XPS Analysis on Chemical Properties of Calcium Phosphate Thin Films and Osteoblastic HOS Cell Responses

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Received January 31, 2006; Accepted April 5, 2006

Abstract: The chemical composition and binding state of calcium phosphate (CaP) thin films formed on silica substrates were investigated using X-ray photoelectron spectroscopy (XPS). The CaP films were prepared at various treatment periods (such as 15, 30, and 60 min) through contact with an ionic solution supersaturated with calcium and phosphate at 37 °C. These CaP films are denoted as CaP15, CaP30, and CaP60, respectively. Examination of XPS spectra demonstrated that carbon, oxygen, calcium, and phosphorus were the major elements constituting the CaP films. For the carbon and oxygen elements, the intensities of the XPS peaks decreased as the treatment time increased. On the other hand, the peak intensities of calcium and phosphorus increased after extended treatment, during which maturation of the CaP film occurred. The continuous variation of the Ca/P ratio of the CaP film illustrates that a variety of active chemical interactions existed. The value of the Ca/P ratio for CaP15 was 1.35, which is very similar to that of octacalcium phosphate. When treatment was continued for over 30 min, the Ca/P values declined to 1.27. Hence, the excess amount of phosphorus was incorporated into the CaP film during prolonged incubation to form calcium-deficient apatite-like structures. The peak analysis of high-resolution XPS spectra for C 1s, O 1s, Ca 2p, and P 2p demonstrates that the prepared films consist of calcium phosphate. For an examination of the applicability of our CaP preparation method to polymeric substrates, we formed CaP films on polystyrene. HOS cell adhesion to the CaP films appears to be significantly faster than that on the control, polystyrene. The increase in electrostatic interactions due to the highly charged nature of both the CaP films and the cell surface may be a plausible cause for this event. The proliferation of HOS cells was most pronounced for both CaP15 and CaP30. According to these experimental results, we believe that the CaP films introduced in this study can be applied for the surface modification of polymeric and metallic substances to improve the functionality of biomaterials.

Keywords: calcium phosphate, thin film, XPS, HOS cell, adhesion, proliferation

Introduction

Biomaterials used as hard tissue substitutes should feature excellent biocompatibility in addition to mechanical strength corresponding to that of the tissues in which the substitutes are to be implanted [1-3]. The biological properties of biomaterials that determine the biocompatibility and osteoconductivity are directly related to the surface properties of the materials. Nevertheless, the biological functions of the such materials' surfaces have not been fully exploited. Hence, extensive investigations are currently directed at improving our understanding on the interactions between surfaces of biomaterials and tissues. In this regard, calcium phosphate-based ceramics display excellent biocompatibility and osteointegration. Calcium phosphates, such as hydroxyapatite and carbonate apatite, are known to improve osteointegration [4-6]. In addition to metals and alloys used for bone substitutes, a variety of polymeric substances are widely used as scaffolds for tissue substitutes, especially for hard tissues [7-9]. The surface properties of some polymeric materials are somewhat problematic because of the hydrophobicity of the surface properties of synthetic polymers [10]. Furthermore, these polymeric substances are also used in controlled drug release systems. A

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number of attempts have been executed to enhance surface functionality, for example, by immobilizing RGD sequences on the surfaces of polymeric materials [11,12]. If we can prepare calcium phosphate coatings on polymeric biomaterials, we can readily improve the degrees of osteointegration and biocompatibility of the polymeric materials because calcium phosphates, such as hydroxyapatite, and octacalcium phosphate, have excellent biological properties [13,14]. Several methods for the preparation of calcium phosphate coatings on solid substrates, such as plasma spraying and sol-gel approaches, have been reported previously [15-17]. However, these methods are not suitable for preparing CaP coatings on polymeric substances because of harsh treatment conditions, such as high temperature, and limited accessibility, e.g., in the case that the surface shape is extremely tortuous or highly porous.

The purpose of this study was to analyze the chemical properties of CaP films on silica surfaces treated with a supersaturated ionic solution. The CaP films prepared at the different treatment times were subjected to X-ray photoelectron spectroscopic analysis to analyze the chemical elements constituting the films and to examine the chemical binding states of the constituent elements. In addition, we formed CaP films on polystyrene substrate to examine whether our method could be applied to the surface modification of polymeric substances. CaP films formed on polystyrene demonstrated considerable improvements in the degrees of adhesion and proliferation of HOS cells.

