

**BUKTI
SUBMIT
MANUSKRIP**

Submission to Biomaterials Advances - manuscript number

1 message

Materials Science & Engineering C <em@editorialmanager.com>
Reply-To: Materials Science & Engineering C <support@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Fri, Sep 16, 2022 at 10:57 PM

This is an automated message.

Manuscript Number: BIOADV-D-22-02327

Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Dear Dr. Permana,

Your above referenced submission has been assigned a manuscript number: BIOADV-D-22-02327.

To track the status of your manuscript, please log in as an author at <https://www.editorialmanager.com/bioadv/>, and navigate to the "Submissions Being Processed" folder.

Thank you for submitting your work to this journal.

Kind regards,
Biomaterials Advances

More information and support

You will find information relevant for you as an author on Elsevier's Author Hub: <https://www.elsevier.com/authors>

FAQ: How can I reset a forgotten password?

https://service.elsevier.com/app/answers/detail/a_id/28452/supporthub/publishing/

For further assistance, please visit our customer service site: <https://service.elsevier.com/app/home/supporthub/publishing/>

Here you can search for solutions on a range of topics, find answers to frequently asked questions, and learn more about Editorial Manager via interactive tutorials. You can also talk 24/7 to our customer support team by phone and 24/7 by live chat and email

#AU_BIOADV#

To ensure this email reaches the intended recipient, please do not delete the above code

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: <https://www.editorialmanager.com/bioadv/login.asp?a=r>). Please contact the publication office if you have any questions.

**BUKTI
REVIEW
DARI
REVIEWERS**

Decision on submission to Biomaterials Advances

1 message

Materials Science & Engineering C <em@editorialmanager.com>
Reply-To: Materials Science & Engineering C <support@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Thu, Oct 6, 2022 at 7:15 AM

Manuscript Number: BIOADV-D-22-02327

Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Dear Dr. Permana,

Thank you for submitting your manuscript to Biomaterials Advances.

I have completed my evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Dec 04 2022 11:59:59:000PM.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

To submit your revised manuscript, please log in as an author at <https://www.editorialmanager.com/bioadv/>, and navigate to the "Submissions Needing Revision" folder.

Biomaterials Advances values your contribution and I look forward to receiving your revised manuscript.

Kind regards,
Changyou Gao
Associate Editor

Biomaterials Advances

Editor and Reviewer comments:

Reviewer #1: In this work, chloramphenicol (CLP), a powerful antibiotic against a broad spectrum of bacteria, was incorporated into the MPs based on whey protein. Then the MPs were embedded into chitosan gel to synthesize a controllable hydrogel to deliver the CLP. Although the topic is interesting, according to their data, this work is not suitable for this journal.

Materials and methods

- 1) It would be clear to add the exact amount of WPI, CPL, and their concentrations in section 2.2.
- 2) Please uniform all the abbreviations; Particles change to MPs.
- 3) Section 2.3.1. line 129. How could you measure the size of dissolved MPs? It should change to dispersed.
- 4) Please add some references for your procedure to calculate entrapment efficiency and drug loading.
- 5) What is the difference between entrapment efficiency and drug loading? Both of them illustrate how much drug was encapsulated in particles. So why you used two different methods to show it?
- 6) Please add the R² related to the standard curve equation in the drug release section.
- 7) It needs to add the amount of the samples in PXRD analysis and under which condition the test was carried out.
- 8) Please add a reference for stimulating wound fluid. Section 2.3.7
- 9) An equation to calculate drug release is missing; please add it, followed by a reference.
- 10) The chitosan hydrogel was formed in the acidic media; could not this media be toxic for cells?

Results and discussion

- 1) Please add a reference at the end of line 329.

Although It has been claimed that CPL was successfully incorporated into the WPI MPs according to the results of DSC, FTIR, and XRD, there are some questions:

- 1) Which functional groups of PWI and glutaraldehyde contribute to the crosslinking reaction? Make it clear with a schematic.
- 2) What is the reason for disappearing the broad peak at around 2500cm⁻¹ in FTIR spectra of whey protein after incorporation CPL into MPs?
- 3) Moreover, why did the decrease in the intensity of the peak relate to the amide at around 1700 in FTIR spectra of whey protein?
- 4) Even by perfect dispersion of CPL into the MPs they should show their crystallinity peaks at XRD and melting point at DSC, unless they were miscible. On the other hand, if they were miscible, they could not have two separate melting points for CPL and whey protein. Therefore, it is possible the CPL was not incorporated into MPs, or the CPL contributed to the crosslinking reaction and converted it to another component. So how can this conflict be described?
- 5) In the drug delivery section, the unincorporated CPL was used as a control and added directly to the release media. In this way, the consecration of the drug once adding the CPL should be 100%. So how is it possible for CPL without any protection release by time so that after six h, the release reaches the maximum?
- 6) Moreover, why was the release investigated just for 24h, while the CPL MPs were released between eight to 24h?
- 7) Although the mathematic kinetic models were fitted to the release profile of CLP, the lack of discussion about the mechanism of release is crucial.
- 8) Please add the K of each kinetic model and also n of the Korsmeyer-Peppas model.

Reviewer #2: The work constitutes a good and valuable work. Despite of such strong points as well-prepared Introduction, well-designed research methodology and an extensive discussion over the results of performed experiments some corrections are recommended, i.e.:

- 1) Language of the paper should be significantly corrected.
- 2) Section 2.1.: the information concerning the average molecular weight of chitosan and its deacetylation degree needs to be added.
- 3) The methodology of the preparation of samples before the analyses should be added. For example, there is no information concerning the drying conditions or the sputtering process before SEM analysis.
- 4) It is strongly suggested to change the samples' names (formulation codes) to facilitate the analysis of the results of performed experiments.
- 5) Table 2.: the given numerical values should contain dots instead of commas.
- 6) Quality of all figures should be significantly improved.
- 7) Final conclusions should be given using more quantified data.
- 8) The notation of References needs to be consistent. Now, some of them contain the whole journal names and some of them contain their abbreviations.

Data in Brief (optional):

We invite you to convert your supplementary data (or a part of it) into an additional journal publication in Data in Brief, a multi-disciplinary open access journal. Data in Brief articles are a fantastic way to describe supplementary data and associated metadata, or full raw datasets deposited in an external repository, which are otherwise unnoticed. A Data in Brief article (which will be reviewed, formatted, indexed, and given a DOI) will make your data easier to find, reproduce, and cite.

You can submit to Data in Brief when you upload your revised manuscript. To do so, complete the template and follow the co-submission instructions found here: www.elsevier.com/dib-template. If your manuscript is accepted, your Data in Brief submission will automatically be transferred to Data in Brief for editorial review and publication.

Please note: an open access Article Publication Charge (APC) is payable by the author or research funder to cover the costs associated with publication in Data in Brief and ensure your data article is immediately and permanently free to access by all. For the current APC see: www.elsevier.com/journals/data-in-brief/2352-3409/open-access-journal

Please contact the Data in Brief editorial office at dib-me@elsevier.com or visit the Data in Brief homepage (www.journals.elsevier.com/data-in-brief/) if you have questions or need further information.

MethodsX (optional)

We invite you to submit a method article alongside your research article. This is an opportunity to get full credit for the

time and money spent on developing research methods, and to increase the visibility and impact of your work. If your research article is accepted, we will contact you with instructions on the submission process for your method article to MethodsX. On receipt at MethodsX it will be editorially reviewed and, upon acceptance, published as a separate method article. Your articles will be linked on ScienceDirect.

Please prepare your paper using the MethodsX Guide for Authors: <https://www.elsevier.com/journals/methodsx/2215-0161/guide-for-authors> (and template available here: <https://www.elsevier.com/MethodsX-template>) Open access fees apply.

More information and support

FAQ: How do I revise my submission in Editorial Manager?

https://service.elsevier.com/app/answers/detail/a_id/28463/supporthub/publishing/

You will find information relevant for you as an author on Elsevier's Author Hub: <https://www.elsevier.com/authors>

FAQ: How can I reset a forgotten password?

https://service.elsevier.com/app/answers/detail/a_id/28452/supporthub/publishing/

For further assistance, please visit our customer service site: <https://service.elsevier.com/app/home/supporthub/publishing/>

Here you can search for solutions on a range of topics, find answers to frequently asked questions, and learn more about Editorial Manager via interactive tutorials. You can also talk 24/7 to our customer support team by phone and 24/7 by live chat and email

#AU_BIOADV#

To ensure this email reaches the intended recipient, please do not delete the above code

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: <https://www.editorialmanager.com/bioadv/login.asp?a=r>). Please contact the publication office if you have any questions.

**BUKTI
SUBMIT
HASIL
REVIEW**

Confirming submission to Biomaterials Advances

1 message

Materials Science & Engineering C <em@editorialmanager.com>
Reply-To: Materials Science & Engineering C <support@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Sun, Oct 16, 2022 at 12:13 AM

This is an automated message.

Manuscript Number: BIOADV-D-22-02327R1

Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Dear Dr. Permana,

We have received the above referenced manuscript you submitted to Biomaterials Advances.

To track the status of your manuscript, please log in as an author at <https://www.editorialmanager.com/bioadv/>, and navigate to the "Revisions Being Processed" folder.

Thank you for submitting your revision to this journal.

Kind regards,
Biomaterials Advances

More information and support

You will find information relevant for you as an author on Elsevier's Author Hub: <https://www.elsevier.com/authors>

FAQ: How can I reset a forgotten password?

https://service.elsevier.com/app/answers/detail/a_id/28452/supporthub/publishing/

For further assistance, please visit our customer service site: <https://service.elsevier.com/app/home/supporthub/publishing/>

Here you can search for solutions on a range of topics, find answers to frequently asked questions, and learn more about Editorial Manager via interactive tutorials. You can also talk 24/7 to our customer support team by phone and 24/7 by live chat and email

#AU_BIOADV#

To ensure this email reaches the intended recipient, please do not delete the above code

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: <https://www.editorialmanager.com/bioadv/login.asp?a=r>). Please contact the publication office if you have any questions.

Biomaterials Advances

Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy --Manuscript Draft--

Manuscript Number:	BIOADV-D-22-02327R1
Article Type:	Research Paper
Keywords:	Chloramphenicol; Hydrogel; Microparticle; Skin Wounds; Whey Protein.
Corresponding Author:	Andi Dian Permana, Ph.D Hasanuddin University INDONESIA
First Author:	Tri Puspita Roska
Order of Authors:	Tri Puspita Roska
	Ardiyah Nurul Fitri Marzaman
	Mukarram Mudjahid
	Nana Novriana Payung Datu
	Sartini Sartini
	Andi Dian Permana, Ph.D
Abstract:	Skin wounds have been reported to increase the number of microbial colonies susceptible to infection. Treatments using oral antibiotics have been limited due to their toxicity and hydrophobic characteristics. In this study, we developed a formulation of chloramphenicol microparticles (CPL MPs), which was modified into chitosan hydrogel to increase treatment efficiency in targeting infections and creating an optimal environment to support the healing process. CPL MPs were prepared by a cross-linker stabilized method using whey protein (WPI) biopolymer, and the CPL MPs hydrogel was designed using chitosan biopolymer. Based on the result, CPL-loaded MPs showed desired physical and encapsulation characteristics. In the <i>in vitro</i> study, drug release of CPL MPs in simulated wound fluid represented approximately $99.40 \pm 7.01\%$ of the system after 24 h. The antibacterial activity of CPL-loaded MPs formulation (MIC value 12.5 mg/ml, MBC 25 mg/ml) was effective as MIC concentration increased. Furthermore, the formulation of CPL MPs into hydrogel showed a better dermatokinetic profile compared to hydrogel with pure CPL. Interestingly, the antibacterial activity of the <i>ex vivo</i> infection model showed that <i>Staphylococcus aureus</i> activity decreased by up to 99.98% after 24 h administration of CPL MPs hydrogel when compared to pure-CPL hydrogel and blank hydrogel. These studies have confirmed that incorporating CPL MPs into hydrogel can provide a promising approach to skin infection treatment.
Suggested Reviewers:	Eneko Larraneta Queen's University Belfast e.larraneta@qub.ac.uk
	Ahmed Faheem University of Sunderland ahmed.faheem@sunderland.ac.uk
	Aaron Courtenay Ulster University a.courtenay@ulster.ac.uk
Response to Reviewers:	



MINISTRY OF EDUCATION CULTURE OF INDONESIA
HASANUDDIN UNIVERSITY
FACULTY OF PHARMACY

Alamat Jalan Perintis kemerdekaan Km.10, Makassar 90245

Telepon (0411) 588556, Faksimili (0411) 590663

Laman: farmasi@unhas.ac.id

The Editor

Journal of Biomaterials Advance

14th September 2022

Dear Sir/Madam,

I wish you to consider our manuscript entitled: **“Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy”** for publication in Journal of Biomaterials Advance.

