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Responsive Calcium (Ca^{2+}) Alginate-Chitosan Based Hydrogel: A Promising Biomaterial for Spinal Cord Injury

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Abstract. Spinal cord injury is damage to the spinal cord which causes lesions in the spinal cord and leads to an increase in extracellular Ca^{2+}. It results in additional neuronal loss which causes temporary/permanent disability or even death. The aim of this study was to determine characteristics and the best composition of alginate – chitosan hydrogel responsive to Calcium (Ca^{2+}) for spinal cord injury. Hydrogel synthesis with its compositions, namely chitosan was dissolved in 0.4% acetic acid, neutralized in pH 7 with 0.5 M NaOH, added some 0.85% NaCl in it, and added 5 alginate variations which were dissolved in 0.85% NaCl, next will centrifugation method. Based on the FTIR test, hydrogel showed stretching vibrations of Chitosan's O–H bonds appeared in 3415.93cm^{-1} wavenumber, while Na groups of alginate isomer appeared in 1413.82 cm^{-1} wavenumber. The results of the cytotoxicity test using the MTT Assay method showed live cell percentage from less than 50% to 52.61% in Sample B and 83.83% in Sample C. The results of the injectability test showed that all samples were injectable with the highest percentage of injectability at 98.283%. The results of the UV-Vis spectrophotometric test showed that all hydrogel samples were able to absorb Ca^{2+}. Hydrogels can be degraded at more than 90% within 14 days. The results of the morphology test (SEM) obtained 84.7-99.6 μm pore sizes.

Introduction

Spinal Cord Injury (SCI) refers to both direct and indirect injury to one's spine and causes lesions in the spinal cord that subsequently elicit neurological disorders, such as temporary/permanent disability or death [1]. Spinal cord injury is a major health problem whose prevalence is relatively low, but its care and rehabilitation attempts require an enormous cost.

Based on its bio-cellular, spinal cord injury is divided into two stages, namely primary and secondary. Primary injury causes cell swelling and lysis, as well as leads to an increase in Ca^{2+} extracellular fluid. Secondary injury (one occurs after the primary one) is caused by over excretion of excitotoxic amino acids, such as glutamate (neurotransmitter excitatory). Excessive activation of glutamate receptors is the beginning of cellular death known as excitotoxicity. An attempt to prevent it can be conducted by removing extracellular fluid of Ca^{2+} to reduce excitotoxicity triggered by glutamate induction [2] research findings reported that excessive Ca^{2+} extracellular fluid can be inhibited by blocking the Na^{+}/Ca^{2+} exchange and reducing neural mortality.

Treatment for spinal cord injury can be conducted through pharmacological therapy, such as corticosteroid and morphine in opioid compounds. However, giving corticosteroid brings about some negative effects for other organs, such as sepsis and bleeding in the stomach. Besides, morphine also possesses suboptimal abilities of Neurotherapy pain. Chronic morphine treatment may even lead to neuron death [3].

Therefore, based on the previous explanation, an alternative treatment to cure spinal cord injuries is essential, namely through biomaterial injection to the trauma site. Injectable hydrogel is adaptable to irregular size and shape of tissue or cell lesions; minimally invasive; able to fill in the void of damaged tissue; and able to mimic the mechanical character of the spinal cord [4,5].

Injectable hydrogels can be synthesized from natural and synthetic polymeric materials. Alginate is a natural polysaccharide normally found in the cell walls of all brown algae species (*Phaeophyceae*). The hydrogel of alginate polymers is a suitable material for the application of neuronal repair in the traumatized spinal cord because it is crosslinked to divalent cations like Ca^{2+} [2]. $CaCl_2$ (*calcium chloride*) is the most commonly used agent for crosslinking process in alginate because it can accelerate the gelation process. Alginate also has been widely used for biomedical applications, due to its low biocompatibility, biodegradability, low cytotoxicity, and inflammatory character [6].

Cellular attachment on the surface of biomaterials is achievable through the adhesion aided by integrins, or through electrostatic interactions between biomaterial surface and cellular membrane. Pure alginate hydrogel exhibits low cell attachment because integrin's and alginate's ions are negative, so they reject each other [7]. Chitosan is an amino polysaccharide containing a positive ion within the amino group, so it is necessary to add it into alginate hydrogel to trigger cellular attachment [2]. Chitosan is employed for hydrogel applications in the biomedical field because it has many advantages such as non-toxic, biocompatible, biodegradable, and mucoadhesive.

