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Electrospinning Pullulan to Nanoencapsulate Milk Derived Peptides

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ABSTRACT

Background: Milk proteins are a source of bioactive peptides, which are concealed until released and activated. There is a growing interest and necessity for the use of biologically active milk protein-derived peptides with health-promoting properties in food. Despite several health benefits and functionalities, consumption of bioactive peptides through foods is poor due to their unfavorable flavour or bitter taste, low solubility, poor stability during processing and poor oral bioavailability. Electrospinning is an adaptable process for encapsulation since it does not involve severe temperature and requirement of chemicals for the preparation of encapsulate in the form of nanofibres.

Methods: The peptide-loaded electrospun nanofibres are characterized for its surface morphology, encapsulation efficiency, zeta potential and interaction between wall material and peptides. *In vitro* release studies by dialysis bag technique and antioxidant property by DPPH method.

Result: Pullulan at 10, 12 and 14% (w/w) concentrations was used for electrospinning of peptides. Scanning electron micrograph revealed the formation of clean beadfree peptides-loaded pullulan nanofibres at 12 and 14% concentrations. Encapsulation efficiency ranged from 73.00-86.00%. FTIR spectrum and XRD diffractograms showed the nonappearance of interactions between the functional groups of biopolymer pullulan and peptides in the electrospun nanofibres. The peptides from electrospun nanofibres showed sustained release. The antioxidant activity of peptides and those which are in the nanofibres was comparable. The physico-chemical and sensory analysis of milk fortified with peptide loaded nanofibres did not show noticeable difference.

Key words: Electrospinning, Encapsulation efficiency, Nanoencapsulation, Peptides.

INTRODUCTION

Milk is a near complete food and one of the most widely consumed nutritious liquid foods worldwide. Cow milk consists of about 87.3% water and 12.7% total solids. The total solids are mainly consists of carbohydrates (lactose, 4.6%), fat (3.9%), proteins (3.25%) and minerals (1%).

The total protein is majorly comprises of casein (2.6%) and whey proteins (0.65%). Thus, casein constitutes approximately 80% of the total protein in cow milk, while whey protein accounts for the remaining 20%. Cow milk also contains minerals to the extent of 0.65% and other minor bioactive compounds (Walstra and Jenness, 1984). In recent years, constituents of milk, particularly proteins, are recognized as functional foods, demonstrating their direct effect on many health benefits (Park and Nam, 2015).

Scientific confirmation revealed that benefits of milk concerned to health are associated to its proteins, for their nutritional value and their biological properties such as, anticarcinogenic, antioxidant, antidiabetic, *etc.* (Davoodi *et al*. 2016). Milk proteins are a good source of peptides. They can be produced *in vitro* by fermentation with proteolytic starter cultures or by enzymatic hydrolysis. There is a growing interest and need for the use of milk protein-derived biologically active peptides with health-promoting properties in food. In particular, the bioactive peptides chain generally comprise of 2-20 amino acids (Haque *et al*. 2006). Despite several health benefits and functionalities, consumption of bioactive peptides in diet is often limited. Designing effective delivery approaches in the form of nanoparticles or nanofibres is therefore necessary to overcome the limitations. The most favourable method to overcome these limitations is by entrapping the bioactive peptide in the form of capsules, fibres, *etc.* In recent years, there is an increasing attention to encapsulate the bioactive peptides so as to provide the above said advantages for consumers. In fact, it is possible to produce value-added food products as well by employing nanoencapsulation (Jafari, 2017). The objective of encapsulating bioactives is to decrease the degradation and undesirable changes during processing and further in the severe digestive conditions encountered in

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the stomach (Fuchs *et al*., 2010; Katouzian and Jafari, 2016). De Marais *et al*. (2022) reported that nanoencapsulation of bioactives for the development of functional foods has a potential to increase the nutritional and bioactive properties of products. Additionally, improves the sensory properties and extends the storage preservation by reducing the hygroscopicity of the peptides (Aguilar-Toalá *et al*. 2022). Electrospinning is a potential technique for fabrication of peptides-loaded nanofibres due to its ability to spin nanofibres at ambient conditions. It imparts the convenience of direct encapsulation of bioactive compounds. Electrospinning of peptides is influenced by polymer concentration, viscosity, surface tension and electrical conductivity of feed solution. Further, electrospinning is equally influenced by machine parameters such as applied voltage, distance between spinneret and collector and feed rate Bhardwaj *et al*. (2010). Therefore, for electrospinning of any polymer, the process and feed solution parameters have to be evaluated to obtain uniform beadfree nanofibres.