Skin wounds are a health care problem that is estimated to occur in about 4–5% of the adult population. Moreover, skin wounds have been reported to increase the number of microbial colonies susceptible to infection. Chloramphenicol (CPL) is one of antimicrobial with a broad spectrum of action which used for skin infections and can maintain its activity against bacterial resistance, but treatments using oral routes have been limited due to their toxicity and hydrophobic characteristics. In this study, we developed a formulation of chloramphenicol microparticles (CPL MPs), which was modified into chitosan hydrogel to increase treatment efficiency in targeting infections and creating an optimal environment to support the healing process. CPL MPs were prepared by a cross-linker stabilized method using whey protein (WPI) biopolymer, and the CPL MPs hydrogel was designed using chitosan biopolymer. Based on the result, CPL-loaded MPs showed desired physical and encapsulation characteristics with particle sizes in the range of $0.56 \pm 0.13 \mu\text{m}$ to $35.71 \pm 5.23 \mu\text{m}$ and Entrapment Efficiency value of $68.78 \pm 1.40\%$. The drug release behavior of CPL compared to CPL MPs formulation can improved the CPL release profile, then the cytotoxicity of MPs chemicals on RBCs can be quickly assessed using the hemolysis test. From the results, it can be concluded that the fabrication in the form of CPL-loaded MPs is compatible with RBCs because it consists of natural polymers. Currently, the goal of building a medication system on the skin's surface is to provide an ideal environment for wound healing. It has a number of advantages, including the ability to localize the medicine at the site of infection. Chitosan were used for the fabrication of hydrogel to enable skin distribution of the microparticle formulation and support antibacterial activity with strength bioadhesive properties. The use of chitosan as natural gelling agents showed a desired characteristic with a good appearance in pH in the range of 5.45 ± 0.04 to 5.55 ± 0.01 , which is very close to normal skin pH of 5 to 6, indicating that the formulation will be tolerated by the skin and will not interfere with the wound healing process.



MINISTRY OF EDUCATION CULTURE OF INDONESIA
HASANUDDIN UNIVERSITY
FACULTY OF PHARMACY

Alamat Jalan Perintis kemerdekaan Km.10, Makassar 90245

Telepon (0411) 588556, Faksimili (0411) 590663

Laman: farmasi@unhas.ac.id

To investigate the kinetics of the release of CPL MPs formulation, dermatokinetic studies of the hydrogel are carried out after application to the skin. We compared the dermatokinetic profile of this study with a hydrogel containing CPL without formulation in the form of MPs as a control to assess whether the release of drugs affects the state of drug particles. Our findings show that the CPL content in dermal layers obtained pure-CPL hydrogel was remarkably lower compared with that of CPL MP hydrogel. It concludes that a responsive CPL MPs hydrogel approach can successfully deliver MPs into *ex vivo* infection models. To test the efficiency of CPL MPs hydrogel as an antibacterial agent, researchers used an *ex vivo* skin infection model. We sought to determine whether CPL MPs hydrogel could reduce bacterial bioburdens produced from SA by counting cells' log CFU/ml number. Analysis of *ex vivo* antibacterial activity showed an enhancement of 100% after 24 hours when CPL is incorporated into MPs in hydrogel form. This indicates that chitosan hydrogel may enhance the antibacterial activity of CPL MPs. The encouraging findings of this study have validated the study's premise, demonstrating that CPL can be efficiently transported to the skin utilizing MPs, and then, once incorporated into the chitosan hydrogel formula, can exhibit its antimicrobial action to target infection.

We believe that this work will be of great interest to the readers of the Journal of Biomaterials Advance, particularly those working on applications of biopolymer, hydrogel formulations, and drug delivery for dermal administration.

The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

I hope that you consider this manuscript worthy of publication in Journal of Biomaterials Advance. We look forward to hearing from you in due course.

Yours sincerely,

Dr. Andi Dian Permana (on behalf of all authors)

Department of Pharmaceutics, Faculty of Pharmacy, Hasanuddin University, Makassar,
Indonesia

Email: andi.dian.permana@unhas.ac.id

Manuscript Number : BIOADV-D-22-02327

Title : Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Response to Reviewer

We are grateful to the expert reviewers for taking the time to read our work and provide helpful comments for improvement and clarification. As a result of these remarks, we have made some changes to the manuscript. We believe that the manuscript is now substantially improved. Below, we have provided a detailed response to each of the reviewers' comments. Importantly, we have made a great effort to improve the English and the discussion parts of our revised manuscript.

Reviewer #1:

In this work, chloramphenicol (CLP), a powerful antibiotic against a broad spectrum of bacteria, was incorporated into the MPs based on whey protein. Then the MPs were embedded into chitosan gel to synthesize a controllable hydrogel to deliver the CLP. Although the topic is interesting, according to their data, this work is not suitable for this journal.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We are glad that the Reviewer thinks that our work is interesting. We have made a number of key changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved and is suitable for the journal. We have addressed each one of the reviewers' comments in detail.

Materials and methods

- 1) It would be clear to add the exact amount of WPI, CPL, and their concentrations in section 2.2.

Response to Reviewer

We thank the Reviewer for the suggestions. We have made a changes to the manuscript as a result of these comments as follows:

CPL MPs were prepared according to a previous study (Jain et al., 2018), with slight modifications. WPI (with concentration of 2.5%, 5%, 10%, 15% and 20% (w/v)) was dispersed in deionized water, and the protein solution's pH was adjusted to 8 using NaOH (1 M). The solution was swirled for one hour with a magnetic stirrer before being refrigerated overnight (4°C) to fully hydrate the biopolymer. The solution was centrifuged at 1000 rpm for 5 minutes, and the supernatant was separated for further use. CPL (with concentration of 12.5, 25, 50, 100, 200 mg/mL) was dissolved in 5 mL methanol and added to 20 mL of protein solution dropwise while continuously stirring at a temperature of 40°C. Immediately after dissolution using methanol, 2 mL of 5% (v/v) glutaraldehyde was added to facilitate cross-linking of the particles. The detailed concentration of formulation is depicted Table 1. The cross-linking procedure was carried out for 24 hours at 40°C with agitation; this also caused any residual organic solvent to evaporate. The obtained dispersion was thoroughly washed with deionized water three times. The mixtures were then freeze-dried.

2) Please uniform all the abbreviations; Particles change to MPs.

Response to Reviewer

We thank the Reviewer for the suggestions. We have made a changes to the manuscript as a result of these comments and uniformed all the abbreviations.

3) Section 2.3.1. line 129. How could you measure the size of dissolved MPs? It should change to dispersed.

Response to Reviewer

We thank the Reviewer for pointing this out. We have made a changes to the manuscript as a result of these comments as follows:

The MPs were first dispersed with deionized water (0.01% v/v), and 1 mL of each sample was used for the analysis process.

4) Please add some references for your procedure to calculate entrapment efficiency and drug loading.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, for providing helpful comments. We have added some references in this part.

5) What is the difference between entrapment efficiency and drug loading? Both of them illustrate how much drug was encapsulated in particles. So why you used two different methods to show it?

Response to Reviewer

We thank the Reviewer for the suggestions. We have included these in the revised manuscript, as follows:

The encapsulation efficiency of CPL MPs (EE) % is the actual amount of the CPL found in MPs divided by the total amount of the CPL added during the preparation. Drug loading of CPL (DL) % is the mass of the CPL MPs in the dried powder divided by the total mass of the MPs recovered from the drying process. The calculation of % EE and DL follows the equations below (Agwa et al., 2022):

$$\% \text{ Entrapment Efficiency (EE)} = \frac{\text{Total drug amount} - \text{unencapsulated drug amount}}{\text{total drug amount}} \times 100\% \quad (1)$$

$$\% \text{ Drug Loading (DL)} = \frac{\text{Mass of encapsulated drug}}{\text{total mass of microparticle}} \times 100\% \quad (2)$$

6) Please add the R² related to the standard curve equation in the drug release section.

Response to Reviewer

We thank the Reviewer for the suggestions. We have included these in the revised manuscript, as follows:

The CPL released was further determined using a UV-Vis spectrophotometer. The amount of CPL in simulated wound fluid was calculated according to the standard curve equation $Y = 0.0368x + 0.011$ with $R^2 = 0.9996$.

7) It needs to add the amount of the samples in PXRD analysis and under which condition the test was carried out.

Response to Reviewer

We thank the Reviewer for the suggestions. We have included these in the revised manuscript, as follows:

The crystallinity pattern of the sample was analyzed with a powder X-ray diffraction (Rigaku Corporation, Kent, England) instrument using 1 gram of sample in very high-resolution Cu-K α radiation diffraction system. The CPL MPs was placed in an aluminium holder at a voltage of 40 kV, current of 30 mA and scanned 2 θ angle range of 10 to 90°.

8) Please add a reference for stimulating wound fluid. Section 2.3.7

Response to Reviewer

We are very thankful to the Reviewer for the suggestion. We have added a reference in this part in the revised manuscript.

9) An equation to calculate drug release is missing; please add it, followed by a reference.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, and for providing helpful comments. We have made changes to the manuscript, as follows:

The CPL released was further determined using a UV-Vis spectrophotometer. The amount of CPL in simulated wound fluid was calculated according to the standard curve equation $Y = 0.0368x + 0.011$ with $R^2 = 0.9996$ and the drug release percentage was calculated using the equation below[22] :

$$\% \text{ Drug released} = \frac{\text{Amount of CPL release}}{\text{Amount of CPL tested (50 mg)}} \times 100\% \quad (3)$$

10) The chitosan hydrogel was formed in the acidic media; could not this media be toxic for cells?

Response to Reviewer

We are very thankful to the Reviewer for pointing this out. In this study, in the preparation of hydrogel CPL MPs, following the solubilization using acetic acid, the chitosan solution was adjusted until the pH hydrogel to 5 with the addition of NaOH 8.3 M. Based on the measurement, the pH of the hydrogel surface for each formula is very close to normal skin pH of 5 to 6 (Ramos et al., 2012), indicating that the formulation could be tolerated by the skin and could not interfere with the wound healing process. Importantly, we also evaluated the initial toxicity potential of our formulation using haemolysis assay, showing that the formulation could not potentially cause any toxicity issue according to the haemolysis percentage values.

Results and discussion

1) Please add a reference at the end of line 329.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, for providing helpful comments. We have made a changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved.

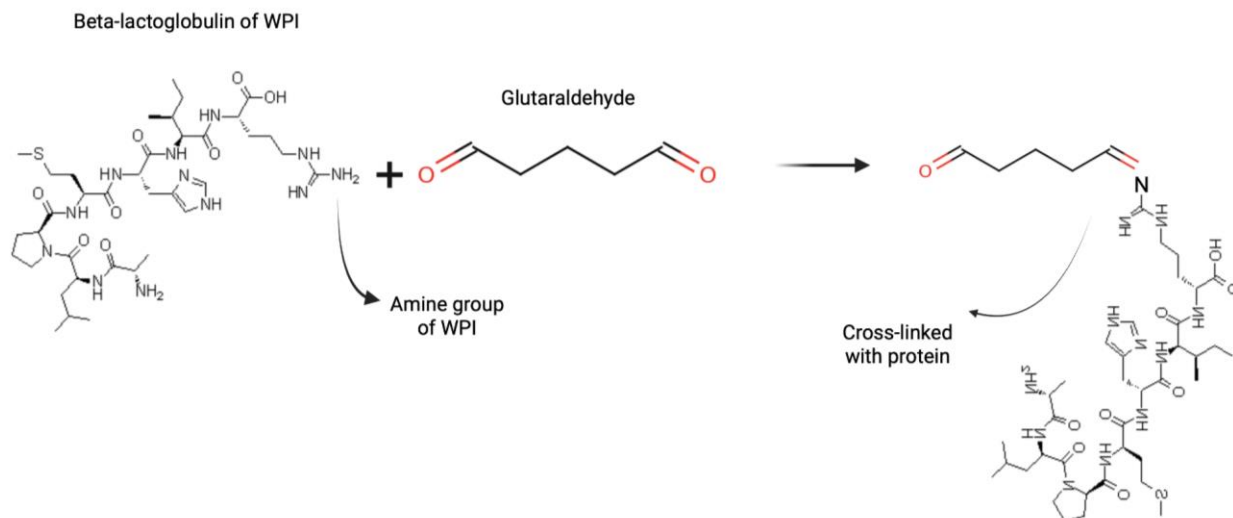
Although It has been claimed that CPL was successfully incorporated into the WPI MPs according to the results of DSC, FTIR, and XRD, there are some questions:

1) Which functional groups of PWI and glutaraldehyde contribute to the crosslinking reaction? Make it clear with a schematic.

Response to Reviewer

We are very thankful to the Reviewer for the suggestions. We have made a changes to the manuscript as follows:

As a cross-linker agent, CPL MPs were prepared by solvent evaporation and stabilized by glutaraldehyde. Moreover, glutaraldehyde has also been reported to introduce intermolecular cross-linking in proteins or to modify adsorbed proteins on amino-glutaraldehyde bonds (Betancor et al., 2006). Glutaraldehyde-mediated cross-linking of whey proteins was formed by aggregates of whey protein by whey protein intermolecular amide bonds (β -lactoglobulin (BLG) bands) and glutaraldehyde as follows the reaction in Fig 1.



2) What is the reason for disappearing the broad peak at around 2500cm⁻¹ in FTIR spectra of whey protein after incorporation CPL into MPs?

