

Diamine-extended Glutaraldehyde- and Carbodiimide Crosslinks Act Synergistically in Mitigating Bioprosthetic Aortic Wall Calcification

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Background and aim of the study: The extension of glutaraldehyde (GA) crosslinks with diamine bridges was shown previously to reduce bioprosthetic heart valve calcification to a significant degree. The aim of the present study was to investigate whether the additional crosslinking of functional carboxyl groups could augment this anticalcific effect at the low glutaraldehyde concentrations typically used in commercial heart valve production.

Methods: Entire aortic roots of medium-sized pigs were fixed after 48 h of cold storage. Crosslinking of amino-functional groups was achieved either by GA fixation alone (0.2% or 0.7%) or with an interim treatment with the diamine L-lysine (25, 50 or 100 mM; 37°C; 2 days). Carboxyl groups were activated with carbodiimide (N'-[3-dimethylaminopropyl]-N-ethyl carbodiimide hydrochloride (EDC), 240 mM) and crosslinked with an oligomeric diamine (polypropylene glycol-bis-aminopropyl ether (Jeffamine™), 60 mM, 230D). By permutation of treatments and combinations thereof, a total of 17 groups was compared. Aortic wall discs (12 mm diameter) were implanted subcutaneously into seven-week-old Long-Evans rats for 60 days. Tissue calcification was determined by histology and atomic absorption spectrophotometry.

With the advent of cardiac surgery, the replacement of diseased heart valves became a reality. However, the demand for prostheses and the shortage of allogenic donor valves soon led to the utilization of porcine and bovine tissue. If untreated, the natural host response to these xenografts is acute rejection with cytotoxic elimination of donor cells and degradation of the extracellular matrix (1).

The most effective treatment to overcome this pro-

Results: There was no significant difference in tissue calcification if either GA or carbodiimide fixation was used alone. Equally, the combined crosslinking with GA and EDC/Jeffamine did not achieve a mitigation of tissue calcification below levels seen in at least one of the two treatments alone. When commercial GA fixation was mildly diamine-enhanced with L-lysine (25 mM), additional EDC/Jeffamine crosslinking of carboxyl groups resulted in a distinct additive effect in both 0.2% (-31%; $p < 0.0002$) and 0.7% (-36%; $p = 0.0073$) GA-fixed tissue. Relative to conventional GA fixation, this combination mitigated aortic wall calcification by 43% ($p < 0.0001$) and 34% ($p = 0.0014$) in 0.2% and 0.7% GA-fixed tissue, respectively. An increase in L-lysine concentration to 100 mM further reduced calcification of 0.7% GA-fixed tissue (18.5%; $p = 0.016$), but had no additional effect on 0.2% GA-fixed tissue (0.6%; $p = 0.463$).

Conclusion: A distinct reduction in bioprosthetic aortic wall calcification can be achieved by combining diamine-extended conventional GA fixation with a diamine-extended carbodiimide based crosslinking step.

The Journal of Heart Valve Disease 2005;14:538-545

hibitive predicament seemed to be crosslinking, which was shown to mask xenogenicity and to mitigate degradation (2). Among a variety of crosslinking reagents, glutaraldehyde (GA) was soon established as the most suitable fixative for bioprosthetic tissue due to the ease with which both of its terminal aldehyde groups formed stable bonds with ϵ -amino groups of proteins (3). On the downside, it became evident that a critical level of GA incorporation is required to induce maximal crosslink efficacy of these ϵ -amino groups (4). Since fixation at higher GA concentrations made the tissue stiff, and thus impaired biomechanical tissue performance, low crosslink efficacies were chosen for commercial fixation regimens. This choice of GA concentrations as low as 0.2 to 0.7% for bioprosthetic heart

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valve production was further motivated by the perception that crosslinks themselves cause calcific tissue degeneration (5).