Very few research works have been reported worldwide on electrospinning of bioactive compounds using biopolymers such as cellulose acetate, gelatin, whey protein isolate (WPI), zein and pullulan and solvents like water, ethanol, acetic acid, *etc*., for food applications. Bovine serum albumin and lysozyme were encapsulated in poly q-caprolactone (PCL) and polyethylene glycol (PEG) as core and shell polymers to attain controlled release (Jiang *et al*., 2005). Similarly, β-carotene and epigallo-catechin gallate (EGCG) have been electrospun into nanofibres as a method of encapsulation (Fernandez *et al*. 2009; Li *et al*. 2009). Brahatheeswaran *et al*. (2012), Bui *et al*. (2014) and Deng *et al*. (2017) produced curcumin-loaded zein electrospun nanofibres and curcumin-loaded zein nanofibres as antibacterial property by electrospinning process, while Mai *et al*. (2012) and Blanco-Padilla *et al*. (2015) developed curcumin-loaded PLA nanofibres and curcumin-loaded amaranth protein isolate (API) in pullulan 20% (w/w/) nanofibres, respectively by electrospinning. Likewise, folic acid loaded in sodium alginate-pectin-poly ethylene oxide (Alborzi *et al*. 2013), folic acid loaded in amaranth protein isolate/ pullulan (Aceituno-Medina *et al*. 2015), anthocyanin-rich red raspberry in soy protein isolate (SPI)-PEO (Wang *et al*. 2013), α-tocopherol in soluble dietary fibre (SDF) (Li *et al*. 2016), quercetin/ cyclodextrin-loaded in 35% (w/w) zein (Aytac *et al*. 2017), carvacrol loaded in zein-poly-lactic acid (PLA) and carotenoids in 25% (w/w) gelatin (Altan *et al*. 2018; Horuz and Belibagh, 2018) have been electrospun into nanofibres. However, there has been no report on electrospinning of casein-derived peptides using biopolymers as encapsulating material.

Once nanofibres are electrospun, their surface morphology, encapsulation efficiency, zeta potential and interaction between wall material and peptides have to be examined. With this background, this present work was carried to evaluate the electrospinning process and preparation peptides-loaded nanofibres and characterizing the electrospun nanofibres for fortification of milk.

MATERIALS AND METHODS

Production of peptides

Casein was enzymatically hydrolyzed at their recommended pH and temperature of 7.5 and 50°C (for alcalase) and 8.0 and 45°C (for chymotrypsin), respectively. The hydrolysates obtained were centrifuged and further ultrafiltered through AMICON membranes with molecular weight cutoff of 10 and 3 kDa.

Antioxidant activity of casein hydrolysates

The antioxidant activity of casein hydrolysates was analyzed based on DPPH (2, 2 diphenyl-1-picrylhydrazyl) radical scavenging activity.

Evaluation of biopolymer for electrospinning

Pullulan was selected as wall material and the solutions were prepared to get concentrations of 8, 10, 12 and 14% (w/w). The solutions viscosity, surface tension and electrical conductivity were determined using the rheometer, stalagmometer and electrical conductivity meter, respectively. The dimensionless quantities such as Deborah (De), Ohnesorge (Oh) and Berry (Be) numbers were computed to evaluate the electrospinnability of feed solutions.

