Hyaluronic Acid - Chitosan / AgNPs Hydrogel Green Synthesis from Curcuma Longa as Antibacterial Anti Intraperitoneal Adhesion

Prihartini Widiyanti[1,2]*, Djoni Izak Rudiardjo[3], Herry Wibowo[4], Aulidya Hanum[1]

1. Biomedical Engineering Study Program, Department of Physics, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia.
2. Institute of Tropical Disease. Universitas Airlangga, Surabaya, Indonesia.
3. Physics Study Program, Department of Physics, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia.
4. Laboratory of Physiology, Department of Biomedical, Faculty of Medicine, Universitas Surabaya, Surabaya, Indonesia.

Abstract

Intraperitoneal adhesions occur 67% -97% after general abdominal and gynecologic pelvic surgery. The purpose of this study revealed the optimal concentrations in AH hydrogels, chitosan, AgNPs from C. longa biosynthesis products through FTIR, XRD, PSA, swelling, degradation, antibacterial, MTT-assay, and SEM. The synthesis method changed AH into aldehydes AH and chitosan to carboxymethyl chitosan, then freeze dry and dissolved used normal saline. After that, vortexed and AgNPs have been synthesized with aqueous turmeric solution. FTIR test results showed that the AH hydrogel and chitosan were formed -CH2COOH at 1404.18 cm-1, -C=O carbonyl group at 1612.49 cm-1, and the aldehyde group at 2887.44 cm-1. The best concentration was obtained at 10-3 M where AgNPs formed at 2θ = 38.140, (111) through the XRD test, and the average size of the PSA test was 23.6 nm. Swelling test was 209.72% and it was degraded on the 9th day by 91.56% based on the intraperitoneal adhesion preventive gene requirements. The results of antibacterial test have sensitive properties resistant to bacteria with a clear zone diameter of 10.29 mm. Meanwhile, MTT-assay test obtained toxic results due to the influence of AgNPs formed at 1.88%. Based on the tests that have been conducted, it was stated that the hydrogel AH, chitosan, AgNPs from C.longa biosynthesis products have the potential as an antibacterial agent for preventing intraperitoneal adhesion.

Experimental article (J Int Dent Med Res 2020; 13(3): 1204-1210)

Keywords: Intraperitoneal adhesion, hyaluronic acid, chitosan, AgNPs.

Received date: 05 April 2020 **Accept date:** 08 June 2020

Introduction

Intraperitoneal adhesion is a complication that occurs seriously and generally performs after surgery such as cholecystectomy, gastrectomy, appendicitis, hysterectomy, colostomy, abdominoperineal resection, and abdominal vascular procedures, with the number likely to range from 67% - 93% after general abdominal surgical procedures. Adhesion events can happen and even up to 97% post gynecologic pelvic surgery.[1] Adhesions begin to develop for 72 hours after surgery. That is due to inflammation, fibroblasts growth, and neovascularization.[2]

Injuries to the peritoneum begin inflammation by exuding fibrinous and forming fibrin. Commonly, activation of the fibrinolytic system, each layer of fibrin formed must be lysis. If fibrinolysis does not occur within 5-7 days, the fibrin matrix will be organized with fibroblasts and can secrete collagen. As a result, intraperitoneal adhesion and new blood vessel growth are formed.[3] Some writers have developed hydrogels as an ideal intraperitoneal adhesion barrier prevention agent to overcome existing problems.[4] Hydrogels were chosen because they were considered to potentially mimic Extracellular Matrix (ECM). That is due to the hydrogel has the ability to swell not only in water but also in biological fluids.

Previous studies have not yet studied the synthesis of hydrogels as a barrier to the prevention of intraperitoneal adhesion agents

Corresponding author:
Prihartini Widiyanti,
Biomedical Engineering Study Program, Department of Physics, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia.
Institute of Tropical Disease Universitas Airlangga Campus C Mulyorejo Surabaya 60115. Indonesia.
E-mail: pwidiyanti@fst.unair.ac.id

with the addition of antibacterial. This happens because the infection will cause inflammation in the peritoneum which will interfere with the process of fibrinolysis.[5] Thus, innovation is needed as an antibacterial agent, silver nanoparticles.

