Modulating protein adsorption onto hydroxyapatite particles using different amino acid treatments

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Hydroxyapatite (HA) is a material of choice for bone grafts owing to its chemical and structural similarities to the mineral phase of hard tissues. The combination of osteogenic proteins with HA materials that carry and deliver the proteins to the bone-defective areas will accelerate bone regeneration. The study investigated the treatment of HA particles with different amino acids such as serine (Ser), asparagine (Asn), aspartic acid (Asp) and arginine (Arg) to enhance the adsorption ability of HA carrier for delivering therapeutic proteins to the body. The crystallinity of HA reduced when amino acids were added during HA preparation. Depending on the types of amino acid, the specific surface area of the amino acid-functionalized HA particles varied from 105 to 149 m² g⁻¹. Bovine serum albumin (BSA) and lysozyme were used as model proteins for adsorption study. The protein adsorption onto the surface of amino acid-functionalized HA depended on the polarities of HA particles, whereby, compared with lysozyme, BSA demonstrated higher affinity towards positively charged Arg-HA. Alternatively, the binding affinity of lysozyme onto the negatively charged Asp-HA was higher when compared with BSA. The BSA and lysozyme adsorptions onto the amino acid-functionalized HA fitted better into the Freundlich than Langmuir model. The amino acid-functionalized HA particles that had higher protein adsorption demonstrated a lower protein-release rate.

Keywords: amino acid-functionalized hydroxyapatite; surface properties; electrostatic interactions; protein adsorption; protein release

1. INTRODUCTION

In the last decade, diverse strategies in surface modification have been applied on implantable biomaterials such as hydroxyapatite (HA) [1], low-density polyethylene (LDPE) [2] and poly(lactic-co-glycolic acid) (PLGA) [3] to improve their intended functions in the body. One of the main purposes for surface modification is to enhance or inhibit protein adsorption on the surface of biomaterials. For example, plasma surface-modification (PSM) techniques such as radio frequency glow discharge have been tested to alter the surface functionalities and, therefore modulate protein adsorption [4] and cell adhesion to biomaterials [5]. Higher reproducibility in surface treatment and flexibility to obtain different surface characteristics, such as surface charge [6], energy [7] and chemistry [8] were achieved using PSM.

The interactions between proteins and surface of biomaterials are mainly energetic. In particular, the van der Waals, electrostatic, hydrogen bonding and hydrophobic interactions are important forces involved in the adsorption of proteins to the surfaces [9,10]. Therefore, the change in surface energy is believed to directly modulate the availability of ‘free charged groups’ on the surface of biomaterials. Immobilization of bisphosphonates [11] and pyrophosphate ions [12] onto HA significantly enhanced protein uptake. However, this conjugation possesses higher affinity towards basic protein (lysozyme) when compared with acidic protein (bovine serum albumin, BSA). As a result, adsorption of lysozyme and BSA onto polyphosphate-anchored HA were shown to be more than 10-fold and threefold increases, respectively, when compared with the untreated HA [13].

Immobilization of molecules containing carboxyl groups on the surface of HA was found to enhance protein adsorption. The amount of adsorbed cytochrome C (basic protein) increased from 25 to 55 μg mg⁻¹ when mercapto-succinic acid (MA) was immobilized onto HA [14]. However, the adsorption of BSA to MA-functionalized HA was significantly suppressed. This could be owing to the increase of negatively charged sites on HA surfaces once MA is immobilized on its surface [14]. Citrate is another candidate that could induce the electronegative potential of HA. The zeta potential value was as low as −31.15 mV at pH 7.0 when 200 mM citrate was added to the solution during HA precipitation [15]. Since citrate has three carboxyl groups, it is likely that citrate-functionalized HA have a high affinity to basic proteins.
The interaction of HA with biological macromolecules such as amino acids is a promising method to control surface charge of HA, therefore to modulate the protein adsorption onto HA carrier. In nature, each amino acid possesses different isoelectric points (IPs) that is directly affected by the number of carboxyl (−COOH) and amine (−NH₂) groups of the amino acid. Immobilization of different amino acids onto the surface of HA could be an effective way in the regulation of its surface polarity. Different degrees of surface polarity could regulate the adsorption of polar (basic or acidic) proteins onto the specific HA-binding sites.

The aim of this work was functionalizing HA particles with different amino acids and to determine the effects on the protein adsorption onto HA particles. In order to evaluate the affinity of amino acid-functionalized HA to proteins, BSA and lysozyme were used, respectively, as an acidic and a basic model protein. The changes in physico-chemical properties of HA particles (e.g. crystallinity, surface energy and specific surface area) in the presence of amino acids were also determined. Four amino acids with different IPs were used in this study: neutral (serine, Ser and asparagine, Asn), acidic (aspartic acid, Asp) and basic (arginine, Arg).

