In Vitro Evaluation for Potential Calcification of Biomaterials Used for Staple Line Reinforcement in Lung Surgery

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Bovine pericardium (BPC) and polytetrafluoroethylene (PTFE) have been widely used to reinforce staple lines in lung resection. Since limited information regarding the calcification of these biomaterials is available, we undertook an *in vitro* study to evaluate their calcification potential. Commercially available BPC and PTFE biomaterials were evaluated and compared with custom-prepared BPC tissue. *In vitro* calcification was performed via submersion in supersaturated solution in a double-walled glass reactor at 37.0°C ± 0.1°C, pH 7.4 ± 0.1, mimicking most ion concentrations of human blood plasma. In processing of calcification, the pH decrease of the solution simulated the addition of consumed H^+, Ca^{2+}, and PO_4^{3-} ions from titrant solutions, the concentrations of which were based on the stoichiometry of octacalcium phosphate. The molar ion addition with time was recorded, and the initial slope of the curve was computed for each experiment. The rate of calcification developed (molar calcium phosphate ion addition rate per time and total surface area) (R) was computed after that with respect to the relative supersaturation (σ) used in each experiment. R for custom-prepared BPC tissues was found to be in the range of 0.19 ± 0.08 to 0.52 ± 0.19 ($n = 17$) in σ range of 0.72 to 1.42. Commercial BPC was found to be 0.016 to 0.052 ($n = 4$), and PTFE was 0.005 to 0.05 ($n = 8$) in the same σ range. Both clinically applied biomaterials, BPC and PTFE, seemed to be calcified with rates of at least one order of magnitude lower than the custom-prepared BPC tissue. This data suggested that BPC and PTFE biomaterials showed a similar, relatively very low tendency for calcification compared with custom-prepared BPC tissue. Although further studies are necessary, staple line reinforcement by these two biomaterials should be considered safe from the calcification point of view. Exp Biol Med 231:1712–1717, 2006

Key words: bovine pericardium; polytetrafluoroethylene; lung-volume reduction; surgical stapling; *in vitro* calcification

Introduction

Lung volume reduction surgery performed on a well-selected subset of patients with severe pulmonary emphysema reduces symptoms, improving lung function and quality of life (1–3). It has been shown that the best method of preventing air leaks is the use of stapling devices armed with strips of either bovine pericardium (BPC) or polytetrafluoroethylene (PTFE). Buttressing the staple line significantly shortens the duration of air leaks and the drainage time in surgery for pulmonary emphysema (4–7).

Biomaterials consisting of BPC are prone to calcification. Various host and implant factors, such as dead cell remnants, lipids, mechanical stress and others, alone or in synergy, may be responsible for the initiation and development of calcification. Despite continuous efforts to develop more effective anticalcification treatments of new biomaterials, including BPC and PTFE, no method so far has proven to be effective in the long term (8–11).

Different methods are used for premarket evaluation of the anticalcification efficacy of new biomaterials. Animal models, although accepted as predictive methods, are expensive and time consuming to use in prescreening evaluation of new biomaterials. Alternatively, the use of *in vitro* models looks promising, provided that solid phases are deposited on biomaterials in a clinically relevant way. In previous works we established a new method for *in vitro* calcification of biomaterials (12, 13).

Today minimal information exists regarding the host reaction to the commercially available strips used to reinforce staple lines. This technique has been applied clinically worldwide for less than 10 years. Although severe long-term complications due to calcification have not been reported so far, it is still too early to safely predict degeneration and calcification because these biomaterials

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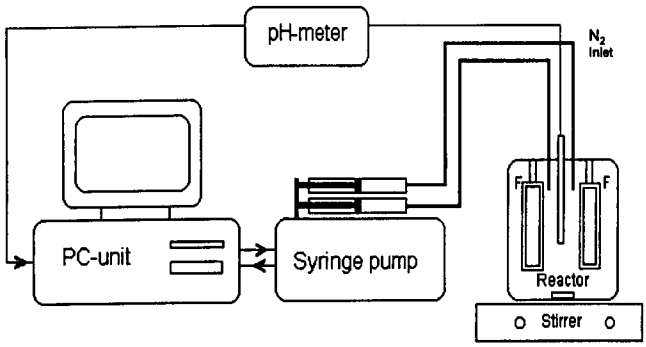

