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Preparation of chitin nanowhiskers to reinforce alginate beads

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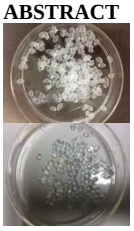
GRAPHICAL ABSTRACT



(a) (b)
(a) Alginate beads crosslinked with CNW and (b) alginate beads without CNW

ABSTRACT

Chitin nanowhiskers (CNW) is a nano-crystalline biopolymer that gained much interest for its reinforcement effect. CNW possess several unique and useful properties including biodegradable and non-toxic. Therefore, this afford exclusivity of CNW in biomedical applications. The aim of this study is to investigate the reinforcement effect of CNW on alginate beads. CNW was prepared from commercial chitin of shrimp shells through acid hydrolysis. Different concentration of CNW were incorporated in the alginate beads and their swelling behaviour were observed. The swelling of CNW/alginate beads decreases as the concentration of CNW embodied in the beads increases. Alginate beads incorporated with 3% CNW showed least swelling. A strength test conducted on CNW/alginate beads indicated that the incorporation of CNW indeed increases the strength as the amount of burst alginate beads were reduced under continuous shaking condition as the concentration of CNW increases. CNW of 3% showed the most minimum swelling which is associated with the probability of the alginate beads to burst. A slow protein release test was also carried out using BSA to determine whether the crosslinking of alginate beads and CNW will lower the protein release. The protein concentration was determined by Biuret Method in which the absorption of protein was measured using UV Spectrometer at 549 nm. The sole alginate beads shows a higher protein release (72.67%) compared to CNW/alginate beads (70.26%). Both chitin and CNW were characterized using Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR).

Keywords: Chitin nanowhiskers, alginate beads, reinforcement, swelling behaviour, strength, protein release

1. INTRODUCTION

Chitin is one of the most abundant organic materials, being second only to cellulose in the amount produced yearly by biosynthesis[1]. It is often found in animals, specifically in crustaceans, molluscs and insects where it is a significant constituent of the exoskeleton, and also in some fungi where it is the principal fibrillary polymer in the cell wall. Chitin possess several unique features, and that includes high tensile strength, biocompatibility, bioactivity, biodegradability, non-antigenicity and non-toxicity, which give them the potential to be used in many application [2].

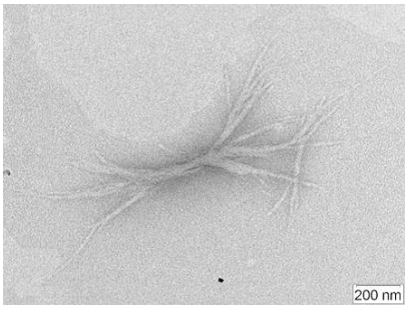


Figure 1 TEM image of chitin nanowhiskers

While chitin is a semi-crystalline biopolymer which forms a microfibrillar arrangements in living organisms[3], chitin nanowhiskers (CNW) is a highly crystalline polymer that are aligned in bundles along the longitudinal axis due to the hydrogen-bonding interactions. These interactions have already been proven to make CNW formed stable, gelled networks in an aqueous condition [4]. The typical morphology and size of average length and width of CNW was observed by TEM as in Figure 1 [5]. The high crystallinity of CNW lead to its excellent mechanical properties such as low thermal expansion and high tensile strength. Currently, CNW are obtained as an aqueous suspension that are being studied and used as a reinforcing additive for high performance environment-friendly biodegradable nanocomposite materials [3]. On the other hand, alginate is a natural polysaccharide that comprises from 30 to 60% of brown algae on dry weight basis. Sodium alginate is very much soluble in water, thus the inclusion of Ca^{2+} that act as a gelling agent managed to form a membrane of alginate beads in the gelling bath, which is $CaCl_2$ solution [6]. The limitations of alginate beads is its high water uptake

capacity which easily swells up the beads and its large pore size which permits easy leakage of entrapped proteins or bacteria.

