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SCANNING ELECTRON MICROSCOPY STUDIES OF THE PREVENTION OF BIOPROSTHETIC HEART VALVE CALCIFICATION WITH CONTROLLED RELEASE POLYMERIC MATRICES

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Abstract:

Pathologic calcification is the principal failure mode of bioprosthetic heart valves fabricated from glutaraldehyde pretreated porcine aortic valves. Bovine pericardial bioprosthetic valves also fail frequently by calcification. This review covers the novel uses of the scanning electron microscope (SEM) for investigating the pathology and prevention of bioprosthetic heart valve calcification. The progression and growth of calcific lesions in the bioprosthetic heart valve tissues has been documented using SEM with elemental localization by energy dispersive X-ray analysis (EDX). Controlled release polymeric matrices consisting of either ethanehydroxydiphosphonate, $FeCl_3$ or $Al(NO_3)_3$ coimplanted with bioprosthetic tissue prevent experimental bioprosthetic calcification. SEM has also been used to study the drug particle distribution in the controlled release matrices. Furthermore, matrix drug release *in vitro* and *in vivo* has also been characterized and quantified using SEM techniques.

Keywords: Bioprosthetic Heart Valve Calcification, Polymeric Matrices, Controlled Release Devices, Anticalcification Agents, EHDP, Aluminum, Iron,

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Introduction

Pathologic calcification is the principal failure mode of bioprosthetic heart valves fabricated from glutaraldehyde pretreated porcine aortic valves. Bovine pericardial bioprosthetic valves also fail frequently by calcification. Currently there is no effective therapy for this disease process (Schoen et al., 1988). Bioprosthetic heart valve calcification has been studied experimentally in either subcutaneous (Levy et al., 1986) or circulatory animal models (Barnhart et al., 1982). These animal model systems yield pathology comparable to clinical materials and reproduce the important pathological features of the intrinsic calcific lesions which lead to valve failure. These features, which include cell and collagen oriented calcification, ultimately lead to bulk mineral deposition (Schoen and Levy, 1986).

Prevention of bioprosthetic heart valve calcification by systemic administration of anticalcification agents such as ethanehydroxydiphosphonate (EHDP) leads to severe side effects on bone formation (Levy et al., 1987). However, local therapy with EHDP via coimplanted controlled release matrices containing these drugs offers a means of avoiding these problems. Controlled release in this context consists of drug dispersions within polymer matrices. The advantages of controlled release therapy are the lower doses required by drug delivery at the site of action, and the effective concentrations far below toxic levels due to constant regional release. Controlled release matrices containing EHDP (Golomb et al., 1987), $FeCl_3$ or $Al(NO_3)_3$ (Pathak et al., In Press) have been shown to be effective in inhibiting bioprosthetic heart valve calcification in subdermal rat model.

The objectives of the present paper are to review the uses of SEM techniques for investigating bioprosthetic heart valve calcification from both the mechanistic point of view, and in terms of understanding controlled release anticalcification therapy. SEM techniques were used to study the initial deposition of calcific deposits in the subdermal bioprosthetic implants with respect to the structure of the calcified valve tissue.

SEM techniques were also used to study the extent and mechanism of in vitro and in vivo drug release from polymeric matrices used for the prevention of bioprosthetic heart valve calcification.

Experimental

Materials and Methods

Polydimethylsiloxane (Silastic 382, Silastic Q-7 4840), and silicone rubber polyurethane copolymer (Silastic 6605-41) were obtained from Dow Corning (Midland, MI). Ethylene vinyl acetate was procured from E.I. DuPont (Wilmington, DE). Polyether polyurethane (Biomer) was obtained from Ethicon (Somerville, NJ). Nonradioactive and [^{14}C] labeled disodium ethanehydroxydiphosphonate (EHDP) were donated by Procter and Gamble (Cincinnati, OH). Reagent grade aluminum nitrate was obtained from Aldrich (Milwaukee, WI), and ferric chloride was obtained from Fisher (Fairlawn, NJ). Glutaraldehyde pretreated bovine pericardium (GPBP) was prepared, as previously described (Golomb et al., 1987): fresh parietal pericardium was obtained at slaughter from 2 to 3 week-old calves and immediately placed in iced sterile saline. Following dissection of superficial fat from the external surfaces, the tissue was crosslinked by incubating in 0.6% glutaraldehyde solution in HEPES buffer (pH 7.4) for 24 hrs and stored in 0.2% glutaraldehyde solution in HEPES buffer (pH 7.4) at 4°C. Glutaraldehyde pretreated porcine aortic valves were also similarly prepared, as described in detail elsewhere (Levy et al., 1983).

