

Chitosan-Hyaluronic Acid Composite Injectable Hydrogel as Open Postoperative Peritoneal Anti-adhesive Agent

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Abstract

Peritoneal Adhesion is considered as pathological healing after an abdominal surgery. Alternative peritoneal adhesion prevention can be done by injecting hydrogel in peritoneal wound site. Synthesis of injectable hydrogel was made by composing chitosan solution with hyaluronic acid. Hydrogel was formed by mixing hyaluronic acid solution and chitosan with concentrations varied from 0, 20, 30, to 40 mg/ml. The results of FTIR test showed that there is a characteristic absorption band of hyaluronic acid, called group of C=O stretching at wave number of 1636.31 cm^{-1} ; while the typical absorption band of chitosan is asym stretch -COO- group at wave number of 1619 cm^{-1} .

The results of swelling test indicated that values of swelling ratio range from 172.57% to 214.43%. Furthermore, the outcome of degradation test illustrates that samples with the best degradation ratio of 20 mg/ml chitosan concentration are able to be degraded by 85% on Day 9. Then, the results of SEM test showed that the samples have good porosity and interconnective tissues. Meanwhile, the outcome of cytotoxicity test indicated that all samples are non-toxic. DSC test results indicated that hydrogel do not yield alteration of thermal properties at 15-45 °C, so this material performed well in terms of body temperature.

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Introduction

Postoperative peritoneal adhesion is very common and can cause serious complications.¹ It is a pathological formation of connective tissues between omentum, bowel, and abdominal wall.² The presence of adhesion due to peritoneal adhesion may cause pain, bowel obstruction, and even infertility or subfertility, if it occurs in parametrium adnexa.³

The prevalence of peritoneal adhesion after intra-abdominal surgery is between 63% and 97%.^{4,5} The incidence of gynaecologic for open post-operative adhesion (laparotomy) reaches from 60% to 90% in women undergoing

major gynaecological surgery procedure.⁶ Overall, about one-third of patients undergoing open surgery on their abdomens or pelvis come back to the treatment center averagely twice in 10 years due to peritoneal adhesion complications.⁷ There have been several researches done to find an effective approach to prevent postoperative adhesion, including chemical drugs and barrier systems. The chemical drugs, such as corticosteroids, antibiotics, fibrinolytic materials, anticoagulants, and hormones, are considered inadequate to prevent adhesion.⁸ Eventhough liquid barriers, such as crystalloid (saline and ringer lactate) and polymer solution (N, O-carboxymethyl chitosan (NOCC), sodium hyaluronate, and carboxymethyl cellulose (CMC)) are used in large numbers, they are rapidly absorbed.⁹ As for solid barriers, it is difficult to use them to cover the wound surface thoroughly and lead them to the damaged tissues. Some can be aggressively attached to a surgeon's gloves during installation.¹⁰

An anti-adhesive agent in the form of hydrogel was invented to overcome the above

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shortcomings. This hydrogel is injected into the injured area; thus, it is called peritoneal injectable hydrogel. The liquid hydrogel covers the wound surface with complex geometry, deforms into gel to form a physical barrier, and prevent direct contact with other surfaces for a certain period.¹¹ It is made of hyaluronic acid (HA) which is commonly used as barrier for adhesion prevention due to its non-toxic character. Additionally, HA is known to increase proliferation in peritoneal mesothelial cells.¹² However, HA-based hydrogel often exhibits weak mechanism with rapid degradation behaviour that decreases its effectiveness to prevent adhesion.¹ The use of the chitosan was found to promote wound healing and induce cell migration and proliferation.¹³ Therefore, through the periodate oxidation method. L.Li et al.¹ made an anti-adhesive agent of HA-based hydrogel which was modified by adding synthesized chitosan. They synthesized hydrogel by modifying and characterizing the composition variation of chitosan on hyaluronic acid.

Materials and methods

This research used Hyaluronic acid/HA, chitosan, monochloroacetic acid (Sigma-Aldrich), normal saline (Otsuka Pharmaceutical), Isopropyl alcohol, methanol, and sodium hydroxide from SAP Chemicals. All chemicals used are pro-analytic without further purification process.

