

An experimental set-up for the in-vitro analysis of polyurethane calcification

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KEYWORDS: BLOOD COMPATIBILITY, CALCIFICATION, IN VITRO TESTS, MECHANICAL PROPERTIES, MECHANICAL STRESS, STRESS-CALCIFICATION RELATIONSHIP

Selected discussion remarks

- 1) Ref.: The solvents, used for casting the samples to measure the thrombogenicity, were dependent on the form of delivery (IMF, THF, DMAC, Dioxane). No effect was seen from the different solvents.

Aud: Surfaces of devices casted from a solution of Dimethylamide are more thrombogenic than those from DMF or THF solutions.

- 2) Ref.: The cast films were dried in nitrogen dry atmosphere.

AN EXPERIMENTAL SET-UP FOR THE IN-VITRO ANALYSIS OF POLYURETHANE CALCIFICATION.

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SUMMARY

A preparatory study is described with regard to the in-vitro analysis of polyurethane calcification, focussed upon the determination of the possible relation between varying bending strain and calcification. A bending loading system and experimental method together with the preliminary results on a commercially available polyetherurethane elastomer are described. The results suggest that varying bending strain alone does not initiate calcification with the material and calcifying solution used so far. Future experiments will have to verify this conclusion for other materials, especially those exhibiting much more hysteresis. There is some evidence that, once initiated, the calcification process is enhanced by varying bending strain.

INTRODUCTION

This research is part of a larger project founded upon the opinion that a detailed analysis of the behaviour of the natural aortic valve may lead to better insights into the relevant design parameters for a valve prosthesis. To that end, in earlier studies, the hydrodynamical (ref. 1), the mechanical (ref. 2) and the kinematical (ref. 3) aspects of natural aortic valve behaviour were studied. In 1980 a study was started to formulate mechanical specifications for the design of an improved artificial leaflet valve prosthesis using the results of these previous studies. A numerical model of a valve prosthesis was developed (ref. 4) to predict stresses and strains in the leaflets of a valve in closed position, as the diastolic loading is one of the unfavourable valve loading situations in a cardiac cycle. The model is based on the finite

element method and incorporates a non-linear relationship between deformations and displacements and viscoelastic properties of frame and leaflets. This valve model was first applied to the asymmetrical Hancock porcine valve prosthesis and the numerical results were verified experimentally. Next, a symmetrical model was developed which comprises fibre reinforcement of the leaflets, viscoelastic properties for frame, fibres and matrix material and a variable geometry of the valve. With this model numerical optimization experiments were carried out and specifications were formulated for geometry and material properties of a synthetic leaflet valve prosthesis. In the next phase of the project the same procedure will be applied to those phases in the cardiac cycle in which the valve prosthesis is in a non-closed position. Then another unfavourable valve loading situation exists, as especially during the opening and closing phase the leaflets are bended and wrinkled, so that high bending strains may arise. Besides of mechanical failure, reduction of bending strains seems also relevant from the point of view of calcification, as several reports (ref. 5-9) indicate some evidence of a relation between calcification of synthetic elastomers and varying bending strain in the materials.

The aim of this part of the research is to develop materials which meet the specifications and exhibit favourable calcification properties. To that end first a number of polyetherurethane rubbers were prepared, based on a prepolymer of poly(oxytetramethylene)glycol (PTMG) of varying average molecular weight and bis(4-isocyanatophenyl)methane (MDI), chain extended with ethylenediamine (EDA) (ref. 10). Also a number of polyurethanes based on coupled polyethers were synthesized, by connecting two or more polyethermolecules with 2,4-toluenediisocyanate (TDI) prior to the prepolymer preparation with MDI. Next, the mechanical material properties were determined by uniaxial tensile and relaxation tests. The results, see table 1, show that the Young's modulus (E_o) and viscoelastic properties, like hysteresis and loss tangent, decrease with increasing average molecular weight of the polyether used. The use of coupled polyethers results in an increase in viscoelastic properties and a considerable decrease in modulus of elasticity in comparison to "normal" polyurethanes with the same average molecular weight of the polyethersegment (table 1). Materials with the required very low modulus of elasticity (1.8 N/mm^2) for the leaflet matrix material show a considerable amount of hysteresis. This may have an effect on calcification of these materials due to heat generated by repeated bending of the material in a valve prosthesis.

