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BIOSCAFFOLD CHITOSAN-ALGINATE FOR TISSUE ENGINEERING APPLICATIONS

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ABSTRACT

This study aims atexamining the condition of chitosan/alginate scaffold processing for tissue engineering applications. Chitosan 90.2% DD and Ca-alginate were both dissolved in 1% acetic acid and varied by volume ratios (v/v) (80:20, 70:30, 50:50, 30:70, 20:80) . The scaffold is printed to a glass mold, allowed to stand at room temperature for 24 hours, and thenheated in an oven at a temperature of 40ºC for 48 hours. The outcome of this research product was in form of non-transparent thin sheets. FTIR test results revealed a number of peaks which indicated a certain level ofinteraction between chitosan, and Ca-alginate. The thermal degradation test established that there is a reduction in the melting point of the scaffold when there is a higher concentration in the material. It also indicated that there is an increase in the decomposition point in line with an increase in chitosan. Scaffold samples are physically superior as regards the easy handling of wet conditions (fixed shape and not easily torn), and providesunexpected results in aspects of biocompatibility.

KEY WORDS: chitosan, alginate, scaffold

INTRODUCTION

Tissue engineering is an interdisciplinary field that applies the principles of engineering and biological sciences to make life parts for the body. The general approach often employed is the *scaffold* which is capable of stimulating the growth of artificial cellular structure, cellular proliferation and differentiation. The studies carried out have introduced new possibilities for building vital autologous tissue *in vitro* using appropriate biomaterials for the repairing of tissues (Arca HC, et. Al 2008, Langer R, et.al, 1993, Peter X Ma., 2006).

Extensive skin tissue damages due to accidents and burns require special and integrated treatments to prevent the occurrence of severe complications asthis can result in death in humans. Rapid healing of skin defects and scar formation due to extensive skin tissue loss can be avoided with the use of several skin replacement cells. For example,*xenografts, allografts, and autografts* are widely used for healing skin wounds. Many studies therefore employ tissue engineering approaches forthe regeneration of tissues, as well asrecovering and maintaining human organ functions. (Sun J et. Al., 2013, Guarino et. Al., 2015, Kumbar SG, et.al, 2015, Jeong IS et.al, 2010, Jeong IS et.al, 2012, Roy T, 2012).

One important factor in skin tissue engineering is the*scaffold* structure which is a matrix or an artificial structure. This structure is needed for the infiltration and physical support of cells which leads to *proliferation,* and cell *differentiation* into functional tissues or human organs. An ideal scaffold utilised for skin tissue engineering must possess excellent biocompatibility characteristics, suitable microstructure within the range of 100-200µm (pore size and porosity above 90%), biodegradability control and suitable mechanical properties. Several biomaterials have been studied in the medical field prior to the advent of tissue engineering. Among them are chitosan, alginate and several others which are materials that can be put to use for making scaffolds, as a result of the have several advantages in their physical and chemical properties (Sun J et. Al., 2013, Guarino et. Al., 2015, Kumbar SG, et. al, 2015, Jeong IS et.al, 2010, Roy T, 2012).

This study will run examinations on the chitosan scaffold processing (DD 90.2%) of shrimp skin and Na-alginate with variations in composition comparisons, FTIR testing, thermal testing, and growth of cell culture (fibroblasts) above the scaffold. This research is expected to produce scaffold designs applicable to the field of medicine (surgery), especially for human skin tissue repair.

METHODS

Alginate (3% b/b) and chitosan 3% b/b (shrimp skin) from Bio Chitosan Indonesia are both completely dissolved in 1% glacial acetic acid (SIGMA) solution, to produce a 0.5% solution (b/v). This mixture is stirred until homogeneous with variations in weight comparison (80:20, 70:30, 50:50, 30:70, 20:80), for 1 hour. Afterwards, the chitosan/alginate scaffold is crosslinked with CaCl2 5% (w/v) solution. The formed

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bubbles are then taken out of the polymer solution. The mixture was centrifuged for 15 minutes at 3000 x g, to eliminate insoluble impurities. The Chitosan/alginate solution was then poured into a glass mold (7.5x7.5) of 5mm thickness, after which it was frozen at room temperature for 24 hours, and finally heated in an oven at 40, 50 and 60 ^{o}C for 48 hours.

