In Vitro Bioactivity Study of Thermoplastic Starch/Bentonite/Hydroxyapatite Composites for Biomedical Applications

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Abstract. Potato starch composites film incorporating hybrid bentonite and hydroxyapatite (HA) were investigated as novel biomaterial for potential biomedical applications. The selected potato starch/bentonite composites were prepared using different ratios of HA (1, 5 and 10 wt%) through solution casting technique. The in vitro bioactivity of potato starch composites film and neat potato starch film to be used as a control were evaluated through immersion in simulated body fluid (SBF) at 37°C for 14 days. The composites films were also subjected to characterization such as morphology, pH evaluation and biodegradability analyses following immersion in SBF. The obtained results showed that the incorporation of HA into starch/bentonite composites improves the bioactivity of the starch hybrid composites by formation of apatite layer on the surface. Increasing HA content in the composite is directly proportional to the quantity of the apatite formed on the scaffolds indicating active surface interactions between the composites and SBF. Furthermore, increasing HA content contributes also to lowering biodegradability of the composite decreases.

Keywords: Bioactivity, biodegradability, potato starch, hybrid composites, simulated body fluid.

1. Introduction
Presently, disease and health problems are the main issue in society that need constant demand for cost effective and innovative biomaterials for biomedical applications. A critical requirement for biomaterial is biocompatibility, which is the ability of a material to function and adapt with an appropriate host response in a specific application. In addition to biocompatibility, several other important properties must be considered for biomaterial which are biodegradable, suitable
permeability and processability for intended application [1]. Thus, research and development of quality biomaterials are therefore vital in order to fulfil the requirements of diverse medical industry.

Polymers are one of the biomaterials which received greater attention in biomedical applications such as coronary stent. This is due to their versatility properties, particularly easy to be shaped, good mechanical characteristics and their biocompatibility is unmatched by other biomaterials, such as metals [2]. Recently, natural polymers such as starch has been introduced as a promising biodegradable material for biomedical applications [3]. Starch has been famously used in food, pharmaceutical and biomedical applications because of its biocompatibility, total degradability without toxic residues, low cost, wide availability, renewability and has thermoplastic behavior [3, 4]. In addition, starch has shown great potential in biomedical fields including tissue engineering scaffolds, bone cements, drug delivery systems and stent [5, 6]. However, to perform as an ideal implantable material, further improvement in starch mechanical properties and biocompatibility is needed. The enhancement in starch performance as implantable material can be achieved by adding small amount of nanofillers as reinforcing fillers to form composites.

Nanoclay such as bentonite is the most frequently employed nanofiller for polymers because they are abundant, cheap and own tailorable surface chemistry [7], is used commonly as nanofiller for starch. Besides, bentonite addition has been proven to provide enhancement in tensile strength, toughness and thermal stability to the host starch [8, 9]. While numerous literature in this area is focused on improving mechanical properties, however bioactivity constraints are not adequately addressed. Therefore, researchers began to employ hybrid fillers in order to obtain composites material with greater combination properties.

Hybrid composites is an alternative method allowing a third phase materials to be integrated into starch based composites system for achieving synergistic effects in mechanical and bioactivity properties compared to single filler. Various types of reinforcement fillers is being used in hybrid composites such as nano hydroxypatite (HA), titanium dioxide and carbon nanotubes. HA has been used widely in biomedical applications, particularly for contact with bone tissue due to its resemblance with mineralized bone. HA exists in natural or synthetic form and possesses favorable properties such as biocompatibility and osteoconductivity [10, 11]. Owing to these properties, HA is capable in promoting fast cell regeneration without intermediate connective tissues.

A common characteristic of HA is the formation of a carbonated apatite layer on their surface when they are in contact with a physiological fluid, which is very similar to the apatite in bone [12]. Kokubo and Takadama confirmed that the in vivo apatite formation can be simulated in vitro using an artificially prepared solution known as simulated body fluid (SBF) [13]. It was initially developed by Kokubo for initial prediction about the bioactivity and dissolution behavior of bioactive materials by examining their apatite-forming ability in the fluid. Thus, this study aims to highlight enhancement of bioactivity of the starch/bentonite composites with the addition of HA through in vitro bioactivity study. The composites are being subjected to characterization such as morphology, pH evaluation and biodegradability analyses following immersion in SBF.

2. Materials and Method

2.1 Materials

Commercially available potato starch, (C\textsubscript{6}H\textsubscript{10}O\textsubscript{5})\textsubscript{n} (HmbG Chemicals) was used as matrix material. Bentonite and hydroxyapatite powder purchased from (Multifilla Sdn. Bhd and Sigma Aldrich, respectively) were used as nanofillers. Glycerol, (C\textsubscript{3}H\textsubscript{8}O\textsubscript{3}) obtained from (HmbG Chemicals) was used as plasticizer.

