

Reduction of Calcification of Carbodiimide-Processed Heart Valve Tissue by Prior Blocking of Amine Groups with Monoaldehydes

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Background and aim of the study: Failure of implanted bioprostheses due to calcification is a commonly occurring phenomenon. In order to prevent calcification, many alternative cross-linking methods to glutaraldehyde (GA) have been developed and evaluated.

Methods: In a novel approach an improved carbodiimide (EDC) cross-linking method that comprises a two-step process was developed. First, the available amine groups in (tissue) collagen were blocked with a monoaldehyde, followed by an EDC-activated cross-linking reaction of the carboxyl groups in the tissue with a poly(propylene glycol) bis 2-(amino-propyl) ether (Jeffamine™).

Results: Samples processed via this method have shown a significantly reduced calcification in a subdermal juvenile rat model compared to samples with standard GA treatment. In the present study, heart

valve tissue was blocked with various monoaldehydes, followed by reaction with Jeffamine using carbodiimide cross-linking chemistry. Leaflet calcification was almost eliminated using different aldehydes, whereas wall calcification was maximally 95% reduced when propionaldehyde was used as blocking agent, as compared to a carbodiimide cross-linked control without Jeffamine and blocked amine groups.

Conclusion: Amine blocking and cross-linking technology appears promising in the design of the next generation of tissue valves. Calcification was significantly reduced compared to GA cross-linking. The mechanistic insight of decreased wall calcification is still unknown, and research investigations are ongoing.

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Calcification of bioprostheses after implantation is a commonly occurring phenomenon that may have several different causative factors. In order to mitigate the local inflammatory responses and associated enzymatic degradation that begins after implantation of the bioprosthesis, the tissue matrix is usually preserved, primarily by the introduction of cross-links between the collagen molecules in the matrix. Glutaraldehyde (GA)-based cross-linking of valvular bioprostheses is the current standard (1), and these prostheses have a low incidence of thromboembolism and a satisfactory hemodynamic performance. Nevertheless, problems of durability have been reported with this process (2-5). Clinical failure due to cuspal calcification has been reg-

istered in many cases (4), with calcium deposition causing valve stiffness, tearing and rupture that result in stenosis and/or insufficiency (6). The exact mechanism of calcification is still not well understood, and numerous hypotheses have been described in the literature. Most theories are based on the loss of components from the matrix, such as proteoglycans and glycosaminoglycans during the reaction with GA (7). These components act as natural inhibitors of calcification. Other factors mentioned are changes in the morphology and charge distribution in the valve after fixation, and the occurrence of local stress in the valve material (2-4,8).

In various studies, post-GA fixation reactions were developed with the aim of reducing calcification, and some of these were successfully introduced into clinical practice (9). During the past few years, research has also focused on the use of alternative cross-linking agents (1). Cross-linking based on water-soluble carbodiimide (EDC) in the presence of *N*-hydroxysuccinimide (NHS) has been reported to be a particularly promising alternative to GA (10-12). Calcification after explantation has been reported to be less than that

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with GA, although it has also been mentioned that direct cross-linking between existing free amine and carboxyl groups (the so-called "zero-length cross-links") in the collagen chain causes stiffening of collagen-based materials. In order to prevent the formation of these zero-length cross-links, a two-step reaction was developed. The first step comprises blocking of the amine groups of the collagen matrix, followed by an EDC- and NHS-activated cross-linking reaction with a poly(propylene glycol)bis 2-(aminopropyl) ether (Jeffamine™) (13,14). The stabilized matrix with improved mechanical behavior showed a significantly decreased calcification of wall material in a subdermal juvenile rat model compared to the standard GA-fixed control (14,15).

The aim of the present study was to follow through on these successful initial results. Various monoaldehyde blocking agents were evaluated for their blocking efficacy. After optimization of the blocking reaction, porcine heart valves were cross-linked with Jeffamine and implanted subdermally in juvenile rats to evaluate calcification after an eight-week period of implantation.

Materials and methods

Tissue preparation

Fresh porcine aortic valve tissue was obtained from a local slaughterhouse (Bleijlevens, Kerkrade, The Netherlands), rinsed free of blood with a buffered *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethane sulfonic acid) (HEPES; 10 mM, pH 7.4; Sigma Aldrich, Zwijndrecht, The Netherlands) physiological saline solution, and trimmed to remove excess myocardium and adventitial tissue. The valves were rinsed for at least 24 h (maximum 48 h) in HEPES-buffered saline (10 mM, pH 7.4, 4°C) before starting the fixation process.

