Calcium phosphate /poly (ethylene glycol) bone cement: Cell culture performance

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Abstract*— Calcium phosphate cement (CPC) for injectable bone cement application has been developed in this study. The CPC was produced using a novel wet chemical precipitation method derived hydroxyapatite (HA) powder. The calcium and phosphorus precursors used to synthesize HA powder were calcium hydroxide, $Ca(OH)_2$, and di-ammonium hydrogen phosphate, $(NH_4)_2HPO_4$. The HA powder was mixed with distilled water at certain powder-to-liquid (P/L) ratios. In this study, the P/L ratios were varied at 1.3 and 1.7. PEG was added into CPC with the P/L ratio of 1.3, and it was adjusted at 1 and 5 wt%. The results of this study revealed that higher P/L ratio contributed to the decreased in porosity of CPC. Meanwhile, the addition of PEG increased the porosity of CPC. This is significant for cells adhesion and proliferation, such that cell proliferate faster and better adhesion with the incorporation of PEG into CPC. The cell culture on CPC has proven that the fabricated CPC shows no toxic reaction and cells grow well.

Keywords: Calcium phosphate cement; Injectable; Polymeric additive; Vero cell culture; Wet chemical precipitation

1. INTRODUCTION

Bone cement has been attracting considerable attention as human hard tissue filler and joint anchorage materials since the second half of the last century. Development of bone cement biomaterials has been an alternative to overcome the limitations of bone grafting; inadequate bone supply and require additional operations for autograft, and high risk of immunological reactions and disease transmission in allograft [1,2].

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Bone cement materials that were clinically available in this present year are polymethylmethacrylate (PMMA), calcium phosphate cement (CPC) and calcium sulfate cement (CSC). PMMA-based cement shows biocompatibility, high mechanical strength, and excellent setting and injectability. However, it has low of bioactivity and resorbability, high stiffness, exothermic setting reaction, as well as monomer toxicity and leakage [3]. On the other hand, CSC is much stronger than CPC, but its degradation rate is much faster which causes the bone regeneration and cement degradation rates to be different [3].

CPC is significant and clinically accepted as injectable bone filling material. CPC able to overcome the limitations of PMMA and CSC bone cement due to its remarkable biological response, injectability and potential to set in vivo [4-6]. CPC is able to be injected and molded to fill and take the shape of bone defect. Injectable CPC is prepared by mixing calcium phosphate powder of various phases and water at certain ratios and set via dissolution-precipitation mechanism at body temperature [5]. The mixture of various calcium phosphate phases such as tetracalcium phosphate (TTCP) and α–tricalcium phosphate (α-TCP) with water formed the apatite cements of hydroxyapatite (HA) [7].

The synthesis method of CPC can be classified into dry and wet methods. Solid-state synthesis and mechanochemical process categorized as dry methods. Meanwhile, chemical precipitation, hydrolysis, hydrothermal, sol-gel, emulsion and sonochemical methods categorized as wet methods [8,9]. Wet-chemical precipitation method is more favourable to be employed in the synthesis of CPC because it offers simple route, low reaction temperature, highly pure end products and inexpensive resources [10]. Poor injectability and low mechanical strength are the critical limitations of CPC which could hinder its clinical applications. The improvement of properties of CPC can be made through the addition of polymeric additives [11,12]. The incorporation of polymeric additives into CPC including chitosan, alginate, poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA) have been addressed in various studies [11].

In the present study PEG was employed as the polymeric additive. PEG is a polyether composed of glycerol monomers. It is non-toxic, water soluble, flexible and

anti-coagulant properties which make it suitable to be used in biomedical fields [13]. PEG has been used in the preparation of premixed CPC, acts as a thickening agent and keeps the cement paste stable [11,13].

The incorporation of PEG into CPC is expected to improve setting time, injectability and anti-washout performance of CPC [11]. The work investigates the effect of powder-to-liquid (P/L) ratio and PEG content on the cell culture performance of CPC derived from wet chemical precipitation method.

