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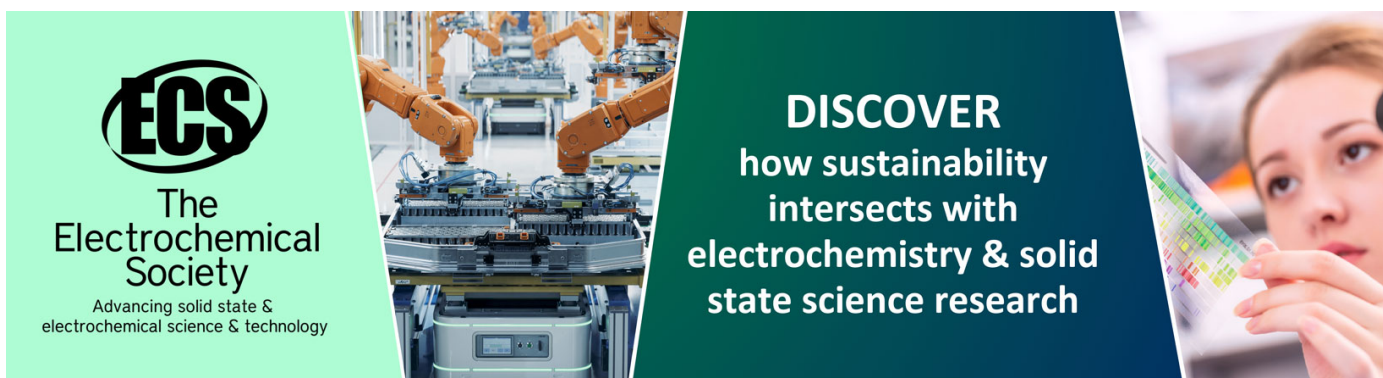
Hyaluronic Acid (HA)-Polyethylene glycol (PEG) as injectable hydrogel for intervertebral disc degeneration patients therapy

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Hyaluronic Acid (HA)-Polyethylene glycol (PEG) as injectable hydrogel for intervertebral disc degeneration patients therapy

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Abstract. Chronic Low Back Pain (CLBP) is one health problem that is often encountered in a community. Inject-able hydrogels are the newest way to restore the disc thickness and hydration caused by disc degeneration by means of minimally invasive surgery. Thus, polymers can be combined to improve the characteristic properties of inject-able hydrogels, leading to use of Hyaluronic Acid (a natural polymer) and Polyethylene glycol (PEG) with Horse Radish Peroxide (HRP) cross linker enzymes. The swelling test results, which approaches were the ideal disc values, were sampled with variation of enzyme concentrations of 0.25 $\mu\text{mol}/\text{min}/\text{mL}$. The enzyme concentrations were 33.95%. The degradation test proved that the sample degradation increased along with the decrease of the HRP enzyme concentration. The results of the cytotoxicity assay with MTT assay method showed that all samples resulted in the 90% of living cells are not toxic. In vitro injection, models demonstrated that higher concentration of the enzymes was less state of gel which would rupture when released from the agarose gel. The functional group characterization shows the cross linking bonding in sample with enzyme adding. The conclusion of this study is PEG-HA-HRP enzyme are safe polymer composites which have a potential to be applied as an inject-able hydrogel for intervertebral disc degeneration.

1. Introduction

Chronic Low Back Pain (CLBP) is one of some health problems, which often encountered in a community. Almost everyone experiences low back pain regardless of age and gender differences. Approximately 60-80% of the world's population has ever experienced low back pain at least once during their lifetime (lifetime prevalence). Pain Study Group of PERDOSSI (Nerve Specialist Doctors Association of Indonesia) conducted a study in 14 teaching hospitals in May 2002. The results showed that the incidence of LBP included 18.37% of all pain cases that had ever been handled. The primary causes of low back pain vary, one of which is intervertebral disc degeneration [1].

There are different ways to cure lower back pain, such as conservative measures (bed rest, anti-pain medication, anaesthesia, medical rehabilitation) and surgical (operative) measures. Total disc replacement surgery can only be performed when conservative therapies fail to overcome the severe pain which resulting limited movements and pain attacks that would occur repeatedly to the patients.