In vivo implant protocols: Pathophysiology studies

Male rats (CD strain, Charles River, Burlington, MA) 21 days old, were anesthetized with an intraperitoneal injection of ketamine and Rompun. Subcutaneous pouches were created over the anterior abdominal wall, an entire valve cusp was implanted into two to four subcutaneous pouches separated by at least 2 cm. At predetermined time intervals, ten specimens were explanted and examined by light microscopy, and chemically assayed for mineral content (Golomb et al., 1987). Two specimens from each time point were analyzed by transmission electron microscopy and four from each time point were examined by scanning electron microscopy with EDX. The EDX was performed on gold-coated glycol-methacrylate sample blocks from which histologic sections were prepared. Regions of interest were compared with those of the associated light histology slides (Schoen et al., 1985). Data for each valve sample consisted of the numbers of Ca and P x-rays detected at each site (counts) during a 1-minute interval minus background spectra obtained from an area of the plastic block without tissue. This method is described in detail elsewhere (Nelson et al., 1985). For each valve, several regions (100x100 μm) of fibrosa and spongiosa, separated by 500 to 1000 μm, were analyzed. The layered architecture of the porcine aortic valve, previously described (Ferrans et al., 1978), is analogous to that of the human cardiac valve (Gross and Kugel, 1931).

Preparation of controlled release polymeric matrices

The ethylene vinyl acetate (EVA) EHDP polymeric matrices were prepared by dispersing EHDP (20% by weight) in EVA (20% weight by volume) and casting as 1 cm diameter hemispherical matrices (Levy et al., 1985). This geometric structure provides nearly constant release rates for various test drugs (Hsieh et al., 1983). Before the release studies, the matrices were coated with ethylene vinyl acetate in methylene chloride (15%, w/v) and air dried; a release aperture, 0.58 mm in diameter, was drilled into the flat face of the hemisphere. Scanning electron micrographs were obtained of the pre- and post- in vitro release polymeric matrices.

Carrier-free radiolabelled disodium [^{14}C] EHDP, appropriately diluted with unlabeled disodium EHDP to yield a specific activity of 1830 dpm/μmol EHDP, was levigated into polymer formulations for determining in vitro release of EHDP (Golomb et al., 1987). For implants, circular (i.d.= 25 mm, o.d.= 31 mm) controlled release matrices were formulated by levigating disodium EHDP (90 to 106 μm particle size) into Silastic 382 or Q-7 4840 at a 20% or 30% concentration.

$AlCl_3$ and $FeCl_3$ polymeric matrices were solvent cast in both Silastic polyurethane copolymer and Biomer (Pathak et al., in press). Either, $AlCl_3$ or $Fe(NO_3)_3$ both 10% by weight per matrix) and the polymer were separately dissolved into dimethylacetamide. These two solutions were mixed thoroughly together and dried in a vacuum oven. The dried films were 0.54 $\pm$ 0.03 mm thick.

In vitro release studies

The in vitro release study of the EHDP silicone rubber drug delivery systems was conducted by incubating the matrices in 20 ml of a physiologic buffer (0.1 M NaCl in 0.05 M HEPES, pH 7.4) and a center, cross-sectional cut of the matrix was obtained at 0,1,2,3,4 and 5 months. The samples taken at various time intervals were allowed to dry at room temperature before the analysis. The samples were mounted on stainless steel stubs and sputter-coated with carbon. Samples were analyzed for phosphorous and silicone. The release profiles of EHDP from the silicone matrices were determined by using energy dispersive x-ray analysis (EDX) coupled with SEM and calculating the diphosphonate-associated phosphorous (P) depleted from the polymer matrix as a function of time. EDX was conducted using a Super 8000 Analyst (Kevex, Foster City, CA) equipped with a Kevex system for elemental localization which was coupled with a scanning electron microscope (Hitachi, Model S-570, Santa Clara, CA). Emitted X-rays for each element were collected for 1.5 min. the counts associated with the area under peak for each element were calculated by a deconvolution routine contained in the software program Quantex 1 (Myklebust et al., 1979). The amount of silicon in the silicone rubber based polymers was used to normalize the phosphorous depletion

by computing the ratio of the counts associated with the phosphorous peak to the counts observed for the silicon (Si) peak for each sample at the above specified time intervals and were compared with a series of precisely formulated standards of known composition (5%, 10%, 15%, 20% and 30% w/w EHDP in silicone rubber). The results obtained from EDX were also correlated with data obtained from parallel radioactive in vitro release studies using [^{14}C] EHDP (Johnston et al., 1989).

