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Calcification of polyurethane-based biomaterials implanted subcutaneously in rats: role of porosity and fluid absorption in the mechanism of mineralization

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The object of this study was to investigate the mineralization of polyurethane in the rat subcutaneous environment, in comparison to collageneous biomaterials, which would facilitate investigation of the calcification mechanism in implantable biomaterials. This model was compared to an *in vitro* model of non-spontaneous formation of calcium phosphate precipitate. To study the role of porosity/fluid absorption capacity, various biomaterials such as polyurethane, hydrophilic polyurethane foam, Type I collagen sponges, and bioprosthetic heart valve tissue were examined in *in vitro* and *in vivo* models. In view of the exceedingly low levels of polyurethane calcification, it seems that the rat subcutaneous model is not suitable for routine studies of the mechanism of polyurethane calcification. The calcification extent of both collageneous and hydrophilic polyurethane, was found to be in good correlation with the water absorption capacity of these biomaterials. It is suggested that the water capacity of the biomaterial determines the bulk level of calcification, which in turn is generated and propagates via the inherent affinity sites of the biomaterial to Ca^{2+}.

1. Introduction

The search for hemocompatible materials, which is several decades old, takes on a new meaning with the discovery that long-term implants will fail or become severely impaired due to calcification. Calcification has limited the durability of trileaflet polymeric cardiac valve prostheses [1–3]. It is the leading cause of failure of contemporary bioprosthetic heart valves [4–6], limits the functional lifetime of experimental blood pumps [7], and is likely to occur in long-term clinical artificial hearts.

Implant factors affecting the rate of calcification which have received the most attention are the effects of local stress concentrations [8, 9], calcium-binding serum protein absorption [10], the presence of surface defects [11, 12], and surface-adhered organic or cellular debris [13]. Although it is generally accepted that the calcification of blood pumps is dystrophic in nature, i.e. caused by degraded-local changes [14, 15], it is important to mention that calcification of polyurethane blood pumps was observed with no evidence of cellular involvement [16–18].

Methods to prevent calcification [19] are dependent on understanding the mechanism of induction and propagation of this disease. Unfortunately, this information is not well defined and data supporting various hypotheses are difficult to obtain in a well-controlled environment. Methods for studying polymeric implant calcification are almost exclusively dependent on large animal circulatory models which are expensive, complicated (open heart surgery), and the data obtained from the limited number of experiments is variable, impeding comparisons.

A new *in vitro* model, consisting of polymer incubation in a calcium phosphate metastable solution, has recently been developed [20]. This model of spontaneous hydroxyapatite formation is adequately sensitive to both diagnose biomaterials' propensity to calcify and as a pre-screening method to examine anticalcification methods. However, simulation of the basic metabolic requirements for mineralization, with similar pathophysiology and morphology of human implants, is achieved only by an animal model.

An attempt to calcify porous polyurethane (Biomer) has been reported [21], by intramuscular implantation in adolescent rats (160–200 g). However, calcification was exhibited only in polymers pre-embeded with calcium. In contrast, subcutaneous implants of bioprosthetic tissue in young rats is a convenient, economical and well-controlled model, which provides reliable quantitative examination of the biochemical and morphologic aspects of the disease [19, 22–26], as well as studies of therapeutic approaches [27–29]. This model has the advantage of an extremely accelerated time course in younger animals (three-week-old rats, 70–80 g), and the accumulation of bulk mineral after several weeks equivalent to several years of human heart valve replacements

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used to study polyurethane-based implants calcification.

The object of this study was to investigate the mineralization of polymeric substrate in the rat subcutaneous environment, in comparison to collageneous biomaterials, which would facilitate investigation of the calcification mechanism in implantable biomaterials. In addition, a comparison was made to the *in vitro* model of non-spontaneous formation of calcium phosphate precipitate. To study the calcification mechanism, various biomaterials (collageneous and polyurethane-based) with different porosity/fluid absorption capacity were examined in *in vitro* and *in vivo* models.

