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Adsorption of recombinant human bone morphogenetic protein rhBMP-2m onto hydroxyapatite

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Abstract

Recombinant human mature bone morphogenetic protein 2 (rhBMP-2m) has been expressed to study its adsorption onto precipitated hydroxyapatite (HA). The influence on the adsorption process of different parameters such as pH and concentrations of calcium, phosphate or NaCl has been investigated. Although the adsorption proceeds rapidly at the initial stages, the maximum adsorbed amount is reached after four hours. The process is notably influenced by adding calcium or phosphate to the system but, while calcium ions increase the adsorption of rhBMP-2m onto HA, phosphate ions inhibit it. The influence of pH and NaCl concentration are notable but less important than those of calcium and phosphate. The adsorption data fit well to a Langmuir adsorption isotherm. The values of the isotherm parameters have been calculated and discussed, and an adsorption mechanism has been proposed.

Keywords: rhBMP-2m; Hydroxyapatite; Adsorption; Langmuir isotherm

1. Introduction

Bone morphogenetic proteins (BMPs) are multifunctional cytokines which are members of the transforming growth factor-β superfamily. They are signalling molecules which can induce de novo bone formation at orthotopic and heterotropic sites [1]. Recombinant human BMP-2 (rhBMP-2) is a two-chain homodimer, each monomer composed of 114 aminoacids, which is involved in the induction of bone formation in a process that mimics endochondral ossification at ectopic sites in a rat model [2,3]. The use of a carrier matrix to deliver the BMPs is sometimes necessary to delay the rapid dispersion of diffusible BMPs from the implant site [4–6], and to avoid the liver uptake and catabolism since their serum half-live is quite short. Among other inorganic matrices implantologists have recently used calcium phosphate ceramics, made of tricalcium phosphate (TCP) or hydroxyapatite (HA), as carriers of BMPs. Those ceramics have the added ability to attract and concentrate the host's endogeneous BMPs further enhancing the osteoconductive response [7,8]. Therefore,

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the study of the surface adsorption of protein rhBMP-2 onto HA is of great interest in order to understand the adsorbate–adsorbent surface interaction at the molecular level, as well as to evaluate the maximum amount of protein which can be loaded by the HA carrier matrix at physiological conditions. From this viewpoint, the present work is aimed to study the influence of different parameters such as pH, and either calcium, phosphate or NaCl concentrations on the adsorption process of recombinant human mature bone morphogenetic protein 2 (rhBMP-2m) onto HA. Several authors have studied protein adsorption onto HA [9–15], but none of them has used BMP.

2. Materials and methods

2.1. rhBMP-2m

PCR cloning and plasmid construction. The partial hBMP-2 cDNA clone (ID 843398) was provided by IMAGE Consortium [16]. The cDNA sequence of mature peptide encoding aminoacids 282–396 was amplified by PCR (polymerase chain reaction) with two specific oligonucleotids especially designed for hBMP-2m. The oligonucleotides primers corresponded to aminoacids 282–291 (5′ CAA GCC AAA CAC AAA CAG CGG AAA CG 3′) and aminoacids 396–388 (3′ CTA GCG ACA CCC ACA ACC CTC C 5′) (TIB MOLBIOL). PCR was performed with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s. After that, the mixture was incubated at 72 °C for 5 min. The 330 bp ADN amplified by PCR was ligated into pGEM-T-Easy vector (Promega) and sequenced. That vector was then digested with *Eco*RI (Novagen) and the 350 bp insert ligated into an *Eco*RI linearized pET28b (+) expression vector. The plasmid produced was termed PET-BMP and the entire coding identity of this construct was confirmed through DNA sequencing. Then, the insert was cloned in the pET28b expression vector in *Eco*RI restriction enzyme site and transformed it into *Escherchia coli* strains BL21 (DE3) cells.

Protein expression. *E. coli* strains BL21(DE3) cells were transformed with the expression plasmid PET-BMP described above and grown in LB supplemented with 50 μg/ml kanamycin to an OD_{595} of 0.6–0.8. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), cultures were grown for a further 1 h. The bacterial cells were then fractionated by sonication in binding buffer (0.005 M imidazole, 0.5 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane hydrochloride, called Tris–HCl, pH 7.52) using standard methods. The recombinant protein was found primarily in the insoluble fraction (pellet) and it required the inclusion of 6 M urea in all buffers for extraction.

