***Journal of the Argentine Chemical Society***

# SYNTHETIC HYDROXYAPATITE AS A SURFACE MODEL OF DENTAL ENAMEL AND DENTINE

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*Received October 8, 2008. In final form December 22, 2008.*

***Dedicated to Dr. Enrique Baran, who taught us the importance of perspiration in scientific work, but failed, in spite of his efforts, to pass on us his methodical attitude.***

## Abstract

The interaction of powdered enamel and dentine with bacteria is compared with the same interplay on synthetic hydroxyapatite (HAP). The surface characteristics of the three materials were

studied using electrophoretic measurements and EDAX. The effect of $Ca^{2+}$ on the mobilities of the biomaterials was measured and explained in terms of a simple surface complexation model involving $\equiv Ca_5(PO_4)_3^+$ and $\equiv Ca_4(PO_4)_3^-$ as the key surface species. The effect of $Ca^{2+}$ on bacterial adhesion was determined and interpreted in terms of the bridging between bacterial and biomaterial surfaces by $Ca^{2+}$.

**Keywords:** hydroxyapatite, enamel, dentine, electrophoretic mobility, bacterial adsorption.

## Resumen

En el presente trabajo se compara la interacción entre bacterias y polvos de esmalte y dentina con la existente entre bacterias e hidroxiapatita sintética (HAP). Se estudiaron las características superficiales de los tres materiales por medio de medidas de movilidad electroforética y EDAX. Se determinó la influencia de $Ca^{2+}$ sobre las movilidades de los biomateriales y se explicó el efecto observado en términos de una simple complejación superficial que involucra las especies $\equiv Ca_5(PO_4)_3^+$ y $\equiv Ca_4(PO_4)_3^-$. Se evaluó el efecto del $Ca^{2+}$ en la adhesión bacteriana y se lo interpretó considerando la formación de puentes de $Ca^{2+}$ entre la superficie bacteriana y la del biomaterial.

**Palabras clave:** hidroxiapatita, esmalte, dentina, mobilidad electroforética, adsorción bacteriana.

## Introduction

The first stage of interaction of a dental piece with the mouth fluids leads to the formation of the *acquired pellicle* through the adsorption onto the enamel surface of inorganic and organic substances (mainly low molecular weight proteins). The *biofilm*, main etiologic caries agent, develops onto this film. The biofilm is a complex structure strongly attached to the dental surface, formed by a large number of microorganisms closely clustered and intermixed with extracellular abiotic substances from bacteria, salivary and dietary sources [1],[2].

Hard tissues (bones and dental pieces) are complex structures built around an inorganic phase closely related, both structurally and chemically, to calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [3]-[7]. Thus, hydroxyapatite becomes a material of choice not only as a potential biomaterial; it is also assumed that HAP is a good model for studying the biogenic hydroxyapatites behavior in the presence of bacteria. In the present study we explore the validity of this assumption.

Several papers in the literature describe the interaction of various bacteria with synthetic biomaterials [8]-[10], but the validity of using synthetic HAP as a model for both enamel and dentine is to be proven. Although HAP accounts for *ca* 95 % of enamel, and 67-70 % of dentine [11],[12], the surface properties of both dentine and enamel may be drastically altered, even though enamel contains a small fraction of other constituents. This paper compares the surface composition, the electrophoretic mobilities, and the bacterial adhesion behavior of synthetic microcrystalline HAP, enamel and dentine.

## Experimental

### *Materials*

Samples of microcrystalline powdered of synthetic hydroxyapatite were provided by the Instituto de Ciencias de Materiales de Barcelona, Spain, and synthesized by the precipitation method. Specific surface area of 23.5 $m^2\ g^{-1}$ was measured by $N_2$ physisorption at liquid nitrogen temperature (BET), using Micromeritics AccuSorb 2100 E equipment.

