Detoxification and Endothelialization of Glutaraldehyde-Fixed Bovine Pericardium With Titanium Coating
A New Technology for Cardiovascular Tissue Engineering
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Background—Endothelial cell seeding of glutardialdehyde-fixed biological heart valves is hypothesized to improve biocompatibility and durability; however, the toxicity of glutardialdehyde prevents its use as a biological coating. Therefore, different detoxification strategies are applied, including surface coating with titanium, before in vitro endothelialization of glutaraldehyde-fixed bovine pericardium as the base material for prosthetic heart valves.

Methods and Results—Bovine pericardium was fixed with 0.25% glutaraldehyde. Detoxification was performed with citric acid, aldehyde dehydrogenase, and plasma deposition with titanium at low temperatures of 30°C to 35°C. Toxic glutaraldehyde ligands were quantified photometrically, and the vitality of seeded cells was tested to validate detoxification methods. Detoxification agents and titanium coating were applied before seeding with human endothelial cells. Endothelial cells were visualized by electron microscopic surface scanning. To evaluate cell adhesion, shear stress was applied by a flow of 5 L/min over 24 hours. Compared with untreated glutaraldehyde-fixed samples, treatment with the different agents reduced free aldehyde groups gradually (citric acid 5% < citric acid 10% < titanium < aldehyde dehydrogenase). A combination of citric acid 10%, aldehyde dehydrogenase, and titanium coating resulted in a reduction of free aldehyde ligands to 17.3 ± 4.6% (P < 0.05) and demonstrated a vitality of seeded cells of 94 ± 6.7% (P < 0.05). This procedure yielded a completely confluent layer of regular human endothelial cells (n = 5). After application of shear stress for 24 hours on these endothelial layers, cell vitality was 81%.

Conclusions—Titanium coating combined with chemical procedures yielded significant detoxification and complete endothelialization of conventional glutaraldehyde-fixed pericardium. This new technique might improve glutaraldehyde-fixed cardiovascular bioimplants for better biocompatibility and longer durability. (Circulation. 2009;119:1653-1660.)

Key Words: collagen • endothelium • surgery • valves • vessels

Biological heart valve prostheses with long durability and function are desirable for the treatment of heart valve disease. At present, 275 000 valve replacements are performed annually worldwide, including more than 120 000 tissue valve prostheses. Biological heart valve prostheses do not require chronic anticoagulation therapy; however, they degenerate prematurely, especially in young patients. It is generally accepted that the breakdown of bioprosthetic heart valves is due to calcification that occurs because of chemical processes and a retained antigenicity, even after glutaraldehyde treatment. Chemical processes occur between free aldehyde groups and phospholipids, and in combination with calcium ions from the circulation, this induces calcification. Thus, detoxification and endothelial cell seeding of glutaraldehyde-fixed biological heart valves have been hypothesized to improve biocompatibility and durability.

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The toxicity of glutaraldehyde, however, prevents its use as a biological coating. Detoxification of glutaraldehyde-fixed scaffolds by amino acids and citric acid (CA) in vitro has been used successfully for partial detoxification, but endothelialization was only possible by preseeding with fibroblasts. Such preseeded layers on porcine heart valves were stable in a sheep model. Because of the fibroblasts, however, the valves showed a thickening of the leaflets that resulted in an impairment of function. In the present investigations, we sought to develop detoxification strategies and

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apply nanotechnological methods such as ultrathin titanium surface coating at temperatures between 30°C and 35°C\textsuperscript{16–18} on glutaraldehyde-fixed bovine pericardium as the base material for prosthetic heart valves.

**Methods**

**Glutaraldehyde-Fixed Bovine Pericardium**

Previously, genuine bovine pericardium was fixed with 0.25% glutaraldehyde solution at room temperature for at least 24 hours. Circular platelets of 9-mm diameter were cut out and sorted by weight. The platelets were selected for a weight between 90 and 100 mg to minimize the risk that a different mass might influence the test results.

**Detoxification**

Detoxification was performed with CA 5% and 10% as described elsewhere.\textsuperscript{8,9} In brief, detoxification was performed by incubating the platelets with 50 mL of CA 5% or 10% per platelet for 30 minutes. Afterward, platelets were rinsed twice with 30 mL of buffer solution (PBS; Invitrogen, Carlsbad, Calif) per platelet for 10 minutes to reestablish a neutral pH. Detoxification was also performed with aldehyde dehydrogenase (ALDH) and buffer solution from a commercially available kit (Enzymatic BioAnalysis; R-Biopharm, Darmstadt, Germany). Pericardial platelets were incubated in a 24-well plate with 1 mL of enzyme solution each for 24 hours. Detoxification was also achieved by plasma deposition for titanium as described below.