In this report a preparatory study is described with regard to the in-vitro analysis of polyurethane calcification, focussed upon the determination of the possible relation between varying bending strain and calcification. A bending loading system and experimental method together with the preliminary results on a commercially available polyurethane material are described.

TABLE 1 Mechanical properties of a number of polyetherurethanes

Material	$E_{o.o}$ (N/mm^2)	Hysteresis (%)
PU650	50.3	14.4
PU1000	36.4	11.8
PU2000	11.7	8.3
PU2*1000	7.6	9.0
PU2*2000	3.3	16.7
PU3*2000		
Biomer	10.0	4.9

GENERAL ASPECTS

With the increased clinical use and the availability of information on long-term implants, the deposition of calcium salts on synthetic surfaces, in particular calcium phosphates, has emerged as a serious and potentially limiting factor in the longevity of prostheses, like heart valves and artificial hearts (ref. 6). The build-up of hard crystalline structures can cause stenosis and/or mechanical rupture of flexible bloodcontacting surfaces. In the majority of studies it was observed that calcification was most likely to occur on moving or otherwise deforming surfaces, especially in areas of varying bending strain. In case of smooth, nontextured surfaces, surface defects appeared to serve as predilection sites for calcific deposits (ref.6-7).

The processes which are involved in the deposition of calcium salts are still not understood. A summary of theories that have been advanced to explain the process is given by Anderson (ref. 11) and Coleman (ref. 12). Most of these theories have concentrated on the metastable solution approach of calcium and phosphate being present physiologically. This means that there is insufficient calcium and phosphate in biological fluids to initiate spontaneous precipitation of hydroxyapatite, the form in which calcium phosphate is most often found in the deposits, but a sufficient amount is present to support crystal proliferation once the process has begun. All mechanisms proposed (ref. 11-12) give possible explanations for the initiation of the process, that is the formation of the first seed crystals of apatite.

Although a number of factors appear to be implicated in the process, like material chemistry, animal species and age, anticoagulation and ab- or adsorption of calcium binding proteins and lipids (ref. 5, 9, 13, 14), mechanical

factors are believed to play a fundamental causative role in the initiation and development of calcific deposits (ref. 5, 7, 9, 12).

In this study three possible calcification mechanisms which are strongly influenced by varying mechanical bending strain will be considered. In the first concept, repeated flexing of the elastomer is accompanied by microscopic deformations of its structural elements giving rise to "openings" and "closings" of pores. This process permits the entry of components, like calcium and phosphate, from the surrounding fluid into the surface of the material. Mechanical strain may further enhance this process by the creation of new pores and micro cracks as a result of mechanical failure. It is well known that surface defects serve as predilection sites for calcification in in-vivo experiments and that the first calcific deposits are formed subsurface (ref. 6). If the material in some way is suitable to act as a heterogeneous nucleator, which may be the case with polyurethanes, a crystallization nucleus that is formed subsurface or in surface defects can be stabilized and will grow further. It is known from thermodynamical considerations (ref. 15) that the potential nucleation and growth rate of such a nucleus in surface imperfections or material pores is much greater than it would be on a flat surface.

A second possible explanation for the calcification process is that initiation takes place as a result of local thermal effects. In high flex areas the local polymer temperature may be slightly increased as a result of mechanical hysteresis. Especially below the surface and in small surface defects from which heat transport to the surface is limited, this effect could be substantial (ref. 16). In-vitro studies have not only demonstrated that calcium phosphates are less soluble at elevated temperatures but also that amorphous calcium phosphate, which is formed first (ref. 5), is converted to the even less soluble crystalline hydroxyapatite more rapidly at elevated temperatures (ref. 17). Here again, surface defects, like micro cracks which result from the varying mechanical strain, may further enhance the process.