Protein purification and analysis. The N-terminal His-tag region of the recombinant protein allowed its purification using a nickel affinity column. The protein extract for a maximum bacterial culture volume of 500 ml was loaded onto Poly-Prep chromatography columns (Bio-Rad) packed with His-band resin (Novagen) that had been previously charged with 0.05 M $NiSO_4$. Protein was eluted by competition with 0.5 M imidazole-containing elution buffer after several lower-concentration imidazole washes. Its analysis was performed by SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) with Mini Protean Kit (BioRad) using 18% gels run for 150 min at 125 mV. Protein was visualized by silver staining and/or Western-blot plus immunoassay developed with ECL technique. Immunoassay was made using as primary antibody (AB) T7 Tag (1:10,000) (Novagen), and as secondary AB antimouse conjugated to horseradish peroxidase (HRP) (1:2000) (Amersham Pharmacia).

2.2. Hydroxyapatite

Synthesis and characterization. HA crystals were prepared by precipitation in a continuous flow mixed suspension mixed product removal (MSMPR) reactor, mixing 0.8 M $CaCl_2$ and 0.48 M K_2HPO_4 solutions at 85 °C under N_2 inert atmosphere [17]. The pH was monitored and adjusted automatically with KOH 1 M by means of a pH-stat system (pH 9.0 ± 0.1). The precipitate obtained at steady state was filtered with 0.1 μm porous size membranes (Millipore), repeatedly washed with deionized water and dried in an oven overnight. The X-ray diffraction patterns of dry powder, analyzed with a Rigaku Rotaflex RU-200B diffractometer in the $5° \leqslant 2\theta \leqslant 60°$ range, coincide with those of mineral HA (ASTM-9-432). The specific surface area (SSA) determined by the BET (Brunauer–Emmett–Teller) method, using a Micromeritics ASAP 2000 apparatus, was 7.0 m^2/g. The chemical Ca/P composition, determined by induced coupled plasma spectroscopy (ICP) with a Thermo Jarrell Ash Mark IV instrument, was 1.68 ± 0.05, that is, stoichiometric HA. Scanning electron microscopy observations with a Hitachi S-570 microscope showed aggregates of crystals. Most HA crystals were plates (0.5 μm × 0.03–0.05 μm) which appeared as needles side on showing the (1 0 0) face. The mean size of these aggregates, characterised by laser light scattering with a Coulter LS 130 instrument was 9 μm.

2.3. Adsorption experiments

The rhBMP-2m solubilized and purified in a TE-U buffer (6 M urea, 0.5 M imidazole, 0.5 M NaCl, 0.02 M Tris–HCl, pH 7.52) was dialysed until 2 M urea and eliminating imidazole. The pH and the concentra-

tion of salt were adjusted as required in each experiment. Prior to each run HA was sterilised at 200 °C for 2–3 h in order to avoid the presence of proteases, which could degrade the protein.

Time profiles. 10.0 ± 0.1 mg of HA were weighed in 2 ml tubes and equilibrated at 37 °C during 1-2 h. 1 ml of protein solution (2 M urea, 0.5 M NaCl, 0.02 M Tris–HCl, pH 7.52) was added to each tube, manually and carefully resuspended, and stirred during a maximum of 360 min in a basculant mixer at 37 °C. Time zero was taken when protein solution and HA were in contact. Tubes were withdrawn after 0, 0.5, 1, 1.5, 2, 3, 4, 5, 10, 15, 20, 30, 45, 60, 120, 180, 240, 300 and 360 min. The samples were then centrifuged at 13,000 rpm. during 30 s, the supernadant was separated with a micropipette and both fractions stored at −80 °C until analysis.

Adsorption isotherms. For each experiment a buffered solution called TA, with the same salt concentration and same pH as the protein solution, was prepared. So, when both solutions were mixed in 2 ml tubes, only protein concentration varied. After mixing, 1 ml of each tube was stored at −80 °C to measure initial protein concentration, and the other 1 ml was equilibrated during 1–2 h at 37 °C. 10.0 ± 0.1 mg of sterilized HA were weighted in 2 ml tubes and equilibrated at 37 °C during 1–2 h. In the following step, 1 ml of the incubated solution was added to each tube containing HA, manually and carefully resuspended, and stirred during 4 h in a basculant mixer at 37 °C. Time zero was taken when the protein solution and the HA were in contact. Tubes were centrifuged at 13,000 r.p.m. during 30 s, supernatant was separated with a micropipette and both fractions stored at −80 °C until analysis. The pHs, salts, and concentrations investigated fall within the following ranges: pH (7.02–8.41), NaCl (171.4-500 mM); $CaCl_2$ (0–100 mM); Na_2HPO_4 (0–100 mM).

