Tunable hydroxyapatite wettability: Effect on adhesion of biological molecules

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Abstract

Surface wettability modifications of biocompatible materials and wettability patterning are attractive methods for directed biological cells immobilization for tissue engineering, drug delivery, gene transfer, etc. Hydroxyapatite is known as an implantable biomimetic material and a substrate for effective adhesion of biological cells of various origins. Here we report the use of a low-energy electron irradiation to achieve tunable wettability of the hydroxyapatite in a wide range of contact angles, from 10° to 100°, with accuracy of ±3°. The incident electrons generate electron/hole pairs resulting in significant variation of the surface potential of the hydroxyapatite semiconductor and give rise to pronounced wettability modification. Tailoring the gradually varied wettability state in the hydroxyapatite nanoceramics enabled the differential binding of biological materials with different surface properties, such as bovine serum albumin (BSA) and deoxyribonucleic acid (DNA).

Keywords: Surface modification; Wettability; Hydroxyapatite; Adhesion molecule

1. Introduction

Controlling solid surfaces wettability is intensively studied for understanding fine mechanisms of biological cell integration and development of biomimetic materials for tissue engineering, drug and gene delivery, etc. Hydroxyapatite is known as an implantable biomimetic material and a substrate for effective adhesion of biological cells of various origins. Here we report the use of a low-energy electron irradiation to achieve tunable wettability of the hydroxyapatite in a wide range of contact angles, from 10° to 100°, with accuracy of ±3°. The incident electrons generate electron/hole pairs resulting in significant variation of the surface potential of the hydroxyapatite semiconductor and give rise to pronounced wettability modification. Tailoring the gradually varied wettability state in the hydroxyapatite nanoceramics enabled the differential binding of biological materials with different surface properties, such as bovine serum albumin (BSA) and deoxyribonucleic acid (DNA).

Among the various biocompatible substrate materials, hydroxyapatite (HAp) is widely used as an implantable bioactive agent that replaces defective bone tissues and also serves as a substrate for effective adhesion of various biological cells. Wettability plays the critical role for all these uses [13,14].

Recently, it has been possible to change surface wettability by the use of diverse techniques on the basis of extrinsic surface modification including deposition of self-assembled monolayers (SAMs) [2,3], electrical [5], light-induced [6] and electrochemical methods [7], as well by changing the environmental conditions with various solvents [8], temperature [9], pH [10] and surface pressure [11]. Among the surface modification methods, the formation of SAMs proved to be a simple and practical technique for controlling wettability [2,6] and biological adhesion [3,12]. However, the aforementioned techniques are accompanied by either surface chemical reactions or form foreign molecules structure on the modified surfaces.

Among the various biocompatible substrate materials, hydroxyapatite (HAp) is widely used as an implantable bioactive agent that replaces defective bone tissues and also serves as a substrate for effective adhesion of various biological cells. Wettability plays the critical role for all these uses [13,14].

Here we present an alternative approach for gradual tuning and on/off switching of the surface wettability of the HAp, induced by a low-electron energy irradiation [15]. The electron/hole charges, generated by the electron beam, are trapped in the vicinity of the HAp surface, and resulting in significant wettability tuning without applying external electric field. This method allows the fabrication of homogenous distribution or patterning of the wettability state on the HAp surface. The differential immobilization of biological materials, such as bovine serum albumin (BSA) and DNA on the wettability-engineered HAp ceramics surface is demonstrated.
2. Materials and methods