2. Materials and methods

2.1. Polyurethane film preparation

Pellethane 80AE (received from Dow Chemicals, USA), a segmented, aromatic, butane diol extended, polyether-based polyurethane, was used. The polymer was washed in soxhlet with methanol for 12 h, and then for an additional 12 h with water to extract impurities and additives [30]. The water was extracted by several washings with acetone and oven dried (24 h, 95 °C). Scanning electron microscopy studies of films prepared from non-washed polymeric beads revealed surface pores, created by impurities and additives which migrates to the film's surface [30].

Films of polyurethane (PU) were cast on glass petri dishes from 10% w/v solutions of polymer in tetrahydrofuran (THF) at room temperature. Various dry film thicknesses of 0.2 and 0.7 mm (measured by micrometer at five points, each measurement deviating less than 10% from the calculated mean), were produced by varying the volume of the polymer casting solutions.

2.2. Porous films

Porous Pellethane films were prepared by dissolving polyethylene glycol 3000 (PEG) and Pellethane in THF, as detailed above. Casted films containing PEG (20% w/w) were extracted with 50% ethanol in H_2O. Dried films were accurately weighed, and complete extraction was determined by 20% ($\pm$ 1%) weight loss. The extraction of the pre-embedded PEG 3000 created uniform pores of 2–5 μm in diameter [20].

2.3. Hydrophilic polyurethane films

To study the role of water capacity of the biomaterial on its calcification, foamable hydrophilic polyurethane polymer (Hypol FHP 2002, Grace, Lexington, MA) has been prepared. The prepolymer is a polyether polyisocyanate which reacts with water yielding CO_2 (foaming), and urea chain extension with crosslinkages. Hypol prepolymer and water in 1:1 ratio were mixed in a plastic petri dish, and were allowed to polymerize in the hood at ambient temperature.

Non-porous Hypol films (non-foamed) were prepared by casting the plain prepolymer solution in dry air at 60 °C for 48 h.

2.4. Collageneous biomaterial preparation

Bioprosthetic heart valve tissue was prepared from bovine pericardial tissue which was pretreated with glutaraldehyde as detailed elsewhere [31]. Fresh parietal pericardium was obtained at slaughter from 1–3-month-old calves and immediately placed in iced sterile saline. After dissection of superficial fat from the external surfaces, pieces of 1×1 cm^2 were cut and were incubated in 0.2% glutaraldehyde (25% for electron microscopy, under nitrogen, Merck, FRG) of 0.05 M HEPES and 0.1 M NaCl, pH = 7.4, for crosslinking (for at least a two-week period) and storage at 4 °C.

Type I collagen sponges were obtained as sterile, plain dry films (Helistat, Helitrex, Princeton, NJ). The crosslinking procedure was similar to the tissue preparation, as above.

2.5. Serum pretreatment

The rationale for this study is the implication of calcium-binding serum protein in calcification [10]. In addition, it has been demonstrated that heat inactivated human sera can serve as a calcifying media for PU and a series of hydrogels [7]. Pre-cut polyurethane films were immersed in human serum for 2 h, and after gently wiping with tissue paper they were implanted.

2.6. Rat subdermal implants

The detailed procedure was described previously [31]. Specimens were rinsed extensively (20 times) with a copious amount of saline. A piece of 1×1 cm^2 tissue or polymer was implanted in each subcutaneous pocket in the abdominal walls of ether-anaesthetized male rats (Sabra strain, 70–80 g, three-week-old, Faculty of Medicine, Hadassah University Hospital, Jerusalem, Israel). The rats were killed by an overdose of ether, and the amount of calcium in the explant was determined by atomic absorption spectroscopy on HCl hydrolysates of dried material. In this experiment the total implantation time was 15, 30, and 60 days. In some experiments re-implantation was performed by re-implanting the 15 day's explant for an additional 15 days in young rat.

