

EFFECT OF DEACETYLATION DEGREES VARIATION ON CHITOSAN NERVE CONDUIT FOR PERIPHERAL NERVE REGENERATION

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EFFECT OF DEACETYLATION DEGREES VARIATION ON CHITOSAN NERVE CONDUIT FOR PERIPHERAL NERVE REGENERATION

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ABSTRAK

Saraf yang rusak dapat diregenerasi jika hanya mengalami cedera yang sederhana dengan menggunakan konduit saraf yang secara fisiologis dan mekanis sesuai dan dapat mendukung regenerasi saraf di sekitar celah trauma. Salah satu biopolimer untuk komposisi konduit adalah kitosan karena biokompatibel, biodegradabel, non toksik dan memiliki kesamaan struktur dengan glikosaminoglikan. Tujuan penelitian ini adalah mensintesis kitosan dengan variasi derajat deasetilasi dan mengkarakterisasi pengaruh derajat deasetilasi terhadap sifat mekanik dan biokompatibilitasnya. Desain penelitian ini adalah observasional prospektif. Kitosan diproses dengan penurunan derajat deasetilasi dan peningkatan temperatur dengan solusi alkalin, dengan konsentrasi solusi NaOH 5%, 20%, 35%, dan 50% dalam 2 jam dengan temperatur pemanasan 95°C. Hasil dari tiap variasi DD adalah 23.24, 46.55, 53.48, dan 55.06. Uji tarik juga telah dilakukan dengan nilai kuat tarik antara 0.25 - 1.18 MPa. Hasil uji degradasi menunjukkan kecenderungan penurunan seiring bertambahnya konsentrasi NaOH menunjukkan bahwa sampel bersifat biodegradabel/dapat terdegradasi. Morfologi permukaan sampel menunjukkan ukuran pori berkisar antara 61.52 µm - 220.3 µm. Hasil terbaik didapatkan dari sampel kitosan dengan 35% NaOH berdasarkan nilai kuat tarik dan ukuran pori yang sesuai dengan standart normal. Kuat tarik sebesar 0.41 MPa - 3.69 MPa dan ukuran pori sebesar 40 µm - 250 µm yang dapat mempercepat regenerasi saraf. Hasil penelitian ini diharapkan dapat menjadi dasar solusi pengembangan konduit saraf untuk defek saraf perifer. (FMI 2017;53:101-107)

Kata kunci: konduit saraf, kitosan, biodegradabel, derajat deasetilasi

ABSTRACT

Broken nerves could regenerate when exposed to simple injuries by using a nerve conduit that has appropriate physiological and mechanical ability to support the nerves regeneration around the fissure of trauma. One of the biopolymer for the conduit composition is chitosan because it is biocompatible, biodegradable, non-toxic, and has similarity structure as natural glycosaminoglycans. The aim of research is to synthesize chitosan with variation of Degrees of Deacetylation (DD) and characterize the DD influence on mechanical properties and biocompatibility. Research design is prospective observational. Chitosan was treated with a decrease in the DD method and an increase in the temperature with the strength of alkaline solution, which was NaOH solution with concentrations of 5%, 20%, 35%, and 50% within 2 hours with a heating temperature of 95°C. The results of each DD variation were 23.24, 46.55, 53.48, and 55.06. It was characterized by tensile test with tensile strength values of 0.25 - 1.18 MPa. The degradation test results tend to decrease with the increasing concentration of NaOH proving that samples are biodegradable. The surface morphology of samples shows a pore range of 61.52 µm - 220.3 µm. The best result is the chitosan sample with 35% NaOH because due to the tensile characteristic and a pore in accordance with normal standard. Tensile strength is around 0.41 MPa - 3.69 MPa and pore size around 40 µm - 250 µm to accelerate nerve regeneration. The results are expected to provide alternative solution of nerve conduit development for peripheral nerve defects. (FMI 2017;53:101-107)

Keywords: nerve conduit, chitosan, biodegradable, degrees of deacetylation

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INTRODUCTION

Peripheral nerve injury is a critical and disabling condition. This injury is great problem for health as well as socioeconomic challenge throughout the world. Each year approximately 100.000 patients in the United States and Europe undergo neurological surgery in order to improve the nerve injury (Schlosshauer et al 2006). Indonesia is a country with high population den-sity and

a high number of accidents with peripheral nerve trauma impact. According the data from Central Statistics Agency (BPS) 69.260 cases of accidents occurred in 2009 and 66.488 cases occurred in 2010 (bps.go.id 2015).