Electrospinning of peptides-loaded nanofibres

Feed solutions for electrospinning of peptides were prepared by dissolving pre-weighed quantity of pullulan and peptide in 9:1 ratio in double-distilled water to get total solids concentrations of 10, 12 and 14% (w/w). The peptides-added pullulan solutions were electrospun at applied voltages of 18, 21 and 24 kV and feed rates of 0.5, 0.75 and 1.0 mL/h. Factorial design was used to determine the effects of each independent parameter on the diameter and encapsulation efficiency of electrospun peptides-loaded nanofibres.

Characterization of peptides-loaded electrospun nanofibres

The morphology of electrospun nanofibres were examined using field emission scanning electron microscope (FESEM). Zeta potential of the nanofibres was determined by electrophoretic light scattering method. The encapsulation efficiency of peptides in the nanofibres was determined by dialysis bag technique. The microstructure and functional groups present in the electrospun nanofibres were examined by Fourier transform infrared (FTIR) spectroscope. X-ray diffraction (XRD) patterns of pullulan, peptides and peptides-loaded pullulan nanofibres were obtained using X-ray diffractometer. The *in-vitro* release of peptides from the electrospun nanofibres under simulated gastric and intestinal pH conditions was determined by dialysis bag technique. The antioxidant activity of peptides from loaded nanofibres was analyzed based on DPPH radical scavenging ability.

Fortification of milk with peptides-loaded nanofibres

The physico-chemical parameters of fortified milk such as fat, solids-not-fat, pH and titratable acidity were determined according to AOAC methods (AOAC, 2012), while the organoleptic quality was assessed using 9-point hedonic scale.

RESULTS AND DISCUSSION

Enzymatic hydrolysis of casein

Casein hydrolysed when enzymes added together resulted in the highest DH of 26.81% after 8 h. When compared to chymotrypsin, alcalase gave higher DH at corresponding time intervals because bacterial enzymes has a broader cleavage sites.

Antioxidant activity of peptides

The <10 and <3 kDa permeate fractions obtained by combined action of both enzymes exhibited antioxidant activity of 58.00% and 62.00%, respectively.

Electrospinning of peptides-loaded nanofibres

Pullulan and peptides were blended in 9:1 ratio and feed solutions of 10, 12 and 14% concentrations were prepared. Pullulan had electrical conductivity of 0.07-0.12 mS/cm and surface tension of 67.18-71.58 mN/m. But, addition of peptides to pullulan increased its electrical conductivity to the range of 1.32-1.40 mS/cm due to the charged ionic groups associated with peptides. The viscosity of pullulan ranged from 54.5 to 302.0 mPa.s.

For smooth electrospinning, the feed solution should meet the inequality conditions of $De \geq Oh > 1$ and $Be > 2$. For 10% pullulan solution, the inequality conditions were not satisfied. In comparison, at 12 and 14% (w/w) concentrations, Oh was >1 and the inequality conditions of $De \geq Oh > 1$ and $Be > 2$ were satisfied. In the initial trials, applied voltages below 14 kV were found to be insufficient to overcome the surface tension of feed solution. Oppositely, at voltages above 25 kV, feed solution was forming multiple unstable liquid jets. From the initial trials, voltage levels of 18, 21 and 24 kV were selected for electrospinning of peptides.

Characterization of peptides-loaded electrospun nanofibres

Scanning electron microscopy of nanofibres

It was observed that beaded nanofibres were formed at 10% concentration. At 12% concentration, clean, uniform and beadfree nanofibres were formed with mean fibre diameter ranging from 60.45 to 99.81 nm. However, with further increase in concentration to 14%, the diameter of nanofibres increased to the range of 106.97 to 133.05 nm (Fig 1). Increase in concentration lead to increase in elastic stress which stops the fluid jet breakup and maintain the stability of a jet with high relaxation time. Also, the increase in concentration aided to continuous entanglement of molecules and thus reduced breakup of chain entanglement during stretching of jet. Furthermore, slight decrease in surface tension with an increased concentration also contributed to overcome the formation of beads. Similarly, Saraf *et al*. (2021) reported the diameter of pullulan nanofibres produced was less than 100 nm at applied voltage and polymer concentration of 20 kV and 18% pullulan, respectively.