Blocking of amine groups with monoaldehydes

Four different aldehydes were evaluated as blocking agents: butanal, 2-methyl-butanal, propional, and glycerol (all from Sigma Aldrich). For the blocking reactions, 2-(morpholino)ethane sulfonic acid (MES) (100 ml, 0.2 M, pH 6.4; Acros, Geel, Belgium) buffer was prepared and the selected aldehyde was added (0.5 M), followed by the addition of a valve. Three equal amounts of NaCNBH₃ (0.1 g; Aldrich) over an 8 h interval were added. After 24 h reaction time, the valves were rinsed in physiological saline solution (three times, each of 30 min). Five valves were treated with each type of aldehyde. The blocking efficacy was determined using the 2,4,6 trinitrobenzene sulfonic acid (TNBS) method (as described below). For each aldehyde group, five samples were taken randomly from the wall, and five samples from the leaflets of one

valve. The remaining valves (*n* = 4 for each aldehyde group) were subsequently immersed in MES buffer (0.2 M, pH 5.5) and Jeffamine 230 (0.06 M; Sigma) for 30 min and subsequently cross-linked using *N'*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) (0.3 M; Aldrich) and NHS (0.12 M; Sigma). The cross-linking reaction was conducted for 24 h at room temperature, after which the valves were rinsed five times (30 min each) in physiological saline and thereafter individually stored in HEPES (100 ml, 10 mM, pH 7.4) containing 20% isopropyl alcohol (IPA).

Because the blocking efficacy of glycerol-processed wall tissue was relatively low, the reactions for this aldehyde were varied with respect to its concentration (0.2 and 0.5 M), temperature (room temperature and 40°C) and time (24 and 48 h). Other conditions were maintained as described previously. Three valves were treated for each combination of variables. The TNBS analysis taken of these valves was carried out on five randomly obtained wall samples.

Further optimization of process conditions

For further optimization of the process it is advantageous to use the lowest buffer and NaCNBH₃ concentrations, which still permit efficient blocking of amine groups and subsequent cross-linking.

First, the MES buffer concentration was varied between 0.01 M and 0.2 M using propional (0.5 M) and NaCNBH₃ added three times (0.1 g, total 0.3 g over a period of 8 h). Three valves per buffer concentration were treated in 100 ml buffer solution each. The reaction was performed for 24 h at room temperature. After the reaction, the valves were rinsed in physiological saline solution (three times for 30 min). The blocking of amine groups of five wall samples taken randomly from these three valves for each buffer concentration was determined using the TNBS method.

Second, while maintaining the MES buffer concentration at 0.05 M, tissue wall samples (diameter 1 cm, randomly punched out of 10 fresh tissue valves) were treated with propional (0.5 M) using different concentrations of NaCNBH₃. NaCNBH₃ was added as one amount, or in three equal amounts over a period of 8 h. The blocking efficacy was determined again by analyzing five samples taken randomly from each concentration group.

Preparation of valves for the implant study

Samples for the implant study were processed (five valves per sample group were prepared) with butanal (0.5 M), 2-methyl-butanal (0.5 M), propional (0.5 M) or glycerol (0.2 M). The reaction was carried out for 48 h at room temperature, using MES buffer (100 ml per valve, 0.05 M, pH 6.5) and NaCNBH₃ (0.3g) was added at the beginning of the blocking experiment. The

blocking efficacy was determined using a TNBS assay of five wall pieces per aldehyde group, punched randomly out of the valve material. The remaining valvular material was rinsed in MES buffer (0.05 M, pH 6.5), subsequently immersed in MES buffer (100 ml per valve, 0.2 M, pH 5.5) containing Jeffamine 230 (0.06 M) for 30 min and cross-linked by adding EDC (0.3 M) and NHS (0.12 M) with a reaction time of 48 h.

The valves were rinsed five times for 30 min in physiological saline, and thereafter stored individually in HEPES buffer (100 ml, 10 mM, pH 7.4) containing 20% IPA until further use.

The obtained sample groups were investigated in more detail by determination of the tissue shrinkage temperature, enzymatic digestion, water uptake and static contact angle measurements. Before the experiments were carried out, the valvular material was extensively washed in HEPES-buffered saline solution (10 mM, pH 7.4).

In this calcification study, two control groups were added; these included an EDC/NHS cross-linked sample group, and a group that was cross-linked in 0.2% GA for one week at 4°C. For the EDC/NHS group, fresh tissue was cross-linked in MES buffer solution (0.2 M, pH 5.5) with the addition of 0.3 M EDC and 0.12 M NHS. Cross-linking was performed for 48 h, after which the samples were rinsed and stored as described above.

