Adsorption and Conformational Change of Myoglobin on Biomimetic Hydroxyapatite Nanocrystals Functionalized with Alendronate

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Adsorption and Conformational Change of Myoglobin on Biomimetic Hydroxyapatite Nanocrystals Functionalized with Alendronate

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The chemical conjugation of bisphosphonates (BPs), specifically alendronate, to hydroxyapatite could be an effective means to impart to it fine-tuned bioactivity. Horse heart myoglobin (Mb), a well-characterized protein, has been adsorbed onto biomimetic hydroxyapatite nanocrystals (nHA) and onto the nHA/alendronate conjugate powdered samples. The obtained materials have potential use in bone implantation and as prospective drug-delivery devices. The kinetic absorption of Mb onto nHA is dramatically affected by its functionalization with alendronate. The covering of the nHA surface by alendronate inhibits the adsorption of myoglobin. The adsorption mechanisms of the protein were studied by spectroscopic techniques (UV−vis and surface-enhanced Raman spectroscopy). The results indicate that the protein changes conformation upon adsorption on the inorganic substrate. In particular, the interaction with nHA alters the coordination state of the iron in the heme through the formation of a hexacoordinated low-spin Mb heme, possibly involving the distal histidine. Instead, the covering of the nHA surface by alendronate does not adsorb the protein but preserves the coordination state of the heme moiety. This study could be of significance either in the field of biomaterials science, in particular, to fine tune a bone-specific drug delivery device and to test nHA as a new support for heterogeneous catalysis, improving the understating of enzyme immobilization.

Introduction

The interaction of proteins with solid inorganic surfaces is not only a fundamental phenomenon but is also the key to several important and novel applications. In the biomaterials field, protein−surface adhesion is the first event in the integration of an implanted device or material with biological tissues. In nanotechnology, protein−surface interactions are pivotal for the assembly of interfacial protein constructs, such as sensors, activators, and other functional components at the biological/electronic junction. The detailed mechanistic understanding of the protein−surface interaction and the ability to tailor protein−surface interaction in nanosized materials would be of value to bionanoassembly technologies. The study of protein−solid surface interaction, which involves both protein binding and unfolding, may also increase our general knowledge of protein biophysics.[1]

The exposure of biomaterials to plasma proteins, blood, or biological fluids normally leads to the adsorption of blood proteins onto the biomaterials surface. The adsorbed protein layer can further mediate additional biological responses, such as cell attachment and activation, and can create unpredicted perturbations to device operation.[2] Although protein adsorption on solid surfaces has been widely studied for decades, its mechanisms are still far from being fully understood. This is because the adsorption of protein on solid surface is a complicated process consisting of many events, such as conformational changes in protein molecules and the coadsorption of ions. In particular, the protein conformational change, which results in entropic gain, is thought to be an important driving forces for the protein surface adsorption.[3] However, the most important forces involved in the molecular adsorption can be expected to be electrostatic, which are associated with exposed charged groups on the protein surface.

In the biomaterial field, calcium phosphates, which are the inorganic components of bone, are widely used as bone grafts. Here, a knowledge of the underlying principles of protein interaction with calcium phosphates is required not only in evaluating their potential application but also in their ability to act as carriers for enzymes. The chemical and biological properties of the latter are strictly linked to their nanodimensions, the regulation of which requires a nanoscientific approach. In fact, the mineral phase of bone and tooth consists of nonstoichiometric carbonated hydroxyapatite (HA) crystals with a length of about 100 nm, a width of 20−30 nm, and a thickness of 3−6 nm.[4] Biomimetic calcium phosphates need to be synthesized with similar nanoscale dimensions and with properties such as low crystallinity, nonstoichiometric composition, crystalline disorder, and the presence of carbonate ions in the crystal lattice.[5] The excellent biological properties of biomimetic hydroxyapatite nanocrystals (nHA), such as the lack of toxicity, inflammatory and immune responses, and high bioresorbibility can be significantly increased by lowering their crystallinity. It is well known that hydroxyapatite shows a high affinity for proteins.[6]

Myoglobin (Mb) can be considered to be a model protein because of its well-known structure and properties, commercial

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availability, and relatively small size. Myoglobin is a globular heme protein with a molecular mass of 17 800 Da, an isoelectric point (pI) of 7.4, and approximate dimensions of 2.5 × 3.5 × 4.5 nm^3. It is found in cardiac and red skeletal muscle tissues, where it acts as an oxygen storage and transporting protein. It contains a single polypeptide chain of 153 amino acid residues and an iron porphyrin IX complex as the prosthetic group.[7] Myoglobin is probably the most investigated protein in the construction of enzymes that catalyze desired and often non-natural reactions, achieving predictable substrate specificity, and is one of the objectives of applied biotechnology.[8] The effective immobilization of enzymes on solid substrates without changing the original conformation and bioactivity has attracted increasing interest among researchers along with rapid advances of biotechnology and biodevices.[9−11] Spectroscopists have investigated heme proteins by means of resonance Raman technique, in which the Raman effect is enhanced by the coupling of vibrational and electronic transitions. This technique uses visible and UV excitation sources that enhance vibration of the porphyrin ring and allow studying the stereochemistry and electronic properties of heme.[12,13]