Complications, caused by the operative measure, include allergies, infection, the increase of motoric deficit, and the failed back syndrome [2]. Inject-able hydrogel is the newest way to restore the disc thickness and hydration caused by disc degeneration by means of minimally invasive surgery [3]. Inject-able hydrogel is easily formed, fills the defect perfectly, and has good permeability [4].

Frith *et al* in 2013 used the enzyme horse radish peroxide (HRP) catalysed in polysaccharides and it was proven to increase the degradation rate linear with the increasing of the concentrations of the enzyme. Tyramine, as a conjugate polymer, was added in the reaction between HRP and polysaccharides, and the HRP catalysed phenol and aniline by tying H_2O_2 chain [5]. This study synthesized Hyaluronic Acid (HA) and Polyethylene Glycol (PEG) by using enzymatic cross linking method by adding enzymes to speed up the formation of the hydrogels and to increase the degradation rate of the hydrogels. The characterization used was the swelling test, degradation test, cytotoxicity assay, an in vitro study of injection models, and functional group test.

2. Materials and methods

2.1. Materials

The materials used were Hyaluronic Acid (HA) 215 KDa, Polyethylene Glycol (PEG) 10000 KDa, enzyme Horse Radish Peroxide (HRP), and tyramine hydrochloride, which derived from Sigma Aldrich. This research also used 3-4-hydroxyphenyl Propionic Acid (HPA) from Sigma Aldrich, Dimethyl Aminopropyl-ethyl carbodiimide hydrochloride (EDC), Sulpho-Hydroxysuccinimide (NHS), MES buffer, NaOH, distilled water, a solution of H_2O_2 , Phosphate Buffered Saline (PBS), and agarose.

2.2. Variables

The control variable of this research is concentration of HA (15 gram) and PEG (16,5 gram). The released variable is concentration of HRP enzyme (0 ; 0,15 ; 0,25 ; 0,35 ; 0,45 $\mu\text{mol}/\text{min}/\text{mL}$). While the dependent variable is characterization of *inject-able hydrogel*.

2.3. Methods

The first solution which was prepared as a stock solution was Hyaluronic Acid (HA) and Tyramine Hydrochloride (Tyr) mixed with a 1:10 ratio in 0.1 MES of Buffer. After that, it was added with Aminopropyl Dimethyl-Ethyl Carbodiimide Hydrochloride (EDC) and Sulpho-Hydroxysuccinimide (NHS) with a composition of 1 M of NHS and 10 M of EDC. The HA-Tyr solution of 1 M of NaOH solution was added to increase the pH from 4.0 to 6.0 and reacted at room temperature with gentle agitation afterwards. Finally, a solution of HA-Tyr was freeze-dried.

The second solution which was prepared as a stock solution was 3-4-hydroxyphenyl Propionic Acid (HPA) dissolved in 0.1 M MES Buffer, added with 50 μM of NHS and 500 μM of EDC to be reacted. Furthermore, Polyethylene Glycol (PEG) was dissolved in 0.1 M of MES Buffer. After all of the solutions were prepared, HPA was reacted with PEG at room temperature with gentle agitation. In the last of it, the solution of PEG-HPA was freeze-dried.

The Freeze-drying result of HA-Tyr was reacted with PEG-HPA in a micro tube. The Horse Radish Peroxide enzyme (HRP) was added to a solution of Phosphate Buffered Saline (PBS) and the concentration ratio which was varied in accordance with the ratio of HA: PEG: HRP (gram: gram: $\mu\text{mol}/\text{min}/\text{mL}$), namely 15: 16.5: 0 ; 15: 16.5: 0.15; 15: 16.5: 0.25; 15: 16.5: 0.35; and 15: 16.5: 0.45. The solution was mixed with a vortex. Then, 2.5 mM of H_2O_2 was added for gelation, and the vortex was performed.

