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Development of Aldehyde Hyaluronic Acid - N,O-Carboxymethyl Chitosan Based Hydrogel for Intraperitoneal Antiadhesion Application

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Abstract. Intraperitoneal adhesion is a serious case that often occurs with a prevalence of 90-97 % after undergoing gynecological surgery and laparotomy. This study aims are to characterized the hydrogel and identified the optimal composition of Hyaluronic acid (HA) - N, O-carboxymethyl chitosan (NOCC) as an anti-adhesion biomaterial barrier. The synthesis method involved firstly the synthesis of aldehyde derivative of hyaluronic acid (AHA) and also the conversion of chitosan into its derivative, N,O-carboxymethyl chitosan. These two compounds were mixed in various compositions and crosslinked to form N, O-carboxymethyl *chitosan (*NOCC) /AHA. Fourier-transform infrared spectroscopy has confirmed that the functional groups found -C = O stretching at 1644 cm^{-1} indicating the hyaluronic acid and carboxymethyl group (-CH2COOH) in 1380 cm^{-1} which indicate the presence of chitosan. The crosslink is evidenced by the group C = N stretching at a wavenumber of about 1630 cm^{-1}. The best composition of intraperitoneal anti-adhesion is the ratio of hyaluronic acid: chitosan at 30:10 mg/ml. The swelling test is showed a swelling ratio of around 211.8 % in accordance with the standard as intraperitoneal anti-adhesion. Hydrogel has a degradation rate up to 86.87 % on day 10, and this is in accordance with the standard as intraperitoneal anti-adhesion. Cytotoxicity assay showed that hydrogel was nontoxic with a percentage of 92.9 % cell viability. The newly developed hyaluronic acid-carboxymethyl chitosan has characteristics that conform to the criteria of an intraperitoneal anti-adhesion.

Introduction

Adhesion intraperitoneal is the emergence of pathological attachment that usually arises between the omentum, bowel, and abdominal wall. The form of adhesion can be a thin film of connective tissue, a thick fibrous bridge containing blood vessels and nerve tissue, or direct contact between the two surfaces [1]. These fibrous bands of tissue adhesions typically begin to form within about 72 hours after laparotomy surgery procedures and will continue to form denser until the 7th day postoperatively [2].

Most of the patients who had intraperitoneal adhesions after laparotomy surgery have a risk of almost 90 % and about 97 % occur in most women who had undergone gynecologic surgery [3]. Obaid research in Malaysia in 2011 showed that the cause of intestinal obstruction in 92 cases found the percentage of them, 38 % of external hernia, adhesions by 25 %, neoplasms of 15.2 %, 8.6 % volvulus, intussusception of 5.4 %, and others 2.17 % lead.

Antiadhesive solutions can be chategorized as pharmacologic agents in the form of solid or liquid intraperitoneal barriers. Barriers are utilized to avoid contact of injured peritoneal surfaces during the healing process so as to evade tissue adherence. In the handling process of intraperitoneal adhesions problem, the hydrogel has been found as an innovation for the antiadhesion barrier. At body temperature, a solution could transform into a hydrogel. These hydrogels can be applied by injection, spray, or laparoscopic [4]. Chitosan has been used for biomedical purposes due to renewable, nontoxic and biodegradable [5]. Due to its capability to be easily processed into different forms, chitosan considered as promising material for the avoidance of postoperative adhesion [6]. Hyaluronic acid (HA) has achieved as corresponding material for a tissue adhesion barrier, due to its biocompatibility and viscosity which can grant homogenous coating around the injury site as a physical barrier [7].

According to the research of Pados [8] material antiadhesion which ideally should have a period of degradation of 7 days post-surgery, biocompatible, safe, not inflamed, not immunogenic, survived during the phase of re-mesothelialization critical, remain in place without sutures, remains active in the presence of blood, and maintain separate peritoneal surfaces to prevent contact between the surface of the serous damaged during 5-7 days for peritoneal epithelialization.

