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To Build or Not to Build: The Interface of Bone Graft Substitute Materials in Biological Media from the View Point of the Cells

Andrey Shchukarev[1], Maria Ransjö[2] and Živko Mladenović[2]
[1]*Department of Chemistry, Umeå University*
[2]*Department of Odontology, Umeå University*
Sweden

1. Introduction

The phenomena at the biomaterial - biological media interface are crucial in maintaining optimal cellular functions, initiating biomineralization and bone tissue regeneration. In contrast to "inert" implants substituting hard and soft tissues, bioactive ceramics used as bone graft substitutes are known to possess a reactive surface with respect to the body liquids and similar model solutions (Hench & Wilson, 1993). It is the interface that cells are approaching and "deciding" to build or not to build a new bone.

At interfacial equilibrium, an electrical double layer (EDL) is formed at the interface. The key feature that determines the EDL structure is the surface charge developed due to chemical interactions between bone graft substitute material and biological media. Three scenarios are possible depending on biomaterial surface chemistry, composition of the media, and the media pH. Positively or negatively charged surfaces encourage electrostatic (long-range forces) attraction of oppositely charged species. For example, ions, protonated/deprotonated aminoacids, peptides, proteins and even, probably, cells. For the particles with zero surface charge, short-range (chemical binding) interactions are preferable. Since body fluids have relatively constant composition and stable buffered pH, it is mainly the bulk and surface chemistry of biomaterial that affect the surface charge development. Electrostatic attraction of inorganic cations and anions from biological media is particularly important to initiate biomineral surface nucleation. The surface charge of biomaterial plays a pivotal role in adsorption of organic species from the media. The adsorption can change hydrophobic/hydrophilic properties of the surface if preferential accumulation of lipids, proteins, and vitamins from one side, or sugars, small organic acids and aminoacids from another side occurs. The adsorbed organic molecules will determine if the cells can attach to the bioactive surface with functional groups on the cell membrane. Moreover, the surface charge will influence the interface pH and bone graft substitute dissolution/re-precipitation processes. The extent of biomaterial dissolution is very important considering the rate of bone regeneration and biomaterial substitution by new bone. Another important factor which can significantly improve initial adhesion of the cell

to the bone graft substitute materials is the surface morphology. Considering the cell size (μm) and the interface (EDL) thickness (nm), it should be the surface morphology which certainly maintains comfortable hosting for the cell to adhere, to fine tune their micro environment, and to communicate with other cells. The main parameters to consider are particles size, shape, porosity, surface (macro and micro) roughness, and availability of natural and artificial scaffolds. Initial morphology is not kept during the formation of new bone; however it can contribute at the first stage of the tissue regeneration process.

"Biomaterial in biological media" system can be separated to three different parts: solid phase (macro), solution phase (macro), and solid-solution interface (nano). Continuous phases are easy to investigate, but the most important information about the interface is almost inaccessible. The formation of new bone can be experimentally observed already at micro level using histological sections of biopsies from patients; changes in composition of biological media are routinely monitoring by standard chemical tools. However, specific characteristics necessary for a potential interfacial "nano-reactor" and the key elements which initiate and control cellular activity are still unknown. To understand these phenomena at a molecular level we need supplementary physico-chemical information about the surface of bone graft substitute material and the immediate interface between the material and the biological media. This information will allow material scientists and bone biologists to improve existing or to create a new tissue substitutes with attractive interface properties, leading the cell towards the decision – "To build or not to build" the bone.

2. Biomaterials and bone cell interactions

Although bone is unique in terms of being able to repair itself (e.g. fracture healing), bone defects caused by trauma and pathological conditions will not always heal spontaneously (Feng & McDonald, 2011; Nakahama, 2010). Autologous bone graft is considered the best treatment option, but has the limitation of donor sites, and the interest for bone graft substitute materials is increasing (Porter et al., 2009). Ideally, bone formation induced by the biomaterial will mimic the physiological process where osteoblasts differentiate from mesenchymal stem cells and produce extracellular proteins that serve as a template for the biomineralization process. It is crucial that the biomaterial does not promote an imbalanced bone remodeling process with uncoordinated activities between bone forming osteoblasts and bone resorbing osteoclasts. Osteoclasts are multinucleated cells with a hematopoietic origin formed by fusion of mono nuclear cells mainly regulated by osteoblasts and stromal cells (Raggatt & Partridge, 2010).

Cell differentiation in general is regulated by complex interacting signaling pathways which eventually lead to altered gene expression and subsequent changes in cell behavior. The interaction between cell surface molecules and extracellular structures is one of the key mechanisms regulating the differentiation and activities of bone cells. Mesenchymal stem cells and stromal cells, including osteoblasts, as well as osteoclasts, are depending on attachment to extra cellular matrix (ECM) proteins. Cellular interactions with adhesion proteins/ECM proteins occur via transmembrane integrins receptors which recognize proteins containing the Arg-Gly-Asp (RGD) amino acid sequence. The RGD sequence can be found within proteins such as fibronectin and vitronectin which are components in ECM and interestingly also among the serum proteins which are adsorbed to implanted surfaces (Kundu & Putnam, 2006; Raggatt & Partridge, 2010; Wilson et al., 2005). Vitronectin is reported to promote osteogenic differentiation of mesenchymal stem cells via integrin-

mediated signals (Kundu & Putnam, 2006). The mitogen activated protein (MAP)-kinase cascade is an important downstream intracellular signaling transduction pathway in integrin-mediated signals. MAP kinase activation leads to extracellular-regulated kinase (ERK) activation via a pathway of downstream protein effectors ultimately regulating gene transcription and cell activities (Kundu & Putnam, 2006; Yee et al., 2008) (Illustrated in Fig. 1. based on: Geiger et al., 2009; Kundu & Putnam, 2006; Lu & Zreiqat, 2010; Milani et al., 2010; Raggatt & Partridge, 2010; Stevens & George, 2005; Wang et al., 2009; Wilson et al., 2005; Yee et al., 2008)