Tissue analysis

TNBS assay

The primary amine group concentration of tissue samples was determined using a colorimetric assay (14). Tissue samples (cut-out valve leaflet, or 6-mm punched disc from valve wall) were exposed to a solution of NaHCO₃ (2%, w/v, pH 9.0) and TNBS (0.5%, w/v; Fluka, Switzerland). The reaction was continued for 4 h at 40°C, after which the samples were rinsed in saline solution using a vortex mixer to remove unreacted TNBS.

Samples were freeze-dried overnight, after which the dry mass was determined. Dry samples were immersed in aqueous hydrochloric acid (2 ml, 6 M, 80°C) until fully dissolved. The obtained solution was then diluted with deionized water (8 ml) and the absorbance measured at 340 nm. The concentration of free amine groups was calculated using the following equation:

$$[\text{NH}_2] = \frac{A \cdot V}{\epsilon \cdot l \cdot m_{\text{tissue}}}$$

where: [NH₂] is the free amine group content (mol/g tissue); A is absorbance (-); V is the solution volume (ml); ε = 14,600 (ml/mmol-cm); l is the path length

(cm); and *m*_{tissue} is the dry weight of the sample (mg).

The obtained value was used to determine the %blocking, which was calculated from the following formula:

$$\% \text{blocking} = \frac{[\text{NH}_2]_{\text{native}} - [\text{NH}_2]_{\text{blocked}}}{[\text{NH}_2]_{\text{native}}}$$

where: %blocking = % of blocked amine groups (%); [NH₂]_{native} = the free amine content of the native material (mol/g tissue); and [NH₂]_{blocked} = the free amine content of the blocked material (mol/g tissue).

Resistance to enzymatic degradation

Tissue resistance to enzymatic degradation was determined using protease (20 U/mg; Aldrich) (14). In this procedure, the wall and leaflet samples (which had been previously weighed after being washed in demineralized (Di) water in order to remove buffer-salt and freeze-dried) were initially soaked in a HEPES-buffered saline solution (10 mM, pH 7.4) containing NaCl (9 g/l) and glycine (7.5 g/l) for 4 h at 37°C. Samples were then transferred to a solution of the same buffer containing the enzyme (3 ml with 12 mg enzyme per sample). In order to ensure optimal enzymatic activity of the solution, the enzyme was added along with CaCl₂ (96 mg to 180 ml solution). Samples were incubated for 4 h, washed in Di-water and freeze-dried. After renewed determination of the dry weight, the actual weight loss due to enzymatic degradation was calculated. The results were presented as remaining weight (in %).

Shrinkage temperature determination

The shrinkage temperature (Ts) of tissue samples was determined using differential scanning calorimetry (Piris 1; Perkin Elmer, Fullerton, USA). Samples of leaflets were obtained by cutting, and samples of walls were punched (6-mm discs) and immersed in 0.05 M NaH₂PO₄ solution for 3 h. Samples were then removed and excess buffer was blotted with a lint-free tissue. An individual sample was placed in the pan and the cover crimped. Three samples were analyzed for each leaflet or wall (n = 5).

The Ts of each sample was determined using a temperature scan between 30 and 95°C, with a scan rate of 2°C per min. Ts was defined as the temperature at the maximum of the endothermic transition.

Water content

To determine the water content of the tissue, 6-mm round discs of fresh, blocked or blocked-and-cross-linked wall samples were punched, blotted on lint-free paper and freeze-dried after weight determination. The

weight was re-determined and the weight loss recorded. Ten samples were used for each aldehyde group.

Contact angle

Contact angle measurements of wall tissue samples were performed at room temperature (using the sessile drop technique) with image analysis. A 10 µm thin slice was cut from each sample, using a cryotome. The material was sliced starting at the adventitia, and after approximately 10 slices, five samples were retrieved (located in the middle of the tissue sample). Using a static contact angle device (Dataphysics Contact Angle System, OCA, Germany), the contact angle of a droplet of water on the surface of each sample was determined. Rinsed samples were placed on a microscope slide and any excess of solution was removed with lint-free tissue. The contact angle was determined 5 s after the water droplet had been positioned. The sample was placed back into the saline solution and a fresh sample taken for the next measurement, allowing the first sample to re-hydrate completely. The experiment was repeated ten times for each sample.

Subdermal implantation

Subdermal implantation of the processed tissue in male rats (Long-Evans, age 5 weeks) was carried out to determine the effect of the chemical modification on calcification. All anesthetic and surgical procedures were approved by the Animal Ethics Committee of the University of Cape Town, and complied with the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals* (NIH 85-23 Rev. 1985).

