nature portfolio

Peer Review File

Facile Engineering of Interactive Double Network Hydrogels for Heart Valve Regeneration

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The manuscript by Li et al. describes a new method for generating interactive materials for heart valve regeneration using TCDI chemistry, applied as a double network hydrogel. This material enables the release of hydrogen sulfide, which has anti-inflammatory properties and induces proliferation of endothelial cells. The resulting hydrogel PEG/PSBMA-DHV showed (1) slow release of hydrogen sulfide; (2) anti-calcification, durable and anti-fouling properties; (3) low protein adhesion; (4) low platelet adhesion and activation; (5) low cytotoxicity; (6) promotion of anti-inflammatory cytokines and macrophage polarisation to M2 subtype; (7) in vivo low degradation; (8) in vivo endothelialisation.

Major comments
1. Fig. 7E. On day 14, the standard deviation of each point is high. This needs to be clarified.

Minor comments
1. Abstract. Please define DHV.
2. Legend to the figures 2, 3, 4, 5, 6, 7. Please confirm that * $P<0.05$. Currently, it reads as * $P<0.1$.
3. Line 498. “As shown in Fig. 6E&G…” should read “…Fig. 6G&H…”.
4. Line 501. “Fig. 6G” should read “Fig. 6H”.
5. Fig. 7E. Please include the days measured (7, 14 and 28) instead of 10, 20, 30. Further, the standard deviation of each point on day 14 is high, which needs to be clarified.

Reviewer #2:
Remarks to the Author:
1-Figure 1 seems complicated; please modify it by simplifying it.

2-In some subheadings in the method section, any reference to “Anti-enzyme degradation test, Anti-protein adsorption test, In Vitro Calcification Experiment, Mechanical test, Free amino content test, In Vitro Hemocompatibility of the Scaffolds, In Vivo Degradation and Remodeling of the Scaffolds by Rat Subdermal Implantation Model” not done. Please add.

3-In which parts of the valves (leaflets, sinus, and arterial wall) were characterization tests performed after decellularization? Please indicate.

4-If in vitro cell seeding was performed on the leaflets of the valves, on which surface was the seeding performed? Please indicate.

5-Please indicate in which layers the recellularity shown in the histological stains is seen.

6-Were any tests performed after decellularization to confirm acellularity (residual DNA content determination, histological staining, DAPI fluorescent staining, etc.)? If it has been done, please add it to the article with the results. Because, especially in in vivo tests, insufficient decellularization causes inflammatory cell infiltration and affects the accuracy of your results. Please include these tests in the study.

7-Please include the limitations of the study in the article.

8-In the discussion section, only the findings are summarized, but they should be included in the discussion by relating them to each other and supported by literature findings, and the discussion section should be rewritten. For example, what do mechanical characterization test findings mean,

and how are they different or similar to findings in the literature? As another example, what kind of different results were encountered between the method you presented and the different cross-linking agents available in the literature (such as genipin)? It is necessary to rewrite and enlighten the readers in a way that emphasizes the importance of your work.

In summary, in this study, which presents a new approach for cross-linking decellularized valve scaffolds, its advantages and disadvantages compared to other cross-linking methods should be examined and discussed. Therefore, this study is not suitable for publication unless the above-mentioned deficiencies are corrected.

Reviewer #3:
Remarks to the Author:
The research is dealing with developing double network hydrogels for replacing the current glutaraldehyde-crosslinked bioprosthetic heart valve. An interesting and complete study has been performed, describing the synthesis and crosslinking of the polymers, the characterization mechanically and in vitro and in vivo work.
I believe this work is significant to the field. I am not familiar enough to currently existing DHV systems to know if it is extremely original, but according to what has been presented it makes sense to move this step forward by such a system.
I believe the work has been presented in a consistent way, however I believe the discussion part could be more extensive, describing more detail and explaining some more unexpected results in further detail.
The methods used are described in a logical way.
Nonetheless, I believe that some things require adjusting or stronger clarification:
-Line 44: explain already here the abbreviation of DHVs
-Line 58: you describe mechanical valves, but what about using the ROSS procedure, using your pulmonary valve and a support structure? This is now also used quite extensively. Describe this as well in the introduction.
-Line 97: when using hydrogels to enhance the mechanical strength, will they not swell and reduce the strength and hinder the valve due to this swelling?
-General comment (a few example on lines 113 and 138): when using abbreviations, be consistent in using them and do not use the full names after abbreviating. Be consistent throughout the manuscript.

Results part:
Throughout the results and discussion it is not clear what percentage of amino vs. hydroxyl groups are crosslinked. You do experiments to measure the remaining amount of amino groups, but this is not done on the hydroxyls and thus makes it difficult to measure the crosslinking density. Identify separately the percentage of hydroxyl groups linked
What about the stability of the TCDI linkage between 2 amines and between amine/hydroxyl or between 2 hydroxyl groups? Can you identify this? (Fig. 2 A)
Line 185-188: this sentence is not grammatically correct
What is the rationale to use Mw of 2 kDa for 4-arm-PEG-NH2? Why not longer or shorter arms are used? This should be clarified.
Is it interesting to also test the relative weight loss after longer times than 24h as shown in Fig. 3 K?
How homogeneous are the sample strips from Fig 4A, especially after looking at the heart valves in Fig 3B and the quite inhomogeneous color differences throughout the sample.
Line 405: did u check for possible remaining traces of solvent which can also explain this strong difference? Is the GV in general causing such high cell death in literature?
Line 532: can the fact that PEG-DHV and PEG/PSBMA-DHV show no cell infiltration even after 7 days be further discussed?
Line 578: what about at 7 days, linking the data to the in vitro work?

