., 2018). Siow and Gan (2013) reported the antioxidative effect of peptides derived from Parkia speciosa seeds with high DPPH radical scavenging activity (2.9 mg GAE/g), reducing power (11.7 mM) and %ACE-inhibitory activity (80.2%) values.

2.3 Carnosine as a Bioactive Peptide

Carnosine (β-alanyl-L-histidine) is a hydroplylic endogeneously sythesized dipeptide in muscular or other tissues and distributes organs such as brain and kindley. Meat and fish are primary dietary sources for carnosine intake (Jackson & Lenney, 1996; Jargin, 2019; Yan et al., 2019). In previous studies, food-derived carnosine was isolated from different species of chicken breast and thigh meats (S.-K. Kim et al., 2014; Kojima et al., 2014; J. Liu et al., 2013), cured beef meat (Marcolini et al., 2015), pork extract (Gil et al., 2016). It was reported that carnosine has numerous biological functions such as antioxidation, anti-glycation, anti-stress hydroxyl radical scavenging, maintenance of pH-balance, metal chelating properties, anti-aging and wound healing effects (Dahl et al., 1988; Decker et al., 1992), therefore protective against diabetics, cardiac deseases and neurodegenerative deseases such as Parkinson and Alzheimer's diseases (Kawahara et al., 2018; Y. Kim et al., 2019).

The antioxidative effect of carnosine was firstly taken into consideration by Kohen et al. (1988)'s study that carnosine scavenging peroxyl radicals, than efficient singlet oxygen quencher property and the ability of inhibits copper-catalyzed oxidative reactions was also reported (Dahl et al., 1988; Decker et al., 1992). Furthermore, carnosine dipeptide as a natural antioxidants is receiving increasing attention due to its remarkable potential as biopreservative in recent food technology (Maherani et al., 2012). Many studies were conducted to evaluate antioxidative effect of carnosine on packaged in modified atmosphere or gamma irradiation applied beefs during storage. It was reported that, carnosine can be successfully used as a natural antioxidant to increase the oxidative stability, decrease colour changes and inhibit lipid oxidation (Badr, 2007; Djenane et al., 2004; Sánchez-Escalante et al., 2001). Wu et al. (2003) compared antioxidant activities of carnosine and anserine dipeptides with free amino acids of histidine, 1-methylhistidine, taurine, glycine, alanine, β-alanine, they determined that the constituent amino acids are not as effective as inhibiting oxidation like carnosine and anserine. They stated that the imidazole groups of histidine-containing compounds and the peptide linkage between amino acids was related to the ability of antioxidation (H.-C. Wu et al., 2003). Apart from all these functions, carnosine was found to have higher emulsifying properties than commercial emulsifiers such as casein, Tween 80, and Triton X-100 (Murase et al., 1993).

There are several studies in the literature which deal with carnosine respect to use as food supplement. Maherani et al. (2012) encapsulated carnosine by nanoliposomal encapsulation technique to overcome the challenges related to the direct addition of these antioxidant peptides in food and evaluated physical properties and encapsulation efficiencies of nanoliposomes. Kim et al. (2014) studied on optimization of carnosine extraction to maximize physiological activities such as anti-glycation and anti-oxidation. On the other hand, Zhu et al. (2016) suggested a new approach, carboxyl group functionalized mesoporous silica, to adsorption and concentration of carnosine to produce nutraceuticals from meat-wastes.

2.4 Encapsulation of Bioactive Peptides

Bioactive peptides from food-derived proteins present great potential for incorporation into nutraceuticals and functional foods due to their putative safety, low toxicity, selectivity and multiple health benefits (Shuhui Zhang et al., 2021). A number of nutraceuticals and functional food products contain food-derived bioactive peptides and claimed health effects (Hartmann & Meisel, 2007). However, there are some restrictions to use of bioactive peptides commercially such as short *in vivo* half-lives, low bioavailability due to poor solubility or low digestibility and unstability against gastrointestinal conditions (Pei et al., 2022; Sun et al., 2020).

Encapsulation of bioactive peptides with non-toxic and biodegradable polymer matrices can enhance stability and bioavailability. There are numerous encapsulation techniques used for protection and delivery of bioactive peptides including, coacervation, ionic gelation, electrospraying, micro-fluidic, emulsification, liposomal encapsulation, spray drying and electrospinning (Hosseini et al., 2019; G.-Q. Huang et al., 2012; Kadam et al., 2015; Sarabandi & Jafari, 2020; B. Zhang & Zhang, 2013). Many studies were conducted to encapsulate bioactive peptides by a number of delivery systems such as microcapsules (Cian et al., 2019), nanoparticles (B. Zhang & Zhang, 2013), liposomes (Ramezanzade et al., 2017), emulsions (Giroux et al., 2019), nanofibers (Hosseini et al., 2019). Antioxidative peptides were encapsulated by several methods and investigated their encapsulation and release properties in previous studies. Liposomal encapsulation and nanoemulsions are the most applied method to encapsulate antioxidative peptide sources (da Rosa Zavareze et al., 2014; Hosseini et al., 2019; J. Huang et al., 2020; L. Wang et al., 2016).

2.4.1 Microparticles

Microparticles are one of the microparticulate delivery systems and may be characterized as either a homogenous or heterogeneous structure depending on the formulation and processing with the sizes range from 1 to 1000 μm (Lengyel et al., 2019). Microparticles are promising encapsulation vehicles for protecting and delivery of bioactive peptides. Antimicrobial peptide, nisin, was successfully encapsulated by alginate-high methoxy peptin microparticles and *in vitro* release behaviour of nisin was followed sustained release profile (Khaksar et al., 2014). In the study of Tvares and colleagues (2017), a commercial bioactive peptide concentrate was encapsulated with beta-lactogloblin microparticles by sol gel transition method combined with high intensity ultrasound treatment. It was proved that, high intensity ultrasound treatment improve production of monodispersed microparticles for delivery of bioactive peptides.

2.4.2 Microcapsules

Microcapsules are heterogeneous microparticulate delivery systems where an exterior shell is surrounding the interior core forming a capsule (He et al., 2009). They are ideal for bioactive peptides delivery applications. Bioactive peptides loaded in microcapsules are resistant to gastrointestinal conditions and prevented from chemical and enzymatic degradation (Malmsten, 2006). There are numerous studies on encapsulation of bioactive peptides by microcapsules as delivery systems (Blanco-Pascual et al., 2014; Cian et al., 2019; Sarabandi & Jafari, 2020; X. Zhang et al., 2017). In the study of Cian et al. (2019), bioactive peptides were protected from the gastrointestinal medium by microencapsulation. Microcapsules were produced by spray drying using maltodextrin and gum arabic as biocompatible polymers and it was reported that bioactive properties of peptides including ACE-inhibitory activity successfully preserved during *in vitro* gastrointestinal digestion (Cian et al., 2019). Similarly, Sarabandi and Jafari (2020) improved the stability of antioxidant flaxseed peptide fraction by microencapsulation by spray drying method (Sarabandi & Jafari, 2020).

2.4.3 Nanoparticles

Nanoparticles are nanoparticulate delivery systems and are referred to particles with at least one dimension < 100 nm (Biswas & Wu, 2005). Nanoparticles are promising

encapsulation vehicle to improve bioavailability and delivery of bioactive peptides (Nourbakhsh et al., 2016). In the study of Intiquilla et al. (2022), antioxidant peptides from Lupinus mutabilis were encapsulated successfully by nanoencapsulation for colonic delivery. Nanoparticles were produced by ionic gelling and spray freeze drying with the maximum encapsulation efficiencies of 63.80 and 71.75%, respectively. It was reported that both system showed a controlled release of the antioxidant peptides, throughout 6 h of study, of 42.9 and 37.4%, respectively, maintaining the antioxidant capacity (>80%) of the peptides after the encapsulation and release process (Intiquilla et al., 2022). Similarly, two food-derived antihypertensive tri-peptides, isoleucine-proline-proline and leucine-lysine-proline, were successfully encapsulated into chitosan based nanoparticles (Danish et al., 2017).

2.4.4 Liposomes

Liposomes, also named as lipid vesicles, are colloidal structures consisting of one or more phospholipid bilayers encapsulating a volume of aqueous media (Akhavan et al., 2018). Liposomes are ideal carriers for bioactive peptides due to the use of food-grade ingredients in their formulas, carrying simultaneously both hydrophilic and lipophilic substances, targeted and controlled delivery (Ghorbanzade et al., 2017; T. Zhang et al., 2019). In recent years, nanoliposomes have been used to incorporate bioactive peptides in a number of studies (Mazloomi et al., 2020; Mohan et al., 2016; Pavlović et al., 2022; Ramezanzade et al., 2021; T. Zhang et al., 2019). In the study of Mosquera et al. (2014), bioactive peptide fraction isolated from sea bream scales collagen were encapsulated in nanoliposomes using partially purified phosphatidylcholine. Nanoliposomes preserved antioxidant activity and ACE inhibitory activity of the loaded peptide fraction during 8 days at 4 °C and it was reported that encapsulation successfully improved stability of bioactive peptides (Mosquera et al., 2014). Similarly, Sarabandi and colleagues (2020) improved the stability of antioxidant flaxseed protein hydrolysates by encapsulation in nanoliposomes and then spray-drying treatment (Sarabandi et al., 2019).

2.4.5 Emulsions

Emulsions are mixtures of two or more immiscible liquids in which one is named as continuous phase and the other one as droplets distributed throughout the continuous phase (Agarwal & Greiner, 2011). Double emulsions, also named as multiple

emulsions, are emulsions of other emulsions. In other words, a double emulsion consist an emulsion present as fine droplets in a continuous phase (Dickinson, 2011; Lamba et al., 2015). Emulsification is among the methods that can be used for encapsulation of bioactive substances and there are many studies in the literature related to encapsulation of bioactive peptides in emulsions and double emulsions (Choi et al., 2020; Lajmi et al., 2019; Ying et al., 2021). Casein-derived bioactive peptides, were successfully encapsulated in water-in-oil-in-water double emulsions and its reported that the kinetics of release in gastrointestinal environment can be controlled by adjusting oil phase composition (Giroux et al., 2016). In the study of Jo et al. (2019), collagen peptides were encapsulated in emulsions of water in oil and double emulsions of water in oil in water. They reported that double emulsions encapsulation demonstrated higher stability and controlled release compared to single emulsions (Y.-J. Jo et al., 2019).

2.4.6 Nanofibers

Nanofibers are ideal vehicles for the delivery of bioactive compounds due to their high surface area to volume ratio, which provide more efficient mass transfer of bioactive compounds. Core-shell nanofibers are more suitable for the delivery of bioactive molecules and controlling the release over a sustained time period (Crespy et al., 2012). Accordingly, nanofibers are promising encapsulation vehicle to improve bioavailability and sustained delivery of bioactive peptides. Hosseini and colleagues (2019) successfully fabricated electrospun chitosan/poly(vinyl alcohol) nanofibrous mat to encapsulate fish-derived antioxidative peptide under the optimum electrospinning process conditions with encapsulation efficiency beyond 94%. It was reported that antioxidative activity of peptides preserved in electrospun fibers and loaded peptides displayed sustained release from nanofibers (Hosseini et al., 2019).

2.5 Electrospinning as an Encapsulation Method

Electrospinning is a simple, cost-effective and flexible technique to produce nanofibers from synthetic or biopolymers. In this process, a high voltage supplier, a capillary tube with a needle of small diameter, and a grounded collecting plate are used to obtain nanofibers. When the electric field is gradually increased, the droplet at the tip of the needle is elongated from a spherical shape to a cone shape, creating a “Taylor

cone". When the applied voltage overcomes the surface tension of feeding solution droplet, a charged jet of solution is ejected from the tip of the Tyler cone and the jet becomes longer and thinner until it reaches to the collector plate (Kulkarni et al., 2010; Okutan et al., 2014). The morphology and structure of the nanofibers depend on numerous factors, including the properties of polymer solution (viscosity, surface tension, dielectric properties, electrical conductivity etc.), parameters of electrospinning process (applied voltage, distance between needle and collector plate, feed rate etc.), type of spinneret (uniaxial/multiaxial geometries) and environmental conditions (temperature, humidity etc. (X. Lu et al., 2009). The type of spinneret is the most important parameter, by the virtue of the multiple configurations can be carried out. The electrospinning process can be divided into different types depending on the spinneret geometries such as needle-less, single (uniaxial), coaxial, side-by-side, and tri-axial electrospinning (Bellan & Craighead, 2009; Pant et al., 2019).

In food science and technology, nanofibers have a great potential to use with their different attributes such as high porosity, water stability, high gas permeability, and large surface area (Kulkarni et al., 2010; Steyaert et al., 2016). Moreover, electrospun nanofibers produced from food grade biopolymers (i.e. proteins and polysaccharides) are promising nanomaterials due to their safety, biodegradability properties. Filtration, edible coating, encapsulation, enzyme immobilization, active/intelligent food packaging and development of biosensors are common applications of electrospinning in food technology (Bhushani & Anandharamakrishnan, 2014; Yue Wang et al., 2022). Electrospinning is considered to be a promising technique to prepare encapsulation systems for the delivery of bioactive compounds due to it has great encapsulation efficiency, enhanced bioavailability, good thermal, light and storage stability, enhanced protection of bioactive compounds from chemical degradation and sustained release of loaded compounds (H. Huang et al., 2022; Rostamabadi et al., 2020). There are a number of studies on electrospinning encapsulation of bioactive peptides in literature (Table 2.1).

Electrospun fibers are ideal vehicles for the delivery of bioactive compounds due to their high surface area to volume ratio, which provide more efficient mass transfer of biomolecules. Electrospun fibers are ideal vehicles for the delivery of bioactive compounds due to their high surface area to volume ratio, which provide more efficient mass transfer of biomolecules. Core-sheath nanofibers are more suitable vehicles to

eliminate the burst release for bioactive molecule delivery applications. In general, coaxial and triaxial spinnerets were used to fabricate core-sheath nanofibers to incorporate the bioactive compounds into the core part of the nanofibers. On the other hand, emulsion electrospinning, is the alternative technique to obtain core – sheath fibers, has attracted growing interests in the last years (Crespy et al., 2012; Pant et al., 2019).

2.5.1 Single / uniaxial electrospinning

Single / uniaxial electrospinning technique, also named as blending electrospinning, is the easiest electrospinning technique to encapsulate bioactive compounds and performed by blending polymer and biomolecule and use as feed solution. The major disadvantages of blending electrospinning technique are tendency of biomolecule to locate on the surface of fiber due to applied electrical force, thus burst release phenomenon in the early stage of release (Y. Yang, Li, Cui, et al., 2008; Zeng et al., 2005).

Several studies were encapsulated nisin by uniaxial electrospinning technique to improve stability and preserve antimicrobial activity (Dheraprasart et al., 2009; Han et al., 2017; Soto et al., 2016; H. Wang et al., 2015). Similarly, Wang et al. (2015) improved the stability and retained the antimicrobial activity of pleurocidin peptide derived from the skin-secreted mucous of the winter flounder by encapsulation in poly(vinyl alcohol) based uniaxial nanofibers (X. Wang et al., 2015). Rajanna and colleagues (2019) successfully fabricated bead-free peptides-loaded pullulan nanofibers to encapsulate casein-derived peptides with mean diameter of 60.45–133.05 nm and encapsulation efficiency of 72.95–86.04%. It was reported that antioxidative activity of peptides preserved in electrospun fibers and loaded peptides displayed more sustained release from nanofibers compared to free peptides (Rajanna et al., 2022). In a recent study, bioactive oyster peptides were encapsulated in electrospun octenylsuccinylated starch-pullulan nanofiber mats for adsorbing off-flavors of fish-derived peptides (Li et al., 2022).

Table 2.1 : Bioactive peptides encapsulated by electrospinning in previous studies.

Bioactive peptide	Bioactivity	Electrospinning technique	Solvent	(Bio) Polymer	Reference
Nisin	Antimicrobial activity	Uniaxial electrospinning	Formic acid	Amaranth protein concentrate/pullulan	(Soto et al., 2016)
		Uniaxial electrospinning	Acetic acid	Gelatin	(Dheraprasart et al., 2009)
		Uniaxial electrospinning	Deionized water	Poly (vinyl alcohol)/wheat gluten	(H. Wang et al., 2015)
		Uniaxial-coaxial-triaxial electrospinning	2,2,2,-trifluoroethanol	Cellulose acetate-poly(ε-caprolactone)-polyvinylpyrrolidone-nylon 6	(Han et al., 2017)
		Uniaxial electrospinning	Formic acid	Amaranth/pullulan	(Soto et al., 2019)
Rana chensinensis skin peptides	Antibacterial and antioxidant activity	Uniaxial – coaxial electrospinning	1,1,1,3,3,3,-hexafluoro-2-propanol	Poly(l-lactide)/zein	(M. Zhang et al., 2016)
Fish-purified antioxidant peptide	Antioxidant activity	Uniaxial electrospinning	Acetic acid – Distilled water	Chitosan/poly(vinyl alcohol)	(Hosseini et al., 2019)
Casein-derived peptides	Antioxidant and ACE inhibitory activity	Uniaxial electrospinning	Distilled water	Pullulan	(Rajanna et al., 2022)
Pleurocidin	Antimicrobial activity	Uniaxial electrospinning	Distilled water	Poly(vinyl alcohol)	(X. Wang et al., 2015)
Oyster peptides	Antioxidant, anticancer and antithrombotic activity	Uniaxial electrospinning	Distilled water	Octenylsuccinylated starch-pullulan	(Li et al., 2022)

2.5.2 Coaxial - triaxial electrospinning

Coaxial or triaxial electrospinning is the characteristic technique used to obtain core-sheath nanofibers to eliminate burst-release. In order to obtain coaxial or triaxial configurations, special spinnerets are required consist of two or more concentric capillaries with different diameters. The core, middle layer (if any) and sheath solutions flow in separate capillaries during the electrospinning process (Pant et al., 2019; X. Qin, 2017). Though this technique provide well controlled fiber morphology, requirements such special apparatus and demands careful selection of the operational conditions to ensure desirable results are adversely affect its feasibility (Jun Hu et al., 2013; Viry et al., 2012; Zdraveva et al., 2015).

Zhang and colleagues (2016) successfully fabricated poly-L-lactic acid /zein nanofibers to encapsulate Rana chensinensis skin peptides demonstrated antibacterial and antioxidant activity by uniaxial and coaxial electrospinning techniques (M. Zhang et al., 2016). In another study, Han and co-workers (2017) developed electrospun triaxial fiber membranes to encapsulate nisin and according to results of release study, they achieved long term – sustained release of nisin from nanofiber. Nisin-loaded nanofibers were also fabricated by uniaxial and coaxial electrospinning. However, triaxial fiber membranes provided more and more sustained antimicrobial activity compared to others (Han et al., 2017).

2.5.3 Emulsion electrospinning

Emulsion electrospinning, is the alternative technique to obtain core – shell fibers, has attracted growing interests in the last years. The major steps of emulsion electrospinning comprises emulsification to form a water-in-oil (W/O) or oil-in-water (O/W), dissolution selected polymer and electrospinning of resulting emulsion. During the electrospinning process; the morphology of electrospun fibers changes from a pearl-on-string like structure to a co-continuous two-phase core – shell structure due to stretching and elongation by high electrical field(Agarwal & Greiner, 2011). This technique is relatively simple and has various other advantageous such as minimize the amount of organic solvents and other hazardous chemicals, thus environmentally friendly and suitable for organic solvent sensitive hydrophilic components (peptides, proteins, DNA) (Crespy et al., 2012).

Even though, the emulsion electrospinning is known as an environmentally friendly approach, due to minimize the amount of organic solvents used during the electrospinning process, some hazardous chemicals and organic solvents may have to be used. Since, the most of fiber forming polymers cannot solubilize bio-solvents such as water, phosphate buffer saline, acetic acid etc. Chloroform, toluene, dicloro metane (DCM), dimethylformamide (DMF), methylene dicloride are most commonly used organic solvents for hydrophobic polymers such as polycaprolactone (PCL), poly [(D,L-lactic acid)-co-(glycolic acid)] (PLGA), poly(ethylene glycol)-poly(L-lactide) (PELA) (Agarwal & Greiner, 2011). Especially, the use of toxic solvents in the production of nutraceuticals and functional food products is not desirable. It is not easy to perform emulsion electrospinning use only green and non-toxic chemicals.

Therefore, using green chemicals and making food grade composites has become a very important issue.