2. MATERIAL AND METHODS

2.1. Materials and chemicals

BSA (minimum 98% electrophoresis grade) and lysozyme (from chicken white egg) were obtained from Sigma and used without further purification. Calcium chloride (CaCl₂) and sodium phosphate dibasic (Na₂HPO₄) were supplied by Sigma. All the amino acids used in this study were purchased from MP Biomedicals. Ammonium acetate (CH₃COONH₄) and ammonium hydroxide (NH₄OH) were obtained from (Ajax, AR grade).

2.2. Preparation of amino acid-functionalized hydroxyapatite

The synthesis of amino acid-functionalized HA particles was performed using modified methods [16,17]. Solutions of 500 ml of 100 mM CaCl₂ and 500 ml of 60 mM Na₂HPO₄ were prepared separately in deionized (DI) water. The pH of the solutions was adjusted to 10 using 28 per cent NH₄OH. 0.2 M solutions of each amino acid were prepared by dissolving the amino acid powder into 1.3 M of CH₃COONH₄ buffer solution. The pH of the amino acid solutions was adjusted to 10 using NH₄OH and they were subsequently heated to 80°C. The Ca²⁺ and PO₄³⁻ precursors were added instantaneously to the amino acid solutions and white precipitation was formed immediately in the solution. The precipitation was aged by stirring at 80°C for 5 h. Amino acid-functionalized HA particles were recovered by centrifugation at 10 000 r.p.m. for 10 min. To ensure the removal of excess CH₃COONH₄ and free amino acids, supernatant-free particles were suspended in DI water (three times), followed by washing in absolute ethanol (three times). The product was dried at 37°C overnight. For control, untreated HA was prepared without the addition of amino acids.

2.3. Analysis methods

2.3.1. X-ray diffraction

The crystal structure of samples was analysed using X-ray diffraction (XRD) (Shimadzu, 6000) with the voltage and current setting at 40 kV and 30 mA, respectively. Data were collected over the 2θ ranging from 20° to 45° at a scan speed of 1.2° min⁻¹ with the step size 0.02° and Cu Kα radiation (1.5406 Å). The diffraction patterns were indexed by comparing them with the reference data JCPDS 09-0432 and with the commercial crystalline HA. The effect of amino acid treatment on HA crystallinity was evaluated by the following formula:

\[ X = \left(1 - \frac{I_{200}}{I_{300}}\right) \times 100\% , \]

where \( I_{200} \) is the intensity of (200) reflection and \( I_{112/300} \) is the intensity of the hollow between (112) and (300) reflections.

2.3.2. Zeta potential measurements

Surface charge measurements were conducted using a Malvern Instrument Nano Series ZS Zetasizer (Nano-ZS). The suspensions of different samples were prepared by adding 0.02 g of the HA particles into 40 ml of KCl (0.01 M). The pH of the solutions was maintained at 7.4 by addition of 0.01 M (HCl) and/or 0.01 M (NaOH). Thirty runs were carried out and the average value was calculated for each measurement.

2.3.3. Specific surface area

The specific average surface area of samples was determined using Micrometres Tristar II gas adsorption analyzer. Approximately 0.6 g of each sample was degassed by heating at 100°C for 12 h under continuous flow of nitrogen. The specific surface area was determined by the Brunauer, Emmett and Teller method. All the measurements were conducted in triplicate.

2.3.4. Morphology of particles

The morphology of samples was characterized using FE-scanning electron microscopy (Zeiss Ultra). All samples were mounted onto adhesive carbon tabs (which were placed on aluminium stubs). HA particles were coated with gold at 15 nm thickness prior to the scanning electron microscopy (SEM) observation.

2.4. Protein uptake and release study

Twenty milligrams of amino acid-functionalized HAs were placed in polypropylene containers and incubated separately in BSA and lysozyme solutions under continuous shaking at 37°C. The concentration of proteins varied between 200 and 1000 µg ml⁻¹. The adsorption profiles for BSA and lysozyme were determined by measuring the concentrations of remaining proteins in the supernatant after 24 h incubation. Aliquots of 1.5 ml were centrifuged at 10 000 r.p.m. for 5 min to sediment HA particles suspended in the
supernatant. The concentration of proteins in the supernatant was measured based on the established standard curves (BSA; \( r^2 = 0.9998 \); lysozyme; \( r^2 = 0.9997 \)) using Quant-iT protein assay kit. The amount of proteins absorbed per unit area of samples was determined using the following equation:

\[
\text{Protein}_{\text{adsorption}} = \frac{\text{Protein}_{\text{I}} - \text{Protein}_{\text{S}}}{\text{TSA}},
\]

where Protein\(_{\text{I}}\) is the initial amount of protein (\( \mu \text{g} \)) in the solution, Protein\(_{\text{S}}\) is the amount of protein (\( \mu \text{g} \)) remaining in the solution after 24 h incubation and TSA is the total surface area (m\(^2\) g\(^{-1}\)) of amino acid-functionalized HA particles.