Discussion: in general this part requires improvement of the English (Line 647: valves, Line 652: thiocarbamate, grammatically...) and should be more extensive as described earlier.
Methods: also this part should be checked for English mistakes.
Line 718: small writing error: '--' -> '-'
Mechanical test: why were long strips and not dogbones used for the tensile test?
Line 752: small writing error: strain at a rate
Line 762: rpm instead of pm
Line 819: by an MTT method instead of by MTT method.
Line 828-836: paragraph is not outlined

Revisions made in reply to the comments of reviewer #1

Q1: Fig. 7E. On day 14, the standard deviation of each point is high. This needs to be clarified.

A1: Thank you for pointing this out. The high standard deviation is caused by an improper data analysis. We originally process the image data by calculating the fluorescent area ratio between CD163 channel and DAPI channel. This method cannot exclude the influence from false positive areas and background fluorescence. Therefore, we modified the analysis method according to other literature (J. Periodontol., 2016, 87, 1092-1102.; Sci Rep,2015, 5, 14273.). We randomly select 6 fields of view for each slice at a 200x magnification. Then, we counted the number of cells (N0) according to DAPI fluorescence staining and the number of M2 phenotype macrophages (N1) according to CD163 fluorescence staining, and then calculated the phenotype proportion of M2 macrophages by the following formula:

Ratio of M2 phenotype macrophages = N1/N0 * 100%

Due to the CD163 positive region and DAPI positive region can be co-located under high magnification field of view, the influence of false positive can be excluded. At the same time, this method directly counts the number of cells, which can reflect the experimental results more accurately than the intensity statistics. This new statistical method is also described in the Methods section of the manuscript, page 37.

The revised Figure 7 was shown below:

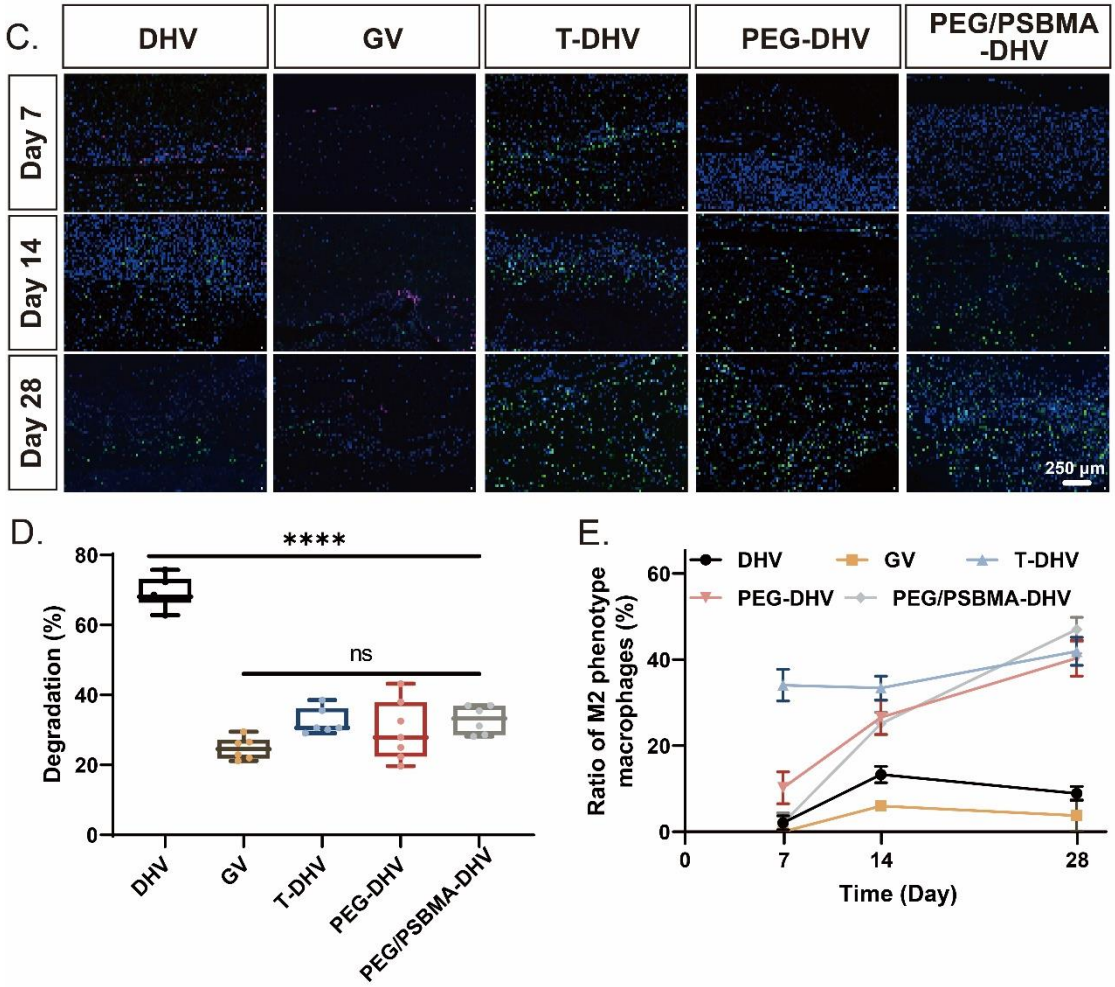


Fig. 7 In vivo degradation and immunomodulatory ability of the samples after subcutaneous implantation. C) Samples were taken at day 7, day 14 and day 28, and CD163 (green) / iNOS (red) /DAPI (blue) staining was performed on the sections to evaluate the immunomodulatory capacity of the sample (n=3). D) The degradation rate of sample was calculated by calculating the percentage of collagen in the Masson stain (n=6). E) The area of CD163 positive area and DAPI positive area were counted by Image J, and their proportion was calculated as the immunomodulatory capacity (n=6). Data were expressed as mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Q2. Abstract. Please define DHV.

