Chitosan-Cellulose Nanocrystal Microencapsulation to Improve Encapsulation Efficiency and Stability of Entrapped Fruit Anthocyanins

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Abstract

For improving stability of fruit anthocyanins (ACN), this study investigated the use of cellulose nanocrystal (CNC) as a macroion crosslinking agent to develop blueberry anthocyanin extract (BB)-loaded chitosan (CH)-CNC microcapsules, and compared with CH-sodium tripolyphosphate (TPP) ones. The yield of microcapsules (~6.9 g) and total monomeric anthocyanin recovery (~94%) were significantly ($P<0.05$) higher in CH-CNC microcapsules than those (~0.3 g and ~33%, respectively) in CH-TPP microcapsules. ACN distribution (%) in CH-CNC microcapsules was 61% on the surfaces, 12% bound with the matrix, and 27% in cores, but that in CH-TPP microcapsules was mostly presented on the surfaces (99%). CH-CNC microcapsules were more stable at pH 7.4 buffer by showing less ACN release (%) than that of CH-TPP, but no difference at pH 1.2. CH-CNC and CH-TPP microcapsules showed different structural and morphological properties. This study demonstrated that CNC is a promising crosslinking agent forming stable BB-loaded CH microcapsules.

Keywords: Ionic gelation encapsulation, chitosan, cellulose nanocrystal, blueberry anthocyanin

1. Introduction

Anthocyanins (ACN) are found in various fruit and vegetables with a broad color scheme from bright red to purple and dark blue, and present in the glycoside forms of anthocyanidin (aglycone) with high antioxidant capacity (Cavalcanti, Santos, & Meireles, 2011a). Blueberries contain a high amount (25–495 mg/100 g FW) of ACN with up to 25 different kinds depending on the aglycone species (delphinidin, cyanidin, peonidin, petunidin and malvidin) (Routray & Orsat, 2011; Barnes, Nguyen, Shen, & Schug, 2009). ACN is a promising substitute for synthetic antioxidants and food colorants. Unfortunately, ACN is very sensitive to environmental conditions, such as pH, temperature, light, and oxygen, and easily degraded. The cationic flavylum form of ACN can be irreversible decomposed into small colorless aldehydes or phenolic acids during processing and/or storage (Cavalcanti, Santos, & Meireles, 2011b; Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Patras, Brunton, O'Donnell, & Tiwari, 2010). Hence, it is necessary to develop effective techniques for improving the stability of ACN under various environmental and processing conditions.

Microencapsulation, a popular drug delivery technique used in pharmaceutical industry, has been used to protect active solid, liquid, or gas core compounds against surrounding conditions by using a suitable polymeric system (Dias, Ferreira, & Barreiro, 2015). Considering the different interactions (entrapping, bounding, and attaching) between the bioactive compounds and polymeric system, microencapsulation can also control the release of the core materials at specific time and targeted location in the digestion system. Various encapsulation methods are available, including 1) physical methods such as spray drying, fluid bed coating, extrusion spheronization, centrifugal extrusion, and processes using the supercritical fluids; 2) physicochemical methods using spray cooling, hot melt coating, ionic gelation, solvent

evaporation extraction, and simple or complex coacervation; and 3) chemical methods through interfacial polycondensation, polymerization, and interfacial crosslinking (Munin & Edwards-Lévy, 2011; Shanthi, Gupta, & Mahato, 2010). Amongst, the method of ionic gelation has attracted great interest due to easy to handle, highly compatible, non-toxic, organic solvent free, convenient and controllable (Abdel-Hafez, Hathout, & Sammour, 2014; Mahajan, Dhillon, Kumar, & Singh, 2014; Yoksan, Jirawutthiwongchai, & Arpo, 2010). In the ionic gelation, polysaccharides undergo ionic gelation and precipitate to form spherical microcapsules due to the complexation between oppositely charged species (Racoviță, Vasiliu, Popa, & Luca, 2009).

