

**(12) PATENT**  
**(19) AUSTRALIAN PATENT OFFICE**

**(11) Application No. AU 199860567 B2**  
**(10) Patent No. 735988**

(54) Title  
**Method of sterilization**

(51)<sup>7</sup> International Patent Classification(s)  
**A61L 002/00**

(21) Application No: **199860567**

(22) Application Date: **1998.02.09**

(87) WIPO No: **WO98/34650**

(30) Priority Data

(31) Number **60/037528** (32) Date **1997.02.10** (33) Country **US**

(43) Publication Date : **1998.08.26**

(43) Publication Journal Date : **1998.10.15**

(44) Accepted Journal Date : **2001.07.26**

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(56) Related Art  
**US 5447536**  
**WO 84/01894**



<b>(51) International Patent Classification 6 :</b> <b>A61L 2/00</b>		<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 98/34650</b> <b>(43) International Publication Date:</b> 13 August 1998 (13.08.98)
<b>(21) International Application Number:</b> PCT/US98/02395 <b>(22) International Filing Date:</b> 9 February 1998 (09.02.98)  <b>(30) Priority Data:</b> 60/037,528 10 February 1997 (10.02.97) US		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	
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<b>(54) Title:</b> METHOD OF STERILIZATION			
<b>(57) Abstract</b> <p>Heart valves or other components for replacement of heart or other bodily organs and tissue prostheses or synthetic prosthetic materials are effectively sterilized by treatment with a coupling agent known to create amide linkages between amines and carboxylic acids in the presence of a lower alkanol. Such treatment has been shown to be bactericidal when carried out at 40 °C for over 1 hour. The sterilization treatment preferably employs EDC as a water-soluble coupling agent, plus an optional coupling enhancer such as sulfo NHS or NHS, in the presence of isopropanol or an equivalent alkanol. Such sterilization treatment is preferably carried out at a temperature above ambient temperature in a buffered aqueous solution. The treatment leaves no residuals other than ones which are nontoxic and biocompatible, does not affect the resistance of the tissue to thermal denaturation and to digestion by proteolytic enzymes, and surprisingly also increases the resistance of fixed biological tissue to calcification.</p>			

### METHOD OF STERILIZATION

This application claims priority from U.S. Provisional Application Serial No. 60/037,528, filed February 10, 1997. The disclosure of this application is incorporated herein 5 by reference.

#### Field of the Invention

This invention relates to methods of sterilization and more specifically to methods of sterilization which are particularly suited for biological materials, such as organ 10 replacements, and which methods exhibit efficacy against difficult-to-kill bacteria and bacterial spores.

#### Background of the Invention

Sterilization techniques are widely used and important in industries such as food processing and health care. 15 Saturated steam at temperatures above 110°C has frequently been used to destroy microorganisms, such as microbial spores. Certain articles, particularly those used for health care, cannot withstand the temperatures and moisture of steam sterilization, and oftentimes such articles are 20 also considered not to be suitable for sterilization by ionizing radiation. As a result, gaseous sterilants have been developed which function at relatively low temperatures and thus offer an attractive alternative. One of the most commonly used gaseous sterilants is ethylene 25 oxide, which is used for medical product sterilization and for other sterilization processes. However, in certain instances, the presence of residual ethylene oxide is considered to be detrimental, even in small quantities, and accordingly improved methods of sterilization, particularly 30 for sterilization of medical products, have continued to be sought.

#### Summary of the Invention

It has now been found that sterilization of items, including biological tissue, replacement organs and 35 synthetic prosthetic materials, including polymers and metals, can be effectively carried out by treatment with a coupling agent known to create amide linkages between

amines and carboxylic acids which has been proven to be bactericidal. The sterilisation treatment may employ an optional coupling enhancer and is preferably carried out at a temperature above ambient in a buffered solution that contains isopropyl alcohol or an equivalent alcohol in an amount effective to achieve penetration of said coupling agent into the cells of the microorganisms. The 5 residuals from such treatment are nontoxic, biocompatible, and water-soluble, so that they can easily be washed off the tissue before implantation in a human body. It was surprisingly found that biological tissue which is sterilised in this manner exhibits enhanced resistance to calcification following implantation within a living body.

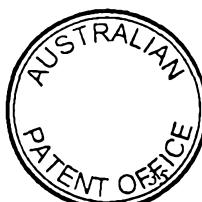
Thus, according to a first embodiment of the invention, there is provided a process for 10 sterilisation of material by effectively killing microorganisms carried by such material, which process comprises treating such material for an effective length of time with an aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds.

According to a second embodiment of the invention, there is provided a process for 15 sterilisation of material by effectively killing microorganisms carried by such material, which process comprises treating such material for an effective length of time with an aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds and an effective amount of an alkanol which achieves penetration of said coupling agent into the cells of microorganisms.

According to a third embodiment of the invention, there is provided an aqueous solution 20 containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds, when used for sterilisation of a material by treating said material with said aqueous solution for an effective length of time for effectively killing microorganisms carried by the material.

According to a fourth embodiment of the invention, there is provided a process for 25 sterilisation of biological tissue intended for implantation by effectively killing bacteria and spores, including *Bacillus subtilis*, carried by such tissue and for increasing the resistance to calcification of such tissue, which process comprises treating such tissue for at least 1 hour at about 35°C or higher with an aqueous solution containing at least about 25mM of a water-soluble carbodiimide coupling agent capable of creating amide bonds, which solution contains at least about 20 volume % of isopropanol.