Characterization. The initial protein concentration in solution (C_0), as well as the equilibrium concentration after adsorption (C), was measured using the Bradford method [18]. The adsorbed amount of protein onto HA, Q (mol/m^2), was calculated by difference between both the initial and equilibrium amount of protein divided by the specific surface area of the adsorbent (HA). Other characterization methods of the adsorption products were those used for HA.

3. Results

3.1. Adsorption kinetics

The adsorption kinetic, Q versus time, of rhBMP-2m onto HA at 37 °C, is represented in Fig. 1. It is seen that adsorption proceeds rapidly during the first minutes, but the plateau or adsorption equilibrium is reached after approximately four hours from the start of the experiments.

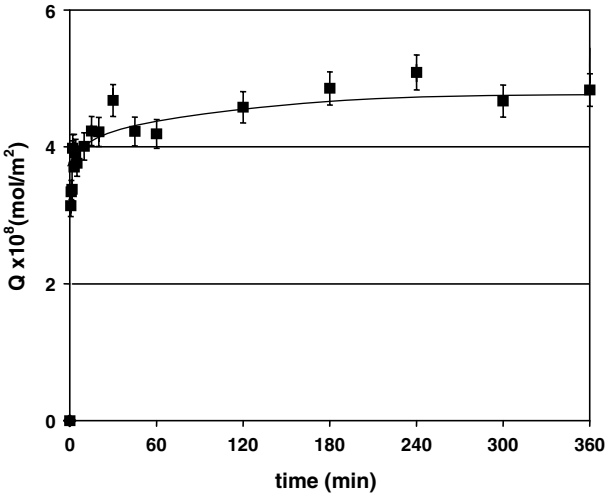

Fig. 1. Adsorption of rhBMP-2m onto HA versus time at 37 °C, pH 7.52 and 0.5 M NaCl (■).

3.2. Influence of NaCl concentration, Ca^{2+} and phosphate

The experiments at different NaCl concentrations (different ionic strength I), exp. nos. 2 and 5–7 in Table 1, show a more rapid tendency to reach the maximum coverage at low values of the NaCl concentration (Fig. 2(a)). The analysis of these experiments indicates that a Langmuir isotherm fit well the adsorption data (Fig. 2(c)). A linearized expression of the Langmuir isotherm is

$$C/Q = C/N + 1/(NK), \quad (1)$$

where Q and C indicate the adsorbed amount of protein per surface unit of adsorbent and the concentration at equilibrium, respectively, and the parameters K and N represent the adsorption constant (affinity constant) and the number of binding sites, respectively. N is dimensionally expressed as adsorbed amount of protein per surface unit. The K and N parameters of this isotherm are tabulated in Table 1. A slight increase in the adsorption constant K is observed when the NaCl concentration increases from 170 to 390 mM, followed by a decrease at the highest value of 500 mM. The influence on N is small, but a slight increase is observed when the NaCl concentration increases.

Experiments at different calcium concentrations in solution when maintaining the same NaCl concentration and the same pH (exp. nos. 2 and 8–10 in Table 1) show a notable influence of Ca^{2+} on the rhBMP-2m adsorption, even at low Ca^{2+} concentrations (Fig. 2(b)). It can also be observed that further increases in Ca^{2+} concentration are of less influence. The adsorption data fit well to a Langmuir isotherm (Fig. 2(d)). The values of

Table 1
Experimental conditions and values of the parameters N and K calculated from the corresponding Langmuir adsorption isotherm at 37 °C

Experiment	pH	NaCl (mM)	$CaCl_2$ (mM)	Na_2HPO_4 (mM)	I (mM)	$N \times 10^8$ (mol/m^2)	$K \times 10^{-6}$ (M^{-1})	Correlation coefficient
1	7.02	500	–	–	500	6.3	0.96	0.991
2	7.52	500	–	–	500	7.8	0.57	0.996
3	8.18	500	–	–	500	9.2	0.28	0.995
4	8.41	500	–	–	500	11.5	0.24	0.994
5	7.52	171.4	–	–	171	6.7	4.4	0.981
6	7.52	281.0	–	–	281	7.4	6.1	0.996
7	7.52	390.5	–	–	390	7.1	8.4	0.997
8	7.52	500	10	–	530	6.4	12.4	0.998
9	7.52	500	50	–	650	6.4	22.6	0.999
10	7.52	500	100	–	800	6.0	18.3	0.999
11	7.52	500	–	10	530		No adsorption	
12	7.52	500	–	50	650		No adsorption	
13	7.52	500	–	100	800		No adsorption	