Circular 12-mm discs punched from the post-sino-tubular junction wall as well as the aortic leaflets were used. Prior to implantation, these were washed three times for 2 min each in sterile saline solution.

Following anesthesia with ketamine hydrochloride:xylazine (i.p.), the ventral abdominal aspect of each rat was shaved and disinfected using an iodine solution. Using a sterile technique, six individual incisions were made in the skin contralateral to the spine, and subcutaneous pockets were created by blunt dissection. The disk and leaflet samples were inserted into individual pockets with the intimal and fibrosal surfaces respectively directed towards the abdominal muscle and away from the skin. The incisions were closed using individual sutures. Six disk and six leaflet replicates were implanted for each treatment group.

Analysis of calcium concentration

Samples were retrieved from rats 60 days postoperatively. The animals were killed by CO₂ asphyxiation and the samples immediately explanted. Disks and leaflets were divided longitudinally into two halves using a microtome blade. The tissue half destined for

Table I: Blocking of amine groups of heart valve tissue with various mono-aldehydes (five valves treated per aldehyde group).

Aldehyde (n = 3)	Blocking wall (%)	Blocking leaflet (%)
Butanal	67 ± 6	87 ± 3
Methyl-butanal	73 ± 2	81 ± 2
Propional	75 ± 3	87 ± 3
Glyceral	58 ± 3	79 ± 4

histological analysis was immediately transferred to 10% formalin, while the remaining half was separated from its surrounding fibrous capsule and a 1 mm-thick rim removed from the outer edge using a 10-mm punch. Samples for atomic absorption spectrometry (AAS) were stored at -20°C until being analyzed. Qualitative analysis of calcium in the explants was performed using von Kossa staining.

For quantitative calcium analysis, the samples were dried at 104°C for 24 h, weighed, ashed in a muffle furnace (560°C for 12 h), and dissolved in hydrochloric acid (20%; 1 ml per 10 mg dry weight). Samples were diluted with lanthanum chloride (0.5%) and absorption measured at 422.7 nm using an atomic absorption spectrophotometer (Varian AA1275).

Statistical analysis

All data were analyzed using Student's *t*-test (two-tailed). A *p*-value ≤0.05 was considered to be statistically significant. Assessment of the calcium-mitigating effect of the presence and type of amine blocker was made using a one-factor analysis of variance. Post-hoc comparisons were made using Student's *t*-test (two-tailed). These were limited to comparisons between control and test samples only, and therefore excluded Bonferonni correction for multiple comparisons.

Table II: Blocking of amine groups of heart valve wall tissue with glycerol as a function of the glycerol concentration, reaction time and temperature (three valves processed for each experimental group).

Concentration (M)	Reaction temperature (°C)	Reaction time (h)	Blocking efficacy (%)
0.2	20	24	42 ± 3
0.5	20	24	64 ± 2
0.2	40	24	64 ± 4
0.5	40	24	64 ± 3
0.5	40	48	69 ± 3
0.2	20	48	74 ± 2
0.5	20	48	74 ± 1
0.2	40	48	75 ± 2

Table III: Blocking of amine groups of wall tissue samples of valves treated with propional in MES buffer with different concentrations.

Concentration (M)	Final pH	Blocking efficacy (%)
0.01	9.2	76 ± 2
0.02	8.6	74 ± 2
0.05	8.0	76 ± 2
0.1	7.6	73 ± 4
0.2	6.9	75 ± 3

Results

Alternative blocking agents

Details of the blocking efficacy for wall and leaflets using different blocking agents are listed in Table I. Typically, leaflets had higher blocking efficacies compared to wall tissue ($p < 0.01$). The use of different aldehydes led to comparable results, with the exception of glycerol, which provided a significantly lower blocking efficacy for the wall ($p < 0.01$).

The blocking process for glycerol was further investigated to increase blocking efficacy; the results are listed in Table II. In order to achieve a high blocking efficacy, at least one of the parameters of reaction temperature, time or glycerol concentration had to be increased ($p < 0.01$). The most optimal reaction conditions were achieved with a long reaction time ($p < 0.01$). For convenience, reaction at room temperature and a glycerol concentration of 0.2 M (the preparation of a 0.5 M solution is difficult) were used in further studies.

In order to further optimize the process, two studies were performed to determine the lowest buffer and NaCNBH_3 concentrations at which blocking of $\pm 75\%$ was achieved; the results of are listed in Table III. Although the initial pH of 6.4 was not maintained under the conditions utilized, the blocking efficacy achieved was not significantly affected by the buffer concentrations used ($p < 0.001$).

