Toxicon

Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebite wound dressings and drug delivery systems inhibit spitting cobra venom-induced cytotoxicity

--Manuscript Draft--

Manuscript Number:	TOXCON-D-25-01026R1
Article Type:	Research Paper
Section/Category:	Other topics in toxinology
Keywords:	Snakebite, Venom, Hydrogel, Heparins, Wound-dressing, Cytotoxicity
Corresponding Author:	Steven Hall, PhD Lancaster University UNITED KINGDOM
First Author:	Matthew J. Gray, MSci
Order of Authors:	Matthew J. Gray, MSci
	Daniel K. Baines, PhD
	Gabrielle De Castro, MEng
	Thomas Cobb, MSci
	Layla A. Tyrrell
	Sam J. Hyam
	Alan M. Smith, PhD
	Nicholas R. Casewell, PhD
	Sarah L. Allinson, PhD
	Timothy E. L. Douglas, PhD
	Steven R. Hall, PhD
Abstract:	Snakebite envenoming affects millions of people annually, with current treatments limited to animal-derived antivenoms. Repurposed drug-inhibitors of toxin families offer an exploitable avenue to improve snakebite treatment, including heparins which can inhibit cytotoxic three-finger toxins. However, to be effective therapies in-the-field such treatments must be engineered into drug delivery devices capable of rapidly administering drug(s) to the envenomation site. Herein we introduce the concept of integrating heparins, specifically unfractionated heparin (H) and its low molecular weight heparinoid variant, tinzaparin (T), into hydrogels composed of whey protein isolate (WPI), an inexpensive byproduct of the dairy industry that is cytocompatible, stiff, sterilizable by autoclaving, and that has the dual function of being able to locally deliver drugs and act as wound dressings. The aims of this research were to investigate whether heparin-containing WPI hydrogels displayed physical characteristics suitable for wound dressings and could effectively release drug in sufficient quantities to inhibit the cytotoxic activity of spitting cobra venom. To do so, five hydrogel prototypes were produced: 40% WPI no drug control, and 5% H, 10% H, 5% T, and 10% T all within 40% WPI hydrogels. It was determined that heparins successfully integrated into WPI hydrogels and heparin-containing WPI hydrogels exhibited improved swelling versus the 40% WPI no drug control, suggesting improved absorption of wound exudate; however, this heparin integration also increased the hydrogel degradation rate in simulated wound environments. Most importantly, sufficiently high concentrations of T were released into phosphate buffered saline from the 10% T hydrogels to neutralise Naja nigricollis venom cytotoxicity in a HaCaT cell cytotoxicity model. Together, our results suggest that the integration and diffusion of venom-inhibiting drugs in WPI hydrogels is possible and that the development of such drug-integrated hydrogels into snakebite wound dressings warrants further research.
Response to Reviewers:	Please note, we have also attached these Comments to Reviewers as a separate word

document, which is better formatted and likely easier for the reviewers and editor to read.

We would like to sincerely thank the editor and reviewers for their time and their detailed commentary. We would also like to thank them for their patience in allowing us an extended deadline to complete further experimentation addressing their concerns with the previous version of this study. We believe that we have adequately tackled the identified issues, and that the suggested edits have been implemented to produce an improved manuscript. Below you can find a point-by-point response to the specific comments made. For clarity, please note that all revisions to the manuscript have been highlighted using red font, including minor errors we noticed in our original writing that we edited of our own accord including italicising all instances of et al.

Reviewer #1
Background: cytotoxins are the prevail components in the venom of this cobra. Heparins bind and neutralize them. The WPI-based hydrogel is capable of including heparin. Methodology: the authors incubated tinzaparin-containing hydrogel with PBS for a 24h, measured the release of tinzaparin (after 24h), and established its neutralizing activity towards the cytotoxicity of cobra venom. Findings: The authors have found that tinzaparin retains its ability to neutralize cobra venom cytotoxicity being released into PBS from WPI-based hydrogel during 24h incubation. To my knowledge, this is the first report on activity of pure WPI-hydrogel against cobra venom cytotoxicity. Roughly, this suggests the possibility of using such a drug to treat a cobra bite. In addition, the authors provided some data (SEM and others) on heparin-containing WPI-hydrogels. Although the latest data are not directly related to toxinology, they are of interest for the pharmacy technology.
In overall, the manuscript is well-written and of interest. Graphical abstract, figures, and tables are relevant.

We would like to thank Reviewer #1 for their thorough evaluation and supportive comments.

However, some questions require answers before recommendation for publication. The main issue is: the effect of cytotoxins develops quite quickly (in the very first minutes of envenomation; and even delayed effects, such as necrotic lesions, are triggered in the first hours after the bite). The questions are: why did the authors not investigate the release of tinzaparin from the hydrogel in the first 1-3-5 hours of its application? Why are they waiting 24 hours? The speed of the onset of the drug's effect is critically important for the successful recovery of a bitten person. After 24 hours, all pathological processes have already started and are in full swing. Will 10%T be effective if applied immediately (does tinzaparin release enough, for example, within an hour or three)? The fact that an effective final concentration of tinzaparin has accumulated in PBS after 24 hours does not guarantee an effect in the early stages of the lesion, while this is important. Therefore, based on the data provided, it is premature to talk about 10%T as a promising treatment for snake bites. Based on the above, I strongly recommend the authors either soften the wording regarding the use of 10%T as a medicine (wound dressing) for cobra bite (including in the title of the manuscript and elsewhere in text, e.g. Lines 539 and 549), or to determine the release of tinzaparin from the hydrogel in PBS during the first hours of incubation.

This is a very fair point, and we agree that showing effective tinzaparin release at more appropriate time points is crucial for demonstrating the potential of WPI hydrogels. To address this, we have conducted additional experimentation to determine the release of tinzaparin from a hydrogel at the more biologically relevant timepoint of 1 hour. This work is summarised in the new Fig. 5 which demonstrates that tinzaparin is released from the gels at high concentrations within the first hour, with no significant difference to the release after 24 hours. Several changes have been made to the manuscript following the acquisition of these results. Sam Hyam and Layla Tyrrell have been added to the list of authors for the creation of a new batch of tinzaparin-containing WPI hydrogel discs and for completing this new set of requested experiments, respectively. Throughout the document, details have been added to include the 1-hour incubation

	and the 24-hour incubation. For the methods section, under subheading 2.2.7., lines 224-233 have been rewritten to detail an updated analysis method for quantifying tinzaparin release, wherein each sample was compared to each standard curve and the average found, which should give a more accurate measurement of how much tinzaparin is within each sample. In addition, detail has been added regarding the new 1-hour incubation time point. This section now reads: “To measure drug release from the tinzaparin-containing WPI hydrogel, 10% T and WPI control discs were soaked in PBS (3 discs / 180 µL PBS) for 1 hour or 24 hours under standard conditions (37 oC, 5% CO2). Over three experimental replicates, 50 microlitres of the resulting solutions were pipetted into separate wells in a half-volume 96-well UV-capable microplate along with serial dilutions of known tinzaparin concentrations (4,000 – 31.25 µg/mL, with a halving of the concentration with each serial dilution). A230 was measured in each well using a Tecan MPLEX plate reader and a standard curve plotted for each known tinzaparin concentration serial dilution, from which the concentration of tinzaparin released from the 10% tinzaparin-containing discs under standard conditions was determined for each of three experimental replicates and the means calculated.” In the results section, a new version of Fig. 5 has been produced, with the absorbance of new tinzaparin standard curves being given in Fig. 5B, and the quantity of tinzaparin released after incubation in PBS for 1 hour compared to 24 hours being given in the new Fig. 5C. The figure legend has also been revised to describe these new figures. The paragraph following this describing the results has also been edited to reflect the new data and experimental methods (lines 396-407). In addition, we then also repeated the venom-inhibition experiments with the 1 hour-soaked 10% T hydrogel solution, which provided statistically significant evidence that this solution was capable of inhibiting the N. nigricollis venom’s cytotoxic activity (Fig. 6B). Finally, Table 3 has been revised to include the 1-hour tinzaparin release concentration (2.80 mg/mL) and that of the tinzaparin concentration released after incubation for 24 hours determined after these new experiments (3.05mg/mL), since new hydrogel discs had to be prepared for their completion. The discussion section has principally been edited between lines 506-523 to discuss the new 1-hour incubations and to utilise the updated tinzaparin concentrations. In addition, we have softened the language at the end of this paragraph regarding the use of these hydrogels as medicines for the treatment of snakebite in line with the reviewer’s suggestion. Additional editing for this purpose has been conducted in the conclusions. Thank you to Reviewer #1 for this comment. We believe the additional experiments that have been undertaken in response address their valid critique.
	Line 104. There's probably a typo here. Heparis is a hepatoprotector food supplement. This is a typo and has now been corrected to “heparin”.
	Line 141 (or Line 208). What the proteases have been used? Human collagenases? The proteases used for this experiment were a dried mixture of proteases produced from bovine pancreas obtained from Sigma-Aldrich, incubated with the hydrogels at a concentration consistent with human blood collagenase levels. For improved clarity, this sentence (starting in line 209) has been altered to read: “Five hydrogel samples were incubated in 200 µL of pH 7.4 PBS containing 13.33 µg/mL protease mixture obtained from bovine pancreas (CAS no. 9001-92-7), concentrations consistent with blood collagenase levels”
	Line 384. Here it states that PBS incubated with 10% WPI was used as a solution for control samples of 10%T. However, the hydrogel used for 10%T was 40% WPI (Table 1). 40% WPI releases protein into solution in 24 hours with an absorption of about 0.3 A at 280 nm (Fig. 4A). Then, taking into account a typical protein spectrum, absorption at 230 nm should be about 1 A, or even more. Therefore, 10% WPI is an unsuitable control for calculating tinzaparin release, since an increase in 0.5-1.5 A absorption at 230 nm will mostly be due to protein loss from the 10%T preparation based on 40%WPI, rather than the release of tinzaparin.

The statement “10% WPI” was a typo. This should instead read “40% WPI”, as the same control gel was used here as with other experiments. As such, there should not be a technical issue to address, and the typo has now been corrected in the manuscript. Thank you for catching this error.

Line 414. What prevented the authors from taking the same venom concentration in A and B? The authors describe a purely technical situation (Lines 525-535). I suggest the authors repeat this important experiment both A and B under the equal conditions; otherwise, these data raise doubts about the validity of the observed difference.

This is again a highly valid critique. The experiments summarised in Fig. 6 have therefore been repeated to ensure that an equal venom concentration (20 µg/mL) could be used for both 10% T and 40% WPI hydrogels, as well as exploring the effect of PBS solutions incubated with either hydrogel type for only 1 hour compared to 24 hours. Surprisingly, whilst this has continued to demonstrate that the solutions generated from 10% T hydrogel incubations, including 1-hour incubations, are able to inhibit spitting cobra venom cytotoxicity, albeit at slightly different concentrations than previously observed, the significant inhibitory effect from the blank 40% WPI hydrogels solutions was no longer seen. As a result of this, changes have been made to this manuscript to reflect these new results which are no longer statistically significant by removing any previous mention of such significance, and instead now focuses on the significant results observed from the 10% T hydrogel solution. We did, however, feel it was important to mention that despite the lack of significance, it was still interesting how the mean % cell viability in cells treated with venom and the blank WPI hydrogel solution was slightly higher than the venom-only control, and that further research is required to determine if this is a real effect that could be exploited in future snakebite therapies or not.
The methods section has been rewritten to accurately detail the methodologies used for the new and repeated experiments. Under subheading 2.2.8., this section has been modified to reflect that the cytotoxicity assays were repeated using the same venom concentration for each condition, and now reads:
“Three discs of both hydrogel types were incubated in the same manner for 1 hour. Serial dilutions of the resulting hydrogel solutions (0.08x to 1.95x10-5x dilutions with a quartering of the concentration with each serial dilution) were mixed with a constant 20 µg/mL N. nigricollis venom or venom vehicle control (PBS) in Low Background Fluorescence Medium.”
Several sections from the results section have also needed to be modified to reflect these new results. Firstly, the title of section 3.6 is now:
“3.6. 10% Tinzaparin WPI Hydrogels Inhibit N. nigricollis Venom Cytotoxicity”
Fig. 6 has now been improved using the new experimental data based on the reviewer’s suggestions, with all experiments being conducted at the same venom concentration (20 ug/mL), as well as additional cytotoxicity assays being conducted on solutions derived from 1-hour hydrogel incubations which should more accurately reflect the realities of treating snakebite in the field (Fig. 6A and 6B). The figure legend has also been updated to describe the new figure. The following paragraph describing the results shown in Fig. 6 and Table 3 has therefore also been rewritten:
“HaCaT cells exposed to N. nigricollis venom and treated with vehicle control for both hydrogel experiments effectively reduced cell viability to close to 0%, confirming that cytotoxic venom concentrations were used in both cases (Fig. 6). The supernatant from the 40% control hydrogels for both incubation periods did not significantly increase cell viability at any concentration, although mean cell viability did increase from the vehicle control at most concentrations (Fig. 6A, 6C). The 1-hour incubation, 10% T hydrogel supernatant dilution series effectively inhibited the venom-induced cytotoxicity only at the 0.02x dilution which contained approximately 56 µg/mL of T (Table 3), resulting in a significant increase in mean cell viability to 52.5% (Fig. 6B). The 24-hour incubation, 10% T hydrogel dilution series was able to inhibit N. nigricollis venom cytotoxicity at a greater concentration range than the 1-hour incubation, significantly increasing cell viability at dilution factors of 0.05, 0.02 and 0.08 (15, 61 and 244 µg/mL [Table 3]), resulting in cell viabilities of 39.5%, 48.8% and 31.5% respectively (Fig. 6D).”
Finally, the discussion section has been amended in several places to reflect the new experimental results and to remove mention of the previously significant cytoprotective effect of 40% WPI hydrogels. The first of these is between lines 514-517, which has

	been altered to read: “As predicted, the 24-hour incubated, 10% T hydrogel-derived solution successfully inhibited N. nigricollis venom-induced skin cell cytotoxicity at several tested serial dilutions (15 – 244 µg/mL of T; Table 3), and at one tested concentration for the solution derived from a 1-hour incubation (61 ug/ml of T; Table 3). These results confirm that T-integrated WPI hydrogels can release drug at sufficiently high concentrations within as little as an hour to inhibit snake venom-induced cytotoxicity – a valuable characteristic since rapid treatment is crucial for necrosis prevention (Gutiérrez et al., 2017a). As such, this evidence suggests that drug-incorporated WPI hydrogels warrant further research and development as potential pre-hospital drug delivery devices for the treatment of snakebite envenoming.” As Reviewer #1 requested, all the new experiments have been repeated using the same concentration of N. nigricollis venom (20 ug/mL). As such, the paragraph which detailed the limitation inherent in using different venom concentrations (previously lines 525-535) has been removed entirely, and the reference found only in this paragraph (Jain et al., 2021) removed from the reference list. Finally, the final paragraph of the discussion as well as the conclusions (have also been edited to accurately reflect the data generated by the new experiments, including the new 1-hour incubation timepoints and the non-significant results from the blank hydrogels. Thank you again to Reviewer #1 for their valid criticism which, after completing these new experiments, has certainly improved our manuscript. Line 547. Baines et al., 2024 used hydrogel but did not use varispladib or another anti-venom drug; Lewin et al., 2016 used varespladib but did not use hydrogels or any similar delivery system. I understand what the authors meant by that, but these references don't complement each other in a reason way, so they seem to be improper here. We thank Reviewer #1 for flagging this. This section has been rewritten to now read: “In addition, a range of other snake venom inhibitors which are currently under investigation such as varespladib, a hydrophobic inhibitor of snake venom PLA2s (Clare et al., 2021; Lewin et al., 2016) , could be incorporated into hydrogels and tested against a range of snake venoms. This could exploit WPI hydrogels’ ability to bind and release hydrophobic drugs (Baines et al., 2024).” Reviewer #2 This research manuscript investigated whether heparin-containing WPI hydrogels displayed physical characteristics suitable for wound dressings and could effectively release drug in sufficient quantities to inhibit the cytotoxic activity of spitting cobra venom. I have read the manuscript and have some suggestions that I recommend to do on it to be accepted: 1- On Introduction on line 87, the authors mentioned that..." Some promising candidates are heparins including unfractionated heparin..." There are previous relevant publications on heparins as antivenom against diferente snakes from different continents, that they should be mentioned in your citations. We thank reviewer #2 for their feedback. This is a fair comment. Additional historic citations about the use of heparins as antidotes against snake venoms across the globe have now been included and the sentence between lines 88-92 reworded to read: “Some promising candidates are heparins including unfractionated heparin (H), a highly sulphated polysaccharide previously explored for hemotoxic snakebite treatment, and its low molecular weight (LMW) heparinoid variant, tinzaparin (T), both of which are anticoagulants traditionally used for thromboembolic indications (Boechat et al., 2001; Christy et al., 1973; Du et al., 2024; Hirsh, 1998).” In addition, the full citations for Boechat et al., 2001 and Christy et al., 1973 have been added to the reference list.

2- On line 104 is the word heparis correct ?

Apologies, this was a typo. It has now been corrected to “heparin”.

3- Which protein composition has the WPI hydrogels? If it contains casein, could explain some results of your data about incorporating the snake venom component. It would be better to add more specific information about the WPI. On line 269 Fig., the morphological changes induced by the polyanions on the surface of WPI indicated its contain charged amino acid, which allowed the incorporation of heparins. This needs a comment to explain it.

Whey protein isolate does not contain casein, as part of the process involved in producing WPI from milk is to remove casein proteins (Khalid et al., 2025). Specific information about the composition of WPI has also been added to the discussion section over Lines 447-449, reading:
“WPI is >97% protein, of which the majority is β-lactoglobulin (75%), however other components include α-lactalbumin, immunoglobulins, bovine serum albumin, bovine lactoferrin and lactoperoxidase (Keppler et al., 2014; Madureira et al., 2007).”
Additionally, β-lactoglobulin has been reported to have an isolelectric point of pH 5.3 in the native state which would make it negatively charged at neutral pH (Wang et al., 2024). However, it is possible that the denaturation of β-lactoglobulin caused by heating may change the isoelectic point resulting in the binding of heparinoid polyanions, however this is only speculative. A comment has been added to explain this in the discussion between Lines 450-454, reading:
“β-lactoglobulin has been reported to be negatively charged in the native state (isoelectric point = pH 5.3), with polyanions such as heparin typically binding patches of positively charged amino acids (Kamerzell et al., 2011; Wang et al., 2024). However, it may be possible that protein denaturation caused by heating may alter the isoelectric point, allowing heparin polyanion binding.”
In addition, the full citations relating to these additional in-text citations have been added to the reference list.
Note that the decision was made to add these further details to the discussion section rather than the results section (i.e. on Line 269) as they are a commentary upon the results found instead of a description of the results.

4- On line 228 topic 2.2. Cytotoxicity Assay, please indicate the temperature that the experiment was performed on.

The temperature under which tinzaparin was released from the gel was 37oC. This has been corrected in the manuscript by adding the term “under Standard Conditions”, defined earlier in the document as 37oC and 5% CO2.

5- On line 452-453 Could the authors explain better this statment?

Thank you to Reviewer #2 for pointing out the ambiguous nature of this statement. This has now been expanded to say:
“The improved swelling properties observed in the WPI hydrogels integrated with heparins is also a desirable characteristic for a snakebite wound dressing, as snakebite envenoming often produces excess wound exudate which should be removed for more effective wound healing (Collier et al., 2014; Ibiapina et al., 2019; Rucavado et al., 2016).”
The additional citation by Collier et al., 2014 details why excess wound exudate should be removed, and this has also been added to the reference list.

6- Line 503-504 - It could interact as a substract for the venom, decreasing the amount of free active toxins

Thank you to Reviewer #2 for this suggestion regarding a possible mechanism of action regarding the inhibition of cytotoxicity by the blank 40% hydrogel-derived solutions. However, as we are no longer observing a significant cytoprotective effect

from these solutions after repeating these experiments, we have stripped back the commentary about the effects of the blank 40% hydrogel-derived solutions from the discussion, as previously discussed in our response to a comment from Reviewer #1, and as such will no longer go into extended detail regarding a possible mechanism of action.

Final comments: I have enjoyed to read this this relevant manuscript that raises an important issue in Toxinology, the development of experimental models. New and good experimental models need time to be reproduced and become relevant. This is an in vitro model that show the relevance of polyanions as antitoxin agents against isnake venom.
After a good revision and adjustment in the citations it will be better suited for acceptance.

We thank Reviewer #2 for their kind words as well as their suggestions for how we could improve our manuscript.

Editorial Comments
Owing to the model limitations, it would seem necessary to tone down the concluding statements on the suitability of this kind of device until more detailed evidence of its translational usefulness is gathered.

Thank you to the editor for their review of our manuscript. Due to the additional experiments undertaken to improve this manuscript, we believe that the translational utility of these heparinoid-incorporated WPI hydrogels is now much less in question, as we have demonstrated that they are able to operate at more relevant time points, as well as improving the validity of the cytotoxicity assay experiments by using the same concentration of venom for each experiment. However, we have also taken the editors suggestion to soften the language used in some of our concluding experiments, as we acknowledge more information is needed before we can confidently state that this methodology could be used for in-the-field treatment of cytotoxic snakebite injury. This includes commentary within the discussion (lines 521-523) and conclusions (lines 560).

The work is certainly important and interesting. The final sentence in the cover letter "...our results do suggest that the integration and diffusion of venom-inhibiting drugs in WPI hydrogels is possible and that the development of such drug-integrated hydrogels into snakebite wound dressings warrants further research." seemed to me even better than the final sentence of the abstract "...these data suggest drug inhibitor-containing WPI hydrogels could be suitable for translation into snakebite wound dressings for field use, and that future work is certainly warranted". Just a personal suggestion.

We thank the editor for their kind words and for their observation on the final sentence of the abstract. This sentence has now been replaced with the conclusion of the cover letter, now reading:
“Together, our results suggest that the integration and diffusion of venom-inhibiting drugs in WPI hydrogels is possible and that the development of such drug-integrated hydrogels into snakebite wound dressings warrants further research.”

Bibliography
Baines, D.K., Wright, K., Douglas, T.E.L., 2024. Preliminary In Vitro Assessment of Whey Protein Isolate Hydrogel with Cannabidiol as a Potential Hydrophobic Oral Drug Delivery System for Colorectal Cancer Therapy. Polymers 16, 3273. https://doi.org/10.3390/polym16233273
Boechat, A.L.R., Paiva, C.S., França, F.O., Dos-Santos, M.C., 2001. Heparin-antivenom association: differential neutralization effectiveness in Bothrops atrox and Bothrops erythromelas envenoming. Rev. Inst. Med. Trop. São Paulo 43, 07–14. https://doi.org/10.1590/S0036-46652001000100002
Castro-Amorim, J., Novo de Oliveira, A., Da Silva, S.L., Soares, A.M., Mukherjee, A.K., Ramos, M.J., Fernandes, P.A., 2023. Catalytically Active Snake Venom PLA2 Enzymes: An Overview of Its Elusive Mechanisms of Reaction. J. Med. Chem. 66, 5364–5376. https://doi.org/10.1021/acs.jmedchem.3c00097

Christy, N.P., Weiss, H.J., Phillips, L.L., Phillips, G.B., Hopewell, W., 1973. Exotic snakebite in an urban setting: heparin therapy of disseminated intravascular coagulation in a patient bitten by a saw-scaled viper (Echis carinatus). Trans. Am. Clin. Climatol. Assoc. 84, 37–48.
Clare, R.H., Hall, S.R., Patel, R.N., Casewell, N.R., 2021. Small Molecule Drug Discovery for Neglected Tropical Snakebite. Trends Pharmacol. Sci. 42, 340–353. https://doi.org/10.1016/j.tips.2021.02.005
Collier, M., Potts, C., Shaw, E., 2014. Use of a coverlet system for the management of skin microclimate. Br. J. Nurs. Mark Allen Publ. 23, S28, S30-35. https://doi.org/10.12968/bjon.2014.23.Sup15.s28
Du, T.Y., Hall, S.R., Chung, F., Kurdyukov, S., Crittenden, E., Patel, K., Dawson, C.A., Westhorpe, A.P., Bartlett, K.E., Rasmussen, S.A., Moreno, C.L., Denes, C.E., Albulescu, L.-O., Marriott, A.E., Mackay, J.P., Wilkinson, M.C., Gutiérrez, J.M., Casewell, N.R., Neely, G.G., 2024. Molecular dissection of cobra venom highlights heparinoids as an antidote for spitting cobra envenoming. Sci. Transl. Med. 16, eadk4802. https://doi.org/10.1126/scitranslmed.adk4802
Hiremath, K., Dodakallanavar, J., Sampat, G.H., Patil, V.S., Harish, D.R., Chavan, R., Hegde, H.V., Roy, S., 2024. Three finger toxins of elapids: structure, function, clinical applications and its inhibitors. Mol. Divers. 28, 3409–3426. https://doi.org/10.1007/s11030-023-10734-3
Hirsh, J., 1998. Low-Molecular-Weight Heparin. Circulation 98, 1575–1582. https://doi.org/10.1161/01.CIR.98.15.1575
Ibiapina, H.N.S., Costa, A.G., Sachett, J.A.G., Silva, I.M., Tarragô, A.M., Neves, J.C.F., Kerr, M.W.A., Santana, M.F., Martins-Filho, O.A., Lacerda, M.V.G., Ferreira, L.C.L., Malheiro, A., Monteiro, W.M., 2019. An Immunological Stairway to Severe Tissue Complication Assembly in Bothrops atrox Snakebites. Front. Immunol. 10, 1882. https://doi.org/10.3389/fimmu.2019.01882
Jain, K., Salamat-Miller, N., Taylor, K., 2021. Freeze–thaw characterization process to minimize aggregation and enable drug product manufacturing of protein based therapeutics. Sci. Rep. 11, 11332. https://doi.org/10.1038/s41598-021-90772-9
Keppler, J.K., Sönnichsen, F.D., Lorenzen, P.-C., Schwarz, K., 2014. Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and fluorescence quenching. Biochim. Biophys. Acta BBA - Proteins Proteomics 1844, 1083–1093. https://doi.org/10.1016/j.bbapap.2014.02.007
Khalid, M., Ghasem, N., Yang, X., Schroen, K., Nazir, A., 2025. Progress in Membrane Technology for the Enrichment of Individual Whey Proteins. Ind. Eng. Chem. Res. 64, 19305–19321. https://doi.org/10.1021/acs.iecr.5c00643
Kilara, A., Vaghela, M.N., 2018. Whey proteins, in: Proteins in Food Processing. Woodhead Publishing, pp. 93–126. https://doi.org/10.1016/B978-0-08-100722-8.00005-X
Lewin, M., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) Appears to Be a Potent, Broad-Spectrum, Inhibitor of Snake Venom Phospholipase A2 and a Possible Pre-Referral Treatment for Envenomation. Toxins 8, 248. https://doi.org/10.3390/toxins8090248
Machado de Avila, R.A., Alvarenga, L.M., Tavares, C. a. P., Molina, F., Granier, C., Chávez-Olórtegui, C., 2004. Molecular characterization of protective antibodies raised in mice by Tityus serrulatus scorpion venom toxins conjugated to bovine serum albumin. Toxicon Off. J. Int. Soc. Toxinology 44, 233–241. https://doi.org/10.1016/j.toxicon.2004.04.015
Madureira, A.R., Pereira, C.I., Gomes, A.M.P., Pintado, M.E., Xavier Malcata, F., 2007. Bovine whey proteins – Overview on their main biological properties. Food Res. Int. Ott. Ont 40, 1197–1211. https://doi.org/10.1016/j.foodres.2007.07.005
Rucavado, A., Nicolau, C.A., Escalante, T., Kim, J., Herrera, C., Gutiérrez, J.M., Fox, J.W., 2016. Viperid Envenomation Wound Exudate Contributes to Increased Vascular Permeability via a DAMPs/TLR-4 Mediated Pathway. Toxins 8, 349. https://doi.org/10.3390/toxins8120349
Tang, L., Wu, J.J., Ma, Q., Cui, T., Andreopoulos, F.M., Gil, J., Valdes, J., Davis, S.C., Li, J., 2010. Human lactoferrin stimulates skin keratinocyte function and wound re-epithelialization. Br. J. Dermatol. 163, 38–47. https://doi.org/10.1111/j.1365-2133.2010.09748.x
Wang, Z.L., Tang, X., Wang, M., She, Y.X., Yang, B.R., Sheng, Q.H., Abd El-Aty, A.M., 2024. β-Lactoglobulin separation from whey protein: A comprehensive review of isolation and purification techniques and future perspectives. J. Dairy Sci. 107, 11785–11795. https://doi.org/10.3168/jds.2024-25321

	Xu, R., 2009. Effect of whey protein on the proliferation and differentiation of osteoblasts. J. Dairy Sci. 92, 3014–3018. https://doi.org/10.3168/jds.2008-1702

Cover Letter

Dr Steven R. Hall
Lecturer in Pharmacology and Director of Studies for Pharmacology
Division of Biomedical and Life Sciences

Lancaster University

Dear Prof. Lomonte and your fellow editors of *Toxicon*, **09/03/2026**

My co-authors and I wish to re-submit an original research manuscript titled, "Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebite wound dressings and drug delivery systems inhibit spitting cobra venom-induced cytotoxicity" for consideration to be published in *Toxicon*. I can confirm that this work is original, has not been written with AI, the results have not been published elsewhere, is currently not being considered for publication by any other academic journals, and that we have addressed the comments and concerns posed by the reviewers.

While the overall findings of the manuscript remain largely unchanged – in that the drug tinzaparin (a) successfully incorporates into whey protein isolate (WPI) hydrogels, (b) can be released from these hydrogels upon soaking in saline solution, and (c) retains its ability to inhibit the cytotoxic activity of *Naja nigricollis* venom – we can confirm that the manuscript has been substantially improved following careful consideration of the reviewers' comments. First and foremost, where we previously only tested whether solution in which tinzaparin-containing hydrogels had been soaked for 24 hours could inhibit venom-induced cytotoxicity, we have now demonstrated that such inhibition is also achievable following a much shorter 1-hour soaking period. Secondly, we have repeated the cell viability experiments in which 12.5 µg/mL of *N. nigricollis* venom was used for the control (drug-free) WPI hydrogels and 20 µg/mL for the tinzaparin-containing WPI hydrogels, correcting this inconsistency by performing both conditions using 20 µg/mL of venom. Thirdly, these repeated experiments, while providing stronger evidence that solution derived from tinzaparin-containing hydrogels can inhibit *N. nigricollis* venom-induced cytotoxicity, no longer show that solutions derived from blank (drug-free) hydrogels produce significant inhibition. While this difference may be attributable to the use of 20 rather than 12.5 µg/mL venom in these control hydrogel experiments, the fact remains that these results are no longer statistically significant and therefore should not be discussed as such. Accordingly, much of the discussion surrounding these findings in the previous version of the manuscript has been removed. Finally, all editorial suggestions made by the reviewers have been addressed in our Response to Reviewers document, and all manuscript edits have been highlighted in red text to ensure clarity where all such changes occurred.

In addition, I would like to mention that we have added two co-authors to the author list: Layla Tyrrell and Sam Hyam. This is because this work was initially completed by students of mine and of Dr Douglas' during the 2024-2025 academic year who have since completed their degrees and are no longer in our lab. Sam, Dr Douglas' current Master of Engineering student, therefore prepared new hydrogel discs and Layla, my current Integrated Masters student, then performed the new cellular assays requested by the reviewers. An Authorship Change Request form has been completed for which I have received all required signatures, all of which are included within the manuscript re-submission documents.

