Nanostructure of bone tissue probed with Ca 2p and O 1s NEXAFS spectroscopy

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Abstract

X-ray absorption spectroscopy is applied to investigate relationships between hierarchical organization of the skeleton and nanostructure of femoral bone in knee compartments and to understand the osteoarthritis (OA) related changes at the subcellular level. Our focus is on local electronic and atomic and molecular architectonics of the medial and lateral condyles of the femur resected during total knee arthroplasty in patients with medial compartmental knee OA. The element-specific and site-dependent peculiarities in spectral distributions of oscillator strength for core-to-valence transitions are revealed. The near Ca 2p and O 1s edges x-ray absorption fine structure (Ca 2p and O 1s NEXAFS) spectra of the saw cuts demonstrate substantial redistributions in intact and OA damaged areas on the proximal side, and on the proximal and distal sides of the samples. Examining the O 1s NEXAFS spectra new chemical bonds are revealed on the proximal surface in the OA areas. Strong intra-atomic intershell Ca2+ 2p3/2, 1/2 3d1 interaction specifies the great similarity of the Ca 2p NEXAFS spectra. Their analysis performed in combination with the x-ray photoelectron data has demonstrated the formation of non-apatite calcium in the OA areas of the samples. It is shown that NEXAFS spectroscopy is a powerful tool for deeper understanding relationship between hierarchical skeletal organization and nanostructure of native bone. Perspectives for development of novel methods for medical imaging and diagnosis of subchondral bone at the nanolevel are discussed.

1. Introduction and general remarks

Bone is the most complex material in nature. The complexity is originated by the interplay of two hierarchical organized organic and mineral subsystems. Each of them includes several hierarchical levels extending from atomic to skeletal and even higher to ecological spheres [1]. Macroscopic skeletal distortions invoke specific site-dependent changes in its nanostructure. Regrettably the mechanisms responsible for these changes are poorly understood.

The main efforts were conventionally directed to investigations of 3D atomic structure of bioapatite and morphology of mineralized bone by applying either the mineralogical (see, e.g. [2–5]) or biomedical (see, e.g. [6–8]) approaches. Electronic structure, molecular architectonics and peculiarities of chemical bonding remain virtually unattended. This knowledge gap blocks solutions of many fundamental such as biomechanics, bio-designing of advanced materials [9–18], fine tuning of ion exchange processes [15], and clinically relevant problems such as the development of new methods of medical imaging at subcellular levels and medical diagnosis of skeletal pathology at the early stage [19].

Bone being a composition of the hard hydroxyapatite (HAP, Ca10(PO4)6(OH)2) with the flexible collagen molecules is more elastic and firm [9–11] in comparison with the parent mineral and collagen. The single crystal HAP and the collagen molecule are respectively taken for the ground levels of the mineral and organic matrices.
Figure 1. Schematic presentation of the lowest hierarchical levels in mineralized bone. Panel (a): the extended rectangular crystallographic cell of HAP. Black arrows point at Ca, P, O and H atoms shown with green, orange, red and grey, respectively; (b): the coplanar assembly of the NHAP (6) separated by the hydrated layers (7). $b_1$, $b_2$ are the basic vectors of superlattice translation; (c) The mineralized plates (5) distributed among the collagen molecules (4) and swirling around a fibril; (d) fibrils (1) and inter- and intra-fibrilar distributions of NHAPs (2) and (3) respectively.

Figure 1 (panel a) displays the extended rectangular crystallographic cell of HAP. The band theory describes the single crystal HAP as the wide-band-gap insulator in the range of $\sim 5$ eV [20, 21]. This crystal is determined by the sets of [PO$_4$]$^{3-}$ and Ca$^{2+}$ channels populated by columns of OH$^-$ anions [21]. Strong ionic conductivity of HAP is conveniently associated with high mobility of hydroxyl ions.