**Experimental**

**Preparation of CaP Surfaces**

The calcium phosphate thin films were prepared on silica and polystyrene substrates using an ionic solution supersaturated calcium and phosphate ions. The ionic solution was filtered through a syringe ceramic filter (pore size: 0.2 µm) and kept at low temperature (~4 °C) prior to use in order to remove any unwanted homogeneous precipitate. The filtered ionic solution was applied to substrates to form CaP films. After the addition of the ionic solution, the samples were kept at 4 °C to promote the formation of CaP nucleates. After incubation for 1 h, the temperature was increased to 37 °C and the sample was incubated for 15, 30, or 60 min. After the samples had been incubated for the prescribed periods, they were rinsed thoroughly with excess amounts of distilled deionized water (ddw) and then dried under ambient conditions while preventing any contamination. The CaP films prepared at incubation times of 15, 30, and 60 min are labeled as CaP15, CaP30, and CaP60, respectively.

**Analysis of Surface Composition by XPS**

XPS is an extremely sensitive technique for detecting the chemical elements constituting the outermost layer of a surface up to ca. 200 Å. Moreover, the binding states of constituent elements can be readily characterized from an XPS spectrum. In this study, the chemical elements of the calcium phosphate films were analyzed using an ESCALAB 250 XPS spectrometer (VG Scientifics). The XPS spectrophotometer employed a monochromatic Al Kα X-ray source and an ambient pressure of 10⁻⁶ torr. Samples were dried completely under ambient conditions prior to XPS analysis. To remove the adventitious contaminants, the sample surface was sputtercleaned using argon ions prior to XPS analysis. The relative atomic concentrations were determined from the C 1s, O 1s, Ca 2p, and P 2p peaks by using the built-in software. The amounts of the surface elements were quantified in terms of molar percentages. XPS narrow survey scan spectra for Ca, P, O, and C were obtained. The aliphatic C 1s peak at 284.6 eV was used as an internal standard to correct for peak shifts that were due to the surface charging effects. The XPS spectra for the chemical elements detected from the CaP films were subjected to peak deconvolution using a peak analysis program in order to locate the binding energies representing the chemical binding states of the elements within the CaP films.

**HOS Cell Culture**

Osteoblastic HOS cells were used to study both the biocompatibility and cytotoxicity of CaP films formed on polystyrene substrates. The HOS cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum and 1 % (v/v) antibiotic antimycotic (cat. no.: 15240-062, Gibco), at 37 °C under a 5 % CO₂ atmosphere. For the adhesion and proliferation assays, HOS cells were harvested from the culture plate under confluent conditions.

**HOS Cell Adhesion Assay**

For the adhesion assay, 1×10⁴ cells per well were seeded on each sample. Each well was filled with 1 mL of DMEM. The samples were incubated under the same conditions as those used for the culture environment. After the HOS cells had been left to adhere to the CaP films for the desired time, i.e., 6 or 12 h, the number of adherent cells was quantified using a crystal violet test kit (Sigma, C-6258) following the manufacturer’s instructions. Briefly, the culture medium was removed, followed by thorough rinsing three times with PBS. The adherent cells were then fixed with 70 % cold ethanol for 10 min, followed by rinsing with PBS. Crystal violet
solution (0.05%, 200 µL) was added to each well and then incubation proceeded for 10 min at room temperature. The crystal violet solution was washed out three times with ddw and then 600 µL of 1 % SDS was added. An aliquot of 300 µL was subjected to determination of the optical density using the microplate reader operated at a wavelength of 570 nm.

HOS Cell Proliferation Assay
To examine the proliferation of HOS cells on the CaP films, 5×10³ cells were seeded on each sample and cultured for 2, 3 or 4 days. During the proliferation assay, the culture media was changed at regular intervals. After the desired culture time, the culture medium was removed and then 300 µL of 10 % CCK-8 solution was added, followed by an incubating period of ca. 4 h. After incubation, an aliquot (100 µL) was taken from each sample for determination of its optical density using a microplate reader operated at a wavelength of 450 nm.

Results and Discussion

XPS Analysis
In this study, we analyzed the chemical properties and binding states of the chemical elements constituting three different CaP films by utilizing XPS. Scanning electron microscopic observations reported elsewhere illustrate how the variation of surface morphology of CaP films is dependent on the treatment duration [18]. XPS wide survey scans indicated that the chemical elements Ca, P, O, and C were present in all of the CaP films. We obtained high-resolution narrow survey scans for these elements from each sample. To remove the effect of charge from the sample films, the aliphatic carbon peak at the binding energy (BE) at 284.6 eV was used to correct all of the XPS spectra. The detection of Ca and P elements in particular after treatment for as little as 15 min indicates that a CaP film was actively forming on the substrate.
Figure 2. XPS spectral decompositions of C 1s to subpeaks: (a) CaP15, (b) CaP30, (c) CaP60. The scale of the y-axis is arbitrary.