The HAp nanopowder was fabricated by several steps using fine mechanical treatment and chemical reactions, $3\text{Ca(HPO}_4\text{)}_2\cdot\text{H}_2\text{O} + 7\text{Ca(OH)}_2 = \text{Ca}_{10}\text{(PO}_4\text{)}_6\text{(OH)}_2 + 15\text{H}_2\text{O}$. Mechanical activation was performed under air environment in a planetary mill containing two steel drums and steel balls. The nanocomposite particles were tested by means of X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FT-IR) and the specific surface analysis. Sizes of particles were defined by means of transmission electron microscopy. The obtained data shows, that all probes are enough crystallized HAp in the form of nanoparticles with sizes in range from 20 up to 100 nm. The HAp particles of typical 40 nm size were extracted from nanopowder mass and used as a raw material for preparation of the ceramic samples. The platelet-like samples (height of 2 mm; diameter of 5 mm) were fabricated using dry pressing HAp powders. A press form greased by rape seed oil was used for two-stage compaction. Pressure of 250 and 375 MPa during the first stage and second one was applied. After pressing the resulting ceramic bodies were sintered with heating rate of $5\, ^\circ\text{C}/\text{min}$ to $1100\, ^\circ\text{C}$ annealing at that temperature for 60 min. Sintered platelets were cooled down to room temperature within an oven. The XRD analysis and FT-IR spectra of the synthesized samples show the good formed crystalline structure of the HAp. A typical formula of the HAp is $\text{Ca}_{10}\text{(PO}_4\text{)}_6\text{(OH)}_2$, where $X$ ranges from 0 to 2, giving a Ca/P atomic ratio of between 1.33 ($X=2$) and 1.67 ($X=0$) [16]. The composition and atomic concentrations of the elements contained in the investigated HAp ceramics were determined from X-ray photoelectron spectroscopy (XPS) measurements. The Ca/P molar ratio obtained from atomic concentration measurements was found to be 1.66, which is the evidence that the fabricated HAp ceramics is close to the stoichiometric composition.

Topography features were studied by atomic force microscopy (AFM) (Multimode, Digital Instruments, USA) in tapping mode. Additionally, the HAp samples were imaged by conventional scanning electron microscopy (SEM) using a Raith 150 ultra high-resolution e-beam tool (Raith, GmbH Germany). The roughness and the porosity analysis were performed using the scanning probe microscopy software.

The low-energy electron irradiation was used to achieve tunable wettability of the HAp in a wide range of contact angles. The electron charging of the studied HAp samples was performed using a commercially available electron gun (EFPG-7, Kimball Physics Inc., USA) in vacuum 10$^{-7}$ Torr with constant excitation energy of $E_e = 100$ eV and electron current density of $J_e = 100$ nA/cm$^2$. The exposure time was varied in the range of $t = 0-3000\, \text{s}$ in accordance to desire surface wettability state.

The HAp surface properties modifications were detected by measurements of a surface electric potential and wettability variation. The XPS and AFM were used to control electrochemical and topographical surface modification induced by a low-energy electron beam. The surface electric potential distribution was studied by the Kelvin probe force microscopy (KPFM) [17]. The method is based on measuring the contact potential difference between a reference electrode with invariable work function (Si-nanometer-size tip with Ti–Pt coating) and a studied sample surface, using modified AFM. The instrument has a high resolution for both the surface potential, $\Delta \Phi$ (better than 5 mV) and the lateral dimension ($<30\, \text{nm}$) and allows the simultaneous imaging of topography and surface potential difference.

The surface hydrophobicity was studied by measuring contact angles with a sessile drop of distilled water deposited on a sample surface. The optical wettability inspection was performed by an inspection microscope (Olympus MX-50, Opelco, USA) combining charge-coupled device camera and digital imaging techniques. The measured contact angle characterizes not only the intrinsic wettability of a solid surface, but also reflects the contribution of the surface roughness and chemical heterogeneity of the observed wettability state [18]. The tilting plate technique measures the left and right sides of a drop when the surface under study is tilted with respect to the optical axis. Therefore, fluid accumulates in the leading (advancing) edge of the drop and drains from the trailing (receding) edge. The difference between advancing receding angles are attached with the drop is tilted to “the point of incipient motion” and the drop is free to move. The hysteresis effect may be absent or negligible in some systems, but in most systems, it is appreciable [19]. The volume of the liquid was kept constant ($2\, \mu\text{l}$) all over the contact angle measurements of different specimens. The wettability investigations were carried out with an accuracy of $\pm1^\circ$ at a temperature of $26\pm1\, ^\circ\text{C}$ and a humidity of $45\pm5\%\, \text{RH}$.