Initially, 1 gram of HA was dissolved into 10 mg/ml-concentrated aquabidest. Then, 0.3g of NaIO₄ liquefied in 5 ml aquabidest was added when HA had perfectly diffused. The admixture was undergoing stirring process for 2 hours at room temperature in the dark. Subsequently, 0.5 ml of ethylene glycol was added, along with NaIO₄, to stop the reaction. The solution stirring lasted for 1 hour. Lastly, the solution was dried by freeze-drying.¹

Chitosan was prepared based on the previous methods with some modifications. Initially, 10g of chitosan was dissolved in 75 ml of Isopropyl Alcohol (IPA) and stirred in a beaker glass at 25°C. Then, 25 ml of NaOH 1N solution, which is divided into 5 parts, was added to the chitosan-IPA in 5 minute intervals. Stirring was performed for 30 minutes using a magnetic stirrer. Next, 20g of monochloroacetic acid was gradually added into the solution over a period of 20 minutes. Subsequently, the solution was then

stirred for 3 hours at 60 °C. Additionally, the solution was filtered and rinsed three times with a mixture of 80% v/v methanol/water and washed it twice with alcohol. Finally, the solution was lyophilized using a freeze dryer.¹

Hyaluronic acid-chitosan hydrogel was made by mixing hyaluronic acid and the prepared chitosan. Hyaluronic acid and chitosan were dissolved in normal saline (NS) at a concentration ratio of hyaluronic acid: chitosan of 30:0, 30:20, 30:30, and 30:40 mg/ml, namely sample A, B, C, and D respectively. Then, they were mixed at a volume ratio of 1:1. Characterization of the injectable hydrogel is conducted through fourier transform infra red (FTIR) test, swelling test, degradation test, scanning electron microscope (SEM) test, cytotoxicity test, and differential scanning calorimetry (DSC) test.

Fourier Transform Infra Red (FTIR) Test

Fourier Transform Infra Red (FTIR) spectroscopy is used for the confirmation and specification of the functional group of the compound. FTIR test in this research using Thermo Scientific Nicolet iS10. Samples can be solid or liquid. For hyaluronic acid (AH)-chitosan hydrogel sample, an example consists of two layers of KBr. Furthermore, the functional group analysis was performed by comparing absorbance bands or transmittance formed on the infrared spectrum by the correlation table and using a spectrum of compound compounds.

Swelling test

Determination of hybrid acid swelling ratio of hyaluronic acid (AH)-chitosan was done by gravimetric method. The swelling ratio is required to determine the level of hydrogel elasticity. The freeze dried hydrogel was formed like a disk and weighed as dry weight (W_0). Then the hydrogel sample was immersed into a vial bottle containing Phospat Buffered Saline (PBS) with pH=7.4. After 24 hours, the hydrogel was removed and the surface was dried by means of suction paper. Then the hydrogel was weighted based on a predetermined time range. The hydrogel mass after the lifting time was calculated as wet weight (W_1).¹ Equilibrium swelling ratio (% ESR) in hydrogel was studied using Equation 1 below:

$$\%ESR = \frac{W_1 - W_0}{W_0} \times 100\%$$

Degradation Test

In vitro degradation test of the hydrogel was done by simulating the sample under physiological conditions. A total of 1 mL of the hydrogel sample was weighed as dry weight (W_0). Then the hydrogel sample was immersed into media i.e. 10 mL PBS (pH=7.4), incubated at 37 °C. Degradation media was replaced daily to ensure routine degradation activities. After 1,3,5,7 and 9 days, the sample was removed from the medium, rinsed with distilled water, frozen, lyophilized and weighed as wet weight (W_t).¹⁴ The degradation rate is expressed by the percentage of weight loss at the hydrogel at each time interval calculated using equation 2 below:

$$\Delta W = \frac{(W_0 - W_t)}{W_0} \times 100\%$$

ΔW = percentage of sample weight lost
 W_0 = initial weight of sample
 W_t = sample weight after degradation test

Scanning Electron Microscopy (SEM) Test

The morphology test was performed using scanning electron microscopy (SEM) to determine the surface and cross-sectional morphology of the hydrogel sample. The preparation procedure of hydrogel samples for morphology test begins with freeze drying the sample. Furthermore, the sample surfaces were coated with gold-palladium first by attaching the coating to the sample surface using a conductive double-sided tape. Surface morphology and cross-sectional samples were observed using SEM with 100x and 300x magnifications.¹

Cytotoxicity test

The cytotoxicity test was used to determine the hydrogel sample were toxic or not. This experiment was carried out by culturing Human Hepatocyte Cell on Eagle medium which has been formed into monolayer removed from the incubator after 24 hours. The media was removed and washed with Dimethyl Sulfoxide (DMSO) to clear the remaining cell metabolism

and residual serum remaining. Thereafter, cell culture was trypsinated with a 0.25% trypsin versence to separate the cells attached to the bottle wall and to prevent the cells from colonizing. Put the cells in 100µl Eagle media into 96-microwell plate according to the number of samples and controls. The MTT reagent was added as much as 25 µl in each hole. After being added with MTT, cell culture was incubated for 4 hours at 37 °C. Then added Dimethyl Sulfoxide (DMSO) as much as 50µl at each hole to stop the reaction and processed in vortex for 5 minutes to mix the DMSO well. Furthermore, formazan optical density was observed with ELISA Reader at 560 nm wavelength.