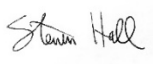
In summary, this manuscript is the first to detail how a drug inhibitor of a snake venom can be incorporated into a hydrogel, retained in that hydrogel, and then later released from that hydrogel at which point the drug retains its venom-inhibiting properties. Whilst much work must be done to confirm if other drugs or antibodies can be used, whether such a biomaterial could be used against other venoms, and whether it would prove effective in a more clinically relevant *in vivo* model of envenoming, it does provide an important initial proof-of-concept for the development of hydrogel-based drug delivery devices that could one day be translated into pre-hospital treatments for the

Dr Steven R. Hall
Lecturer in Pharmacology and Director of Studies for Pharmacology
Division of Biomedical and Life Sciences

local effects of snakebite. My collaborators and I will continue pursuing this line of research in the coming years, and we hope to share further developments with you in future submissions to *Toxicon*.

Thank you kindly for your time and for considering our manuscript re-submission.

Yours sincerely,



Dr Steven R. Hall
T: +44 (0) 7495 809945 | E: s.r.hall@lancaster.ac.uk
Lecturer in Pharmacology and Director of Studies for Pharmacology
Division of Biomedical and Life Sciences | Lancaster University | United Kingdom

Authorship change request

Important information. Please read before completing this form.
This form is to request any change in authorship (additions, removals, or reordering) after the submission of a manuscript, including changes in corresponding authors, if any. This form should not be used for changes requested *after* publication or for name changes or corrections.

Prior to completing this form, all authors should carefully review the 'Duties of Authors' section of the Elsevier publishing ethics policy, and in particular, the sections on:

- Authorship of the paper
- The use of generative AI and AI-assisted technologies in scientific writing and in figures, images and artwork

Please also carefully review the journal's guide for authors (this might also be referred to as 'instructions for authors') as some journals may have additional authorship criteria (e.g., the ICMJE guidelines for authorship).

The publisher and editor cannot investigate or mediate any authorship disputes. If you are unable to obtain agreement from all authors, including those you intend to remove, we recommend seeking guidance from your institution. We will not consider your change request and will not proceed with the publication of your manuscript until all outstanding authorship disputes are resolved.

If your manuscript is still under consideration, this completed form should be submitted in Editorial Manager for consideration as part of your revision submission (use the 'cover letter' file type).

If your manuscript has already been accepted and is in the proofing or production stages, please return this completed form to the Journal Manager.

If the final version of your manuscript has already been published, a corrigendum will be required. Please see the Article Correction, Retraction and Removal Policy.

Section 1. Submission information

To be completed by the corresponding author.

Submission information	
Journal title	Toxicon
Manuscript number and/or article number	TOXCON-D-25-01026R1
Manuscript title	Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebi

Change(s) requested (indicate as appropriate)

☑ Add new author(s)	☐ Remove author(s)
☐ Change the corresponding author	☐ Change the order of authors

Section 2. Author(s) added or removed

Complete one table for each author to be added or removed. Please include as much detail as possible in the "Reason for change" section so that we can evaluate if the change is necessary. At a minimum, this should include an explanation for why the change is being requested and why the author was/was not included in the original author list.

If the form is not provided, incomplete, or the reasons provided are insufficiently detailed or do not address the points above, your request will be denied and your submission may be rejected.

Unless instructed by the editor, further changes to the author list without an approved authorship change request will result in the rejection of your submission, or retraction, if the article has already been published.

2.1 Author information

Given/first name(s)	Layla A.
Family/last name	Tyrrell
Email address	l.tyrrell@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☑ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

As Matt Gray is now a PhD student at another university, Layla (my [Steve Hall's] current MSci student) was the one who repeated the original assays that Matt had previously done and which are now included within Figures 5 and 6. Therefore she should be included as a co-author.

2.2 Author information

Given/first name(s)	Sam J.
Family/last name	Hyam
Email address	s.hyam@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

The original hydrogels were produced by Daniel Baines and Gabrielle de Castro, who have since graduated after this work was initially submitted. For the experiments that had to be repeated for Figures 5 and 6, new hydrogels had to be made. Sam (Tim Douglas' current Masters student) is the one who made these for us to test in our biological assays, and therefore he should also be included as a co-author in the final paper.

2.3 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.4 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.5 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

*Add additional page(s) as needed for more requested changes.

Section 3. Author order and agreement

Provide the author list in the order that you would like it to be published.

The form must be signed individually by each author, including any added/removed authors. In cases of consortia group authorship, the corresponding author may sign on behalf of the group.

While handwritten signatures are acceptable, we highly encourage the use of electronic signature software (DocuSign, Adobe Sign, HelloSign, or similar) with valid e-signatures. These signatures should reflect your institutional information and email, as provided in the author list below. **Typed signatures or images of signatures will not be accepted**.

By signing this form all authors agree:

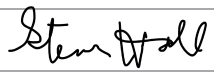
1) that they have read and acknowledge the publishing ethics policies linked in the "Important Information" section of this form;
2) agree to the addition and/or removal of the authors listed in section 2 and to the revised order of the author list in this section 3, and;
3) that all information provided accurately reflects the authorship of the article.

Agreement of removed author(s)

Full name	Email address	Signature	Date

*Add additional page(s) as needed.

Proposed author list

Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		06/03/2026
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request form signature - Author 1

Proposed author list

Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		06/03/2026
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request form signature - Author 2

Proposed author list

Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk	D.Baines	06/03/2026
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request

Important information. Please read before completing this form.
This form is to request any change in authorship (additions, removals, or reordering) after the submission of a manuscript, including changes in corresponding authors, if any. This form should not be used for changes requested *after* publication or for name changes or corrections.

Prior to completing this form, all authors should carefully review the 'Duties of Authors' section of the Elsevier publishing ethics policy, and in particular, the sections on:

- Authorship of the paper
- The use of generative AI and AI-assisted technologies in scientific writing and in figures, images and artwork

Please also carefully review the journal's guide for authors (this might also be referred to as 'instructions for authors') as some journals may have additional authorship criteria (e.g., the ICMJE guidelines for authorship).

The publisher and editor cannot investigate or mediate any authorship disputes. If you are unable to obtain agreement from all authors, including those you intend to remove, we recommend seeking guidance from your institution. We will not consider your change request and will not proceed with the publication of your manuscript until all outstanding authorship disputes are resolved.

If your manuscript is still under consideration, this completed form should be submitted in Editorial Manager for consideration as part of your revision submission (use the 'cover letter' file type).

If your manuscript has already been accepted and is in the proofing or production stages, please return this completed form to the Journal Manager.

If the final version of your manuscript has already been published, a corrigendum will be required. Please see the Article Correction, Retraction and Removal Policy.

Section 1. Submission information

To be completed by the corresponding author.

Submission information	
Journal title	Toxicon
Manuscript number and/or article number	TOXCON-D-25-01026R1
Manuscript title	Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebi

Change(s) requested (indicate as appropriate)

☑ Add new author(s)	☐ Remove author(s)
☐ Change the corresponding author	☐ Change the order of authors

Section 2. Author(s) added or removed

Complete one table for each author to be added or removed. Please include as much detail as possible in the "Reason for change" section so that we can evaluate if the change is necessary. At a minimum, this should include an explanation for why the change is being requested and why the author was/was not included in the original author list.

If the form is not provided, incomplete, or the reasons provided are insufficiently detailed or do not address the points above, your request will be denied and your submission may be rejected.

Unless instructed by the editor, further changes to the author list without an approved authorship change request will result in the rejection of your submission, or retraction, if the article has already been published.

2.1 Author information

Given/first name(s)	Layla A.
Family/last name	Tyrrell
Email address	l.tyrrell@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate)

- ☑ Add new author
- ☐ Remove author
- ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☑ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

As Matt Gray is now a PhD student at another university, Layla (my [Steve Hall's] current MSci student) was the one who repeated the original assays that Matt had previously done and which are now included within Figures 5 and 6. Therefore she should be included as a co-author.

2.2 Author information

Given/first name(s)	Sam J.
Family/last name	Hyam
Email address	s.hyam@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

The original hydrogels were produced by Daniel Baines and Gabrielle de Castro, who have since graduated after this work was initially submitted. For the experiments that had to be repeated for Figures 5 and 6, new hydrogels had to be made. Sam (Tim Douglas' current Masters student) is the one who made these for us to test in our biological assays, and therefore he should also be included as a co-author in the final paper.

2.3 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.4 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.5 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

*Add additional page(s) as needed for more requested changes.

Section 3. Author order and agreement

Provide the author list in the order that you would like it to be published.

The form must be signed individually by each author, including any added/removed authors. In cases of consortia group authorship, the corresponding author may sign on behalf of the group.

While handwritten signatures are acceptable, we highly encourage the use of electronic signature software (DocuSign, Adobe Sign, HelloSign, or similar) with valid e-signatures. These signatures should reflect your institutional information and email, as provided in the author list below. **Typed signatures or images of signatures will not be accepted**.

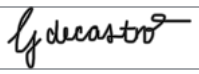
By signing this form all authors agree:

1) that they have read and acknowledge the publishing ethics policies linked in the "Important Information" section of this form;
2) agree to the addition and/or removal of the authors listed in section 2 and to the revised order of the author list in this section 3, and;
3) that all information provided accurately reflects the authorship of the article.

Agreement of removed author(s)

Full name	Email address	Signature	Date

*Add additional page(s) as needed.

Proposed author list				
Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		06/03/2026
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request

Important information. Please read before completing this form.
This form is to request any change in authorship (additions, removals, or reordering) after the submission of a manuscript, including changes in corresponding authors, if any. This form should not be used for changes requested *after* publication or for name changes or corrections.

Prior to completing this form, all authors should carefully review the 'Duties of Authors' section of the Elsevier publishing ethics policy, and in particular, the sections on:

- Authorship of the paper
- The use of generative AI and AI-assisted technologies in scientific writing and in figures, images and artwork

Please also carefully review the journal's guide for authors (this might also be referred to as 'instructions for authors') as some journals may have additional authorship criteria (e.g., the ICMJE guidelines for authorship).

The publisher and editor cannot investigate or mediate any authorship disputes. If you are unable to obtain agreement from all authors, including those you intend to remove, we recommend seeking guidance from your institution. We will not consider your change request and will not proceed with the publication of your manuscript until all outstanding authorship disputes are resolved.

If your manuscript is still under consideration, this completed form should be submitted in Editorial Manager for consideration as part of your revision submission (use the 'cover letter' file type).

If your manuscript has already been accepted and is in the proofing or production stages, please return this completed form to the Journal Manager.

If the final version of your manuscript has already been published, a corrigendum will be required. Please see the Article Correction, Retraction and Removal Policy.

Section 1. Submission information

To be completed by the corresponding author.

Submission information	
Journal title	Toxicon
Manuscript number and/or article number	TOXCON-D-25-01026R1
Manuscript title	Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebi

Change(s) requested (indicate as appropriate)

☑ Add new author(s)	☐ Remove author(s)
☐ Change the corresponding author	☐ Change the order of authors

Section 2. Author(s) added or removed

Complete one table for each author to be added or removed. Please include as much detail as possible in the "Reason for change" section so that we can evaluate if the change is necessary. At a minimum, this should include an explanation for why the change is being requested and why the author was/was not included in the original author list.

If the form is not provided, incomplete, or the reasons provided are insufficiently detailed or do not address the points above, your request will be denied and your submission may be rejected.

Unless instructed by the editor, further changes to the author list without an approved authorship change request will result in the rejection of your submission, or retraction, if the article has already been published.

2.1 Author information

Given/first name(s)	Layla A.
Family/last name	Tyrrell
Email address	l.tyrrell@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☑ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

As Matt Gray is now a PhD student at another university, Layla (my [Steve Hall's] current MSci student) was the one who repeated the original assays that Matt had previously done and which are now included within Figures 5 and 6. Therefore she should be included as a co-author.

2.2 Author information

Given/first name(s)	Sam J.
Family/last name	Hyam
Email address	s.hyam@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

The original hydrogels were produced by Daniel Baines and Gabrielle de Castro, who have since graduated after this work was initially submitted. For the experiments that had to be repeated for Figures 5 and 6, new hydrogels had to be made. Sam (Tim Douglas' current Masters student) is the one who made these for us to test in our biological assays, and therefore he should also be included as a co-author in the final paper.

2.3 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.4 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.5 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

*Add additional page(s) as needed for more requested changes.

Section 3. Author order and agreement

Provide the author list in the order that you would like it to be published.

The form must be signed individually by each author, including any added/removed authors. In cases of consortia group authorship, the corresponding author may sign on behalf of the group.

While handwritten signatures are acceptable, we highly encourage the use of electronic signature software (DocuSign, Adobe Sign, HelloSign, or similar) with valid e-signatures. These signatures should reflect your institutional information and email, as provided in the author list below. **Typed signatures or images of signatures will not be accepted**.

By signing this form all authors agree:

1) that they have read and acknowledge the publishing ethics policies linked in the "Important Information" section of this form;
2) agree to the addition and/or removal of the authors listed in section 2 and to the revised order of the author list in this section 3, and;
3) that all information provided accurately reflects the authorship of the article.

Agreement of removed author(s)

Full name	Email address	Signature	Date

*Add additional page(s) as needed.

Proposed author list				
Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		06/03/2026
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request

Important information. Please read before completing this form.
This form is to request any change in authorship (additions, removals, or reordering) after the submission of a manuscript, including changes in corresponding authors, if any. This form should not be used for changes requested *after* publication or for name changes or corrections.

Prior to completing this form, all authors should carefully review the 'Duties of Authors' section of the Elsevier publishing ethics policy, and in particular, the sections on:

- Authorship of the paper
- The use of generative AI and AI-assisted technologies in scientific writing and in figures, images and artwork

Please also carefully review the journal's guide for authors (this might also be referred to as 'instructions for authors') as some journals may have additional authorship criteria (e.g., the ICMJE guidelines for authorship).

The publisher and editor cannot investigate or mediate any authorship disputes. If you are unable to obtain agreement from all authors, including those you intend to remove, we recommend seeking guidance from your institution. We will not consider your change request and will not proceed with the publication of your manuscript until all outstanding authorship disputes are resolved.

If your manuscript is still under consideration, this completed form should be submitted in Editorial Manager for consideration as part of your revision submission (use the 'cover letter' file type).

If your manuscript has already been accepted and is in the proofing or production stages, please return this completed form to the Journal Manager.

If the final version of your manuscript has already been published, a corrigendum will be required. Please see the Article Correction, Retraction and Removal Policy.

Section 1. Submission information

To be completed by the corresponding author.

Submission information	
Journal title	Toxicon
Manuscript number and/or article number	TOXCON-D-25-01026R1
Manuscript title	Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebi

Change(s) requested (indicate as appropriate)

☑ Add new author(s)	☐ Remove author(s)
☐ Change the corresponding author	☐ Change the order of authors

Section 2. Author(s) added or removed

Complete one table for each author to be added or removed. Please include as much detail as possible in the "Reason for change" section so that we can evaluate if the change is necessary. At a minimum, this should include an explanation for why the change is being requested and why the author was/was not included in the original author list.

If the form is not provided, incomplete, or the reasons provided are insufficiently detailed or do not address the points above, your request will be denied and your submission may be rejected.

Unless instructed by the editor, further changes to the author list without an approved authorship change request will result in the rejection of your submission, or retraction, if the article has already been published.

2.1 Author information

Given/first name(s)	Layla A.
Family/last name	Tyrrell
Email address	l.tyrrell@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☑ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

As Matt Gray is now a PhD student at another university, Layla (my [Steve Hall's] current MSci student) was the one who repeated the original assays that Matt had previously done and which are now included within Figures 5 and 6. Therefore she should be included as a co-author.

2.2 Author information

Given/first name(s)	Sam J.
Family/last name	Hyam
Email address	s.hyam@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

The original hydrogels were produced by Daniel Baines and Gabrielle de Castro, who have since graduated after this work was initially submitted. For the experiments that had to be repeated for Figures 5 and 6, new hydrogels had to be made. Sam (Tim Douglas' current Masters student) is the one who made these for us to test in our biological assays, and therefore he should also be included as a co-author in the final paper.

2.3 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.4 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.5 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

*Add additional page(s) as needed for more requested changes.

Section 3. Author order and agreement

Provide the author list in the order that you would like it to be published.

The form must be signed individually by each author, including any added/removed authors. In cases of consortia group authorship, the corresponding author may sign on behalf of the group.

While handwritten signatures are acceptable, we highly encourage the use of electronic signature software (DocuSign, Adobe Sign, HelloSign, or similar) with valid e-signatures. These signatures should reflect your institutional information and email, as provided in the author list below. **Typed signatures or images of signatures will not be accepted**.

By signing this form all authors agree:

1) that they have read and acknowledge the publishing ethics policies linked in the "Important Information" section of this form;
2) agree to the addition and/or removal of the authors listed in section 2 and to the revised order of the author list in this section 3, and;
3) that all information provided accurately reflects the authorship of the article.

Agreement of removed author(s)

Full name	Email address	Signature	Date

*Add additional page(s) as needed.

Proposed author list				
Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		06/03/26
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Proposed author list

Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk	Sam Hyam	6/3/26
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request

Important information. Please read before completing this form.
This form is to request any change in authorship (additions, removals, or reordering) after the submission of a manuscript, including changes in corresponding authors, if any. This form should not be used for changes requested *after* publication or for name changes or corrections.

Prior to completing this form, all authors should carefully review the 'Duties of Authors' section of the Elsevier publishing ethics policy, and in particular, the sections on:

- Authorship of the paper
- The use of generative AI and AI-assisted technologies in scientific writing and in figures, images and artwork

Please also carefully review the journal's guide for authors (this might also be referred to as 'instructions for authors') as some journals may have additional authorship criteria (e.g., the ICMJE guidelines for authorship).

The publisher and editor cannot investigate or mediate any authorship disputes. If you are unable to obtain agreement from all authors, including those you intend to remove, we recommend seeking guidance from your institution. We will not consider your change request and will not proceed with the publication of your manuscript until all outstanding authorship disputes are resolved.

If your manuscript is still under consideration, this completed form should be submitted in Editorial Manager for consideration as part of your revision submission (use the 'cover letter' file type).

If your manuscript has already been accepted and is in the proofing or production stages, please return this completed form to the Journal Manager.

If the final version of your manuscript has already been published, a corrigendum will be required. Please see the Article Correction, Retraction and Removal Policy.

Section 1. Submission information

To be completed by the corresponding author.

Submission information	
Journal title	Toxicon
Manuscript number and/or article number	TOXCON-D-25-01026R1
Manuscript title	Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebi

Change(s) requested (indicate as appropriate)

☑ Add new author(s)	☐ Remove author(s)
☐ Change the corresponding author	☐ Change the order of authors

Section 2. Author(s) added or removed

Complete one table for each author to be added or removed. Please include as much detail as possible in the "Reason for change" section so that we can evaluate if the change is necessary. At a minimum, this should include an explanation for why the change is being requested and why the author was/was not included in the original author list.

If the form is not provided, incomplete, or the reasons provided are insufficiently detailed or do not address the points above, your request will be denied and your submission may be rejected.

Unless instructed by the editor, further changes to the author list without an approved authorship change request will result in the rejection of your submission, or retraction, if the article has already been published.

2.1 Author information

Given/first name(s)	Layla A.
Family/last name	Tyrrell
Email address	l.tyrrell@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☑ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

As Matt Gray is now a PhD student at another university, Layla (my [Steve Hall's] current MSci student) was the one who repeated the original assays that Matt had previously done and which are now included within Figures 5 and 6. Therefore she should be included as a co-author.

2.2 Author information

Given/first name(s)	Sam J.
Family/last name	Hyam
Email address	s.hyam@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

The original hydrogels were produced by Daniel Baines and Gabrielle de Castro, who have since graduated after this work was initially submitted. For the experiments that had to be repeated for Figures 5 and 6, new hydrogels had to be made. Sam (Tim Douglas' current Masters student) is the one who made these for us to test in our biological assays, and therefore he should also be included as a co-author in the final paper.

2.3 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.4 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.5 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

*Add additional page(s) as needed for more requested changes.

Section 3. Author order and agreement

Provide the author list in the order that you would like it to be published.

The form must be signed individually by each author, including any added/removed authors. In cases of consortia group authorship, the corresponding author may sign on behalf of the group.

While handwritten signatures are acceptable, we highly encourage the use of electronic signature software (DocuSign, Adobe Sign, HelloSign, or similar) with valid e-signatures. These signatures should reflect your institutional information and email, as provided in the author list below. **Typed signatures or images of signatures will not be accepted**.

By signing this form all authors agree:

1) that they have read and acknowledge the publishing ethics policies linked in the "Important Information" section of this form;
2) agree to the addition and/or removal of the authors listed in section 2 and to the revised order of the author list in this section 3, and;
3) that all information provided accurately reflects the authorship of the article.

Agreement of removed author(s)

Full name	Email address	Signature	Date

*Add additional page(s) as needed.

Proposed author list				
Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk	Alan M Smith	06/03/2026
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request

Important information. Please read before completing this form.
This form is to request any change in authorship (additions, removals, or reordering) after the submission of a manuscript, including changes in corresponding authors, if any. This form should not be used for changes requested *after* publication or for name changes or corrections.

Prior to completing this form, all authors should carefully review the 'Duties of Authors' section of the Elsevier publishing ethics policy, and in particular, the sections on:

- Authorship of the paper
- The use of generative AI and AI-assisted technologies in scientific writing and in figures, images and artwork

Please also carefully review the journal's guide for authors (this might also be referred to as 'instructions for authors') as some journals may have additional authorship criteria (e.g., the ICMJE guidelines for authorship).

The publisher and editor cannot investigate or mediate any authorship disputes. If you are unable to obtain agreement from all authors, including those you intend to remove, we recommend seeking guidance from your institution. We will not consider your change request and will not proceed with the publication of your manuscript until all outstanding authorship disputes are resolved.

If your manuscript is still under consideration, this completed form should be submitted in Editorial Manager for consideration as part of your revision submission (use the 'cover letter' file type).

If your manuscript has already been accepted and is in the proofing or production stages, please return this completed form to the Journal Manager.

If the final version of your manuscript has already been published, a corrigendum will be required. Please see the Article Correction, Retraction and Removal Policy.

Section 1. Submission information

To be completed by the corresponding author.

Submission information	
Journal title	Toxicon
Manuscript number and/or article number	TOXCON-D-25-01026R1
Manuscript title	Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebi

Change(s) requested (indicate as appropriate)

☑ Add new author(s)	☐ Remove author(s)
☐ Change the corresponding author	☐ Change the order of authors

Section 2. Author(s) added or removed

Complete one table for each author to be added or removed. Please include as much detail as possible in the "Reason for change" section so that we can evaluate if the change is necessary. At a minimum, this should include an explanation for why the change is being requested and why the author was/was not included in the original author list.

If the form is not provided, incomplete, or the reasons provided are insufficiently detailed or do not address the points above, your request will be denied and your submission may be rejected.

Unless instructed by the editor, further changes to the author list without an approved authorship change request will result in the rejection of your submission, or retraction, if the article has already been published.

2.1 Author information

Given/first name(s)	Layla A.
Family/last name	Tyrrell
Email address	l.tyrrell@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☑ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

As Matt Gray is now a PhD student at another university, Layla (my [Steve Hall's] current MSci student) was the one who repeated the original assays that Matt had previously done and which are now included within Figures 5 and 6. Therefore she should be included as a co-author.

2.2 Author information

Given/first name(s)	Sam J.
Family/last name	Hyam
Email address	s.hyam@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

The original hydrogels were produced by Daniel Baines and Gabrielle de Castro, who have since graduated after this work was initially submitted. For the experiments that had to be repeated for Figures 5 and 6, new hydrogels had to be made. Sam (Tim Douglas' current Masters student) is the one who made these for us to test in our biological assays, and therefore he should also be included as a co-author in the final paper.

2.3 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.4 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.5 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

*Add additional page(s) as needed for more requested changes.

Section 3. Author order and agreement

Provide the author list in the order that you would like it to be published.

The form must be signed individually by each author, including any added/removed authors. In cases of consortia group authorship, the corresponding author may sign on behalf of the group.

While handwritten signatures are acceptable, we highly encourage the use of electronic signature software (DocuSign, Adobe Sign, HelloSign, or similar) with valid e-signatures. These signatures should reflect your institutional information and email, as provided in the author list below. **Typed signatures or images of signatures will not be accepted**.

By signing this form all authors agree:

1) that they have read and acknowledge the publishing ethics policies linked in the "Important Information" section of this form;
2) agree to the addition and/or removal of the authors listed in section 2 and to the revised order of the author list in this section 3, and;
3) that all information provided accurately reflects the authorship of the article.

Agreement of removed author(s)

Full name	Email address	Signature	Date

*Add additional page(s) as needed.

Proposed author list

Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		9th March 2026
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Authorship change request

Important information. Please read before completing this form.
This form is to request any change in authorship (additions, removals, or reordering) after the submission of a manuscript, including changes in corresponding authors, if any. This form should not be used for changes requested *after* publication or for name changes or corrections.

Prior to completing this form, all authors should carefully review the 'Duties of Authors' section of the Elsevier publishing ethics policy, and in particular, the sections on:

- Authorship of the paper
- The use of generative AI and AI-assisted technologies in scientific writing and in figures, images and artwork

Please also carefully review the journal's guide for authors (this might also be referred to as 'instructions for authors') as some journals may have additional authorship criteria (e.g., the ICMJE guidelines for authorship).

The publisher and editor cannot investigate or mediate any authorship disputes. If you are unable to obtain agreement from all authors, including those you intend to remove, we recommend seeking guidance from your institution. We will not consider your change request and will not proceed with the publication of your manuscript until all outstanding authorship disputes are resolved.

If your manuscript is still under consideration, this completed form should be submitted in Editorial Manager for consideration as part of your revision submission (use the 'cover letter' file type).

If your manuscript has already been accepted and is in the proofing or production stages, please return this completed form to the Journal Manager.

If the final version of your manuscript has already been published, a corrigendum will be required. Please see the Article Correction, Retraction and Removal Policy.

Section 1. Submission information

To be completed by the corresponding author.

Submission information	
Journal title	Toxicon
Manuscript number and/or article number	TOXCON-D-25-01026R1
Manuscript title	Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebi

Change(s) requested (indicate as appropriate)

☑ Add new author(s)	☐ Remove author(s)
☐ Change the corresponding author	☐ Change the order of authors

Section 2. Author(s) added or removed

Complete one table for each author to be added or removed. Please include as much detail as possible in the "Reason for change" section so that we can evaluate if the change is necessary. At a minimum, this should include an explanation for why the change is being requested and why the author was/was not included in the original author list.

If the form is not provided, incomplete, or the reasons provided are insufficiently detailed or do not address the points above, your request will be denied and your submission may be rejected.

Unless instructed by the editor, further changes to the author list without an approved authorship change request will result in the rejection of your submission, or retraction, if the article has already been published.

2.1 Author information

Given/first name(s)	Layla A.
Family/last name	Tyrrell
Email address	l.tyrrell@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☑ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

As Matt Gray is now a PhD student at another university, Layla (my [Steve Hall's] current MSci student) was the one who repeated the original assays that Matt had previously done and which are now included within Figures 5 and 6. Therefore she should be included as a co-author.

2.2 Author information

Given/first name(s)	Sam J.
Family/last name	Hyam
Email address	s.hyam@lancaster.ac.uk
Institution	Lancaster University

Change(s) requested (indicate as appropriate) ☑ Add new author ☐ Remove author ☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☑ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☑ Validation	☐ Visualization
☐ Writing – original draft	☑ Writing – review & editing	

Reason for the change

The original hydrogels were produced by Daniel Baines and Gabrielle de Castro, who have since graduated after this work was initially submitted. For the experiments that had to be repeated for Figures 5 and 6, new hydrogels had to be made. Sam (Tim Douglas' current Masters student) is the one who made these for us to test in our biological assays, and therefore he should also be included as a co-author in the final paper.

2.3 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.4 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

2.5 Author information

Given/first name(s)			
Family/last name			
Email address			
Institution			
Change(s) requested (indicate as appropriate)	☐ Add new author	☐ Remove author	☐ Make the corresponding author

Individual contributions per CRediT Contributor Roles Taxonomy (required for author additions only)

☐ Conceptualization	☐ Data curation	☐ Formal analysis
☐ Funding acquisition	☐ Investigation	☐ Methodology
☐ Project administration	☐ Resources	☐ Software
☐ Supervision	☐ Validation	☐ Visualization
☐ Writing – original draft	☐ Writing – review & editing	

Reason for the change

*Add additional page(s) as needed for more requested changes.

Section 3. Author order and agreement

Provide the author list in the order that you would like it to be published.

The form must be signed individually by each author, including any added/removed authors. In cases of consortia group authorship, the corresponding author may sign on behalf of the group.

While handwritten signatures are acceptable, we highly encourage the use of electronic signature software (DocuSign, Adobe Sign, HelloSign, or similar) with valid e-signatures. These signatures should reflect your institutional information and email, as provided in the author list below. **Typed signatures or images of signatures will not be accepted**.

By signing this form all authors agree:

1) that they have read and acknowledge the publishing ethics policies linked in the "Important Information" section of this form;
2) agree to the addition and/or removal of the authors listed in section 2 and to the revised order of the author list in this section 3, and;
3) that all information provided accurately reflects the authorship of the article.

Agreement of removed author(s)

Full name	Email address	Signature	Date

*Add additional page(s) as needed.

Proposed author list

Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		06/03/26
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk		
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Proposed author list				
Order	Full name	Email address	Signature	Date
01	Matthew J. Gray	mgray20@qub.ac.uk		
02	Daniel K. Baines	d.baines3@lancaster.ac.uk		
03	Gabrielle De Castro	gdc22650@gmail.com		
04	Thomas Cobb	cobbthomas35@gmail.com		
05	Layla A. Tyrrell	l.tyrrell@lancaster.ac.uk		
06	Sam J. Hyam	s.hyam@lancaster.ac.uk		
07	Alan M. Smith	a.m.smith@hud.ac.uk		
08	Nicholas R. Casewell	nicholas.casewell@lstmed.ac.uk		
09	Sarah L. Allinson	s.allinson@lancaster.ac.uk		
10	Timothy E. L. Douglas	t.douglas@lancaster.ac.uk	Timothy E.L. Douglas	6/3/2026
11	Steven R. Hall	s.r.hall@lancaster.ac.uk		
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

*Add additional page(s) as needed.

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Steven Hall reports financial support was provided by Friends of Lancaster University in America. Steven Hall reports financial support was provided by Nicholas Bone (philanthropic donation). Given Prof Casewell's role as part of Toxicon's Editorial Council, he had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

No experiments involving human participants or live animals were performed for this project. The venom used in this project was collected from snakes maintained in the Centre for Snakebite Research and Interventions' Herpetarium facility, whose animal protocols are approved by the UK Home Office, the Liverpool School of Tropical Medicine and the University of Liverpool's Animal Welfare and Ethical Review Boards.

Response to Reviewers

We would like to sincerely thank the editor and reviewers for their time and their detailed commentary. We would also like to thank them for their patience in allowing us an extended deadline to complete further experimentation addressing their concerns with the previous version of this study. We believe that we have adequately tackled the identified issues, and that the suggested edits have been implemented to produce an improved manuscript. Below you can find a point-by-point response to the specific comments made. For clarity, please note that all revisions to the manuscript have been highlighted using red font, including minor errors we noticed in our original writing that we edited of our own accord including italicising all instances of *et al.*

Reviewer #1

Background: cytotoxins are the prevail components in the venom of this cobra. Heparins bind and neutralize them. The WPI-based hydrogel is capable of including heparin. Methodology: the authors incubated tinzaparin-containing hydrogel with PBS for a 24h, measured the release of tinzaparin (after 24h), and established its neutralizing activity towards the cytotoxicity of cobra venom. Findings: The authors have found that tinzaparin retains its ability to neutralize cobra venom cytotoxicity being released into PBS from WPI-based hydrogel during 24h incubation. To my knowledge, this is the first report on activity of pure WPI-hydrogel against cobra venom cytotoxicity. Roughly, this suggests the possibility of using such a drug to treat a cobra bite. In addition, the authors provided some data (SEM and others) on heparin-containing WPI-hydrogels. Although the latest data are not directly related to toxinology, they are of interest for the pharmacy technology.