According to a fifth embodiment of the invention, there is provided a process for 30 sterilisation of cross-linked biological tissue by effectively killing microorganisms carried by such tissue, which process comprises treating tissue that has previously been subjected to a chemical fixation by exposing for 4 hours or more at a temperature of at least 35°C with an aqueous solution containing a concentration of 40mM to 100mM of a water-soluble coupling agent capable of creating amide bonds.



### Detailed Description of the Preferred Embodiments

The term "coupling agent" is herein used to refer to a chemical reagent that facilitates the formation of amide bonds. Such bonds may be formed between reactive amines and reactive carboxyls on enzymes and proteins as well as with the reactive carboxyl or amine moieties located on and within bioprosthetic tissue. Those having skill in peptide synthesis and related arts will be familiar with such reagents, e.g. water-soluble carbodiimides and succinimides. When this coupling is carried out in the presence of a C<sub>2</sub> to C<sub>4</sub> alkanol, or other equivalent alcohol, sterilisation occurs, destroying bacteria and spores.

The coupling agent and the optional coupling enhancer are preferably water-soluble so the treatment can be effected in aqueous solution, particularly when biological tissue materials are being sterilised. The preferred coupling agent is 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide hydro-chloride (EDC); alternative suitable coupling agents include N-hydroxysuccinimide and other water-soluble carbodiimides. When biological tissue is being treated, a water-soluble coupling agent is preferably used. When EDC is used as the coupling agent, the preferred enhancer is N-hydroxysulfosuccinimide (sulfo-NHS), although other suitable enhancers, such as N-hydroxysuccinimide (NHS), can alternatively be used. The concentrations of the coupling



agent and of the enhancer (when employed) can be varied somewhat; however, appropriate concentrations are readily determinable by those of skill in the art. Although lower concentrations are effective if the concern is only with 5 certain categories of bacteria, the coupling agent is preferably used in a concentration between about 5 millimolar (mM) and about 100 mM, more preferably between about 15 mM and about 50 mM, and most preferably at between about 20 mM and about 40 mM, in order to be certain of 10 destroying all commonly encountered bacteria and spores. The optional enhancer is preferably employed at a concentration between 0.5 mM and about 30 mM, and more preferably at about 1 mM to about 5 mM.

All solutions used are preferably filtered through 15 0.45  $\mu\text{m}$  or smaller filters before use. The solution preferably contains at least about 10 volume % and more preferably contains between about 10 and about 30 volume % of a  $\text{C}_2$  to  $\text{C}_4$  alkanol or an equivalent alcohol; by volume % is meant volume of alcohol relative to volume of solution. 20 Other alcohols which might be employed include ethanol, propanol, 1-butanol, 2-butanol, tert-butyl alcohol, 1-pentanol, 2-pentanol and tert-pentyl alcohol. A lower alkanol is generally used in an amount which effectively assists the coupling agent in penetrating the cell walls of 25 the bacteria, spores or other microorganisms. Preferably, isopropanol is used in an amount equal to at least about 10g per 100 ml of solution; more preferably, the solution contains between about 15% and about 25% of isopropanol and most preferably contains about 20 volume % of isopropanol. 30 Of course, higher alcohol concentrations may be used so long as compatible with the material to be sterilized and may reduce the duration of treatment needed. The treatment not only effects sterilization without risk of damage to biological tissue but also may make some contribution to 35 fixation of certain tissue to be implanted; surprisingly, the treatment enhances the resistance of biological tissue to undergo calcification within a living body.

Reaction conditions for the sterilization may vary somewhat depending on the specific coupling agent employed. In general, the sterilization treatment is carried out in an aqueous buffer solution selected from among those that 5 are well known to those of ordinary skill in this art. Examples of suitable buffers include, but are not limited to, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES) and 3-(N-morpholino)propanesulfonic acid (MOPS), and the like.

10 The pH and concentration of the buffered solution also may vary, again depending upon the coupling agent employed. Preferably, the buffer concentration and pH are chosen to provide an effective sterilization environment while being the least harmful to bioprosthetic material or the like.

15 For example, with EDC as the coupling agent, with or without sulfo-NHS or NHS as an enhancer, the pH of the solution employed is about 6.0 to about 7.0. The temperature of the sterilizing solution may be maintained between about 25°C and 55°C, although higher temperatures 20 may be used so long as compatible with the materials being sterilized. Preferably, the sterilization is carried out between 35°C and 45°C for at least about 1 hour, preferably for at least about 5 hours and more preferably for at least about 12 hours. For polymeric or metallic materials, 25 temperatures higher than 55°C may be used so long as not harmful to the material being sterilized and may shorten duration of treatment.

Although the sterilization treatment method is considered useful for a wide variety of prosthetic and 30 bioprosthetic materials, it is considered to be particularly useful for sterilizing replacement organ components, such as heart valves, which have been made from animal tissue that has been suitably fixed. It may be desirable to rinse such material first with cold saline 35 prior to sterilization. Inasmuch as sterilization is usually a final step, tissue fixation is normally first carried out. Although glutaraldehyde or other fixation techniques, e.g. polyepoxide crosslinking or photo-

oxidation may be carried out, a fixation process of the types detailed in U.S. Patent No. 5,447,536 (September 5, 1995) and in Patent Application WO 95/22361, filed February 16, 1995, is preferably used.

5        The material being sterilized is usually maintained in contact with the sterilization solution for about 5 to 72 hours, and it is found that such treatment effectively inactivates even hard-to-kill bacteria and spores, thus proving to be potently bactericidal. Moreover, this  
10      sterilization treatment does not adversely affect bioprosthetic tissue, as by possibly lowering the shrinkage temperature of such sterilized material or by lowering its resistance to proteolytic degradation by collagenase or by proteases, and has surprisingly been found to increase  
15      resistance to calcification.