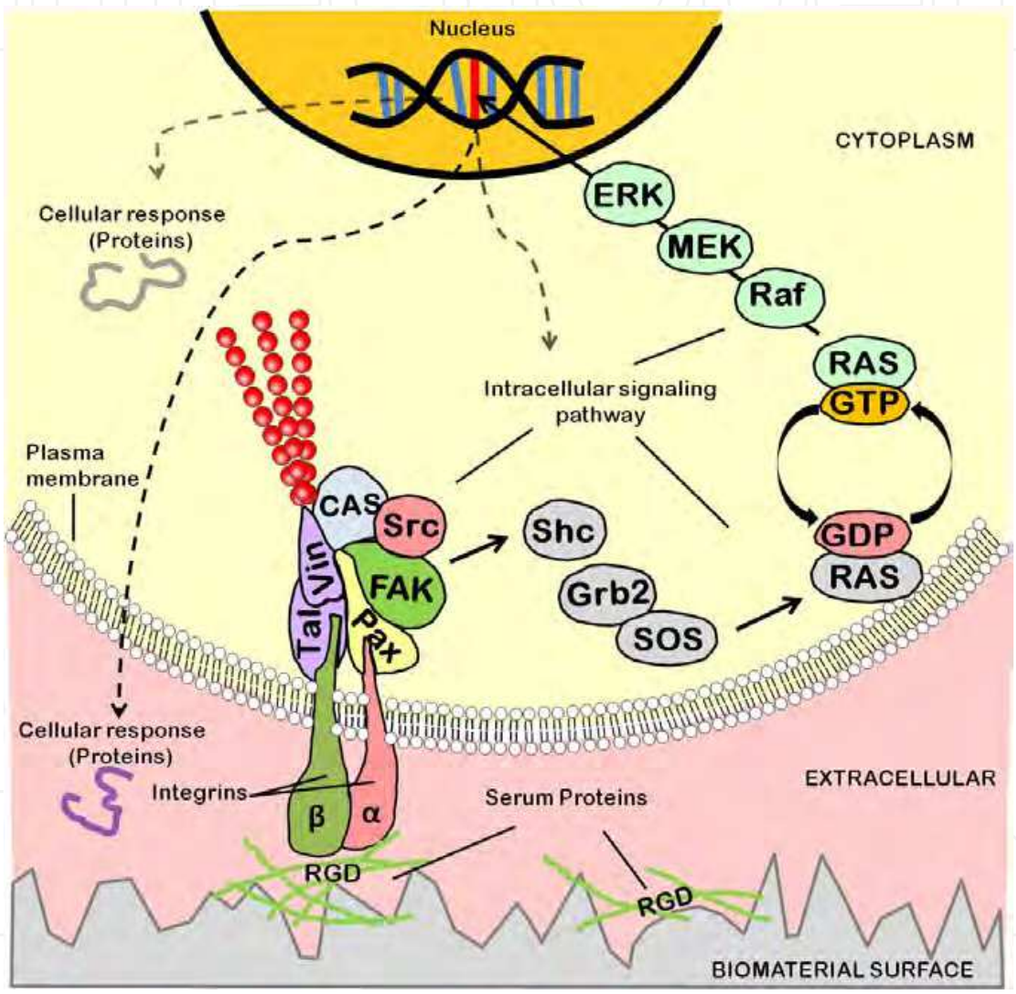

Fig. 1. RGD-integrin mediated cell attachment to biomaterial surface which leads to intra cellular signaling and change of cell functions. Figure was produced using Servier Medical Art.

It has also been proposed the inorganic components of bone could support the attachment and interactions between cells and bone (Feng, 2009). Moreover, it has recently been demonstrated that bone-specific extracellular matrix proteins could promote the osteogenic differentiation of embryonic stem cells (Evans et al., 2010). In addition to the control of cell activities by matrix proteins and signaling molecules, it is becoming evident that nanoscale

alterations in topography will influence osteoblast differentiation and formation of a mineralized matrix (Diniz et al., 2002; Stevens & George, 2005). The mechanisms by which biomaterials support bone formation are largely unknown but will certainly be determined by cell-surface interactions and the interface between the biomaterial and cells. Bone graft materials may regulate cell adhesion and functions through chemical and physical properties, dissolution and precipitation reactions, surface charge, protein adsorption and surface topography. A schematic illustration of the hypothetical time dependent development of the biomaterial-tissue interface leading to bone formation is presented in Fig. 2.

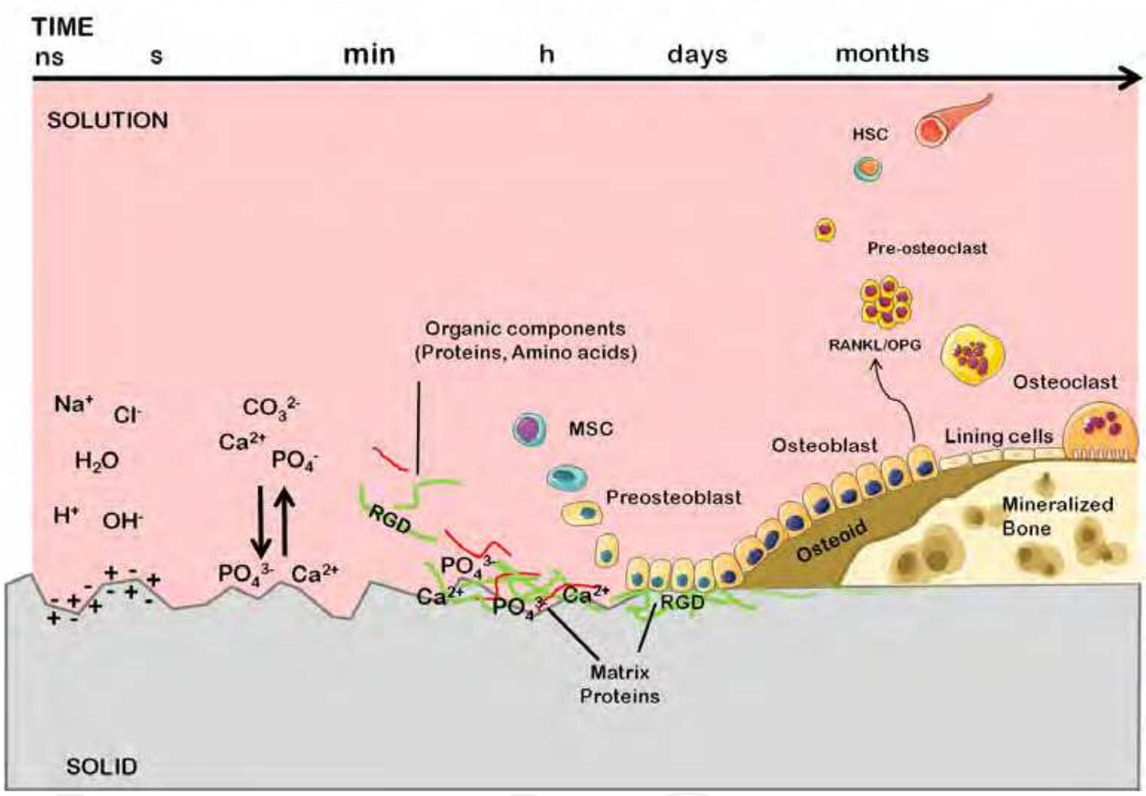

Fig. 2. Time dependent development of the biomaterial-tissue interface leading to bone formation. Figure was produced using Servier Medical Art.

When the bone graft substitute has been implanted in the patient, the biomaterial and surrounding tissue fluids will start interacting within nanoseconds (ns). After minutes (min) to hours (h), depending on material and solution properties, equilibrium will be reached, organic components will be attracted. The development of a biomaterial-tissue interface will be started and this interface will facilitate (pre)osteoblast attachment. A possible attachment mechanism could be the integrin-RGD sequence binding leading to activation of intracellular signaling and change of cellular functions. Activated and differentiated osteoblasts will then produce the bone matrix (osteoid), which needs to be mineralized to become bone. The mature mineralized bone will undergo bone remodeling involving both osteoclast and osteoblast activity in response to physiological changes in the environment. (Fig. 2 based on: Hamilton & Brunette, 2007; Hing, 2004; Hoppe et al., 2011; Jell & Stevens, 2006; Raggatt & Partridge, 2010; Roach et al., 2007; Shekaran & Garcia, 2011; Zambuzzi et al., 2011).

3. Bone graft substitute materials and biological media

Biomaterials have different origins and may be grouped into natural materials, metals, ceramics and polymers. Chemical and physical properties of the material might differ between the bulk and the surface. The material bulk will determine the mechanical and other physical characteristics, while the surface is responsible for the interaction with the surrounding environment. In the present study, three commercially available biomaterials (described below) were used as model systems to analyze the surface reactions which may promote cell adhesion and bone formation. The materials investigated have different origin and are already used in the clinic to repair dentoalveolar bone defects in patients. Frios® Algipore® (Algipore), is a biological fluoro-hydroxyapatite derived from calcifying marine algae. Bio-Oss® (Bio-Oss), is a deproteinized sterilized cancellous bovine bone chemically and structurally comparable to mineralized human bone. Bioactive glass 45S5 (45S5), a synthetic material, is a four-component glass. Bulk chemical composition and some important properties of these three bone graft substitutes are summarized in Table 1.

Biomaterial properties			
Biomaterial	Algipore	Bio-Oss	45S5
Particle size(μm)	500–1000	250–1000	70–700
Chemical composition	$CaCO_3$ transformed into $Ca_5(PO_4)_3OH_xF_{1-x}$†	n/a (deproteinized bone)	45wt.% SiO_2, 24.5wt.% CaO, 24.5wt.% Na_2O & 6wt. % P_2O_5 ‡
Surface area (m^2/g)	10.4	85.5	0.16
Pore Volume (cm^3/g)	0.06	0.42	0.0009
Average pore size (nm)	23.6	19.5	23.3

Table 1. Biomaterial properties for Algipore (†Ewers, 2005), Bio-Oss & Bioactive glass 45S5 (‡ Hench, 2006).