**Dehydration**

Dehydration of the biological material was completed before a chemical vapor deposition process was performed (plasma-activated chemical vapor deposition). This extraction of water was performed under a low pressure of 3.8\times10^{-10} Pa by a slow drying process over 3 hours at 32°C.

**Plasma-Activated Chemical Vapor Deposition for Titanium**

Plasma-activated chemical vapor deposition is a coating technology (Gesellschaft für Elektrometallurgie GmbH, Nürnberg, Germany; patent No. EP 0 897 997 A1) that transfers the so-called precursor, tetraakis(dimethylamido)titanium [Ti[N(CH\textsubscript{3})\textsubscript{2}]]\textsubscript{4}, into the gas phase and brings it into the reactor by a carrier gas, such as nitrogen gas,\textsuperscript{16} under vacuum conditions (Figure 1).

Within nonthermal plasma with high electron temperatures but with neutrons and ions at room temperature, only the electrons can follow a quickly changing electrical field with a typical frequency of 13.56 MHz. The plasma is able to supply the precursor with high energy, while the temperature during deposition can be kept low, at approximately 30°C to 35°C.\textsuperscript{16,17} Under these conditions, the precursor is fractionalized, and parts of the precursor are precipitated on the plasma-activated collagen surface (Figure 1). The carbon atoms that are in contact with the titanium are then able to perform paired electron bindings with carbon atoms of other ligands that have titanium bindings and with the carbon atoms of the collagen. Thus, titanium is irremovably bound to collagen in an extremely thin layer of 30 nm.

**X-Ray Photoelectron Spectroscopy**

X-ray photoelectron spectroscopy (XPS), described elsewhere,\textsuperscript{19} was performed in 5 pairs of noncoated and titanium-coated glutaraldehyde-fixed bovine pericardium platelets after treatment with CA 10% and ALDH. XPS demonstrates the composition of elements in the coating layer for uncoated and coated pericardium. The applied sputter time is related to the argon sputter for removal 1 layer after the other and is proportional to the analysis depth of the substrate.\textsuperscript{18,19} The XPS analysis showed a maximal surface atom fraction of 21.1±3.8% titanium atoms (Figure 2A) within a removal time of ~300 seconds, which translates to a titanium thickness of 30 nm. The presence of oxygen (Figure 2A) indicates that titanium has been changed by subsequent oxidation into titanium dioxide, with an electron-binding energy for titanium of 463.2±2.4 eV (Figure 2B). The 2 peaks for titanium represent titanium bound with carbon (small peak) and titanium bound with oxygen as titanium oxide (high peak).

**Rehydration**

After plasma-activated chemical vapor deposition, the substrate was rehydrated in physiological saline solution (0.9%) for 30 minutes and regained its original shape.

**Determination of Free Aldehyde Groups**

The following method of evaluating free aldehyde groups was developed and validated in our laboratories. To determine free aldehyde ligands, a procedure used to determine the number of free aldehyde groups in specimens of glutaraldehyde-fixed pericardium was developed with ALDH. In the presence of ALDH, aldehydes are oxidized to carbon acids, while a reduction equivalent (here, NAD\textsuperscript{+}; Figure 3) is reduced.

The amount of NADH is proportional to the original amount of aldehyde; thus, statements on the remaining toxicity of the fixed pericardium can be made (Figure 1). Enzyme, NAD\textsuperscript{+} (tablets), and buffer solution from a commercially available kit, originally developed to determine acetaldehyde concentrations in a variety of materials (acetaldehyde, Enzymatic BioAnalysis; R-Biopharm, Darmstadt, Ger-
many) were used. Pericardial platelets were incubated in a 24-well plate with 1 mL of enzyme solution each for 24 hours. Thereafter, 100/L from each well was examined photometrically at a wavelength of 340 nm (Figure 4). Native pericardium served as control.

Of particular interest was the determination of the time course of oxidizing aldehydes to carbon acids. To determine the optimal duration of incubation, the degradation kinetics of ALDH and glutaraldehyde were evaluated. Twenty-four hours (1440 minutes) proved to be a practical and reliable time, because the reaction had reached its maximum, and no further processing of aldehyde was anticipated. A time curve of the photometric extinction of NADH after 24 hours showed values with a high accuracy of evaluating the total aldehyde (data not shown).