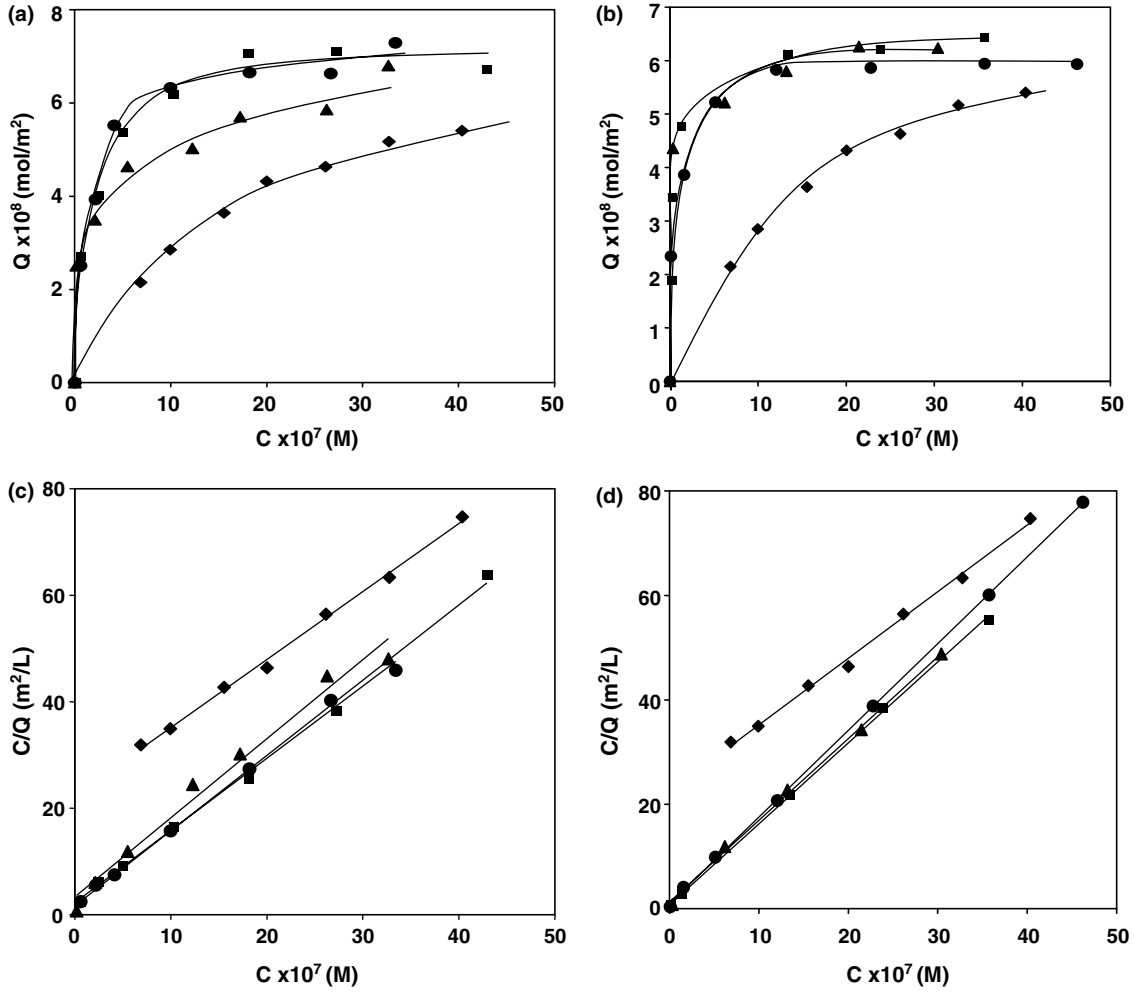

Fig. 2. Adsorption isotherms Q vs C of rhBMP-2m onto HA. (a) Variation of NaCl concentration: ▲, 171.43 mM NaCl; ●, 280.95 mM NaCl; ■, 390.48 mM NaCl; ◆, 500 mM NaCl. (b) Variation of calcium, at constant NaCl concentration of 500 mM: ◆, 0 mM $CaCl_2$; ▲, 10 mM $CaCl_2$; ■, 50 mM $CaCl_2$; ●, 100 mM $CaCl_2$. Plots of the corresponding Langmuir isotherms C/Q vs C: (c) variation of NaCl concentration, (d) variation of calcium. The experiments were carried at 2 M urea, 0.02 M Tris–HCl, pH 7.52 and 37 °C.

the parameters K and N are shown in Table 1. The K values present an important increase in the presence of added Ca^{2+}, when maintaining the same NaCl concentration and the same pH, meanwhile its influence on N is small, with a slight decrease when the calcium concentration increases.