The present invention is further described by the examples that follow. These examples are not to be construed as limiting in any way either the spirit or the scope of the present invention.

20      Devices to be implanted in the human body are required to be sterilized in a manner to effectively destroy all microorganisms. Due to the unique applications of liquid chemicals for use in sterilization processes, it is necessary to be vigilant in detecting, screening and  
25      testing microorganisms which could pose significant resistance to the sterilization process. Examples of reference microorganisms which have previously demonstrated high resistance to liquid chemical sterilants are: the spores of *Bacillus subtilis*, *Clostridium sporogenes*, *Bacillus pumilus*,  
30      *Chaetomium globosum* and *Microascus cinereus* and representative vegetative cells, such as *Mycobacterium chelonae*, *Methylbactrium extorquens*, and *Trichosporon aquatile*. Of the foregoing, the most resistant may be the spores of *Bacillus subtilis*. The coupling agent used in the following examples is 1-ethyl-3(3-  
35      dimethyl aminopropyl) carbodiimide hydrochloride (EDC), and when used, the optional enhancer is either N-hydroxy

sulfosuccinimide (sulfo-NHS) or hydroxysuccinimide (NHS), which are all commercially available. Peptone water was prepared by dissolving 1g of Bacto Peptone in 1 liter of de-ionized water. The solution was then filtered into 5 sterile bottles using sterile 0.2 micron filters. All agents are solubilized in 10 mM HEPES buffer containing 0.85% of sodium chloride, pH 6.5 (HEPES buffer). Concentrations are expressed as mM (number of millimoles of chemical for each liter of solution), or as % (grams per 10 100 ml of solution). Temperatures are in °C (degrees Celsius), with room temperature being about 20-25°.

Porcine aortic roots are fixed by cross-linking according to the method described in U.S. Patent No. 5,447,536. After fixation, the valves are stored in 10 mM 15 HEPES, 0.85% NaCl, 20% isopropyl alcohol, pH 7.4, at 4°C. The sterility tests described in the following examples were in most cases conducted in the presence of 20 bioprosthetic heart valve tissue, but when such tissue was not present, the solutions were filtered through a 0.45 micron filter attached to a funnel (filter funnel). The filters were then rinsed with peptone water to eliminate residual chemicals on the membrane that may prevent growth of the organisms tested. The membrane filters were then incubated on TSA plates at about 32° to 33°C, e.g. 32.5°C. 25 When an aortic valve was inoculated with microorganisms for test purposes and then submitted to sterilization, the solution was filtered as described above. The aortic valve tissue was then washed for 20 minutes in a reciprocating shaker in the presence of peptone water containing Tween 80 30 in order to extract all indigenous spores or microorganisms from the tissue. This solution was filtered and then incubated as described above. All microbiological testing is performed in a biological laminar flow hood to prevent contamination. The shrinkage temperature and the 35 proteolytic (collagenase and protease) degradation tests are conducted as previously described in the '536 patent. Resistance to calcification is assessed by subdermal

implantation of sterilized leaflets and aortic wall coupons in young rats, as also described in the '536 U.S. patent.

Example 1

*Mycobacterium Cheloneae* ATCC 35752 (~10<sup>5</sup>) was inoculated in 5 sterile cups. A 10 mM HEPES, 0.85% NaCl, pH 6.5 solution containing 10 mM EDC and 1 mM Sulfo-NHS in the presence or absence of 20% isopropyl alcohol was maintained in contact with the bacteria in the cups for various periods of time (treatment duration). The cups were maintained at room 10 temperature, and the solutions were then filtered. The filters were then incubated at about 32-33°C for up to 6 weeks (incubation duration) using either Trypticase Soy Agar (TSA) plates or Trypticase Soy Broth (TSB). All inoculations were done in duplicate.

15

RESULTS

NO ISOPROPYL ALCOHOL

	Treatment Duration	Incubation Duration				
		1 Day	1 Week	3 Week	4 Week	6 Week
	0 hour	+,+	+,+	+,+	+,+	+,+
20	12 hours	+,+	+,+	+,+	+,+	+,+
	24 hours	-,-	+,+	+,+	+,+	+,+
	48 hours	-,-	-,-	-,-	-,+	-,+
	72 hours	-,-	-,-	-,-	-,-	-,+
		TSA, TSB	TSA, TSB	TSA, TSB	TSA, TSB	TSA, TSB

25 (+) Indicates growth while (-) indicates no growth.

20% ISOPROPYL ALCOHOL

	Treatment Duration	Incubation Duration				
		1 Day	1 Week	3 Week	4 Week	6 Week
	0 hour	-,-	-,-	+,+	+,+	+,+
30	12 hours	-,-	-,-	-,-	-,-	-,-
	24 hours	-,-	-,-	-,-	-,-	-,-
	48 hours	-,-	-,-	-,-	-,-	-,-
	72 hours	-,-	-,-	-,-	-,-	-,-
		TSA, TSB	TSA, TSB	TSA, TSB	TSA, TSB	TSA, TSB

35 (+) Indicates growth while (-) indicates no growth or complete kill.

The results indicate that, in the presence of 20% isopropyl alcohol, room temperature treatment with EDC plus Sulfo-NHS kills all *Mycobacterium chelonae* within 12 hours of sterilization treatment.

5

Example 2

A sterilization process similar to Example 1 is carried out using *Bacillus Subtilis* spores (~ 10<sup>6</sup>) inoculated in sterile cups some of which contain cross-linked heart valves that had been fixed by a process according to U.S.