Hydrodynamic diameter and zeta potential

The electrospun nanofibres with the highest encapsulation efficiency of 86.04% had mean fibre diameter of 113.19 nm, hydrodynamic diameter of 220.3 nm, zeta potential of -23.6 mV and PDI of 25.2%. Thus, the peptides-loaded nanofibres are expected to be quite stable in liquid food systems.

Encapsulation efficiency

The encapsulation efficiency of peptides in pullulan nanofibres ranged from 73.00 to 86.00%. During electrospinning, as the liquid jet is drawn from the spinneret tip, evaporation of solvent takes place within a few seconds. Hence, some peptides might not have been encapsulated within but distributed on the surface of pullulan matrix, which reduced the encapsulation efficiency. Seethu *et al*. (2020) achieved encapsulation efficiency in the range of 74.0-96.7% for resveratrol-loaded pullulan-WPI uniaxial electrospun nanofibres.

Fourier transform infrared spectroscopy

The spectrum of peptides-loaded pullulan nanofibre is shown in Fig 2. In comparison, the spectrum of peptides-loaded nanofibre was almost similar to that of pullulan. The absence of peptide nitrogen peaks in loaded nanofibres suggested that the peptides could be well-encapsulated within pullulan used as encapsulating polymer. This proved that the encapsulation process was more of a physical incorporation of peptides into pullulan rather than chemical interaction. The peaks obtained for pullulan were closely comparable with those reported by Drosou *et al*. (2018).

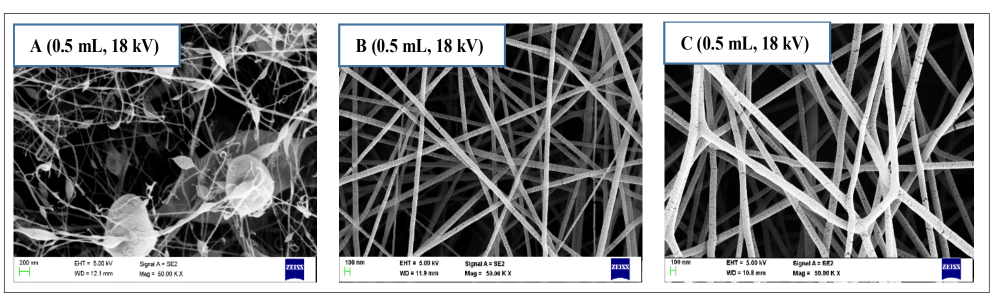

Fig 1: FESEM images of electrospun peptides-loaded nanofibres obtained at 10% (A), 12% (B) and 14% (C) pullulan concentrations.

X-ray diffractometry

A broad 2θ peak was observed for pullulan and for peptides (Fig 3). These broad peaks revealed that the structure of pullulan and peptides are amorphous in nature Qin *et al*. (2019). In comparison, the electrospun nanofibres exhibited a broad peak at 2θ angle between 10 and 25°C. Thus, the amorphous structure of electrospun peptides-loaded nanofibres was similar to those of peptides and pullulan. The molecular chains of pullulan were stretched and solidified rapidly during electrospinning could be the reason for the nanofibres to remain amorphous.

In vitro release study

The release of free peptides in simulated GI pH at 2, 4, 6 and 8 h was 70.41, 92.64, 98.06 and 98.63%, respectively. Whereas the release of peptides from nanofibres was lower at 31.3, 58.2, 70.1 and 75.3%, respectively, thereby demonstrating longer and sustained release. Therefore, dissolution of peptides in the simulated gastric and intestinal pH was a relatively slow process, resulting in sustained release of peptides. Another reason for reduced burst release was that most peptides were encapsulated within the pullulan matrix and were not completely aggregated on the surface of nanofibres.

Antioxidant activity

Antioxidant activity of free peptides was 58.16%, however the peptides from electrospun nanofibres expressed antioxidant activity of 54.10%. Therefore, it could be stated that the antioxidant property of peptides was not hindered by the application of high voltage during electrospinning. Faki *et al*. (2019) and Locilento *et al*. (2019) reported that the encapsulated grape seed extract retained its antioxidant property after electrospinning as nanofibres.