2. EXPERIMENTAL

2.1. Synthesis of powder

Wet chemical precipitation method was employed to synthesize CPC based on the procedure reported elsewhere [14]. In this case, the HA powder was prepared using calcium hydroxide, $Ca(OH)_2$, and diammonium hydrogen phosphate, $(NH_4)_2HPO_4$, as the calcium and phosphorus precursors respectively, and the solvent used was distilled water. Calcium-to-phosphate (Ca/P) ratio was fixed at 1.67. Each precursor was dissolved in distilled water to produce calcium and phosphorus solution. Then, it was followed by drop wise addition of phosphorus solution into the calcium solution. 25% ammonia solution was added until pH 11 was achieved. The mixture was then refluxed at 90ºC, followed by aging overnight at room temperature, washing with distilled water and filtration. Afterwards, drying the precipitate overnight in an oven at 85ºC, and finally crushed.

2.2. Preparation of CPC

Preparation of CPC was done by mixing the as-synthesized HA powder and liquid phase at certain ratios. The P/L ratio was varied at 1.0, 1.3, 1.5 and 2.0. The P/L ratio of 1.3 was selected to prepare CPC incorporated with PEG (MW300, Sigma) based on its optimized properties. PEG addition into the liquid phase was varied at 1, 2, 3, 4 and 5 wt%.

2.3. Porosity

The measurement of density was done using a densitometer. The resultant apparent density was then used to calculate the porosity of CPC using the following equations:

$$\%\ porosity\ = 100\% - relative\ density \quad (1)$$

$$relative\ density = \frac{\rho_{app}}{\rho_{th}} \times 100\% \quad (2)$$

Where,

ρ_{app} = apparent density measured by densitometer

ρth = theoretical density of HA taken as 3.156 g/cm^3 [15,16].

2.4. Cell culture

A Vero cell line was used throughout the study. The cell line was procured from Cell Lines Services (CLS, Germany). The culture media was prepared by mixing Dulbecco's Modified Eagle Medium (DMEM) with Fetal Bovine Serum (FBS). The culture media prepared comprising of 10% FBS and 90% DMEM.

Thawing is the process to revive the cells and cultivate the cell in 2D cultured T-flask. The method suggested by Freshney [17] was employed in the thawing and subculture of Vero cells. CPC were subjected to sterilization using the standard autoclaving method. Meanwhile, CPC with PEG underwent sterilization via UV method because of low melting point of PEG.

Cells were grown on samples by using the same method in subculture of cells, by leaving the cement in 25 cm2 T-flask containing cells with 10 ml culture media. The flask was then left in CO2 incubator supplied with 5% CO2 at 37ºC for 7 days. The cells attachment on CPC was observed after 3, 5 and 7 days culture period. The samples were dried through air drying by the evaporation of hexamethyldisilazane (HMDS) prior to SEM (JEOL JSM-IT100) observation. Cells counting have been done for 3, 5 and 7 days culture periods by utilizing haemacytometer. Then, cells concentration (cells/ml) was calculated using the following equations:

$$c = n\ x\ 10^4 \quad (3)$$

$$c = n\ x\ dilution\ factor\ x\ 10^4 \quad (4)$$

Where,

c = cells concentration (cells/ml)

n = number of cells counted by using haemacytometer.

3. RESULTS AND DISCUSSION

3.1. Porosity

The effect of P/L ratio on the porosity of CPC has been investigated. The result is presents in Figure 1. The fabricated CPC with the P/L ratio of 1.0, 1.3, 1.5 and 1.7 has porosity of 47.1%, 45.2%, 40.8% and 47.7%, respectively. This result shows decreasing trend of porosity with the increase in P/L ratio. This is attributed to increasing powder content which contributed to the formation of HA crystals during setting via dissolution and reprecipitation mechanisms. However, this is applicable for CPC with the P/L ratio less than 1.7. When P/L ratio 1.7 is used, the porosity started to increase due to the slowed down of apatite crystals formation rate. The insufficient water content in the paste halt the dissolution and reprecipitation setting mechanisms.

