(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 December 2002 (05.12.2002)

PCT

(10) International Publication Number
WO 02/096476 A1

(51) International Patent Classification[7]: A61L 27/36, A01N 1/02

(21) International Application Number: PCT/GB02/02341

(22) International Filing Date: 20 May 2002 (20.05.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0112586.3 24 May 2001 (24.05.2001) GB

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF LEEDS** [GB/GB]; Leeds LS2 9JT (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FISHER, John** [GB/GB]; School of Mechanical Engineering, University of Leeds, Leeds LS2 9JT (GB). **BOOTH, Catherine** [GB/GB]; Department of Microbiology, University of Leeds, Leeds LS2 9JT (GB). **INGHAM, Eileen** [GB/GB]; Department of Microbiology, University of Leeds, Leeds LS2 9JT (GB).

(74) Agent: **HARRISON GODDARD FOOTE**; Belgrave Hall, Belgrave Street, Leeds LS2 8DD (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/096476 A1

(54) Title: DECELLULARISATION OF MATRICES

(57) Abstract: A method of preparing matrices or tissue engineered biomaterials for implantation, and in particular to a method of improving decellularisation of matrices or tissue engineered biomaterials prior to implantation. The method employs a single anionic detergent in combination with protease inhibitors.

Decellularisation of Matrices

The present invention relates to a method of preparing matrices or tissue engineered biomaterials for implantation, and in particular to a method of improving decellularisation of matrices or tissue engineered biomaterials prior to implantation.

Background to the Invention

A large variety of body implants are known for medical uses such as substitute vascular prostheses, skin dressings and coverings, and for other purposes. The implant material can be synthetic or body tissues from the same species or other species as the species to be implanted. When body tissues and structures are to be implanted, they may be used fresh from the donor but in many cases, it is preferred to have some means of preserving the implant tissue for later use. There are several preservation techniques currently available including cryopreservation and chemical fixation with cross-linking agents such as glutaraldehyde, polyglycidyl ether and carbodiimide. In order to prepare the implant tissue for later use it is desirable to decellularise the tissue prior to storage whilst minimising any damage to the physical structure of the tissue matrix itself. This decellularisation can be important in improving the biocompatability and reducing the immunological reaction in the tissue graft.

It is known from the prior art to use anionic detergents such as sodium dodecyl sulphate (SDS) for the extraction of cellular components. SDS extraction was first described in US4323358 as a method of preventing or delaying the calcification of glutaraldehyde-fixed Hancock heart valve bioprosthesis, the method is referred to as the "Hancock T6 treatment". In this method, fixed tissue is contacted with SDS so as to retard calcification. However, serious limitations of the method have been reported (Bodnar E et al, *Thorac. Cardiovasc. Surgeon.*1985 **34:** 82-85; Courtman D W et al, *J Biomed Mater Res.* 1994 **28**: 655-666; Wilson G J et al. *ASAIO Trans* 1990 36: M340-343). These researchers all report that SDS has a deleterious effect on

heart valve extracellular matrix (ECM) and in particular on the collagen and elastin fibre components.

In order to mitigate the effects of SDS on ECM, US4776853 describes the use of an earlier pre-treatment with other non-ionic detergents, such as Triton X-100 so that SDS is only employed as the second detergent in a multistage detergent decellularisation program.

A further problem associated with decellularising tissue implant is to minimise the degradation to the ECM during the process. It is known to use protease inhibitors to prevent such degradation during incubation with a non-ionic detergent in the first stage of the multistage detergent decellularisation program and also to use them to prevent naturally occurring proteases from attacking collagens. There are a number of different proteases that reside within the tissue matrix, either in direct association with the cells themselves or bound within the ECM. One of the largest of the protease families, the matrix metalloproteases (MMPs), has a wide range of substrate specificities including collagen, laminin, fibronectin and elastin. Another important family of matrix-degrading proteases are the plasminogen activators, which generates the broad specificity protease plasmin from the abundant zymogen plasminogen. As well as proteolytic activity, plasmin has the further ability of activating members of the MMP family. However, most of the protease inhibitors are inherently toxic which is undesirable if the matrix is to be seeded with living cells and implanted into a human or animal. Moreover, some of the protease inhibitors used so far, for example PMSF, are extremely unstable in solution having a half life of less than 1 hour, and since decellularisation is a lengthy process i.e. several days, this limits the choice of inhibitors that have sufficient half lives.