Both electron microscopy and x-ray diffraction distinguish the inter- and intra-fibrilar associations of nanocrystallites of HAP (NHAPs). The panel d shows the interfibrilar associations. Their disordering increases with an increase of distance from the fibrils. The intra-fibrilar associations are composed from coplanar assemblies of the NHAPs. They form a series of parallel and spiral wound mineralized plates shown on the panels c and d [6, 10, 18]. The mineralized plates are distributed among the collagen fibrils (see, panel c) and reproduce their spiral shape [9–14, 14]. Schematically a fragment of the NHAPs coplanar assembly is exhibited in panel b. The NHAPs are separated one from the other by nanolayers of saturated aqueous solution containing mainly the anions OH$^-$, [PO$_4$]$^{3-}$ and [CO$_3$]$^{2-}$ and the dications Ca$^{2+}$ [12]. The thickness of the nanolayers is about 2 nm.

In addition to the apatite and collagen subsystems water goes all through bone tissue [11, 22, 23]. Amorphous calcium phosphate is another basic component in mineralized phase [24].

This morphological picture is a result of the structural and functional relations between adjacent hierarchical levels [23, 25]. According to Wolff’s paradigm [26] spatial organization of bone structures and their orientations are determined by mechanical loads that arise during the execution of locomotors functions against the gravity.
force. The electron microscopic studies [6, 27–31] confirm a correspondence between the directions of long axes of collagen fibrils in mineralised matrix and the force lines in the skeleton space. A question arises: in what extent the paradigm is applicable to bone nanostructure?

2. Goals and objectives

The recent study of health and osteoarthritis (OA) damaged bone [19] has shown that Wolff’s biomechanics encounters with difficulties at the nanoscale. By using x-ray photoelectron spectroscopy (XPS) and x-ray diffraction (XRD) the distinct site-dependent changes in both crystallinity and core electron binding energies (BEs) of atoms on the proximal side of OA-damaged areas in human femoral bone were revealed [19, 32]. The apatite calcium bonds dominate in intact area, however, the situation is changing dramatically in OA-damaged areas where the fraction of new non-apatite bonds becomes substantial. The non-apatite bonds were assigned with both biochemical reactions occurring on the bone—cartilage interface and rupture of the sacrificial bonds [33] in the regions of abnormal mechanical loads. Presumably, the non-apatite bonds are originated from carbonization of nanocrystallites and their coupling with the fragments of broken collagen molecules on the cartilage—bone interface.

To examine the non-apatite bonding in more detail the near edges x-ray absorption fine structure (NEXAFS) spectroscopy is applied to the bone samples studied in [19]. This spectroscopy is an element and site specific tool providing spectra of different bonding sites for the same element. This method is a widely used for the investigations of core-to-valence transitions in molecules, free clusters, liquids and solids (see, e.g. [34–37]). Strong dynamic spatial localization of core excitations is conditioned by femtosecond core-hole lifetime [35]. This probing is expected as a prospective way for obtaining of quantitative information about local atomic geometry, spectral and spatial distributions of valence shells.

The Ca 2p and O 1s NEXAFS spectra are measured by using synchrotron radiation and analyzed by applying the 3DSL model [18]. The total electrons yield (TEY) mode [38–40] used here for the registration of the NEXAFS spectra, provides a sensitive testing of surface layers ≤ 10 nm on the bone—cartilage interface. We keep our main emphasis on (i) non-apatite bonds and their characterization and (ii) relationships between the hierarchical skeletal organization and bone nanostructure.

3. Experimental

As samples of bone tissue in intact and damaged areas the medial and lateral condyles of the femur resected during total knee arthroplasty in patients with medial compartmental knee OA are used. Figure 2 presents the radiograph of the OA damaged knee joint. The bone sample of damaged bone is a saw cut of the femoral condyle, subjected to excessive mechanical stress due to the development of arthritis and deformity in the joint, with full-layer loss of cartilage. The sample of healthy bone is a saw cut of the condyle of the same femoral bone, not subjected to excessive load, with intact cartilage. The bone samples are exhibited in figure 3 too. The regions where measurements were taken are designated as 1 (intact area), 2 (sclerotic area), and 3 (the vicinities of sclerotic area).