Hydroxyapatite is also known for its binding capability to a wide variety of molecules (HA is nowadays widely applied for separation of various proteins in a high performance liquid chromatograph system).[14,15] Moreover, most therapeutic agents intended for bone diseases have a particular affinity to bone hydroxyapatite under physiological conditions.[16]

A class of molecules known as bisphosphonates (BPs) exhibit a strong affinity to bone mineral under physiological conditions. This because BPs are characterized by two phosphonate groups attached to a single carbon atom and have strong affinity for the Ca^{2+} ions of bone apatite. This property is the basis for their use as inhibitors of ectopic calcification and of bone resorption. In fact, calcium deficient synthetic apatites has been investigated as devices for the controlled delivery of geminal bisphosphonates.[17] Chemical surface binding of bisphosphonates to hydroxyapatite could represent an effective means to impart it fine-tuned bioactivity.[18] Hydroxyapatite, which is insoluble and nonreactive with many biochemical compounds, could be an elective material for supporting immobilized proteins, which can interact properly with the charged groups on the HA surface.

The aim of this research is to compare myoglobin affinity versus the BPs surface functionalization of biomimetic nHA in respect to unfunctionalized hydroxyapatite. Moreover, using by UV−vis and SERS[1919] (surface-enhanced raman spectroscopy) spectroscopic techniques, it has been pointed out that the apatitic nanostructured biomimetic substrate affects the conformation of the protein. The Mb analysis recognizing and assembling on drug-loaded apatite crystals not only allows a better understanding of the protein adhesion mechanism but could also aid in the development of surface coatings to improve the biocompatibility of bone-implantable biomaterials and for hard-tissue engineering and regeneration technologies. This study could improve the understanding of enzyme immobilization in the field of biomaterials science, in particular, to fine tune a bone-specific drug-delivery device, and to test nHA as a new support for heterogeneous catalysis.

Experimental Section

Materials. Myoglobin (from horse heart) was obtained from Sigma and used without further purification ($A_{Soret}/A_{280} = 4.7$). Sodium alendronate was a kind gift from Merck Sharp & Dohme, Italy. All common high-purity chemical reagents were supplied by Sigma. Ultrapure water (0.22 μS, 25 °C) was used.

Preparation and Characterization of HA. HA nanocrystals with a nearly stoichiometric Ca/P ratio (1.67) were synthesized in order to produce a plate-shaped bonelike apatite phase. The powder fraction of nanocrystals having granular dimensions ranging from 100 to 150 μm was selected for this study. The surface Ca/P ratio determined by X-ray photoemission spectroscopy (XPS) analysis was significantly lower (1.45) as a result of surface disorder.[20] Specific surface-area measurement was undertaken by using a Carlo Erba Sorpty 1750 instrument by measuring N_2 adsorption at 77 K and adopting the well-known BET procedure.[21] The specific surface area of nHA was 120 ± 6 m^2/g. Details of its synthesis, as well as the other physicochemical properties, are reported elsewhere.[17]

Alendronate Adsorption. An aliquot (1.5 mL) of alendronate solution (1 mg/mL) was added to 10 mg of nHA in a 2 mL conical polyethylene Eppendorf tube. After 15 s of treatment in a vortex apparatus, the nHA suspension was maintained in a bascule bath at 37 °C. The adsorption profile for alendronate was determined by measuring the concentration of drug remaining in the supernatant solution as function of time. At scheduled times, aliquots (100 μL) of the supernatant that was well separated from the solid phase by 3 min of centrifugation at 10 000 rpm (12 675*g*) on a micro Centrifugette 4214 were removed for drug quantification and replaced with fresh water. The concentration of alendronate was determined by UV−vis spectroscopy analysis. The quantity of the drug was measured by a colorimetric method based on the reaction of the primary amino group with ninhydrin in a methanolic medium in the presence of 0.05 M sodium bicarbonate. The colored product was measured at 568 nm against the reagent blank[22] ($\epsilon = 29\ M^{-1}\ cm^{-1}$).

Myoglobin Immobilization. Samples of adsorbed Mb were prepared by mixing 10 mg of nHA with 1.5 mL of protein dissolved in Hepes buffer (0.01 M Hepes, 0.5 M NaCl) at pH 7.4 at different concentrations (ranging from 0.2 to 2 mg/mL) in a 2 mL conical polyethylene Eppendorf tube. The mixture was maintained in a bascule bath at 37 °C for 24 h. The solid, for the Raman spectroscopic investigation, was recovered by centrifuging at 12 675*g* for 3 min and then freeze drying at −60 °C under vacuum (3 mbar) for 12 h. The supernatant solution was assayed for protein content by UV spectroscopy ($\lambda = 409$ nm, $\epsilon = 188\ 000\ M^{-1}\ cm^{-1}$). The amount of adsorbed protein was calculated from the difference between the concentrations of the initial solution and the supernatant.

The adsorption data were fitted to the Langmuir (1) and Freundlich (2) equations[23,24]

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$$Q_e = \frac{Q_{max} C_e}{(1/a_L) + C_e} \quad (1)$$

$$Q_e = K_F C_e^{1/n} \quad (2)$$

where C_e is the concentration of Mb in solution at equilibrium (mg/mL), Q_e is the amount of Mb adsorbed onto the support (mg/g of support), Q_{max} is the maximum adsorption capacity of the support (mg/g of support), a_L is the Langmuir constant (mL/mg), K_F is the Freundlich adsorption coefficient ((mg/g of support)/(mg/mL)$^{-1/n}$), and $1/n$ is the heterogeneity factor (a-dimensional).