Experiments at different phosphate concentrations in solution (exp. nos. 11–13 in Table 1) show that the pres-

ence of this anion in solution at the studied concentrations inhibits the rhBMP-2m adsorption at detectable levels. These effects of calcium and phosphate ions in solution have been observed for the adsorption of other proteins onto HA [9,13,15,19] as well as for aminoacids onto calcium phosphates [20]. However, in our experiments the influence of both calcium and phosphate is stronger.

3.3. Influence of pH

The analysis of the experiments carried out at different pH values (exp. nos. 1–4), ranging from 7 to 8.4, indicates that the Langmuir isotherm can also be used to predict the adsorption data (Fig. 3(a)), the isotherm parameters being indicated in Table 1. It is observed that the parameter K decreases when the pH increases, while N increases (Fig. 3(b)).

In Fig. 4 are plotted the values of free energy change, ΔG, enthalpy, ΔH, and entropy, ΔS, of the adsorption process calculated according to the expressions used by Moreno et al. [14] and using the Langmuir isotherm. These values indicate that adsorption of rhBMP-2m onto HA is an exothermic process, being less favourable when Q increases. Considering that the calculated $\Delta S < 0$, we can affirm that the adsorption process is driven by the change of enthalpy. The values of ΔH (around -60 kcal/mol) also point to a chemisorption process (usually $\Delta H < -10$ kcal/mol) rather than a physical adsorption (usually $\Delta H > -10$ kcal/mol).

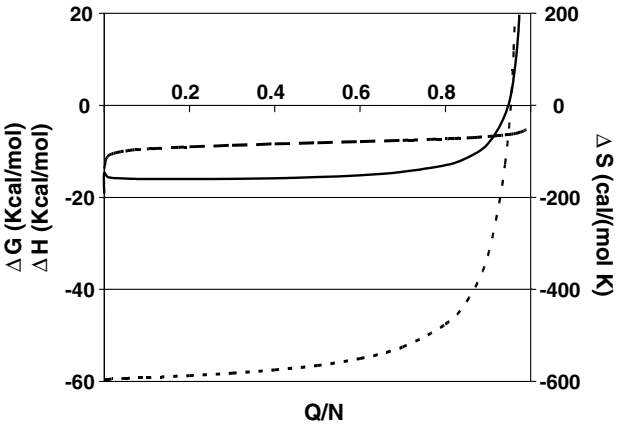

Fig. 4. Plot of ΔG (kcal/mol) ––, ΔH (kcal/mol) ---, and ΔS (cal/(mol K)) —, for the adsorption of rhBMP-2m onto HA at 37 °C.

4. Discussion

The results of the adsorption experiments of rhBMP-2m onto HA reported in the present work show a dependence of the adsorption data on pH, NaCl concentration, and especially on the addition of calcium and phosphate ions in solution. The adsorption data fit well to a Langmuir isotherm, a behaviour similar to that presented by other proteins and aminoacids. In Table 2 we show the values of N and K of the Langmuir isotherm reported for several proteins. It is observed that the affinity constants K for rhBMP-2m onto HA 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 slightly lower than the affinity constant of poly-L-ASP, and lower than those of statherin and PRP1, a salivary proline-rich phosphoprotein, adsorbed onto HA under similar experimental conditions to those used in the present work. On the other hand, the values of K for rhBMP-2m onto HA are higher than