Cell Culture

Endothelial cells were isolated from human saphenous veins harvested from coronary bypass surgery. Ethical approval was obtained from the Ethical Commission on Research on Humans of the University of Schleswig Holstein, Campus Lübeck, Germany (registration No. 05-097). Patients had been informed before surgery that parts of their veins were to be used for research purposes. The vein pieces were cannulated, rinsed with cell medium, filled with 0.5% dispase, and incubated for 20 minutes at 37°C and 5% CO2. The solution was then centrifuged at 1200 rpm for 5 minutes; the cell pellet was resuspended in endothelial growth medium (EGM-2; Cambrex Corp, Walkersville, Md) and plated on cell culture flasks. For passaging, endothelial cells were trypsinized after reaching confluence, centrifuged at 1200 rpm for 5 minutes, then resuspended in endothelial cell medium and plated again. Cell counting was performed by incubating 20/L of the cell suspension with an equal amount of trypan blue and then counting with a Neubauer counting chamber.

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\text{H}_3\text{C}-\text{CHO} + \text{NAD}^+ \rightarrow \text{H}_2\text{C} = \text{C}-\text{O} + \text{H} + \text{H} + \text{NADH}
\]

Figure 3. Principle of evaluating free aldehyde groups. Aldehydes are oxidized to carbon acids while NAD+ is reduced to NADH, which can be quantified photometrically.

Figure 2. A, XPS of glutaraldehyde-fixed bovine pericardium before (left) and after (right) titanium coating. Element composition of glutaraldehyde-fixed bovine pericardium demonstrated more than 90% carbon atoms from the surface into the substrate (analyses are performed from left to right within the diagram), minimal oxygen atoms of 5%, and a <5% nitrogen atom concentration (left diagram). After titanium coating, XPS analysis (n=5) for titanium atoms showed a maximal surface atom fraction of 21.1±3.8% (shaded area), which decreased toward the inner part of the substrate, which was characterized by a high concentration of carbon atoms. Nitrogen was derived from the precursor. Integration of oxygen occurred via oxidation as titanium oxide after the titanium coating was applied. During plasma deposition, there was no oxygen present. B, Binding energies (eV) between elements evaluated during XPS of titanium-coated pericardium for oxygen, titanium, nitrogen, and carbon. The titanium-coated surface is in the front, and the substrate is in the back. Binding energy of titanium is 463.2±4.3 eV.
Cell Seeding
Detoxified platelets of glutaraldehyde-fixed pericardium were dabbed with sterile swabs, clamped into V4A steel rings specifically customized for the cell seeding procedure, and arranged on a 24-well plate. Human endothelial cells were seeded on the clamped platelets with endothelial growth medium (EGM-2) and incubated for 24 hours at 37°C and 5% CO2. The samples were then removed from the plate, and pericardial platelets were extracted from the rings to perform scanning electron microscopy.

Flow Chamber
A flow chamber made of V4A steel and glass incorporated 6 detoxified and endothelialized platelets in its bottom within V4A steel rings. It was constructed such that no significant turbulence occurred, and the laminar shear stress of 30 dyne/cm² and flow of 5 L/min were similar to the intra-aortic fluid dynamic.

MTS Test
The MTS test is a photometric method used to evaluate vitality and number of living cells. MTS (methosulfate) is a salt of tetrazolium that can become incorporated into vital cells. In the presence of the electron-coupling molecule PMS (phenazine methosulfate), MTS is reduced by mitochondrial dehydrogenases into soluble formazan, which is distributed within the surrounding medium. Formazan concentration is evaluated photometrically by a wavelength of 492 nm and is proportional to the vitality and number of living cells in culture when calibrated to a positive control with a distinct number of comparable vital cells 48 hours after cell seeding.

Scanning Electron Microscopy
Differently detoxified pericardial platelets (n=3 groups of 5 samples each) were examined by scanning electron microscopy after being

Figure 4. Photometric evaluation of glutaraldehyde concentration of glutaraldehyde-fixed pericardial platelets after ALDH treatment by measurement of optical density (extinction) of NADH at a wavelength of 340 nm. Results show values of different glutaraldehyde concentrations (A–C). Native pericardium served as control (D).