A method which could simplify the decellularisation process whilst minimising damage to ECM would offer significant advantage over current practices.

Statement of the Invention

In its broadest aspect, the present invention provides a method of decellularising a tissue matrix using an anionic detergent at a concentration sufficient to effect decellularisation but at a concentration which maintains the histoarchitecture of the ECM, the sole anionic detergent being used in conjunction with protease inhibitors.

According to a first aspect of the invention there is provided a method of preparing biological material for implantation comprising the steps of:

(i) incubating the biological material with a buffer solution at a mild alkaline pH which includes active amounts of a proteolytic inhibitor;

(ii) incubating the biological material with an anionic detergent at a mild alkaline pH at a concentration which is sufficient to effect decellularisation but which maintains the histoarchitecture of the biological material ;

(iii) washing the biological material with a buffer solution at a mild alkaline pH both with and without active amounts of proteolytic inhibitors;

(iv) incubating the biological material with one or more enzymes selected from the group comprising DNase Type I, DNase Type II, and/or Rnase and optionally;

(v) placing the biological material in a cryoprotectant medium.

Preferably the method does not include any additional detergent incubation steps.

Reference herein to decellularisation is intended to include the removal of cellular membranes, nucleic acids, lipids, cytoplasmic components and retaining an ECM having as major components collagens and elastins.

Preferably, the buffer solution is hypotonic or isotonic. It will be appreciated that each may be used either as the sole buffer or in combination at different stages of the

method and that use of hypotonic or isotonic buffer is not intended to limit the scope of the present application.

The method may include the further step of cryopreserving the biological material in a cryogen such as liquid nitrogen until required.

Preferably, the proteolytic inhibitors are ethylene diamine tetraacetic acid (EDTA) and Aprotinin.

We have found Aprotinin particularly effective as a proteolytic inhibitor and of particular utility because of its low toxicity, stability in solution at different pHs and stability at a variety of different temperatures.

Typically, EDTA is used at a concentration range of 1 to 100 mM or 0.01-1.0 % (w/v) and typically at 10mM or 0.1% and Aprotinin at a concentration range of 1-100 KIU and typically at 10KIU.

Preferably, the mild alkaline conditions of step (i) are in the range of pH above 7.0 and up to pH 10.0, and more preferably are at pH 8.0.

Preferably, the incubation period of step (i) of the method is for between 8 to 20 hours and more preferably is for 14 hours.

Preferably, the anionic detergent is sodium dodecyl sulphate (SDS) or sodium deoxycholate.

Preferably, SDS is used at a concentration equal to or below 0.1% (w/v), and equal to or above 0.03% (w/v).

Reference herein to the term % (w/v) refers to the percentage in weight (grams) per unit volume (100 ml), thus 0.1% w/v is equivalent to 0.1 gm dissolved in 100 ml.

Prior art methods in which SDS has been suggested for decellularisation use concentrations of SDS equal to or greater than 1% (w/v) in order to effect decelluarisation. We have found that using anionic detergents at this concentration results in destabilisation of protein interactions and/or solubilisation thus leading to degradation of ECM proteins. It has been the prevailing wisdom that SDS would not be effective below a concentration of 1% (w/v). However, our results have shown surprisingly, that a concentration of 0.1% or below it is effective for decellularisation when conducted in the presence of protease inhibitors and that there is no damage to the ECM.