Chitosan is one of the most abundant polymeric materials in nature, good antibacterial, and hemostatic. [6] Chitosan as product with high molar mass results from the deacetylation of chitin has many functions for healing such as homeostasis, accelerate tissue regeneration. Chitosan have many properties which beneficial for healing recovery such biocompatible, biodegradable, non-toxic and anti-bacterial content.[7]

Silver nanoparticles were obtained from the synthesis of $AgNO_3$. Although, there are many methods of silver nanoparticles synthesis and biosynthesis products with plants (green synthesis) are reported to be the most effective because they require shorter reaction[8] and inexpensive to produce.[9] The green synthesis resulted in various shapes and sizes dominated by spherical, polydisperse and have been found to be efficient for silver nanoparticles formation.[8] Based on the previous explanation, the synthesis and characterization of physical barrier hydrogels based on hyaluronic acid-chitosan acid by innovating antibacterial nano-silver (AgNPs) comes from the Curcuma longa biosynthesis product.

Materials and methods

Synthesis of AgNPs Curcuma longa Biosynthesis Products

Preparation of AgNPs samples, deionized water mixed with Curcuma longa powder produced a turbid yellow solution. The solution was centrifuged for 10 minutes at a speed of 200 rpm to separate the precipitate to produce a clear yellow solution. The solution was mixed with $AgNO_3$ solution that has been dissolved in aqua bides according to variations in the concentration of 0 M (F0), 10^{-3} M (F1), 10^{-4} M (F2), and 10^{-5} M (F3). Moreover, the solution was stirred for 24 hours in dark conditions. The final result of the solution was a brownish yellow solution where the higher the concentration of $AgNO^3$ was used, the more brownish yellow color

Synthesis of Hyaluronic Acid Hydrogel-Chitosan / AgNPs Curcuma longa Biosynthetic Products

Hyaluronic acid sample preparation, 100 ml of aqua bides was mixed with 1 gram of Hyaluronic acid. The solution was mixed with 0.32 grams of sodium metaperiodate ($NaIO_4$) which has been dissolved into 10 ml aqua bides first in dark conditions and room temperature. The periodic oxidation reaction was stopped by adding 0.5 methylene glycol. The result was clear and slightly viscous. After the sample preparation was completed, freeze dry stage was conducted.

Chitosan sample preparation, 75 ml isopropyl alcohol was mixed with 10 grams of 85% chitosan DD powder. The solution was mixed with 25 ml of 1N NaOH, the solution was mixed with 20 grams of monochloroacetic acid. Then, it was stirred at 60 ^oC for 3 hours. The result was a supernatant so the solution needed to be filtered with filter paper. The solid product was filtered from chitosan and purified by using methanol and water concentrations of 80% v / v (using 8 ml of ethanol with 10 ml of water) and 2x with alcohol. After that, it was the freeze dry stage.

At the hydrogel formation stage, initially each sample was formed by using a mixture of hyaluronic acid: chitosan solution at a ratio of 30: 10 mg / ml. Dry samples from Hyaluronic Acid were dissolved with normal saline at a concentration of 30 mg / ml to become clear. Dry samples from chitosan were dissolved with normal saline at a concentration of 10 mg / ml into a yellow solution. The hyaluronic acid, chitosan, and AgNPs solution was divortex thus the homogeneous solution became turbid and formed a gel-like lump.

Characterization

Fourier Transform Infrared (FTIR)

Hyaluronic acid-chitosan / AgNPs composite hydrogel samples in gel form were mixed with KBr powder and placed on a platinum pan that could be penetrated by infrared light.

Swelling Test

Swelling test was used to measure the amount of sample absorption that was tested through immersion of the sample. The swelling test used the gravimetric method.

X-Rays Diffraction

Analysis of the XRD test results obtained through the software Match! Examination of the AgNPs formation was conducted by using

analysis of the Joint Committee on Powder Diffraction Standards (JCPDS) cards produced, where the JCPDS card for $AgNO_3$ samples showed 2θ of 38,128 with crystal lattice (111) which meant that the crystal lattice was one of the lattices of AgNPs with cubic crystal structure system.