2.7. *In vitro* calcification experiment

In this system the calcium concentration (10.28 mg/100 ml) was similar to mean total serum levels (10 mg/100 ml), and the ratio of Ca/PO_4 was 1.67, as in hydroxyapatite (HAP). The concentration product of calcium ($CaCl_2 \cdot 2H_2O$) and phosphate (K_2HPO_4) in the incubation solution was 3.95 mM^2, 2.57 mM calcium and 1.54 mM phosphate. Methyl paraben (0.03%) and propyl paraben (0.01%) were used as preservatives. Each salt solution was prepared in 0.05 M Tris buffer, pH = 7.4. Equal volumes of doubled concentrations of 2.57 mM calcium and 1.54 mM phosphate were mixed in Eppendorf vials, containing the polymer disc (0.5 cm in diameter). The vials were placed in a shaker (100 revolutions per

minute) at 37 °C. At specified time points the solution was filtered (0.22 μm), and the discs were rinsed three times with water to remove excess solution and the loosely attached deposits. The discs were oven dried (2 h, 110 °C), accurately weighed and hydrolysed in 2 ml HCl 6 N (Ultrex II, J. T. Baker) for 24 h at 50 °C. The amount of calcium was determined by atomic absorption spectroscopy [31] on aliquots which were diluted with lanthanum solution (La^{+3} 5%, HCl 3.0 N). A standard curve was obtained by using a calcium standard solution (Sigma) in lanthanum solution (La^{+3} 0.5% and HCl 0.6 N), having a correlation coefficient of 0.99–1. Sensitivity was 0.5 μg ml^{-1} calcium for 10% absorption, and the coefficient of variance was lower than 2.5.

2.8. Water absorption

Pieces of bioprosthetic tissue and collagen incubated in glutaraldehyde solution ($n = 10$) were rinsed, excess water was gently and quickly wiped with a filter paper, and the specimens were accurately weighed (it was difficult to accurately measure the amount of absorbed water in the floppy and spongy collagen sponges). The amount of water absorbed was determined from the loss of weight after drying (vacuum oven, 60 °C) to constant weight. Freeze-dried pieces of non-crosslinked collagen sponges, polyurethane and Hypol polymers (dried to constant weight in vacuum oven, 60 °C) were placed in water for 24 h, gently wiped with a filter paper, and accurately weighed for water weight gain determination.

For scanning electron microscopy (SEM) and energy dispersive X-ray examinations, representative films were rinsed several times with double distilled water and then oven dried. Sections were made with a scalpel, and sputter-coated with gold.

2.9. Data analysis

Data are expressed as means ± standard deviation (SD). The amount of calcium was expressed as μg calcium per cm^2 of polyurethane film's surface area, since most of calcium deposits were on the surface. In cases where calcification was within the material, the data are expressed as μg calcium per mg material dry weight. The significance of differences between measurements was assessed with the non-parametric Mann-Whitney U test, and the Spearman rank correlation test.

3. Results

3.1. Calcification of polyurethane implants

The calcification of polyurethane films implanted for various periods, and the effects of films thickness and porosity is detailed in Table I. As can be seen, the re-implanted films (15 + 15 days) exhibited similar low calcification levels as in films implanted for the same total period. No significant differences were found in the calcific deposits of films implanted for a 60 day period, or in films pretreated in serum. The introduction of porosity in polyurethane (by extracting pre-embedded polyethylene glycol 20% w/w, see Fig. 1) resulted in significant increase in calcification of both the thin and thick films, implanted or re-implanted for 30 days. However, it should be noted that intra-pores surface area was not included in the calculation of biomaterial's surface area.

TABLE I The effects of re-implantation, film thickness, and porosity on the calcification of Pellethane in rat subcutaneous implants (μg (cm^{-2} calcium) (± SD), $n = 8$)

	Film type and implant duration (days)			
	Plain		Porous[a]	
	30	15 + 15	30	15 + 15
Thickness (mm)				
0.2	1.8 (0.97)	1.93 (1.1)	4.3 (0.3)	6.56 (4.7)
0.7[b]	2.2 (1.2)	2.5 (1.1)	9.5 (4.0)	24.5 (5.5)

[a] All calcium levels in porous films were significantly higher than in plain films ($p < 0.05$).
[b] Significantly different from thinner films data ($p < 0.05$).