Response to Reviewer

We are very thankful to the Reviewer for pointing this out. We have made a changes to the manuscript as a result as follows:

Major interesting peaks are observed in 3200–3600 cm⁻¹, indicating the presence of O–H and N–H bonds. The broad peak at around 2800-2500 cm⁻¹ is also associated with O-H stretching vibration. O–H stretching was remarkably shifted after CPL was encapsulated in WPI microparticles due to the interaction of CPL and WPI. These changes suggested that hydrogen bonding was formed between β -lactoglobulin and CPL. In the infrared spectrum, major interesting peaks are observed in 3200–3600 cm⁻¹, indicating the presence of O–H and N–H bonds (Abbasi et al., 2014).

3) Moreover, why did the decrease in the intensity of the peak relate to the amide at around 1700 in FTIR spectra of whey protein?

Response to Reviewer

We are very thankful to the Reviewer for the question. We have made a changes to the manuscript as a result as follows:

The C=O stretching vibration carbonyl groups are primarily responsible for the Amid I observed in the encapsulated CPL at 1600-1700 cm^{-1} . The amide I band mainly confirms the presence of C=O stretch vibration and N–H bending. The middle absorption band II shown correlates with the structural combination group of C–N stretch with N–H deformation, while the amide III bands are associated with C–O bending vibration (Goyal et al., 2015). Amide I in the spectrum of 1745 cm^{-1} , amide II at 1451 cm^{-1} , and amide III at 1241 cm^{-1} are present in CPL-loaded MPs and physical mixtures.

4) Even by perfect dispersion of CPL into the MPs they should show their crystallinity peaks at XRD and melting point at DSC, unless they were miscible. On the other hand, if they were miscible, they could not have two separate melting points for CPL and whey protein. Therefore, it is possible the CPL was not incorporated into MPs, or the CPL contributed to the crosslinking reaction and converted it to another component. So how can this conflict be described?

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. While we determined that the encapsulation of CPL into microparticles was successfully accomplished by the DSC and XRD tests. We believe that CPL is incorporated in whey protein which is also supported by the DSC, XRD and FTIR data because there has been a finding of the wavelength of CPL after the manufacture of microparticles. Wang et al. reported similar outcomes (Wang et al., 2019) that the encapsulation procedure was successfully carried out in 3,3'-Diindolylmethane in β -lactoglobulin of WPI. These findings also align with DIM-based polymerized whey protein nanoparticles (Khan et al., 2019) and vitamin D3 encapsulated in WPI nanoparticles (Abbasi et al., 2014).

5) In the drug delivery section, the unincorporated CPL was used as a control and added directly to the release media. In this way, the consecration of the drug once adding the CPL should be

100%. So how is it possible for CPL without any protection release by time so that after six h, the release reaches the maximum?

Response to Reviewer

We thank the Reviewer for the suggestions. We have improved the results and discussion section by explaining the behaviour drug release of pure CPL as follows:

The experiment showed that $99.40 \pm 7.01\%$ CPL is released from the microparticle formulation after 24 hours. Meanwhile, the amount of drug release from pure CPL is around $95.43 \pm 2.41\%$ after 6 h and then CPL reached its maximum release.

6) Moreover, why was the release investigated just for 24h, while the CPL MPs were released between eight to 24h?

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made changes to the manuscript as a result as follows:

The drug release of CPL MPs was examined as the time in which the drugs needed to be completely soluble and migrate from the initial form as in the polymeric system to the simulated wound fluid until attaining maximum drug accumulation. The experiment showed that $99.40 \pm 7.01\%$ CPL was released from the microparticle formulation after 24 hours. Meanwhile, the amount of drug release from pure CPL was around $95.43 \pm 2.41\%$ after 6 h and then CPL reached maximum release. For simulated wound fluid, the estimated drug release during the first hour was $4.76 \pm 0.16\%$. After that, approximately $10.31 \pm 0.23\%$, $24.81 \pm 1.03\%$, $42.04 \pm 4.21\%$ and $62.39 \pm 1.68\%$ of CPL were released from MPs within 2 h, 4 h, 6 h, and 8h, respectively. By comparing both CPL and CPL MP's, a quick release lasting up to only 6 hours was observed in pure CPL, while that in CPL MP's needed 24 hours to completely release, showing the contribution of particle encapsulation and the sustained released drug was achieved.

7) Although the mathematic kinetic models were fitted to the release profile of CLP, the lack of discussion about the mechanism of release is crucial.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made a changes to the manuscript as follows:

The regular area of a collection of particles was found by Hixson and Crowell to be proportional to the volume cube root. The Hixson-Crowell cube root law describes particle release from a system where the surface area and diameter of the particles change (Reddy et al., 2018). In this work, the applicability of these equations was tested. Hixson and Crowell recognized that the rate of dissolution depends on the surface contacting with the applied solvent. Increased surface area leads to faster dissolution (Bradford et al., 2009). Because of the differences in surface area and diameter, this model corresponds well to drug release from MPs.

8) Please add the K of each kinetic model and also n of the Korsmeyer-Peppas model.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made a changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved.

Reviewer #2:

The work constitutes a good and valuable work. Despite of such strong points as well-prepared Introduction, well-designed research methodology and an extensive discussion over the results of performed experiments some corrections are recommended, i.e.:

Response to Reviewer

We are grateful to the expert reviewers for taking the time to read our work and provide helpful comments for improvement and clarification. As a result of these remarks, we have made some changes to the manuscript. We believe that the manuscript is now substantially improved. Below, we have provided a detailed response to each of the reviewers' comments. Importantly, we have made a great effort to improve the English and the discussion parts of our revised manuscript.

1) Language of the paper should be significantly corrected.

Response to Reviewer

We thank the Reviewer for the suggestion. We have re-read the manuscript and have made a great effort to improve the English throughout.

2) Section 2.1.: the information concerning the average molecular weight of chitosan and its deacetylation degree needs to be added.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made a changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved.

3) The methodology of the preparation of samples before the analyses should be added. For example, there is no information concerning the drying conditions or the sputtering process before SEM analysis.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made a changes to the manuscript as follows:

CPL MPs were freeze-dried under vacuum by lyophilizing. The CPL MPs were directly attached to the carbon tape and observed with the following operating conditions: accelerating voltage 15 kV, probe current 45 nA, and counting time 60 s.

4) It is strongly suggested to change the samples' names (formulation codes) to facilitate the analysis of the results of performed experiments.

Response to Reviewer

We are very thankful to the Reviewer for the suggestions. We have made a changes to the manuscript as a result of these comments.

5) Table 2.: the given numerical values should contain dots instead of commas.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made a changes to the manuscript as a result of these comments.

6) Quality of all figures should be significantly improved.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have improved the quality of all figures in the revised manuscript.

7) Final conclusions should be given using more quantified data.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have added more quantified data in the revised manuscript.

8) The notation of References needs to be consistent. Now, some of them contain the whole journal names and some of them contain their abbreviations.

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made significant changes in this part in the revised manuscript.

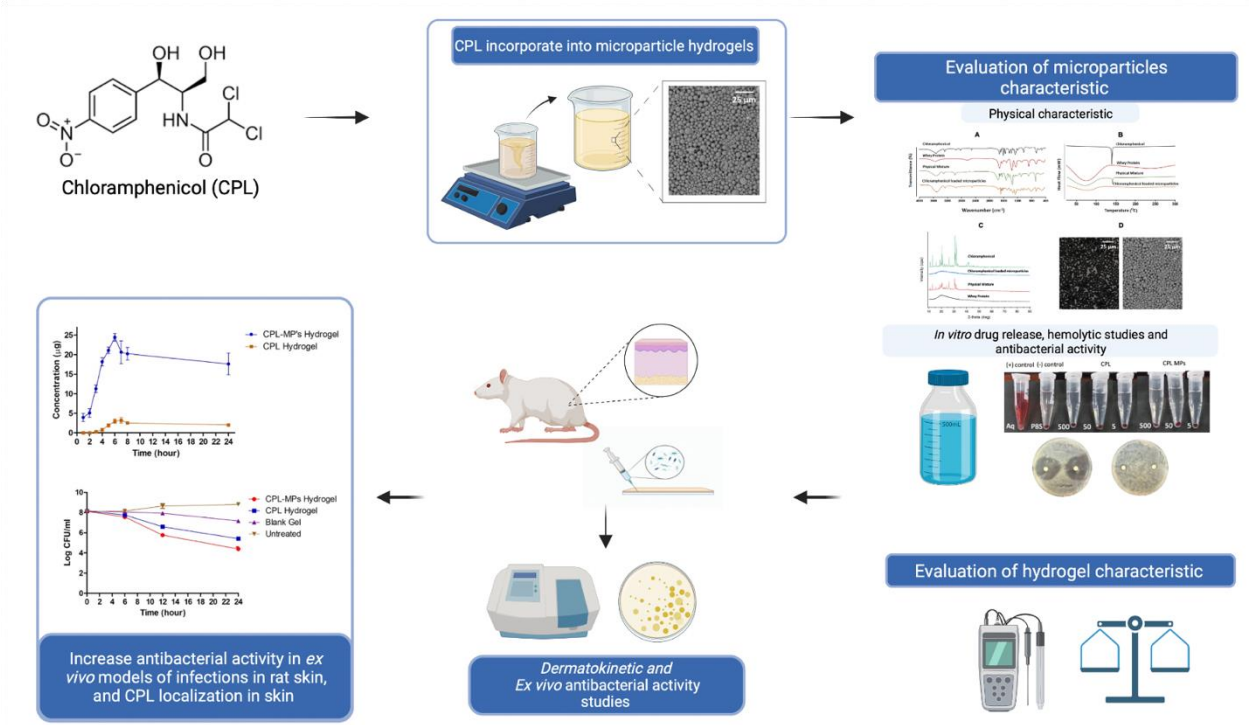
References

- Abbasi, A., Emam-Djomeh, Z., Mousavi, M. A. E., & Davoodi, D. (2014). Stability of vitamin D3 encapsulated in nanoparticles of whey protein isolate. *Food Chemistry*, 143, 379–383. <https://doi.org/10.1016/j.foodchem.2013.08.018>
- Agwa, M. M., Sabra, S., Atwa, N. A., Dahdooh, H. A., Lithy, R. M., & Elmotasem, H. (2022). Potential of frankincense essential oil-loaded whey protein nanoparticles embedded in frankincense resin as a wound healing film based on green technology. *Journal of Drug Delivery Science and Technology*, 71, 103291. <https://doi.org/10.1016/j.jddst.2022.103291>
- Betancor, L., López-Gallego, F., Hidalgo, A., Alonso-Morales, N., Mateo, G. D.-O. C., Fernández-Lafuente, R., & Guisán, J. M. (2006). Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions. *Enzyme and Microbial Technology*, 39(4), 877–882. <https://doi.org/10.1016/j.enzmictec.2006.01.014>
- Bradford, C., Freeman, R., & Percival, S. L. (2009). In Vitro Study of Sustained Antimicrobial Activity of a New Silver Alginate Dressing. *The Journal of the American College of Certified Wound Specialists*, 1(4), 117–120. <https://doi.org/10.1016/j.jcws.2009.09.001>
- Goyal, A., Sharma, V., Sihag, M. K., Tomar, S. K., Arora, S., Sabikhi, L., & Singh, A. K. (2015). Development and physico-chemical characterization of microencapsulated flaxseed oil powder: A functional ingredient for omega-3 fortification. *Powder Technology*, 286, 527–537. <https://doi.org/10.1016/j.powtec.2015.08.050>
- Jain, A., Sharma, G., Ghoshal, G., Kesharwani, P., Singh, B., Shivhare, U. S., & Katare, O. P. (2018). Lycopene loaded whey protein isolate nanoparticles: An innovative endeavor for

- enhanced bioavailability of lycopene and anti-cancer activity. *International Journal of Pharmaceutics*, 546(1–2), 97–105. <https://doi.org/10.1016/j.ijpharm.2018.04.061>
- Khan, A., Wang, C., Sun, X., Killpartrick, A., & Guo, M. (2019). Preparation and Characterization of Whey Protein Isolate–DIM Nanoparticles. *International Journal of Molecular Sciences*, 20(16), 3917. <https://doi.org/10.3390/ijms20163917>
- Ramos, Ó. L., Fernandes, J. C., Silva, S. I., Pintado, M. E., & Malcata, F. X. (2012). Edible Films and Coatings from Whey Proteins: A Review on Formulation, and on Mechanical and Bioactive Properties. *Critical Reviews in Food Science and Nutrition*, 52(6), 533–552. <https://doi.org/10.1080/10408398.2010.500528>
- Reddy, B. B. K., Nagoji, K. E. V., & Sahoo, S. (2018). Preparation and in vitro & in vivo evaluation of cephalexin matrix tablets. *Brazilian Journal of Pharmaceutical Sciences*, 54(3). <https://doi.org/10.1590/s2175-97902018000317277>
- Wang, C., Zhou, X., Wang, H., Sun, X., & Guo, M. (2019). Interactions between β -Lactoglobulin and 3,3'-Diindolylmethane in Model System. *Molecules*, 24(11), 2151. <https://doi.org/10.3390/molecules24112151>

