Polymer Degradation and Stability 147 (2018) 35–40

Contents lists available at ScienceDirect

Polymer Degradation and Stability

journal homepage: www.elsevier.com/locate/polydegstab

Design of low molecular weight pectin and its nanoparticles through combination treatment of pectin by microwave and inorganic salts

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ARTICLE INFO

Keywords:
Calcium acetate
Microwave
Nanoparticle
Pectin
Sodium chloride

ABSTRACT

This study examined primarily the molecular weight and degree of esterification profiles of pectin in response to treatment by microwave as a function of irradiation duration in combination with monovalent (sodium chloride) or divalent (calcium acetate) inorganic salt. The possibility of formation and physical attributes of nanoparticles prepared form these pectins were assessed against their suitability for use as nanocarrier in cancer therapy. The pectin was treated by microwave (900 W) with inorganic salts as the promoter of superheating at liquid state. Its molecular weight, degree of esterification, viscosity, particle size, zeta potential and elemental content were determined. The pectin was subjected to nanospray drying with the size, zeta potential and morphology of the formed nanoparticles examined. The use of calcium acetate translated to the formation of pectin with lower molecular weight and degree of esterification, but higher solution viscosity than that of sodium chloride. Fourier transform infrared spectroscopy, particle size and elemental content analysis indicated such pectin had its molecules crosslinked by soluble calcium at COO^- moiety in liquid phase. It experienced a higher heat transfer through salt bridges and chain breakdown propensity particularly with prolonged duration of treatment. The formed nanoparticles were characterized by a mean size smaller than 600 nm and were envisaged appropriate for use as nanocarrier of cancer therapeutics with respect to permeation and retention attributes of tumour vasculature. The combination of microwave with multivalent inorganic salt is a viable approach for use to convert the pectin into matrix material of nanoparticles.

1. Introduction

Pectin is a cell wall polysaccharide with polymeric chain consisting of repeating units of α-1,4-linked-D-galacturonic acid [1]. It is first discovered by a French chemist and pharmacist, Henry Braconnot in 1825 [2]. The primary sources of pectin for commercial applications are citrus peel and apple pomace, which are waste products from fruit juice manufacturing and are readily available at low costs [3]. Depending on their degree of esterification (DE), pectins are commonly categorized as high methoxyl pectin (HMP) or low methoxyl pectin (LMP), with DE > 50% and < 50% respectively [4]. Molecular weight and degree of esterification of pectin are important attributes that define its functional properties such as viscosity [5,6].

Pectin is biodegradable, biocompatible and safe for human consumption [7]. Pharmaceutically, pectin has been widely applied as the matrix or coat materials of drug delivery systems specifically in oral colon-specific carrier development [8–15]. Pectin, as a dietary fibre, plays a role in preventing colon cancer [2,16]. Ginseng pectin, citrus pectin, and apple pectin, subjected to heat and/or physical treatments, have demonstrated apoptotic, cell cycle arrest, galectin-3 inhibitory activities that are beneficial in cancer prevention and treatment [17–21].

Polysaccharides such as pectin, chitosan and alginate have been advocated for use in design of nanoparticulate drug delivery system for the treatment of cancer [22–24]. The nanoparticles are considered as a favourable vehicle due to its small size and large specific surface area attributes. They can be decorated with ligands for specific cancer cell targeting and are suitable for receptor targeting at the cellular level [25–27]. The size of nanoparticles is known to be the governing parameter that dictates their interaction with the cell membrane and eventually cellular internalization into cytoplasm and nucleus where the drug targets reside [28].

The size of the polysaccharide nanoparticles is directly governed by the molecular weight and size of the matrix polymer [29]. Typically,

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https://doi.org/10.1016/j.polymdegradstab.2017.11.011
Received 9 October 2017; Received in revised form 9 November 2017; Accepted 17 November 2017
Available online 21 November 2017

low molecular weight or small size polysaccharide is preferred in the development of nanoparticles. With reference to pectin, its physical constructs have been modified by means of chemical, enzymatic and heat approaches [18,30–32]. Lately, the dynamic high pressure microfluidization technique is used to reduce the molecular weight, size and viscosity of pectin [7]. The high pressure homogenization and ultrasound techniques are likewise used to reduce the chain length of pectin [3,33,34]. The modification of pectin via the physical approach could lead to minimal changes to the polysaccharide as a function of the source of pectin [33]. Using chemical and enzymatic approaches, it can however cause environmental pollution and is a costly process [3,35].