Biological materials with different surface properties, such as BSA and DNA, were used to confirm the wettability modification of the treated HAp surface. BSA and single stranded salmon sperm DNA (Sigma–Aldrich Corporation, St. Louis, MO, USA) were dissolved in distilled water at a concentration of 1 mg/ml and applied to the treated HAp ceramic surface (the volume of the solution was 100 nl). Since the DNA and BSA concentration was quiet low the pH was that of the water ($\sim 5.5$). The samples were incubated 15 min at room temperature with no vibrations, washed several times with distilled water and then Giemsa stained (Sigma–Aldrich Corporation) to visualize the biomolecules.

3. Results

Figs. 1 and 2 illustrate SEM and AFM surface topography images of the studied HAp ceramics, respectively. The SEM and AFM images of the HAp are presented at approximately the same lateral magnification. The two images show similar surface structure, however, they differ in the other types of information that can be acquired on this sample. The three
dimensional nature of the AFM can be used to calculate changes in roughness and surface area variations. Statistical analysis gave the average size of the ceramic grains around 1.0 μm with dispersion of 0.3 μm. The fabricated samples were also characterized by porosity and root mean square roughness around 10% and 0.16 μm, respectively. The SEM and AFM measurements did not reveal any differences in topographic features between studied samples.

The untreated HAp samples demonstrate a profoundly hydrophilic state with a contact angle of θ = 10° (Fig. 3a). The electron irradiation changes the contact angle in a very wide range of θ = 10–100° leading to a deep wettability modulation. These measured angles appear to be stable and closely reproducible. Fig. 3 shows a gradual increase of the contact angle for invariable electron beam current density, Jₑ = 100 nA/cm², and electron energy, Eₑ = 100 eV, versus the varied electron irradiation exposure time, t = 0–3000 s. Fig. 4 demonstrates the relation of contact angle versus exposure time. The accuracy of the wettability tailoring may be as high as Δθ = ±3° by adjusting the duration of exposure time. It was found that the increase of the irradiation time up to t = 3000 s using the chosen density of electron current leads to the contact angle, θ, saturation that runs up to θ = 100° (Fig. 4). It should be noted that the increasing of the incident electron energy up to Eₑ = 1 keV results in larger penetration depth of electrons and reduction of electron-induced contact angle variation.

Influence of heterogeneity (chemical or physical) of studied sample surfaces was examined by contact angle hysteresis measurements using the tilting plate technique. It was found from the data obtained from the tilting plate measurements that hysteresis between the advancing and receding contact angles was 2 ± 1° for hydrophilic state (θ = 10°) and 14 ± 3° when the surface was modified to the hydrophobic state (θ = 100°). We attribute this hysteresis to the roughness of the HAp surfaces (Fig. 2). Thus, the main contribution of the induced wettability may be ascribed to the variation of the surface potential.

The KPFM measurements showed that the induced average value of the surface electric potential (Fig. 4) gradually grows with the irradiation time exposition from ΔΦ = 0 V (t = 0 s) to 0.65 V (t = 3000 s) leading to the corresponding enlargement of the contact angle in a wide range of θ = 10–100° (Fig. 3). Fig. 5 demonstrates highly inhomogeneous electric potential distribution (ΔΦ ~ 0.1 V) in nanoscale, observed by the KPFM for both the charged and untreated HAp samples.