The natural HAPs samples were obtained by abrasion of two healthy dental elements, free of cavities and not subjected to dental repair. Diamond stones and low speed drills operated at low pressure were used to obtain more uniform and smaller particles. Further size reduction was achieved by grinding in an agate mortar and sorting the particles using a bronze mesh 100 sieve to produce particles less than 150 μm and a stainless steel mesh 400 sieve to obtain particles smaller than 36 μm.

Bacterial adhesion was studied using *Lactobacillus casei* ATCC27304 from the oral cavity.

### *Surface observation and composition*

Twenty five μL aliquots of suspensions of natural and synthetic HAP samples in deionized water were placed on an aluminium holder and gently evaporated under warm air. After metallization with Ag, the samples were observed in a Philips 515 scanning electron microscope; elementary surface composition was explored with an EDAX Falcon PV 8200 probe.

### *Electrophoretic mobilities*

Aqueous suspensions of HAP were conditioned in the dark in a closed vessel free of $CO_2$ for 15 days under soft stirring at different pH values, maintaining a constant ionic strength. The pH values were adjusted at 48 h intervals. If required, adequate amounts of calcium, lactate or fluoride were added to diluted suspensions, and the electrophoretic mobilities were measured in a Rank Brothers apparatus (Cambridge, UK) with a micro cylindrical cell immersed in a thermostatic bath at 25ºC.

### *Bacterial adhesion*

Twenty mg synthetic HAP or powdered enamel were suspended in 1 mL of $KNO_3$ solution (0.01 mol $dm^{-3}$); after equilibration for 15 h, further equilibration for 2 h, either with sterile natural diluted (1:1) saliva or with aqueous solutions of adequate composition. Equal volumes of HAP suspensions and bacterial culture (12 h in steady growth phase, containing about $2 \times 10^8$ CFU), were mixed for 2 h at 28ºC. The suspension was centrifuged (600 *g*), separating the solid composed of HAP and adhered bacteria; the supernatant containing non-adhered bacteria was discarded. The solid was re-suspended in 0.1 % peptone water and stirred slowly for one minute. The number of adsorbed cells was determined by the successive dilutions and count in agar media.

## Results and discussion

### *Surface composition*

Figure 1 shows the EDAX spectra of the powered samples of synthetic HAP, enamel and dentine. The peaks at 2.0 and 3.7 keV demonstrate that the relationships Ca/P for the synthetic sample (1.65) and enamel (1.66) are very close to the theoretical value (1.67), whereas the ratio is much higher in dentine (1.99). This high value can be attributed to the substitution facility (especially by carbonates). It is accepted that the structural materials in organisms can accept rather large degrees of ionic substitution without changing appreciably their structure and crystal chemistry [4]. The large peak at 3 keV is due to Ag used in the metallization.