3. Results and discussion

On this research, there has been successfully synthesized hydrogel made from Hyaluronic Acid (HA) and Polyethylene Glycol (PEG) by the method of enzyme using enzymatic cross linking Horse Radish

Peroxide Type I. Samples carried out five variations of composition of enzymes(Figure1), (a) HA-PEG-0 HRP, (b) HA-PEG-0.15 HRP, (c) HA-PEG-0.25 HRP, (d) HA-PEG-0.35 HRP, and (e) HA-PEG-0.45 HRP. And thus the hydrogel is white.

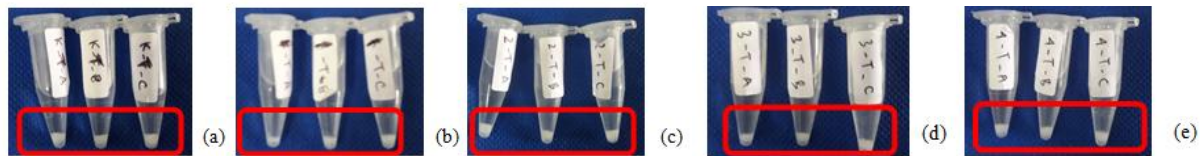


Figure 1.Hydrogel Research Results

3.1. Swelling test

In the swelling test, hydrogels were freeze-dried, and then the initial weight of all samples were weighed and soaked in a solution of Phosphate Buffer Saline (PBS) for 24 hours to determine the ability of the material to absorb the PBS solution. Inject-able hydrogels that were qualified to be applied to the intervertebral disc ranged from 21-27% to meet the requirements of occupying disc defect and that it would not spread to the avascular tissues around the disc nucleus pulposus (Firth *et al*, 2013). The result in Figure 2, obtained after weighing the samples, was that those three samples demonstrated decreasing swelling graphs when the concentration of HRP enzyme increased. The result that was nearly ideal was the concentration of the enzyme at 0.25 $\mu\text{mol}/\text{min}/\text{mL}$ was 33.95%.

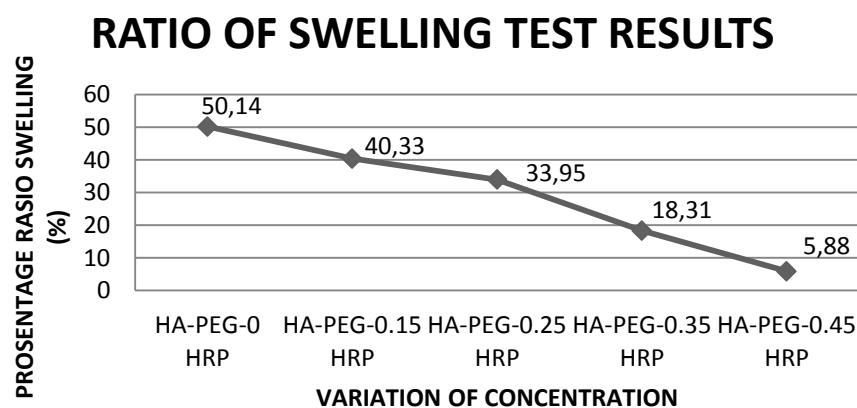


Figure 2. Line Graph of Swelling Test Results

3.2. Degradation test

Hydrogel in degradation testing PBS during the time period 1, 7 and 14 days in vitro simulation was done as for the observed degradation hydrogel in body fluids on the physiological PH with normal body temperature; 37°C. Soaking solution of PBS is the simulation of bioactive material. Based on data analysis (Figure 3), the best results are in a composite sample of HA-PEG-0.45 HRP. The rate of degradation is decreased if there is addition of enzyme concentration cross linking bonds due to the presence of hydrogel. This sample has a steady degradation rate and total degradation during long 8.5 weeks (2.5 months). Long degradation of total on this sample varies with the time of maximum 3-month hydrogel according to Firth *et al* (2013) due to the difference in the use of the polymer on the research. In the previous research used, the polymer molecular weight was PEG 40000 KDa, where as on this research, used molecular weight was PEG 10000 KDa. This caused a difference in the strength of the bond that formed between polymers and enzymes. The larger molecular weights polymer, then

ties the chain which is formed and will be getting longer, so the bond with the other ingredients will be more powerful and hard-degraded [6].