The bone saw cuts were cleaned of cartilage tissue using a gentle mechanical treatment with a scalpel, to subchondral bone plate. Then, to degrease the cuts and delete the myeloid contents from the trabeculae of the spongy layer, the samples were kept for 4 days in a bath with an aqueous 33% hydrogen peroxide solution (H2O2) mixed in a 1:1 ratio with hot water (60 °C) and with the addition of 5 ml of 10% aqueous ammonium hydroxide solution (NH4OH). This mixture was replaced daily. To complete the cleaning process the samples were placed for one day in distilled water, changing it every 6 h. Before measuring the NEXAFS spectra the samples were subjected to heating at 200 °C for 24 h in thermostat. The bone samples are prepared in Vreden Russian Research Institute of Traumatology and Orthopedics.

The Ca 2p and O 1s NEXAFS spectra of HAP and native cortical bone of rats were also measured. The native bone samples are prepared from middle third of the femur, tibia and humerus white mongrel adult male rats. Cortex has been (i) thoroughly cleaned of soft tissue, (ii) washed in saline, and (iii) dried with blotting paper. The HAP of almost stoichiometric composition (a = 9.416 (2), c = 6.880 (1)) was synthesized by applying the reverse precipitation method in an ammonium-containing solution as it is described in [41, 42].

The Ca 2p and O 1s NEXAFS spectra were obtained by monitoring the TEY and measuring current from the sample at BEAR station at the Elettra-sincroton-Trieste lightsource. The spectra were normalized to current from mirror with gold coating located directly before analytical chamber. Currents from the samples and from the mirror were measured simultaneously, that excluded effect of the own structure in the incident radiation in the region of the O 1s absorption edge. Each spectrum was measured after 10 min waiting before start of scanning that excluded effect of sample charging on the shape of TEY-spectra.
The energy resolution was better than 0.2 eV. Light spot size was about 300 * 300 mkm, the shape of NEXAFS spectra measured at different points (at the same intact/sclerotic area) was well reproducible. No sample charging or decomposition effects due to the intense beam of soft x-ray radiation were detected for samples during the measurements. The spectra were recorded several times and their fine structures have usually shown a good reproducibility. The energy scale was referenced to the Ca 2p and O 1s spectra of the reference sample HAP [41]. The spectra of HAP were measured every 2 h. The accuracy of the energy scale was about 0.05 eV (half value of energy step).

3.1. Ca 2p NEXAFS spectra
The Ca 2p NEXAFS spectra are plotted in figure 4. They are measured on the proximal side of the subchondral femur saw cuts in the intact (1) and sclerotic (2) areas and just outside (3) the sclerotic area and the distal side (trabecular bone). The areas location is exhibited in figures 2 and 3. For comparison the Ca 2p NEXAFS spectra of HAP (green) and rat cortex (dark green) [18] are also shown in figure 4. The most pronounced resonant features in the spectra are labeled as a, A, b and B. The close similarity of the Ca 2p NEXAFS spectra is seen clearly. The measured energy positions (E) and full-width-at-half-maximum (FWHM) of the ‘white’ lines A and B are given in table 1.
3.2. O 1s NEXAFS spectra

The O 1s NEXAFS spectra of subchondral femoral bone measured in the same areas are plotted in figure 5. The strong site-dependent changes in the spectral distributions are seen. For comparison the O 1s NEXAFS spectra of native rat cortex and HAP are also presented. One may see that the observed spectral distribution for the O 1s-to-valence transitions in the bone samples is determined by the low energy band A and the broad band B centered at 532 and 537 eV, respectively. The positions and widths of the band A are shown in table 2. The spectra of liquid water [43] and peptides [44] in collagen molecules are also plotted in the figure.

