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Characterization and Formulation of the Bivalve *Anodonta*'s Chitosan–Platelet Rich Plasma-Mesenchymal Stem Cellsas a Composite Scaffold

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Abstract: Various studies have been conducted to obtain an ideal scaffold for mesenchymal stem cells. The types of scaffold studied included those of ceramic group, tricalcium phosphate, collagen, gelatin, cellulose and agarose. The results varied due to many factors. Recently, the hydroxyapatite scaffold constitutes the most promising scaffold for bones. The bivalve *Anodonta* are freshwater mussels that have shells with the smallest toxicity. The contents of chitin in the shells' nacreous layer are expected to be a good biomaterial for the life of mesenchymal stem cells because, when deacetylated, it will become an active compound called chitosan. Chitosan is a potential natural biopolymer class for a biomaterial for bones. The purpose of this study to explore the use of the mussels Bivalve *Anodonta* a*s a* base material for scalfold**.** In this study, platelet-rich plasma (PRP) as a growth factor was added to the bivalve *Anodonta*'s chitosan, which was expected to be a good scaffold for mesenchymal stem cells through several screening for biomaterial requirements. The screening included: characterization using scanning electron microscopy (SEM) and infra-red spectrophotoscopy (FTIR), toxicity tests using MTT assay and growth tests of mesenchymal stem cells in the bivalve *Anodonta*'s chitosan scaffold and observation under an electron microscope. SEM examination showed pores of150–200μm in size, numerous and even pore interconnectivity and wall thickness. FTIR showed spectra IR graph patterns corresponding to the standard chitosan for the functional groups OH, NH, CH, NH_2 and COC, but with a better degree of deacetylation (68%). Toxicity tests using MTT assays with one-way ANOVA tests (p> 0.05) showed the bivalve *Anodonta*'schitosan had no toxicity to mesenchymal stem cells. The growth tests of mesenchymal stem cells in the bivalve *Anodonta*'s chitosan–PRP scaffold showed that the cells could be grown on a porous bivalve *Anodonta*'s chitosan. In conclusion, the composite bivalve *Anodonta's* chitosan–PRP –mesenchymal stem cell scaffold can be used as a biomaterial.

Keyword: Mesenchymal stem cells, platelet-rich plasma, bivalve*Anodonta*, chitosan, scaffold.

Introduction

Biomaterials research progresses quite rapidly from year to year. A variety of sources have been used as basic materials in tissue engineering. These biomaterials can be derived from nature such as plants, animals and humans themselves. But, many people have developed biomaterials from synthetic materials [1]. As one of the important components in tissue engineering, biomaterials have three main functions: providing the construction for regeneration of the appropriate tissues or organs, transmitting molecular and mechanical signals to cells and optimizingcellular functions in the composite. The current biomaterials are categorized into six domains: (1)the origin of materials, such as tissue derivatives (allograft bone matrix), polymers (collagen, hyaluronan), ceramics (tricalcium phosphate, hydroxyapatite), metals and composites of the above materials;(2) three-dimensional architecture and porosity, wherein the pores serve as the placesfor cells to form new tissue, neovascularization, and transport of nutrients;(3) the mechanical properties, which can serve as the load-bearing; (4) surface chemistry, such as cell–biomaterial interactions on the surface will form a unique chemical environment, in which adhesion will occur between cells that have hundreds of surface receptors and tens of integrin cell-matrix receptors after cellular contact with biological fluids of proteins;(5) physiological pH and osmolarity (initial environment);(6) degradation are not toxic and perfectly in the whole cell environment (final environmental) [2].

The main function and the category of biomaterials are used as reference for researching new biomaterials that possibly have the same or even better effectiveness than the former ones; thus, it can be used as an alternative to hydroxyapatite biomaterials that are widely used today. Chitosan is a biomaterial that began to be frequently studied since it is economical and easy to obtain, despite the unpromising effectiveness. Chitosan is obtained from the deacetylation of chitin previously deproteinized and demineralized from natural resources (shrimp, crab, clams). Chitosan of various basic materials has been introduced for a variety of benefits ranging from those in engineering, electrical, agriculture, property to the health sector. This biomaterial combines good resorptivity and osteoconductivity and osteoinductivity. In this context, chitosan is a polysaccharide with a large biocompatible molecular weight [3]. In terms of chemical properties, chitosan is reminiscent of the glycosaminoglycans, the extracellular matrix components essential for bone formation. In the same way, chitosan is capable of binding to several different components of the extracellular matrix as growth factors and cytokines [4]. For tissue engineering, chitosan is capable of concentrating on the factors implanted around the scaffold in the growth sites *in vivo*, decreasing the compound with hydrolase, especially lysozyme, that will break down chitosan to produce a non-toxic oligomeric enzyme. Recent studies showed that chitosan derivative products are anti-inflammatory drugs and support angiogenesis [5].Chitosan is regarded as a material with promising prospects in regenerative medicine [6,7].