In vivo implant protocols: Controlled release

To study the prevention of bioprosthetic heart valve calcification by controlled release polymeric matrices, in a separate set of animal experiments, subcutaneous pouches were created in the rat abdomen as described above. In these pouches 1x1 cm pieces of either the GPBP alone or attached to the controlled release drug matrices containing EHDP or $Al(NO_3)_3$ or $FeCl_3$ were implanted. After 21 days, rats were sacrificed with CO_2 asphyxiation and the GPBP's explanted, lyophilized and hydrolyzed in 6N HCl, using established procedures. Calcium levels were determined on aliquots of the hydrolysates by atomic absorption spectroscopy. The explanted polymeric matrices were studied by SEM to elucidate the possible in vivo mechanism of release of the drug from the matrices.

Results and Discussion

Semi-quantitive assessment of morphology and distribution of mineralization

In the rat subdermal model, the onset of calcification was noted by 48 hours with both the light and electron microscopy (Schoen et al., 1985). Mineral deposits were not observed in specimens implanted for only 24 hrs. Early calcific deposits were small and punctate; they appeared to be associated with porcine aortic valve connective tissue cells diffusely in both the valve spongiosa and fibrous elements. The number of sites calcified, as demonstrated by EDX as well as the size of individual deposits, increased with time, principally in the spongiosa. Ultrastructural examination revealed both cell-associated and collagen-associated mineralization. Early calcium deposits were noted in devitalized porcine connective tissues, in the cytoplasm, or associated with the plasma membrane itself. Early deposits did not involve collagen bundles. Cell associated calcific deposits became widespread and prominent over the course of the first 21 days.

Although early mineral deposits occurred in both valvular fibrosa and spongiosa, EDX confirmed that the spongiosa was the preferential site of localization for advanced calcific deposits. The concentrations of both calcium and phosphorous in the spongiosa and fibrosa are shown in Figure 1. With increasing duration of implantation, there was little change apparent in the concentration of both Ca and P within the fibrous elements, but the spongiosa continued to accumulate mineral. EDX demonstrated early saturation of fibrosa with calcium by 7 days of implantation. In contrast, calcium in the spongiosa increased markedly with

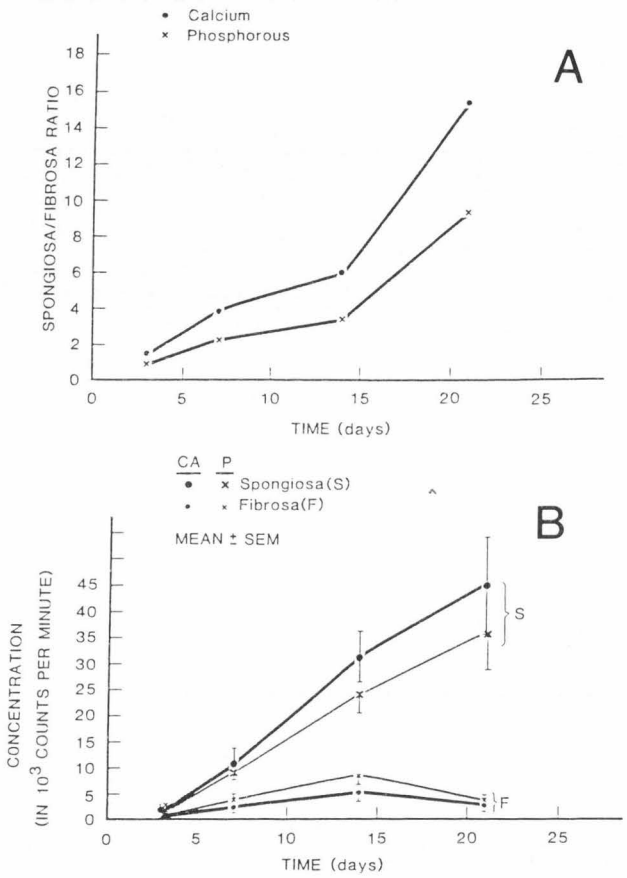
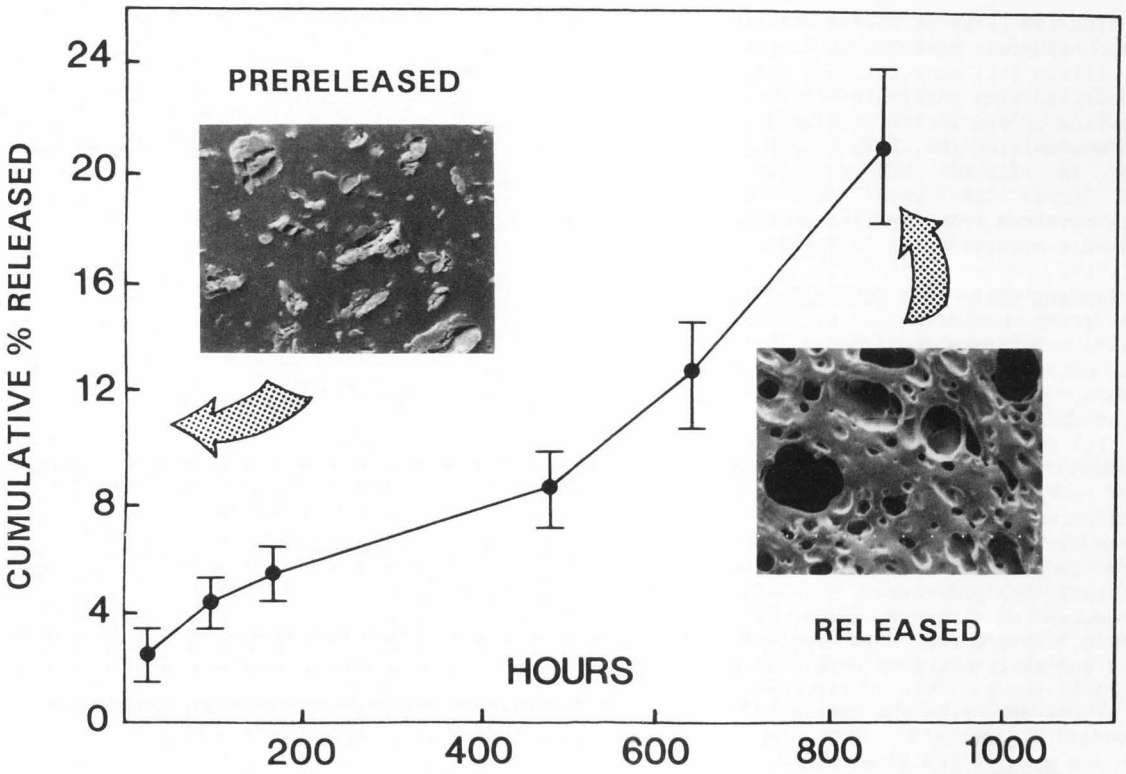