Dulbecco's Modified Eagle Medium (DMEM) Sigma-Aldrich Co., Missouri, USA. Bovine serum (FBS) Invitrogen, California, USA. Human dermal fibroblasts (HDF, Integrated Laboratory FK YARSI, Indonesia). Penicillin-Streptomycin is 1% and Fungizone is 1ug/ml. Making use of the above material, the test was conducted using a culture plate with 12 wells (4cm2/well). The assessment was performed by examining the attachment ability, morphology and cell viability qualitatively based on photomicrograph, which was taken at 1, 24 and 72 hours after seeding.

RESULTS AND DISCUSSION

This study was conducted in 3 stages: (1) the stage of processing raw materials, (2) that of processing chitosan/alginate scaffold which had been crosslinked with CaCl2 and used in various compositions (3) and the chitosan/alginate scaffold testing stage. Comparison of chitosan/alginate (v/v) composition in 100mL acetic acid, resulting in more transparent, thin, flexible and flat scaffold layers can be seen illustrated in Figure 1.

The scaffold dried at a temperature of 40^{o}C with a chitosan/alginate ratio of 50:50 and the addition of 5% CaCl2 (w/v) displayed better characteristics (homogeneous, flexible and transparent) compared to other variations. The use of chitosan as a small portion (up to 50%) of the ingredients, produces a better scaffold than that with more alginate in the ingredients. A higher variation of chitosan/alginate concentration produces a transparent, thick, hard, tough and uneven scaffold (Figure not shown).

Figure 1. Chitosan / alginate scaffold images with comparative variations in the composition of thin, transparent, flexible, flat and homogeneous layers.

FTIR TEST

The interaction between scaffold constituents can be realised by conducting a FTIR test. Information on structural influences can be known in FT-IR analysis making use of Elmer GX Perkin Spectrum (FT-IR system). A scaffold with a thickness of about 10µM is put to use in infrared tests. The FT-IR analysis is based on identifying the absorption of the band concerned with the vibration of the functional group shown in macromolecules (Kumbar S.G, 2015, Jeong IS et. Al, 2010, Archana D et.al, 2013). The FTIR spectra combined scaffolds from chitosan/alginate with variations in composition comparisons, as illustrated in Figure 2 to Figure 9 below:

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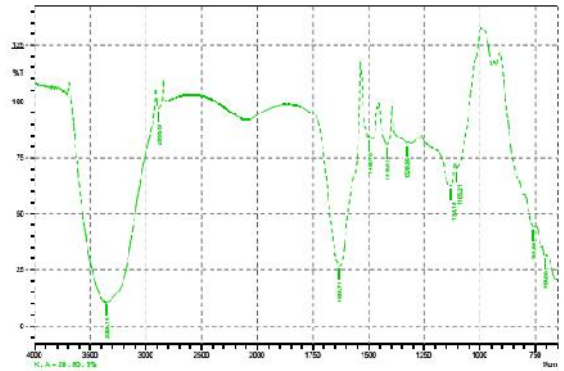

Figure 2 FT-IR spectrum of chitosan / alginate v / v (20/80) scaffold.

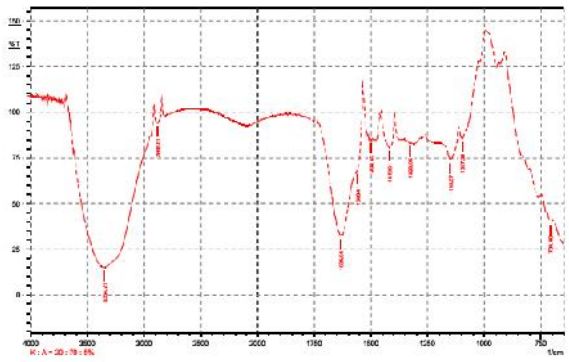

Figure 3 FT-IR spectrum of chitosan / alginate v / v (30/70) scaffold.

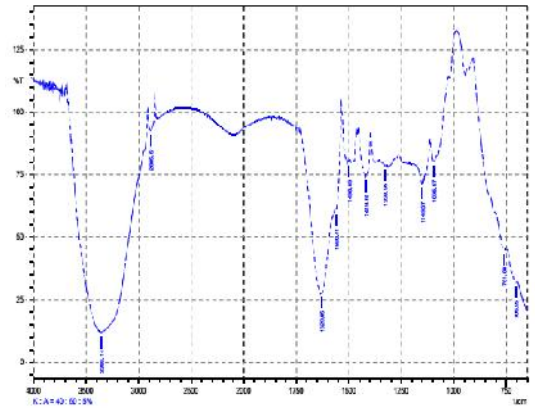

Figure 4 FT-IR spectrum of chitosan / alginate v / v (40/600) scaffold in the temperature of 40ºC.