Highlights

- CPL was formulated into microparticles (CPL-MPs) using Whey Protein (WPI) biopolymer and then modified into chitosan hydrogel.
- CPL-loaded MPs show desired physical and encapsulation characteristics with no hemolytic potential to RBCs.
- CPL MPs hydrogel produced suitable characteristics with excellent bioadhesive strength
- The hydrogels exhibited good antibacterial activity in the rat skin infection model by decreasing bacterial bioburden



Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Tri Puspita Roska¹, Sartini¹, Mukarram Mujahid¹, Ardiyah Nurul Fitri Marzaman¹, Nana Novriana Payung Datu¹, Andi Dian Permana^{1*}

1. Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia

***Corresponding author:**

Andi Dian Permana

Faculty of Pharmacy, Hasanuddin University, Indonesia

Email: andi.dian.permana@farmasi.unhas.ac.id

Abstract

Skin wounds have been reported to increase the number of microbial colonies susceptible to infection. Treatments using oral antibiotics have been limited due to their toxicity and hydrophobic characteristics. In this study, we developed a formulation of chloramphenicol microparticles (CPL MPs), which was modified into chitosan hydrogel to increase treatment efficiency in targeting infections and creating an optimal environment to support the healing process. CPL MPs were prepared by a cross-linker stabilized method using whey protein (WPI) biopolymer, and the CPL MPs hydrogel was designed using chitosan biopolymer. Based on the result, CPL-loaded MPs showed desired physical and encapsulation characteristics. In the *in vitro* study, drug release of CPL MPs in simulated wound fluid represented approximately $99.40 \pm 7.01\%$ of the system after 24 h. The antibacterial activity of CPL-loaded MPs formulation (MIC value 12.5 $\mu\text{g/ml}$, MBC 25 $\mu\text{g/ml}$) was effective as MIC concentration increased. Furthermore, the formulation of CPL MPs into hydrogel showed a better dermatokinetic profile compared to hydrogel with pure CPL. Interestingly, the antibacterial activity of the *ex vivo* infection model showed that *Staphylococcus aureus* activity decreased by up to 99.98% after 24 h administration of CPL MPs hydrogel when compared to pure-CPL hydrogel and blank hydrogel. These studies have confirmed that incorporating CPL MPs into hydrogel can provide a promising approach to skin infection treatment.

Keywords: Chloramphenicol; Hydrogel; Microparticle; Skin Wounds; Whey Protein.

1. Introduction

A skin wound is a condition in which the skin has impaired integrity, anatomical structure and tissue function, and may be caused by chronic disease, burns or postoperative trauma. Skin wounds are a health care problem that is estimated to occur in about 4–5% of the adult population [1]. Damage to the integrity of the skin due to injury causes colonization by microorganisms and may result in infection complications, which can prolong patient treatment time and increase drug use [2]. When bacteria infect a wound, they stimulate the release of inflammatory cells, certain enzymes and ROS, which make the wound worse [3]. The most common aerobic microorganisms causing wound infections include *S. aureus*, *S. pyogenes*, *P. aeruginosa*, MRSA, *S. epidermidis*, *Enterococcus faecalis*, coliforms, and *Acinetobacter baumannii* [4]. Wound infections can cause bacteremia, sepsis, and organ dysfunction, so an effective method is needed to prevent wound infections, one of which is the administration of appropriate topical antibiotics [5].

One antimicrobial with a broad spectrum of action is chloramphenicol (CPL), which is used for skin infections and can maintain its activity against bacterial resistance, with a bacteriostatic mechanism specifically inhibiting bacterial protein synthesis [6]. However, the oral route of CPL is still limited due to its toxicity [7], and the physicochemical properties of hydrophobicity make it difficult for the drug to penetrate the skin and inhibit drug transport to the target of infection [8]. This limitation can be overcome via the dermal route, which facilitates the loading of hydrophobic drugs with direct action activity to the target of infection.

Drug delivery systems targeting local infections could be developed by designing drugs into microparticles [9]. Microparticles (MPs) are widely preferred because they release drugs in a responsive manner, increase the effectiveness of the drug on the target, and provide local effects on the skin. In addition, drug-containing MPs offer high drug loading flexibility and good physical and chemical stability [10]. MPs have also been reported to be designed for local administration with desired effects, including wound care. The MPs approach has also been widely developed for the treatment of infectious diseases to increase efficiency at selectively targeting infections through specific stimulus responses [11].

The drug substance can be incorporated into MPs through various methods such as ionotropic gelation, single or double emulsification, polymerization, and stabilization with cross-linkers [12].

MPs prepared by a cross-linker method using natural biopolymers as drug carriers offer optimal drug loading. Hydrophobic drug encapsulation using natural biopolymers was chosen because of its biodegradability, biocompatible, and suitability for skin delivery system formulation [13]. Whey protein is a high-quality protein from cheese processing or cow's milk and is primarily consisted of major proteins including β -lactoglobulin, α -lactalbumin, immunoglobulin, and lactoferrin [14]. A study has shown that the binding of whey protein biopolymers with hydrophobic drugs can increase drug solubility and dissolution [15]. Liu et al. used whey protein for curcumin encapsulation by employing the spray-drying method to increase curcumin solubility and bioavailability [16]. This can be chosen as a strategy to increase the effectiveness of drugs in treating skin infections. However, there are limitations in using whey protein in formulations because it tends to have a weak protein bond, is easily damaged by heating, and may be susceptible to degradation. Therefore, hydrogel formulation is one of the preparations to overcome this problem.

Hydrogels are polymer networks with hydrophilic properties, which are needed in wound treatment because they have excellent biocompatibility and provide an optimal environment for wound healing. Hydrogels can be applied in various physical forms, such as nanoparticles, MPs, and films [17]. Chitosan, the natural polymers in the manufacture of hydrogels, contain many hydroxyl and amine groups typically used as functional groups to react with cross-linking agents, which stabilize the protein structure [18]. In the treatment of wound infections, there are several reported benefits of using chitosan as a biopolymer, including good antibacterial activity [19], anti-inflammatory and hemostatic properties, enhanced skin regeneration; superior biocompatibility and biodegradability; good water absorption and skin occlusivity properties; and due to the presence of amino and hydroxyl groups in the molecular chain, many biological activities can be improved by grafting on additional groups and chemical elements [19,20].

For the potential enhancement of wound infection therapy, in this study, we provide a novel combination delivery method of chitosan hydrogel and chloramphenicol microparticles (CPL MPs). CPL MPs are formed and optimized by solvent evaporation, and they are stabilized using a cross-linker. Additionally, tests were done on drug loading, entrapment effectiveness, zeta potential, particle size, polydispersity index, and antibacterial activity. The fabrication capacity to deliver CPL intradermally was next tested in an *ex vivo* dermatokinetic study on healthy rat skin. Lastly, the penetration and antibacterial activity of the formulation were evaluated in an *ex vivo* skin infection

model. The major findings of this study have the potential to provide new insights for solving problems in the treatment of skin wound infections.

2. Materials and Methods

2.1 Materials

Whey protein (WPI) containing 80% was gifted by Glanbia Nutritionals, Inc, Fitchburg, USA. Chloramphenicol, chitosan, glutaraldehyde, 2-alpha naphthol, tryptone soya agar (TSA) medium, nutrient broth (NB) medium, phosphate-buffered saline (PBS), and sodium hydroxide were purchased from Sigma–Aldrich Pte Ltd., Singapore, Singapore. The reagents utilized in this investigation were all analytical grade.

2.2 Preparation of CPL MPs

CPL MPs were prepared according to previous studies [21], with slight modifications. WPI was dispersed in deionized water, and the protein solution's pH was adjusted to 8 using NaOH (1 M). The solution was swirled for one hour with a magnetic stirrer before being refrigerated overnight (4°C) to fully hydrate the biopolymer. The solution was centrifuged at 1000 rpm for 5 minutes, and the supernatant was separated for further use. CPL was dissolved in methanol and added to 20 mL of protein solution dropwise while continuously stirring at a temperature of 40°C. Immediately after dissolution using methanol, 2 mL of 5% (v/v) glutaraldehyde was added to facilitate cross-linking of the particles. The cross-linking procedure was carried out for 24 hours at 40°C with the dispersion agitated; this also causes any residual organic solvent to evaporate. The obtained dispersion was thoroughly washed with deionized water multiple times. The mixtures were then freeze-dried.

2.3 Characterization of CPL MPs

2.3.1 Evaluation of particle size, zeta potential and polydispersity index (PDI)

The particle size, polydispersity index (PDI) and zeta potential of formulation were determined using the size analyzer (Malvern Instruments, Malvern, UK). The MPs were first dissolved with deionized water (0.01% v/v), and 1 mL of each sample was used for the analysis process. All samples were analyzed at 25°C and a scattering angle of 90°.

2.3.2 Measurement of entrapment efficiency (EE) and drug loading (DL) percentages

Microparticle EE was measured using the indirect method. CPL MPs suspension was centrifuged at 1000 rpm for 20 minutes to separate the supernatant. The supernatant was then analyzed using UV-Vis spectrophotometer at a wavelength of 488.7 nm after being derivatized with 2- alpha naphthol (0.01 M) and sodium bicarbonate (0.01 M). A derivatization procedure was performed on CPL to produce peaks that did not overlap with WPI and showed accurate measurement data. The reduced CPL MPs solution was created by dissolving the CPL MPs in methanol, then reacting it with zinc powder and HCl (12 M) while stirring it for 5 minutes in bath water at a temperature of 50° C. The reduced solution was filtered into a flask and diluted with deionized water. In a volumetric flask containing the CPL MPs solution, 1 ml of 2-alpha naphthol and 0.5 mL of sodium bicarbonate (0.1 M) were added to complete the reduced process. To create an orange-red result, the solution was heated at 70° C in bath water for 10 min. The amount of CPL was calculated according to the standard curve equation $Y = 0.0769x - 0.0097$. The calculation of % EE and DL follows the equations below:

$$\% \text{ Entrapment Efficiency (EE)} = \frac{\text{Total drug amount} - \text{unencapsulated drug amount}}{\text{total drug amount}} \times 100\% \quad (1)$$

$$\% \text{ Drug Loading (DL)} = \frac{\text{Mass of encapsulated drug}}{\text{total mass of microparticle}} \times 100\% \quad (2)$$

2.3.3 Powder X-ray diffraction (PXRD) analysis

The crystallinity pattern of the sample was analyzed with a powder X-ray diffraction (Rigaku Corporation, Kent, England) instrument.

2.3.4 Differential scanning calorimetry (DSC) analysis

The materials were thermally examined using a DSC 2920 TA instrument (Surrey, UK). The detection parameters for this measurement were a 5 mg powder sample, a temperature range of 25° to 300°C, a heating rate of 10°C/min, and a nitrogen cleaning rate of 50 mL/min.

2.3.5 Fourier-transform infrared spectroscopy (FT-IR) analysis

Chemical interaction between chemical components in CPL MPs in WPI polymer was analyzed by FT-IR spectrophotometer instrument (Accutrac FT/IR-4100™ Series, Perkin Elmer, USA) in the wavenumbers range of 4000–400 cm⁻¹.

2.3.6 Scanning electron microscope analysis

A scanning electron microscope (TM3030 microscope, Hitachi, Krefeld, Germany) was used to examine the morphologies of CPL MPs.

2.3.7 *In vitro* release studies of CPL MPs

In vitro drug release of CPL MPs and CPL using simulated wound fluid. Simulated wound fluid was composed of 0.36 g of sodium hydrogen carbonate, 5.84 g of sodium chloride, 0.28 g of calcium chloride, 30.30 g of potassium chloride, and 33.00 g of bovine albumin in 1000 mL of deionized water. Two Erlenmeyers contained 50 mL of simulated wound fluid for each formula. CPL MPs equivalent to 50 mg of CPL was added into simulated wound fluid with 5 mL of samples taken at predetermined time intervals (0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 24 h) and replaced with the same volume of new release media. The test was carried out at a temperature of 37°C and a speed of 100 rpm. The CPL was further determined drug percentages using a UV-Vis spectrophotometer and following procedure section 2.3.2 (Shimadzu® FTIR-8400).

2.3.8 *Mathematical modeling of the kinetics of drug release*

The principal mathematical models of drug release were tested, along with the relevant equations and parameters, using the Excel® (Microsoft Corporation, Redmond, USA) Add-In-DDSolver to model the systems' drug release data. The adjustment of the theoretical models to the experimental data was compared using the adjusted coefficient of determination (adjusted- R^2). The kinetics model follows the equations below [22]:

Zero-order:

$$Q_t = Q_0 + K_0 t \quad (3)$$

First-order:

$$\ln Q_t = \ln Q_0 + K_1 t \quad (4)$$

Higuchi:

$$Q_t = K_H \sqrt{t} \quad (5)$$

Hixson-Crowell:

$$Q_0^{1/3} - Q_t^{1/3} = K_s t \quad (6)$$

Korsmeyer-Peppas:

$$Q_t = K_t n \quad (7)$$

where Q_0 is the preliminary value of Q , t is the time, n is the exponent of diffusion release, K_0 , K_1 , K_H , K_t , and K_s are the coefficients of release according to the relevant kinetic model, and Q_t (%) is the percentage of CPL released at time t .