Emulsion electrospinning requires a stable emulsion in order to confirm the successful electrospinning to take place (Jun Hu et al., 2013). Emulsions consist of immiscible liquids, the input of energy in the form of mechanical agitation during the preparation process is required to disperse the separate phases. Mechanical agitation technique and duration, surfactants added to formulations, ratio of polymer and biomolecule are main effective parameters on fiber morphology and release kinetics of bioactive compounds (Briggs & Arinzeh, 2014; Zdraveva et al., 2015). Even though, emulsion electrospinning is an effective technique for synthesizing core-sheath nanofibers, several challenges are encountered. Because of the different rheological behaviours of the continuous and dispersed phase in emulsion, these two phase are exposed to different stresses during the Taylor cone formation stage of electrospinning process. Thus, it is not easy to control the fiber morphology during electrospinning process due to the possible modifications of the emulsion composition over long electrospinning times (Crespy et al., 2012).

Double emulsions, also named as multiple emulsions, are emulsions of other emulsions. Double emulsions consist two different oil-water interface, so two different emulsifying agents are necessary to obtain a double emulsion (Dickinson, 2011; Lamba et al., 2015). Two different techniques have been proposed in the literature to obtain double emulsions. The first one is single-step emulsification and the second one is two-step emulsification techniques (Lamba et al., 2015). Matsumoto (1983) was explained single-step technique as heating of an emulsion consisting of a non-ionic emulsifier or a mixture of different emulsifiers, causing phase inversion which leads to the formation of double emulsion (Matsumoto, 1983). The second, also the most common technique in the literature, two step emulsification technique; initially making the primary emulsion involves the use of a low hydrophilic-lipophilic balance (HLB) surfactant in case of $W_1/O/W_2$ emulsion or a high HLB surfactant in case of $O_1/W/O_2$ emulsion. In the second step, W/O emulsion or O/W emulsion is dispersed in an aqueous continuous phase containing high HLB surfactant to form $W_1/O/W_2$ double emulsion or dispersed in an oil continuous phase containing a low HLB surfactant to obtain an $O_1/W/O_2$ double emulsion (Dickinson, 2011; Lamba et al., 2015; Rajesh et al., 2012). Double emulsions are among the methods that can be used for encapsulation

of bioactive substances such as anthocyanin, bioactive extracts, probiotic microorganisms, essential oils etc. There are many studies in the literature related to encapsulation of hydrophilic or hydrophobic bioactive compounds in double emulsions. $W_1/O/W_2$ were mostly used to encapsulate hydrophilic bioactive substances like peptides. On the other hand very limited studies were conducted to electrospinning of double emulsions (Carrillo et al., 2016; Foraida et al., 2017). Foraida et al. (2017) were produced a composite nanofiber scaffold which optimized to act as a sustained growth factor delivery system using epidermal growth factor as a model protein by double emulsion electrospinning (Foraida et al., 2017).

3. FABRICATION OF PEPTIDE-LOADED GREEN COMPOSITE NANOFIBERS BASED ON CARBOHYDRATE POLYMERS

3.1 Abstract

To realize encapsulation of peptides and easily evaluation of encapsulation efficiency after electrospinning standard peptide mixture (SPM), as a model peptide mixture, encapsulated via uniaxial electrospinning of green feed solutions based on pullulan (Pul) and/or sodium alginate (NaAlg) with/without $CaCl_2$. The effects of solution properties such as electrical conductivity, surface tension, viscosity and dielectrical properties on electrospinnability were investigated. In addition, scanning electron microscopy and zeta potential measurements were performed to characterize the nanofibers. Electrospun Pul-NaAlg-$CaCl_2$ composite nanofibers encapsulating SPM were successfully fabricated under the certain conditions (flow rate: 0.50 ml/h, applied voltage: 12 kV and distance between tip and collector plate: 12 cm). Scanning electron microscopy results revealed that the SPM-loaded electrospun fibers exhibited fine, uniform and bead-free nanostructures with mean diameter of 88.7 ± 10.85 nm. Results showed that decreased dielectrical constant of feed solutions by incorporating $CaCl_2$ reduced fiber diameter and thus improved stability of electrospun fibers. Encapsulation efficiency of SPM by uniaxial electrospinning procedure were found to be 77.97 ± 1.65 % in terms of total peptide content. These results indicate that suggested technique is suitable for the green encapsulation of bioactive peptides with potential use for food purposes.

Keywords: encapsulation; green composite nanofiber; electrospinnability; peptide; pullulan

3.2 Introduction

Electrospinning is a simple and all-purpose technique to produce nanofibers from synthetic or biopolymers. Besides, it is a cost-effective technique with controllability in fiber diameter and versatility in making fibers with multicomponent cross-sectional

configurations (Senthamizhan et al., 2017). In this process, a high voltage supplier, a capillary tube with a needle of small diameter and a grounded collecting plate are used to obtain nanofibers. When the applied voltage overcome the surface tension of feeding solution droplets, a charged jet of solution is ejected and the jet become longer and thinner until it reach to the collector plate (Kulkarni et al., 2010). Nanofibers have a great potential to use with their different attributes such as high porosity, water stability, high gas permeability and large surface area (Kulkarni et al., 2010). In recent years, electrospinning have various applications regarding food science and technology such as filtration; fabrication of active and smart packaging and biosensors for food analyses; improvement of food ingredients; encapsulation and delivery of bioactive compounds (Fuenmayor et al., 2014; Lemma et al., 2015; Perez Espitia et al., 2012; Xu et al., 2015). Electrospun fibers are ideal vehicles for encapsulation and delivery of bioactive compounds due to their high surface area to volume ratio, which provide more efficient mass transfer of biomolecules (Okutan et al., 2014). Uniaxial electrospinning is the easiest electrospinning technique and performed by blending polymer and biomolecule in a suitable solvent, then using as feed solution (Senthamizhan et al., 2017).

Bioactive peptides are biologically active peptides in the sequence of proteins that remain inactive in precursor protein. Foodstuff derived bioactive peptides regulate immunological, gastrointestinal, hormonal and neurological responses, thereby playing vital role in the prevention of cancer, osteoporosis, hypertension and some other disorders. These naturally occurring biomolecules are advantageous compared to synthetic chemicals as pharmaceuticals (Maestri et al., 2016). Due to these advantages, bioactive peptides have great interest in recent years. However, bioactive peptides isolated from natural sources are more instable against environmental stresses such as light, oxygen, metal catalysts, and pH etc. (Maestri et al., 2016). There are many studies have been performed related to fractionation, identification and physiologically characterization of novel bioactive peptides (P. Zhang et al., 2020). However, several studies have been performed in order to protection and release of bioactive peptides to overcome potential challenges associated with their potential usage in pharmaceutical and/or nutraceutical foods. Encapsulation of bioactive compounds, which provide stability of bioactive molecules in their isolated /pure forms, has recently become important to obtain functional foods due to latest demands

and tendencies of consumers towards healthy food (Flores & Kong, 2017). Bioactive peptides were encapsulated by several methods and investigated their encapsulation and release properties in previous studies. Liposomal encapsulation is the most applied method to encapsulate bioactive peptide sources (da Rosa Zavareze et al., 2014; Ramezanzade et al., 2017). Furthermore, double emulsion encapsulation, microencapsulation in micro hydrogels, micro emulsion gelling and high intensity ultrasound microparticulation were also performed to encapsulate various bioactive peptides (Giroux et al., 2016; Tavares et al., 2017; X. Zhang et al., 2017). Electrospinning is an alternative method to encapsulate bioactive peptides. Fish-purified antioxidant peptides have been successfully loaded into chitosan/poly(vinyl alcohol) nanofibrous mat by electrospinning to develop active food packaging (Hosseini et al., 2019).

Synthetic polymers are more suitable materials to electrospinning procedure and high encapsulation efficiencies can be obtained by using them as polymer. However, they can be toxic or not biodegradable. Thus, natural food-grade biopolymer such as proteins and polysaccharides must be used for food technology applications, especially to fabricate healthy food supplements. However, most of natural biopolymers, particularly polysaccharides, cannot be electrospinnable, due to their various physical and chemical properties. Previous studies have focused on make polysaccharides electrospinnable by adding proteins and synthetic polymers to feeding solutions or use harmful chemical solvents to make them electrospinnable. Pullulan is a non-toxic, biodegradable and biocompatible carbohydrate polymer produced from the industrial fermentation of starch syrup caused by *Aureobasidium pullulans* that used in pharmaceuticals, cosmetics, and the food industry (Román et al., 2019). On the other hand, alginate is an edible, biodegradable, relatively low cost and non-toxic polysaccharide, extracted from brown seaweed, its sodium alginate form has been widely used in food industry (Zhong et al., 2010). The surface active property of sodium alginate as well as high water solubility of pullulan and sodium alginate makes it possible to obtain nanofiber without using any hazardous solvents.

The aim of this study was produce peptide-loaded Pul-NaAlg-$CaCl_2$ composite nanofibers, without using any synthetic polymers or any hazardous solvents via green electrospinning. To realize encapsulation of peptides and easily evaluation of encapsulation efficiency after fabrication SPM was used as model peptides. To assess

the morphology of the fibers, Scanning Electron Microscopy (SEM) and image analysis were used. Besides, effects of electrical, rheological and surface properties of feed solutions on electrospinnability were investigated. Finally, to determine the efficiency of encapsulation, HPLC analysis were carried out.

3.3 Materials and Methods

3.3.1 Materials

Standard peptide mixture (SPM) including Gly-Tyr, Val-Tyr-Val, Methionine Enkephalin Acetate, Leucine Enkephalin, Angiotensin II Acetate used as a source of peptide and for HPLC analysis were supplied as HPLC grade from Sigma-Aldrich (St. Louis, USA). Pullulan was donated by Hayashibara Co. Ltd. (Japan). Sodium alginate was purchased from a local company. $CaCl_2$, methanol (HPLC grade), acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, USA).

3.3.2 Preparation of polymer solutions

Polymer solutions were prepared as described in Table 3.1. Pul and NaAlg contained solutions were mixed over night at room temperature and 200 rpm. Briefly, 2.5 mg of SPM (0.5 mg of each different peptide) was reconstituted in 1 mL of distilled water to obtain a final concentration of 2.5 µg/ µL of SPM stock solution. A volume of 200 µL of SPM stock solution, contains 500 µg of SPM, was added to 1 g polymer solutions shown in Table 3.1 to yield a final concentration of 500 µg SPM/g of polymer solution. SPM and $CaCl_2$ were added to formulations 1 hour and 30 min before the electrospinning process, respectively then mixed at same conditions.

Table 3.1 : Description of solutions.

Solution no	Polymer concentrations	Solvent	Analysis Applied
1	Pullulan 10% (w/w)	Water	- Solution Properties - Characterization of electrospun samples
2	Pullulan 10%, NaAlg 2% (w/w)	Water	
3	Pullulan 10%, NaAlg 2%, $CaCl_2$ (30 ppm) (w/w)	Water	

3.3.3 Characterization of polymer solutions

Electrical conductivities of polymer solutions were measured by a conductometer (WTW LF95, Germany) and surface tension values were measured by a tensiometer

(Dataphysics DCAT 11 E, Germany), both at 25 °C, in triplicate for each sample. Furthermore, dielectric constant (ε') of feed solutions were measured by a network analyzer (Agilent E5061B, USA) equipped with a dielectric probe kit (Agilent 5070E) at 25 °C, in triplicate for each sample. Dielectric constants of the solutions were investigated from 1 MHz to 3 GHz.

Rheological properties of polymer solutions were measured using a rheometer (Haake Rheostress 1, Germany) using the plate-plate sensor (35 mm diameter, 1 mm gap) configuration at 25 °C, in duplicate for each sample. The shear rate range was between 0 and 300 s^{-1} in 120 s. The results were modeled using the software (SPSS Version 20, IBM Statistics) according to the power-law equation:

$$\tau = \mathrm{K}\,\dot{\gamma}^{\mathrm{n}} \tag{3.1}$$

where τ is the shear stress (Pa) ,K is the consistency index (Pa s^n), n is the flow behavior index and $\dot{\gamma}$ is the shear rate (s^{-1}). Taking K and n values into account, η the apparent viscosity (Pa s) can be calculated using the power-law equation for viscosity:

$$\eta = K.\dot{\gamma}^{n-1} \tag{3.2}$$

The shear rates (maximum shear rate) were found from the equation 3.3 which is used for power-law materials in tubular geometry:

$$\dot{\gamma}_{max} = \left(\frac{3n+1}{4n}\right)\frac{4\mathrm{Q}}{\pi R^3} \tag{3.3}$$

where Q is the volumetric flow rate of the material and R is the inside radius of the tube (Steffe, 1996). In present study, feed rate of polymer solutions was 0.5 mL/h, which can be expressed as 1.39 10^{-10} m^3/s. The diameter of the tip where the solutions sprayed into the electric field is ~ 0.72 mm (21 G diameter needle). According to the equation 3.3, maximum shear rates of polymer solutions at the tip were calculated as ~ 4 s^{-1} after n values were determined. The apparent viscosities were calculated at the shear rate of 4 s^{-1} by the help of Equation 3.2.

3.3.4 Electrospinning process

The electrospinning process was conducted with the equipment of Inovenso NE100 (Turkey) at room temperature. The equipment has the syringe pumps (New Era Pump Systems Inc., NE-300, USA), the needle, the voltage supply (Nanofen, Turkey) and the collector plate. The operation conditions were given in Table 3.2. The electrospun nanofibers were collected on an aluminium foil which was used for covering the collector plate. In present study, uniaxial electrospinning was performed to encapsulate the SPM. The moisture content of the electrospun samples was accepted to be completely removed during electrospinning process, so the results of all analyses were expressed on a dry weight basis.

Table 3.2 : Process conditions.

Sample no	Solution no	Encapsulation method	Flow rate (ml/h)	Loaded substance	Diameter of needle (G)	Distance (cm)	Applied voltage (kV)
1	1	Uniaxial Electrospinning	0.50	-	21	12	10
2	2		0.50	-	21	12	12
3	3		0.50	-	21	12	12
4	3		0.50	SPM (500 µg/g)	21	12	12

3.3.5 Characterization of electrospun fibers

The morphology and surface characteristics of the electrospun fibers were carried out by scanning electron microscopy (SEM - Tescan Vega 3, Czech Republic). Briefly, 1 cm^2 electrospun samples were coated with gold-palladium to minimize sample charging by using a sputter coater (Quorum, SC7620). Microscopy was performed at high vacuum with an accelerating voltage. Images were taken at 1000, 12500 and 30000 magnifications. Mean diameter of fibers was calculated according to data obtained from analyses of SEM images by ImageJ software (National Institutes of Health, USA).

A dynamic light scattering instrument (Malvern Zetasizer NanoZS, UK) was utilized for zeta potential (ξ) measurements of electrospun fibers. Samples were first dispersed at 0.1:100 dilution ratio with ethanol to prevent multiple scattering effects. They were then mixed at 15000 rpm for 1 minute. Three replicates were conducted for each sample at constant temperature of 25°C.

3.3.6 HPLC analysis

Analyses were operated by a High Performance Liquid Chromatography system (Agilent 1260 Infinity, USA) coupled to a PDA detector (Agilent G1315D, USA). Compounds were separated using a ACE 5 C18 column (150 x 4.6 mm, 5 μm; Advanced Chromatography Tech., Scotland). Injection volume was 10 μl and samples were injected by an auto sampler (Agilent 1329B, USA). Column temperature was adjusted to 50°C. The gradient system consist of a phase A (5% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) contained distilled water) and phase B (75% ACN and 0.1% TFA contained distilled water) was used as mobile phase. The gradient was adjusted as follows: 0 to 2. Min, 0 to 5% B as linear gradient; up to 5. min, 5% B as constant; 5. to 10. min, 5 to 10% B as linear gradient; 10. to 15. min, 10 to 20 % B as linear gradient; up to 20. Min, 20% B as constant; 20. to 30. min, 20 to 30% B as linear gradient; up to 35. min, 30% B as constant; 35. to 40. min, 30 to 5% B as linear gradient; 40. to 45. min, 5 to 0% B as linear gradient. The flow rate of mobile phase was 1 mL/min. The detection of eluted peaks was achieved at 220 nm. Calibration curves were drawn for each peptide by using 20 - 100 mg/L dilutions of SPM. The samples were eluted with distilled water and filtered through 0.45 μm membrane filters. The measurements were performed as three parallel for each concentration.

3.3.7 Encapsulation efficiency

Encapsulation efficiency of electrospun fibers were calculated according to HPLC analysis results. Encapsulation efficiency was calculated by the following equations:

$$\text{Encapsulation efficiency } (\%) = \frac{amount\ of\ peptide\ in\ electrospun\ fiber}{amount\ of\ bioactive\ compounds\ fed\ initially} \times 100 \quad (3.4)$$

3.3.8 Statistical analysis

The means and standard deviations of replicated measurements were calculated and significance of differences between samples were determined with the help of analysis of variance (ANOVA) were performed by using a statistical software (SPSS, IBM Statistics).

3.4 Results and Discussion

3.4.1 Electrospinnability of polymer solutions

SEM images of electrospun samples were given in Figure 3.1. Results shows that Sample 1 (containing only "Pul" as a polymer) has not a good and uniform fibrous structure (Figure 3.1-a). Although some fine fiber formation was observed, a large part of the sample was not in fiber form. Additionally, beady fiber formation was observerd (Figure 3.1-b). Results shows that Sample 2 and Sample 3 (composites of Pul-NaAlg and Pul-NaAlg-$CaCl_2$, respectively) have uniform and fine fiber formation (Figure 3.1-c, d, e and Figure 3.1-f, g, and h). Mean diameters calculated by image analyses of SEM micrographs were found to be 109.2 ± 13.26 nm and 64.2 ± 10.30 nm for Sample 2 and Sample 3, respectively. According to the results, significantly finer fibers were obtained with the addition of $CaCl_2$ to the formulation ($P < 0.05$). Moreover, while sample 4 (containing SPM in composite of Pul-NaAlg-$CaCl_2$) has an uniform and fine fiber formation (Figure 3.1-i,j,k), the mean diameter of the obtained fibers (88.7 ± 10.85 nm) was found to be significantly higher than that of sample 3, but lower than that of sample 2 ($P < 0.05$). The intermolecular interactions of NaAlg were enhanced by Ca^{2+} cations via increasing the ionic interactions (Stokke et al., 2000). It could be inferred that the increase of the intermolecular interactions of NaAlg chains with the addition of $CaCl_2$ could improved the electrospinnability of feed solutions.

Some of the solution properties including electrical conductivity (EC), surface tension, dielectrical constants and solution rheology should be taken into consideration to evaluate the electrospinnability of polymer solutions. The properties of the solutions whose compositions were described in Table 3.1 were given in Table 3.3. The electrical conductivity of the polymer solutions must be higher than zero, to start the electrospinning process. Because, conductive solutions have high repulsion to overcome surface tension of droplets at the top of the needle (S. Wang et al., 2013). Surface tension is one of the effective parameters on electrospinning and related to chemical composition and concentration of polymer solution. It is preferred that the polymer solution has a low surface tension to obtain bead-free electrospun fibers (Okutan et al., 2014). Results shows that the conductivity of Solution 1 (0.07 mS/cm) was quite lower than others (2.15-2.17 mS/cm), thereby it is not enough to overcome surface tension to form uniform and bead-free fibers and it is consistent with literature

(Román et al., 2019). They reported that the pullulan concentration was lower than 15% w/w, the nanofbers exhibited bead formation after the electrospinning process. When NaAlg was added to pullulan solution (Solution 1) the conductivity of the mixed solutions (Solution 2 and 3) increased probably the higher conductivity value of NaAlg.