Figure 5. Aldehyde concentration of glutaraldehyde-fixed pericardium after application of diverse treatment protocols (n=6). Titan indicates titanium.
seeded in vitro with human endothelial cells. For each specimen, 10 visual fields were evaluated under ×370 magnification. Both cell morphology and confluence of the cell layer were assessed semi-quantitatively by different examiners.

Statistical Analysis
Data are presented as mean±SD. For comparisons between groups, a Mann–Whitney U test was used for unpaired samples, and a Wilcoxon signed-rank test was applied for paired samples. Differences were considered significant at values of $P\leq0.05$. All statistical calculations were performed with the software WinSTAT 3.0 (Kal-mia Co, Cambridge, Mass).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Determination of Concentration From Free Aldehyde Groups
The different detoxification levels attained by different detoxification procedures are presented in Figure 5. The combination of CA 10%, ALDH, and a titanium coating showed the most favorable results, with a reduction of the aldehyde concentration’s respective toxicity to $17.3\pm4.6\%$ ($P\leq0.05$).

Scanning Electron Microscopy
Scanning electron microscopy (Figure 6) of human endothelial cells on glutaraldehyde-fixed bovine pericardium showed sporadic cell cadavers (left column, group I). GA indicates solely glutaraldehyde fixed. Cells treated with CA (10%) demonstrated malformed endothelial cells (middle column, group II). A confluent epithelial cell layer was visualized after ALDH, CA 10%, and the nanotechnologically promoted procedure with titanium plasma administration in group III (right column).

Vitality of Seeded Cells
Human endothelial cells on glutaraldehyde-fixed pericardial platelets showed a vitality of $21.9\pm3.7$ (Figure 7). The greatest endothelial cell survival of $94.1\pm9.7\%$ was found in nanotechnologically promoted detoxification by a titanium coating of glutaraldehyde-fixed pericardial probes combined with CA 10% and ALDH.
Cell Adhesion Under Flow Conditions
Adhesion of human endothelial cells on glutaraldehyde-fixed pericardial platelets treated by CA 10%, ALDH, and a titanium coating was expressed as the vitality of the remaining cells after a flow of 5 L/min for 24 hours within a flow chamber. Vitality was measured by extinction of the MTS test of 72% (n=6) after a preseeding period of 15 minutes and 81% (n=6) after a preseeding period of 60 minutes (Figure 8).

Discussion
The present study provides evidence that titanium coating in combination with treatment with CA10% and ALDH removed most of the free aldehyde ligands of glutaraldehyde-fixed pericardium in vitro and yielded a living, confluent cell layer of human endothelial cells with a high adhesion capacity.

Titanium Coating
Titanium has been the leading structured metallic biomaterial for 50 years. One reason for its widespread use is the excellent biocompatibility of the metal and its alloys. Theoretically, surface coating of the less biocompatible glutaraldehyde-treated tissue with titanium was desirable for improving biocompatibility of this kind of implant material. However, a titanium coating on biomaterials did not appear to be possible until now owing to the high temperatures needed for commonly used sputtering techniques. Therefore, a novel coating method was developed for a biological substrate that used plasma-activated chemical gas deposition at temperatures of 30°C to 35°C. Within this nonthermal plasma with high electron temperatures but with neutrons and ions at room temperature, only the electrons can follow a quickly changing electrical field with a typical frequency of 13.56 MHz under low-pressure plasma, as described in detail elsewhere.

Another technique that applied titanium to polytetrafluoroethylene was reported to result in a thicker manifold and a removable titanium layer but was not transferable to collagen because of the need for high temperatures, which denature collagen. The applied method in the present study is...
performed at 30°C to 35°C. The precursor containing titanium is electron-pair bound with molecules of the amino acids of the collagen, is irremovable, and has a thickness of 30 nm (Figures 1 and 2B).

**Dehydration**

As part of the coating process, dehydration of the bovine pericardium was performed before the plasma-activated chemical vapor deposition process over a 3-hour period at 32°C under low pressure. In a pilot study, we applied dehydration and rehydration in commercially available biological glutaraldehyde-fixed heart valves with no loss of function. However, this process of dehydration and rehydration is not yet well understood and requires continued research in the future.