Based on McKay research findings, they made injectable hydrogels from alginate composites (0.50% w/v)-chitosan (0.25% w/v)-genipin (0.01% w/v) which demonstrated an ability to absorb Ca^{2+} extracellular fluid. The ability can be seen from significant changes in crosslinking bond structures when it is incubated in $CaCl_2$ agents via rheological tests. Adding chitosan aims to increase matrix attachment's interaction to tissue or cell and bio-absorbable. Adding genipin as natural crosslinking to chitosan with low concentration did not result in a significant effect in the healing process of spinal cord injury. Research synthesized hydrogel from alginate for the healing application of spinal cord injury [4] by implanted it in mouse experiments and clarified the optimization of alginate hydrogel in treating injury; however, the hydrogel is less favourable in the cellular attachment process and easily degradable.

The aim of this study was to determine characteristics and the best composition of alginate – chitosan hydrogel responsive to Calcium (Ca^{2+}) for spinal cord injury. For this research, the alginate-chitosan hydrogel is made by combining alginate composition. It is intended to find out the most optimal alginate concentration to respond to Ca^{2+}. Several characterizations are performed in this research to test the hydrogel, namely Fourier Transform InfraRed (FTIR) test, cytotoxicity test, injectability test, UV-Vis spectrophotometer test, degradation test, and Scanning Electron Microscopy (SEM) test.

Materials and Methods

The materials and equipments that used were sodium alginate, chitosan, 0.85% NaCl, $CaCl_2$, 0.4% acetic acid, 0.5 M NaOH, aquods, artificial cerebrospinal solution (aCSF), and Eriochrome black T (EBT), FTIR (8400 Shimadzu), MTT-Assay ELISA Reader, UV-Vis Spectrophotometer (Shimadzu, UV-1800), and Scanning Electron Microscopy (SEM).

Chitosan was weighed on a 0.75-gram digital balance and then dissolved in 0.4% acetic acid of 6 ml using a magnetic stirrer until it is homogeneous. The homogeneous chitosan solution was neutralized until it reached pH 7 using 0.5 M NaOH. Neutral chitosan solution was then added by 0.85% NaCl until its volume reached 10 ml. The chitosan solution was then incubated for 24 hours at 37ºC. The incubation process aimed to induce the formation of polymerization in chitosan solution. After the incubation process was completed, the dissolved alginate in 0.85% NaCl of 5 ml was added to the chitosan solution and stirred again using a magnetic stirrer until it was homogeneous. The

mixed solution was then inserted into a centrifuge tube to be processed for 30 minutes at 3500 rpm and room temperature (27ºC), then separated the results with the supernatant part. Sedimentation results from this centrifugation process were the formation of a hydrogel. Several hydrogel samples were characterized by FTIR test, cytotoxicity test, and injection test. Several other hydrogel samples were adjoined to 20 mm $CaCl_2$ and were stirred for 30 min using a magnetic stirrer. Those samples also went through the centrifugation process and were separated with supernatant part to be characterized by UV-Vis spectrophotometry test, degradation test, and SEM test [2].

Results

Functional Group Test with FTIR (Fourier Transform Infrared)

The FTIR test is used to determine functional groups contained within the synthesized sample. FTIR test on alginate-chitosan hydrogels samples was carried out from 4000 to 500 cm^{-1}. In this study, the functional group test was performed on all five samples. The spectrum of alginate-chitosan hydrogel is presented in Figure 1.