Our further studies using a second non-ionic detergent such as Triton X-100, n-hexyl-β-D-glucopyranoside, TWEEN 20 and MEGA 10 and the zwitterionic detergent CHAPS showed no effect on the decellularisation produced by a low concentration of SDS alone. The results showed that use of a second detergent had no significant effect on decellularisation of porcine heart valves even after a period of 72 hrs. Thus, the present invention is of particular advantage in that we have shown that there is no requirement for a second non-ionic detergent. Accordingly, the present invention has obviated the need for a multistage detergent process.

Preferably, sodium deoxycholate is used at a concentration equal to or below 2.0% (w/v) and equal to or above 0.5% (w/v).

Preferably, the incubation period of step (ii) of the method is for between 20 to 28 hours and more preferably is for 24 hours.

Preferably, the mild alkaline conditions of step (ii) are in the range of pH above 7.0 and up to pH 10.0, and more preferably are at pH 8.0.

Preferably, the washing step (iii) of the method involves multiple washes, typically X3 washes with tris buffered saline (preferably 0.15M NaCl, 0.05M tris in distilled water) containing protease inhibitors (0.1% EDTA and 10KIU/ml Aprotinin), and

further, multiple washes, typically X3 washes with tris buffered saline without the protease inhibitors.

Preferably, the mild alkaline conditions of step (iii) are in the range of pH above 7.0 and up to pH 10.0, and more preferably are at pH 8.0.

Preferably, the incubation step (iv) of the method is for 4-6 hours at 37°C.

The DNase Type I, DNase Type II or Rnase are employed as low ionic strength solutions in an amount effective so as to eliminate nucleic acids and provide a tissue matrix of limited immunogenicity. Accordingly any other agents which are capable of the same function are included within the scope of the present invention.

Preferably, DNAse I is used at a concentration range of 5.0-100 μg/ml and typically at 20μg/ml and RNAse A at a concentration range of 0.1-10 μg/ml and typically at 1μg/ml.

Preferably, the biological tissue is prepared in step (v) of the method for storage by placement in a cryoprotectant, such as and without limitation, Dulbecco's modified eagles medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS) and 10% (v/v) dimethyl sulphoxide (DMSO).

According to a further aspect of the invention there is provided a tissue implant comprising ECM from which have been removed cellular membranes, nucleic acids and other cytoplasmic components the tissue implant having been produced by the method of the present invention.

In summary, preferred embodiments of the present invention provides a method of decellularisation of a tissue matrix which results in no significant deleterious effects to the ECM proteins and subsequent histoarchitecture of the aortic valve, as assessed by both histological and biomechanical techniques and wherein a single anionic

detergent such as SDS or sodium deoxycholate is used at a concentration sufficient to cause decellularisation;

- as a single stage detergent;
- at low concentrations that effect decellularisation whilst maintaining the ECM in good condition;
- in combination with the protease inhibitors EDTA (as an inhibitor of MMPs) and Aprotinin (inhibitor of serine family of plasminogen activators);
- for a period of about 24 hours;

According to a yet further aspect of the invention there is provided a product comprising a combination of an anionic detergent at a concentration hereinbefore described and a proteolytic inhibitor as hereinbefore described and optionally including a set of instructions for use thereof for use in the method of the present invention.

Alternatively the product comprising may comprise concentrates for dilution and use in a method.

The present invention will now be described by way of example only with reference to the following Figures wherein:

Figure 1A illustrates a cross section of heart valve leaflet treated with 0.05% SDS solution at x 10 magnification;

Figure 1B illustrates Figure 1A at x 40 magnification;

Figure 2A illustrates a cross section of heart valve leaflet treated with 0.02% SDS solution at x 10 magnification;

Figure 2B illustrates Figure 2A at x 40 magnification;

Figure 3A illustrates a photomicrograph at x400 magnification of fresh porcine patellar tendon stained with heamatoxylin and eosin and;

Figure 3B illustrates a photomicrograph at x400 magnification of porcine patellar tendon following the de-cellularisation treatment according to the present invention.