Figure 1. Drawing of the experimental set-up used for the development of calcific deposits on implant materials *in vitro*. The computer receives a signal of pH drop from the pH meter, stimulates the syringe pump, and records the quantity of the solutions ejected from the two syringes into the reactor.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

may become problematic as long as 10 years after implantation. Our study aims to evaluate the potential *in vitro* for calcification of biomaterials used in staple line reinforcement.

Materials and Methods

Commercially available BPC patches (Peri-Strips; Bio-Vascular, St. Paul, MN) and PTFE patches (Seamguard; W.L. Gore & Associates, Flagstaff, AZ), both used clinically for staple line reinforcement in lung surgery, were evaluated and compared with custom-prepared animal-harvested BPC tissue, which was subsequently fixed with glutaraldehyde and stored in formaldehyde solution. To the best of our knowledge, this fixation procedure is the most well-established and standardized method (13).

The BPC tissues were obtained from the local slaughterhouse immediately after the deaths of the animals and were transported to the laboratory in iced saline medium. Within 3 to 4 hours pericardium was trimmed by removing the external excess fatty tissue. The trimmed tissue samples were then stored in Ringer's solution at 4°C.

The samples were fixed the next day in 0.15 *M* phosphate buffer solution (pH 7.4) into which 0.625% glutaraldehyde was added (25% w/v solution; Serva Feinbiochemica GmbH, Heidelberg, Germany). The tissues were immersed in the solution for 1 hour at room temperature. A slight mechanical prestress was applied to the BPC tissues during glutaraldehyde fixation. The tissues were prestressed biaxially by suturing and slightly tensioning rectangular samples (7 × 7 cm) into 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 (13).

***In Vitro* Calcification.** The detailed theoretic and technical descriptions of our method of *in vitro* calcification have been previously reported (12). A brief description is presented here.

Physicochemically, the driving force for the formation of a crystalline mineral phase of a type A_mB_n $(m+n=\nu)$ in a solution of positively and negatively charged ions, A^{n+} and B^{m-}, is the change in Gibbs free energy, ΔG, for going from the supersaturated solutions to equilibrium:

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

where a represents the activities of the respective ions, R_g the gas constant, T the absolute temperature, and K_s^0 the thermodynamic solubility product of the mineral phase considered (14). The supersaturation with respect to the mineral phase forming may be expressed as:

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

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:

$$\sigma = \Omega^{\frac{1}{\nu}} - 1.$$

Figure 1 shows a schematic diagram of the experimental set-up used for the *in vitro* experiments on calcification of biomaterials. All experiments were done in a thermostable double-walled water-jacked Pyrex glass reactor, 50 ml total volume. The reactor was filled with a simulation plasma solution containing similar electrolytes (K^+, Na^+, Ca^{2+}, and PO_4^{3-}) and concentration levels to human plasma. The temperature was maintained at 37.0°C ± 0.1°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 m*M* and 1.2 m*M* total calcium equivalent concentration of total phosphate and at ionic strength 0.15 *M* adjusted with NaCl. Under these conditions, the supersaturated medium was stable practically indefinitely. In all experiments the homogeneity of the working solutions was ensured by magnetic stirring, and water vapor–presaturated nitrogen maintained an inert atmosphere. The drop in the solution pH signaled the initiation of the calcification process. A drop in the working solution pH by as small as 0.005 pH units triggered the addition of titrant solutions from the computer-controlled syringes. To keep the working solution composition constant, one of the syringes contained $CaCl_2$ solution and the second mixed NaH_2PO_4, NaOH, and NaCl solutions. The stoichiometry of the titrant solutions with respect to calcium and phosphate depended upon the expected stoichiometry of the precipitat-

