Screening biomaterials with a new *in vitro* method for potential calcification: Porcine aortic valves and bovine pericardium

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Calcification is still a major cause of failure of implantable biomaterials. A fast and reliable *in vitro* model could contribute to the study of its mechanisms and to testing different anticalcification techniques. In this work, we attempted to investigate the potential calcification of biomaterials using an *in vitro* model. We purposed to test the ability of this model to screening possible anticalcification efficacy of different biomaterials. Porcine heart valve (PAV) and bovine pericardial (BP) tissues, fixed with glutaraldehyde were immersed into biological mimicking solution, where the pH and the initial concentrations of calcium and phosphoric ions were kept stable by the addition of precipitated ions during calcification. Kinetics of calcification was continuously monitored. The evaluation of biomaterials was carried out by comparing the kinetic rates of formation of calcific deposits. After 24 h, the calcific deposits on PAVs were found to be developed at significant higher rates (ranged from 0.81×10^{-4}–2.18×10^{-4} mol/min m^2) than on BP (0.19×10^{-4}–0.52×10^{-4} mol/min m^2) (one-way ANOVA, $p < 0.05$) depending on the experimental conditions (supersaturation of the solution). Parallel tests for similar biomaterials implanted subcutaneously in animal (rat) model showed after 49 days that significant higher amounts of total minerals deposited on PAV (236.73 ± 139.12, 9 animals mg minerals/g dry net tissue) (mean $\pm$ standard deviation) compared with that formed on BP (104.36 ± 79.21, #9 mg minerals/g dry net tissue) (ANOVA, $p < 0.05$). There is evidence that *in vitro* calcification was correlated well with that of animal model and clinical data.

Introduction

Calcification is one of the major causes for dysfunction of native connective tissues like heart valves and blood vessels, as well as many implantable biomaterials like bioprosthetic heart valves and polyurethane vascular grafts [1–7].

A number of host and implant factors, alone or in synergy may contribute in the initiation and further development of calcification. Dead cell remnants, lipids, inadequate crosslinking and mechanical stress are some of the alternative explanations for the initiation of calcification [8–11]. In designing new methods of production for new biomaterials, a large number of anticalcification treatments have been proposed so far [12–17]. However, until now no method has proven its effectiveness in long-term applications *in vivo* [6, 18].

Attempts to develop novel implantable biomaterials have led to the development of a number of different *in vitro* and *in vivo* models for the evaluation of the efficiency of anticalcifying treatments [9, 19]. Subcutaneous implantation in rats and orthotopic or heterotopic implantation in the blood circulatory system of sheep are some examples of animal models used for screening or pre-clinical studies. The *in vivo* models have been accepted as a predictive method for evaluation of potential calcification of biomaterials. However, the need for the development of reliable tests for the various anticalcification methods render the use of animal

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models for screening new biomaterials as an expensive and time-consuming method. An additional limitation of the *in vivo* tests is their inability to isolate possible factors hypothesised for implication in calcification, thus making the exclusive use of these models inappropriate for a complete understanding of the initiation and growth of the formation of calcific deposits. Last, but not least, ethical reasons suggesting the development of alternative methods reducing the needs for use of animal models, impose limitations on the *in vivo* experiments.

In vitro models are important alternative methods for the investigation of the calcification process. A significant number of *in vitro* investigation models have been proposed [12, 20–22]. Before, however, such a model is accepted as a reliable tool for the screening of anticalcification processes, its relevance to animal and clinical investigations should be proven [6].

In a previous study, we presented a novel *in vitro* model for the study of calcification in implantable biomaterials [21]. This model showed that it was possible *in vitro* and at controlled conditions of fluid temperature, pH and composition to monitor the calcification process. The calcium phosphate deposits formed *in vitro* exhibited similar morphology with those found in parallel animal model experiments. Moreover, solid deposits formed at similar sites of the tissues [23]. The morphology of the calcium phosphate crystallites formed in the *in vitro* experiments correlated morphologically closely with the calcific deposits from explanted calcified natural human and porcine bioprosthetic heart valves [24].