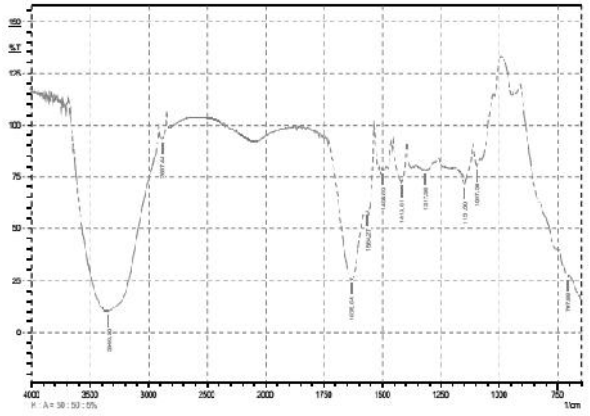

Figure 5 FT-IR spectrum of chitosan / alginate v / v (50/50) scaffold.

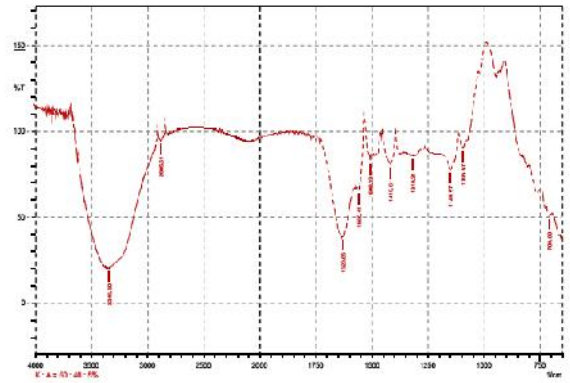

Figure 6 FT-IR spectrum of chitosan / alginate v / v (60/40) scaffold.

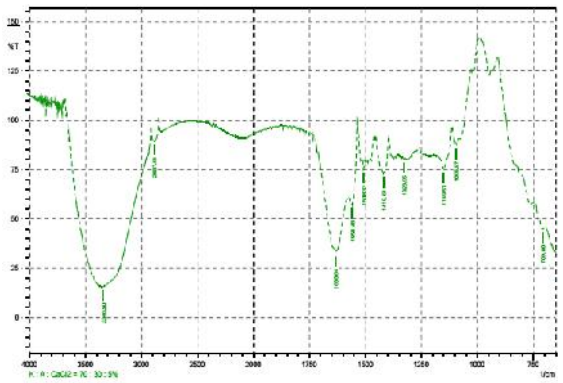

Figure 7 FT-IR spectrum of chitosan / alginate v / v (70/30) scaffold.

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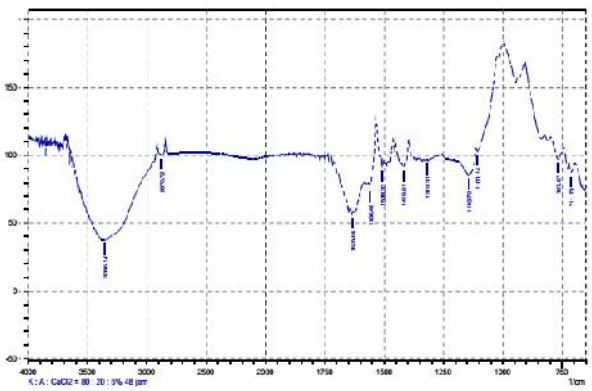

Figure 8 FT-IR spectrum of chitosan / alginate v / v (80/20) scaffold.

Figure 3 through Figure 8 The FT-IR spectrum of chitosan/alginate scaffolds displayed significant differences with different composition variations. The wavelength ranges from 750 to 4000. There is a peak shift along with the addition of a new peak between the graphs which indicates the interaction between chitosan/alginate and CaCl2 groups. The difference in composition is obviously illustrated on the graph. In other words, there are significant peak shifts at 3350 wavelengths (–OH groups), 1650 (N-H groups) and 1150 (C-O groups).