Initially, there was widely held consensus that such low-grade fixation was still capable of preventing the adverse effects of GA treatment without any risk of insufficiently masking tissue xenogenicity. For more than two decades this consensus represented the generally accepted paradigm. During the 1990s, this paradigm became increasingly creaky when a number of studies began to demonstrate the detrimental consequences of insufficiently disguised immunogenicity (6,7). Resulting efforts to optimize xenograft-fixation through increased crosslink density led to a surprising observation: contrary to long-held beliefs, fixation at high concentrations of GA mitigated not only immune- and inflammatory responses (8,9) but also calcification (10-12). This paradigm shift and re-emphasis on crosslink efficacy, however, also meant a re-confrontation with tissue stiffness. A remaining challenge, therefore, is to create 'smart' crosslinks with all the benefits of high crosslink densities but without concomitant stiffening of the tissue. One emerging approach in this direction was the 'engineering' of novel, non-aldehyde-based crosslinks (13). Given the longstanding experience with GA, however, regulatory aspects would make it attractive to improve rather than to replace this well-established treatment that formed the backbone of fixation regimens of perhaps more than two million clinically implanted tissue valves. The goal

for the latter option would be to modify GA crosslinks in order to achieve better immune masking and mitigation of calcification, without adversely affecting the biomechanical function of the tissue.

As a first step in this direction, L-lysine-based diamine bridges were previously introduced on top of mild commercial GA fixation, and this resulted in a distinct suppression of calcification and inflammation (14-16).

In a further attempt to optimize conventional low-grade fixation, the present study aimed at evaluating a possible synergistic effect between such mild dialdehyde-based amino-group crosslinks and carboxyl-group crosslinks. Aortic wall tissue was chosen because of its known resilience towards conventional anticalcification treatments.

Materials and methods

Tissue fixation

Porcine aortic roots were collected at the abattoir, immediately after animal slaughter. Following thorough rinsing in phosphate-buffered saline (PBS; 0.1 M, pH 7.4), the roots were transferred into cold (4°C) GA solutions (Saarchem Holpro Analytic, Krugersdorp, RSA) at the two commercially used concentrations (0.2% and 0.7%, v/v in PBS; 4°C). After 30 h, circular discs of 12 mm diameter were punched out from the supra-commissural aortas and transferred into fresh GA (weight:volume ratio = 1 g per 30 ml fixative solu-

Table I: A complete listing of tissue treatment groups and calcification results obtained in the 60-day rat subcutaneous model.

Sample no.	[GA] (%)	[L-Lysine] (mM)	[EDC/Jeffamine] (mM)	Calcium content (µg/mg)	sem
1	0	0	240/60	87.6	6.4
2	0.2	0	0	103.7	3.2
3	0.2	25	0	85.1	1.5
4	0.2	50	0	80.8	5.2
5	0.2	100	0	70.5	4.1
6	0.2	0	240/60	82.2	4.9
7	0.2	25	240/60	58.7	4.3
8	0.2	50	240/60	61.1	4.6
9	0.2	100	240/60	58.0	5.2
10	0.7	0	0	87.1	3.3
11	0.7	25	0	89.0	7.2
12	0.7	50	0	62.8	4.6
13	0.7	100	0	47.8	5.9
14	0.7	0	240/60	81.5	4.6
15	0.7	25	240/60	57.4	6.0
16	0.7	50	240/60	55.0	5.2
17	0.7	100	240/60	41.2	2.5

Values are mean ± SEM.

[] indicates concentration.