Peripheral nerve injury can cause paralysis either motor and sensory palsy which disrupt daily activities and reduce the quality of life of the individual in the long

run. A slightly damage of peripheral nerve tissue has the potential to regenerate, however severe peripheral nerve damage caused by a rupture on nerve tissue bobbin leading to a crack or gap in the nerve fibers has low regeneration ability. Approximately 5% of all cases of peripheral nerve damage has a gap in the nerve fiber axons (Ijkema, et al 2005). Both axons severed by a distance of more than 1-2 cm require grafts or pipes connecting nerves to improve their function (Deumens et al 2010). Peripheral nerve transection (damage of some or all segments of the nerves) is always difficult to improve. The clinical method includes suturing the epineurium (peripheral nerve fiber wrapping composed of dense ir-regular connective tissue) performed on the injured nerves as well as nerve autografts performed on severely damaged nerves. However, both methods have the disadvantage of nerve distortion (aberration), increased tensions, difficulty in operation treatment, unsatisfactory operating results, and so (Jianchun et al 1999).

Biomedical research experts are now conducting more intensive searches for an effective method for nerve recovery. One of the most promising methods is to bridge a nerve stump with a nerve conduit. The nerve conduit is an artificial nerve channel that has ability to guide axonal regrowth in facilitating nerve regeneration and is one of several clinical treatments for nerve injury. It has appropriate mechanical and physiological ability to help nerve growth through the trauma gap and to restore the motoric function and sensation to certain extent (Den Dünnen et al 1993).

In the last few years, there has been an increasing interest in biomedical research and tissue engineering application to use natural biopolymer chitosan derived from chitin. Chitosan is a non-toxic substance, which is a natural polysaccharide consisting of a copolymer of glucosamine and N-acetyl glucosamine, and obtained from deacetylation of chitin (Khan et al 2002). It has biocompatibility, biodegradability, non-toxicity, and structural similarity as natural glycosaminoglycans. The latest in vitro research demonstrates the suitability of chitosan membrane as a substrate for the survival and growth of Schwann cells (Yuan et al 2004) as well as for supporting the continuity and differentiation of the nerve cells. The substrate acts as an extracellular matrix providing growth antigens and migration medium for Schwann cells and cells that have not differentiated (Freier et al 2005, Simoes et al 2011).

The development of technology is able to overcome the weakness of mechanical forces on the chitosan conduit, which is one of the main factors limiting the use of nerve conduits for clinical applications to date. Optimization of the biodegradation process and biocompa-

tibility of the cells is an important factor in tissue engineering, which must be tailored to the different degrees of chitosan acetylation. Chemical characteristics that are critical to chitosan interaction with the surrounding environment are degrees of deacetylation (henceforth DD) and molecular weight (MW) (Freier et al 2005).

Based on the background, we propose the manufacture of a nerve conduit based on nano-chitosan with variation of DD of 5%, 15%, and 25% as candidates for the peripheral nerve regeneration conduit. Chitosan is chosen as the base material for the manufacture of nerve conduit as it has a fast degradation rate in accordance with the period of a neural matrix formation process (Jianchun et al, 1999). The objective of this study is to conduct research on the effects of nano-chitosan DD as candidates for the peripheral nerve regeneration conduit.

MATERIALS AND METHODS

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Equipment and materials

The equipment used in this study includes a Fourier Transform Infrared Spectrometry (FTIR), an Elmar Spectrum One Perin instrument, a Scanning Electron Microscopy (SEM) (Inspect S50) and an MTT Assay (Elisa reader). The materials used were chitosan, acetate acid (CH₃COOH), Glycerol, distilled water, and natrium hydroxide (NaOH).