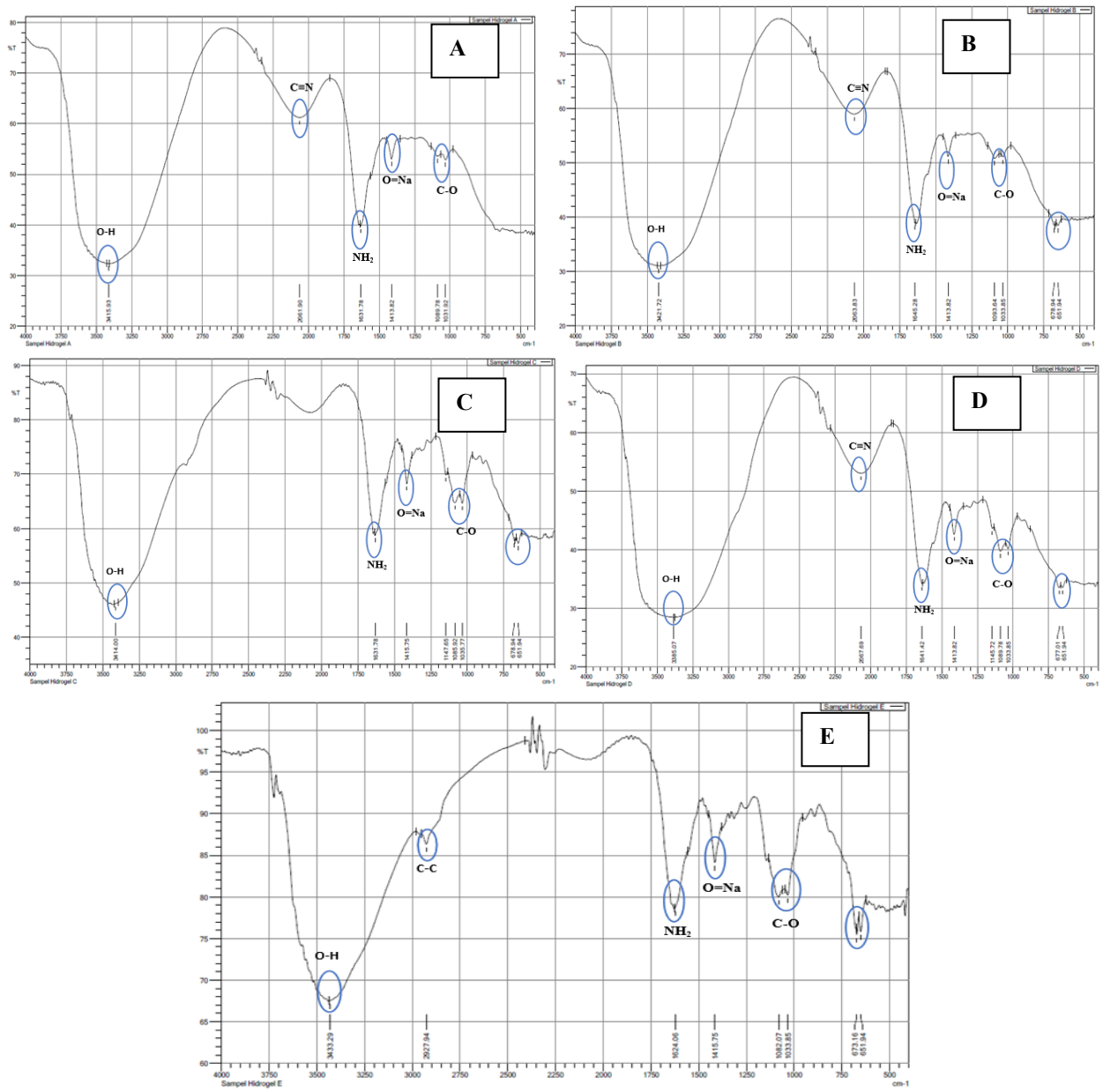


Figure 1. FTIR spectrum results of samples with the composition of alginate : chitosan as follows (a) 0.250: 0.125; (b) 0.375: 0.125, (c) 0.500: 0.125; (d) 0.625: 0.125; (e) 0.750: 0.125

Cytotoxicity Test

The cytotoxicity test aims to determine the direct toxic effects of a substance on cellular structure [5]. In this research, the test was performed using the MTT Assay method and using BHK-21 cell culture (Figure 2).

