FIELD OF THE INVENTION:

This invention relates to a process for the production of remodelable decellularised animal tissue.

This invention further relates to the method of production of remodelable decellularised animal tissue such as and not limited to bovine pericardium, bovine jugular vein or bovine saphenous vein with covalently linked heparin using a single step process for providing antithrombotic surface for better performance during *in vivo* blood contact applications.

Decellularised tissues produced by this method have obvious advantages in blood contact applications such as cardiac patch, vascular conduits, cardiac valves or cardiac valve sewing rings because of remodelable nature of the decellularised matrix as well as its thrombo-resistant nature.

BACKGROUND OF THE INVENTION:

Decellularised tissue is mostly a collagenous material and hence it may invariably activate platelets through the interaction between glycoprotein la/2a of platelets and RGD peptide sequence in the collagen. The fibrin forming effect of increased local concentration of coagulation factors released from activated platelets will be attenuated by surface bound heparin by way of binding with anti-thrombin, thereby inhibiting thrombin formation and resultant thrombus formation on decellularised tissue surface. In addition to this, heparin is known to bind growth factors such as VEGF, FGF, thereby increasing the local concentration of these factors in the decellularised tissue thereby inducing a site appropriate remodeling process. Hence an heparin grafted decellularised

tissue will perform better in the short term by having a thrombo-resistant surface as well as in the long term by having a site appropriate positive remodeling response in a blood contacting application such as vascular conduit, cardiac patches, heart valve leaflet or for heart valve sewing ring applications. Positive remodeling may accrue a growth potential to the above decellularised tissue resulting in structural and physiological integration of the decellularised tissue to the host organ, which is of paramount importance in pediatric patients.

Indian Patent No. 191945 discloses a process for preparing heparin-immobilized pericardium is disclosed. The method involves subjecting bovine pericardium to decellularisation by treatment with detergent and heparin, to obtain decellularized bovine pericardium; cross-linking the decellularized pericardium with glutaraldehyde for 5-24 hours followed by polyethylene glycol (PEG) grafting, to obtain the heparin-immobilized pericardium. The method involves extensive crosslinking with glutaraldehyde thereby rendering it resistant matrix metalloproteinase in the tissue, thereby rendering it resistant to any remodeling process in *in vivo*. *In vivo* efficacy of this process is not available.

According to Patent Number(s) WO2009044407-A1 & IN200702222-14, the process involves cleansing harvested vein grafts in balanced salt solution with antifungal agent followed by treatment of vein grafts for 20 to 50 hours in a solution of deoxycholic acid in aqueous solvent of suitable pH along with ribonucleic enzyme treatment. The collagen matrix is reorganized with heparin in glucose solution. Photooxidation physico chemical is cross linked with methylene blue and UV radiation for specific duration, where the cross linking is effected in 0.01 to 10

percentage range of methylene blue. The process uses a detergent based decellularisation process and does covalently link heparin to the decellularised tissue thereby reducing the duration of available heparin, thus nullifying all the benefits that may accrue on account of heparin grafting. The ability of the decellularised tissue to undergo *in vivo* remodeling is also not mentioned.

Implantable xenograft prepared from a non-human tissue portion' according to US patent application no. 13/263,111 discloses a xenograft decellularised by deoxycholic acid treatment followed by DNAase and RNAase followed by cross-linking with heparin, treatment with formaldehyde and aldehyde capping with Glutamic acid.

The process described in this patent uses detergent based decellularisation and there is also the use of formaldehyde which will also extensively cross-link the decellularised tissue.

In the invention entitled 'Decellularized vascular prosthesis resistant to thrombus occlusion and immunologic rejection' according to US Patent No. 6,689,161B2 and 7,060,02282, the patents disclose tissue decellularised by hypotonic exposure followed by sequential treatment using trypsin-EDTA, lipase, micrococcal nuclease, triton X100, dehydrocholic acid, and SDS. The decellularised tissue is stored in 70% ethanol. This is followed by biotynilation of graft using N-hydroxy succinimidyl-biotin or biotin-amino di caproate N-hydroxy succinimide ester for studying in vivo remodeling. Heparin is grafted by treatment with 1M hydroxylamine sulphate followed by treatment with 1:1 ratio of

cross-linking agent 1-ethyl 3(3 dimethyl amino propyl) carbodimide (EDC) and heparin at pH 1.5. The graft is sterilized and stored in 70% ethanol at 4°C. bFGF was attached to grafted heparin just before implantation by treating it for 5 minutes.