Table 1. Energy positions (E) and FWHM of the main absorption peaks A and B.

| Line     | E [eV] | FWHM [eV] | E [eV] | FWHM [eV] |
|----------|--------|-----------|--------|-----------|
| Intact   | 349.3  | 0.48      | 352.7  | 0.52      |
| Sclerotic| 349.25 | 0.45      | 352.56 | 0.53      |
| Around sclerotic | 349.3 | 0.48 | 352.6 | 0.53 |
| Trabecular| 349.25 | 0.51   | 352.55 | 0.59      |
| Cortex   | 349    | 0.39      | 352.23 | 0.53      |
| HAP [41] | 349.27 | 0.27      | 352.58 | 0.33      |
4. Data analysis and discussion

The spectral dependences of x-ray absorption in figures 4 and 5 show the element-specific and site-dependent distributions of oscillator strength for the transitions from the Ca 2p and O 1s levels to unoccupied valence shells in bone. Examining the NEXAFS spectra we see their similarity near the Ca 2p edges and strong site-dependence.

**Figure 5.** The O 1s NEXAFS spectra of subchondral femoral bone in intact and inside and around sclerotic areas of the proximal side and of trabecular bone on the distal side of the saw cuts are presented (current work). The spectra of the reference compounds (liquid water [43], peptides [44], HAP) are highlighted with light yellow and bone samples in OA areas with rose respectively. The energy 532.7 eV of the line 2 in the O 1s NEXAFS spectrum of HAP was used to calibrate the spectra.

**Table 2.** Energy positions (E) and FWHM of the absorption band A in the Ca 2p NEXAFS spectra.

|            | E [eV] | FWHM [eV] |
|------------|--------|-----------|
| Cortex     | 531.64 | 1         |
| Intact     | 532.1  | 2.1       |
| Sclerotic  | 532.2  | ≈3        |
| Around sclerotic | 532.4 | >3        |
| Trabecular | 532.32 | ~2.1      |

* FWHM of the band A in the spectra inside and outside the sclerotic area is determined rather crudely due to its complicated shape.
distortion near the O 1s edge. The element-specific behavior is opposite to that observed in x-ray linear dichroism. Maximal and minor changes are detected respectively at the Ca 2p and O 1s edges in bone [45].

The resonance structure $a-A-b-B$ in the Ca 2p NEXAFS spectra of bone was attributed to the multiplet splitting of the core-excited Ca$^{2+}$ 2p$^3$3d$^1$ ionic state in the anisotropic surroundings potential [18]. The recent experimental and theoretical investigations of Ca$^{2+}$ 2p excited calcium solutions [46] agree with the assignment. The peak separation $a-A$ and $b-B$ characterizes the crystal field effect and the small ratio $a/A$ and $b/B$ its weakness compared to the intra-atomic intershell 2p$^3$3d$^1$ interaction. It is the superiority of the intra-atomic interaction over the crystal field effect that explains the observed Ca 2p NEXAFS similarity in bone, solvated Ca$^{2+}$ in water [46, 47], CaCO$_3$ [48], CaCO$_3$ in invertebrates [49] and other calcium compounds [48].

According to the measurements the intensity ratios $a/A$, $b/B$ and the separation energies $a-A$, $b-B$, and $A-B$ are not constant. But as for the quantitative characterization of the crystal field, their variations cannot be used as the anisotropy of the field is not exactly determined. Table 1 shows the $A-B$ separation varies from 3.23 eV in cortex up to 3.4 eV in healthy femur and does not coincide with the reference Ca$^{2+}$ 2p$_{3/2}$$-$$2p_{1/2}$ spin–orbit splitting $\approx$ 3.55 eV [50]. Note the measured separation between the Ca$^{2+}$ 2p$_{3/2}^{-1}$ and 2p$_{1/2}^{-1}$ PE lines in the samples [19] coincides with the reference energy. So, we infer that the deviations in the $A-B$ separation display different spatial localization of Ca$^{2+}$ 3d electron density in the areas.

