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XPS Study of elastin-solubilized peptides binding onto apatite in orthopaedic biomaterials

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The adsorption behaviour of elastin-solubilized peptides (ESP) on hydroxyapatite (HA) was studied by X-ray photoelectron spectroscopy (XPS). The analysis of the data indicated a modification of both the apatitic surface and the peptide moieties. The aliphatic and aromatic carbon peak increased with respect to the hydrophilic carbon peak indicating a preferential orientation of the peptide molecules interacting essentially with the apatite surface by their hydrophilic groups. A deprotonation of the amine groups of adsorbed peptides was also observed, revealing a possibly chemical interaction with the mineral surface. Simultaneously a decrease of the Ca/P ratio of the mineral surface occurred. The orientation of ESP adsorbed on the mineral surface was related to the modification of their reactivity with collagens I + III in the process of formation of composite biomaterials for orthopaedic applications.

1. Introduction

A new artificial connective matrix based on the polar association between elastin peptides-ESP and type I + III collagens has recently been developed by one of the authors [1]. Modifications of these matrices have allowed various biomaterials to be obtained [2, 3]. The association of the matrices with calcium phosphate should lead to interesting bone substitutes presenting acceptable mechanical properties and being biodegradable. Moreover the presence of apatite gives hope for osteoconductive properties for such new bone substitutes. However, the various components of the material have to be linked together. To do this, one solution is to adsorb ESP onto apatite (the adsorption properties of proteins onto calcium phosphate are well known), and then to perform the reaction with collagen. Previous studies have shown that ESP are adsorbed irreversibly onto apatite [4].

A surface analysis method has been used to study the mechanisms which take place during the adsorption of ESP onto apatite. The apatite and ESP were studied before and after adsorption onto apatite. The study concerned both the quantitative aspect of the adsorption and the change in orientation which could result.

2. Materials and methods

Apatitic calcium phosphate samples were prepared using a direct method of double decomposition between a solution of calcium chloride and a solution of sodium hydrogenophosphate at 10 mM/l concentration, in basic medium [4]. The sample was examined by chemical analysis, Fourier transform infrared analysis (FTIR) and X-ray diffraction (XRD). The prepared sample is an apatite of atomic Ca/P ratio equal to 1.50, containing small amounts of hydrogenophosphate.

ESP were obtained as described previously [4], by alkaline hydrolysis of insoluble elastin (Sigma). ESP were used in solution in PBS buffer (pH 7.4).

The adsorption of ESP onto apatite was performed at 37 °C by adding the mineral to a solution containing 20 mg of ESP/ml of PBS buffer. Two ESP/apatite ratios were used: 250 and 400 μg of ESP/mg of apatite. After stirring for 30 min, the reaction was stopped, and the samples were washed several times with PBS solution.

All the samples were analysed on adhesive tape using an ESCALAB MK II (V.G.) spectrometer with an AlK_α X-ray source (1486.6 eV of photon energy) and a three-channel detector. In order to avoid any degradation under radiation, X-ray source power was limited to 100 W (10 mA, 10 kV) and the analyser pass energy fixed at 50 eV. The pressure in the spectrometer was about 10^{-7} Pa. Since apatite and ESP are insulators, the spectra were shifted towards higher binding energies due to the accumulation of positive charge on the surface. In the case of apatite, calibration was done by referencing to P_{2p} (133.6 eV) because the binding energy of this element does not change very much in apatite due to the screening of the P atom by oxygen atoms, and to a close crystalline potential. The value determined for $Ca_{2p3/2}$, using this

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correction was 347.5 eV, which was consistent with that usually observed in these compounds. In the case of ESP, calibration was done by referencing to the aliphatic carbon obtained after decomposition of the C_{1s} peak into its components. In the same way, it was checked that the main component of N_{1s} was at a binding energy consistent with those reported by several authors for the nitrogen atom of the protein peptide backbone (near 400 eV). From the integrated intensities of the peaks, and with Scofield's relative cross-section [5], a quantitative evaluation of the XPS data was performed. In the case of apatite, the Ca/P ratio was refined by using an additional factor determined in the case of pure apatite: in this case, the correct ratio was obtained by multiplying Scofield's raw ratio, by 1.12.