A great advantage is that histological studies in patients treated with these biomaterials have been published. It is clear from the clinical studies that new bone is formed around all three materials. However, the materials react very different when they are placed in patients. Algipore is resorbed and replaced by newly formed bone (Ewers, 2005), Bio-Oss is neither resorbed by osteoclasts nor degraded (it can still be seen after several years) but new bone is formed in close contact around the Bio-Oss particle (Mordenfeld et al., 2010). 45S5 glass particles corrode and bone formation seems to start inside the particle which is completely replaced by bone after approximately 16 months (Tadjoedin et al., 2000). The ability of these three materials to promote bone formation in vivo taken together with the different material properties provides the concept for our experimental investigation of their surfaces in equilibrium with biological medium. The formation of new bone at the biomaterial surface implies a reaction regulating bone cell functions at the interface. 45S5 has been analyzed in many studies but very little is known concerning the surface reactions and dissolution of the other two materials. We used a-MEM cell culture medium, instead of water, ethanol or simulated body fluid (SBF), which is common in several studies, because the composition of cell culture medium (Table 2) mimics the extra cellular tissue fluid more closely.

α-MEM Component		mM
Inorganic Salts		
Calcium Chloride	$CaCl_2 \cdot 2H_2O$	1.8
Magnesium Sulfate	$MgSO_4$ (anhyd.)	0.814
Potassium Chloride	KCl	5.33
Sodium Bicarbonate	$NaHCO_3$	26.19
Sodium Chloride (ionic medium)	NaCl	117.24
Sodium Phosphate monobasic	$NaH_2PO_4 \cdot 2H_2O$	1.01
Organic		
Amino Acids	21 Amino Acids (Main L-Glutamine)	8.503 (2)
Vitamins	11 Vitamins (Main Ascorbic Acid)	0.324 (0.284)
Other Components	4 Compounds (Main D-Glucose/Dextrose)	6.588 (5.56)

Table 2. α-MEM cell culture medium composition (Gibco®, obtained from Invitrogen. Cat.nr: 22561021).

If analyzing the materials surface reactions using a non-physiological solution, the results will not be relevant when relating to clinical reactions. Furthermore, using a-MEM for incubation of the materials gives us the possibility to use the incubation medium for cell culture experiments which otherwise is not possible.

Our first series of experiments were performed without addition of serum to the incubation medium which means that amino acids but no proteins are present (Mladenovic et al., 2010). In 1991, Kokubo et al., suggested that prediction of an artificial materials bioactivity (i.e. the ability of a material to form a bone-like hydroxyapatite on the surface) is essential and could be evaluated in vitro with simulated body fluid (SBF) (Kokubo & Takadama, 2006). This method has since then been widely used for assessment of bioactivity in vitro. Recently, the use of SBF for testing bioactivity has been questioned and suggestions have been made for improvement of the test in general (Bohner & Lemaitre, 2009). In line with this, Lee et al. (2011) suggested that cell culture medium would be a better option for bioactivity testing of biomaterials. In contrast to Lee et al. (2011) we believe that the presence of serum/proteins is essential when studying the interface and therefore in our second set of experiments we added 10% fetal bovine serum (FBS) to the cell culture medium.

Our two series of experiments correspond to the initial stages of bone regeneration process before cell attachment (Fig. 2.). What interfacial processes can experimentally be observed? What phenomena dominate at the interface? Why do cells find the interface attractive to initiate osteogenesis? We will try to answer some of these questions by applying one of the most powerful surface analysis techniques – X-ray photoelectron spectroscopy (XPS).

4. X-ray Photoelectron Spectroscopy (XPS) with fast-frozen samples

X-Ray Photoelectron Spectroscopy (XPS) is one of the most widely used principal techniques that probe the surface of materials (Ratner & Castner, 2009). The attractiveness of XPS is explained by its relatively simple theoretical background, applicability to virtually any type of samples, very high and sound information output, as well as sufficient availability of commercially manufactured laboratory spectrometers and experienced personal.

XPS experiment is based on the photoelectric effect. When X-rays (usually monochromatic) with known, well-defined, photon energy ($h\nu$) strike the sample, photoelectrons from the

constituting atoms are emitted. By measuring the kinetic energy (KE) of the emitted electrons, their binding energy (BE) can be calculated in accordance with the energy conservation law:

$$BE = h\nu - KE - \varphi \quad (1)$$

where φ is the spectrometer work function (constant for given instrument).
All electrons with binding energies less than the energy of the X-ray beam (e.g. mono Al K_α 1486.6 eV) are excited and can be counted. The binding energies for core level electrons (1s, 2s, 2p, 3s, 3p, 3d, etc.) are unique for each chemical element in the Periodic Table. Thus, the determination of surface elemental composition is straightforward from the BE values obtained by XPS. Typical XPS spectrum (Fig. 3.) is recorded as a plot of the number of detected electrons (counts per second) on the ordinate and their binding energy (electron-Volts, eV) on the abscissa.

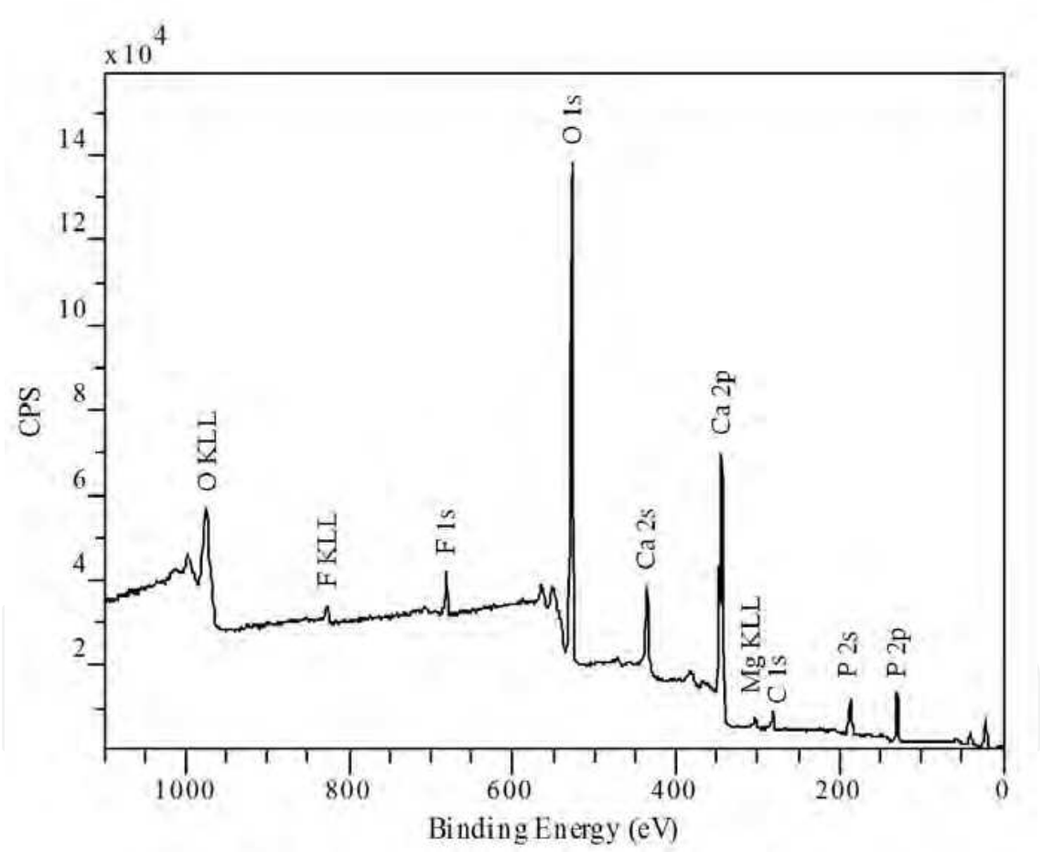

Fig. 3. Survey XPS spectrum of initial (dry) Algipore biomaterial.