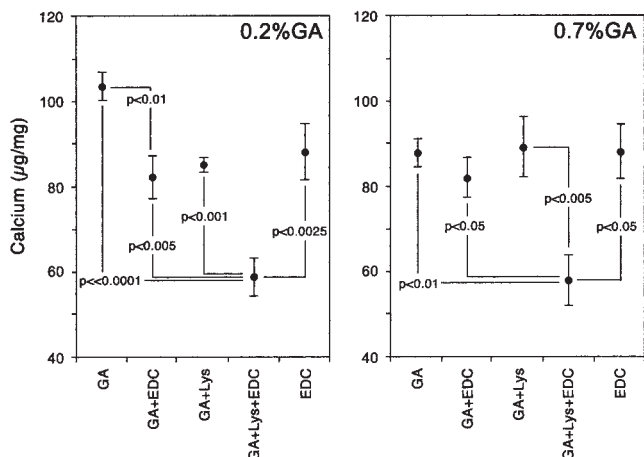


Figure 1: Calcification values and statistical significances between various fixation treatments of aortic wall tissue. The underlying fixation was either 0.2% or 0.7% glutaraldehyde (GA). No synergism occurred between GA and carbodiimide (EDC) fixation alone. If GA crosslinks were extended with diamine bridges (L-lysine, 25 mM), the synergistic effect of GA and EDC on the mitigation of aortic wall calcification was highly significant.

tion). Following 48 h of initial fixation, samples were placed in diamine solutions (L-lysine (Sigma): 0 mM, 25 mM; 50 mM and 100 mM in PBS; pH 7.6, 37°C; 30 ml/g tissue; 48 h). This diamine step was followed by a final fixation step in GA (37°C; 5 days; 1 g tissue/30 ml fixative - 0.2% GA if the initial fixation was in 0.2% GA and 0.7% if the initial fixation was at 0.7% GA). Tissue undergoing carboxyl group crosslinking (either fresh or GA-treated) was transferred into sterile-filtered Jeffamine (poly{propylene glycol}-bis-amino-propyl ether (Aldrich) 230D: 60 mM in morpholino-ethane-sulfonic acid buffer (Aldrich): 0.27 M, pH 5) on an orbital shaker for 30 min at room temperature. Samples were then immediately transferred into freshly prepared Jeffamine solution containing N'-[3-dimethylaminopropyl]-N-ethyl carbodiimide hydrochloride (Aldrich) 0.24 M (EDC) and NHS (N-hydroxysuccinimide (Aldrich) 0.1 M) and allowed to react for 2.5 h at room temperature on an orbital shaker. The tissue was subsequently washed five times with sterile double-distilled deionized water. Altogether, 17 groups were compared by permutation of treatments and combinations thereof (see Table I).

Rat implants

All anesthetic and surgical procedures were approved by the animal research and ethics committee of the University of Cape Town, and complied with the *Principles of Laboratory Care* and the *Guidelines for the Care and Use of Laboratory Animals* (NIH publication no. 86-23). Seven-week-old Long-Evans rats (200-250 g

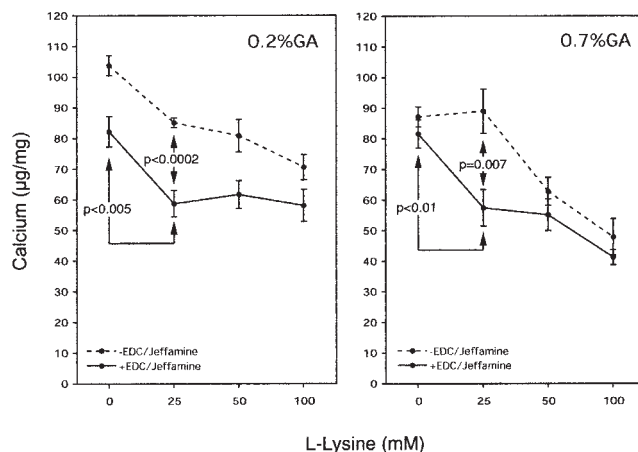


Figure 2: Tissue calcium levels (mean ± SEM) as a function of L-lysine concentration and combined carbodiimide-based crosslinking of carboxyl groups as determined by atomic absorption spectroscopy in 0.2% and 0.7% glutaraldehyde (GA)-fixed porcine aortic wall samples implanted subdermally in rats for 60 days.

bodyweight) were used for implantations. Samples were inserted into abdominal subcutaneous/supra-muscular pouches. Each of the 43 rats received four samples assigned on a rotational basis whereby two of the four samples implanted into the 43rd rat were not included in the analysis. Ten replicates were implanted per group. After 60 days of implantation, the animals were euthanased and the implants retrieved for quantitative and qualitative tissue calcium analysis. Each retrieved tissue sample was cut into two equal halves; one half was fixed in 10% formalin for histology, and the other was processed for atomic absorption spectrophotometry.

