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INVESTIGATION OF BONE AND CALCIUM PHOSPHATE COATINGS AND CRYSTALLINITY DETERMINATION USING RAMAN MICROSPETROSCOPY

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(Received for publication April 29, 1996 and in revised form December 29, 1996)

Abstract

Conventional spontaneous Raman micro-spectroscopy was used for the investigation of bone and calcium phosphate coatings on bone-implant surfaces. Chemical and structural changes at the bone-coating interface could be monitored on a microscopic scale. It was shown that the crystallinity did not vary within the crystalline apatite and amorphous calcium phosphate coatings, while the density within each coating did vary. Different degrees of crystallinity in coatings were investigated for a series of plasma-sprayed apatite coatings. It is concluded that Raman microspectroscopy is an easy non-destructive way to obtain information about the apatite structure and the degree of crystallinity.

Key Words: Hydroxy apatite, Raman spectroscopy, crystallinity, calcium phosphate, bone, implant

Introduction

Calcium phosphate (CP) coatings are used on metal implants mainly because of their ability of bone bonding to enhance bone formation at the interface. The ease with which bone forms bonds with calcium phosphates depends on the ability of the material to form a surface apatite layer (de Groot, 1981; Jarcho, 1981). The formation of this layer is dependent on the dissolution rate of the calcium phosphate and consequently on the crystallinity of the layer (LeGeros et al., 1991; De Bruijn et al. 1992). It was found that amorphous CP coatings result in an earlier bone formation response compared to highly crystalline coatings (De Bruijn et al., 1994; Van Blitterswijk et al., 1993a). Since the structure of calcium phosphates is of great importance for the bioactivity of the coating it is of importance to be able to characterize coating materials before and after implantation. Raman spectroscopy provides chemical data about the composition and conformation of the sample. Samples can be investigated in situ without the need for extensive or degenerative sample preparation. In this study we used spontaneous Raman microspectroscopy. Using a microscope setup, in contrast to for example X-ray diffraction, chemical information was obtained, in a non destructive way, with a typical resolution in the order of a few cubic micrometers.

Materials and Methods

Samples

Hydroxyapatite (HAP) from CAM-Implants (Leiden, The Netherlands) was plasma sprayed onto commercially pure titanium (cpTi) rods. An amorphous CP coating was obtained with a layer thickness ranging from 30 to 70 μm. A highly crystalline coating was produced by gradually heating the amorphous coating to 600°C (100°C per hour) and after one hour at 600°C cooling down in air to room temperature with a temperature drop of 100°C per hour. This results in a homogeneous highly crystalline coating (Van Blitterswijk et al., 1993b). For the degree of crystallinity determination, various degrees of crystallinity of the apatite coating

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were obtained by thermal treatment of the amorphous coating at temperatures between 400 an 600°C followed by slowly cooling down the samples. Sections of approximately 10 μm thickness were cut and measured without further treatment.

Titanium rods with amorphous coating and rods with a crystalline coating were implanted by press fit insertion in rat femora and recovered after 24 weeks. The excised implants were fixed in Karnovsky’s fixative, dehydrated using a graded series of ethanol and embedded in methyl methacrylate.

**Raman microspectroscopy**

Raman measurements were performed on a Confocal Raman Microscope (Puppels et al., 1990), either with spot illumination or in a line-scan mode. A laser beam of 660 nm wavelength from a DCM operated dye laser (Spectra Physics 375B, Mountainview, CA) was focused onto the sample through a microscope objective. The sample can be viewed simultaneously in bright light and the position which is to be measured is selected by positioning it under the laserspot. A focus before the objective ensures that the entrance pupil of the objective is always completely filled and at the same time makes it possible to insert a pinhole for confocality. The 63x (numerical aperture, NA = 0.8) objective used in combination with a 100 μm pinhole results in a spot size of less than 1 μm diameter. After passing through a notch filter for laser light suppression, the back scattered signal is dispersed using one grating and focused onto a liquid nitrogen cooled charge coupled device (CCD) camera (back-thinned TBK512, 512x512 pixels, Wright Instruments, Cambridge, UK).

In the line-scan mode the laser beam is scanned over the sample using a small piezo driven mirror. The Raman scattered light is then lead through a slit (50 μm wide and 3 mm long) and focused, using two cylindrical lenses, via a grating onto the CCD camera. In this way the spatial information from the scanned line on the sample is projected on the camera in the horizontal direction while the spectral information is contained in the vertical direction. The lateral resolution for this configuration was determined to be between 0.9 and 1.9 μm depending on the optical penetration depth of the sample.