In overall, the manuscript is well-written and of interest. Graphical abstract, figures, and tables are relevant.

We would like to thank Reviewer #1 for their thorough evaluation and supportive comments.

However, some questions require answers before recommendation for publication.

The main issue is: the effect of cytotoxins develops quite quickly (in the very first minutes of envenomation; and even delayed effects, such as necrotic lesions, are triggered in the first hours after the bite). The questions are: why did the authors not investigate the release of tinzaparin from the hydrogel in the first 1-3-5 hours of its application? Why are they waiting 24 hours? The speed of the onset of the drug's effect is critically important for the successful recovery of a bitten person. After 24 hours, all pathological processes have already started and are in full swing. Will 10%T be effective if applied immediately (does tinzaparin release enough, for example, within an hour or three)? The fact that an effective final concentration of tinzaparin has accumulated in PBS after 24 hours does not guarantee an effect in the early stages of the lesion, while this is important. Therefore, based on the data provided, it is premature to talk about 10%T as a promising treatment for snake bites. Based on the above, I strongly recommend the authors either soften the wording regarding the use of 10%T as a medicine (wound dressing) for cobra bite (including in the title of the manuscript and elsewhere in text, e.g. Lines 539 and 549), or to determine the release of tinzaparin from the hydrogel in PBS during the first hours of incubation.

This is a very fair point, and we agree that showing effective tinzaparin release at more appropriate time points is crucial for demonstrating the potential of WPI hydrogels. To address this, we have conducted additional experimentation to determine the release of tinzaparin from a hydrogel at the

more biologically relevant timepoint of 1 hour. This work is summarised in the new **Fig. 5** which demonstrates that tinzaparin is released from the gels at high concentrations within the first hour, with no significant difference to the release after 24 hours. Several changes have been made to the manuscript following the acquisition of these results. Sam Hyam and Layla Tyrrell have been added to the list of authors for the creation of a new batch of tinzaparin-containing WPI hydrogel discs and for completing this new set of requested experiments, respectively.

Throughout the document, details have been added to include the 1-hour incubation and the 24-hour incubation. For the methods section, under subheading 2.2.7., lines 224-233 have been rewritten to detail an updated analysis method for quantifying tinzaparin release, wherein each sample was compared to each standard curve and the average found, which should give a more accurate measurement of how much tinzaparin is within each sample. In addition, detail has been added regarding the new 1-hour incubation time point. This section now reads:

"To measure drug release from the tinzaparin-containing WPI hydrogel, 10% T and WPI control discs were soaked in PBS (3 discs / 180 µL PBS) for 1 hour or 24 hours under standard conditions (37 oC, 5% CO2). Over three experimental replicates, 50 microlitres of the resulting solutions were pipetted into separate wells in a half-volume 96-well UV-capable microplate along with serial dilutions of known tinzaparin concentrations (4,000 – 31.25 µg/mL, with a halving of the concentration with each serial dilution). A230 was measured in each well using a Tecan MPLEX plate reader and a standard curve plotted for each known tinzaparin concentration serial dilution, from which the concentration of tinzaparin released from the 10% tinzaparin-containing discs under standard conditions was determined for each of three experimental replicates and the means calculated."

In the results section, a new version of **Fig. 5** has been produced, with the absorbance of new tinzaparin standard curves being given in **Fig. 5B**, and the quantity of tinzaparin released after incubation in PBS for 1 hour compared to 24 hours being given in the new **Fig. 5C**. The figure legend has also been revised to describe these new figures. The paragraph following this describing the results has also been edited to reflect the new data and experimental methods (lines 396-407).

In addition, we then also repeated the venom-inhibition experiments with the 1 hour-soaked 10% T hydrogel solution, which provided statistically significant evidence that this solution was capable of inhibiting the *N. nigricollis* venom's cytotoxic activity (**Fig. 6B**). Finally, **Table 3** has been revised to include the 1-hour tinzaparin release concentration (2.80 mg/mL) and that of the tinzaparin concentration released after incubation for 24 hours determined after these new experiments (3.05mg/mL), since new hydrogel discs had to be prepared for their completion.

The discussion section has principally been edited between lines 506-523 to discuss the new 1-hour incubations and to utilise the updated tinzaparin concentrations. In addition, we have softened the language at the end of this paragraph regarding the use of these hydrogels as medicines for the treatment of snakebite in line with the reviewer's suggestion. Additional editing for this purpose has been conducted in the conclusions.

Thank you to Reviewer #1 for this comment. We believe the additional experiments that have been undertaken in response address their valid critique.

Line 104. There's probably a typo here. Heparis is a hepatoprotector food supplement.

This is a typo and has now been corrected to "heparin".

Line 141 (or Line 208). What the proteases have been used? Human collagenases?

The proteases used for this experiment were a dried mixture of proteases produced from bovine pancreas obtained from Sigma-Aldrich, incubated with the hydrogels at a concentration consistent with human blood collagenase levels. For improved clarity, this sentence (starting in line 209) has been altered to read:

"Five hydrogel samples were incubated in 200 µL of pH 7.4 PBS containing 13.33 µg/mL protease mixture obtained from bovine pancreas (CAS no. 9001-92-7), concentrations consistent with blood collagenase levels"

Line 384. Here it states that PBS incubated with 10% WPI was used as a solution for control samples of 10%T. However, the hydrogel used for 10%T was 40% WPI (Table 1). 40% WPI releases protein into solution in 24 hours with an absorption of about 0.3 A at 280 nm (Fig. 4A). Then, taking into account a typical protein spectrum, absorption at 230 nm should be about 1 A, or even more. Therefore, 10% WPI is an unsuitable control for calculating tinzaparin release, since an increase in 0.5-1.5 A absorption at 230 nm will mostly be due to protein loss from the 10%T preparation based on 40%WPI, rather than the release of tinzaparin.

The statement "10% WPI" was a typo. This should instead read "40% WPI", as the same control gel was used here as with other experiments. As such, there should not be a technical issue to address, and the typo has now been corrected in the manuscript. Thank you for catching this error.

Line 414. What prevented the authors from taking the same venom concentration in A and B? The authors describe a purely technical situation (Lines 525-535). I suggest the authors repeat this important experiment both A and B under the equal conditions; otherwise, these data raise doubts about the validity of the observed difference.

This is again a highly valid critique. The experiments summarised in **Fig. 6** have therefore been repeated to ensure that an equal venom concentration (20 µg/mL) could be used for both 10% T and 40% WPI hydrogels, as well as exploring the effect of PBS solutions incubated with either hydrogel type for only 1 hour compared to 24 hours. Surprisingly, whilst this has continued to demonstrate that the solutions generated from 10% T hydrogel incubations, including 1-hour incubations, are able to inhibit spitting cobra venom cytotoxicity, *albeit* at slightly different concentrations than previously observed, the significant inhibitory effect from the blank 40% WPI hydrogels solutions was no longer seen. As a result of this, changes have been made to this manuscript to reflect these new results which are no longer statistically significant by removing any previous mention of such significance, and instead now focuses on the significant results observed from the 10% T hydrogel solution. We did, however, feel it was important to mention that despite the lack of significance, it was still interesting how the mean % cell viability in cells treated with venom and the blank WPI hydrogel solution was slightly higher than the venom-only control, and that further research is required to determine if this is a real effect that could be exploited in future snakebite therapies or not.

The methods section has been rewritten to accurately detail the methodologies used for the new and repeated experiments. Under subheading 2.2.8., this section has been modified to reflect that the cytotoxicity assays were repeated using the same venom concentration for each condition, and now reads:

"Three discs of both hydrogel types were incubated in the same manner for 1 hour. Serial dilutions of the resulting hydrogel solutions (0.08x to 1.95x10^{-5}x dilutions with a quartering of the concentration with each serial dilution) were mixed with a constant 20 µg/mL N. nigricollis venom or venom vehicle control (PBS) in Low Background Fluorescence Medium."

Several sections from the results section have also needed to be modified to reflect these new results. Firstly, the title of section 3.6 is now:

"3.6. 10% Tinzaparin WPI Hydrogels Inhibit N. nigricollis Venom Cytotoxicity"

Fig. 6 has now been improved using the new experimental data based on the reviewer's suggestions, with all experiments being conducted at the same venom concentration (20 µg/mL), as well as additional cytotoxicity assays being conducted on solutions derived from 1-hour hydrogel incubations which should more accurately reflect the realities of treating snakebite in the field (**Fig. 6A** and **6B**). The figure legend has also been updated to describe the new figure. The following paragraph describing the results shown in **Fig. 6** and **Table 3** has therefore also been rewritten:

"*HaCaT cells exposed to N. nigricollis venom and treated with vehicle control for both hydrogel experiments effectively reduced cell viability to close to 0%, confirming that cytotoxic venom concentrations were used in both cases (Fig. 6). The supernatant from the 40% control hydrogels for both incubation periods did not significantly increase cell viability at any concentration, although mean cell viability did increase from the vehicle control at most concentrations (Fig. 6A, 6C). The 1-hour incubation, 10% T hydrogel supernatant dilution series effectively inhibited the venom-induced cytotoxicity only at the 0.02x dilution which contained approximately 56 µg/mL of T (Table 3), resulting in a significant increase in mean cell viability to 52.5% (Fig. 6B). The 24-hour incubation, 10% T hydrogel dilution series was able to inhibit N. nigricollis venom cytotoxicity at a greater concentration range than the 1-hour incubation, significantly increasing cell viability at dilution factors of 0.05, 0.02 and 0.08 (15, 61 and 244 µg/mL [Table 3]), resulting in cell viabilities of 39.5%, 48.8% and 31.5% respectively (Fig. 6D)*."

Finally, the discussion section has been amended in several places to reflect the new experimental results and to remove mention of the previously significant cytoprotective effect of 40% WPI hydrogels. The first of these is between lines 514-517, which has been altered to read:

"As predicted, the 24-hour incubated, 10% T hydrogel-derived solution successfully inhibited N. nigricollis venom-induced skin cell cytotoxicity at several tested serial dilutions (15 – 244 µg/mL of T; Table 3), and at one tested concentration for the solution derived from a 1-hour incubation (61 □g/ml of T; Table 3). These results confirm that T-integrated WPI hydrogels can release drug at sufficiently high concentrations within as little as an hour to inhibit snake venom-induced cytotoxicity – a valuable characteristic since rapid treatment is crucial for necrosis prevention (Gutiérrez et al., 2017a). As such, this evidence suggests that drug-incorporated WPI hydrogels warrant further research and development as potential pre-hospital drug delivery devices for the treatment of snakebite envenoming. "

As Reviewer #1 requested, all the new experiments have been repeated using the same concentration of *N. nigricollis* venom (20 µg/mL). As such, the paragraph which detailed the limitation inherent in using different venom concentrations (previously lines 525-535) has been removed entirely, and the reference found only in this paragraph (Jain *et al.*, 2021) removed from the reference list.

Finally, the final paragraph of the discussion as well as the conclusions (have also been edited to accurately reflect the data generated by the new experiments, including the new 1-hour incubation timepoints and the non-significant results from the blank hydrogels.

Thank you again to Reviewer #1 for their valid criticism which, after completing these new experiments, has certainly improved our manuscript.

Line 547. Baines et al., 2024 used hydrogel but did not use varispladib or another anti-venom drug; Lewin et al., 2016 used varespladib but did not use hydrogels or any similar delivery system. I understand what the authors meant by that, but these references don't complement each other in a reason way, so they seem to be improper here.

We thank Reviewer #1 for flagging this. This section has been rewritten to now read:

"In addition, a range of other snake venom inhibitors which are currently under investigation such as varespladib, a hydrophobic inhibitor of snake venom PLA2s (Clare et al., 2021; Lewin et al., 2016) , could be incorporated into hydrogels and tested against a range of snake venoms. This could exploit WPI hydrogels' ability to bind and release hydrophobic drugs (Baines et al., 2024)."

Reviewer #2

This research manuscript investigated whether heparin-containing WPI hydrogels displayed physical characteristics suitable for wound dressings and could effectively release drug in sufficient quantities to inhibit the cytotoxic activity of spitting cobra venom.
I have read the manuscript and have some suggestions that I recommend to do on it to be accepted:

1- On Introduction on line 87, the authors mentioned that..." Some promising candidates are heparins including unfractionated heparin..." There are previous relevant publications on heparins as antivenom against diferente snakes from different continents, that they should be mentioned in your citations.

We thank reviewer #2 for their feedback. This is a fair comment. Additional historic citations about the use of heparins as antidotes against snake venoms across the globe have now been included and the sentence between lines 88-92 reworded to read:

"Some promising candidates are heparins including unfractionated heparin (H), a highly sulphated polysaccharide previously explored for hemotoxic snakebite treatment, and its low molecular weight (LMW) heparinoid variant, tinzaparin (T), both of which are anticoagulants traditionally used for thromboembolic indications (Boechat et al., 2001; Christy et al., 1973; Du et al., 2024; Hirsh, 1998)."

In addition, the full citations for Boechat *et al.*, 2001 and Christy *et al.*, 1973 have been added to the reference list.

2- On line 104 is the word heparis correct ?

Apologies, this was a typo. It has now been corrected to "heparin".

3- Which protein composition has the WPI hydrogels? If it contains casein, could explain some results of your data about incorporating the snake venom component. It would be better to add more specific information about the WPI. On line 269 Fig., the morphological changes induced by the polyanions on the surface of WPI indicated its contain charged amino acid, which allowed the incorporation of heparins. This needs a comment to explain it.

Whey protein isolate does not contain casein, as part of the process involved in producing WPI from milk is to remove casein proteins (Khalid *et al.*, 2025). Specific information about the composition of WPI has also been added to the discussion section over Lines 447-449, reading:

"WPI is >97% protein, of which the majority is β-lactoglobulin (75%), however other components include α-lactalbumin, immunoglobulins, bovine serum albumin, bovine lactoferrin and lactoperoxidase (Keppler et al., 2014; Madureira et al., 2007)."

Additionally, β-lactoglobulin has been reported to have an isolelectric point of pH 5.3 in the native state which would make it negatively charged at neutral pH (Wang *et al.*, 2024). However, it is possible that the denaturation of β-lactoglobulin caused by heating may change the isoelectic point resulting in the binding of heparinoid polyanions, however this is only speculative. A comment has been added to explain this in the discussion between Lines 450-454, reading:

"β-lactoglobulin has been reported to be negatively charged in the native state (isoelectric point = pH 5.3), with polyanions such as heparin typically binding patches of positively charged amino acids (Kamerzell et al., 2011; Wang et al., 2024). However, it may be possible that protein denaturation caused by heating may alter the isoelectric point, allowing heparin polyanion binding."

In addition, the full citations relating to these additional in-text citations have been added to the reference list.

Note that the decision was made to add these further details to the discussion section rather than the results section (i.e. on Line 269) as they are a commentary upon the results found instead of a description of the results.

4- On line 228 topic 2.2. Cytotoxicity Assay, please indicate the temperature that the experiment was performed on.

The temperature under which tinzaparin was released from the gel was 37°C. This has been corrected in the manuscript by adding the term "under Standard Conditions", defined earlier in the document as 37°C and 5% CO_2.

5- On line 452-453 Could the authors explain better this statment?

Thank you to Reviewer #2 for pointing out the ambiguous nature of this statement. This has now been expanded to say:

"The improved swelling properties observed in the WPI hydrogels integrated with heparins is also a desirable characteristic for a snakebite wound dressing, as snakebite envenoming often produces excess wound exudate which should be removed for more effective wound healing (Collier et al., 2014; Ibiapina et al., 2019; Rucavado et al., 2016)."

The additional citation by Collier *et al.*, 2014 details why excess wound exudate should be removed, and this has also been added to the reference list.

6- Line 503-504 - It could interact as a substract for the venom, decreasing the amount of free active toxins

Thank you to Reviewer #2 for this suggestion regarding a possible mechanism of action regarding the inhibition of cytotoxicity by the blank 40% hydrogel-derived solutions. However, as we are no longer observing a significant cytoprotective effect from these solutions after repeating these experiments, we have stripped back the commentary about the effects of the blank 40% hydrogel-derived solutions from the discussion, as previously discussed in our response to a comment from Reviewer #1, and as such will no longer go into extended detail regarding a possible mechanism of action.

Final comments: I have enjoyed to read this this relevant manuscript that raises an important issue in Toxinology, the development of experimental models. New and good experimental models need time to be reproduced and become relevant. This is an in vitro model that show the relevance of polyanions as antitoxin agents against isnake venom.

After a good revision and adjustment in the citations it will be better suited for acceptance.

We thank Reviewer #2 for their kind words as well as their suggestions for how we could improve our manuscript.

Editorial Comments

Owing to the model limitations, it would seem necessary to tone down the concluding statements on the suitability of this kind of device until more detailed evidence of its translational usefulness is gathered.

Thank you to the editor for their review of our manuscript. Due to the additional experiments undertaken to improve this manuscript, we believe that the translational utility of these heparinoid-incorporated WPI hydrogels is now much less in question, as we have demonstrated that they are able to operate at more relevant time points, as well as improving the validity of the cytotoxicity assay experiments by using the same concentration of venom for each experiment. However, we have also taken the editors suggestion to soften the language used in some of our concluding experiments, as we acknowledge more information is needed before we can confidently state that this methodology could be used for in-the-field treatment of cytotoxic snakebite injury. This includes commentary within the discussion (lines 521-523) and conclusions (lines 560).

The work is certainly important and interesting. The final sentence in the cover letter "...our results do suggest that the integration and diffusion of venom-inhibiting drugs in WPI hydrogels is possible and that the development of such drug-integrated hydrogels into snakebite wound dressings warrants further research." seemed to me even better than the final sentence of the abstract "...these data suggest drug inhibitor-containing WPI hydrogels could be suitable for translation into snakebite wound dressings for field use, and that future work is certainly warranted". Just a personal suggestion.

We thank the editor for their kind words and for their observation on the final sentence of the abstract. This sentence has now been replaced with the conclusion of the cover letter, now reading:

"Together, our results suggest that the integration and diffusion of venom-inhibiting drugs in WPI hydrogels is possible and that the development of such drug-integrated hydrogels into snakebite wound dressings warrants further research."

Bibliography

Baines, D.K., Wright, K., Douglas, T.E.L., 2024. Preliminary In Vitro Assessment of Whey Protein Isolate Hydrogel with Cannabidiol as a Potential Hydrophobic Oral Drug Delivery System for Colorectal Cancer Therapy. Polymers 16, 3273. https://doi.org/10.3390/polym16233273

Boechat, A.L.R., Paiva, C.S., França, F.O., Dos-Santos, M.C., 2001. Heparin-antivenom association: differential neutralization effectiveness in Bothrops atrox and Bothrops erythromelas envenoming. Rev. Inst. Med. Trop. São Paulo 43, 07–14. https://doi.org/10.1590/S0036-46652001000100002

Castro-Amorim, J., Novo de Oliveira, A., Da Silva, S.L., Soares, A.M., Mukherjee, A.K., Ramos, M.J., Fernandes, P.A., 2023. Catalytically Active Snake Venom PLA2 Enzymes: An Overview of Its Elusive Mechanisms of Reaction. J. Med. Chem. 66, 5364–5376. https://doi.org/10.1021/acs.jmedchem.3c00097

Christy, N.P., Weiss, H.J., Phillips, L.L., Phillips, G.B., Hopewell, W., 1973. Exotic snakebite in an urban setting: heparin therapy of disseminated intravascular coagulation in a patient bitten by a saw-scaled viper (Echis carinatus). Trans. Am. Clin. Climatol. Assoc. 84, 37–48.

Clare, R.H., Hall, S.R., Patel, R.N., Casewell, N.R., 2021. Small Molecule Drug Discovery for Neglected Tropical Snakebite. Trends Pharmacol. Sci. 42, 340–353. https://doi.org/10.1016/j.tips.2021.02.005

Collier, M., Potts, C., Shaw, E., 2014. Use of a coverlet system for the management of skin microclimate. Br. J. Nurs. Mark Allen Publ. 23, S28, S30-35. https://doi.org/10.12968/bjon.2014.23.Sup15.s28

Du, T.Y., Hall, S.R., Chung, F., Kurdyukov, S., Crittenden, E., Patel, K., Dawson, C.A., Westhorpe, A.P., Bartlett, K.E., Rasmussen, S.A., Moreno, C.L., Denes, C.E., Albulescu, L.-O., Marriott, A.E., Mackay, J.P., Wilkinson, M.C., Gutiérrez, J.M., Casewell, N.R., Neely, G.G., 2024. Molecular dissection of cobra venom highlights heparinoids as an antidote for spitting cobra envenoming. Sci. Transl. Med. 16, eadk4802. https://doi.org/10.1126/scitranslmed.adk4802

Hiremath, K., Dodakallanavar, J., Sampat, G.H., Patil, V.S., Harish, D.R., Chavan, R., Hegde, H.V., Roy, S., 2024. Three finger toxins of elapids: structure, function, clinical applications and its inhibitors. Mol. Divers. 28, 3409–3426. https://doi.org/10.1007/s11030-023-10734-3

Hirsh, J., 1998. Low-Molecular-Weight Heparin. Circulation 98, 1575–1582. https://doi.org/10.1161/01.CIR.98.15.1575

Ibiapina, H.N.S., Costa, A.G., Sachett, J.A.G., Silva, I.M., Tarragô, A.M., Neves, J.C.F., Kerr, M.W.A., Santana, M.F., Martins-Filho, O.A., Lacerda, M.V.G., Ferreira, L.C.L., Malheiro, A., Monteiro, W.M., 2019. An Immunological Stairway to Severe Tissue Complication Assembly in Bothrops atrox Snakebites. Front. Immunol. 10, 1882. https://doi.org/10.3389/fimmu.2019.01882

Jain, K., Salamat-Miller, N., Taylor, K., 2021. Freeze–thaw characterization process to minimize aggregation and enable drug product manufacturing of protein based therapeutics. Sci. Rep. 11, 11332. https://doi.org/10.1038/s41598-021-90772-9

Keppler, J.K., Sönnichsen, F.D., Lorenzen, P.-C., Schwarz, K., 2014. Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and fluorescence quenching. Biochim. Biophys. Acta BBA - Proteins Proteomics 1844, 1083–1093. https://doi.org/10.1016/j.bbapap.2014.02.007

Khalid, M., Ghasem, N., Yang, X., Schroen, K., Nazir, A., 2025. Progress in Membrane Technology for the Enrichment of Individual Whey Proteins. Ind. Eng. Chem. Res. 64, 19305–19321. https://doi.org/10.1021/acs.iecr.5c00643

Kilara, A., Vaghela, M.N., 2018. Whey proteins, in: Proteins in Food Processing. Woodhead Publishing, pp. 93–126. https://doi.org/10.1016/B978-0-08-100722-8.00005-X

Lewin, M., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) Appears to Be a Potent, Broad-Spectrum, Inhibitor of Snake Venom Phospholipase A2 and a Possible Pre-Referral Treatment for Envenomation. Toxins 8, 248. https://doi.org/10.3390/toxins8090248

Machado de Avila, R.A., Alvarenga, L.M., Tavares, C. a. P., Molina, F., Granier, C., Chávez-Olórtegui, C., 2004. Molecular characterization of protective antibodies raised in mice by Tityus serrulatus scorpion venom toxins conjugated to bovine serum albumin. Toxicon Off. J. Int. Soc. Toxinology 44, 233–241. https://doi.org/10.1016/j.toxicon.2004.04.015

Madureira, A.R., Pereira, C.I., Gomes, A.M.P., Pintado, M.E., Xavier Malcata, F., 2007. Bovine whey proteins – Overview on their main biological properties. Food Res. Int. Ott. Ont 40, 1197–1211. https://doi.org/10.1016/j.foodres.2007.07.005

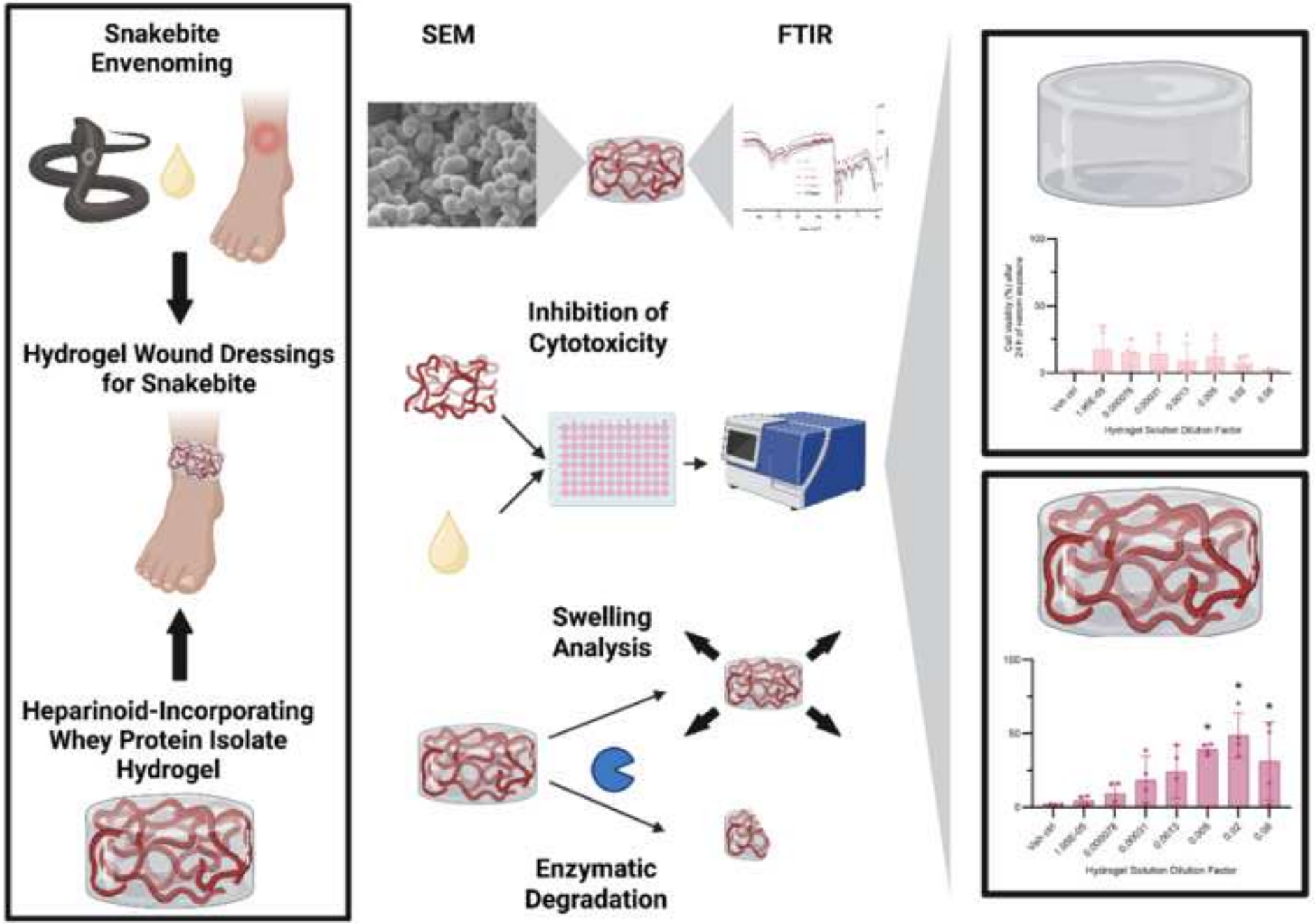
Rucavado, A., Nicolau, C.A., Escalante, T., Kim, J., Herrera, C., Gutiérrez, J.M., Fox, J.W., 2016. Viperid Envenomation Wound Exudate Contributes to Increased Vascular Permeability via a DAMPs/TLR-4 Mediated Pathway. Toxins 8, 349. https://doi.org/10.3390/toxins8120349

Tang, L., Wu, J.J., Ma, Q., Cui, T., Andreopoulos, F.M., Gil, J., Valdes, J., Davis, S.C., Li, J., 2010. Human lactoferrin stimulates skin keratinocyte function and wound re-epithelialization. Br. J. Dermatol. 163, 38–47. https://doi.org/10.1111/j.1365-2133.2010.09748.x

Wang, Z.L., Tang, X., Wang, M., She, Y.X., Yang, B.R., Sheng, Q.H., Abd El-Aty, A.M., 2024. β-Lactoglobulin separation from whey protein: A comprehensive review of isolation and purification techniques and future perspectives. J. Dairy Sci. 107, 11785–11795. https://doi.org/10.3168/jds.2024-25321

Xu, R., 2009. Effect of whey protein on the proliferation and differentiation of osteoblasts. J. Dairy Sci. 92, 3014–3018. https://doi.org/10.3168/jds.2008-1702

Graphical Abstract



- Whey protein isolate (WPI) hydrogels incorporating heparin or tinzaparin were designed to act as snakebite wound dressings.
- Heparin-integration improves WPI hydrogels' swelling properties but reduces their longevity in model wound environments.
- WPI hydrogels released tinzaparin into solution at high concentrations following incubation at both 1 and 24 hours
- 1 and 24-hour tinzaparin-containing solutions inhibited *N. nigricollis* venom cytotoxicity in a dermonecrosis cell model.

Title

Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebite wound dressings and drug delivery systems inhibit spitting cobra venom-induced cytotoxicity

Author Names and Affiliations

Matthew J. Gray[a,1], Daniel K. Baines[b], Gabrielle De Castro[b], Thomas Cobb[a], Layla A. Tyrrell[a], Sam J. Hyam[a], Alan M. Smith[c], Nicholas R. Casewell[d,e], Sarah L. Allinson[a], Timothy E. L. Douglas[b†], and Steven R. Hall[a†]

[a]Division of Biomedical and Life Sciences, Lancaster University, Bailrigg, Lancaster LA1 4YW, UK. [b]School of Engineering, Lancaster University, Bailrigg, Lancaster LA1 4YW, UK. [c]Department of Pharmacy, University of Huddersfield, Queensgate, Huddersfield HD1 3DH, UK. [d]Centre for Snakebite Research & Interventions, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. [e]Centre for Drugs & Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK.

[†]Co-corresponding authors. TELD email: t.douglas@lancaster.ac.uk, SRH email: s.r.hall@lancaster.ac.uk

Present address: [1]School of Biological Sciences, Queen's University Belfast, University Road, Belfast BT7 1NN, UK.