X-rays penetrate deep into the sample (tens of microns) and generate photoelectrons over the entire penetration depth. Of interest in XPS are the electrons that do not lose their kinetic energy travelling through the sample. The escape depth of the photoelectrons from solids limits the analysis depth to 2 – 10 nm, providing very high surface sensitivity of the technique. The depth of analysis is dependent on the KE of the measured photoelectron, the sample density and the type of elements present. Typically, the depth of analysis is

determined to be 2-3 nm for metals, 3-6 nm for inorganic oxides, and 6-10 nm for organic compounds.

The key feature of XPS making the technique exclusively important for surface chemistry is a direct measurement of a chemical shift. Specific chemical information is obtained on the principle that the binding energies of core electrons of an atom are affected by the valence electrons. Shifts in binding energies of core levels, therefore, occur (and are routinely measured) due to changes in electron density around the atom of a sample. As a result, a change in oxidation state, ligand electronegativity, coordination, protonation, etc. can be immediate experimentally observed. To illustrate the chemical shift effect, high resolution C 1s spectrum showing different functional groups of α-MEM fast-frozen drop without serum is given in Fig. 4. Specific BE values, which have been determined experimentally for different chemical compounds, have been tabulated and can be easily found in handbooks (e.g. Handbook of X-ray Photoelectron Spectroscopy) and databases (e.g. NIST X-ray Photoelectron Spectroscopy Database & The XPS of Polymers Database (Beamson & Briggs, 2001)).

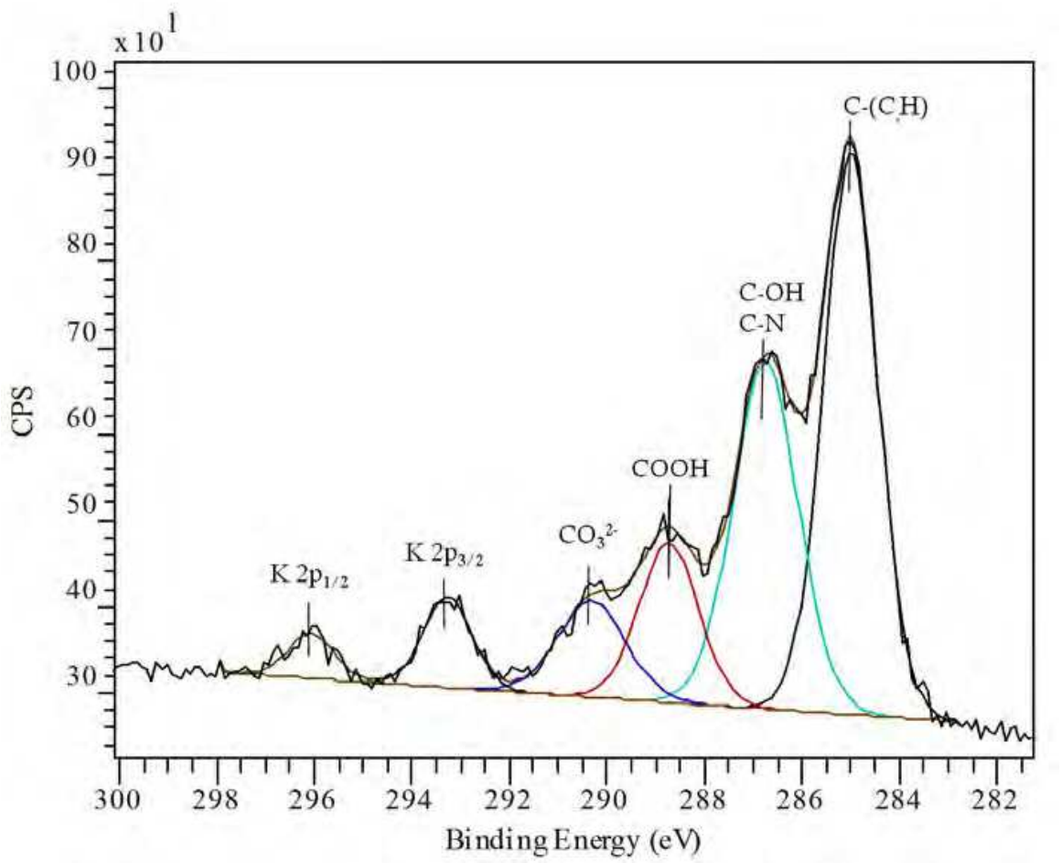

Fig. 4. XPS C 1s spectrum of a-MEM fast-frozen drop without serum.

Intensity of the photoelectron line is proportional to the numbers of atoms at the surface. An error of 10% is typically quoted for routinely determined XPS atomic concentrations using standard relative sensitivity factors. However, the intensities measured from similar samples are reproducible with good precision. Since many surface related problems

involve monitoring changes in samples, the precision of XPS makes the techniques quantitatively very powerful. To avoid uncertainties in absolute intensities measurements, atomic ratios calculated from the obtained atomic concentrations are implemented. It is the ratios, which directly relate to the formulas of chemical compounds, that are quantitative values in XPS. Analysis area can be varied in the range of tens μm^2 up to 1 cm^2, therefore small spot analysis and surface mapping are available. Analytical sensitivity of XPS is exceptionally high, and 10% of monolayer (or 0.1 atomic % concentration) being routinely detected and measured in reasonable time with acceptable signal-to-noise spectral ratio.

Modern achievements in XPS applications, including special sample treatment and handling, provide insight to intimate information about chemistry, vertical and lateral distribution of chemical species, and even topography and morphology of a surface. Detailed description of XPS principles, instrumentation, practice, and developing aspects can be found in "XPS Bible" (Briggs & Grant, 2003). Some biomedical (Castner & Ratner, 2002) and bioengineering (McArthur, 2006) applications of XPS were earlier reviewed.

In respect to real solid (biomaterial) - aqueous solution (biological media) interface, there has always been a major disadvantage in XPS that the technique cannot operate in ambient conditions. To detect the photoelectrons, ultra-high vacuum (UHV) is necessary. It is the reason why the technique is traditionally considered to be *ex situ*. Indeed, conventional XPS has very limited application to real wet samples which possess very high vapor pressure under ambient conditions and are not compatible with the UHV environment of electron spectrometer. However, inelastic scattering of photoelectrons on gas phase water molecules can be avoided in two general ways: (i) decrease the distance between the sample and detector or/and (ii) reduce the vapor pressure by lowering the sample temperature or/and reduce the size of evaporating surface. Experimental approaches to real solid-aqueous solution interfaces along these lines were shortly reviewed by Shchukarev (2006a; b), including recent developments for humid conditions (Salmeron & Schlögl, 2008).

Considering conventional laboratory electron spectrometers, only two approaches can be regarded as adequate sample preparation and handling methods which do not significantly alter the real interface. On the basis of well-known "freeze-drying" technological process, a deep freezing (*ex situ*) of hydrated samples followed by ice sublimation (*in situ*) was proposed (Ratner et al, 1978) and developed (Ratner, 1995) aimed towards the application for polymers used in biology and medicine. The freezing of hydrated polymer sample is performed at 160 or 113 K, *ex situ*. The frozen sample is then placed onto precooled sample holder in the spectrometer and pumped down to UHV. XPS spectra taken with the frozen polymer indicate only O 1s photoelectron line corresponding to the water (ice). The sample is then heated *in situ* up to 200 K until C 1s line from the polymer appears, with consecutive XPS measurements performed at 160 K. The final dehydration of the sample is carried out at 303 K, also *in situ*, followed by XPS investigation of dehydrated sample. In spite of important information obtained about the processes at polymer-water interface, the technique is only applicable to pure water or aqueous solutions of very low concentrations. Ice sublimation from natural frozen biological media will "precipitate" all non-volatile solution components, like inorganic salts and organic compounds, thus altering the interface and its real chemical composition. Taken into account the analysis depth of XPS, it is very realistic that only solution components will be seen in the spectra. Moreover, the "freeze-drying" technique

has not been widely used because most commercial laboratory instruments are not equipped with accessories necessary to perform controlled ice sublimation, and the experiment is complicated and time consuming (Lukas et al, 1995).