Table 1. XPS Binding Energies and Corresponding Elements Observed on CaP Films Prepared Using Different Incubation Times

<table>
<thead>
<tr>
<th>Elements</th>
<th>CaP15</th>
<th>CaP30</th>
<th>CaP60</th>
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<tbody>
<tr>
<td>Cs1</td>
<td>284.6</td>
<td>284.5</td>
<td>284.6</td>
</tr>
<tr>
<td></td>
<td>286.3</td>
<td>286.3</td>
<td>286.5</td>
</tr>
<tr>
<td></td>
<td>288.3</td>
<td>288.5</td>
<td>288.6</td>
</tr>
<tr>
<td>O1s</td>
<td>530.5</td>
<td>530.6</td>
<td>530.6</td>
</tr>
<tr>
<td></td>
<td>532.5</td>
<td>532.5</td>
<td>532.6</td>
</tr>
<tr>
<td>Ca2P</td>
<td>346.9</td>
<td>346.7</td>
<td>346.8</td>
</tr>
<tr>
<td></td>
<td>350.5</td>
<td>350.2</td>
<td>350.3</td>
</tr>
<tr>
<td>P2p</td>
<td>132.3</td>
<td>132.4</td>
<td>132.4</td>
</tr>
<tr>
<td></td>
<td>133.3</td>
<td>133.5</td>
<td>133.3</td>
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</tbody>
</table>

Figures 1(a) ~ d) display the XPS spectra of the C 1s, O 1s, Ca 2p, and P 2p signals, respectively, identified from each CaP film sample. In Figure 1(a), all of the peaks for C 1s are asymmetric and a small shoulder appearing at higher BE. The intensity of the peak decreased when the samples were incubated for longer times in the supersaturated ionic solution. Incubation times longer than 30 min did not give rise to significant variation of the C 1s spectral intensity.

Figure 1(b) illustrates the O 1s XPS spectra of the CaP films. Comparison of the spectra obtained after different treatment times indicates an increase in the O 1s XPS peak as a function of incubation time up to 30 min. For incubations longer than 30 min, however, no significant variation in the intensity of the O 1s peak was observed. In addition, because both the substrate and the CaP film contain oxygen, the corresponding XPS peak may not be differentiated accurately.

Figure 1(c) displays the Ca 2p XPS spectra detected from all of the CaP films, they imply the formation of CaP films on the substrates. Each of the Ca 2p peaks displays a doublet band, which is typical for calcium oxide in inorganic calcium-oxygen compounds [19]. The presence of different chemical states for the calcium elements within the CaP film indicates their various chemical binding states. The intensity of the XPS peak for calcium appeared to increase when the incubation time was extended. This result indicates that the amount of calcium incorporated into the film increased upon increasing the incubation time. At the same time, this result may indicate an increase of the CaP film thickness with increasing incubation time.

The P 2p XPS spectra are presented in Figure 1(d). The P 2p XPS peak is already visible on the surface prepared after treatment for 15 min. The peak intensities of the P 2p XPS spectra obtained from the CaP films display similar changes to those for calcium. The shape of the P 2p peak appears to be broad, implying that the P 2p band comprises, multiple subpeaks. The presence of the P 2p XPS spectra in addition to that of calcium provides evidence for the formation of CaP films. The intensity of the P 2p peak increased for treatment times up to 30 min. However, treatment times longer than 30 min did not appear to bring about any significant change in peak intensity.

The BE values of subpeaks for an element characterize its binding states. XPS high-resolution survey scans for elements of interest were subjected to decomposition to locate the subpeaks underlying the main peak. The positions of the BE of the subpeaks obtained from curve fitting are summarized in Table 1. The detailed results of peak decomposition of each element are presented in Figures 2 ~

Figure 2 shows the peak deconvolution of the C 1s main peak. The C 1s main peaks of all of the CaP films were deconvoluted into three subpeaks characterizing the chemical states of carbon. The BE positions of these decomposed C 1s subpeaks are 284.6±0.1, 286.4±0.1, and 288.5±0.2 eV. These subpeak positions remained consistent for all of the CaP films, irrespective of the
incubation time, while the intensity of the main peak decreased with time. The C 1s peak at 284.6 eV is attributed to the aliphatic C-H. The peaks at 286.3 and 288.5 can be assigned to CO bonds [20] and organic C 1s [21], respectively. A BE of 286.2±0.2 eV, which may be equivalent to the BE of 286.4±0.1 in our case, has been assigned to carbon atoms bound to phosphate groups [22].