Sequences of HAp samples were electron irradiated using increasing exposure time resulting in wettability modification in the range of θ = 10–100°. Fig. 6 illustrates that the variation of the HAp wettability results in selective adhesion of the

![Fig. 4. The dependence of the measured contact angle, θ (dotted line), average surface potential difference, ΔΦ (solid line), on the hydroxyapatite surface and electron exposition time, t. The electron irradiation energy is Eₑ = 100 eV and electron current density is Jₑ = 100 nA/cm².](image1)

![Fig. 5. Surface potential difference profile measurements of the hydrophilic (black line, θ = 10°) and hydrophobic (grey line, θ = 100°) states of the hydroxyapatite surface. The hydrophobic state was achieved by a low-energy electron beam irradiation.](image2)
biological molecules, such as BSA and DNA. The obtained results are highly reproducible after each repetitive experimental cycle, which included electron irradiation and molecules binding.

4. Discussion

The observed flexible tuning of the HAp ceramics from the initial superhydrophilic state $\theta = 10^\circ$ (Fig. 3a) to a pronounced hydrophobic surface, $\theta = 100^\circ$ (Fig. 3c, $t = 3000$ s) may be unanimously ascribed to increasing trapped electron/hole charge in the vicinity of the HAp surface layer. Our recent experimental studies [20] showed that the HAp bioceramics represents a P-type wide band gap semiconductor with energy gap $E_g \approx 4$ eV and originally positive surface band bending. The optical spectroscopy performed by photoluminescence and surface photovoltage methods enabled to study fine electronic states structure of the HAp ceramics. Found trap spectrum revealed five bulk local centers and one surface state. The implemented electron energy state spectroscopy studies [20] show the way for variation of charged states near the surface by electron/hole populating of the found surface and bulk levels. The presented experimental data (Figs. 3 and 4) illustrate a strong influence of the irradiated region in the vicinity of the HAp substrate. That occurs due to the charge trapping in the vicinity of the HAp surface. Our estimations implemented by the Monte Carlo simulation method gave the penetration depth of the incident electrons with the used energy of $E_e = 100$ eV, around 20 Å that is consisted with the analytical method [21] solution.

The energy of primary electrons significantly exceeds the mobility gap, $E_g \approx 4$ eV, of the HAp samples, resulting in electron–hole pairs generation [20]. The traps of different origin located in the irradiated region in the vicinity of the surface layer capture primary and generated secondary electrons and holes after their thermalization at the length of about dozens of angstroms [22]. The applied electron current density, $J_e = 100$ nA/cm$^2$, and highest exposition time, $t = 3000$ s, gives the maximum injected electron charge of $Q = 6 \times 10^{-5}$ C. If the depth of electron/hole charge scattering and thermalization is around 20 Å then the concentration of one-fold charged electron/ hole traps is $n \approx 10^{22}$ 1/cm$^3$. Such a value seems reasonable for the highly defective HAp ceramic material produced from enormously deformed nanoparticles following by high pressure at the stage of the ceramics fabrication.

The experimental data presented in Figs. 3 and 4 demonstrate the obvious correlation between the measured surface potential, $\Delta \Phi$, of the HAp sample versus the irradiation time, $t$. Thus, the injected charge, $Q$, which is proportional to the total number of injected electrons, allows monotonous variation of $\Delta \Phi$. The observed continuous enlargement of $\Delta \Phi$ with the absorbed charge is the evidence of the growth of the HAp band bending in $\Delta \Phi = 0.65$ V resulting from electron/hole filling traps (Fig. 4). It should be noted that the KPFM technique enabled observing highly inhomogeneous electric potential distribution in nanoscale (Fig. 5). The KPFM image of charge appears as a protrusion on the surface, due to the electrostatic interaction between the image charge in the grounded tip and the charge in the sample. Thus, the electric potential fluctuations for the electron-treated and virgin HAp ceramics samples are significant and may reach $\Delta \Phi \sim 0.1$ V (Fig. 5).