The processes of deacetylation

Chitosan samples weighing 2 grams were added to a NaOH solution at concentrations of 5%, 20%, 35%, and 50% as much as 100 ml. The mixture was stirred and heated at 95°C for 2 hours. The solution was separated and then filtered by using wolfram filter paper. Samples in an alkaline state, were washed with distilled water until reaching a neutral pH of 6-7.

Preparation of materials

The wet samples were then dried by using an oven with a temperature of 60°C for 24 hours. The dried samples on each of the various concentrations of NaOH were subsequently dissolved in a solution of 2% acetic acid for 3 hours. A Chitosan solution was mixed with glycerol. The solution was then were put in a container in accordance to the mold. Freeze drying was then performed for 24 hours in a temperature of -80°.

Characterization of Fourier Transform Infrared Spectroscopy (FTIR)

The prepared samples were characterized by an FTIR to calculate the percentage of the DD. Determination of the DD by an IR spectroscopy was conducted by using a base line method. There were two base lines used in determining the DD: the base line (a) as proposed by Domzy & Robert and the base line (b) as proposed by Baxter et al (Khan et al 2002). The calculation method formula of the DD with the base line (a) method was as follows:

$$DD = 100 - \left[\left(\frac{A_{1655}}{A_{3450}} \right) \times \frac{100}{1,33} \right]$$

Notes:

DD : degree of deacetylation ⁶

A1655 : The absorbance at wave number 1655 cm-1 indicating the absorption of the amide carbonyl.

A3450 : The absorbance at wave number 3450 cm-1 indicating the absorption of hydroxyl and is used as an internal standard.

Factor 1.33 : comparison value of $\frac{A_{1655}}{A_{3450}}$ for chitosan which is 100% deacetylated.

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Characterization of Scanning Electron Microscopy (SEM)

Characterization of the surface morphology with a microstructure scale and a conduit structure analysis was performed by using a Scanning Electron Microscopy (SEM). POC samples were inserted into a Sputter Coater that has two sides. They were then coated with Au-Pd (Aurum Palladium). The coated samples were observed by using a SEM at a voltage of 10 kV, a pressure of 60 Pa and emission current from 0.1 to 98.6 uA. The resulting image is a topography image with all protrusions, indentations and surface holes which were observed on the screen at 50 and 250 time magnification.

Tensile test

The testing of mechanical properties of chitosan-based samples of the nerve conduit was performed with tensile strength characterization. The film samples were made into a dog bone shape based on ASTM D412a (26 mm x 4 mm x 1.5 mm, length x width x thickness).

Degradation characterization

Chitosan samples that had been freeze-dried were shaped in squares with a size of 10 mm x 10 mm in accordance to the concentration of NaOH variation with

the percentage of 5, 20, 35 and 50 wt.%. The samples were placed into Simulated Body Fluid (SBF), pH 7.4 at 37°C in 3 weeks in a static condition. The chitosan conduit was expected to degrade during the incubation in the solution. The SBF solution was necessarily measured to ensure that the pH did not fall below 7. Before weight calculation, samples had been extensively rinsed with deionized water and dried. Weight was calculated by comparing the initial weight (W₀) with the weight measured at weeks 1, 2, and 3 (W_t), as shown in the following equation.

$$\text{Degradation ratio (\%)} = \frac{W_0 - W_t}{W_0} \times 100\%$$

Cytotoxicity characterization

Diluted 25 mL MTT reagent of 5 mg/ml was added into PBS in the media for each pit. Moreover, incubation was performed in an incubator for 4 hours at 37°C. A 50 µL DMSO solvent was added to every pit and then centrifuged to 30 rpm for 5 minutes. Formazan optic value was then calculated by the Elisa reader at a wavelength of 630 nm. The more intense the color, the higher the value of its absorbance and the greater the number of cells were.