2.2 Preparation of Thermoplastic Potato Starch Composites Film

The hybrid composites film (potato starch/glycerol/bentonite/hydroxyapatite) were prepared according to the formulation design in Table 1. Potato starch hybrid composite films were prepared by mixing two different solutions which is bentonite/hydroxyapatite and starch solutions. Specified amount of
dried bentonite and HA were first dispersed at room temperature in 40 ml deionized water with the following mixing modes:

I. Mechanical mixing with 5 cm conventional impeller at 1000 rpm for 30 minutes, using a RW 20 mixer (IKA, USA) in a 100 ml beaker.

II. Sonication mixing for 30 minutes, perform on a Bandelin HD 2200 ultrasonic homogenizer with the high frequency power at a frequency of 20 kHz and 6 mm probe diameter.

Meanwhile, 5 g of potato starch was dispersed in 100 ml deionized water with (30 wt% relative to dry starch) of glycerol content. Subsequently, the hybrid nanofillers suspension were added to the aqueous dispersion of starch and the suspension was then heated to 85 °C for 30 minutes under constant stirring. Afterwards, the mixture was poured homogeneously onto 14 cm diameter petri dish. The mixture were then dried at 45 °C in an oven with air force circulation overnight. The dry films were removed from the petri dish and stored in a desiccator before being tested to ensure the stabilization of films. The films were then subjected to in vitro bioactivity test using simulated body fluid (SBF) and characterized through morphology (SEM), pH evaluation, thermal stability and biodegradability analyses.

Table 1. Formulation design of potato starch hybrid composites film.

| SAMPLE MIXTURE | RATIO | ACRONYM | POTATO STARCH | AMOUNT |
|----------------|-------|---------|---------------|--------|
| Potato starch/Gly/Bentonite/HA | 5:30:15:1 | SBHA1 | 5 g | 30 wt% | 15 wt% | 1 wt% |
| Potato starch/Gly/Bentonite/HA | 5:30:15:5 | SBHA5 | 5 g | 30 wt% | 15 wt% | 5 wt% |
| Potato starch/Gly/Bentonite/HA | 5:30:15:10 | SBHA10 | 5 g | 30 wt% | 15 wt% | 10 wt% |

2.3 In Vitro Bioactivity Test

The bioactivity of the composites was analyzed using Simulated Body Fluid (SBF). The SBF with ion concentrations nearly equal to those of human blood plasma was prepared according to Kokubo method [13]. The composites films were incubated in the SBF solution by using the film to liquid ratio of 10 mg/ml (w/v). The film samples were soaked with SBF solution in a 50 ml conical sterile polypropylene centrifuge tubes. The film samples were incubated in the incubator shaker IKA KS4000, USA at 37 °C with 100 rpm for 1 day, 4, 7 and 14 days. At each interval, the film samples were removed from the SBF solution and rinsed with deionized water and dried at room temperature for 24 hours. Morphology, pH level and biodegradability analyses were performed on the film samples.

2.4 Morphology Analysis

Scanning electron microscopy (SEM) model JEOL JSM-6460LA was used to characterize the formation of phosphate layer and apatite on starch hybrid composites film following in vitro bioactivity test. Before examining using SEM, the samples were first sputter-coated with platinum to avoid charging effect during characterization.
2.5 pH Evaluation
At designated time points of 1 day, 4, 7 and 14 days, pH of the SBF solution containing the potato starch/glycerol film and potato starch hybrid composites films for in vitro bioactivity test was evaluated by using pH meter (Fisher Scientific Accumet AB150). The pH meter was calibrated with buffer solution at pH of 4, 7 and 10 prior to pH evaluation of the samples.

2.6 Biodegradability Analysis
The potato starch/glycerol film and potato starch hybrid composites films were cut into the same weight of 250 mg (Wi) and immersed in 25 mL SBF maintained at 37°C. After 14 days, films were taken out, washed with deionized water and dried at room temperature and weighed (Wd). The biodegradability of the films were assessed by weight loss percentage of each specimen calculated using the equation 1:

\[
\text{Weight loss} (\%) = \frac{(W_i - W_d)}{W_i} \times 100
\]

Where:

Wi = Initial weight of the film  
Wd = Dried weight of the film

3. Results and Discussion
3.1 Morphological Analysis
Figure 1 (a, c, e and g) shows SEM micrographs of the surface of S-control, SBHA1, SBHA5 and SBHA10 films after 1 day of immersion in SBF solution. Observation from the SEM demonstrated that smaller cauliflower-like spherical crystals is formed on the surface of the films especially for potato starch films containing bentonite and HA, while no spherical crystal formation was observed on the surface of S-control. The presence of the spherical crystals noticeably increased proportional to the HA content. After 7 days immersion, the larger cauliflower-like spherical crystals that were connected to each other had grown and formed on the surface of all SBHA films as shown in Figure 1 (b, d, f and h). Furthermore, the spherical crystals grew more rapidly and almost covering on the surface of SBHA5 and SBHA10.