Figure 1. Site specific time dependence of calcium and phosphorus concentrations as determined by EDX on explanted porcine bioprosthetic heart valve leaflets retrieved from rat subdermal implants. A) Absolute EDX counts of calcium and phosphorus; B) EDX count ratio of spongiosa to fibrosa, for both calcium and phosphorus (reproduced with permission from Schoen et al., 1985)

time. The EDX count ratio of elemental concentrations in spongiosa to those in fibrosa increased progressively throughout the 21 days of implantation.

Assessing the mechanism of polymeric matrix drug release in vitro

SEM has been valuable for elucidating the in vitro progression of drug release from polymeric matrices. Prior work has demonstrated the utility of SEM imaging of a variety of matrix substrates and dispersed drugs. For example, studies of ethylene vinylacetate-disodium ethane hydroxy diphosphonate (EVA-EHDP) revealed that uniform sustained release of EHDP was observed for more than 600 hours in vitro, with only 12% cumulative release by this time (Figure 2) (Levy et al., 1985). Scanning electron micrographs of unimplanted matrices of EVA-EHDP showed a uniform distribution of EHDP particles ranging from 1 to 10 μm. After release of EHDP in vitro, scanning electron microscopy showed formation of cavities and


CUMULATIVE % RELEASED
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200
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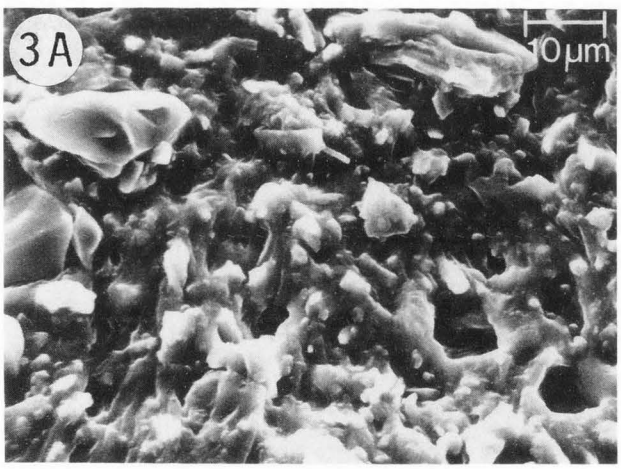
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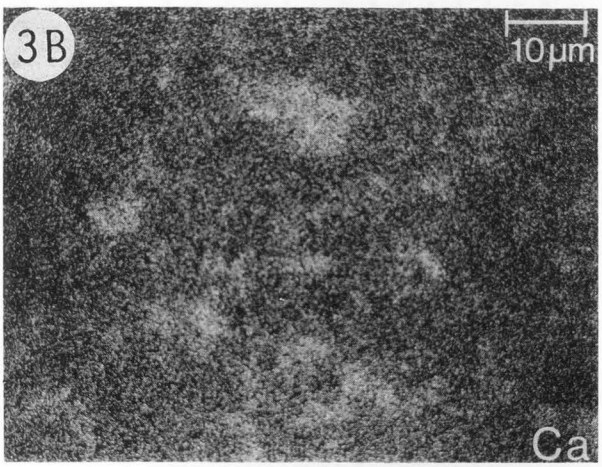
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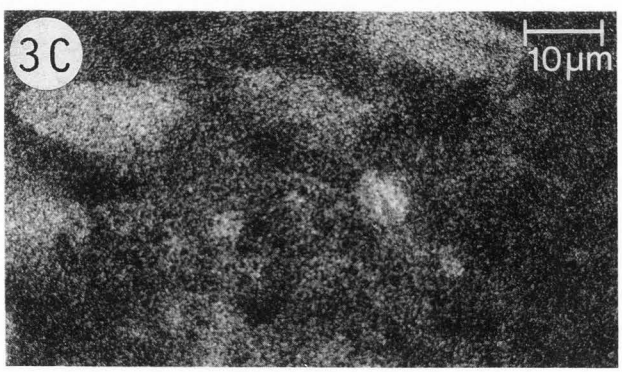
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3D
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Figure 2. Cumulative percentage of EHDP released from an ethylene-vinyl acetate EHDP matrix. Polymer matrices containing [^{14}C]EHDP were incubated at 37° C in 0.1 M NaH_2PO_4, pH 7.4. **Inserts**: scanning electron microscopy was performed on specimens before release and after 42 days of release in vitro. Drug particles (disodium EHDP) ranged in size from 1 to 10 μm (reproduced with permission from Levy et al., 1985).