Materials and Methods

The materials used in this study were Hyaluronic acid (HA), Chitosan (Mw 327 kDa), Sodium meta periodate ($NaIO_4$), Isopropyl Alcohol (IPA), NaOH, Monochloroacetic Acid, Methanol, Alcohol, Distilled water, Ethylene Glycol, Normal Saline (NS), and Phosphate Buffered Saline (PBS).

Fourier Transform Infra Red (FTIR) Test

The FTIR test is used to determine the functional groups of the compounds in sample. This test uses the FTIR tool. Testing this functional group with looking for carboxymethyl amino and hydroxyl group substitutions from the chitosan – hyaluronic acid group. In this test using a Shimadzu IR Tracer-100 FTIR. In this study using an acid hydrogel sample hyaluronate-chitosan in the form of a gel to be mixed with KBr powder and placed on a platinum pan then the sample will be penetrated by light infrared. The results of the functional group testing using this FTIR produces a graph with the x-axis which is a number the wave on the compound and on the y-axis is the percentage of transmittance. Graph analysis of functional groups by comparing the transmittance bands at infrared spectrum with correlation table and using the spectrum of compounds comparison.

Synthesis of Aldehyde - Hyaluronic Acid (AHA)

AHA which were purchased from SigmaAldrich was prepared by mixing the powder with distilled water to give a clear and viscous solution. Further reacted with sodium meta periodate ($NaIO_4$) in dark conditions for periodic oxidation reaction to obtain an aldehyde group. Then the periodic oxidation reaction was stopped by the addition of Ethylene glycol and produced a clear solution and little lumpy. Then the dried product was obtained by freeze dry.

Synthesis N, O-carboxymethyl chitosan (*NOCC)*

N, O-carboxymethyl chitosan (NOCC) which were purchased from SigmaAldrich was prepared by mixing the ingredients between chitosan powder with isopropyl alcohol produces a yellowish turbid solution. Then proceeded with the addition of NaOH as pH control in the solution. Furthermore, the addition of acid monochloroacetate little by little as the formation of carboxymethyl chitosan. If the ingredients have been incorporated and stir at 60 °C for about 3 hours for all of the ingredients for success are mixed homogeneously. The final result was obtained with a chitosan solution supernatant. After the solution was filtered using filter paper. The dried product of chitosan was then purified or washed with methanol and 70 % alcohol. While the solution was rinsed with methanol and alcohol give a very thick yellow solution. Then the dried product was obtained by freeze dry.

Synthesis N, O-carboxymethyl chitosan *(NOCC) /AHA*

NOCC / AHA created by crosslinking between the AHA with NOCC at concentrations of 30: 0, 30:10, 30:20, 30:30 mg/ml. crosslink hydrogel is made by mixing the AHA and NOCC solution in a volume ratio of 1: 1.

Characterization of AHA, NOCC

Equilibrium swelling

Swelling test was conducted to determine the level of elasticity and increase the volume of the hydrogel samples. This swelling test using gravimetric methods. Then the dried samples were soaked in a solution of PBS with a *pH* of 2.4 incubator for approximately 24 hours at 37 °C. After that, the hydrogel surface was dried using blotting paper to remove moisture on the surface. After gaining weight before and after soaking the swelling ratio percentage obtained using the Eq. (1).

$$\%.SR = (W_{wet} - W_{dry}) / W_{dry} \times 100\ \% \quad (1)$$

%SR = Percentage of swelling ratio
Wwet = Weight wet (weight after sample immersion)
Wdry = Weight dry (initial weight before immersion)

In vitro degradation of hydrogels

Degradation test is a test carried out in vitro in the hydrogel to determine the weight lost in each sample quantitatively by soaking samples at predetermined time intervals. Soaking using liquid representing the physiological condition of the peritoneal cavity in PBS. The level of degradation of samples expressed as a percentage weight loss at each time interval was calculated using Eq. (2).

$$\Delta W\ (\%) = (W_o\text{-}W_t) / W_o \text{ x } 100\ \% \quad (2)$$

ΔW = Percentage of sample weight lost
Wo = Initial weight before sample immersion
Wt = Weight after sample immersion

In vitro test cytocompatibility

In vitro cytotoxicity assay was performed using cell cultures to determine the toxic effects of a substance directly. This test can provide information about the possibility that the cells could still survive. This test using the MTT Assay method with plate 96. This method relies on the ability of living cells to reduce MTT salt. This test used hepatocyte cell cultures were incubated for 48 hours at Eagle media. After the results were read by an ELISA reader, then calculated using Eq. (3).