The third possible explanation of the process is that, in case of polyurethanes, basic compounds eluting from the material are responsible for initiation. Leaching of amine containing oligomeric compounds is enhanced by high mechanical strain which allows rapid diffusion out of the material (ref. 18). In micro-bubble defects these amine containing compounds may cause a local rise in pH. Such an increase would also decrease the solubility of calcium phosphates.

It is expected that the viscous material properties will have the strongest effect in the second mechanisms because the amount of heat generated strongly depends on the amount of hysteresis. To enable interpretation of the results with respect to the other two possible mechanisms Biomer (Ethicon Inc.), a material which is known to calcify in in-vivo experiments (ref. 6) and which

exhibits only a small amount of hysteresis (ref. 10), was chosen for the experiments shown in this paper. In later experiments polyurethanes showing much more hysteresis will be used.

MATERIALS AND METHODS

An equipment has been designed in which material strips can be loaded with a well determined varying bending strain. This equipment (Figure 1) is constructed around a transparent lucite reservoir filled with a calcifying medium. The calcifying medium employed in this preparatory study consisted of 15 mM barbital buffer (pH= 7.4), 100 mM sodium chloride, 1.7 mM potassium phosphate and 1.7 mM calcium chloride. This metastable calcium phosphate solution has been extensively used in calcification research in the past (ref. 19).

The equipment essentially exists of 4 lucite plates which are driven by a small electrical motor (Engel, GNM2130), a gear wheel transmission (Engel, G4) and a construction to transform the rotational movement into a translational one. The outer two plates move 180° out of phase with the inner two, to eliminate the fluid mass inertia effects. Between those plates the polyurethane test specimens are clamped and submitted to a bending movement by which a specimen lies straight against one plate during one part of the loading cycle, and curved circularly during another part of that cycle (Figure 2). This results in a varying bending strain in the specimen. The moving plates can be positioned slantingly to each other so that the test specimens between two plates have a different radius of curvature during the curved phase of the loading cycle, resulting in bending strains of different magnitude.

The magnitude of the maximum bending strain in the circularly curved part of the specimen may be roughly estimated by:

$$\epsilon_b = \frac{d}{D-d} \quad (1)$$

in which d is the specimen thickness and D the distance between two plates, while the bending strain in the straight part of the specimen equals zero. The thickness d of the polyurethane specimens used, is controlled during the solution casting process in our laboratory and is adjustable between 0.1 and 0.6 mm. The plate distance D can be adjusted within a range of 5 to 25 mm. The maximum bending strain in the specimens ranges then from about 1% to 14%. This is in agreement with the order of magnitude of bending strains found in leaflet valve prostheses by Thubrikar et al. (ref. 20).