These observations indicated that the potato starch containing bentonite and HA nanocomposites films showed better bioactivity than neat potato starch film. This finding is in accordance with a study which reported that the addition of HA in the polymer composites could improve the bioactivity of the composites [14]. The S-control film did not show formation of spherical crystals and after 7 days immersion some voids were noticed on the surface of the films which may be due to degradation process. On the other hand, the cauliflower-like spherical crystals formed on the surface of the SBHA films, were attributed to the formation of calcium-phosphate (Ca-P) layer as reported by other researchers [15, 16]. Besides, it was also reported that the composite containing larger amount of HA had greater ability to induce the formation of minerals in vitro.

3.2 pH Evaluation
The pH changes of S-control, SHA1, SBHA5 and SBHA10 films in SBF solution with different immersion time (days 1, 4, 7 and 14) is shown in Figure 2. The pH behavior of the SBF solution for all films showed similar trends throughout 14 days immersion period. The starting pH of SBF was set to 7.4 which is similar to human blood plasma condition. Observation from the graph shows that, at the first 24 hours of immersion the pH of all films was rapidly increased to pH value of 7.6 and followed by significant decrease during 4 days of immersion in SBF solution. The pH value of S-control and SBHA1 were slowly decreasing until day 14 and stabilized at pH value of 7.3. Meanwhile, the pH of SBHA5 and SBHA10 exhibited a slightly increase at day 7 and then slowly decreased until day 14, which posted a pH value of 7.4.
Figure 1. SEM micrographs of (a,b) S-control, (c,d) SBHA1 (e,f) SBHA5 and (g,h) SBHA10 after immersed in SBF solution for 1 and 7 days.

The pH values of the SBF solution upon immersion of the nanocomposites films showed rapid increase corresponding with the dissolution behavior of HA in SBF solution which maybe contributed by the formation of Ca-P. Fast release of calcium (Ca) ions from the nanocomposites films containing bentonite and HA coupled with hydrogen ions (OH) from SBF solution may cause high concentrations of Ca, thus resulted in a pH increase [17]. Meanwhile, the pH of SBF solution significantly dropped at day 4 that might be the results of radical hydroxyl release from the films surface. A recent study described that Ca-P compounds precipitated spontaneously when the pH value of the physiological solution was over 7.4. During the prolonged immersion period, Ca and phosphorus (P) ions are consumed largely due to the formation of abundant apatite and simultaneously, OH- is consumed correspondingly. This will cause a decline in the SBF pH value [18].
3.3 Biodegradability Analysis

The *in vitro* biodegradability analysis of starch/bentonite/HA hybrid composites film at various HA content (SBHA1, SBHA5 and SBHA10) compared with neat starch (S-control) were performed through weight loss measurement in SBF solution at 37°C for 14 days. Figure 3 shows the percentage of weight loss for all composites and neat starch film. Observation from the graph shows that all samples experienced weight loss after 14 days exposure in SBF fluid. Besides, the results showed a decreasing trend in weight loss with increasing HA content. Neat starch (S-control) exhibits the highest weight loss of 33.52 %, followed by SBHA1, SBHA5 and SBHA10 with 31.74 % and 30.82 % respectively.

The presented results revealed that the incorporation of HA in starch matrix affected the biodegradation properties of the composites film. It was found that increases HA content lead to decreased SBHA composites film weight loss compared to S-control. This reason is attributed to the low degradation rate of HA. Furthermore, the decreased weight of SBHA composites film is also correlated to the significant increase in apatite layer formation on the surface of the SBHA composites film. This phenomenon was proven by morphological analysis (section 4.7.1) in which spherical crystals grew more rapidly on the SBHA5 and SBHA10 surface. This finding is in accordance with the previous reported research [19].

Meanwhile, S-control film displayed the highest weight loss percentage. This is mainly due to hydrophilic properties of the potato starch which contributed to the highest weight loss during exposure in SBF fluid. Besides, glycerol utilized in preparation of S-control film also possess hydrophilic properties. Although glycerol is known to be a good plasticizer for potato starch, however the fact that glycerol domains for being hydrophilic really do affects the degradation rate of the starch when in contact with SBF fluid. This finding was in line with R. Shi et al. [20], which reported that the degradation of starch is initiated by the separation of glycerol among the starch chains out of the sample during degradation in SBF. The cleavage of the linkages of glycerol resulted in the destruction of crystallinity of starch sample during degradation.
4. Conclusion
The effect of hybridized bentonite and HA with various HA content in potato starch composites films with 1, 5 and 10 wt% on the bioactivity of potato starch films were examined through immersion in SBF solution. The result is supported by surface morphological, pH evaluation and biodegradability analyses. The current finding shows that the increasing HA incorporation into starch/bentonite composites improves the bioactivity of the starch hybrid composites indicating its potential to be used in biomedical applications.

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