Figure 3 presents the results of deconvolution of the O 1s XPS spectra. The O 1s peaks are divided into two subpeaks at 530.6±0.1 and 532.5±0.1 eV, which are assigned to oxide and hydroxide, respectively [23]. The peak at lower binding energy is attributed to PO$_4^{3-}$ oxygen atoms and the peak at higher BE to OH$^-$ oxygen atoms [24]. The subpeak at lower BE has been assigned to hydroxyapatite in the literature [19]. In comparison to carbon, the peak intensity for the O 1s XPS spectrum increased with the incubation time, indicating that a greater amount of oxygen was incorporated into the CaP film.

The Ca 2p XPS spectra of CaP films display doublets that can be deconvoluted into two subpeaks at BEs of ca. 346.8±0.1 and 350.3±0.2 eV. The deconvoluted spectra are shown in Figure 4. The subpeak at a BE of 350.3 eV is attributed to Ca 2p$_{1/2}$. The subpeak located at a BE of 346.7 eV is assigned to Ca 2p$_{1/2}$ and possibly to CaCO$_3$ [21].

The deconvoluted P 2p XPS spectra are presented in Figure 5. The P 2p main peak is divided into two subpeaks at BE values of 132.4±0.1 and 133.3±0.1 eV. The subpeak at 133.3 eV is attributed to the P 2p$_{1/2}$ BE of pyrophosphate groups, characterizing the phosphorus binding state [23]. Pyrophosphate that occurs in natural body fluids is known to play a physiological role in regulation of calcium phosphate precipitation [25]. The subpeak at the BE of 132.4 eV can be assigned to the P 2p peak corresponding to the existence of phosphate within the CaP film [23,26]. Together with our XPS spectral analysis of calcium, this result confirmed that calcium phosphate was formed on the substrate.
Figure 5. XPS spectral decompositions of P 2p to subpeaks: (a) CaP15, (b) CaP30, (c) CaP60. The scale of the y-axis is arbitrary.

Figure 6. Compositional variation of CaP films prepared on the solid substrate, determined from XPS spectra in terms of relative atomic percentages.

The Ca/P ratios of the CaP films have been reported as 1.35, 1.36, and 1.27 for CaP15, CaP30, and CaP60, respectively [18]. For the CaP15 and CaP30 samples, the Ca/P ratios are close to that of octacalcium phosphate, but lower than that of hydroxyapatite (1.66). These Ca/P ratios indicate that the chemical environment of the CaP films formed during the initial treatment was similar to that of octacalcium phosphate. The Ca/P ratio decreased after incubation for over 30 min, which indicates the excessive incorporation of phosphorus, with respect to calcium, in the CaP films. Therefore, it is believed that the prepared CaP films obtained after 30 min of incubation have the structure of calcium-deficient apatite. Based on the finding that the XPS spectra of Ca 2p and P 2p were visible for CaP15, we believe that the CaP films formed rapidly during the initial stages of treatment.

Figure 6 displays the variation of relative atomic concentrations as a function of the treatment time. The atomic concentrations of oxygen and carbon decreased after 30 min of treatment, but remained constant thereafter. On the other hand, the calcium and phosphorus concentrations showed different behavior: they increased up to 30 min of treatment and then remained relatively constant. The O/C ratios were 2.34, 3.37, and 3.89 for CaP15, CaP30, and CaP60, respectively, indicating that the O/C ratio increased continuously. These measurements indicate that the incorporation rate of oxygen atoms into the CaP films was considerably faster than that of carbon atoms.

HOS Cell Adhesion
The number of adherent cells was obtained by measuring the optical density of the cell lysate treated with crystal violet solution (Figure 7). In all cases, at least four measurements were performed on each sample. The error bar indicated on the plot represents the standard deviation of the average value of the measurements. The results of the adhesion assay are somewhat surprising, demonstrating that the optical density of adherent HOS cells on both the CaP30 and CaP60 samples were two times greater than that of the control, which was the surface of a commercial culture plate. The HOS adhesion on CaP15 was lower than those of CaP30 and CaP60, but still it was considerably higher than that of the control. These results suggest that the rate of HOS cell adhesion was extremely rapid on the CaP films, and much higher than that on the commercial culture plate. This result is probably due to the charges of the CaP films, which interact strongly with the highly charged membranes of the HOS cells. Interestingly, HOS cell adhesion on CaP films measured after 12 h of culturing was slightly lower than that after 6 h, implying that adherent cells were taken apart from the CaP films. For the control, the number of adherent cells was almost equivalent for both the 6 and 12 h adhesion assays. We speculate that a culture time of 6 h is sufficiently long for all of the cells.
HOS cell adhesion: HOS cells were allowed to adhere to CaP films for 6 and 12 h. The optical density for adherent cells was determined at culture times of 6 and 12 h. CTL represents the control polystyrene surface. The OD values from the background were subtracted from the raw data.