Particle Size Analyzer (PSA)

PSA (Particle Size Analyzing) test was used to measure the particle size and size distribution of silver nanoparticles (AgNPs) that have been formed. Analysis of PSA test data obtained through Zetasizer software to determine the size of the formed AgNPs. A material can be said as nano material if a large range of particle size was <100nm.

Degradation Test

The degradation test was used to determine the degradation ability of the hydrogel sample which was tested through immersion of the sample into a solution that represented intraperitoneal physiological conditions, namely Phosphate Buffer Saline (PBS) solution. This degradation test was performed by preparing 1 ml of each test sample as a Wo value (initial weight before immersion). Then, put it into the test media and added 10 ml of PBS solution. After that, the media performed the incubation process with a temperature of 37 °C for 1, 3, 5, 7, and 9 days.

Anti-bacterial Assay

Antibacterial test using agar media made from the dissolution of 4 grams of Tryptic Soy Agar (TSA) and 100 ml of distilled water performed autoclave for 15 minutes, 121°C, and incubated for 24 hours. On the other hand, isolate bacteria with gram-positive bacteria, Staphylococcus Aureus, were activated first by mixing one bacterial eye in a 2.5 gram Tryptic Soy Broth (TSB) solution and 100 ml of distilled water which was incubated for 24 hours. Then, the formation of bacterial colonies was performed by rubbing the TSB solution with the eye of the bacteria that has been grown into agar media. After that, the finished bacterial colony was mixed with 10 ml of 0.9% NaCl to form a solution with a turbidity level of 0.5 Mc Farland. Next, the solution was flattened to the agar medium and the hydrogel sample was placed on top then incubated for 24 hours. The final result was a clear zone formed around the sample.

Results

X-Rays Difraction (XRD)

Figure 1 and Figure 2 that was given a green circle were the XRD results of AgNPs formation from $AgNO^3$ solution. Table 1 and Table 2 showed the value of 2θ AgNPs and the crystal lattice index Ag.

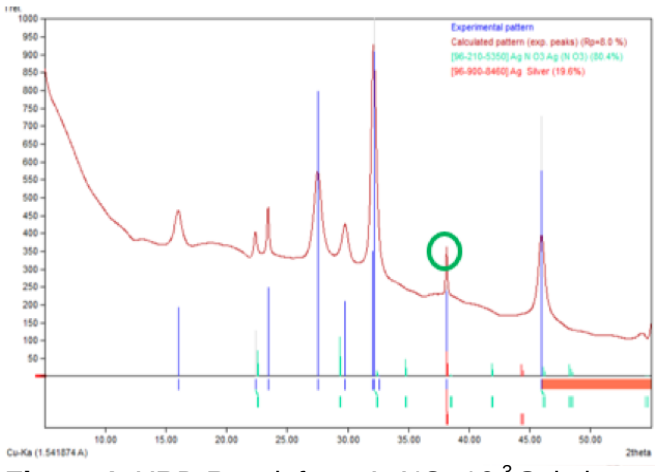

Figure 1. XRD Result from $AgNO_3$ 10^{-3} Solution.

2θ	Index	Sample
38.14	(111)	Ag (*Silver)*

Table 1. 2θ value, Index, and Sample of AgNPs from $AgNO_3$ 10^{-3} Solution.

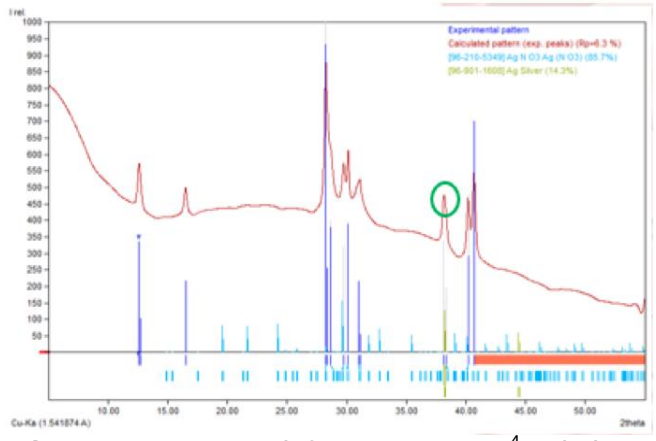

Figure 2. XRD Result from $AgNO_3$ 10^{-4} Solution.