Highlights

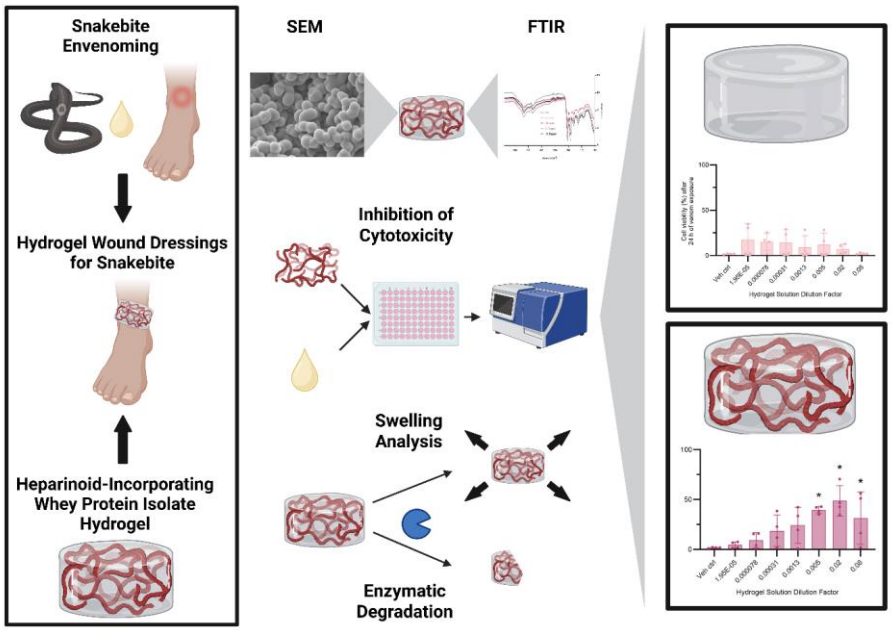
- Whey protein isolate (WPI) hydrogels incorporating heparin or tinzaparin were designed to act as snakebite wound dressings.
- Heparin-integration improves WPI hydrogels' swelling properties but reduces their longevity in model wound environments.
- WPI hydrogels released tinzaparin into solution at high concentrations following incubation at both 1 and 24 hours
- 1 and 24-hour tinzaparin-containing solutions inhibited *N. nigricollis* venom cytotoxicity in a dermonecrosis cell model.

Abstract

Snakebite envenoming affects millions of people annually, with current treatments limited to animal-derived antivenoms. Repurposed drug-inhibitors of toxin families offer an exploitable avenue to improve snakebite treatment, including heparins which can inhibit cytotoxic three-finger toxins. However, to be effective therapies in-the-field such treatments must be engineered into drug delivery devices capable of rapidly administering drug(s) to the envenomation site. Herein we introduce the concept of integrating heparins, specifically unfractionated heparin (H) and its low molecular weight heparinoid variant, tinzaparin (T), into hydrogels composed of whey protein isolate (WPI), an inexpensive byproduct of the dairy industry that is cytocompatible, stiff, sterilizable by autoclaving, and that has the dual function of being able to locally deliver drugs and act as wound dressings. The

aims of this research were to investigate whether heparin-containing WPI hydrogels displayed physical characteristics suitable for wound dressings and could effectively release drug in sufficient quantities to inhibit the cytotoxic activity of spitting cobra venom. To do so, five hydrogel prototypes were produced: 40% WPI no drug control, and 5% H, 10% H, 5% T, and 10% T all within 40% WPI hydrogels. It was determined that heparins successfully integrated into WPI hydrogels and heparin-containing WPI hydrogels exhibited improved swelling versus the 40% WPI no drug control, suggesting improved absorption of wound exudate; however, this heparin integration also increased the hydrogel degradation rate in simulated wound environments. Most importantly, sufficiently high concentrations of T were released into phosphate buffered saline from the 10% T hydrogels to neutralise *Naja nigricollis* venom cytotoxicity in a HaCaT cell cytotoxicity model. Together, our results suggest that the integration and diffusion of venom-inhibiting drugs in WPI hydrogels is possible and that the development of such drug-integrated hydrogels into snakebite wound dressings warrants further research.

Graphical Abstract



Created in BioRender. Gray, M. (2026) https://BioRender.com/uf60qur

Keywords

Snakebite, Venom, Hydrogel, Heparins, Wound-dressing, Cytotoxicity

Abbreviations

3FTx – 3-Finger Toxin

ANOVA – Analysis of Variance

CSRI – Centre for Snakebite Research & Interventions

DMEM - Dulbecco's Modified Eagles Medium

FBS – Foetal Bovine Serum

H - Heparin

LMW – Low Molecular Weight

LSTM – Liverpool School of Tropical Medicine

NTD – Neglected Tropical Disease

PBS – Phosphate Buffered saline

PLA_2 – Phospholipase A_2

SEM – Scanning Electron Microscopy

SVMP – Snake Venom Metalloprotease

T - Tinzaparin

WPI – Whey Protein Isolate

1. Introduction

Snakebite envenoming is classified by the World Health Organization (WHO) as a neglected tropical disease (NTD), responsible for up to 131,000 deaths and 400,000 cases of long-term disability every year (Afroz *et al.*, 2024; J. M. Gutiérrez *et al.*, 2017a). Compounding this public health issue is the fact that the only currently available specific treatments are animal-derived antivenoms, which have remained broadly unchanged for over a century (Lalloo and Theakston, 2003). These conventional antivenoms suffer numerous limitations: often exhibiting poor pan-snake species efficacy, high expense of treatment, limited effectiveness at neutralising severe local envenoming caused by venom cytotoxicity, the requirement to be administered intravenously in a hospital environment, the need to be kept cold, and potentially serious adverse events that frequently develop (Alangode *et al.*, 2020; Clare *et al.*, 2021; Rivel *et al.*, 2016).

As such, other possible treatment options have begun to be explored, including the use of repurposed drug inhibitors targeting the main classes of toxins found in many different snake venoms across different species and genera (Clare *et al.*, 2021). Some promising candidates are heparins including unfractionated heparin (H), a highly sulphated polysaccharide previously explored for hemotoxic snakebite treatment, and its low molecular weight (LMW) heparinoid variant, tinzaparin (T), both of which are anticoagulants traditionally used for thromboembolic indications (Boechat *et al.*, 2001; Christy *et al.*, 1973; Du *et al.*, 2024; Hirsh, 1998). These drugs are inhibitors of cytotoxic three-finger toxins (3FTxs), a toxin class especially prevalent in the venoms of many cobra species and have been shown to markedly reduce venom cytotoxicity in both cell and animal models (Du *et al.*, 2024; Tasoulis and Isbister, 2017). Such venom-induced cytotoxicity often causes severe and life-altering morbidity in snakebite victims, with spitting cobras (*Naja* spp.) being largely responsible for such injury in Africa (Du *et al.*, 2024; Rivel *et al.*, 2016).

However, for any new toxin-inhibitor to be effective against snakebite-induced tissue damage it must first reach the venom in and around the envenomation site sufficiently quickly (Bartlett *et al.*, 2024). This is problematic with IgG-based and intravenously delivered antivenoms due to their limited extravasation and tissue permeability (Clare *et al.*, 2021; Conner *et al.*, 2020; J. M. Gutiérrez *et al.*, 2017b) while promising oral drug candidates currently in development (Abouyannis *et al.*, 2025; Gerardo *et al.*, 2024) are limited by the time it takes for them to be absorbed by the gut and to adequately distribute from the blood stream into the affected tissues (Bartlett *et al.*, 2024; Bashiardes and Christodoulou, 2024). One potential option to aid rapid local drug delivery is the use of hydrogels in which venom toxin-inhibiting drugs, such as heparins, are integrated and used as snakebite wound dressings. Hydrogels are three-dimensional hydrophilic polymers crosslinked to form a lattice which possess numerous benefits over traditional wound dressings including the ability to retain moisture, crucial for the wound healing process, and to absorb fluids such as wound exudate (Bashir *et al.*, 2020; Brumberg *et al.*, 2021; Sweeney *et al.*, 2012). Importantly, hydrogels can be incorporated with drugs into their structure which can later diffuse out of the gel in solution or when applied to a wound, allowing them to be used for targeted drug delivery (Kesharwani *et al.*, 2021). As such, it was hypothesised that by incorporating H or T into hydrogels, a unique material could be engineered to be later translated into a snakebite wound dressing that would simultaneously protect the physical site of a snakebite injury whilst also releasing drug into the wound to reduce the effects of cytotoxic 3FTx-containing snake venoms.

Many different materials can be used to make hydrogels, however a promising one for this purpose is WPI (Bashir *et al.*, 2020). WPI, a byproduct of the dairy industry, contains 97% protein, 75% of which

is β-lactoglobulin, the primary compound making up the hydrogel lattice (Baines *et al.*, 2025). The benefits of WPI hydrogels include low cost, high cytocompatibility, and ease of gelation (Genç *et al.*, 2023). In addition, WPI hydrogels can incorporate and deliver both hydrophobic and hydrophilic drugs for a variety of biomedical applications including facilitating tissue engineering, managing conditions such as cancers, and the treatment of bacterial infections (Baines *et al.*, 2025, 2024; Platania *et al.*, 2021). Both H and T have been incorporated into WPI hydrogels to explore their potential as tissue engineering scaffolds (Pawlak-Likus *et al.*, 2025). Additionally, studies exploring H-incorporated non-WPI hydrogels as wound dressings for diabetic, excisional, and burn injuries have demonstrated beneficial effects of H on wound healing (Uzunalli *et al.*, 2017; Yergoz *et al.*, 2017; Zhang *et al.*, 2018). However, whilst studies have investigated the use of hydrogels incorporating snake venoms into their lattice as haemostatic agents (Yegappan *et al.*, 2022), no studies have yet explored H- or T-integrated hydrogels for use as wound dressings for snakebite.

Therefore, the aims of this study were to investigate as proof-of-concept whether heparin-containing WPI hydrogels could be engineered that (a) exhibit physical properties suitable to translation into wound dressings and (b) release their contained drug in sufficient quantities to inhibit spitting cobra venom-induced cell death. To date, no study has proposed or explored the potential utility of toxin inhibiting drug-integrated hydrogels as a pre-hospital snakebite wound treatment. However, the results contained herein suggest that this is a promising approach and that further work on this topic is warranted.

2. **Materials and Methods**

2.1. Materials

2.1.1. Chemicals, drugs and biological materials

WPI was purchased as the product BiPro from Davisco Foods International (Eden Prairie, MN, USA). Trypan blue stain (0.4%) was acquired from Logos Biosystems, resazurin and proteases were purchased from Sigma-Aldrich (Merck), and PBS purchased from Gibco, Corning or Sigma-Aldrich. Unfractionated heparin (H) sodium salt ($M_w \approx$ 20,000 g/mol) and tinzaparin (T; $M_w \approx$ 8,000 g/mol) derived from porcine mucosa were supplied by LEO Pharma (Ballerup, Denmark).

2.1.2. Venoms

Naja nigricollis venom (Nigeria) was provided by the Liverpool School of Tropical Medicine's (LSTM) Centre for Snakebite Research and Interventions (CSRI). The venom was a pool collected from multiple milkings of four wild-caught snakes maintained in the CSRI Herpetarium facility, whose animal protocols are approved by the UK Home Office, the LSTM and the University of Liverpool's Animal Welfare and Ethical Review Boards. The venoms were lyophilised and stored at 4 °C before reconstitution in PBS and storage at –80 °C until use.

2.1.3. Cells

Immortalised human epidermal keratinocytes (HaCaT cells) were provided by Prof Sarah Allinson (Lancaster University; originally sourced from AddexBio) and cultured in Dulbecco's Modified Eagles Medium (DMEM) (Gibco) augmented with 9.5% Foetal Bovine Serum (FBS) (LabTech), 2% sodium pyruvate (2 mM; Cytiva), and 1% penicillin (100 IU/mL) and streptomycin (250 µg/mL) (Gibco), hereafter referred to as Standard Medium. Cellular assays were then conducted in low background

fluorescence medium, made up of FluoroBrite DMEM (Gibco), 1% FBS, 2% sodium pyruvate, 1% penicillin and streptomycin and 1% GlutaMax (Gibco), hereafter referred to as Low Background Fluorescence Medium (Hall *et al.*, 2023). All 96-well plates were purchased from Greiner.

2.2. Data Collection

2.2.1. Hydrogel Formation

Five whey protein isolate (WPI) hydrogel types were produced (**Table 1**), formed as previously described (Pawlak-Likus *et al.*, 2025). The solutions were homogenised for 24 hours using an IKA Loopster (IKA England LTD, Oxford, UK), before the mixture was sonicated to remove bubbles that could compromise gel uniformity. Solutions were injected into a hydrogel dressing-gelation device (Biomat Ltd, UK) and heated for 20 minutes at 70˚C to encourage efficient gelation. Hydrogel samples were then cut into 0.03 g discs, hereafter referred to simply as discs, using a metal corer to ensure uniform volume and surface area. Experiments were conducted using discs of all five hydrogel types unless stated otherwise.

Table 1 – Hydrogel samples and their compositions.

Sample group name	WPI concentration (% w/v)	Heparin or tinzaparin concentration (% w/v)
40% WPI	40	0
5% H	40	5
10% H	40	10
5% T	40	5
10% T	40	10

2.2.2. Scanning Electron Microscopy

Hydrogel samples were imaged using scanning electron microscopy (SEM), conducted with a JSM-6390 LV, JEOL Ltd. (Welwyn Gardens, UK) scanning electron microscope, an accelerating voltage of 15 kV, and using magnifications of 100x, 3,000x and 10,000x. Samples were taken from the centre of each hydrogel disc, dehydrated in a desiccator, and gold-coating was performed with a Quorum Technologies, Q150RES, sputter coater to a thickness of ca. 5 nm before imaging took place.

2.2.3. Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was used to determine chemical linkages present within the gel. Discs of each hydrogel type were dehydrated and the resultant IR spectra analysed using a Cary 630 FTIR spectrophotometer (Agilent, Santa Clara, CA, USA) in attenuated total reflection (ATR) mode and using a spectral range of between 650 and 4000 cm^{-1}. Experiments were completed in triplicate.

2.2.4. Hydrogel Swelling Analysis in Dulbecco's Modified Eagles Medium and Phosphate Buffered Saline

To determine how the incorporation of the H or T concentrations in the hydrogel affected the polymer's swelling behaviour, samples of each hydrogel type were weighed before being incubated at 37°C for 7 days in either Dulbecco's Modified Eagles Medium (DMEM) or pH 7.4 Phosphate Buffered Saline

(PBS). Five samples per hydrogel were individually placed into 200 µL of pH 7.4 PBS, and ten samples were individually placed into 200 µL of DMEM. Following incubation, water excess was removed from the surface of each disc using a paper towel, and each sample reweighed. The % mass change (M%) was calculated from the following formula:

$$M\% = \frac{M_W - M_i}{M_i} \times 100\%$$

where M_w is the wet mass and M_i is the initial mass.

2.2.5. Protein Release

To determine how H or T incorporation altered the release of protein from the 3D polymer lattice, as a measure of general hydrogel degradation, hydrogel samples were submerged in 1 mL pH 7.4 PBS and incubated at 37°C. Following 24 and 48 hours, 1 µL of this solution was removed and analysed using a NanoDrop at A_{280} (NanoDrop 2000c, ThermoScientific) (Pawlak-Likus *et al.*, 2025). Experiments were completed in triplicate.

2.2.6. Enzyme Degradation

To better evaluate how the hydrogels may degrade in protease-containing biological fluids, enzyme degradation experiments were conducted (Lee *et al.*, 2005). Five hydrogel samples were incubated in 200 µL of pH 7.4 PBS containing 13.33 µg/mL protease mixture obtained from bovine pancreas (CAS no. 9001-92-7), concentrations consistent with blood collagenase levels (Manicourt *et al.*, 1994; Pawlak-Likus *et al.*, 2025), at Standard Conditions for seven days. The samples were weighed before and after the incubation period, with excess solution being removed with a paper towel, and the % mass change (M%) calculated:

$$M\% = \frac{M_a - M_i}{M_i} \times 100\%$$

where M_a is the mass after incubation and M_i is the initial mass.

2.2.7. Tinzaparin Release Quantification

Absorbance determination: An absorbance scan (230-400 nm with 2 nm increments) was completed on 50 µL of 4 mg/mL tinzaparin in PBS and compared to a PBS-only control in a half-volume 96-well UV-capable microplate using a Tecan MPLEX plate reader over three independent experimental replicates to confirm an absorbance wavelength at which tinzaparin could be quantified in solution.

Tinzaparin release from hydrogels determination: To measure drug release from the tinzaparin-containing WPI hydrogel, 10% T and WPI control discs were soaked in PBS (3 discs / 180 µL PBS) for 1 hour or 24 hours under standard conditions (37 °C, 5% CO_2). Over three experimental replicates, 50 microlitres of the resulting solutions were pipetted into separate wells in a half-volume 96-well UV-capable microplate along with serial dilutions of known tinzaparin concentrations (4,000 – 31.25 µg/mL, with a halving of the concentration with each serial dilution). A_{230} was measured in each well using a Tecan MPLEX plate reader and a standard curve plotted for each known tinzaparin concentration serial dilution, from which the concentration of tinzaparin released from the 10% tinzaparin-containing discs under standard conditions was determined for each of three experimental replicates and the means calculated.

2.2.8. Cytotoxicity Assay

Day 1: HaCaT cells (10,000/well) were seeded in black-sided, clear-bottomed, half-volume 96-well microplates and incubated for 24 hours under standard conditions. Three discs of the WPI control hydrogel or the 10% T hydrogel were added to 180 µl PBS and incubated for 24 hours at standard conditions. *Day 2:* Three discs of both hydrogel types were incubated in the same manner for 1 hour. Serial dilutions of the resulting hydrogel solutions (0.08x to 1.95×10^{-5}x dilutions with a quartering of the concentration with each serial dilution) were mixed with a constant 20 µg/mL *N. nigricollis* venom or venom vehicle control (PBS) in Low Background Fluorescence Medium. The following control solutions were also prepared: the WPI control and 10% T hydrogel solutions with no venom, venom only, medium only, venom with 2,000 µg/ml T, and the 0% cell viability control using 0.1% Ecosurf™. All solutions were kept at standard conditions for 30 minutes prior to cell treatments, after which the cells were exposed to these solutions for 24 hours under standard conditions. *Day 3:* Resazurin cell viability assays were used as a model of cell viability as described previously (Du *et al.*, 2024). Briefly, all wells were treated with 90 µg/mL resazurin and incubated for 1.5-2 hours. Fluorescence (Ex_{544}/Em_{590}) was quantified using a Tecan MPLEX plate reader. Percent cell viability within each experimental well was calculated relative to the mean within all untreated 100% cell viability positive control wells following subtraction of the average fluorescence response from all background control wells which contained no cells:

$$\%\ Cell\ Viability = \frac{Em_{590\ (treatment\ well)} - Average(Em_{590\ (blank\ well)})}{Average(Em_{590\ (positive\ control\ well)}) - Average(Em_{590\ (blank\ well)})} \times 100$$

2.3. Data Analysis

All data are presented as mean ± standard deviation (SD) unless stated otherwise. 'n' is defined as an independent experimental replicate completed at a separate time from other replicates. At least three independent experimental replicates were completed for each statistically analysed set of data. Statistical analyses of data and graph creation were performed using GraphPad Prism unless stated otherwise. The specific statistical tests performed are stated in the figure legends. A difference was considered significant if $P \leq 0.05$.

3. **Results**

Analysis of the effectiveness of hydrogels as heparin delivery vehicles was conducted along several avenues, beginning with the determination of their physical and mechanical properties, followed by investigating how they may act in the biological environment of a wound such as those caused by snakebite, before finally confirming both the release of the drug from the gel and the effectiveness of the hydrogel delivery vehicle in preventing *N. nigricollis* venom-induced cytotoxicity. This began with the generation of one control 40% WPI hydrogel with no drug and four drug-integrated hydrogels containing the same percentage of WPI: 5% H, 10% H, 5% T, and 10% T.

3.1. Scanning Electron Microscopy Reveals Heparin Incorporation Alters Hydrogel Structure

To generate a detailed image of its surface characteristics and three-dimensional structure of each of the hydrogels, SEM was conducted on a dried sample of each hydrogel type at 100x, 3,000x and 10,000x magnification (**Fig. 1**).

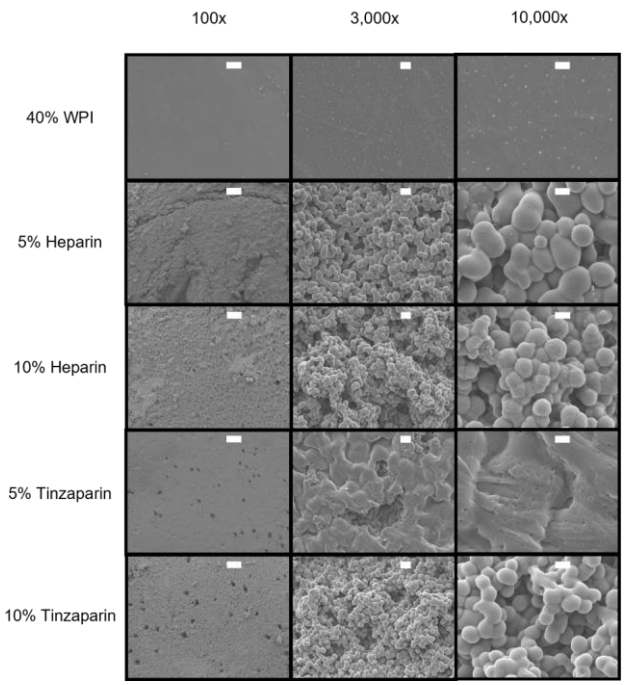


Fig. 1 – SEM images reveal structural differences between dried samples of the five tinzaparin types. SEM images of dried samples of control 40% WPI hydrogel with no drug, and WPI hydrogels integrated with 5% unfractionated heparin (H), 10% H, 5% tinzaparin (T), and 10% T. Moving left to right, images were taken at magnifications of 100x, 3,000x, and 10,000x, and the corresponding scalebars are equal to: 100 µm, 4 µm, and 1 µm.

As can be seen in **Fig. 1**, the control 40% WPI hydrogel contains very few pores, even at high magnifications. In comparison, the surface structure appears rougher and more textured in the hydrogel modified with 5% H, even more so than the hydrogel modified with 10% H. At 3,000x and 10,000x it becomes apparent that this is due to the presence of clusters of spherical and semi-spherical structures. In the 10% H hydrogel, these structures seem more tightly packed together than in the 5% H hydrogel. Differences are also apparent in the tinzaparin-containing hydrogels. At 100x magnification, dark pores can be seen on the surfaces of both T-containing hydrogels. However, at higher magnifications, the physical structure of the 5% T hydrogel appears disrupted and slightly porous but still relatively smooth compared to that of the hydrogel incorporated with 10% T, where similar spheroid structures to the H hydrogels are present.

3.2. Fourier Transform Infrared Spectroscopy Confirms the Presence of Heparins

Following SEM, FTIR was conducted to identify the chemical bonds within each of the hydrogel samples and to determine if there were any major differences amongst these bonds between different hydrogel types resulting from heparin incorporation (**Fig. 2**) (Hameed *et al.*, 2024).

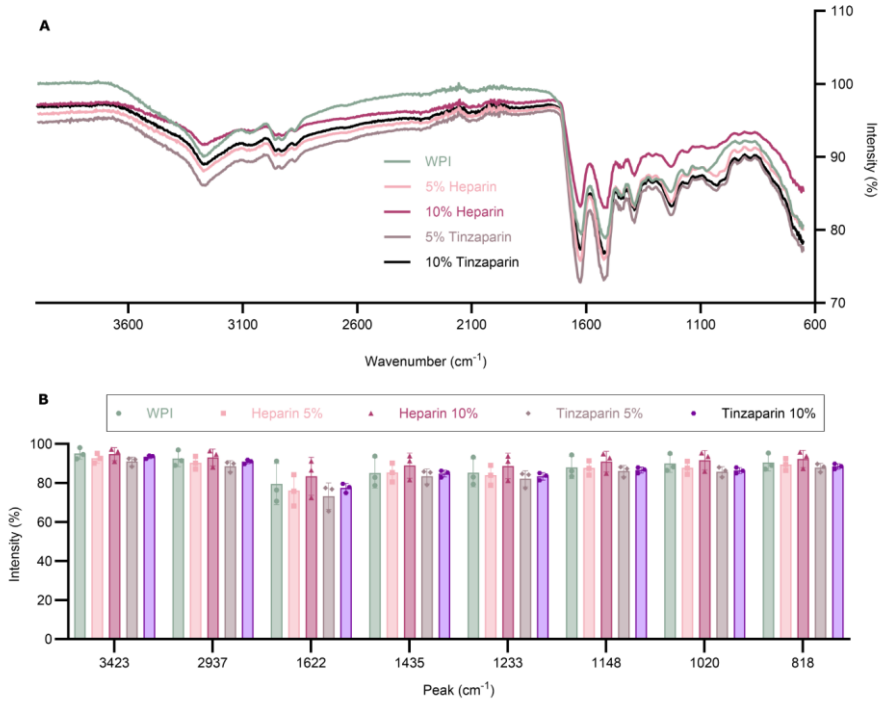


Fig. 2 – (A) FTIR spectra of WPI hydrogels loaded with heparins and (B) analysis of the intensity of specific peaks characteristic of heparins at select wavenumbers. FTIR spectra were gathered for each of the five hydrogel types and the intensity of the most prominent peaks from each spectrum compared against one another. A) The FTIR spectra of the five hydrogel types were acquired on a Cary 630 FTIR spectrophotometer using a spectral range of 650-4000 cm^{-1}. Data are presented as mean values. B) The % intensity of specific heparin-defining peaks (818 cm^{-1}, 1020 cm^{-1}, 1148 cm^{-1}, 1233 cm^{-1}, 1435 cm^{-1}, 1622 cm^{-1}, 2937 cm^{-1}, 3423 cm^{-1}) from the five hydrogel types (Ivory-Cousins *et al.*, 2023; Mecozzi *et al.*, 2011). Data are presented as mean values ± SD, with data from individual trials presented as dots within each bar (n = 3). No significant differences were calculated as determined by a 2-way ANOVA followed by Tukey's multiple comparisons test.

Table 2 – FTIR peak wavenumber and their region/potential interactions.

Wavenumber (cm^{-1})	**Region/Potential Interactions**
3260-3270	Symmetric stretching O-H
2952	Asymmetric stretching CH_3
2925	Asymmetric stretching CH_2
2870	Symmetric stretching CH_3 Stretching N-H, C-H Symmetric stretching CH_3 of acyl chains
1625	Amide I region
1517	Amide II
1446	Stretching C=O

1386	Bending CH_3 Stretching C-O Deformation C-H, N-H
1306	C-N amide III band
1228	Sulfate vibrations of S=O bonds
1157	C-O-C polysaccharide
1070	C-O carbohydrate
965	Unassigned
870	Bending C=O inorganic carbonate

FTIR analysis revealed many peaks, including main bands for all gels at 3260-3270 cm^{-1}, 2952cm^{-1}, 2925 cm^{-1}, 2870 cm^{-1}, 1625 cm^{-1}, 1517 cm^{-1}, 1446 cm^{-1}, 1386 cm^{-1}, 1306 cm^{-1}, 1228 cm^{-1}, 1157 cm^{-1}, 1070 cm^{-1}, 965 cm^{-1}, and 870 cm^{-1} (**Fig. 2A**). An overview of the potential underlying interactions of these bands is shown in **Table 2** (Ivory-Cousins *et al.*, 2023; Mecozzi *et al.*, 2011). The peaks in the spectra generated at 818 cm^{-1}, 1020 cm^{-1}, 1148 cm^{-1}, 1233 cm^{-1}, 1435 cm^{-1}, 1622 cm^{-1}, 2937 cm^{-1} and 3423 cm^{-1} (**Fig. 2B**) were comparable to heparin-defining FTIR peaks (Devlin *et al.*, 2023; Shen *et al.*, 2019). The intensity of all peaks did not significantly increase as the concentration of both H and T increased from 5% to 10%. In the spectra of samples containing heparins, a combination of bands specific for WPI and heparins were also observed, as expected. No noticeable shifts in the bands specific for WPI were detected.

3.3. Heparin Integration Increases Hydrogel Swelling Ability

The analysis of the hydrogels' physical properties was followed by an evaluation of their potential as wound dressings. Performance was assessed based on the extent of swelling in solution, which modelled wound exudate absorption, and the rate of degradation in simulated wound environments (Pawlak-Likus *et al.*, 2025; Zhu *et al.*, 2022).

To determine the extent to which the hydrogels swell by absorbing liquid from their environments samples of each hydrogel type were weighed before and after incubation in PBS or DMEM for seven days and the % change in their mass calculated (**Fig. 3**).

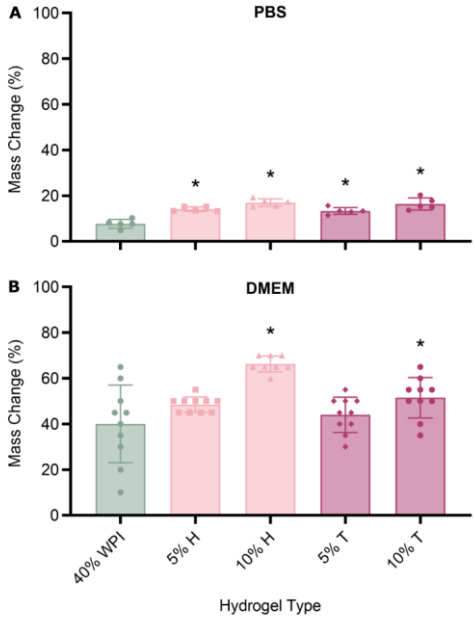
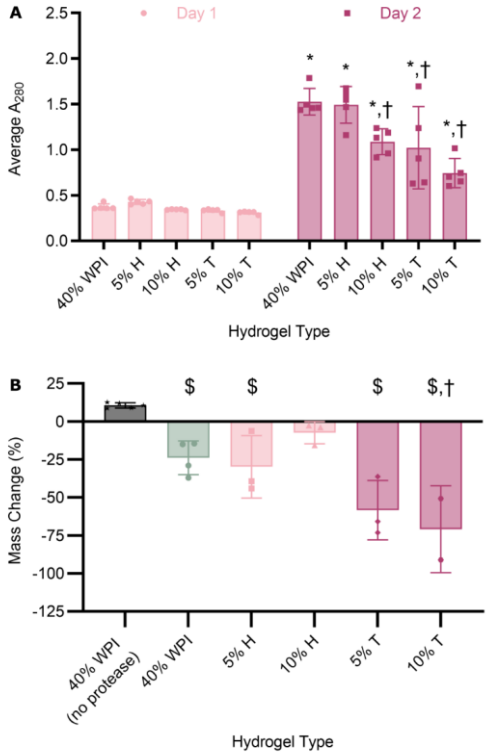


Fig. 3 – Incorporation of heparins into WPI hydrogels can increase their ability to swell in PBS and DMEM. Hydrogel samples were weighed and then incubated for seven days in (A) PBS (n=5) or (B) DMEM (n=10) after which samples were again weighed and the % mass change calculated. Data are presented as mean values ± SD, with data from each trial presented as individual data points within each bar. * Signifies the value is significantly different to that of the 40% WPI control as determined by a one-way ANOVA followed by Dunnett's multiple comparisons test in which the mean of all test columns was compared to a single control (40% WPI) (P<0.05).

In PBS (pH 7.4), which approximately replicates the pH and osmolarity of human cells and was therefore used in the initial model of exudate absorption from an open wound (Martin *et al.*, 2006), all heparin-integrated hydrogels, 5% H (14.1%), 10% H (17.0%), 5% T (13.3%), and 10% T (16.4%), swelled to a greater extent than the 40% WPI control hydrogel (7.7%). In DMEM, basal cell medium that more closely resembles the environment of mammalian tissue than PBS and which was therefore used in the second model of exudate absorption, all hydrogel types swelled more than what was observed in PBS though only the 10% H (66.2%) and 10% T (51.5 %) swelled to a greater extent than the 40% WPI control hydrogel (40.0%).

3.4. Simulated Wound Environments Degrade WPI-based Hydrogels

To help assess a hydrogel's suitability as a wound dressing it is important to determine its biodegradability as fast degradation can lead to quicker drug dispersal but lower stability over time (Kharkar *et al.*, 2013).