The results of the blocking efficacy of wall tissue samples achieved with different concentrations of added NaCNBH_3 added in a single step or divided in three portions over an 8-h interval are shown in Figure 1. Maximal blocking efficacy in wall tissue was shown to require a minimal NaCNBH_3 concentration of 50 mM; no significant difference was obtained by adding the NaCNBH_3 at the start of the experiment or divided into three equal additions ($p < 0.001$). In subsequent studies, 50 mM NaCNBH_3 was used, added at the start of the blocking reaction.

Characterization of samples for the implant study

Details of the sample groups prepared for further analysis, and the results obtained, are listed in Table IV.

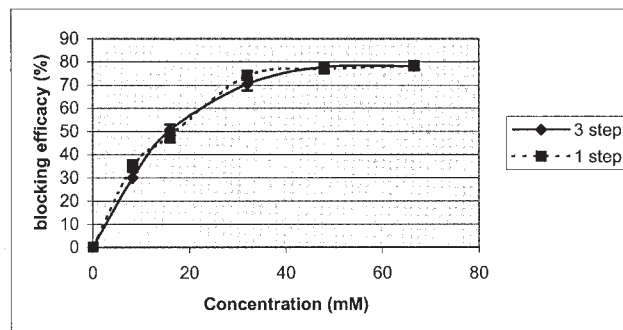


Figure 1: Blocking efficacy compared to total concentration of NaCNBH_3 added to the reaction mixture in a single addition or added in three steps over an 8-h interval.

The results showed that, for most aldehydes, both wall and leaflet could be blocked by ca. 80%, though for glycerol the values were significantly lower ($p < 0.01$). The T_s was determined after cross-linking, and increased for all groups compared to the fresh sample group ($p < 0.001$). The highest values were obtained with glycerol-blocked and EDC/NHS sample groups ($p < 0.01$). For both the propional and glycerol groups, the T_s -values for wall and leaflet were almost equal ($p < 0.01$), while for the 2-methyl butanal and butanal groups the leaflets had a higher T_s compared to the wall ($p < 0.01$).

Finally, the enzymatic digestion data indicated that all groups were more resistant to enzymatic degradation than the fresh sample group. The wall was found to be more resistant compared to the leaflets of all groups ($p < 0.001$). The glycerol-treated group showed the highest degradation for the leaflets ($p < 0.01$). The EDC/NHS control group had the highest T_s -values, and degradation of both the wall and leaflets was lowest ($p < 0.01$).

Water content and contact angle

The contact angles of blocked material (left) and blocked-and-cross linked wall material (right) are illustrated graphically in Figure 2. The contact angles of materials from the different groups varied, depending upon the treatment. Samples became more

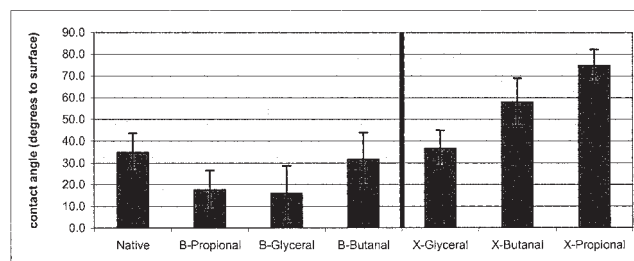


Figure 2: Contact angle of native, blocked (B) and blocked-and-cross-linked (X) wall tissue slices. The analysis was performed after the blocking step and after completion of the cross-linking.

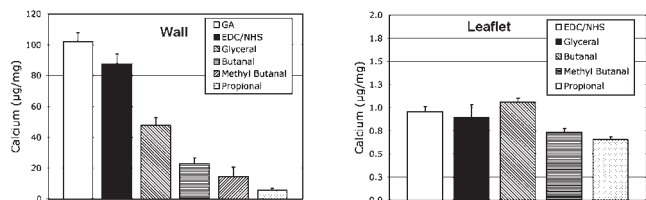


Figure 3: Atomic absorption spectrometry calcification data of the implanted wall (left) and leaflet (right) samples. Types of aldehyde used are as indicated. The EDC/NHS control group was cross-linked without prior blocking of the amine groups. A second GA control group was added; wall calcification data are depicted; leaflet calcification data not reported as this value was >100 µg/mg tissue

hydrophilic after being blocked with aldehydes compared to a native fresh control ($p < 0.05$; the exception was butanal, where the difference was not significant). On completion of the cross-linking reaction, the contact angle measurements indicated that the materials became more hydrophobic in the order glycerol > butanal > propional ($p < 0.05$ if subsequently compared with each other). Furthermore, all samples had water contents of 70-75%, and no significant inter-group differences were observed ($p < 0.01$).