For protein-release study in both untreated HA and amino acid-functionalized HA, 20 mg of samples were suspended into 20 ml of PBS buffer and incubated at 37°C and were subjected to shaking over 14 days. An aliquot (0.5 ml) was withdrawn every 24 h and replaced with fresh PBS. The amount of protein released into the supernatant was measured by Quant-iT kit.

### 2.5. Statistical analysis

Statistical analysis of data was performed using the SPSS Statistic 19 software package. All data were collected in independent triplicate experiments and the mean values and standard deviations were calculated. The statistical differences between groups were determined by analysis of variance (ANOVA) test. The pairwise comparisons of individual group means were performed using the Tukey test. Values of \( p < 0.05 \) were considered statistically significant.

### 3. RESULTS

#### 3.1. Physico-chemical properties of amino acid-functionalized hydroxyapatite

The effect of amino acids on the physical and chemical properties of HA was studied and compared with untreated HA. Figure 1a shows the specific (total) surface area of both untreated and synthesized HA in the presence of different amino acids. Functionalizing HA particles with amino acids resulted in a remarkable change in their surface area. The untreated HA had the lowest surface area at 64 m\(^2\) g\(^{-1}\), while more than twofold increase in the specific surface area was noted for Asp-HA (149 m\(^2\) g\(^{-1}\)) followed in the descending order by Ser-HA (134 m\(^2\) g\(^{-1}\)) > Asn-HA (110 m\(^2\) g\(^{-1}\)) > Arg-HA (105 m\(^2\) g\(^{-1}\)).

The change in specific surface area of amino acid-functionalized HA was closely related to the samples’ crystallinity as shown in figure 1b. Samples with a higher crystallinity demonstrated a lower specific surface area, which is owing to a bigger crystallite size in the highly crystalline HAs. Among five different tested samples, untreated HA possessed the highest degree of crystallinity (crystallinity index of 67%). By introducing a small amount of amino acids (0.2 M) during HA preparation, at least twofold decrease in crystallinity was observed for both Asn-HA (25%) and Arg-HA (24%). The treatment of HA with Asp and Ser resulted in the formation of poorly crystalline HA, where the hollow between (112) and (300) reflections did not appear in the XRD patterns of Asp-HA and Ser-HA (figure 2); therefore, the crystallinity index was not calculated for these two groups.

The surface charge of HA particles depended directly on the types of the amino acids used. Figure 1c shows the zeta potential value at pH 7.4 for different amino acid-functionalized HAs as well as the control. The untreated HA samples (control) leaned towards slightly negative charge (−1.4 mV). The zeta potential value was further decreased (−7.3 mV) when Ser was added and the lowest value (−13.4 mV) was obtained when Asp was added during HA preparation. However, the polarity of samples shifted towards positive charge (+6.7 mV) when HA was prepared in the presence of Arg.
The morphology of untreated HA and amino acid-functionalized HA was observed using SEM (figure 3). From SEM images, a combination of rod and flat shape crystallites was observed in untreated HA samples. Functionalizing HA with amino acids reduced the particle size and altered the morphology to very small rod-like shape.

3.2. Adsorption of bovine serum albumin and lysozyme

The adsorption studies of two model proteins (BSA and lysozyme) onto amino acid-functionalized HA are shown in figure 4 and tables 3 and 4. The interactions between BSA or lysozyme and HA particle surface determine the adsorption rate of these proteins onto HA. In comparison with other amino acid-functionalized HAs, Arg-HA that had positive surface charge demonstrated the highest affinity towards BSA with the BSA adsorption per unit area of 0.269 mg m\(^{-2}\) after incubation in 1000 \(\mu\)g ml\(^{-1}\) BSA solution for 24 h (table 3). On the other hand, negatively charged Asp-HA showed the lowest BSA adsorption at 0.129 mg m\(^{-2}\) in which the differences in BSA adsorption between Asp-HA and Arg-HA were statistically significant \((p < 0.05)\) (table 3). The adsorption of BSA onto the different samples were in the following order: Arg-HA \((0.269 \text{ mg m}^{-2})\) > Asn-HA \((0.209 \text{ mg m}^{-2})\) > Ser-HA \((0.157 \text{ mg m}^{-2})\) > Asp-HA \((0.129 \text{ mg m}^{-2})\). Both untreated HA \((0.101 \text{ mg m}^{-2})\) and negatively charged surface Asp-HA \((0.129 \text{ mg m}^{-2})\) exhibited the significantly \((p < 0.05)\) lowest BSA adsorption rates compared with the other groups, in which the difference between their BSA adsorption isotherms was statistically significant (table 3). When low concentrations of BSA was used \((200–800 \mu\text{g ml}^{-1})\), all amino acid-functionalized HA (Ser-HA, Arg-HA, Asn-HA) except Asp-HA had significantly higher BSA adsorption per unit area when compared with untreated HA (table 3).