Calcium analysis

To avoid the complications of an 'edge effect', a 1 mm-wide circumferential ring was removed from all explanted coupons destined for analysis of tissue calcium by atomic absorption spectroscopy. Tissue was dried at 104°C for 24 h, weighed, ashed in a muffle furnace at 560°C for 12 h, and dissolved in a 20% hydrochloric acid solution (10 mg dried tissue per ml HCl). The final dilution was achieved in a 0.5% lanthanum chloride solution, and absorption measured at 422.7 nm on an atomic absorption spectrophotometer (Varian AA1275). Calcium levels were expressed as µg/mg dry mass of tissue. Histologically, von Kossa stains with van Gieson counterstaining were prepared from paraffin sections of the tissue.

Statistical analysis

Calcium data from atomic absorption spectroscopy analysis of explanted tissue samples were expressed as mean \pm SEM. Inferential statistical analyses were performed using the Tukey-Kramer HSD (honestly significant difference) test (JMP version 5.0 software; SAS). The Tukey-Kramer HSD test is sized for all differences among the means, and is thus able to accommodate multiple inferences. A significance level of 0.05 (two-tailed) or less was accepted as being statistically significant.

Results

Quantitative calcium analysis

When tissue was fixed exclusively with GA, calcification could be reduced by 16.0% ($p = 0.0048$) by increasing the GA concentration from 0.2% to 0.7% (Figs. 1 and 2). If GA crosslinks were not further combined with EDC/Jeffamine crosslinks, the effect of additional diamine bridges was concentration-dependent ($p = 0.0348$). While low concentrations of 25 mM L-lysine achieved a significant mitigation of calcification only in 0.2% GA fixation ($p = 0.0004$), higher concentrations (50 mM and 100 mM) of the diamine exerted a significant anticalcification effect in both 0.2% and 0.7% GA. If fixation was enhanced by 100 mM L-lysine treatment, calcification decreased by as much as 32.0% ($p < 0.0001$) and 45.1% ($p < 0.0002$) in 0.2% and 0.7% GA fixation, respectively. Tissue fixed entirely on the basis of carboxyl group crosslinks (EDC/Jeffamine; 240 mM/60 mM) showed almost identical calcium levels to tissue fixed in 0.7% GA only. If GA fixation was combined with EDC/Jeffamine treatment, tissue calcium levels of the combined treatment were not significantly less than the lower one of the two components. In contrast, combined GA and EDC/Jeffamine fixation showed a synergistic effect if GA crosslinks were extended with L-lysine. The most pronounced synergistic effect of combined fixation was found in mildly diamine-enhanced GA fixation: EDC/Jeffamine based carboxyl-group crosslinking achieved a distinct reduction in calcification at both GA concentrations if the L-lysine concentration was 25 mM (-31%; $p < 0.0002$ and -36%; $p = 0.0073$ at 0.2% GA and 0.7% GA, respectively). Relative to GA fixation alone, this combination mitigated aortic wall calcification by 43% ($p < 0.0001$) and 34% ($p < 0.01$) in 0.2% and 0.7% GA-fixed tissue. Additional carboxyl-group crosslinking failed to further diminish calcification at L-lysine concentrations higher than 50 mM in 0.2% GA fixation and 25 mM L-lysine at 0.7% GA fixation. By analogy, if GA fixation was not diamine-enhanced by L-lysine, the effect of EDC/Jeffamine treatment was equally confined to low-grade fixation. While addi-