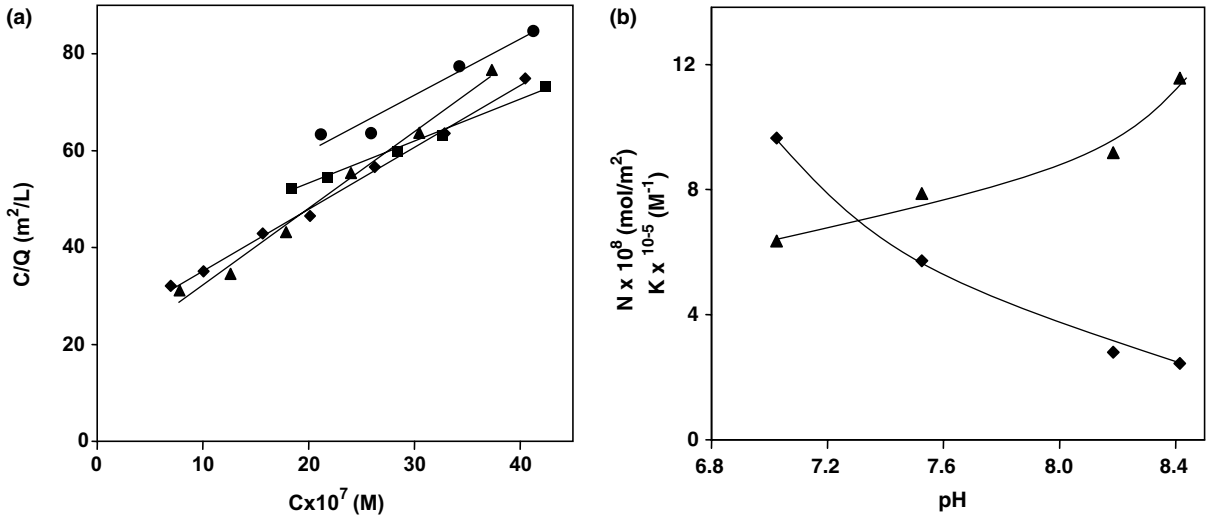

Fig. 3. (a) Plots of the Langmuir isotherms C/Q vs C for adsorption of rhBMP-2m onto HA at different pHs: ▲, pH 7.02; ◆, pH 7.52; ●, pH 8.18; ■, 8.41. (b) Variation of K (◆) and N (▲) for the adsorption of rhBMP-2m onto HA at different pHs.

Table 2
Values of parameters N and K for the adsorption of several biomolecules

Adsorbent	Adsorbate	N (mol/m^2)	K (M^{-1})	Reference
HA	rhBMP-2	$3.8–11.5\times10^{-8}$	$2.4–230\times10^{5}$	This work
HA	BSA	$23.4–42.1\times10^{-9}$	$0.3–160\times10^{5}$	[12]
HA	BSA	$6.0–7.1\times10^{-7}$ mol/g HA*		[9]
HA	BSA	$8.7–26.1\times10^{-9}$	$0.1–1160\times10^{5}$	[15]
HA	Catalase	50–84 mg/g*		[19]
HA	Succinylated LSZ	$1.2–8.2\times10^{-8}$	$0.1–10\times10^{5}$	[13]
HA	Poly-L-ASP	9×10^{-8}	15.7×10^{5}	[14]
HA	Statherin	80×10^{-8}	211×10^{5}	[14]
HA	PRP1	20×10^{-8}	260×10^{5}	[14]
TiO_2	Cyt-C		1.0×10^{5}	[22]
TiO_2	Hb		1.7×10^{5}	[22]

those of biomolecules such as cytochrome-*c* (Cyt-C) or hemoglobin (Hb) onto TiO_2 films, a different adsorbent.

Several kinds of adsorbate–adsorbent interactions can take place during protein adsorption, for instance covalent bond, H-bond, electrostatic or hydrophobic. The relative predominance of one of them over the others will depend on the protein structure and the nature of the surface of the adsorbent. For pure HA, it seems reasonable that hydrophobic interactions can be discarded and that covalent and electrostatic interactions [23] will take place.

The test of Scatchard [21] has been performed to observe the presence or absence of cooperative effects on the protein adsorption [24,25]. The plots of Q/C vs Q for the different experimental conditions indicate good linearity, and consequently no appreciable cooperative effects. As an example, Fig. 5 shows the plots of the Scatchard test for the experiments carried out at different pHs.

The HA surface contains Ca and P sites, related to emerging calcium and phosphate ions in the surface