Microwave is an electromagnetic radiation with frequency ranges between 300 MHz and 300 GHz [36]. It is a green technique advocated in chemical reactions and polymer modifications for its evident advantages over conventional heating [37]. The microwave technology offers lower activation energies, higher reaction rates, reduced reaction times, extremely fast temperature increment and hazardous chemical-free operations [37,38]. It has been used to modify the physicochemical properties of polymers such as poly(alkyl methacrylates) [39], chitosan [35,40–47], and poly(ethylene oxide) [38]. The polymer modification by microwave is commonly assisted by inorganic salt in liquid state via its superheating action above normal boiling point. Thus far, the monovalent salt such as sodium chloride is primarily used as the promoter of superheating [41,42,47]. This study aims to modify the pectin by means of combination treatment using microwave and inorganic salt. The divalent salt, namely calcium acetate, is employed with the outcome of pectin modification compared against that of sodium chloride. It is hypothesised that the soluble dicationic calcium can crosslink with the polyanionic pectin chains. The microwave is inclined to interact with water and electrolytes [48]. The heat generated from the interaction between microwave, water and electrolytes could possibly transmit to pectin chains in a more effective manner if these polymeric backbones are held by electrolytes while surrounded by the water molecules in comparison to cases where monovalent salt is used. In the latter, the pectin chains are free from crosslinkages by the electrolyte. The heat transmission is not aided by pectin-electrolyte crosslinkages.

2. Experimental

2.1. Materials

Pectin from citrus peels (Herbstreith & Fox KG Pektin-Fabrik, Germany) was used as the polymer of interest. Calcium acetate (Sigma-Aldrich, USA) and sodium chloride (Merck, Germany) were used as the inorganic salts in pectin modification. Nitric acid 65%, ethanol 96%, acetone, sodium azide and sodium nitrate were obtained from Merck, Germany. Dextrans with molecular weights of 1000, 12000, 50000, 80000, 150000, 270000, 410000, 670000 and 1400000 Da (Sigma-Aldrich, Denmark) were used as the standard in molecular weight analysis of pectin.

2.2. Pectin modification

One gram of pectin was added into 49 g deionized water under continuous magnetic stirring to produce 2 %w/w pectin solution. Two ml of 0.9 %w/w sodium chloride solution or 2.4 %w/w calcium acetate solution, at an equivalent mole of sodium and calcium ionic species, were introduced into the pectin solution under stirring and further mixed for 1 h. The pectin-inorganic salt mixture was subjected to microwave treatment at 900 W for 5, 10, 20 and 40 min using a microwave oven (NN-CD997S, Panasonic, Japan). Following microwave irradiation, the mixture was cooled to 25 ± 1 °C. The pectin was precipitated using ethanol 96%, filtered, washed with acetone and left to dry in hot air oven (Memmert, Germany) at 40 °C for 24 h. The dried pectin was conditioned in silica gel desiccator at 25 ± 1 °C.

2.3. Nanoparticles preparation

Twenty five mg of pectin were dissolved in 50 ml deionized water under continuous magnetic stirring. It was subjected to nanospray drying process using the nanospray dryer B-90 (Büchi, Switzerland) by means of the following conditions: spray mesh size = 4 μm, pectin solution flow rate = 12 ml/min, inlet temperature = 90 °C, outlet temperature = 44 °C, drying air flow rate = 120 L/min, spray rate = 100%, atomization pressure = 44–52 mbar. The spray-dried nanoparticles were collected from the electrostatic particle collector. They were kept in amber glass vials and stored in a silica gel desiccator at 25 ± 1 °C.