DEGRADATION TEST RESULT

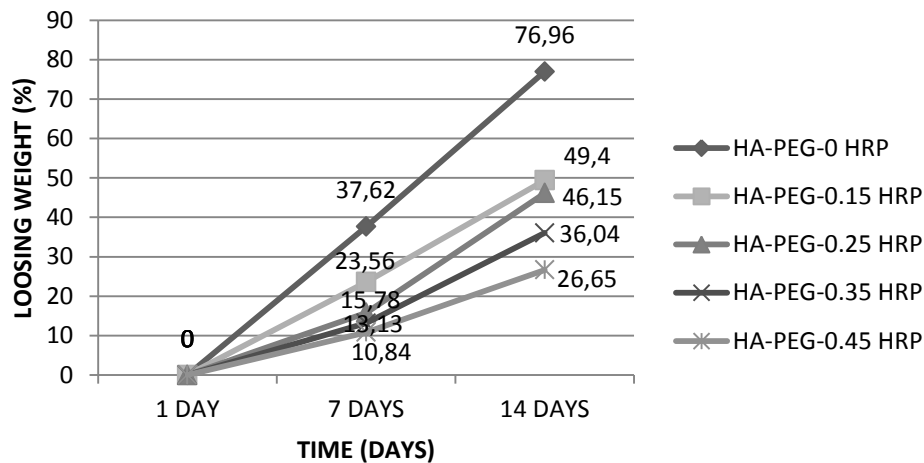


Figure 3. Line Graph of Degradation Test Results

3.3. Cytotoxicity assay

Cytotoxicity assay using [3-(4,5-Dimethylthiazol-2-Yl) -2,5-Diphenyltetrazolium Bromide] method (MTT Assay) with liver cells Hepatosite (Dhuha-7). Samples were incubated at Dhuha-7 cells in Eagle medium for 24 hours, and then, the absorbance wavelength used ELISA Reader.

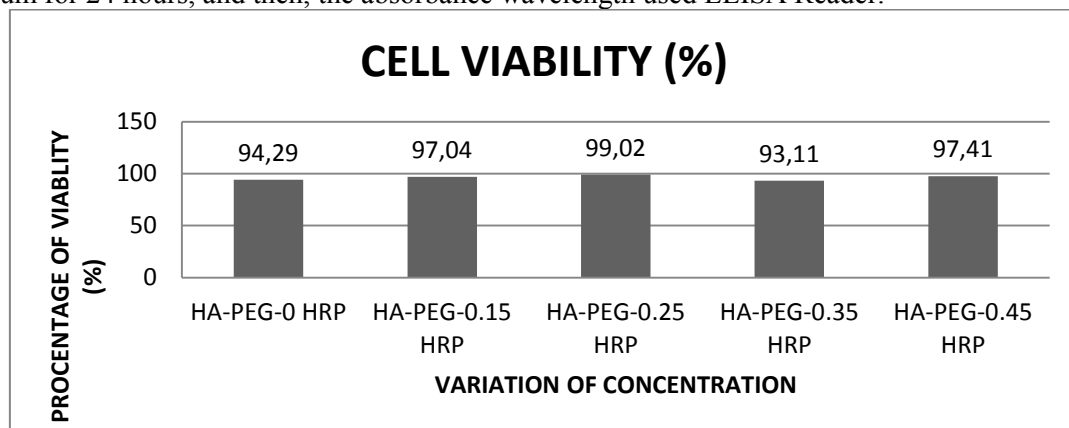


Figure 4. Bar Graph of Cytotoxicity Assay Results

From the results of cytotoxicity testing (Figure 4), they against the samples of all the variations, the percentage of live cells obtained above 90%, the highest living cell was found in HA-PEG-0.25 HRP which was 99.02% and the lowest living cell about 93.11% when concentration of HRP was 0.35 $\mu\text{mol/min/mL}$. The results showed that all types of sample hydrogel are not toxic because it was far above the limit of the number of toxic substances, i.e. 50% [7]. In the sample, the covalent bond intermolecular with phenol polymer cluster which does not cause damage to living cells mitochondria so that resulting test results proving the old purple creature compounds are not toxic.