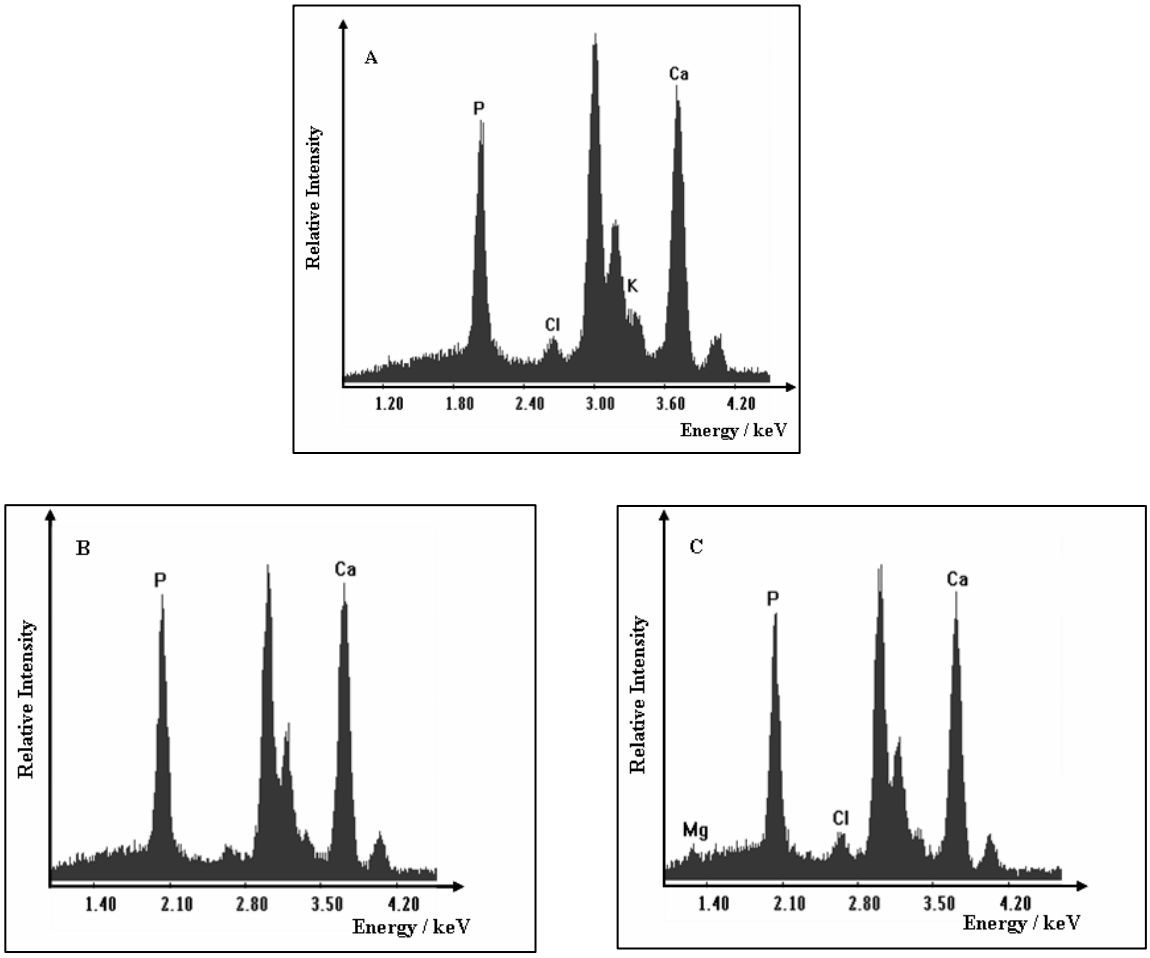


**Figure 1.** The EDAX spectrum of synthetic hydroxyapatite (A), dental enamel (B) and dentine (C).

### *Electrophoretic mobilities*

The HAP/solution interface has been the subject of many studies [13]-[16]; Kambara *et al.* reported measurements of electrophoretic mobilities of enamel [17]. Somasundaran *et al.* [13] demonstrated early in 1968 that $Ca^{2+}$, $PO_4^{3-}$ and $HO^-$ are the potential determining ions of HAP, and Saleeb and de Bruyn [14] lead to a whole set of possible points of zero charge. García Rodenas *et al.* [18] described the electrophoretic behavior of HAP suspensions using a simple surface complexation model. It was assumed that, except at high pH, surface $HO^-$ are rapidly and totally released, leaving a basic surface structure described as $\equiv Ca_5(PO_4)_3^+$ species. It was further assumed that phosphate ions are not labile, and that the surface charge is defined by the equilibria involving calcium exchange, phosphate protonation and, at high pH, $HO^-$ fixation, as described in equations (1)-(3).

$$\equiv Ca_5(PO_4)_3^+ = \equiv Ca_4(PO_4)_3^- + Ca^{2+} \quad ;K_{Ca} \quad (1)$$

$$\equiv Ca_4(PO_4)_3^- + H^+ = \equiv Ca_4(PO_4)_2(PO_4H) \quad ;K^{-1}_{H} \quad (2)$$

$$\equiv Ca_5(PO_4)_3^+ + HO^- = \equiv Ca_5(PO_4)_3(OH) \quad ;K^{-1}_{OH} \quad (3)$$