Subdermal implantation

Samples were implanted subdermally in rats for 60 days and, after explantation, were further processed for histological or quantitative determination of calcium content (Fig. 3). The calcium content of wall sam-

ples was reduced in those groups with blocked amine groups. Decreasing calcium contents were found in the order glycerol ($p < 0.01$, compared to GA control), butanal ($p < 0.0001$), 2-methyl-butanal ($p < 0.005$) and propional ($p < 0.0001$). Among the carbodiimide-processed sample groups, the EDC/NHS control group showed the highest level of calcification.

In leaflet tissues, calcium levels were very low for all groups, with only the propional ($p < 0.02$) and 2-methyl-butanal ($p < 0.005$) groups showing significant reductions in calcification compared to the EDC/NHS group (see Fig. 3). The calcium content of the GA-fixed leaflet samples was >100 µg per mg tissue (data not shown).

Qualitative calcium contents of wall tissue as determined by von Kossa staining are shown in Figure 4. In the control EDC/NHS tissue group calcification occurred throughout the material, with two bands of complete calcification at the outer edges of the wall. Moreover, the glycerol group showed the highest incidence of calcification among the aldehyde-treated sample groups, with small calcium spots seen throughout the sample. These condensed calcium spots were observed for both the butanal and 2-methyl-butanal groups, but they occurred less frequently than the small calcium spots in the glycerol group (based on image analysis, $p < 0.01$). Finally, calcification was almost absent in the propional group. The results for leaflet material were not depicted since calcification as measured with AAS could not be visualized with von Kossa staining.

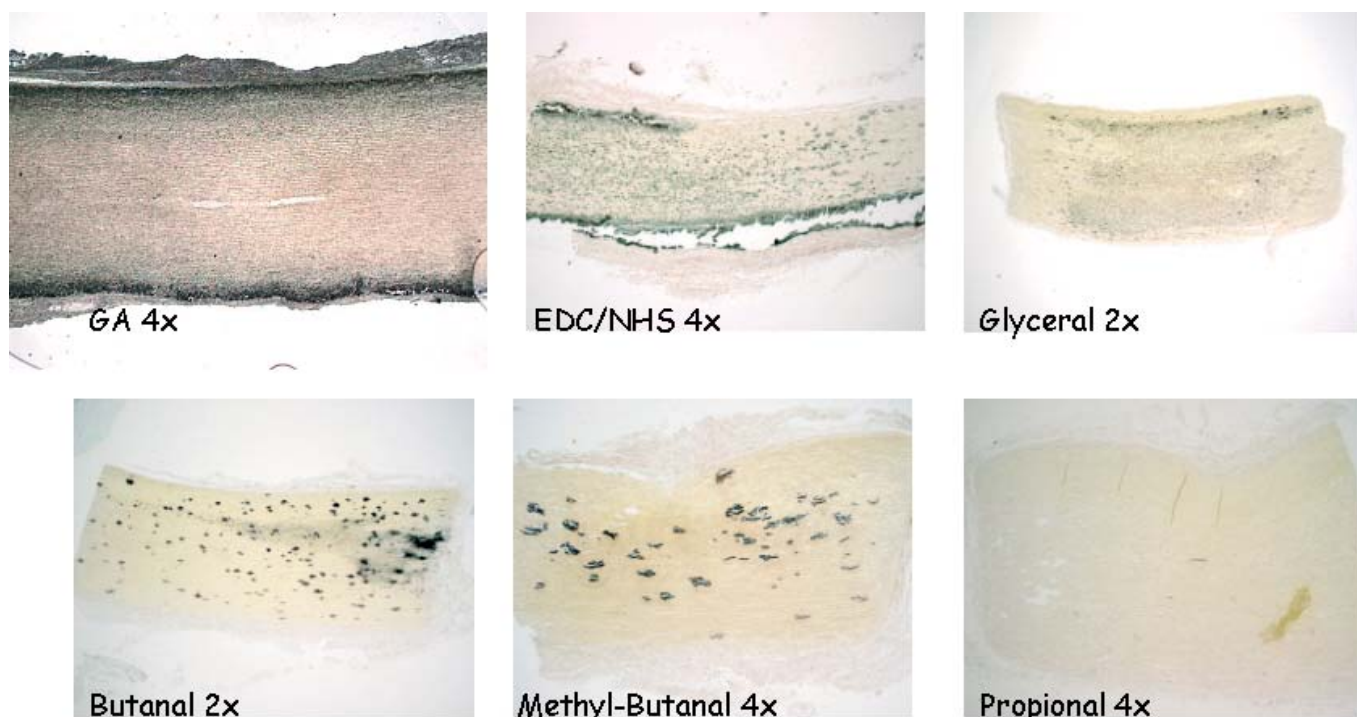


Figure 4: Histology of wall samples depicted in Figure 3 (von Kossa staining). A) GA; B) DC/NHS; C) glycerol; D) butanal; E) 2-methyl-butanal; F) propional. (Original magnification: A, C, D $\times 2$; B, E, F $\times 4$.)