Above method is a detergent based method, which is likely to have residues of toxic detergents. Furthermore extensive use of detergent is mentioned in this process which might remove all the essential glycosaminoglycan content making it vulnerable for calcification.

OBJECTS OF THE INVENTION:

It is therefore an object of this invention to propose a process for the production of remodelable decellularised animal tissue, which avoids the use of detergents.

It is a further object of this invention to propose a process for the production of remodelable decellularised animal tissue, which is non-toxic.

Another object of this invention is to propose a process for the production of remodelable decellularised animal tissue, which makes the decellularised tissue susceptible to matrix metalloproteinase in vivo and rendering it remodellable.

Yet another object of this invention is to propose a process for the production of remodelable decellularised animal tissue, which has improved biological response and is less immunogenic.

A still further object of this invention is to propose a process for the production of remodelable decellularised animal tissue, the tissue showing heparin activity even after storage for considerable duration.

These and other objects and advantages of the invention will be apparent from the ensuing description.

BRIEF DESCRIPTION OF THE INVENTION:

Thus according to this invention is provided a process for the production of remodelable decellularised animal tissue.

In accordance with this invention, a decellularised animal tissue with covalently linked heparain is produced in a single step. Heparin is covalently linked to the matrix by acetal formation. The decellularisation is effected using a non-delegent based process. There is a mild crosslinking of decellularised tissue thereby making it susceptible to matrix metalloproteinase in vivo, thereby rendering it remodelable. Short duration glutaraldehyde crosslinking of decellularised bovine pericardium improves biological response. The proposed method also covalently links heparin to decellularised tissue and retention of heparin activity is shown even after storage for considerable duration.

a. Decellularisation is achieved using a non-detergent based method (Indian patent application no. 2113/CHE/2008). The decellularised tissue produced by this method is observed to be non-toxic.

Glutaraldehyde treatment elicits toxic response compared to decellularisation in bovine pericardium (Toxicology International 2012; 19(l):51-58.))

b. Mild glutaraldehyde crosslinking:

Glutaraldehyde at the concentration of 0.1% to 0.6% more preferably at 0.2% is prepared in phosphate buffered saline (PBS) at pH 7.4 or in 50Mm HEPES at pH 7.4 and the decellularised tissue is exposed for 1 minute to 60 minutes preferably for 10 minutes at 37°C. The mildly fixed tissue is thoroughly washed in at least 3 changes of sterile normal saline containing antibiotics (Ampicillin-Cloxacillin at Img/mL), with one washing cycle lasting for a minimum of 10 minutes.

Mild cross-linking has resulted in a decellularised tissue with better tissue compatibility, low immunogenicity, and better host tissue incorporation and less calcification.

c.**Single step covalent linking of heparin to decellularised tissue** :

The mildly crosslinked decellularised tissue is treated with heparin solution containing 0.2 to 2g, and preferably in 0.5 to lg and more preferably in 0.95g in 0.05M 200 ml HCL pH 1.5) for 48 hrs in shaker at R.T. Following this, the tissue is rinsed in distilled water and incubated in 70% alcohol at 37°C for 30 days, followed by storage at 4°C.

Mild glutaraldehyde cross-linking will result in free aldehyde groups on the decellularised tissue, without affecting susceptibility of decellularised tissue to matrix metalloproteinase in the body. These free aldehyde groups will interact with OH groups of heparin producing hemiacetals. Since this reaction is taking place at acidic pH, there will be formation of acetals. Acetals are stable and are reversible only under acidic conditions and not under alkaline conditions. Since physiological pH is in the alkaline side (pH 7.35-7.45), the acetal formed between OH of Heparin and

CHO of glutaraldehyde crosslinked decellularised tissue will be stable in the body following implantation. Since the ant-thrombin binding activity of heparin is attributed to highly negative sulphate groups, acetal formation as mentioned above do not interfere with the anticoagulant activity of bound heparin.

d. Confirmation of covalent linking of heparin

Heparin linking was demonstrated by presence of metachromatic reaction evidenced as purplish staining on heparin treated decellularised tissue (Test) following staining with 0.2% Ortho Toluidine blue solution. Control, heparin non-grafted sample had only a light bluish color following staining.

e.**Estimation of covalently bound heparin in the modified decellularised tissue**

As the direct estimation of covalently bound heparin is not feasible, an indirect method was adopted, which is a modification of what is reported in 'Smith PK, Mallia AK, Hermanson GT. Colorimetric method for assay of heparin content immobilized heparin preparations. Anal Biochem 1980; 109:466-73'.