to adhere on the samples. After adhesion, some of the adherent cells that might adhere to the surface mainly via electrostatic attraction, due to the strongly charged CaP films, would be disjoined during extended culturing. Despite the reduction of adherent cells at the extended culture time, the overall trend of adhesion to the sample surfaces was relatively consistent with that of the 6-h adhesion assay. According to these results, the rate of HOS cell adhesion on CaP films was increased significantly when compared with that on the commercial culture plate. The adhesion rate was most prominent on the CaP30 and CaP60 samples.

HOS Cell Proliferation
Assays on HOS cell proliferation were performed to examine the biocompatibility and cytotoxicity of the prepared CaP film samples. HOS cells were grown for 2, 3, and 4 days on the CaP films and the control. The cells present on the surface of each sample were quantified using a CCK-8 proliferation kit. The results of the proliferation assay are shown in Figure 8. For each sample, at least four measurements were performed. The average values for the measurements were used for plotting. The error bar marked on the plot represents the standard deviation of the measurements. At a culture time of 2 days, all of the samples displayed similar growth rates. However, after culturing for 3 days, the proliferation behavior was slightly different: a slightly reduced proliferation occurred on CaP60. The HOS cell proliferation rate on the control surface was slightly lower than those on CaP15 and CaP30, but higher than that on CaP60. At a culture time of 4 days, the proliferation trends on the samples were similar to those obtained after 3 days, but the difference between the samples became more pronounced. Both CaP15 and CaP30 had the highest proliferation yields, followed by the control. The CaP60 sample had the lowest proliferation rate. These results clearly demonstrate that the CaP films prepared in this investigation possess excellent biocompatibility and very low cytotoxicity, as confirmed through comparison with the results obtained from the commercial culture plate.

Conclusion
In this investigation, XPS spectral analysis of the chemical elements constituting films formed on silica substrates were performed. Carbon, oxygen, calcium, and phosphorus atoms were detected as the major elements of the films. Detection of calcium and phosphorus atoms as early as 15 min into the treatment process confirms the formation of a CaP layer on the substrate. The intensity of the C 1s XPS peak was inversely proportional to the treatment time. On the other hand, the intensities for the O 1s, Ca 2p, and P 2p XPS peaks increased with respect to the treatment time. Therefore, we can conclude that the prepared films consist of calcium phosphate in addition to air-born calcium carbonate. The spectral analysis also suggested that the CaP layer became thicker when the treatment time was extended. Peak deconvolutions of the various BE values of each element suggested that the constituent elements existed in a diverse array of chemical binding
states. We confirmed that the prepared films consisted of calcium phosphate according to the analysis on the binding states of calcium and phosphorus.

In addition to the CaP formed on the silica, we prepared CaP films on a polymeric substance, polystyrene, in particular, and performed adhesion and proliferation assays to examine the cellular responses to the CaP films. As shown in Figure 7, HOS cells adhered onto the CaP films, CaP30 and CaP60 in particular, much faster than they did on the control surface. We observed that a small number of adherent cells detached from the CaP films after prolonged incubation, which might indicate that some cells adhered to the CaP films merely via electrostatic effects, arising from the highly charged nature of the CaP films and the cellular surface, without forming firm bonds between cell membrane-embedded integrin and adhesion molecules present on the surface of substrate. However, the electrostatic interaction also plays a major role in the enhanced adhesion of HOS cells on CaP films, based on the substantial increase in the number of adherent cells on CaP films when compared to those on polystyrene. The proliferation of HOS cells was most pronounced for the CaP15 and CaP30 samples. Therefore, we are certain that the CaP coatings investigated in this study can provide favorable environments for the adhesion and proliferation of HOS cells. These results strongly suggest that CaP coating is a useful surface modification tool for improving the biological functionality of materials. Another advantage of our method that we emphasize is that the preparation of CaP films is extremely simple and economically competitive.

Acknowledgment

The present research was conducted using the research funds of Dankook University in 2004.

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