The bivalve *Anodonta*'schitosan is a freshwater mussel-based one, which has never been studied as a biomaterial. The species *Anodonta* has a sturdy structure and is resistant to heating up to 600°C without the fragility and has low toxicity [8,9].The bivalve *Anodonta*'s chitosan is identified and characterized using a scanning electron microscope (SEM) and infra-red spectrophotoscopy (FTIR). SEM examination is capable of observing pores and the size, interconnectivity among pores and thickness of the wall construction, where the pore structures and sizes are often different among chitosans depending on the habitats [11]. Characterization by FTIR method will show special patterns on graphs showing the wavelength of the functional groups OH, NH, CH, NH_2 and COC. Toxicity is examined using the MTT Assay method. This examination is to test the toxicity of chitosan to mesenchymal stem cells [12].

A therapy modality with mesenchymal stem cells implantation into bones defects still needed a construction to serves asscaffold between bone tissues. Construction of scaffold is conventionally in the form of graft [13]. Implantation of mesenchymal stem cells into chitosan scaffolds will lead to an interesting chemical process, in which chitosan scaffolds will produce biological fluids of proteins that will bind to cell surface receptors attached by the integrin cell-matrix receptors. Thus, it is expected that cells will grow in the scaffold pores [2,13].

In this study,platelet-rich plasma (PRP) was added to the scaffold formulation of the bivalve *Anodonta*'s chitosan and mesenchymal stem cells to provide optimal efficacy in order to support the growth and angiogenesis. PRP contains many growth factors, among others, BMP2, TGF-β, IGF-1, FGF-2, PDGF, KGF, IL-8 and, most importantly, VEGF which stimulates neovascularization [14].

Materials and Methods

Biomaterials:

The bivalve *Anodonta*'s chitosan was produced from freshwater shells by for preparing fine crystal of BA.Initially, the fine crystals were deproteinized by soaking into NaOH solution for 10 h and then demineralized with HCl for 1 h. Then It washed again and dried. Subsequently, it was decolorized, so that only chitin left. Finally, it was deacetylated by adding NaOH solution for producing chitosan from chitin.

Cells:

Mesenchymal stem cells were isolated from bone marrow stem cells using the Ficol method and cultured in medium culture. The microsture cells were characterized by fluorescent microscopy (Data not shown)

Growth Factor:

Platelet-rich plasma (PRP) was generated by centrifugation of venous blood.

Procedures:

(1)microscopic examination using a scanning electron microscope (SEM) in order to observe the structure and pore size, wall architecture and pore interconnectivity; (2) characterization of the bivalve *Anodonta*'s chitosan using infra-red spectrophotoscopy (FTIR) in order to determine the validity of chitosan by revealing its chemical functional groups; (3) toxicity test using the MTT Assay method in order observe the toxicity of the scaffold to mesenchymal stem cells using the tetrazolium salt MTT;(4) growth test of mesenchymal stem cells in the scaffold in order to observe the growth of mesenchymal stem cells in the pores of the bivalve *Anodonta*'s chitosan using a scanning electron microscope (SEM).

Results

Microscopic structure of the bivalve Anodonta'schitosan by SEM

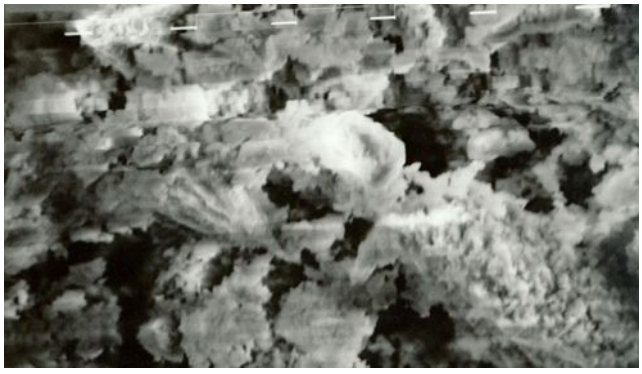


Figure 1.Microscopic structure of the bivalve *Anodonta*'s chitosan observed using an electron microscopeat 750 x magnification.
Note:Pores of 150–200µm insize appear with many interconnected pores.

