Alginate/PVA/chitosan injection composites as scaffold material for nucleus pulposus regeneration

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Abstract. Lower Back Pain (LBP) is common health problem in society with prevalence 80-85%. One of the causes of LBP is Herniated Nucleus Pulposus (HNP). This disease occurs in intervertebral discs (IVD), supporting the spine, found a gel fluid called Nucleus pulposus (NP) at the center surrounded by 15-20 concentric thin layers of the Annulus fibrosus (AF). This gel that will come out through AF and nerve pinched behind which makes sufferer feel pain and numb. Handling HNP with physical therapy only reduce the pain and not totally cured. While the surgery use discectomy method by eliminating the protruding parts NP or in whole, but has some drawbacks, such as: high cost, wide surgical incisions, probably occurrence of bleeding or other neurological disorders. Solving problem with find new methods that are faster, easier, and more affordable, we analyze using of alginate/PVA composite with chitosan suspense as a reinforcement as hydrogel injectable material to replace NP. Independent variable is chitosan percentage each sample, as much 5; 10; 15%. This study as a purpose to know mechanical properties, biocompatible, injectable performance, viscosity of alginate/PVA/chitosan, SEM-EDX test and FTIR test. The best result got is addition chitosan 15% appropriate as NP replacement.

1. Introduction

Lower Back Pain (LBP) is very common, with an estimated 40% to 80% of individuals experiencing LBP over the course of their lives [1]. At least, in 2013 the back pain sufferers in Indonesia reached 37% of total population. Lower back pain began to be felt when the human reaches adulthood from age 25 to 65 years [2]. Lower back pain can be categorized into two parts, the pain due to degradation of the disc (DDD) and Herniation Nucleus Pulposus (HNP) or know as a pinched nerve [3]. HNP disease is condition where nucleus pulposus is supposed to be in the middle and covered by the AF, be out into the AF and pinched nerves behind Intervertebral Disc (IVD) [4]. So, the patient will feel pain, numbness, tingling around the nerve pressure that can interfere with daily activities. HNP disease can occur due to aging which cause degenerative changes in the NP [5]. In addition, because of the trauma caused by the mechanical load on the annulus fibrosus IVD tearing and obese [6]. This time, solution for treating HNP is divided into two ways, physical therapy and surgery. For therapy, such as TENS therapy, laser, and using a lumbar pillow can only reduce pain and sometimes patients feel pain repeatedly [7]. While the surgery is performed through discectomy method by removing the prominent or overall NP portion, but has some drawbacks, such as: high cost, wide surgical incisions, allowing bleeding or other neurological repair. In general, this method cannot be used as curative and only acts to overcome neurological problems resulting from HNP [8].
Research is needed to find new methods that are faster, easier, and more affordable for this treatment such as injection method [9]. Research conducted in 2006, NuCoreTM was made from silk and elastin copolymers, capable of recovering biomechanical, biocompatible disc by curing in 5 until 30 minutes [10]. However, until now only limited use in US, affordable price and implantable materials such as these cannot be degraded.

So, we need another material (composite) which can be used as an alternative to injectable NP, has the appropriate mechanical properties, biocompatible and biodegradable. The first candidate that PVA is a polymer synthesis has a high swelling ratio and biocompatible [11]. However, PVA has a low mechanical properties requiring other material that can cover the shortcomings that alginate [12]. Alginate is a natural polysaccharide exhibiting excellent biocompatibility and biodegradability of composites [13]. However, alginate is not scaffold cells, so that the necessary additional material that is chitosan [8]. Chitosan has biocompatible properties, bio-degradable, and scaffolding materials [14]. Wirongrong’s study in 2011 stated that the addition of chitosan by 30% to reach the maximum value of tensile strength, so in this study we choose the addition of chitosan 5; 10; 15% as the independent variable as injectable composite alginate / PVA / Chitosan as alternative to the nucleus pulposus.

2. Materials and Methods

2.1 Materials

The materials used in this study are Na₂HPO₄, CaSO₄, polyvinyl alcohol (PVA), distilled water, sodium alginate (SA), vanillin pa 99%, tripolyphosphate (STPP), acetic acid (CH₃COOH), chitosan suspension (HIMEDIA) and Phosphate-buffered Saline.

2.2 Equipments

Equipment used in this study are magnetic stirrer, glass beaker, thermometer, pH meter, analytical balance, syringe, SEM-EDX, Universal Testing Machine (UTM), FTIR, Elisa Reader, Optical Microscopy and Oswald viscometer.

