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Calcification of porcine and human cardiac valves: testing of various inhibitors for antimineralization

S. KOUTSOPOULOS, A. KONTOGEORGOU, J. PETROHEILOS‡, E. DALAS*
Department of Chemistry and ‡Medical School, University of Patras, GR-26500, Patras, Greece

Despite distinct advantages over mechanical cardiac valve prostheses, the use of bioprosthetic valves remains limited, due to poor long-term durability, primarily as a result of tissue calcification. A novel *in vitro*, anticalcification process based on treatment of porcine and human heart valves with metallocene dichlorides, as well as with amino acids (phenylalanine, aspartic acid, glutamic acid), has been developed. This anticalcification process reduced mineralization of porcine and human valves up to 32% and 28%, respectively.

1. Introduction

Development of prosthetic cardiac valves has evolved mainly along two lines: mechanical and bioprosthetic [1–3]. The main advantage of biological valve prostheses over mechanical valve prostheses is the low rate of thromboembolism without administration of anticoagulants. Calcification is the most frequent cause of the clinical failure of cardiac valve bioprostheses fabricated from porcine aortic valves [4, 5]. Therapy with diphosphonate compounds [2] or 2-amino oleic acid [6] to retard calcification process has been proposed as a means for alleviating bioprostheses calcification.

The mineral deposits of the human atherosclerotic aorta consisted mainly of calcium apatite (71%), carbonate (9%) and contained a relatively high percentage of protein (15%) [3, 7]. The average Ca/P ratio was ~1.7 in mature atherosclerotic plaque biomineral and mature skeletal biomineral, both of which approximate hydroxyapatite (HAP, $Ca_5(PO_4)_3OH$) in composition.

In the present work, an attempt was made to evaluate the antimineralization activity *in vitro* of three amino acids and four metallocene dichlorides used as drugs for various therapeutic applications (antitumor, antiinflammatory and antiarthritic agents) [8–11] on human and porcine heart valves by the constant composition approach [12–14].

2. Experimental procedure

All experiments were performed at 37 ± 0.1 °C in a thermostatted double-walled water-jacketted Pyrex vessel, volume totalling 0.250 dm^{-3}. Solid reagent-grade (Merck) calcium chloride, potassium dihydrogen phosphate, sodium chloride and triply distilled CO_2-free water were used in the preparation of the solutions. Potassium hydroxide solutions were prepared from concentrated standards (Merck, Titrisol). Prior to standardization, all solutions were filtered through membrane filters (0.22 μm, Sartorious). The standardization of the stock solutions is described in detail elsewhere [12–14]. The supersaturated solutions were prepared in the thermostatted vessel by mixing the appropriate volumes of calcium chloride and potassium dihydrogen phosphate under a nitrogen atmosphere which was ensured by bubbling water-vapour-saturated prepurified nitrogen (Linde Hellas) through the supersaturated solution. The ionic strength of the solutions was adjusted to 0.15 mol dm^{-3} by the addition of sodium chloride. The solution pH was measured by a glass/saturated calomel pair of electrodes (Metrohm, 6.0101.100 and 6.0726.100, respectively) standardized before and after each experiment by NBS buffer solutions [15]. Following pH adjustment, by the addition of dilute potassium hydroxide (Ck, M), all solutions in this work were stable for at least 3 d, as indicated from the constancy of pH and the solution composition. The solutions were stirred by a magnetic stirrer with a Teflon-coated stirring bar at ~350 r.p.m. After a waiting period of 4 h for each experiment, a quantity of cardiac valve (10 cm^2 total geometric surface area) was introduced in the supersaturated solution along with the inhibitor. Porcine hearts were obtained from a local Packing Company and were transported to the laboratory on ice. Valve leaflets were dissected, rinsed in cold sterile saline and after ultraviolet sterilization were stored in 0.15 M NaCl solution at 2 °C for a maximum of 4 wk before each experiment. Human grossly normal cardiac valves were obtained at autopsy (from body-organ donors) and handled by the same above-described procedure. The cardiac valve leaflets were

*Author to whom all correspondence should be addressed.