Detailed Description of the Invention

With reference to Figures 1A and 1B there is shown a cross section of a heart valve leaflet treated with concentrations of 0.03% SDS. Total leaflet decellularisation was observed at this concentration. However, at concentrations below 0.05% (Figures 2A and 2B), whole cells or cell fragments were found to have been retained by the matrix. Cross section of a heart valve leaflet treated with 0.02% SDS is therefore a concentration below which decellularisation does not occur. It can be seen that cell fragments and whole cells have been retained within the matrix (Blue/black pigment). Following the method of the present invention, patella tendons may also be successfully decellularised (Figure 3B).

Example 1:- Porcine Aortic Valves

Porcine hearts were procured from a local abattoir within 2 hours of slaughter and transported on ice to the laboratory. On arrival at the laboratory, aortic valve roots were dissected from the heart and washed in transport solution [Hanks' balanced salt solution (HBSS), 10KIU/ml Aprotinin, 10u/ml penicillin, 100µg/ml streptomycin, 100U/ml Nystatin, 10mM HEPES pH7.6). The aortic valves were incubated overnight (14 hours) in hypotonic tris buffer (10mM tris pH8, 0.1% (w/v) ethylene diamine tetraacetic acid (EDTA), 10KIU Aprotinin in distilled water DW).

Subsequently, the aortic valves were incubated for 24 hours with shaking at ambient temperature in (0.05%-0.1%) (w/v) sodium dodecyl sulphate (SDS) or 0.5% sodium deoxycholate in hypotonic tris buffer. They were then washed (X3) with tris buffered

saline (0.15M NaCl, 0.05M tris pH 7.6 in DW) containing protease inhibitors (0.1% w/v EDTA and 10KIU/ml Aprotinin). They were then subjected to a further wash (X3) with tris buffered saline (TBS) without protease inhibitors.

The aortic valves were then incubated for 4-6 hours at 37°C with DNAse I (20μg/ml) and RNAse A (1μg/ml). After this they were washed (X3) in TBS containing protease inhibitors. Finally, in preparation for storage they were placed in cryoprotectant [Dulbecco modified eagles medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS) and 10% (v/v) dimethyl sulphoxide (DMSO) and cryopreserved in liquid nitrogen until they were required for implantation.

Example 2:- Porcine Patella Tendons

Porcine patella tendons were dissected and then washed in PBS. The tendons were incubated overnight (24 hours) in hypotonic Tris buffer (10mM Tris pH 8, 0.1% ethylene diamine tetraacetate (EDTA), 10KIU Aprotinin in distilled water (DW)]. Tendons were subsequently incubated for a further 24 hours with shaking at ambient temperature in 0.03 – 0.1% w/v sodium dodecyl sulphate (SDS) or 0.5% sodium deoxycholate in hypotonic Tris buffer. They were then washed (X3) with PBS containing protease inhibitors (0.1% EDTA and 10KIU/ml Aprotinin).

With reference to Figure 3A there is shown a photomicrograph of fresh porcine patella tendon, stained with heamatoxylin and eosin (x400). Figure 3B shows a photomicrograph of porcine patellar tendon following de-cellularisation treatment as described above and also stained with heamatoxylin and eosin (x400). It is apparent from comparing the Figures that decellularisation has been achieved whilst maintaining the histoarchitecture of the material.

CLAIMS

1. A method of preparing biological material for implantation comprising the steps of:

(i) incubating the biological material with a buffer solution at a mild alkaline pH which includes active amounts of a proteolytic inhibitor;

(ii) incubating the biological material with an anionic detergent at a mild alkaline pH at a concentration which is sufficient to effect decellularisation but which maintains the histoarchitecture of the biological material ;

(iii) washing the biological material with a buffer solution at a mild alkaline pH both with and without active amounts of proteolytic inhibitors;

(iv) incubating the biological material with one or more enzymes selected from the group comprising DNase Type I, DNase Type II, and/or Rnase and optionally;

(v) placing the biological material in a cryoprotectant medium.