Thermal Test with DSC (Differential Scanning Calorimetry)

The DSC test was conducted to analyse the endothermic peak temperature of scaffolds chopped into small pieces (2mm3). These scaffolds are sliced in order for 4 to 6 small pieces to weigh about 5mg, which are then placed in an aluminum pan. The analysis was carried out in DSC (2010; TA Instrument, New Castle, DE) from 50ºC to 250ºC with a heating rate control of 5ºC/min. Tests were conducted on all samples using various compositions.

The DSC Test (Differential Scanning Calorymeter)was carried out to discover the melting point of the scaffold towards heat, and also to determine the intensity of heat required to attain that point. The melting point depicts the phase of changing from solid to liquid form without undergoing any change in composition. The melting temperature is that level of temperature at which the polymer loses its crystallinity. The curve has a typical model with two endothermic peaks. The melting point and decomposition temperature of chitosan/alginate scaffold is not a single price but lies within a certain range. In Figure 10, the first peak relates information regarding the melting point of the material, while the second provides information regarding the decomposition temperature. Scaffold material has a lowered melting point attributable to the addition of crosslinking materials. This happens because the polymer chain is physically degraded. This means, the polymer chain is pressed by a crosslinking material which damages the molecular chain of the polymer.

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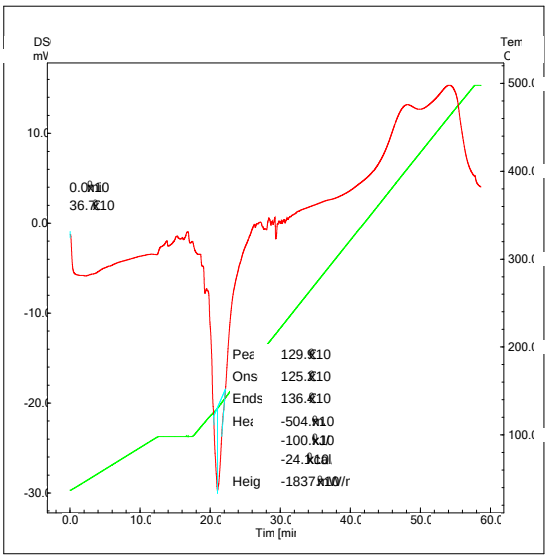

Figure 9 Graph of heat value of chitosan / alginate v / v (20/80) scaffold DSC test results.

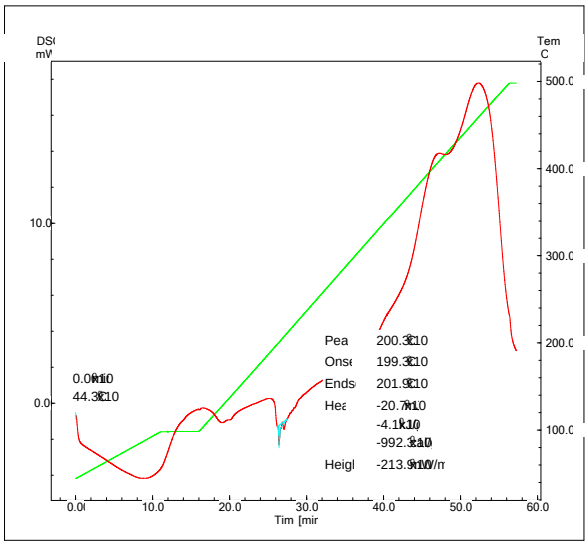

Figure 10 Graph of heat value of chitosan / alginate v / v (30/70) scaffold DSC test results.

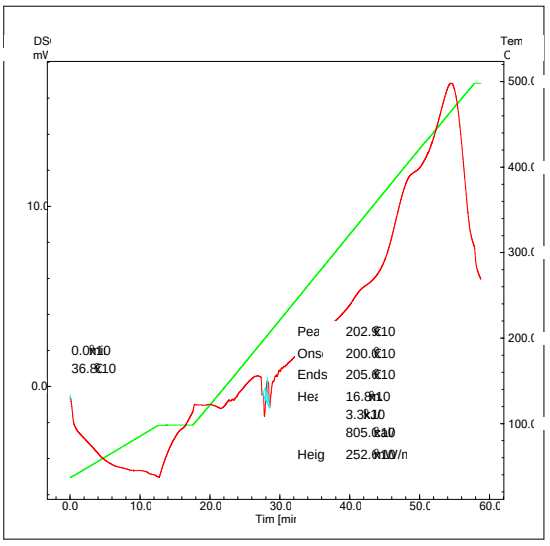

Figure 12 Graph heat value of DSC scaffold test results (a) Chitosan / alginate 40/60, at 40°C