The same procedure previously reported for alendronate adsorption was used to evaluate the kinetic adsorption of Mb onto nHA, and the concentration was determined by UV−vis spectroscopy (λ = 409 nm, ϵ = 188 000 M^{-1} cm^{-1}).

Morphological Investigation. Transmission electron microscopy (TEM) investigations were carried out using a Philips CM 100 instrument (80 kV). The powdered samples of unfunctionalized nHA were sonically dispersed in water, whereas the sonic dispersion has not been used with the functionalized samples. A few droplets of the slurry have been deposited on holey-carbon foils supported on conventional copper microgrids.

Spectroscopic Investigation. The UV−vis spectra of Mb solutions were recorded between 200 and 800 nm using a Varian Cary 5 UV−vis−NIR spectrophotometer (Varian, Palo Alto, CA) against a blank buffer solution. The measurements were performed using a 1 cm quartz cell.

Surface-enhanced Raman spectroscopy spectra were collected between 400 and 1750 cm^{-1} using a Jasco NRS-2000C instrument with a macro-objective of 20× magnification. All spectra were recorded under backscattering conditions with 4 cm^{-1} spectral resolution using the 514.5 nm excitation line (Innova Coherent 70) with a power of ca. 10 mW. The detector was a 160 K frozen digital CCD (Spec-10: 100B, Roper Scientific Inc.). Native myoglobin powder (used as a control) was analyzed after a treatment of 24 h in Hepes buffer at 37 °C in a bascule bath and freeze drying. The solid samples were dissolved in a silver−hydroxylamine colloid prepared using the Leopold and Lendl method[25] at a concentration of about 10^{-5} M.

Statistic Analysis. Adsorption experiments were performed in triplicate, and results were plotted as mean values ± standard deviation (SD). Data obtained from adsorption experiments were compared by a two-tailed t test. Differences were considered to be statistically significant at a significance level of 90%.

Results and Discussion

Interaction of Myoglobin with Hydroxyapatite. The results of myoglobin adsorption onto nHA are reported as an isotherm in Figure 1, where the adsorbed amount, Γ_{Mb} (mg/m^2), is plotted against the protein concentration after adsorption, $C_{myoglobin}$ (mg/mL). The plot is characterized by an initial slope indicating high protein affinity for the nHA surface. The adsorption saturation yields a plateau value corresponding to the maximum amount of Mb surface immobilization of about 0.7 mg/m^2. This finding is in agreement with the data reported by Kandori et al.[14] They observed a Mb adsorption limit on different carbonate−hydroxyapatite particles in the range of 0.65−0.82 mg/m^2. The increase in Mb concentration in the buffer solution increases the surface coverage, until completion takes place using a Mb starting concentration higher than 2 mg/mL onto 10 mg of nHA. The solubility limit of myoglobin under these experimental conditions avoids the investigation of more concentrated solutions. When the plateau value is reached in the adsorption isotherm, probably a monolayer of Mb molecules has been deposited.

Both the Langmuir and the Freundlich models have been utilized to describe the Mb adsorption isotherm. The values of

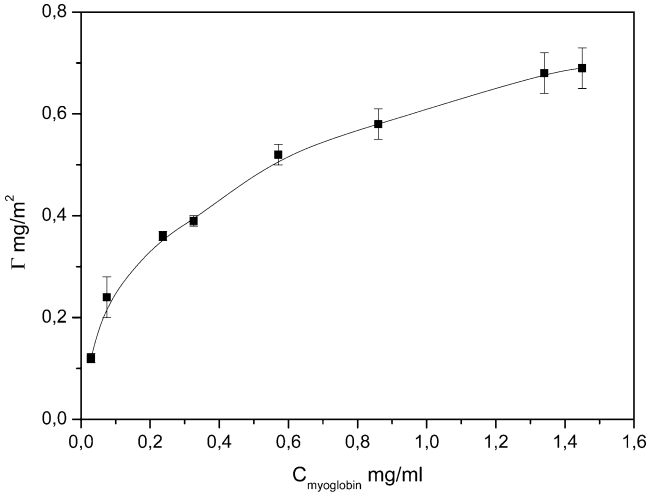

Figure 1. Adsorption isotherm of myoglobin on biomimetic hydroxyapatite nanocrystals at pH 7.4.