Figure 3. Post in vitro release scanning electron micrograph (A) and X-ray elemental microanalysis maps (B, C and D respectively for calcium, phosphorus and silicon) of a matrix containing 30% (W/W) drug, 50:50 Ca_2EHDP: Na_2EHDP demonstrating element-specific drug particle and polymer spatial distribution (reproduced with permission from Golomb et al., 1987).

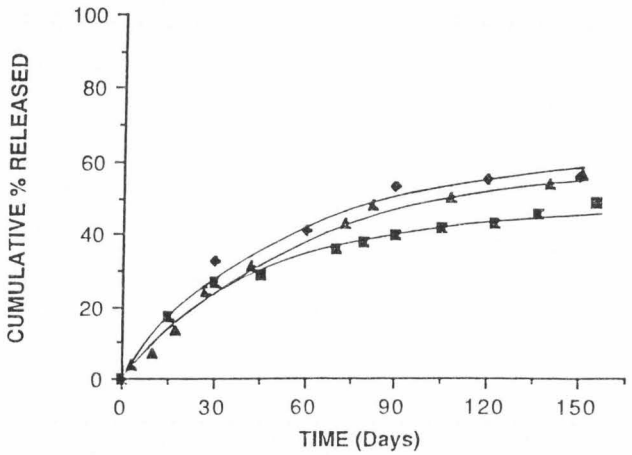

Figure 4. Typical in vitro release profiles of EHDP from a silicone rubber-EHDP circular drug delivery matrix (30% w/w Na_2EHDP and Ca_2EHDP 1:1) ,
(♦) Determination by electron microprobe analysis,
(▲) determination by release of Na_2 [^{14}C]EHDP, and
(■) determination by following overall decline in matrix weight.
(reproduced with permission from Johnston et al., 1989).

tortuous canals due to the dissolution and release of EHDP particles from the matrices.

SEM has also been useful for elucidating the controlled release mechanism of a mixture of disodium EHDP and the less soluble salt, CaEHDP from a silicone rubber matrix. A post release SEM (Fig. 3A) demonstrates cavitation, but a Ca-EDX map shows sparing of the CaEHDP particles (Fig. 3B) in comparison with the P-EDX (Fig. 3C), which demonstrates predominantly the CaEHDP phosphorus emissions. The Si-EDX (Fig. 3D) map is useful for demonstrating the background polymer matrix substrate.

Scanning electron microscopy in quantifying and evaluating controlled release matrices: In vivo results

SEM coupled with EDX has also been useful for documenting the extent of in vivo drug release using elemental specific x-ray emissions in a semiquantitative method. For example, residual drug remaining in silicone rubber can not be determined through any extraction or dissolution procedures. However, the in vitro drug release of EHDP from silicone rubber monolithic matrices was also studied using EDX analysis by calculating the diphosphonate -associated phosphorus (P) depleted from the polymer matrix as a function of time. Studies of matrices containing equal molar amounts of Na_2EHDP and CaEHDP(1:1) demonstrated in vitro release in complete agreement with control data obtained by matrix weight change, and release of Na_2[^{14}C] EHDP. Figure 4 illustrates cumulative release profiles obtained by each of these three methods; these document the reliability of the EDX approach.