In this work, we attempted to compare two biomaterials, porcine bioprosthetic heart valves (PAVs) and bovine pericardial (BP) tissues, with respect to their potential for calcification using the *in vitro* model we have presented earlier. Both biomaterials have been prepared with the same fixation procedure, without any anticalcification treatment. The kinetics of calcium phosphate deposits formation on the biomaterials tested was used as the relative evaluation factor. The results were compared with those obtained from parallel subcutaneous implantation of the biomaterials tested in animal (rat) model.

Materials and methods

The tested materials were animal harvested tissues, elaborated chemically in our laboratory: PAV and BP. The porcine hearts and BP tissues were obtained from the local slaughterhouse, immediately after the death of the animals and were transported to the laboratory in iced saline medium. Within a few hours following the dissection of the hearts, the PAVs were extracted and trimmed for removing the excess myocardial tissue. BP was trimmed for removing the external excess fatty tissue. All trimmed tissue samples were then stored in Ringers' solution at 4 °C.

Next day, they were fixed in 0.15 M, pH 7.4 phosphate buffer solution into which 0.625% glutaraldehyde was added (25% w/v solution; Serva Feinbiochemica). The tissues were immersed in the solution for 1 h at room temperature. A slight mechanical prestress was applied on both tissues during glutaraldehyde fixation. For PAVs a small hydrostatic overpressure was applied on the aortic side, sufficient to keep the valve leaflets closed during the fixation process [25]. BP tissues were prestressed biaxially by suturing and slightly tensioning rectangular samples $7 \times 7\,cm^2$ onto rigid plastic frames. After fixation, the tissues were washed with normal saline and stored in a formaldehyde solution (4%, pH 5.6) at 4 °C [25]. A total number of 86 PAV and 18 BP tissue samples were used.

In vitro calcification

The detailed theoretical and technical description of the used *in vitro* method has been presented previously [21]. A brief description will be presented here. Physicochemically, the driving force for the formation of a crystalline mineral phase of a type $A_mB_n(m+n=v)$ in a solution of positive and negative charged ions A^{n+} and B^{m-} is the change in Gibbs free energy, ΔG, for going from the supersaturated solutions to equilibrium:

$$\Delta G = -\frac{R_g T}{v} \ln \frac{(a_{A^{n+}})^m (a_{B^{m-}})^n}{K_s^0}$$

where a represents the activities of the respective ions, R_g is the gas constant, T the absolute temperature and K_s^0 the thermodynamic solubility product of the mineral phase considered [26]. The supersaturation with respect to the mineral phase forming may be expressed as:

$$\beta = \frac{\Delta G}{R_g T} = -\frac{1}{v} \ln \frac{(a_{A^{n+}})^m (a_{B^{m-}})^n}{K_s^0}$$

The ratio Ω is the supersaturation ratio of the corresponding mineral phase, defined as

$$\Omega = \frac{(a_{A^{n+}})^m (a_{B^{m-}})^n}{K_s^0}$$

The relative supersaturation (σ) is defined as:

$$\sigma = \Omega^{\frac{1}{v}} - 1$$

Fig. 1 shows a schematic diagram of the experimental set-up used for the *in vitro* calcification experiments of biomaterials. All experiments were carried out in a thermostated double walled water jacked Pyrex® glass reactor, volume totaling 50 ml. The reactor was filled with a simulation plasma solution containing similar