The present study characterized chitosan by using a scanning electron microscope (SEM) and infra-red spectrophotometer (FTIR) to determine the pore structure and the degree of deacetylation of chitosan. Scanning electron microscope (SEM) examination showed the microstructure of the bivalve *Anodonta*'chitosan powder with pores and interconnected pores in large numbers. The size of pores was 150–200 µm, which was within the biomaterials pore requirement of 150–500 µm. This was different from the microstructure of some other biomaterials used as scaffolds. The difference was in accordance with the habitat, in which the pore size, interconnectivity among pores and the wall structure of chitosan are very diverse depending on the habitat [10,15]. The microscopic features of freshwater mussel-basedchitosan analyzed using a scanning electron microscope can be seen in Fig. 1.

Characterization of the bivalve *Anodonta*'schitosan

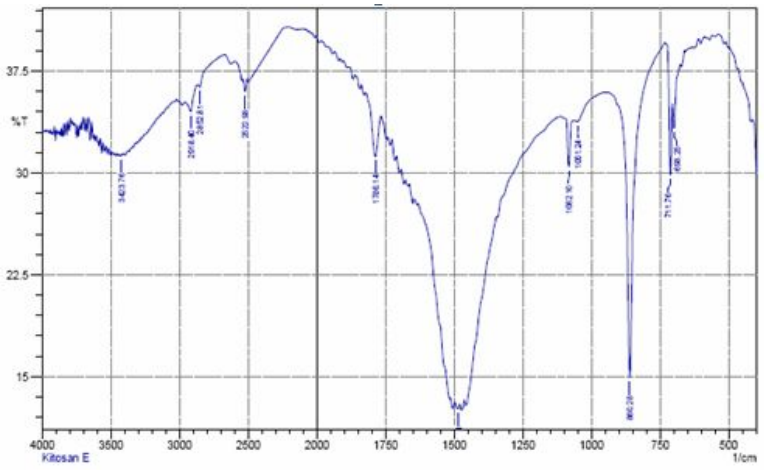
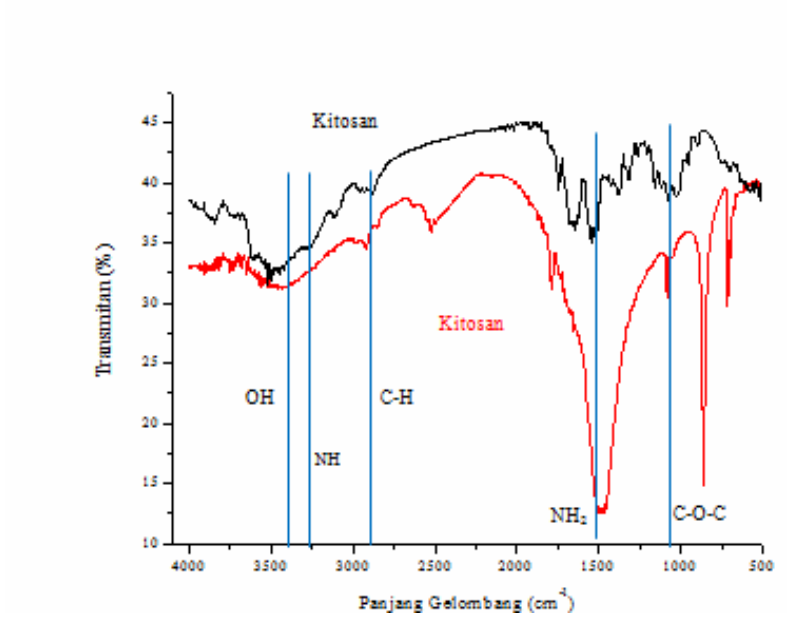


Figure 2.FTIR Spectra of the bivalve *Anodonta*'s chitosan



Black: standard chitosan Red: bivalve *Anodonta*'s chitosan
Figure 3. Comparison of FTIR spectra of the bivalve *Anodonta*'s chitosan and standard chitosan

The bivalve *Anodonta*'schitosan was characterized using a Fourier Transform Infra-Red Spectrophotometer (FTIR). This instrument demonstrated the presence of the group $(C_6H_{11}NO_4)_n$, which is the chemical structure of chitosan (Fig. 2). Comparison of the bivalve *Anodonta*'schitosan and standard chitosan is shown by the FTIR spectralgraph in Fig.3, indicating similarities in the wavelength of functional groups: OH group at a wavelength of 3450 cm^{-1}, NH group at a wavelength of 3335cm^{-1}, CH at a wavelength of 2891cm^{-1}, NH_2group at a wavelength of 1655cm^{-1} and COC group at a wavelength of 1072cm^{-1} (Table 1).