A2. The definition of DHV was added in abstract.

Q3. Legend to the figures 2, 3, 4, 5, 6, 7. Please confirm that * P<0.05. Currently, it reads as * P<0.1.

A3. Thank you for the expert comment. We apologize for this mistake. The *p from the analysis software (GraphPad Prism 9) is indeed indicate * P<0.05. We have corrected this value for all figures.

Q4. Line 498. “As shown in Fig. 6E&G…” should read “…Fig. 6G&H…”.

A4: Thank you for this careful review! We have corrected this mistake, page 23.

Q5. Line 501. “Fig. 6G” should read “Fig. 6H”.

A5: Thank you for this careful review! We have corrected this mistake, page 23.

Q6. Fig. 7E. Please include the days measured (7, 14 and 28) instead of 10, 20, 30. Further, the standard deviation of each point on day 14 is high, which needs to be clarified.

A6: Thanks for the suggestion. We have revised the X-axis of Figure 7E as suggested, as shown in Answer 1. As for the standard deviation of each point on day 14, we have reprocessed the data and a detailed discussion has been made in Answer 1.

Revisions made in reply to the comments of reviewer #2

Q1. Figure 1 seems complicated; please modify it by simplifying it.

A1: Thanks for the suggestion. We have simplified the Figure 1, only display the most essential mechanism in the figure.

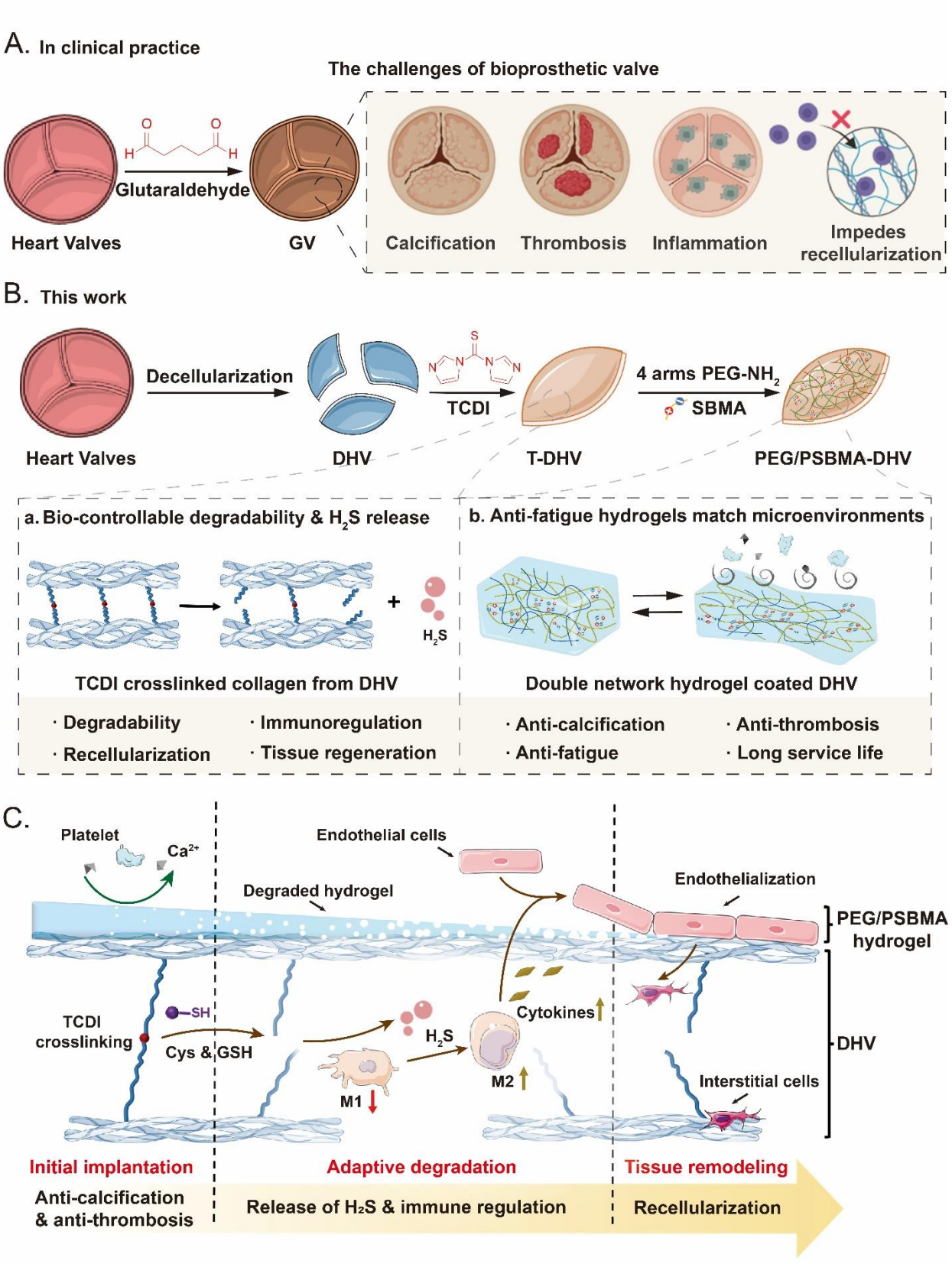


Fig. 1 A schematic overview of the fabrication process, structure, and function of the DHVs coated with PEG/PSBMA double network hydrogel (PEG/PSBMA-DHV). A) The challenge of bioprosthetic valves.

B) A schematic overview of the fabrication process and structure of PEG/PSBMA-DHV. C) A schematic overview of the function of PEG/PSBMA-DHV.