Chitosan (CH) is a natural polysaccharide, and can form a matrix by itself and/or with other crosslinking agents due to the presence of amino (NH_2) groups (Risbud, Hardikar, Bhat, & Bhonde, 2000). The complexation between positively charged CH in acidic solution and anionic crosslinking agent creates active compounds-loaded microcapsules, and the type of crosslinking agent significantly impacts the stability (e.g. loading capacity and release ratio) of the loaded active compounds in the microcapsules (Devi & Maji, 2009; Kumbar, Kulkarni, & Aminabhavi, 2002). As an anionic crosslinking agent, sodium tripolyphosphate (TPP), has been used to form inter- and intra-crosslinking with the amino groups of CH for various purposes (Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013). However, CH-TPP encapsulation produced a metastable structure that is easily affected by the surrounding conditions, such as pH and ionic strength (Tsai, Chen, Bai, & Chen, 2011). Another organic macro-ion, cellulose nanocrystal (CNC), has recently appeared as a potential substitute for sodium TPP. This rod-like, crystalline nanoparticle is produced by the acid hydrolysis of cellulose fiber from wood pulp using sulfuric acid, thus having negatively charged sulfate (SO_4^{2-}) groups on the cellulosic backbone for ionic interaction. CNC exhibits good mechanical properties, such as low density, high specific strength and

modulus, and has highly charged surface. CNC has also been used as a filler for strengthening polymeric matrix (Huq et al., 2012). Therefore, CNC can function as both crosslinking agent and filler for CH-based microcapsule matrix to protect fruit ACN from the surrounding attack. This study was thus hypothesized that CH-CNC microcapsules can effectively entrap fruit ACN with improved stability through generating rigid cellulosic wall matrix in comparison with the CH-TPP microcapsules. No study has yet been conducted for loading the active ACN compounds using CH-CNC microcapsules.

In this study, chitosan based microcapsules using two different ionic crosslinking agents (CNC vs. TPP) were utilized to encapsulate blueberry anthocyanin extracts. The yield of microcapsules (YOM), total monomeric anthocyanin (TMA) recovery, and sizes of the microcapsules from the two systems were compared by analyzing the formed microcapsules and supernatants. The optimum level of the crosslinking agents was selected based upon YOM and TMA recovery of the microcapsules and the total phenolic content (TPC) and DPPH radical scavenging capacity in the supernatants. ACN distribution (%) of CH-based microcapsules was also compared between the two different crosslinking agents, CNC and TPP, to reveal the different encapsulation mechanisms. The structural and morphological differences between CH-CNC and CH-TPP microcapsules were investigated using Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) analyses. In addition, *in vitro* ACN releasing test of the lyophilized microcapsules was conducted at 0.2% NaCl solutions with pH 1.2 and 0.05 M phosphate buffer with 7.4 to investigate the stability of microcapsules under different pH conditions. It was anticipated that this study will provide new insights on the use of CNC as an anionic crosslinking agent and filler to form more stable CH-based microcapsules to preserve fruit ACN extracts from the environmental attack.

2. Materials and methods

2.1. Materials

Sodium tripolyphosphate (TPP) (nominal purity = 98%) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Chitosan was obtained from Primex ehf (Siglufjordur, Iceland) with degree of deacetylation (DDA) of 97.5% and molecular weight (Mw) of 149 kDa. Sulfuric acid hydrolyzed CNC was produced from softwood kraft pulp with a final concentration of 11.8% (Process Development Center at the University of Maine, ME, USA). Folin-Ciocalteu (FC) reagent and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), acetic acid and L-ascorbic acid from Avantor Performance Materials (Center Valley, PA, U.S.A.), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (95%) from Alfa Aesar (Ward Hill, MA, U.S.A.). Hydrochloric acid, sodium acetate, potassium chloride, and sodium carbonate were all obtained from EMD Chemicals (Gibbstown, NJ, U.S.A.).

2.2. Microencapsulation process

Blueberry (BB) extracts were prepared by using the ultrasound-assisted extraction procedures from our previous study (Wang, Jung, Tomasino, & Zhao, 2016). The extracts were diluted with pH 1.0 potassium chloride (KCl) buffer to retain cationic flavylum form of anthocyanins.