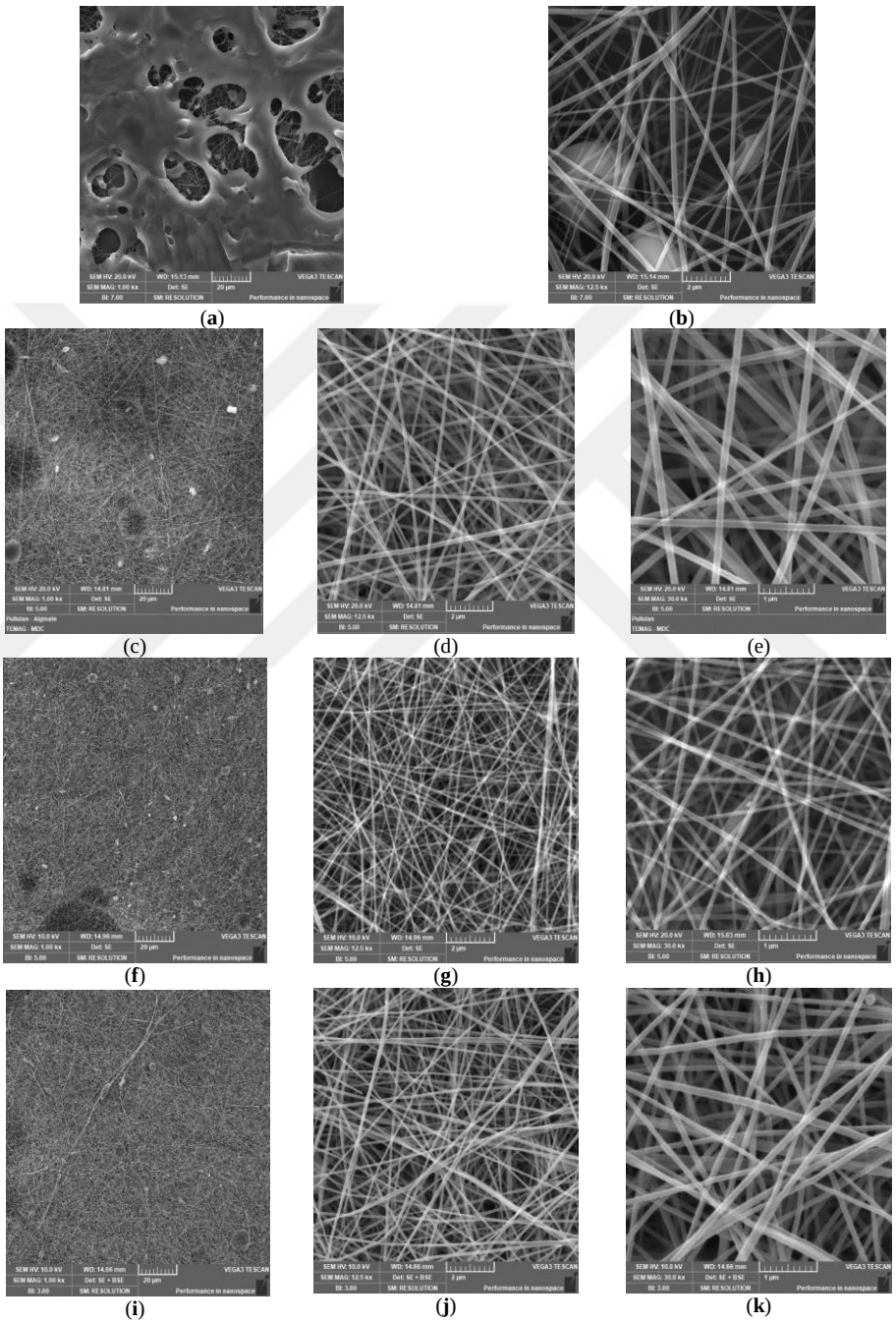

Figure 3.1 : SEM images of electrospun samples obtained from defined solutions. Sample 1 (a- 1000 and b-12500 magnification); Sample 2 (c- 1000, d-12500 and e-30000 magnification); Sample 3 (f- 1000, g-12500 and h-30000 magnification); Sample 4 (i- 1000, j-12500 and k-30000 magnification).

The results were in accordance with the literature. A study on electrospinning of pure sodium NaAlg reported that conductivity of 2% (w/w) NaAlg in distiled water was 4.18 mS/cm (Nie et al., 2008). According to the results, it is clear that higher conductivity values (Solution 2 and 3) leaded to the uniform and fine fiber formation. Whereas addition of low amount of $CaCl_2$ slightly decreased EC of solutions, presence of SPM did not affect it, significantly ($P < 0.05$). On the other hand, high conductivity value alone is not adequate for electrospinning. To obtain nanofiber morphology by electrospinning, repulsion force has to overcome the surface tention (Ki et al., 2005).

Table 3.3 : Properties of polymer solutions.

Solution No	Electrical conductivity (mS/cm)	Surface tension (mN/m)	*K* (Pa.s)	*n*	η (Pa.s) at 4 s^{-1}
1	0.07 ± 0.01^{a}	72.42 ± 0.19^{c}	0.08 ± 0.01^{a}	0.97 ± 0.03^{b}	0.08 ± 0.01^{a}
2	2.17 ± 0.01^{c}	51.68 ± 0.68^{ab}	2.70 ± 0.10^{c}	0.79 ± 0.01^{a}	2.01 ± 0.08^{c}
3	2.15 ± 0.01^{b}	53.15 ± 1.24^{b}	2.51 ± 0.14^{b}	0.79 ± 0.01^{a}	1.87 ± 0.10^{b}
3*	2.16 ± 0.01^{bc}	50.16 ± 1.94^{a}	2.58 ± 0.05^{bc}	0.80 ± 0.00^{a}	1.96 ± 0.03^{bc}

*SPM added solution
Data given in this table represents average amounts ± standard deviation of three replications for each sample. Data was analyzed by the one-way ANOVA and different letters (a–b) in the same column indicate significant differences by the Duncan test ($P < 0.05$).

The results showed that the surface tention was found to be the highest for Solution 1 (72.42 ± 0.19 mN/m). It could be deduced that lower EC of the Solution 1 was not sufficient to overcome the relatively higher surface tension for the fiber formation. When NaAlg was added to pullulan solution (Solution 1) the surface tention of the mixed solutions (Solution 2 and 3) decreased, and it is consistent with literature (J.-W. Lu et al., 2006). They reported that the incorporation of a small amount of NaAlg into poly(ethylene oxide) (PEO) solutions had resulted in decreased surface tention of the mixed solutions due to the surface-active properties of alginate. No significant difference was observed between surface tensions of Solution 2 and 3, therefore the addition of $CaCl_2$ did not effect the surface tention.

The electrospinning process has been characterized according to Saville's leaky dielectric model, which considers fluid motion in electric fields due to the charges on the surface of the fluid (Saville, 1997). The leaky dielectrics have enough free charges to move onto the surface of the fluid form a Taylor cone at the tip of the fluid droplet, and the electrospinning process then start making nanofibers (Angammana & Jayaram,

2011). Dielectrical constants of polymer solutions were demonstrated in Figure 3.2 as a function of frequency. The dielectrical properties of Solution 1 and Solution 2 are so close to each other, while their electrospinnabilities are quite different. Due to the fact that dielectric properties alone are not sufficient to explain the solution electrospinnability the effects of conductivity as well as surface tension should be considered for a comprehensive evaluation. It was also stated that the dielectric constants of Solution 3 significantly lower than that of Solution 2. As mentioned before, significantly finer fibers with lower mean diameter fabricated with the addition of $CaCl_2$ to Pul and NaAlg contained polymer solutions. However, this situation cannot be explained with the electrical conductivity or surface tension of solutions. Dielectric materials diminish the magnitude of electrical field when placed in an electrical field (Isik et al., 2018). Thus, solutions with higher dielectric constant inhibit electrospinning process. On the other hand, the differences between fiber characteristics and mean diameter of Solution 2 and Solution 3 can be explained by lower dielectric constants of the Solution 3, possibly due to the ionic interactions between NaAlg and $CaCl_2$ (Gutiérrez-Jara et al., 2020).

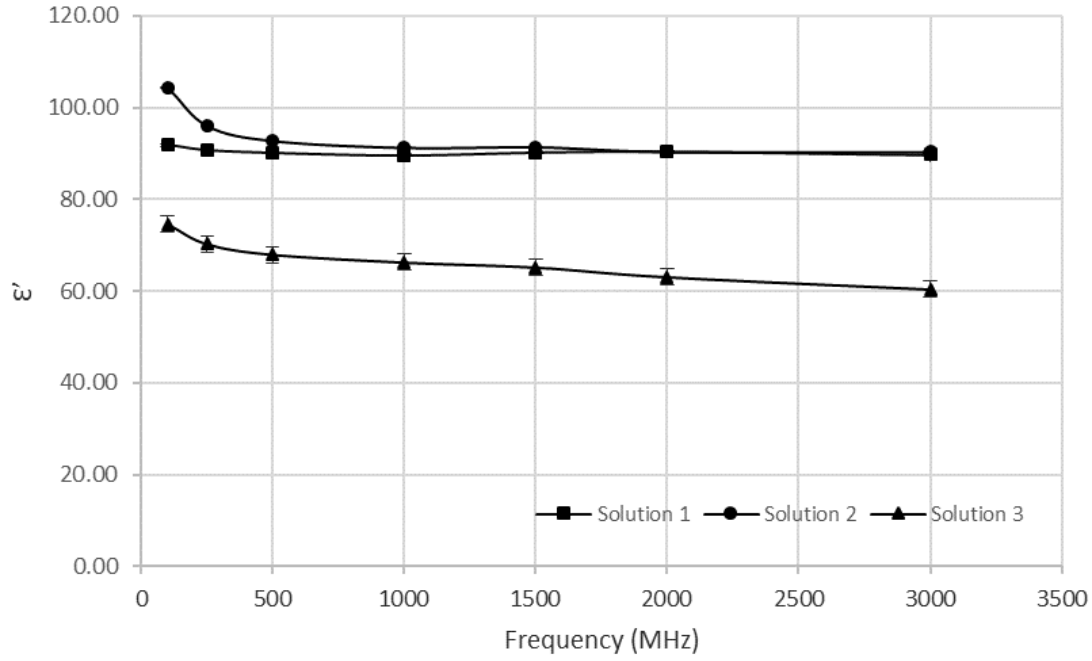

Figure 3.2 : Dielectrical properties of polymer solutions.

Viscosity is another important factor that affect the electrospinnability of solutions to be used for fiber formation. According to the results of rheological measurements, all feed solutions exhibited non-Newtonian shear-thinning / pseudoplastic behaviour with a flow behaviour index lower than one. The power-law equation is the most common

model to explain pseudoplastic behaviour, according to the consistency index (*K*) and the flow behaviour index (*n*). The viscosity of the pseudoplastic solutions were decreased with increasing shear rates (Steffe, 1996). Therefore, if the polymer solution exhibite non-Newtonian behaviour, the apparent viscosity must be given with a corresponding shear rate. Maximum shear rate value of solution at the ejection point of needle was chosen as the corresponding shear rate and apparent viscosities were calculated according to that maximum shear rate. The maximum shear rate at the ejection point were calculated as ~ 4 s^{-1} from Equation 3. As expected, the incorporation of NaAlg into pullulan solutions had resulted in increased viscosity of the mixed solutions due to the increased polymer concentration. According to literature, the increase in viscosity favors the formation of bead-free smooth fibers, while the increase in surface tension and the decrease in conductivity show opposite effect (J.-W. Lu et al., 2006). When the results regarding the solution properties measurements are evaluated as a whole, it can be said that they are considerably consistent with the literature.

3.4.2 Characterization of electrospun fibers

Zeta potential measurements were performed in order to characterize electrospun fibers and results obtained as 5.86 ± 1.23, -4.65 ± 0.43, -7.13 ± 0.71 and -7.18 ± 0.72 for Sample 1, 2, 3 and 4; respectively, resulting with negative surface charges for all the samples. Isik et al. (2017) studied the encapsulation of sour cherry concentrate by electrospinning using gelatin and lactalbumin as wall materials. They found that the zeta potentials of sour cherry concentrate loaded nanofibers were ranging from -3.19 to -9.38 (Isik et al., 2018). Zeta potential values of Sample 3 and 4 are relatively lower than others and there are no differences between them, significantly ($P < 0.05$). Therefore, zeta potential was not change with the addition of SPM to the formulation. Results indicate that addition of $CaCl_2$ reduced the zeta potential value but increased absolute value of zeta potential. It's proved that the intermolecular interactions were enhanced by introducing $CaCl_2$ to the solutions. Koo et al. (2014) interpreted the increase of absolute value of zeta potential as the increase of stability. Therefore, considering the zeta potential values, it is possible to deduce that the addition of $CaCl_2$ has a stabilizing effect on the nanofibers.

3.4.3 Encapsulation efficiency of electrospun fibers

Encapsulation efficiency of the electrospun samples were calculated based on the ratio between total peptide content in the electrospun and the initial solution. First of all, the calibration curves were drawn using SPM standard solution and the calibration equations for each compounds, y=ax+b, were constructed using the peak area versus peptide concentration (μg/mL) data. The correlation coefficients, r, were calculated between 0.969 - 0.998. The encapsulation efficiency results were calculated for each peptide in SPM and given in terms of total peptide content. Encapsulation efficiency of SPM by uniaxial electrospinning procedure was calculated as 77.97 ± 1.65%.

Similar to the results obtained in the present work, encapsulation efficieny of peptides varied from 42% to 95%, depending on the encapsulation method, polymers and other process parameters (Hosseini et al., 2019; Ramezanzade et al., 2017; Z. Wang et al., 2015; X. Zhang et al., 2017). For instance, Wang et al. (2015) obtained encapsulation efficiencies ranging from 87.1 to 94.7% for rapeseed peptides encapsulated using spray drying with rapeseed protein isolate. Addition to this, Ramezanzade et al. (2017) obtained encapsulation efficiency of rainbow trout skin-derived antioxidant peptides of chitosan-coated nanoliposomes ranged between 46.1 – 80.2%. In another study investigating the encapsulation of oyster peptide extract in microcapsules formed with microemulsion gelling method, encapsulation efficiencies ranging from 71.46 to 75.37% (X. Zhang et al., 2017). Furthermore, Hosseini et al. (2019) studied the production of chitosan/poly(vinyl alcohol) nanofibrous mat by electrospinning. Fish-purified antioxidant peptides weresuccessfully loaded into the nanofibrous mat with high encapsulation efficiency, beyond 94%.

3.5 Conclusion

This study showed that uniaxial electrospinning was successfully applied for encapsulation of peptides with 77.97% encapsulation efficiency value via green electrospinning. Thus, biodegradable and non-toxic nanofibers made only of food-derived polysaccharides could be produced for encapsulation of peptides without using any synthetic polymers and /or harmful solvents. NaAlg and $CaCl_2$ improved electrospinnability of pullulan with enhanced the solution properties such as viscosity, surface tention and electrical conductivity; thus SPM loaded Pul-NaAlg-$CaCl_2$ composite nanofibers were successfully fabricated under pre-determined proper

conditions. Additionally, incorporation of $CaCl_2$ decreased mean diameter and improved stability of electrospun fibers possibly due to intermolecular interactions between NaAlg and $CaCl_2$. Considering all the results obtained in this study, Pul-NaAlg-$CaCl_2$ composite nanofibers can be considered as a promising material to encapsulate various bioactive peptides with several health effects for food purposes.

4. INCORPORATING ANTIOXIDATIVE PEPTIDES WITHIN NANOFIBROUS DELIVERY VEHICLES: CHARACTERIZATION AND *IN VITRO* RELEASE KINETICS

4.1 Abstract

L-carnosine (Car), an antioxidative dipeptide, is a promising health-promoting bioactive agent, which can be isolated from animal waste. In the present study, Car loaded pullulan (Pul)-sodium alginate (NaAlg) based nanofibrous delivery vehicles were fabricated by uniaxial, coaxial and emulsion electrospinning. The $CaCl_2$ crosslinking was applied after electrospinning process to evaluate the effect on release behavior of Car. Results showed that Car was successfully loaded in water-in-oil-in-water ($W_1/O/W_2$) double emulsion to produce nanofibers by emulsion electrospinning. Encapsulation efficiencies were found to be 74.11% and 81.69% for the uniaxial (UENFs) and coaxial nanofibers (CENFs), respectively. Encapsulation efficiency was determined for different formulas of emulsions, whereas among the samples that could form nanofibers (NFs) and encapsulate Car, the highest value was obtained for nanofibers from emulsion-VIII (EE-VIII NFs) at 68.63%. DPPH and CUPRAC assays revealed that all electrospinning methods demonstrated protective effect on the antioxidant activity of Car and thus helped in enhancing its antioxidant potential significantly. According to *in vitro* digestion results, the release of Car from all electrospun NFs was predominantly controlled by Fickian diffusion mechanism. The $CaCl_2$ crosslinking treatment improved water resistance of NFs and enhanced sustained release of Car in the gastrointestinal tract. The initial burst release of Car from EE-VIII NFs was significantly lower than for UENFs and CENFs in the gastric phase, and the release from EE-VIII NFs in the intestinal phase was followed by sustained release, with/without crosslinking treatment. It can therefore be said that the simultaneously encapsulation of Car in double emulsion and in Pul-NaAlg based electrospun NFs can provide sustained release.

Keywords: encapsulation; electrospinning; double emulsion; antioxidative peptide; *in vitro* release

4.2 Introduction

Bioactive peptides, biologically active amino acid groups in protein sequence, regulate immunological, gastrointestinal, hormonal and neurological responses, thus playing an important role in the prevention of cancer, osteoporosis, hypertension and some other disorders (Maestri et al., 2016). L-carnosine (β-alanyl-L-histidine) (Car) is a hydrophilic endogenously synthesized dipeptide in muscular and other tissues being distributed to organs. Meat and fish are primary dietary sources for Car intake (Jackson & Lenney, 1996). It is reported that Car has numerous biological functions such as antioxidation, anti-glycation, anti-stress hydroxyl radical scavenging, maintenance of pH-balance, metal chelating, anti-aging and wound healing effects (Dahl et al., 1988; Decker et al., 1992). The antioxidative effect of Car was first taken into consideration by Kohen et al. (1988)'s study on Car scavenging peroxyl radicals.

The current relevance of health-promoting ingredients and food supplements in human nutrition and healthy living has led to an increased interest in developing encapsulation technologies to carry, protect and deliver bioactive agents. Encapsulation is a concept by which one bioactive compound or a complex containing bioactive compounds is coated with or entrapped within another complex body, which provides physical and chemical stability to bioactive compounds and sustained delivery into the gastrointestinal tract (Risch, 1995). Like many bioactive compounds, bioactive peptides are sensitive against gastrointestinal tract hostile conditions, such as the stomach acidic environment and high proteolytic activity, and may lose their biological activity (Fabiano et al., 2018; Fernández-Tomé et al., 2019). Furthermore, colon is a convenient organ for the absorption of proteins and peptides, due to its reduced proteolytic enzyme activity, neutral pH and prolonged transit time (Choonara et al., 2014; de Oliveira Cardoso et al., 2020).

Antioxidative peptides, potentially natural, safe and healthy agents for use as food supplement, have been encapsulated by several methods, such as liposomal encapsulation and nanoemulsions in previous studies (J. Huang et al., 2020; Y.-H. Wang et al., 2016). On the other hand, there are limited studies in the literature which deal with encapsulating Car with respect to use as a food supplement (Maherani et al., 2012; J. Zhu et al., 2016). In the study of Intiquilla et al. (2022), antioxidant peptides from *Lupinus mutabilis* were encapsulated successfully by nanoencapsulation for

colonic delivery and they were controlled release from resulting nanocarriers with maintaining their antioxidant capacity (Intiquilla et al., 2022).

Electrospinning is a simple, flexible and promising encapsulation technology because of the controlled release possibilities based on high encapsulation efficiency, enhanced bioavailability, good thermal, light and storage stability, advanced protection of bioactive compounds from chemical degradation and sustained release of loaded compounds (H. Huang et al., 2022; Rostamabadi et al., 2020). Recently, electrospinning technique has been used to encapsulate bioactive peptides to increase the bioaccessibility and improve sustained release (Han et al., 2017; Hosseini et al., 2019; Rajanna et al., 2022). Han et al. (2017) developed electrospun triaxial fiber membranes to encapsulate nisin and achieved long term – sustained release of nisin from nanofiber (Han et al., 2017). Furthermore, it was reported that antioxidative activity of peptides preserved in electrospun fibers and loaded peptides displayed more sustained release from nanofibers compared to free peptides (Hosseini et al., 2019; Rajanna et al., 2022). The release data were analyzed kinetically using various mathematical models (Mehrnia et al., 2017). The Higuchi, Korsmeyer-Peppas, Kopcha, Peppas-Sahlin, Weibull and Gompertz models are commonly used kinetic models to evaluate release data (Charpashlo et al., 2021; Kaboli et al., 2021).