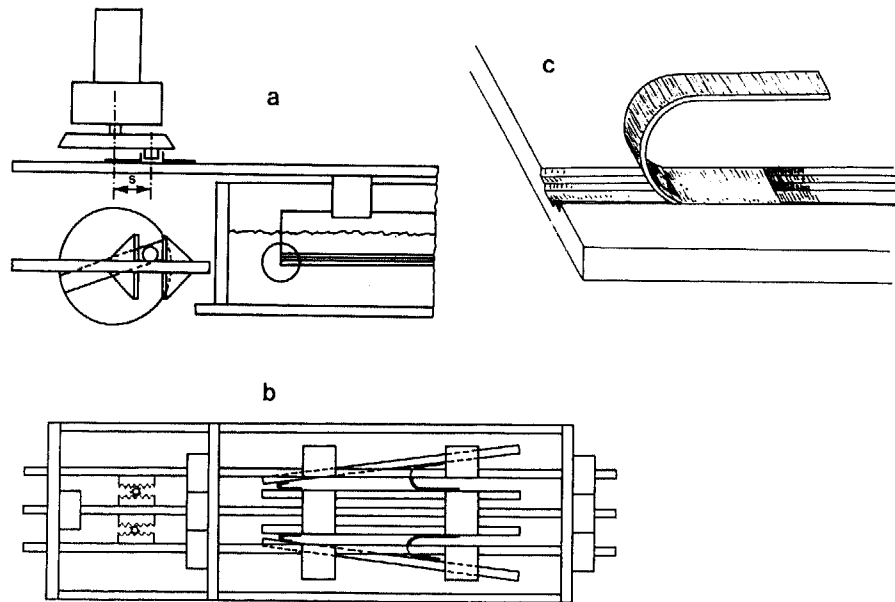


Fig.1 Bending equipment for polymer or biological strips
 a. side view, showing the driving system
 b. upper view (the driving system is not drawn)
 c. a schematic outline of the material strip positioned between the plates.

They found in porcine leaflet valve prostheses bending strains in the leaflets of 5-6% in circumferential direction and of 10-12% in radial direction. In the moving plates grooves are present to ensure that the backside of the leaflet strip, when lying parallel to the plate, remains in contact with the fluid (Figure 1). The loading frequency is adjustable between 0 and 8 Hz and the amplitude of the sinusoidal movement (the strike s , see Figure 1 and 2) between 0 and 40 mm. The number of cycles during an experiment is recorded with a mechanical counter. The test specimens are 10 mm wide and have a minimum length according to (see Figure 2):

$$l_{sp} = 2s + \frac{1}{2}\pi D + 2l_{cl} \quad (2)$$

where s is the strike of the moving plates, D is the plate distance and l_{cl} is the clamp length.

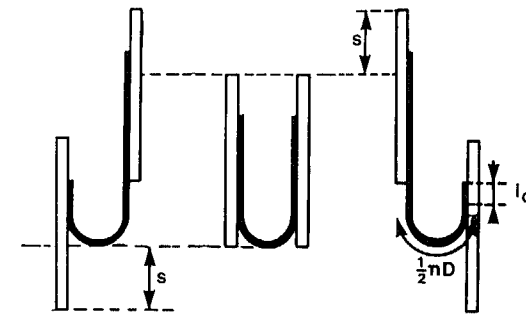


Fig.2 The movement of the plates and the deformations of the material strips.

In the reservoir a number of blanks (no bending strain) are positioned at the same height in the reservoir as the loaded strips. During the experiment the solution is kept at 37 °C by a heat exchanger operated with water of the same temperature to avoid calcific deposits on the heat exchanger and instability of the solution. The calcifying solution is renewed every 24 hours. In one experiment 10 strips can be loaded at the same time.

After removal from the loading system the strips are washed with barbital buffer twice, twice with distilled water and finally with acetone. After drying, half the number of the strips was analysed quantitatively for phosphate. To that end, 10 mm were cut off both ends of the strips to eliminate the clamp effects. To the resulting strips 7.0 ml of 0.1 M hydrochloric acid is added to dissolve the deposits. After 48 hours of stirring the amount of phosphate is determined photospectrometrically as the molybdo-vanadate complex (ref. 21). The equipment was calibrated with standard solutions containing 1 to 10 µg of phosphate per ml. The other material strips are analysed qualitatively. After staining according to von Kossa calcific deposits can be detected with reflected light microscopy. To see if the deposits are associated with surface irregularities of the material Nomarski interference contrast is used at magnifications of up to 500X. Scanning electron microscopy was used to achieve higher magnifications. After collection of the deposits, the composition is examined by X-ray diffraction and X-ray fluorescence. Besides, the presence of leachables is investigated by extraction of material strips with methanol for two weeks, followed by infrared spectroscopic analysis of the extract.

In this preparatory study the following two series of experiments were carried out at the maximum frequency of 8.3 Hz with Biomer strips. The first series was performed in the set-up as it was originally designed. In the second series all metal parts of the set-up have been coated with plastic or replaced

by plastic parts (table 2). Both series comprised three experiments at various maximum values of bending strain and for two numbers of loading cycles.