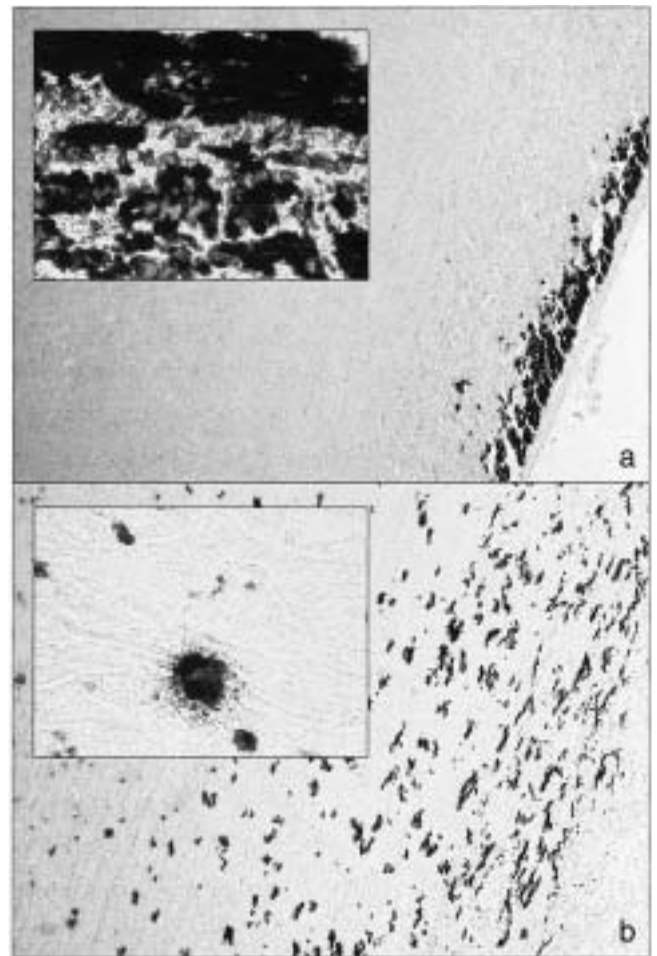


Figure 3: Typical band-like calcification pattern of L-lysine-enhanced (25 mM) glutaraldehyde (GA)-fixed (0.2%) tissue without EDC/Jeffamine treatment (a) with its coalescing mineralization (insert a) as opposed by the more diffuse distribution of calcium conglomerates throughout the aortic wall (b) and the spherical appearance of these conglomerates (insert b) in combined GA (0.2%)-L-lysine (25 mM)-EDC/Jeffamine fixation. (Von Kossa staining; original magnifications $\times 10$ and $\times 60$, respectively.)

tional carboxyl-group crosslinking led to a 21% reduction in 0.2% GA-fixed tissue ($p = 0.0045$), it was only 6% ($p = 0.346$) in tissue fixed at 0.7% GA. Relative to EDC/Jeffamine fixation alone, additional 25 mM L-lysine-enhanced GA fixation resulted in a similar reduction of calcification in 0.2% GA- (-28.6%; $p < 0.005$) and 0.7% GA- (-29.6%; $p < 0.01$) fixed tissue. The complete data set is listed in Table I.

Qualitative calcium analysis

If aortic wall tissue was fixed in GA without any additional treatment, the typical triple-layered calcification pattern of GA fixation was found, with a preferential location in the layers close to the blood surface

and to a slightly lesser extent associated with the adventitial surface. The morphology of calcium was mostly band-like (Fig. 3a), with the dense calcification zones typical for GA fixation (Fig. 3a, see insert). No difference between 0.7% and 0.2% GA-fixed tissue could be detected morphologically. With increasing concentrations of L-lysine, the extent of calcification in both the blood and adventitial surfaces diminished while the pattern remained unchanged. Alternative fixation in EDC/Jeffamine showed a similar (albeit mitigated) pattern on the luminal surface as GA fixation. On the adventitial side, the surface band of calcification was often fractured into conglomerates rather than occur as continuous bands. In the groups that were crosslinked with both GA and EDC/Jeffamine, this trend towards a pattern of calcium conglomerates rather than dense surface bands became more obvious. If GA crosslinks of these combined GA/EDC-Jeffamine groups were extended with L-lysine diamine bridges, a pronounced shift in calcification patterns was observed, predominantly on the adventitial side (Fig. 3b). As a trend, the sandwich pattern of surface bands was replaced by spherical calcium conglomerates reaching into the depth of the media. At the higher concentrations of L-lysine, the mineralization bands had sometimes even completely disappeared and were replaced by only a few of the round conglomerates (Fig. 3b, see insert). In contrast to the superficial position of the calcium depositions in GA-fixed tissue, these round and relatively large particles were scattered throughout the aortic wall. Overall, only half of the L-lysine/EDC-Jeffamine-treated samples showed these drastically changed calcification patterns, mostly in the 0.7% GA-fixed groups.