Regarding comparison of BSA and lysozyme adsorptions onto different samples, opposite trends were observed for Asp-HA and Arg-HA groups (table 4). Asp-HA had the highest lysozyme adsorption \((0.206 \text{ mg m}^{-2})\) after incubation for 24 h in 1000 \(\mu\)g ml\(^{-1}\) of lysozyme solution, significantly lower \((p < 0.05)\) than that of Arg-HA \((0.133 \text{ mg m}^{-2})\). The BSA adsorption for Asp-HA and Arg-HA groups was opposite that of lysozyme: higher BSA adsorption was observed in Arg-HA than that of Asp-HA. At a lower concentration of lysozyme solution \((200 \mu\text{g ml}^{-1})\), there was no significant differences in lysozyme adsorptions between untreated HA and amino acid-treated HA. When higher concentrations of lysozyme were used \((600–1000 \mu\text{g ml}^{-1})\), the adsorption affinity of lysozyme towards untreated HA was significantly lower when compared with Asp-HA, Ser-HA and Asn-HA (table 4).

It can be seen from figure 4\(a,b\) that the protein adsorption trends for amino acid-functionalized HA samples follow the same general pattern. All samples showed a gradual increase in BSA and lysozyme adsorption and did not show a plateau phase over the concentration studied. This suggests that the maximum adsorption level of the protein has yet to be reached. Further increase in BSA concentrations was found to enhance protein adsorption. When 600 \(\mu\text{g ml}^{-1}\) of BSA was used, the adsorption of Arg-HA and Asn-HA was 0.188 mg m\(^{-2}\) and 0.155 mg m\(^{-2}\), respectively. However, the adsorption affinity for Arg-HA \((0.269 \text{ mg m}^{-2})\) and Asn-HA \((0.209 \text{ mg m}^{-2})\) has increased by 30–40% when samples were incubated at a higher concentration \((1000 \mu\text{g ml}^{-1})\) of BSA solution. The isotherm of adsorbed lysozyme also presented similar curves to those of BSA adsorption, a gradual increase in protein concentration led to a higher adsorption rate.

3.3. Adsorption of bovine serum albumin and lysozyme: Langmuir and Freundlich models

Langmuir and Freundlich models were applied to elucidate the protein adsorption behaviour onto HA particle surfaces. From the Langmuir model, the maximal amount of proteins bound onto the surface of particles and the equilibrium constant for the specific
adsorption can be estimated. The Langmuir equation is expressed as:

\[
\frac{C}{Q} = \frac{1}{bQ_m} + \frac{C}{Q_m},
\]

where \(C\) is the concentration of protein at equilibrium (\(\mu g/ml^{-1}\)), \(Q\) and \(Q_m\) are the adsorption amounts of proteins (\(\mu g/m^2\)) and the maximum adsorption amount of protein, respectively. \(b\) is the Langmuir’s equilibrium constant, indicating the strength of interaction between proteins and particles’ surface [18]. \(Q_m\) and \(b\) can be calculated by plotting \(C/Q\) as a function of \(C\).

The Freundlich model is suitable for the heterogeneous systems and different equilibrium adsorption constant can be determined. The \(C\) and \(Q\) values are as in Langmuir equation, while \(n\) is the Freundlich constant and \(K_F\) is the binding-energy constant. The Freundlich equation is as follows:

\[
\log Q = \log K_F + \frac{1}{n} \log C.
\]

Figure 3. SEM images of amino acid-treated HAs and untreated HA. The presence of amino acids during HA formation reduced the crystallite size of HA.

Figure 4. Adsorption isotherms of (a) BSA and (b) lysozyme onto the amino acid-functionalized HA. The amount of absorbed proteins was expressed as weight of the adsorbed proteins divided by the specific surface area of sample (mg m^{-2}). Basic protein (lysozyme) demonstrated a higher affinity to the negatively charged particles such as Asp-HA, while BSA (acidic protein) adsorbed better onto the positively charged surfaces such as Arg-HA. Circles, untreated HA; squares, Asn-HA; triangles, Arg-HA; inverted triangles, Asp-HA; diamonds, Ser-HA.
1 when compared with that obtained for the Langmuir model. The 1/n values from the Freundlich model are generally higher for the samples with a higher protein-loading capacity. For example, the BSA adsorption onto Arg-HA showed the highest 1/n value (0.836), which is in agreement with the high rate of BSA adsorption on the samples of this group.