TABLE 2 Preparatory experiments with Biomer strips

	cycles *10 ⁶	time (days)	bending strain (%)	loaded strips	PO ₄ ³⁻	blancs	PO ₄ ³⁻
First	1.5	2.5	0-8.4	10	+	2	0
series (1)	3.3	5.0	0-12.4	10	+	3	0
	3.3	5.0	0-11.8	10	+	3	0
Second	1.5	2.5	0-7.2	10	0	5	0
series (2)	3.3	5.0	0-8.5	10	0	5	0
	3.3	5.0	0-7.0	10	0	5	0

1 original set-up + calcified

2 metal contact avoided 0 calcification not detectable

RESULTS

The experimental set-up proved to be reliable at bending rates up to 8.3 Hz. Loading numbers up to 3.5 million (5 days) can easily be reached without mechanical damage to the equipment or instability of the calcifying solution.

In the first series of experiments (see table 2) in the original set-up calcification of the strips did occur as became clear from the quantitative analysis. The quantitative results of the first series are presented in table 3. Relatively large amounts of phosphate were detected on the loaded strips in contrast to the blancs. The amounts detected on the blancs lie within the detection limit of the spectrophotometric analysis. There is no apparent relation between the amount of phosphate and the magnitude of the bending strain. The results also show that the duration of the experiment or the number of loading cycles has an effect on phosphate deposition. The analysis of the calcifying solution after 24 hours shows that the phosphate concentration decreases from 162 µg/ml to values around 148 µg/ml.

TABLE 3 Phosphate deposits per unit area on the loaded Biomer strips in the first series

1.47 * 10 ⁶ cycles time= 51 hours		3.3 * 10 ⁶ cycles time= 109 hours		3.3 * 10 ⁶ cycles time= 109 hours	
strain %	phosphate µg/cm ²	strain %	phosphate µg/cm ²	strain %	phosphate µg/cm ²
0.0	0.016	0.0	0.9	0.0	0.2
3.8	1.6	4.6	3.3	4.2	3.2
4.8	0.8	5.8	3.3	5.6	3.6
5.5	1.4	7.0	3.5	7.0	4.3
6.5	1.4	7.5	3.2	7.7	3.6
8.4	1.0	12.4	3.2	11.8	3.4

Light microscopy of calcified strips reveals that the deposits appear as plaques randomly distributed over the surface of the strips and that there is no clear difference in calcification between the two sides of the strip. An association with surface defects was not clearly visible. Figure 3 shows scanning electron micrographs of loaded and unloaded strips. The formation of microcracks as a result of mechanical loading could not be detected microscopically.

X-ray diffraction of collected deposits indicates that it is mainly amorphous material. Phosphorus and calcium were shown to be the main components of the deposits by X-ray fluorescence. Trace amounts of a number of metals, especially aluminium could be detected as contaminants. These metals apparently dissolve in the calcifying solution from metal parts of the construction.

In the second series of experiments (see table 2), all the metal parts were coated with plastic or replaced by plastic parts. In contrast to the first series calcification did not occur anymore after these improvements. The results of the analysis of both loaded and unloaded strips lie within the detection limit, which proves that metal ions can play an important role in the initiation of calcification. Analysis of the calcifying solution after 24 hours shows that the phosphate concentration remains unchanged (162 µg/ml) confirming that initiation did not take place in these experiments.