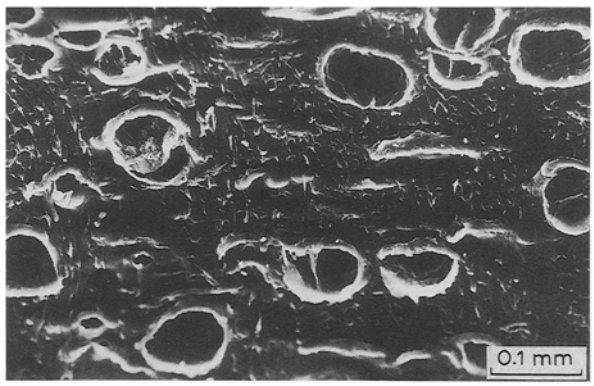

Figure 1 Scanning electron micrograph of cross-sectioned porous Pellethane film (20% PEG 3000 extracted film).

3.2. Effect of water capacity on calcification *in vivo*

The calcification of various biomaterials implanted subcutaneously in rats is described in Fig. 2. Polyurethane films (thick and porous) exhibited exceedingly low amounts of calcification (less than 1 μg mg^{-1}). Similarly, non-porous Hypol film (non-foamed) calcified only the surface (0.83 ± 0.2 μg (mg calcium)$^{-1}$). Marked calcification was observed in crosslinked collagen sponge, bioprosthetic tissue and Hypol foam (48.4, 53.2 and 37.2 μg (mg calcium)$^{-1}$), respectively). As can be seen, the amount of water absorbed was strongly associated with the calcification extent ($p < 0.01$, Spearman rank correlation test).

Representative SEM micrographs of Hypol sponges are depicted in Fig. 3. In calcified Hypol polymers (Fig. 3b–d), the calcification was mostly on the surface, and the crystals were deposited as a plaque of spherulles, in rosette-like structure. Corresponding X-ray dispersion analyses confirmed the presence of high levels of calcium.

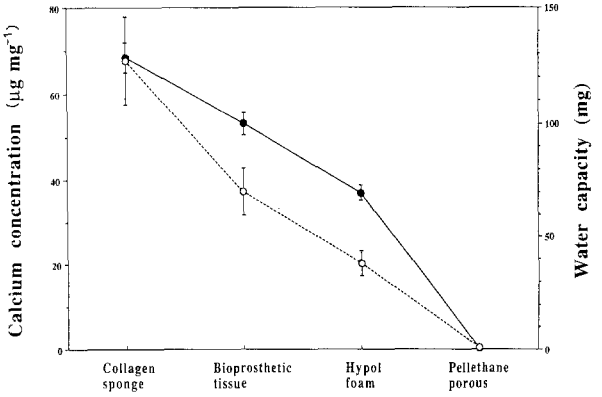

Figure 2 The water absorption capacity and calcification (± SD) of various biomaterials implanted subcutaneously in rats ($n = 10$) for 14 days. A correlation was found between calcification and water capacity of the various biomaterials ($p < 0.01$, Spearman rank correlation test). (●) Calcium concentration, (○) water capacity.