Table 1. Adsorption Parameters of Myoglobin onto Biomimetic Hydroxyapatite Nanocrystals Calculated According to Langmuir and Freudlinch Models

	Langmuir			Freudlinch		
	Q_{max} mg/g of support	a_L mL/mg	r^2	K_F mg/g of support/ (mg/mL)$^{-1/n}$	$1/n$	r^2
Mb/nHA	76	8.22	0.994	75	0.42	0.989

the Langmuir and Freundlich constants calculated by linear fitting for Mb immobilization onto the nHA surface are compared in Table 1, revealing that both models can well describe the Mb monolayer on nHA. Probably the two models represent a simplification of the real adsorption systems, which would be a combination of both. In fact, the Langmuir isotherm assumes an energetically homogeneous support surface with identical adsorption sites throughout. Therefore, these sites are expected to hold equal numbers of Mb molecules, and a monolayer is formed. Moreover, the Langmuir formula implies highly favorable and irreversible adsorption. The calculated Q_{max} value (maximum adsorption capacity of the support) of 76 mg/g is higher than that (35 mg/g) reported by Bellezza et al.[11] for Mb immobilization on zirconium benzenephosphonate. The affinity of nanohydroxyapatite for biomolecules is strictly related to its high surface area. These two materials have different surface areas (120 vs 8 m^2/g), but considering the uptake of myoglobin per mass unit, the hydroxyapatite is more loaded than zirconium benzenephosphonate. This fact is remarkable regarding the utilization of biomimetic hydroxyapatite nanocrystals as an innovative support for the immobilization of proteins with catalytic activity. However, according to the Freundlich isotherm, the adsorbent surface is considered to be energetically heterogeneous with nonidentical adsorption sites. When the term $1/n$ (heterogeneity factor) is close to 1.0, the surface is characterized by a high degree of homogeneity. The calculated value of 0.42 shows a high level of surface heterogeneity, in agreement with the typical surface of the biomimetic hydroxyapatite nanocrystals at low crystallinity having a nonstoichiometric surface Ca/P molar ratio.

Myoglobin molecules are electrostatically neutral at pH 7.4. Previous studies have demonstrated that no remarkable relationship with the bulk Ca/P molar ratio of the particles was detected for neutral myoglobin adsorption.[26]

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Table 2. Values of *N* and *K* Langmuir Isotherm Parameters Calculated for the Adsorption of Several Biomolecules on Different Hydroxyapatites

adsorbent	adsorbate	*N* mol/m^2	*K* M^{-1}	ref
HA	Mb	4.41×10^{-8}	0.7×10^5	this work
HA	rhBMP-2	$(3.8-11.5) \times 10^{-8}$	$(2.4-230) \times 10^5$	27
HA	BSA	$(23.4-42.1) \times 10^{-8}$	$(0.3-160) \times 10^5$	28
HA	BSA	$(8.7-26.1) \times 10^{-9}$	$(0.1-1160) \times 10^5$	29
HA	succinylated LSZ	$(1.2-8.2) \times 10^{-8}$	$(0.1-10) \times 10^5$	30
HA	poly-L-Asp	9×10^{-8}	15.7×10^5	31
HA	statherin	80×10^{-8}	211×10^5	31
HA	PRP1	20×10^{-8}	260×10^5	31

The Mb adsorption data on biomimetic hydroxyapatite nanocrystals was well fit to a Langmuir isotherm, a behavior similar to that presented by other proteins and amino acids. In Table 2 we show the values of *N* and *K* of the Langmuir isotherm reported for several proteins. Parameters *K* and *N*, calculated by linear fitting, represent the adsorption constant (affinity constant) and the number of binding sites, respectively. *N* is dimensionally expressed as the adsorbed amount of protein per surface unit. It is observed that the affinity constants *K* for myoglobin onto nHA fall within the range of *K* values reported for the adsorption of bovine serum albumin (BSA) and succinylated lysozyme (LSZ) onto HA, under similar experimental conditions. However, they are lower than those of bone morphogenetic protein (rhBMP-2), poly-L-Asp, statherin, and PRP1, a salivary proline-rich phosphoprotein adsorbed onto HA under similar experimental conditions. The *K* value obtained in this work is consistent with the biological function of proteins considering that myoglobin and albumin are serum proteins whereas poly-L-Asp, statherin, PRP1, and rhBMP 2 are involved in biomineralization processes. Several kinds of adsorbed−adsorbent interactions can take place during protein adsorption on an inorganic surface, for instance, covalent or electrostatic chemical bonds, hydrogen and van der Waals bonds, and hydrophobic and hydrophilic interactions. The relative predominance of one of them over the others is closely related to the protein structure and the chemical−physical characteristic of the inorganic surface. For HA, it seems reasonable that hydrophobic interactions can be discarded and that electrostatic interactions between Mb and the HA surface could take place.

The test of Scatchard[32] has been performed in order to observe the presence or absence of cooperative effects in Mb protein adsorption. The plot of *C*/*Q* versus *C* (where *Q* and *C* indicate the adsorbed amount of protein per adsorbent surface unit and the protein concentration at equilibrium, respectively) indicates no appreciable cooperative effects. The plot of the Scatchard test carried out for Mb adsorption on nHA (Figure 2) suggests the presence of independent noninteracting sites conforming to the Langmuir model. The correlation coefficient for the data in Figure 2 is 0.993.