It should be noted that the observed variation of the surface potential $\Delta \Phi$ as well the contact angle (wettability) are totally reversible. Annealing of the preliminary electron irradiated HAp led to complete restoration of both the surface potential and wettability in the studied HAp samples. Thus, one can assume that found variation of the surface potential and wettability modulation (Figs. 3 and 4) occurs without generation or modification of bulk and surface defects. The XPS and AFM surface measurements of the irradiated or annealed HAp samples did not show any significant changes in

Fig. 6. Adhesion of bovine serum albumin (BSA) (upper row) and DNA (lower row) to the hydroxyapatite surfaces with different wettability state ($\theta$ is the contact angle). The ceramic samples were Giemsa stained and the biomolecules are stained in red.
comparison to the untreated samples. Hence, the applied method allows dynamically modify the surface potential by controlling the number of incident electrons, resulting in a wide range variation of contact angles (Figs. 3 and 4) without applying external electric field or interlayers deposition. The injected and generated carriers of charge trapped on localized centers leads to electric field generation near the surface. The surface charge density is determined by the density of the surface states $n_s \sim 10^{12}$ $1/cm^2$ and in the case of their complete filling the surface charge density is $Q_s \sim 10^{-7}$ $C/cm^2$. Thus, the electric field induced by the applied electron irradiation may reach the magnitude at the flat surface of $E \sim 10^6$ V/cm. The studied HAp samples possess pronounced inhomogeneous topography (Fig. 2) that may lead to significant local field enhancement by one to two orders of magnitude near surface protrusions. Such values of the induced field intensity are close to the field stress used in conventional electrowetting effect [23]. However, the electrowetting effect is based on using an external electrical field and therefore cannot be applied in vivo experiments. The proposed method uses preliminary electrical charging of the surface by electron beam and may be used both in vivo and in vitro studies.

A low-energy electron irradiation leads to pronounced reversible modulation of wettability in the HAp biomimetic material. Among various types of surface interactions the origin of proteins adhesion on biomaterial surface in orthopedic implants and engineered tissues is mainly ascribed to wettability of the biological substrates considering hydrophobic/hydrophilic properties as a leading mechanism responsible for the cells immobilization [2,4]. Experimental evidence suggests that the hydrophobic interaction is the major determinant of protein adsorption [3,24], whilst DNA prefers the more hydrophilic substrates. The observed tunable wettability modulation in the wide range of the contact angles allows engineering of any wettability state needed for adhesion of biological cells.

The wettability of the series of the HAp samples was tailored in the range of $\theta = 10–100^\circ$. The differences in wettability resulted in differential binding of biological molecules, in correlation to their level of hydrophobicity. Thus, DNA, a very hydrophilic molecule due to the phosphate groups in the sugar-phosphate backbone, bound preferentially to the high wettability surface ($\theta < 50^\circ$) (Fig. 6, bottom row). In contrast, the binding of BSA, a protein, which contains hydrophobic domains was more pronounced at low wettability ($\theta > 50^\circ$) (Fig. 6, top row).

Development of biological cells immobilization techniques is based on stable cell/HAp interface [25]. Investigations of the HAp sample retention, after applying the developed wettability engineering method, shows that tailored hydrophilic and hydrophobic states remain stable at least during the month in different environment conditions, such as air and water.

The electron-induced wettability modulation method allows both flexible homogenous wettability tuning and wettability patterning, which may be used for fabrication of a spatially arrayed HAp for biological cells immobilization, gene transfer, etc. [26]. This method may be applied to other solid state materials including metals which are always coated by thin oxide films. Our experiments with titanium and alumina substrates, as well as amorphous silicon nitride and silicon dioxide films and silicon nanocrystals showed identical variation of the wettability under a low-energy electron irradiation [15].

5. Conclusions

A low-energy electron irradiation of the HAp bioceramics leads to reversible wettability modification without generating bulk and surface defects, and without modifying surface topography or the phase state of the material. The method described here allows tailoring of the wettability state of HAp surfaces by variation of injected and trapped electron charge. The wettability state changed in a wide range of contact angles with an accuracy of $\pm 3^\circ$. The resulting surface potential variation of the HAp ceramics enabled selective adhesion of biological molecules such as proteins and DNA.

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