In this way, for a given pH value, the surface charge is only a function of the solution $Ca^{2+}$ concentration. By using the Gouy-Chapman model [19] of the double layer, García Rodenas *et al.*

[18] could calculate surface potential of synthetic HAP as a function of the pH and $Ca^{2+}$ concentration, and obtained an excellent agreement with the experimental curves in the range $5.5 \leq pH \leq 8.5$. The values for the constants, taken from our earlier work, are $K_{Ca} = 4.38 \times 10^{-4}$ mol dm$^{-3}$, $K_H = 1.78 \times 10^{-7}$ mol dm$^{-3}$, $K_{OH} = 2.82 \times 10^{-5}$ mol dm$^{-3}$.

Figure 2 shows the ζ-potentials, derived from values of the measured mobilities in enamel and dentine suspensions. The continuous curve is taken from the earlier work on synthetic HAP; the very good agreement demonstrates that the García Rodenas *et al.* model [18] applies also to both enamel and dentine. The ζ-potentials, and hence the mobilities, are consistently negative; in terms of equation (1), $\equiv Ca_4(PO_4)_3^-$ prevails over $\equiv Ca_5(PO_4)_3^+$, as expected from the value of $K_{Ca}$ for moderate values of calcium concentrations.

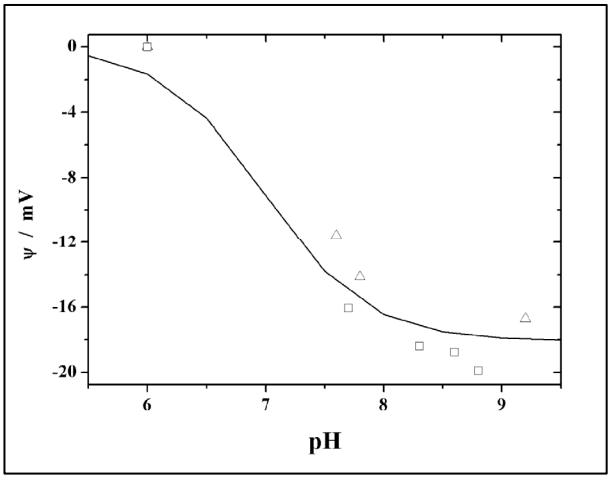


**Figure 2.** ψ potential of enamel (△) and dentine (□). Continuous curve was calculated from ref. [18].

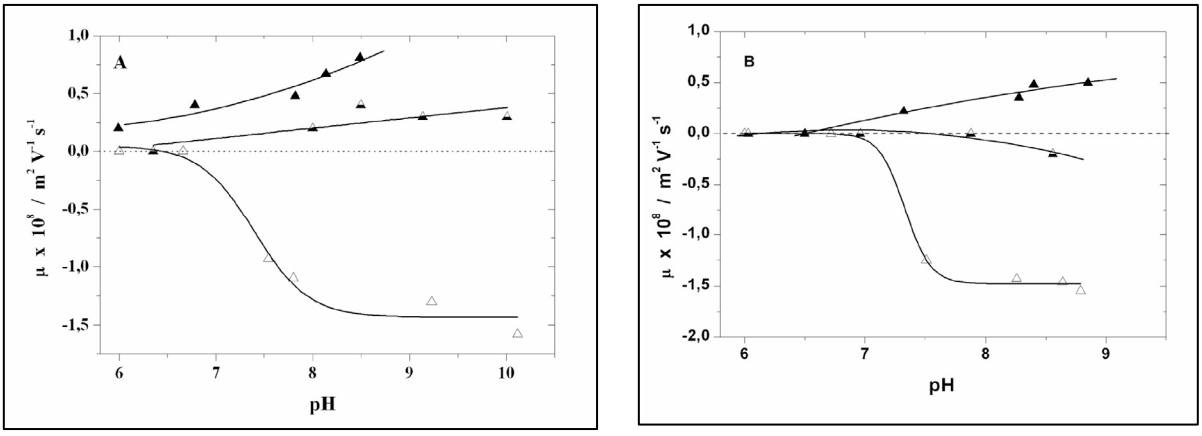


**Figure 3.** Electrophoretic mobility vs pH profiles of dental enamel (A) and dentine (B) at various $Ca^{2+}$ concentrations. $I = 1 \times 10^{-2}$ mol dm$^{-3}$. $[Ca^{2+}] = 0$ (△), $1 \times 10^{-3}$ (▲) and $1 \times 10^{-2}$ (▲) mol dm$^{-3}$. The data correspond to three measurements with a SD ≤ 3 %.