345

346 **Fig. 4 – WPI hydrogels degrade over time in PBS, a process exacerbated by proteases.** (A) Measurement of
347 protein released from WPI hydrogel types into PBS. Hydrogel samples were submerged in pH 7.4 PBS and
348 incubated at Standard Conditions. Supernatant samples were taken at 24 & 48 hours and analysed via NanoDrop
349 (A_{280}) as a measure of total protein concentration in the solution (Pawlak-Likus *et al.*, 2025). (B) Calculated %
350 mass change of each hydrogel type following incubation with protease solution. Hydrogel samples were weighed
351 before being incubated in pH 7.4 PBS solution containing 13.33 µg/mL protease (Manicourt *et al.*, 1994 Pawlak-
352 Likus *et al.*, 2025) for seven days at Standard Conditions. As an additional control, 40% WPI hydrogels were
353 incubated without protease to act as a negative control and are labelled '40% WPI (no protease)'. All hydrogels
354 were then reweighed, and the % mass change calculated. 'n' is equal to a maximum of 5 and a minimum of 2, as
355 not all samples could be reweighed due to gel degradation. Data are presented as mean values ± SD, with data
356 from each trial presented as points within each bar. *Signifies that the value is significantly greater than the same
357 hydrogel type from Day 1, † signifies the value is significantly lower than the 40% WPI control from the same
358 time point, and $ signifies the value is significantly lower than the 40% WPI (no protease) negative control as
359 determined by (A) a two-way ANOVA followed by Tukey's multiple comparisons test in which cell means were
360 compared with others in its row and column or (B) a one-way ANOVA followed by Tukey's multiple comparisons
361 test in which the mean of each column was compared with the mean of every other column (P<0.05).

362

363 To model the general biodegradability the hydrogels, the total protein release of each hydrogel type into
364 PBS after 24 and 48 hours of incubation was measured (Zustiak and Leach, 2011) (**Fig. 4A**). The
365 average A_{280} (Pawlak-Likus *et al.*, 2025) of each supernatant was measured and compared, revealing

that all five hydrogels released more protein into the PBS by day two versus day one. Variation between hydrogel types also increased by day two compared with day 1, as the A_{280} readings remained steady (0.30 – 0.45) on day one whilst significant differences emerged by day two with 40% WPI control (1.50) having released significantly more protein into the PBS solution than 10% H (1.09), 5% T (1.02), and 10% T (0.74).

Following this, a model of enzymatic degradation where the change in each hydrogel's weight was determined after incubation in protease-containing PBS was utilised (Lee *et al.*, 2005) (**Fig. 4B**). The 40% WPI (no protease) control increased in mass by 10.6%, in comparison to which 40% WPI, 5% H, 5% T, and 10% T, all of which were incubated with proteases, decreased significantly in mass by 23.9, 29.8, 58.4, and 70.9%. The only hydrogel type for which mass decreased significantly more than that of 40% WPI in protease-containing solution was 10% T, *albeit* these results should be interpreted with caution as only two 10% T hydrogel samples could be weighed after the seven days of incubation due to the other three degrading to such an extent they could not be accurately measured.

3.5. WPI Hydrogels Release Tinzaparin after Incubation in PBS

The final steps of this project were to determine if the heparins could release from the WPI hydrogels sufficiently to inhibit a snake venom's cytotoxic properties. The 10% T hydrogel was selected for these experiments due to T's previously reported superiority to H against spitting cobra venom-induced cytotoxicity (Du *et al.*, 2024).

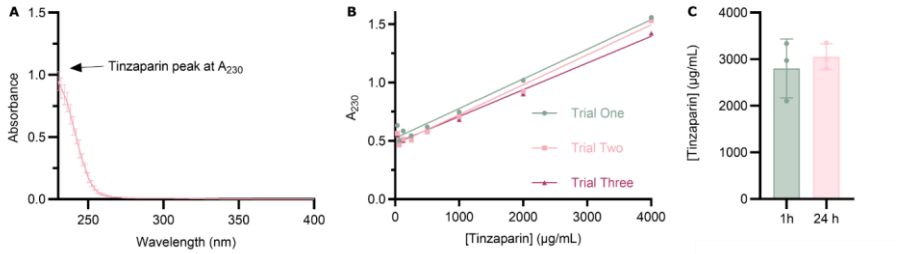


Fig. 5 – The T 10% hydrogel releases tinzaparin into PBS. (A) An absorbance scan (230-400 nm in 2 nm increments) of 4 mg/mL T in PBS versus PBS-only control shows an absorbance peak at A_{230}. Data are presented as mean values ± SD (n = 3). (B) Standard curves (31.25 – 4,000 µg/ml T) to determine the linear relationship between T concentration and A_{230}. T was dissolved in PBS that had been incubated for 24 h with 40% WPI control hydrogel and the A_{230} read at each concentration. Linear regressions were performed on the data from each trial to produce separate standard curves for each (n = 3). (C) The concentration of T released into solution following incubation for 1 or 24 hours. The 10% T hydrogel samples were incubated for either 1 or 24 hours in PBS at Standard Conditions. The A_{230} of each trial was measured and plotted against each standard curve to calculate the concentration of T released from T 10% hydrogels. Statistical analysis was performed using an unpaired t-test with Welch's correction. Data are presented as mean values ± SD (n = 3).

First, the optimal absorbance wavelength at which T could be detected and quantified was determined by measuring the absorbance of 4,000 µg/mL of T in PBS at a range of wavelengths (230-400 nm, 2 nm increments), from which it was determined that the most intense absorbance occurred at 230 nm

(**Fig. 5A**). Following this, standard T curves were created in triplicate at A_{230} using a serial dilution from 4,000 – 31.25 µg/ml (with a halving of subsequent serial dilutions). The data from the three trials were described by the equations $y = 0.0002540x + 0.5237$, $y = 0.0002567x + 0.4683$, and $y = 0.0002293x + 0.4802$ for trials 1-3, respectively (**Fig. 5B**). The A_{230} of samples of supernatant from T 10% hydrogel incubated in PBS at Standard Conditions for 1 and 24 hours was measured. From this, the concentration of T within each sample could be determined using the standard curve equations and calculating the mean. After three experimental replicates, it was determined that the mean concentration of T released from the hydrogels was 2.80 mg/mL after a 1-hour incubation and 3.05 mg/mL after a 24-hour incubation, with no significant difference between the two timepoints (**Fig. 5C**).

3.6. 10% Tinzaparin WPI Hydrogels Inhibit *N. nigricollis* Venom Cytotoxicity

Finally, to examine whether T could be effectively delivered to cells and inhibit venom function *via* a WPI hydrogel system, the effects of PBS solutions incubated for 1 or 24 hours with either 40% WPI control or 10% T hydrogel on the viability of HaCaTs treated with a constant concentration of *N. nigricollis* venom were compared.

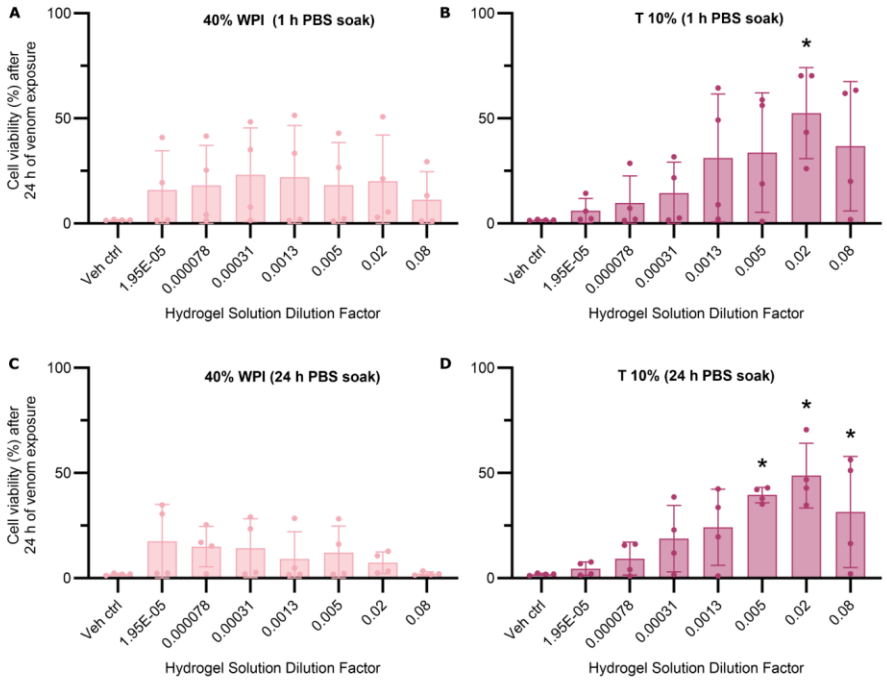


Fig. 6 – Solutions prepared from both incubation timepoints of the T 10% hydrogels inhibited *N. nigricollis* venom cytotoxicity. Hydrogels were incubated for 1 or 24 hours in PBS under Standard Conditions after which the resulting supernatants were serially diluted by quarters to create experimental cell treatment solutions that ranged between 0.08x – $1.95x10^{-5}$x of the original supernatant concentration. These serial dilutions, or a PBS vehicle control, were pre-incubated for 30 minutes with 20 µg/mL *N. nigricollis* venom before being administered to the HaCaT cells for 24 hours. Cell viability was then determined using resazurin assays. (A) The effects of the

40% WPI hydrogel 1-hour incubation solutions. (B) The effect of the 10% tinzaparin hydrogel 1-hour incubation solutions. (C) The effects of the 40% WPI hydrogel 24-hour incubation solutions. (D) The effect of the 10% tinzaparin hydrogel 24-hour incubation solutions. Data are presented as mean values ± SD, with data from each trial presented as points within each bar (n=4). * Signifies the % cell viability is significantly higher than that of the vehicle control as determined by a one-way ANOVA followed by Dunnett's multiple comparisons test (P<0.05).

Table 3 – Concentration of tinzaparin in each T 10% hydrogel serial dilution. The concentration of T released into 180 µL PBS by three 30 mg T 10% hydrogel discs after 1 or 24 hours of incubation at Standard Conditions was calculated to be 2.80 mg/mL and 3.05 mg/mL, respectively. This figure was multiplied by the dilution factor to determine the concentration of tinzaparin in each treatment used in Fig. 6B and 6D.

Dilution Factor	$1.95x10^{-5}x$	$7.81x10^{-5}x$	0.00031x	0.00125x	0.005x	0.02x	0.08x
1-hour incubation [Tinzaparin] (µg/mL)	0.05	0.22	0.88	3.5	14	56	224
24-hour incubation [Tinzaparin] (µg/mL)	0.06	0.24	0.95	3.8	15	61	244

HaCaT cells exposed to *N. nigricollis* venom and treated with vehicle control for both hydrogel experiments effectively reduced cell viability to close to 0%, confirming that cytotoxic venom concentrations were used in both cases (**Fig. 6**). The supernatant from the 40% control hydrogels for both incubation periods did not significantly increase cell viability at any concentration, although mean cell viability did increase from the vehicle control at most concentrations (**Fig. 6A, 6C**). The 1-hour incubation, 10% T hydrogel supernatant dilution series effectively inhibited the venom-induced cytotoxicity only at the 0.02x dilution which contained approximately 56 µg/mL of T (**Table 3**), resulting in a significant increase in mean cell viability to 52.5% (**Fig. 6B**). The 24-hour incubation, 10% T hydrogel dilution series was able to inhibit *N. nigricollis* venom cytotoxicity at a greater concentration range than the 1-hour incubation, significantly increasing cell viability at dilution factors of 0.05, 0.02 and 0.08 (15, 61 and 244 µg/mL [**Table 3**]), resulting in cell viabilities of 39.5%, 48.8% and 31.5% respectively (**Fig. 6D**).

4. Discussion

The primary aims of this study were to investigate whether heparin-integrated WPI hydrogels would exhibit the physical and venom-inhibitory properties required to warrant their further translation into snakebite wound dressings.

Firstly, the structural properties of each WPI hydrogel type were assessed using SEM, with clear structural differences being observed between 40% WPI control hydrogels and those hydrogels containing heparins (**Fig. 1**). WPI is >97% protein, of which the majority is β-lactoglobulin (75%), however other components include α-lactalbumin, immunoglobulins, bovine serum albumin, bovine lactoferrin and lactoperoxidase (Keppler *et al.*, 2014; Madureira *et al.*, 2007). β-lactoglobulin has

been reported to be negatively charged in the native state (isoelectric point = pH 5.3), with polyanions such as heparin typically binding patches of positively charged amino acids (Kamerzell *et al.*, 2011; Wang *et al.*, 2024). However, it may be possible that protein denaturation caused by heating may alter the isoelectric point, allowing heparin polyanion binding. The addition of heparins resulted in the formation of spherical structures (~1 µm in diameter) which clustered to a greater extent at the higher concentrations of either heparin, likely due to the heparins interacting with the WPI polymers with greater frequency at higher concentrations. Such structures have been noted previously when adding the biopolymer poly-γ-glutamic acid to WPI hydrogels (Baines *et al.*, 2025). Furthermore, the increased porosity caused by the clustering of these spheroidal structures may increase the release rate of drug from these hydrogels (Hoare and Kohane, 2008). The peaks observed *via* the completed FTIR analysis (**Fig. 2**) also correlate with what would be expected of heparin and tinzaparin (Devlin *et al.*, 2023; Shen *et al.*, 2019), further evidencing the successful integration and dispersion of both drugs into the WPI hydrogel structure.

The swelling analysis assays in PBS and DMEM (**Fig. 3**), used to model each hydrogel type's ability to absorb wound exudate, showed that the higher concentrations of either heparin increased hydrogel swelling, a phenomenon which may be due to the negative charges of both H and T, resulting in greater hydrophilicity with increasing heparin concentration, thereby resulting in greater water absorption and therefore swelling (Ganji *et al.*, 2010; Pawlak-Likus *et al.*, 2025). The improved swelling properties observed in the WPI hydrogels integrated with heparins is also a desirable characteristic for a snakebite wound dressing, as snakebite envenoming often produces excess wound exudate which should be removed for more effective wound healing (Collier *et al.*, 2014; Ibiapina *et al.*, 2019; Rucavado *et al.*, 2016).

Several studies have shown it takes an average of 5-9 hours for a snakebite victim to reach hospital in rural tropical communities (Abouyannis *et al.*, 2023; Ogunfowokan, 2012; Sharma *et al.*, 2005), and any prehospital-administered wound dressing should remain structurally and functionally stable throughout this prehospital period. However, the biodegradability of hydrogel wound dressings must be balanced between their stability, which maximises the length of time for which the wound site can be protected, and degradation, which improves drug distribution and minimises environmental harm (Khan *et al.*, 2023; Ribeiro *et al.*, 2024). To quantify this, protein release was first measured from each hydrogel type into PBS (**Fig. 4A**) (Baines *et al.*, 2025) which evidenced that heparin-incorporation diminishes WPI lattice breakdown, which may be the result of bonding between WPI proteins and heparins within the hydrogel (He *et al.*, 2019). As such, heparin integration could increase the longevity of a WPI-derived wound dressing. However, wounds also contain enzymatic components such as proteases, crucial elements of tissue remodelling and repair, which could catalyse WPI hydrogel polypeptide degradation (Baines *et al.*, 2025; McCarty and Percival, 2013). As such, the enzymatic degradation of hydrogel samples in solution containing proteases was also determined (**Fig. 4B**). As expected, incubation with proteases did result in significantly greater degradation of most hydrogel types in comparison to the 0% protease negative control; however, a weakness of these data should be noted, as while five hydrogel samples were initially prepared for each data set, due to excessive degradation of some of the samples rendering them impossible to weigh accurately, the final number of experimental replicates was diminished thereby reducing the statistical power within this experiment. Regardless, it was still determined that the hydrogel samples reduced in mass when incubated with proteases, suggesting they would also likely degrade in a real-world snakebite wound environment, especially during the remodelling stage of tissue repair (McCarty and Percival, 2013). Additionally, the data suggest that the presence of heparins, especially T, in the hydrogel matrix amplifies enzymatic degradation. Increased swelling in the presence of heparins (**Fig. 3**) could conceivably increase the pore size in the hydrogel polymer network, facilitating the entry of enzymes into the network by diffusion.

However, such discussion must remain speculative at the present time. As hydrogel degradation within protease-containing solution is more reminiscent of a real wound environment (McCarty and Percival, 2013), versus the trials completed in PBS, this suggests that the addition of heparins into WPI hydrogels would likely facilitate their biodegradability upon administration to a snakebite wound.

Following the experiments designed to describe the physical characteristics of heparin-containing WPI hydrogels, their ability to inhibit snake venom-induced cytotoxicity was investigated. It was decided that 10% T hydrogels would be tested against *N. nigricollis* venom for these experiments as T has previously exhibited potent inhibition of spitting cobra venom-induced cytotoxicity (Du *et al.*, 2024).

First, to confirm T would release from the hydrogel in quantities sufficient to inhibit *N. nigricollis* venom-induced cytotoxicity in cell-based assays its concentration in PBS was determined following a 1-hour or 24-hour incubation at standard conditions (**Fig. 5**), from which its final mean concentration was determined to be 2,803 µg/mL following a 1-hour incubation and 3,054 µg/mL following a 24-hour incubation; sufficiently concentrated in both cases for venom inhibition (Du *et al.*, 2024). No significant difference was found between incubation time periods, suggesting that the majority of tinzaparin able to diffuse into solution within 24 hours does so rapidly, leaving the gel within the first hour. Then, the ability of these 10% T hydrogel-derived versus 40% WPI hydrogel negative control-derived solutions to inhibit *N. nigricollis* venom-induced cytotoxicity was evaluated using a human epidermal keratinocyte (HaCaT) cell model (Hall *et al.*, 2023) (**Fig. 6**). As predicted, the 24-hour incubated, 10% T hydrogel-derived solution successfully inhibited *N. nigricollis* venom-induced skin cell cytotoxicity at several tested serial dilutions (15 – 244 µg/mL of T; **Table 3**), and at one tested concentration for the solution derived from a 1-hour incubation (61 µg/ml **of T; Table 3**). These results confirm that T-integrated WPI hydrogels can release drug at sufficiently high concentrations within as little as an hour to inhibit snake venom-induced cytotoxicity – a valuable characteristic since rapid treatment is crucial for necrosis prevention (Gutiérrez *et al.*, 2017a). As such, this evidence suggests that drug-incorporated WPI hydrogels warrant further research and development in the context of improving the treatment of snakebite envenoming.

The 40% WPI hydrogel-derived solution, prepared for use as the negative control, did not result in significant increases in % cell viability in comparison to the vehicle control**.** However, despite this lack of statistical significance, it is still intriguing how the mean cell viability trended higher for almost all serial dilutions of the 40% WPI hydrogel-derived solutions for both the 1-hour and 24-hour incubations (**Fig. 6A** and **6C**). This could potentially indicate a minor cytoprotective effect from these hydrogel-derived solutions resulting from the WPI itself. Prior investigations have shown that proteins similar to those in WPI can inhibit the functioning of other snake venom toxins, such as buffalo colostrum β-lactoglobulin inhibiting viper snake venom metalloproteinases (SVMPs) (Arpitha *et al.*, 2017). Therefore, a possible explanation is that a component of the WPI hydrogel, such as β-lactoglobulin (Keppler *et al.*, 2014), dissolved into the solution and interacted with one or more of the toxins within *N. nigricollis* venom to affect its functioning. However, further studies will be required to confirm or disprove these observed data trends.

Whilst questions remain about T-incorporated WPI hydrogels as wound dressings for snakebite, the inhibition of *N. nigricollis* venom-induced cytotoxicity by heparin-containing WPI hydrogels and their physiochemical properties discussed herein are the first evidence that such biomaterials could be translated into wound dressings for snakebite envenoming. Further research should aim to both expand the applicability of heparin-incorporated WPI hydrogels as snake venom inhibitor vehicles, as well as confirming their effectiveness in more realistic situations, e.g. using *ex vivo* or *in vivo* models of

envenoming. In expanding their applicability, a greater range of snake venoms should also be explored to ensure that there is the potential for pan-species efficacy. In addition, a range of other snake venom inhibitors which are currently under investigation such as varespladib, a hydrophobic inhibitor of snake venom PLA_2s (Clare *et al.*, 2021; Lewin *et al.*, 2016), could be incorporated into hydrogels and tested against a range of snake venoms. This could exploit WPI hydrogels' ability to bind and release hydrophobic drugs (Baines *et al.*, 2024). Through further research, WPI hydrogels integrated with heparins or other venom inhibiting drugs may prove themselves as effective prehospital wound dressings that slow the tissue destroying effects of cytotoxic snake venoms.

5. Conclusions

This research aimed to address if WPI hydrogels containing heparins could function as wound dressings for snakebite envenoming by assessing their physiochemical properties and determining if they could inhibit *N. nigricollis* venom-induced cytotoxicity. As described above, these physiochemical properties reveal that heparin-containing WPI hydrogels may be appropriate for snakebite wound dressings, being able to absorb wound exudate, deliver drugs swiftly, and biodegrade. In addition, the hydrogels containing 10% T delivered sufficient concentrations of drug into solution to inhibit *N. nigricollis*-induced cytotoxicity at 1/50th of the original solution's concentration following incubation for 1 hour, and up to 1/200th following incubation for 24 hours. This is the first time the ability of drug-containing WPI hydrogels to inhibit snake venoms has been tested, and the data contained herein suggest that such biomaterials could prove valuable as future snakebite wound dressings following further development that could be easily translated for prehospital use, thereby shortening time to initial treatment.

6. CRediT authorship contribution statement

Matthew J. Gray: Validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualisation. **Daniel K. Baines**: Conceptualisation, methodology, investigation, resources, writing – original draft, writing – review & editing. **Gabrielle De Castro**: Formal analysis, investigation, resources, data curation, writing – review & editing, visualisation. **Thomas Cobb**: Methodology, formal analysis, investigation, data curation, writing – review & editing, visualisation. **Layla A. Tyrrell**: Validation, investigation, data curation, writing – review & editing. **Sam J. Hyam**: Validation, investigation, writing – review & editing. **Alan M. Smith:** Resources, writing – review & editing. **Nicholas R. Casewell**: Resources, writing – review & editing. **Sarah L. Allinson**: Methodology, writing – review & editing. **Timothy E. L. Douglas**: Conceptualisation, methodology, resources, data curation, writing – original draft, writing – review & editing, supervision, project administration, funding acquisition. **Steven R. Hall**: Conceptualisation, methodology, formal analysis, investigation, resources, data curation, writing – original draft, writing – review & editing, visualisation, supervision, project administration, funding acquisition.

7. Acknowledgements

We would like to give thanks to Paul Rowley and Edouard Crittenden for maintaining the snakes at the LSTM herpetarium and for routine venom extractions.

8. Funding

This work was supported by (i) a startup grant provided by Lancaster University's Division of Biomedical and Life Sciences to SRH, (ii) a Global Impact Small Grant from Friends of Lancaster University in America to SRH, (iii) a Lancaster University Faculty of Health and Medicine Catalyst

Grant to SRH, (iv) a philanthropic donation from donor Nicholas Bone to Lancaster University allocated to SRH, and (v) a PhD stipend awarded by Lancaster University's Faculty of Science and Technology to DKB.

9. Competing Interests

The authors have no competing interests to declare.

10. References

Abouyannis, M., Boga, M., Amadi, D., Ouma, N., Nyaguara, A., Mturi, N., Berkley, J.A., Adetifa, I.M., Casewell, N.R., Lalloo, D.G., Hamaluba, M., 2023. A long-term observational study of paediatric snakebite in Kilifi County, south-east Kenya. PLoS Negl Trop Dis 17, 1–20. https://doi.org/10.1371/journal.pntd.0010987

Abouyannis, M., Nyambura, Y.K., Ngome, S., Riako, D., Musyoki, J., Muiruri, C., Orindi, B., Else, L., Amara, A., Dickinson, L., Clare, R.H., Albulescu, L.-O., Westhorpe, A.P., Kool, J., Adetifa, I., Ndungu, F.M., FitzGerald, R., Khoo, S., Lalloo, D.G., Casewell, N.R., Hamaluba, M., 2025. Development of an oral regimen of unithiol for the treatment of snakebite envenoming: a phase 1 open-label dose-escalation safety trial and pharmacokinetic analysis in healthy Kenyan adults. EBioMedicine 113, 105600. https://doi.org/10.1016/j.ebiom.2025.105600

Afroz, A., Siddiquea, B.N., Chowdhury, H.A., Jackson, T.N., Watt, A.D., 2024. Snakebite envenoming: A systematic review and meta-analysis of global morbidity and mortality. PLoS Negl Trop Dis 18, e0012080. https://doi.org/10.1371/journal.pntd.0012080

Alangode, A., Rajan, K., Nair, B.G., 2020. Snake antivenom: Challenges and alternate approaches. Biochem Pharmacol 181, 114135. https://doi.org/10.1016/j.bcp.2020.114135

Arpitha, A., Sebastin Santhosh, M., Rohit, A.C., Girish, K.S., Vinod, D., Aparna, H.S., 2017. Inhibition of Snake Venom Metalloproteinase by β-Lactoglobulin Peptide from Buffalo (Bubalus bubalis) Colostrum. Appl Biochem Biotechnol 182, 1415–1432. https://doi.org/10.1007/s12010-017-2407-6

Baines, D.K., Pawlak-Likus, Z., Tavernaraki, N.N., Platania, V., Parati, M., Cheung, T.N.W.W., Radecka, I., Domalik-Pyzik, P., Chatzinikolaidou, M., Douglas, T.E.L., 2025. Effect of Poly-γ-Glutamic Acid Molecular Weight on the Properties of Whey Protein Isolate Hydrogels. Polymers (Basel) 17, 1605. https://doi.org/10.3390/polym17121605

Baines, D.K., Wright, K., Douglas, T.E.L., 2024. Preliminary In Vitro Assessment of Whey Protein Isolate Hydrogel with Cannabidiol as a Potential Hydrophobic Oral Drug Delivery System for Colorectal Cancer Therapy. Polymers (Basel) 16, 3273. https://doi.org/10.3390/polym16233273

Bartlett, K.E., Hall, S.R., Rasmussen, S.A., Crittenden, E., Dawson, C.A., Albulescu, L.O., Laprade, W., Harrison, R.A., Saviola, A.J., Modahl, C.M., Jenkins, T.P., Wilkinson, M.C., Gutiérrez, J.M., Casewell, N.R., 2024. Dermonecrosis caused by a spitting cobra snakebite results from toxin potentiation and is prevented by the repurposed drug varespladib. Proc Natl Acad Sci U S A 121. https://doi.org/10.1073/pnas.2315597121

Bashiardes, S., Christodoulou, C., 2024. Orally Administered Drugs and Their Complicated Relationship with Our Gastrointestinal Tract. Microorganisms 12, 242. https://doi.org/10.3390/microorganisms12020242

Bashir, S., Hina, M., Iqbal, J., Rajpar, A.H., Mujtaba, M.A., Alghamdi, N.A., Wageh, S., Ramesh, K., Ramesh, S., 2020. Fundamental Concepts of Hydrogels: Synthesis, Properties, and Their Applications. Polymers (Basel) 12, 2702. https://doi.org/10.3390/polym12112702

Boechat, A.L.R., Paiva, C.S., França, F.O., Dos-Santos, M.C., 2001. Heparin-antivenom association: differential neutralization effectiveness in Bothrops atrox and Bothrops erythromelas envenoming. Rev. Inst. Med. Trop. São Paulo 43, 07–14. https://doi.org/10.1590/S0036-46652001000100002

Brumberg, V., Astrelina, T., Malivanova, T., Samoilov, A., 2021. Modern Wound Dressings: Hydrogel Dressings. Biomedicines 9, 1235. https://doi.org/10.3390/biomedicines9091235

Christy, N.P., Weiss, H.J., Phillips, L.L., Phillips, G.B., Hopewell, W., 1973. Exotic snakebite in an urban setting: heparin therapy of disseminated intravascular coagulation in a patient bitten by a saw-scaled viper (Echis carinatus). Trans. Am. Clin. Climatol. Assoc. 84, 37–48.

Clare, R.H., Hall, S.R., Patel, R.N., Casewell, N.R., 2021. Small molecule drug discovery for neglected tropical snakebite. Trends Pharmacol Sci 42, 340–353. https://doi.org/10.1016/j.tips.2021.02.005

Collier, M., Potts, C., Shaw, E., 2014. Use of a coverlet system for the management of skin microclimate. Br. J. Nurs. Mark Allen Publ. 23, S28, S30-35. https://doi.org/10.12968/bjon.2014.23.Sup15.s28

Conner, K.P., Devanaboyina, S.C., Thomas, V.A., Rock, D.A., 2020. The biodistribution of therapeutic proteins: Mechanism, implications for pharmacokinetics, and methods of evaluation. Pharmacol Ther 212, 107574. https://doi.org/10.1016/j.pharmthera.2020.107574

Devlin, A.J., Mycroft-West, C.J., Turnbull, J.E., Lima, M.A. de, Guerrini, M., Yates, E.A., Skidmore, M.A., 2023. Analysis of Heparin Samples by Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy in the Solid State. ACS Cent Sci 9, 381–392. https://doi.org/10.1021/acscentsci.2c01176

Du, T.Y., Hall, S.R., Chung, F., Kurdyukov, S., Crittenden, E., Patel, K., Dawson, C.A., Westhorpe, A.P., Bartlett, K.E., Rasmussen, S.A., Moreno, C.L., Denes, C.E., Albulescu, L.-O., Marriott, A.E., Mackay, J.P., Wilkinson, M.C., Gutiérrez, J.M., Casewell, N.R., Neely, G.G., 2024. Molecular dissection of cobra venom highlights heparinoids as an antidote for spitting cobra envenoming. Sci Transl Med 16. https://doi.org/10.1126/scitranslmed.adk4802

Ganji, F., Vasheghani-Farahani, S., Vasheghani-Farahani, E., 2010. Theoretical Description of Hydrogel Swelling: A Review. Iranian Polymer Journal 19, 375–398.