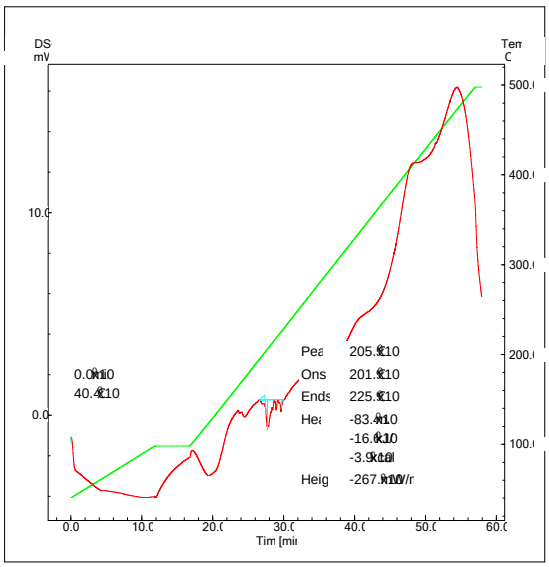

Figure 12 Graph heat value of DSC scaffold test results (a) Chitosan / alginate 50/50, at 40°C

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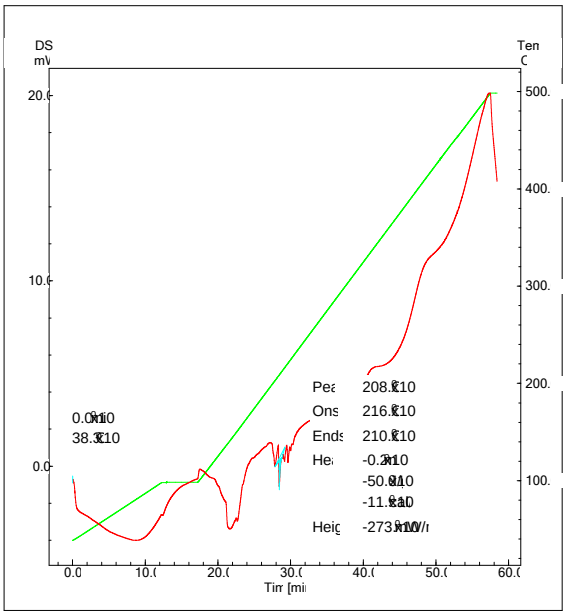

Figure 14 Graph of heat value of chitosan / alginate v / v (60/ 40) scaffold DSC test results.

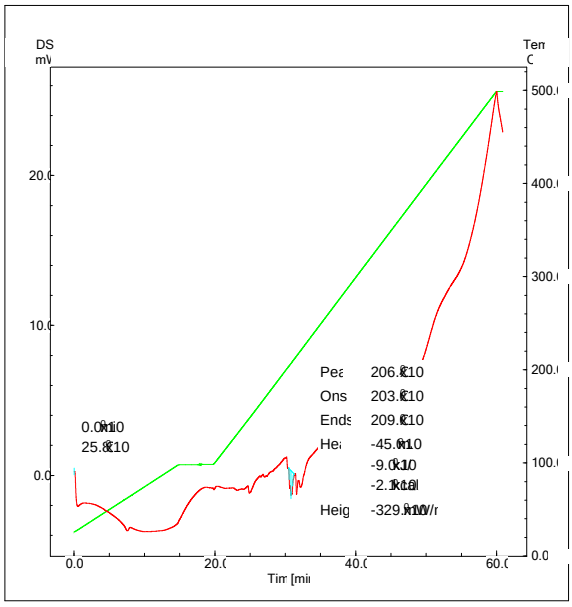

Figure 15 Graph of heat value of chitosan / alginate v / v (70/30) scaffold DSC test results.