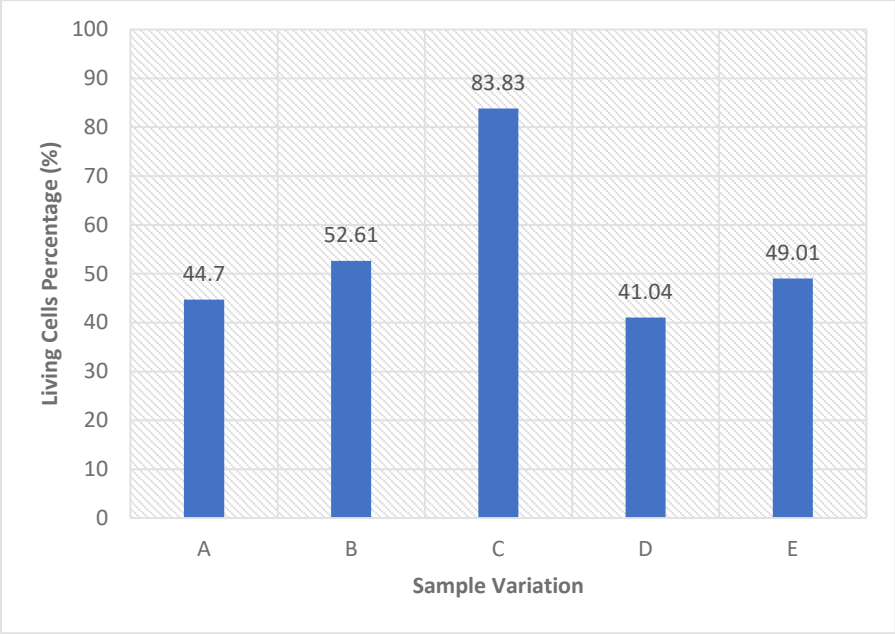


Figure 2. Cytotoxicity test results chart

Injectability Test

Injectability test aims to discover a material's ability with high viscosity to escape from a syringe at a certain time (Figure 3).

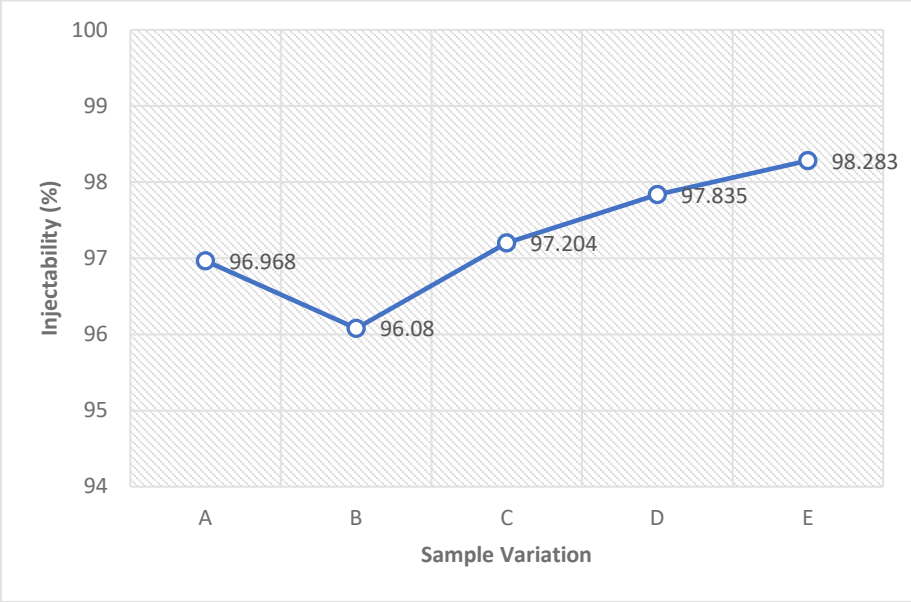


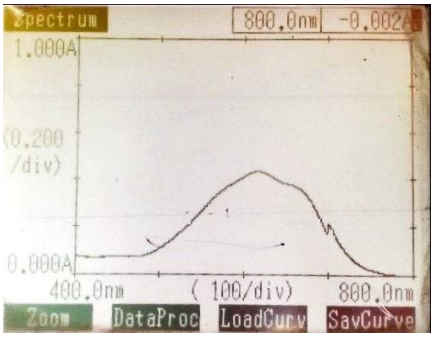
Figure 3. Injectability test results chart

UV-Vis Spectrophotometric Test

This test aims to determine the absorbance of Ca^{2+} responded by the alginate-chitosan hydrogel. The sample used was the supernatant solution resulted from the centrifugation process by mixing alginate-chitosan hydrogel with $CaCl_2$ (Figure 4).