Genç, H., Friedrich, B., Alexiou, C., Pietryga, K., Cicha, I., Douglas, T.E.L., 2023. Endothelialization of Whey Protein Isolate-Based Scaffolds for Tissue Regeneration. Molecules 28, 7052. https://doi.org/10.3390/molecules28207052

Gerardo, C.J., Carter, R.W., Kumar, S., Shirazi, F.M., Kotehal, S.D., Akpunonu, P.D., Bhalla, A., Schwartz, R.B., Bammigatti, C., Manikath, N., Mukherjee, P.P., Arnold, T.C., Wolk, B.J., S Sheikh, S., Sollee, D.R., Vearrier, D.J., Francis, S.J., Aizenberg, A., Kumar, H., Ravikumar, M.K., Sarkar, S., Haston, T., Micciche, A., Oomman, S.C., Owen, J.L., Ritter, B.A., Samuel, S.P., Lewin, M.R., Platts-Mills, T.F., 2024. Oral varespladib for the treatment of snakebite envenoming in India and the USA (BRAVO): a phase II randomised clinical trial. BMJ Glob Health 9, e015985. https://doi.org/10.1136/bmjgh-2024-015985

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017a. Snakebite envenoming. Nat Rev Dis Primers 3, 1–21. https://doi.org/10.1038/nrdp.2017.63

Formatted: English (United Kingdom)

667 Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Vargas, M., Villalta, M., Sánchez, A.,
668 Sanz, L., Lomonte, B., León, G., Calvete, J., 2017b. Preclinical Evaluation of the Efficacy of
669 Antivenoms for Snakebite Envenoming: State-of-the-Art and Challenges Ahead. Toxins (Basel)
670 9, 163. https://doi.org/10.3390/toxins9050163

671 Hall, S.R., Rasmussen, S.A., Crittenden, E., Dawson, C.A., Bartlett, K.E., Westhorpe, A.P., Albulescu,
672 L.O., Kool, J., Gutiérrez, J.M., Casewell, N.R., 2023. Repurposed drugs and their combinations
673 prevent morbidity-inducing dermonecrosis caused by diverse cytotoxic snake venoms. Nat
674 Commun 14. https://doi.org/10.1038/s41467-023-43510-w

675 Hameed, H., Faheem, S., Paiva-Santos, A.C., Sarwar, H.S., Jamshaid, M., 2024. A Comprehensive
676 Review of Hydrogel-Based Drug Delivery Systems: Classification, Properties, Recent Trends,
677 and Applications. AAPS PharmSciTech 25, 64. https://doi.org/10.1208/s12249-024-02786-x

678 He, C., Ji, H., Qian, Y., Wang, Q., Liu, X., Zhao, W., Zhao, C., 2019. Heparin-based and heparin-
679 inspired hydrogels: size-effect, gelation and biomedical applications. J Mater Chem B 7, 1186–
680 1208. https://doi.org/10.1039/C8TB02671H

681 Hirsh, J., 1998. Low-Molecular-Weight Heparin. Circulation 98, 1575–1582.
682 https://doi.org/10.1161/01.CIR.98.15.1575

683 Hoare, T.R., Kohane, D.S., 2008. Hydrogels in drug delivery: Progress and challenges. Polymer
684 (Guildf) 49, 1993–2007. https://doi.org/10.1016/j.polymer.2008.01.027

685 Ibiapina, H.N.S., Costa, A.G., Sachett, J.A.G., Silva, I.M., Tarragô, A.M., Neves, J.C.F., Kerr,
686 M.W.A., Santana, M.F., Martins-Filho, O.A., Lacerda, M.V.G., Ferreira, L.C.L., Malheiro, A.,
687 Monteiro, W.M., 2019. An Immunological Stairway to Severe Tissue Complication Assembly in
688 Bothrops atrox Snakebites. Front Immunol 10. https://doi.org/10.3389/fimmu.2019.01882

689 Ivory-Cousins, T., Nurzynska, A., Klimek, K., Baines, D.K., Truszkiewicz, W., Pałka, K., Douglas,
690 T.E.L., 2023. Whey Protein Isolate/Calcium Silicate Hydrogels for Bone Tissue Engineering
691 Applications—Preliminary In Vitro Evaluation. Materials 16, 6484.
692 https://doi.org/10.3390/ma16196484

693 Kamerzell, T.J., Esfandiary, R., Joshi, S.B., Middaugh, C.R., Volkin, D.B., 2011. Protein–excipient
694 interactions: Mechanisms and biophysical characterization applied to protein formulation
695 development. Adv. Drug Deliv. Rev., Formulating biomolecules: mechanistics insights in
696 molecular interactions 63, 1118–1159. https://doi.org/10.1016/j.addr.2011.07.006

697 Keppler, J.K., Sönnichsen, F.D., Lorenzen, P.-C., Schwarz, K., 2014. Differences in heat stability and
698 ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and
699 fluorescence quenching. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1844,
700 1083–1093. https://doi.org/10.1016/j.bbapap.2014.02.007

701 Kesharwani, P., Bisht, A., Alexander, A., Dave, V., Sharma, S., 2021. Biomedical applications of
702 hydrogels in drug delivery system: An update. J Drug Deliv Sci Technol 66, 102914.
703 https://doi.org/10.1016/j.jddst.2021.102914

704 Khan, M.U.A., Aslam, M.A., Bin Abdullah, M.F., Hasan, A., Shah, S.A., Stojanović, G.M., 2023.
705 Recent perspective of polymeric biomaterial in tissue engineering– a review. Mater Today Chem
706 34, 101818. https://doi.org/10.1016/j.mtchem.2023.101818

707 Kharkar, P.M., Kiick, K.L., Kloxin, A.M., 2013. Designing degradable hydrogels for orthogonal
708 control of cell microenvironments. Chem. Soc. Rev. 42, 7335–7372.
709 https://doi.org/10.1039/C3CS60040H

Lalloo, D.G., Theakston, R.D.G., 2003. Snake antivenoms. J Toxicol Clin Toxicol 41, 277–290. https://doi.org/10.1081/CLT-120021113

Lee, S., Miller, J.S., Moon, J.J., West, J.L., 2005. Proteolytically Degradable Hydrogels with a Fluorogenic Substrate for Studies of Cellular Proteolytic Activity and Migration. Biotechnol Prog 21, 1736–1741. https://doi.org/10.1021/bp0502429

Lewin, M., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) appears to be a potent, broad-spectrum, inhibitor of snake venom phospholipase A2 and a possible pre-referral treatment for envenomation. Toxins (Basel) 8, 1–16. https://doi.org/10.3390/toxins8090248

Madureira, A.R., Pereira, C.I., Gomes, A.M.P., Pintado, M.E., Xavier Malcata, F., 2007. Bovine whey proteins – Overview on their main biological properties. Food Res. Int. Ott. Ont 40, 1197–1211. https://doi.org/10.1016/j.foodres.2007.07.005

Manicourt, D., Fujimoto, N., Obata, K., Thonar, E.J. -M. A., 1994. Serum levels of collagenase, stromelysin-1, and timp-1. Arthritis Rheum 37, 1774–1783. https://doi.org/10.1002/art.1780371211

Martin, N.C., Pirie, A.A., Ford, L.V., Callaghan, C.L., McTurk, K., Lucy, D., Scrimger, D.G., 2006. The use of phosphate buffered saline for the recovery of cells and spermatozoa from swabs. Science & Justice 46, 179–184. https://doi.org/10.1016/S1355-0306(06)71591-X

McCarty, S.M., Percival, S.L., 2013. Proteases and Delayed Wound Healing. Adv Wound Care (New Rochelle) 2, 438–447. https://doi.org/10.1089/wound.2012.0370

Mecozzi, M., Pietroletti, M., Tornambè, A., 2011. Molecular and structural characteristics in toxic algae cultures of Ostreopsis ovata and Ostreopsis spp. evidenced by FTIR and FTNIR spectroscopy. Spectrochim Acta A Mol Biomol Spectrosc 78, 1572–1580. https://doi.org/10.1016/j.saa.2011.02.002

Ogunfowokan, O., 2012. Bite-to-hospital time and morbidity in victims of viper bite in a rural hospital in Nigeria. Afr J Prim Health Care Fam Med 4, 1–7. https://doi.org/10.4102/phcfm.v4i1.371

Pawlak-Likus, Z., Baines, D.K., Tavernaraki, N.N., Platania, V., Smith, A.M., Chatzinikolaidou, M., Domalik-Pyzik, P., Douglas, T.E.L., 2025. Physicochemical Properties and Angiogenic Potential of Whey Protein Isolate Hydrogels Modified with Heparin or Tinzaparin. J Polym Environ 33, 4130–4146. https://doi.org/10.1007/s10924-025-03626-0

Platania, V., Douglas, T.E.L., Zubko, M.K., Ward, D., Pietryga, K., Chatzinikolaidou, M., 2021. Phloroglucinol-enhanced whey protein isolate hydrogels with antimicrobial activity for tissue engineering. Materials Science and Engineering: C 129, 112412. https://doi.org/10.1016/j.msec.2021.112412

Ribeiro, M., Simões, M., Vitorino, C., Mascarenhas-Melo, F., 2024. Hydrogels in Cutaneous Wound Healing: Insights into Characterization, Properties, Formulation and Therapeutic Potential. Gels 10, 188. https://doi.org/10.3390/gels10030188

Rivel, M., Solano, D., Herrera, M., Vargas, M., Villalta, M., Segura, Á., Arias, A.S., León, G., Gutiérrez, J.M., 2016. Pathogenesis of dermonecrosis induced by venom of the spitting cobra, Naja nigricollis: an experimental study in mice. Toxicon 119, 171–179. https://doi.org/10.1016/j.toxicon.2016.06.006

Rucavado, A., Nicolau, C., Escalante, T., Kim, J., Herrera, C., Gutiérrez, J., Fox, J., 2016. Viperid Envenomation Wound Exudate Contributes to Increased Vascular Permeability via a

Formatted: Spanish (Spain)

DAMPs/TLR-4 Mediated Pathway. Toxins (Basel) 8, 349. https://doi.org/10.3390/toxins8120349

Sharma, N., Chauhan, S., Faruqi, S., Bhat, P., Varma, S., 2005. Snake envenomation in a north Indian hospital. Emergency Medicine Journal 22, 118–120. https://doi.org/10.1136/emj.2003.008458

Shen, X., Liu, Z., Li, J., Wu, D., Zhu, M., Yan, L., Mao, G., Ye, X., Linhardt, R.J., Chen, S., 2019. Development of low molecular weight heparin by H2O2/ascorbic acid with ultrasonic power and its anti-metastasis property. Int J Biol Macromol 133, 101–109. https://doi.org/10.1016/j.ijbiomac.2019.04.019

Sweeney, I.R., Miraftab, M., Collyer, G., 2012. A critical review of modern and emerging absorbent dressings used to treat exuding wounds. Int Wound J 9, 601–612. https://doi.org/10.1111/j.1742-481X.2011.00923.x

Tasoulis, T., Isbister, G.K., 2017. A review and database of snake venom proteomes. Toxins (Basel) 9, 1–23. https://doi.org/10.3390/toxins9090290

Uzunalli, G., Mammadov, R., Yesildal, F., Alhan, D., Ozturk, S., Ozgurtas, T., Guler, M.O., Tekinay, A.B., 2017. Angiogenic Heparin-Mimetic Peptide Nanofiber Gel Improves Regenerative Healing of Acute Wounds. ACS Biomater Sci Eng 3, 1296–1303. https://doi.org/10.1021/acsbiomaterials.6b00165

Wang, Z.L., Tang, X., Wang, M., She, Y.X., Yang, B.R., Sheng, Q.H., Abd El-Aty, A.M., 2024. β-Lactoglobulin separation from whey protein: A comprehensive review of isolation and purification techniques and future perspectives. J. Dairy Sci. 107, 11785–11795. https://doi.org/10.3168/jds.2024-25321

Yegappan, R., Lauko, J., Wang, Z., Lavin, M.F., Kijas, A.W., Rowan, A.E., 2022. Snake Venom Hydrogels as a Rapid Hemostatic Agent for Uncontrolled Bleeding. Adv Healthc Mater 11. https://doi.org/10.1002/adhm.202200574

Yergoz, F., Hastar, N., Cimenci, C.E., Ozkan, A.D., Tekinay, T., Guler, M.O., Tekinay, A.B., 2017. Heparin mimetic peptide nanofiber gel promotes regeneration of full thickness burn injury. Biomaterials 134, 117–127. https://doi.org/10.1016/j.biomaterials.2017.04.040

Zhang, L., Ma, Y., Pan, X., Chen, S., Zhuang, H., Wang, S., 2018. A composite hydrogel of chitosan/heparin/poly (γ-glutamic acid) loaded with superoxide dismutase for wound healing. Carbohydr Polym 180, 168–174. https://doi.org/10.1016/j.carbpol.2017.10.036

Zhu, W., Dong, Y., Xu, P., Pan, Q., Jia, K., Jin, P., Zhou, M., Xu, Y., Guo, R., Cheng, B., 2022. A composite hydrogel containing resveratrol-laden nanoparticles and platelet-derived extracellular vesicles promotes wound healing in diabetic mice. Acta Biomater 154, 212–230. https://doi.org/10.1016/j.actbio.2022.10.038

Zustiak, S.P., Leach, J.B., 2011. Characterization of protein release from hydrolytically degradable poly(ethylene glycol) hydrogels. Biotechnol Bioeng 108, 197–206. https://doi.org/10.1002/bit.22911

Formatted: German (Germany)

Title

Heparin-incorporated whey protein isolate-derived hydrogels with an intended dual function as snakebite wound dressings and drug delivery systems inhibit spitting cobra venom-induced cytotoxicity

Author Names and Affiliations

Matthew J. Gray[a]1, Daniel K. Baines[b], Gabrielle De Castro[b], Thomas Cobb[a], Layla A. Tyrrell[a], Sam J. Hyam[a], Alan M. Smith[c], Nicholas R. Casewell[d,e], Sarah L. Allinson[a], Timothy E. L. Douglas[b†], and Steven R. Hall[a†]

[a]Division of Biomedical and Life Sciences, Lancaster University, Bailrigg, Lancaster LA1 4YW, UK. [b]School of Engineering, Lancaster University, Bailrigg, Lancaster LA1 4YW, UK. [c]Department of Pharmacy, University of Huddersfield, Queensgate, Huddersfield HD1 3DH, UK. [d]Centre for Snakebite Research & Interventions, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. [e]Centre for Drugs & Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK.

[†]Co-corresponding authors. TELD email: t.douglas@lancaster.ac.uk, SRH email: s.r.hall@lancaster.ac.uk

Present address: 1School of Biological Sciences, Queen's University Belfast, University Road, Belfast BT7 1NN, UK.

Highlights

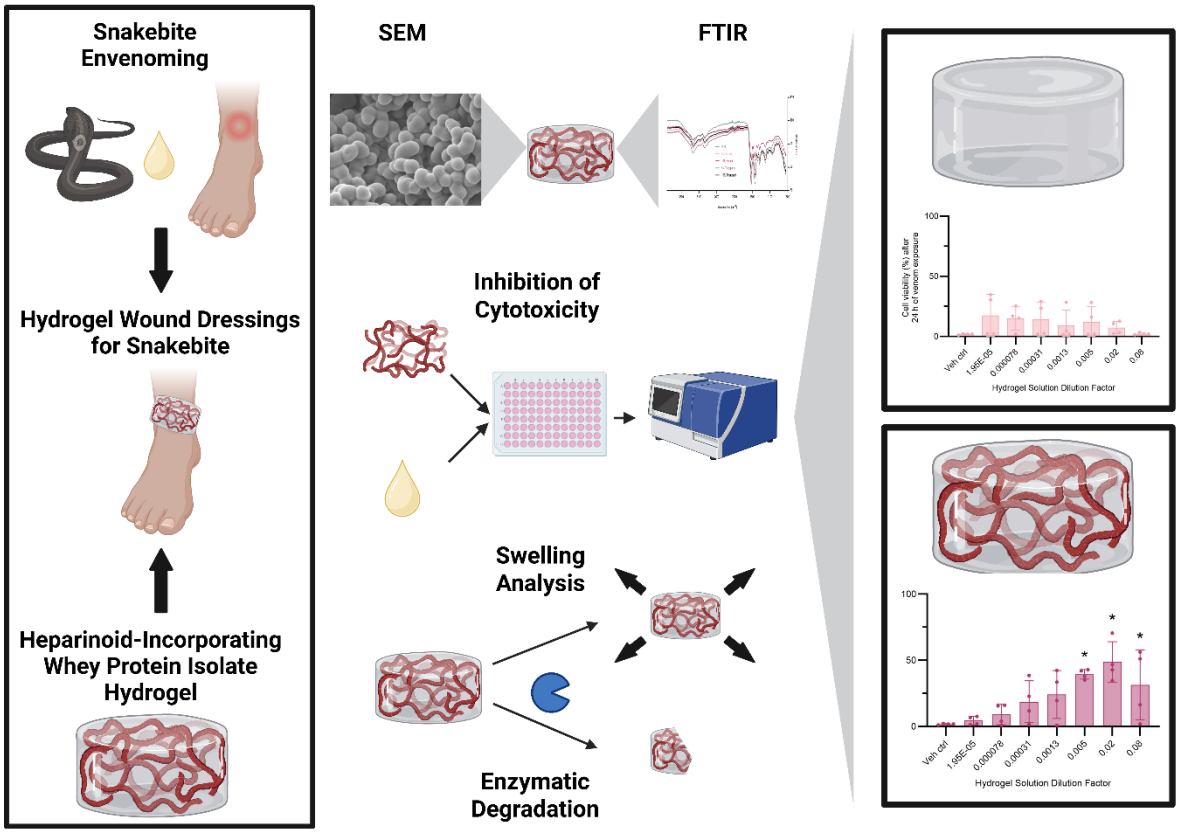
- Whey protein isolate (WPI) hydrogels incorporating heparin or tinzaparin were designed to act as snakebite wound dressings.
- Heparin-integration improves WPI hydrogels' swelling properties but reduces their longevity in model wound environments.
- WPI hydrogels released tinzaparin into solution at high concentrations following incubation at both 1 and 24 hours
- 1 and 24-hour tinzaparin-containing solutions inhibited *N. nigricollis* venom cytotoxicity in a dermonecrosis cell model.

Abstract

Snakebite envenoming affects millions of people annually, with current treatments limited to animal-derived antivenoms. Repurposed drug-inhibitors of toxin families offer an exploitable avenue to improve snakebite treatment, including heparins which can inhibit cytotoxic three-finger toxins. However, to be effective therapies in-the-field such treatments must be engineered into drug delivery devices capable of rapidly administering drug(s) to the envenomation site. Herein we introduce the concept of integrating heparins, specifically unfractionated heparin (H) and its low molecular weight heparinoid variant, tinzaparin (T), into hydrogels composed of whey protein isolate (WPI), an inexpensive byproduct of the dairy industry that is cytocompatible, stiff, sterilizable by autoclaving, and that has the dual function of being able to locally deliver drugs and act as wound dressings. The

aims of this research were to investigate whether heparin-containing WPI hydrogels displayed physical characteristics suitable for wound dressings and could effectively release drug in sufficient quantities to inhibit the cytotoxic activity of spitting cobra venom. To do so, five hydrogel prototypes were produced: 40% WPI no drug control, and 5% H, 10% H, 5% T, and 10% T all within 40% WPI hydrogels. It was determined that heparins successfully integrated into WPI hydrogels and heparin-containing WPI hydrogels exhibited improved swelling versus the 40% WPI no drug control, suggesting improved absorption of wound exudate; however, this heparin integration also increased the hydrogel degradation rate in simulated wound environments. Most importantly, sufficiently high concentrations of T were released into phosphate buffered saline from the 10% T hydrogels to neutralise *Naja nigricollis* venom cytotoxicity in a HaCaT cell cytotoxicity model. Together, our results suggest that the integration and diffusion of venom-inhibiting drugs in WPI hydrogels is possible and that the development of such drug-integrated hydrogels into snakebite wound dressings warrants further research.

Graphical Abstract



Created in BioRender. Gray, M. (2026) https://BioRender.com/uf60qur

Keywords

Snakebite, Venom, Hydrogel, Heparins, Wound-dressing, Cytotoxicity

Abbreviations

3FTx – 3-Finger Toxin

ANOVA – Analysis of Variance

CSRI – Centre for Snakebite Research & Interventions

DMEM - Dulbecco's Modified Eagles Medium

FBS – Foetal Bovine Serum

H - Heparin

LMW – Low Molecular Weight

LSTM – Liverpool School of Tropical Medicine

NTD – Neglected Tropical Disease

PBS – Phosphate Buffered saline

PLA_2 – Phospholipase A_2

SEM – Scanning Electron Microscopy

SVMP – Snake Venom Metalloprotease

T - Tinzaparin

WPI – Whey Protein Isolate

1. Introduction

Snakebite envenoming is classified by the World Health Organization (WHO) as a neglected tropical disease (NTD), responsible for up to 131,000 deaths and 400,000 cases of long-term disability every year (Afroz *et al.*, 2024; J. M. Gutiérrez *et al.*, 2017a). Compounding this public health issue is the fact that the only currently available specific treatments are animal-derived antivenoms, which have remained broadly unchanged for over a century (Lalloo and Theakston, 2003). These conventional antivenoms suffer numerous limitations: often exhibiting poor pan-snake species efficacy, high expense of treatment, limited effectiveness at neutralising severe local envenoming caused by venom cytotoxicity, the requirement to be administered intravenously in a hospital environment, the need to be kept cold, and potentially serious adverse events that frequently develop (Alangode *et al.*, 2020; Clare *et al.*, 2021; Rivel *et al.*, 2016).

As such, other possible treatment options have begun to be explored, including the use of repurposed drug inhibitors targeting the main classes of toxins found in many different snake venoms across different species and genera (Clare *et al.*, 2021). Some promising candidates are heparins including unfractionated heparin (H), a highly sulphated polysaccharide previously explored for hemotoxic snakebite treatment, and its low molecular weight (LMW) heparinoid variant, tinzaparin (T), both of which are anticoagulants traditionally used for thromboembolic indications (Boechat *et al.*, 2001; Christy *et al.*, 1973; Du *et al.*, 2024; Hirsh, 1998). These drugs are inhibitors of cytotoxic three-finger toxins (3FTxs), a toxin class especially prevalent in the venoms of many cobra species and have been shown to markedly reduce venom cytotoxicity in both cell and animal models (Du *et al.*, 2024; Tasoulis and Isbister, 2017). Such venom-induced cytotoxicity often causes severe and life-altering morbidity in snakebite victims, with spitting cobras (*Naja* spp.) being largely responsible for such injury in Africa (Du *et al.*, 2024; Rivel *et al.*, 2016).

However, for any new toxin-inhibitor to be effective against snakebite-induced tissue damage it must first reach the venom in and around the envenomation site sufficiently quickly (Bartlett *et al.*, 2024). This is problematic with IgG-based and intravenously delivered antivenoms due to their limited extravasation and tissue permeability (Clare *et al.*, 2021; Conner *et al.*, 2020; J. M. Gutiérrez *et al.*, 2017b) while promising oral drug candidates currently in development (Abouyannis *et al.*, 2025; Gerardo *et al.*, 2024) are limited by the time it takes for them to be absorbed by the gut and to adequately distribute from the blood stream into the affected tissues (Bartlett *et al.*, 2024; Bashiardes and Christodoulou, 2024). One potential option to aid rapid local drug delivery is the use of hydrogels in which venom toxin-inhibiting drugs, such as heparins, are integrated and used as snakebite wound dressings. Hydrogels are three-dimensional hydrophilic polymers crosslinked to form a lattice which possess numerous benefits over traditional wound dressings including the ability to retain moisture, crucial for the wound healing process, and to absorb fluids such as wound exudate (Bashir *et al.*, 2020; Brumberg *et al.*, 2021; Sweeney *et al.*, 2012). Importantly, hydrogels can be incorporated with drugs into their structure which can later diffuse out of the gel in solution or when applied to a wound, allowing them to be used for targeted drug delivery (Kesharwani *et al.*, 2021). As such, it was hypothesised that by incorporating H or T into hydrogels, a unique material could be engineered to be later translated into a snakebite wound dressing that would simultaneously protect the physical site of a snakebite injury whilst also releasing drug into the wound to reduce the effects of cytotoxic 3FTx-containing snake venoms.

Many different materials can be used to make hydrogels, however a promising one for this purpose is WPI (Bashir *et al.*, 2020). WPI, a byproduct of the dairy industry, contains 97% protein, 75% of which

is β-lactoglobulin, the primary compound making up the hydrogel lattice (Baines *et al.*, 2025). The benefits of WPI hydrogels include low cost, high cytocompatibility, and ease of gelation (Genç *et al.*, 2023). In addition, WPI hydrogels can incorporate and deliver both hydrophobic and hydrophilic drugs for a variety of biomedical applications including facilitating tissue engineering, managing conditions such as cancers, and the treatment of bacterial infections (Baines *et al.*, 2025, 2024; Platania *et al.*, 2021). Both H and T have been incorporated into WPI hydrogels to explore their potential as tissue engineering scaffolds (Pawlak-Likus *et al.*, 2025). Additionally, studies exploring H-incorporated non-WPI hydrogels as wound dressings for diabetic, excisional, and burn injuries have demonstrated beneficial effects of H on wound healing (Uzunalli *et al.*, 2017; Yergoz *et al.*, 2017; Zhang *et al.*, 2018). However, whilst studies have investigated the use of hydrogels incorporating snake venoms into their lattice as haemostatic agents (Yegappan *et al.*, 2022), no studies have yet explored H- or T-integrated hydrogels for use as wound dressings for snakebite.

Therefore, the aims of this study were to investigate as proof-of-concept whether heparin-containing WPI hydrogels could be engineered that (a) exhibit physical properties suitable to translation into wound dressings and (b) release their contained drug in sufficient quantities to inhibit spitting cobra venom-induced cell death. To date, no study has proposed or explored the potential utility of toxin inhibiting drug-integrated hydrogels as a pre-hospital snakebite wound treatment. However, the results contained herein suggest that this is a promising approach and that further work on this topic is warranted.

2. **Materials and Methods**

2.1. Materials

2.1.1. Chemicals, drugs and biological materials

WPI was purchased as the product BiPro from Davisco Foods International (Eden Prairie, MN, USA). Trypan blue stain (0.4%) was acquired from Logos Biosystems, resazurin and proteases were purchased from Sigma-Aldrich (Merck), and PBS purchased from Gibco, Corning or Sigma-Aldrich. Unfractionated heparin (H) sodium salt ($M_w \approx$ 20,000 g/mol) and tinzaparin (T; $M_w \approx$ 8,000 g/mol) derived from porcine mucosa were supplied by LEO Pharma (Ballerup, Denmark).

2.1.2. Venoms

Naja nigricollis venom (Nigeria) was provided by the Liverpool School of Tropical Medicine's (LSTM) Centre for Snakebite Research and Interventions (CSRI). The venom was a pool collected from multiple milkings of four wild-caught snakes maintained in the CSRI Herpetarium facility, whose animal protocols are approved by the UK Home Office, the LSTM and the University of Liverpool's Animal Welfare and Ethical Review Boards. The venoms were lyophilised and stored at 4 °C before reconstitution in PBS and storage at –80 °C until use.

2.1.3. Cells

Immortalised human epidermal keratinocytes (HaCaT cells) were provided by Prof Sarah Allinson (Lancaster University; originally sourced from AddexBio) and cultured in Dulbecco's Modified Eagles Medium (DMEM) (Gibco) augmented with 9.5% Foetal Bovine Serum (FBS) (LabTech), 2% sodium pyruvate (2 mM; Cytiva), and 1% penicillin (100 IU/mL) and streptomycin (250 µg/mL) (Gibco), hereafter referred to as Standard Medium. Cellular assays were then conducted in low background

fluorescence medium, made up of FluoroBrite DMEM (Gibco), 1% FBS, 2% sodium pyruvate, 1% penicillin and streptomycin and 1% GlutaMax (Gibco), hereafter referred to as Low Background Fluorescence Medium (Hall *et al.*, 2023). All 96-well plates were purchased from Greiner.

2.2. Data Collection

2.2.1. Hydrogel Formation

Five whey protein isolate (WPI) hydrogel types were produced (**Table 1**), formed as previously described (Pawlak-Likus *et al.*, 2025). The solutions were homogenised for 24 hours using an IKA Loopster (IKA England LTD, Oxford, UK), before the mixture was sonicated to remove bubbles that could compromise gel uniformity. Solutions were injected into a hydrogel dressing-gelation device (Biomat Ltd, UK) and heated for 20 minutes at 70°C to encourage efficient gelation. Hydrogel samples were then cut into 0.03 g discs, hereafter referred to simply as discs, using a metal corer to ensure uniform volume and surface area. Experiments were conducted using discs of all five hydrogel types unless stated otherwise.

Table 1 – Hydrogel samples and their compositions.

Sample group name	WPI concentration (% w/v)	Heparin or tinzaparin concentration (% w/v)
40% WPI	40	0
5% H	40	5
10% H	40	10
5% T	40	5
10% T	40	10

2.2.2. Scanning Electron Microscopy

Hydrogel samples were imaged using scanning electron microscopy (SEM), conducted with a JSM-6390 LV, JEOL Ltd. (Welwyn Gardens, UK) scanning electron microscope, an accelerating voltage of 15 kV, and using magnifications of 100x, 3,000x and 10,000x. Samples were taken from the centre of each hydrogel disc, dehydrated in a desiccator, and gold-coating was performed with a Quorum Technologies, Q150RES, sputter coater to a thickness of ca. 5 nm before imaging took place.

2.2.3. Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was used to determine chemical linkages present within the gel. Discs of each hydrogel type were dehydrated and the resultant IR spectra analysed using a Cary 630 FTIR spectrophotometer (Agilent, Santa Clara, CA, USA) in attenuated total reflection (ATR) mode and using a spectral range of between 650 and 4000 cm^{-1}. Experiments were completed in triplicate.

2.2.4. Hydrogel Swelling Analysis in Dulbecco's Modified Eagles Medium and Phosphate Buffered Saline

To determine how the incorporation of the H or T concentrations in the hydrogel affected the polymer's swelling behaviour, samples of each hydrogel type were weighed before being incubated at 37°C for 7 days in either Dulbecco's Modified Eagles Medium (DMEM) or pH 7.4 Phosphate Buffered Saline

(PBS). Five samples per hydrogel were individually placed into 200 µL of pH 7.4 PBS, and ten samples were individually placed into 200 µL of DMEM. Following incubation, water excess was removed from the surface of each disc using a paper towel, and each sample reweighed. The % mass change (M%) was calculated from the following formula:

$$M\% = \frac{M_W - M_i}{M_i} \times 100\%$$

where M_w is the wet mass and M_i is the initial mass.

2.2.5. Protein Release

To determine how H or T incorporation altered the release of protein from the 3D polymer lattice, as a measure of general hydrogel degradation, hydrogel samples were submerged in 1 mL pH 7.4 PBS and incubated at 37ºC. Following 24 and 48 hours, 1 µL of this solution was removed and analysed using a NanoDrop at A_{280} (NanoDrop 2000c, ThermoScientific) (Pawlak-Likus *et al.*, 2025). Experiments were completed in triplicate.

2.2.6. Enzyme Degradation

To better evaluate how the hydrogels may degrade in protease-containing biological fluids, enzyme degradation experiments were conducted (Lee *et al.*, 2005). Five hydrogel samples were incubated in 200 µL of pH 7.4 PBS containing 13.33 µg/mL protease mixture obtained from bovine pancreas (CAS no. 9001-92-7), concentrations consistent with blood collagenase levels (Manicourt *et al.*, 1994; Pawlak-Likus *et al.*, 2025), at Standard Conditions for seven days. The samples were weighed before and after the incubation period, with excess solution being removed with a paper towel, and the % mass change (M%) calculated:

$$M\% = \frac{M_a - M_i}{M_i} \times 100\%$$

where M_a is the mass after incubation and M_i is the initial mass.

2.2.7. Tinzaparin Release Quantification

Absorbance determination: An absorbance scan (230-400 nm with 2 nm increments) was completed on 50 µL of 4 mg/mL tinzaparin in PBS and compared to a PBS-only control in a half-volume 96-well UV-capable microplate using a Tecan MPLEX plate reader over three independent experimental replicates to confirm an absorbance wavelength at which tinzaparin could be quantified in solution.
Tinzaparin release from hydrogels determination: To measure drug release from the tinzaparin-containing WPI hydrogel, 10% T and WPI control discs were soaked in PBS (3 discs / 180 µL PBS) for 1 hour or 24 hours under standard conditions (37 ºC, 5% CO_2). Over three experimental replicates, 50 microlitres of the resulting solutions were pipetted into separate wells in a half-volume 96-well UV-capable microplate along with serial dilutions of known tinzaparin concentrations (4,000 – 31.25 µg/mL, with a halving of the concentration with each serial dilution). A_{230} was measured in each well using a Tecan MPLEX plate reader and a standard curve plotted for each known tinzaparin concentration serial dilution, from which the concentration of tinzaparin released from the 10% tinzaparin-containing discs under standard conditions was determined for each of three experimental replicates and the means calculated.