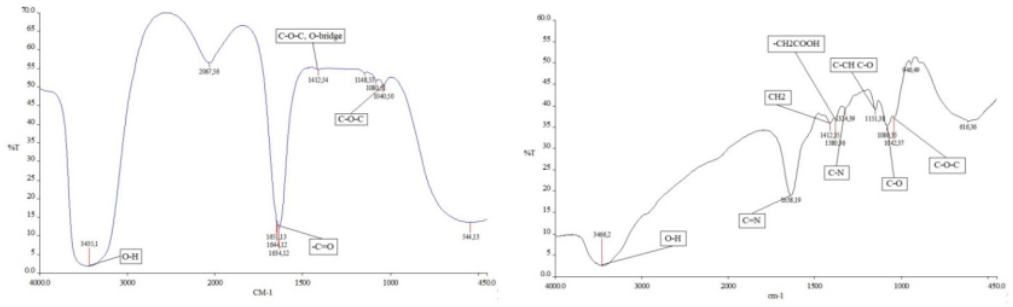
$$(\%\ \text{living cells}) = \frac{absorbance\ sample}{\Sigma\ absorbance\ cell\ control} \times 100\ \% \quad (3)$$

Results

Fourier Transform Infra Red

AHA is derived from hyaluronic acid so that the spectrum is not much different from the spectrum of hyaluronic acid. In all samples, the band wave absorbing wave numbers around 3400 cm^{-1} indicates the presence of OH groups. Furthermore, it is also known to the C = O stretching group at a wavenumber of about 1630 cm^{-1} in all samples. In the wavenumber of about 1400 cm^{-1} indicated as a carboxylic acid group (C(=O)OH) (Fig 1A).

NOCC characteristics can be seen from their group NH stretches that are present in wavenumbers around 3400 cm^{-1}, which shows the process of polymerization occurs. The occurrence of cross-linking between hyaluronic acid and chitosan seen from the group stretching C = N, namely the absorption wavenumber of the absorption band at around 1630 cm^{-1}. Next comes a group -COOH that shows the process carboxymethylation the amino group of chitosan located at wave number 1400 cm^{-1} (Fig 1B).



(A) (B)

Figure 1. FTIR spectrum
(A. Results of the sample spectrum AHA-NOCC 30: 0 mg/ml,
B. The results of the sample spectrum AHA-NOCC 30:10 mg/ml)

Equilibrium swelling

The results of the swelling analysis using gravimetric formula. Sample A did not experience swelling because sample A in liquid form so that the fluid sample was directly hydrolyzed by PBS. In samples B, C, and D respectively produce the percentage of swelling 211.8353 % 160.8237 % and 91.9938 %. Hydrogel swelling effective standards for the application of intraperitoneal adhesions is 123-225 % [9]. So of all the samples according to standard literature is a sample B and C. As for sample D still below the standard literature which can be seen in Fig. 2.