Furthermore the SEM EDX technique just described has been most useful in assessing residual drug in estimating the in vivo release from the matrices explanted from the circulation. Silicone rubber controlled release matrices containing Na_2 EHDP were coimplanted in the sheep circulation as tricuspid valve replacements, to assess the efficiency of this agent in inhibiting bioprosthetic tissue calcification. These matrices were efficacious without adverse effects and their residual drug was readily determined with EDX. In vivo explant analysis of EHDP matrices using EDX revealed 79.0± 4.82 cumulative percent EHDP released for matrices containing Na_2EHDP (1:1) after 120-150 days. These results were comparable to the in vitro data reported elsewhere (Johnston et al., 1989).

Al^{3+} or Fe^{3+} controlled release from polyurethane or polyurethane-silicone rubber copolymer matrices was also effective for inhibition of GPBP calcification in the rat subdermal model, with no adverse effects (Pathak et al., In Press). Hydrophilic polymers such as polyurethanes and silicone rubber-polyurethane copolymers have unique controlled release matrix properties, which can be elucidated with SEM. For example, the prerelease study of the $Al(NO_3)_3$ in Biomer matrices and $FeCl_3$ in silicone rubber-polyurethane matrices revealed that the $Al(NO_3)_3$ had formed a solution in the polymer, while the $FeCl_3$ was uniformly dispersed as particles in the polymeric matrix (Figure 5). However, post in vivo release SEM of each of the two types of matrices demonstrated comparable cavitation and surface erosion (Figure 5). Thus, the comparatively faster release rates from the polyurethane polymers are most likely due to the greater water permeability of this material compared to silicone rubber-polyurethane copolymer.

Conclusions

It is concluded that SEM coupled with EDX is useful for investigating the pathogenesis and prevention of bioprosthetic heart valve calcification. SEM-EDX has provided semi-