This research will focus on how CNW could reinforce the properties of alginate beads. The alginate beads is deemed to increase in strength when CNW is incorporated with it. The swelling of the alginate beads may also be reduced when they is crosslinked with CNW. The increase in strength of alginate beads with the combination of CNW might be due to well-known high crystallinity of CNW [3]. The incorporation of CNW in the alginate beads also been deem to increase slow protein release. Thus, the microcapsule of calcium alginate coated with polycation has been vastly studied for its significant application in drug release system and immunoprotective containers in cell transplantation enzyme immobilization [2]. That being said, the combination of CNW with alginate beads will also potentially enhance the immobilization of enzyme and ions and will be very beneficial in the biomedical and pharmaceutical field.

2. EXPERIMENTAL

The experiment was divided into three main stages. The first stage was focused on the preparation of chitin nanowhiskers (CNW) through acid hydrolysis. The next step was the preparation of sole calcium alginate beads and calcium alginate beads that has been crosslinked with 1%, 2% and 3% CNW as shown in Figure 2. The last step was the tests conducted on the CNW/alginate beads on its swelling behaviour, mechanical strength and also slow protein release. As for the swelling test, equal amounts of beads were placed in a beaker containing distilled water at room temperature. The beads were removed from distilled water after 48 hours and the weight of the swollen beads were recorded. The strength was carried out by placing equal amount of CNW/alginate beads in a beaker containing distilled water and placed in an orbital shaker at 120 rpm. Each of the beaker were covered with aluminium foil. Observation was made every three days until the ninth days to identify whether the beads will burst with continuous shaking. The amount of burst beads were expressed as percentages ((No. of burst beads / No of original bead) x 100). As for the protein release test, *Bovine Serum Albumin* (BSA) was immobilized first in the mixture of CNW and sodium alginate solution before it was dropped into $CaCl_2$ solution under gentle stirring. The CNW/alginate beads of equal amount were placed in a beaker containing Tris-HCl buffer solution at pH 7.4 for 1 hour. This step was repeated for other CNW concentrations (2% and 3%). Calcium alginate beads without CNW was used as the control. The biuret method was used to determine the concentration of protein release in supernatant using UV-VIS Spectrometer. The result was reported as percentage release of protein.

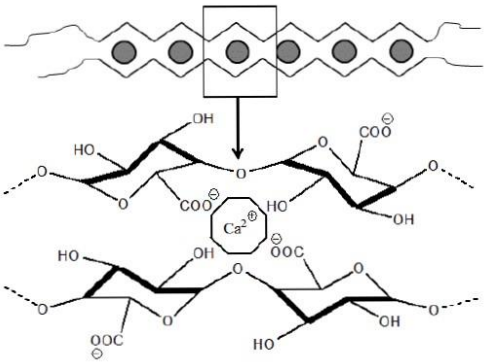
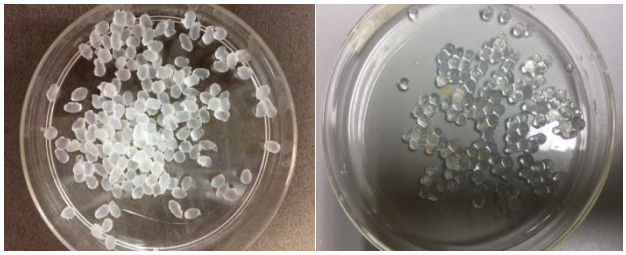


Figure 2 The formation of calcium alginate beads complex

3. RESULTS AND DISCUSSION

3.1. Preparation of CNW/Alginate Beads

The CNW/alginate beads were prepared by dropping sodium alginate containing CNW into $CaCl_2$ solution under magnetic stirring. Alginate beads crosslinked with CNW were found to be white in colour compared to the alginate beads without CNW which is colourless as shown in Figure 3.



(a) (b)

Figure 3 (a) Alginate beads crosslinked with CNW and (b) alginate beads without CNW

3.2 Swelling Test on CNW/Alginate Beads

Swelling test was carried out to observe how the incorporation of CNW affect the degree of swelling of the alginate beads. Figure 4 shows water uptake of CNW/alginate beads at various concentration of CNW. Increasing amount of CNW will decrease the swelling properties of alginate beads, where the adsorption of water will be lower and thus the beads can better retain their shape.