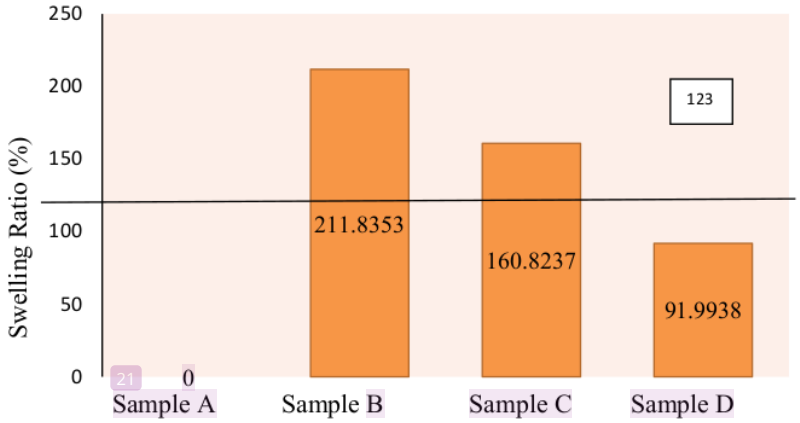


Figure 2. The result of samples swelling test
(A. 30: 0 mg/ml, B. 30:10 mg/ml, C. 30:20 mg/ml, D. 30:30 mg/ml)

In vitro degradation

Characterization of in vitro degradation test is done using a solution of PBS by incubation process at a temperature of 37 ^{o}C. Degradation test performed on days 3, 7, and 14. It can be seen that sample A totally degraded on the first day because it was a liquid sample and obviously no cross-link. While the samples B, C, and D were relegated respectively 93.6 %, 90.7 %, 86.15 % on the 14th day. This is shown in Fig. 3.

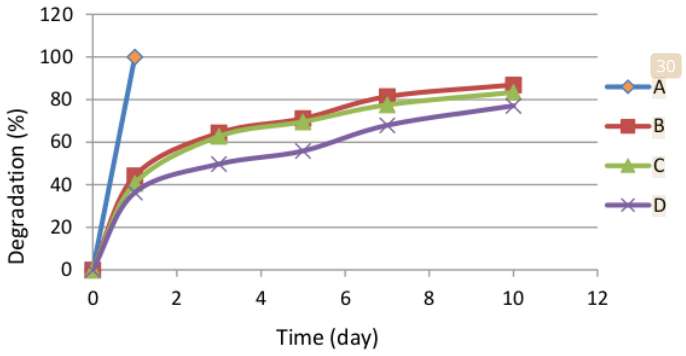


Figure 3. The result of samples degradation rate
(Ratio AHA : NOCCC A. 30: 0 mg/ml, B. 30:10 mg/ml, C. 30:20 mg/ml, D. 30:30 mg/ml)

In vitro test cytocompatibility

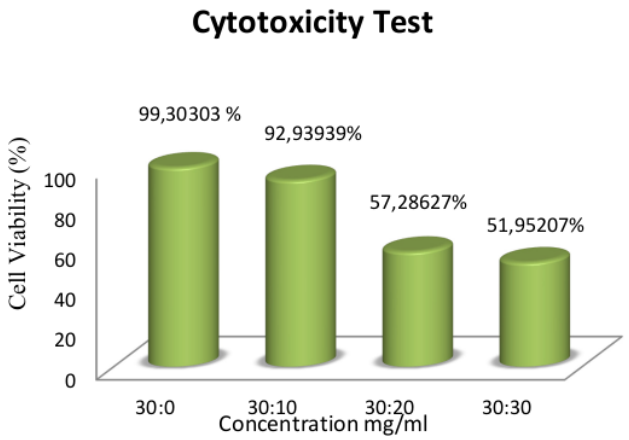


Figure 4. Result of Samples Cytotoxicity Test
(Ratio AHA: NOCCC A. 30: 0 mg/ml, B. 30:10 mg/ml, C. 30:20 mg/ml, D. 30:30 mg/ml)

The percentage of cell viability of living in each sample showed 99.3 %, 92.9 %, 59.3 %, and 51.9 %. The test is in accordance with ISO 10993-5:2009 :Biological evaluation of medical devices, Part 5: Tests for in vitro cytotoxicity. This results show that samples A, B, C, and D categorized as non-toxic materials because they have cell viability more than 50 % [10]. This is shown in Fig. 4.