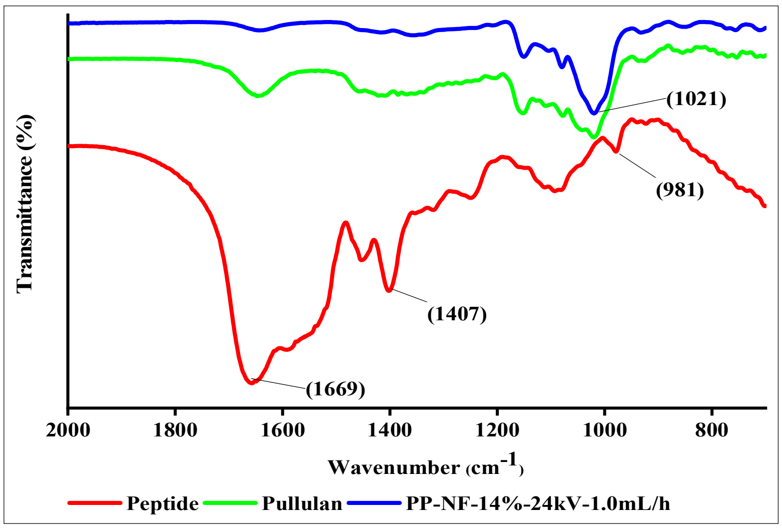

Fig 2: FTIR spectra of peptide, pullulan and pullulan-peptide nanofibres.

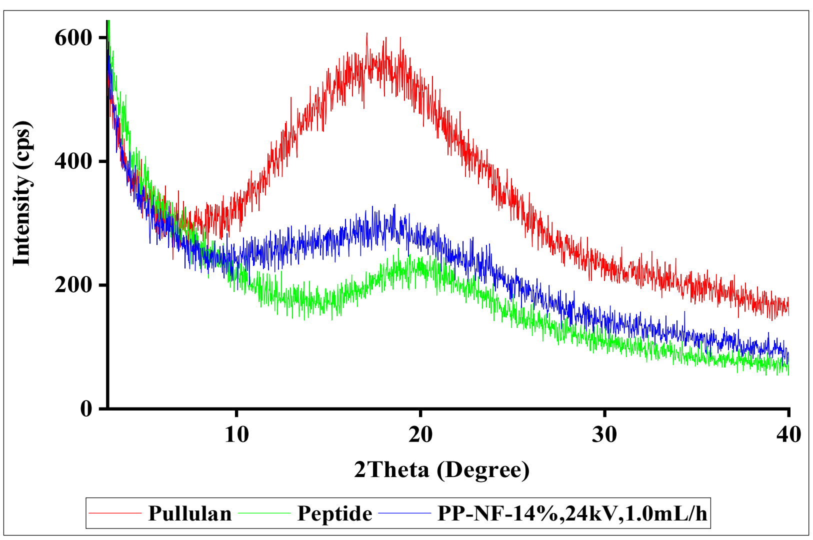

Fig 3: XRD spectra of peptide, pullulan and pullulan-peptide nanofibres.

Fortification of nanofibres into milk

Milk was fortified with peptides-loaded electrospun nanofibres and its physico-chemical and organoleptic qualities were evaluated. There was no marked difference (p>0.05) in the colour, pH and titratable acidity of milk after fortification with peptides-loaded pullulan nanofibres. Therefore, the overall acceptability of milk fortified with nanofibres was slightly higher (p<0.05) at 8.7, while it was 8.5 for control. Fathi *et al*. (2017) fortified cheese with electrospun vitamin E-loaded dextran nanofibres and did sensory evaluation using 7-point hedonic scale. From the sensory data, the authors reported that cheese fortified with nanofibres was better than that of direct fortification of vitamin E.

CONCLUSION

The electrospun peptides-loaded pullulan-based nanofibres had smooth surface morphology, with mean diameter below 100 nm and maximum encapsulation efficiency of about 86.04%. From the encapsulation efficiency and antioxidant activity, it could be inferred that electrospinning could be viable and effective technique of nanoencapsulation of casein-derived peptides.