3.4. Release profile of bovine serum albumin and lysozyme from amino acid-functionalized hydroxyapatite

Figure 5 shows the release rate of BSA and lysozyme from untreated HA and amino acid-functionalized HA samples incubated in PBS for 14 days. The release of both proteins drastically increased for all samples during the first 24 h of incubation and reached the plateau state. The proteins released from untreated HA were lower than the amino acids containing HA. The BSA release rate from Asp-HA was 80 per cent and was the highest among all samples loaded with BSA, while Asp-HA loaded with lysozyme showed the release rate of only 40 per cent. Conversely, the BSA and lysozyme release rates from Arg-HA showed an opposite trend: 85 and 39 per cent of lysozyme and BSA, respectively, were released in 14 days.

4. DISCUSSION

4.1. Physico-chemical properties of amino acid-functionalized hydroxyapatite

The presence of amino acids during HA preparation has greatly affected the physico-chemical properties of HA. In comparison with the untreated HA, the specific surface area increased and the crystallinity decreased in amino acid-functionalized HA. The presence of Asp and Ser during HA preparation substantially reduced the crystallinity of HA (Figure 2). Our results are in good agreement with the data reported by Uddin et al. [19] in which the surface area increased twofold when 0.2 M Asp was added during HA preparation and the size of particles had an inverse relationship with their specific surface area. In a separate study, Gonzalez-McQuire et al. [20] showed that the presence of amino acids inhibited the crystal growth of HA. Based on the observations of Gonzalez-McQuire et al., the length and the width of amino acid-functionalized HA nanorods were smaller when compared with the untreated HA. Results from both XRD and TEM studies on amino acid-treated HA have confirmed the reduction of the width of HA crystallites by at least 50 per cent, suggesting that amino acids have a strong constraining effect on the a and b axes of the HA lattice [20]. It should be noted that in general the specific surface area of HA materials can vary based on the HA preparation method [21,22]. For example, the specific surface area of HA particles increased about sevenfold when the synthesis temperature reduced from 80°C to 40°C [23]. Broadening in XRD peaks indicated a reduction in the crystalline domains within the amino acid-functionalized HA particles. This was further confirmed by Palazzo et al. [24] as significant changes were noted in the primary crystalline domain along the c direction in the amino acid-treated calcium-deficient HA. The arrangements of the crystalline domains of the amino acid-functionalized HA crystallites might also be more perturbed when compared with the untreated HA [25].

In a solution with pH 7.4 (physiological condition), different amino acids lean towards either positive or negative total charge depending on the pKa of their −COO− and −NH3+ groups. As observed by Jack et al. [25], the addition of amino acids to carbonated

### Table 1. Langmuir and Freundlich parameters for BSA adsorption onto amino acid-functionalized HA.

<table>
<thead>
<tr>
<th>samples</th>
<th>Langmuir parameters</th>
<th>Freundlich parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r²</td>
<td>Qm (mg g⁻¹)</td>
</tr>
<tr>
<td>Asp-HA</td>
<td>0.9509</td>
<td>16.23</td>
</tr>
<tr>
<td>Asn-HA</td>
<td>0.9373</td>
<td>18.97</td>
</tr>
<tr>
<td>Arg-HA</td>
<td>0.9182</td>
<td>28.51</td>
</tr>
<tr>
<td>Ser-HA</td>
<td>0.9387</td>
<td>17.71</td>
</tr>
<tr>
<td>untreated HA</td>
<td>0.9041</td>
<td>6.69</td>
</tr>
</tbody>
</table>

### Table 2. Langmuir and Freundlich parameters for lysozyme adsorption onto amino acid-functionalized HA.

<table>
<thead>
<tr>
<th>samples</th>
<th>Langmuir parameters</th>
<th>Freundlich parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r²</td>
<td>Qm (mg g⁻¹)</td>
</tr>
<tr>
<td>Asp-HA</td>
<td>0.9712</td>
<td>29.91</td>
</tr>
<tr>
<td>Asn-HA</td>
<td>0.8479</td>
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<tr>
<td>Arg-HA</td>
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</tr>
<tr>
<td>Ser-HA</td>
<td>0.8338</td>
<td>20.72</td>
</tr>
<tr>
<td>untreated HA</td>
<td>0.9397</td>
<td>7.48</td>
</tr>
</tbody>
</table>
Amino acid-functionalized hydroxyapatite  W.-H. Lee et al.