2.4 In vitro antibacterial activity of CPL MPs

2.4.1 Preparation of bacterial cultures

The bacterial strain used was *Staphylococcus aureus* (ATCC®25923), which was stored at 4°C and sub-cultured in fresh media. Bacterial culture was prepared by growing bacteria on TSB media with a temperature of 37°C. Test bacteria are made equivalent to 0.5 mc Farland equivalent to 2×10^8 CFU / mL.

2.4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluation

The MIC and MBC from CPL and CPL MPs were determined using microtiter dilution techniques in accordance with the Clinical and Laboratory Standards Institute protocol [23]. CPL and CPL MPs solution with a concentration of 1000 µg/mL was made in a 10 mL volumetric flask. The sample was entered into 96-well plates with a concentration of 100 µg/mL. Next, 100 µL of bacterial suspension was added to the wells, then homogenized using micropipettes. TSB with a concentration of 100 µL medium was added to the wells. Wells containing only pure CPL and TSB media alone were used as the positive and negative control, respectively. All filled wells were closed and incubated at 37°C for 24 hours. To determine MBC value, 20 µL from 96-well plates with selected MIC was cultured on a Petri dish containing TSA medium and incubated at 37°C for 24 h. After that, the number of bacteria in the Petri dish colony was then determined. MBC was defined as the lowest concentration that eliminates 99.9% of bacterial growth.

2.4.3 Time-kill assay of CPL MPs

Time-kill assay of CPL and CPL MPs against *Staphylococcus aureus* (SA) was determined. CPL, blank MPs, and CPL MPs were added to the bacterial solution in the same quantities as MIC, 2 x MIC, and 4 x MIC, resulting in 2×10^5 CFU/mL of bacteria. After that, bacterial cultures were cultured at 37°C. 20µL aliquot from the culture was collected at specified time intervals and inoculated aseptically into a Petri dish containing TSA medium. The Petri dish was then incubated at 37°C for 24 hours. The procedure was performed with 3 replications and created a log CFU/mL

curve against time. CPL, blank MPs, and CPL MPs were added to the bacterial solution in the same quantities as MIC, 2 x MIC, and 4 x MIC, resulting in 2×10^5 CFU/mL of bacteria.

2.4.4 Evaluation of hemolytic activity

By incubating the sample with red blood cells (RBC) and quantifying the percentage of hemoglobin produced from lysed erythrocytes, the overall hemolysis of the formulation was examined. *In vitro* hemolytic assays were carried out in accordance with a previously described method to determine the hemolytic activity of CPL MPs hydrogel [24]. First, EDTA tubes containing freshly drawn rat blood were centrifuged for 10 minutes at 3000 rpm to separate the RBCs. After scraping off the plasma layer at the top of the tube, the remaining cells were further washed twice with PBS and diluted to 1:10, v/v. We mixed 100 μ L of the prepared cell suspension with 900 μ L of the sample containing CPL and CPL MPs (serially dissolved in PBS) at concentrations ranging from 5 to 500 mg/L. After 60 minutes of incubation at 37°C, the mixture was centrifuged at 3000 rpm for 10 minutes. Free hemoglobin was estimated using UV-visible spectroscopy measurements by analysing the supernatant taken at wavelength 540 nm. Minimal (negative) and maximum (positive) hemolytic controls were performed using phosphate-buffered saline and distilled water, respectively. Blood sample hemolysis was analyzed by visually inspecting for colour changes in the serum and plasma. For each concentration, the experiments were repeated three times. In the following equation, we included the hemolysis percentage calculation:

$$\text{Hemolysis (\%)} = \frac{\text{test sample} - \text{negative control} \times 100}{\text{positive control} - \text{negative control}} \quad (8)$$

2.5 Formulation of chitosan hydrogel from CPL MPs

Chitosan was dissolved in 2% acetic acid solution at 60°C to produce the CPL MP hydrogel. The chitosan solution was combined with glycerin (10% w/w) and DMDM hydantoin (0.01% w/w) while being stirred with a magnetic stirrer. CPL MPs (1% w/w) were then added to the mixture. Foaming components of the preparation were eliminated using a sonicator for 30 minutes at a temperature of 50°C, thereby adjusting the hydrogel pH to 5 with the addition of NaOH 8.3 M. As a control, a hydrogel containing CPL was created.

2.6 Characterization of CPL MPs hydrogel

2.6.1 pH measurement

A pH meter was used to determine the pH of CPL MPs hydrogel by inserting the electrode into the gel and recording the result after two minutes. All measurements are carried out 3 times to obtain the average value and standard deviation.

2.6.2 Evaluation of viscosity and rheology

With the use of a Brookfield viscometer with a seven-speed spindle running at 50 rpm, the viscosity of the CPL hydrogel was measured. The rheology of the CPL hydrogel was also determined using the same seven-speed spindle tool. Measurements are carried out at speeds of 5, 10, 20, 50, and 100 rpm.

2.6.3 Spreadability

Two sets of standard-sized glass slides were taken in this study. The spreadability of the hydrogel was evaluated by applying 0.5 g of the gel to a circle that had been previously marked on a glass plate with a diameter of 2 cm and then again using a second glass plate. After 1 minute, the diameter of the hydrogel was measured. The spreadability measurement was carried out with loads of 500 g.

2.6.4 Skin occlusivity examination

The previous technique was used to test *in vitro* skin occlusivity [25]. The 100 mL beaker was filled with 50 mL of deionized water, which was covered carefully with Whatman filter paper. Whatman filter paper with a size of 2.5 mm was applied with 250 mg of hydrogel. As a control, filter paper without CPL MPs hydrogel was used. All samples were stored at a temperature of 32°C, and the weight of Whatman paper was calculated at 0 h, 6 h, 24 h and 48 h. Occlusivity (O) was calculated through Eq:

$$\text{Occlusivity percentage (O)} = \frac{\text{mass of control} - \text{mass of sample}}{\text{mass of control}} \times 100\% \quad (9)$$

2.6.5 Extrudability percentage

About 10 g of hydrogel were sealed inside a collapsible aluminum tube, the end of which was crimped, and a clamp was used to keep the tube from unwinding. The gel was released after the cap was removed, and the quantity of gel that was extruded was measured. The amount of gel that was forced out of the extruder was determined as follows:

$$\% \text{ Extrudability} = \frac{\text{total mass of extruded hydrogel}}{\text{Total mass of hydrogel}} \times 100\% \quad (10)$$

2.6.6 Bioadhesivity test

Part of the rat skin tissue was cut and immediately secured with the mucosa side out into each glass vial via rubber bands and aluminum caps. Phosphate-buffered saline (PBS) was applied to vials containing rat skin tissue for 10 minutes. The test was carried out by gluing the skin onto the upper and lower vial bases, after which the hydrogel preparation was placed between the rat skin on the scale's left arm. The hydrogel was moistened with PBS and then attached to the skin. The vial's height was then altered to accommodate the application of hydrogel between the mucosal tissues of both vials. Once two vials were linked, the weights were held at that level. The detachment stress (dyne/cm^2) between two vials was calculated using the adhesive forces presented below [26]

$$\text{Bioadhesive strength: } \frac{m \cdot g}{A} \quad (11)$$

where m is the weight required to remove the gel from the skin, g is the acceleration due to gravity (980 cm/s^2), and A is surface area of the exposed mucosa (cm^2).

2.6.7 Ex vivo dermatokinetic study

Dermatokinetic assays using Franz diffusion cells were carried out following the previous method [27]. Rat skins were cut and soaked in PBS pH 7.4 for 30 minutes before being attached to Franz diffusion cells. The medium used in this study was simulated wound fluid, which was arranged as a receiver compartment. The receiver compartment was heated to $37 \pm 1^\circ\text{C}$ and agitated at 600 rpm. Parafilm® was used to close off the donor area. A 1 g sample of CPL MPs hydrogel was put into the skin using a syringe needle for 30 seconds, and a 5 g cylindrical weight was placed on top of the skin to keep the formula in place during the experiment.

The samples were taken at intervals of 1, 2, 3, 4, 5, 6, 8, and 24 hours. After removing the hydrogel, the skin was cleansed with deionized water to remove any residue particles. The biopsy incision was 5 mm in diameter and was made in the skin. Next, the skin was transferred to a 1.5 mL Eppendorf® tube and heated in a water bath at 60°C for 2–3 minutes. The epidermal layer was separated from the dermis layer using forceps. The extraction process of the drug on the skin layer was carried out by adding 1 mL of methanol separately, and each mixture was homogenized for 10 minutes. The sample was then centrifuged at 3000 rpm for 15 minutes. Supernatants were collected and then measured using a UV-Vis spectrophotometer and following procedure section 2.3.2. C_{max} , T_{max} and

AUC_{0-24} were determined using PK Solver software (China Pharmaceutical University, Nanjing, China).

2.6.8 Ex vivo antibacterial activity test using infection skin model

2.6.8.1 Ex vivo test preparation on rat skin infection model

The skin was sterilized by immersing it in 70% ethanol for one hour. The skin was sensitized to laminar airflow (LAF) for 20 minutes prior to the experiment. Furthermore, the sterilized skin was administered an intradermal injection of 50 μ L of BSA solution, which was equal to 0.5 mg Farland. The skin piece was next positioned aseptically on a Petri plate filled with TSA media. The skin was then aseptically transferred to a new TSA Petri dish, and the Petri dish was incubated at 37°C for 7 days.

2.6.8.2 Antibacterial activity test of ex vivo hydrogel preparations on skin infection model

The antibacterial activity of the *ex vivo* skin infection model was carried out using diffusion Franz diffusion cells. In this study, rat skin of average thickness was used, and a skin infection model was placed on diffusion cells. Before the study, a dilution of CPL MPs hydrogel and CPL hydrogel was prepared by dissolving in DMSO. The skin was collected and 1.5 mL of sterile water was added to a 1.5 mL Eppendorf tube after hydrogel treatments had been left on for 6, 12, and 24 hours, respectively. Next, the mixture was homogenized and TSA medium was added to a Petri dish with 20 mL of homogenous samples. The sample with the medium was incubated for 24 hours at 37°C. Additionally, the same method was used to apply both blank hydrogel and CPL hydrogel to infected skin as a control. We determined the log CFU/mL of the samples.

2.7 Analytical statistic

All data obtained for this study were reported as means of the standard deviation (SD) and computed using Microsoft Excel® 2016. (Microsoft Corporation, Redmond, USA). The data were analyzed using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA) and the SPSS version with a significance value of $p = 0.05$.

3 Result & Discussion

3.1 Particle size, zeta potential and polydispersity index (PDI) of CPL MPs

All ingredients used in the preparation of CPL MPs have been reported to be non-toxic and safe for clinical purposes. As a cross-linker agent, CPL MPs were prepared by solvent evaporation and stabilized by glutaraldehyde. Moreover, glutaraldehyde has also been reported to introduce intermolecular cross-linking in proteins or to modify adsorbed proteins on amino-glutaraldehyde bonds [28]. Glutaraldehyde-mediated cross-linking of whey proteins was formed by aggregates of whey protein by whey protein intermolecular amide bonds (β -lactoglobulin (BLG) bands) and glutaraldehyde. This approach successfully fabricated MPs in this study by designing formulations with a variation of CPL, WPI concentration and WPI:CPL ratio. The MPs' physical characteristics were then evaluated in Table 1 and Figure 1A.

The results show that particle sizes of CPL MPs were produced in the range of $0.56 \pm 0.13 \mu\text{m}$ to $35.71 \pm 5.23 \mu\text{m}$. Conversely, samples with variation in CPL concentration show significant particle size variation between formulations. Moreover, the formulation with a variation of WPI concentration also shows significant particle size variation between formulations ($p < 0.05$) with an increase of $3.62 \mu\text{m}$. It concludes that the concentration of WPI can produce a more compact protein structure so that the higher the protein concentration, the more it will be able to increase particle size [29]. The same trend also occurs in variations in the ratio of WPI and CPL (1:1, 2:1, 8:1, 16:1), of which a 1:1 ratio was found to yield significantly larger particle size compared to the rest ($p < 0.05$). This increase may be due to increased hydrophobic interactions between encapsulated and absorbed CPL in the main component of the protein isolate β -lactoglobulin with an increase in WPI concentration. Solution at pH 8.0 allows ligands to enter the hydrophobic nucleus and bind to many hydrophobic molecules, causing an increase in particle size [30]. These findings also showed that the CPL/WPI microparticle size was dependent on the ratio of WPI: CPL in the system (core: coating ratio), and that this ratio could be raised by increasing the WPI content in the formulations [31].

Table 1. Details of the different formulation characteristics utilized to create CPL MPs, including their PDI, zeta potential, and particle size (means $\pm$ SD, n = 3).