2.3 Preparation of Hydrogel Composite

2.3.1 Synthesis matrix PVA/alginate

Synthesis matrix of alginate / PVA begins by dissolving 0.63 grams of PVA in 15 mL of distilled water at a temperature of 75 °C - 80°C using a magnetic stirrer until dissolved. The solution that has a homogenous PVA added as much as 1.5 grams of sodium alginate and 40 mL of distilled water, the mixture was stirred using a magnetic stirrer speed of 200 rpm for 1 hour. Then, in the homogeneous solution was added as much as 0.3 grams Na₂HPO₄ as retarding agent and as much as 5 mL of distilled water then stirred using a magnetic stirrer speed of 200 rpm for 1.5 hours.

2.3.2 Synthesis chitosan suspension

Synthesis of chitosan suspension begins by dissolving 10.33 mg of chitosan in 1000 mL of 1% acetic acid to a pH 5-5.5. To the solution was added to 1,000 mL (2 mg mL⁻¹) STPP solution drop by drop and the mixture was stirred for 2 hours at room temperature. Then vanillin as much as 4 grams of a chemical cross-linking agent was dissolved in 10 mL of 1% acetate acid and slowly put dropwise into the chitosan solution while stirring for 12 hours.

2.3.3. Synthesis of composite alginate/PVA/chitosan

Having obtained a matrix of alginate / PVA, chitosan suspension is added with varying amounts of 5%, 10% and 15%. CaSO₄ is then added as much as 0.6 grams as a curing agent, 60 mL of distilled water and homogenized using a magnetic stirrer with a speed of 450 rpm for 45 minutes. Then the composite is injected using a syringe into the container and wait how long curing time that happened.

2.4 Characterization
SEM-EDX testing to determine levels of composite elements. Characterization of composite functional groups using FTIR to assess the functional groups of the composite alginate / PVA with chitosan additional variation of 5%, 10% and 15%.

2.5 Compression Test
Press testing use UTM with a cylindrical composite to determine the mechanical properties of the composite.

2.6 Biocompatibility
The test performed to knowing the compatibility of the composite when injected in living organisms or humans. Composites tested by MTT Assay method, which is based on the reduction of testing the compound MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan. Tetrazolium dye that is soluble in water will become purple formazan crystals, this was due to the dehydrogenase activity of the mitochondria of cells. Then the formazan product was analyzed in the spectrophotometer (550 nm) with Elisa Reader. MTT Assay test conducted at the Center for Veterinary Farma Surabaya.

2.7 Injectable Performance Test
Injectable performance testing conducted to determine the ability of the composite to be injected using a syringe which was then put into a container and wait until the composite hardened (solid) to determine the curing time of the composite.

2.8 Viscosity Test
Testing is done by immersing the composite in PBS with a temperature of 37°C for 4 days to determine the ability of degradation through the measured viscosity values.

3. Results and Discussions

3.1 Characterizations
Chitosan has the properties are insoluble in water but soluble in acidic solution with a pH of less than 6, such as acetic acid. The presence of carboxyl group in acetic acid will facilitate the dissolution of chitosan for the hydrogen interaction between the carboxyl group of the amino group of chitosan [15]. Thus, chitosan suspensions have been successfully synthesized. Composite alginate / PVA / Chitosan has been successfully synthesized with the addition of chitosan variation of 5%, 10%, 15%. Chitosan has a chemical formula (C₆H₁₁NO₄)ₙ where n is a specific element of the chitosan [16]. Based on Table 1 seen that the levels of N ranges from 0.79% increase to 1.61%. Along with the addition of chitosan, the N content increases, it is proving synthesis performed properly and composite alginate / PVA / Chitosan been formed. Figure 1 show SEM-EDX result that composite physically soluble.

| Composites | Elements (% Wt) |
|------------|----------------|
|            | C  | N  | O   |
| APC-5      | 16.31 | 0.79 | 53.90 |
| APC-10     | 14.42 | 1.34 | 53.28 |
| APC-15     | 17.04 | 1.61 | 57.45 |

Chitosan, alginate / PVA, APC-5, APC-10 APC-15 were characterized using FTIR to determine the functional groups. The results of such characterization FTIR spectra are shown in Figure 2. In chitosan known at the peak 1,097-1,650 cm⁻¹ shows the CN functional groups, 2,924.18 cm⁻¹ shows the functional groups hydroxyl (OH) and NH groups. In alginate / PVA peak of 3,244.25 cm⁻¹, which is the functional groups OH, NH, CH, and 1,628.42 cm⁻¹ represents NH and C = C. On the results of characterization matrix of alginate / PVA with the addition of chitosan led to a new peak widening the catchment area 1,024-1,320 cm⁻¹ indicating CN stretching vibration of functional groups. Where CN is
a specific functional group-owned chitosan [17]. So that FTIR characterization results indicate successful composite formed by the specific functional group CN.