Figure 5. Cumulative (a) BSA and (b) lysozyme release from different particles incubated in PBS buffer. The released protein was calculated as a percentage of initially adsorbed protein on the different particles. The burst release of proteins was observed for all groups within the first 24 h of incubation. The release of both BSA and lysozyme from the untreated HA was the lowest (approx. 25%) compared with the amino acid-treated HA samples. Circles, untreated HA; squares, Asn-HA; triangles, Arg-HA; inverted triangles, Asp-HA; diamonds, Ser-HA.

apatite led to noticeable changes in the magnitude of zeta potential. In our study, the addition of an acidic amino acid (Asp) that is negatively charged at the working pH (7.4) enhanced the negative charge of HA particles, while the addition of a basic amino acid (Arg) that is positively charged at pH 7.4 resulted in the positively charged HA particles. The changes in the surface charge of amino acid-functionalized HAs reflect the complex interactions between the HA surface Ca\(^{2+}\) and PO\(_4\)\(^{3-}\) sites with the positive and negative charges associated with NH\(_3\)+ and COO\(^-\) termini, as well as the charges correlated to the side groups of amino acids. Palazzo et al. [24] suggested that negatively charged amino acids (e.g. Asp) bind to the HA surface Ca sites through its COO\(^-\) groups, thus exposing its positively charged NH\(_3\)+ groups at the Asp-HA surface. It should be noted that Asp has two acidic groups (COO\(^-\)), therefore the other COO\(^-\) that is not bound to the HA surface is also exposed at the Asp-HA surface, shifting the charge of Asp-HA particles towards negative. Furthermore, binding of COO\(^-\) groups of Asp to the HA surface Ca sites leading to a higher PO\(_4\)/Ca sites’ ratio, promoting the total negative charge of Asp-HA particles. The same model can be applied for the positively charged amino acids such as Arg that preferentially bind to the HA surface PO\(_4\)\(^{3-}\) through its NH\(_3\)+, exposing Arg COO\(^-\) and the two unbound NH\(_3\)+ groups on the surface of Arg-HA particles. As mentioned above, the availability of Ca\(^{2+}\) and PO\(_4\)\(^{3-}\) ions on the surface of HA affects the charge of amino acid-functionalized HAs. The addition of Arg (positively charged amino acid at pH 7.4) into HA preparation solution containing Ca\(^{2+}\) and PO\(_4\)\(^{3-}\) ions leads to the preferential binding of Arg NH\(_3\)+ terminus to PO\(_4\)\(^{3-}\) free ions, inevitably increasing the number of free Ca\(^{2+}\) ions when compared with PO\(_4\)\(^{3-}\) in the solution. This results in PO\(_4\)\(^{3-}\) deficiency on the surface of HA, therefore enhancing the positive value of Arg-treated HA surface charge. Likewise, COO\(^-\) groups from negatively charged amino acids such as Asp preferentially bind to Ca\(^{2+}\) in the HA preparation solution, decreasing the number of free Ca\(^{2+}\) in the solution, which will then contribute to the negative magnitude of the zeta potential value of prepared HA.

4.2. Protein adsorption on amino acid-functionalized hydroxyapatite

Protein adsorption onto HA surfaces relies on three factors: (i) the inherent surface properties of materials, such as degree of crystallinity, surface area [21], surface energy and hydrophobicity [26]; (ii) physiological conditions including ionic strength [27], pH of working solutions [28]; and (iii) protein–material interactions such as specific binding at Ca\(^{2+}\) and PO\(_4\)\(^{3-}\) sites [29], non-specific binding through hydrogen bonding, electrostatic interactions, and so on. The mechanism of protein adsorption onto HA is initiated with the formation of a Stern layer of anions, such as H\(_2\)PO\(_4\)\(^-\) and OH\(^-\) on the HA surface, followed closely by the dispersive electrical double layer around the surface [28]. Protein molecules are then adsorbed through the specific electrostatic interactions between charged groups of proteins and Ca\(^{2+}\). Meanwhile, hydrogen bonding (random interaction) might also take place between the neighbouring protein molecules with polar surfaces [30]. For acidic proteins such as BSA, the carbonyl group is adsorbed to Ca\(^{2+}\) through the displacement of PO\(_4\)\(^{3-}\). Upon reaching the HA surface, the conformation of BSA would change by exposing NH\(_3\)+, which will form hydrogen bonds with the phosphate group of HA [31].