Determination of the number of surviving cells on membrane damage parameters, synthetic disorders and macromolecular degradation, metabolic modification, and cell morphology changes. Guidelines for toxicity based on the damage of membrane membranes that take (up take) or with dye materials such as tripan blue. From the toxicity test can provide information on the percentage of cells that can survive.

Differential Scanning Calorimetry (DSC) test

The DSC test aims to determine melting temperature and crystallization temperature of the material. DSC testing standards refer to American Standard Testing Materials (ASTM) D3895. The operating conditions of the apparatus were at a humidity of about 50±5% with the test temperature at room temperature. A total of 5-15 mg samples were placed in aluminum containers (cruchible) for later incorporated into DSC devices. Next activate the DSC which started with the preeliminary thermal history. Samples were heated gradually from room temperature to a temperature of 250 °C at an average heat of 10 °C/min then cooled to room temperature using cooling fan. The heating or cooling cycle is done in duplo.¹⁵

Results

Fourier Transform Infra Red (FTIR) test

Based on the FTIR spectrum of Sample A in Figure 1, there was an absorption band at wave number of 1636.31 cm^{-1} , indicating

characteristic peaks of hyaluronic acid, namely C=O stretching group. Meanwhile, in Sample B, the absorption band of C=O stretching group was not identified in the spectrum. It was due to the existence of chitosan carboxylic acid salt group which affected the reading for C=O stretching group. The sharp absorption band of the carboxylic acid salt group (-COO-*asym* stretch) at wave number of 1619 cm^{-1} in Sample B indicated the emergence of carboxymethyl group on the chitosan. The thorough FTIR test results are shown in Table 1.

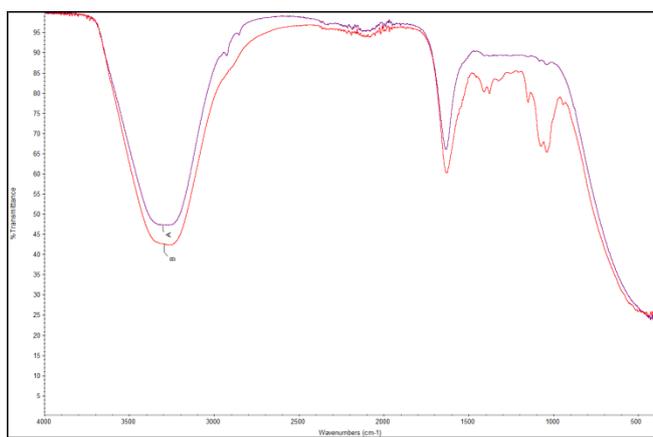


Figure 1. FTIR Spectrum of Sample A (top) and B (bottom).

Sample A (cm ⁻¹)	Sample B (cm ⁻¹)	Explanation
3284.83	3257.31	O-H stretching characteristic peak of A-HA
1636.31	-	C=O stretching
1410.03	1406.86	-COOH <i>asym</i> stretch
-	1378.19	C-CH and O-CH stretching
1039.58	1040.49	C-O-C bending
-	3373.09	N-H stretching characteristic peak of NOCC
-	1457.52	NH ₂
-	1619	-COO- <i>asym</i> stretching
-	1147.23	C-O-C stretching
-	1074.41	C-O stretching

Table 1. FTIR Test Results of Hydrogel Samples.

Swelling test results

Values of the hydrogel swelling ratio for preventive applications against peritoneal adhesion in the abdomen were ranging from 123% to 225%.¹⁶ Out of the four samples, Sample B, C, and D met the swelling ratio of hydrogel for preventive applications against peritoneal adhesion. As for sample A, researchers did not get data of sample weight after the swelling test, as it directly under went degradation.