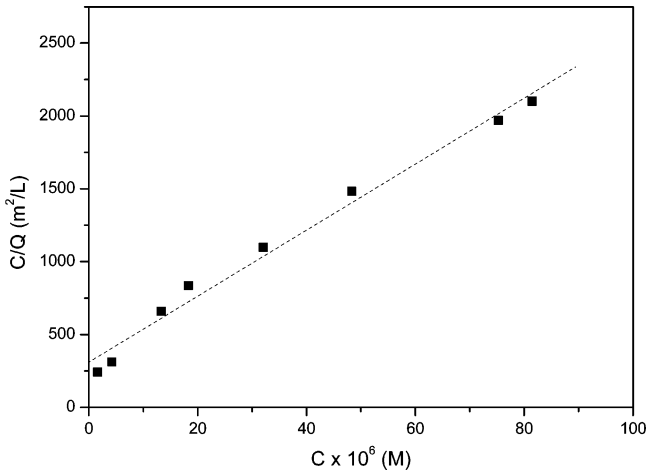

Figure 2. Plot of the Scatchard test, *C*/*Q* vs *C*, for Mb adsorption onto biomimetic hydroxyapatite nanocrystals.

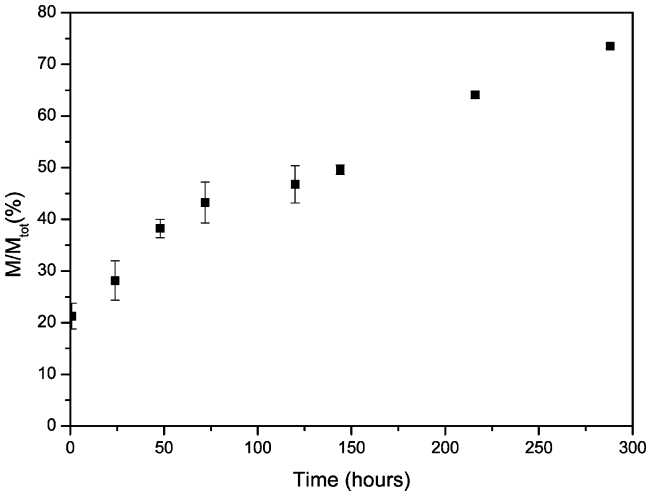

Figure 3. Adsorption kinetic profile of a 2 mg/mL myoglobin solution onto biomimetic hydroxyapatite nanocrystals: M/M_{tot} (%) (mass percentages of myoglobin adsorbed onto nHA with respect to the initial amount of protein) vs time.

The adsorption kinetic profile of a 2 mg/mL myoglobin solution on nHA is reported in Figure 3. The plot shows an almost linear profile, and the percentage of myoglobin mass adsorbed onto the nHA with respect to the initial amount of protein (M/M_{tot} (%)) was 75% after 300 h. This value corresponds to an nHA loading level of 13%, indicating the Mb weight with respect to the composite (Mb−nHA) weight ($100 = W_{Mb-nHA}$).

At the scheduled time, the Mb supernatant solution, upon adsorption onto nHA, was analyzed by UV−vis spectroscopy in order to appreciate protein conformational changes. The spectrum of the Mb solution after 28 h of interaction with nHA (Figure 4, solid line) is superimposable with that of the native Mb solution. In fact, it shows a sharp Soret band at 409 nm, *Q* bands at 504 and 535 nm, and charge-transfer band 1 (CT1 band) at 632 nm and is characteristic of a six-coordinate high-spin (6-cHS) heme with a histidine residue (His-93) and a water molecule bound at the fifth and the sixth coordination positions of the iron atom, respectively.[33] With increasing interaction time, the Mb spectrum shows appreciable changes compared to the native protein. After 72 h of interaction, the HA surface induces the formation of a new species at the expense of the 6-cHS heme, as is evident from the intensity decrease and red shift of the Soret band and the

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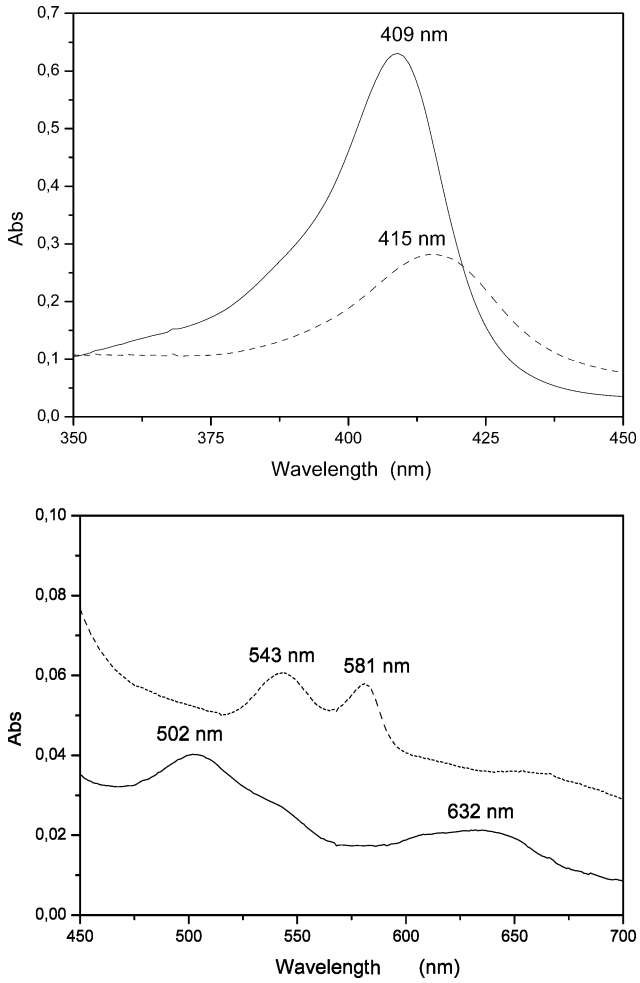