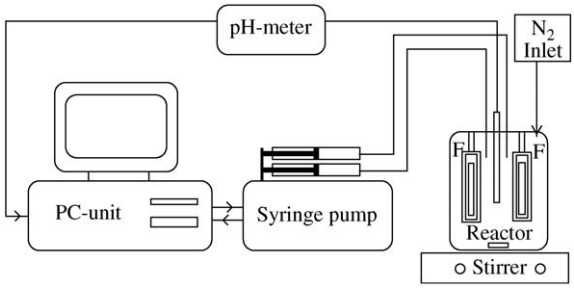

Figure 1 Drawing of the experimental set-up used for the development of calcific deposits on implant materials *in vitro*. The PC-unit receives the signal of pH drop from the pH-meter, stimulates the syringe pump and records the quantity of the solutions ejected from the two syringes into the reactor. F: Plexiglas frames with pericardial samples attached on. The procedure was performed under inert N_2 atmosphere.

electrolytes (K^+, Na^+, Ca^{2+} and PO_4^{3-}) and concentration levels with human plasma. The temperature was maintained at $37.0 \pm 0.1\,^{\circ}C$. The pH of the solution was maintained at 7.4 ± 0.1 throughout the experiments by restoring the consumed ions with the help of a computer-controlled double syringe pump. The composition of the working supersaturated solution varied (depending on the relative supersaturation examined) between 0.8 and 1.2 mM total calcium equivalent concentration of total phosphate and at ionic strength 0.15 M adjusted with NaCl. At these conditions, it was established that the supersaturated medium was stable practically indefinitely. In all experiments, the homogeneity of the working solutions was ensured by magnetic stirring and water vapor pre-saturated nitrogen ensured an inert atmosphere. The drop of the solution pH signaled the initiation of the calcification process. A drop of the working solution pH by as small as 0.005 pH units triggered the addition of titrant solutions from the computer-controlled syringes. In order to keep the working solution composition constant, one of the syringes contained $CaCl_2$ solution and the second with mixed NaH_2PO_4, NaOH and NaCl solutions. The stoichiometry of the titrant solutions with respect to calcium and phosphate depends upon the expected stoichiometry of the precipitating salt. Earlier work on the system has shown that octacalcium phosphate [$Ca_4H(PO_4)_3$] (OCP) is among others in the predominant calcium phosphate phase formed at the first stage of calcification [23, 24]. It was so decided to use the stoichiometry of OCP in our titrant solution added by the syringe pumps.

According to this method applied in our experiments:

- The pH of the working solution in which calcification takes place was maintained constant without the use of buffer solutions.
- The addition of the consumed ions of Ca^{2+} and PO_4^{3-} due to calcification maintained the initial physiological concentrations, permitting the continuation of the process for long time scale, sufficient for the identification of the mineral phase formed.
- Kinetics of formation of the mineral phase formed was measured accurately, through the profiles of the added volumes of the titrant solutions, as a function of time.
- Maximum reproducibility was achieved through the solution composition maintenance.

For each experiment, four BP rectangular specimens $1.5 \times 7\,cm^2$ or 12 leaflets from four valves (with approximately similar total surface area with BP) were mounted on Plexiglas® frames (Fig. 1), immersed in the working solution and subjected to calcification for 24 h. After the immersion of the mineralising substrates in the working solutions, samples were withdrawn randomly in order to verify the constancy of the solution composition. The samples were filtered from membrane filters (0.22 μm: Millipore) and the filtrates were analysed for calcium and phosphate [21]. A total of 19 experiments were performed for testing PAV, and 17 for BP in five values of supersaturation σ.

In vivo calcification

We used male rats weighting 150–200 g. Thirty leaflets from 10 PAVs were bisected radially, giving so a total of 60 semi-leaflet specimens. BP was cut as square pieces with sides of 1 cm. The tissue specimens were implanted subcutaneously in the abdominal wall of ether anesthetised rats. Two groups, one for PAV and one for BP, each of 10 animals were used for a period of 49 days. Six PAV or four BP specimens were implanted in each animal, keeping so, approximately, the same total surface area of the implants per animal. At the end of the period, the rats were sacrificed with ether overdose and the tissues were explanted, rinsed with saline and stored in formalin solution. All experiments were performed at the Experimental Surgery Unit of the University Hospital, University of Patras. EC directive 86/609 as well as national guidelines (13 and 19, P.D 160/91) for the care and use of laboratory animals have been observed.