Results from figure 4, tables 3 and 4 show that the adsorption profiles of BSA and lysozyme are different on each amino acid-functionalized HA. The IPs of BSA and lysozyme are 4.5 and 11.1, respectively. Therefore, BSA and lysozyme are negatively and positively charged, respectively, on their surface when dissolved in PBS buffered at pH 7.4. In addition to the surface charge of HA particles, the dissolution rate of materials also affects the protein adsorption [28]. The lower the crystallinity of HA, the higher would be its dissolution rate [23,32]. Higher solubility of materials leads to a significant increase in ionic strength in the working solution. It has been reported that a higher ionic strength causes conformational changes in proteins by exposing more polar charges, leading to a higher protein adsorption rate on the surface of materials [33].

Our study showed that untreated HA had a lower affinity to BSA and lysozyme compared with the amino acid-treated HAs. This might be owing to both the high crystallinity and the near-zero zeta potential.
Table 3. Adsorptions of BSA at different concentrations onto amino acid-functionalized HA and untreated HA. Values (mean ± s.d. of three replicates) in the same column and not having the same superscript are significantly different at $p < 0.05$ (Tukey post hoc test).

<table>
<thead>
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<th>sample</th>
<th>BSA concentration ($\mu$g ml $^{-1}$)</th>
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<tbody>
<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td>Asn-HA</td>
<td>0.066 ± 0.005$^a$</td>
</tr>
<tr>
<td>Arg-HA</td>
<td>0.085 ± 0.012$^d$</td>
</tr>
<tr>
<td>Asp-HA</td>
<td>0.030 ± 0.003$^{a,b}$</td>
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<tr>
<td>Ser-HA</td>
<td>0.044 ± 0.004$^{a}$</td>
</tr>
<tr>
<td>untreated HA</td>
<td>0.020 ± 0.001$^a$</td>
</tr>
</tbody>
</table>

Table 4. Adsorptions of different concentrations of lysozyme onto amino acid-functionalized HA and untreated HA. Values (mean ± s.d. of three replicates) in the same column and not having the same superscript are significantly different at $p < 0.05$ (Tukey post hoc test).

<table>
<thead>
<tr>
<th>sample</th>
<th>lysozyme concentration ($\mu$g ml $^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Asn-HA</td>
<td>0.041 ± 0.015$^a$</td>
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<tr>
<td>Arg-HA</td>
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<tr>
<td>Asp-HA</td>
<td>0.050 ± 0.013$^a$</td>
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<tr>
<td>Ser-HA</td>
<td>0.068 ± 0.009$^a$</td>
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<tr>
<td>untreated HA</td>
<td>0.038 ± 0.016$^a$</td>
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</tbody>
</table>

value of the untreated HA. Matsumoto et al. [23] have confirmed that highly crystalline HA samples were poor protein adsorbents. The higher the crystallinity, the lower the surface area would be, contributing to fewer binding sites for protein adsorption. Another factor that contributes to the low adsorption of both BSA and lysozyme to untreated HA was the low electrostatic interaction between the adsorbent surface and proteins [34]. Based on our results, the polarity of protein (basic or acidic) is not the main factor contributing to its binding capability to HA, as the adsorption profiles of both BSA and lysozyme to the untreated HA were similar. In this study, to improve the performance of HA as a protein carrier, various types of amino acids were immobilized onto its surfaces. Arg-HA and Asn-HA were compared for their protein adsorption abilities because of their comparable crystallinity and surface area (figure 1). Under physiological conditions, Arg-HA showed excellent adsorption ability towards BSA while exhibiting poor affinity to lysozyme. Meanwhile, Asn-HA showed the opposite trend as higher amount of lysozyme was adsorbed on to Asn-HA in comparison with the BSA adsorption. As Arg has three $\text{NH}_2^+$ groups, at pH 7.4, Arg-HA is positively charged, resulting in a higher affinity towards BSA. If adsorption is assumed to occur through the electrostatic attraction between $\text{Ca}^{2+}$ of HA and $-\text{COO}^-$ groups of BSA, then addition of Arg provides a pool of positive regions ($-\text{NH}_3^+$) on HA particle surfaces that increases the binding sites to $-\text{COO}^-$ groups of BSA. Conversely, lysozyme molecules are always positively charged at pH 7.4. In order for successful adsorption, lysozyme had to overcome strong repulsive forces to Arg-HA with positively charged areas. This might explain the poor loading capacity of Arg-HA with lysozyme.