2.4. Molecular weight

The molecular weight of pectin and its distribution were determined using the gel permeation chromatography technique equipped with a refractive index detector (1100 series, Agilent Technologies, Germany). A PL aquagel-OH mixed column (7.5 mm × 300 mm, 8 μm particle size; Agilent Technologies, USA) was used as the stationary phase with mobile phase constituting of 0.05 %w/w sodium azide and 50 mM sodium nitrate dissolved in deionized water. The flow rate of the mobile phase was 0.5 mL/min and the column temperature was set at 30 °C. The dextran standard solution (1 mg/ml) and pectin solution (1.5 mg/ml), dissolved in deionized water, were filtered through a nylon membrane (Whatman, UK) before analysis. At least triplicates were carried out for each sample and the results were averaged.

2.5. Degree of esterification

The pectin was mixed with potassium bromide powder (2.5 %w/w pectin; FTIR grade, Sigma-Aldrich, USA) and compressed into a pellet using a hydraulic press (Specac, UK). It was subjected to FTIR analysis using an IR spectrometer (Cary 630, Agilent Technologies, USA). The absorbance spectra of pectin were recorded in the wavenumber region of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}. At least triplicates were conducted for each sample. The degree of esterification (*DE*) of pectin was calculated in accordance to the following equations [49]:

$$DE = 124.7R + 2.2013 \quad (1)$$

$$R = A_{1740}/[A_{1740} + A_{1630}] \quad (2)$$

where A_{1740} and A_{1630} were defined as the absorbance intensities of bands ascribing to esterified and non-esterified carboxyl groups of pectin at 1740 cm^{-1} and 1630 cm^{-1} respectively.

2.6. Specific viscosity

The specific viscosity of the pectin solution was measured via characterizing its flow time in an Ubbelohde dilution viscometer (size A, Poulten Selfe & Lee Ltd, UK) at 37 ± 0.5 °C in a temperature-controlled water bath (Memmert, Germany). The pectin was dissolved in deionized water to produce 0.1 %w/w pectin solution. The pectin solution was filtered using a nylon membrane filter (pore size = 0.45 μm; Whatman, UK) prior to viscosity measurements. The flow time of pectin solution (t) and deionized water (t_0) were determined. The specific viscosity (η_{sp}) of the pectin solution was calculated using the following equation:

$$\eta_{sp} = (t - t_0)/t_0 \quad (3)$$

Triplicates of experiment were conducted for each sample and the results were averaged.

2.7. Elemental analysis

The calcium level in pectin was quantified by inductively coupled

plasma mass spectrometry (ICP-MS; 8800 series, Agilent Technologies, Japan). Ten mg of pectin was dissolved in 5 ml of nitric acid and digested using microwave digestion system (Ethos Up, Milestone, Italy) for 30 min at 200 °C. The digested sample was then diluted to 100 ml with deionized water. The calcium content of pectin was quantified by using external calcium standards (Ultra Scientific, USA) prepared in 5% nitric acid solution. Triplicates were conducted and the results averaged.

2.8. Size and zeta potential

The particle size and zeta potential of pectin and nanoparticles were determined using Zetasizer Nano ZS (Malvern Instruments Ltd, UK) based on dynamic light scattering at 25 °C in a folded capillary cell with a detect angle of 90°. The sample was dispersed in deionized water and subjected to brief vortex prior analysis. Triplicates were conducted and the results averaged.

2.9. Surface morphology

The surface morphology of nanoparticles was examined by means of scanning electron microscopy (Helios Nanolab G3 UC, FEI, USA). The nanoparticles were first fixed onto the adhesive carbon tape mounted on a stub. They were then subjected to platinum coating at a current intensity of 20 mA for 30 s by means of an auto fine coater (JEC-3000FC, JEOL, Japan). The sputter-coated nanoparticles were examined microscopically at 10 kV. Representative sections were photographed.

2.10. Statistical analysis

The data were expressed as mean and standard deviation. Pearson correlation, Student's *t*-test and analysis of variance (ANOVA)/post hoc analysis by Tukey HSD were conducted using Minitab software 16.0. Statistical significance was denoted by $p < 0.05$ unless otherwise stated.