10 Patent No. 5,447,536. A 10 mM HEPES, 0.85% NaCl, pH 6.5 solution containing 20 mM EDC and 1 mM Sulfo-NHS in the presence of 20% isopropyl alcohol was added for various periods of time (treatment duration). The cups and the heart valves were maintained at 40°C for the term of the  
 15 treatment. The solutions were then filtered and the filters incubated for up to 7 days (incubation duration) at about 32-33°C using Trypticase Soy Agar (TSA) plates. All inoculations were done in duplicate.

RESULTS

	Treatment Duration	Incubation	Duration
		1 Day	7 Days

WITHOUT VALVES

24 hours	-	-
48 hours	-	-
25 72 hours	-	-

WITH VALVES

48 hours	-	-
72 hours	-	-

(+) indicates growth, while (-) indicates no growth or  
 30 complete kill.

The results demonstrate that the spores of *Bacillus subtilis* are inactivated with this method of sterilization in the absence or presence of porcine aortic valve tissue.

Example 3

35 In the experiments described above, a coupling enhancer (Sulfo-NHS) was added to EDC during the sterilization process. The following experiment was

designed to test the efficacy of EDC in the presence or absence of an enhancer. A sterilization test process was carried out employing about  $5.7$  to  $6.6 \times 10^5$  *Bacillus subtilis* ATCC 9372 spores, inoculating them in sterile cups for 10 minutes. A solution of 10 mM HEPES, 0.85% NaCl, 20% isopropyl alcohol, pH 6.5 was then added to each of the cups, which solution contains 20 mM EDC and either 1 mM Sulfo-NHS or 1 mM NHS or no enhancer. Incubation was carried out for 72 hours (treatment duration) with the cups at about  $40^{\circ}\text{C}$ , and the solutions from the cups were then filtered. The filters were rinsed with a 0.1% peptone water solution to eliminate any residual EDC and/or enhancer and were then incubated on Trypticase Soy Agar (TSA) plates. All inoculations were done in duplicate, and the results are set forth in Table A.

Table A

		NUMBER OF COLONY FORMING UNITS (SURVIVORS)		
CONDITIONS	SAMPLES	ENHANCER		
		SULFO-NHS	NHS	NONE
CONTROLS	negative	1	0	0
		2	0	0
	positive	1	$5.7 \times 10^5$	$6.1 \times 10^5$
		2	$5.7 \times 10^5$	$6.5 \times 10^5$
TESTS	1	0	0	0
	2	0	0	0

The results demonstrate that EDC and isopropanol, either in the presence or absence of enhancer such as Sulfo-NHS or NHS, is a potent bactericide against the spores of *Bacillus subtilis*.

Example 4

The following experiment was designed to test the effect of EDC concentration, of temperature and of duration of incubation on the inactivation of spores of *Bacillus subtilis*.  
 30 The EDC concentrations tested were 5, 12.5 and 20 mM; the

temperatures were 25, 32.5 and 40°C for 4, 24 and 44 hours of incubation. The tests were carried out under the conditions as set forth in Table B. Spores of *Bacillus subtilis* (about  $2.5 \times 10^5$  per sample) were inoculated on tissue that 5 had been cross-linked using the fixation method described in the '536 patent. The spores were allowed to contact the tissue for 10 minutes, after which time 50 ml of a solution of EDC at the concentration indicated above were added to each cup containing the tissue plus the spores, and 10 incubation was carried out at their respective temperatures and for the respective lengths of time. After such incubation for either 4, 24 or 44 hours, the solutions were filtered to recover the spores, and these were incubated on TSA plates for 2 weeks at 32 to 33°C. The tissue samples 15 were washed with a solution containing a surfactant to fully extract the spores from the tissue, and the resulting solutions were filtered. After washing with 0.1% peptone water, the filters were incubated on TSA plates for 2 weeks at 32 to 33°C in order to determine the number of survivors 20 on or in the tissue. The colonies were enumerated for both the EDC solutions and the tissue wash solutions, and the results (numbers of colony forming units) were added. The positive and negative controls were shown to be valid for the test. The results are presented in Table B.

Table B

CONDITION	EDC (mM)	TEMP. °C	TIME (hrs)	SURVIVORS # CFUs	LOG REDUCTION
5	1	5	25	4	135000
	2	5	25	44	1300
	3	5	32.5	24	208
	4	5	40	4	1100
	5	5	40	44	21
	6	12.5	25	24	225
10	7	12.5	32.5	4	5900
	8	12.5	32.5	24	57
	9	12.5	32.5	24	34
	10	12.5	32.5	24	94
	11	12.5	32.5	24	44
	12	12.5	32.5	44	33
15	13	12.5	40	24	27
	14	20	25	4	31400
	15	20	25	44	16
	16	20	32.5	24	24
	17	20	40	4	5
	18	20	40	44	0
20					5.4

The results demonstrate that the bactericidal activity of EDC is dependent on the concentration of EDC, the temperature of incubation and the duration of incubation. In addition, it can be seen that EDC at 20 mM concentration kills all the spores on cross-linked tissue between 4 and 44 hours at 40°C. A logarithmic reduction of about 4.7 was obtained after 4 hours of incubation at 40°C, and no survivors were present after 44 hours of incubation. In addition, a logarithmic reduction of about 4 was achieved at 40°C after 44 hours of incubation in the presence of only 5 mM EDC, indicating that EDC is a potent bactericidal agent.