Discussion

Postoperative intraperitoneal adhesion is common event which might occur in post-laparotomy and surgical gynecology. One of solution is by anti adhesion application. Anti-adhesion agent have been refined to inhibit post-operative adhesion. All biomaterials which will be applied in human body must be safe for clinical use in order to avoid the adverse events. Based on the results of the cytotoxic test on all samples can be considered that all the samples are materials that are safe and non-toxic in the body. This is because the percentage of cell viability showed above 50 %. Known the results in the diagram above, indicate that the concentrations of chitosan higher in the material will result in a lower percentage of survival of living cells. And conversely, the lower the concentration of chitosan in the hydrogel, the percentage viability of living cells is high. This is supported by Montembault et

al. [11] and Hernández et al. [12] who stated that differences in consistency and rigidity of chitosan gel-based which were previously demonstrated by rheological studies related to the higher gel rigidity with increases in chitosan concentration. Rheological studies have proved that increased chitosan concentrations affect gel viscosity [11,12] and rigidity [13]. The higher concentration the higher viscosity of gel, this condition will affect the viability of cells. The principle of MTT assay is to break tetrazolium MTT ring, (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide), with yellow color because of dehydrogenation in active mitochondria, and to produce insoluble blue-purple formazan product. If dehydrogenizes are not active because of cytotoxic effects, the formazan is not produced. The formazan production can be measured by dissolving it and measuring the optic density of the solution produced. The lower the percentage of optic density is, the fewer the number of active metabolic cells that can reduce MTT [14,15]. The biological properties of chitosan depend on their physicochemical parameters, especially their solubility. The characteristic of chitosan is tightly connected with its molecular weight [16]. Chitosan with high molecular weight powder become chitosan gel with good physical characteristic and higher viscosity. Powder with high-molecular weight has greater particle size, due to the larger size of the solute molecules solvent molecules surrounding more increasingly difficult to dissolve. It becomes tenuous bonds by the force of molecular attraction [17]. The molecular weight is also directly related to the length of the molecular chain, longer the molecular chain, the viscosity higher and higher average formazan optic density value. The molecular weight is also directly related to the length of the molecular chain, longer the molecular chain, the viscosity higher and higher average formazan optic density value [17, 19]. Alteration in chitosan concentration affected the ultrastructure of gels, which were contributed to a greater number of polymer chains in the chitosan [18]. Cell growth is favored by the surface characteristics of the material [19].

In a swelling test theoretically, the higher the concentration of the chitosan in a hydrogel, the more crosslinking amino groups with an aldehyde group HA, resulting in less reaction residue and low swelling ability. The hydrophilic groups enable the hydrogel to absorb water and watery fluids that result in hydrogel expansion and occupation of larger volume, the process which is known as swelling [20]. In samples A, B, and C have entered the range of swelling ratio in accordance with standard applications ideal adhesion barrier. Because if swollen beyond tolerance limits of literature is likely to cause pain and pressing the wound so that worsen the condition of the wound.

According to research by Pados [8] antiadhesive ideal materials must experience 7 days post-surgery degradation. Hydrogels have to endure cover the area of the wound until the wound can be healed and then gradually degraded so as not to be residue in the body. From the above test results, it is shown that the higher concentration of chitosan, the rate of degradation is getting longer. This is due to a greater concentration of chitosan that causes crosslinking between the amino groups of NOCC and aldehyde groups in A-HA is greater. Chitosan degradation is dependent on variations in the distribution of acetamide groups in the chitosan molecule [21]. It causes the density of the sample to increase and low porosity. If the porosity is low, the PBS will be more difficult to get into the hydrogel and slow the rate of degradation. Degradation properties of chitosan-based hydrogel were also dependent on the degree of deacetylation of chitosan used and it decreased with an increase in its deacetylation.

Conclusion

The AHA:NOCC hydrogel with the composition of 30:10 mg/ml showed the best properties. This newly developed AHA-NOCC hydrogel is safe and can be considered for anti intraperitoneal adhesion barrier.

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