Q2. In some subheadings in the method section, any reference to “Anti-enzyme degradation test, Anti-protein adsorption test, In Vitro Calcification Experiment, Mechanical test, Free amino content test, In Vitro Hemocompatibility of the Scaffolds, In Vivo Degradation and Remodeling of the Scaffolds by Rat Subdermal Implantation Model” not done. Please add.

A2: We have cited references for the experiments mentioned above and revised the manuscript.

Q3. In which parts of the valves (leaflets, sinus, and arterial wall) were characterization tests performed after decellularization? Please indicate.

A3: In this study, only the valve leaflets of fresh porcine aortic valves were cut out and decellularized according to the method described in the previous article of our group (J.Tissue Eng. Regener. Med., 2018, 12(2): e828-e840.). This information is now added at the method-decellularization section, page 32. We have also performed HE, Masson, EVG, DAPI, and DNA content tests to characterize the heart valve leaflets before and after decellularization as discussed in details in Answer 6. The results are presented in Fig. S1.

Q4. If in vitro cell seeding was performed on the leaflets of the valves, on which surface was the seeding performed? Please indicate.

A4: Thank you for your comment. In this study, we did not involve experiments related to cell seeding. Except for the cell migration experiment (Figure 6G) and the transwell experiment (Fig. S8), which used conditioned medium, the other cell experiments were carried out by co-culture method. The cells were first planted in the plate overnight, and then different DHV samples were added for co-culture. These experimental details were now clearly mentioned in the method section, and relevant references were added, page 36,37.

We also noticed that the surface roughness of the ventricular layer and the fiber layer was very different. In order to exclude the influence of this factor on the experimental results, in vitro experiments such as blood compatibility test, calcification experiment, protein adhesion, etc., the flatter ventricular layer was selected for experiment and observation.

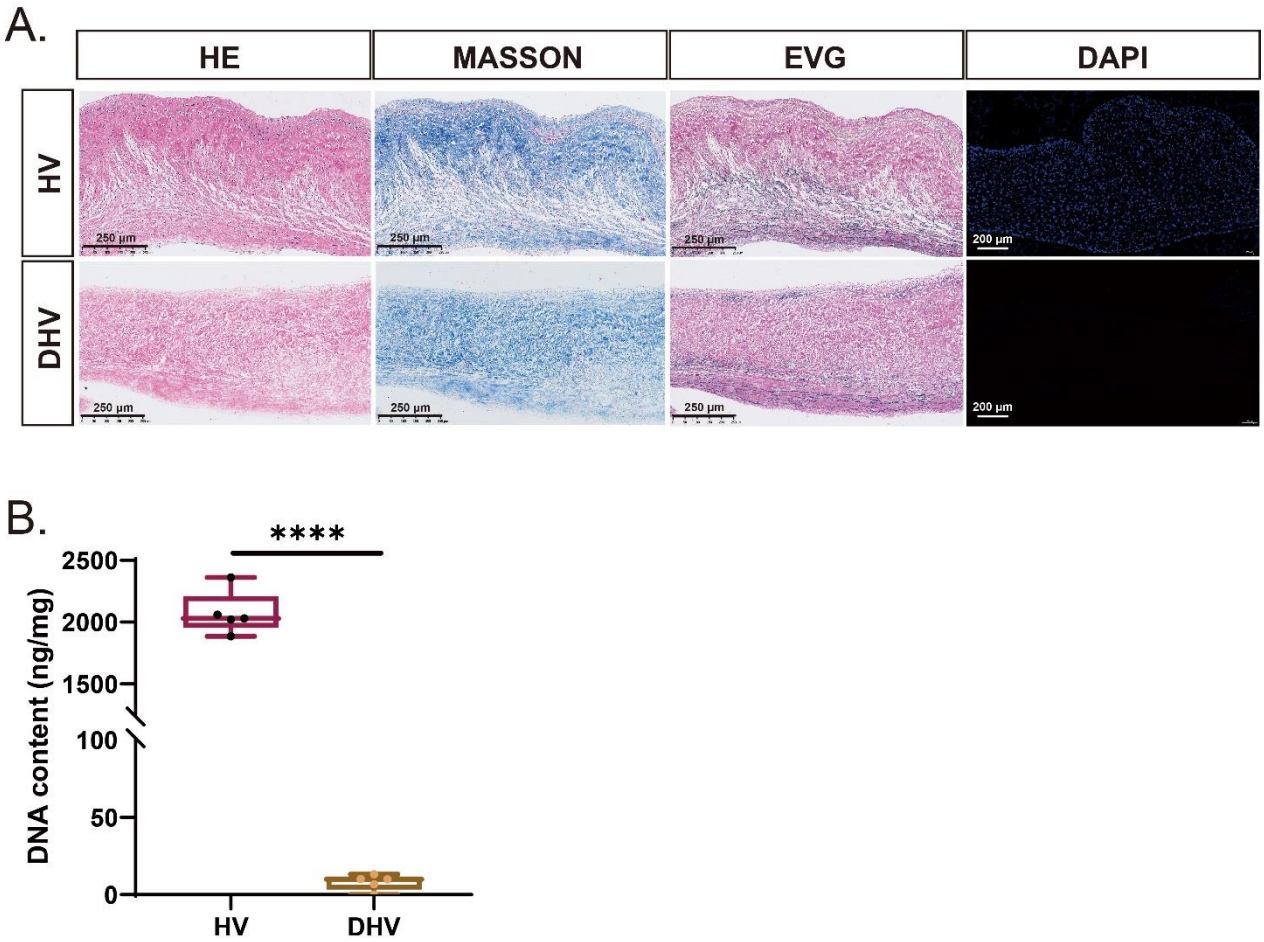
Q5. Please indicate in which layers the recellularity shown in the histological stains is seen.