3. Results and discussion

3.1. Pectin

3.1.1. Specific viscosity

The specific viscosity of the pectin solution decreased with pectin subjected to prior microwave treatment (Fig. 1). It was lower when the pectin was prior treated with microwave for a longer duration of irradiation (Fig. 1; ANOVA, $p < 0.05$). The relationship of specific viscosity of pectin solution with microwave treatment duration was governed by the type of inorganic salt employed in pectin modification by microwave. Apparently, the use of sodium chloride in the process of microwave treatment was accompanied by a greater reduction extent in the specific viscosity of the pectin solution than that of calcium acetate (Fig. 1; Student's-t-test, $p < 0.05$).

3.1.2. Molecular weight

The specific viscosity of a polymer solution is well known to be directly related with the molecular weight of the polymer [50,51]. The present study adopted dextran, the available commercial standard, in pectin molecular weight characterization as it is a complex branched glucan with similar polysaccharide build-up as pectin. The molecular weight of the pectin was lower when it was subjected to a longer duration of microwave irradiation (Fig. 1; ANOVA, $p < 0.05$). Pearson correlation study indicated that a reduction in pectin molecular weight, as a function of microwave irradiation duration, brought about a reduction in the specific viscosity of the pectin solution (sodium chloride: $r = 0.9601$, $p = 0.04$; calcium acetate: $r = 0.9495$, $p = 0.04$). Under the same microwave treatment conditions, the molecular weight of pectin produced in the presence of calcium acetate was nevertheless lower than that of sodium chloride (Student's-*t*-test; $p < 0.05$), though the specific viscosity of the pectin solution demonstrated otherwise (Fig. 1). The molecular weight distribution of pectin was quantified as polydispersity index. A narrower molecular weight distribution was denoted by a smaller value of polydispersity index. The polydispersity index of pectin decreased with prolonged treatment of pectin by microwave (Fig. 1). Lower molecular weight pectin with a narrow molecular weight distribution was attainable through treating the pectin for a prolonged duration.

Previous research studies showed that microwave can reduce the molecular weight of a polymer via heating [35,40,41,44–47]. It was able to cleave the parent chain into shorter fragments and/or execute debranching on the side chains of the polymer, thereby leading to reduced polymer solution viscosity. The extent of polymer molecular weight reduction was greater with longer durations of microwave irradiation. The metal halide such as sodium chloride has been used as the promoter of polymer degradation via its ability to induce superheating in conjunction with microwave or heat treatment of the polymer solution [42,46,47]. Preliminary trials showed that there were no marked differences in the temperature of the pectin solution with respect to the type of inorganic salt added, under the influences of microwave. Both inorganic salts brought about a similar degree of superheating (microwave irradiation duration = 40 min, pectin solution temperature detected by infrared thermometer = 101.0 ± 0.1 °C). With reference to calcium acetate, the dicationic nature of the soluble calcium was envisaged to enable it to crosslink with the adjacent anionic pectin chains in a solution. This was supported by FTIR findings of pectin solution, against the introduction of sodium chloride and calcium acetate, where the band ascribed to non-esterified carboxyl group at 1615.7 ± 0.7 cm^{-1} was reduced to 1600.9 ± 0.6 cm^{-1} when the calcium acetate was employed as the promoter (Fig. 2). This resulted in improved heat transfer between the pectin chains via the electrolyte, and a greater reduction in the molecular weight of the pectin.

The solution specific viscosity of pectin, modified by the combination of microwave and calcium acetate, was higher than that of sodium