Figure 4. UV−vis spectra of Mb solutions after 28 h (−) and 72 h (---) following the interaction with biomimetic hydroxyapatite nanocrystals.

concomitant appearance of *Q* bands at 581 and 543 nm (Figure 4, dashed line). This spectrum, characteristic of a six-coordinate low-spin (6-cLS) heme, is very similar to that of ferric cytochrome b5, which has histidine residues as the fifth and sixth axial ligands.[34] The escape of water from the sixth position may result in a conformational change at the heme distal pocket: the histidine residue at the E7 helical position (His-64) moves toward the central iron and coordinates to it through the nitrogen donor. The simultaneous biomolecule adsorption and release on the nHA surface is a dynamic process. The protein conformational modifications appear to be relevant at times longer than 72 h because after this time of interaction with the nHA surface the concentration of native myoglobin in solution decreases and the concentration of altered molecules increases. The conformational changes seem to be irreversibile until myoglobin is coupled in solution with nHA. However, the protein comes back to its native conformational state when the solution is separated from nHA. These findings lead us to assume that the Mb conformational changes are dynamic. The time needed to reach this refold in the native status ranges from 2 to 10 h, which is higher than the operation time requested to perform UV−vis analysis. This ensured us of observing a "frozen" conformation.

In the solid state, the protein conformational modifications have been analyzed by Raman spectra. The myoglobin and nHA−myoglobin conjugate powder SERS (hydroxylamine−silver colloid) spectra by which the chemical and electromagnetic effects due to the interaction of the heme group with a silver colloid allow a 10^{14}-fold magnification of the vibrational bands, making it possible to reach the limit of single-molecule detection,[35] are reported in Figure 5. Raman bands in the region of1300−1700 cm^{-1} are sensitive to the dimensions of the porphyrin core: the main factors that influence the dimensions are the coordination, spin, and oxidation state of heme iron. The vibration of the C−N group at 1360−1370 cm^{-1} is sensitive to the oxidation state of the heme iron: ferric heme shifts this band to higher wavenumbers. In the 1500−1630 cm^{-1} region, vibrations of C−C of the porphyrin ring can be found, with the band near 1565 cm^{-1} being sensitive to the spin state of the heme iron and the band at 1620 cm^{-1} (stretching of vinylic C═C) being sensitive to both the spin and the oxidation state.[33] The relative intensity of these bands increases if the content of low-spin iron increases. After interaction with nHA, the 1596 cm^{-1} band increases in intensity, showing a higher content of low-spin iron. Another marker band

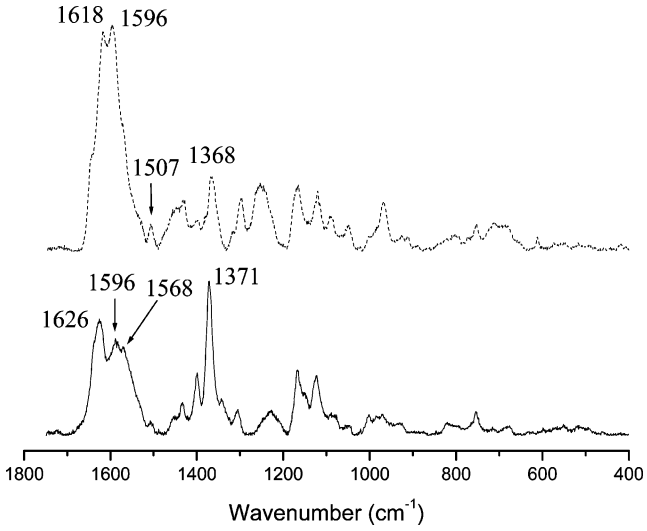

Figure 5. SERS (hydroxylamine−silver colloid) spectra of solid Mb (−) and solid Mb−nHA (---).

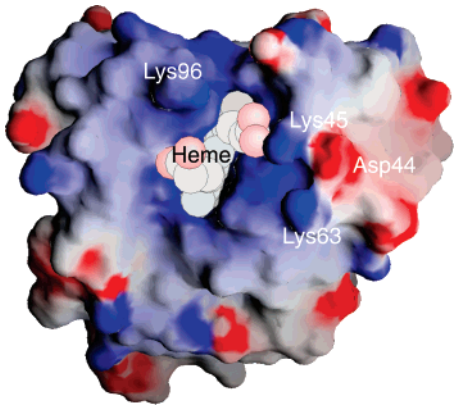

Figure 6. GRASP (graphical representation and analysis of structural properties) representation of the distribution of the electrostatic surface potential[37] of the myoglobin (PDB ID 1YMB). The heme group atoms are represented as spheres. The positions of relevant residues are indicated. The values of surface potentials are expressed as a spectrum ranging from $+10kT/e$ (deep blue) through $0kT/e$ (white) to $-10kT/e$ (deep red). At a temperature of 298.15 K, $kT/e = 25.7$ mV.