A5: Thank you for your question. In order to maintain the consistency of the experiment, we used the ventricular layer as the inner wall of the tube when sewing the valve tube. Therefore, the recellularization shown in Figure 8C&9 is located in the ventricular layer, which is the side where the valve is in contact with the blood. We have indicated the side that contact with blood and explained this information in the figure captions.

Q6. Were any tests performed after decellularization to confirm acellularity (residual DNA content determination, histological staining, DAPI fluorescent staining, etc.)? If it has been done, please add it to the article with the results. Because, especially in in vivo tests, insufficient decellularization causes inflammatory cell infiltration and affects the accuracy of your results. Please include these tests in the study.

A6: Thank you for this valuable suggestion. In this study, the valve leaflets of fresh porcine aortic valves were cut out and decellularized according to the method described in the previous article of our group (J. Tissue Eng. Regener. Med., 2018, 12(2): e828-e840.). According to your suggestion, we have

performed HE, Masson, EVG, DAPI, and DNA content tests on heart valves before and after decellularization, and added as Fig. S1 (See below). From HE, MASSON and EVG staining, it can be seen that decellularization does not destroy the original ECM structure of the heart valve, nor does it cause damage to elastin. Meanwhile, HE and DAPI staining results showed that there were almost no residual cells and DNA in ECM after decellularization, indicating that the decellularization strategy could effectively remove porine-derived cells and DNA (Fig. S1A). Similar trend was also observed in the DNA content test, the quantity of DNA decreased from 2071.77 ± 156.84 ng/mg (heart valve before decellularization, HV) to 10.04 ± 2.36 ng/mg (DHV) (Fig. S1B). Therefore, the influence of residual heterologous cells and DNA on the experimental results can be excluded in this study, and the efficiency of the decellularization strategy used in this study is also verified. These data were also discussed in the main text, page 7.



Supplementary Fig. 1 Characterization of the effect of decellularization strategies. A) Before and after decellularization, sections were stained with HE, Masson, EVG and von Kossa. HE for cell and extracellular matrix, Masson stain for collagen, EVG stain for elastin, DAPI (blue) for cell. B) DNA content detection. Data were expressed as mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Q7. In the discussion section, only the findings are summarized, but they should be included in the discussion by relating them to each other and supported by literature findings, and the discussion section should be rewritten. For example, what do mechanical characterization test findings mean, and

how are they different or similar to findings in the literature? As another example, what kind of different results were encountered between the method you presented and the different cross-linking agents available in the literature (such as genipin)? It is necessary to rewrite and enlighten the readers in a way that emphasizes the importance of your work.

A7: Thank you for this valuable suggestion. According to your suggestion, we have rewritten the discussion. We compared the TCDI crosslinkers used in this study with existing DHV crosslinkers and also highlighted the importance of the interactive degradation feature of this work. The comparison of different crosslinking methods was also summarized in Supplementary Table S1.

The traditional DHV crosslinkers, such as glutaraldehyde, genipin and EDC/NHS systems, could enhance the mechanical robustness and durability of heart valves. However, they lack bioactivity hindering the promotion of valve regeneration. On the other hand, some natural substances like polyphenols could provide non-covalent crosslinking with beneficial bioactivity (e.g antioxidant properties), however they suffer from inadequate protection of the DHVs resulting in significant collagen degradation to 14-40.85% within 24 hours under collagenase treatment. In contrast, the collagen loss in DHVs crosslinked with TCDI under similar conditions was remarkably lower, at a mere 0.65±2.29%. In a striking contrast, TCDI crosslinking offers excellent biocompatibility and substantial mechanical strength comparable to other covalent crosslinking, providing improved stability to DHVs post-implantation. The thiourea and thiocarbamate linkages formed by TCDI crosslinking gradually degrade upon encountering internal thiol-containing molecules like cysteine and glutathione, releasing H_2S for immune modulation. This unique property offers a facile approach to develop interactive materials that can adapt to tissue remodeling processes. Following implantation, the intact hydrogel layer offers anti-calcification and anti-thrombosis characteristics for DHVs in the early stages. In vivo findings demonstrate that this protection can persist for over 7 days without observed cell infiltration. Over a span of two to three weeks, the gradual degradation of crosslinking points leads to continuous H2S release, stimulating the repolarization of macrophages and promoting endothelial cell proliferation and migration. Subsequently, hydrogel degradation coupled with re-endothelialization on the DHV surface and infiltration of M2 macrophages and interstitial cells into the DHV can be clearly observed after 28 days of implantation. These discussions were added to the manuscript.

Revisions made in reply to the comments of reviewer #3

Q1. Line 44: explain already here the abbreviation of DHVs

A1: The DHV has been explained as suggested.

Q2. Line 58: you describe mechanical valves, but what about using the ROSS procedure, using your pulmonary valve and a support structure? This is now also used quite extensively. Describe this as well in the introduction.

A2: Thank you for your valuable suggestion. We have included the discussion of ROSS operation to the introduction, page 2.

Q3. Line 97: when using hydrogels to enhance the mechanical strength, will they not swell and reduce the strength and hinder the valve due to this swelling?

A3: Thank you for your valuable comment. In this study, the mechanical strength of DHV was already enhanced by TCDI crosslinking. The PEG/PSBMA hydrogel mainly provide anti-calcification and anti-

thrombotic functions, without further improving the mechanical strength of the DHVs (Figure 3E&F). Moreover, the mechanical tests of all samples were carried out after complete swelling. Both PEG-DHV and PEG/PSBMA-DHV do not show significant difference with T-DHV, indicating the hydrogel layer do not enhance or reduce the strength of the DHV.