Discussion

In contradiction of a longstanding paradigm, higher GA concentrations were shown previously to mitigate rather than to augment bioprosthetic calcification and inflammation (10-12). This beneficial effect of increasing GA concentrations proved to be somehow limited because its anti-calcific potential reached an early plateau, while the stiffening effect on tissue pliability continued. When additional long-range diamine bridges between terminal aldehyde groups were introduced, aortic wall calcification and inflammatory infiltration further diminished (14,15). However, as much as these diamine bridges dramatically potentiated the effect of fixation at high GA concentrations, they occurred against the background of tissue that had lost part of its pliability and mechanical functionality. In a first attempt to achieve most of the beneficial effects of enhanced GA fixation without the need to increase GA concentrations, diamine concentrations were titrated

for the commercially used 0.2% and 0.7% GA treatment (16). A distinct effect on calcification, together with preserved tissue pliability, encouraged a deeper probe into the combination of different crosslinks with spacer-molecule bridges, without abandoning the underlying conventional GA fixation.

In a subsequent step, it could be shown that additionally introduced crosslinks between carboxyl groups rather than the ϵ -amino groups to which GA binds achieved a synergistic mitigation of aortic wall calcification in the presence of α -amino-oleic acid (AOA®) (17). Without AOA, which is believed to bind to both terminal glutaraldehydes - either attached to ϵ -amino groups of the tissue or free - and EDC/Jeffamine-based carboxyl group crosslinks, no synergism between GA- and EDC crosslinks could be detected. The synergistic 60% reduction in aortic wall calcification seen in the presence of AOA was again observed on the basis of GA concentrations used in commercial heart valve production.

The results of the present study suggest that a similar principle seems to apply to a situation whereby the 'spacer molecule' is the simple diamine L-lysine. Yet again, the basic background fixation was GA crosslinking at the commercial concentrations of either 0.2% or 0.7%. If used on its own, without any additional treatment, the mild increase in concentration from 0.2% to 0.7% led to a significant 17% reduction in aortic wall calcification, confirming the previously observed beneficial effect of higher GA concentrations even for the commercial range of fixation conditions.

The main objective of the present investigation was, however, to find a degree of synergism between carboxyl group crosslinks and amino group crosslinks at low commercial fixative concentrations. In analogy to a previous AOA study, only mild synergism was found between GA and EDC/Jeffamine without the insertion of a 'spacer-molecule' into the GA crosslinks. At concentrations of 0.7% GA even this mild synergism seen at 0.2% GA had disappeared. Since the calcium levels in the combined GA/EDC-Jeffamine groups were similar to those achieved with EDC/Jeffamine fixation alone, carboxyl group bonds on their own did not seem to introduce any beneficial effect beyond that of pure GA crosslinks. When the diamine L-lysine was introduced as a spacer molecule between GA crosslinks, however, a strong synergism between EDC-based and GA-based fixation was found that led to a mitigation of more than one-third of aortic wall calcification at the low L-lysine concentration of 25 mM (16). It remains speculative where the commonalities lie between the two 'spacer molecules' AOA and L-lysine in their ability to trigger this synergism. It is widely believed that the chemical binding of the α -amino oleic acid into GA-fixed tissue occurs through the formation