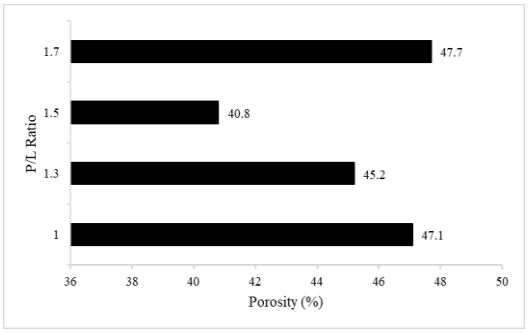

Fig. 1: The effect of P/L ratio on porosity of CPC

Figure 2 presents the result of CPC/PEG with the P/L ratios of 1.0, 1.3 and 1.5. The results revealed that the fabricated CPCs are of high porosity. The high porosity of CPC/PEG is presumably due to water entrapped between the

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crystals and porosity introduced during cement preparation [18]. CPC/PEG with the P/L ratio of 1.0 has porosity in the range of 49.8% to 58%. The porosity of CPC/PEG with the P/L ratio of 1.3 is in the range of 39.6% to 51.4%. Whereas, CPC/PEG with the P/L ratio of 1.5 has porosity between 40.8% and 46.7%. The result of porosity for CPC/PEG with the P/L ratio of 1.7 demonstrates by Figure 3. The addition of PEG into CPC with P/L ratio of 1.7 resulted in porosity in the range of 47.7% to 52.4%. These results indicate that incorporation of PEG into CPC increases porosity of CPC. This might be attributed to the formation of sub-micrometric pores between particles when PEG was added [19]. High volume of submicrometric pores lead to the increase in porosity of the fabricated cement.

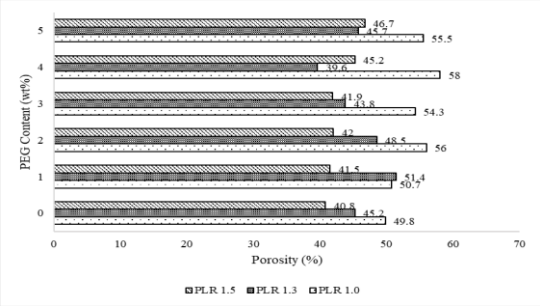

Fig. 2: Porosity of CPC/PEG

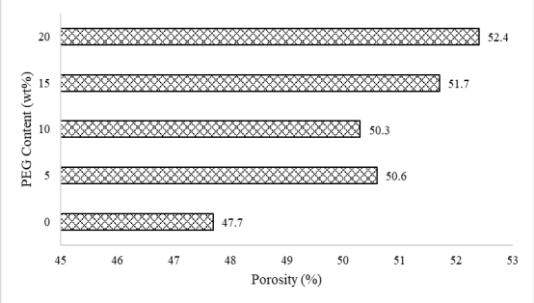

Fig. 3: Porosity of CPC/PEG with the P/L ratio of 1.7

3.2. Vero cell culture on CPC

3.2.1. Qualitative cells attachment

Qualitative cells attachment for cytotoxicity test was done through the immersion of CPC in Vero cells containing media (10% FBS and 90% DMEM). The sample was kept in the CO2 incubator at 37℃ with 5% CO2 for 7 days culture periods. Figures 4, 5 and 6 show the SEM image of cell attachment on CPC after 1, 4 and 7 days culture periods respectively. All figures demonstrate that cells are able to attach and then proliferate on all cement compositions.

Figure 4 (a) and (b) show the cells attached on CPC with the P/L ratios of 1.3 and 1.7 respectively after 1 day culture period. In Figure 4(a), it demonstrates that cells only started to attach on the cement. Meanwhile, Figure 4(b) demonstrates that the cells have already attached and proliferate on the cement surface. This explains that high amount of CaP powder aids the cells adhesion and subsequently spreading on the cement surface. Next, Figs. 4 (c) and (d) present the cells attachment on CPC with the P/L ratio of 1.3 containing 1% PEG and 5% PEG respectively. Both compositions reveal good cells adhesion and proliferation when compared to CPC without PEG in Figure 4(a). This proves that cells are able to grow, attach and proliferate better with the addition of PEG.