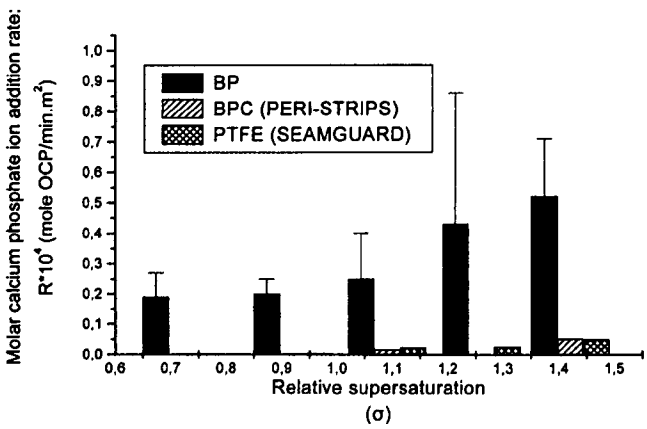

Figure 2. Diagram of the precipitated calcium phosphate quantity expressed in moles OCP per time and per total surface area with respect to the relative supersaturation σ on the tested BPC and PTFE patches *in vitro* (mean ± SD).

ing salt. Earlier work on the system showed that octacalcium phosphate (OCP) is the predominant calcium phosphate phase formed at the first stages of calcification (15, 16). It was decided to use the stoichiometry of OCP in the titrant solution added by the syringe pumps.

By this method:

1. The pH of the working solution in which calcification takes place was maintained constant without the use of buffer solutions.
2. The addition of the consumed ions of Ca^{2+} and PO_4^{3-} due to calcification maintained the initial physiologic concentrations, permitting the continuation of the process for a long time, sufficient for the identification of the mineral phase formed.
3. The kinetics of mineral phase formation were measured accurately through the profiles of the added volumes of the titrant solutions as a function of time.
4. Maximum reproducibility was achieved through the solution composition maintenance (13).

For each experiment, four specimens (1.5 × 7 cm each) of the biomaterial being tested were mounted on Plexiglas frames by sutures (Fig. 1), immersed in the working solution of a specific supersaturation σ value, and subjected to calcification for 24 hours. After immersion of the mineralizing substrates in the working solutions, samples were withdrawn randomly to verify the constancy of the solution composition. The samples were filtered through membrane filters (0.22 μm; Millipore, Billerica, MA), and the filtrates were analyzed for calcium and phosphate (13). A total number of 17 experiments were performed for custom-prepared BPC tissue at five different σ values, four for BPC at two σ values, and eight for PTFE samples at four σ values.

Data Analysis. Results were expressed as the mean ± the standard error of the mean. For comparison of the two groups, the Mann-Whitney *U* test was used. Analyses were performed using SPSS for Windows, release 11.0.1 (SPSS Inc., Chicago, IL).

Results

In each experiment, the comparison between the different substrates was conducted at similar solution conditions. The volume of the titrant solutions added with time was recorded. From these plots, the initial rates of the process were calculated for the different degrees of the solution supersaturation σ tested.

The rates of precipitation (R) were computed as moles (equivalent to) OCP per unit time and surface area. For the area calculation, the geometric area of the substrates multiplied by two (two surfaces) was taken into account.

Figure 2 presents the added calcium phosphate molar quantity R per tissue surface area and per time for different σ values exceeding the physiologic range of blood plasma. All ions from the titrant solutions added to the tested materials did not remain in the solution or on other surfaces in contact with it. Both clinically applied biomaterials, PTFE and BPC, seemed to calcify with rates approximately one order of magnitude lower than the custom-prepared BPC tissue.

Discussion

Prolonged air leak is the major limiting factor in early hospital discharge following pulmonary resection. Although Miller *et al.* (17) in a randomized trial failed to prove a statistically significant difference between buttressed versus nonbuttressed staple lines in pulmonary resection, among most thoracic surgeons staple line reinforcement with BPC or expanded polytetrafluoroethylene (ePTFE) has become widely accepted in lung resections (18).