In order to improve the stability of hydrogel coating, we modified DHV with hydrogel coating based on TCDI reaction and free radical polymerization strategy, which is more stable and safe than non-covalent bonding. In addition, we also further evaluated the biosafety and regenerative performance of PEG/PSBMA-DHV in the blood environment using an abdominal aorta transplantation model. The blood flow status of the abdominal aorta was also monitored by Doppler ultrasound (Figure 8B). These in vivo results showed no observed clog of blood vessels, suggested the PEG/PSBMA-DHV would not uncontrolled swell or drop any fragments to block the vessels (Figure 9).

Q4. General comment (a few example on lines 113 and 138): when using abbreviations, be consistent in using them and do not use the full names after abbreviating. Be consistent throughout the manuscript.

A4: The abbreviations were all checked and ensured consistency throughout the manuscript.

Q5. Throughout the results and discussion it is not clear what percentage of amino vs. hydroxyl groups are crosslinked. You do experiments to measure the remaining amount of amino groups, but this is not done on the hydroxyls and thus makes it difficult to measure the crosslinking density. Identify separately the percentage of hydroxyl groups linked.

A5: Thank you for your valuable suggestion. We employed dansyl chloride assay to estimate the content of hydroxyl groups on the valves surface. Briefly, dansyl chloride (2 mg/mL) and diisopropylethylamine (1.17 mg/mL) are dissolved in the diethylether. Subsequently, the samples were immersed in this solution for 3 h, and then washed with ethanol three times (changing every hour), and left overnight for the fourth time. Finally, the fluorescence intensity (F) of the samples was analyzed with a fluorescence spectrophotometer (Ex=350nm, Em=467 nm). After the test, the samples were rinsed by ultrasonic cleaning with deionized water and freeze-dried to constant weight (W). The relative free hydroxyl content on the valve surface is calculated by the following formula:

Relative free hydroxyl content = $(F_{sample}/W_{sample})/ (F_{DHV}/W_{DHV}) \times 100\%$

Where, F_{sample} represents the fluorescence intensity of the samples treated with different concentrations of TCDI. F_{DHV} indicates the fluorescence intensity of DHV. W_{sample} and W_{DHV} represent the dry weight of TCDI crosslinked samples and original DHV respectively

It can be seen from the results below that the free hydroxyl groups on the valve surface also decreased gradually with the increase of TCDI concentration. This phenomenon shows that TCDI could realize the cross-linking of both amino groups and hydroxyl groups. At 18 mg/mL TCDI concentration which was used in the subsequent experiments, 59±4% amino groups and 87±2% hydroxyl groups were reacted according to the measurements, indicating sufficient crosslinking. This data and experimental method has been updated in the manuscript, page 7, 9 and 33.

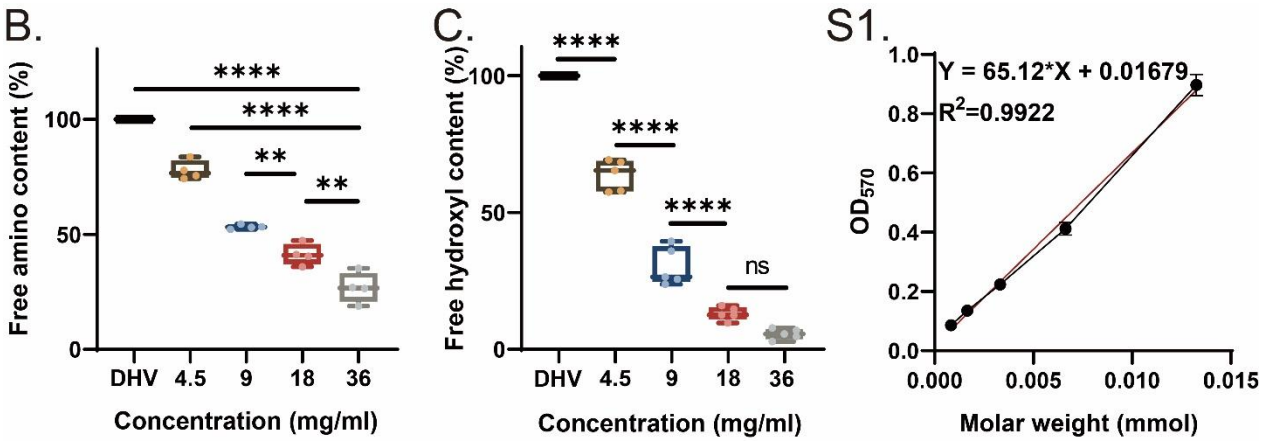


Fig. 2 Construction of the TCDI crosslinked DHV (T-DHV) and the mechanism of H_2S release. B) The content of free amino group on samples' surface determined by ninhydrin method (n=4).C) The content of free hydroxyl group on samples' surface determined by dansyl chloride method (n=5). S1) Amino standard curve by ninhydrin method (n=3). Data were expressed as mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Q6. What about the stability of the TCDI linkage between 2 amines and between amine/hydroxyl or between 2 hydroxyl groups? Can you identify this? (Fig. 2 A)

A6: According to the literature (Tetrahedron Lett.,2002, 43, 6167-6168; J. Am. Chem. Soc.,1989, 111, 1396-1408.), the reaction temperature required for TCDI to cross link the two hydroxyl groups is relatively high, usually around 100 ℃. Therefore, we expect only thiocarbamate (the TCDI linkage between amine/hydroxyl) and thiourea (the TCDI linkage between 2 amines) existed in TCDI crosslinked DHV. For this reason, thiocarbonate (reaction between two hydroxyl groups) was not investigated for H_2S release mechanism. For the stability of thiocarbamate and thiourea, we have carefully analyzed their release kinetics in PBS and cysteine solution in Figure 2G. To simplify the system, N, N-diethyl thiourea (DETU) and O-(1-Methylethyl) N-ethylcarbamothioate (MECT) were taken as model (Figure 2F&G). It can be seen from the results that compared with MECT, DETU shows more stable chemical properties and almost do not release H_2S in PBS. When cysteine was added, the hydrolysis rate was significantly increased for both, but DETU still showed slower release kinetic than MECT. This result indicates that thiocarbamate formed between amine and hydroxyl groups degrade faster than thiourea (formed between two amine groups). These discussions were already mentioned in the manuscript.