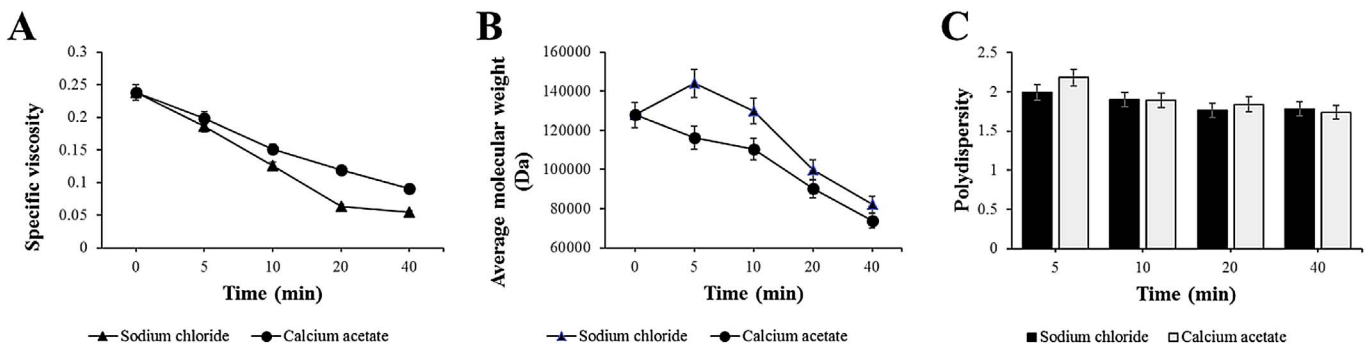

Fig. 1. Effects of ionic salt type on (A) specific viscosity, (B) average molecular weight and (C) polydispersity index of pectin.

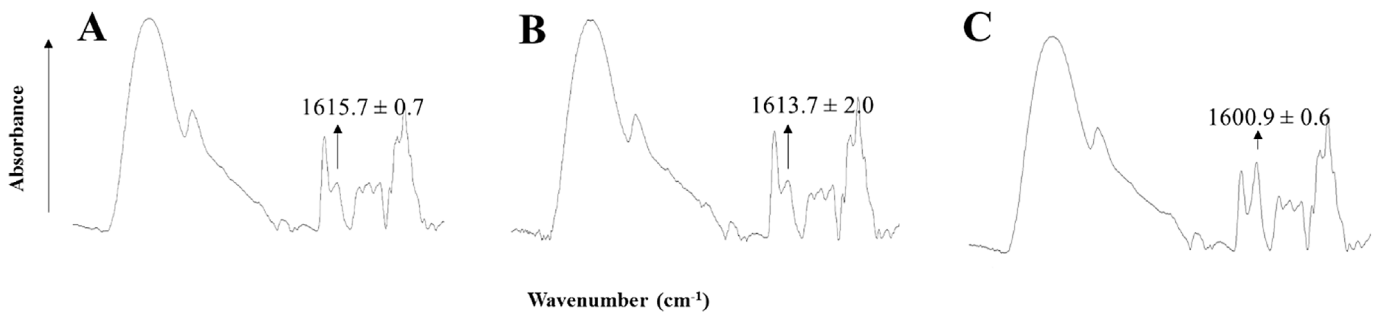

Fig. 2. FTIR spectra of pectin solution (A) without inorganic salt, with (B) sodium chloride and (C) with calcium acetate.

chloride with microwave (Fig. 1). One possible reason was that the pectin chains were crosslinked by the residual calcium ions into egg-box structure [52]. Pectin crosslinkages could induce chain aggregation. This in turn led to a rise in the solution viscosity in accordance to Chen et al. (2014) [53]. Elemental analysis by ICP-MS tests showed that the pectin modified by microwave with calcium acetate as the promoter was characterized by a residual calcium fraction at 2.2 ± 0.1%. The treatment of pectin via the combination of microwave and inorganic salt translated to the formation of particles with reduced physical sizes (untreated pectin: 950.3 ± 33.4 nm; microwave 40 min treatment: 687.0 ± 105.6 nm; combined microwave 40 min and sodium chloride treatment: 341.7 ± 5.9 nm; combined microwave 40 min and calcium acetate treatment: 509.4 ± 11.2 nm). The size of pectin particles, modified by the combination of microwave and calcium acetate, was larger than that of sodium chloride with microwave. It implied that this pectin, though lower in molecular weight, could be aggregative due to the presence of residual calcium ions. This aptly explained the higher solution specific viscosity attribute of such pectin.

3.1.3. Degree of esterification

The FTIR spectrum of untreated pectin was characterized by a broad area of absorbance between 3000 cm^{-1} and 3600 cm^{-1}, of which corresponded to the O-H group (Fig. 3). The FTIR peak at 2937 cm^{-1} was ascribed to C-H group. Stronger bands occurring at 1746-1749 cm^{-1} and 1632-1636 cm^{-1} represented the C=O and COO^{-} functional groups of pectin respectively. The absorption pattern between 1000 cm^{-1} and 1400 cm^{-1} was referred to as the fingerprinting region where C-H bending (pyranoid ring), C-O-C stretching (glycosidic bond), C-O stretching (alcohol), and CH_3 bending ($COOCH_3$) were found.