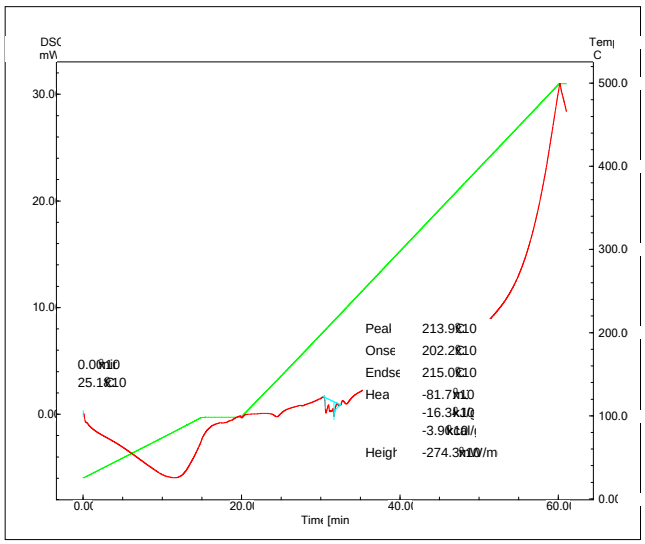

Figure 15 Graph of heat value of chitosan/alginate v/v (80/20) scaffold DSC test results.

Table 1 belowpresents the thermal test data of chitosan/alginate scaffold with composite variations.

Table 1 The thermal test data of chitosan/alginate scaffold with composite variation

Composite comparison	Melting point (°C)	Decomposition point (°C)
Chitosan / alginate (20/80)	36,72	129,92
Chitosan / alginate (30/70)	44,34	200,33
Chitosan / alginate (40/60)	36,82	202,95
Chitosan / alginate (50/50)	40,42	205,53
Chitosan / alginate (60/40)	38,37	208,26
Chitosan / alginate (70/30)	25,82	206,47
Chitosan / alginate (80/20)	25,16	213,97

It can be observed in the table above, that the melting point is relatively low and there is also an increase in the decomposition point which is inline with an increasing chitosan in the material.

Before the cell is grown above the scaffold, the nature of thermal degradation is required for the

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scaffold sterilisation process (by heating). The fluctuation in the melting point of the material is attributable to the addition of CaCl2 crosslinking material. This is as a result of the polymer chain being physically degraded or affected by crosslinking materials which inflict damage on the molecular chain of polymer bonds.

Cell Growth Test in Scaffold

Cell culture tests conducted on membranes or *scaffolds*are aimedat evaluating biocompatibility *in vitro*. The ability of membranes to support cell adhesion, mitosis and proliferation signifies the suitability of membrane *microenviroment* to cell growth. This test also reveals the membrane's resistance to a water-based environment, easy manipulation and *handling*, and the interaction between the membrane and the cells planted on it.

Human Dermal Fibroblast (HDF) culture on the membrane was tested using the DMEM culture medium (gibco), 10% FBS supplementation (gibco), 1% antimicotics (gibco) and 35mm dish. An assessment is carried out by examining the attachment ability, morphology and qualitative viability based on photomicrograph taken 2 hours after seeding. This is performed with a low density (20,000 viable cells/sqcm). The pH check makes use of a pH test strip for the range 6.4-8.0 (Hadi et al Hospital, 2010, Shariati PRS, 2009).

An evaluation 2 hours after seeding revealed that all test membranes did not possess biocompatibility properties. This is characterised by the absence of fibroblast cells that attach and take a spindle-shaped characteristic for fibroblasts when compared to controls. Figure 17 illustrates the entire test membrane, revealing that the cell unattached to the membrane surface after 2 hours of incubation. The control group grown above the standard culture plate is then attached after 2 hours of incubation. Thiscan be observed in Figure 5.16. All test groups also exhibited changes in the color of the medium from red to yellow which clearly indicates a reduction in the pH of the medium. An examination of pH shows a reduction in its concentration in the test group to as low as 6.8. Figure 19 is a representation of the conditions found in all test groups (a blebbing picture). *Blebbing* is a cell membrane protrusion caused by the loss of interaction between actinkortical and membrane filaments in one part. This happening is commonly observed in the process of cell apoptosis or non-apoptotic events such as cell migration, cell division and physical and chemical stress. Based on the above results, the change in pH of the medium is estimated to become a form of chemical stress which instigates the failure of cell attachment to the test membrane. Nevertheless, the physical quality of the membrane is very suitable for testing biocompatibility, especially in handling and aspects of membrane manipulation, except for its thickness which makes it difficult observing its details through a microscope. 24 hours post-planting observation also showed signs of fungal/bacterial contamination which wasnot observed in the control group.