3.4. In vitro injection model

The testing of In Vitro Injection Model was qualitative to see the changes of hydrogel shapes before and after being put into agarose as an intervertebral disc simulation [8]. The result shows the following in Figure 5 which are hydrogel when injected into the agarose will (a) occupy the space of agarose or (b) a portion of the whole, while the form of hydrogel when released from the agarose hydrogel (c) intact or (d) rupture.

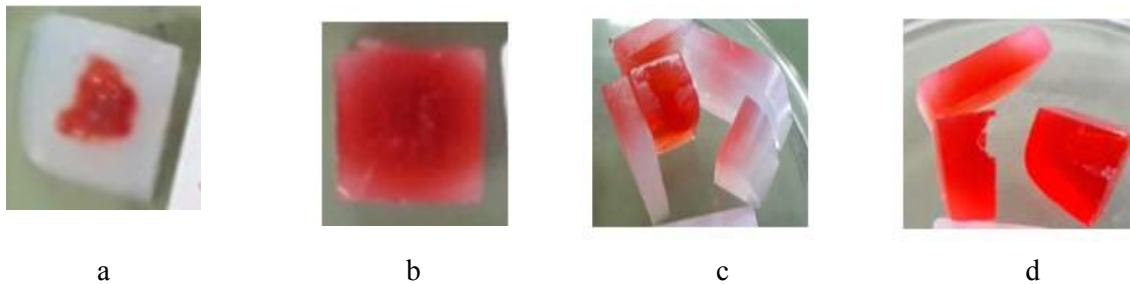


Figure 5. In Vitro Injection Model Results

The higher the concentration of HRP enzyme, the better the hydrogels occupied rooms in agarose and the shape of the hydrogels would be whole when released from agarose (Table 1). This shows that there was an increase in the cross linking bonding by Horse Radish Peroxide enzyme towards Hyaluronic Acid and Polyethylene Glycol.

Table 1. In Vitro Injection Model Testing Results

Variation	Samples	Hydrogel			
		Occupy the Space of Agarose		Form Of Hydrogel When Released From The Agarose	
		Portion	Whole	Intact	Rupture
HA-PEG-0 HRP	K-M-1		√		√
	K-M-2		√		√
	K-M-3		√	√	
HA-PEG-0.15 HRP	1-M-1		√		√
	1-M-2		√		√
	1-M-3		√		√
HA-PEG-0.25 HRP	2-M-1	√			√
	2-M-2	√		√	
	2-M-3		√	√	
HA-PEG-0.35 HRP	3-M-1	√		√	
	3-M-2		√	√	
	3-M-3	√		√	
HA-PEG-0.45 HRP	4-M-1	√		√	
	4-M-2	√		√	
	4-M-3	√		√	

3.5. Functional groups testing

Confirmation tests on functional groups that were conducted using Fourier Transform Infra-Red (FTIR) in both samples (samples with a concentration of 0.45 $\mu\text{mol}/\text{min}/\text{ml}$ could not be tested due to

constrained availability of materials) demonstrated that an indication of cross linking was successful because a functional group was found on both samples, which was a functional group of enzyme Horse Radish Peroxide. The characterization of functional groups aim to find out the number and percentage of transmittance wave at each hydrogel. On the spectrum of Figure 6, it retrieved on absorption peak wave number 1348 cm^{-1} . The absorption intensities of the functional groups is carboxyl, the result of a cross link between HA and PEG. Spectrum formed indicative of cross-linking of polymer bonds as covalent bonds and ties are reversible.