It could be seen from figure 4b and table 4 that lysozyme had the highest adsorption on Asp-HA, followed by Ser-HA. In general, the adsorption of lysozyme increased by enhancing the negativity of the surface charge of samples (figure 1c versus figure 4b). This could be explained in terms of favourable electrostatic interaction of the enhanced negative charge sites of Asp-HA ($\text{PO}_4^{3-}$ and additional two $-\text{COO}^-$ groups on Asp-HA surface) towards the positive region ($-\text{NH}_2^+$) of lysozyme. A similar approach was applied to increase the adsorption of basic protein (cytochrome C) by adding Asp into HA, whereby more than twofold increase of cytochrome C binding was reported [19]. Uddin et al. [19] further investigated the influence of $-\text{COO}^-$ groups on protein-binding by blocking the $-\text{COO}^-$ groups on Asp-HA surface with the excess of $-\text{NH}_3^+$. Consequently, the binding of cytochrome C was significantly reduced. In another study, Ishihara et al. [14] introduced MA, which contains two $-\text{COO}^-$ groups, onto HA to increase the loading capacity of HA for basic proteins.

It is known that the Langmuir isotherm is based on the assumption of a monolayer protein adsorption onto a homogeneous adsorbent surface with identical adsorption sites. In contrast, the modified Freundlich model takes into consideration the multi-layer protein adsorption and interactions on a heterogeneous surface [11]. Based on our results, the Freundlich model could better predict the adsorption of proteins onto the
surfaces of amino acid-functionalized HAs as demonstrated in higher $r^2$ values when compared with the Langmuir model. This indicates that the protein adsorption in our study was a heterogeneous (multi-layer) adsorption system. $1/n$ refers to the heterogeneity index, which varies from 0 to 1, whereby for a homogeneous surface, the $1/n$ value is 1. For example, the calculated $1/n$ for the lysozyme adsorption onto Asp-HA is 0.848 (table 2), which shows a relatively high degree of homogeneity. For the BSA adsorption on Asp-HA, the $Q_m$ value was 16.23 mg g$^{-1}$ (table 1), which is higher than that reported by Uddin et al. [19] (6.72 mg g$^{-1}$). This can be owing to the higher total surface area of our Asp-HA samples (149 m$^2$ g$^{-1}$) compared with that of the work of Uddin et al. (20 m$^2$ g$^{-1}$).

The extent of the first adsorbed protein layer (both BSA and lysozyme) onto HA depends on the strength of proteins–surface electrostatic interactions. As the thickness of protein layer increases on the surface, the strength of electrostatic interactions weakens, resulting in loosening of outer protein layers from the surface [28]. Our data are in good agreement with the earlier observations, whereby a rapid initial or burst release of proteins took place for all samples within the first 12 h of incubation. In addition, we found that negatively charged Asp-HA particles demonstrated a lower release of basic proteins (lysozyme) than acidic proteins (BSA). At the same time, the release rate of BSA from Arg-HA (positively charged) was much slower than the release of lysozyme from Arg-HA under the same conditions. Functionalizing HA with amino acids changes the amount of available Ca$^{2+}$ or PO$_4^{3-}$ on the surface of HA. Our results demonstrated that the release of a negatively charged protein (e.g. lysozyme) is lower when it is adsorbed on a positively charged surface and vice versa for a positively charged protein.

5. CONCLUSIONS

The presence of amino acids during HA preparation affected HA’s crystallinity and total surface area. Zeta potential of amino acid-functionalized HA particles changed according to the functional groups of amino acid molecules. The adsorption of proteins onto amino acid-functionalized HA particles was mainly governed by the surface charge of the particles. In addition, the adsorption of proteins onto the particles was also dependent on the crystallinity and the total surface area of adsorbent materials. Proteins such as BSA and lysozyme possess different IP, resulting in different charges once dissolved in a working solution. In this study, the immobilization of different amino acids onto HA has successfully tailored the surface charge of particles and thus provided selective binding sites for specific proteins. Varying the type of amino acids during precipitation of HA could be an effective strategy to control the adsorption rate of different proteins by providing different charged groups on the surface of amino acid-functionalized HA particles. The adsorption of BSA and lysozyme was fitted better in the Freundlich (multi-layer protein adsorption) than in the Langmuir model. Our results showed that the electrostatic forces between proteins and HA surface influenced the protein adsorption to a higher extent than the crystallinity and the total surface area of HA particles. The higher the adsorption rate of proteins on the surface of amino acid-functionalized HA, the lower or slower the protein release would be from the materials within the 14 day incubation period.

The authors would like to thank Mr Adam Sikorski for his valuable advise and help in XRD. This work was supported by the Australian Research Council Discovery grant DP0986230.

REFERENCES


11 Iafisco, M., Palazzo, B., Falini, G., Di Foggia, M., Bonora, S., Nicolis, S., Casella, L. & Roveri, N. 2008 Adsorption and conformational change of myoglobin on biomimetic hydroxyapatite nanocrystals functionalized...