The percentage of living cells was calculated by using the following formula:

$$\% \text{ living cell} = \frac{OD_{\text{treatmet}} + OD_{\text{Media}}}{OD_{\text{Cell}} + OD_{\text{Media}}} \times 100\%$$

Where as

% living cell = percentage of the number of living cells after testing.

OD Treatment = formazan optical density value in each sample after testing.

OD Media = formazan optical density value on media control

OD Cells = formazan optical density value in the control cells.

RESULTS

Degrees of deacetylation

Table 1. Values of degrees of deacetylation percentage

Concentration Chitosan	Deacetylation Degree
5%	23.24
20%	46.55
35%	53.48
50%	55.06

Pore Size

The morphology analysis shows a hollow-shaped sample with a size of 3.988 mm and the pore size in the range of 61.52 μm - 220.3 μm , which is consistent with previous research by Yang et al (2004) with diameter range of 40-250 μm .

Tensile Test

The results of tensile test as shown in Table 2.

Table 2. Results of the tensile test

Concentration Chitosan	Elongation (%)	Tensile strength (MPa)
5%	2.29	0.44
20%	2.09	0.31
35%	1.14	1.18
50%	2.31	0.25

Degradation Test

The results of degradation test within 3 weeks as shown in Table 3.

Table 3. Results of the degradation test

Concentration Chitosan	Degradation Ratios (%)
5%	30.56%
20%	27.87%
35%	23.68%
50%	38.09%

Cytotoxicity Assay

The results of cytotoxicity assay as shown in Table 4.

Table 4. Results of the cytotoxicity assay

Concentration Chitosan	Cell Viability (%)
5%	81.78%
20%	85.99%
35%	87.69%
50%	141.72%

DISCUSSION

The nerve conduit biomaterial was made with the main base material of chitosan which was derived from red snapper fish scale extracts. Chitosan was treated with a decrease in the DD method and an increase in the temperature and strength of the alkaline solution by using the sodium hydroxide (NaOH) solution with concentrations of 5%, 20%, 35%, and 50% for 2 hours with a heating temperature of 95°C. Samples were rinsed until the normal pH ranged from 6 to 7 to remove the alkalinity due to immersion in NaOH. Physically, sample forms changed according to the increase in the concentration of NaOH particles. Indeed chitosan formed very smooth particles because of excessive release of the acetylation chain, so that the resulting chitosan was dissolved in NaOH solution (Apriani et al 2012).

The samples were then characterized by using a Fourier Transform Infrared Spectrometry (FTIR) to determine the extent of the DD on samples. Through the IR spectrum, determination of the DD was conducted using the base line b proposed by Khan et al (2002). Thus, the data obtained are shown in Figure 1.