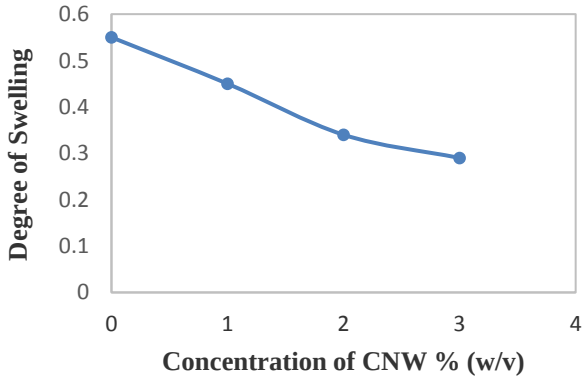


Figure 4 Water uptake of a sole alginate beads and CNW/alginate beads at various CNW concentration

The sole alginate beads showed the highest degree of swelling due to its natural hydrophilic properties that are capable to absorb more water molecules. The highest water absorption by alginate beads without the incorporation of CNW has been predicted since the hydrophilicity nature of alginate beads which consist of easily accessible hydroxyl group (-OH) which in a way gave a remarkable hydrophilic character to alginate. The incorporation of CNW into the alginate beads somehow reduced the affinity of alginate beads towards water. The decrease perhaps due to the high crystallinity of CNW which is known to be more towards the hydrophobic side than the solely alginate as well as the strong interaction that exist between CNW and alginate that reduced the swelling properties. Swelling degree is very much related to crosslinking degree, thus, the alginate beads with a low crosslinking density have a larger pore size and greater ability to swell compared to those with high crosslinking with CNW.

3.3 Strength Test on CNW/Alginate Beads

The strength of alginate beads is closely related to its stability. The alginate beads pose the risk to burst when the mechanical strength of the beads could not withstand the osmotic swelling pressure inside the beads. When that happens, the cells or protein entrapped within the beads will be released at a greater pace which is an unfavorable condition. The CNW/alginate beads that were placed in continuous shaking condition using an orbital shaker at 120 rpm exhibited a distinctive results based on the different concentration of CNW incorporated in the alginate beads which were observed every three days for nine days.

By the ninth day, the degree of swelling of sole alginate beads showed the highest value compared to the alginate beads that were incorporated with CNW. This also indicated that the burst of 0% CNW alginate beads was the greatest. The percentage of burst beads for sole alginate beads was 15% followed by 3.75% and 1.25% for 1% CNW and 2% CNW respectively while there was no burst of beads observed for 3% CNW based on Figure 4.7. When the mechanical strength of the beads cannot bear the osmotic pressure, the probability of the beads to burst is high. This effect will severely affect protein or drug release encapsulated in the beads and thus, facilitate the release. The alginate beads that were crosslinked with 3% CNW retained their shape possibly due to the compensation of higher mechanical strength of beads.

The shape of alginate beads crosslinked with CNW also maintained better compared to the sole alginate beads. The strong electrostatic interaction between the positively charged CNW and negatively charged alginate also contributed to the mechanical enhancement [7]. Compared to the reported compressive strength of calcium alginate hydrogel [8], the composite hydrogel prepared from the crosslinking of CNW and alginate showcased an excellent mechanical properties.