Example 5

An experiment was designed to test the bactericidal activity of the EDC treatment using two sequential inoculations of at least  $1 \times 10^6$  spores of *Bacillus subtilis* per sample. In the first step,  $1.43 \times 10^6$  spores ( $\text{Log}_{10}=6.2$ ) were inoculated in triplicate for 10 minutes onto 45 porcine valves which had been cross-linked using a method as described in the above-mentioned patent and patent application. Solutions of 25 mM EDC in 10 mM HEPES, 0.85% NaCl, 20% isopropyl alcohol, at pH 6.5 were poured into the cups containing the valve samples. After 2, 4, 6, and 8 hours of incubation at 40°C., the total surviving spores from 12 samples (solution plus tissue) were counted. After 8 hours of incubation, an additional  $1.2 \times 10^6$  ( $\text{Log}_{10}=6.1$ ) spores were added to the remaining 33 samples.

Summarizing, these remaining 33 samples were thus inoculated at  $t=0$  with  $1.43 \times 10^6$  spores and at  $t=8$  with  $1.2 \times 10^6$  spores. After various durations of incubation (see Table C for details), the surviving spores in solution and on the valve tissue were removed by filtering and incubated on TSA plates as described above. The positive and negative controls were determined to be valid for the test.

The results are presented in Table C which follows. The results demonstrate that a 6.2 log reduction (no survivors) of *Bacillus subtilis* spores is achieved within 6 hours of incubation with EDC in the presence of isopropanol and that another 6.1 log reduction is achieved within 6 hours after rechallenge. Complete sterilization of the tissue valve samples and the solution was achieved in this manner.

Table C

Incubation		Mean of Three Samples		
	Duration Hours @ 40°C	Total Survivors # CFUs	Total Survivors Log 10	LOG Reduction
5	0	1.43x10 <sup>6</sup>	6.2	0
	2	58	1.7	4.5
	4	4	0.5	5.6
	6	0	0	6.2
	8	0	0	6.2
10	8	1.2x10 <sup>6</sup>	6.1	n/a
	11	300	2.5	3.7
	14	0	0	6.1
	20	0	0	6.1
	24	0	0	6.1
15	28 to 56	0	0	6.1

Example 6

The foregoing experiments have generally shown that

20 the sterilization effect of EDC plus a lower alkanol could be equally demonstrated by testing biological tissue which had been inoculated with bacteria or by simply testing similar amounts of bacteria which have been inoculated into sterile cups. In view of the foregoing verification, it

25 was decided to test the effectiveness of the sterilization process against other bacteria using sterile cups. Added to each of the cups is 20 ml of 10 mM HEPES, 0.85% NaCl, 20% isopropanol, pH 6.5, containing 25 mM EDC without any coupling enhancer, before the cups are placed in an

30 incubator at 40°C. When the solution temperature reached about 38°C., approximately 10<sup>5</sup>-10<sup>6</sup> organisms were inoculated into the solution. The tests were carried out in triplicate. The following four isolates were tested in the form of sporous suspensions: *Clostridium sporogenes* ATCC 3584,

35 *Bacillus pumilus* ATCC 27142, *Chaetomium globosom* ATCC 6205, and

*Microascus cinereus* ATCC 16594. The following three isolates were tested as vegetative cells: *Mycobacterium chelonae* ATCC 35752, *Methylbacterium extorquens* ATCC 43645 and *Trichosporon aquatile* ATCC 22310. The inoculated systems were allowed to 5 incubate for 1 hour, for 5 hours or for 24 hours, and the solutions were then filtered through a 0.45 micron filter. After rinsing, the filter was placed on a TSA plate as described in Example 1. Negative and positive controls indicate that the tests were valid. The results are set 10 forth in Table D which follows wherein the number of CFUs in the inoculum is expressed as its log to the base 10, e.g.  $3.4 \times 10^5 = 5.5$ :

Table D

ORGANISM	INOCULUM Log 10	Log REDUCTION		
		1 Hour	5 Hours	24 Hours
<b>SPORES</b>				
<i>Clostridium sporogenes</i>	4.6	2.12	3.02	
<i>Bacillus pumilus</i>	5.45	3.68	4.41	
<i>Chaetomium globosum</i>				
<i>Micorascus cinereus</i>				
<b>VEGETATIVE CELLS</b>				
<i>Methylbacterium extorquens</i>				
<i>Trichosporon aquatile</i>	4.3	4.3	4.3	—
<i>Mycobacterium chelonae</i>	5.15	5.15	—	—

The results indicate that, after a single hour of 25 incubation, certain bacteria had been completely killed while others had illustrated a very substantial log reduction. The tests show that, against many forms of bacteria, EDC in the presence of isopropanol is a very good sterilant showing time-dependent inactivation of spores at 30 about 38°C.

Example 7

To determine any potentially adverse effect this sterilization may have on shrinkage temperature of the tissue, porcine aortic valves, which had been cross-linked

using the method described above in the pending WO 95/22361 patent application by treatment with EDC in the presence of either sulfo-NHS or NHS as a coupling enhancer, were sterilized using 25 mM EDC in 10 mM HEPES, 0.85% NaCl, 20% isopropyl alcohol, pH 6.5, at 40°C for 24 hours. This duration of incubation has been shown in Table C to twice achieve a logarithmic reduction of spores of *Bacillus subtilis* of about 6 in the sequential testing. The leaflets were dissected and the thermal denaturation temperature was determined for each as described in the '536 patent. The results are presented in Table E and demonstrate that this sterilization method has no adverse effect on the shrinkage temperature of the tissue regardless of which fixation process had been used.

15

Table E

SAMPLES	DENATURATION TEMPERATURE (°C)			
	NHS	STERILIZED	sulfo-NHS	sulfo-NHS STERILIZED
LEAFLETS	85.6 ± 0.2	85.0 ± 0.2	87.5 ± 0.3	87.0 ± 0.2

It can be seen from Table E that the shrinkage temperature does not change after sterilization.