Figure 3 shows the changes in the mobility of enamel and dentine brought about by the addition of $Ca^{2+}$ salts. High levels of $Ca^{2+}$ are required to reverse the mobility sign (and hence the surface charge). At $[Ca^{2+}]$ = 0.001 mol $dm^{-3}$, the mobilities are very low, close to zero. Another interesting feature is the increase of the mobility with increasing pH in suspensions containing $[Ca^{2+}]$ = 0.01 mol $dm^{-3}$. This feature is qualitatively described by the simple model developed earlier for HAP [18]. The differences with synthetic HAP are probably due to the more passive surfaces of the biomaterials, which make slow conditioning phenomena more important.

The influence of other ions was also explored. Figure 4 shows the mobilities of all samples in the presence of lactate (a typical metabolic product of oral micriobota) and fluoride (used commonly in topic treatments). Except for high lactate concentrations with synthetic HAP and enamel (where negative charge increases slightly), the mobility is not influenced by these ions, demonstrating that electrostatic interactions prevail and define a low affinity.

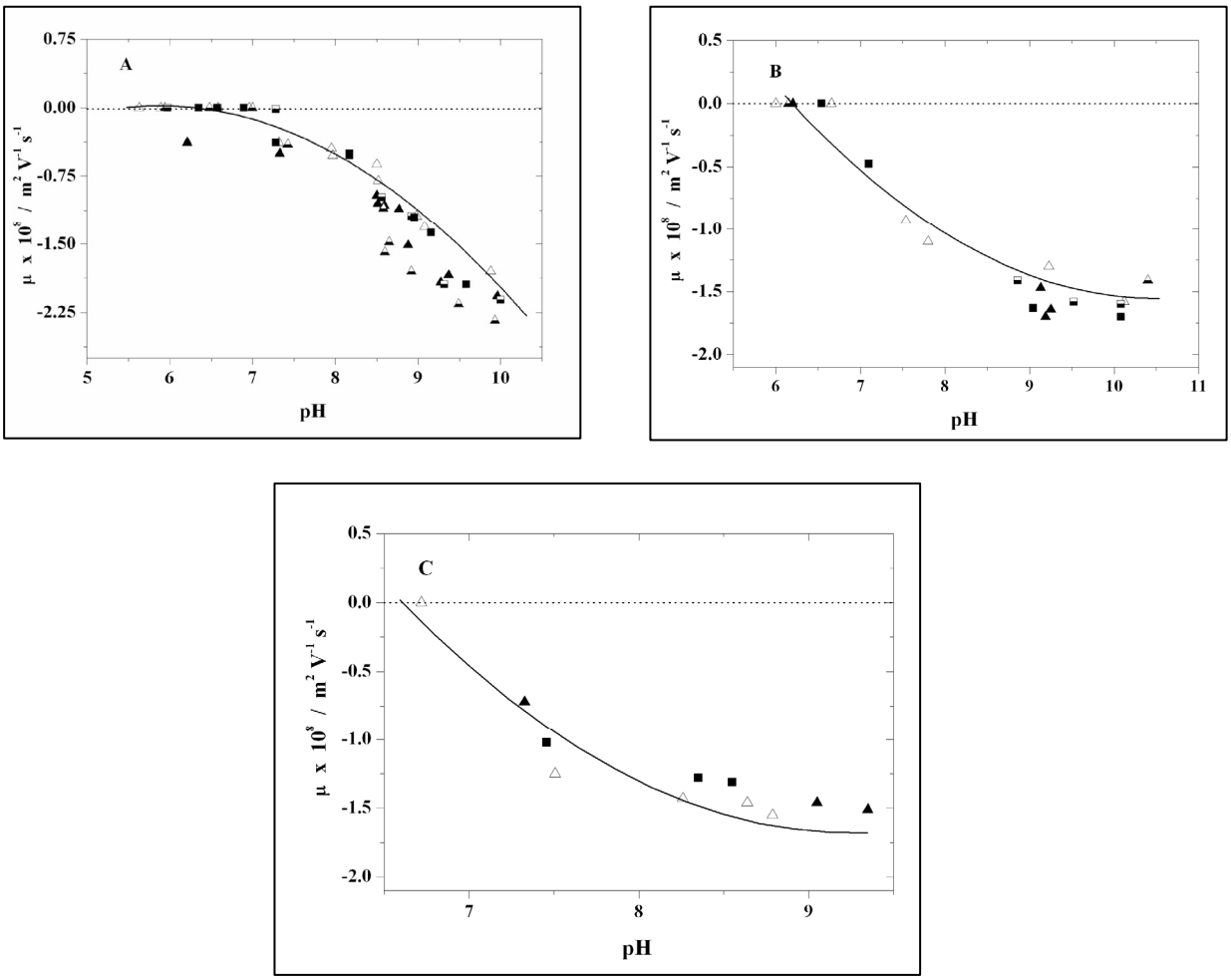