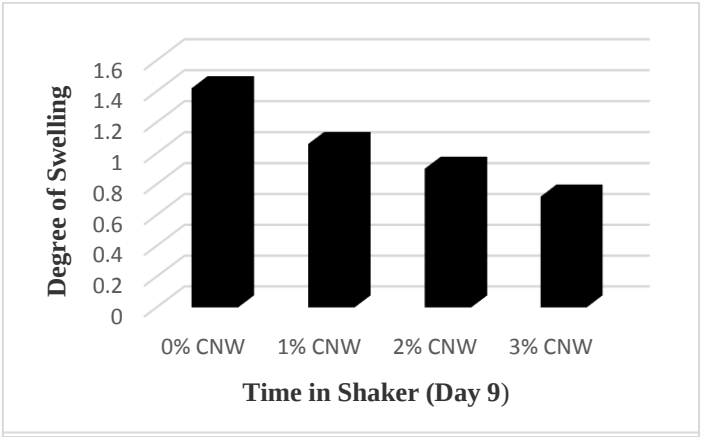


Figure 5 The degree of swelling for sole alginate beads and CNW/alginates beads at various CNW concentration on day 9

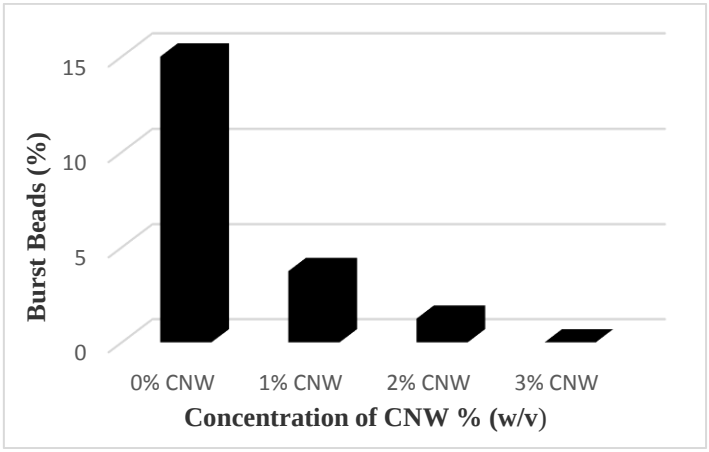


Figure 6 The percentage of burst beads of CNW/alginate beads at various concentration of CNW after nine days

3.4 The Preparation of Immobilized BSA in CNW/Alginate Beads

The incorporation of CNW into the alginate beads was carried out to investigate its influence in slow protein release. *Bovine Serum Albumin* (BSA) was used as a protein of study due to its low cost and the availability. Moreover, it can be handled easily and stable over a long period of time. Alginate gels were formed by ionic-network formation in the presence of calcium ions or other divalent ions.

The first step in achieving the crosslinked CNW/alginate beads was to mix the two water soluble polymers in an aqueous solution. The electrostatic interaction between the carboxyl group on alginate with the amino group that are present in CNW resulted in the formation of strong interactions between the two polymers. This electrostatic interaction between the carboxyl group in alginate with the amino group in CNW assisted the cross-linking process. The incorporation of CNW, which is a polycation into alginate beads can facilitate in controlling the strength of the surface coating and also the porosity of the capsule.

The incorporation of a second component into the hydrogel system at different dosages will change the structure morphology of the network and subsequently controlling their diffusion properties. The higher density and the homogenous, smaller pores in CNW nanocomposites allow not only a reinforcing structure but also a slow release rate of protein or drug, thus avoiding burst release [9]. In controlled release drug delivery system, the properties of CNW could conceivably allow the control rate of drug administration and prolong the duration of therapeutic effects besides targeting of drug to specific sites.

3.5 The Effect of Concentration of Chitin Nanowhiskers on BSA Release

The effect of concentration of chitin nanowhiskers (CNW) to BSA release from calcium alginate beads are shown in Figure 7. The results showed that as the concentration of CNW increases, the percentage of protein released declined. The lowest percentage of protein release was from calcium alginate beads with 3% CNW which was 66.58%. As for calcium alginate with 2% CNW, the protein released was 68.36% while for calcium alginate beads with 1% CNW, the percentage release was 70.26%. The highest percentage of protein release was from calcium alginate beads without the incorporation of CNW with 72.76%.