Alginate: Chitosan	Absorbance
0.250%: 0.125% (w/v)	0.266
0.375%: 0.125% (w/v)	0.290
0.500%: 0.125% (w/v)	0.396
0.625%: 0.125% (w/v)	0.435
0.750%: 0,125% (w/v)	0.436

(A)



(B)

Figure 4. (A). UV-Vis spectrum results, (B). Ca^{2+} absorbance rate

Degradation Test

In-vitro degradation test aims to determine the samples' weight loss before and after they were immersed into a physiological solution quantitatively. This test was performed by simulating the samples into the physiological solution found in the medulla spinal area, such as Artificial Cerebrospinal Fluid (aCSF) solution. The solution was incubated at a normal body temperature of 37ºC. This test was carried out over a certain period: 1, 5, 7, and 14 days (Figure 5).

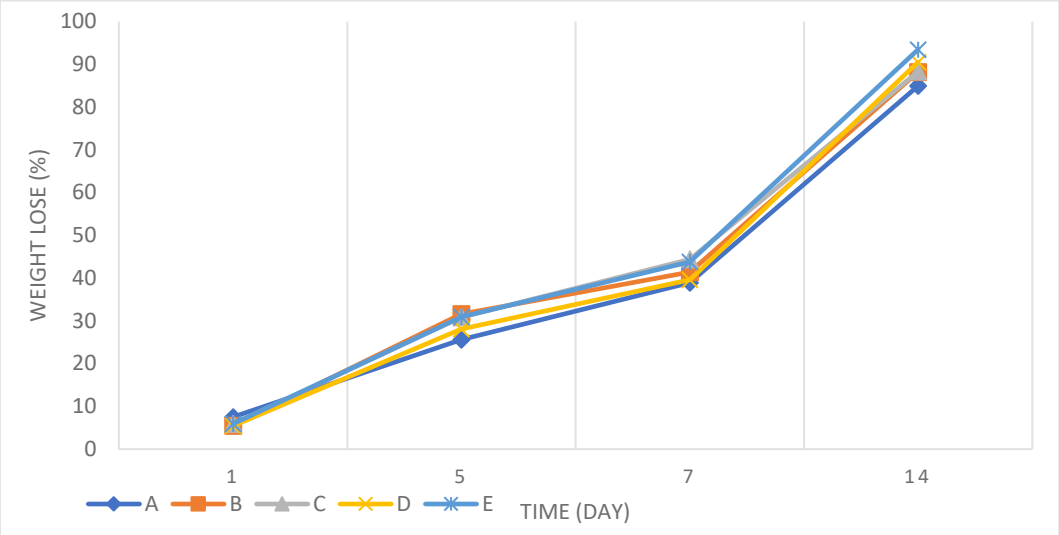


Figure 5. Weight Loss Percentage over Degradation Period

Scanning Electron Microscopy (SEM) Test

Morphological structure analysis of alginate-chitosan's hydrogel surface was conducted through SEM (Scanning Electron Microscopy) test. In this study, the hydrogel surface was represented by Sample C (ratio composition of alginate: chitosan 0.500: 0.125) only based on the best result of the cytotoxicity test. Sample C (ratio composition of alginate: chitosan 0.500 : 0.125) also recorded a good value in the injectability test, UV-Vis spectrophotometry test, and degradation test (Figure 6).