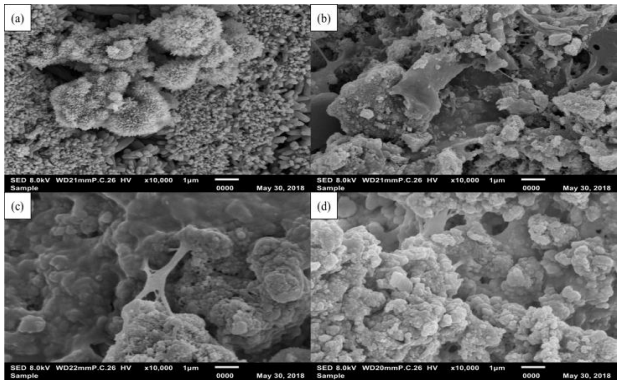

Fig. 4: SEM micrograph of cell attached on (a) CPC with the P/L ratio of 1.3, (b) CPC with the P/L ratio of 1.7, (c) CPC with 1% PEG for the P/L ratio of 1.3, and (d) CPC with 5% PEG for the P/L ratio of 1.3 after 1 day culture period

Figure 5 (a) and (b) show the cells attached on CPC with the P/L ratios of 1.3 and 1.7 respectively after 4 days culture period. In Figure 5(a), it demonstrates that some surface of the cement are still not been covered by cells. Meanwhile, Figure 5(b) shows the multiple layer of cells that fully covered the surface of CPC. This indicates that CPC with the P/L ratio of 1.7 provides better cells attachment and proliferation than that of CPC with the P/L ratio of 1.3. The cells attachment after 4 days culture on CPC/PEG composite for the P/L ratios of 1.3 with 1% PEG and 5% PEG is presented in Figure 5 (c) and (d) respectively. Both figures show the ability of cells to attach and proliferate on the surface of CPC/PEG composite. 1% PEG addition in Figure 5(c) shows that the cement surface is not fully covered by the cells. On the other hand, the surface of CPC with 5% PEG addition have been fully covered by the cells as shown in Figure 5(d). The observation suggests that PEG addition could improve cells attachment and proliferation on CPC. This result indicates that PEG addition does not give negative effect on the cells growth.

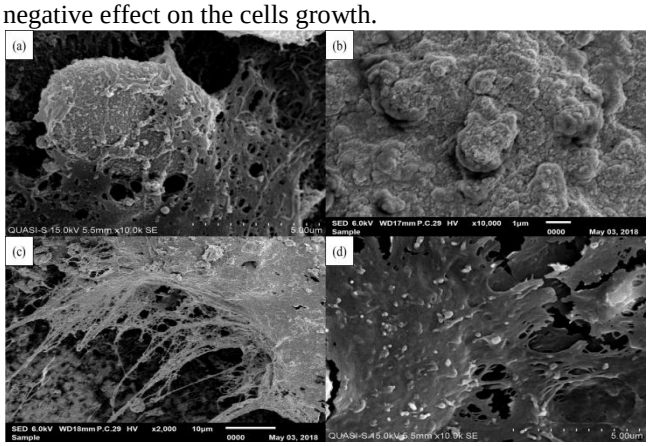

Fig. 5: SEM micrograph of cell attached on (a) CPC with the P/L ratio of 1.3, (b) CPC with the P/L ratio of 1.7, (c) CPC with 1% PEG for the P/L ratio of 1.3, and (d) CPC with 5% PEG for the P/L ratio of 1.3 after 4 days culture period