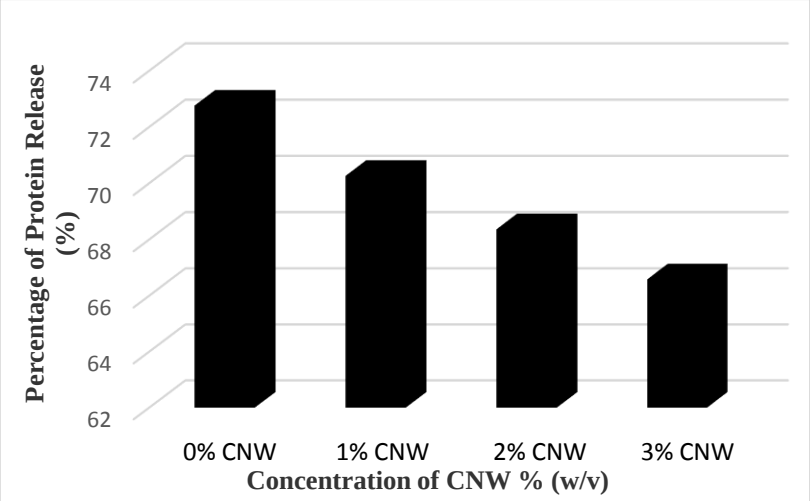


Figure 7 The effect of concentration of CNW on BSA release

The results showed that higher protein release was from calcium alginate beads with no crosslinking of CNW. This happened due to the porosity of the alginate. Thus, the porosity of the alginate can be reduced by incorporating CNW as well as improving the stability.

The incorporation of CNW clearly has an impact in reducing the release of protein from the alginate beads. The gelation process also was quicker and the retention of BSA in CNW/alginate beads was more efficient compared to the one in sole alginate beads. The higher concentration of CNW resulted in lower protein release. This happened due to the high crystallinity of CNW which enhance the barrier properties of alginate beads, which prohibit the protein release at quicker rate. Therefore, the protein release can be controlled by equipped skin coating with CNW in accordance to the requirements.

The presence of polysaccharide nanocrystal such as CNW increased the stability of alginate beads framework and inhibited the diffusion of BSA entrapped within the beads. BSA commonly used as model protein drug in the nanocomposite hydrogels because it showed a sustained release profiles. It was proven that the incorporation of CNW did not increase the cytotoxicity in comparison with the native hydrogel [3]. CNW also has the capacity to induce more than 55% encapsulation due to the creation of 3D network that behaved as barrier, thus, retaining more quantity of BSA [5].

3.6 Characterization of Chitin and Chitin Nanowshikers (CNW)

Both chitin and chitin nanowhiskers (CNW) were characterized using Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR) and the spectrum is shown in Figure 8. The vibration of amides I can be observed in the region 1660 – 1620 cm^{-1}. The absorption of chitin for C=O (Amide I) is detected at 1621.26 cm^{-1} and 1632.10 cm^{-1}. The presence of two types of amide can be explained in which half of the carbonyl groups are bonded through hydrogen bonds to the amino acid inside the same chain (C=O···H-N) that is responsible for the vibration mode at 1632.10 cm^{-1}. The additional bond produce a reduction in amide I bond at 1621.26 cm^{-1}.

In the corresponding region of the OH and NH (3600 – 3000 cm^{-1}) groups, chitin has a significant absorption at 3432.12 cm^{-1} which indicated O-H stretching that was attributed to the intramolecular hydrogen bonding involving O-H(6)··· O=C. A band appeared in chitin at 3258.16 cm^{-1} corresponds to the intramolecular hydrogen bond O(3)H···O(5) from the ring.

As for CNW, the band absorption for O-H stretching can be observed at 3434.73 cm^{-1}, which is the main absorbance region while the N-H group band was significant at 3104.30 cm^{-1}. The characteristic of carbonyl (C=O) stretching at 1653.56 cm^{-1} corresponds to the carbonyl vibrations of amide bond. The existence of amide group is further intensified by the appearance of C-N absorption at 1552.92 cm^{-1}. Meanwhile, the frequency

observed at 1023.00 cm^{-1} and 1070.34 cm^{-1} is assigned to the bending vibration C-O inside the chitin ring and of hydroxyl group (OH).