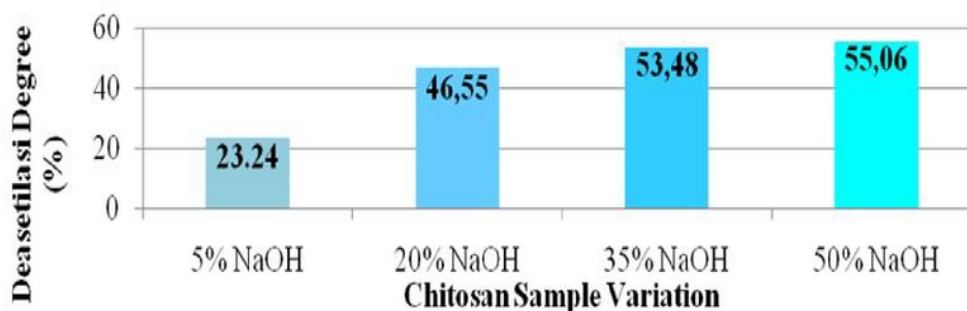


Fig. 1. Values of degrees of deacetylation percentage

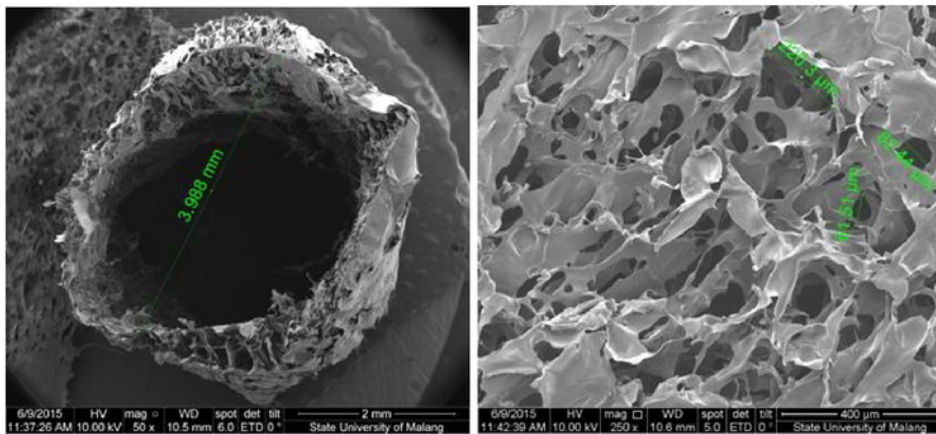


Fig. 2a. Morphology of nerve conduit hollow diameter, 2b. Pore size

Degrees of deacetylation

Figure 1 shows the influence of sodium hydroxide (NaOH) concentration treatment on the increasing number of hydrolyzed acetamide groups. At first, addition reaction which is entrance of the OH- group into the NHCOCH_3 group occurs. Then there is elimination of CH_3COO^- resulting in an amide. On the other hand, the acetate group adjacent to cis hydroxyl group could undergo N-deacetylation under strong alkaline condition but the trans-group is more resistant (Suhardi 1997). The higher the concentration of alkaline solution, the higher the percentage value of the DD is. The higher the degrees of chitosan deacetylation, the lower the chitosan acetyl group is, so that the interaction among the ions and hydrogen bonding is stronger (Apriani et al 2012). The DD is then used as a foothold of the characterization analysis.

Chitosan samples that have appropriate DD values were dissolved in a solution of acetic acid with a concentration of 2% for 3 hours. The samples were made into films and hollow by using the freeze drying method.

The SEM results indicate that the surface of the conduit comprises interconnected porous microstructure. The morphology analysis shows a hollow-shaped sample with a size of 3.988 mm (Figure 2A) and the pore size in the range of $61.52 \mu\text{m} - 220.3 \mu\text{m}$ (Figure 2B), which is consistent with a research (Yang et al 2004) that pore diameter is in the range of 40-250 μm . The pore size in the micro size improves nerve regeneration and the interconnected pore structure reforms vascularization, helping the exchange of nutrients between the conduits

and the outside environment around, accelerating the degradation process conduit and integrating with the surrounding tissue. The conduit pore structure is controlled by varying the parameters of freeze drying and temperature (Wang et al 2007).

The testing of mechanical properties of chitosan-based samples of the nerve conduit was performed with tensile strength characterization to determine the properties of material elasticity. Samples were prepared in the form of film and tensile tested to determine the properties of the material elasticity when implanted into the body. The film samples were made into a dog bone shape based on ASTM D412a (26 mm x 4 mm x 1.5 mm, length x width x thickness). The values of tensile strength are presented in Table 2, in which the results are in accordance with the results of the study (Wang et al 2007) having tensile strength of 0.75 - 0.95 MPa with elongation of $5.8 \pm 0.2\%$ for the porous chitosan conduit.

According to Table 2, it shows that the higher the concentration of NaOH in the immersion of chitosan leads to an increase in tensile strength of the samples followed by a decrease in the percentage of elongation as more and more amide bond is formed. However, on the sample with the percentage of 50% NaOH concentration, there is a significant decrease in tensile strength of 0.25 MPa. This occurs due to the process of deacetylation with the NaOH concentration of 50% experiencing excessive release of acetylation chain so that chitosan produced is dissolved in NaOH solution (Wang et al 2007).