Formulation code	WPI in water (%)	CPL in 5 mL ethanol (mg)	Ratio WPL:CPL	Particle size (μ m)	PDI	Zeta potential (mV)
F1	5	12.5	4:1	1.33 $\pm$ 0.09	0.127 $\pm$ 0.005	-28.27 $\pm$ 0.81
F2	5	25	4:1	1.40 $\pm$ 0.14	0.129 $\pm$ 0.005	-29.25 $\pm$ 1.01
F3	5	50	4:1	1.44 $\pm$ 0.05	0.13 $\pm$ 0.003	-29.44 $\pm$ 0.67
F4	5	100	4:1	1.45 $\pm$ 0.11	0.14 $\pm$ 0.007	-29.14 $\pm$ 1.06
F5	5	200	4:1	1.47 $\pm$ 0.10	0.13 $\pm$ 0.010	-29.21 $\pm$ 1.65
F6	2.5	50	4:1	0.56 $\pm$ 0.13	0.13 $\pm$ 0.004	-24.67 $\pm$ 0.71
F7	10	50	4:1	2.92 $\pm$ 0.20	0.13 $\pm$ 0.005	-31.95 $\pm$ 0.87
F8	15	50	4:1	7.65 $\pm$ 0.99	0.14 $\pm$ 0.005	-34.23 $\pm$ 1.14
F9	20	50	4:1	11.42 $\pm$ 1.07	0.14 $\pm$ 0.009	-35.17 $\pm$ 0.84
F10	10	50	1:1	134.62 $\pm$ 10.29	0.63 $\pm$ 0.029	-27.73 $\pm$ 1.43
F11	10	50	2:1	33.03 $\pm$ 4.08	0.40 $\pm$ 0.006	-28.36 $\pm$ 0.91
F12	10	50	8:1	1.29 $\pm$ 0.05	0.13 $\pm$ 0.007	-32.32 $\pm$ 2.00
F13	10	50	16:1	1.29 $\pm$ 0.07	0.12 $\pm$ 0.009	-32.56 $\pm$ 2.04

PDI was carried out to characterize the molecular weight of a polymer. Based on the results (Figure 1B), samples with a variation of CPL WPI concentrations show no significant results between formulations ($p > 0.05$). While the different outcomes are noticeably seen in the ratio of WPI:CPL, the higher solution of WPI in formulation shows decreased PDI value. The PDI value indicates the same trend as the particle size. PDI smaller than 0.5 is produced when the mass of WPI in WPI:CPL is within the range of 2:1 to 16:1, indicating high particle aggregation with a broad particle size distribution [32].

Measurement of zeta potential can predict the colloidal solution's stability, indicating the repulsive force between particles. The zeta potential graphic of the CPL MPs is shown in Figure 1C. CPL MPs have a relatively high zeta potential (i.e., close to around -30 mV), indicating good stability of MPs exhibited in this study. Furthermore, increasing the concentration of CPL in the formulation has no significant effect ($p > 0.05$) on the zeta potential of MPs, indicating that the significant amount of drugs trapped in the core has no effect on the surface area of β -lactoglobulin. All formu-

lation have extremely negative zeta potentials, which improve the physical stability of many systems by increasing the repulsive force between particles [33]. In addition, The negative zeta potential found in this investigation may be attributable to the structure of the whey protein opening during heating at the microparticle preparation, which reveals more polar groups [34]. Furthermore, β -Lg of WPI has an isoelectric point in the range of 4.9–5.4. The second significant constituent of whey protein is α -La, with an isoelectric point of 4.6. When pH value increases above the isoelectric point, the protein produces a negative charge, which facilitates negative zeta potential. Additionally, the isoelectric point of β -Lg is in the 4.9–5.4 range. Whey protein's second important component (α -La) has an isoelectric point of 4.6. Proteins create a negative charge when the pH level rises above the isoelectric point, which supports the negative zeta potential [35,36]

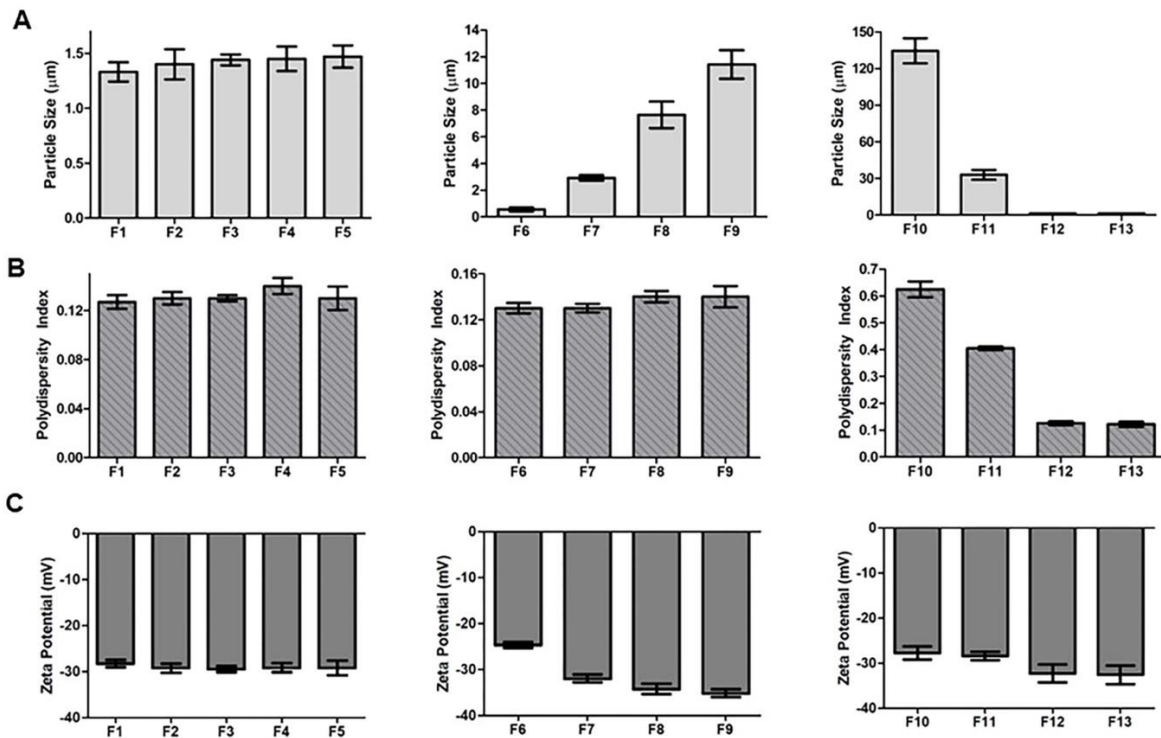


Figure 1. Particle size (A), Polydispersity Index (B) and Zeta Potential (C) of CPL MPs prepared with Whey Protein (WPI) (means $\pm$ SD, n = 3).

3.2 EE and DL percentages

Entrapment efficiency (%EE) and drug loading (%DL) of CPL MPs are shown in Figure 2A. CPL MPs have a higher EE value ($68.78 \pm 1.40\%$) when the mass of WPI is in the range of 10–20%, but there is no noticeable difference between F7, F8 and F9 ($p > 0.05$). The same trend occurred in the

WPI: CPL ratio (8:1 and 6:1), experiencing an increase in EE ($55.67 \pm 3.55\%$ and $45.98 \pm 1.47\%$). This value decreased significantly to $13.50 \pm 2.67\%$ when the WPI:CPL ratio was 1:1. WPI has an amphiphilic structure that can self-assemble into different forms, thereby increasing drug encapsulation. However, this ability can be reduced if DL has been maximized. According to Li et al., the increased surface hydrophobicity of WPI aggregates can explain their higher CPL loading capacity [37]. Li et al. investigated whether the enhanced contact between hydrophobic groups during the chemical cross-linking procedure was causing the surface hydrophobicity of WPI to rise. They found that the more active hydrophobic patches among the generated WPI aggregates for binding to CPL accounted for their increased %EE and %DL.

Drug loading of CPL MPs increased significantly from $3.40 \pm 1.68\%$ to $20.43 \pm 2.15\%$ (Figure 2B). Similar results have also been reported in utilizing α -lactalbumin-based nanoparticles to encapsulate resveratrol, in which the DL value increased significantly as drug content was increased, indicating a higher microparticle loading capacity with the addition of drugs [38]. Moreover, methanol as the solvent to dissolve CPL at higher concentrations modifies the protein solution's dielectric constant and induces the transition into aggregates [39]. Consequently, The total WPI aggregate formed depends on CPL concentration and the amount of solvent in the formulation. The DL of WPI aggregates produced in this preparation was much higher than what Zhang et al. reported for protein based-nanoparticles formulation ($12.69 \mu\text{g}/\text{mg}$ nanoparticles) [40].

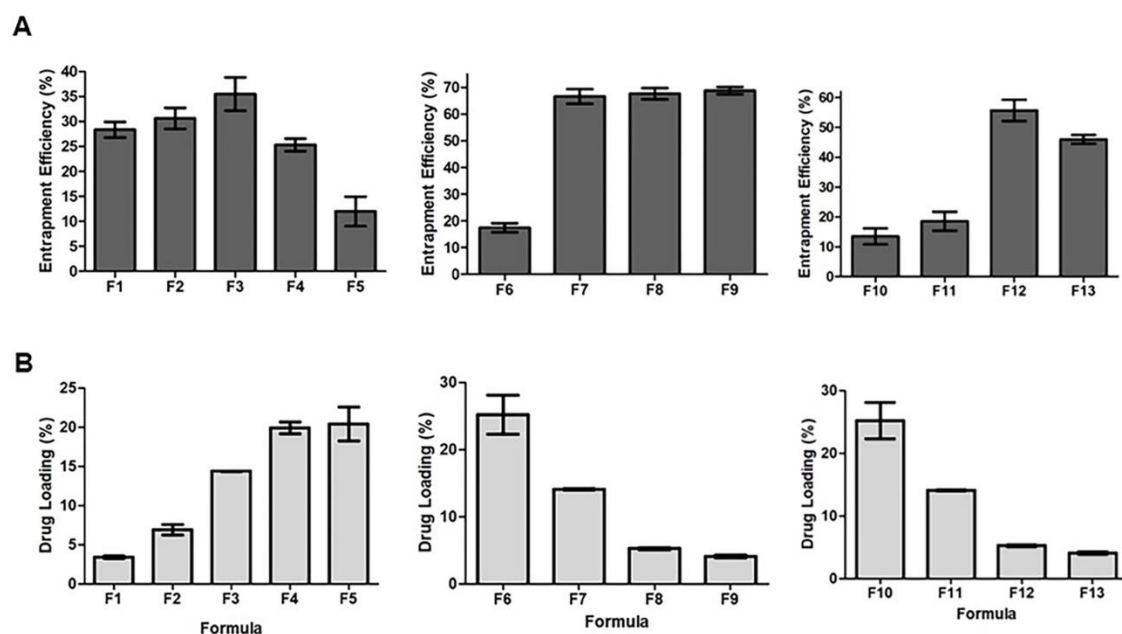


Figure 2. EE (A) and DL of (B) diagram of CPL MP with a variation of CPL, WPI and ratio of CPI: WPI concentration (means $\pm$ SD, n = 3).

3.3 Powder X-ray diffraction (PXRD)

X-ray diffraction (PXRD) is essential to identify the physical state of the drug and the effect of homogenization on the crystalline structure of active substances. XRD patterns of the pure WPI, pure CPL, physical mixture (1.5:1), and CPL MPs have been compared in Figure 3C. The sharp peaks are associated with the crystalline fraction, whereas the amorphous part is represented as broad peaks [41]. It can be observed that firm crystalline peaks are observed in CPL and physical mixture diffraction. There are two sharp dominant peaks at $2\theta = 31.82^\circ$ and $2\theta = 26.1^\circ$ in CAP and $2\theta = 30.64^\circ$ and $2\theta = 25.98^\circ$ in the physical mixture (1.5:1). Two broad peaks were also observed at around 21.5° in CPL-loaded MPs and 21.46° in WPI. The same pattern was observed previously for WPI studies [41]. However, the crystalline peaks almost disappeared in the XRD pattern of CPL MPs and WPI, indicating the disruption of the crystalline form of CPL after encapsulation and entrapment of CPL into WPI-based MPs.

3.4 Differential scanning calorimetry (DSC)

To assess the physicochemical interactions between the formulation chemicals and to ascertain whether crystallinity was preserved during the encapsulation procedure, a thermal analysis of the DSC was carried out. Thermograms of pure CPL, pure WPI, physical mixture and CPL-loaded MPs are presented in Figure 3B. The DSC curve of pure WPI exhibits a broad endothermic peak between 40 and 120 °C, centred at 85°C. The primary whey protein components (β -lactoglobulins and α -lactalbumin) undergo heat-induced transitions, which are what cause this distinctive endotherm. Similar characteristics were also seen in the physical mixture WPI:CPL (1.5:1) and in prior literature [42]. The endothermic point remains sharp at 159°C, representing CPL's melting point and crystallinity (pure CPL and physical mixture). The endothermic point at 159°C is not investigated in the pure WPI and CPL-loaded MPs. However, in CPL MPs, an endothermic peak is observed only in pure WPI, which shows that CPL is distributed molecularly in the polymer matrix and hence properly encapsulated in WPI MPs. Endothermic peaks and melting points were identified following the encapsulation of DIM with protein nanoparticles, providing strong evidence of nanoencapsulation [43].