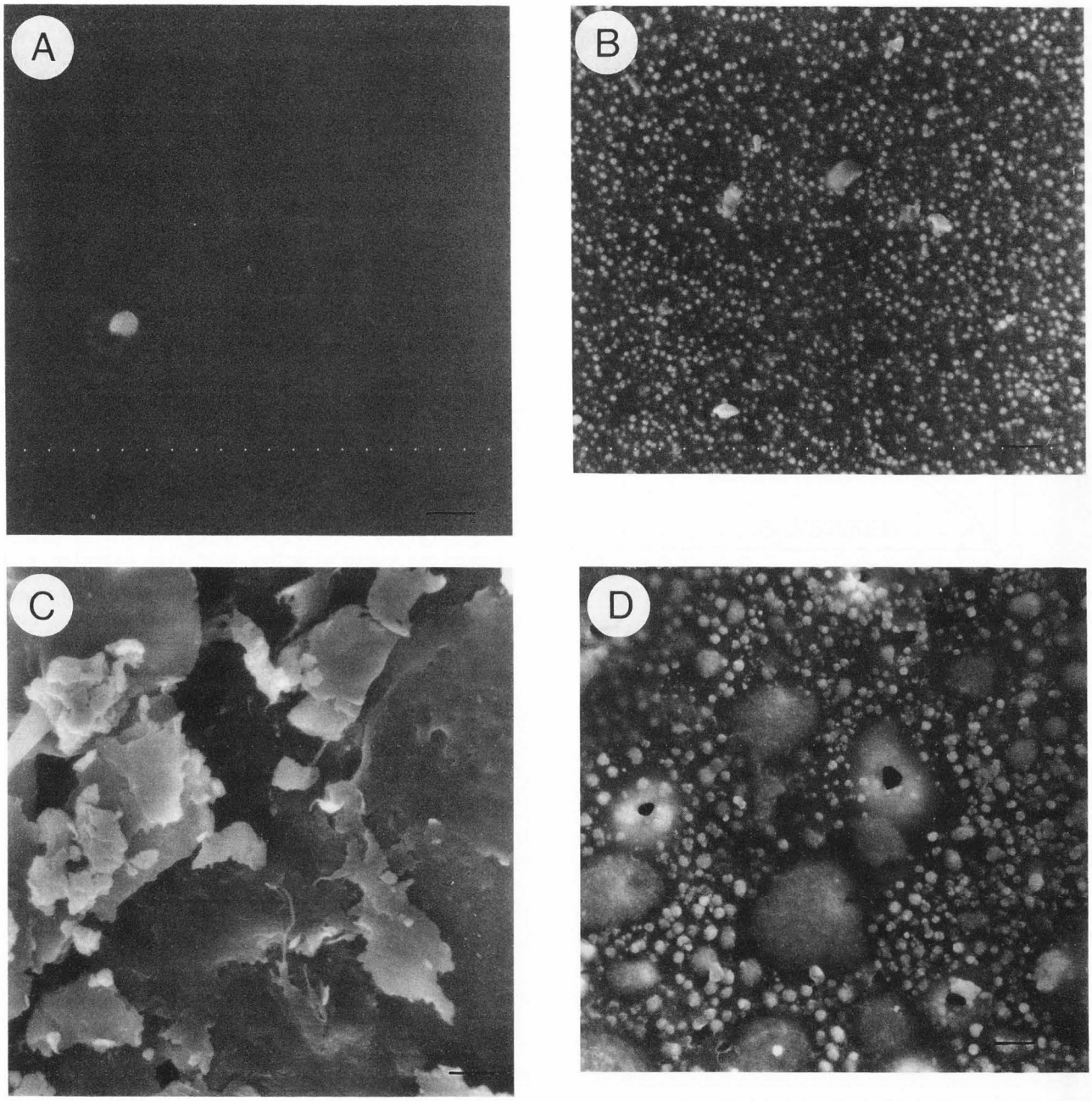

Figure 5. Scanning electron micrograph of pre (A) and post (B) in vivo release $Al(NO_3)_3$ polymeric matrices (in polyether polyurethane polymer: Biomer) showing the complete solution of the drug in the matrix material (A); the post in vivo release micrograph (B) shows the particle swelling, drug dissolution and cavitation phenomena during release. The $FeCl_3$ polymer (in silicone rubber-polyurethane copolymer) scanning electron micrographs show the uniform particle dispersion of the drug in the matrix pre release (C), while a comparable release mechanism (D) to $Al(NO_3)_3$ (B) is observed in the post in vivo release polymer (Bar=2 micrometers).

quantitative documentation of the progression of GPBP calcification. In addition SEM and SEM-EDX have helped to elucidate the mechanism of drug release from controlled release polymeric matrices.

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References

Barnhart GR, Jones M, Ishihara T, Chavez AM, Rose DM, Ferrans VJ (1982) Bioprosthetic valvular failure. Clinical and Pathological observations in an experimental animal model. J Thorac Cardiovas Surg, 83, 618-631.

Ferrans VJ, Spray TL, Billingham ME, Roberts WC (1978) Structural changes in glutaraldehyde treated porcine heterografts used as substitute cardiac valves: transmission and scanning electron microscopic observations in 12 patients. Am J Cardiol, 41, 1159-1184.

Golomb G, Dixon M, Smith MS, Schoen FJ, Levy RJ (1987) Controlled release drug delivery of diphosphonates to inhibit bioprosthetic heart valve calcification: Release rate modulation with silicone matrices via drug solubility and membrane coating, J Pharm Sci, 76, 271-276.

Gross L, Kugel MA (1931) Topographic anatomy and histology of the valves in the human heart. Am J Pathol, 7, 445-473.

Hsieh DST, Rhine WD Langer R (1983) Zero-order controlled release polymer matrices for micro and macromolecules, J Pharm Sci, 72, 17-22.

Johnston TP, Bove EL, Bolling SF, Boyd JA, Ciesliga BL, Amidon GL, Schoen FJ, Levy RJ (1989) Controlled release of 1-hydroxyethylidene diphosphonate: in vitro assessment and effects on bioprosthetic calcification in sheep tricuspid valve replacements. Int J Pharm, 52, 139-148.

Levy RJ, Schoen FJ, Levy JT, Nelson AC, Howard SL, Oshry LJ (1983) Biologic determinants of dystrophic calcification and osteocalcin deposition in glutaraldehyde-preserved porcine aortic valve leaflets implanted subcutaneously in rats. Am J Pathol, 113, 142-155.

Levy RJ, Wolfrum J, Schoen FJ, Hawley MA, Lund SA Langer R (1985) Inhibition of calcification of bioprosthetic heart valves by local controlled release diphosphonates. Science, 228, 190-192.

Levy RJ, Schoen FJ, Golomb G (1986) Bioprosthetic heart valve calcification: clinical features, pathobiology, and prospects of prevention. CRC Critical Reviews in Biocompatibility, 2 (2), 147-187.

Levy RJ, Schoen FJ, Lund SA, Smith MS (1987) Prevention of leaflet calcification of bioprosthetic heart valves with diphosphonate injection therapy. Experimental studies of optimal dosages and therapeutic durations. J Thorac Cardiovas Surg, 94, 551-557.

Myklebust RL, Fiore CE, Heinrich KFJ (1979) Frame-C: a compact procedure for quantitative energy dispersive electron probe X-ray analysis. NBS Technical Note, 1106.