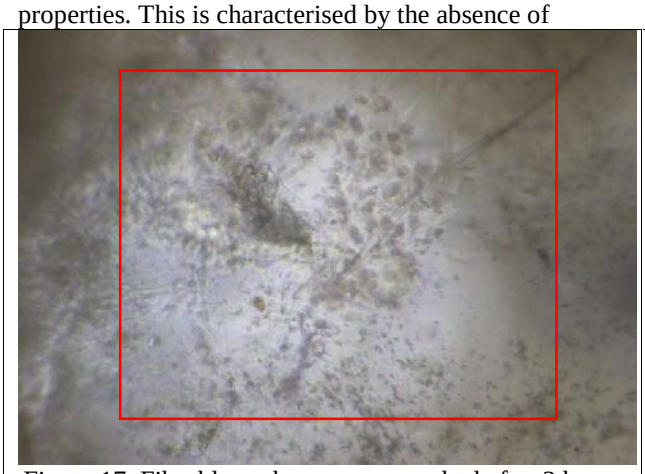

Figure 17. Fibroblasts that are not attached after 2 hours of seeding(red box), Nikon 10x.

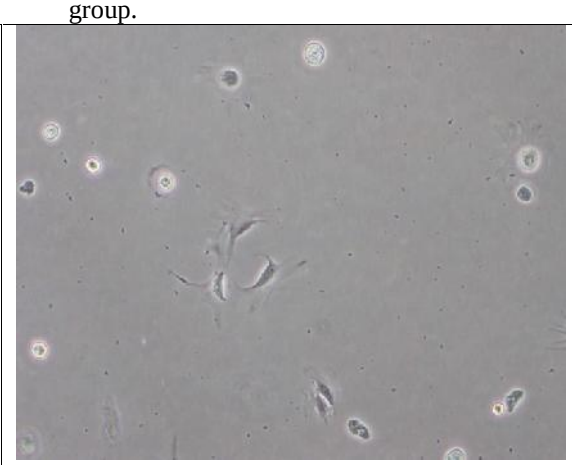

Figure 18 Adhesion of fibroblast cells to 2-hour post-seeding culture plates, Nikon 10x

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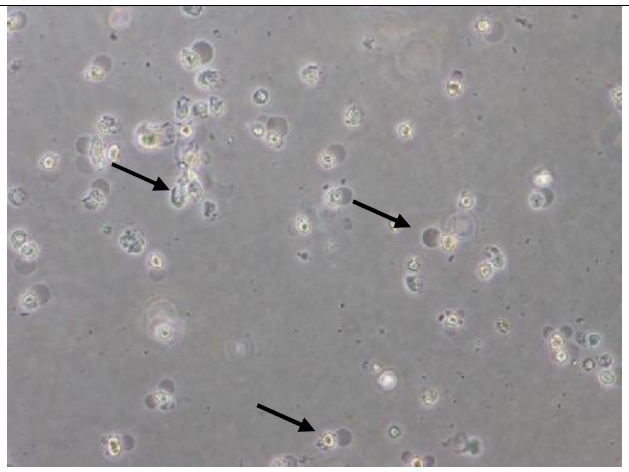

Figure 19. Cell Blebbing attached to the plastic cup of the test group around the scaffold, 2 hours post seeding in the test group (arrow).

In the picture above, this scaffold sample is physically superior in terms of easy handling and manipulation. However, the results as regards biocompatibility is not in accordance with the initial expectations. As for the membrane in general, the cell test is not attached to the membrane surface after 2 hours of incubation. Physically, this membrane is still thick, making it difficult to observe. The decrease in pH of the medium along with an observation 24 hours after planting show signs of fungal/bacterial contamination.

CONCLUSION

The results of this study indicated that the chitosan/alginate scaffold with a low composition ratio was better and more homogeneous compared tothose with high concentrations. FTIR test results revealed a number of peaks which showed interaction between chitosan, alginate and CaCl2 in the scaffold. Peak shifts are significant at wavelengths 3350 (-OH groups), 1650 (N-H groups) and 1150 (C-O groups). The results of thermal degradation (DSC) melting point on chitosan/alginate scaffold decreased,while the decomposition point increased with an increasing chitosan in the material. Scaffold samples are physically superior as regards easy handling in wet conditions (fixed form and not easily torn), however, they have not provided results in accordance with expectations for biocompatibility aspects.

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