Electrospun fibers are ideal vehicles for the delivery of bioactive compounds due to their high surface area to volume ratio, which provides more efficient mass transfer of biomolecules. There are three electrospinning techniques for loading bioactive compouns into fiber; blending (uniaxial), coaxial, and emulsion electrospinning (Briggs & Arinzeh, 2014; Jue Hu et al., 2015). In the blending (uniaxial) electrospinning technique is the easiest electrospinning technique to encapsulate bioactive compounds and performed by blending polymer and biomolecule to use as feed solution. The major disadvantages of blending electrospinning technique is tendency of biomolecule to locate on the surface of fiber due to applied electrical force, thus resulting in burst release phenomenon, decomposition of biomolecule and loss of its biological functions due to the challenging conditions of the digestive system, in the early stage of release (Y. Yang, Li, Qi, et al., 2008; Zeng et al., 2005). Core-shell fibers are more suitable for the delivery of bioactive molecules and controlling the release over a sustained time period (Crespy et al., 2012). The coaxial electrospinning is the characteristic technique used to obtain core-shell fibers and provides higher

controlled release property (López-Rubio et al., 2012); though this technique provides well controlled fiber morphology, requirements, such as special apparatus, demand careful selection of the operational conditions to ensure desirable results without adversely affecting feasibility (Jun Hu et al., 2013; Viry et al., 2012).

Emulsion electrospinning is the alternative technique to obtain core-shell fibers (Y. Yang, Li, Qi, et al., 2008) and has attracted growing interest in recent years (Falsafi et al., 2022; L. Liu et al., 2021; Zhan et al., 2021; T. Zhang et al., 2019). This technique is relatively simple and has various advantages, such as minimizing the amount of organic solvents and other hazardous chemicals, thus being environmentally friendly and suitable for organic solvent sensitive hydrophilic components (peptides, proteins, DNA) (Crespy et al., 2012). Double emulsions, also known as multiple emulsions, are among the methods that can be used for encapsulation of bioactive compounds. There are many studies in the literature related to encapsulation of hydrophilic or hydrophobic bioactive compounds in double emulsions. On the other hand, very limited studies have been conducted on electrospinning of double emulsions (Carrillo et al., 2016; Foraida et al., 2017).

Synthetic polymers can be toxic or non-biodegradable (López-Rubio et al., 2012). Thus, natural food-grade biopolymer such as proteins and polysaccharides must be preferred for food technology applications, especially to fabricate healthy food supplements. Pullulan (Pul) is a non-toxic, biodegradable and biocompatible carbohydrate polymer produced from the industrial fermentation of starch syrup caused by *Aureobasidium pullulans* and is used in pharmaceuticals, cosmetics, and the food industry (Román et al., 2019). On the other hand, alginate is an edible, biodegradable, relatively low cost and non-toxic polysaccharide, extracted from brown seaweed, and its sodium alginate (NaAlg) form has been widely used in the food industry (Zhong et al., 2010). The surface active property of NaAlg, as well as high water solubility of Pul and NaAlg, make it possible to obtain nanofiber without using any hazardous solvents (Brovko et al., 2023; Román et al., 2019; Zhong et al., 2010).

Hence, this study aims to fabricate a nanofibrous delivery vehicle for Car antioxidative dipeptide to maintain its integrity and biological activity against gastrointestinal tract hostile conditions, such as the stomach acidic environment and high proteolytic activity until they reach the intestinal wall. Pul-NaAlg-Car composite nanofibers were produced by uniaxial, coaxial and emulsion electrospinning techniques without using

any synthetic polymers or any hazardous solvents via green electrospinning. Ionic crosslinking was applied to improved water-resistant properties. Emulsion properties were investigated to assess the role of emulsion parameters on electrospun fibers. The effects of electrical, rheological and surface properties of feed solutions / emulsions on electrospinnability were also investigated. The encapsulation efficiencies, antioxidant activities and the release behavior of Car were investigated and its release kinetics were analyzed by establishing a corresponding mathematical model.

4.3 Materials and Methods

4.3.1 Materials

L-carnosine (98%, HPLC grade) was purchased from Sigma-Aldrich (St. Louis, USA). Pullulan (Pul) was donated by Hayashibara Co. Ltd. (Japan). Sodium alginate (NaAlg), sunflower oil (SO), medium-chain triglycerides (MCT) was purchased from a local company. Lecithin (soybean, 90%) was purchased from Alfa Aesar Chemicals (USA). Polyglycerol polyricinoleate (PGPR) was donated by Aspek Chemical Co., Ltd, Turkey. Tween 80 and $CaCl_2$ were purchased from Merck KGaA (Darmstadt, Germany). 1, 1-dipheny l-2-picrylhydrazyl (DPPH), methanol (HPLC grade), acetonitrile (HPLC grade), trifluoroacetic acid (TFA) (HPLC grade), NaOH, HCl, porcine pepsin, pancreatin, and bile salt were purchased from Sigma-Aldrich (St. Louis, USA).

4.3.2 Preparation of $W_1/O/W_2$ double emulsions

A series of emulsions containing different ratios of Car, and different combinations surfactants and oily phases were used for emulsion electrospinning (Table 4.1). The emulsions were prepared by two step emulsification technique (Dickinson, 2011).

Primary W_1/O single emulsions were prepared using 30% (w/w) of the inner aqueous phase (W_1) and 70% (w/w) of the continuous oily phase (O). Dispersed phase was prepared by dissolving Car in distilled water at room temperature by mixing on a magnetic stirrer for 30 min. Two different ratios of Car and distilled water in dispersed phase were 1:2 (w/w) and 1:5 (w/w). SO and MCT were used as continuous oily phases containing PGPR as a hydrophobic emulsifier at certain concentration, previously dissolved by stirring at 50°C for 30 min. PGPR is a commonly used hydrophobic surfactant with low hydrophilic–lipophilic balance value (HLB, 3.0) in

food formulation and has been demonstrated to be highly effective for stabilizing W_1/O emulsions (Matos et al., 2014). The ratio of PGPR and oil in continuous phase was 1:13 (w/w). Therefore, 0.30 g of dispersed phase containing Car was added drop wise to 0.70 g of corresponding continuous oily phase and mixed by a high shear homogenizer (Heidolph SilentCrusher S, Germany) at 20000 rpm for 5 minutes to yield a creamy emulsion. The final compositions of W_1/O emulsions are also described in Table 4.1.

$W_1/O/W_2$ double emulsions were prepared using 20% (w/w) of primary emulsions (W_1/O) and 80% (w/w) of external continuous phase (W_2). Distilled water was used as external continuous phases containing Tween 80, Lecithin and Tween 80-Lecithin mixture (2:1; w/w) at certain concentration, previously dissolved by stirring at room temperature for an hour. W_1/O primary emulsions were introduced drop wise to continuous aqueous phases (W_2). The emulsion was further mixed by a high shear homogenizer (Heidolph Silent Crusher S, Germany) at 15000 rpm for 5 minutes. The final compositions of $W_1/O/W_2$ double emulsions were also described in Table 4.1.

Table 4.1 : Compositions of differently formulated polymer solutions and process conditions of electrospinning.

	Role	Composition	Solutions				Emulsions											
			Control	UE	CE		EE											
					Core	Shell	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
W_1/O emulsions	Dispersed phase	Car	-	-	-	-	10	10	10	10	10	10	5	5	5	5	5	5
		Deionized water	-	-	-	-	20	20	20	20	20	20	25	25	25	25	25	25
	Continuous phase	PGPR	-	-	-	-	5	5	5	5	5	5	5	5	5	5	5	5
		Sunflower oil	-	-	-	-	65	65	65	65	65	65	-	-	-	-	-	-
		MCT	-	-	-	-	-	-	-	-	-	-	65	65	65	65	65	65
$W_1/O/W_2$ emulsions	Dispersed phase	Primary W_1/O emulsion	-	-	-	-	10	10	10	10	10	10	10	10	10	10	10	10
	Continuous phase	Deionized water	-	-	-	-	87	87	87	87	87	87	87	87	87	87	87	87
		Tween 80	-	-	-	-	3	-	2	3	-	2	3	-	2	3	-	2
		Soy lecithin	-	-	-	-	-	3	1	-	3	1	-	3	1	-	3	1
Feed solutions	Solvent	$W_1/O/W_2$ double emulsion	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100
		Deionized water	100	100	100	100	-	-	-	-	-	-	-	-	-	-	-	-
	Polymer	Pul	10	10	20	10	10	10	10	10	10	10	10	10	10	10	10	10
		NaAlg	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2
	Active component	Car	-	0.54	3	-												
	Charge density enchanger	$CaCl_2$ (mg/kg)	30	30	-	30	30	30	30	30	30	30	30	30	30	30	30	30
	Process conditions																	
	Flow rate (ml/h)		0.7	0.7	0.6	0.15	1	0.7	0.7	1	0.7	0.7	1	0.7	0.7	1	0.7	0.7
	Diameter of needle (G)		19	19	22	16	19	19	19	19	19	19	19	19	19	19	19	19
	Applied voltage (kV)		15	15	15		15	19	15	15	19	15	15	19	15	15	19	15

All the values of emulsions are expressed as % (w/w); the ratio of polymers and active component are g basis, and based on the amount of 100 g $W_1/O/W_2$ double emulsion or deionized water; the ratio of $CaCl_2$ is based on the amount of $W_1/O/W_2$ double emulsion.

4.3.3 Characterization of emulsions

A dynamic light scattering instrument (Malvern Zetasizer NanoZS, UK) with a He-Ne laser (633 nm) was utilized for mean droplet size and zeta potential (ξ) measurements of $W_1/O/W_2$ double emulsions before and after polymer addition for electrospinning (described here as feed emulsions). Each sample was diluted 1000 times using deionized water at room temperature prior to measurement to prevent multiple scattering effects. The angle of scattered light was 173°, and the detection temperature was maintained at 25°C. All measurements were performed in triplicate for each sample.

The morphology of the W_1/O primary emulsion and $W_1/O/W_2$ double emulsion was obtained with a binocular microscope equipped with a camera (Nicon Eclipse NiU, USA). Fresh prepared emulsion samples were examined with 40 × magnification lens at room temperature.

4.3.4 Preparation of feed solutions / emulsions

Feed solutions were prepared as described in Table 4.1. Pul and NaAlg containing solutions were mixed over night at room temperature and 200 rpm. Certain amounts of Car were added to polymer solutions for uniaxial electrospinning (UE) and completely dissolved by stirring at room temperature and 500 rpm for two hours. $CaCl_2$ was added to formulations 30 min before the electrospinning process then mixed under the same conditions. The control solution was prepared following the same procedure without the addition of Car.

To obtain core and shell polymer solutions of coaxial electrospinning (CE) Pul and NaAlg containing solutions were mixed over night at room temperature and 200 rpm. Car were added to core feed solution at the ratio of 3 g/100 g deionized water and completely dissolved by stirring at room temperature and 500 rpm for two hours. $CaCl_2$ was added to shell feed solution 30 min before the electrospinning process then mixed under the same conditions.

Pul (10 g/100 g emulsion) and NaAlg (2 g/100 g emulsion) were added to already prepared $W_1/O/W_2$ double emulsions for emulsion electrospinning and completely dissolved by stirring at room temperature and 500 rpm for two hours. $CaCl_2$ was added to formulations 30 min before the electrospinning process then mixed under the same conditions.

4.3.5 Characterization of feed solutions

Electrical conductivities of polymer solutions were measured by a conductometer (WTW LF95, Germany) and surface tension values were measured by a tensiometer (Dataphysics DCAT 11E, Germany) by the Wilhelmy plate method, both at 25 °C, in triplicate for each sample. Furthermore, dielectric constant (ε') of feed solutions was measured by a network analyzer (Agilent E5061B, USA) equipped with a dielectric probe kit (Agilent 5070E) at 25 °C, in triplicate for each sample. Dielectric constants of the solutions were investigated from 1 MHz to 3 GHz. All measurements were performed in triplicate for each sample.

Rheological properties of polymer solutions were measured using a rheometer (Haake Rheostress 1, Germany) using the plate-plate sensor (35 mm diameter, 1 mm gap) configuration at 25 °C, in duplicate for each sample. The shear rate range was between 0 and 300 s-1 in 120 s. The results were modeled using the software (Haake RheoWin3 Data Manager, Germany) according to the power-law equation 4.1:

$$\tau = \mathrm{K}\,\dot{\gamma}^{\mathrm{n}} \tag{4.1}$$

where τ is the shear stress (Pa), K is the consistency index (Pa s^n), n is the flow behavior index and $\dot{\gamma}$ is the shear rate (s^{-1}).

4.3.6 Electrospinning process

The electrospinning process was conducted with the equipment of Inovenso NE100 (Turkey) at room temperature. The equipment includes syringe pumps (New Era Pump Systems Inc., NE-300, USA), the needle, the voltage supply (Nanofen, Turkey) and the collector plate. The distance between the tip of needle and the collector plate was kept constant at 15 cm. The applied voltages were determined according to the preliminary tests, and voltages that allowed continuous fiber formation from the drops at the tip of the needle throughout electrospinning were chosen. The operation conditions are given in Table 4.1. The electrospun samples were collected on an aluminum foil which was used for covering the collector plate. In the current study, uniaxial, coaxial and emulsion electrospinning were performed to encapsulate the Car. The moisture content of the electrospun samples was accepted to have been completely removed during the electrospinning process, so the results of all analyses were expressed on a dry weight basis.

4.3.7 Crosslinking of electrospun nanofibers

Surface crosslinking was performed to enhance mechanical stability and to control the release of Car from NFs according to the procedure of Baek et al. (2020), with some modifications. To stabilize Pul-NaAlg NFs $CaCl_2$ was used as a crosslinking agent. Crosslinking process was conducted by putting nanofiber films into 5% $CaCl_2$ solution (w/v in 95% ethanol) for 2 hours. Then, to remove the residual $CaCl_2$, the films were rinsed several times in 95% ethanol. Finally, the films were exposed to open air until the solvent evaporated.

4.3.8 Characterization of electrospun nanofibers

The morphology and surface characteristics of the electrospun samples were ascertained by scanning electron microscopy (SEM) (Zeiss Evo LS10, Germany). Briefly, 1 cm^2 electrospun samples were coated with Au-Pd to minimize sample charging by using a sputter coater (Quorum, SC7620). Microscopy was performed at high vacuum with an accelerating voltage. Images were taken at certain magnifications. Mean diameter of fibers was calculated according to data obtained from analyses of SEM images by ImageJ software (National Institutes of Health, USA). Two different micrographs were analyzed to calculate the mean diameters of each sample and twenty-five measurements were made from each micrograph.

The mechanism of crosslinking and possible other interaction between components involved in the emulsion structure was investigated by Fourier-transform infrared spectroscopy (FTIR). FTIR spectra of electrospun samples were obtained by a FTIR spectrometer (PerkinElmer Spectrum Two, USA) with an attenuated total reflection (ATR) unit attached. Scans were conducted over a spectral range of 450 - 4000 cm^{-1}.

Contact angle measurements were performed to characterize the hydrophobicity and stability of the resulting nanofiber mats. The contact angles of the electrospun nanofibers were measured in triplicate for each sample by a tensiometer (Dataphysics DCAT 11 E, Germany).

4.3.9 HPLC analysis

Analyses were operated by a reversed phase High Performance Liquid Chromatography system (Agilent 1260 Infinity, USA) coupled to a PDA detector (Agilent G1315D, USA). Car and other compound such as polymer and surfactants

were separated using a Waters Symmetry C18 column (150 x 4.6 mm, 5 µm; Waters Corporation, Milford, MA), then the content of Car was quantified. Injection volume was 10 µl and samples were injected by an auto sampler (Agilent 1329B, USA). Column temperature was adjusted to 50°C. The isocratic elution system consisted of a phase A (5% acetonitrile [ACN] and 0.1% trifluoroacetic acid [TFA] contained distilled water) used as mobile phase. The flow rate of the mobile phase was 1 mL/min. The detection of eluted peaks was achieved at 210 nm. Dose-response curve of Car (50 – 600 mg/L) was used to quantify the compounds in the samples. The samples were eluted with distilled water and filtered through 0.45 µm membrane filters. Measuring was performed in three parallel measurements for each concentration.

Before the HPLC analysis, *in vitro* gastrointestinal digestion samples (prepared as explained in 2.2.11) were centrifuged (Hettich Zentrifugen Universal 32 R refrigerated centrifuge, Germany) at 15000 g and 4 °C for 5 min. The supernatants were filtered through 0.45 µm membrane filters. Two different dose-response curves of Car (5 – 40 mg/L and 200 – 1000 mg/L) were used to quantify the compounds in the digestion samples.

4.3.10 Encapsulation efficiency

Encapsulation efficiency of electrospun fibers was calculated according to HPLC analysis results using the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{amount\ of\ peptide\ in\ electrospun\ fiber}{amount\ of\ bioactive\ compounds\ fed\ initially} \times 100 \quad (4.2)$$

4.3.11 Antioxidant capacity assays

4.3.11.1 DPPH radical scavenging activity assay

The DPPH radical scavenging activity was determined according to Hara et al. (2018). 50 µl sample was mixed with 200 µl 0.01 mM DPPH solution. After 30 min in the dark, the absorbance was read at 517 nm against methanol.

4.3.11.2 Cupric ion reducing antioxidant capacity (CUPRAC)

The CUPRAC antioxidant capacity was determined according to Apak et al. (2004). 5 µl sample was mixed with 50 µl 0.01 mM $CuCl_2$ solution, 50 µl 7.5 mM neocuproine

solution, 50 µl NH_4Ac buffer at pH: 7 (19.27 g NH_4Ac was dissolved in 250 ml distilled water) and 50 µl distilled water. After 30 min in the dark, the absorbance was read at 450 nm against distilled water.

4.3.12 *In vitro* release study

The *in vitro* digestion studies were carried out following the methodology adapted from Minekus et al. (2014). Simulated gastric fluid (SGF) containing 6.9 ml 0.5 M KCl, 0.9 ml 0.5 M KH_2PO_4, 12.5 ml 1M $NaHCO_3$, 0.4 ml 0.15 M $MgCl_2(H_2O)_6$, 0.5 ml 0.5 M $(NH_4)_2CO_3$ and 1.3 ml 6 M HCl was diluted with distilled water to reach a final volume of 400 ml and acidified to pH 3 using 1 M HCl under constant stirring. Simulated intestinal fluid (SIF) containing 6.8 ml 0.5 M KCl, 0.8 ml 0.5 M KH_2PO_4, 42.5 ml 1M $NaHCO_3$, 9.6 ml 2 M NaCl, 1.1 ml 0.15 M $MgCl_2(H_2O)_6$ and 0.7 ml 6 M HCl was diluted with distilled water to reach a final volume of 400 ml and adjusted to pH 7 using 1 M NaOH under constant stirring.

Briefly, 10 mg of fibers (10 tubes with 10 mg of sample each) was diluted with 5 ml of distilled water and the mixture was incubated at 37 °C in a shaking water bath (Classic C76, New Brunswick Scientific, USA) for 2 min. For the gastric digestion, 3.35 mL of SGF, 0.8 mL of pepsin solution (25000 U/mL), 2.5 µL of 0.3 M $CaCl_2$ were added to mixture. Afterwards, the total volume of the mixture was increased to the full 10 mL with the addition of distilled water and the mixture was incubated in a shaking water bath at 37 °C for 2 h. For the intestinal digestion, 7.7 mL of SIF, 2.5 mL of pancreatin (800 U/mL), 1.25 mL 160 mM bile salt and 20 µL 0.3 M $CaCl_2$. The pH of the mixture was adjusted to 7.0 using 1 M NaOH. Then the total volume was brought up to 20 mL using distilled water and the mixture was incubated in a shaking water bath at 37 °C for another 2 h. The blank (without the added electrospun samples) was also incubated under the same conditions described above and used for the correction of interferences caused by digestive fluids.

At different time intervals (10, 30, 60, 90 and 120 min) during gastric and intestinal digestion periods, one of the tubes was collected and centrifuged (Hettich, Tuttlingen, Germany) at 15000 g and 4 °C for 5 min, and the supernatants were used to analyze the amount of released Car through HPLC. *In vitro* digestion procedure was performed in duplicate to Car and uniaxial, coaxial and emulsion electrospun samples. One of the twelve different emulsion electrospinning samples was selected for use in the *in vitro*

digestion study, taking into account desired fiber morphology, lowest fiber diameter, and highest encapsulation efficiency criteria.

4.3.13 Mathematical modelling of release kinetics

The Car release data were kinetically evaluated by Higuchi (Eq. (3)), Kopcha (Eq. (4)), Korsmeyer-Peppas (Eq. (5)), Peppas-Sahlin (Eq. (6)), Weibull (Eq. (7)) and Gompertz (Eq. (8)) models. The release data fitting was performed using a software (Matlab R2018b (Curve Fitting Toolbox 3.5.8.), The MathWorks Inc.), then the validity of all models was verified according to their regression coefficients.