Briefly, colorimetric estimation of heparin in the eluting media was done utilizing the properties of the metachromatic dye Toluidine blue. The method involved monitoring the dye depletion in the supernatant at 631 nm as Toluidine blue is adsorbed onto the heparin covalently linked upon the decellularised tissue following overnight incubation at 37°C. A calibration curve was plotted with different concentrations of heparin and a polynomial equation defining the relation between OD and heparin

concentration was made by curve fitting. The unknown concentration of heparin was calculated from this equation.

The covalently bound heparin to approximately 500 mg heparin grafted decellularised tissue (bovine saphenous vein in triplicates) was estimated to be 1.302±0.0076mg/500mg tissue.

f.Experiments on stability of heparin bonded to decellularised tissue (bovine saphenous vein)

i. Estimation of heparin released from heparin modified decellularised tissue (bovine saphenous vein) following sonication for 5 minutes in PBS (pH 7.4)

Approximately 500mg of heparin bonded decellularised bovine saphenous vein (in triplicates) was placed in 1ml of PBS (pH 7.4) and subjected to sonication at 33MHz for 5 minutes. Following this, the heparin eluted into the PBS was estimated using the method given earlier.

The heparin released was estimated to be 0.139±0.01mg/ml, which was 10.65% of the total bonded heparin in the decellularised tissue (bovine saphenous vein).

The heparin remaining in the sample was demonstrated by ortho toluidine blue staining reaction.(see Fig 2)

ii. Estimation of heparin released from heparin modified decellularised tissue (bovine saphenous vein) following incubation in PBS (pH 7.4) at 37°C for different periods, upto 7 days.

Approximately 500mg of heparin bonded decellularised bovine saphenous vein was placed in 1ml of PBS (in triplicates and at pH 7.4) and incubated for 60 minutes to 7 days at 37°C. PBS was replaced with fresh ones at the end of each incubation period. Heparin eluted into the PBS at each incubation period was estimated using the method given earlier.

The heparin released was estimated and is presented below.

Heparin released (mg/ml) in 1ml PBS (pH 7.4) at 37°C

	Bound Heparin in 500 mg sample	60 min in PBS	1Day in PBS	2Days in PBS	3Days in PBS	7Days in PBS
Mean± SD	1.302±0.0076mg /500mg tissue	0.04487±0.00249	0.0205±0.0072	0.007±0.006	-0.0017±0.005	0.00015±0.00026

The heparin remaining in the sample after 7 days incubation was demonstated by ortho toluidine blue staining reaction.

The metachromatic purple staining of heparin grafted indicating retention of bound heparin after incubation in PBS (pH 7.4) for 7 days.

iii. Demonstration of anticoagulant effect of bound heparin

Triple washed (in PBS, pH7.4) heparin grafted 0.5x 0.5cm pieces of decellularised bovine saphenous vein were exposed to 3ml of freshly drawn non-anticoagulated pig blood for 60 minutes at 37°C in a clean test tube(in triplicates). Ungrafted decellularised bovine saphenous vein

exposed to another set of 3ml of freshly drawn non-anticoagulated pig blood for 60 minutes at 37°C (in triplicates) was considered as control. Similar volume of un-coagulated blood alone taken in a separate set of test tubes (in triplicates) acted as normal control. Blood coagulation was observed in both normal control and control samples within 10 minutes. Blood in test samples did not clot even at the end of 60 minutes.

The uncoagulated blood in the Test group (marked 'T') seen free flowing in slanted test tube compared to clotted blood in control (marked 'C') and normal control (unmarked) which is seen confined to bottom of the test tube.

The above test was repeated on heparin grafted decellularised bovine saphenous vein which was stored for 2 months in 70% ethanol. Heparin grafted samples showed no coagulation of pig blood even at the of 60 minutes incubation.

The uncoagulated blood in the Test group (marked 'T1, 2 and 3') seen free flowing in slanted test tube compared to clotted blood in control (marked 'C' 1,2 and 3) and normal control (marked 'B1,2 and 3) which is seen confined to bottom of the test tube.

DATED THIS 29th DAY OF OCTOBER, 2012

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