2. A method according to claim 1 which does not include any additional detergent incubation steps.

3. A method according to either preceding claim wherein the buffer is hypotonic and/or isotonic.

4. A method according to either preceding claim further including the step of cryopreserving the biological material in a cryogen such as liquid nitrogen until required.

5. A method according to any preceding claim wherein the proteolytic inhibitor is ethylene diamine tetraacetic acid (EDTA) and Aprotinin.

6. A method according to claim 5 wherein the EDTA is used at a concentration in the region of of 1 to 100 mM or 0.01-1.0 % (w/v).

7. A method according to either claim 5 or 6 wherein EDTA is used at a concentration of 10mM or 0.1% (w/v).

8. A method according to claim 5 wherein Aprotinin is used at a concentration range of 1-100 KIU.

9. A method according to either claim 5 or 8 wherein Aprotinin is used at 10 KIU.

10. A method according to any preceding claim wherein the mild alkaline conditions of step (i) are in the pH range of above 7.0 and up to pH 10.0.

11. A method according to claim 10 wherein the pH is 8.0.

12. A method according to any preceding claim wherein the incubation period of step (i) is for between 8 to 20 hours.

13. A method according to claim 12 wherein the incubation period is 14 hours.

14. A method according to any preceding claim wherein the anionic detergent is sodium dodecyl sulphate (SDS) or sodium deoxycholate.

15. A method according to claim 14 wherein SDS is used at a concentration equal to or below 0.1% (w/v) and equal to or above 0.03% (w/v).

16. A method according to claim 14 wherein sodium deoxycholate is used at a concentration equal to or below 2.0% (w/v) 0.5% (w/v) and equal to or above 0.5% (w/v).

17. A method according to any preceding claim wherein the incubation period of step (ii) is between 20 to 28 hours.

18. A method according to claim 17 wherein the incubation period is 24 hours.

19. A method according to any preceding claim wherein the alkaline conditions of step (ii) are in the region of are in the pH range of above 7.0 and up to pH 10.0.

20. A method according to claim 19 wherein the pH is 8.0.

21. A method according to any preceding claim wherein the washing step (iii) comprises multiple washes with tris buffered saline containing protease inhibitors and multiple washes with tris buffered saline without the protease inhibitors.

22. A method according to claim 21 wherein the buffer is 0.15M NaCl, 0.05M tris in distilled water with or without EDTA and Aprotinin.

23. A method according to any preceding claim wherein the alkaline conditions of step (iii) are in the region of are in the pH range of above 7.0 and up to pH 10.0.

24. A method according to claim 23 wherein the pH is 8.0.

25. A method according to any preceding claim wherein the incubation of step (iv) is for 4-6 hours at 37°C.

26. A method according to any preceding claim wherein DNAse I is used at a concentration in the range of 5-100 μg/ml and RNAse A at a concentration in the range of 0.1-10 μg/ml.

27. A method according to any preceding claim wherein the biological tissue is prepared in step (v) for storage by placement in a cryoprotectant comprising Dulbecco's modified eagles medium (DMEM) containing between 10-20% (v/v) foetal bovine serum (FBS) and 5-15% (v/v) dimethyl sulphoxide (DMSO).

28. A method of decellularising a tissue matrix using an anionic detergent at a concentration sufficient to effect decellularisation but at a concentration which maintains the histoarchitecture of the ECM, the anionic detergent being used in conjunction with EDTA and Aprotinin protease inhibitors.

29. A method according to claim 28 further including any one or more of the features of claims 2 to 27.

30. A tissue implant comprising ECM from which has been removed cellular membranes, nucleic acids and other cytoplasmic components the tissue implant having been produced by the method according to any preceding claim.

31. A product comprising a combination of an anionic detergent, optionally as defined by any one of claims 14 to 16, and a proteolytic inhibitor, optionally as defined in any one of claims 5 to 9 and optionally including instructions for use thereof to prepare a method of any one of claims 1 to 29.