Let us consider now the hierarchy-induced energy shift of electron bands in bone compared to the relevant bands in HAP. The HAP-to-bone red band shift $\delta E_{\text{HAP}}$ was revealed by comparing the energies of the Ca$^{2+}$ 2p$_{3/2,1/2}$ $\rightarrow$ 3d transitions in HAP, rat cortex [18, 32, 41] and fish bone [51]. Our Ca 2p NEXAFS spectra in figure 4 show the red shift exists in cortical bone and is not seen in intact, trabeculae and OA-damaged areas.

To understand the disappearance we focus on the BE of the initial Ca$^{2+}$ 2p$_{3/2,1/2}$ state of the transitions. The XPS studies [19] have demonstrated that the Ca$^{2+}$ 2p$_{3/2}$ BEs differ. They are equal to 347.2, 347.1 and 347.0 eV in HAP, rat cortex and intact area of human femur, respectively.

More than that in OA areas the non–apatite calcium fraction becomes substantial. The contribution of the weakly bound non–apatite Ca$^{2+}$ (X) is 47% inside and 78% outside the sclerotic area [19]. The corresponding Ca$^{2+}$ (X) 2p$_{3/2}$ BE is $\approx$ 346.5 eV. Taking the BEs into account we obtain the Ca$^{2+}$ 3d band positions $\approx$1.8 eV, 2.1 eV, 2.2 eV and 3.1 eV with error $\pm$ 0.2 eV above the bottom of conduction band in cortex, HAP, intact area and around the sclerotic area.

Comparing the band energies we see that the HAP-to-bone-band-shift agrees with the red shift $\approx$ 0.3 eV in cortex [18] and demonstrates the opposite shift $\approx$1 eV in OA areas. This unexpected blue shifting can be originated neither by coplanar aggregation of NHAP crystallites nor by their disordering. It is reasonably to suppose that the concept of the HAP-to-bone red shift is not applicable to the interface because non-apatite calcium bonds play dominate role in electronic structure in OA areas. Hence the blue shift is not connected with the band structure of HAP. Thus, the combination of the Ca 2p XPS and NEXAFS spectra confirms the non–apatite origin of chemical bonding in OA area.

In intact area where apatite bonds are found dominating, another situation takes place. The HAP-to-bone red shift of the Ca$^{2+}$ 3d band can be described as

$$\delta E_{3d} \approx 2\gamma E_{3d} \frac{d}{L} 0$$

where $\gamma$ is the fraction of Ca$^{2+}$ ions forming the superlattice to their total number in bone, $L$ is the mean size of the effective NHAP, $d$ is the electro–optical thickness of the inter–crystallite region and $E_{3d}$ is the band energy in HAP [41]. This means that Ca$^{2+}$ in interribular regions (see, figure 1, panel d) and amorphous calcium phosphates does not contribute to the $\delta E_{\text{HAP}}$ shift. So, its minor value in intact area can be primary attributed to a low probability of the coplanar aggregations of NHAP crystallites in it.

The absorption line $A$ at 532 eV and the broad band $B$ at 537 eV are the major resonance features in the O 1s NEXAFS spectra in figure 5. One may see that their shapes demonstrate substantial site-dependent changes associated with the redistribution of O 1s-to-valence transitions in mineral, organic and water subsystems. The reference O 1s NEXAFS spectra of liquid water [43], peptides [44] and HAP included in the figure, confirm the composite origin of the band $B$.

The line $A$ has a complicated origin too. According to [18] it is mainly populated by (i) the O 1s $\rightarrow$ $\pi^*$ transitions in peptides, carboxyl and carbonyl groups in collagen (see, e.g. [44, 52, 53]) and (ii) the O 1s $\rightarrow$ $\sigma^*$ transitions in hydroxyl anions in aqua solutions (see, e.g. [54–56]). One can observe the noticeable changes in the FWHM and relative intensity of the line in figure 5. The line broadens at transition from cortex to intact and trabecular bone and then, to the OA areas in femur. The intensity is maximal in spectra of intact femoral and cortical bone and minimal for trabecular one.