Conflict of interest: None.

REFERENCES

Aceituno-Medina, M., Mendoza, S., Lagaron, J.M. and López-Rubio, A. (2015). Photoprotection of folic acid upon encapsulation in food-grade amaranth (*Amaranthus hypochondriacus* L.) protein isolate-Pullulan electrospun fibers. LWT-Food Science and Technology. 62: 970-975.

Aguilar-Toalá, J.E., Quintanar-Guerrero, D., Liceaga, A.M. and Zambrano-Zaragoza, M.L. (2022). Encapsulation of bioactive peptides: A strategy to improve the stability, protect the nutraceutical bioactivity and support their food applications. RSC Advances. 12: 6449-6458.

Alborzi, S., Lim, L.T. and Kakuda, Y. (2013). Encapsulation of folic acid and its stability in sodium alginate-pectin-poly (ethylene oxide) electrospun fibres. Journal of Microencapsulation. 30: 64-71.

Altan, A., Aytac, Z. and Uyar, T. (2018). Carvacrol loaded electrospun fibrous films from zein and poly (lactic acid) for active food packaging. Food Hydrocolloids. 81: 48-59.

AOAC. (2012). Official Methods of Analysis. Washington, DC, USA: Association of Official Analytical Chemists.

Aytac, Z., Ipek, S., Durgun, E., Tekinay, T. and Uyar, T. (2017). Antibacterial electrospun zein nanofibrous web encapsulating thymol/cyclodextrin-inclusion complex for food packaging. Food Chemistry. 233: 117-124.

Bhardwaj, N. and Kundu, S.C. (2010). Electrospinning: A fascinating fibre fabrication technique. Biotechnology Advances. 28: 325-347.

Blanco-Padilla, A., López-Rubio, A., Loarca-Pina, G., Gómez-Mascaraque, L.G. and Mendoza, S. (2015). Characterization, release and antioxidant activity of curcumin-loaded amaranth-pullulan electrospun fibers. LWT-Food Science and Technology. 63: 1137-1144.

Brahatheeswaran, D., Mathew, A., Aswathy, R. G., Nagaoka, Y., Venugopal, K., Yoshida, Y. and Sakthikumar, D. (2012). Hybrid fluorescent curcumin loaded zein electrospun nanofibrous scaffold for biomedical applications. Biomedical Materials. 7: 1-16.

Bui, H.T., Chung, O.L. and Park, J.S. (2014). Fabrication of electrospun antibacterial curcumin-loaded zein nanofibres. Polymer. 38: 744-751.

Davoodi, S.H., Shahbazi, R., Esmaeili, S., Sohrabvandi, S., Mortazavian, A., Jazayeri, S. and Taslimi, A. (2016). Health-related aspects of milk proteins. Iranian Journal of Pharmaceutical Research. 15: 573-591.

De Morais, M.G., Alvarenga, A.G.P., Da Silva Vaz, B. and Costa, J.A.V. (2022). Nanoencapsulation of Spirulina biomass by electrospraying for development of functional foods- A review. Biotechnology Research and Innovation Journal. 5: 1-6.

Deng, L., Kang, X., Liu, Y., Feng, F. and Zhang, H. (2017). Effects of surfactants on the formation of gelatin nanofibres for controlled release of curcumin. Food Chemistry. 231: 70-77.

Drosou, C., Krokida, M. and Biliaderis, C.G. (2018). Composite pullulan-whey protein nanofibers made by electrospinning: Impact of process parameters on fiber morphology and physical properties. Food Hydrocolloids. 77: 726-735.

Faki, R., Gursoy, O. and Yilmaz, Y. (2019). Effect of electrospinning process on total antioxidant activity of electrospun nanofibers containing grape seed extract. Open Chemistry. 17: 912-918.

Fathi, M., Nasrabadi, M.N. and Varshosaz, J. (2017). Characteristics of vitamin E-loaded nanofibres from dextran. International Journal of Food Properties. 20: 2665-2674.