20 To determine any effect this sterilization may have on susceptibility of the tissue to proteolytic degradation, porcine aortic valves similarly cross-linked using the method described in the pending patent application in the presence of either sulfo-NHS or NHS as a coupling enhancer 25 were sterilized, using 25 mM EDC in 10 mM HEPES, 0.85% NaCl, 20% isopropyl alcohol, pH 6.5, at 40°C for 24 hours. Aortic valve leaflets and aortic wall coupons were dissected, and they were then submitted to standard collagenase and protease degradation testing. Such testing 30 is described in detail in the previously mentioned patent and patent application. The results of collagenase digestion testing are expressed as nanomoles of amine released per mg of dry tissue and are presented in Table F.

Table F

SAMPLES	AMINES RELEASED (nmol/mg dry tissue)			
	NHS	NHS STERILIZED	sulfo- NHS	sulfo-NHS STERILIZED
LEAFLETS	12.0 ± 0.3	12.4 ± 1.2	14.5 ± 1.5	14.9 ± 1.3
AORTIC WALL	12.3 ± 0.6	10.9 ± 0.5	13.1 ± 0.8	10.0 ± 0.5

5        The results are presented as means ± SEM of six samples. There is no significant difference for the leaflets before and after sterilization,  $p = 0.718$  and  $p = 0.994$  for NHS and sulfo-NHS respectively. For the aortic wall coupons, there is a significant difference  
10      which indicates that they exhibit greater resistance to collagenase after sterilization, i.e.,  $p = 0.046$  and  $p = 0.0099$  for NHS and sulfo-NHS, respectively. Thus, not only is the resistance to collagenase digestion not adversely affected by this EDC sterilization, in some instances, it  
15      may be improved. For comparison, previous experiments conducted under similar conditions showed that the level of amines released from fresh tissue were approximately 2150 and 430 nanomoles/mg of tissue for leaflets and aortic wall coupons, respectively.

20      The results of protease digestion testing are presented in Table G; results for glutaraldehyde-fixed tissue are also shown for comparison purposes. They indicate that there is no significant difference as a result of this sterilization of tissue cross-linked  
25      according to the method described in the WO 95/22361 patent application when either NHS or sulfo-NHS is used as a coupling enhancer; the results obtained following the use of EDC for sterilization for either leaflets or aortic wall coupons show no adverse effect and a resistance equal to  
30      that of glutaraldehyde fixation.

Table G

SAMPLES	% ORIGINAL WEIGHT DIGESTED				
	GLUTARALDE-HYDE-FIXED	NHS	NHS STERILIZED	sulfo-NHS	sulfo-NHS STERILIZED
LEAFLETS	31.6 ± 5.7	28.1 ± 1.7	32.3 ± 1.3	29.5 ± 2.6	31.9 ± 2.2
AORTIC WALL	74.5 ± 1.2	73.8 ± 1.0	75.1 ± 0.9	75.1 ± 0.9	75.6 ± 2.2

5 To determine the effect this sterilization may have on resistance to calcification, samples of (a) glutaraldehyde-fixed porcine aortic valve leaflets and of (b) porcine aortic valve leaflets cross-linked according to the method of the '076 patent application, which were sterilized using  
10 EDC in the presence of isopropanol as described above for the shrinkage temperature experiment, were implanted subdermally in young rats for four weeks. The samples were then retrieved, and quantitative calcium analysis was conducted using Atomic Absorption Spectrophotometry. The  
15 results are presented as means ± SEM of six samples per condition in the following Table H.

Table H

SAMPLES	CALCIUM (mg/g dry sample)				
	Glutaraldehyde-fixed (n=4)	NHS before ster. (n=6)	NHS after ster. (n=6)	sulfo-NHS before ster. (n=6)	sulfo-NHS after ster. (n=6)
LEAFLETS	195±8.9	23.7±11.4	2.9±1.4	25.2±10.9	0.9±0.1
AORTIC WALL	66.8±4.5	53.2±4.7	35.4±6.1	54.2±1.5	43.6±3.5

20 The results demonstrate that the sterilization method using EDC at 25 mM in the presence of 20% isopropyl alcohol at 40°C has no adverse effect on the resistance of the porcine aortic valve tissue to calcification. Moreover, it surprisingly shows that the sterilized samples are more  
25 resistant to calcification than the samples that were not sterilized. In addition, all the samples cross-linked according to the above-identified patent application are significantly less calcified than samples that had been  
30 cross-linked according to the standard glutaraldehyde method.

The results indicate that a solution of EDC in the presence of 20% isopropyl alcohol at about 40°C, with or without NHS or sulfo-NHS, is a powerful bactericide against spores of *Bacillus Subtilis* and other bacteria. Vegetative cells 5 of *Mycobacterium Chelonae* were effectively inactivated at room temperature by EDC + Sulfo-NHS in the presence of 20% isopropyl alcohol, and subsequent tests with a variety of other vegetative cells showed that 25 mM EDC in 20% isopropanol is an effective sterilant for biological 10 tissue. It is believed that treatment with a coupling agent in the presence of isopropyl alcohol or an equivalent alkanol and at slightly elevated temperature, and optionally with a coupling enhancer, has a potent bactericidal effect and is excellently suited for treatment 15 of tissue valves, and is also considered suitable for sterilizing polymers, metals and the like. In addition, not only are the denaturation temperature and the resistance to proteolytic degradation of tissue valves not adversely affected by such sterilization treatment of 12 20 hours or more, but surprisingly, samples which have been sterilized using this process appear to be significantly more resistant to calcification, the leading cause of tissue valve failure. Compared to tissue fixed by the standard glutaraldehyde method, sterilization of heart 25 valve tissue using the present method results in an unexpected increase in calcification resistance which should be quite important commercially.