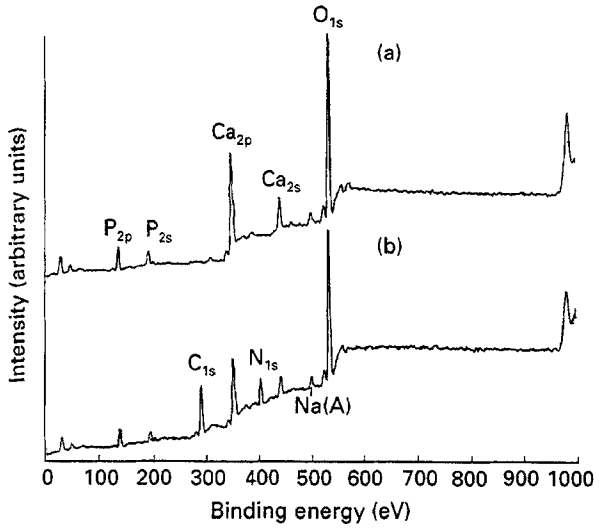

Figure 1 XPS wide scan spectra of apatite sample (a) and ESP adsorbed onto apatite sample (b).

3. Results

Samples of apatite, ESP and ESP adsorbed onto apatite were examined by XPS.

3.1. Apatite

A survey spectrum (0–1000 eV) of the apatitic sample used (Fig. 1a) presents the photoelectronic peaks of their constituents, i.e. calcium, phosphorus, oxygen. The peak near 500 eV with low intensity corresponds to an Auger transition of sodium, an impurity probably due to the synthesis method (use of Na_2HPO_4 as starting reagent).

The spectra of Ca_{2p}, P_{2p}, O_{1s} were performed. The O_{1s} peak is assymetric. It was decomposed into two components (Fig. 2). The main component at 531.3 eV corresponds to the O linked only to a phosphorus atom as in $P-O_4$ ions. The second at higher energy (533.1 eV) of lower intensity, corresponds to the O linked both to phosphorus and hydrogen atoms, such as P–O–H [6]. The presence of HPO_4 ions is in agreement with the low value of Ca/P ratio corresponding to non-stoichiometric apatite.

From the integrated intensities, a quantitative evaluation of the XPS data was made. The experimental ratios are given in Table I. The results show that the Ca/P ratio, equal to 1.54, is close to the value determined by chemical analysis, 1.50.

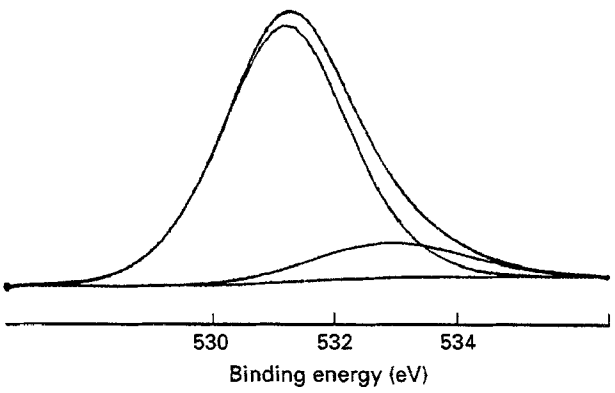

Figure 2 O_{1s} peak of apatite sample.

TABLE I Atomic ratios in apatite, ESP and ESP adsorbed onto apatite samples

Experimental	Ca/P	O/P	C/P	C/N	C/O
Apatite	1.54	3.31	–	–	–
ESP	–	–	–	4.6	4.11
ESP adsorbed at 250 μg/mg	1.50	3.75	3.6	4.3	1.15
ESP adsorbed at 400 μg/mg	1.46	3.94	4.5	4.5	1.14
Theoretical					
ESP fraction of high MW				4.13	4.17
ESP fraction of low MW				3.62	3.53

3.2. ESP

The survey spectrum reveals the photoelectronics peaks of carbon, nitrogen and oxygen. The spectra of C_{1s}, N_{1s} and O_{1s} were obtained and allowed the atomic ratios C/O, C/N and O/N to be determined. They are compared in Table I with those calculated from the amino-acid compositions of the highest ($> 1.5 \times 10^5$) and the lowest molecular weight (10–32 000) of hydrolysis fractions of elastine isolated. These compositions were determined by Pelletier-Lebon [7]. The observed values are in agreement with those of the highest MW fraction.