The ionic gelation was performed by following the procedures of Domaratzki & Ghanem (2013) with slight modification (Fig. 1). Briefly, chitosan (0.1% w/v) was dissolved in 1.0% (v/v) acetic acid solution (pH 2.6). For minimizing the structural modification and/or degradation of BB ACN during encapsulation process, the crosslinking agents (TPP and CNC) were adjusted to pH 2.6 using 0.1 N hydrochloride acid, where 1 mL of BB ACN extract (pH 1.0) was added into

20 mL of 0.1% (w/v) chitosan solution (pH 2.6), followed by dropwise titrating the mixture into 20 mL of 0.025–2.5% (w/v) TPP or CNC bath (pH 2.6) with vigorous agitation. After another 3 min stirring, the encapsulation suspensions were centrifuged at 10,000 g and 4 °C for 10 min (International Equipment Co., Chattanooga, TN, U.S.A.). Both microcapsules and supernatants were collected for further analysis.

2.3. Characteristics of the microcapsules and supernatants

2.3.1. Size and yield of microcapsules

To determine the microcapsule size, the collected microcapsules were re-suspended in distilled (DI) water at the ratio of 1:12.5 (v/v), and analyzed using the Particle Size Analyzer (ZetaPALS, Brookhaven Instr. Co., Holtsville, NY). The re-dispersed samples were filtered through a 0.20 µm filter to prevent the aggregation of the microcapsules and dust from air.

For analyzing the yield of microcapsules (YOM), the weight (m_0) of 50 mL empty centrifuge tube (VWR International Inc., Bridgeport, NJ, U.S.A.) was precisely recorded, and the encapsulation suspension was transferred to the tube. After centrifugation at 10,000 g (4 °C) for 10 min, the supernatants were collected for further analysis, whereas the microcapsules left at the bottom of tube were weighed with the tube (m). YOM obtained from the encapsulation suspensions was calculated as: $YOM (g) = m - m_0$.

2.3.2. Recovery of total monomeric anthocyanins (%) in the microcapsules

Total monomeric anthocyanin (TMA) content in the BB extracts or the collected supernatants was determined using the pH differential method (Wrolstad, Durst, & Lee, 2005). For measuring TMA, 1 mL of BB extract and supernatant were diluted with 3 mL of 0.025 M KCl buffer

(pH=1.0) and 0.4 M sodium acetate buffer (pH=4.5), respectively. The mixture was vortexed and then kept at room temperature for 15 min. The absorbance difference between pH 1.0 and pH 4.5 was determined at both 520 nm and 700 nm, respectively using a Shimadzu UV160U spectrometer (Shimadzu Co., Kyoto, Japan).

The amount of TMA was expressed as cyanidin-3-glucoside equivalent (Cy-3-Glu) mg since Cy-3-Glu is the predominant ACN species in BB. It was calculated as: TMA (mg) =

$\frac{A \times Mw \times DF \times 10^3}{1 \times \epsilon} \times V$, where A = (A520 nm – A700 nm) at pH 1.0 – (A520 nm – A700 nm) at pH 4.5; Mw = 449.2 g/mol for Cy-3-Glu; DF = dilution factor; l = length of light path in cm; ϵ = 26,900 molar extinction coefficient (L mol⁻¹cm⁻¹) for Cy-3-Glu; and 10³ = conversion factor from g to mg; V = volume of diluted test sample solution.

TMA recovery (%), indicating the encapsulation efficiency of ACN in the microcapsules, was then estimated as: $\frac{\text{TMA in 1 mL of BB ACN extract} - \text{TMA in supernatant of rncapsulation suspension}}{\text{TMA in 1 mL BB ACN extract}} \times 100$ (%).

2.3.3. Total phenolic content (TPC) in the supernatants

Considering that a direct analysis of TPC on the microcapsules requires harsh hydrolysis process (e.g. strong acid and high temperature) before the chemical assay, TPC was analyzed for supernatants instead of microcapsules to prevent the TPC degradation. TPC entrapped in the microcapsules could be thus estimated by the difference of TPC in the supernatants among the samples (i.e. higher TPC in the supernatants meant less TPC in the microcapsules).