Although the invention has been described with regard to certain preferred embodiments which constitute the best 30 mode presently known by the inventors for carrying out the invention, it should be understood that changes and modifications that would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the 35 claims appended hereto. For example, although the invention has been described with regard to the

sterilization of porcine aortic valves and the like, it may also be used to sterilize polymeric or metal heart valve components or other components for implantation within the human body. Although the use of an alkanol, such as 5 isopropanol, is preferred, other organic solvents that are not deleterious to the material being sterilized may alternatively be used, e.g. toluene, and are considered to be equivalents.

**The claims defining the invention are as follows:**

1. A process for sterilisation of material by effectively killing microorganisms carried by such material, which process comprises treating such material for an effective length of time with an aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds.

2. The process for sterilisation according to claim 1, wherein the aqueous solution further comprises a coupling enhancer that enhances the amide-creating action of said coupling agent.

3. The process for sterilisation according to claim 2, wherein said coupling enhancer is NHS or sulfo-NHS.

4. The process for sterilisation according to any one of claims 1 to 3 wherein said treatment is carried out at a temperature of about 25° to about 55°C.

5. The process for sterilisation according to any one of claims 1 to 3, wherein said treatment is carried out at a temperature of about 35° to about 45°C.

6. The process for sterilisation according to any one of claims 1 to 5, wherein said treatment is carried out for at least about 1 hour.

7. The process for sterilisation according to any one of claims 1 to 5, wherein said treatment is carried out for at least about 5 hours.

8. The process for sterilisation according to any one of claims 1 to 7, wherein said coupling agent is present in said solution at a concentration of at least about 5 mM.

9. The process for sterilisation according to any one of claims 1 to 7, wherein said coupling agent is present in said solution at a concentration of at least about 20 mM.

10. The process for sterilisation according to any one of claims 1 to 9, wherein said coupling agent is a water-soluble carbodiimide.

11. The process for sterilisation according to any one of claims 1 to 9, wherein said coupling agent is EDC.

12. The process for sterilisation according to claim 11 wherein EDC is present at a concentration of at least about 20 mM.

13. The process for sterilisation according to claim 12 wherein said solution contains at least about 1 mM sulfo-NHS as a coupling enhancer.

14. The process for sterilisation according to claim 12 wherein said solution contains at least about 1 mM NHS as a coupling enhancer.

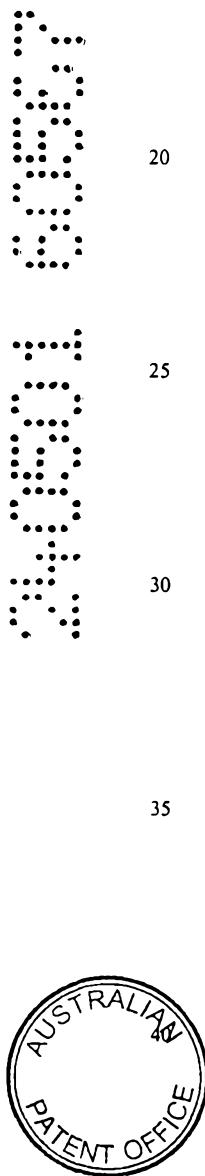
15. The process for sterilisation according to any one of claims 1 to 14, wherein the aqueous solution further comprises an effective amount of an alkanol which achieves penetration of said coupling agent into the cells of microorganisms.

16. The process for sterilisation according to claim 15, wherein said alkanol is a C<sub>2</sub> to C<sub>4</sub> alkanol and is included in the solution in an amount of at least about 10 volume percent.

17. The process for sterilisation according to claim 15 or claim 16, wherein said alkanol is isopropanol.

18. The process for sterilisation according to claim 15 wherein said solution contains at least 20 volume percent of isopropanol and said treatment is carried out at a temperature of between 35° and 45°C.

19. A process for sterilisation of material by effectively killing microorganisms carried by such material, which process comprises treating such material for an effective length of time with



an aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds and an effective amount of an alkanol which achieves penetration of said coupling agent into the cells of microorganisms.

20. The process for sterilisation according to claim 19, wherein the aqueous solution further comprises a coupling enhancer that enhances the amide-creating action of said coupling agent.

5 21. The process for sterilisation according to claim 20, wherein said coupling enhancer is NHS or sulfo-NHS.

10 22. The process for sterilisation according to any one of claims 19 to 21, wherein said treatment is carried out at a temperature of about 25° to about 55°C.

23. The process for sterilisation according to any one of claims 19 to 21, wherein said treatment is carried out at a temperature of about 35° to about 45°C.

15 24. The process for sterilisation according to any one of claims 19 to 23, wherein said treatment is carried out for at least about 1 hour.

25. The process for sterilisation according to any one of claims 19 to 23, wherein said treatment is carried out for at least about 5 hours.

26. The process for sterilisation according to any one of claims 19 to 25, wherein said coupling agent is present in said solution at a concentration of at least about 5 mM.

20 27. The process for sterilisation according to any one of claims 19 to 25, wherein said coupling agent is present in said solution at a concentration of at least about 20 mM.

28. The process for sterilisation according to any one of claims 19 to 27, wherein said coupling agent is a water-soluble carbodiimide.

29. The process for sterilisation according to any one of claims 19 to 27, wherein said coupling agent is EDC.

30 30. The process for sterilisation according to claim 29 wherein EDC is present at a concentration of at least about 20 mM.

31. The process for sterilisation according to claim 30 wherein said solution contains at least about 1 mM sulfo-NHS as a coupling enhancer.

32. The process for sterilisation according to claim 30 wherein said solution contains at least about 1 mM NHS as a coupling enhancer.