The simplest technique to study aqueous solutions in the form of solid transparent ice was proposed in 1969 (Kramer & Klein, 1969), and developed during 1970's by Burger's group (Burger & Fluck, 1974; Burger et al, 1975; Burger, 1978; Burger et al, 1977). A fast-frozen sample was prepared by direct injection of solution drop inside the spectrometer onto "cold finger" holder precooled by liquid nitrogen. XPS spectra were then acquired also at liquid nitrogen temperature. Freezing rate is so high (15-20 ^{o}C/s) that a frozen drop coming from a pipette nozzle often has a shape of ball or even onion. It is extremely important for the technique that fast-freezing has been proven to preserve the chemical speciation of solutes, and prevents solutions from crystallization.

Recently, the fast-freezing procedure was modified and applied to study the mineral- (Shchukarev & Sjöberg, 2005; Shchukarev, 2006b), bacteria- (Leone et al, 2006; Ramstedt et al, 2011), and biomaterial- (Mladenović et al, 2010) aqueous solution interfaces. Instead of a solution drop, a wet paste (alternatively gel, sediment etc) is applied to the sample holder, usually outside the spectrometer and at room temperature (T). The wet paste is obtained by centrifuging from diluted equilibrated powder (bacterial, colloidal) suspension. If the suspension is sensitive to oxygen, a glove box attached to the entry lock of spectrometer can be easily used. After applying the wet paste, the sample holder is immediately placed onto precooled claw (-170 ^{o}C) of sample transfer rod in the spectrometer entry lock. After a few seconds, the paste becomes visibly frozen and is kept at atmospheric pressure of dry nitrogen for another 30-45 s to be sure that it is cold enough to prevent water losses during the pumping. The pumping and XPS measurements are performed under liquid nitrogen cooling. To investigate the changes of the interface due to water loss, a fast-frozen sample can be left at the analysis position inside the spectrometer overnight without cooling. Slow increase in the sample temperature up to room T causes sublimation of water and other possible volatile species into the vacuum system of the spectrometer. Corresponding changes in composition and chemical speciation at the "dry" surface are monitored and quantified next day by routine XPS measurements.

Cryogenic XPS with fast-frozen samples was shown to be indispensable in the determination of electrolyte interface concentrations, their pH and ionic strength dependences, and particularly the particles surface charge using the atomic ratio of electrolyte counter-ions. As an example, the atomic ratio of Na^{+} and Cl^{-} (in the case of NaCl ionic media) at the interface of neutral particles is equal to one. A significant excess of sodium (chloride) ions is observed for negatively (positively) charged surfaces. Practically important for bioceramics, XPS determination of the surface charge is suitable for large particles where traditional zeta-potential measurements are impossible to perform due to immediate sedimentation of the suspension. Finally, XPS measurements repeated the next day at room temperature, with the same sample, have provided even more direct experimental evidences for the important interfacial phenomena, such as specific adsorption, ion pair formation, protonation of amine group as well as remarkable interfacial shifts in protonation constants (Ramstedt et al, 2004; Shimizu et al, 2011). In addition, pH dependent interface ligand exchange reaction, $M\text{-}OH + Cl^{-} \leftrightarrow M\text{-}Cl + OH^{-}$, was found and described; a surface potential drop due to collapse of electrical double layer was observed and measured; and the thickness of interface solution layer about 5 Å was experimentally estimated.

Cryogenic XPS with fast-frozen samples, as a "snapshot" technique, is very promising in describing even dynamic processes at the interface of biomaterial in biological media by changing contact time or/and one of solution parameters (T, concentration, pH, composition, etc.). In this fashion, XPS was used to study dry surfaces of three biomaterials described in previous section as well as their wet pastes obtained from α-MEM solution with and without serum addition. In parallel with XPS measurements, the supernatant is also analyzed, and both sets of experimental data are combined together to obtain chemically self-consistent insights into the phenomena at the interface of bone graft substitute (45S5, Algipore and Bio-Oss) materials in biological (α-MEM) media.

5. Three bone graft material interfaces in biological media

Formation of new bone in close proximity of implanted particles, described in section 3 and shown in Fig 5, indicates that bone formation process is facilitated by the surface of the biomaterial. In the case of a bone graft substitute, it occurs at the interface of the biomaterial which is in equilibrium with surrounding body fluid.

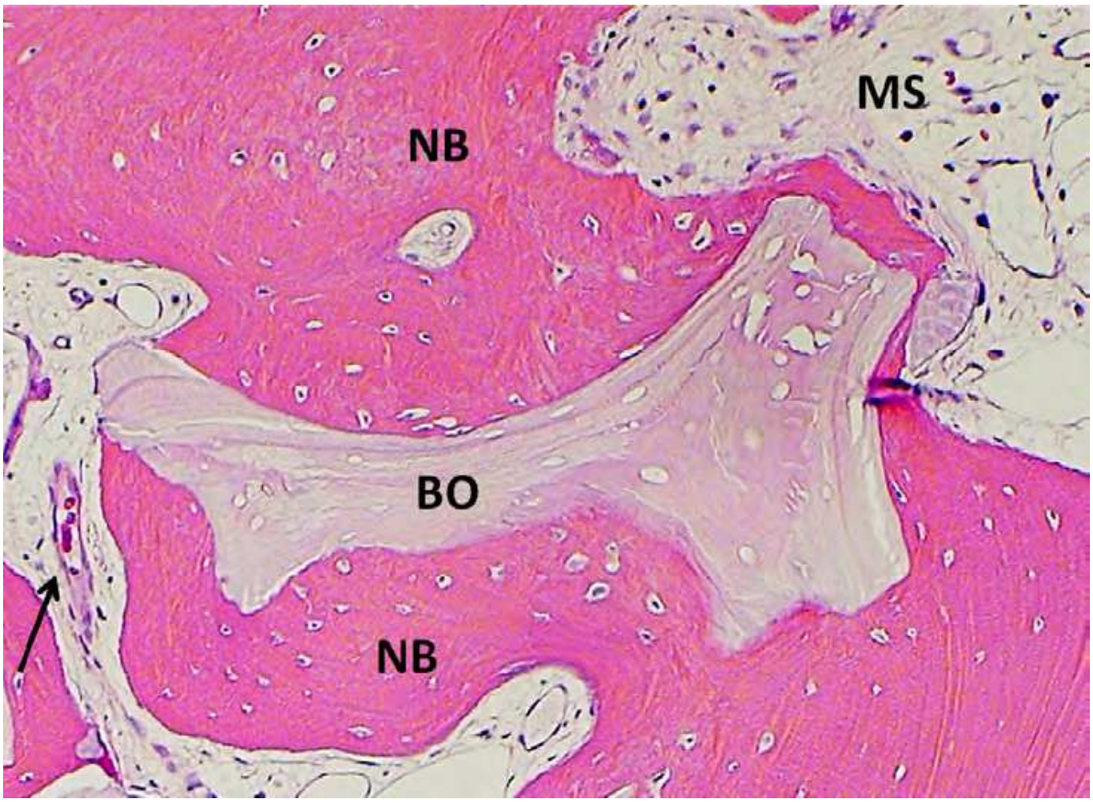

Fig. 5. Light microphotograph of biopsy taken from patient at implant installation, 12 months after bone augmentation with Bio-Oss (without addition of autogenous bone). The histological section shows newly formed bone (NB) in close relation to the Bio-Oss (BO) interface. Marrow space (MS) surrounding NB and BO indicates bone formation growing "out" from the bone graft substitute. Black arrow (↑) points out a blood vesicle. Htx-Eosin staining, original magnification 10x. (Image courtesy of Dr A. Sahlin-Platt).