Table IV: Characterization of valves used for the implant study. Valves (five per group) were treated using optimized conditions.

Aldehyde	Tissue	Concentration (M)	Blocking (%)	Ts (°C)	Digest remaining weight (%)
Fresh	Wall	-	0	61 ± 2.1	5 ± 2
EDC/NHS	Wall	NA	NA	80.1 ± 2.1	87 ± 2
Butanal	Wall	0.5	79 ± 2	68.8 ± 2.3	83 ± 4
2-Methyl-butanal	Wall	0.5	79 ± 3	70.3 ± 1.8	75 ± 5
Propional	Wall	0.5	81 ± 3	72.7 ± 2.8	64 ± 2
Glyceral	Wall	0.2	72 ± 4	79.9 ± 2.4	63 ± 3
Fresh	Leaflet	-	0	58.2 ± 3.1	0
EDC/NHS	Leaflet	NA	NA	82.0 ± 0.3	60 ± 3
Butanal	Leaflet	0.5	84 ± 2	75.8 ± 1.2	50 ± 5
2-Methyl-butanal	Leaflet	0.5	83 ± 3	78.7 ± 0.5	30 ± 4
Propional	Leaflet	0.5	84 ± 3	74.5 ± 0.5	50 ± 6
Glyceral	Leaflet	0.2	75 ± 2	81.9 ± 0.1	22 ± 2

NA: Not applicable; Ts: Shrinkage temperature.

Discussion

Before commencing the present study, a broad range of aldehydes was screened and evaluated as blocking agents for amine groups of dermal collagen in sheep. The results obtained showed that, for many aldehydes, almost 100% of the primary amine groups could be blocked. Based on these data, a selection of monoaldehydes was further investigated for the blocking of amine groups of tissue valves. Since all primary amine groups can be detected using the TNBS method, and tissue material contains more compounds with free amine groups, which may be less accessible to the blocking agent than in sheep dermal collagen, the theoretical maximal blocking efficacy would be expected to be less than 100%.

In the present study, glycerol, 2-methyl-butanol, propional and butanol were evaluated in detail. These aldehydes were selected based on their different hydrophobicity and the fact that they have a relatively low molecular weight. In this way, diffusion limitations were minimized and the original valve matrix morphology could be maintained as much as possible.

Following a feasibility study which showed that all four aldehydes could be successfully used, glycerol was evaluated in more detail because the degree of blocking was lower than for the other aldehydes. Studies with glycerol showed that extending the reaction time, increasing the temperature or increasing its concentration during the reaction, all led to a higher degree of blocking. Since glycerol was relatively insoluble in the medium, a concentration of 0.2 M was used to ensure that no solid glycerol particles were present in the blocking reaction. The reaction time for the

process was increased from 24 to 48 h to ensure also that all chemicals reached and reacted in the middle section of the tissue (wall) material (1). This was confirmed by the presence of a single sharp peak in the calorimetry thermogram, from which the Ts of treated samples was determined.

In order to further optimize the reaction conditions, studies were performed to: (i) determine the lowest NaCNBH₃ concentration that would provide the highest blocking efficacy; and (ii) reduce the concentration of buffer that would allow efficient blocking of amine groups. The results showed the minimal NaCNBH₃ concentration to be 50 mM, added at the start of the experiment. As the buffer pH did not significantly affect the degree of blocking, a study was conducted to show that the MES buffer concentration during the blocking reaction could be reduced to 0.05 M; previous studies with such buffer had shown the pH to rise, but to stay within an acceptable range. When optimization studies had been completed, a wall blocking-efficacy close to 80% was achievable.

An analysis of sample groups prepared for implantation showed the glycerol-blocked samples to have the lowest degree of blocking, despite the process having been optimized.

The high Ts-values of the glycerol-blocked and cross-linked samples may have indicated that free, unreacted amine groups were still present and that zero-length cross-links may have been formed between the amine and carboxyl groups. Enzymatic digestion of the glycerol leaflet material was greater than that of leaflets blocked with other aldehydes, though this may have been due to a better accessibility of enzymes to the collagen structure, which had

become more hydrophilic following glycerol treatment. Further studies are required, however, to investigate these findings.