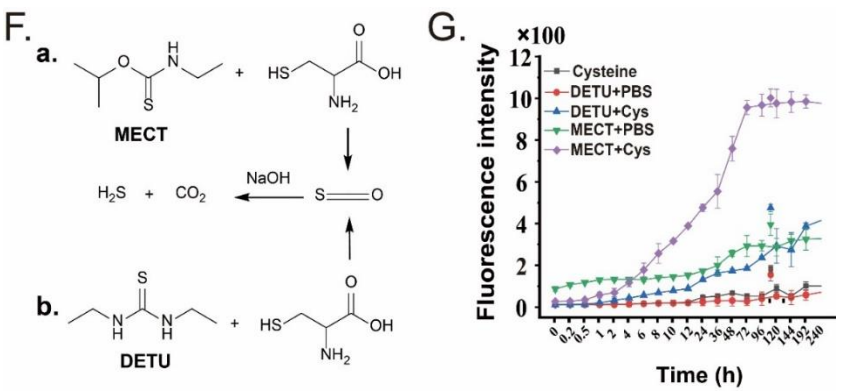


Fig. 2 Construction of the TCDI crosslinked DHV (T-DHV) and the mechanism of H_2S release. F) H_2S release equations of DETU and MECT with cysteine. G) The release kinetics of DETU and MECT (n=3).

Q7. Line 185-188: this sentence is not grammatically correct

A7: Thank you for your valuable suggestion. We have already made corrections, page 8.

Q8. What is the rationale to use Mw of 2 kDa for 4-arm-PEG-NH2? Why not longer or shorter arms are used? This should be clarified.

A8. According to the literature, the short-chain PEG was better than long-chain PEG in inhibiting non-specific adsorption of proteins (Langmuir, 2012, 28, 15743-15750.). It has been reported that long-chain PEG is insufficient to form a dense layer to resist protein due to large steric hindrances (Chinese J. Polym. Sci., 2023, 41, 1879-1888; Biomater. Surf. Sci., 2013, 45-61.). In this study, the PEG/PSBMA hydrogel was desired to inhibit the protein deposition and calcification on the valve surface. Therefore, we chose the 2 kDa 4-arm-PEG-NH2 (Huateng PHARMA, Hunan, China) with the smallest molecular weight that we could purchase. As can be seen from Fig. 4, PEG-DHV can effectively inhibited calcium deposition and protein adhesion before and after fatigue test.

Q9. Is it interesting to also test the relative weight loss after longer times than 24h as shown in Fig. 3 K?

A9: Thank you for your suggestion. In this revision, we have tested the weight loss over 7 days. All samples were treated with type I collagenase and their weight loss were statistically analyzed on day 1, day 4 and day 7 respectively. We found that GV barely degrade within seven days, due to the non-degradability of glutaraldehyde crosslinking. In contrast, DHV without modification degrades rapidly, only 31.13 ± 3.5% of total weight was remaining on the first day and almost complete degradation was observed on day 7. All TCDI crosslinked samples showed improved stability comparing to DHV. T-DHV retained 50.53 ± 5.07% of the total mass on the day 7, whereas PEG-DHV and PEG-PSBMA-DHV revealed 68.13 ± 6.63% and 76.95 ± 2.82% remaining mass respectively. These results suggested that the crosslinking and hydrogel coating would prevent the fast degradation of DHV and facilitate slow degradation over a longer regenerative period. This data was updated in Figure 3K, and page 12.

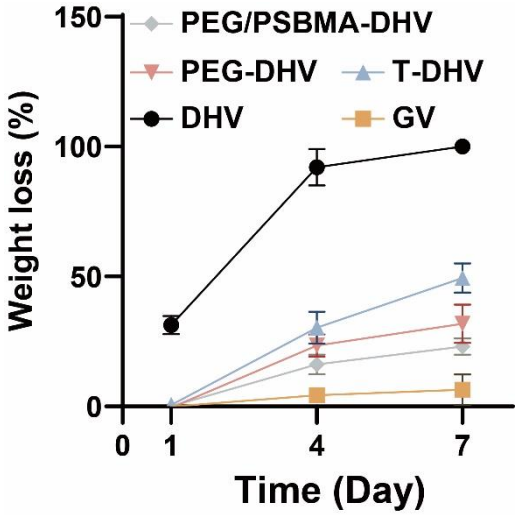
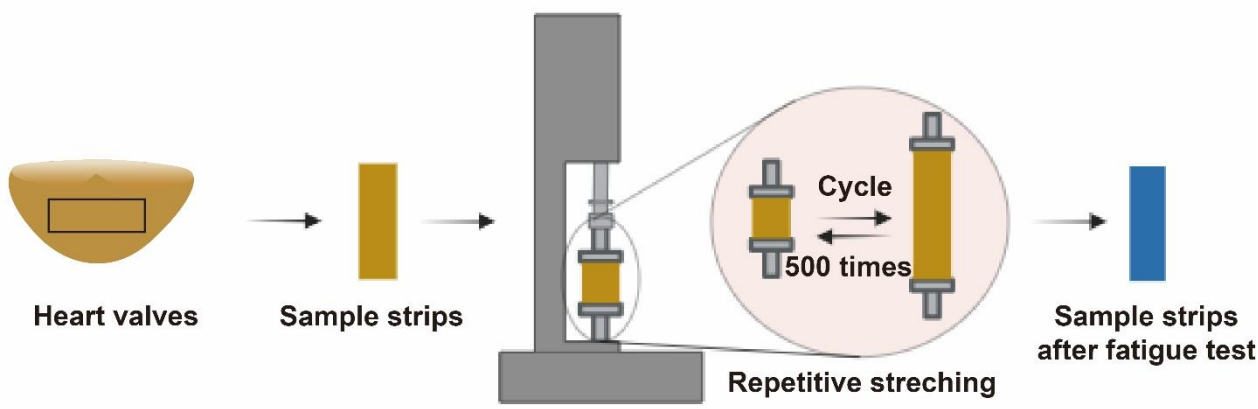