The equilibrium, in turn, is achieved in the process of initial (dry) particle surface interaction with water and other components of the biological medium. Reference XPS data on initial surface chemical composition of all three bone graft substitutes and both of the biological media (as a fast-frozen drops) are given in Table 3.

Surface composition (Atomic %) of initial biomaterials and α-MEM biological media					
	α-MEM	α-MEM + serum	Algipore	Bio-Oss	45S5
Photoelectron line					
C 1s					
Organic	14.72	33.88	6.79	8.39	12.3
Surface carbonate	0.92	1.67	0.82	1.61	16.78
O 1s	70.48	47.03	56.54	57.66	49.27
N 1s	1.94	7.76	-	-	-
Ca 2p	-	-	19.87	19.49	2.91
P 2p	-	-	12.91	11.59	0.25
Si 2p	-	-	-	-	2.23
F 1s	-	-	2.92	0.82	-
K 2p	0.24	0.33	-	-	-
Na 1s	5.8	4.77	0.61	0.45	16.27
Cl 2p	5.91	4.57	-	-	-

Table 3. Surface composition of initial Algipore, Bio-Oss, Bioactive glass 45S5 and α-MEM biological media.

The chemical compositions of the surface of Bio-Oss and Algipore are very similar to each other, only differing in fluorine content. Except for surface organic contaminations, their surface chemistry is in good agreement with bulk apatite formulation in respect to Ca/P atomic ratio. Conversely, the surface of Bioactive glass particles is covered by sodium bicarbonate (Mladenovic et al., 2010), probably due to increased surface reactivity and prolonged storage of the material in air. Upon the interaction with biological medium, this water soluble surface $NaHCO_3$ phase will dissolve, enriching the medium by additional sodium and carbonate ions and probably causing a momentarily change in the local pH. Due to very low surface area of 45S5 Bioactive glass (Table 1) and the remarkable buffer capacity of the medium with a pH of 7.3, the α-MEM composition will not be noticeably influenced. XPS data, taken from fast-frozen drops of both α-MEM solutions (Table 3), demonstrates a predominance of sodium and chloride in the inorganic part of the medium. It is important to notice that the Na/Cl atomic ratio equal to one (Na/Cl = 5.8/5.91 for pure α-MEM, and 4.77/4.57 for α-MEM with serum). This would indicate a neutral (non-charged) surface of the fast-frozen drops. The amino acids are the dominating contributors to the organic part of pure α-MEM as evident by N 1s spectrum (Fig. 6, a), showing both protonated and neutral amino groups with BE of 402.1 and 400.2 eV, respectively. Serum addition to the biological medium results in the dominance of protein which can be clearly

seen by the increased carbon and nitrogen concentrations (Table 3), and also evidenced by the N 1s spectrum (Fig 6, b) being similar to the one observed for bacterial cell walls (Leone et al., 2006).

XPS investigation of fast-frozen biomaterial samples, conditioned in original α-MEM medium (without serum), shows that equilibrium is reached at the interface within only 1 day (Mladenovic, et al., 2010). Corresponding interfacial chemical compositions are given in Table 4.

Surface composition (Atomic %) of biomaterials in original α-MEM after 1 day of equilibration				
Photoelectron line	α-MEM	Algipore	Bio-Oss	45S5
C 1s				
Organic	14.72	35.99	8.33	43.05
Surface carbonate	0.92	2.6	1.5	2.11
O 1s	70.48	34.82	68.56	38.96
N 1s	1.94	3.38	-	2.79
Ca 2p	-	5.7	9.74	0.58
P 2p	-	4.69	7.02	0.16
Si 2p	-	-	-	0.17
F 1s	-	-	-	-
K 2p	0.24	0.46	0.06	0.34
Na 1s	5.8	5.62	3.08	5.04
Cl 2p	5.91	6.76	1.71	6.8
Atomic ratio Na/Cl	0.98 : 1	0.83 : 1	1.8 : 1	0.74 : 1

Table 4. Surface composition of biomaterials in α-MEM after 1 day of equilibration.

The Bio-Oss - α-MEM medium interface does not show any adsorption of organic components from the biological medium therefore similar in composition to the initial (dry) surface, except for the contributions from water and Na^+ and Cl^- ions from the solution. An excess of sodium over chloride, providing the atomic ratio Na/Cl 1.8:1, indicates negative charge of the Bio-Oss particles' surface. Assuming the Bio-Oss is similar to hydroxyapatite (HAP), the isoelectric point of the biomaterial can be expected to have a pH value close to 8.1 (Bengtsson, et al., 2009). Therefore, the surface in α-MEM solution has to be substantially neutral with the atomic ratio Na/Cl close to one. The observed excess of sodium ions may indicate a re-adsorption of the phosphate-related solution species, like HPO_4^{2-}, producing outer-sphere coordination sites for the Na^+. Such occurrences were confirmed for synthetic HAP by the solution analysis and surface complexation modeling (Bengtsson, et al., 2009). Additional adsorption of hydro phosphate ions is indirectly observed by a decrease in the atomic ratio Ca/P at the Bio-Oss - α-MEM medium interface, compared to that at the dry surface.

The interfacial composition of both Algipore and 45S5 is dominated by water, sodium and chloride, and adsorbed organic molecules. The amino acids are the key players in the adsorbed layer, as evident by N 1s spectra (Fig. 6, c and 7, c) which similar to that of the

pure α-MEM (Fig. 6, a, and 7, a). As it was noted by Mladenovic et al. (2010), the adsorption of amino acids on the surface of Algipore and 45S5 leads to a partial deprotonation of $–NH_2^+$ group, which can be clearly seen by a decrease in intensity of the N 1s component with BE 402.1 eV. Amine group deprotonation would mean an increased pH value at the interface, compared to that in the solution. The influence of the biomaterial surface on the acid-base equilibria at the interface can be important for the expected attachement of cells. In particular, an increase in interfacial pH from physiological value of 7.4 to 8 significantly enhances both the proliferation and the alkaline phosphatase activity of osteoblasts (Shen, et al., 2011; Liu et al., 2011).

The atomic ratio Na/Cl is lower than 1, and this clearly indicates that the surface of both the Algipore and the Bioglass is positively charged. The positive charge seems to be related to the pronounced adsorption of organic species from the α-MEM solution. Unfortunately, it is difficult to distinguish whether the generation of the surface charge is followed by the formation of electrical double layer, which precedes the adsorption, or vice versa. Since no adsorption occurred at the negatively charged surface of Bio-Oss, the surface charge generation seems to be a first principal phenomenon responsible for the formation of interface's organic (mainly amino acids) layer with specific acid-base properties. We have to emphasize that, had the samples been suspended in SBF, this adsorption would never have been detected, as was shown by Mahmood & Davies, 2000 for the Bioactive glass 45S5 material.

Serum addition to the α-MEM causes significant changes in the interfaces discussed above. Interfacial chemical composition (Table 5) and XPS N 1s spectra, typical for serum (Fig. 6 - 8), demonstrate an immediate adsorption of the protein at the surface of all three biomaterials after only 1 day of equilibration.

Surface composition (Atomic %) of biomaterials in α-MEM with 10% serum after 1 day of equilibration				
Photoelectron line	α-MEM + serum	Algipore	Bio-Oss	45S5
C 1s				
Organic	33.88	52.04	51.0	55.74
Surface carbonate	1.67	2.0	-	2.13
O 1s	47.03	29.42	30.0	26.68
N 1s	7.76	7.17	9.62	5.37
Ca 2p	-	0.73	3.03	0.44
P 2p	-	0.7	3.54	0.21
Si 2p	-	-	-	0.28
F 1s	-	-	-	-
K 2p	0.33	0.32	-	0.29
Na 1s	4.77	2.5	0.6	3.08
Cl 2p	4.57	5.09	0.74	5.76
Atomic ratio Na/Cl	1.04 : 1	0.49 : 1	0.81 : 1	0.53 : 1

Table 5. Surface composition of biomaterials in α-MEM with 10% serum after 1 day of equilibration.