Fernandez, A., Torres-Giner, S. and Lagaron, J.M. (2009). Novel route to stabilization of bioactive antioxidants by encapsulation in electrospun fibres of zein prolamine. Food Hydrocolloids. 23: 1427-1432.

Fuchs, S., Kutscher, M., Hertel, T., Winter, G., Pietzsch, M. and Coester, C. (2010). Transglutaminase: New insights into gelatin nanoparticle cross-linking. Journal of Microencapsulation. 27: 747-754.

Haque, E., Chand, R. and Kapila, S. (2006). Biofunctional properties of bioactive peptides of milk origin. Food Reviews International. 25: 28-43.

Horuz, T.Ý. and Belibaðlý, K.B. (2018). Nanoencapsulation by electrospinning to improve stability and water solubility of carotenoids extracted from tomato peels. Food Chemistry. 268: 86-93.

Jafari, S.M. (2017). An overview of nanoencapsulation techniques and their classification. In: Nanoencapsulation Technologies for the Food and Nutraceutical Industries, [Jafari, S.M (ed.)], (pp. 1-34). Academic Press, Cambridge, USA.

Jiang, H., Hu, Y., Li, Y., Zhao, P., Zhu, K., and Chen, W. (2005). A facile technique to prepare biodegradable coaxial electrospun nanofibers for controlled release of bioactive agents. Journal of Controlled Release. 108: 237-243.

Katouzian, I. and Jafari, S.M. (2016). Nano-encapsulation as a promising approach for targeted delivery and controlled release of vitamins. Trends in Food Science and Technology. 53: 34-48.

Li, J., Chotiko, A., Narcisse, D.A. and Sathivel, S. (2016). Evaluation of alpha-tocopherol stability in soluble dietary fibre based nanofibre. LWT-Food Science and Technology. 68: 485-490.

Li, Y., Lim, L.T. and Kakuda, Y. (2009). Electrospun zein fibres as carriers to stabilize epigallocatechin gallate. Journal of Food Science. 74: 233-240.

Locilento, D.A., Mercante, L.A. andre, R.S., Mattoso, L.H., Luna, G.L., Brassolatti, P. and Correa, D.S. (2019). Biocompatible and biodegradable electrospun nanofibrous membranes loaded with grape seed extract for wound dressing application. Journal of Nanomaterials. 2019: 1-11.

Mai, T.T.T., Nguyen, T.T.T., Le, Q.D., Nguyen, T.N., Ba, T.C., Nguyen, H.B. and Park, J.S. (2012). A novel nanofibre Cur-loaded polylactic acid constructed by electrospinning. Advances in natural sciences. Nanoscience and Nanotechnology. 3(2): 1-4.

Park, Y.W. and Nam, M.S (2015). Bioactive peptides in milk and dairy products: A review. Korean Journal of Food Science and Animal Resource. 35: 831-840.

Qin, Z. Y., Jia, X. W., Liu, Q., Kong, B.H. and Wang, H. (2019). Fast dissolving oral films for drug delivery prepared from chitosan/pullulan electrospinning nanofibers. International Journal of Biological Macromolecules. 137: 224-231.

Saraf, K. and Nadanathangam, V. (2021). Production of nanofibers of pullulan biopolymer by electrospinning process. Academia Letters. 1206: 1-8.

Seethu, B.G., Pushpadass, H.A., Emerald, F.M.E., Nath, B.S., Naik, N.L. and Subramanian, K.S. (2020). Electrohydrodynamic encapsulation of resveratrol using food-grade nanofibres: Process optimization, characterization and fortification. Food and Bioprocess Technology. 13: 341-354.

Walstra, P. and Jenness, R. (1984). Dairy Chemistry and Physics. John Wiley and Sons, New Jersey, US.

Wang, S., Marcone, M.F., Barbut, S. and Lim, L.T. (2013). Electrospun soy protein isolate-based fibre fortified with anthocyanin-rich red raspberry (*Rubus strigosus*) extracts. Food Research International. 52: 467-472.