Stoichiometric chemical analysis was performed for the samples calcified *in vivo*. For this purpose, the tissues were dried to a constant weight and weighed. Subsequently, the tissues were subjected to acid hydrolysis (0.1 N HCl) for three days and then they were dried and weighed again. The total mineral deposited (expressed in mg of total mineral per g of net dry tissue) was then determined from the weight difference of the samples. The residual solution was analysed for calcium (Ca^{2+}) with atomic absorption (PerkinElmer 305A) and for phosphate (PO_4^{3-}) with UV-visible spectroscopy (Spectronics Genesys 5). Next, the molar ratio of Ca^{2+} to PO_4^{3-} (Ca/P) was calculated.

Results

In vitro calcification

The results of the addition of the titrant solutions showed that the rate of addition of calcium and phosphate ions, depends strongly on the solution supersaturation. Chemical analyses of samples of the working solution during the process showed that the initial concentration of calcium and phosphate ions in the solution remained constant. Careful examination ensured that calcium phosphate deposits were not formed on the reactor walls or on other surfaces in contact with the working supersaturated solutions. All calcium and phosphate ions added through the titrant solutions resulted in the replacement of the solution ions transferred to the mineral deposits through a heterogeneous crystal growth process, onto the tested tissue substrates.

In each experiment, the comparison between the different substrates was carried out at similar solution conditions. The volume of the titrant solutions added with time was recorded as shown in Fig. 2. From this type of plots, the initial rates of the process were calculated for the various degrees of the solution supersaturation σ tested.

The rates of precipitation (R) were computed as moles OCP per unit time and surface area. For the area calculation, the geometrical area of the substrates multiplied by two (two surfaces) was considered. Fig. 3 presents a diagram with the results of the precipitated calcium phosphate quantity R as moles OCP per time and per total surface area with respect to σ for the tested PAV

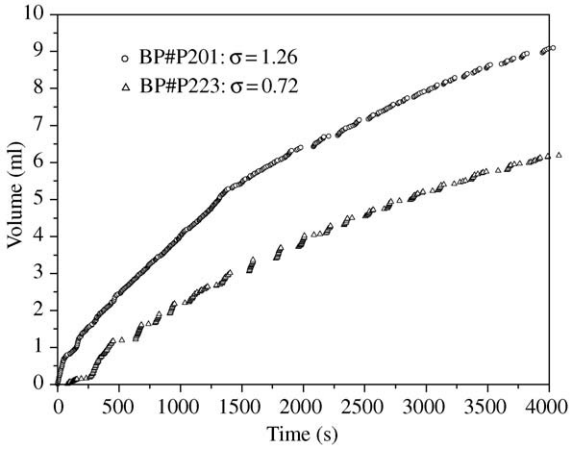

Figure 2 Kinetic diagrams of the added titrant solutions' volume with time from two *in vitro* calcification experiments of glutaraldehyde-treated BP tissue at different relative supersaturation σ values.

and BP tissue samples *in vitro*. R of PAV was found to be significantly greater than that of BP for each σ value (one-way ANOVA, $p < 0.05$).

Calcification in animal model

After 49 days of implantation, the tissue samples of both PAV and BP were calcified. Histological sections showed the development of calcific deposits at the interior of the tissues, associated with dense collagen fibres (e.g. at fibrosa of PAVs) (optical microscopy, von Kossa stain). Results from two animals, one of PAV and one of BP groups were excluded because of (a) loss of some implants (PAV) (possibly because of biological degradation) and (b) folding of some implants (BP). The quantity of total minerals developed on PAVs was found to be at average 236.73 $\pm$ 139.12, 9 animals mg minerals/g dry net tissue (mean $\pm$ SD). It was significantly greater than the quantity found in BP (104.36 $\pm$ 79.21, 9 animals mg minerals/g dry net tissue) (ANOVA, $p < 0.05$).