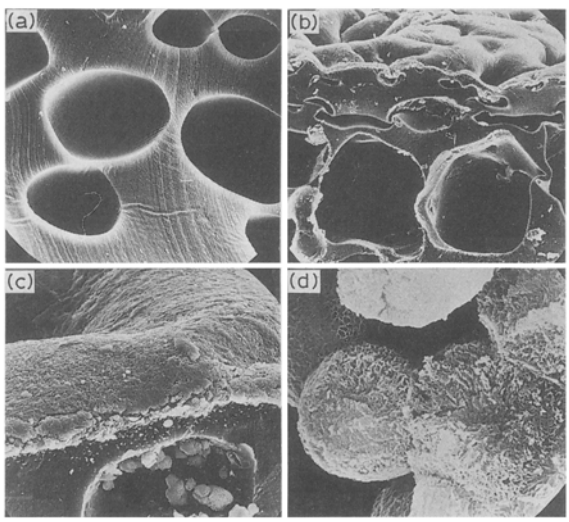

Figure 3 Scanning electron microscopy micrographs of (a) cross-section of Hypol (magnification × 500); (b)–(d) calcified Hypol after 14 day subcutaneous implantation; (b), (c) surface and cross-section view (magnification × 110 and × 500, respectively); (d) representative micrographs of calcium phosphate deposits (magnification × 2750).

3.3. Effect of water capacity on calcification *in vitro*

The role of material type (plain collagen, crosslinked collagen, bioprosthetic tissue, and hydrophilic polyurethane – Hypol), and the effect of biomaterial's water capacity on calcification rate and extent was studied *in vitro* (Fig. 4). In general, the calcium concentration in the collageneous material was significantly higher than in Hypol, at all incubation time periods. The collageneous materials exhibited similar levels of calcification after 4 and 8 h incubation time; ranging from 4.4 to 5.5 μg (mg calcium)$^{-1}$. Moderate increase in calcium concentration was observed after incubation for 30 h, but only in bioprosthetic tissue (6.1 μg (mg calcium)$^{-1}$). In contrast, the calcification profile of Hypol was different. Thus, significantly higher calcification values were observed after 8 and 30 h, in comparison to the those obtained after 4 h incubation in the calcium phosphate solution (1.7 and 0.096 μg mg^{-1}, respectively).

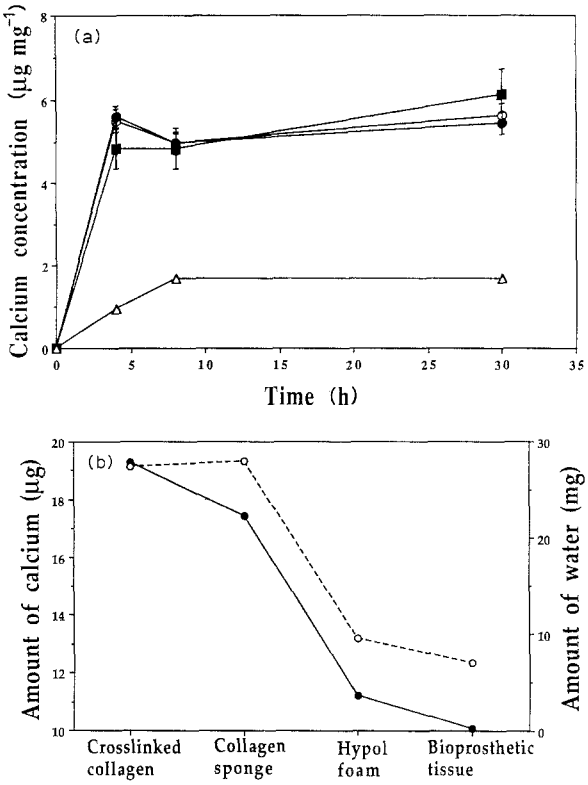

Figure 4 (a) The calcification (μg (cm^{-2} calcium) ± SD) of various biomaterials incubated in 3.95 mM^2 calcium phosphate solution. (b) The correlation between water absorption capacity and extent of calcification ($p < 0.01$, Spearman rank correlation test). (a) (○) Plain collagen sponge, (●) crosslinked collagen sponge, (■) bioprosthetic tissue, (△) Hypol foam; (b) (●) calcium, (○) water.

A correlation ($p < 0.01$, Spearman rank correlation test) was found between the amount of water absorbed and the calcification of the various biomaterials (Fig. 4b).