It is evident that the line $A$ appears in sclerotic area (blue line) as a superposition of three components labeled as $A', A''$ and $A'''$ in figure 5. This complication, on one hand, confirms the composite origin of the absorption
line [18], and on the other hand, it demonstrates the formation of new valence states. The features $A'''$ and $C$ specify the substantial redistribution of the O 1s-to-valence transitions in the sclerotic area. Note that according to our preliminary study of the O 1s excited HAP the low lying absorption bands $i$–$i$ in figure 5 (green line) can be assigned with one-electron O 1s $\rightarrow \sigma^*$, O 1s $\rightarrow \psi$(Ca$^{2+}$ 3d$-O$ 2p) and O 1s $\rightarrow a_i^o$(P 3s$-O$ 2p) transitions dynamically localized on OH$^-$, [Ca$^{2+}$ O$_n$]$^{X-}$ and [PO$_4$]$^{3-}$ quasi-molecular anions, respectively. Taking this assignment into account we can link the resonant features $A'''$ and $C$ with the bands $i$ and $i$ in HAP and conclude that the Ca–O and phosphate bonds increase in the sclerotic area. The thin solid lines in figure 5 serve to demonstrate the linkage.

The observed site-dependence of the Ca 2p and O 1s NEXAFS spectra makes evident (i) new valence bonds appearance and (ii) interplay of biochemical reactions and biomechanics in OA areas. Relationships between the hierarchical organization of the skeleton and chemical bonding in bone matter demand further theoretical and experimental research. The standard statistics in the case does not work as nanostructure of bone tissue is strongly personalized. It depends on fatigue damages, age, ecology and so on. The identification of common patterns and personal characteristics as well as their interactions at the nanoscale require detailed studies.

To develop new methods for medical imaging of bone at subcellular levels and diagnosis of bone pathology at the early stage the patient dependence research in hierarchy-induced effect on local electronic and atomic structure of bone is required. In particular, measuring the Ca 1s and 2p, P 1s and 2p and O 1s NEXAFS spectra and aligning them in the single energy scale by using the relevant XPS data we have a chance to visualize the redistribution of unoccupied valence orbitals in intact and OA-damaged areas, which opens the possibility of 3D mapping of chemical bonding and targeted medical impact.

5. Conclusion

The Ca 2p and O 1s NEXAFS studies of human femoral bone were performed for intact and OA damaged areas of human femoral bone. It is shown that NEXAFS spectroscopy provides a powerful probing of relationships between hierarchical skeletal organization and nanostructure and chemical bonding in bone tissue. The site-dependent changes in the x-ray absorption spectra are revealed near the both edges. We stated the agreement between the NEXAFS changes and those obtained with XPS and XRD approaches to the bone samples: the most essential deviations from healthy bone are revealed inside and just outside the sclerotic area and new valence states are observed in OA damaged area.

It is shown that (i) the aggregation of nanocrystallites is disordered on the proximal surface on the bone–cartilage interface and (ii) the non-apatite chemical bonds are formed on the surface, (iii) the superiority of intra-atomic Ca$^{2+}$ 2p$^53d^1$ intershell interaction over the crystal field effect controls the spectral distribution of oscillator strength for the Ca$^{2+}$ 2p$^5$ $\rightarrow$ 2p$^53d^1$ transitions in bone tissue.

The combination of XRD, XPS, and NEXAFS spectroscopic methods opens up promising prospects for the development of new approaches to (i) quantitative characterization of local electronic and atomic structure and medical imaging of bone tissue at the nanoscale and (ii) the elaboration of biomaterials with predetermined dynamic and kinematic properties.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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