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introduced into the supersaturated solution on a special holder [16] and after 1 h (in the experiments in the presence of metallocene complexes and the amino acids) the crystallization process was started by the addition of calcium chloride without any appreciable induction period. When following the above procedure, adsorption phenomena do not interfere with the kinetic measurements. Metallocene complexes were prepared and purified according to literature methods [8–11]. The initiation of the precipitation process resulted in proton release which, when lowering the solution pH by 0.003 pH unit, triggered the addition of calcium chloride and potassium dihydrogen phosphate from two mechanically coupled burettes of an appropriately modified pH-stat (Metrohm, 625 dosigraph, 614 Impulsomat and 632 pH-meter). The concentration of the titrant in the two burettes was calculated as follows:

burette 1: $(10\,Ca_t + 2Ca_t)$ M $CaCl_2$

$$+ [0.3 - (20\,Ca_t + 30\,P_t + 10/5\,Ca_t)] \text{ M NaCl}$$

burette 2: $(10\,P_t + 2\,P_t)$ M KH_2PO_4

$$+ (20\,P_t + 10/5\,Ca_t + 2\,Ck) \text{ M KOH}$$

where $Ca_t:P_t:OH = 5:3:1$ and subscript t indicates total concentrations.

The rates of HAP formation were taken from the plots of titrant addition as a function of time. In the present work, the rates at the time of the onset of precipitation were used for the kinetic treatment in order to avoid taking into account the fact that the HAP crystals formed may serve as seed crystals, thus accelerating the rate of precipitation. The reproducibility of the measured rates was $\pm 4\%$ a mean of five experiments. During the course of the experiments, samples were withdrawn at random times and filtered (0.22 μm Sartorius membrane filters), and the filtrates were analysed for calcium by atomic adsorption spectroscopy (Varian 1200) and for the phosphate, as vanadomolybdate complex, spectrophotometrically [17] (Varian Cary 219). The analysis confirmed the constancy of calcium and phosphate concentrations throughout the precipitation process within 2% [13]. The solid phases were analysed by Fourier transform–infrared (FT–IR) spectroscopy (Perkin–Elmer 16-PC), X-ray diffraction (Phillips PW 1830/1840) and scanning electron microscopy (Jeol GSM 5200). The stoichiometric ratio Ca:P experimentally determined was 1.67 ± 0.01.

3. Results and discussion

The experimental conditions are summarized in Table I. The solution speciation in all experiments was calculated from the proton dissociation and ion-pair formation constants for calcium and phosphate, the mass balance and the electroneutrality conditions by successive approximations for the ionic strength [12]. The driving force for the HAP formation is the change in Gibbs free energy, ΔG, for the transfer from the supersaturated solution

TABLE I Initial conditions for the crystallization of HAP on cardiac valves at 37 °C; pH 7.4; ionic strength 0.15 M NaCl; molar ratio of total calcium T_{ca} to total phosphate $T_P = 1.67$; $T_{ca} = 5 \times 10^{-4}$ M; total volume of working solution 200 ml; $\Delta G_{HAP} = -4.43$ kJ mol^{-1}

Experiment	Cardiac valve	Inhibitor (10^4 mol dm^{-3})	R (10^{-9} mol min^{-1})
5	Porcine		1.36
20	Human		1.80
8	Porcine	Cp_2ZrCl_2 (1)	1.03
10	Porcine	Cp_2VCl_2 (1)	1.18
11	Porcine	Cp_2HfCl_2 (1)	0.92
21	Human	Cp_2HfCl_2 (1)	1.35
12	Porcine	$(MeC_5H_4)_2TiCl_2$ (1)	1.29
13	Porcine	Phenylalanine (10)	0.90
14	Porcine	Aspartic acid (10)	1.25
19	Porcine	Glutamic acid (10)	1.26
22	Porcine	Cp_2HfCl_2 (1)	0.84

to equilibrium

$$\Delta G = -\frac{R_g T}{9} \ln \frac{IP}{K_s^0}$$

$$= -\frac{R_g T}{9} \ln \frac{(Ca^{2+})^5 (PO_4^{3-})^3 (OH^-)^1}{K_s^0} \quad (1)$$