The comprehensive TPC analysis was conducted for supernatant using the Folin-Ciocalteu (FC) assay (Singleton & Rossi, 1965). Briefly, 0.5 mL of supernatants and gallic acid at 0.1, 0.3, 0.5, and 0.7 mg/mL (for the standard curve) were mixed with 0.5 mL of FC reagent and 7.5 mL

of DI water, respectively. A 0.5 mL of DI water was used as a control. The mixtures were vortexed at room temperature for 20 min, and then transferred into a 40 °C water bath for 20 min with the addition of 3 mL of 20% sodium carbonate (w/v). The mixtures were immediately transferred into an ice bath for 3 min for cooling down to room temperature. The absorbance was measured at 765 nm using a Shimadzu UV160U spectrometer (Shimadzu Co., Kyoto, Japan). Results were expressed as gallic acid equivalent (GAE) mg/mL.

2.3.4. DPPH radical scavenging (DPPH) activity in the supernatants

Similar to TPC assay, DPPH activity assay of the collected supernatants was also conducted to reflect the antioxidant capacity in the microcapsules (Brand-Williams, Cuvelier, & Berset, 1995). Briefly, 1 mL of supernatants or ascorbic acid standard (a series of concentration at 0.005, 0.01, 0.05, and 0.1 mg/mL) were mixed with 3 mL of DPPH agent (9 mg DPPH in 100 mL of methanol), respectively. The mixtures were vortexed at room temperature and kept in the dark for 20 min. The absorbance was read at 517 nm using the UV/Vis spectrometer (Shimadzu Co., Kyoto, Japan), and the results were reported as ascorbic acid equivalent (AAE) mg/mL. The ideal microcapsules were aimed to possess higher YOM and TMA recovery (%) in the microcapsules and lower TPC and DPPH activity in the supernatants.

2.3.5. ACN distribution in the microcapsules

Due to different crosslinking patterns, ACN could present in any segment of the entire microcapsules, including 1) weak attachment and interaction on the surfaces of the capsules, 2) freely in the cores, and 3) bound with the matrix of wall materials. For monitoring the ACN

distribution on these three segments, TMA on the microcapsules at each of the continuous treatment steps was determined as illustrated in Fig. 3.

Microcapsules were firstly washed by 30 mL of DI water and centrifuged to collect the supernatants. This process was conducted twice, and the collected supernatants were combined together to analyze TMA. The estimated TMA from this process indicated ACN attached and/or interacted on the surface of microcapsules. The microcapsules were continuously immersed in 30 mL of 0.1 N HCl buffer (pH 1.5) for 24 h to release free ACN from the cores of the microcapsules. Samples were centrifuged, and the supernatants were collected for measuring TMA (the presence of ACN) in the core part of the microcapsules (Sahasathian, Praphairaksit, & Muangsin, 2010). Lastly, the amount of ACN bound with the wall matrix was estimated by subtracting the combined amount of TMA from the first and second processes from the amount of TMA in the freshly-made microcapsules, and multiplied by 100. ACN distribution (%) in the microcapsules was calculated as the percentage of TMA existed on the surface, bound in the matrix, and in the core of the microcapsules.

2.3.6. *In vitro* releasing test

In vitro releasing test of ACN from the microcapsules was conducted in two stimulated fluid systems with different pH (0.2% NaCl with pH 1.2 and 0.05 M phosphate buffer with pH 7.4), following the method of Yoo, Song, Chang, & Lee (2006). Microcapsules produced from the optimized encapsulation processing conditions were lyophilized at -55°C and 17.33 Pa vacuum (Model 651 m-9WDF20, Hull Corp., Hatboro, PA, U.S.A.). A 150 mg of lyophilized samples was suspended at 0.2% NaCl solution with pH 1.2 adjusted by 0.05 M HCl and 0.05 M phosphate buffer with pH 7.4, respectively. Samples were placed in a 37°C of incubator with the

continuous shaking at 100 rpm, and a 1 mL of aliquot was taken every 1 or 2 h to monitor ACN amount released from the microcapsules for 24 h. The releasing ratio was calculated as the TMA in the collected sample at a given time divided by the initial TMA in the microcapsules, and multiplied by 100.