2θ	Index	Sample
38.14	(111)	Ag (*Silver)*

Table 2. 2θ Value, Index, and Sample of AgNPs from $AgNO_3$ 10^{-4} Solution.

Particle Size Analyzer (PSA)

The samples tested were AgNPs samples from Curcuma longa biosynthesis products.

- PSA results from $AgNO_3$10-3 M solution:
- PSA results from $AgNO_3$ 10-4 M solution:
- PSA results from 10-5 M AgNO3 solution:

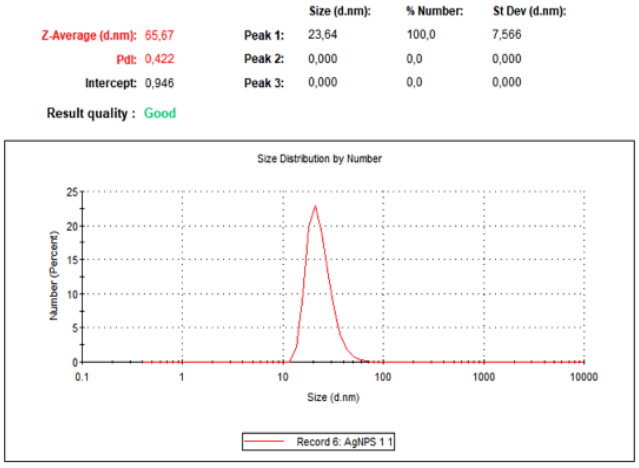

Figure 3. Distribution of $AgNO_3$ 10^{-3} M Size.

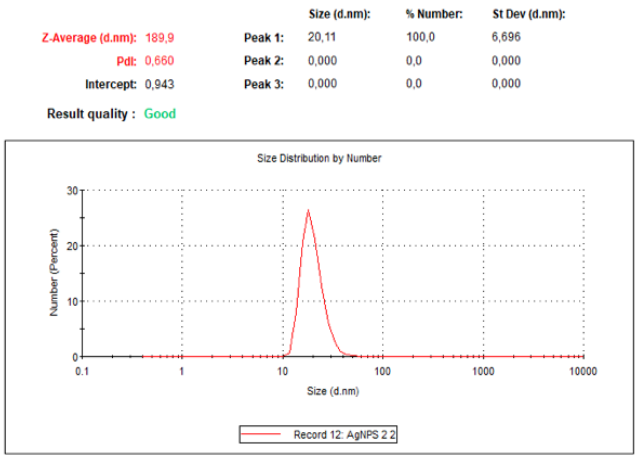

Figure 4. Distribution of $AgNO_3$ 10^{-4} M Size.

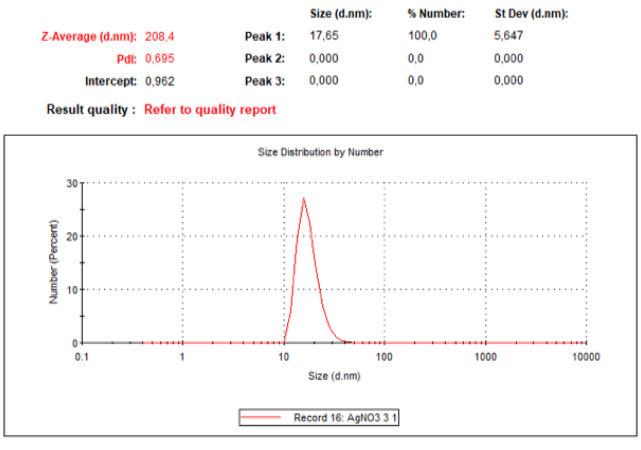

Figure 5. Distribution of $AgNO_3$ 10^{-5} M Size.

Figure 3, Figure 4, and Figure 5 showed that the particle sizes formed from the three different variations of $AgNO_3$ concentration were all in the size range <100nm.

Fourier-transform Infrared Spectroscopy (FTIR)

FTIR test results can be seen through Figure 6 for a sample of 0 M $AgNO^3$ solution and Figure 7 for a 10-3 $AgNO^3$ solution sample.