The sum of the atomic concentrations of organic carbon and nitrogen is about 60 atomic %, independent on the bone graft substitute. However, the atomic ratio C/N, calculated for Bio-Oss (5.3:1), is very close to one for the serum-containing α-MEM (4.4:1, Table 5). This may indicate that solution structure of the protein is not noticeably altered upon adsorption. Significant increase in the C/N ratio observed for Algipore (7.3:1) and 45S5 (10.4:1) would mean possible changes in secondary protein structure resulting in the preferential orientation of nitrogen-containing functional groups towards the biomaterial surface and the carbon-enriched polymeric chains towards the solution. The adsorption-induced orientation effect correlates with the different surface charge gained by the biomaterial particles in the original α-MEM. It is important to notice that the adsorption of serum at the surface of Bio-Oss unexpectedly causes the charge reversal at the interface and thereby the positive charge of the particles, as evidenced by the change in the atomic ratio Na/Cl (1.8 → 0.81, Tables 4 and 5). The protein layer formed at the interface could serve as a template for the recruitment of cells, production of bone extracellular matrix, biomineralization, and finally, as an initiator of osteogenesis.

Using cryogenic XPS with fast-frozen samples, two dominating phenomena common for all three biomaterials can clearly be distinguished at the interface with biological media. In the absent of proteins (serum), the particles gain different surface charge. It is the charge that seems to be pivotal for the adsorption of organic molecules from the medium. Significant adsorption of organic species, in particular amino acids, at the positively charged surfaces of Bioactive glass 45S5 and Algipore (completely different in the bulk and surface chemical composition) results in the formation of a thin organic interface layer. Chemically and structurally similar to Algipore, Bio-Oss particles in α-MEM gain negative surface charge which suppresses the adsorption. Even protonated $-NH_3^+$ end members of amino acids are not attracted by the negatively charged surface. An exception to the rule is protein adsorption. Serum adsorbs immediately at the surface of all three biomaterials, independent of their surface charge. Moreover, the protein adsorption at the surface of Bio-Oss results in the charge reversal at the interface and the positive charge of the particles. Serum adsorption cannot be explained by pure electrostatics; an underlying mechanism should include weak hydrophobic/hydrophilic interactions, hydrogen bonding, and change in the protein primary and secondary structures. The protein adsorption at the abiotic materials, independent on their surface charge and chemical composition, seems to be major prerequisite for subsequent cell recognition and biomineralization.

Besides the template formation for bone regeneration, fast adsorption of organic molecules and serum can serve as a dissolution/precipitation barrier, controlling the biomaterial degradation and resorbing processes. In this respect, time dependence of the Ca/P atomic ratios, measured in biological media and at the interface, can indicate the extent of bone graft substitute dissolution and re-adsorption of calcium and phosphate ions. We have monitored the Ca/P atomic ratio in pure α-MEM (Mladenovic, et al., 2010). Since the availability of Ca and P from the medium in our batch experiments is limited, compared to circulating natural body fluids, the results might not be relevant and therefore are not discussed here. Regardless, biomaterial degradation and biomineralization are long-term processes and can be considered as an effect of "secondary order" at the first stages of biomaterial – biological medium interface evolution.

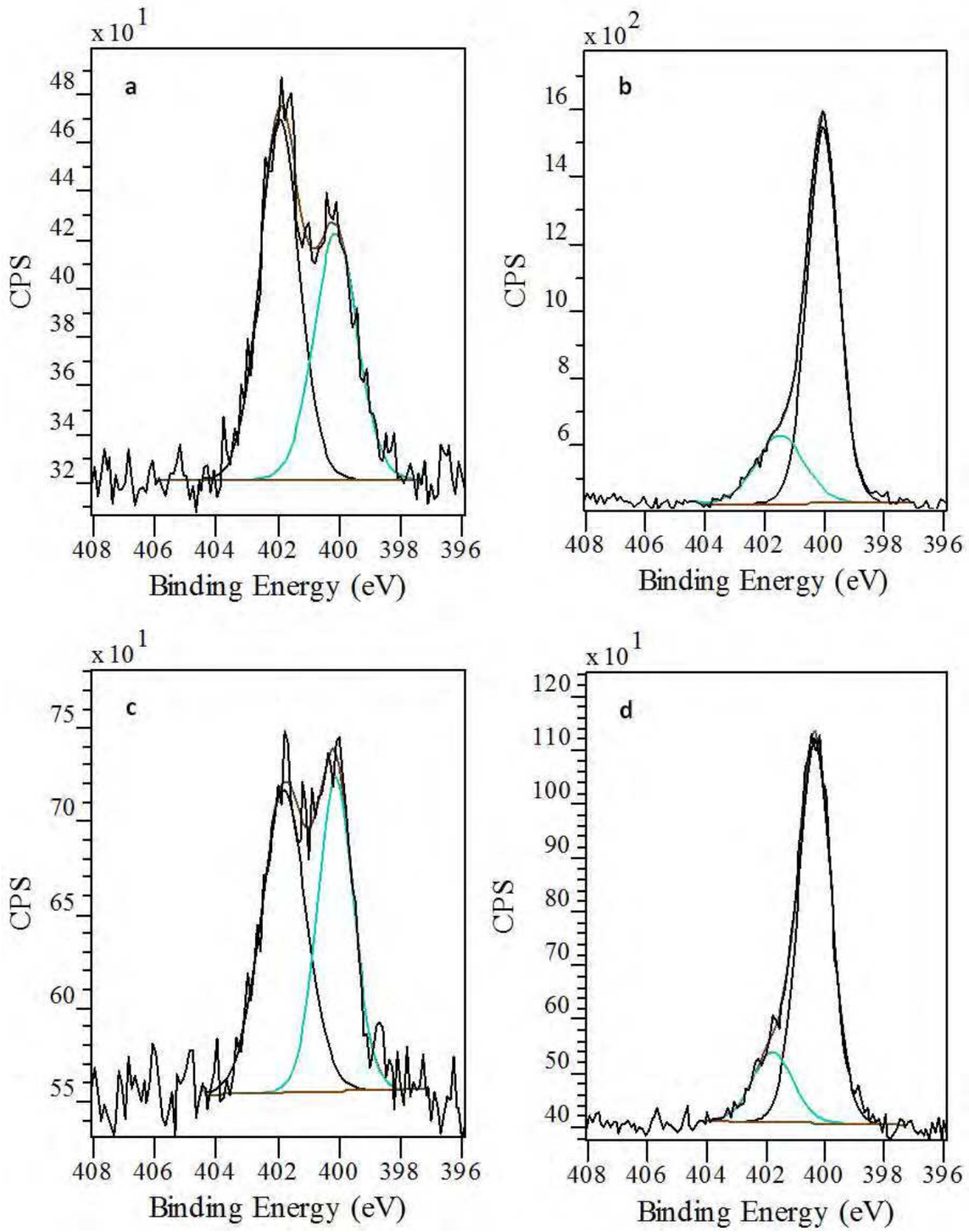

Fig. 6. N 1s spectra of fast-frozen samples: (a) a-MEM without serum, (b) a-MEM with serum, (c) Algipore in a-MEM without serum (1 day), (d) Algipore in a-MEM with serum (1 day).