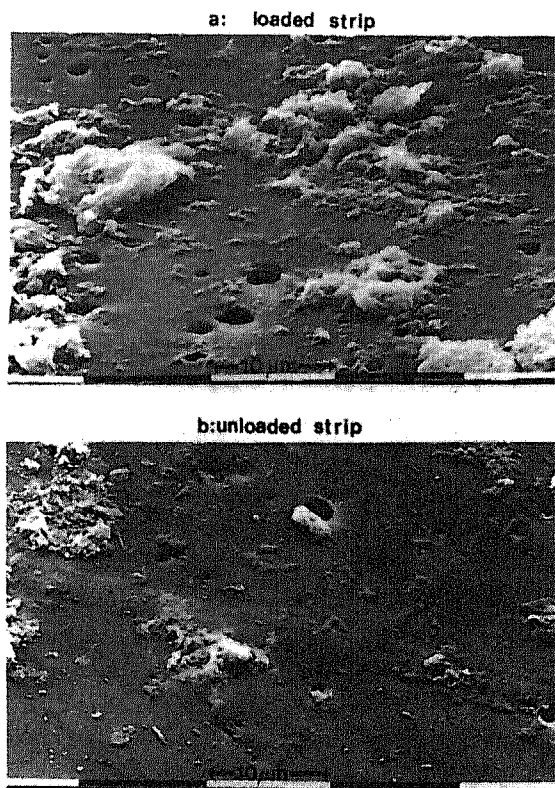


Fig. 3 Scanning Electron Micrographs of loaded and unloaded (blanc) strips

Extraction of Biomer strips with methanol yields a small amount (< 1 %) of a dark brownish, viscous fluid, indicating the presence of a relatively large amount of oligomeric compounds. Infrared analysis of the extract reveals that it contains amines.

CONCLUDING DISCUSSION

An experimental set-up has been designed which enables the testing of materials under varying bending strain in a calcifying medium. In the two series of experiments, using a buffered, metastable calcium phosphate solution, initia-

tion of the calcification process only occurred when using the original set-up. Initiation of the process in this case is probably due to contamination of the calcifying solution with metal ions dissolving from parts of the driving system. It is known from in-vivo experiments that metals can cause initiation of the calcification process (ref. 22, 23). Although initiation in this first series of experiments is not according to one of the proposed mechanisms, the results of the quantitative analysis support the idea that calcification is enhanced by varying bending strain. The magnitude of the bending strain appears to have no effect on the rate of calcification. There is a marked influence of the duration of the experiment or the number of bending cycles on the amount of deposited phosphate. So for the design of a valve prosthesis it may be important to minimize the bending strain in the leaflet material during a cardiac cycle.

The second series of experiments, in the absence of metals, shows that initiation of the calcification process does not occur. Heterogeneous nucleation in surface pores or defects apparently does not take place or at such a slow rate that the duration of the experiments is too short for the build up of detectable amounts of phosphate. Long-term experiments and an improved quantitative analysis will have to verify this hypothesis.

Although the presence of a relatively large amount of oligomeric amine containing compounds in the material was established, leaching of these compounds did not initiate calcification during the experiments. Here again, long-term experiments may be necessary to verify this result.

Hence, varying bending strain alone seems not enough to initiate the process in this calcifying solution and with this material. Future experiments will have to verify this hypothesis for other materials, especially those materials exhibiting much more hysteresis like the polyurethanes with the required low Young's modulus developed in our laboratory (ref. 10).

Future research will also have to include the investigation of other factors on the calcification process. It may be necessary to use another calcifying medium like blood or serum to initiate mineralization in the set-up. The effect of material composition and surface properties, like surface charge and hydrophobicity should also be investigated. A more detailed study on the possible relation between surface defects or material porosity and calcification is also important. Furthermore, the relation between leaflet calcification and the magnitude of varying bending strain has to be verified for total leaflet valve prostheses. For this purpose, an equipment has been designed (ref. 24) in which a total leaflet valve prostheses can be loaded under physiological conditions at increased frequencies up to 10 Hz.

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KEYWORDS: CALCIFICATION, IN VITRO TESTS, ION COMPLEXATION
MECHANICAL STRESS, STRESS-CALCIFICATION RELATIONSHIP

Selected discussion remarks

- 1) Ref.: Polyurethane was taken because of its versability and the large range of the mechanical properties.
- 2) Ref.: Amino extended polyurethane shows superior mechanical properties to those of the glycol extended one. On the other hand, the synthesis with glycol runs slower than the synthesis with amines and is better to control therefore.