The treatment of pectin by the combination of microwave and sodium chloride did not appear to affect its degree of esterification. There was minimal change to the FTIR spectra of the modified pectin with reference to the untreated sample (Fig. 3). Conversely, the intensity of the FTIR absorption band at 1632-1636 cm^{-1} increased with pectin subjected to the combination treatment of microwave and calcium acetate (Fig. 3). The degree of esterification of pectin was markedly reduced from 70.0 ± 1.0% to 64.4 ± 0.2% when the sample was treated for 40 min. The use of calcium acetate reduced the molecular weight of pectin to a higher degree than sodium chloride. In addition to parent chain cleavage, the calcium acetate caused de-esterification of the pectin side chains. The ability of calcium acetate to induce de-esterification might be associated with the tendency of soluble calcium interacting with the carboxyl/carboxylate moieties of pectin. This exerted a more effective heat transfer to the immediate vicinity of the ester moiety and subsequent bond breakages.

Zeta potential analysis of pectin indicated that both unmodified (−17.4 ± 0.8 mV) and modified pectins (sodium chloride: −18.5 ± 1.3 mV; calcium acetate: −19.6 ± 2.3 mV) had similar surface charges. The pectin treated by microwave with calcium acetate, though undergoing a higher degree of deacetylation, did not exhibit a higher value of negative zeta potential. This was probably due to pectin chains crosslinked by residual calcium ions at the carboxyl moiety.

3.2. Pectin nanoparticles

Fig. 4 showed the scanning electron microscopy images of nanoparticles made of untreated pectin and pectin treated with microwave and inorganic salts. The nanoparticles were largely spherical and had scaly surfaces. Their particle sizes were 484.5 ± 9.4 nm, 488.1 ± 14.0 nm and 267.7 ± 5.4 nm with reference to matrices made of untreated pectin, and pectin modified by the combination treatment of microwave with calcium acetate and sodium chloride respectively. Nanospray drying of pectin tend to result in the formation of

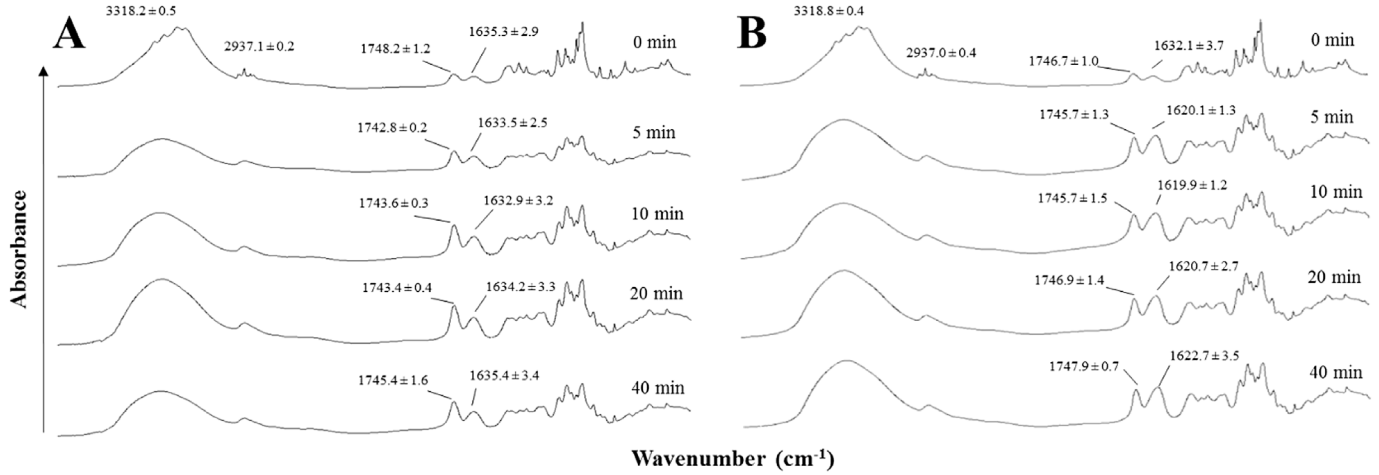

Fig. 3. FTIR spectra of pectin treated by microwave as a function of irradiation time using (A) sodium chloride and (B) calcium acetate as the superheating promoter.