4. Discussion

Subdermal implants of bioprosthetic tissue in young rats provide a well-controlled model, yielding after a short duration comparable calcification levels to those noted in clinico-pathologic material after several years, with similar pathophysiology [19, 22–24, 16]. The pre-incubation of polyurethane films in serum did not result in increased calcification, although protein deposition on film surfaces was noted. This finding was also observed in *in vitro* studies [20], and it is in accord with the findings of Urry *et al.* [32], who showed that the calcification of crosslinked polypentapeptide of tropoelastin in serum is mediated only by calcium and phosphorous.

To obtain increased calcium concentrations, we hypothesized that re-implantation, which repeatedly exposes the polymer to the rapid calcification rate of the young growing rat [23, 24], would provide calcific deposits enabling reliable quantitative comparisons. Indeed, the highest calcification values were observed in polyurethane films which were re-implanted (Table I). However, even the highest calcification value of polyurethane (24.5 μm cm^{-2} or 2.8 μg (mg Ca^{2+})$^{-1}$), which was obtained with the combination of several factors, i.e. re-implantation of thick and porous films,

is exceedingly small in comparison to the 42.7 ± 21 μg (mg calcium)$^{-1}$ observed in polyurethane trileaflet cardiac valve prostheses that had been implanted in sheep for 17–21 weeks [3]. Subcutaneous implants of bioprosthetic tissue exhibit even higher calcification levels of 50–90 μg mg^{-1} [19, 23, 26]. It is well known that circulatory implants of tissue-derived biomaterials are markedly more prone to calcification (including both rate and extent) than synthetic, polymeric-derived biomaterials (for a review see [24]). In view of the higher propensity of BHV tissue to calcify both in circulation and in the rat subdermal model, it is important to note that the same tendency was also demonstrated in a recently developed *in vitro* model [20].

Our rat subdermal studies demonstrate that by increasing film thickness and porosity, significantly higher levels of calcification were obtained (Table I). The same phenomenon of increased calcification levels of thick and porous films has been also observed in *in vitro* studies [20]. The increased porosity and thickness of the polymeric film results in a larger surface area and volume, which facilitate increased permeation of calcium/phosphate ions yielding greater calcification. Studies on vascular graft materials demonstrate the important role of material porosity in calcification [17, 33, 34]. The increased calcification levels of thick and porous films found in this study are likely to be due to the increased diffusion via surface pores, as was found in subdermal implants of porous poly hydroxyethyl methacrylate hydrogels [35]. It has been suggested that the cardiovascular calcification of smooth polyurethane surfaces, not expected to produce pseudo-neointima, is attributed to surface defects and subsurface voids [11, 12]. Coleman [12] suggested that microbubbles or surface fractures may act like porous sites to induce mineralization of smooth elastomers.

Based on the findings of enhanced calcification of porous polyurethane, which was also observed in our previous *in vitro* studies [20], we postulated an hypothesis which could explain the different propensity of prostheses (polyurethane) and bioprostheses (bioprosthetic tissue) to calcify, in circulation and in the rat subdermal milieu. We hypothesized that material hydrophilicty, i.e. the ability of the implanted biomaterial to adsorb water is an important determinant of the calcification rate and extent. To test this hypothesis, the calcification of various biomaterials with different capabilities to absorb water was examined. As can be seen in Fig. 2, while the highest calcification was exhibited by collagen sponge and bioprosthetic tissue, substantial calcification was also observed in Hypol, an hydrophilic polyurethane foam. In contrast, porous polyurethane, which absorbs a markedly lower amount of water (28% w/w in comparison to water sorption in the range of 137–900% w/w), exhibited exceedingly low amounts of calcification. The calcific deposits noted in Hypol foam were mostly on the surface (see Fig. 3b and c), probably due to the restricted diffusion into deeper sections of the polymer, which is constructed of many closed cells (see Fig. 3a).