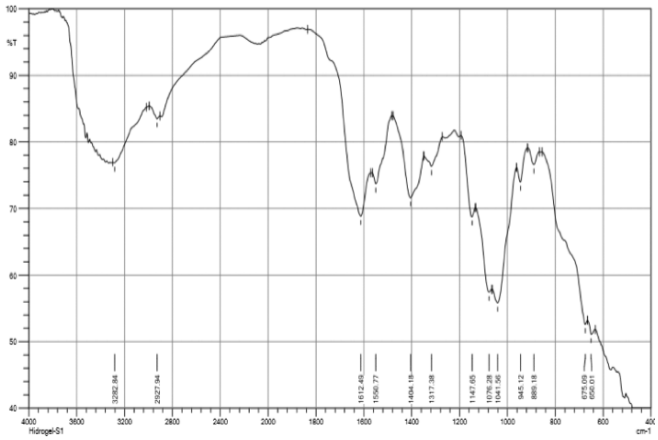

Figure 6. FTIR Result from $AgNO_3$ 0 M Solution.

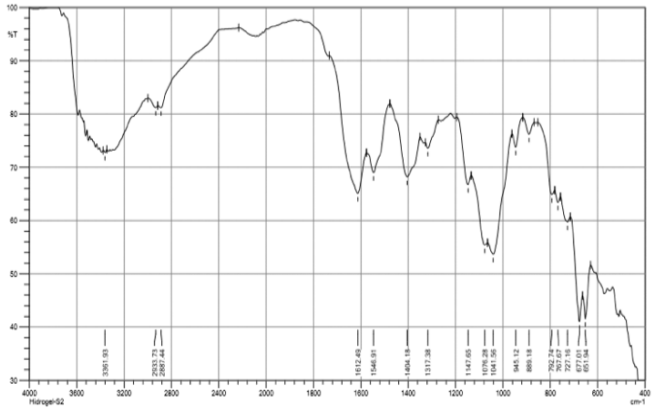

Figure 7. FTIR Result from $AgNO_3$ 10^{-3} Solution.

Swelling Test

Table 3 showed the results that the higher the concentration of $AgNO_3$, the more increasing the swelling ratio. It means that the increase in $AgNO_3$ concentration was in line with the increase / number of AgNPs content formed in accordance with observations from the previous XRD test results.

Sample	Mean (%)
F0	124.99 ± 14.66
F1	209.72 ± 1.13
F2	196.50 ± 3.53
F3	151.85 ± 6.63

Table 3. Mean of Swelling Percentage.

Degradation Test

Table 4 showed that each sample produced a different rate of degradation which the degradation process that occurs was not a process of degradation that occurred quickly but gradually.

Sample	Mean				
	1	3	5	7	9
F0	11.88 ± 3.26	20.64 ± 1.46	42.81 ± 3.44	64.44 ± 0.32	81.64 ± 1.01
F1	20.67 ± 6.43	31.89 ± 5.27	60.01 ± 5.23	81.06 ± 2.01	91.56 ± 9.08
F2	15.70 ± 3.98	28.15 ± 4.73	57.63 ± 1.57	76.41 ± 0.81	89.18 ± 0.96
F3	13.34 ± 4.19	23.37 ± 1.16	55.94 ± 5.03	74.14 ± 2.54	86.55 ± 3.10

Table 4. Mean of Weight Lost Percentage (Wt%).

Antibacterial Test

Table 5 explained that the higher the concentration of AgNO3, the greater the diameter of the clear zone formed.

Sample	Clear Zone	Bacterial Inhibitory Power
F0	4.98 mm	Resistent
F1	10.39 mm	Sensitive
F2	10.2 mm	Sensitive
F3	7.75 mm	Less sensitive

Table 5. Mean of Clear Zone Antibacterial Test.