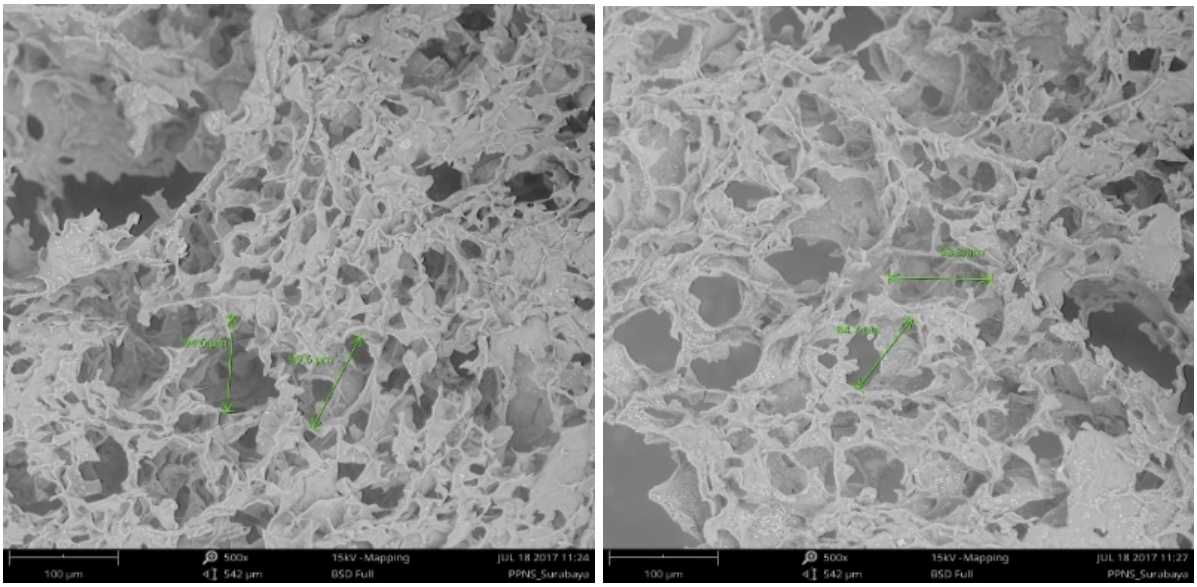


Figure 6. Scanning electron microscopy test result on hydrogel with ratio composition of alginate: chitosan 0.500: 0.125

Discussion

Functional group test results using Fourier Transform Infra-Red (FTIR) illustrated that alginate-chitosan hydrogel contained a functional group representing its constituent materials. For chitosan, amine function groups ($-NH_2$) appeared at the wavenumbers of each sample 1631.78; 1645.28; 1631.78; 1641.42; and 1624.06 cm^{-1} [8]. While the formation of alginate emerged at a wavenumber of 1413.82; 1413.82; 1415.75; 1413.82; and 1415.75 cm^{-1} which characteristically illustrated the Na group in alginate isomer [9] and proved that only physical interaction occurred.

Moreover, there were other chitosan peaks within the hydroxyl groups (-OH) range of 2700-3800 cm^{-1} waves in the form of widening bands due to the presence of hydrogen bonds between molecules. The other alginate peaks appeared on the wavenumber 2927.94 cm^{-1} as an aliphatic C-C functional group; carbonyl functional group (C=O) within 1650-1600 cm^{-1} wave range as an aromatic group. Meanwhile, the C-O bond emerged at a wavenumber of 1300-1000 cm^{-1} and an absorption peak occurred at wavelength area of 673.16 cm^{-1} and 651.94 cm^{-1}, indicating fingerprint area.

The cytotoxicity test results as seen in Figure 2 showed the highest average viability was obtained in Sample B and C with average values of viability at 52.61% and 83.83%. While the other three samples (A, D, and E) were found toxic, as they recorded below the standard rate of maternal toxicity. A material is successfully deemed as non-toxic if the percentage of living cells is more than 50% [10]; therefore, it can be concluded that two out of five samples were not toxic and safely applicable to the human body. The A, D and E samples' low living cells was because of two factors: First, the higher volume compared with B and C samples making the cells physically damaged while it's being poured to a microwell plate; Second, the multiple clean-up process can also wash out the living cells, which making Elisa Reader unable to give a good result [11].

Due to injectability test results of Figure 3, it showed that the five hydrogel samples of chitosan-alginate possessed an excellent injectability capability close to 100% [12] and the resulting force did not exceed 100 N. Thus, measuring it with human strength, this alginate-chitosan hydrogel material is feasible to use [9]. The reason why it could not achieve a 100 % rate was that there were some residues on the syringe while being injected, factorized by the high viscosity structure of the samples [13]. The higher the viscosity of the samples created a viscous fluid, which made the samples unable to be fully injected through the syringe [14].