where IP is the ionic product of the precipitating salt, K_s^0 the solubility product equal to 2.35×10^{-59} [18], 9 is the number of ions, R_g the gas constant and T the absolute temperature. Examination of the precipitated solids by X-ray diffraction, FT–IR spectroscopy, scanning electron microscopy and elemental analysis confirmed the exclusive formation of HAP [14]. Well-grown HAP crystals on valve leaflets substrate, both porcine and human, may be seen in the scanning electron micrographs in Figs 1 and 2. Doubling or tripling the amounts of cardiac valve substrate introduced in the supersaturated solutions had no effect on the initial rates normalized per unit surface area of the substrate. Also, changes in the stirring rate (between 60 and 500 r.p.m.) had no effect on the kinetics parameters. It may therefore be suggested that the HAP overgrowth was induced by the biological matrix by heterogeneous nucleation [19–21].

The relative reduction of HAP crystal growth rates (%) $(R_0 - R_i)/R_0$, where R_0 is the rate in the absence of any additive and R_i is the rate in the presence of the inhibitor i, in the presence of three amino acids (phenylalanine, aspartic acid, glutamic acid) and four metallocene dichlorides (Cp_2HfCl_2, Cp_2ZrCl_2, Cp_2VCl_2, $(MeC_5H_4)_2TiCl_2$, where Cp = cyclopendadienylring and Me = CH_3) on porcine cardiac valves are shown in Fig. 3. Hafnocene dichloride from the metallocene dichlorides and phenylalanine from the amino acids examined showed better antimineralization activity *in vitro* for the porcine valves.

The relative reduction of HAP crystal growth rate in the presence of hafnocene dichloride (HC) was found to follow the trend blank, human heart valve > blank, porcine heart valve > HC, human heart valve > HC, porcine heart valve, and

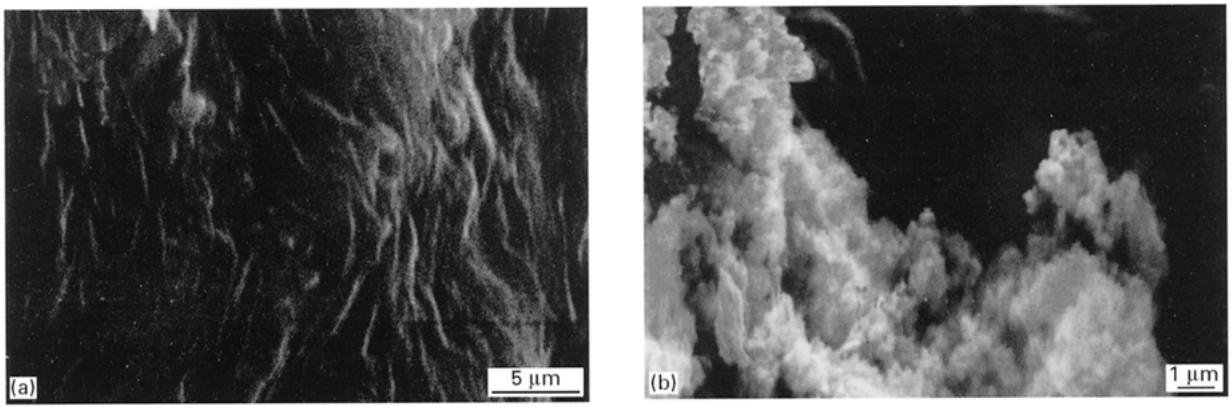

Figure 1 Scanning electron micrographs of (a) porcine valve substrate, and (b) HAP crystals on porcine valve leaflets.