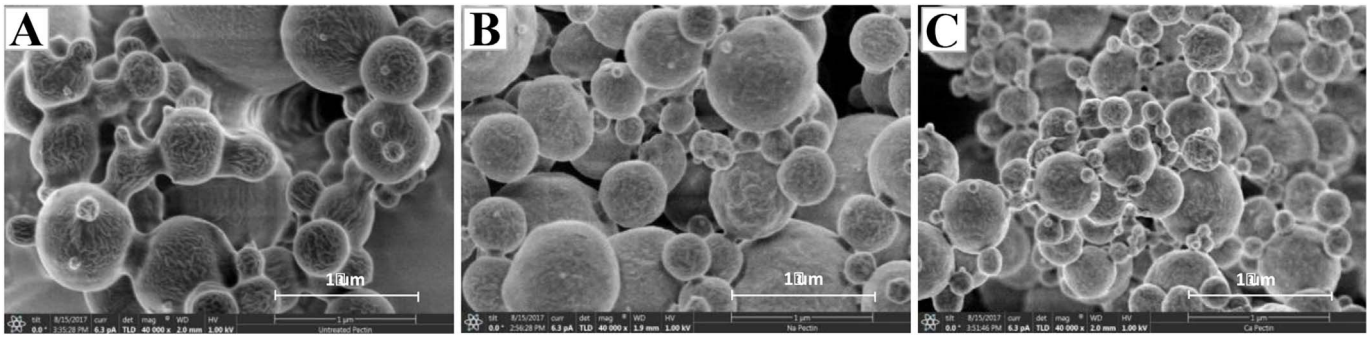

Fig. 4. Scanning electron microscopy images of nanoparticles made of (A) untreated pectin, pectin treated with microwave using (B) sodium chloride and (C) calcium acetate as the superheating promoter.

nanoparticles that had a smaller physical size than the pectin itself. This bottom-up assembly process induced pectin chain clustering and densification into spherical nanomatrices. Tumour vasculature was reported to have a different construct from normal tissue. The angiogenic blood vessels in tumour tissues have gaps as large as 600 nm–800 nm between adjacent endothelial cells [54]. The vasculature pore cut-off size of a human colon carcinoma was found to be between 400 nm and 600 nm [55]. The vasculature permeability to a molecule had also been reported to be independent of its pore cut-off size as long as the diameter of the molecule is less than the pore diameter. In conjunction with the present investigation, the pectin treated by combination of microwave and inorganic salts was concluded to be beneficial for use in nanoparticulate drug delivery system design for the treatment of cancer. Their nanogeometry was smaller than the pore size or pore cut-off size of the tumour vasculature.

4. Conclusions

Combination of microwave with calcium acetate brought about the formation of pectin with lower molecular weight and degree of esterification, but higher solution viscosity than that of with sodium chloride. This was attributed to pectin molecules were crosslinked by soluble calcium at COO^- moiety in the liquid state, which allowed a higher heat transfer through salt bridges and chain breakdown propensity specifically with prolonged duration of treatment. The formed nanoparticles from pectin treated by microwave in the presence of inorganic salts had a mean size smaller than 600 nm. They were deemed suitable for use to encapsulate cancer therapeutics with respect to permeation and retention attributes of tumour vasculature. The combination of microwave with inorganic salt is a viable approach for use to convert the pectin into matrix material of nanoparticles. The smaller pectin molecules can be nanospray-dried into nanoparticles with an appropriate geometry as cancer drug carrier. Future study shall include drug encapsulation and have drug release, bioavailability and pharmacodynamics action examined.

Acknowledgements

The authors wish to thank Universiti Teknologi MARA, and Ministry of Higher Education of Malaysia for fund (0141903) and facility support.

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