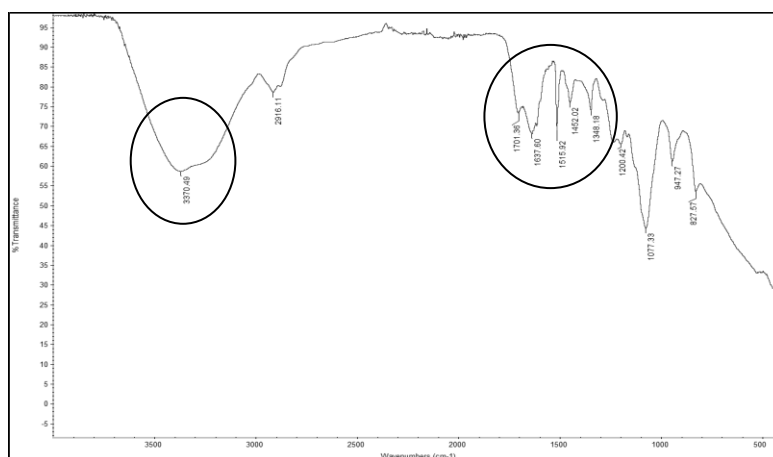


Figure 6. IR Spectrum of Hydrogel With Variations of HA-PEG-0 HRP

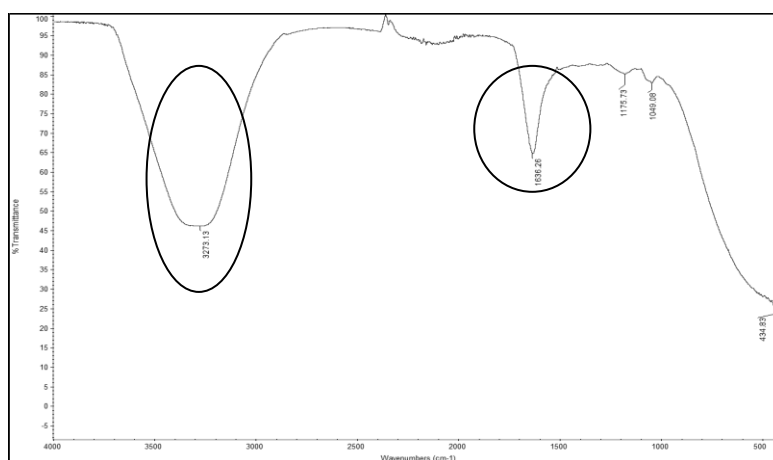


Figure 7. IR Spectrum of Hydrogel With Variations of HA-PEG-0.25 HRP

Based on the analysis of functional groups in the IR spectrum of the enzyme composition variations with hydrogel $0\mu\text{mol/min/mL}$ (Figure 6) and $0.25\mu\text{mol/min/mL}$ (Figure 7), indicate that the existence of the new cluster experienced a chemical cross linking (Table 2), i.e., hydrocarbons, amine, amide II and carboxyl. HRP in the presence of a catalyst of hydrogen peroxide of hydrogen atom undergoes on a cluster of phenol hydroxyl resulting in oxygen atoms on the phenol hydroxyl HA cluster frees electrons and have the nature of reactive free radicals, evidenced by the widening of the area transmittance absorption on the 3300 cm^{-1} , resulting in a molecular results between cross linking HA with a PEG. The formation of free radicals results in polymer molecular distances are relatively short HRP and polymer, so that it can bind with the addition of the HRP enzyme, stronger cross linking. Cross linking which occurs on intermolecular protein crosslinking hydrogel is so functional group generated between before and after the addition of the enzyme will be different [8].

Table 2.Results of Functional Group Test

Samples	Wave Number (cm ⁻¹)						
	Hydroxyl	Hydrocarbon	Ether	Amide I	Amide II	Amino	Carboxyl
	O-H	C-H	C-O-C	C=O	C=O	NH ₂	COOH
HA-PEG-0 HRP	3370.49	2916.11	1200.42	1637.6	1452.02	1515.92	1348.18
HA-PEG-0.25 HRP	3273.13		1175.73	1636.26			

4. Conclusion

The combination of Hyaluronic Acid (HA) -Polyethylene Glycol (PEG) has a potential to be the candidate for inject-able hydrogels for intervertebral disc degeneration. This is proven through swelling test, degradation test, cytotoxicity assay, an in vitro study of injection models, and functional group test.

Acknowledgments

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