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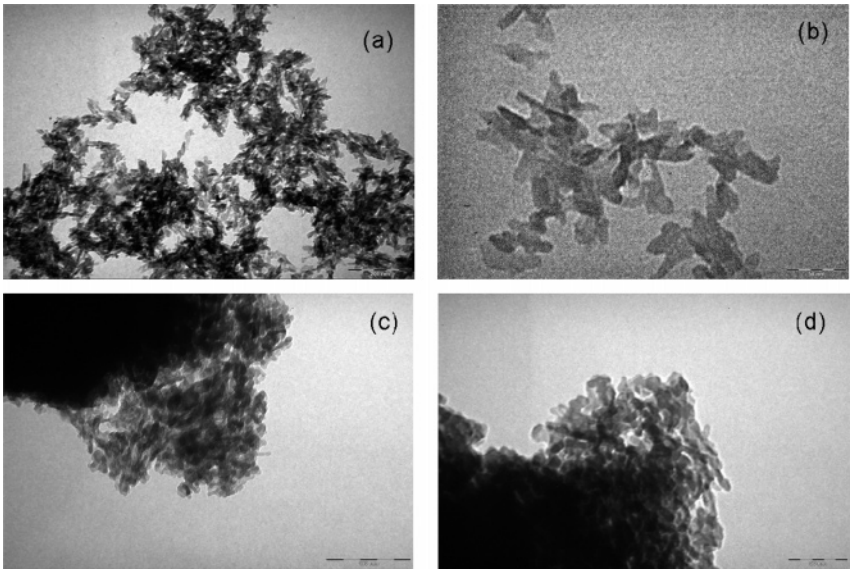

Figure 7. TEM images of synthetic biomimetic hydroxyapatite nanocrystals after interaction with Mb (a, b) and of synthetic biomimetic hydroxyapatite nanocrystals not interacting with the protein (c, d). Scale bars are 200 (a), 50 (b), 100 (c), and 50 (d) nm.

of low-spin heme is at 1507 cm^{-1} [36] and can be observed after the interaction with nHA. The presence of the bands at 1596 and 1568 cm^{-1} in the myoglobin sample indicates that the powder is a mixture of two spin states. It is reasonable that the modification of spin in a small native myoglobin sample is due to the freeze-drying process. Instead, all myoglobin molecules adsorbed onto the nHA show a low-spin state. Another important consideration is that the interaction with nHA does not affect the oxidation state of iron in the heme moiety. The spin state of myoglobin is relevant in relation to the catalytic activity of the protein because only the five-coordinate high-spin form of the heme iron can activate exogenous species such as hydrogen peroxide by coordination to the free sixth octahedral position. Besides the protein form containing six-coordinate low-spin heme, a lack of activity by the immobilized myoglobin can arise from limited accessibility of the substrates to the active site of the protein bound on the solid surface.

The assumption that during the interaction with nanocrystals the heme and the residues near it are principally exposed to modification is supported by the examination of the surface electrostatic potential of myoglobin at pH 7.4 (Figure 6). The electrostatic interaction is one of the important factors that determine the extent of conformational changes. The exposed surface close to the heme moiety is largely positive; furthermore, it is the zone in which a large number of charges are present. This region could be the effective part of the molecule that interacts with nHA, and because of its positive charge, it has a high affinity for the negative phosphates of the calcium-deficient surface. Therefore, the adsorption of myoglobin onto nHA might follow a preferable orientation with respect to the random side-on or end-on adsorption mechanism onto titania and zirconia particles.[3]

The TEM images of biomimetic hydroxyapatite nanocrystals interacting with Mb (Figure 7 a,b) show that the nanocrystals are more monodisperse than are the unfunctionalized ones[17] (Figure 7c,d) as a result of the electrostatic repulsion between their covered charged surfaces. The protein molecules adsorbed onto the nHA surface are able to disaggregate the nanoparticles and, consequently, to increase the reactivity toward the biological environment.

Interaction of Alendronate with Hydroxyapatite. The adsorption profiles of alendronate using aqueous solutions containing an initial drug concentration of 1 mg/mL (3×10^{-3} M) in the presence of nHA are shown in Figure 8. The plot showed that the maximum uptake of alendronate was significantly fast reaching a maximum value after about 24 h. The percentage of drug mass adsorbed onto the nHA nanoparticles with respect to the initial drug mass was about 90%. This value corresponds to an nHA loading level of 8.3%, as the alendronate weight with respect to the composite (alendronate−nHA) weight ($100 = W_{\text{alendronate-nHA}}$). The high adsorption of alendronate, however, despite its negative charge, suggests that the interaction with the nHA surface takes place by ligand exchange in which the two phosphonate groups of the drug molecule replace two surface phosphate groups.[17]

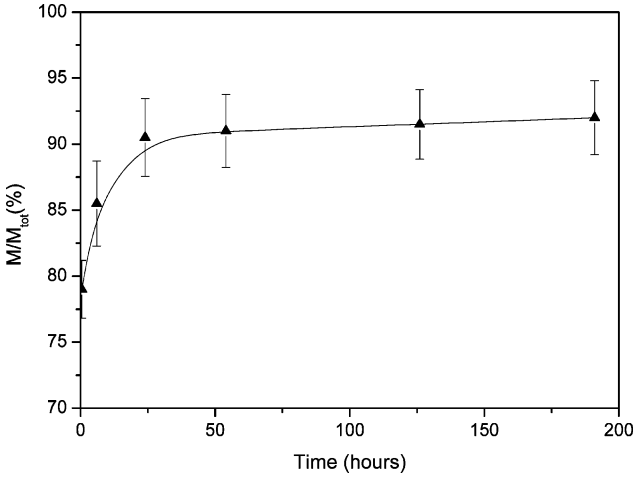

Figure 8. Kinetics of the adsorption of alendronate onto synthetic biomimetic hydroxyapatite nanocrystals: M/M_{tot} (%) (mass percentages of alendronate adsorbed onto nHA with respect to the initial amount of drug) vs time.