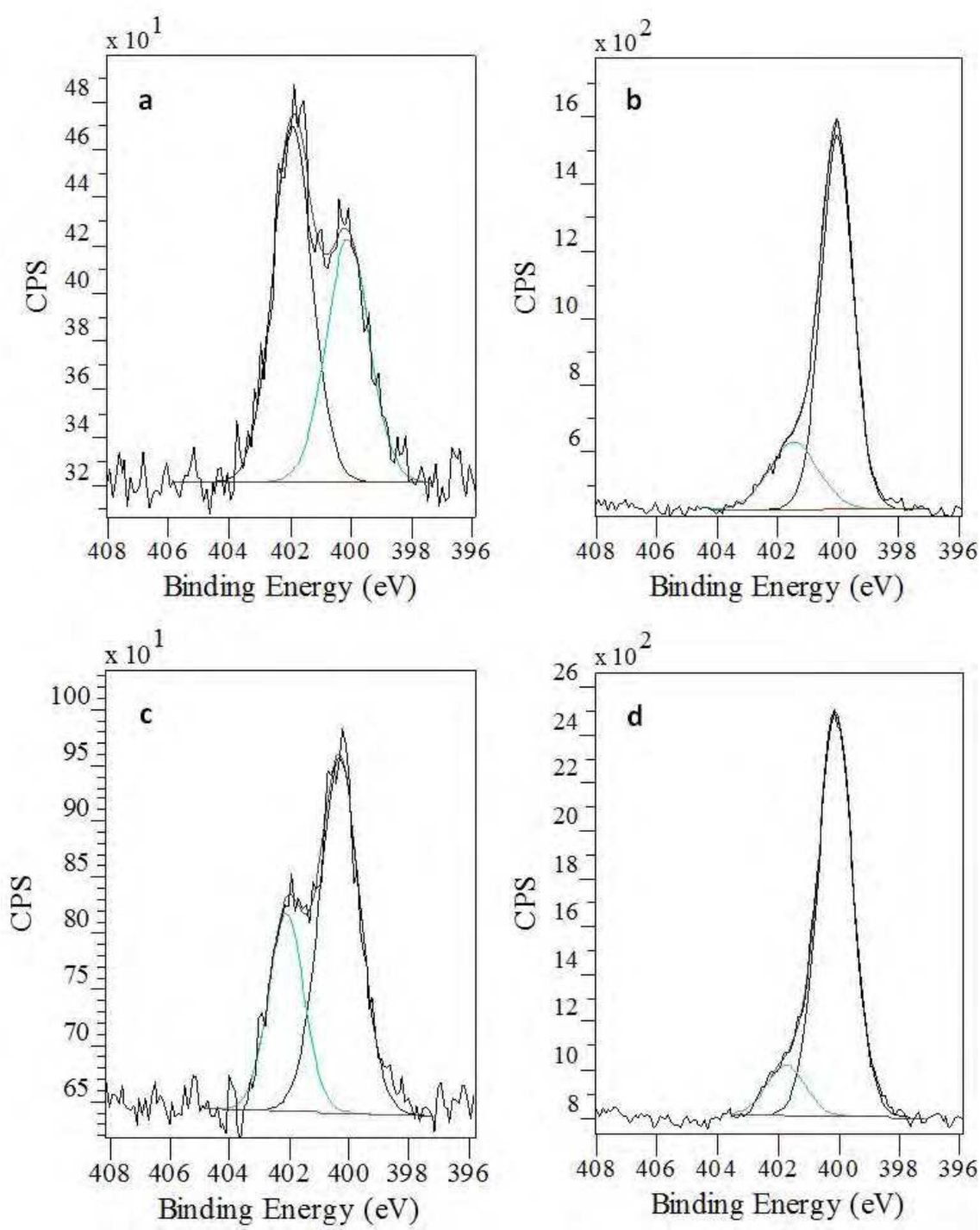

Fig. 7. N 1s spectra of fast-frozen samples: (a) a-MEM without serum, (b) a-MEM with serum, (c) Bioactive glass 45S5 in a-MEM without serum (1 day), (d) Bioactive glass 45S5 in a-MEM with serum (1 day).

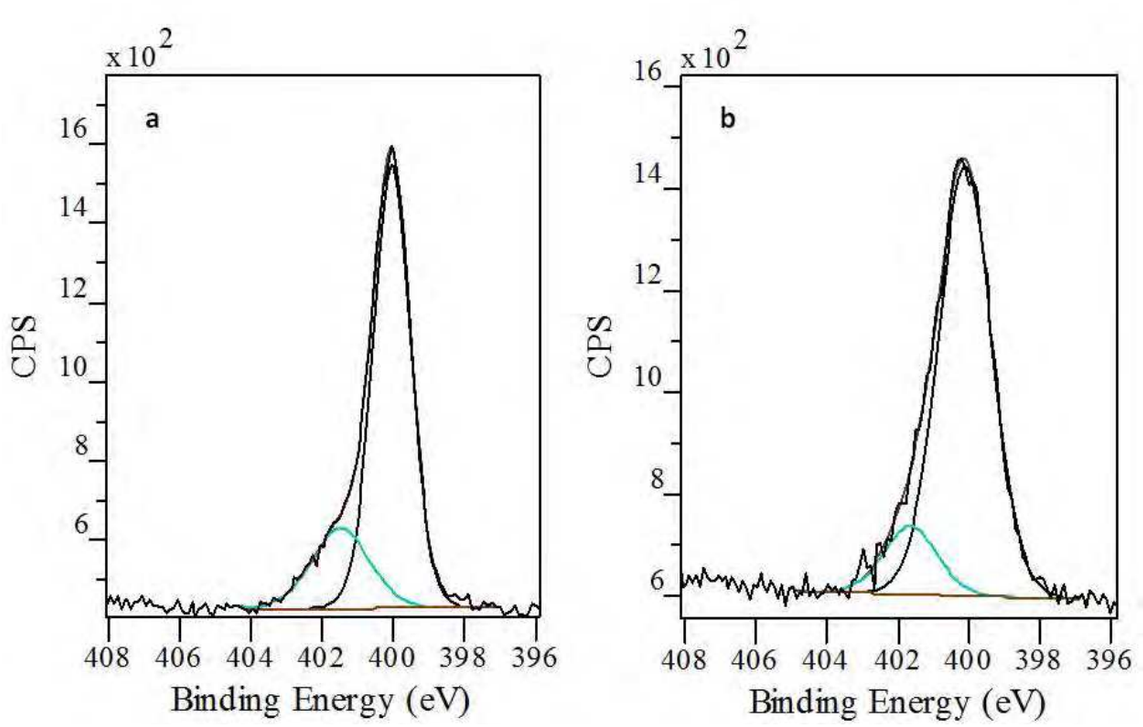

Fig. 8. N 1s spectra of fast-frozen samples: (a) a-MEM with serum, (b) Bio-Oss in a-MEM with serum (1 day).

6. Conclusion

From the cells viewpoint, it is the interface that will determine "to build or not to build" bone. In this respect, cryogenic XPS demonstrates the great potential of fast-freezing technique to study the real biomaterial interface in cell culture medium. Physico-chemical information about the interface, important for cell adherence, such as chemical composition, surface charge, and absorbing molecules can directly be obtained. The next step in this direction would be to acquire nanoscale information on surface structures and adsorption of specific proteins at the biomaterial interface that could have an effect on cellular function. Surface science techniques like Atomic Force Microscopy (*in vivo* and *in vitro*) and Mass Spectrometry (*in vitro*) can be laterally applied to investigate areas occupied by a single cell. The cell surface can be studied in the same manner to elucidate membrane functions responsible for the cell attachment.

The use of bone graft substitutes in clinical practices is still limited by insufficient knowledge of biomaterial interactions with the living tissue. This makes the treatment outcome unpredictable or even unreliable. To improve the knowledge, the great scientific potential existing in biology and material science must be combined thus creating important "interface" between respective scientists. The interactions within this "interface" can result in design of new cost effective biomaterials with improved tissue regenerative potential.

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These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentials of different synthetic and engineered biomaterials. Contributions were not selected based on a direct market or clinical interest, than on results coming from very fundamental studies which have been mainly gathered for this book. This fact will also allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessarily imposed by industrial or profit concerns. The book collects 22 chapters related to recent researches on new materials, particularly dealing with their potential and different applications in biomedicine and clinics: from tissue engineering to polymeric scaffolds, from bone mimetic products to prostheses, up to strategies to manage their interaction with living cells.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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