2.3.7. Structural and morphological properties of the microcapsules

A single bound attenuated total reflection (ATR)-FTIR spectrometer (PerkinElmer, USA) was operated for studying the structural differences of the microcapsules from CNC and TPP, and analyzed as the absorption mode with Omnic 7.4 software (Thermo Fisher Inc. USA). The collected microcapsules after centrifugation were analyzed under the operating conditions of 32 scans at a 4 cm^{-1} resolution using air as the reference.

Microcapsules (1 g) were re-dispersed in 100 mL of pH 4.5 buffer, and sonicated (Branson Ultrasonics Co., Danbury, CT, U.S.A.) for 10 min to prevent microcapsule aggregation. The suspension was placed on a glass slide, and observed under x 40 magnification using a Nikon Eclipse 50i microscope (Nikon Corporation, Japan) fitted with an Infinity 1-3C camera (Lumenera Co., Atlanta, GA, U.S.A.). Lyophilized microcapsules after centrifugation were further analyzed using a scanning electron microscopy (SEM, the AmRay 3300 field emission (FE)-SEM, AmRay, Bedford, MA, U.S.A.). Samples were mounted on aluminum stubs and coated by a gold–palladium alloy sputter coater (Edwards model S150B sputter coater, BOC Edwards Vacuum, Ltd., West Sussex, U.K.) to improve the interface conductivity. Digital images were collected at an accelerating voltage of 5 kV.

2.4. Experimental design and statistical analysis

A completely randomized design was applied for investigating the effect of the type of crosslinking agent (CNC vs. TPP) and their concentration (0.25%-2.5%) on the characteristics of the formed microcapsules. All measurements were conducted in triplicate, and mean values and standard deviation were reported. Data were analyzed for statistical significance via least significant difference (LSD) *post hoc* testing using SAS v9.2 (The SAS Institute, Cary, NC, U.S.A.). Results were considered to be significantly different at $P < 0.05$.

3. Results and discussions

3.1. Comparison of encapsulation characteristics between CNC and TPP as crosslinking agent

Encapsulation characteristics, including total volume and pH of the supernatants, and yield, TMA recovery, and size of the microcapsules separated from the encapsulation suspensions were compared between CH-CNC and CH-TPP systems (Table 1). CH-CNC encapsulation system resulted in significantly ($P < 0.05$) lower total volume and pH of the supernatant than that of CH-TPP system. The lower pH could attribute to the crystal structure of CNC generating more free hydrogen ions in the suspensions by forming less hydrogen bonds (Nii & Ishii, 2005). CH-CNC encapsulation also produced significantly ($P < 0.05$) higher yield (6.87- 8.13 g) and TMA recovery (~94%), and much smaller particle sizes (~65 nm) in comparison with CH-TPP encapsulation. The higher yield of CH-CNC encapsulation could be attributed by more hydration of the microcapsules due to the hydroxyl groups in CH and CNC, thus increasing both mass and density of CH-CNC microcapsules compared with the CH-TPP encapsulation (Shu & Zhu, 2001). In addition, the size of the CH-CNC microcapsules in this study was smaller than that reported in the previous study by Wang & Roman (2011) (around 265-969 nm with different added mass

ratios of CNC to CH). Correspondingly, significantly larger size of CH-TPP microcapsules (~34 μm) might be due to two possible reasons: 1) the presence of relatively large gaps within formed microcapsules, which also indicated weaker complex (Shu & Zhu, 2001) and 2) the aggregation of formed microcapsules with less surface charges (Hassani, Laouini, Fessi, & Charcosset, 2015). This study thus suggested two different encapsulation mechanisms as illustrated in Fig. 1: CH-CNC formed a more hydrated, but smaller microcapsules with CNC as a sort of fillers forming rigid matrix, while CH-TPP system formed larger microcapsules with inner gaps, making less tight complex.