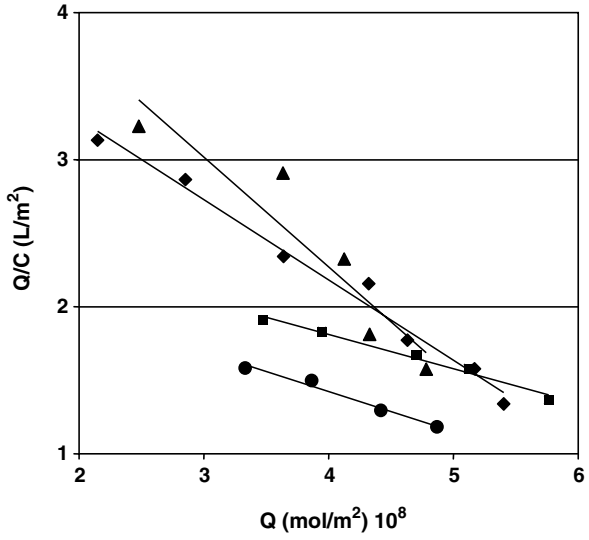

Fig. 5. Plots of the scatchard test Q/C vs Q for adsorption of rhBMP-2m onto HA at different pHs: ▲, pH 7.02; ◆, pH 7.52; ●, pH 8.18; ■, 8.41.

structure. In aqueous solution hydroxylated species form on these sites [26,27], with the corresponding surface equilibrium given by Eqs. (2) and (3), where S(HA) represents the HA surface

$$S(HA)–CaOH + H^+ \leftrightarrows S(HA)–Ca(OH_2)^+ \quad (2)$$

$$S(HA)–POH \leftrightarrows S(HA)–PO^- + H^+ \quad (3)$$

Since the ionic species in solution such as H^+, OH^-, Na^+, Cl^-, Ca^{2+} and HPO_4^{2-}/PO_4^{3-} influence these surface equilibria as well as the surface complexation, the net surface charge of the HA will depend basically on the surface structure, pH and the concentration of the mentioned ions in solution. There exist a wide range of point of zero charge, zpc, values reported in the literature. Nevertheless, most of them are situated within the acidic and nearly neutral pH range [24,26,28].

Yin et al. [9] found that, for HA of similar characteristics to that used in the present work, the ξ-potential, at whatever explored pH, always exhibited negative values, except when the authors added calcium ions to the solution. Kawasaki et al. [29] also reported a negative ξ-potential value for HA, even in the presence of adsorbed proteins. These results can be interpreted by the predominance of phosphate or $–PO^-$ species in the surface.

To better explain the HA surface behaviour as a function of pH, we can draw the surface structure as a mixture of the model of surface complexation (Fig. 6(a)) and the model of surface hydroxylation (Fig. 6(b)). Calculations using the values reported by Wu et al. [26] for the equilibrium (2) and (3), indicate that at the pHs used in our work an small predominance of negative surface phosphate over positive calcium species exist. Then, a small net negative surface charge is expected.

From the point of view of the adsorbate, the rhBMP-2m presents acidic and basic residues, whose ionization depends on the solution pH. The isoelectric point (IEP) of rhBMP-2m is around pH 7.9, calculated with the program GCG (Genetics Computer Group, Inc. Madison, WI, USA). The fact that the working pH values are close to the IEP of the protein leads to small net charge

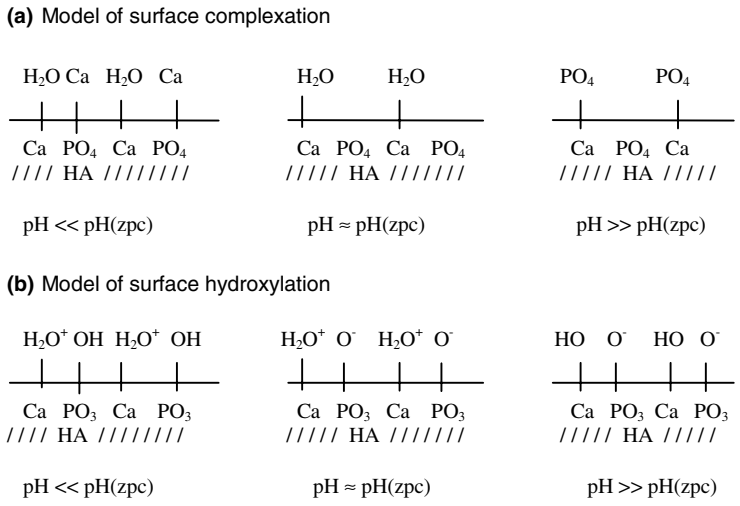

Fig. 6. Models for HA surface: (a) model of surface complexation, (b) model of surface hydroxylation.

in the protein, and then the electrostatic interactions will be of less importance.