Chemical analysis showed that the Ca/P molar ratio ranged from 1.43 to 2.19. It was found at average to be 1.8 $\pm$ 0.23 (PAV), significantly lower than the value found in BP (2.07 $\pm$ 0.14) (mean $\pm$ SD). The high values of the molar ratios of Ca/P obtained for the mineral deposits *in vivo*, compared with the corresponding theoretical ratios of pure crystalic phases of different calcium phosphates (1.00–1.67) may be attributed to the substitution of the phosphate ions by other ions (e.g. sodium, magnesium and/or carbonate) [23].

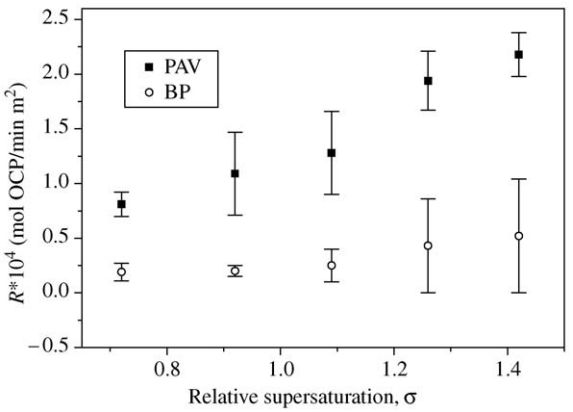

Figure 3 Diagram of the precipitated calcium phosphate quantity (R) expressed in moles OCP per time and per total surface area with respect to the relative supersaturation σ of the tested PAV and BP tissue samples *in vitro* (mean $\pm$ SD).

Discussion

Despite the fact that a number of biological, biochemical, mechanical or pathological factors may initiate or control calcification, it may be basically considered as a physicochemical process [27].

Previous studies have shown that calcific deposits developed in biological implants, detected *in vitro*, in animal models and *in vivo*, comprises mainly different phases of calcium phosphate crystals, like dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) (DCPD), OCP and hydroxyapatite [$Ca_5(PO_4)_3OH$] (HA) [22, 23, 28–30]. DCPD and OCP are thermodynamically less stable than HA, considered to be its precursor phases forming transiently [23, 24, 31]. The precursor phases have been encountered in higher content ratios in the calcific deposits formed in immature formations, studied at early animal implantation or at bioprosthetic heart valves following implantation in humans, as compared with more mature formations like the deposits of calcified natural human valves, where HA seems to be the predominant phase [23, 24, 30, 32].

In this work, we compared the results of *in vitro* calcification of PAVs and BP. The rates of the development of the calcific deposits in PAVs were found to be significantly higher than the respective rates measured on BP substrates, tested over a wide range of relative supersaturation σ values, which include the corresponding respective values in the blood serum of humans.

A number of questions could be raised, concerning the validity of the *in vitro* calcification models developed to serve as methods for the evaluation of the anti-calcification efficacy of new biomaterials. The questions are mainly related to the correlation of the histological and ultra-structural results of *in vitro* calcification with the results obtained in the animal models and in humans [6]. Some critical points to be examined are

- Is the calcification extrinsic (surface) or intrinsic (interior of the tissue)?
- Is it associated with specific elements (collagen fibers, cells, lipids), where potentially calcification initiated *in vivo*?
- Is there any morphological similarity of the crystalic phases developed *in vitro*, in animal models and in humans?
- Do the results of the *in vitro* comparative evaluation of the extent of calcification confirm that of similar materials implanted in animal models and in humans? [6, 9, 19].