A theoretic problem involves the use of BPC and the risk for transmissible spongiform encephalopathy. The World Health Organization has recommended that whenever possible cattle sources should be avoided for medical products (19). However, there has been no evidence of transmission of this fatal disease through medical materials derived from a bovine source. Alternative and/or complementary techniques to staple line reinforcement to reduce air leaks from the lung parenchyma have been reported, including the use of a collagen-reinforced polyglycolic mesh and a liquid sealant (20, 21). Neither technique appears to be superior nor complementary to either BPC or ePTFE (18). Buttressing the staple line with BPC or ePTFE has become one of the most popular variations in surgical technique (4, 18, 22).

The issue of biocompatibility needs to be considered with any prosthetic material. Calcification remains a severe cause of dysfunction following implantation of various biomaterials, especially those which are in direct contact with blood circulation. The ability of biomaterials to resist calcification has to be tested using both *in vitro* and animal

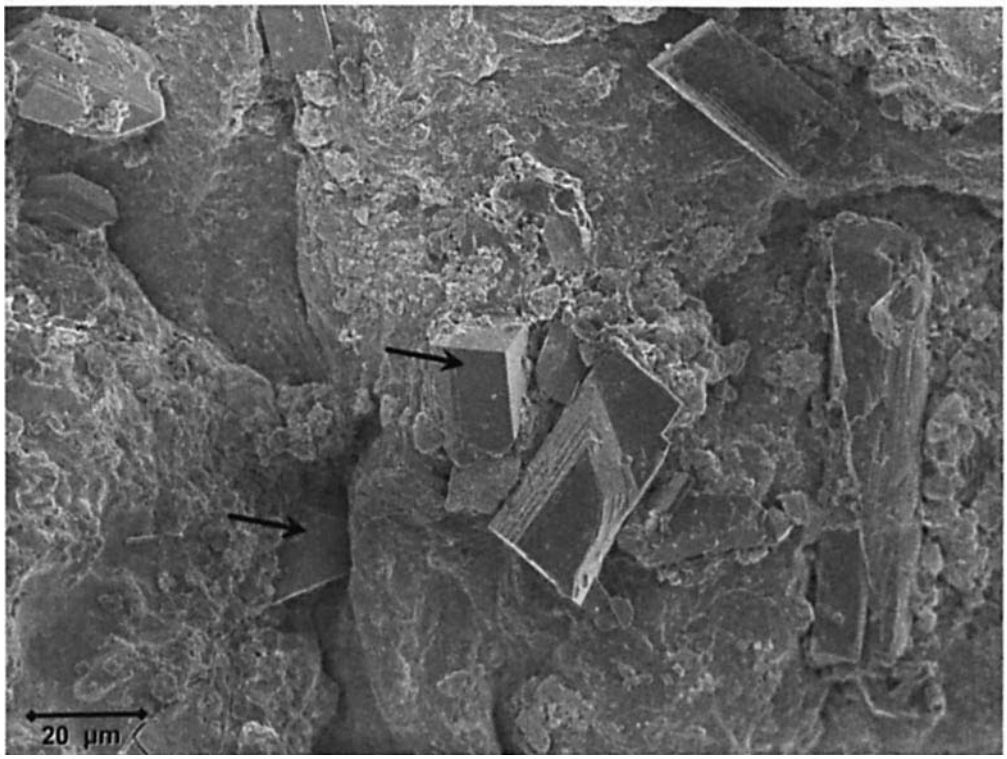

Figure 3. Scanning electron microscopy photograph of a calcified BPC. Arrows indicate, large, sharp crystals penetrating and damaging the BPC tissue. Magnification: ×627.

models prior to their final acceptance for clinical applications. Direct contact of the implant material with extracellular fluids of the tissues may lead to biomaterial calcification. From a physicochemical point of view, crystal calcific deposits develop in biomaterials due to a precipitation mechanism from the dilution fluid (blood or other tissue fluid with calcium and phosphate ions in supersaturating conditions), stimulated by different host and implant factors (9, 10, 15). Initial crystal formation in biomaterial may, with synergistic factors, trigger calcification in contacting tissues (Fig. 3). In the case of buttressing biomaterials, the contacting lung parenchyma tissue may be calcified. Moreover, calcification may play a role of an inciting agent in foreign body granuloma formation (23). Currently little information is available regarding host reactions in lung resection procedures with buttressing materials (24).