2.2.8. Cytotoxicity Assay

Day 1: HaCaT cells (10,000/well) were seeded in black-sided, clear-bottomed, half-volume 96-well microplates and incubated for 24 hours under standard conditions. Three discs of the WPI control hydrogel or the 10% T hydrogel were added to 180 µl PBS and incubated for 24 hours at standard conditions. *Day 2:* Three discs of both hydrogel types were incubated in the same manner for 1 hour. Serial dilutions of the resulting hydrogel solutions (0.08x to 1.95×10^{-5}x dilutions with a quartering of the concentration with each serial dilution) were mixed with a constant 20 µg/mL *N. nigricollis* venom or venom vehicle control (PBS) in Low Background Fluorescence Medium. The following control solutions were also prepared: the WPI control and 10% T hydrogel solutions with no venom, venom only, medium only, venom with 2,000 µg/ml T, and the 0% cell viability control using 0.1% Ecosurf™. All solutions were kept at standard conditions for 30 minutes prior to cell treatments, after which the cells were exposed to these solutions for 24 hours under standard conditions. *Day 3:* Resazurin cell viability assays were used as a model of cell viability as described previously (Du *et al.*, 2024). Briefly, all wells were treated with 90 µg/mL resazurin and incubated for 1.5-2 hours. Fluorescence (Ex_{544}/Em_{590}) was quantified using a Tecan MPLEX plate reader. Percent cell viability within each experimental well was calculated relative to the mean within all untreated 100% cell viability positive control wells following subtraction of the average fluorescence response from all background control wells which contained no cells:

$$\%\ Cell\ Viability = \frac{Em_{590\ (treatment\ well)} - Average(Em_{590\ (blank\ well)})}{Average(Em_{590\ (positive\ control\ well)}) - Average(Em_{590\ (blank\ well)})} \times 100$$

2.3. Data Analysis

All data are presented as mean ± standard deviation (SD) unless stated otherwise. 'n' is defined as an independent experimental replicate completed at a separate time from other replicates. At least three independent experimental replicates were completed for each statistically analysed set of data. Statistical analyses of data and graph creation were performed using GraphPad Prism unless stated otherwise. The specific statistical tests performed are stated in the figure legends. A difference was considered significant if $P \leq 0.05$.

3. **Results**

Analysis of the effectiveness of hydrogels as heparin delivery vehicles was conducted along several avenues, beginning with the determination of their physical and mechanical properties, followed by investigating how they may act in the biological environment of a wound such as those caused by snakebite, before finally confirming both the release of the drug from the gel and the effectiveness of the hydrogel delivery vehicle in preventing *N. nigricollis* venom-induced cytotoxicity. This began with the generation of one control 40% WPI hydrogel with no drug and four drug-integrated hydrogels containing the same percentage of WPI: 5% H, 10% H, 5% T, and 10% T.

3.1. Scanning Electron Microscopy Reveals Heparin Incorporation Alters Hydrogel Structure

To generate a detailed image of its surface characteristics and three-dimensional structure of each of the hydrogels, SEM was conducted on a dried sample of each hydrogel type at 100x, 3,000x and 10,000x magnification (**Fig. 1**).

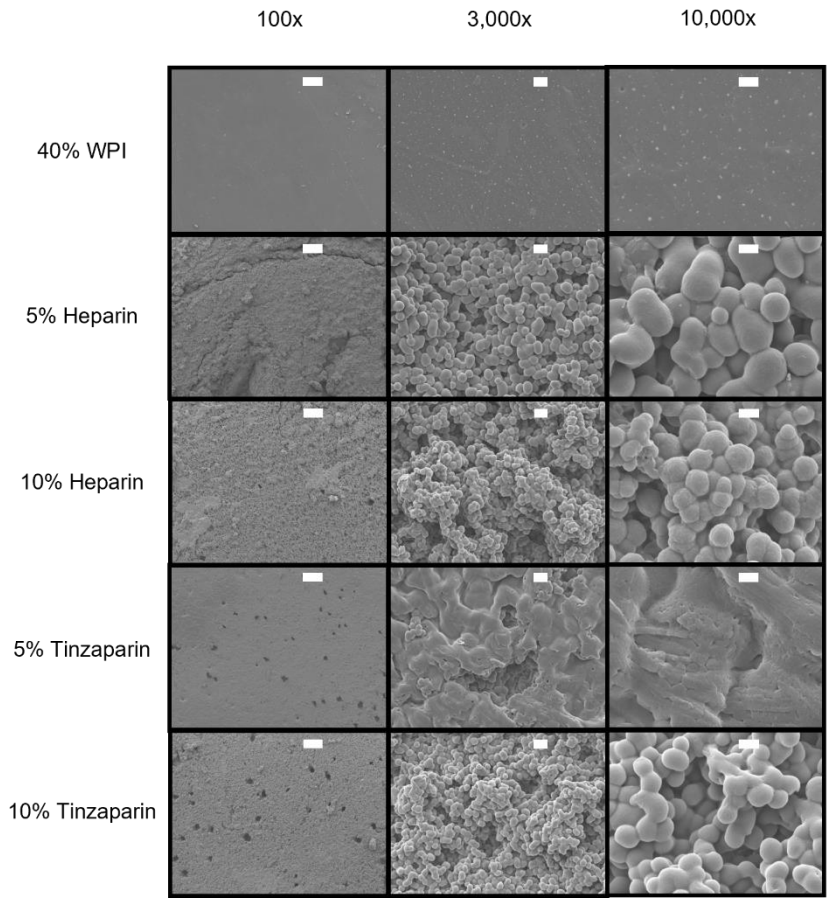


Fig. 1 – SEM images reveal structural differences between dried samples of the five tinzaparin types. SEM images of dried samples of control 40% WPI hydrogel with no drug, and WPI hydrogels integrated with 5% unfractionated heparin (H), 10% H, 5% tinzaparin (T), and 10% T. Moving left to right, images were taken at magnifications of 100x, 3,000x, and 10,000x, and the corresponding scalebars are equal to: 100 µm, 4 µm, and 1 µm.

As can be seen in **Fig. 1**, the control 40% WPI hydrogel contains very few pores, even at high magnifications. In comparison, the surface structure appears rougher and more textured in the hydrogel modified with 5% H, even more so than the hydrogel modified with 10% H. At 3,000x and 10,000x it becomes apparent that this is due to the presence of clusters of spherical and semi-spherical structures. In the 10% H hydrogel, these structures seem more tightly packed together than in the 5% H hydrogel. Differences are also apparent in the tinzaparin-containing hydrogels. At 100x magnification, dark pores can be seen on the surfaces of both T-containing hydrogels. However, at higher magnifications, the physical structure of the 5% T hydrogel appears disrupted and slightly porous but still relatively smooth compared to that of the hydrogel incorporated with 10% T, where similar spheroid structures to the H hydrogels are present.

3.2. Fourier Transform Infrared Spectroscopy Confirms the Presence of Heparins

Following SEM, FTIR was conducted to identify the chemical bonds within each of the hydrogel samples and to determine if there were any major differences amongst these bonds between different hydrogel types resulting from heparin incorporation (**Fig. 2**) (Hameed *et al.*, 2024).

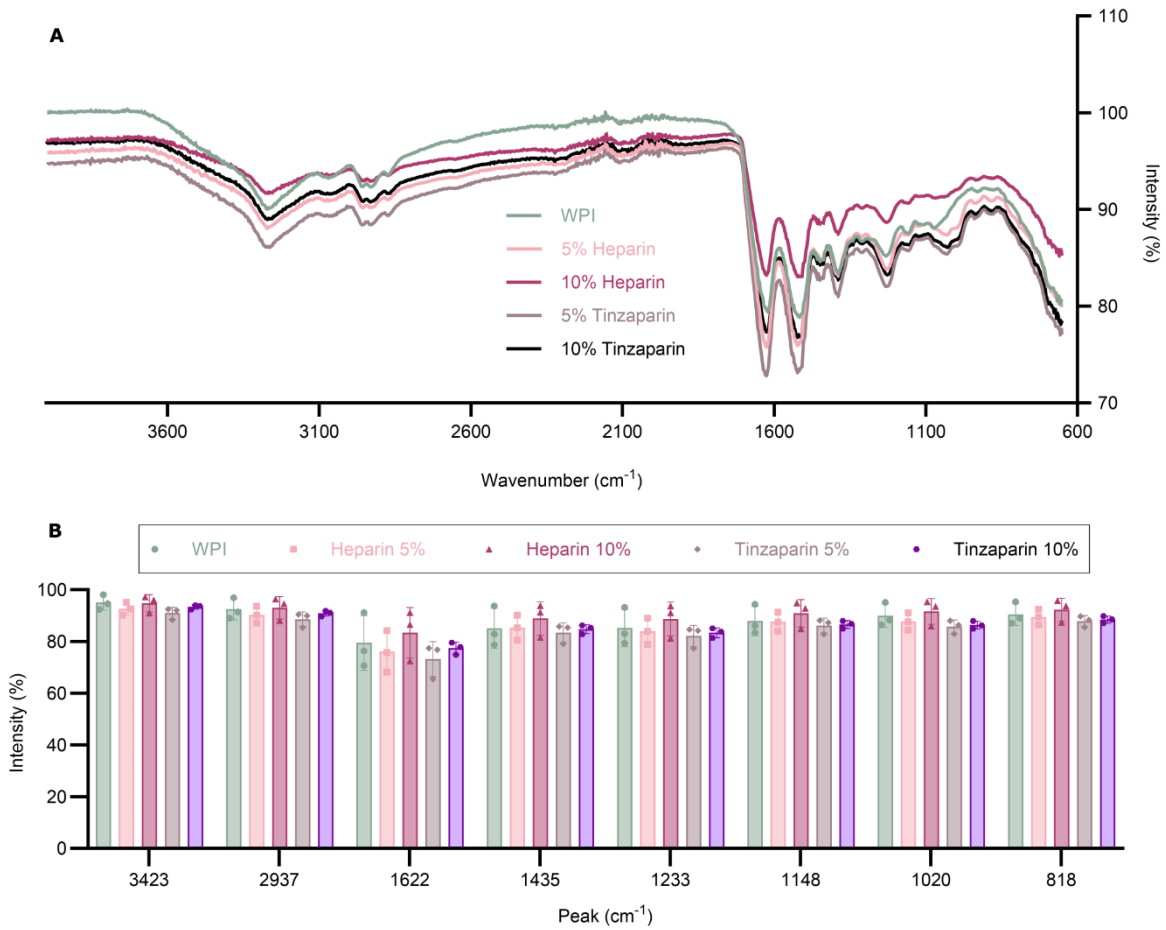


Fig. 2 – (A) FTIR spectra of WPI hydrogels loaded with heparins and (B) analysis of the intensity of specific peaks characteristic of heparins at select wavenumbers. FTIR spectra were gathered for each of the five hydrogel types and the intensity of the most prominent peaks from each spectrum compared against one another. A) The FTIR spectra of the five hydrogel types were acquired on a Cary 630 FTIR spectrophotometer using a spectral range of 650-4000 cm^{-1}. Data are presented as mean values. B) The % intensity of specific heparin-defining peaks (818 cm^{-1}, 1020 cm^{-1}, 1148 cm^{-1}, 1233 cm^{-1}, 1435 cm^{-1}, 1622 cm^{-1}, 2937 cm^{-1}, 3423 cm^{-1}) from the five hydrogel types (Ivory-Cousins *et al.*, 2023; Mecozzi *et al.*, 2011). Data are presented as mean values ± SD, with data from individual trials presented as dots within each bar (n = 3). No significant differences were calculated as determined by a 2-way ANOVA followed by Tukey's multiple comparisons test.

Table 2 – FTIR peak wavenumber and their region/potential interactions.

Wavenumber (cm^{-1})	**Region/Potential Interactions**
3260-3270	Symmetric stretching O-H
2952	Asymmetric stretching CH_3
2925	Asymmetric stretching CH_2
2870	Symmetric stretching CH_3 Stretching N-H, C-H Symmetric stretching CH_3 of acyl chains
1625	Amide I region
1517	Amide II
1446	Stretching C=O

1386	Bending CH_3 Stretching C-O Deformation C-H, N-H
1306	C-N amide III band
1228	Sulfate vibrations of S=O bonds
1157	C-O-C polysaccharide
1070	C-O carbohydrate
965	Unassigned
870	Bending C=O inorganic carbonate

FTIR analysis revealed many peaks, including main bands for all gels at 3260-3270 cm^{-1}, 2952cm^{-1}, 2925 cm^{-1}, 2870 cm^{-1}, 1625 cm^{-1}, 1517 cm^{-1}, 1446 cm^{-1}, 1386 cm^{-1}, 1306 cm^{-1}, 1228 cm^{-1}, 1157 cm^{-1}, 1070 cm^{-1}, 965 cm^{-1}, and 870 cm^{-1} (**Fig. 2A**). An overview of the potential underlying interactions of these bands is shown in **Table 2** (Ivory-Cousins *et al.*, 2023; Mecozzi *et al.*, 2011). The peaks in the spectra generated at 818 cm^{-1}, 1020 cm^{-1}, 1148 cm^{-1}, 1233 cm^{-1}, 1435 cm^{-1}, 1622 cm^{-1}, 2937 cm^{-1} and 3423 cm^{-1} (**Fig. 2B**) were comparable to heparin-defining FTIR peaks (Devlin *et al.*, 2023; Shen *et al.*, 2019). The intensity of all peaks did not significantly increase as the concentration of both H and T increased from 5% to 10%. In the spectra of samples containing heparins, a combination of bands specific for WPI and heparins were also observed, as expected. No noticeable shifts in the bands specific for WPI were detected.

3.3. Heparin Integration Increases Hydrogel Swelling Ability

The analysis of the hydrogels' physical properties was followed by an evaluation of their potential as wound dressings. Performance was assessed based on the extent of swelling in solution, which modelled wound exudate absorption, and the rate of degradation in simulated wound environments (Pawlak-Likus *et al.*, 2025; Zhu *et al.*, 2022).

To determine the extent to which the hydrogels swell by absorbing liquid from their environments samples of each hydrogel type were weighed before and after incubation in PBS or DMEM for seven days and the % change in their mass calculated (**Fig. 3**).

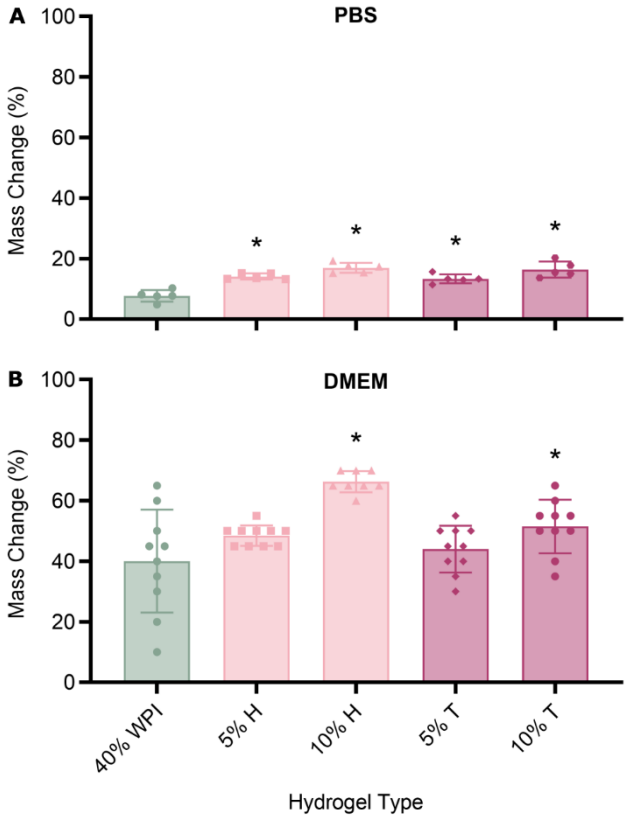
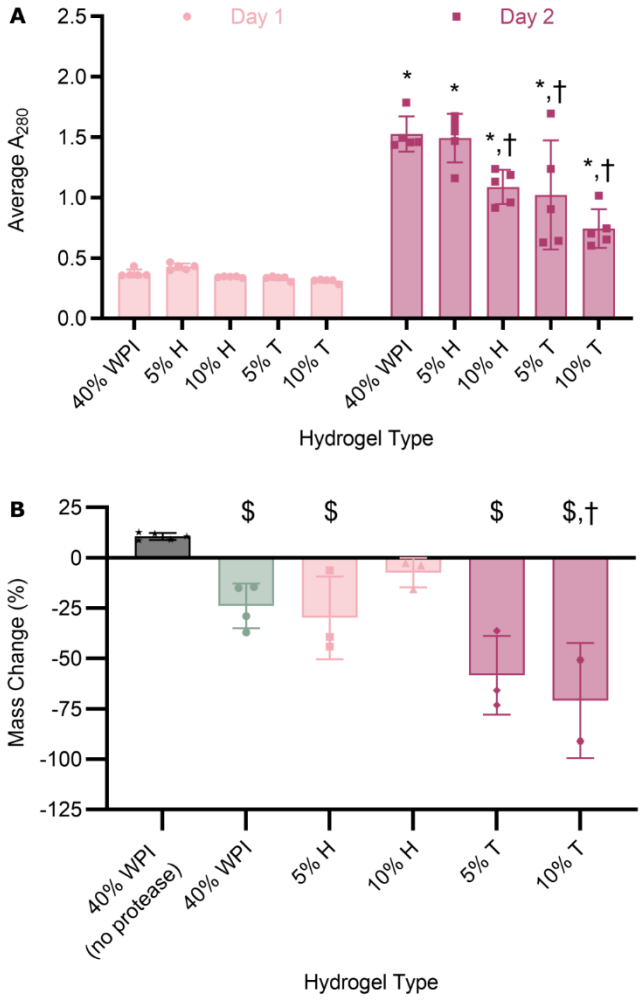


Fig. 3 – Incorporation of heparins into WPI hydrogels can increase their ability to swell in PBS and DMEM. Hydrogel samples were weighed and then incubated for seven days in (A) PBS (n=5) or (B) DMEM (n=10) after which samples were again weighed and the % mass change calculated. Data are presented as mean values ± SD, with data from each trial presented as individual data points within each bar. * Signifies the value is significantly different to that of the 40% WPI control as determined by a one-way ANOVA followed by Dunnett's multiple comparisons test in which the mean of all test columns was compared to a single control (40% WPI) (P<0.05).

In PBS (pH 7.4), which approximately replicates the pH and osmolarity of human cells and was therefore used in the initial model of exudate absorption from an open wound (Martin *et al.*, 2006), all heparin-integrated hydrogels, 5% H (14.1%), 10% H (17.0%), 5% T (13.3%), and 10% T (16.4%), swelled to a greater extent than the 40% WPI control hydrogel (7.7%). In DMEM, basal cell medium that more closely resembles the environment of mammalian tissue than PBS and which was therefore used in the second model of exudate absorption, all hydrogel types swelled more than what was observed in PBS though only the 10% H (66.2%) and 10% T (51.5 %) swelled to a greater extent than the 40% WPI control hydrogel (40.0%).

3.4. Simulated Wound Environments Degrade WPI-based Hydrogels

To help assess a hydrogel's suitability as a wound dressing it is important to determine its biodegradability as fast degradation can lead to quicker drug dispersal but lower stability over time (Kharkar *et al.*, 2013).



345

346 **Fig. 4 – WPI hydrogels degrade over time in PBS, a process exacerbated by proteases.** (A) Measurement of
347 protein released from WPI hydrogel types into PBS. Hydrogel samples were submerged in pH 7.4 PBS and
348 incubated at Standard Conditions. Supernatant samples were taken at 24 & 48 hours and analysed via NanoDrop
349 (A_{280}) as a measure of total protein concentration in the solution (Pawlak-Likus *et al.*, 2025). (B) Calculated %
350 mass change of each hydrogel type following incubation with protease solution. Hydrogel samples were weighed
351 before being incubated in pH 7.4 PBS solution containing 13.33 µg/mL protease (Manicourt *et al.*, 1994 Pawlak-
352 Likus *et al.*, 2025) for seven days at Standard Conditions. As an additional control, 40% WPI hydrogels were
353 incubated without protease to act as a negative control and are labelled '40% WPI (no protease)'. All hydrogels
354 were then reweighed, and the % mass change calculated. 'n' is equal to a maximum of 5 and a minimum of 2, as
355 not all samples could be reweighed due to gel degradation. Data are presented as mean values ± SD, with data
356 from each trial presented as points within each bar. *Signifies that the value is significantly greater than the same
357 hydrogel type from Day 1, † signifies the value is significantly lower than the 40% WPI control from the same
358 time point, and $ signifies the value is significantly lower than the 40% WPI (no protease) negative control as
359 determined by (A) a two-way ANOVA followed by Tukey's multiple comparisons test in which cell means were
360 compared with others in its row and column or (B) a one-way ANOVA followed by Tukey's multiple comparisons
361 test in which the mean of each column was compared with the mean of every other column (P<0.05).

362

363 To model the general biodegradability the hydrogels, the total protein release of each hydrogel type into
364 PBS after 24 and 48 hours of incubation was measured (Zustiak and Leach, 2011) (**Fig. 4A**). The
365 average A_{280} (Pawlak-Likus *et al.*, 2025) of each supernatant was measured and compared, revealing

that all five hydrogels released more protein into the PBS by day two versus day one. Variation between hydrogel types also increased by day two compared with day 1, as the A_{280} readings remained steady (0.30 – 0.45) on day one whilst significant differences emerged by day two with 40% WPI control (1.50) having released significantly more protein into the PBS solution than 10% H (1.09), 5% T (1.02), and 10% T (0.74).

Following this, a model of enzymatic degradation where the change in each hydrogel's weight was determined after incubation in protease-containing PBS was utilised (Lee *et al.*, 2005) (**Fig. 4B**). The 40% WPI (no protease) control increased in mass by 10.6%, in comparison to which 40% WPI, 5% H, 5% T, and 10% T, all of which were incubated with proteases, decreased significantly in mass by 23.9, 29.8, 58.4, and 70.9%. The only hydrogel type for which mass decreased significantly more than that of 40% WPI in protease-containing solution was 10% T, *albeit* these results should be interpreted with caution as only two 10% T hydrogel samples could be weighed after the seven days of incubation due to the other three degrading to such an extent they could not be accurately measured.

3.5. WPI Hydrogels Release Tinzaparin after Incubation in PBS

The final steps of this project were to determine if the heparins could release from the WPI hydrogels sufficiently to inhibit a snake venom's cytotoxic properties. The 10% T hydrogel was selected for these experiments due to T's previously reported superiority to H against spitting cobra venom-induced cytotoxicity (Du *et al.*, 2024).

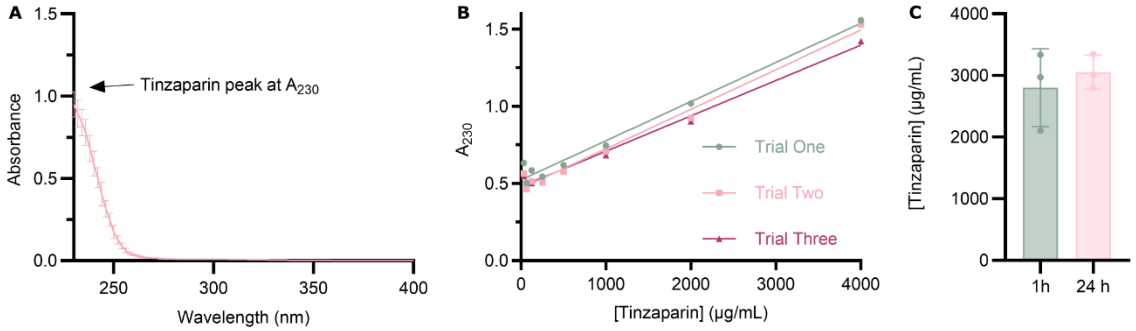


Fig. 5 – The T 10% hydrogel releases tinzaparin into PBS. (A) An absorbance scan (230-400 nm in 2 nm increments) of 4 mg/mL T in PBS versus PBS-only control shows an absorbance peak at A_{230}. Data are presented as mean values ± SD (n = 3). (B) Standard curves (31.25 – 4,000 µg/ml T) to determine the linear relationship between T concentration and A_{230}. T was dissolved in PBS that had been incubated for 24 h with 40% WPI control hydrogel and the A_{230} read at each concentration. Linear regressions were performed on the data from each trial to produce separate standard curves for each (n = 3). (C) The concentration of T released into solution following incubation for 1 or 24 hours. The 10% T hydrogel samples were incubated for either 1 or 24 hours in PBS at Standard Conditions. The A_{230} of each trial was measured and plotted against each standard curve to calculate the concentration of T released from T 10% hydrogels. Statistical analysis was performed using an unpaired t-test with Welch's correction. Data are presented as mean values ± SD (n = 3).

First, the optimal absorbance wavelength at which T could be detected and quantified was determined by measuring the absorbance of 4,000 µg/mL of T in PBS at a range of wavelengths (230-400 nm, 2 nm increments), from which it was determined that the most intense absorbance occurred at 230 nm

(**Fig. 5A**). Following this, standard T curves were created in triplicate at A_{230} using a serial dilution from 4,000 – 31.25 µg/ml (with a halving of subsequent serial dilutions). The data from the three trials were described by the equations $y = 0.0002540x + 0.5237$, $y = 0.0002567x + 0.4683$, and $y = 0.0002293x + 0.4802$ for trials 1-3, respectively (**Fig. 5B**). The A_{230} of samples of supernatant from T 10% hydrogel incubated in PBS at Standard Conditions for 1 and 24 hours was measured. From this, the concentration of T within each sample could be determined using the standard curve equations and calculating the mean. After three experimental replicates, it was determined that the mean concentration of T released from the hydrogels was 2.80 mg/mL after a 1-hour incubation and 3.05 mg/mL after a 24-hour incubation, with no significant difference between the two timepoints (**Fig. 5C**).

3.6. 10% Tinzaparin WPI Hydrogels Inhibit *N. nigricollis* Venom Cytotoxicity

Finally, to examine whether T could be effectively delivered to cells and inhibit venom function *via* a WPI hydrogel system, the effects of PBS solutions incubated for 1 or 24 hours with either 40% WPI control or 10% T hydrogel on the viability of HaCaTs treated with a constant concentration of *N. nigricollis* venom were compared.

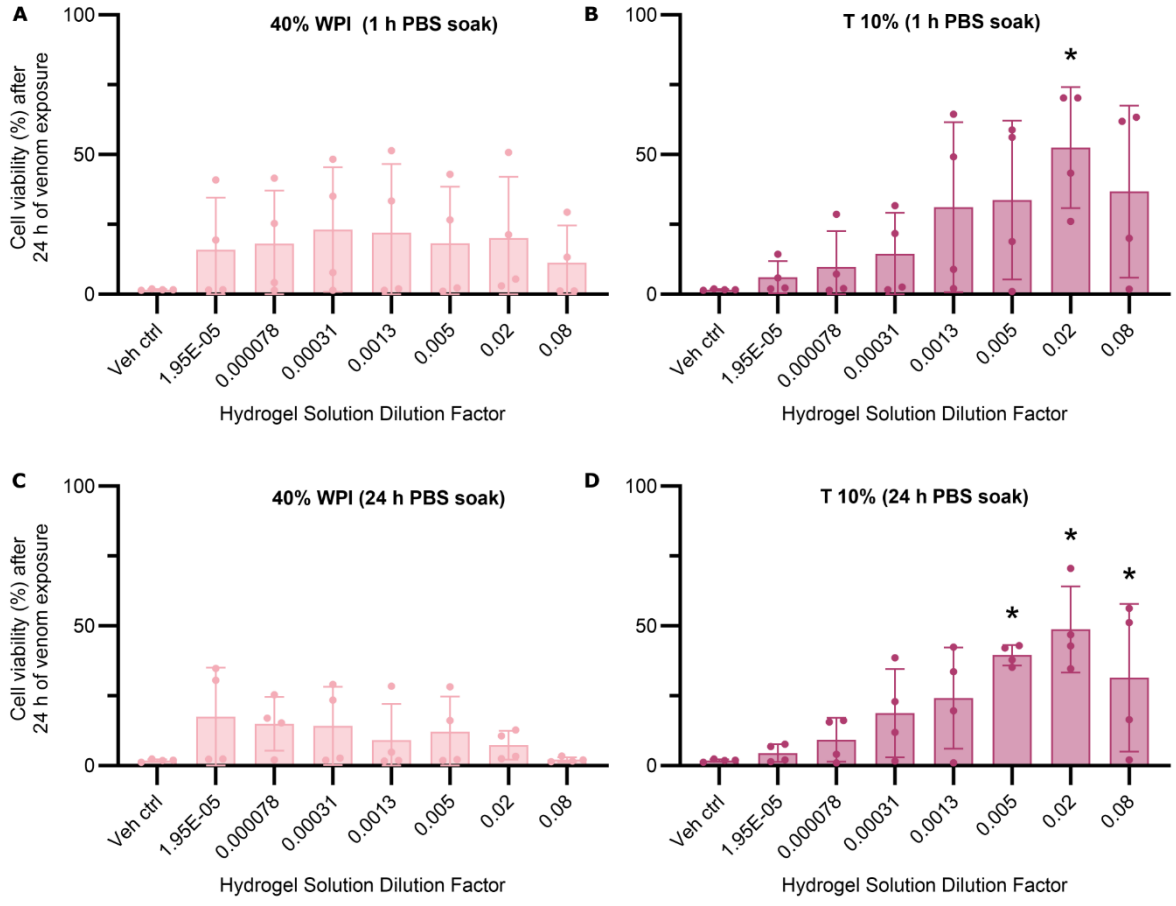


Fig. 6 – Solutions prepared from both incubation timepoints of the T 10% hydrogels inhibited *N. nigricollis* venom cytotoxicity. Hydrogels were incubated for 1 or 24 hours in PBS under Standard Conditions after which the resulting supernatants were serially diluted by quarters to create experimental cell treatment solutions that ranged between 0.08x – $1.95x10^{-5}$x of the original supernatant concentration. These serial dilutions, or a PBS vehicle control, were pre-incubated for 30 minutes with 20 µg/mL *N. nigricollis* venom before being administered to the HaCaT cells for 24 hours. Cell viability was then determined using resazurin assays. (A) The effects of the

40% WPI hydrogel 1-hour incubation solutions. (B) The effect of the 10% tinzaparin hydrogel 1-hour incubation solutions. (C) The effects of the 40% WPI hydrogel 24-hour incubation solutions. (D) The effect of the 10% tinzaparin hydrogel 24-hour incubation solutions. Data are presented as mean values ± SD, with data from each trial presented as points within each bar (n=4). * Signifies the % cell viability is significantly higher than that of the vehicle control as determined by a one-way ANOVA followed by Dunnett's multiple comparisons test (P<0.05).

Table 3 – Concentration of tinzaparin in each T 10% hydrogel serial dilution. The concentration of T released into 180 µL PBS by three 30 mg T 10% hydrogel discs after 1 or 24 hours of incubation at Standard Conditions was calculated to be 2.80 mg/mL and 3.05 mg/mL, respectively. This figure was multiplied by the dilution factor to determine the concentration of tinzaparin in each treatment used in Fig. 6B and 6D.

Dilution Factor	$1.95x10^{-5}x$	$7.81x10^{-5}x$	0.00031x	0.00125x	0.005x	0.02x	0.08x
1-hour incubation [Tinzaparin] (µg/mL)	0.05	0.22	0.88	3.5	14	56	224
24-hour incubation [Tinzaparin] (µg/mL)	0.06	0.24	0.95	3.8	15	61	244

HaCaT cells exposed to *N. nigricollis* venom and treated with vehicle control for both hydrogel experiments effectively reduced cell viability to close to 0%, confirming that cytotoxic venom concentrations were used in both cases (**Fig. 6**). The supernatant from the 40% control hydrogels for both incubation periods did not significantly increase cell viability at any concentration, although mean cell viability did increase from the vehicle control at most concentrations (**Fig. 6A, 6C**). The 1-hour incubation, 10% T hydrogel supernatant dilution series effectively inhibited the venom-induced cytotoxicity only at the 0.02x dilution which contained approximately 56 µg/mL of T (**Table 3**), resulting in a significant increase in mean cell viability to 52.5% (**Fig. 6B**). The 24-hour incubation, 10% T hydrogel dilution series was able to inhibit *N. nigricollis* venom cytotoxicity at a greater concentration range than the 1-hour incubation, significantly increasing cell viability at dilution factors of 0.05, 0.02 and 0.08 (15, 61 and 244 µg/mL [**Table 3**]), resulting in cell viabilities of 39.5%, 48.8% and 31.5% respectively (**Fig. 6D**).