Phosphate anions in solution compete with carboxylate groups of the protein for adsorption on Ca sites. When phosphate is added, it adsorbs on Ca sites replacing hydroxylated species (Fig. 6(b)), and the surface charge becomes more negative (Fig. 7(b)), and though the global charge of the protein is slightly positive, the adsorption is inhibited. Smith et al. [30] have observed by chemical force microscopy (CFM) that in the presence of phosphate ions the adsorption of BSA onto HA is inhibited. However, they observed that some molecules remain adsorbed, although not detectable macroscopically, in specific sites. On the other hand, when Ca^{2+} ions are added in solution, they adsorb on phosphate sites of the HA surface (Fig. 7(a)) allowing a more favorable protein adsorption through the carboxylate groups. Although the HA surface charge becomes positive [9] and the global charge of the protein is slightly positive, so the global electrostatic interaction is unfavorable, an increase in adsorption is observed. Then, the adsorption results indicate that rhBMP-2m adsorbs or binds preferably on the Ca sites, and through the carboxylate groups of the acidic residues. This type of interaction has also been proposed by other authors [11] dealing with protein adsorption on HA.

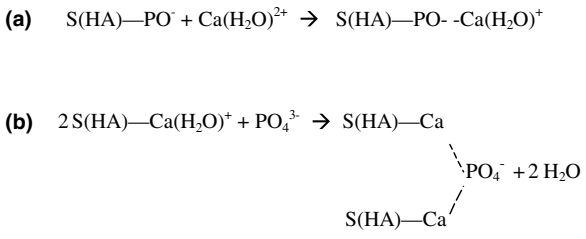

Fig. 7. Adsorption of calcium and phosphate ions onto HA surface.

The influence of the NaCl concentration, at pH 7.52, on the adsorption process can be related to the double layer structure around the HA surface and the protein. At this pH, there is a positive diffuse layer surrounding the negative HA surface, and a negative diffuse layer surrounding the positive protein. When the NaCl concentration increases (the ionic strength I increases) there is a thinning of the double layer and consequently a more favorable protein–surface interaction. But at high I the diffuse layer practically disappears, and it must be expected that the influence of the layer of chloride anions specifically adsorbed on both the HA surface and the protein would increase. Consequently, the repulsion effects tend to be higher, leading to a lower value of the affinity constant K.

With reference to the influence of pH on the adsorption process, the values of the K parameter indicate a stronger favorable interaction at lower pHs. Since the IEP for the expressed rhBMP-2m is situated at a pH around 7.9, rhBMP-2m presents a positive charge for the two lower pHs, and a negative charge for the two higher ones. At the same time, HA presents a negative surface charge, which is increasing with the rise of pH [9]. According to Eq. (2) and Fig. 6(b), at higher pHs there is a decrease in the proportion of positive surface species $-Ca(OH_2)^+$ as well as an increase in the proportion of negative ones $-PO^-$, which leads to a higher negative surface charge. Then, at the lower pH values there is a more favorable electrostatic interaction between rhBMP-2m and HA, which is reflected in the higher K values.

The rhBMP-2m molecule contains a region with a high concentration of basic residues (Fig. 8). At pH < IEP these residues are protonated and therefore

MGSS**HHHHHH**SSGLVP**R**GS**H**MASMTGGQQMG**R**<u>**D**</u>PNSIQA**KHK**Q
RKRL**K**SSC**KRH**PLYV<u>**D**</u>FS<u>**D**</u>VGWN<u>**D**</u>WIVAPPGY**H**AFYC**H**G<u>**E**</u>CPFPL
A<u>**D**</u>**H**LNSTN**H**AIVQTLVNSVNS**K**IP**K**ACCVPT<u>**E**</u>LSAISMLYL<u>**DE**</u>N<u>**E**</u>**K**
VVL**K**NYQ<u>**D**</u>MVV<u>**E**</u>GCGC**R***

H, K, R: free amino group
***D*, *E*: free carboxyl group**

Fig. 8. Aminoacid sequence of the protein rhBMP-2m showing aminoacids with both residual $-COO^-$ groups (***D***, ***E***) and $-NH_3^+$ groups (**H**, **K**, **R**).

can form H-bridges with the HA surface. These interactions are favored when the protein displays a more extended conformation. As a consequence, this fact can also contribute to the observed increase of K value at the lower pHs.

The observed effects of the studied parameters on the adsorption process could give insights on how to prepare rhBMP2-HA composites able to release the protein fraction at the implant site. However, more experiments must be carried out to study the effects of urea on rhBMP-2 conformation, and then on the adsorption of rhBMP-2 on HA under physiological conditions. Nevertheless, BMPs extracted with a chaotropic agent have proved to be able to induce new bone formation [31–33].

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