Figure 1. SEM-EDX picture of composite with addition chitosan 5% (a), chitosan 10% (b), chitosan 15% (c)

Figure 2. Result of FTIR characterization

3.2 Compression Test
Table 2 shows that the addition of chitosan lowers the mechanical properties of the composite, this is caused by the mechanical properties of chitosan are low, so with the addition of chitosan to the composite as reinforcement will lead to the mechanical properties of composites will decrease [18].

| Composites | Modulus Young (kPa) |
|------------|---------------------|
| APC-5      | 121.442             |
| APC-10     | 78.239              |
| APC-15     | 51.606              |

3.3 Biocompatibility Test
Biocompatibility testing performed testing using MTT Assay in DMEM media (Dulbecco's Modified Eagle Medium). In this test MTT yellow will turn into a purple formazan while in living cells. Color is analyzed to determine how BHK fibroblast cells surviving 21 on a composite sample for 1 day. Because this test lasted only one day, the number of living cells was little. Analyses result the number cell viability was 19.18% for addition chitosan 5%, 24.76% for addition chitosan 10%, and 27.08% for addition chitosan 15%. Increase chitosan concentration will improve cell viability of
hydrogel composite. Appropriate with chitosan properties that scaffold and biocompatible. Finally, Figure 3 shows test result of MTT Assay.

![Figure 3](image)

**Figure 3.** Cell viability picture read on Elisa machine show result of composit with chitosan 5% (a), chitosan 10% (b), chitosan 15% (c)

| Composites | Cell Viability (%) |
|------------|--------------------|
| APC-5      | 19.18              |
| APC-10     | 24.76              |
| APC-15     | 27.08              |

### Table 3. Viability cell composites

3.4 Injectable Performance Testing

Injectable performance testing performed by inject composite through syringe, placed on container, and calculated the curing time. Based on Figure 4, composites have ability to be injected and solidified in accordance with the shape. While in Table 3, shows that the shorter the curing time with the addition of chitosan. From Table 4, curing time result still affordable to do surgery NP replacement. Thus, this composite could be alternative material as NP replacement.

![Figure 4](image)

**Figure 4.** Injectable performance testing: (a) injection process, (b) injected into the container, (c) composite solidified

| Addition of Chitosan (%) | Curing Time (min) |
|--------------------------|-------------------|
| 5                        | 70                |
| 10                       | 62                |
| 15                       | 53                |

### Table 4. Curing time composites

3.5 Viscosity Test

On the first day of immersion, the composite is stable in PBS (Phosphate Buffer Saline). After 4 days of immersion, to determine the ability of the composite degradation can be tested viscosity. Based on Table 5, viscosity increases from 1.985 mL/g to 4.328 mL/g with the addition of chitosan, this proves
that the composite can be degraded. By the nature of the bio-degradable chitosan led to the increasing number of composite addition of more and more degraded as indicated by the increase in viscosity.

| Table 5. Viscosity composite |
|-------------------------------|
| Composites | Viscosity (mL/g) |
|-------------|-----------------|
| APC-5       | 1.985           |
| APC-10      | 2.299           |
| APC-15      | 4.328           |

4. Conclusions
Based on characteristic result of composite alginate/PVA/chitosan successfully made cause by SEMEDX test profiled N element increase along chitosan addition. Then, on FTIR test, C-N which is specific group functions of chitosan found on APC-5; APC-10; APC-15 characteristic result. Sign that composite has been made successfully. The results of injectable performance testing showed all three variations of chitosan composition can be injected through the syringe, can form the hydrogel composite has been made successfully. The results of injectable performance test showed the composition with the best biocompatible of 27.08% cell viability. From the explanation above, it is concluded that the composite composition with the best result is alginate/PVA/Chitosan 15%.

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