Nelson AC, Schoen FJ, Levy RJ (1985) SEM methodology for study of the pathophysiology of calcification in bioprosthetic heart valves. Scanning Electron Microscopy, 1, 209-213.

Pathak YV, Boyd J, Schoen FJ, Levy RJ. (1990) Prevention of calcification of glutaraldehyde pretreated bovine pericardium through controlled release polymeric implants: studies of Fe^{3+}, Al^{3+}, Protamine sulfate and Levamisole. Biomaterials (in press).

Schoen FJ, Levy RJ, Nelson AC, Bernhard WF, Nashef A, Hawley M (1985) Onset and progression of experimental bioprosthetic heart valve calcification. Lab Invest., 52, 523-532.

Schoen FJ, Harasaki H, Kim KM, Anderson HC, Levy RJ (1988) Biomaterial-associated calcification: pathology, mechanisms, and strategies for prevention. J Biomed Mater Res,: Applied Biomaterials, 22, 11-36.

Schoen FJ, Levy RJ (1986) Biomaterial-associated pathology of cardiac valve prostheses: Clinical explant analysis and studies of tissue valve calcification. Mat Res Symp Proc, 55, 29-36.

Webb CL, Flowers WE, Boyd J, Rosenthal EL, Schoen FJ, Levy RJ (1990) Al^{3+} binding studies and metallic cation effects on bioprosthetic heart valve calcification. Trans ASAIO, 36, 56-59.

Discussions with reviewers

F. M. Lupinetti: How would the author's methods for ameliorating GPBP calcification, i.e. controlled release matrices, compare with other methods, e.g. surfactants?

Authors: Controlled release drug delivery has the advantage of providing high local levels of effective pharmacologic agents, which would either not be effective systemically or would be associated with prohibitive side effects. Specifically, controlled release of diphosphonates, or ferric chloride, or aluminum salts, has advantages over surfactants, in that constant sustained levels of drug are available whereas the possibility exists for the surfactants to wash out. In addition, surfactants used as a biomaterial pretreatment undoubtedly extract or alter the bioprosthetic heart valve tissue, thereby potentially compromising its material properties.

F. M. Lupinetti: Other studies have suggested that diphosphonates increase calcification of GPBP in large animal models of tricuspid valve replacement. To what extent are these differences in results attributable to the differences in models and are there other factors that explain the discrepancy?

Authors: We are not aware of any substantiated published results demonstrating systemic diphosphonate therapy to be effective for bioprosthetic heart valve calcification in

circulatory studies. Diphosphonates are well known anticalcification agents and, if used at effective doses, systemically will always be efficacious for preventing pathologic calcification but would certainly adversely effect skeletal calcification as well.

F. M. Lupinetti: Do the treatment methods for prevention of calcification decrease the ultimate quantity of calcium deposits on the valves, or merely shift the time course of calcification to a certain degree?
Authors: The treatment and methods described would prevent calcification according to their various specific mechanisms of action. For example, diphosphonates are known to restrict hydroxyapatite crystal growth. Therefore, the ultimate bulk quantity of calcium phosphates deposited on the valves would be limited. Furthermore, Al^{3+} and Fe^{3+} are thought to inhibit calcification by blocking cellular initiation sites. Therefore, the ultimate quantity of calcification would also be limited, rather than a mere shifting of the time course.

H.C. Anderson: What was the rationale for adding aluminum nitrate versus ferric chloride? Are these substances active in preventing prostheses calcification or do they simply mimic the dissolution pattern of EHDP?
Authors: The rationale for studying both aluminum and ferric salts was based upon recent results in our laboratory demonstrating that both metallic salts have comparable efficacy for inhibiting bioprosthetic heart valve calcification in preincubation studies (Webb et al., 1990). Preliminary results demonstrate thus far that their mechanism of action has to do with a high affinity association with the cell membranes of the devitalized cells within the bioprosthetic heart valve tissue. The reported controlled release results, using either ferric or aluminum salts, demonstrate the first successful efforts toward sustained drug delivery of these compounds. Their mechanism of action is undoubtedly very different from ethanehydroxydiphosphonate (EHDP), since this compound most likely inhibits calcium phosphate crystal growth, and has not been shown to have any specific affinity or association with cell membranes.

H.C. Anderson: Although EM probes microanalysis of the release of phosphorous seems to be a quantitatively adequate method to describe the kinetics of release, I should think that the simple weighing would be far simpler and therefore the more expeditious approach.
Authors: Our data presented in Figure 4 conclusively showed that the EDX results agree very nicely with results obtained by weighing. However, the weighing technique is only suitable when an entire drug delivery matrix can be retrieved intact from either an experiment or a clinical retrieval. For a variety of reasons, this was almost never possible in our series of circulatory explants, therefore the EDX microanalysis approach proved to be exceedingly useful.