**Figure 4.** Electrophoretic mobility vs pH profiles of synthetic HAP (A), dental enamel (B) and dentine (C) at various lactate and fluoride concentrations. I = 1 × $10^{-2}$ mol $dm^{-3}$. $[Lac^-]$ and $[F^-]$ = 0 (△), $[Lac^-]$ = 1 × $10^{-3}$ (▲) and 1 × $10^{-2}$ (▲) mol $dm^{-3}$, $[F^-]$ = 1 × $10^{-3}$ (⬓) and 1 × $10^{-2}$ mol $dm^{-3}$ (■). The data correspond to three measurements with a SD ≤ 3%.

### *Bacterial adhesion to HAP surface*

In a previous work [20], the influence of $Ca^{2+}$and bacterial hydrophobicity on synthetic HAP adhesion was studied. Now, we present a comparison of the adsorption of a hydrophilic bacterium

(*L. casei* ATCC27304) onto synthetic HAP and powdered enamel. In ref [20] we have demonstrated, by means of tests of electrophoretic mobility, that the negative surface charge of several bacteria became more positive, and the sign can be reverted by the addition of ion $Ca^{2+}$. This behavior is also shown in the new strain, but in this case the sign is negative in all the studied concentrations (Figure 5). This effect must be attributed to a greater presence of carboxilate groups in the bacterial membrane. The importance of the charge surface in the adsorption process will be discussed below.

Table 1 shows the ratios of (adsorbed)/(non adsorbed) bacteria, in absence and in presence of exogenous $Ca^{2+}$ ($10^{-3}$ mol $dm^{-3}$).