**Results and Discussion**

The spectra of bone, amorphous and highly crystalline coatings, which had been implanted, are shown in Figure 1. All the peaks in the spectra from the coatings are due to the PO₄³⁻ phosphate vibrations also reported by others (Dasarathy et al., 1993; De Mul et al., 1986; Rehman et al., 1995). Band assignments are: 431 and 450 cm⁻¹ ($v_3$ bending), 581, 592 and 608 cm⁻¹ ($v_4$ bending), 962 cm⁻¹ ($v_1$ symmetric stretch) and 1029, 1048 and 1076 cm⁻¹ ($v_3$ asymmetric stretch). A peak at 3575 cm⁻¹ found in spectra of hydroxyapatite arises from the $v_1$ stretching vibration of OH. This band is present in the spectra of the crystalline HAP powder which was used to produce the coatings (spectra not shown). OH is known to partially disappear from hydroxyapatite forming oxyapatite because of the heat during the plasma spraying. In the spectrum of the coating no activity is found around 3575 cm⁻¹ meaning that this OH conformation is not present in the coating. The spectrum of amorphous CP shows features at similar positions as crystalline apatite but with a larger peak widths than found in crystalline apatite. Since the Raman signal from all similar vibrations add up to one peak, the more uniform the crystal structure the more narrow and

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**Figure 1.** Raman spectra of plasma sprayed amorphous calcium phosphate (CP), 100% crystalline apatite (AP) both after implantation, and bone of rat femora. Applied excitation powers 25 mW, 25 mW and 70 mW, respectively, with a 20 seconds measuring time.
the resulting Raman peak will be. In amorphous material, compared to crystalline, there is a larger variation in inter-molecular and intra-molecular distances in the calcium phosphate coming from differences in local environment. This variation results in a range of slightly different wave numbers for the vibrations and therefore in broadened Raman bands.

Looking at the bone spectrum in Figure 1, many Raman peaks can be identified. The main feature is still the phosphate peak at 961 cm\(^{-1}\). This peak has an asymmetry towards lower wave numbers indicating additional peaks appearing on that side. With a resolution of 0.8 cm\(^{-1}\) separate peaks were not resolved, indicating a sort of continuum may exist between 961 to 942 cm\(^{-1}\). Peaks at 1240-1270 cm\(^{-1}\) (amide III) 1450-1455 cm\(^{-1}\) (CH\(_2\) deformation) and 1665-1670 cm\(^{-1}\) (C=O stretch, C-N stretch, NH bending amide I) correspond to protein vibrations due to collagen present in the bone. The presence of proteins is confirmed by the peaks at 2882, 2939 and 2984 cm\(^{-1}\) (CH\(_2\) stretch) which are known to diminish when bone is deproteinated (Ducheyne et al., 1990). The \(v_3\) PO\(_4\) peaks as found in the crystalline spectrum, cannot account for the sharp peak at 1072 cm\(^{-1}\) in the bone spectrum. Since CO\(_3\)\(^{2-}\) is known to be a constituent of bone it is likely that the 1072 cm\(^{-1}\) peak must come from the CO\(_3\)\(^{2-}\) stretch vibration at this position. We found no distinct peak at 3575 cm\(^{-1}\) for the OH stretch of HAP. The broad band around 3340 with a shoulder at 3450 in the bone spectrum is due to water OH stretching vibrations. The vibration of free water has a different shape with maxima at 3240 and 3425 cm\(^{-1}\). This means that the water present in the bone is in a different configuration than in bulk liquid. It is probably in some bound state which might interfere with, and alter, the 3575 cm\(^{-1}\) OH vibration, as found in HAP. The width of the 961 cm\(^{-1}\) peak is a measure for the crystallinity of the material, as explained above. The full widths at half maximum (FWHM) found for amorphous CP, crystalline apatite and bone are 33.7 ± 0.3, 7.5 ± 0.3 and 18.6 ± 0.5 cm\(^{-1}\) respectively. This corresponds well to the findings of (Leung et al., 1992) who reports 35 ± 1 for amorphous CP and 19 ± 1 cm\(^{-1}\) for bone from dog femur. From this it can be concluded that the crystallinity of the apatite in bone is in a lower crystalline state than highly crystalline apatite. In bone apatite, part of the PO\(_4\) ions are replaced by CO\(_3\)\(^{2-}\) ions causing local changes in the apatite structure and therefore a Raman line broadening (De Mul et al., 1986; LeGeros, 1990). Other bone constituents like ions and proteins are likely to contribute to this line broadening.

Spectra from other calcium phosphate structures like tricalcium phosphate and tetracalcium phosphate (spectra not shown) differ significantly from the apatite spectra. No evidence for these structures was found in the spectra of crystalline apatite and bone. The spectra of amorphous and crystalline coatings before and after implantation were not significantly different. This can be seen by comparing Figures 1 and 4. The spectra in Figure 1 were taken from coatings which had been implanted and in Figure 4 from coatings which had not been implanted.