An additional support to the direct relationship between water absorption capacity and calcification was provided by the *in vitro* studies (Fig. 4). This model, which is characterized by non-spontaneous formation of calcium phosphate precipitate but triggered by the biomaterial, enabled us to examine the relationship between water capacity of the biomaterial and its calcification at the initial stage of the deposits formation. The extent of calcification was found to be in good correlation with the water sorption capacity of the material (Fig. 4b). It is generally accepted that the circulating fluids of vertebrates are supersaturated with respect to hydroxyapatite precipitation to allow for the continued crystal growth of this material, but undersaturated to allow for the *de novo* formation of the inorganic phase [14, 36–38]. Thus, a specific material property such as the enhanced affinity of glutaraldehyde-crosslinked tissue/collagen to Ca^{2+} [26], the ability of collagen fibrils to nucleate hydroxyapatite [39], or to a lesser extent, the affinity of polyurethane for metal ions/calcium [40–43)], generates the calcification cascade in the biomaterial, which due to its absorbed water facilitates an increased diffusion of calcium/phosphate ions.

This explanation implies that a biomaterial having an increased affinity sites (quantitatively and/or qualitatively) to Ca^{2+} would result in enhanced calcification concerning both, rate and extent. Indeed, as was found in our *in vivo* and *in vitro* studies (Figs 2 and 4, respectively), significantly higher calcium concentrations are exhibited by bioprosthetic implants in comparison to prosthetic implants. Nucleation sites for hydroxyapatite formation could be an inherent property of the biomaterial (for example, glutaraldehyde-induced crosslinks in collageneous material), or conferred by the contacting fluid to the implant (for example, devitalized cells entrapped in the pseudo-intima formed on the rough surface of blood pumps).

It is suggested that the water capacity of the biomaterial determines the bulk level of calcification, which in turn is generated and propagates via the inherent affinity sites of the biomaterial to Ca^{2+}. This explanation is also supported by other studies. It has been shown [44] that calcification of bioprosthetic heart valves is enhanced in regions of dynamic mechanical stress associated with void formation, which could imbibe water facilitating the permeation of calcium/phosphate ions. A seemingly opposite phenomenon is the finding that compressive mechanical deformation inhibits calcification of bioprosthetic tissue [45]. This finding is readily explained by our theory, namely, the decreased space between the collagen bundles resulting from the compressive deformation is less accessible to fluid and ion permeation, as was speculated by those researchers. The site of calcific deposits observed in both bioprosthetic heart valves and polymeric-based medical devices further supports our hypothesis. Although the predominant calcific deposits responsible for valves failure are within tissue-collagen, calcification rapidly forms in fluid containing sites such as within insudated plasma constituents, termed "sponge phenomenon" [46], in cuspal hematomas, or in tissue overgrowth [47]. Similarly, the calcification of fabric-covered caged ball

heart valves is frequently present in the tissue associated with the cloth. The pseudointima formed on the rough surface of blood pumps calcifies when its thickness exceeds several hundred micrometres [48], initiated by the devitalized trapped cells [13]. However, it is conceivable that the thicker pseudointima, which contains more fluid, is more permeable to calcium/phosphate ions nucleating in the devitalized cells. Another example is the calcification of polyurethane-derived implants which is frequently associated with subsurface voids and surface defects [7]. Calcium deposits are found in these regions which imbibe fluid, as well as, occurring within the fluids imbibed by the biomaterial, or in the tissue growing into interstices of porous materials [49].

5. Summary and conclusions

The calcification of bioprosthetic tissue exhibited in the rat subdermal model was not reproduced by polyurethane implants, although increased calcification was obtained by re-implanting porous polymer. In view of the exceedingly low levels of polyurethane calcification, it seems that the rat subdermal model is not suitable for routine studies of the mechanism of polyurethane calcification. The calcification extent of both collageneous and hydrophilic polyurethane, was found to be in good correlation with the water absorption capacity of these biomaterials. It is suggested that the water capacity of the biomaterial determines the bulk level of calcification, which in turn is generated and propagates via the inherent affinity sites of the biomaterial to Ca^{2+}. This explanation implies in turn that a biomaterial having increased number and/or higher affinity sites to Ca^{2+} would result in enhanced calcification concerning both rate and extent.

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