32. A product comprising concentrates for dilution and use in a method of any one of claims 1 to 29 comprising an SDS or sodium deoxycholate concentrate and a proteolytic inhibitor concentrate of which the active ingredients are EDTA and Aprotinin, the SDS or sodium deoxycholate and EDTA and Aprotinin being suitable for dilution into the relative amounts defined in any one of claims 13 to 16 or 6 to 9.

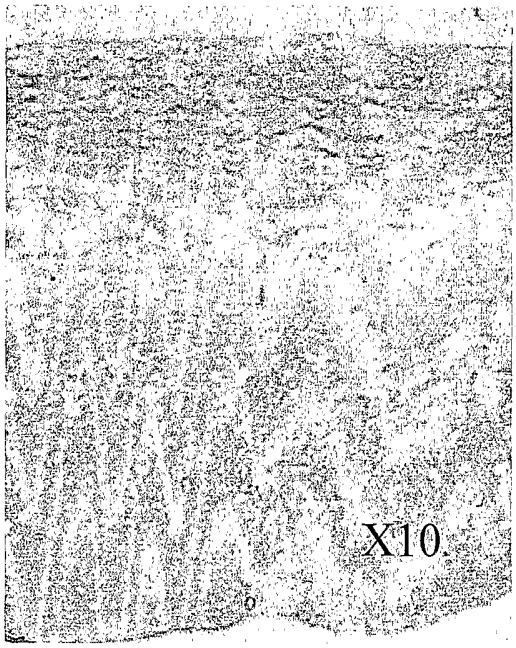


Fig1A

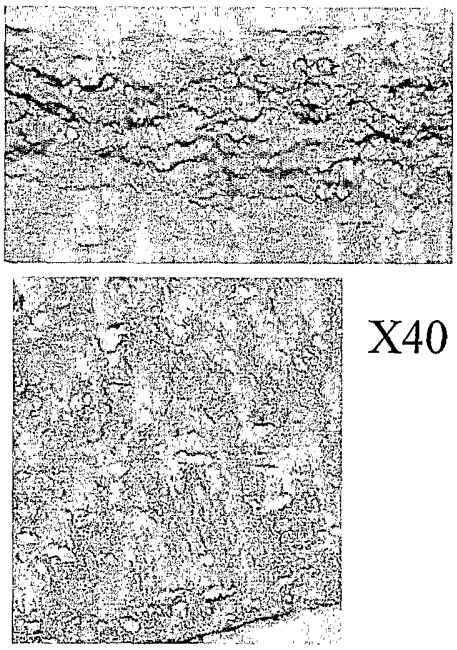


Fig1B

Fig 2A

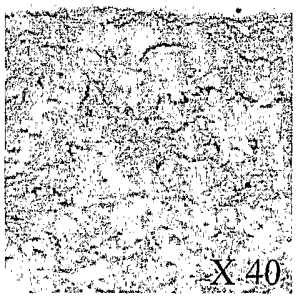


Fig 2B.