**Line-scan Raman**

By illuminating the sample with a line the spectral variation along this line can be imaged on the camera. In this way, the biochemical changes, obtained from the spectral distribution observed along the line, can be monitored on a microscopic scale. Figure 2A shows a white light image of an apatite coating-bone interface. The measuring line as indicated starts in the apatite at approximately 20 \(\mu\)m from the titanium-apatite interface and crosses the coating-bone interface halfway. In Figure 2B, the measured spectra of the crystalline apatite-bone interface are presented. The full width at half peak maximum (FWHM) and the peak intensity, of the phosphate peak around 960 cm\(^{-1}\) are plotted in Figure 3. The change in intensity and the broadening of the peak indicates the coating-bone interface. The Raman spectra also provide an interpretation for the pattern of darker and lighter patches in the whitelight image of the coating. The variation in signal intensity, as seen especially in the coating, follows the appearance of the sample when compared with the white light image; darker regions in the coating, correlate with a higher Raman signal. No variation in full width at half maximum is observed in the HAP coating. A higher Raman signal, with an unchanged crystallinity, means a higher concentration of material in the sample volume. This means that, although the density of the material changes, the crystallinity does not. In Figure 3, similar profiles are plotted which were measured on an amorphous CP-bone interface. The FWHM and intensity increase when entering the coating, indicating a higher CP concentration and a lower crystallinity. Here also the change in CP density does not correlate with a change in crystallinity.

**Degree of crystallinity**

The degree of crystallinity of the apatite is of importance for the bioactivity and firm bonding of the bone to the coating (De Bruijn et al., 1994). We investigated the possibility to determine the crystallinity ratio, of a given CP coating, from its Raman spectrum. A way to produce an increase of crystallinity of amorphous CP is by heat treatment. Six different samples were held at temperatures of 400, 450, 500, 525, 575 and 600°C for six hours and cooled down as described in materials and methods. Raman spectra were measured at random positions on each sample and some examples are presented in Figure 4. By comparing these spectra with those of amorphous CP and crystalline apatite in Figure 1, it is
clear these coatings consist of mixtures of crystalline apatite and amorphous CP and not of some sort of semi-crystalline state. The spectra were fitted with spectra from 100% crystalline and 100% amorphous CP. From the ratio of the fit spectra necessary to fit the measured spectra, follows the degree of crystallinity which is presented in Figure 5. The accuracy with which the crystallinity ratios could be determined is ± 0.7%. The crystallinity of the samples was also determined by powder X-ray diffraction (XRD). From the sample some material was collected, ground to powder, measured with XRD and compared with data from 100% crystalline material. The XRD crystallinity ratio of the samples is indicated in Figure 5. The Raman measurements coincide to a reasonable degree with the XRD data. The spread in the Raman measurements is due to sample inhomogeneity. In each separate measuring point, a volume of a few μm³ was probed. In temperature treated coatings, like the ones measured here, it has been observed that in the amorphous material hexagonal crystallites and small needle-shaped crystals start to grow with sub micrometer dimensions (De Bruijn, 1992). This is

Figure 2. (A) Bright light photograph of the crystalline apatite coating-bone interface as seen under the microscope. The white line (with a length of 26 μm) indicates the scanned laserline. (B) Linescan Raman image with the spectral information of a line over the sample as indicated in (A). Measuring time 5 minutes, excitation power 48 mW.
Bone and calcium phosphate coatings

Figure 3. (A) Integrated Raman intensity of the 960 cm\(^{-1}\) phosphate band for the crystalline apatite - bone measurement shown in Figure 2, and for a similar sample with an amorphous coating. (B) FWHM of the 960 cm\(^{-1}\) peak from the corresponding data in (A).

Figure 4. Examples of spectra recorded of three samples which had undergone a different temperature treatment: 500°, 525°, and 575°C. The spectra consist of a broad band stemming from the amorphous material and a sharp peak which comes from the crystalline material. Spectra measured with an illumination spot diameter of 0.9 μm. Excitation power: 69 mW, 60 seconds measuring time.

Figure 5. Crystallinity ratios on six different temperature treated apatite coatings (V), Raman measurements, (-) XRD data. Number of separate Raman measurements for each temperature from 425-600°C: 7, 17, 20, 15, 14 and 6 measurements.

Illustrated in Figure 6. Since the size of the measuring spot is of the same order the variation in crystallinity can be accounted for by more or less crystallites being probed. This is especially evident in the 525°C sample which apparently is not homogeneous. The samples at 425°C and above 575°C are much more homogeneous. If one is interested in the averaged sample crystallinity it could be advantageous to use an objective with a lower magnification, thereby averaging over a larger sample volume and obtaining a stronger Raman signal in the same measuring time.

Conclusions

We have shown that Raman measurements of calcium phosphate (CP) coatings on bone implants give high resolution information about the structure, being (oxy)apatite or amorphous CP, and crystallinity of the sample. Line-scan Raman shows that in both crystalline and amorphous CP coatings there can exist a variation in material density without a significant change in crystallinity. Heat treated CP coatings consist of a mixture of crystalline apatite and amorphous CP. The local degree of crystallinity of several of these coatings can determined with a precision of 0.7%.

References


Editor’s Note: All of the reviewer’s concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.