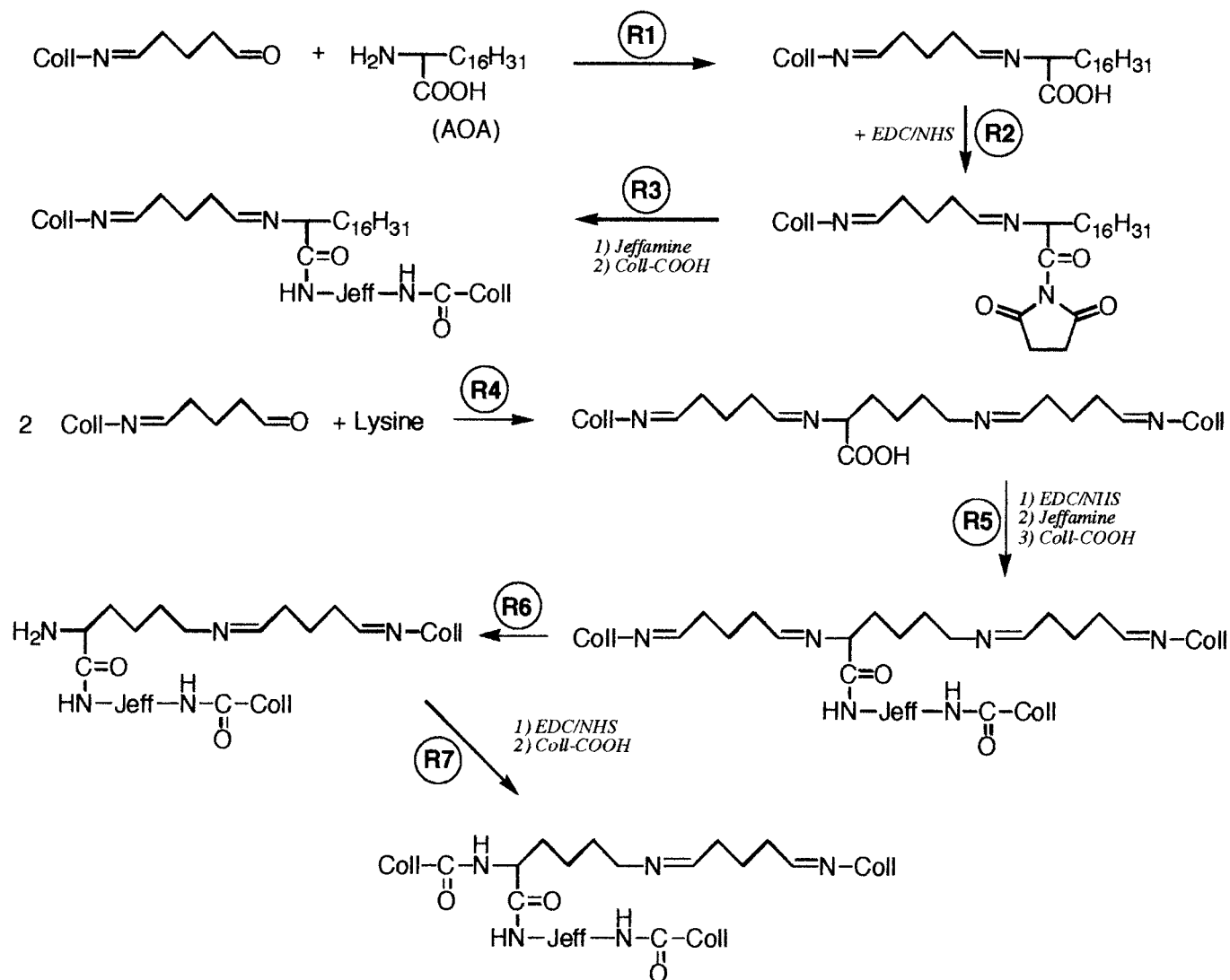


Figure 4: Possible pathways for reactions between glutaraldehyde (GA)-treated tissue and amino-oleic acid (AOA) (R1) followed by carboxylic acid activation (R2) and further crosslinking to tissue carboxyls via the AOA molecule and Jeffamine (R3). Reaction with L-lysine (R4), followed by carboxyl activation in the presence of Jeffamine, leads to trifunctional incorporation of (R5). Even if the imino-bond linking the GA terminal to the α -amino group is severed (R6), the resultant amine may participate in amide formation with a tissue carboxyl (R7), thereby restoring the trifunctional crosslink.