Discussion

The hydrogel as physical barrier could be considered as the prevention strategy for intraperitoneal adhesion. 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.[10] It is made of hyaluronic acid (HA) which is commonly used as barrier for adhesion prevention due to its non-toxic character.[11] However, HA-based hydrogel often exhibits weak mechanism with rapid degradation behaviour that decreases its effectiveness to prevent adhesion. They synthesized hydrogel by modifying and characterizing the composition variation of chitosan on hyaluronic acid 4 Based on the research of Witantri R et al[12] which used hyaluronic acid and chitosan at a ratio of 30:0, 30:20, 30:30, and 30:40 mg/ml, it showed that 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 concentration of chitosan, the lower degradation rate is, because chitosan concentration positively correlates the likelihood of crosslinking between amine group of chitosan and aldehyde group of hyaluronic acid.[12]

Figure 1 and Figure 2 illustrate the XRD results of $AgNO^3$ solution that shows the crystal lattice (111) is the most dominant crystal lattice in AgNPs formation. This shows that the synthesized AgNPs are highly crystalline. Besides that, the crystal lattice becomes more dominant as an increase in the concentration of $AgNO^3$ precursors which means it shows an increase in the number of biosynthetic AgNPs 9 where in addition to being viewed than AgNPs particle size, the presence of crystal lattice (111) from AgNPs is a major area which also contributes to the strength of antibacterial properties.[13] Table 1 and Table 2 show the value of AgNPs is 38.[14] which is close to the standard silver diffract gram data that is 38.128 with crystal lattice (111). It results that the crystal lattice is one of the lattices of AgNPs with a cubic crystal structure system according to the Match database! Phase Analysis Report.

The analysis data in Figure 3, Figure 4, and Figure 5 show that the AgNPs formed are at most 21 nm at 23% for the $AgNO^3$ PSA yield of 10-3M, 18.1 nm at 26.4% for the PSA $AgNO^3$ result at 10-4 M, and 15.6 nm at 27.2% for the PSA $AgNO^3$ 10-5 M. This shows that the higher the concentration of $AgNO^3$, the greater the particle size. This is due to the high concentration

of $AgNO^3$ causing the increasing amount of Ag + that must be reduced. Figure 6 and Figure 7 show the carboxymethyl group (a typical group showing NOCC formation) giving rise to OH absorption at a wave number around 1404.18 cm-1 resulting in -CH2COOH. There is a -C=O carbonyl group at wave number 1612.49 cm^{-1}. The aldehyde group is located at wave number 2887.44 cm^{-1}.

Table 3 states that the addition of silver metal yield an increase of hydrogel swelling properties due to silver metal ability to adhere to hydrogel which caused by increased porosity and empty space between the hydrogel network. Hence, it can make hydrogel capable to absorb more lots of water. Chitosan based hydrogel showed higher porosity and swelling degree.[14] The swelling properties of porous hydrogels are also strongly influenced by the ratios of components.[15] Table 4 shows the result with higher concentration of $AgNO^3$, the higher rate of degradation in line with increase $AgNO^3$ concentration is in accordance with the increase / number of AgNPs content formed as can be observed from the previous XRD test results. This phenomena due to the ability of $AgNO^3$ to induce oxidative stress as indicated by histochemical staining of superoxide radical and hydrogen peroxide that was manifested in terms of DNA degradation and cell death.[16]

Table 5 shows that the sample has better inhibitory properties against bacteria. It reveals the increase of $AgNO^3$ concentration which in line with the increase / number of AgNPs content in accordance with the observations from the previous XRD test results. The results of this study are in accordance with the results of study from Ferfera-Harrar et al 9 which states that the higher the concentration of $AgNO^3$, the greater percent reduction of bacteria formed. It could be stated that the properties are sensitive to bacteria.

Conclusions

The optimal composition of AgNO3 concentration used for hydalurel hyaluronic acid-chitosan / AgNPs Biosynthetic products of Curcuma longa as a preventive agent intraperitoneal adhesion is 10-3 M.

Acknowledgements

The authors thank to the Institute of Tropical Disease for all facilities.

Declaration of Interest

The authors report no conflict of interest.