Degradation test results

Degradation test showed that Sample B, C, and D were degraded by 52.97%, 45.2%, and 30.6% respectively on Day 4. On the other hand, Sample A was perfectly degraded by 100% at the first day. Then, on Day 9, sample B was reduced to about 85%. These results indicated that hyaluronic acid-chitosan hydrogel has good biodegradability, so that it has the potential to be used for adhesion prevention applications.

Scanning Electron Microscopy (SEM) test result

The results of swelling and degradation test showed that Sample B had the best characteristics. Therefore, Scanning Electron Microscopy (SEM) Test in this study is focused on Sample B. The result can be showed in Figure 4 below.

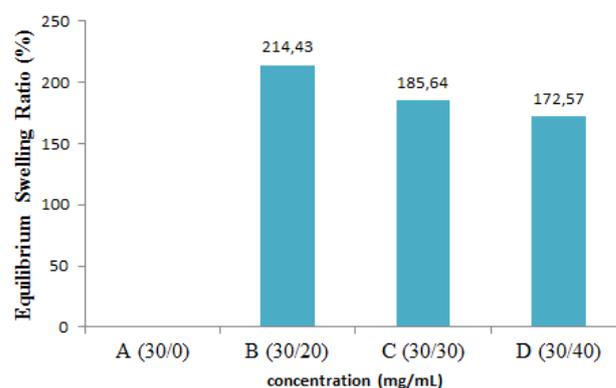


Figure 2. Swelling Test Result Chart on Various Concentrations of Chitosan.

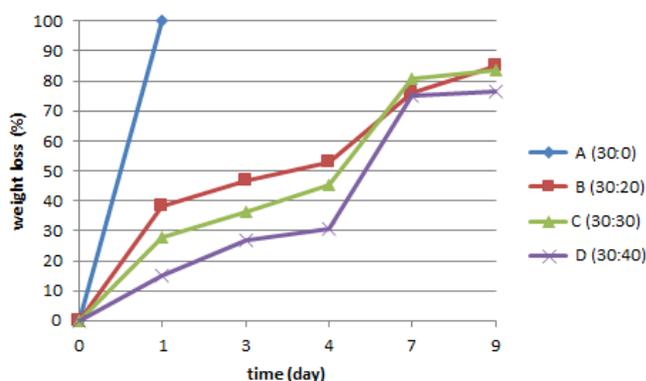


Figure 3. Weight Loss Percentage Against Degradation Time.

Cytotoxicity test results

The injected hyaluronic acid-chitosan hydrogel is treated as a foreign object by the body. Therefore, it is important to know the toxicological properties of the samples through MTT Assay Test. This test was performed using reagents of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT). The cytotoxicity assay results are depicted in Figure 5. Figure 5 illustrates that percentage of living cells of Samples A, B, C, and D are 58.67%, 57.62%, 58.02%, and 58.35% respectively.

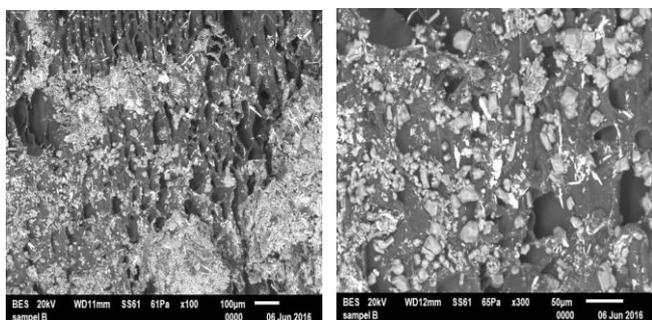


Figure 4. SEM Test Results of Sample B, Magnified 100x (left) and 300x (right).

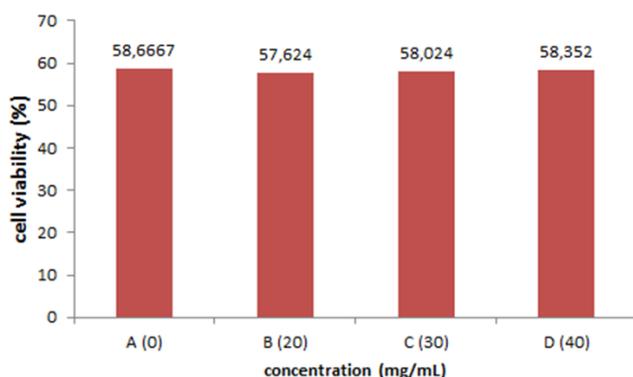


Figure 5. Cytotoxicity Assay.