Table 1. Comparison of wavelength of functional groups of standard chitosan and the bivalve *Anodonta*'s chitosan

Functional Group	Wavelength (cm^{-1})	
	Standard chitosan	bivalve *Anodonta's* chitosan
OH	3450	3450
NH	3335	3335
CH	2891	2891
NH_2	1655	1655
COC	1072	1072

Toxicitytests of the bivalve *Anodonta* and PRP Scaffold to mesenchymal stem cells

MTT [3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] assaywas performed by tetrazolium salt MTT to examine cell viability upon exposure to a substance. Mesenchymal stem cells were examined as follows. Trypsinization was performed to remove the stem cell layerson petridishes. Suspension of mesenchymal stem cells was prepared by adding5 ml of 20% CCM. Subsequently, 50 µl/well of cell suspension was put into a 96-well plate, which contained approximately 2 x 10^4 cells. Then,100 µd of 20% CCM was added to each well. The 96-wellplate that already contained mesencymal stem cells was incubated in CO_2 incubator at 37°C for 24 h and the prepared biomaterial was added. The biomaterials tested were the bivalve *Anodonta*, platelet-rich plasma and bone allograft. The 96-wellplate that was already contained mesenchymal stem cells and biomaterials was re-incubated for 19 h and then 25 µd of MTT solution were added to each well.Subsequently, it was incubated for 5 h. Then, the medium was removed and replaced with 200 µd of DMSO/well.

MTT assay was used to determine the toxicity of biomaterials to stem cells. The biomaterial tested was the bivalve *Anodonta*'s chitosan. One-way ANOVA tests found no significant difference between the hydroxyapatite–PRP–mesenchymal stem cell group andthe bivalve *Anodonta*'s chitosan–PRP–mesenchymal stem cellgroup (*P*-value ≥0.05).The tests showed no toxicity of the biomaterial bivalve *Anodonta*'schitosan to mesenchymal stem cells (data not shown).

Growth tests of mesenchymal stem cells in the composite bivalve *Anodonta*'s chitosan–PRP scaffold

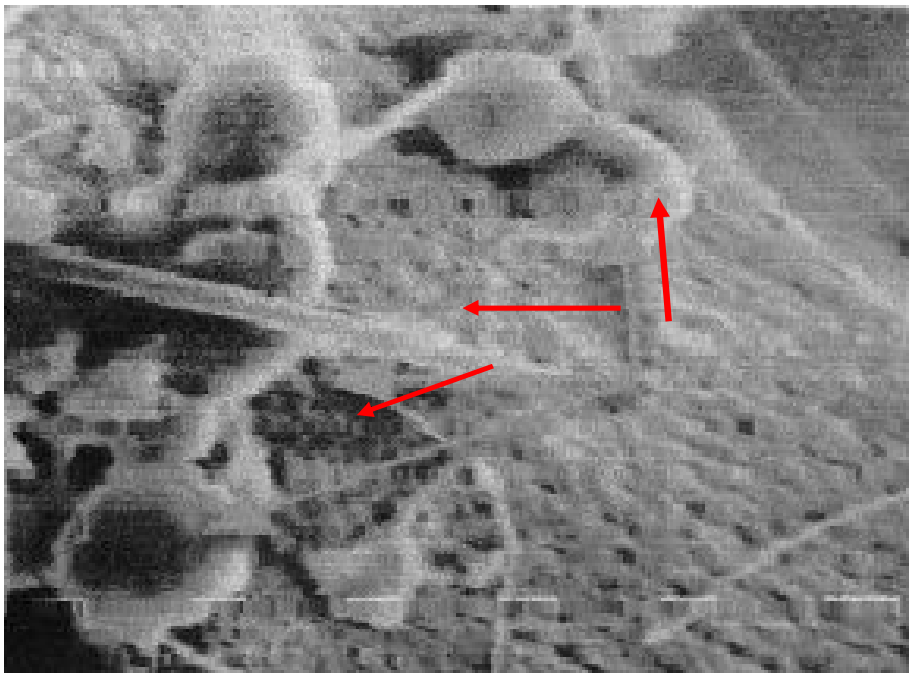


Figure 4. The result of the growth of mesenchymal stem cells ina chitosan composite scaffold bivalve Anodonta-PRP.
Note: (SEM, 1000x magnification), mesenchymal stem cells grown on porous chitosan bivalve Anodonta indicated by red arrows.