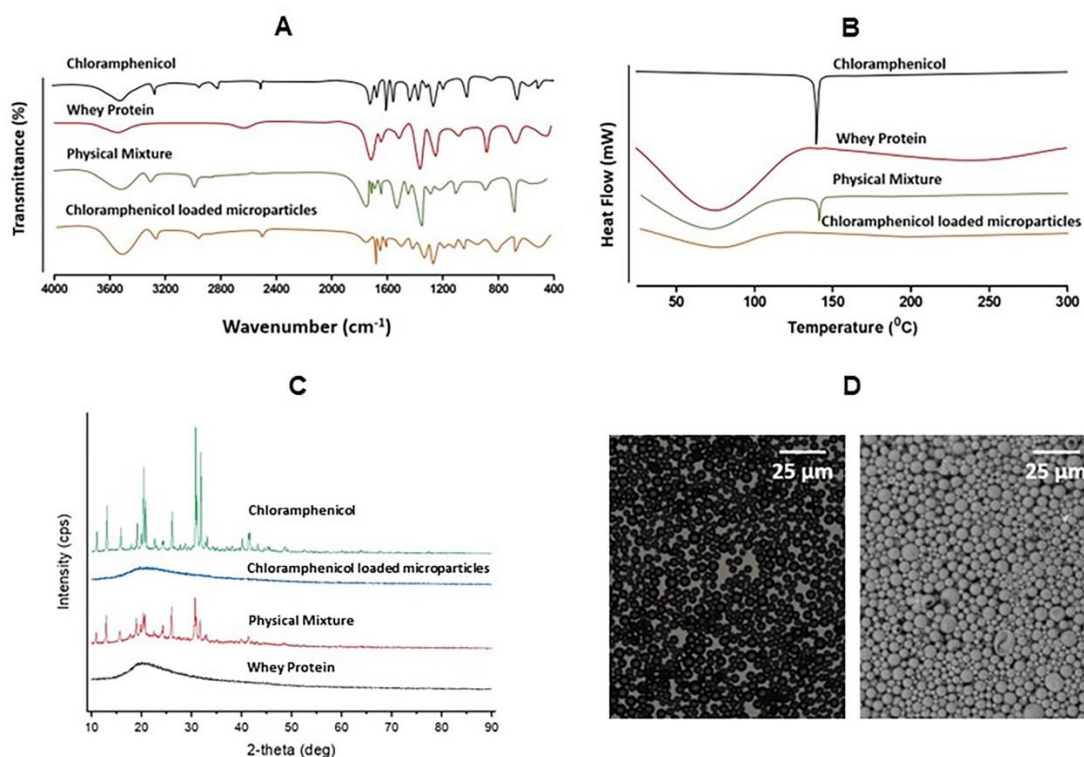


Figure 3. FT-IR Spectra of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (A). DSC thermogram of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (B). X-ray diffractogram of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (C). SEM images of CPL-loaded MPs (D).

3.5 Fourier-transform infrared spectroscopy (FT-IR) analysis

FT-IR analysis was used to determine the functional groups and interactions between pure WPI, pure CPL, physical mixture, and CPL-loaded MPs. The results are presented in Figure 3A. In the infrared spectrum, interesting peaks are observed in 3200–3600 cm^{-1} , indicating the presence of O–H and N–H bonds. The presence of strong peaks appeared on the CPL spectrum at 3493 cm^{-1} , which is associated with O–H stretching vibration; spectrum 3218 cm^{-1} is associated with N–H stretching vibration bond; spectrum 2978 cm^{-1} is associated with aromatic C–H stretching vibration, spectrum 1668 cm^{-1} with C=O stretching vibrations; and spectrum 1561 cm^{-1} is associated with NO₂ stretching peak [44]. The entire spectrum was detected in CPL encapsulated into MPs, indicating that encapsulation had been successfully performed on the system. Amides I, II, and III are regarded as typical WPI spectra, indicating the presence of WPI's primary components, β -lactoglobulin and α -lactalbumin. The amide I band mainly confirms the presence of C=O stretch vibration and N–H bending. The middle absorption band II shown correlates with the structural combination group of C–N stretch with N–H deformation, while the amide III bands are associated with C–O bending vibration [45]. Amide I in the spectrum of 1745 cm^{-1} , amide II at 1451 cm^{-1} and amide III at 1241 cm^{-1} are present in CPL-loaded MPs and physical mixture. The binding of CPL to β -lactoglobulin was demonstrated by comparing the WPI spectrum with the encapsulated CPL. Wang et al. reported similar outcomes [46]. The spectrum of CPL-loaded MPs shows most of the characteristic peaks identical to the spectrum of WPI and CPL, which indicates the encapsulation procedure was successfully carried out. The change in intensity indicates the hydrophobic interaction of CPL and lactoglobulin complexes. These findings align with DIM-based polymerized whey protein nanoparticles [33].

3.6 SEM analysis

Morphology of CPL-loaded microparticle formulation was observed using SEM. As shown in Figure 3D, the CPL MPs show the spherical shape of the particles. The size of CPL MPs found in this experiment was similar to that observed in the DLS analysis ($\sim 25\ \mu\text{m}$). Particle size is closely related to drug penetration into target tissues; Figure 3D shows that CAP MPs meet the particle size criteria to penetrate the stratum corneum lipid layer through the lipidic intercellular route or aqueous pores, which can penetrate to a depth of $> 2\ \text{mm}$ efficiently into hair follicles. Another study also used the solvent evaporation method and prepared MPs, resulting in particle sizes of 6–25 μm [47]. Similar

studies have also been described by previous studies [48], in which nanoparticle formulation of frankincense essential oil-loaded whey with a particle size of 321 ± 22.42 nm showed a good healing effect for skin wounds. This capability can be supported if the particle size is within the range of 10–100 nm based on confirmation by studies on human, porcine or murine skin [49]. Moreover, the permeability of MPs into the skin is influenced by the shape of the particles. A spherical shape has an excellent ability to penetrate the skin because it is symmetrical in all three dimensions; thus, it can be located deep in the epidermis and dermis layers.

3.7 *In vitro* microparticle drug release of CPL MPs

The drug release behaviour of CPL compared to CPL MP formulation is presented in Figure 4A. To mimic the physiological condition of the skin, *in vitro* drug release was prepared using simulated wound fluid. In this study, encapsulation of the drug into microparticle form improved the drug release profile. The experiment showed that $99.40 \pm 7.01\%$ CPL is released from the microparticle formulation after 24 hours. Meanwhile, the amount of drug release from pure CPL is around $95.43 \pm 2.41\%$ after 6 h. The CPL MP formulations showed sustained release patterns up to 24 h. Statistically, the drug release of pure CPL at 6 h was higher ($p < 0.05$) than that of CPL-loaded MPs. This behaviour is related to an enlarged CPL surface area, leading to an increased drug release, with a controlled release profile [50]. For simulated wound fluid, the estimated drug release during the first hour was 4.76%. After that, approximately 10.31%, 24.81%, and 42.04% of CPL were released from MPs within 2 h, 4 h, and 6 h, respectively. Following that, a quick release lasting up to 24 hours was observed; weakly bound CPL that was released and attached to the surface of the microparticle may have caused the drug release. The solubility of WPI in simulated wound fluid can be attributed to the release profile of WPL from MPs, which predicts prolonged release by causing erosion of the microparticle matrix.

3.8 *Mathematical modeling of the kinetics of drug release*

The experimental drug release was employed in multiple kinetic models to further understand the process of CPL release from MPs. The most acceptable mathematical model is chosen based on the correlation coefficient value. Based on Table 2, It was concluded that the best results of CPL MPs were observed with R^2 value of 0.91877. According to the results, the release behavior of the CPL-loaded MPs formulation followed the Hixson–Crowell kinetic model. The drug release from these

spherical sustained-release dosage forms depends on matrix drug diffusion and polymeric matrix erosion during polymer degradation [51].

Table 2. Kinetic modeling of pure-CPL and CPL-loaded MPs (means $\pm$ SD, n = 3).

Formulation	R value of Kinetic model				
	Zero-order	First-order	Higuchi	Krosmeier-Peppas	Hixson-Crowell
Pure CPL	0,9814	0,7495	0,7623	0,9658	0,91877
CPL MPs	0,8481	0,6279	0,8677	0,2097	0,9642

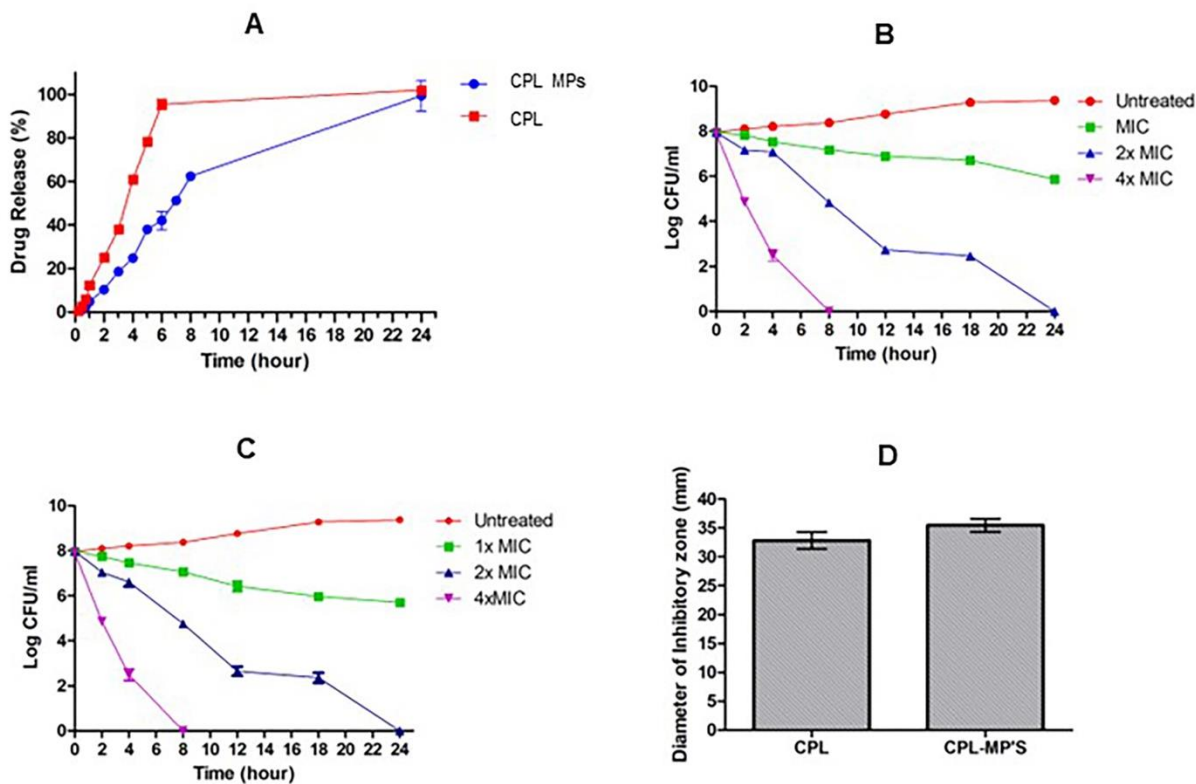


Figure 4. In vitro release profiles of CPL-loaded MPs in comparison with the pure CPL (A). Time-kill assay of CPL-loaded MPs (B) and pure CPL (C) against *Staphylococcus aureus* (means $\pm$ SD, n = 3). Diameter of inhibitory zone of CPL-loaded MPs against *Staphylococcus aureus* (means $\pm$ SD, n = 3) (D).

3.9 Determination of MIC and MBC

This work compared the bactericidal properties of pure CPL and CPL MPs. The MIC and MBC values of CPL MPs and pure CPL are 12.5 μ g/mL and 25 μ g/mL, respectively. The result shows that the MIC value at the lowest concentration indicates strong antibacterial activity. Next, the MBC value is determined. The MBC value was greater than the MIC value, indicating that a larger concentration of CPL was required to kill bacterial cultures. Moreover, MIC values of samples were

lower than MBC values. The ratio of MBC to MIC is < 4 . A ratio of 4 indicates bactericidal activity, and a ratio > 4 shows bacteriostatic activity [52]. Accordingly, a higher concentration of CPL MPs was required to kill the SA. Our study also suggests that CPL should be in solution form or MPs formulation to exhibit bactericidal activity.

The results showed that the inhibition zones of pure CPL and CPL MPs were 35.45 ± 1.14 mm and 32.82 ± 1.48 mm, respectively (Figure 5C). This might be because the substance disperses in the media, improving its capacity to penetrate the SA cell membrane. Previous research has shown that CPL prevents bacterial ribosomes from producing peptidyl transferase. The 50S ribosomal subunit's 23S rRNA contains residues A2451 and A2452, which this activity preferentially binds to [53]. CPL's ability to successfully permeate SA cell membranes may have contributed to its strong antibacterial action against SA when used in solution form. Notably, our control study using DMSO alone did not show antibacterial activity. Moreover, the formulation in the form of MPs showed higher antibacterial activity compared with CPL solutions. This may be due to the increased water solubility of CPL once in the MPs, enhancing CAP's capacity to efficiently permeate SA cell membranes.