Higuchi model describes the release of bioactive compounds from an insoluble matrix, controlled by Fickian diffusion mechanism.

$$M_t/M_\infty = kt^{1/2} \tag{4.3}$$

Where k is the Higuchi rate constant (Higuchi, 1963). M_t and M_∞ in all equations are the amount of Car released at time t and the total amount initially present in the electrospun fibers.

The Kopcha kinetic model define release of bioactive substance based on the mechanisms of diffusion and erosion.

$$M_t/M_\infty = A \times t^{1/2} + B \times t \tag{4.4}$$

Where A and B are the expressed diffusion rate constant, and the erosion rate constant respectively. Accordingly, if the ratio is |A/B|>1, release is controlled by diffusion mechanism; while for |A/B|<1, release is controlled by erosion mechanism. However, |A/B|=1 reveals the cooperation of both mechanisms to control release (Kopcha et al., 1991)

Korsmeyer- Peppas model describes release of bioactives from a polymeric system based on diffusion (Fickian) and erosion (non-Fickian).

$$M_t/M_\infty = kt^n \tag{4.5}$$

Where k is the Korsmeyer-Peppas rate constant related to the properties of delivery matrix and the encapsulated substance, n is the release exponent that gives information about the release mechanism of loaded substance; if n ≤ 0.45 represents a Fickian

diffusion mechanism; 0.45 < n < 0.89 represents an anomalous (non-Fiction) diffusion mechanism; n ≥ 0.89 up to 1 erosion mechanism controls the release of loaded substance from the cylindrical geometries like electrospun fibers (Korsmeyer et al., 1983; Rezaeinia et al., 2020).

The Peppas-Sahlin model is another simple equation used to investigate the release mechanism of encapsulated substance.

$$M_t/M_\infty = K_1 \times t^m + K_2 \times t^{2m} \quad (4.6)$$

Where K_1, K_2 and m are expressed as the Fickian-diffusion rate constant, the erosion rate constant, and Fickian-diffusion exponent, respectively. Accordingly, if the ratio is $|K_1/K_2|>1$, release is controlled mostly by diffusion mechanism; while for $|K_1/K_2|<1$, release is controlled mostly by erosion mechanism. Besides, the ratio of $|K_1/K_2|=1$ reveals the cooperation of both diffusion and erosion mechanisms to control release (Peppas & Sahlin, 1989).

The Weibull model describes release of substance demonstrating "regular" dissolution curves.

$$M_t/M_\infty = 1 - \exp\left[-\frac{(t-T_i)^b}{a}\right] \quad (4.7)$$

Where M_t and M_∞ are the amount of Car dissolved at time t and the total amount initially present in the electrospun fibers. T is lag time of the release process (mostly taken as zero), a is scale parameter, b is shape parameter (Ramteke et al., 2014). Accordingly, if shape parameter b ≤ 1, release follows the Fickian diffusion mechanism, while b in the range between 0.75 and 1 is associated with combined mechanisms of Fickian diffusion and swelling. Besides, the shape parameter b > 1, reveals a complex mechanism of control transport process (Papadopoulou et al., 2006).

The Gompertz model is another kinetic model used to describe *in vitro* dissolution profile.

$$X(t) = X_{max} \exp[-a \exp(b\ logt)\ ] \quad (4.8)$$

Where $X(t)$ and X_{max} are the percent of dissolved Car at time t and the maximum dissolution, respectively. Also, a is scale parameter and b is shape parameter (Paarakh et al., 2018).

4.3.14 Statistical analysis

The means and standard deviations of replicated measurements were calculated and significance of differences between samples was determined with the help of analysis of variance (ANOVA) using statistical software (SPSS, IBM Statistics).

4.4 Results and Discussion

4.4.1 Droplet size and zeta potential of emulsions

The droplet size distribution and zeta potential of the feed solutions are important factors to consider for emulsion electrospinning since they may affect the oily phase distribution and entrapment of bioactive molecules within the nanofibers. Thus, the effect of polymer addition to emulsions on droplet size distribution and zeta potential values were evaluated (Table 4.2). Droplet size (z-average in intensity) of double emulsions was measured between 0.21 – 0.42 μm. On the other hand, droplet sizes of feed emulsions were measured at between 0.32 – 0.61 μm after 2 hours mixing with polymers. The results were in accordance with the literature. A study on electrospinning of W/O emulsions for protein encapsulation reported that droplet size of feed emulsions was between 0.35 and 0.60 μm. According to results, It was determined that the effect of emulsion parameters on droplet size of emulsions is not statistically significant ($P < 0.05$). However, after blending, the droplet size distribution of feed emulsions was closer to the original emulsions stabilized by soy lecithin. This is in agreement with the studies of Q.-Q. Yang et al. (2021) which indicated that adding lecithin stabilized W/O emulsions and significantly increased solubility and stability of the oily phase.

Zeta potential is an important parameter to determine stability of emulsions (Koo et al., 2014). All emulsions had negative surface charges. Zeta potential of emulsions was measured between (-46.43) – (-60.97) mV. After polymer addition, the absolute values of zeta potentials increased to (-50.23) – (-83.57) mV levels. The value of ± 30 mV is critical to explain stability of emulsions. If the absolute value of zeta potential is more than 30 mV, the emulsion is considered stable. However, when the absolute value of

zeta potential decreases from 30 to 0 mV, the particles become unstable and coagulation or flocculation accelerates (Ahn et al., 2018).

Accordingly, both double emulsions and feed emulsions can be considered stable, since the absolute values of zeta potentials were higher than 30 kV. On the other hand, results indicate that incorporation of soy lecithin increased the absolute value of zeta potential of feed emulsions. The interactions between emulsion droplets and other charged compounds such as surfactants, proteins, and polysaccharides can be affected by the electrostatic interactions and enhanced stability of emulsions (McClements & Jafari, 2018). Soy lecithin, a natural surfactant, had anionic functional groups which caused the particles to become negatively charged at neutral pH and previous studies demonstrated the potential of soy lecithin to form stable O/W emulsions (Chung et al., 2017). Therefore, considering the zeta potential values, it is possible to conclude that the soy lecithin present in the emulsions has a stabilizing effect on the nanofibers by interacting with Pul and NaAlg.

Table 4.2 : Droplet size and zeta potentials of $W_1/O/W_2$ emulsions and feed emulsions.

Sample	$W_1/O/W_2$ emulsions		Feed emulsions	
	z-average (μm)	Zeta potential (mV)	z-average (μm)	Zeta potential (mV)
EE-I	0.28 ± 0.05^{bcd}	-55.40 ± 1.15^{cd}	0.34 ± 0.03^{ab}	-60.33 ± 0.71^{d}
EE-II	0.32 ± 0.00^{cde}	-59.27 ± 2.00^{abc}	0.34 ± 0.03^{ab}	-79.30 ± 1.55^{abc}
EE-III	0.21 ± 0.02^{a}	-53.73 ± 1.29^{de}	0.34 ± 0.02^{ab}	-83.57 ± 1.75^{a}
EE-IV	0.38 ± 0.01^{fg}	-50.47 ± 1.07^{ef}	0.61 ± 0.02^{e}	-53.20 ± 0.26^{e}
EE-V	0.25 ± 0.00^{ab}	-57.43 ± 2.14^{abcd}	0.32 ± 0.00^{ab}	-83.33 ± 6.12^{a}
EE-VI	0.27 ± 0.00^{bc}	-56.33 ± 2.12^{bcd}	0.55 ± 0.01^{d}	-82.20 ± 1.77^{ab}
EE-VII	0.24 ± 0.01^{ab}	-49.00 ± 1.10^{f}	0.59 ± 0.02^{e}	-52.27 ± 1.33^{e}
EE-VIII	0.35 ± 0.02^{ef}	-60.97 ± 1.91^{a}	0.36 ± 0.02^{b}	-77.03 ± 1.45^{c}
EE-IX	0.38 ± 0.05^{fg}	-54.60 ± 4.61^{d}	0.44 ± 0.02^{c}	-82.80 ± 2.08^{ab}
EE-X	0.42 ± 0.03^{g}	-50.20 ± 2.57^{ef}	0.33 ± 0.01^{ab}	-50.23 ± 1.01^{e}
EE-XI	0.31 ± 0.01^{cde}	-59.70 ± 1.61^{ab}	0.32 ± 0.01^{a}	-80.53 ± 2.20^{abc}
EE-XII	0.33 ± 0.04^{de}	-46.43 ± 0.29^{f}	0.32 ± 0.03^{a}	-78.77 ± 1.60^{bc}

Data given in this table represents average amounts ± standard deviation of three replications for each sample. Data was analyzed by the one-way ANOVA and different letters (a–g) in the same column indicate significant differences by the Duncan test ($P < 0.05$).

4.4.2 Emulsion morphology

Emulsions were visualized using a polarization microscope in order to gain a better understanding of their microstructure and Figure 4.1 shows the microphotographs of the primary and double emulsion of EE-VIII, which were selected for further analyses. In Figure 4.1-b, oil globules containing small droplets inside (inner phase (W_1)) can be clearly identified, indicating that the double emulsion had formed. Double emulsions were classified into three different types depending on the disposition of the inner water droplets inside the oil globules. For $W_1/O/W_2$ double emulsions, type A double emulsion has only one large inner water droplet, known as "core-shell" globules, whereas type B and type C double emulsions contain several and a large number of small internal droplets, respectively (Florence & Whitehill, 1981). In the present work, the type B double emulsions were dominant and several small inner water droplets (less than 50) were successfully encapsulated in the relatively larger oil globules (Iqbal et al., 2015).

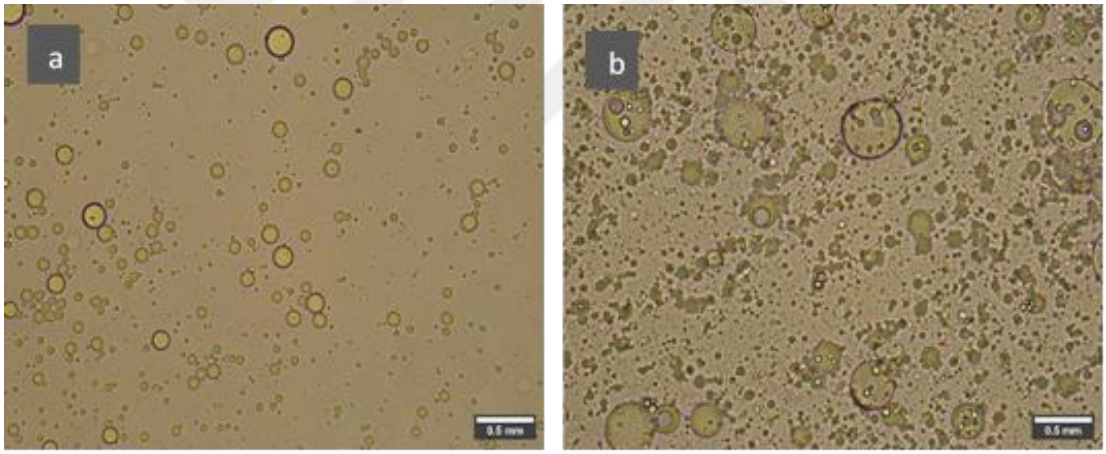

Figure 4.1 : Micrographs of EE-VIII primary (a) and double (b) emulsions obtained with use of polarization microscope.

4.4.3 Properties of feed solutions

Various parameters have been reported to influence fiber formation and morphology, such as solution properties, environmental factors and process conditions (Chronakis, 2010; Mohanty et al., 2016). Some of the solution properties, including electrical conductivity, surface tension, dielectric constant, and solution rheology, were investigated to evaluate the effects on the electrospinnability of feed solutions. The properties of the solutions whose compositions were described in Table 4.1 are given in Table 4.3.

The electrical conductivity of the feed solutions must be higher than zero, to start the electrospinning process. Because conductive solutions have high repulsion to overcome the surface tension of droplets at the top of the needle during the electrospinning process (S. Wang et al., 2013). Among all blends, EE-II, EE-V, EE-III and EE-XI emulsions, which contain soy lecithin in the formulation of parent emulsion, had the highest electrical conductivities (3.08 ± 0.01, 3.08 ± 0.01, 2.89 ± 0.01 and 3.16 ± 0.02 mS/cm, respectively). Thus, addition of soy lecithin instead of Tween 80 increased electrical conductivities of feed emulsions. Electrical conductivity of CE-Core solutions (0.16 ± 0.01 mS/cm) was somewhat lower than others, and insufficient on its own to overcome surface tension to form uniform and bead-free fibers but shell solution drags the core solution along with it. Furthermore, statistically significant differences were observed between the Control and UE feed solutions, meaning that the addition of Car slightly increased the electrical conductivity of the solutions ($P < 0.05$). The results were in accordance with the literature. A study on encapsulation of casein-derived peptides by electrospinning reported that conductivity of Pul solution increased with addition of peptide (Rajanna et al., 2022). On the other hand, high conductivity value alone is not adequate for electrospinning. To obtain proper nanofiber morphology by electrospinning, repulsion force has to overcome the surface tension (Ki et al., 2005).

The results showed that the surface tension was found to be the highest for CE-Shell solution (51.95 ± 0.44 mN/m). Besides, feed solutions from emulsions have lower surface tension between 31.26 and 40.60 mN/m. This situation can be interpreted as the feed solutions obtained from emulsions being able to form fibers more easily since they have high electrical conductivity and low surface tension. According to results, it was determined that the effect of emulsion parameters on surface tension of solutions is not statistically significant, except in the oily phase ($P < 0.05$). When MCT was added to emulsions instead of SO as oily phase, the surface tension of the obtained polymer solutions decreased. It was determined that the surface tension of solutions containing MCT (31.26 - 36.31 mN/m) was lower than that of solutions containing SO (36.37 - 40.60 mN/m). According to the literature, the reduction of surface tension is beneficial to the preparation of bead-free fibers (Yuanduo Wang et al., 2019). However, low surface tension is not adequate for emulsion electrospinning. Single nozzle formation of core-shell structure is not produced by the structure of the utilized

spinneret as in coaxial electrospinning but by electrospinning of stable emulsion of two or more immiscible liquids. Therefore, in order for emulsion electrospinning to be carried out, the emulsion must first have the stability to withstand spinning conditions (Buzgo et al., 2018).

Table 4.3 : Properties of polymer solutions. Electrical conductivity, surface tension, dielectic constant consistency index and flow behavior index of feed solutions and emulsions.

Sample	Electrical conductivity (mS/cm)	Surface tention (mN/m)	Dielectic constant (ε') at 3 GHz	K (Pa.s)	n
UE	2.53 ± 0.00^{f}	43.61 ± 0.31^{j}	70.73 ± 0.12^{h}	1.09 ± 0.03^{a}	0.90 ± 0.01^{e}
Control / CE-Shell	2.49 ± 0.01^{e}	42.83 ± 0.42^{ij}	72.88 ± 0.17^{h}	0.97 ± 0.04^{a}	0.90 ± 0.00^{e}
CE-Core	0.16 ± 0.01^{a}	51.95 ± 0.44^{k}	76.69 ± 0.01^{i}	0.59 ± 0.09^{a}	0.94 ± 0.01^{f}
EE-I	2.48 ± 0.01^{e}	36.37 ± 0.41^{ef}	61.44 ± 2.07^{ef}	2.46 ± 0.06^{b}	0.80 ± 0.00^{d}
EE-II	3.08 ± 0.01^{l}	40.60 ± 1.46^{i}	41.57 ± 1.26^{ab}	5.55 ± 0.44^{e}	0.75 ± 0.01^{b}
EE-III	2.78 ± 0.01^{h}	38.02 ± 0.27^{gh}	58.87 ± 1.20^{e}	3.55 ± 0.33^{cd}	0.77 ± 0.01^{c}
EE-IV	2.43 ± 0.01^{d}	34.14 ± 0.16^{c}	63.13 ± 1.29^{f}	3.84 ± 0.28^{d}	0.74 ± 0.02^{ab}
EE-V	3.08 ± 0.01^{l}	31.26 ± 0.32^{a}	54.60 ± 0.41^{d}	6.42 ± 0.51^{f}	0.72 ± 0.02^{a}
EE-VI	2.62 ± 0.01^{h}	36.31 ± 0.43^{ef}	52.29 ± 0.46^{d}	3.55 ± 0.41^{cd}	0.78 ± 0.01^{cd}
EE-VII	2.35 ± 0.00^{c}	37.89 ± 0.30^{gh}	60.12 ± 0.34^{e}	3.92 ± 0.42^{d}	0.74 ± 0.02^{ab}
EE-VIII	2.89 ± 0.01^{k}	37.15 ± 0.72^{fg}	67.51 ± 0.40^{g}	6.42 ± 0.52^{f}	0.74 ± 0.01^{ab}
EE-IX	2.66 ± 0.01^{j}	38.46 ± 0.22^{h}	54.72 ± 1.60^{d}	3.68 ± 0.34^{d}	0.78 ± 0.01^{cd}
EE-X	2.14 ± 0.02^{b}	34.33 ± 0.09^{cd}	47.70 ± 1.36^{c}	2.82 ± 0.02^{bc}	0.78 ± 0.00^{c}
EE-XI	3.16 ± 0.02^{m}	33.10 ± 0.17^{b}	39.51 ± 3.20^{a}	6.64 ± 1.12^{f}	0.73 ± 0.02^{ab}
EE-XII	2.59 ± 0.01^{g}	35.32 ± 0.14^{de}	46.82 ± 2.20^{c}	3.11 ± 0.70^{bcd}	0.78 ± 0.00^{cd}

Data given in this table represents average amounts ± standard deviation of three replications for each sample. Data was analyzed by the one-way ANOVA and different letters (a–g) in the same column indicate significant differences by the Duncan test ($P < 0.05$).

The results showed that the dielectric constants of the feed solutions vary depending on the frequency up to 1 GHz and remain constant at higher frequencies (see Supplementary material Figure 1S). The results were concluded at 3 GHz. It has been determined that the dielectric constant of the CE-Shell solution and the uniaxial solution are very close to each other (70.73 ± 0.12 and 72.88 ± 0.17, respectively), while the dielectric constant of the solutions obtained from the emulsions is quite low by comparison (between 39.51 and 67.51). The results were in accordance with the literature. The dielectric constant gives information about the polarity of the solutions. High dielectric constant of a solution means that high net charge density has in the solution (Atik et al., 2022). The dielectric constants of solutions depend on water content, chemical composition, and the physycal structure of the materials (Cetinkaya

et al., 2022). Accordingly, the solutions obtained from emulsions are less polar than others. Among all the solutions obtained from emulsions, EE-VIII emulsion had the highest dielectric constant (67.51 ± 0.40) at 3 GHz. According to Saville's leaky dielectric model, the leaky dielectrics have enough free charges to move onto the surface of the fluid forming a Taylor cone at the tip of the droplet, and the electrospinning process then begins to produce nanofibers (Saville, 1997). However, dielectric materials diminish the magnitude of electrical field when placed in an electrical field, thus solutions with higher dielectric constant may inhibit the electrospinning process (Gutiérrez-Jara et al., 2020; Isik et al., 2018). Due to the fact that dielectric properties alone are not sufficient to explain the solution electrospinnability the effects of conductivity as well as surface tension should be considered for a comprehensive evaluation (Angammana & Jayaram, 2011).

Rheological properties, another important factor, affect the electrospinnability of solutions to be used for fiber formation. The increase in viscosity favors the formation of bead-free smooth fibers, while the increase in surface tension and the decrease in conductivity show opposite effect (Lu et al., 2006). According to the results of rheological measurements, all feed solutions exhibited non-Newtonian shear-thinning / pseudoplastic behavior due to lower flow behavior index than one. The shear thinning behavior of feed solutions was in accordance with the other studies on Pul containing polymer blends (Z. Qin et al., 2019; Shao et al., 2018; Xiao & Lim, 2018). The power-law equation is the most common model to explain pseudoplastic behavior, according to the consistency index (K) and the flow behavior index (n). The results showed that the consistency index was the lowest (0.59 ± 0.09) and flow behavior index was found to be the highest (0.94 ± 0.01) for CE-Core solution due to low polymer content and absence of sodium alginate. Furthermore, the consistency indices of EE emulsions were found to be higher than those of UE and CE solutions, which is thought to affect the electrospinnability. As expected, the incorporation of lecithin into emulsions resulted in increased consistency index and decreased flow behavior index of the feed solutions due to the increased dry matter ratio. Furthermore, viscosity is an effective parameter on the stability of emulsions. According to Stoke's law equation, destabilization of an emulsion is directly proportional to droplet size and inversely proportional to viscosity (Lamba et al., 2015). The zeta potential and average droplet size results mentioned in the previous sections are mostly consistent with this rule. As

such, EE-V, EE-VIII and EE-XI emulsions had the highest viscosities and the lowest droplet size, thus the absolute values of zeta potential which indicate stability were noticeably high. The oily phase type and Car content did not affect rheological properties significantly ($p<0.05$).