3.2. Effect of different concentrations of crosslinking agents

YOM and TMA recovery of the formed microcapsules and TPC and DPPH of the supernatants were evaluated for investigating the effectiveness of the crosslinking agent concentration on CH-CNC and CH-TPP encapsulations (Fig. 2). The lower TPC and DPPH values in the supernatants indicated the high efficient encapsulation process with more BB ACN entrapped in the microcapsules. As shown in Fig. 2, CH-CNC encapsulation induced significantly higher YOM (1.3–12.4 g) and TMA recovery (40–96%) along with increasing amount of added CNC in comparison with CH-TPP encapsulation (0.2–1.0 g YOM and 11.3–19.0% TMA recovery). The YOM values were greatly increased when CNC concentration reached to 1.0% (w/v), and further increased (up to ~12.4 g) at 2% CNC (w/v). For CH-TPP microcapsules, the highest YOM (1.0 g) was observed at 1% TPP. Based upon the DPPH activity result, CH-CNC supernatants had lower DPPH activity than that of CH-TPP ones, which was consistent with the results of TMA recovery in the microcapsules. Meanwhile, CH-CNC supernatants retained higher TPC than that of CH-TPP supernatants. The contradicted result

between TPC and DPPH in the supernatants might be due to the interference of CNC with TPC assay (using FC reagent under alkali condition). According to Singleton & Rossi (1965), the crystalline white precipitate could be formed due to the sodium complex in alkali condition after the reaction, and the addition of sulfate could facilitate the dissolving of white precipitate, thus increasing the reading value. In addition, the reducing ends of cellulose could also possibly interact with FC reagent. Hence, the colorimetric TPC assay for the supernatant could be overestimated due to the remained CNC after encapsulation process. A sharp reduction of TPC and DPPH in the CH-CNC supernatants was observed at 1.0% CNC, indicating more TPC and/or CNC incorporated into the microcapsules. At 1% concentration, CNC was more able to interact with CH, possibly due to the less aggregation of CNC through the electrostatic repulsions of sulfate ions in comparison with lower concentration of CNC. Hence, 1% CNC and 1% TPP was chosen as the optimal concentration of crosslinking agent for BB ACN-loaded CH-based encapsulation to produce microcapsules with higher encapsulation efficiency of anthocyanins.

3.3. ACN distribution in the microcapsules

The microcapsules are usually divided into two categories due to their structure: core-shell type and matrix type (Zuidam & Shimoni, 2010), depending on the ACN distributions which are either entrapped in the cores, bound with the matrix, or weakly attached and/or interacted on the surfaces. The type of ACN distribution could also indicate the stability of the microcapsules, i.e., degradation and/or release of ACN. Fig. 3 illustrates the method used in this study to evaluate the ACN distribution through a continuous treatment phase.

The CH-CNC microcapsules were close to the core-shell type since ACN was mostly detected on the surfaces (~61%) and cores (~27%) (Fig. 3), while the CH-TPP microcapsules

had most ACN attached and/or interacted on the surfaces (~99.42%), which was close to a matrix type. CH-TPP microcapsules mainly loaded ACN on the surface matrix since it had relatively weaker matrix with larger gaps within the matrix, but was not able to carry active ACN within the cores. These results demonstrated that CH encapsulation mechanisms were distinguished depending on the type of crosslinking agent applied, and CH-CNC encapsulation could form more stable microcapsules with high core loading efficiency.

3.4. Releasing profile of ACN from the microcapsules

For testing the release of ACN from the microcapsules, the microcapsules were produced by using the optimized level of crosslinking agent (1% for both CNC and TPP), and then lyophilized. The prepared samples were dispersed in buffer solutions at pH 1.2 and 7.4 for 24 hr. At pH 7.4 environment, ACN releasing ratio from CH-CNC microcapsules was significantly ($P<0.05$) lower than that from CH-TPP system (Fig. 4). At lower pH, however, there was no significant difference in ACN release between CNC and TPP systems, and both reached up to 90% release by 24 hr. Less stability of CH-CNC microcapsules in pH 1.2 might be related to the aggregation of formed microcapsules since strong anion sulfate groups ($-O-SO_3^-$) in CNC could be screened by hydrogen, thus forming $-O-SO_3H_2$ (Zhong et al., 2012). These results indicated that both microcapsules are pH responsive, but CH-CNC microcapsules are more stable in neutral pH than CH-TPP microcapsules. Therefore, both CH-CNC and CH-TPP microcapsules were suitable for ACN delivery to human digestion system as the target release of ACN can be induced at stomach with acidic pH environment as well as increasing its absorption through stomach. The stability of CH-CNC microcapsules needs to be further tested under various processing and/or environmental conditions for expanding its application within food system.