Calcification developed in our model seems to be intrinsic. No calcific deposits were detected on the vessel walls or at any surface in contact with the working solution before, during and after the end of the

experiments. Histological sections examined by optical microscopy after von Kossa staining showed at high magnification no calcified surfaces of PAV tested *in vitro*. However, it was shown that sparse sites of calcification were developed deep into the rich-collagen fibrosa in contact with collagen fibers. This region of the PAV tissue is considered as a main site of nucleation of calcific deposits *in vivo* [6, 9, 18]. These findings were confirmed by SEM (equipped with energy dispersive spectroscopy (EDS) microanalysis) observations of sequential parallel, non-stained, carefully deparaffinised histological sections of the same tissue samples, placed on aluminium sample holders and sputtered with gold (Fig. 4) [23]. The morphology and the deposition layout of the calcium phosphate phases developed *in vitro* were also found to be similar to that of animal testing of the same materials, as well as to deposits examined at explanted calcified natural and bioprosthetic heart valves [23, 24, 30, 32].

Published data comparing porcine and bovine pericardial bioprosthetic heart valves gave evidence about the superiority of BP against PAVs [33–36]. However, other studies have shown that superiority depends not only on the tissue selection, but also on the whole tissue preparation procedure and the design of the valves [37–40].

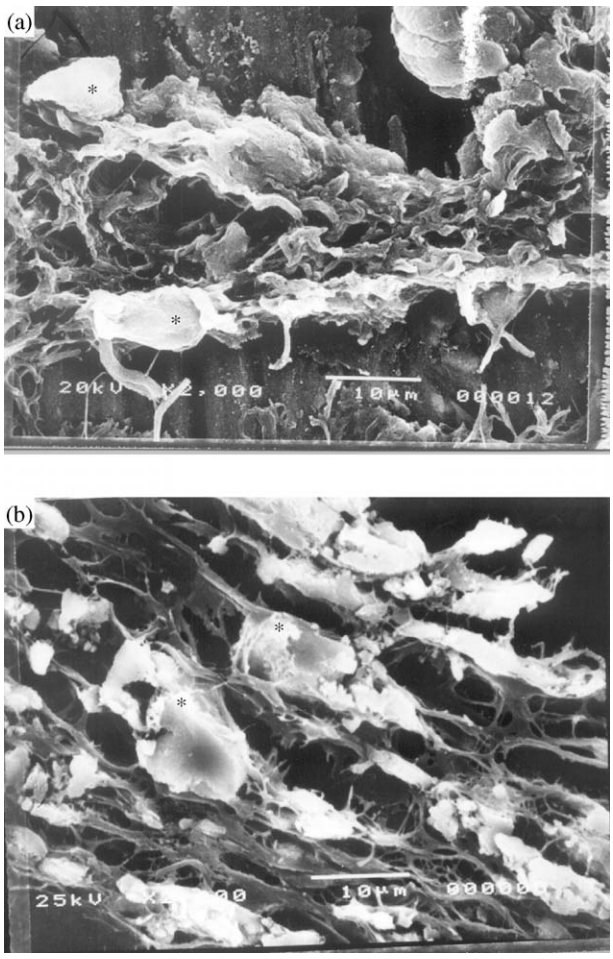

Figure 4 SEM micrographs of tissue sections from porcine aortic leaflets (PAV) treated with glutaraldehyde showing the development of calcific deposits (*) closely associated with collagen fibers of fibrosa. (A) *In vitro* after 24 h. (B) In animal model after 56 days of implantation. Calcium phosphate deposits were verified with built-in EDS microanalysis.

Conclusions

In this work, we attempted to evaluate two biomaterials, PAV and BP, for potential calcification using an *in vitro* model. The results showed that the rate of formation of calcific deposit on BP was significantly less than that of PAV samples. This superiority of BP was confirmed with parallel testing of similar biomaterial samples in animal model. Further testing of reference biomaterials, prepared with special anticalcification treatments have to be carried out before this *in vitro* model is suggested for an accurate method for screening anticalcification efficacy of new biomaterials.

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