4.4.4 Morphology of electrospun nanofibers

The surface morphologies of UENFs, CENFs and EENFs are shown in Figure 4.2. According to the SEM micrographs all samples demonstrated fibrous structure, except sample EE-I (Figure 4.2-d). Additionally, some beady fiber formation was observed at UENFs which had the finest fibers (Figure 4.2-b) of all. However, results revealed that the differences between mean diameters of control, UENFs and CENFs were not statistically significant ($P < 0.05$).

CENFs demonstrated fine and uniform fiber structure with a mean diameter of 132.19 ± 30.35 nm (Figure 4.2-c). Although some fine fiber formation was observed for EE-II – EE-VII and EE-X samples (Figure 4.2-e – j and m), a large part of the samples were not in fiber form (see Supplementary material Figure 4.2S). In addition, although the electrospun samples EE-IV, EE-V, EE-VI and EE-X showed fiber formation, the surface of the samples was covered with a layer of oil during the electrospinning process (Figure 4.2-g – i and m). It could be inferred that the emulsion breaks down with the harsh conditions of the electrospinning process and coating the fiber surface by exposing the oil phase out of the fiber. Accordingly, it was concluded that emulsions containing SO as oily phase tended to deteriorate during the electrospinning process and the addition of soy lecithin and decreasing Car concentration enhanced the resistance of the emulsions to harsh conditions during the electrospinning process (Figure 4.2-n-o). The results were consistent with the emulsion stability results mentioned Section 4.3.1. As mentioned, incorporation of soy lecithin increased the absolute value of zeta potential of feed solutions and enhanced the stability of emulsions.

Mean diameters of electrospun fibers obtained by emulsion electrospinning were calculated to be between 219.27 ± 65.38 and 633.94 ± 201.50 nm. According to the results, significantly finer fibers were obtained by uniaxial electrospinning. However, while higher mean diameter fibers were obtained by coaxial electrospinning, nanofibers were more uniform and beadless than UENFs. Fibers obtained by emulsion

electrospinning were thicker compared to others. Among them, the SO-containing ones, EE-VIII and EE-IX NFs, had finer fiber formation (219.27 ± 65.38 and 273.16 ± 79,46 nm, respectively), while MCT containing ones, E-XI and E-XII NFs, had significantly higher fiber diameters (340.60 ± 129.67 nm and 358.17 ± 95.60 nm, respectively).

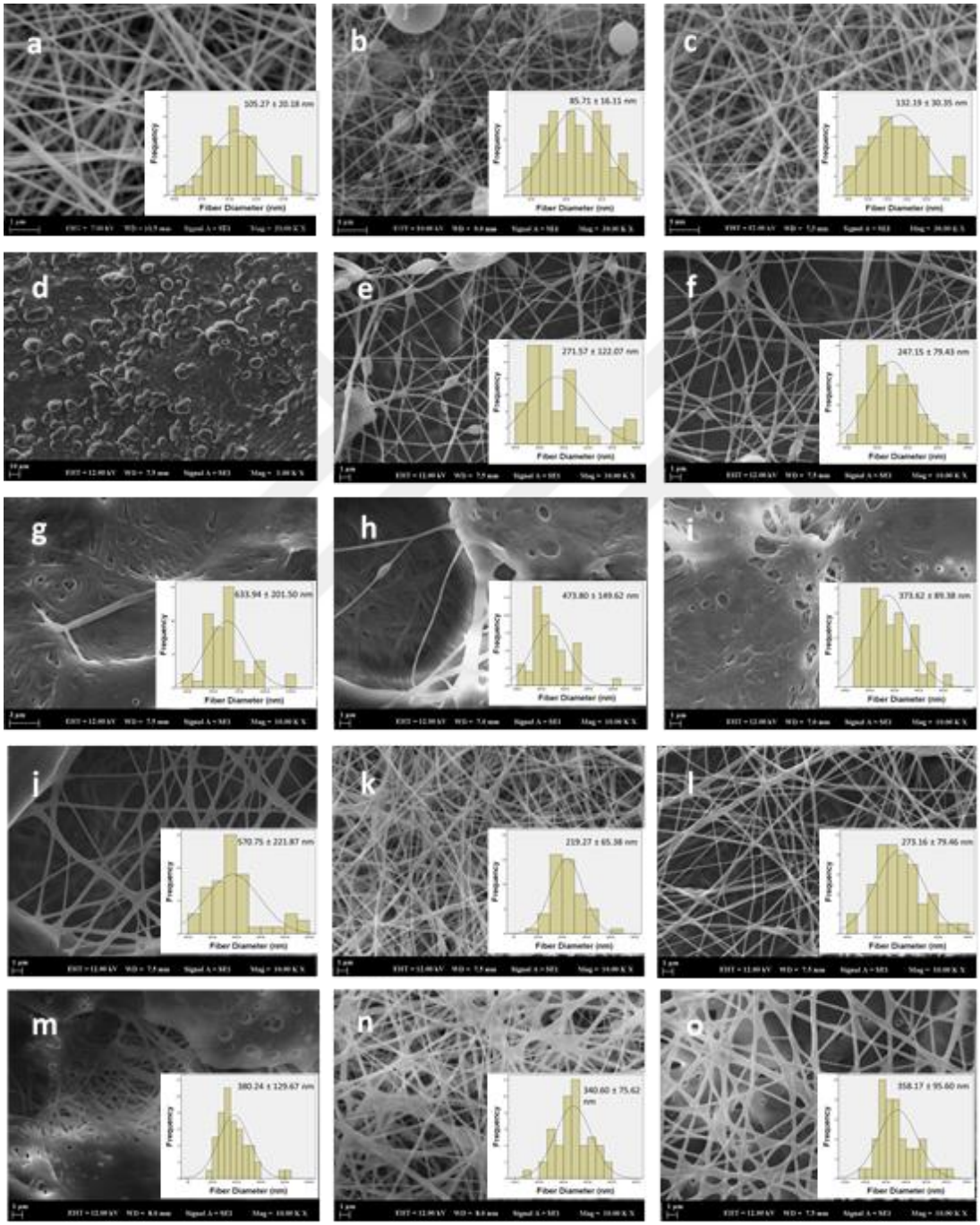

Figure 4.2 : SEM images of electrospun samples obtained from defined solutions. Control (a), UE (b) and CE (c) samples at 30000 magnification; EE-I (d), EE-II (e), EE-III (f), EE-IV (g), EE-V (h),E E-VI (i), EE-VII (j), EE-VIII (k), EE-XI (l), EE-X (m), EE-XI (n) and EE-XII (o) samples at 10000 magnification.

According to the literature, without changing the composition or concentration of a solvent or a polymer, adding a surfactant to the feed solution can reduce the surface tension of the solution, thus the lower conductivity that is required for spinning. Therefore, finer fibers can be produced with more uniform diameter distribution (X. Liu et al., 2019). S. A. Park et al. (2010) state that lecithin as natural surfactant improved the fiber structure of NaAlg – polyethylene oxide (PEO) electrospun fibers and removed the bead of NFs.

In this work, an ionic crosslinking was applied to improve the water resistance ability and enhance release characteristics of the Pul-NaAlg NFs. By applying ionic crosslinking with $CaCl_2$, the use of toxic chemicals in chemical crosslinking methods is also prevented (Bonino et al., 2011). In fact the SEM analysis evidences that, among all fibers obtained from emulsions, the E-VIII NFs were characterized by fine, beadless and uniform nanofibrous structure. Therefore, Control NFs, UENFs, CENFs and EE-VIII NFs were selected to apply crosslinking and some further characterizations.

The morphologies of crosslinked NFs (Figure 4.3) are quite different from the uncurving shape of the non-treated ones (Figure 4.2-a,b,c and k). Essentially, crosslinked nanofibers, except for the CENFs, were able to largely preserve their well-defined nanofibrous structure after crosslinking. As shown in Figure 4.3-c, after crosslinking, the CENFs curved and coalesced with each other, thus losing their high surface-to-volume ratio. This was probably due to the high non-crosslinked pullulan / crosslinked alginate ratio of CENFs that could provide the polymer matrix more hydrophilic and crosslinking-sensitive solutions containing 95% ethanol. The results were in accordance with the literature. Q. Wang et al. (2019) stated that while non-crosslinked NFs have circular fiber morphology without mixed-random bonds, some interconnections were formed between the fibers after crosslinking with $CaCl_2$ and these connections caused the fibers to have a more compact and flat structure. A study on ionic crosslinking of alginate-polyethylene oxide composite NFs by $CaCl_2$ reported that electrospun fibers with increasing ratios of polyethylene oxide were largely disintegrated after crosslinking (S. A. Park et al., 2010).

Furthermore, the effect of crosslinking on mean diameters of NFs was investigated. According to the results, the mean diameters of Control NFs, UENFs and CENFs were significantly increased after crosslinking treatment. In contrast, EE-VIII NFs retained their original fiber diameter after crosslinking ($P < 0.05$). The results were consistent

with the literature. It was reported that after $CaCl_2$ crosslinking, average diameters of alginate-based electrospun nanofibers increased significantly (Qian Wang et al., 2019).

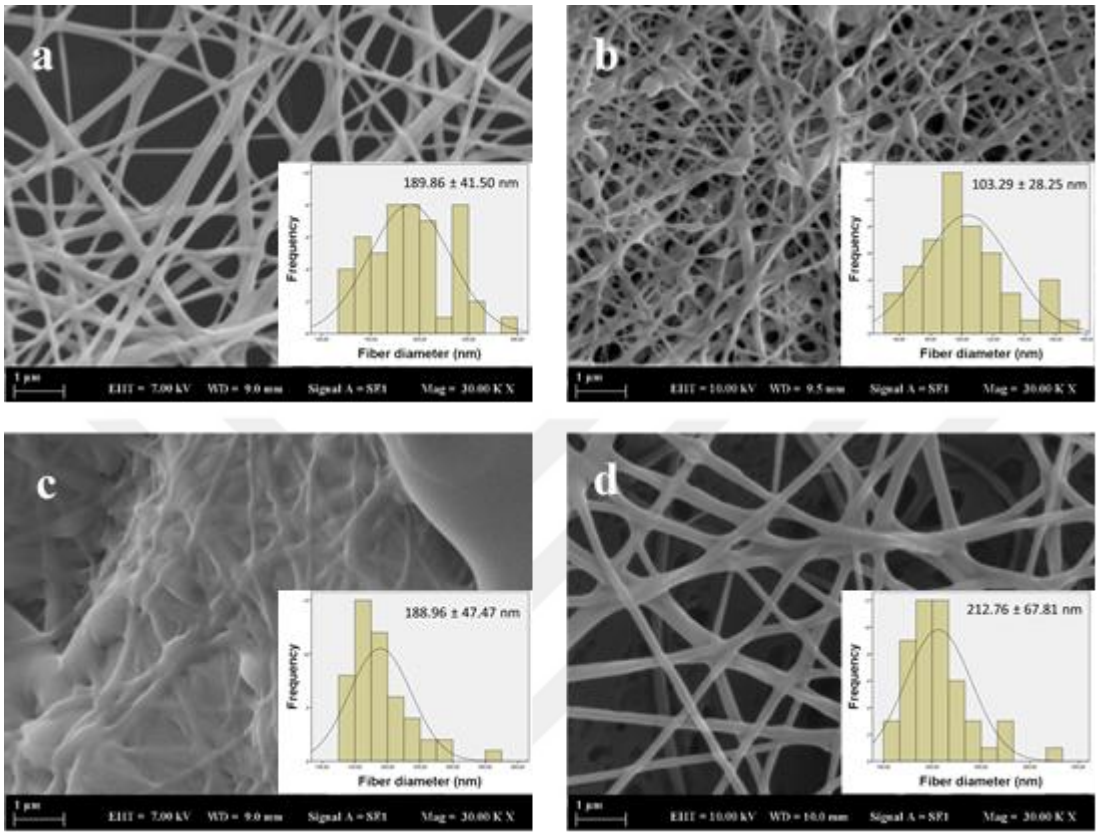

Figure 4.3 : SEM images of electrospun samples obtained from defined solutions. Control (a), UE (b) and CE (c) samples at 30000 magnification; EE-I (d), EE-II (e), EE-III (f), EE-IV (g), EE-V (h),E E-VI (i), EE-VII (j), EE-VIII (k), EE-XI (l), EE-X (m), EE-XI (n) and EE-XII (o) samples at 10000 magnification.

4.4.5 FTIR analysis

FTIR analysis was used to confirm the presence of chemical structures and any possible interactions between Pul-NaAlg fiber matrix and encapsulated Car and to investigate the effects of crosslinking on chemical structures of fibers. FTIR spectra of Car, Pul, NaAlg, Pul-NaAlg nanofiber without loaded Car (Control NFs), UENFs, CENFs and EE-VIII-NFs before and after crosslinking were given in Figure 4.4.

In the infrared spectra of Pul powder, a broad band centered at 3327 cm^{-1} corresponded to hydrogen bonded OH stretching vibration and the peaks at 2923 cm^{-1} and 2856 cm^{-1} were assigned to symmetric and asymmetric stretching vibrations of C-H groups (R. S. Singh et al., 2009). Moreover, the peak at 1636 cm^{-1} was attributed to -OCO- and the consecutive peaks at 1152 cm^{-1}, 1104 cm^{-1}, 1078 cm^{-1} and 1000 cm^{-1} were related

to C-O-C, C-O, C-C and C-O-H vibrations of Pul molecules (Z. Qin et al., 2019; Xiao et al., 2014). The additional peaks at 930 cm-1, and 726 cm-1 were assigned to the characteristic α-(1,6) and α-(1,4) glycosidic bonds and 843 cm^{-1} was attributed to α-glucopyranoside unit (Z. Qin et al., 2019).For NaAlg powder, a broad band was observed centered at 3327 cm^{-1} assigned to hydrogen bonded OH stretching vibration and the peaks at 1575 cm^{-1} and 1406 cm^{-1} corresponded to asymmetric and symmetric stretching vibrations of COO^- groups (Papageorgiou et al., 2010). The peaks at 1079 cm^{-1}, 1023 cm^{-1} and 944 cm^{-1} were attributed to the vibrations of mannuronic unit, guluronic unit and uronic acid, respectively (Sellimi et al., 2015). The spectrum of the Control NFs revealed absorbance bands of higher peak intensities than Pul and NaAlg powder for OH stretching vibration at 3294 cm^{-1}, asymmetric and symmetric stretching vibrations of COO^- groups at 1602 cm^{-1} and 1409 cm^{-1}, C-O-C, C-O, C-C and C-O-H vibrations at 1149 cm^{-1}, 1090 cm^{-1}, 1078 cm^{-1} and 998 cm^{-1}, and α-(1,6) glycosidic bond, α-glucopyranoside unit and α-(1,4) glycosidic bond at 932 cm^{-1}, 822 cm^{-1} and 719 cm^{-1}, and C–N stretching at 1452 cm^{-1}. At the peaks of Control NFs, a shift to lower wavenumber was observed compared to Pul powder with the addition of NaAlg and $CaCl_2$. The peaks corresponding to the vibration of asymmetric stretching of C-H groups at 2856 cm^{-1} were coated by symmetric stretching of C-H group present in the pullulan powder and a single broad C-H stretching peak at 2944 cm^{-1} in the Pul-NaAlg nanofibers spectrum was observed. Furthermore, at the peaks of Control NFs at 1602 cm^{-1} and 1409 cm^{-1}, a shift to considerably higher wavenumber was observed compared to NaAlg powder. This shifting and coating confirmed the interactions of polymers during electrospinning (Luo et al., 2011).

The characteristic peaks of Car are detected at 3239 cm^{-1} and 3048 cm^{-1} for the NH_2 and (NH_3^+) stretching vibrations, respectively (Abdelkader et al., 2016; Durmus et al., 2011). The peaks at 2857 cm^{-1} and 2740 cm^{-1} are related to symmetric and asymmetric stretching vibrations of C-H groups (Branham et al., 2011). The observed band at the 2165 cm^{-1} in the spectrum of Car corresponded to the vibrational bending of imidazole (N-H) stretching (Abdelkader et al., 2016). In addition, a wider band with higher intensity was observed at 1644 cm^{-1} for the vibration of amide group stretching (Gholibegloo et al., 2018). Furthermore, the asymmetric and symmetric stretching frequencies of carboxylate (COO^-) were detected at 1564 cm^{-1} and 1404 cm^{-1} (Abdelkader et al., 2016). Regarding the Car loaded electrospun nanofibers, the

characteristic NH_2 and (NH_3^+) stretching vibration peaks of Car observed at 3239 cm^{-1} and 3048 cm^{-1} had been coated with the broad band observed centered between 3650 - 3000 cm^{-1} assigned to hydrogen bonded OH stretching vibration. The absence of the other characteristic peaks of Car (the vibrational bending of imidazole (N-H) stretching and amide group stretching at 2165 cm^{-1} and 1644 cm^{-1}, respectively) in loaded nanofibers suggested that the Car could be well encapsulated within Pul-NaAlg nanofibers.

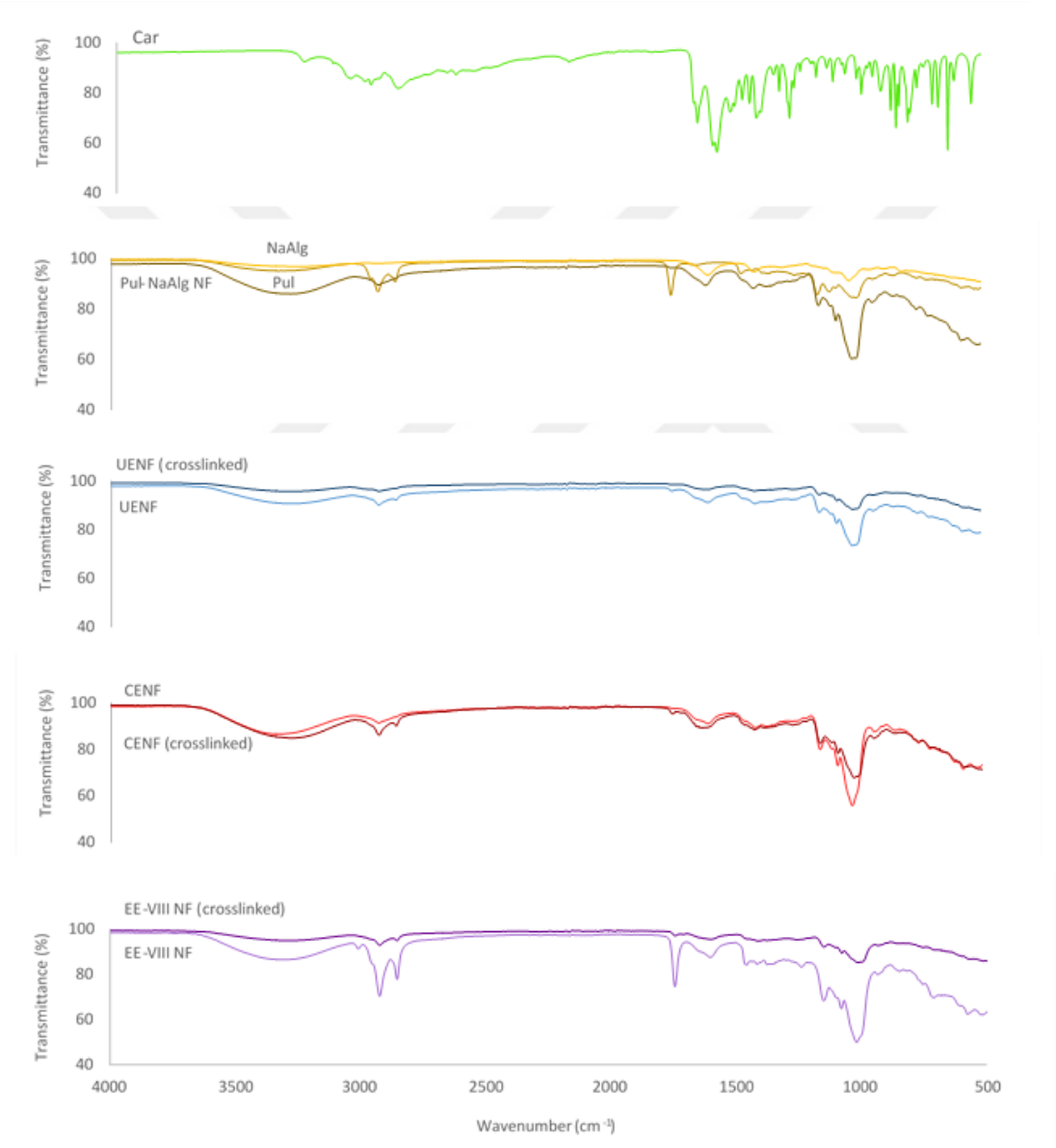

Figure 4.4 : FTIR spectra of Car, Pul, NaAlg, Pul-NaAlg nanofiber without loaded Car (Control NF), UENF, CENF and EE-VIII-NF before and after crosslinking.