Figure 3A

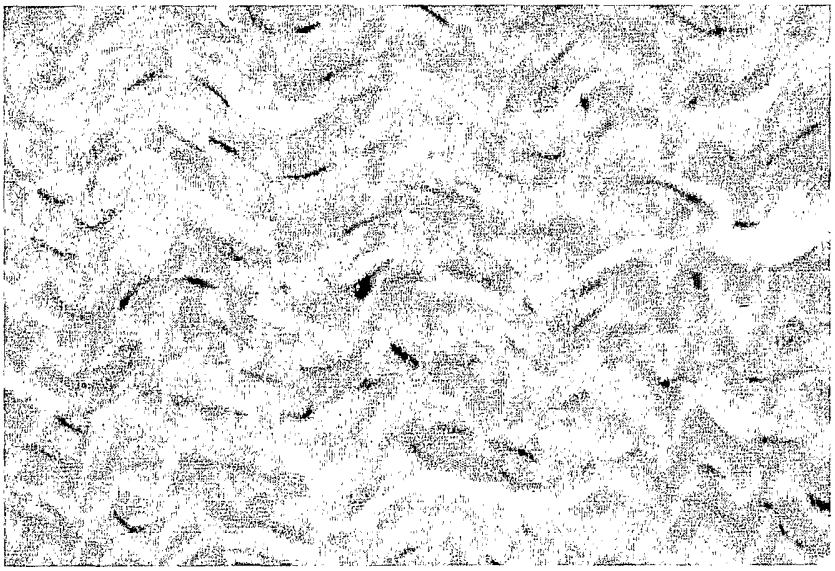
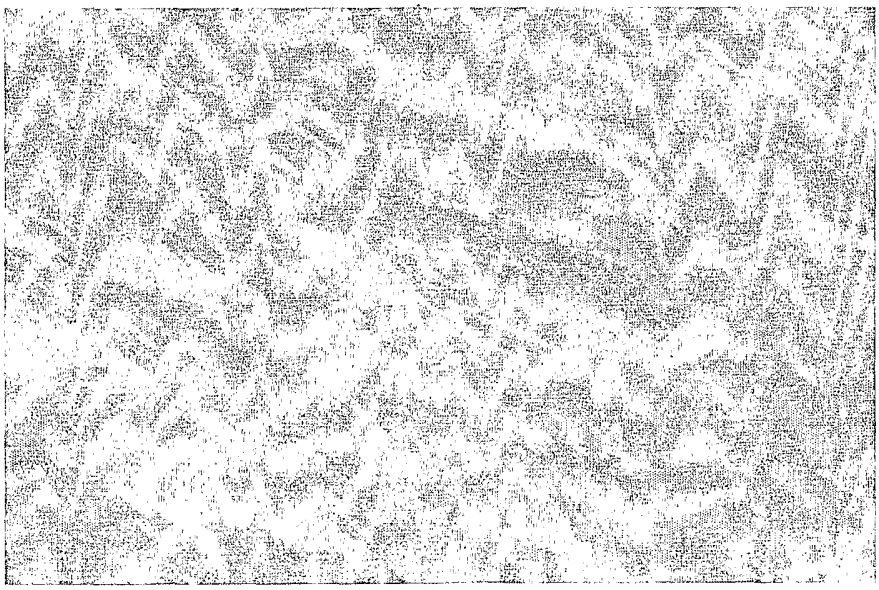


Figure 3B



INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/GB 02/02341

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L27/36 A01N1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, BIOSIS, EPO-Internal, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 4 776 853 A (KLEMENT PETR ET AL) 11 October 1988 (1988-10-11) cited in the application example 1 claims	30 28
X	WO 99 41981 A (BOERBOOM LAWRENCE E ;COLEMAN CHRISTOPHER L (US); LIVESEY STEPHEN A) 26 August 1999 (1999-08-26) page 4, line 13 -page 5, line 20 page 6, line 1 - line 6 claims	30
X	US 6 147 080 A (SALITURO FRANCESCO GERALD ET AL) 14 November 2000 (2000-11-14) column 44, line 22 - line 59	31,32

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2002	24/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fey-Lamprecht, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 100 064 A (BURKE RAE LYN ET AL) 8 August 2000 (2000-08-08) column 22, line 56 - line 61	31,32
X	US 6 194 153 B1 (ROMMENS JOHANNA M ET AL) 27 February 2001 (2001-02-27) column 57, line 53 - line 58	31,32
Y	EP 0 564 786 A (LIFECELL CORP) 13 October 1993 (1993-10-13) page 7, line 28 -page 8, line 9	28
P,A	WO 02 14480 A (UNIV DUKE ;KOH JENNIFER (US); PRABHAKAR VIKAS (US); MITCHELL SHANN) 21 February 2002 (2002-02-21) page 29, line 23 -page 31, line 20	1-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/02341