Following explantation, von Kossa staining of the wall samples highlighted differences in calcification patterns among the groups. In the EDC/NHS group, high calcification was seen at the edges and throughout the wall samples, while in the aldehyde groups calcification was concentrated in nodes throughout the material. It remains to be proven whether there is any relationship between these nucleation sites and the reactions that occurred in the tissue during blocking.

In the case of leaflets, calcification in all groups was found to be very low. Indeed, von Kossa staining of the leaflet samples failed to show any calcification, though AAS showed calcium levels to be <1 mg per g tissue. A GA-fixed control sample was added to the implantation study in order to compare results with carbodiimide-processed sample groups to those of a 'standard' fixed sample group. The results showed that, indeed, a significant decrease in calcification could be obtained in comparison to the GA-fixed group.

As discussed previously (14), the experimental model selected to evaluate calcification *in vivo* does not predict the real outcome. Therefore, a propional-processed group was evaluated, using a juvenile sheep descending thoracic aorta model, and this achieved a similar significantly decreased calcification compared to a GA-processed control group (unpublished results).

Contact angle experiments demonstrated variations in the hydrophobicity of the different cross-linked samples after blocking, together with decreasing calcification as hydrophobicity increased ($p < 0.01$). Clearly, further studies are required with differently processed samples in order to verify whether this observation might hold for alternative non-carbodiimide cross-linked valve samples.

The available literature has suggested two possible hypotheses that might explain the present findings. The first hypothesis was a reduction in the transport of calcification-promoting agents (e.g., Ca^{2+} -ions) through the tissue with increased hydrophobicity (15). As a relationship exists between the hydrophobicity of a surface and the surface charge, it was also proposed that there might be a link in that each blocking agent has a different influence on the final surface charge of the matrix. Golomb and Ezra (16) proved that an impaired balance between positively and negatively charged amino acid residues resulted in affinity sites for Ca^{2+} . Zilla et al. (17) observed that the content of free aldehyde groups and polymeric GA cross-links must be reduced in order to obtain low calcification. In their process, the impaired balance after cross-linking was essentially restored.

The use of compounds such as bisphosphonate (18),

aluminum chloride (19), sodium dodecyl sulfate (SDS) (20) and other metallic salts (21) bound to the tissue or present in the matrix, all change the surface charge of the tissue, and each of these treatments has been successful in mitigating calcification. Since the observation of the relationship between calcification and hydrophobicity was based on four aldehyde samples only, a more detailed investigation is required before any final conclusions can be reached.

The second hypothesis was the removal of antigenic substances such as cellular elements and soluble proteins or potential nucleation sites for hydroxyapatite crystals, as this might be effective in reducing calcification. Various extraction methods have been used, including SDS, trypsin and dimethylsulfoxide (DMSO) (20,22-24). DMSO, which acts as a cryoprotective agent for tissues, appears to extract compounds, and may also nullify the nucleation sites that can attract Ca^{2+} (24). Based on the present findings, this leads to the second hypothesis that the blocking reaction components are extracted from the matrix, or that the aldehyde reacts with amine groups of compounds which are subsequently removed from the matrix. Initial extraction studies on processed tissue have indeed indicated that compounds such as proteins and lipids are removed during the blocking reaction, and that the amount varies with the type of aldehyde used to generate the blocking reaction.

Although the low calcification of the heart valve tissues achieved in the present study was promising, the biomechanical properties were found to lie between those of the first generation of GA cross-linked valves and valves processed as per the Freestyle™ (Medtronic, Minneapolis, USA). Thus, reduced tissue stiffness is desirable as the biomechanical properties should, preferably, be better or at least equal to those of the last-mentioned commercial product.

It is expected that mechanical properties would improve if valves were to be processed using root-pressure or zero-pressure fixation techniques (25). Two alternative approaches which have been investigated are to: (i) increase the chain-length of the Jeffamine used (14); and/or (ii) reduce the number of cross-links formed, for example by blocking a predefined number of carboxyl groups prior to cross-linking with Jeffamine. However, reducing the amount of EDC in order to decrease the number of cross-links formed leads to incomplete cross-linking and to cross-links that are not well distributed within the matrix, and this approach is not desirable.

In conclusion, these new approaches to blocking and cross-linking technology appear promising in the design of the next generation of tissue valves, notably as calcification was significantly reduced compared to

GA cross-linking. The mechanistic insight regarding decreased wall calcification remains unknown and, based upon the two hypotheses made here, research investigations are ongoing. In addition, the mechanical properties of the material need to be improved, and the results of these studies will be available shortly.

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