Based on UV-Vis spectra of Figure 4 aimed to discover the responsiveness of alginate-chitosan hydrogels in absorbing Ca^{2+}, it can be deduced based on the table above that alginate-chitosan

hydrogel samples were able to absorb Ca^{2+}. Associating it with trauma media of spinal cord (Ca^{2+} extracellular fluid), the higher absorbance rates the more capable alginate-chitosan hydrogel to respond Ca^{2+} content will be [15]. In normal condition, the concentration of Ca^{2+} extracellular liquid could be 1 mM, but on trauma, it can be increased to be 6mM [2]. Based on the test, it can be understood that the hydrogel of alginate-chitosan can absorb Ca^{2+} in accordance to the traumatic media from the medulla spinalis, where the higher the absorbance capability, the hydrogel to respond to the Ca^{2+} will also be increased. This is because the Ca^{2+} ion on $CaCl_2$ can interact with the carboxyl (-COO-) and cross-banded to the alginate molecules. The higher the concentration of alginate, the chemical bond will be more stable, because there will be no free electrons and the possibility of the cross will also be higher [15].

Based on daily observations as seen in Figure 5, there were some physical changes of hydrogel materials. The changes were marked by indirect and gradual processes. This study obtained degradation test respectively from Sample A, B, C, D, and E at 84.94%, 88.19%, 88.29%, 90, 51%, and 93.45% on the 14^{th} day.The interaction with ACSf illustrated that alginate hydrogels were able to degrade faster than alginate-chitosan-genipin's hydrogel at approximately 95% within 14 days; while alginate-chitosan-genipin's hydrogel composites were degraded less than 90% within 14-21 days [2].

The measurement results of pore diameters using SEM as seen as in Figure 6 showed it was obtained that Sample C (ratio composition of alginate : chitosan 0.500 : 0.125) diameter pores were recorded within 84.7-99.6 μm. This pore size was almost identical to the pore size obtained by McKay CA et al [2] which recorded that alginate-chitosan hydrogen mixed with genipin (crosslinker) was obtained within the range of 100 μm. This small difference was caused by genipin crosslinking's nature of the chitosan which resulted in larger pore size and influenced pore distribution number. The addition of chitosan to alginate hydrogels unquestionably influences with the numer of astrocytes attached to hydrogel surface as well as affects the event of astrocyte colony [2]. Cellular attachment to a biomaterial surface may occur through incorporation of integrin-mediated focal adherence complexes or materials that empower electrostatic interactions between the surface and the cellular membrane. However, due to the inadequacy of intrinsic integrin binding sites and its negatively charged character, unmodified alginate exhibits low cellular attachment [7]. Chitosan, an amine-containing, positively charged polysaccharide polymer has been used to modify hydrogel charge within an agarose/methylcellulose hydrogel blend to promote cellular attachment [16]. Alginate-chitosan hydrogel has viscoelastic behaviour which can reduce impact transmitted to spinal cord. The linear viscoelastic limit (LVE) of a viscoelastic material is characterized as the highest strain value that can be applied to a material before showing change in elastic modulus. In term of hydrogel materials, the magnitude of LVE limit is related with structural stability of material, the changeover from solid to liquid like phase and material break down. An alteration in the magnitude of LVE limit express an alteration in internal cross-linking structure of hydrogel material [2]. We can adjust or arrange the ratio composition and crosslink condition in hydrogel in order to obtain the best characteristic of hydrogel. We can adjust all the concentration or ratio composition, adding some additive / reinforcement/ crosslinker agent to optimize hydrogel characteristic. We can determine the type of material and the composition/ concentration in order to obtain hydrogel which can functionate correctly but do not yield unwanted compression. Alginate has polyelectrolyte complexes [17]. This polyelectrolyte can change hydrogel mechanical behaviour by advancing chain entanglements and diminishing structural cross linking [2].

Conclusions

Alginate-Chitosan hydrogel's characteristics for spinal cord therapy is indicated the presence of amine function group ($-NH_2$) which represented chitosan, while alginate was represented the emergence of Na groups within alginate isomer, safe to be applied on the human body, able to inject, degradation rate increased along with the addition of alginate concentration, the pore sizes were

approaching the standard size to be applied to spinal cord injuries' patients, and were capable of absorbing Ca^{2+}. The best hydrogel composition is ratio composition of alginate : chitosan 0.500 : 0.125).

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Conflict of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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