3.5. Structural and morphological properties of the microcapsules

FTIR spectrum of CH-CNC and CH-TPP microcapsules were investigated to compare their structural property (Fig. 5). For CH-CNC microcapsules, the bands of -OH and -NH stretching were much wider and higher than that of CH-TPP microcapsules, which could be the presence of more hydrated microcapsules with entrapped ACN. The bands of -SH and C-O-C stretching were only appeared in the CH-CNC microcapsules, indicating sulfate ions and cellulosic linkage from incorporated CNC. The CH-TPP microcapsules showed the additional bands for -NH bending vibration, indicating free amino groups from CH, and PO_4^{3-} functional groups from TPP. It thus showed the clear structural differences of CH-based microcapsules depending on the type of crosslinking agent applied.

The morphological property of the microcapsule suspensions and lyophilized samples were analyzed using optical microscope and SEM (Fig. 6). The sizes of CH-CNC and CH-TPP microcapsules were clearly distinguished in the microscope images (left column), consistent with the results from microcapsule size measurements (Table 1). Although CH-TPP microcapsules had larger sizes than that of CH-CNC microcapsules, both microcapsules formed similar mononuclear morphology. SEM images also distinguished the structures of the lyophilized CH-CNC and CH-TPP microcapsules. The CH-CNC ones exhibited the cellulosic matrix since the centrifugation before lyophilization could generate highly viscous aqueous phase, and form polymeric matrix during lyophilization process. Although the original mononuclear morphology of CH-CNC microcapsules could disappear during centrifugation and lyophilization processes, the CH-CNC cellulosic matrix looked strong enough to entrap ACN while it formed

microcapsule globules in comparison with lyophilized CH-TPP microcapsules with loose structure and rough surface.

4. Conclusions

This study successfully demonstrated the superior benefits of using cellulose nanocrystal (CNC) as the macro-ion crosslinking agent in chitosan-based microencapsulation for improving the encapsulation efficiency and stability of blueberry anthocyanin extracts, compared with the usage of chitosan-sodium tripolyphosphate (TPP) microcapsules. Results revealed that CNC incorporation into chitosan functioned not only as macro-ion crosslinking agent on the positively charged amino groups in chitosan, but also as fillers for chitosan matrix, thus generating more rigid and stable microcapsules. As a stronger vehicle, chitosan-CNC microencapsulation could be extended to load other bioactive compounds and active food ingredients for various applications.

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Table 1. Effects of different anionic crosslinking agents (cellulose nanocrystal (CNC) and sodium tripolyphosphate (TPP)) on the characteristics of supernatants and microcapsules from blueberry extract (BB)-loaded chitosan (CH)-based encapsulation⁺

Crosslinking agent	Supernatant [*]		Microcapsule		
	Total volume (mL)	pH	Yield (g)	TMA recovery (%) ^{**}	Size (nm)
CNC	30.67 ^{b++}	2.23 ^b	6.87 ^a	94.02 ^a	64.80 ^b
TPP	38.67 ^a	2.53 ^a	0.27 ^b	32.54 ^b	33887.00 ^a

⁺ Encapsulation suspension was prepared with 1 mL of BB extracts, 20 mL of CH solution (0.1% CH in 1% acetic acid, pH 2.6), and 20 mL of 2% TPP or CNC solution (pH 2.6).

^{*} Both supernatant and microcapsules were collected for analysis after centrifugation

^{**} TMA recovery (%) = $\frac{\text{TMA in 1 mL of BB extract} - \text{TMA in supenatant after encapsulation process}}{\text{TMA in 1 mL BB extract}} \times 100$ (%)

⁺⁺ Means preceded by the same lowercase superscript letter within the same column were not significantly different with each other ($P>0.05$) (n=3).

Fig. 1 The schematic diagram showing the preparation of blueberry anthocyanin extract (BB ACN) loaded microcapsules [A] and proposed mechanisms of forming ionic gelation between chitosan (CH) and cellulose nanocrystals (CNC) or tripolyphosphate (TPP) [B]; TMA, TPC, DPPH and YOM indicated total monomeric anthocyanin, total phenolic content, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and yield of microcapsules, respectively.