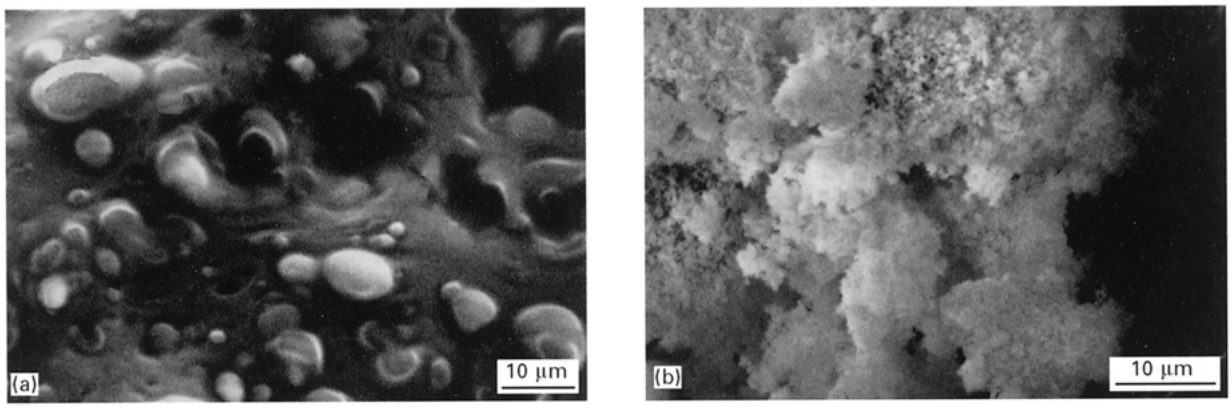

Figure 2 Scanning electron micrographs of (a) human valve substrate, and (b) HAP crystals on human valve leaflets.

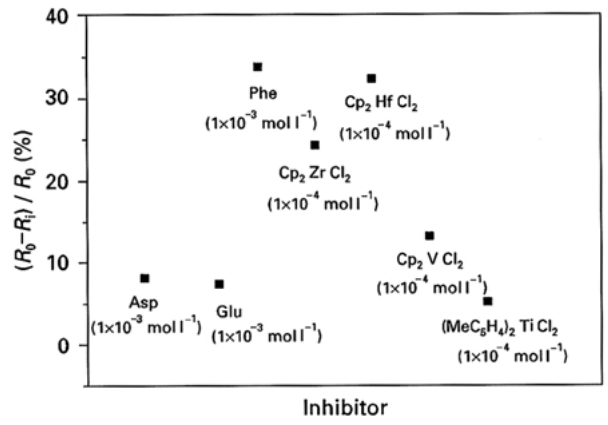

Figure 3 The effects of the inhibitors examined on the reduction of the rate of HAP crystal growth on porcine cardiac valves.

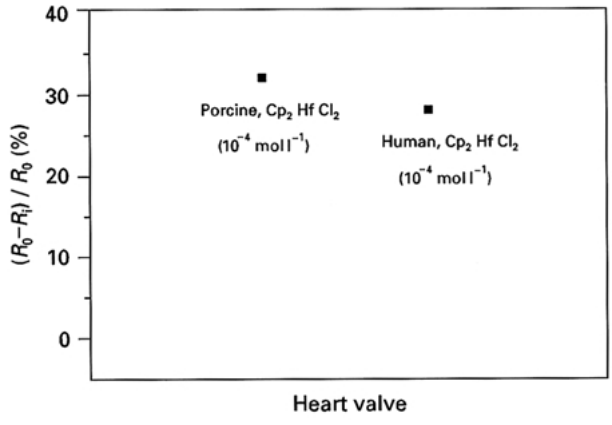

Figure 4 Relative reduction of HAP crystal growth rate in the presence of hafnocene dichloride on porcine and human cardiac valve, respectively.

the relative reduction of growth rates are shown in Fig. 4.

Finally, if the crystallization started 4 h after hafnocene dichloride introduction, the rate measured was 0.84×10^{-9} mol min^{-1} and showed an additional effect of the hafnocene hydrolysis products on the inhibition of crystal growth [9–11].

In conclusion, both porcine and human cardiac valves are substrates on which calcium phosphate (HAP) may nucleate and subsequently grow. The formation of HAP may be initiated via adsorption of inorganic phosphate on the biological polymer matrix of the valve leaflets [20, 21]. Metallocene dichlorides and amino acids reduced the rates of crystal growth of HAP by 5%–32%. This inhibitory effect may be explained by the adsorption and subsequent blocking of the active growth sites [9–11, 22].

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