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4776853 A	11-10-1988	CA 1261290 A1	26-09-1989
WO 9941981 A	26-08-1999	AU 2775399 A	06-09-1999
		CA 2320887 A1	26-08-1999
		EP 1056335 A1	06-12-2000
		JP 2002503678 T	05-02-2002
		WO 9941981 A1	26-08-1999
US 6147080 A	14-11-2000	US 5945418 A	31-08-1999
		AU 738000 B2	06-09-2001
		AU 5610598 A	15-07-1998
		BG 103575 A	30-06-2000
		BR 9714415 A	18-04-2000
		CN 1244867 A	16-02-2000
		CZ 9902163 A3	15-09-1999
		EE 9900252 A	15-12-1999
		EP 0951467 A1	27-10-1999
		HU 0001125 A2	28-10-2000
		JP 2001506266 T	15-05-2001
		NO 992960 A	17-08-1999
		NZ 336146 A	29-09-2000
		PL 334133 A1	14-02-2000
		SK 80599 A3	18-01-2000
		TR 9902194 T2	21-06-2000
		WO 9827098 A1	25-06-1998
US 6100064 A	08-08-2000	US 5244792 A	14-09-1993
		AU 3103195 A	04-03-1996
		CA 2196097 A1	15-02-1996
		EP 0802979 A1	29-10-1997
		WO 9604382 A2	15-02-1996
		US 6190860 B1	20-02-2001
		US 5834307 A	10-11-1998
		AU 641121 B2	16-09-1993
		AU 3041389 A	25-08-1989
		DK 179290 A	28-09-1990
		EP 0436537 A1	17-07-1991
		EP 0609580 A1	10-08-1994
		JP 9176190 A	08-07-1997
		JP 2607712 B2	07-05-1997
		JP 3503478 T	08-08-1991
		WO 8907143 A1	10-08-1989
		US 5547834 A	20-08-1996
		US 5648079 A	15-07-1997
		US 5759814 A	02-06-1998
		AT 118353 T	15-03-1995
		CA 1337181 A1	03-10-1995
		DE 3587991 D1	23-03-1995
		EP 0175787 A1	02-04-1986
		EP 0628633 A1	14-12-1994
		HK 107595 A	14-07-1995
		WO 8504587 A1	24-10-1985
		US 5747039 A	05-05-1998
		US 5171568 A	15-12-1992
		US 5750114 A	12-05-1998
US 6194153 B1	27-02-2001	US 5986054 A	16-11-1999
		US 6210919 B1	03-04-2001

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/GB 02/02341

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6194153 B1		AU 732508 B2	26-04-2001
		AU 1299297 A	20-08-1997
		WO 9727296 A1	31-07-1997
		EP 0876483 A1	11-11-1998
		JP 2000506375 T	30-05-2000
		US 6395960 B1	28-05-2002
		US 6117978 A	12-09-2000
		US 6020143 A	01-02-2000
		US 5840540 A	24-11-1998
		AU 716307 B2	24-02-2000
		AU 5394096 A	18-11-1996
		BR 9608140 A	07-12-1999
		CA 2219214 A1	31-10-1996
		WO 9634099 A2	31-10-1996
		CZ 9703390 A3	18-02-1998
		EP 0826042 A2	04-03-1998
		HU 9801639 A2	28-10-1998
		JP 11504214 T	20-04-1999
		NO 974964 A	29-12-1997
		NZ 305822 A	25-08-2000
		NZ 335945 A	29-09-2000
		PL 323109 A1	16-03-1998
		SK 145397 A3	09-09-1998
EP 0564786 A	13-10-1993	US 5336616 A	09-08-1994
		AU 668703 B2	16-05-1996
		AU 3293493 A	19-08-1993
		CA 2089336 A1	13-08-1993
		EP 0564786 A2	13-10-1993
		JP 6261933 A	20-09-1994
WO 0214480 A	21-02-2002	AU 8496801 A	25-02-2002
		WO 0214480 A2	21-02-2002
		US 2002115208 A1	22-08-2002