of a Schiff base between the α -amino group of the AOA and a terminal aldehyde residue of GA (Fig. 4, R1). The subsequent EDC/Jeffamine treatment is likely to activate not only tissue carboxyl groups but also the carboxyl group of the α -amino oleic acid, thereby additionally tying the AOA molecule to a tissue carboxyl group via the Jeffamine (Fig. 4, R2 and R3). Similarly, the ϵ - and α -amino groups of L-lysine offer themselves to the formation of Schiff bases with free GA terminals (Fig. 4, R4) and at the same time possess a carboxyl group, which is accessible to EDC activation and eventually to an additional crosslink with a tissue-carboxyl group via Jeffamine (Fig. 4, R5). In both instances, mixed long-range bonds are created

between a carboxyl group and the α -amino group of the tissue - the opposite of zero-length crosslinks between adjacent carboxyl and ϵ -amino groups. In the case of L-lysine, the 'spacer molecule' itself is shorter than the AOA molecule, but it offers itself to a trifunctional incorporation with two imine and one EDC-activated amide bonds (Fig. 4, R5). Even if there is a certain probability that the Schiff base between one terminal aldehyde and the α -amino group of the L-lysine breaks when the carboxyl is activated (Fig. 4, R6), there is still a chance that the L-lysine molecule ends up as a trifunctional crosslinker rather than a bifunctional one. Once the α -amino group is dissociated from the second terminal aldehyde, it may become available for a reac-

tion with an activated carboxyl group - either of another free L-lysine or the tissue (Fig. 4, R7).

This phenomenon highlights once more that, even with deeper insight into crosslink chemistry, the ability to explain the actual mechanisms of mitigation of mineralization remains limited. Previous observations in connection with modifications of crosslink chemistry have suggested a role for intermolecular space obliteration. The inhibitory effect of high crosslink density of calcification and its augmentation through the introduction of additional long-range bonds suggests that some sort of spatial inhibition of crystal growth could play a role. The concomitant utilization of both carboxyl and amino groups in the present study may well have a similar effect. On the other hand, this space-filling effect may be of a functional nature. In a recent study it was shown that, rather than the actual intermolecular space being occupied by crosslinking or 'capping' molecules, it was hydrophobicity that determined the degree of inhibition of calcification (18). Similarly, the changes in hydrophilicity caused by the introduction of various crosslinking agents in the present study may also play a role in the tissue mineralization.

The possibility that a 'spacer molecule' concurrently binding to the terminal ends of amino- and carboxyl group crosslinks represents a hinge element for the beneficial effect of both L-lysine and AOA is further supported by the similar change found in the calcification pattern. In both cases, the switch from the typical band-like surface mineralization of aortic wall tissue to more randomly distributed spherical 'nodules' was not so much dependent on the introduction of carboxyl group crosslinks but on the presence of a 'bridge molecule' which has the theoretical capability of binding to both crosslink types.

In conclusion, the subtle step-by-step mitigation of calcification through three chemically distinct crosslink formations is encouraging for those who believe that the eventual engineering of a smart crosslink chemistry may hold the key to future bioprosthetic research. The fact that a 43% reduction in tissue calcification was obtainable against the background of mild commercial rather than high-concentration GA-fixation indicates that 'smart' crosslinks may be created without necessarily jeopardizing crucial tissue pliability and without abandoning the well-established GA fixation as a basic treatment. Although tissue softness appeared to be preserved, the fine-titration of 'smart' GA-based crosslinks will depend on an assessment of the influence of various crosslink types on the biomechanical behavior of bioprosthetic tissue.

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