**Detoxification Processes**

Detoxification of glutaraldehyde has been performed by other investigators with CA 5%13 and later with more efficacy with CA 10%.15 but without any quantification of the remaining free aldehyde groups. As demonstrated in Figure 5, with the procedure used by Gulbins et al,13,15 the concentration of free aldehyde groups ranged between 68% and 76% compared with the concentration of the untreated glutaraldehyde-fixed pericardium as a control with 100%. After partial detoxification, most of the extremely vulnerable endothelial cells did not survive (Figure 6). This may be why Gulbins first seeded the blood-contacting surface of CA-treated valves with a layer of less vulnerable fibroblasts to protect the added endothelium. The disadvantage of an additional fibroblast layer is that it induced a thickening and thinning of these valves.15

An initial nanotechnological procedure of plasma deposition with titanium using a high-frequency current for detoxification of glutaraldehyde-treated collagen dismissed most of the free aldehyde ligands. A reduced number of aldehyde groups should result in fewer chemical processes between free aldehyde groups and phospholipids, which are able to create calcification in combination with calcium ions from the circulation.2–5 The expected prevention of calcification should prolong the durability of the implants.

**Cytotoxicity**

The cytotoxicity of the coating material and process is of major relevance, because nanotechnology is used. In particular, 2 aspects are important for toxicity. First is the cytotoxicity of the material and the process itself. The present results with a confluent layer of living endothelial cells (Figures 6 and 7), the 6-month experience in a goat model that also used a living confluent endothelial surface,23 and clinical results over the years with the same titanium coating on hernia meshes24,25 provide evidence that cytotoxicity may be of minor clinical impact. Furthermore, titanium has been used as a biomaterial with excellent biocompatibility in recent decades.20,21 Second, nanoparticle release may cause remote pathology.26 However, titanium is incorporated into the fragments of the precursor that are chemically bound to the substrate, and furthermore, the coating is covered by endothelial cells that counteract the release of nanoparticles. Nevertheless, various other parameters may be of influence in this regard and need to be integrated into further complementary cytotoxicity tests according to US Food and Drug Administration requirements.

**Future Prospects**

Although our primary aim in the present study was to evaluate the possibility of using titanium coating and detoxification of biological material, many questions related to the coating process remain unanswered. One of these concerns the long-term effect of this coating technique on the elasticity and compliance of the valve leaflets. One sign of unchanged material characteristics may be gained from our 6-month experience in a goat model,23 in which we found no macroscopic impact in this regard.

Not only is preservation of the functional characteristics of the biological materials, such as elasticity and compliance, of great importance, but so is its structural integrity over time. From the literature, it is known that the NO-React detoxification process27–29 may lead to conduit failure or peel formation at the distal anastomosis, which may cause severe clinical problems. The mechanisms involved in these pathological processes are generally unknown, whereas immunologic reactions are suspected.30 We speculate that the detoxification process presented here, which includes titanium coating, may reduce immunologic reactions, because the xenologous material is completely covered with autologous endothelial cells and as such is less reactive. Because this topic is of major importance, further studies on the influence of the described treatment process on long-term preservation of structural integrity and immunology are under way.

Another item of major importance is the redistribution of nanoparticles. Although the high-energy electron-pair binding (Figure 2B) and the complete endothelial cell layer on the coating used here provide a type of sealing effect and potentially protect the implant from redistribution of nanoparticles, further studies on this subject are required.

Analysis of endothelial cell binding to the nanocoating surface is of major importance. The shear stress data demonstrate that most of the endothelial cells adhere to the nanocoating surface even under in vitro conditions. Furthermore, the first in vivo case in animals provides some evidence that a self-seeding process with endothelial cells over time is possible with this coating technology.16,17,24,25 If these results can be confirmed by future studies, preseeding would appear to be unnecessary, which would be a great improvement in tissue engineering. However, more refined analyses that include adhesion proteins are desirable.

**Conclusions**

Tissue engineering of heart valves is considered a promising concept to generate improved substitute bioprostheses. Because there currently is no realizable blueprint for the construction of semilunar valves,31 our aim was to refine conventional proven bioprostheses using innovative nanotechnological techniques such as plasma deposition with the most biocompatible titanium. Titanium coating in combination with chemical agents has been shown to support detoxification and endothelialization of biological glutaraldehyde-fixed tissue. Although these results show a reasonable perspective for tissue engineering, several questions must be addressed, such as the influence of dehydration and rehydr-
tion on tissue structure, the in vivo function of such constructs, the function of the seeded cells, and the influence of titanium coating on thrombogenicity and immunogenicity. Nevertheless, this new technique might improve glutaraldehyde-fixed cardiovascular biomposites from the standpoint of better biocompatibility and longer durability.

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**Disclosures**

None.

**References**


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