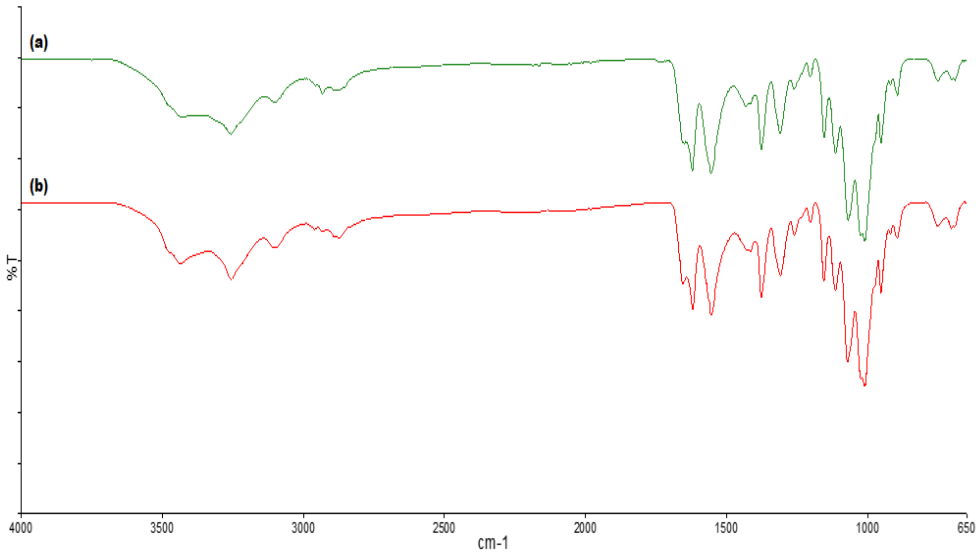


Figure 8 ATR-IR spectra of (a) chitin and (b) chitin nanowhiskers

Table 1 ATR-IR absorption bands of chitin and chitin nanowhiskers

Absorptions Bands (cm^{-1})	Chitin	CNW
O-H stretching	3432.12, 3258.16	3434.73, 3256.22
N-H stretching	3101.3	3104.3
C-H stretching	2934	2865.4
C=O (Amide I)	1632.10, 1621.26	1653.56
C-N (Amide II)	1553.26	1552.92
C-O	1014.25, 1068.08	1023.00, 1070.34
CH_3	951.81	952.17

The IR spectra of CNW showed no significant difference in comparison to chitin as shown in Figure 8 and in Table 1. This marked that the acid hydrolysis process did not alter the chemical structure of the chitin. The process only involved the breaking down of glycosidic bond that linked the chitin groups. The process does not associated with carbonyl (amide) bonds breaking despite the fact that the treatment of chitin in boiling HCl could possibly dissolve the amorphous domain as well as ether and amide linkages. Thus, by controlling the concentration and reaction time, hydrolysis of amide bond can be prevented. This indicated that the chemical groups of the resulting chitin were stable and no strong chemical reaction took place.

4. CONCLUSION

In this research, chitin nanowhiskers (CNW) was prepared through acid hydrolysis of chitin which enhanced the properties of the alginate beads. The formation of CNW/alginate beads involved the dropping of CNW/alginate mixture into $CaCl_2$ solution. The presence of CNW in alginate beads lowers the capability of alginate beads to absorb water due to the hydrophobic structure of CNW thus, reduce the degree of swelling of the alginate beads. Besides that, the incorporation of CNW increase the mechanical strength of alginate beads. The strength of alginate beads is relatively related to its stability. The embodiment of CNW in the alginate beads reduced the amount of burst beads that is caused by the osmotic swelling pressure in the beads. The high crystallinity properties of CNW act as a barrier that limited the water uptake of alginate beads thus reduced the swelling and the possibility for them to burst. The incorporation of CNW also play a role in lowering protein (BSA) release entrapped within the beads. The higher concentration of the CNW used, the slower the protein release. This indicated that the protein could be retained longer in the beads which is very useful in the biomedical applications such as drug delivery system.

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