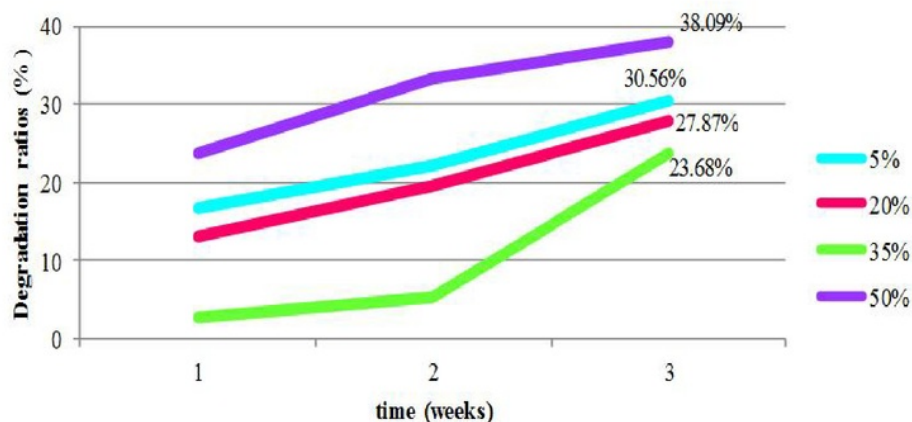


Fig. 3. Results of the degradation test

The graph shows the results of the three-week degradation process of 30.56%; 27.87%; 23.68%; 38.09% for a degradation ratio percentage of each of the samples is with varied NaOH concentrations of 5%, 20%, 35% and 50%, respectively. The results indicate that the greater the concentration of NaOH, the lower the rate of degradation is due to the growing number of the amide bond which causes degradation ratio to decrease. This is also associated with tensile strength results in the NaOH concentration of 50%. The degradation ratio increases due to weak tissue inter-connection (Lee et al 2011).

The implanted nerve conduit, as a connecting bridge of the broken nerves, is recognized by the body as a foreign object, so that the material must be bio-

compatible with the body. Indeed the cytotoxicity test is performed to determine the toxicological properties of samples. This test was performed by using MTT reagents (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide). The cells used were BHK-21 cells (Baby Hamster Kidney-21). Results of the cytotoxicity assay is shown in Figure 4.

Fig. 4. shows the percentage of living cells per sample. The best result is shown by the chitosan sample with the 50% NaOH concentration which has a degree of deacetylation of 55.06% with 141.72% of living cells. All chitosan with DD variation is included in the non toxic area because it has more than 50% cell viability (Spielmann et al 2007).



Fig. 4. Cytotoxicity assay

CONCLUSIONS

Synthesis of the chitosan-based neural conduit was conducted with a decrease in the DD method and an increase in the temperature as well as the strength of alkaline solution, which is sodium hydroxide solution with concentrations of 5%, 20%, 35%, and 50% for 2 hours with a heating temperature of 95°C. It was dissolved with a solution of acetic acid with a concentration of 2% for 3 hours. The samples were prepared in films and hollow formed by a drying method using freeze drying.

The FTIR test results show absorption peaks of the amide I and absorption of hydroxyl groups resulting in a variation of DD as 23.24%; 46.55%; 53.48% and 55.06%. Then, the SBF test results indicate degraded samples which are characterized by mass reduction when measured in 3 weeks. Next, the tensile test obtains tensile strength in the range of 0.25 to 1.18 MPa. Furthermore, the SEM analysis shows the size of hollow diameter of 3.988 mm and the pore size on the walls of 61.51 μm - 220.3 μm , so that it is potential to be applied as a nerve conduit. At last, the MTT Assay obtains living fibroblast cell percentages above 50% so that the samples can be said to be non toxic. The best value of tensile strength is around 0.41 MPa - 3.69 MPa and pore size around 40 μm - 250 μm .

The best result is the chitosan sample with 35% NaOH due to the tensile strength and pore size in accordance with normal standard which could accelerate nerve regeneration. The result research could be baseline of nerve conduit development for peripheral nerve defects.

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