Figure 6 shows the cells attached on CPC and CPC/PEG composite after 7 days culture period. Figure 6(a) demonstrate that after 7 days, the cells still attached on the surface of CPC with the P/L ratio of 1.3. Meanwhile, the cells on the surface of CPC with the P/L ratio of 1.7 after 7 days culture started to detached from the cement surface as demonstrated in Figure 6(b). Comparing between CPC without and with PEG demonstrate that CPC with PEG also shows cells detachment from the cement surface as shown in Figure 6(c). The cells detachment occurs as demonstrated in Figure 6 (b) and (c) are deduced to happen because of the unchanged media which contain the waste from cells. The presence of hydrophilic PEG also contributed to the cells detachment as the dissolved PEG could affected the culture media, in addition to the waste from cells.

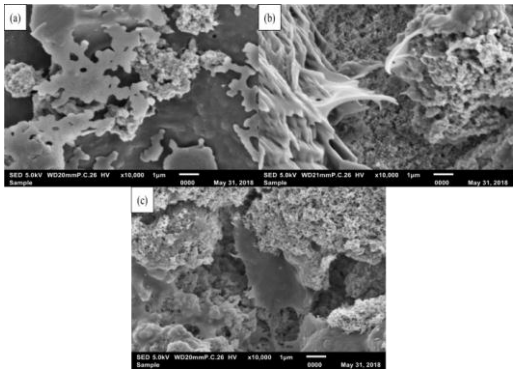

Fig. 6: SEM micrograph of cell attached on (a) CPC with the P/L ratio of 1.3, (b) CPC with the P/L ratio of 1.7, and (c) CPC with 1% PEG for the P/L ratio of 1.3 after 7 days culture period

3.2.2. Quantitative cells count

Quantitative analysis for cytotoxicity was done by cells counting using haemacytometer with the aid of trypan blue. The cell counting was done to calculate the number of cells able to attach on the fabricated CPC. The counting was done for 3, 5 and 7 day culture period to investigate the cell growth over each culture period.

The number of cells attached on CPCs with different P/L ratios is presented in Figure 7. The graph demonstrates that the increase in P/L ratio increases the number of cells attached on CPC. This is attributed to the high calcium phosphate powder which formed biological apatite during setting reaction. The formation of biological apatite during the dissolution and precipitation process led to faster cell growth. CPC with a higher P/L ratio has a higher concentration of calcium-phosphate pair ions and hence increased ionic activity that make cell proliferate faster.

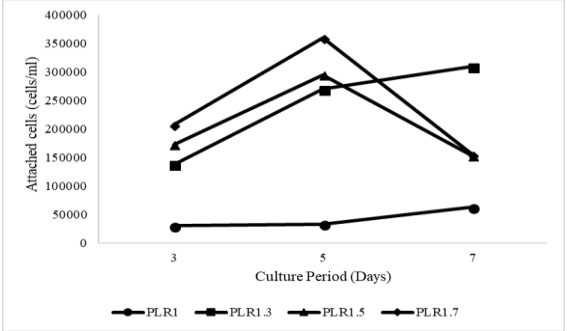

Fig. 7: Effect of different P/L ratios on the cells attachment on CPC

The result of cells counting for CPC with different P/L ratios is tabulated in Table 1. Figure 4, 5 and 6 (a and b) are the evidences which have proven this cell counting results, such that the surface of CPC with the P/L ratio of 1.7 has been fully covered by the cells compared to that of P/L ratio of 1.3. However, the number of cells attached on CPC with the P/L ratios of 1.5 and 1.7 started to decrease after 5 days culture period. This could be attributed to the unchanged culture media, which might contain waste produced by the cells. The unchanged culture media could not provide fresh nutrients to the cells, which could negatively affect the cells adhesion.