The C_{1s} peak was decomposed (Fig. 3) using the three main components corresponding to aliphatic carbon atoms, α-carbon atoms and peptidic carbon atoms, as already done by several authors in XPS studies of various proteins [8, 9]. The amount of each type is listed in Table II, with the amounts calculated from the amino-acid composition of the highest and lowest MW fraction of elastine. Indeed, it was shown that it is possible to determine, from a protein composition in aminoacids and the formula of each of them, the content in C atoms which corresponds to a given environment, for example aliphatic carbon $-CH_2-$, C–OH or C(=O)N [10]. Only three kinds

of C atoms play a leading role: the aliphatic or aromatic, the α-carbons and amide carbons. The other carbon atom contributions can be shared in these three components according to their chemical environment. It can be seen that the observed amounts are in agreement with the highest MW fraction of hydrolysed elastin.

The N_{1s} peak was decomposed into two components (Fig. 4). The main one, near 400 eV, corresponds to the nitrogen atom of the peptide backbone. The second, at higher binding energy (near 401.7 eV), is due to the protonated nitrogen atoms present in the desmosine, histidine and lysine residues. The amounts of each component are indicated in Table II, and compared with the values which can be calculated from the chemical composition of the low or high MW fractions of hydrolysed elastine. In this case too, the observed amount of protonated nitrogen atoms in ESP is close to the calculated values for the high MW fraction.

All the results show that the ESP used is closer to the elastin hydrolysis fraction of high MW than to that of low MW.

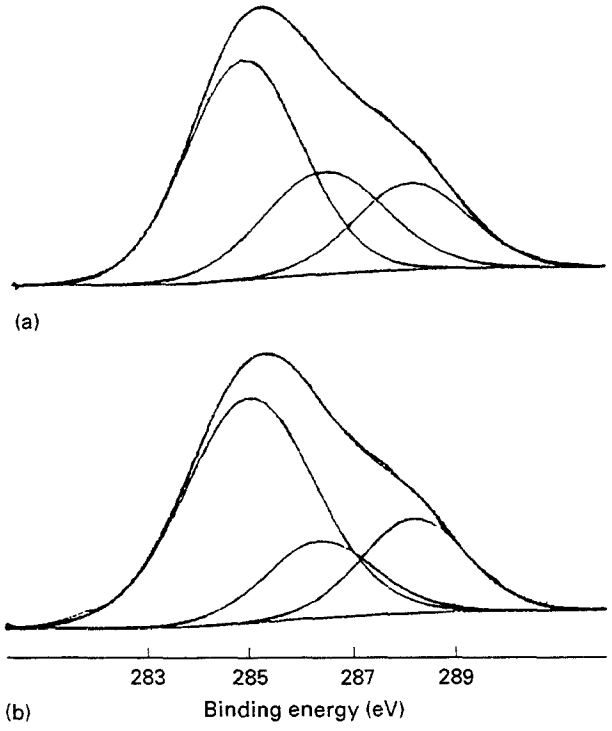

Figure 3 C_{1s} peak of initial ESP (a) and ESP after adsorption onto apatite (b).

3.3. ESP adsorbed onto apatite

The survey spectra (for example Fig. 1b, at 400 μg/mg), present both the elements of apatite and ESP. In this case too, the small Auger peak of sodium near 500 eV, can be seen. This contamination comes from the PBS buffer.

The spectra of Ca_{2p}, P_{2p}, O_{1s}, C_{1s} and N_{1s} were obtained. They allowed a quantitative analysis to be made. The different atomic ratios are gathered in Table I, with the values obtained previously with the starting compounds.

The **Ca/P ratio** is characteristic of calcium phosphate. It decreases when ESP is adsorbed. This decrease can be due to a slight change in the composition of apatite during the adsorption of ESP in PBS buffer.

The **C/P ratio** corresponds to the binding of ESP onto apatite. It increases with the concentration of ESP in the starting solution. XPS appears to be a very convenient method for the determination of the amount of protein adsorbed onto a surface. In this way, radioactivity labelling can be avoided.

The **C/N ratio** of between 4.3 and 4.5 is close to the initial value observed in ESP before adsorption, equal to 4.6.

The C_{1s} was decomposed as previously into three components (Fig. 3). As can be seen in Table II, the aliphatic and aromatic carbon content increases when ESP is adsorbed onto apatite, whereas the α-component including hydrophilic carbon atoms, decreases. The amount of peptidic carbon remains the same. Due to the high sensitivity of the XPS technique to surface atoms, these observations indicate that an orientation of ESP occurs during its adsorption onto apatite. Aliphatic or aromatic amino-acid residues of ESP are turned preferentially towards the outer surface, whereas the hydrophilic residues are turned inwards, towards the interface with calcium phosphate. These latter seem to be involved in the adsorption mechanisms.