Interaction between the alendronate solution (1 mg/mL) and biomimetic hydroxyapatite nanocrystals was performed for 48 h, when the maximum uptake of alendronate was reached (Figure 8) and the surface of the support was partially covered by a layer of bisphosphonate molecules.

Interaction of Myoglobin with the Alendronate−Hydroxyapatite Conjugate. The adsorption kinetic of myoglobin onto the alendronate−nHA conjugate is dramatically affected by the alendronate functionalization of nanocrystals (Figure 9). The percentage of myoglobin adsorbed onto the nHA/alendronate

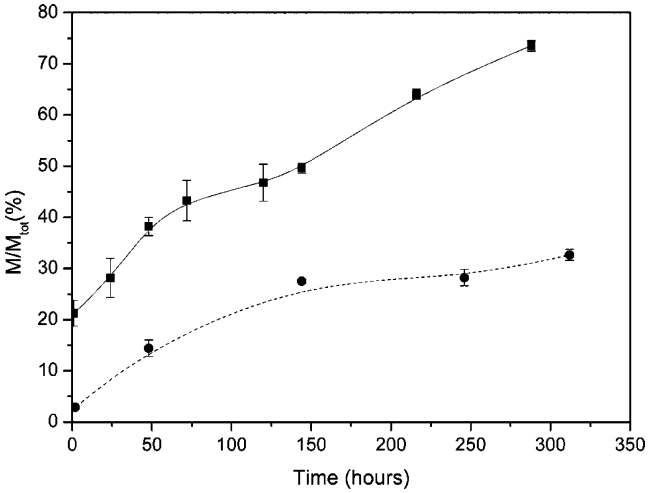

Figure 9. Comparison of adsorption kinetics of Mb onto nHA nanocrystals (−) and Mb onto nHA/alendronate (---).

conjugates with respect to the initial protein concentration after 300 h was about 25% against 75% on the free support. The mass percentage of myoglobin adsorbed onto the conjugate is probably due to adsorption onto the partially free surface. The surface is able to undergo the adsorption of protein because of the insufficient quantity of alendronate covering the surface during functionalization and because of the small amount of alendronate released from the nanocrystals. After 24 h, the mass percentage of alendronate released in Hepes buffer from hydroxyapatite is about 30%.[17] Mb adsorption is in agreement with the contemporary alendronate desorption and the resulting nHA free-site enhancement. After that time, an almost steady state it has been reached in which the alendronate release is very slow and almost absent. This means that a stable alendronate−HA conjugate interacting with myoglobin is present. The supernatants were analyzed by UV−vis spectroscopy, and in all of the samples any relevant shifts of the Soret band from 409 nm and of any other band have been observed. Therefore, it can be assumed that the surface functionalization of nHA with alendronate avoids the adsorption of myoglobin and protects the protein from the modification of the coordination and the change in the spin of the heme moiety.

Conclusions

Biomimetic hydroxyapatite nanocrystals represent an ideal bone-substitute material; the clarification of its interaction with proteins is central to understanding the integration of an implanted device or material with tissue. The nHA−myoglobin interaction mechanism has been elucidated, pointing out that the maximum adsorption capacity of the support is higher than that of other inorganic phosphates, the adsorption isotherm can be described by both the Langmuir and Freundlich models, and the inorganic substrate affects the conformation of protein with respect to its adsorbed status. In particular, using UV−vis and SERS, we found that the spin state of the myoglobin heme moiety changes from the six-coordinate high-spin native state to the six-coordine low-spin state as a consequence of the interaction with biomimetic hydroxyapatite nanocrystals. The spin state of myoglobin is relevant in relation to the catalytic activity of the protein. The surface electrostatic potential map of the protein allows us to hypothesize a preferential interaction mechanism through one defined region of the protein surface in spite of the random adhesion mechanism toward other inorganic supports. The heme moiety is attracted to the apatitic surface as a consequence of the surface disorder of the nanocrystals, which is connected to a negative surface charge with respect to the crystalline core. The immobilization of bisphosphonates onto nHA is an important strategy for setting up a bone-specific drug-delivery device. Considering that the interaction of protein with nHA−biomolecule conjugates tailored for specific therapeutic applications plays a key role as a biological probe, we tested the myoglobin affinity for nHA−alendronate bioconjugates. We found that alendronate avoids both the adsorption and the conformational changes of the myoglobin heme moiety. Myoglobin behavior toward alendronate-grafted nHA crystals shows that this functionalization imprints surface selectivity to nHA and drives the biological environmental response toward them.

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