The band at 3294 cm^{-1} (the O-H stretching vibrations) of Control NFs shifted toward lower values (3269 cm^{-1}) in UENFs, suggesting the formation of hydrogen bond

between peptide and polymers (Wen, et al., 2017). In addition, the absorption peaks of asymmetric and symmetric stretching vibrations of COO^- groups which are found at 1602 cm^{-1} and 1409 cm^{-1} for Pul-NaAlg nanofibers slightly shifted to 1593 cm^{-1} and 1403 cm^{-1} in UENFs, respectively. These shifts indicated convenient interaction and compatibility between Car and the composite nanofibers. Comparing with non-crosslinked UENFs, the intensities of peaks were decreased, indicating that some of the Pul content could be removed during the crosslinking treatment in the FTIR spectra of crosslinked nanofibers (Mokhena & Luyt, 2017). The spectrum of the CENFs revealed absorbance bands at higher wavenumbers with higher peak intensities than Control NFs nanofibers for OH stretching vibration at 3336 cm^{-1}, as a corollary to the difference in the concentration of polymers arising from core-sheath structure. Unlike uniaxial ones, the peak intensities of non-crosslinked and crosslinked CENFs were observed to be quite close to each other, indicating that the CENFs were more stable against crosslinking. Furthermore, the asymmetric and symmetric stretching vibrations of COO^- were shifted from 1592 cm^{-1} and 1408 cm^{-1} to 1614 cm^{-1} and 1416 cm^{-1}, respectively, and demonstrated increased intensities compared to non-crosslinked nanofibers. These shiftings and changes in intensity were explained by an increase of the interaction between COO^- groups of alginate and Ca^{2+} ions (G. Ma et al., 2012). In comparison, some extra peaks were observed at the IR spectrum of EE-VIII NFs due to the presence of soy lecithin and SO in emulsion-based feed solutions. The strong band absorptions were observed in the region of 3000 - 2800 cm^{-1} corresponding to C–H stretching vibrations. In addition, a characteristic peak at 1740 cm^{-1} was assigned to C=O double bond stretching vibration of esters (Rohman & Che Man, 2012; Whittinghill et al., 2000). Comparing with non-crosslinked ones, the intensities of these peaks were decreased dramatically in the spectra of crosslinked nanofibers, indicating that the soy lecithin and SO could be removed during the crosslinking treatment due to the presence of ethanol as solvent.

4.4.6 Contact angle of electrospun nanofibers

The contact angle measurements were performed on UENFs, CENFs and EE-VIII NFs, before and after crosslinking. The water contact angle values of non-crosslinked samples could not be measured because these samples were immediately dissolved on contact with water, indicating the nanofibers were highly hydrophilic prior to crosslinking treatment. The results were consistent with Z. Qin et al. (2019). The water

contact angle values of all crosslinked samples were lower than 90° (67.95 ± 0.12°, 63.72 ± 0.36° and 79.22 ± 0.20° for UENFs, CENFs and EE-VIII NFs, respectively), indicating that all samples had hydrophilic character. According to results, EE-VIII NFs were demonstrated to have the highest water contact angle, due to the hydrophobic components in its structure, such as SO and PGPR. The results showed that the $CaCl_2$ crosslinking treatment improved water resistance of nanofibers, consistent with the literature (W.-C. Lu et al., 2020).

4.4.7 Encapsulation efficiency

The encapsulation efficiencies of Car loaded electrospun nanofibers were calculated according to HPLC analysis results and the results are given in Table 4.4. The highest encapsulation efficiency value was calculated for CENFs as 81.69 ± 0.32 %. Encapsulation efficiency of UENFs was significantly lower than CENFs, while significantly higher than EENFs. Encapsulation efficiency values of Car loaded electrospun nanofibers obtained by emulsion electrospinning (EENFs) were between 60.33 ± 0.14 and 73.32 ± 0.13%. Among them, the highest encapsulation efficiency values were calculated for EE-VI and EE-VII NFs (73.04 ± 0.19 and 73.32 ± 0.13%, respectively. However, although EE-VI NFs showed fiber formation, the surface of the sample was covered with a layer of oil during the electrospinning process (Figure 4.1– i). This could be attributed to emulsion breakdown with the harsh conditions of electrospinning process, as mentioned in previous sections. In addition, although some fine fiber formation was observed for EE-VII (Figure 4.1- j), a large part of the sample was not in fiber form, as mentioned in Section 4.4.4. This discrepancy between fiber morphology and encapsulation efficiency results indicates that encapsulation efficiency alone is not sufficient to demonstrate successful encapsulation. On the other hand, according to SEM results, emulsion electrospinning was carried out successfully for sample EE-VIII, EE-IX, EE-XI and EE-XII NFs, especially. Among the samples that could successfully encapsulate Car by emulsion electrospinning, the highest encapsulation efficiency value was calculated for E-VIII NFs (68.63 ± 0.45%).

Similar to the results obtained in the present work, encapsulation efficiency of peptides varied from 42% to 95%, depending on the encapsulation method, polymers and other process parameters (Hosseini et al., 2019; Ramezanzade et al., 2017; X. Zhang et al., 2017). For instance, rainbow trout skin-derived antioxidant peptides were

encapsulated by chitosan-coated nanoliposomes with the, encapsulation efficiencies ranged between 46.1 – 80.2% (Ramezanzade et al., 2017). In another study investigating the encapsulation of oyster peptide extract in microcapsules formed with microemulsion gelling method, encapsulation efficiencies ranged from 71.46 to 75.37% (X. Zhang et al., 2017). Furthermore, (Hosseini et al., 2019) studied the production of chitosan/poly(vinyl alcohol) nanofibrous mat by electrospinning. Fish-purified antioxidant peptides were successfully loaded into the nanofibrous mat with high encapsulation efficiency, beyond 94%.

Table 4.4 : Encapsulation efficiency and antioxidant capacity of electrospun nanofibers.

Sample	Encapsulation efficiency (%)	Antioxidant capacity	
		DPPH (mg TE/g)	CUPRAC (mg TE/g)
Car	-	$0.159 \pm 0.013^{3.}$	3.750 ± 0.000c
UE	74.11 ± 0.20^{h}	0.108 ± 0.000^{ef}	1.660 ± 0.631^{b}
CE	81.60 ± 0.32^{i}	0.121 ± 0.000^{f}	1.702 ± 0.383^{b}
EE-I	68.66 ± 0.48^{e}	0.066 ± 0.000^{a}	1.116 ± 0.072^{a}
EE-II	69.18 ± 0.35^{e}	0.078 ± 0.007^{abc}	1.576 ± 0.072^{b}
EE-III	71.00 ± 0.39^{f}	0.074 ± 0.000^{ab}	1.367 ± 0.145^{ab}
EE-IV	62.19 ± 0.31^{b}	0.066 ± 0.000^{a}	1.116 ± 0.217^{a}
EE-V	65.39 ± 0.22^{c}	0.070 ± 0.007^{ab}	1.242 ± 0.145^{ab}
EE-VI	73.04 ± 0.19^{g}	0.074 ± 0.000^{ab}	1.409 ± 0.192^{ab}
EE-VII	73.32 ± 0.13^{g}	0.083 ± 0.007^{bc}	1.074 ± 0.125^{a}
EE-VIII	68.63 ± 0.45^{e}	0.100 ± 0.013^{de}	1.618 ± 0.125^{b}
EE-IX	66.44 ± 0.22^{d}	0.070 ± 0.007^{ab}	1.473 ± 0.403^{ab}
EE-X	68.35 ± 0.20^{e}	0.066 ± 0.007^{a}	1.283 ± 0.072^{ab}
EE-XI	60.33 ± 0.14^{a}	0.091 ± 0.019^{cd}	1.367 ± 0.072^{ab}
EE-XII	65.79 ± 0.65^{c}	0.078 ± 0.007^{abc}	1.367 ± 0.072^{ab}

Data given in this table represents average amounts ± standard deviation of three replications for each sample. Data was analyzed by the one-way ANOVA and different letters (a–g) in the same column indicate significant differences by the Duncan test ($P < 0.05$).

4.4.8 Antioxidant capacities of carnosine loaded electrospun nanofibers

To investigate the antioxidant capacity of Pul-NaAlg-Car electrospun fibers the DPPH and CUPRAC analysis were carried out and the results are presented in Table 4.4. According to the results, the antioxidant capacity of Car evaluated by the DPPH method was 0.159 ± 0.013 mg TE/g Car. Results were also calculated according to the following equation: DPPH scavenging capacity (%) = $\left(\frac{A_C - A_S}{A_C}\right) \times \mathbf{100}$, where A_C and A_S are the absorbances of the control and sample, respectively (data not shown). Accordingly, the DPPH scavenging capacity of 10 mg/ml CAR was calculated as 11.11 ± 1.11%. J. Liu et al. (2013) reported that DPPH radical scavenging capacity of carnosine is 7.7 ± 0.3% for 10 mg/ml concentration. DPPH radical scavenging capacities of UENFs (0.108 ± 0.000 mg TE/g sample) and CENFs (0.121 ± 0.000 mg TE/g sample) were significantly higher than EENFs (between 0.066 ± 0.007 and 0.100 ± 0.013 mg TE/g sample). The results are consistent with encapsulation efficiencies of electrospun samples. Among EENFs, although DPPC scavenging capacity values were very close to each other, EE-VIII and EE-XI NFs demonstrated significantly higher capacities compared to others.

Luisi et al., (2018) reported that the experimental results obtained with different methods may differ from each other and that this deviation is due to many factors such as different chemotypes, mechanisms and light, oxygen, pH and nature of solvent. According to the CUPRAC assays results, Car showed 3.750 ± 0.000 mg TE/g Car antioxidant activity. The highest antioxidant capacity was obtained for pure Car. Contrary to DPPH results, antioxidant activities of UENFs and CENFs were not found to be significantly higher than for EENFs. Among EENFs, the highest antioxidant activities were detected for EE-II and EE-VIII NFs, in terms of the CUPRAC results. In addition, the antioxidant capacities of all electrospun fibers were determined to be higher with the CUPRAC assay compared to the DPPH assay. This could be attributed to the analysis of mostly hydrophilic compounds in the DPPH method, while both hydrophilic and hydrophobic antioxidants can be analyzed with the CUPRAC method (Apak et al., 2004). According to the overall results, it is clearly confirmed that all electrospinning methods demonstrate the protective effect on the antioxidant activity of Car and thus help in enhancing its antioxidant potential significantly. Similarly, Shah et al. (2016) reported that the radical scavenging capacity of curcumin increased by encapsulation of curcumin in nanoparticles obtained from nanoemulsions.

4.4.9 *In vitro* release of Car

The behavior of Car released from electrospun nanofibers in the simulated gastric fluid and simulated intestinal fluid was investigated and is presented in Figure 4.5. As can be seen in Figure 4.5, for Car release in SGF, all nanofibers non-crosslinked and crosslinked showed initial burst release in the first 10 min of approximately between 50-80% and 30-60%, respectively. This can be related to the high solubility of Pul and NaAlg polymers into the SFG. Furthermore, the burst release can be associated with the surface-loaded Car which has high diffusion tendency and weak bond with the surface of the nanofiber (Rezaei et al., 2019). The release of Car was followed by sustained release until the end of the first 120 min gastric phase. All of the nanofibers demonstrated lower burst release percentage (near 100%) compared to pure Car in SGI. The initial burst release of Car from EE-VIII NFs (50%) was significantly lower than UENFs (83%) and CENFs (78%) during gastric digestion, before crosslinking. Similarly, an initial burst release occurred in the first 30 min during incubation of non-crosslinked nanofibers in SIF, except for EE-VIII NFs, then followed by sustained release until the end of the 240 min SGI tract. However, the release of Car from EE-VIII NFs was followed by sustained release during 120 min intestinal phase. At the end of the 240 min SGI tract, nearly 100% of Car was released from all nanofibers.

Figure 4.5-b shows the behavior of Car released from electrospun nanofibers in the SGF and SIF, after crosslinking. Contrary to release results of nanofibers before crosslinking, the release profile of Car from all nanofibers was more sustained, especially during intestinal digestion. The initial burst release of Car from crosslinked EE-VIII NFs (29%) was significantly lower than crosslinked UENFs (59%) and CENFs (41%) during gastric digestion. The release of Car from all crosslinked NFs was followed by sustained release during 120 min intestinal phase. However, crosslinked treatment decreased bioceessibility of Car during in vitro digestion. At the end of the 240 min SGI tract, released Car level from non-crosslinked nanofibers were higher than crosslinked ones.

It can be concluded that simultaneously encapsulation of Car in double emulsion and in Pul-NaAlg NFs can provide sustained release. The results indicated that the crosslinking of Pul-NaAlg NFs via $CaCl_2$ could potentially be applied to protect the system against the early release of Car in the gastrointestinal tract.

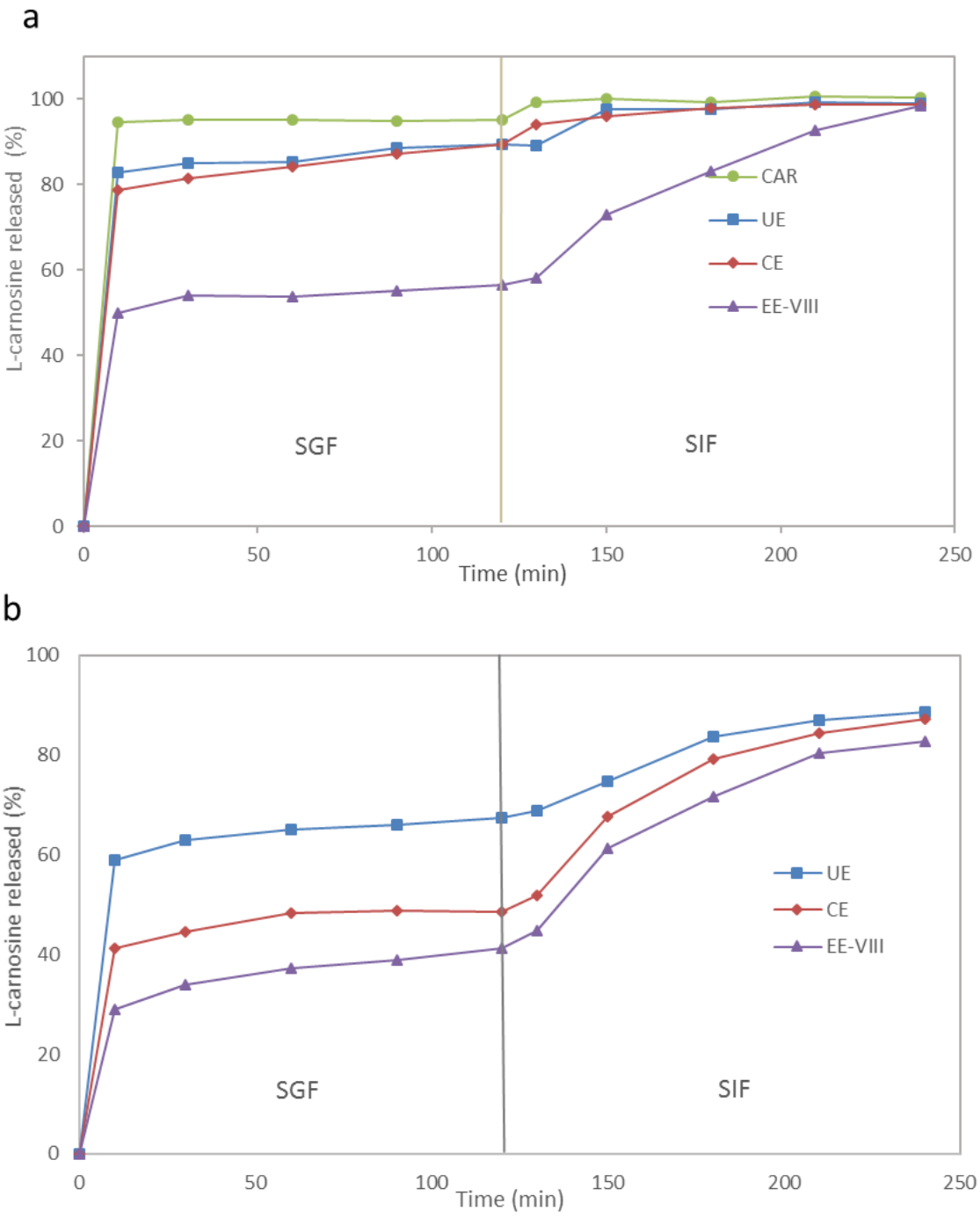

Figure 4.5 : Release profile of Car from electrospun nanofibers in simulated gastric fluid and simulated intestinal fluid before (a) and after (b) crosslinking.

4.4.10 Release kinetics of Car in the SGI tract

To reveal the release mechanism of Car from all NFs in the SGF and SIF, the release profile data were fitted with various kinetic models (Higuchi model, Kopcha kinetic model, Korsmeyer-Peppas model, Peppas-Sahlin model, Weibull model and Gompertz model) and the results are summarized in Table 5.

According to the correlation coefficient (R^2), the Higuchi model, showed the lowest fitting performance in comparison to the other selected models. The Higuchi kinetic model define time-dependent release from insoluble matrix based on Fickian diffusion mechanism and indicates the sustained release of the loaded substance (Kaboli et al., 2021). Results showed that the correlation coefficient values of Higuchi model in SIF ($R^2 > 0.888$) were considerably higher than in SGF ($R^2 > 0.356$) and correlation coefficient values of crosslinked NFs were slightly higher than non-crosslinked ones. It can be concluded that more sustained release occurred in SIF in comparison to the SGF, and crosslinking application can provide sustained release of Car.

Korsmeyer-Peppas model was also studied to investigate the mechanism of release based on the diffusion (Fickian) and erosion (non-Fickian) from a polymer matrix. As seen in Table 5, one of the models has the highest values of regression coefficient obtained for Korsmeyer-Peppas model. The Car release profile data were better fitted with Korsmeyer-Peppas model in SGF ($R^2 > 0.999$) than in SIF ($R^2 > 0.942$). For all NFs in SGF and for non-crosslinked UENFs and CE in SIF, the release exponent was $n<0.45$, indicating a Fickian diffusion mechanism. Dede & Altay (2019) stated that Peppas equation expressed the release behavior of limonene from electrospun fibers produced from various biodegradable polymers and the release mechanism was diffusion controlled due to the values of the release exponent lower than 0.5. On the other hand, for the remaining NFs (non-crosslinked EE-VIII NFs and crosslinked UENFs, CENFs and EE-VIII NFs) the release exponent $0.45 < n < 0.89$, represents an anomalous (non-Fickian) diffusion mechanism, which corresponded to Car dissolution and polymer erosion (Shuang Zhang et al., 2022). The difference between n values of non-crosslinked EE-VIII NFs and the other non-crosslinked NFs can be the difference in their digestibility and degradation when exposed to the enzymes in the SIF due to having different formulations. Our results were consistent with

Table 4.5 : Kinetic models parameters of L-carnosine released from electrospun nanofibers.