In the clinical setting the nature of the tissue response to strips used for staple line reinforcement in lung surgery is characterized by early chronic inflammation, minimal fibroplasia and encapsulation, and little to no resorption—even at 6 months after implantation and adhesion formation (25). The chronic inflammatory process may lead eventually to calcification after a longer period of time than the current 5 years of observation (26).

The use of BPC in cardiac surgical experiments has been associated with stiffening due to calcification, inflammatory reactions, and formation of fibrosis (27), leading to loss of the use of the bioprosthesis. Tissue damage due to the formation of large calcium phosphate crystals, like that observed in biologic heart valves, may also aggravate implant failure (Fig. 3). The calcification process is more extensive in cardiac surgery compared with lung resections after mechanical stress so we can safely assume that the impact of calcification has not yet been determined at the present available observation time.

Recently reports have proposed several ramifications of staple line reinforcement in lung surgery; for example, patients in whom these strips have been used may be at an increased risk of separation of the staple line and resultant pneumothorax or bacterial colonization of the exposed prosthesis, especially in the presence of a chest infection (28, 29).

Since human studies require a prolonged follow-up time, the available reports regarding calcification of BPC and PTFE strips are limited. Similarly, animal studies would require prolonged observations. *In vivo* models testing biomaterials are well established as a method for predicting calcification. However, *in vivo* studies have limitations: animal studies are associated with ethical problems, are time consuming, costly, and occasionally are unable to isolate specific factors implicated in calcification. Therefore, *in vitro* models may alternatively be used (9, 10).

In previous studies of biomaterial calcification, we introduced a model that showed that it was possible to estimate the calcification process *in vitro* under controlled

conditions of fluid temperature, pH, and composition. The calcium phosphate deposits formed *in vitro* exhibited similar morphology to those found in parallel animal model experiments. Moreover, the solid deposits formed at similar sites of the tissues (14). 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 (15).

Using the above model, we tested PTFE and BPC and compared these biomaterials to custom-prepared BPC tissues. Custom-prepared BPC, made from the same natural material, has demonstrated a different calcification resistance than that of commercially available BPC due to the method of preparation and storage. It seems that although glutaraldehyde treatment is common (based on basic information provided with the commercial material), possible differences in methodology result in a higher calcification resistance in the commercially manufactured BPC biomaterial. BPC and PTFE are totally different materials. Despite their differences, we showed that both presented a very low tendency for calcification *in vitro*, compared with custom-prepared BPC.

However, in animal experiments, these biomaterials have shown different behaviors. Vaughn *et al.* (30) evaluated the tissue response to PTFE and BPC in a canine model. They found a focal chronic inflammation and thin tissue coverage for BPC compared with thick tissue coverage and no focal inflammation for PTFE 30 days after application. Tissue coverage was further increased in the PTFE group at 167 days.

The fact that BPC is related to some inflammatory reaction (18) is consistent with the clinical observation of dense adhesion formation at the site of previous lung biopsy in patients undergoing lung transplantation whenever BPC was used (25).

There has been no clinical trial comparing these biomaterials in the long term. Our study has been based on an *in vitro* model, effectively testing the potential for calcification. Based on our results, we can predict a low potential for calcification for both PTFE and BPC. Therefore, both can be considered safe in preventing early air leakage, as has been well established, and considered safe from the biocompatibility point of view as far as long-term calcification is concerned. In conclusion, these data suggested that PTFE and BPC biomaterials show a relatively lower tendency for calcification compared with custom-prepared BPC tissue. Although further studies are necessary to elucidate these findings, staple lines reinforced by these two commercially available biomaterials may be considered safe from the calcification point of view.

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