N_{1s} peak fitting (Fig. 4) reveals only one type of nitrogen atom, corresponding to the peptide backbone. The second component due to protonated residues atoms disappeared. It can be deduced that binding of ESP onto apatite involves the deprotonation of these residues.

These observations cannot be due to a selective binding of particular peptides of ESP, characterized by a high content in aliphatic carbons, and the absence of protonated nitrogen atoms. In this case, the C/N ratio would increase whereas it decreased slightly after adsorption of ESP onto apatite.

TABLE II Decomposition of the C_{1s} and N_{1s} peaks

Experimental	Aliph. C 285.0 eV (%)	α–C 286.4 eV (%)	Peptidic C 288.0 eV (%)	Peptidic N 399.9 eV (%)	Proton. N 401.7 eV (%)
ESP	53	26	21	95	5
ESP adsorbed at 250 μg/mg	59	20	21	100	0
ESP adsorbed at 400 μg/mg	61	17	22	100	0
Theoretical					
ESP fraction of high MW	54	24	22	95	5
ESP fraction of low MW	45	28	27	99	1

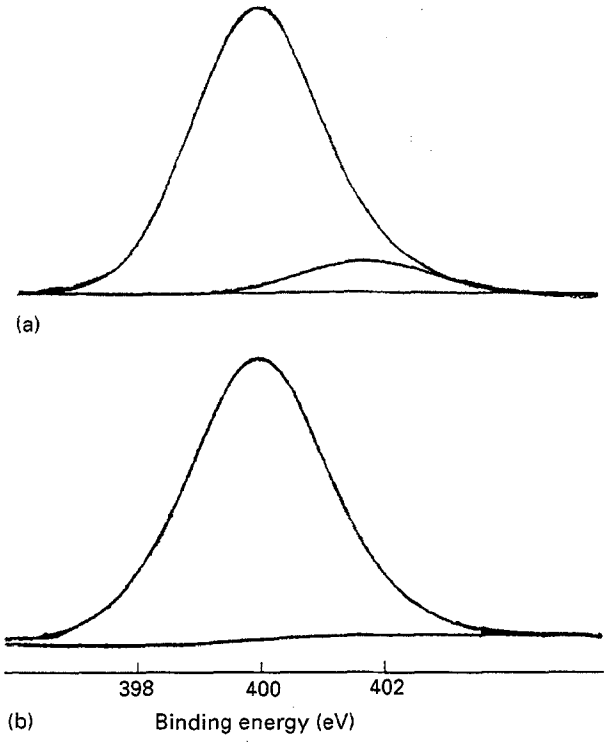

Figure 4 N_{1s} peak of initial ESP (a) and ESP after adsorption onto apatite (b).

4. Discussion

Without apatite, ESP react easily with collagens I + III to give a connective matrix used as a bioresorbable material. This reaction is mainly due to the interaction between the polar groups of ESP and collagens. After adsorption onto apatite, ESP keep their reactivity with the collagens. But the reaction is noticeably delayed in this case. This change in reactivity of ESP can be explained by the results of this study. It has been shown that preferential orientation of residues and deprotonation of diaminoacids take place during the adsorption of ESP onto apatite. This change in orientation, corresponding to a modification of the molecular conformation of ESP, and the loss of the positive charge on the diaminoacid residues, leads to a decrease in the number of reactive sites available, and consequently in the reactivity.

Several authors have demonstrated by XPS studies, such preferential orientations in proteins. Thus, Shard *et al.* [11] used XPS to examine the surface of solvent cast films of hyaluronic acid esters. They deduced that these compounds preferentially present the ester chain at the surface in the dry state. Also, it was shown [10] that in the case of tensioactive proteins, preferential orientation of the hydrophobic or hydrophilic residues is observed depending on the method of elaboration used. When in the form of a Langmuir Blodgett film on glass substrate, the hydrophobic residues of this protein are turned towards the outer surface, whereas obtained by crystallization from solution, the hydrophobic residues are located inside, and the hydrophilic ones turned outside. Ratner [8] analysed protein film on solid surfaces by XPS and concluded that the organization of hemoglobin films on platinum and PTFE are markedly different. According to these authors, the nitrogen present in the protein structure is preferentially oriented towards the surface of the hemoglobin. The deprotonation of amino groups during adsorption onto apatite has also been observed by the authors [6] and by Barrough in the case of glycine adsorption [12].

All these studies show that it is possible, by choosing the nature of the support, to give a preferential orientation to the residues. XPS spectroscopy allowed identification of the residues which are turned outside. Knowing them is important because they give the protein its reactivity. It allows the structure-activity relations to be better understood.

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