References

1. Song L, Li L, He T, et al. Peritoneal adhesion prevention with a biodegradable and injectable N,O-carboxymethyl chitosanaldehyde hyaluronic acid hydrogel in a rat repeated-injury model. Sci Rep 2016; 6:37600.
2. Chen CH, Chen SH, Mao SH, et al. Injectable Thermosensitive Hydrogel Containig Hyaluronic Acid and Chitosan as a Barierr for Prevention of Postoperative Peritoneal. Carbohydr Polym 2017; 173:721-731.
3. Arung W, Michel M, dan Olivier D. 2011. Pathophysiology and Prevention of Postoperative Intraperitoneal Adhesions. World J Gastroenterol 2011;17(41): 4545–53.
4. Li L, Wang N, Jin X, et al. Biodegradable and Injectable in Situ Cross-Linking Chitosan-Hyaluronic Acid Based Hydrogels for Postoperative Adhesion Prevention. Biomaterials 2014; 35(12):3903-17.
5. Sahbaz A, Isik H, Aynioglu O, Gungorduk K, Gun BD. Effect of Intraabdominal Administration of Allium Sativum (Garlic) Oil on Postoperative Peritoneal Adhesion. Eur J Obstet Gynecol Reprod Biol 2014; 177: 44–47.
6. Evlyn Anggraini Santoso, Prihartini Widiyanti, Fulky A'yunni, Fathania Nabilla,Novita Putri Rahayu, Adita Wardani Rahmania. Citric Acid-Polyurethane-Chitosan-Based Innovative Biocomposites as Candidates of Antibacterial Dialyzer Membrane. Journal of International Dental and Medical Research 2018; 11(2) : 718-722
7. Imroatus Solikhah, Prihartini Widiyanti, Aminatun. Composition Variation of Chitosan-Gelatine Scaffolds with Glutaraldehyde Cross linker for Skin Tissue Engineering in Burn Wound Cases. Journal of International Dental and Medical Research 2018; 11(3) : 778-785
8. Reem Hassan Ahmed, Damra Elhaj Mustafa.Green synthesis of silver nanoparticles mediated by traditionally used medicinal plants in Sudan.International Nano Letters 2020; 10 : 1–14
9. Ferferra-Harrar H, Dalila B, Tayeb B. Hydrogel Nanocomposites Based on Chitosan-Gpolyacrylamide and Silver Nanoparticles Synthesized Using Curcuma loga for Antibacterial Applications. Polymer Bulletin 2018 75(7): 2819–2846.
10. Sakai S, Ueda K, Taya M. Peritoneal Adhesion Prevention by a Biodegradable Hyaluronic Acid-Based Hydrogel Formed In Situ Trough a Cascade Enzyme Reaction Initiated by Contact with Body Fluid on Tissue Surfaces. Actabiomaterialia. 2015;24:152-158.
11. Reijnen MM, Falk P, van Goor H, Holmdahl L. The Antiadhesive Agent Sodium Hyaluronate Increases the Proliferation Rate of Human Peritoneal Mesothelial Cells. Fertil Steril. 2000;74:146-51.
12. Retno Witantri, Prihartini Widiyanti, Jan Ady. Chitosan-Hyaluronic Acid Composite Injectable Hydrogel as Open Postoperative Peritoneal Anti-adhesive Agent. Journal of International Dental and Medical Research 2018; 11(3) : 1130-1136
13. Yixis Z, Dapeng Y, Yifei K, Xiansong W, Omar P, dan Guo G. Synergetic Antibacterial Effects of Silver Nanoparticles@Aloe Vera Prepared via a Green Method. Nano Biomed Eng 2010;2(4):252–257.

14. Ecaterina Stela Dragan, Maria Valentina Dinu. Advances in porous chitosan-based composite hydrogels: Synthesis and applications. Reactive and Functional Polymers 2020; 146 : 104372
15. M.T. Khorasani, A. Joorabloo, A. Moghadam, H. Shamsi, Z.M. Moghaddam, Incorporation of ZnO nanoparticles into heparinized polyvinyl alcohol/chitosan hydrogels for wound dressing application, Int. J. Biol. Macromol 2018 ; 114 : 1203–1215
16. Kanchan Vishwakarma, Shweta, Neha Upadhyay, Jaspreet Singh, Shiliang Liu, Vijay P. Singh, Sheo M. Prasad, Devendra K. Chauhan, Durgesh K. Tripathi,Shivesh Sharma. Differential Phytotoxic Impact of Plant Mediated Silver Nanoparticles (AgNPs) and Silver Nitrate (AgNO3) on Brassica sp. Frontiers in Plant Science 2017; 8 : 1501