Fig. 3 Construction and characterization of PEG/PSBMA-DHV. K) Relative weight loss of scaffolds after collagenase digestion (n=5). Data were expressed as mean ± SD.

Q10. How homogeneous are the sample strips from Fig 4A, especially after looking at the heart valves

in Fig 3B and the quite inhomogeneous color differences throughout the sample.

A10: Thanks for your comment. Indeed, the whole piece of heart valve is inhomogeneous as shown in Figure 3B. To ensure the area we tested is representative, we always cut the sample strip (length of 10 mm, width of 5 mm) from the thickest central area of the valve belly which is the most homogeneous area in the valve. In order to further maintain the consistency between different samples, the thickness of the samples was measured by vernier caliper before the cyclic tensile test to ensure that different samples have the same size. In addition, we noticed that the surface roughness of the ventricular layer and the fiber layer was very different. In order to exclude the influence of this factor on the experimental results, the flatter ventricular layer was selected for the calcification experiment, protein adhesion in vitro. We have revised the Figure 4A to clearly demonstrate the strip cutting area, and the related description has also been added to the main text, page 14.



Q11. Line 405: did u check for possible remaining traces of solvent which can also explain this strong difference? Is the GV in general causing such high cell death in literature?

A11: Thanks for the question. Before cytotoxicity tests, all samples were rinsed with PBS, sterilized with peracetic acid for 3h, again washed with PBS for 1h*3 times and further soaked in PBS overnight. After the intensive wash procedure, we believe no toxic solvent should remain in the sample, and this method is also widely used in the literature (Chem. Eng. J., 2021, 410, 128244; Adv. Funct. Mater., 2022, 33, 2211267.). Concerning the toxicity of GV, there are a large number of literature reports showing strong cytotoxicity. Our observation is consistent with the literature reports. It is also a common understanding that the toxicity caused by glutaraldehyde crosslinking is one of the important reasons impeding regeneration of the biological valves in clinic. (Chem. Eng. J., 2021, 410, 128244; Adv. Funct. Mater., 2022, 33, 2211267; ACS Biomater. Sci. Eng., 2019, 5, 1452-1461; ACS Biomater. Sci. Eng., 2019, 5, 1822-1832.)

Q12. Line 532: can the fact that PEG-DHV and PEG/PSBMA-DHV show no cell infiltration even after 7 days be further discussed? Line 578: what about at 7 days, linking the data to the in vitro work?

A12: Thank you for noticing the difference of cell infiltration at the early stage of implantation. Indeed, this is an important phenomenon for our system, which is consistent in vitro and in vivo. In the updated Figure 3K, we have demonstrated that the PEG-DHV and PEG/PSBMA-DHV after hydrogel coating showed slow degradation within 7 days. This phenomenon is likely due to the slow cleavage kinetic of the thiourea crosslinking in the hydrogel formed by TCDI reaction, as investigated in Figure 2G. Such slow release ensured sufficient anti-calcification and anti-thrombotic function of the DHV at the initial stage after implantation. Meanwhile, this effect also prevent the cell infiltration before hydrogel

degradation. However, at day 14 and day 28, significant immune cells infiltration was observed in the DHV, which is consistent with the in vitro tested degradation rate of the hydrogel layer. During this slow degradation process, the continuous release of H_2S modulate the repolarization of macrophage and resulting the M2 macrophages detected in the DHV eventually. The complete endothelialisation and tissue remodelling after 28 days also correlate well with the rate of hydrogel degradation. This discussion has been added in the manuscript, page 24.

Q13. Discussion: in general this part requires improvement of the English (Line 647: valves, Line 652: thiocarbamate, grammatically...) and should be more extensive as described earlier.
A12: More extensive discussion was added and the writing was improved.

Q14. Methods: also this part should be checked for English mistakes.
A14: This part has been carefully checked and revised.

Q16. Line 718: small writing error: '--' -> '-'
A16: Corrected

Q17. Mechanical test: why were long strips and not dogbones used for the tensile test?
A17: Thank you for the suggestion. However, the DHV is very soft and difficult to cut into the same size dogbones shape. Therefore, in order to maintain uniformity between different samples, we choose a long strip that is easier to control the size. In order to prevent the sample from slipping during the stretching process, we place sandpaper at both ends of the sample. In addition, the samples which the fracture occurred between the upper and lower fixtures were selected as valid samples for the analysis of the mechanical properties. The description of this part has also been updated in the Methods-Mechanical test, page 34.

Q18. Line 752: small writing error: strain at a rate

Line 762: rpm instead of pm

Line 819: by an MTT method instead of by MTT method.

Line 828-836: paragraph is not outlined
A18: All the grammatical errors are corrected.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Thank you for the answers. I have no further comments.

Reviewer #2:
Remarks to the Author:
It has been observed that the authors have taken our critiques seriously and responded to each one individually. All the critiques we proposed have been addressed. We have no further critiques. We believe the publication is ready to be published in its current form.

Reviewer #3:
Remarks to the Author:
I believe all comments from the first round of reviewing have been handled in depth.
I am satisfied with the answers