Differential Scanning Calorimetry (DSC) test results

Figure 6 showed endothermic transition at 83.73 °C and 198.18 °C. This phenomenon is similar with Ramona, Polexe, and Delair research¹⁶ in which thermogram of hyaluronic acid-chitosan experienced twice endothermic transition. Peak of 83.73 °C was indicated by water molecules evaporation in the sample. Meanwhile, the peak of 198.18 °C indicated endothermic transition related to sample melting temperature (T_m).

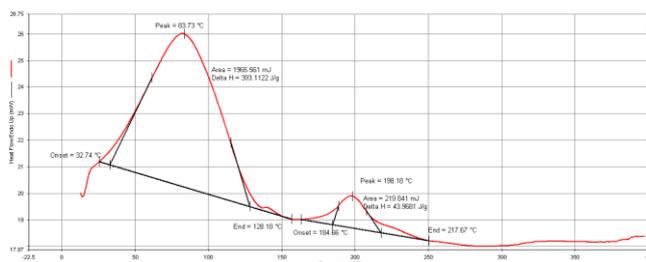


Figure 6. DSC Thermogram of Sample B with HA:Chitosan 30:20 mg/mL.

Sample C Thermogram on Figure 7 displayed shifted peak compared to Sample B thermogram. Endothermic peak of sample is between 90.99 °C and 195.75 °C. Widening T_m in the sample occurs due to the strong bond between the amino group of chitosan and the aldehyde of hyaluronic acid; thus, to change the samples' forms require considerable reaction. The stronger the polymer molecules bound to one another, the greater thermal energy needed to yield movement.¹⁷

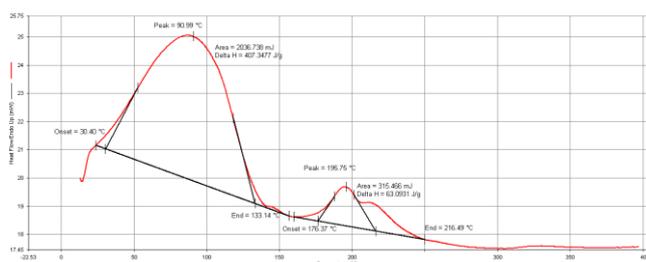


Figure 7. DSC Thermogram of Sample C with HA:Chitosan 30:30 mg/mL.

During the manufacturing sample process and storage, range of melting temperature (T_m) of the sample should be avoided. If not, it could conduct to break polymer bond and decrease the samples' qualities.¹⁸

Discussion

Synthesis of hydrogel hyaluronic acid-chitosan was made in 4 groups. Samples A, B, C, and D are samples with variations in HA:Chitosan concentrations 30:0, 30:20, 30:30, and 30:40 mg / mL respectively. Subsequently, sample characterization was done through Fourier Transform Infra Red (FTIR) test, swelling test, degradation test, morphology test, cytotoxicity test and Differential Scanning Calorimetry (DSC) test.

The swelling ratio (Equilibrium Swelling Ratio) in hydrogel was calculated gravimetrically.

The higher concentration of chitosan in hydrogel results in smaller percentage of swelling. This is due to higher concentration of chitosan which makes the distance among the molecules in hydrogel becomes closer. As a result, it is difficult for water to diffuse into the material, causing small deployment capability.

The higher the concentration of chitosan, the lesser the degradation rate is, because chitosan concentration positively correlates the likelihood of crosslinking between amine group of chitosan and aldehyde group of hyaluronic acid.¹ As a result, the density of the resulting material would even be more enormous. High density material has low porosity which impedes PBS solution to infiltrate into the pores of the hydrogel and slows down the rate of degradation. Results from the degradation test indicated that hyaluronic acid-chitosan hydrogel has good biodegradability, so that it has the potential to be used for adhesion prevention applications.

The hydrogel has fairly high porosity and good interconnective tissues. Based on those characteristics, it can be concluded that it has good permeability for nutrients and certain substances to support the process of peritoneum remesothelialization. Samples are not considered toxic materials since they exceed 50% of cell

viability.¹⁹ From the DSC test result, the sample thermogram was at 15°C-45°C temperature range, showed no change of thermal properties mean. Thus, samples of hyaluronic acid-chitosan hydrogel are capable to maintain good performance within the range of body temperature.

Conclusions

The hydrogel of hyaluronic acid chitosan composite is suitable to be used as an anti-adhesive agent due to its biodegradability, good interconnecting pore and its non toxic status.

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Declaration of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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