Fig 2. Effect of **concentrations of each type of** anionic crosslinking agents (cellulose nanocrystal (CNC) or sodium tripolyphosphate (TPP)) on the yield of microcapsules (YOM), total monomeric anthocyanin (TMA) recovery (%) of microcapsules, total phenolic contents (TPC) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of supernatants in blueberry extract (BB)-loaded chitosan (CH)-based encapsulation process; Encapsulation process was conducted as: 1 mL of BB extracts added to 20 mL of CH solution (0.1% in 1% acetic acid, pH 2.6) were dropwise transferred to the flask containing 20 mL of TPP or CNC (pH 2.6); Yield of microcapsules indicated the total weight of microcapsules obtained from the encapsulation process; TMA (total monomeric anthocyanin) recovery (%) = $\frac{\text{TMA in 1 mL of BB ACN extract} - \text{TMA in supernatant after encapsulation process}}{\text{TMA in 1 mL BB ACN extract}} \times 100$ (%); Total phenolic content (TPC, mg gallic acid equivalent (GAE)) and DPPH radical scavenging activity (mg ascorbic acid equivalent (AAE)) were measured for the remained supernatant after the encapsulation process.

Fig. 3 Measurement of anthocyanin distribution (%) in blueberry anthocyanin extract loaded chitosan (CH)-cellulose nanocrystal (CNC) microcapsules or CH- tripolyphosphate (TPP) microcapsules; The loaded anthocyanin amount for CH/CNC and CH/TPP encapsulations was optimized with 26.06 mg cyanidin-3-glucose and 14.23 mg cyadinin-3-glucose based upon the preliminary study (data not shown), respectively; Encapsulation process was conducted as: 1 mL of blueberry (BB) extract added to 20 mL of 0.1% CH (pH 2.6) was dropwise titrated into 20 mL of 1.0% anionic crosslinking agent (pH 2.6).

Fig. 4 Releasing ratio of anthocyanins from lyophilized blueberry anthocyanin extract loaded chitosan (CH)-cellulose nanocrystal (CNC) and CH-tripolyphosphate (TPP) microcapsules in pH 1.2 and pH 7.4. The loaded anthocyanin amount for CH-CNC and CH-TPP encapsulation was optimized with 26.06 mg cyanidin-3-glucose and 14.23 mg cyadinin-3-glucose based upon the preliminary study (data not shown); Encapsulation process was conducted as: 1 mL of blueberry (BB) extract added to 20 mL of 0.1% CH (pH 2.6) was dropwise titrated into 20 mL of 1.0% anionic crosslinking agent (pH 2.6).

Fig. 5 Fourier-Transform Infrared (FTIR) spectra of blueberry anthocyanin extract loaded chitosan (CH)-cellulose nanocrystal (CNC) and CH-tripolyphosphate (TPP) microcapsules; The loaded anthocyanin amount for CH-CNC and CH-TPP encapsulation was optimized with 26.06 mg cyanidin-3-glucose and 14.23 mg cyanidin-3-glucose based upon the preliminary study (data not shown), respectively; Encapsulation process was conducted as: 1 mL of blueberry (BB) extract added to 20 mL of 0.1% CH (pH 2.6) was dropwise titrated into 20 mL of 1.0% anionic crosslinking agent (pH 2.6).

Microcapsules in suspensions
(x40 magnification)

Freeze-dried microcapsules

CH-CNC

CH-TPP

Fig. 6 Microscopic images of blueberry anthocyanin extract loaded chitosan (CH)-cellulose nanocrystal (CNC) and CH-tripolyphosphate (TPP) microcapsule suspensions using the optical microscope and the morphology of lyophilized samples collected from the centrifugation using the scanning electron microscopy (SEM); The loaded anthocyanin amount for CH-CNC and CH-TPP encapsulation was optimized with 26.06 mg cyanidin-3-glucose and 14.23 mg cyadinin-3-glucose based upon the preliminary study (data not shown), respectively; Encapsulation process was conducted as: 1 mL of blueberry (BB) extract added to 20 mL of 0.1% CH (pH 2.6) was dropwise titrated into 20 mL of 1.0% anionic crosslinking agent (pH 2.6).