Discussion

Several previous studies have suggested that chitosan could become a biomaterial for bone growth. Chitosan is derived from a variety of animals that contain chitinin their skeleton in different levels. Researchers usechitosan derived from shells of freshwater mussels (the bivalve *Anodonta*) based on previous studies that show thebivalve *Anodonta* has several advantages, among others, no toxicity to the environment and higher protein content than other species [16]. Microstructure and macroanatomyof aquatic organisms are affected by physical and chemical properties of water, among others, current velocity, suspended solid, water temperature and ammonia [17]. The present study began with preparation of the active compound chitosan from the shells.The Fourier Transform Infra-Red Spectrophotometer (FTIR) was used to characterize the powder for chitosan. FTIR proved the presence of the group $(C_6H_{11}NO_4)_n$, which is the chemical structure of chitosan [(1,4) -2-amine-2-deoxy-β-D-glucose]. Comparison of the bivalve *Anodonta*'s chitosan and standard chitosan is shown by the FTIR spectral graph, indicating similarities in the wavelength of functional groups: OH group at a wavelength of 3450 cm^{-1}, NH group at a wavelength of 3335cm^{-1}, CH at a wavelength of 2891cm^{-1}, NH_2 group at a wavelength of 1655cm^{-1} and COC group at a wavelength of 1072cm^{-1}.

The loss of acetyl group is chitin, which is convertedto -NH_2 (amine), determine the degree of deacetylation.The degree of deacetylation of the bivalve *Anodonta*'schitosanwas 68%. Characterization of the chemical structure of chitosanandcomparison with the standardchitosanshowed a similar chemical structure. However, the degree of deacetylation ofthe standard chitosanwas only 52%.

The bivalve *Anodonta*'schitosanwas observed using the JEOL JSM T-100 electron microscope for shell architecture, shell wall density, pore size and interconnectivity between pores. Scanning electron microscopy showed a solid architectural structure, thick structural wall, pore size of 150–200 μm and numerous interconnectivity among pores, compared to chitosan derived from other species

The toxicity of the bivalve *Anodonta*'s chitosan to mesenchymal stem cells was tested using the method of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.One-way ANOVA of MTT assayperformed for the bivalve *Anodonta*'s chitosan, platelet-rich plasma and bone allograftshowed a $p = 0.100$ (> 0.05), meaning that there was no toxicity of the bivalve *Anodonta*'s chitosanto mesenchymal stem cells.

Mesenchymalstem cellswere implanted into tubes containing the scaffold consisting of the bivalve *Anodonta*'schitosan added with platelet-rich plasma.This study showed that the bivalve *Anodonta*'schitosanwas dissolved in the medium, so that centrifugation of 50μg of chitosanin the α-MEM mediumwill dissolve it.Then,$2x10^6$ of mesenchymal stem cellswere seeded by putting it in the incubator for 16 h and added with 1.5 mL of platelet-rich plasma. Formulation of the bivalve *Anodonta*'schitosan–PRP–mesenchymal stem cellswas observed by using a scanning electron microscope, which indicated that the mesenchymal cells lived between pores of the bivalve *Anodonta*'s chitosan.

Results demonstrated that the bivalve *Anodonta*'schitosan and platelet-rich plasma can be used as a composite scaffold.The requirement for biocompatibilityhas been confirmed by the MTT assay, which showed that the bivalve *Anodonta*'s chitosan was not toxic to the mesenchymal stem cells.The requirement for bioactivitywas fulfilled by the living mesenchymal cells in the pores of the bivalve *Anodonta*'s chitosan.The requirements for mechanical properties, porosity and pore sizewere met by the structural featuresas shown by the scanning electron microscopy in which there was a strong shell architecture, the presence of interconnected pores and anideal pore size of 150–200μm. The requirement for biodegradability was fulfilled at the final stage in which the shells will be shed and completely fused with cells and matrices. Osteoconductivitywas demonstrated by the scaffold construction of the bivalve *Anodonta*'s chitosan and the protein chitosan that would secrete biological fluids which bind to cell surface receptors and growth factors.

Conclusion

In conclusion, the bivalve *Anodonta*'s chitosan scaffold was not toxic to the mesenchymal stem cells and PRP, the cells could grow properly in the pores of the bivalve *Anodonta*'s chitosan and PRP, so the bivalve *Anodonta*'s chitosan–PRP–mesenchymal stem cells have possibility used for a composite scaffold and can be used for *in vivo* studies.

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