33. The process for sterilisation according to any one of claims 19 to 32, wherein said alkanol is a C<sub>2</sub> to C<sub>4</sub> alkanol and is included in the solution in an amount of at least about 10 volume percent.

34. The process for sterilisation according to claim 33, wherein said alkanol is isopropanol.

35 35. The process for sterilisation according to any one of claims 19 to 31, wherein said solution contains at least 20 volume percent of isopropanol and said treatment is carried out at a temperature of between 35° and 45°C.

36. An aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds, when used for sterilisation of a material by treating said material with said aqueous solution for an effective length of time for effectively killing microorganisms carried by the material.



37. The aqueous solution, when used for sterilisation according to claim 36, wherein the aqueous solution further comprises a coupling enhancer that enhances the amide-creating action of said coupling agent.

5 38. The aqueous solution, when used for sterilisation according to claim 37, wherein said coupling enhancer is NHS or sulfo-NHS.

39. The aqueous solution, when used for sterilisation according to any one of claims 36 to 38 wherein said treatment is carried out at a temperature of about 25° to about 55°C.

10 40. The aqueous solution, when used for sterilisation according to any one of claims 36 to 38, wherein said treatment is carried out at a temperature of about 35° to about 45°C.

10 41. The aqueous solution, when used for sterilisation according to any one of claims 36 to 40, wherein said treatment is carried out for at least about 1 hour.

42. The aqueous solution, when used for sterilisation according to any one of claims 36 to 40, wherein said treatment is carried out for at least about 5 hours.

15 43. The aqueous solution, when used for sterilisation according to any one of claims 36 to 42, wherein said coupling agent is present in said solution at a concentration of at least about 5 mM.

44. The aqueous solution, when used for sterilisation according to any one of claims 36 to 42, wherein said coupling agent is present in said solution at a concentration of at least about 20 mM.

20 45. The aqueous solution, when used for sterilisation according to any one of claims 36 to 44, wherein said coupling agent is a water-soluble carbodiimide.

46. The aqueous solution, when used for sterilisation according to any one of claims 36 to 44, wherein said coupling agent is EDC.

25 47. The aqueous solution, when used for sterilisation according to claim 46 wherein EDC is present at a concentration of at least about 20 mM.

48. The aqueous solution, when used for sterilisation according to claim 47 wherein said solution contains at least about 1 mM sulfo-NHS as a coupling enhancer.

30 49. The aqueous solution, when used for sterilisation according to claim 47 wherein said solution contains at least about 1 mM NHS as a coupling enhancer.

50. The aqueous solution, when used for sterilisation according to any one of claims 36 to 49, wherein the aqueous solution further comprises an alkanol in an amount which is effective to achieve penetration of said coupling agent into the cells of microorganisms.

35 51. The aqueous solution, when used for sterilisation according to claim 50, wherein said alkanol is a C<sub>2</sub> to C<sub>4</sub> alkanol and is included in the solution in an amount of at least about 10 volume percent.

52. The aqueous solution, when used for sterilisation according to claim 50 or claim 51, wherein said alkanol is isopropanol.

53. The aqueous solution, when used for sterilisation according to claim 50 wherein said solution contains at least 20 volume percent of isopropanol and said treatment is carried out at a temperature of between 35° and 45°C.

54. A process for sterilisation of biological tissue intended for implantation by effectively killing bacteria and spores, including *Bacillus subtilis*, carried by such tissue and for increasing the resistance to calcification of such tissue, which process comprises treating such tissue for at least 1



hour at about 35°C or higher with an aqueous solution containing at least about 25mM of a water-soluble carbodiimide coupling agent capable of creating amide bonds, which solution contains at least about 20 volume % of isopropanol.

5. 55. A process for sterilisation of cross-linked biological tissue by effectively killing microorganisms carried by such tissue, which process comprises treating tissue that has previously been subjected to a chemical fixation by exposing for 4 hours or more at a temperature of at least 35°C with an aqueous solution containing a concentration of 40mM to 100mM of a water-soluble coupling agent capable of creating amide bonds.

10. 56. The process for sterilisation according to claim 55 wherein said treatment is with an aqueous solution of EDC as said coupling agent at a concentration of at least 50mM.

15. 57. A process for sterilisation of material by effectively killing microorganisms carried by such material, which process comprises treating such material for an effective length of time with an aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds, said process being substantially as hereinbefore described with reference to any one of the examples.

20. 58. A process for sterilisation of material by effectively killing microorganisms carried by such material, which process comprises treating such material for an effective length of time with an aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds and an effective amount of an alkanol which achieves penetration of said coupling agent into the cells of microorganisms, said process being substantially as hereinbefore described with reference to any one of the examples.

25. 59. An aqueous solution containing an effective amount of a coupling agent capable of facilitating the creation of amide bonds, when used for sterilisation of a material by treating said material with said aqueous solution for an effective length of time for effectively killing microorganisms carried by the material substantially as hereinbefore described with reference to any one of the examples.

30. 60. A process for sterilisation of biological tissue intended for implantation by effectively killing bacteria and spores, including *Bacillus subtilis*, carried by such tissue, said process being substantially as hereinbefore described with reference to any one of the examples but excluding the comparative examples.

61. A process for sterilisation of cross-linked biological tissue by effectively killing microorganisms carried by such tissue, said process being substantially as hereinbefore described with reference to any one of the examples but excluding the comparative examples.

35. 62. Sterilised material obtained by a process according to any one of claims 1 to 35, 57 or 58.

63. Sterilised biological tissue obtained by a process according to any one of claims 54 to 56, 60 or 61.

**Dated 22 May 2001**

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