4. Discussion

The primary aims of this study were to investigate whether heparin-integrated WPI hydrogels would exhibit the physical and venom-inhibitory properties required to warrant their further translation into snakebite wound dressings.

Firstly, the structural properties of each WPI hydrogel type were assessed using SEM, with clear structural differences being observed between 40% WPI control hydrogels and those hydrogels containing heparins (**Fig. 1**). WPI is >97% protein, of which the majority is β-lactoglobulin (75%), however other components include α-lactalbumin, immunoglobulins, bovine serum albumin, bovine lactoferrin and lactoperoxidase (Keppler *et al.*, 2014; Madureira *et al.*, 2007*).* β-lactoglobulin has

been reported to be negatively charged in the native state (isoelectric point = pH 5.3), with polyanions such as heparin typically binding patches of positively charged amino acids (Kamerzell *et al.*, 2011; Wang *et al.*, 2024). However, it may be possible that protein denaturation caused by heating may alter the isoelectric point, allowing heparin polyanion binding. The addition of heparins resulted in the formation of spherical structures (~1 μm in diameter) which clustered to a greater extent at the higher concentrations of either heparin, likely due to the heparins interacting with the WPI polymers with greater frequency at higher concentrations. Such structures have been noted previously when adding the biopolymer poly-γ-glutamic acid to WPI hydrogels (Baines *et al.*, 2025). Furthermore, the increased porosity caused by the clustering of these spheroidal structures may increase the release rate of drug from these hydrogels (Hoare and Kohane, 2008). The peaks observed *via* the completed FTIR analysis (**Fig. 2**) also correlate with what would be expected of heparin and tinzaparin (Devlin *et al.*, 2023; Shen *et al.*, 2019), further evidencing the successful integration and dispersion of both drugs into the WPI hydrogel structure.

The swelling analysis assays in PBS and DMEM (**Fig. 3**), used to model each hydrogel type's ability to absorb wound exudate, showed that the higher concentrations of either heparin increased hydrogel swelling, a phenomenon which may be due to the negative charges of both H and T, resulting in greater hydrophilicity with increasing heparin concentration, thereby resulting in greater water absorption and therefore swelling (Ganji *et al.*, 2010; Pawlak-Likus *et al.*, 2025). The improved swelling properties observed in the WPI hydrogels integrated with heparins is also a desirable characteristic for a snakebite wound dressing, as snakebite envenoming often produces excess wound exudate which should be removed for more effective wound healing (Collier *et al.*, 2014; Ibiapina *et al.*, 2019; Rucavado *et al.*, 2016).

Several studies have shown it takes an average of 5-9 hours for a snakebite victim to reach hospital in rural tropical communities (Abouyannis *et al.*, 2023; Ogunfowokan, 2012; Sharma *et al.*, 2005), and any prehospital-administered wound dressing should remain structurally and functionally stable throughout this prehospital period. However, the biodegradability of hydrogel wound dressings must be balanced between their stability, which maximises the length of time for which the wound site can be protected, and degradation, which improves drug distribution and minimises environmental harm (Khan *et al.*, 2023; Ribeiro *et al.*, 2024). To quantify this, protein release was first measured from each hydrogel type into PBS (**Fig. 4A**) (Baines *et al.*, 2025) which evidenced that heparin-incorporation diminishes WPI lattice breakdown, which may be the result of bonding between WPI proteins and heparins within the hydrogel (He *et al.*, 2019). As such, heparin integration could increase the longevity of a WPI-derived wound dressing. However, wounds also contain enzymatic components such as proteases, crucial elements of tissue remodelling and repair, which could catalyse WPI hydrogel polypeptide degradation (Baines *et al.*, 2025; McCarty and Percival, 2013). As such, the enzymatic degradation of hydrogel samples in solution containing proteases was also determined (**Fig. 4B**). As expected, incubation with proteases did result in significantly greater degradation of most hydrogel types in comparison to the 0% protease negative control; however, a weakness of these data should be noted, as while five hydrogel samples were initially prepared for each data set, due to excessive degradation of some of the samples rendering them impossible to weigh accurately, the final number of experimental replicates was diminished thereby reducing the statistical power within this experiment. Regardless, it was still determined that the hydrogel samples reduced in mass when incubated with proteases, suggesting they would also likely degrade in a real-world snakebite wound environment, especially during the remodelling stage of tissue repair (McCarty and Percival, 2013). Additionally, the data suggest that the presence of heparins, especially T, in the hydrogel matrix amplifies enzymatic degradation. Increased swelling in the presence of heparins (**Fig. 3**) could conceivably increase the pore size in the hydrogel polymer network, facilitating the entry of enzymes into the network by diffusion.

However, such discussion must remain speculative at the present time. As hydrogel degradation within protease-containing solution is more reminiscent of a real wound environment (McCarty and Percival, 2013), versus the trials completed in PBS, this suggests that the addition of heparins into WPI hydrogels would likely facilitate their biodegradability upon administration to a snakebite wound.

Following the experiments designed to describe the physical characteristics of heparin-containing WPI hydrogels, their ability to inhibit snake venom-induced cytotoxicity was investigated. It was decided that 10% T hydrogels would be tested against *N. nigricollis* venom for these experiments as T has previously exhibited potent inhibition of spitting cobra venom-induced cytotoxicity (Du *et al.*, 2024).

First, to confirm T would release from the hydrogel in quantities sufficient to inhibit *N. nigricollis* venom-induced cytotoxicity in cell-based assays its concentration in PBS was determined following a 1-hour or 24-hour incubation at standard conditions (**Fig. 5**), from which its final mean concentration was determined to be 2,803 μg/mL following a 1-hour incubation and 3,054 μg/mL following a 24-hour incubation; sufficiently concentrated in both cases for venom inhibition (Du *et al.*, 2024). No significant difference was found between incubation time periods, suggesting that the majority of tinzaparin able to diffuse into solution within 24 hours does so rapidly, leaving the gel within the first hour. Then, the ability of these 10% T hydrogel-derived versus 40% WPI hydrogel negative control-derived solutions to inhibit *N. nigricollis* venom-induced cytotoxicity was evaluated using a human epidermal keratinocyte (HaCaT) cell model (Hall *et al.*, 2023) (**Fig. 6**). As predicted, the 24-hour incubated, 10% T hydrogel-derived solution successfully inhibited *N. nigricollis* venom-induced skin cell cytotoxicity at several tested serial dilutions (15 – 244 μg/mL of T; **Table 3**), and at one tested concentration for the solution derived from a 1-hour incubation (61 μg/ml **of T; Table 3**). These results confirm that T-integrated WPI hydrogels can release drug at sufficiently high concentrations within as little as an hour to inhibit snake venom-induced cytotoxicity – a valuable characteristic since rapid treatment is crucial for necrosis prevention (Gutiérrez *et al.*, 2017a). As such, this evidence suggests that drug-incorporated WPI hydrogels warrant further research and development in the context of improving the treatment of snakebite envenoming.

The 40% WPI hydrogel-derived solution, prepared for use as the negative control, did not result in significant increases in % cell viability in comparison to the vehicle control**.** However, despite this lack of statistical significance, it is still intriguing how the mean cell viability trended higher for almost all serial dilutions of the 40% WPI hydrogel-derived solutions for both the 1-hour and 24-hour incubations (**Fig. 6A** and **6C**). This could potentially indicate a minor cytoprotective effect from these hydrogel-derived solutions resulting from the WPI itself. Prior investigations have shown that proteins similar to those in WPI can inhibit the functioning of other snake venom toxins, such as buffalo colostrum β-lactoglobulin inhibiting viper snake venom metalloproteinases (SVMPs) (Arpitha *et al.*, 2017). Therefore, a possible explanation is that a component of the WPI hydrogel, such as β-lactoglobulin (Keppler *et al.*, 2014), dissolved into the solution and interacted with one or more of the toxins within *N. nigricollis* venom to affect its functioning. However, further studies will be required to confirm or disprove these observed data trends.

Whilst questions remain about T-incorporated WPI hydrogels as wound dressings for snakebite, the inhibition of *N. nigricollis* venom-induced cytotoxicity by heparin-containing WPI hydrogels and their physiochemical properties discussed herein are the first evidence that such biomaterials could be translated into wound dressings for snakebite envenoming. Further research should aim to both expand the applicability of heparin-incorporated WPI hydrogels as snake venom inhibitor vehicles, as well as confirming their effectiveness in more realistic situations, e.g. using *ex vivo* or *in vivo* models of

envenoming. In expanding their applicability, a greater range of snake venoms should also be explored to ensure that there is the potential for pan-species efficacy. In addition, a range of other snake venom inhibitors which are currently under investigation such as varespladib, a hydrophobic inhibitor of snake venom PLA_2s (Clare *et al.*, 2021; Lewin *et al.*, 2016), could be incorporated into hydrogels and tested against a range of snake venoms. This could exploit WPI hydrogels' ability to bind and release hydrophobic drugs (Baines *et al.*, 2024). Through further research, WPI hydrogels integrated with heparins or other venom inhibiting drugs may prove themselves as effective prehospital wound dressings that slow the tissue destroying effects of cytotoxic snake venoms.

5. Conclusions

This research aimed to address if WPI hydrogels containing heparins could function as wound dressings for snakebite envenoming by assessing their physiochemical properties and determining if they could inhibit *N. nigricollis* venom-induced cytotoxicity. As described above, these physiochemical properties reveal that heparin-containing WPI hydrogels may be appropriate for snakebite wound dressings, being able to absorb wound exudate, deliver drugs swiftly, and biodegrade. In addition, the hydrogels containing 10% T delivered sufficient concentrations of drug into solution to inhibit *N. nigricollis*-induced cytotoxicity at $1/50^{th}$ of the original solution's concentration following incubation for 1 hour, and up to $1/200^{th}$ following incubation for 24 hours. This is the first time the ability of drug-containing WPI hydrogels to inhibit snake venoms has been tested, and the data contained herein suggest that such biomaterials could prove valuable as future snakebite wound dressings following further development that could be easily translated for prehospital use, thereby shortening time to initial treatment.

6. CRediT authorship contribution statement

Matthew J. Gray: Validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualisation. **Daniel K. Baines**: Conceptualisation, methodology, investigation, resources, writing – original draft, writing – review & editing. **Gabrielle De Castro**: Formal analysis, investigation, resources, data curation, writing – review & editing, visualisation. **Thomas Cobb**: Methodology, formal analysis, investigation, data curation, writing – review & editing, visualisation. **Layla A. Tyrrell**: Validation, investigation, data curation, writing – review & editing. **Sam J. Hyam**: Validation, investigation, writing – review & editing. **Alan M. Smith:** Resources, writing – review & editing. **Nicholas R. Casewell**: Resources, writing – review & editing. **Sarah L. Allinson**: Methodology, writing – review & editing. **Timothy E. L. Douglas**: Conceptualisation, methodology, resources, data curation, writing – original draft, writing – review & editing, supervision, project administration, funding acquisition. **Steven R. Hall**: Conceptualisation, methodology, formal analysis, investigation, resources, data curation, writing – original draft, writing – review & editing, visualisation, supervision, project administration, funding acquisition.

7. Acknowledgements

We would like to give thanks to Paul Rowley and Edouard Crittenden for maintaining the snakes at the LSTM herpetarium and for routine venom extractions.

8. Funding

This work was supported by (i) a startup grant provided by Lancaster University's Division of Biomedical and Life Sciences to SRH, (ii) a Global Impact Small Grant from Friends of Lancaster University in America to SRH, (iii) a Lancaster University Faculty of Health and Medicine Catalyst

Grant to SRH, (iv) a philanthropic donation from donor Nicholas Bone to Lancaster University allocated to SRH, and (v) a PhD stipend awarded by Lancaster University's Faculty of Science and Technology to DKB.

9. **Competing Interests**

The authors have no competing interests to declare.

10. **References**

Abouyannis, M., Boga, M., Amadi, D., Ouma, N., Nyaguara, A., Mturi, N., Berkley, J.A., Adetifa, I.M., Casewell, N.R., Lalloo, D.G., Hamaluba, M., 2023. A long-term observational study of paediatric snakebite in Kilifi County, south-east Kenya. PLoS Negl Trop Dis 17, 1–20. https://doi.org/10.1371/journal.pntd.0010987

Abouyannis, M., Nyambura, Y.K., Ngome, S., Riako, D., Musyoki, J., Muiruri, C., Orindi, B., Else, L., Amara, A., Dickinson, L., Clare, R.H., Albulescu, L.-O., Westhorpe, A.P., Kool, J., Adetifa, I., Ndungu, F.M., FitzGerald, R., Khoo, S., Lalloo, D.G., Casewell, N.R., Hamaluba, M., 2025. Development of an oral regimen of unithiol for the treatment of snakebite envenoming: a phase 1 open-label dose-escalation safety trial and pharmacokinetic analysis in healthy Kenyan adults. EBioMedicine 113, 105600. https://doi.org/10.1016/j.ebiom.2025.105600

Afroz, A., Siddiquea, B.N., Chowdhury, H.A., Jackson, T.N., Watt, A.D., 2024. Snakebite envenoming: A systematic review and meta-analysis of global morbidity and mortality. PLoS Negl Trop Dis 18, e0012080. https://doi.org/10.1371/journal.pntd.0012080

Alangode, A., Rajan, K., Nair, B.G., 2020. Snake antivenom: Challenges and alternate approaches. Biochem Pharmacol 181, 114135. https://doi.org/10.1016/j.bcp.2020.114135

Arpitha, A., Sebastin Santhosh, M., Rohit, A.C., Girish, K.S., Vinod, D., Aparna, H.S., 2017. Inhibition of Snake Venom Metalloproteinase by β-Lactoglobulin Peptide from Buffalo (Bubalus bubalis) Colostrum. Appl Biochem Biotechnol 182, 1415–1432. https://doi.org/10.1007/s12010-017-2407-6

Baines, D.K., Pawlak-Likus, Z., Tavernaraki, N.N., Platania, V., Parati, M., Cheung, T.N.W.W., Radecka, I., Domalik-Pyzik, P., Chatzinikolaidou, M., Douglas, T.E.L., 2025. Effect of Poly-γ-Glutamic Acid Molecular Weight on the Properties of Whey Protein Isolate Hydrogels. Polymers (Basel) 17, 1605. https://doi.org/10.3390/polym17121605

Baines, D.K., Wright, K., Douglas, T.E.L., 2024. Preliminary In Vitro Assessment of Whey Protein Isolate Hydrogel with Cannabidiol as a Potential Hydrophobic Oral Drug Delivery System for Colorectal Cancer Therapy. Polymers (Basel) 16, 3273. https://doi.org/10.3390/polym16233273

Bartlett, K.E., Hall, S.R., Rasmussen, S.A., Crittenden, E., Dawson, C.A., Albulescu, L.O., Laprade, W., Harrison, R.A., Saviola, A.J., Modahl, C.M., Jenkins, T.P., Wilkinson, M.C., Gutiérrez, J.M., Casewell, N.R., 2024. Dermonecrosis caused by a spitting cobra snakebite results from toxin potentiation and is prevented by the repurposed drug varespladib. Proc Natl Acad Sci U S A 121. https://doi.org/10.1073/pnas.2315597121

Bashiardes, S., Christodoulou, C., 2024. Orally Administered Drugs and Their Complicated Relationship with Our Gastrointestinal Tract. Microorganisms 12, 242. https://doi.org/10.3390/microorganisms12020242

Bashir, S., Hina, M., Iqbal, J., Rajpar, A.H., Mujtaba, M.A., Alghamdi, N.A., Wageh, S., Ramesh, K., Ramesh, S., 2020. Fundamental Concepts of Hydrogels: Synthesis, Properties, and Their Applications. Polymers (Basel) 12, 2702. https://doi.org/10.3390/polym12112702

Boechat, A.L.R., Paiva, C.S., França, F.O., Dos-Santos, M.C., 2001. Heparin-antivenom association: differential neutralization effectiveness in Bothrops atrox and Bothrops erythromelas envenoming. Rev. Inst. Med. Trop. São Paulo 43, 07–14. https://doi.org/10.1590/S0036-46652001000100002

Brumberg, V., Astrelina, T., Malivanova, T., Samoilov, A., 2021. Modern Wound Dressings: Hydrogel Dressings. Biomedicines 9, 1235. https://doi.org/10.3390/biomedicines9091235

Christy, N.P., Weiss, H.J., Phillips, L.L., Phillips, G.B., Hopewell, W., 1973. Exotic snakebite in an urban setting: heparin therapy of disseminated intravascular coagulation in a patient bitten by a saw-scaled viper (Echis carinatus). Trans. Am. Clin. Climatol. Assoc. 84, 37–48.

Clare, R.H., Hall, S.R., Patel, R.N., Casewell, N.R., 2021. Small molecule drug discovery for neglected tropical snakebite. Trends Pharmacol Sci 42, 340–353. https://doi.org/10.1016/j.tips.2021.02.005

Collier, M., Potts, C., Shaw, E., 2014. Use of a coverlet system for the management of skin microclimate. Br. J. Nurs. Mark Allen Publ. 23, S28, S30-35. https://doi.org/10.12968/bjon.2014.23.Sup15.s28

Conner, K.P., Devanaboyina, S.C., Thomas, V.A., Rock, D.A., 2020. The biodistribution of therapeutic proteins: Mechanism, implications for pharmacokinetics, and methods of evaluation. Pharmacol Ther 212, 107574. https://doi.org/10.1016/j.pharmthera.2020.107574

Devlin, A.J., Mycroft-West, C.J., Turnbull, J.E., Lima, M.A. de, Guerrini, M., Yates, E.A., Skidmore, M.A., 2023. Analysis of Heparin Samples by Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy in the Solid State. ACS Cent Sci 9, 381–392. https://doi.org/10.1021/acscentsci.2c01176

Du, T.Y., Hall, S.R., Chung, F., Kurdyukov, S., Crittenden, E., Patel, K., Dawson, C.A., Westhorpe, A.P., Bartlett, K.E., Rasmussen, S.A., Moreno, C.L., Denes, C.E., Albulescu, L.-O., Marriott, A.E., Mackay, J.P., Wilkinson, M.C., Gutiérrez, J.M., Casewell, N.R., Neely, G.G., 2024. Molecular dissection of cobra venom highlights heparinoids as an antidote for spitting cobra envenoming. Sci Transl Med 16. https://doi.org/10.1126/scitranslmed.adk4802

Ganji, F., Vasheghani-Farahani, S., Vasheghani-Farahani, E., 2010. Theoretical Description of Hydrogel Swelling: A Review. Iranian Polymer Journal 19, 375–398.

Genç, H., Friedrich, B., Alexiou, C., Pietryga, K., Cicha, I., Douglas, T.E.L., 2023. Endothelialization of Whey Protein Isolate-Based Scaffolds for Tissue Regeneration. Molecules 28, 7052. https://doi.org/10.3390/molecules28207052

Gerardo, C.J., Carter, R.W., Kumar, S., Shirazi, F.M., Kotehal, S.D., Akpunonu, P.D., Bhalla, A., Schwartz, R.B., Bammigatti, C., Manikath, N., Mukherjee, P.P., Arnold, T.C., Wolk, B.J., S Sheikh, S., Sollee, D.R., Vearrier, D.J., Francis, S.J., Aizenberg, A., Kumar, H., Ravikumar, M.K., Sarkar, S., Haston, T., Micciche, A., Oomman, S.C., Owen, J.L., Ritter, B.A., Samuel, S.P., Lewin, M.R., Platts-Mills, T.F., 2024. Oral varespladib for the treatment of snakebite envenoming in India and the USA (BRAVO): a phase II randomised clinical trial. BMJ Glob Health 9, e015985. https://doi.org/10.1136/bmjgh-2024-015985

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017a. Snakebite envenoming. Nat Rev Dis Primers 3, 1–21. https://doi.org/10.1038/nrdp.2017.63

Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Vargas, M., Villalta, M., Sánchez, A., Sanz, L., Lomonte, B., León, G., Calvete, J., 2017b. Preclinical Evaluation of the Efficacy of Antivenoms for Snakebite Envenoming: State-of-the-Art and Challenges Ahead. Toxins (Basel) 9, 163. https://doi.org/10.3390/toxins9050163

Hall, S.R., Rasmussen, S.A., Crittenden, E., Dawson, C.A., Bartlett, K.E., Westhorpe, A.P., Albulescu, L.O., Kool, J., Gutiérrez, J.M., Casewell, N.R., 2023. Repurposed drugs and their combinations prevent morbidity-inducing dermonecrosis caused by diverse cytotoxic snake venoms. Nat Commun 14. https://doi.org/10.1038/s41467-023-43510-w

Hameed, H., Faheem, S., Paiva-Santos, A.C., Sarwar, H.S., Jamshaid, M., 2024. A Comprehensive Review of Hydrogel-Based Drug Delivery Systems: Classification, Properties, Recent Trends, and Applications. AAPS PharmSciTech 25, 64. https://doi.org/10.1208/s12249-024-02786-x

He, C., Ji, H., Qian, Y., Wang, Q., Liu, X., Zhao, W., Zhao, C., 2019. Heparin-based and heparin-inspired hydrogels: size-effect, gelation and biomedical applications. J Mater Chem B 7, 1186–1208. https://doi.org/10.1039/C8TB02671H

Hirsh, J., 1998. Low-Molecular-Weight Heparin. Circulation 98, 1575–1582. https://doi.org/10.1161/01.CIR.98.15.1575

Hoare, T.R., Kohane, D.S., 2008. Hydrogels in drug delivery: Progress and challenges. Polymer (Guildf) 49, 1993–2007. https://doi.org/10.1016/j.polymer.2008.01.027

Ibiapina, H.N.S., Costa, A.G., Sachett, J.A.G., Silva, I.M., Tarragô, A.M., Neves, J.C.F., Kerr, M.W.A., Santana, M.F., Martins-Filho, O.A., Lacerda, M.V.G., Ferreira, L.C.L., Malheiro, A., Monteiro, W.M., 2019. An Immunological Stairway to Severe Tissue Complication Assembly in Bothrops atrox Snakebites. Front Immunol 10. https://doi.org/10.3389/fimmu.2019.01882

Ivory-Cousins, T., Nurzynska, A., Klimek, K., Baines, D.K., Truszkiewicz, W., Pałka, K., Douglas, T.E.L., 2023. Whey Protein Isolate/Calcium Silicate Hydrogels for Bone Tissue Engineering Applications—Preliminary In Vitro Evaluation. Materials 16, 6484. https://doi.org/10.3390/ma16196484

Kamerzell, T.J., Esfandiary, R., Joshi, S.B., Middaugh, C.R., Volkin, D.B., 2011. Protein–excipient interactions: Mechanisms and biophysical characterization applied to protein formulation development. Adv. Drug Deliv. Rev., Formulating biomolecules: mechanistics insights in molecular interactions 63, 1118–1159. https://doi.org/10.1016/j.addr.2011.07.006

Keppler, J.K., Sönnichsen, F.D., Lorenzen, P.-C., Schwarz, K., 2014. Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and fluorescence quenching. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1844, 1083–1093. https://doi.org/10.1016/j.bbapap.2014.02.007

Kesharwani, P., Bisht, A., Alexander, A., Dave, V., Sharma, S., 2021. Biomedical applications of hydrogels in drug delivery system: An update. J Drug Deliv Sci Technol 66, 102914. https://doi.org/10.1016/j.jddst.2021.102914

Khan, M.U.A., Aslam, M.A., Bin Abdullah, M.F., Hasan, A., Shah, S.A., Stojanović, G.M., 2023. Recent perspective of polymeric biomaterial in tissue engineering– a review. Mater Today Chem 34, 101818. https://doi.org/10.1016/j.mtchem.2023.101818

Kharkar, P.M., Kiick, K.L., Kloxin, A.M., 2013. Designing degradable hydrogels for orthogonal control of cell microenvironments. Chem. Soc. Rev. 42, 7335–7372. https://doi.org/10.1039/C3CS60040H

Lalloo, D.G., Theakston, R.D.G., 2003. Snake antivenoms. J Toxicol Clin Toxicol 41, 277–290. https://doi.org/10.1081/CLT-120021113

Lee, S., Miller, J.S., Moon, J.J., West, J.L., 2005. Proteolytically Degradable Hydrogels with a Fluorogenic Substrate for Studies of Cellular Proteolytic Activity and Migration. Biotechnol Prog 21, 1736–1741. https://doi.org/10.1021/bp0502429

Lewin, M., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) appears to be a potent, broad-spectrum, inhibitor of snake venom phospholipase A2 and a possible pre-referral treatment for envenomation. Toxins (Basel) 8, 1–16. https://doi.org/10.3390/toxins8090248

Madureira, A.R., Pereira, C.I., Gomes, A.M.P., Pintado, M.E., Xavier Malcata, F., 2007. Bovine whey proteins – Overview on their main biological properties. Food Res. Int. Ott. Ont 40, 1197–1211. https://doi.org/10.1016/j.foodres.2007.07.005

Manicourt, D., Fujimoto, N., Obata, K., Thonar, E.J. -M. A., 1994. Serum levels of collagenase, stromelysin-1, and timp-1. Arthritis Rheum 37, 1774–1783. https://doi.org/10.1002/art.1780371211

Martin, N.C., Pirie, A.A., Ford, L.V., Callaghan, C.L., McTurk, K., Lucy, D., Scrimger, D.G., 2006. The use of phosphate buffered saline for the recovery of cells and spermatozoa from swabs. Science & Justice 46, 179–184. https://doi.org/10.1016/S1355-0306(06)71591-X

McCarty, S.M., Percival, S.L., 2013. Proteases and Delayed Wound Healing. Adv Wound Care (New Rochelle) 2, 438–447. https://doi.org/10.1089/wound.2012.0370

Mecozzi, M., Pietroletti, M., Tornambè, A., 2011. Molecular and structural characteristics in toxic algae cultures of Ostreopsis ovata and Ostreopsis spp. evidenced by FTIR and FTNIR spectroscopy. Spectrochim Acta A Mol Biomol Spectrosc 78, 1572–1580. https://doi.org/10.1016/j.saa.2011.02.002

Ogunfowokan, O., 2012. Bite-to-hospital time and morbidity in victims of viper bite in a rural hospital in Nigeria. Afr J Prim Health Care Fam Med 4, 1–7. https://doi.org/10.4102/phcfm.v4i1.371

Pawlak-Likus, Z., Baines, D.K., Tavernaraki, N.N., Platania, V., Smith, A.M., Chatzinikolaidou, M., Domalik-Pyzik, P., Douglas, T.E.L., 2025. Physicochemical Properties and Angiogenic Potential of Whey Protein Isolate Hydrogels Modified with Heparin or Tinzaparin. J Polym Environ 33, 4130–4146. https://doi.org/10.1007/s10924-025-03626-0

Platania, V., Douglas, T.E.L., Zubko, M.K., Ward, D., Pietryga, K., Chatzinikolaidou, M., 2021. Phloroglucinol-enhanced whey protein isolate hydrogels with antimicrobial activity for tissue engineering. Materials Science and Engineering: C 129, 112412. https://doi.org/10.1016/j.msec.2021.112412

Ribeiro, M., Simões, M., Vitorino, C., Mascarenhas-Melo, F., 2024. Hydrogels in Cutaneous Wound Healing: Insights into Characterization, Properties, Formulation and Therapeutic Potential. Gels 10, 188. https://doi.org/10.3390/gels10030188

Rivel, M., Solano, D., Herrera, M., Vargas, M., Villalta, M., Segura, Á., Arias, A.S., León, G., Gutiérrez, J.M., 2016. Pathogenesis of dermonecrosis induced by venom of the spitting cobra, Naja nigricollis: an experimental study in mice. Toxicon 119, 171–179. https://doi.org/10.1016/j.toxicon.2016.06.006

Rucavado, A., Nicolau, C., Escalante, T., Kim, J., Herrera, C., Gutiérrez, J., Fox, J., 2016. Viperid Envenomation Wound Exudate Contributes to Increased Vascular Permeability via a

DAMPs/TLR-4 Mediated Pathway. Toxins (Basel) 8, 349. https://doi.org/10.3390/toxins8120349

Sharma, N., Chauhan, S., Faruqi, S., Bhat, P., Varma, S., 2005. Snake envenomation in a north Indian hospital. Emergency Medicine Journal 22, 118–120. https://doi.org/10.1136/emj.2003.008458

Shen, X., Liu, Z., Li, J., Wu, D., Zhu, M., Yan, L., Mao, G., Ye, X., Linhardt, R.J., Chen, S., 2019. Development of low molecular weight heparin by H2O2/ascorbic acid with ultrasonic power and its anti-metastasis property. Int J Biol Macromol 133, 101–109. https://doi.org/10.1016/j.ijbiomac.2019.04.019

Sweeney, I.R., Miraftab, M., Collyer, G., 2012. A critical review of modern and emerging absorbent dressings used to treat exuding wounds. Int Wound J 9, 601–612. https://doi.org/10.1111/j.1742-481X.2011.00923.x

Tasoulis, T., Isbister, G.K., 2017. A review and database of snake venom proteomes. Toxins (Basel) 9, 1–23. https://doi.org/10.3390/toxins9090290

Uzunalli, G., Mammadov, R., Yesildal, F., Alhan, D., Ozturk, S., Ozgurtas, T., Guler, M.O., Tekinay, A.B., 2017. Angiogenic Heparin-Mimetic Peptide Nanofiber Gel Improves Regenerative Healing of Acute Wounds. ACS Biomater Sci Eng 3, 1296–1303. https://doi.org/10.1021/acsbiomaterials.6b00165

Wang, Z.L., Tang, X., Wang, M., She, Y.X., Yang, B.R., Sheng, Q.H., Abd El-Aty, A.M., 2024. β-Lactoglobulin separation from whey protein: A comprehensive review of isolation and purification techniques and future perspectives. J. Dairy Sci. 107, 11785–11795. https://doi.org/10.3168/jds.2024-25321

Yegappan, R., Lauko, J., Wang, Z., Lavin, M.F., Kijas, A.W., Rowan, A.E., 2022. Snake Venom Hydrogels as a Rapid Hemostatic Agent for Uncontrolled Bleeding. Adv Healthc Mater 11. https://doi.org/10.1002/adhm.202200574

Yergoz, F., Hastar, N., Cimenci, C.E., Ozkan, A.D., Tekinay, T., Guler, M.O., Tekinay, A.B., 2017. Heparin mimetic peptide nanofiber gel promotes regeneration of full thickness burn injury. Biomaterials 134, 117–127. https://doi.org/10.1016/j.biomaterials.2017.04.040

Zhang, L., Ma, Y., Pan, X., Chen, S., Zhuang, H., Wang, S., 2018. A composite hydrogel of chitosan/heparin/poly (γ-glutamic acid) loaded with superoxide dismutase for wound healing. Carbohydr Polym 180, 168–174. https://doi.org/10.1016/j.carbpol.2017.10.036

Zhu, W., Dong, Y., Xu, P., Pan, Q., Jia, K., Jin, P., Zhou, M., Xu, Y., Guo, R., Cheng, B., 2022. A composite hydrogel containing resveratrol-laden nanoparticles and platelet-derived extracellular vesicles promotes wound healing in diabetic mice. Acta Biomater 154, 212–230. https://doi.org/10.1016/j.actbio.2022.10.038

Zustiak, S.P., Leach, J.B., 2011. Characterization of protein release from hydrolytically degradable poly(ethylene glycol) hydrogels. Biotechnol Bioeng 108, 197–206. https://doi.org/10.1002/bit.22911