Release media	Sample	Higuchi model		Kopcha model			Korsmeyer-Peppas model			Peppas-Sahlin model				Weibull model			Gompertz model		
		k	R^2	A	B	R^2	k	n	R^2	K_1	K_2	m	R^2	a	b	R^2	a	b	R^2
SGF	UE	0.104	0.356	0.252	-0.016	0.897	0.770	0.030	0.999	0.520	0.252	0.022	0.999	0.706	0.088	0.999	0.295	-0.183	0.999
	CE	0.102	0.423	0.239	-0.015	0.908	0.695	0.050	0.999	0.482	0.213	0.377	0.999	0.902	0.135	0.999	0.450	-0.256	0.999
	EE-VIII	0.065	0.395	0.156	-0.010	0.915	0.457	0.043	0.999	0.587	-0.148	0.098	0.999	1.656	0.065	0.999	0.804	-0.069	0.999
	UE (crosslinked)	0.077	0.423	0.184	-0.012	0.924	0.524	0.052	1.000	0.653	0.151	0.109	1.000	1.377	0.090	1.000	0.686	-0.114	1.000
	CE (crosslinked)	0.056	0.479	0.131	-0.008	0.944	0.352	0.071	0.999	0.385	-0.075	0.179	0.999	2.341	0.098	0.999	1.087	-0.091	0.999
	EE-VIII (crosslinked)	0.045	0.668	0.092	-0.005	0.961	0.212	0.138	1.000	0.167	0.050	0.103	0.999	4.342	0.172	0.999	1.677	-0.130	1.000
SIF	UE	0.009	0.888	0.018	-0.001	0.964	0.025	0.292	0.942	0.013	-0.001	0.589	0.967	39.57	0.304	0.943	3.967	-0.116	0.949
	CE	0.009	0.924	0.017	-0.001	0.998	0.027	0.268	0.993	0.019	-0.001	0.472	0.998	36.61	0.278	0.993	3.814	-0.104	0.995
	EE-VIII	0.042	0.951	0.018	0.002	0.978	0.013	0.738	0.982	0.004	0.000	1.070	0.992	44,06	0.679	0.987	12.44	-0.558	0.997
	UE (crosslinked)	0.022	0.940	0.012	0.001	0.958	0.009	0.689	0.965	0.001	0.000	1.224	0.996	35.50	0.754	0.969	8.526	-0.368	0.985
	CE (crosslinked)	0.040	0.955	0.034	0.001	0.956	0.026	0.590	0.961	0.007	0.000	1.011	0.991	51.64	0.703	0.969	7.915	-0.459	0.990
	EE-VIII (crosslinked)	0.042	0.961	0.033	0.001	0.966	0.024	0.613	0.970	0.007	0.000	1.012	0.997	56.70	0.739	0.978	8.691	-0.491	0.994

Data given in this table represents average amounts ± standard deviation of three replications for each sample. Data was analyzed by the one-way ANOVA and different letters (a–g) in the same column indicate significant differences by the Duncan test ($P < 0.05$)

Mehdi et al. (2021) who stated that anomalous (non-Fickian) diffusion mechanism controlled release of rizatriptan from Pul based nanofibers, according to the Korsmeyer-Peppas model due to the release exponent, $n > 0.45$.

The Kopcha and Peppas-Sahlin models describe release of encapsulated substance based on the mechanisms of diffusion and erosion. These two models are quite similar to each other and therefore the results were obtained in the same direction. Release profile data were better fitted with Peppas-Sahlin model ($R^2 > 0.941$) than Kopcha model ($R^2 > 0.897$). According to the Kopcha model, for all NFs in both SGF and SIF, Fickian diffusion mechanism was able to control release, predominantly ($|A/B|>1$). Results show that the correlation coefficient values of Peppas-Sahlin model in SGF ($R^2 > 0.999$) were considerably higher than in SIF ($R^2 > 0.991$ except for non-crosslinked UENFs ($R^2 = 0.967$). Unlike the Kopcha model, the purely Fickian diffusion exponent, m, appears in the Peppas-Sahlin model allowing better fitting of the release data in the present study. According to the Peppas-Sahlin model, for all NFs in both SGF and SIF, Fickian diffusion mechanism controlled release, predominantly ($|K_1/K_2|>1$). Charpashlo et al. (2021) similarly applied Kopcha and Peppas-Sahlin models and found the main release mechanism of lycopene from electrospun fibers, diffusion transport mechanism in SGF and SIF. Furthermore, the results show that the values of $|K_1/K_2|$ increased after crosslinking for all electrospun fibers, indicating the effectiveness of diffusion mechanism increased via crosslinking.

Weibull and Gompertz models were studied to describe *in vitro* dissolution profile and good fits were obtained with quite high correlation coefficient values ($R^2 > 0.943$ and $R^2 > 0.949$, respectively). Altay & Okutan (2015) stated that dissolution of the wall material is a triggering mechanism that is effective in initiating the release. Additionally, the correlation coefficient values of both models in SGF ($R^2 > 0.999$) were considerably higher than in SIF ($R^2 > 0.943$). According to the Weibull model, for all NFs in both SGF and SIF, Fickian diffusion mechanism controlled the release, at predominantly ($b \leq 1$). Korsmeyer-Peppas, Peppas-Sahlin, Weibull and Gompertz models had the highest correlation coefficient values in SGF, while Peppas-Sahlin and Gompertz models had the highest correlation coefficient values in SIF. Consequently, it can be stated that Fickian diffusion mechanism was predominant in releasing Car from Pul-NaAlg NFs in the gastrointestinal tract.

4.5 Conclusion

The fabrication and characterization of Car loaded Pul-NaAlg NFs by uniaxial, coaxial and emulsion electrospinning procedures were successfully achieved, with encapsulation efficiencies calculated as 74.11, 81.69 and 68.63%, respectively. The zeta potential results of emulsions demonstrated that all emulsions can be considered as stable and incorporation of soy lecithin increased the absolute value of zeta potential, thus enhancing the stability of emulsions. The microscopy results indicated that the type B double emulsion was successfully obtained and several small inner water droplets were encapsulated in the relatively larger oil globules. DPPH and CUPRAC assays revealed that all electrospinning methods demonstrated the protective effect on the antioxidant activity of Car and thus helped in enhancing its antioxidant potential significantly. In fact, the SEM analysis provides evidence that, among all samples obtained from emulsion electrospinning, the EE-VIII NFs were characterized by fine, beadless and uniform nanofibrous structure, and were thus selected to apply crosslinking and some further characterizations. Crosslinked NFs, except for the coaxial sample were able to largely preserve their well-defined nanofibrous structure after crosslinking, while the morphologies are quite different from the uncurving shape of the non-threated ones. Additionally, fiber diameters of NFs were increased after crosslinking treatment, except for the EE-VIII NFs which retained their original size. The $CaCl_2$ crosslinking treatment improved water resistance of NFs and enhanced sustained release of Car in the gastrointestinal tract. According to *in vitro* digestion results, it can be stated that Fickian diffusion mechanism was predominant in releasing Car from Pul-NaAlg NFs in the gastrointestinal tract. The initial burst release of Car from EE-VIII NFs was significantly lower than UENFs and CENFs in both gastric phases, besides the release from EE-VIII NFs in intestinal phase was followed by sustained release, with/without crosslinking treatment. Results indicate that simultaneously encapsulation of Car in double emulsion and in Pul-NaAlg NFs can provide sustained release. This study may provide further development of double emulsion-based nanofibers as edible protectors to encapsulate various bioactive agents for food applications.

5. CONCLUSIONS AND RECOMMENDATIONS

Bioactive peptides with a range of biological activities are considered as promising substances for health-promoting ingredients and functional foods and have drawn the considerable interest in developing encapsulation technologies to carry, protect and delivery of them. Electrospinning is a simple, flexible and cost-effective delivery approach when compared to conventional encapsulation techniques. In this Ph.D. thesis, to fabricate a nanofibrous delivery vehicles for bioactive peptides, without using any synthetic polymers or any hazardous solvents was aimed and standard peptide mixture (SPM) as a model peptide was encapsulated to evaluate the encapsulation performance of produced uniaxial nanofibers, as start step (Chapter 3). As a result, Pul-NaAlg based uniaxial electrospun fibers was successfully fabrricated and SPM was encapsulated with 77.97 % encapsulation efficiency . Thus, biodegradable and non-toxic nanofibers made only of food-derived polysaccharides could be produced for encapsulation of peptides without using any synthetic polymers and /or harmful solvents.

On the other hand, the effect of formulation and properties of feed solutions including viscosity, surface tention, dielectrical constant and electrical conductivity on electrospinnability in terms of fiber morphology and stability were investigated. Results showed that, NaAlg and $CaCl_2$ improved electrospinnability of pullulan with enhanced the solution properties such as viscosity, surface tention and electrical conductivity; thus SPM loaded Pul-NaAlg-$CaCl_2$ composite nanofibers were successfully fabricated under pre-determined proper conditions. Additionally, incorporation of $CaCl_2$ decreased mean diameter and improved stability of electrospun fibers possibly due to intermolecular interactions between NaAlg and $CaCl_2$. Considering all the results obtained in this study, Pul-NaAlg-$CaCl_2$ composite nanofibers can be considered as a promising material to encapsulate various bioactive peptides with several health effects for food purposes.

At the second step (Chapter 3), carnosine (Car), an antioxidative peptide, loaded Pul-NaAlg based composite fibers designed in previous chapter, were fabricated

successfully by uniaxial (blending), coaxial and emulsion electrospinning techniques and characterized to evaluate the effect of solution properties and the role of emulsion parameters on electrospun nanofibers with encapsulation efficiencies calculated as 74.11, 81.69 and 68.63%, respectively. Carnosine-loaded $W_1/O/W_2$ double emulsions were prepared with different formulations including three different surfactant combination, surfactant, two different oily phase and two different carnosine level to evaluate the effect of formulation on electrospinnability and used as feed emulsion in emulsion electrospinning study. The zeta potential results of double emulsions demonstrated that all emulsions can be considered as stable and incorporation of soy lecithin increased the absolute value of zeta potential, thus enhancing the stability of emulsions. The microscopy results indicated that the type B double emulsion was successfully obtained and several small inner water droplets were encapsulated in the relatively larger oil globules. Addition to these, the effect of encapsulation on antioxidant activity of Car was investigated and DPPH and CUPRAC assays results revealed that all electrospinning methods demonstrated the protective effect on the antioxidant activity of Car and thus helped in enhancing its antioxidant potential significantly.

Subsequently, ionic crosslinking was applied by $CaCl_2$ as crosslinking agent to improve the stability of electrospun fibers. In fact, the SEM analysis provides evidence that, among all samples obtained from emulsion electrospinning, the EE-VIII NFs were characterized by fine, beadless and uniform nanofibrous structure, and were thus selected to apply crosslinking and some further characterizations. According to the results, crosslinked nanofibers, except for the coaxial sample were able to largely preserve their well-defined nanofibrous structure after crosslinking, while the morphologies are quite different from the uncurving shape of the non-threated ones. Additionally, fiber diameters of nanofibers were increased after crosslinking treatment, except for the EE-VIII NFs which retained their original size.

Lastly, the effects of electrospinning encapsulation and crosslinking on release behavior of Car was investigated by *in vitro* digestion and the release kinetics was analyzed by establishing corresponding mathematic models. According to *in vitro* digestion results, it can be stated that the $CaCl_2$ crosslinking treatment enhanced sustained release of Car in the gastrointestinal tract. The initial burst release of Car from EE-VIII NFs was significantly lower than UENFs and CENFs in both gastric

phases, besides the release from EE-VIII NFs in intestinal phase was followed by sustained release, with/without crosslinking treatment. Therefore, emulsion electrospinning technique can be suggested compared to other techniques for sustained delivery of Car in the gastrointestinal tract. The results provided by mathematical modelling of release kinetics revealed that, Fickian diffusion mechanism was predominant in releasing Car from Pul-NaAlg NFs in the gastrointestinal tract. Finally, the results indicated that simultaneously encapsulation of Car in double emulsion and in Pul-NaAlg NFs can provide sustained release. To conclude, the results of these part of the thesis provided promising perspectives for further development of double emulsion-based nanofibers as edible protectors to encapsulate various bioactive agents for food applications.

As a future aspect, the properties of emulsions may be examined in detail throughout the electrospinning process in order to understand the effect of process conditions and duration on electrospinnability and encapsulation of bioactive compounds. Moreover, Furthermore, it could be suggested that more comprehensive *in vitro* and *in vivo* studies should be conducted to stimulate the digestion process and examining physicochemical changes of bioactive peptide-loaded nanofibers in the gastrointestinal tract.

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APPENDICES

APPENDIX A: Supplemental materials for chapter 3.

APPENDIX B: Supplemental materials for chapter 4.

APPENDIX A

Table A.1 : Zeta potentials of electrospun fibers.

Sample no	Zeta potential (mV)
1	-5.86 ± 1.23^{ab}
2	-4.65 ± 0.43^{b}
3	-7.13 ± 0.71^{a}
4	-7.18 ± 0.72^{a}

Data given in this table represents average amounts ± standard deviation of three replications for each sample. Data was analyzed by the one-way ANOVA and different letters (a–b) in the same column indicate significant differences by the Duncan test ($P < 0.05$).

APPENDIX B

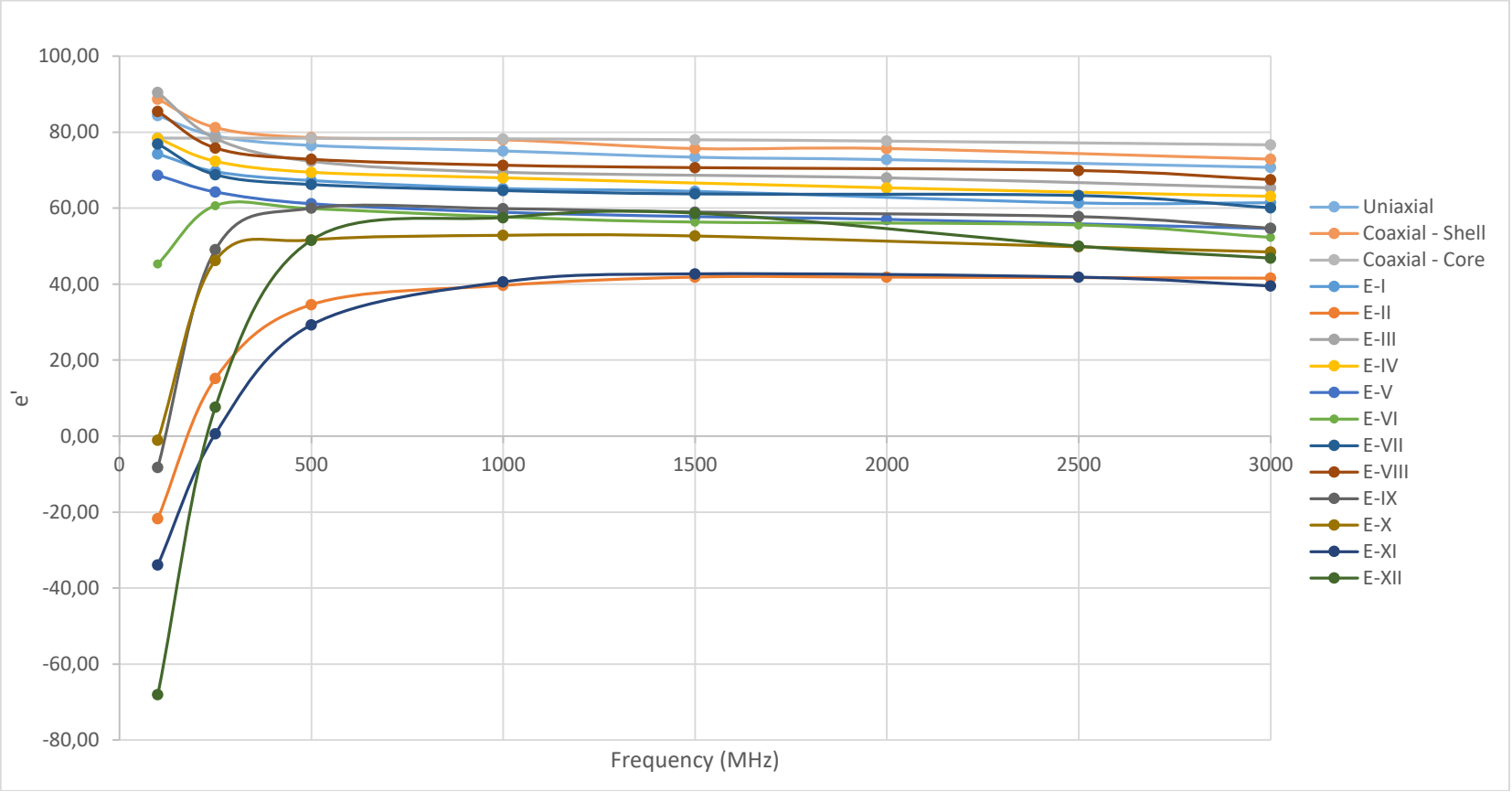

Figure B.1 : Dielectric properties of the feed solutions and emulsions.

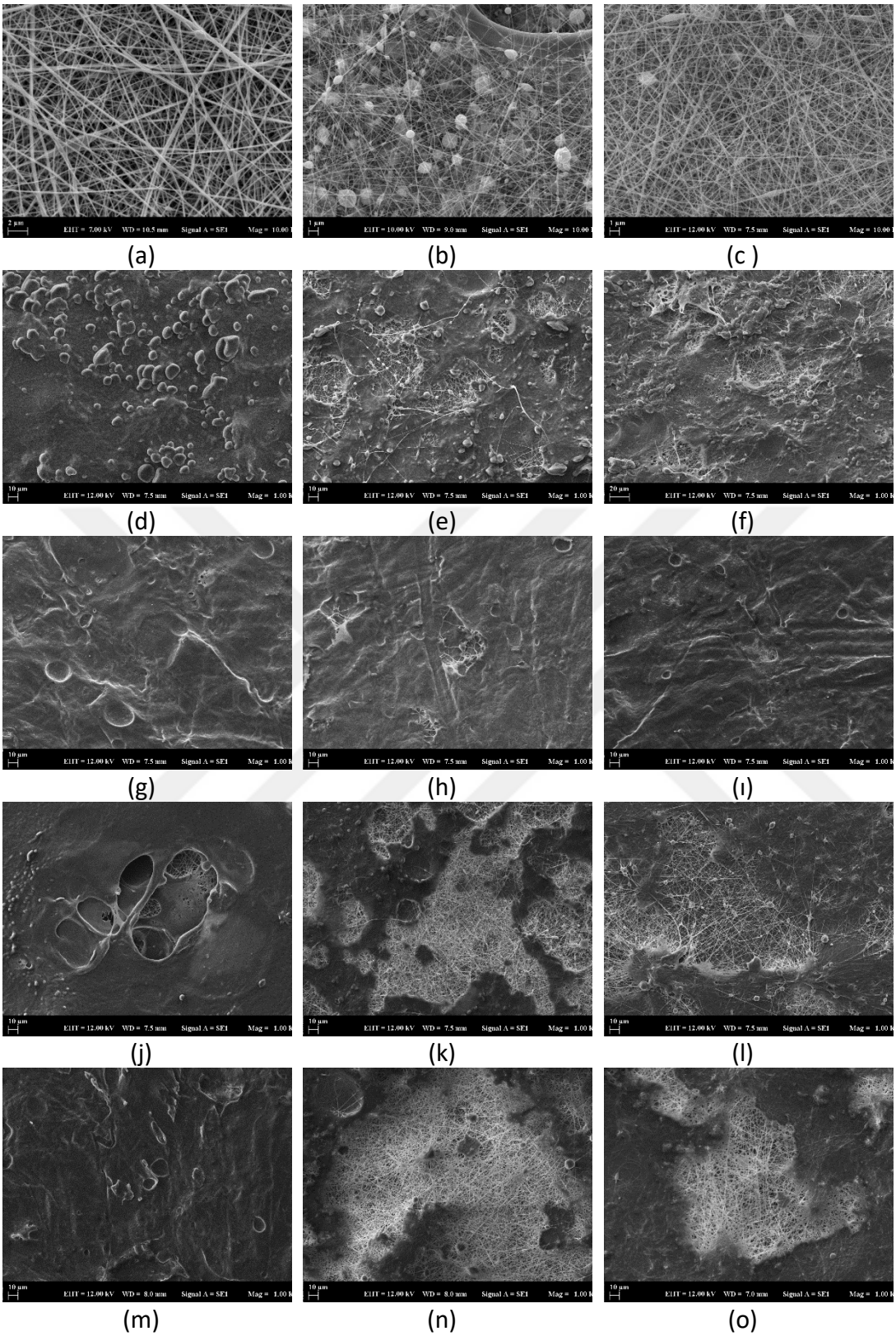

Figure B.2 : SEM images of electrospun samples obtained from defined solutions. Control (a), UE (b) and CE (c) samples (10000 magnification); EE-I (d), EE-II (e), EE-III (f), EE-IV (g), EE-V (h), EE-VI (i), EE-VII (j), EE-VIII (k), EE-XI (l), EE-X (m), EE-XI (n) and EE-XII (o) (1000 magnification).

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