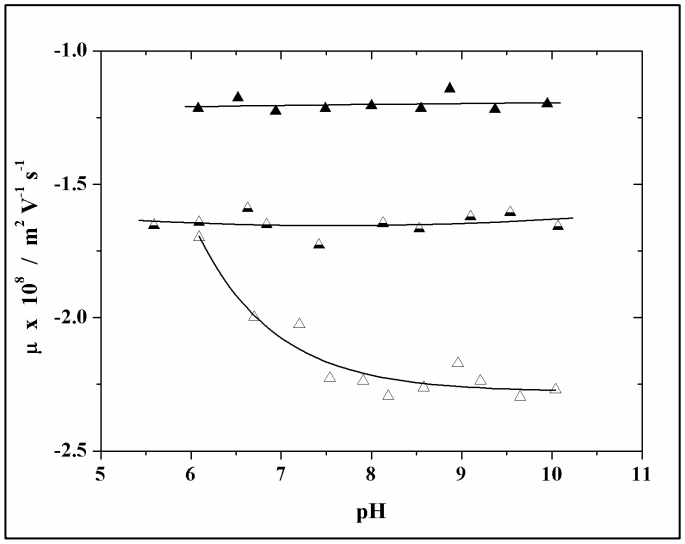


**Figure 5.** Electrophoretic mobility vs pH profiles of *L. casei* ATCC27304 at various $Ca^{2+}$ concentrations. I = 1 × $10^{-2}$ mol $dm^{-3}$. [$Ca^{2+}$] = 0 (△), 1 × $10^{-3}$ (⛰) and 1 × $10^{-2}$ (▲) mol $dm^{-3}$. The data correspond to three measurements with a SD ≤ 3 %.

**Table 1.** Ratios of adsorbed and non adsorbed bacteria, and of bacterial adsorption percentage on synthetic HAP and powdered enamel in absence and presence of exogenous $Ca^{2+}$.

| **Substrate** | **Adsorbed / non Adsorbed Bacteria** | | **Adsorbed Bacteria Percentage** | |
|---|---|---|---|---|
| | **No added $Ca^{2+}$** | **$[Ca^{2+}]_{added}$ 1 × $10^{-3}$ mol $dm^{-3}$** | **No added $Ca^{2+}$** | **$[Ca^{2+}]_{added}$ 1 × $10^{-3}$ mol $dm^{-3}$** |
| **Synthetic HAP** | 1.6 | 1.6 | 61.5 | 61.5 |
| **Enamel** | 1.3 | 1.9 | 55.5 | 65.5 |

The affinity is high in both cases, as expected for a hydrophilic strain [20]**.** The main difference between adhesion on synthetic HAP and on enamel, is the effect of $Ca^{2+}$. Whereas added $Ca^{2+}$ does not influence the adhesion on synthetic HAP, it modulates the extent of adhesion on enamel. The lack of influence of $Ca^{2+}$ in the first case was traced earlier to a high availability of this ion in the interface of a hydrophilic bacterium and water. In the case of enamel, the effect of $Ca^{2+}$ on adhesion suggests that the much more passive enamel surface is modified by this ion, providing more anchorage sites of the type $\equiv Ca_5(PO_4)_3^+$ that may bind the terminal groups of the bacterial membrane molecules. This fact can be explained by the postulate of Venegas *et al.* [20]**:** adsorption is modulated by the presence of Ca through the formation of bridges HAP-Ca-Bacteria. Actually, both surfaces (HAP and bacteria) are negatively charged and they bind $Ca^{2+}$; at low concentrations, the low availability of $Ca^{2+}$ promotes bridge formation, but at larger concentrations HAP and the bacterium may bind different $Ca^{2+}$, and other effects become important, such as repulsion and even autoaggregation, as illustrated in Figure 6. Note that $Ca^{2+}$ binding may reverse the surface charge of enamel at pH 7, whereas, the bacterial charge remains negative.