Table 1: Number of cells attached on CPC after 7 days culture period

Culture Period (Days)	Number of Cells Attached on CPC (cells/ml)			
	PLR1.0	PLR1.3	PLR1.5	PLR1.7
3	3.00×10^4	1.38×10^5	1.73×10^5	2.07×10^5
5	3.33×10^4	2.70×10^5	2.95×10^5	3.60×10^5
7	6.29×10^4	3.10×10^5	1.53×10^5	1.55×10^5

The effect of PEG addition on the cells attachment and proliferation on CPC is demonstrated in the Figure 8. The graph shows that the increase in PEG content increased the number of cells attached on CPC. This is because of the increase in porosity of CPC with the increase in PEG content as presented in Figure 2 and 3. CPC/PEG which have higher porosity than CPC, is more soluble with higher surface roughness led to faster cell growth. The formation of micropores provide surface texture for more propitious cell adhesion. In addition, the hydrophilic properties of PEG also contributed to the improvement of cells attachment on the CPC.

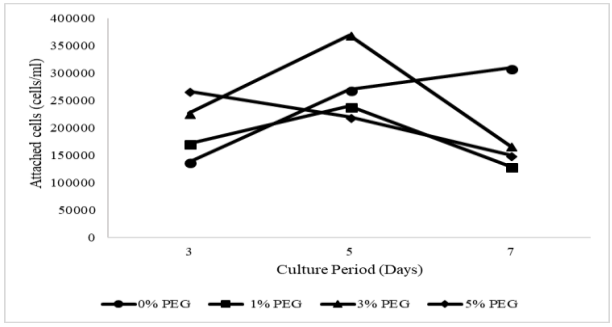

Fig. 8: Effect of PEG addition on the cells attachment on CPC

The result for cells counting presented in Table 2 is in line with the SEM observation as demonstrated in Figure 4, 5 and 6 (c and d). Both results proved that the addition of PEG was able to enhance cells adhesion and spreading on CPC, such that the cement surface with 5% PEG content has been fully covered by cells compared to that of 1% PEG content. However, the number of cells attached decreased after 5 days culture for 1% and 3% PEG.

Meanwhile, decreasing number of cells attached on CPC is observed for 5% PEG content. This is attributed to the hydrophilic properties of PEG, which dissolves in the culture media and produced waste that could hamper cells adhesion. The cytotoxicity and excellent bioactivity of CPC and CPC/PEG on the cell have been proven since cells are able to attach and grow well on the fabricated cement with no toxic indication has been observed throughout the culturing time. Evidently, PEG addition was able to enhance cells attachment and it does not negatively affected cells growth.

Table 2: Number of cells attached on CPC/PEG for the P/L ratio of 1.3 after 7 days culture period

Culture Period (Days)	Number of Cells Attached on CPC (cells/ml)			
	0% PEG	1% PEG	3% PEG	5% PEG
3	1.38×10^5	1.72×10^5	2.27×10^5	2.67×10^5
5	2.70×10^5	2.40×10^5	3.70×10^5	2.20×10^5
7	3.10×10^5	1.30×10^5	1.67×10^5	1.50×10^5

CONCLUSION

A novel calcium phosphate cement (CPC) has been fabricated via wet chemical precipitation derived HA powder. In this method, calcium hydroxide and di-ammonium hydrogen phosphate have been used as the precursors to synthesize HA powder. Subsequently, the wet chemical precipitation derived HA powder was mixed with distilled water at various P/L ratios. The effect of PEG on the porosity and biological properties of CPC was investigated by incorporating PEG into the liquid phase at different concentrations. It was found that the increase in P/L ratio reduced porosity. Meanwhile, PEG addition increased porosity of CPC.

The bioactivity of the CPC was evaluated using culture of VERO cells. It was proven that CPC with and without PEG show no toxic reaction to the cells and the cells grow well. The cultured cells are able to attach on the fabricated CPC. CPC with higher P/L ratio shows more active cells proliferation and adhesion in the culture media and more cells are able to grow. The incorporation of PEG into CPC improved cells proliferation and adhesion on CPC, with more cells attached on the CPC/PEG compared to CPC without PEG. The results presented in this study has elucidate that the fabricated CPC could become one of the promising material for injectable bone cement applications in the future.

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