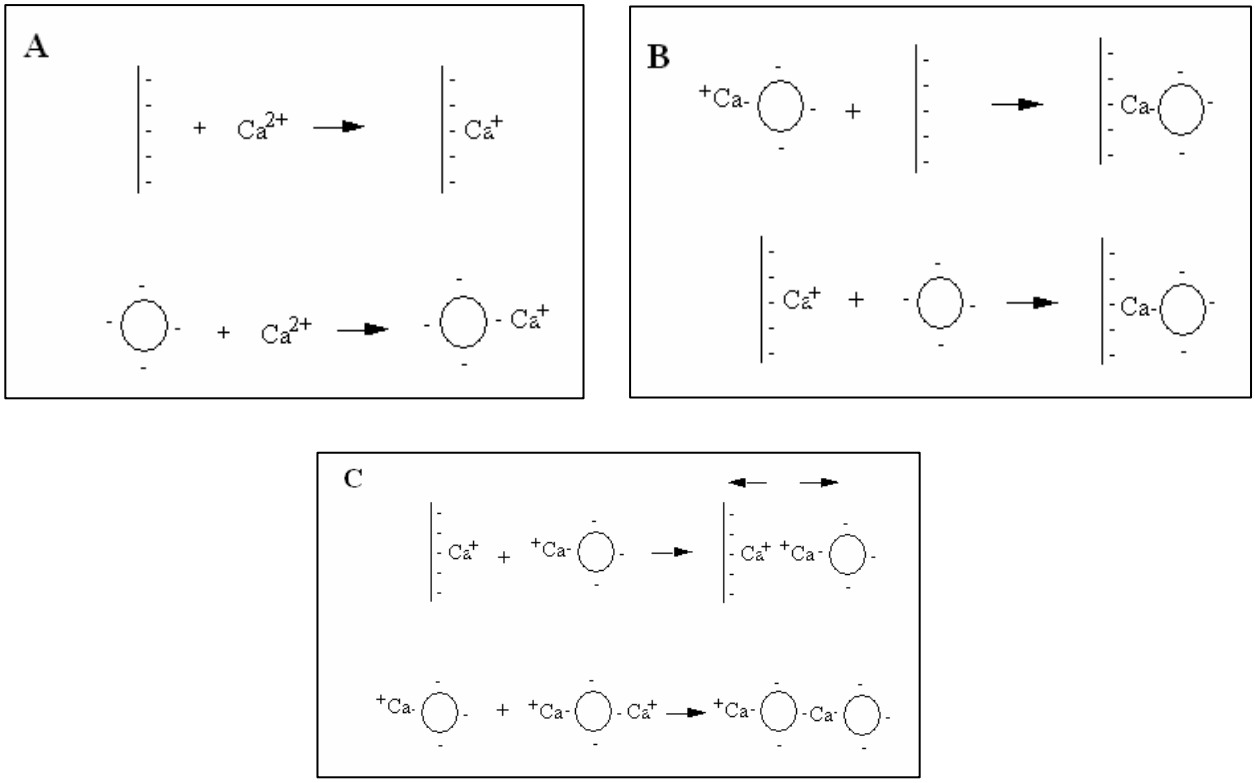


**Figure 6.** Simplified picture of the interaction of $Ca^{2+}$ with biomaterials and bacteria (A), its effect on adhesion at low $Ca^{2+}$ levels , repulsion and auto autoaggregation brought about by high [$Ca^{2+}$] and by high ionic strengths (C).

## Conclusions

The surface composition of enamel and dentine are appreciably different, the latter containing larger amounts of carbonate. Powdered HAP may be synthesized also with various degrees of substitution, and this factor must be taken into account when using HAP as a model for the biomaterial. In the present case, surface properties of teeth, determined by the properties of

enamel, were more important and, accordingly, a synthetic HAP was chosen with a surface composition close to that of enamel.

The electrophoretic measurements prove that the gross features of enamel and dentine generation of surface charges are similar to those of HAP. However, the influence of the levels of dissolved $Ca^{2+}$ brings about charge reversal on enamel at pH 7, whereas the surface charge of dentine at this pH remains very low.

Adhesion of *L. casei* on enamel is quantitatively similar to adhesion on synthetic HAP; however, in the former case dissolved $Ca^{2+}$ modulates the interaction, whereas this effect is negligible on synthetic HAP. Thus, even though the surface compositions are similar, the extent of Ca-rich sites on enamel can be increased by the addition of calcium salts. Since calcium provides the bridges responsible for strong bacterial adhesion and formation of the biofilm, it is concluded that colonization of the passive enamel surfaces is sensitive to the levels of $Ca^{2+}$, defined by dietary habits and the possible ingestion of supplements and anti-acid tablets.

**Aknowledgments.** This work was supported by CONICET (PIP Nº 5978), CIUNT (G-305) and ANPCYT (PICT Nº 06-13534). SCV thanks CONICET for fellowships.

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