3.10 Time–kill assay

The time–kill assay was performed to examine the time required for the CPL to completely kill the SA. Time–kill curves of free CPL and CPL MPs in DMSO versus SA are depicted in Figure 4B–C. The viable SA colony-forming unit increased by about 9.37 ± 0.05 log CFU/ml after 24 hours of cultivation in the control (untreated) group. With MIC values, CPL solutions and CPL MPs were unable to kill 99.99% of SA cultures after 24 hours. When 2x MIC values were observed, no feasible SA cultures were found after 24 hours in the case of either CPL solutions or CAP MPs. Furthermore, the time needed to eliminate 100% of CA cultures was decreased to 8 hours after incubation with 4x MIC values of both CPL and CPL MPs solutions. The results achieved in this work indicate that the killing rate of CPL in the microparticle-based formulation is based on how much CPL is present in the bacterial culture medium.

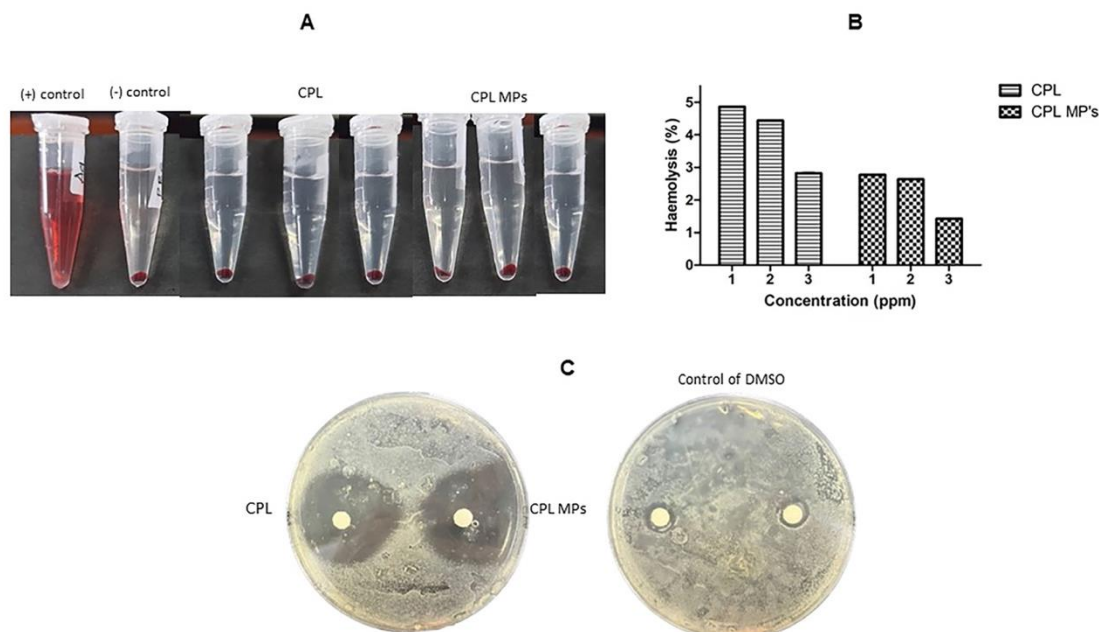


Figure 5. Hemolysis result of CPL-loaded MPs and pure CPL with concentrations of 500, 50 and 5 µg/mL (Aquadest and PBS as positive and negative controls, respectively) (A). Hemolysis percentage of CPL-loaded MPs and pure CPL (B). Inhibitory zone of CPL-loaded MPs against *Staphylococcus aureus* compared with control (means $\pm$ SD, n = 3) (C).

3.11 Hemolysis test

The cytotoxicity of chemicals on RBCs can be quickly assessed using the hemolysis test. These RBCs will burst open entirely in the presence of water, releasing their hemoglobin content. In a similar vein, changes in osmotic pressure on the surface of RBCs caused by exposure to external elements can cause significant lysis. When RBCs are damaged or lysed, they emit adenosine diphosphate, which stimulates platelet assembly on the foreign body surface, speeds up the coagulation and thrombotic cascade pathways, and disrupts the body's natural ability to heal the wound [54]. Therefore, a good wound dressing membrane should not compromise the coagulation pathway or the integrity of the circulating RBC. By spectrophotometrically lysing RBC at 540 nm, the hemolytic potential of CPL MPs was calculated. As shown in Figures. 5A–B, different concentrations of MP (5–500 µg/mL) showed hemolysis percentages in the range of 1.42–2.76%, whereas pure CPL showed a higher hemolysis percentage of 2.82–4.86%. According to the standard, materials exhibiting hemolysis greater than 5% are thought to be hemolytic, those between 2 to 5% are thought to be slightly hemolytic, and those less than 2% are thought to be non-hemolytic [48]. From

the results above, it can be concluded that the fabrication in the form of CPL-loaded MPs is compatible with RBCs because it consists of natural polymer.

3.12 Characteristics of hydrogel

3.12.1 pH measurement

Because the application of CPL MPs hydrogel should not irritate the skin, the pH of the formulation is very important. The pH of the hydrogel surface for each formula is in the range of 5.45 ± 0.04 to 5.55 ± 0.01 , as shown in Figure 6B, which is very close to normal skin pH of 5 to 6 [55], indicating that the formulation will be tolerated by the skin and will not interfere with the wound healing process.

3.12.2 Viscosity and rheological evaluation

Viscosity evaluation in this study used a Brookfield viscometer with spindle 7. The viscosity of CPL MP hydrogel preparation can be seen in Figure 6G, which shows that the viscosity of preparations obtained in this study for F1 to F5, respectively, were 2266.66 ± 230.94 cPs, 3200 ± 173.20 cPs, 6900 ± 173.21 cPs, 9433.33 ± 152.75 cPs, and 23066.66 ± 832.67 cPs. Then, the viscosity test results from statistical analysis data showed that F1, F2 and F3 with respective chitosan concentrations of 1% (w/w), 1.25% (w/w) and 1.5% (w/w) have no significantly different values ($p > 0.05$). Meanwhile, the F4–F5 formulation has a significantly different value ($p < 0.05$) from F1. The good viscosity range in gels is in the field of 2000–4000 cPs, and other studies state that the viscosity of gels can reach up to 16000 cPs; therefore, F1, F2 and F3 are within the required viscosity range [56,57]. More importantly, chitosan has an intrinsic viscosity of 1.2×10^5 to 3.2×10^5 Da with molecular weight (MW) in the range of 3.2 to 6.52 dL/g [58], in which the amount of chitosan concentration in the formulation can increase the value of hydrogel viscosity. Based on the results of rheological measurements (Figure 5G), all sample flow behaviors exhibited non-Newtonian or pseudoplastic behavior ($n < 1$). Pseudoplastic flow is a viscosity flow that continues to decrease as the rate of shear increases. Thus, it can be estimated that an Increase in spreadability will occur at higher shear speeds or after dilution with liquids [56].

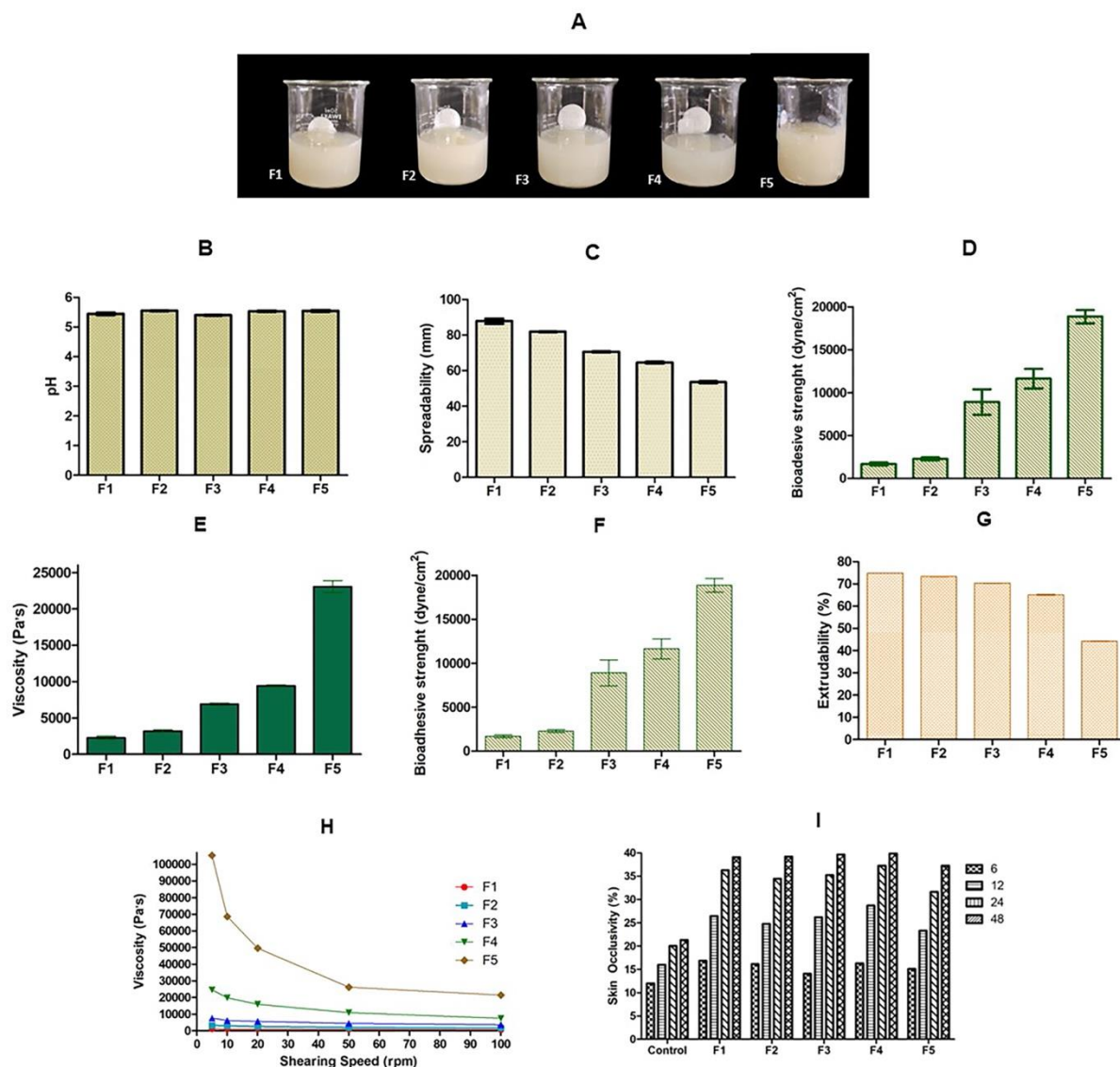


Figure 6. Formulation of CPL MPs Hydrogel with variation concentration of chitosan (F1: 1%; F2: 1,25%; F3: 1,5%; F4: 1,75%; F5: 2%) (A). pH (B), Spreadability (C), Extrudability (D) and Viscosity (E), Bioadhesive strength (F), Extrudability (G), Rheology (H) and Skin occlusivity (I) of CPL MP hydrogel (means $\pm$ SD, n = 3).

3.12.3 Spreadability

Poor spreadability can cause inhomogeneous distribution of particles, which can in turn affect the amount of dose applied as well as the efficiency of the active ingredients penetrating the skin [59]. The spreadability of hydrogel preparation was determined by the hydrogel's ability to spread on the skin's surface. Good spreadability can ensure optimal distribution of hydrogel when applied to the skin. The spread test results of CPL MPs Hydrogel preparations reveal a value between $53.52 \pm$

0.50 mm to 87.84 ± 1.45 mm (Figure 6C), which indicates that CPL MP hydrogel has good spreadability. The results of the ANOVA analysis show that the spreadability of CPL MP hydrogel is not significant between all formulations ($p > 0.05$), with the exception of F4 and F5 to F1. Spreadability is inversely proportional to viscosity [60]. Good spreadability of the preparation is in the range of 50–70 mm; therefore F3, F4 and F5 are qualifying criteria, and F3 is the most optimal formulation. The results of the measurement in Figure 6C show that the greater the concentration of chitosan, the smaller the spreadability of the hydrogel.

3.12.4 Skin occlusivity test

The capacity of the formulation to preserve skin moisture properties was predicted using an *in vitro* model of skin occlusivity. Over 48 hours, the percentage of occlusion factors (Figure 6H) was evaluated for each formulation (F1–F5), and a filter paper without formulation was utilized as a reference. The occlusion factor values of all hydrogel formulas showed a significant difference ($p < 0.05$) compared to the control, and chitosan represented a good occlusion factor. This indicates that incorporating CPL MPs into the hydrogel can increase the occlusive value. Due to the lowering of the corneocyte slits, higher occlusion may improve skin moisture for 48 hours, allowing drugs to penetrate to deeper layers of the skin [25]. According to statistical analysis, there was no difference between any CPL MP hydrogel formulation's occlusive value ($p > 0.05$).

3.12.5 Extrudability percentage

Hydrogel extrusion from the tube is essential during application. A sufficient consistency is necessary to remove the gel from the tube because hydrogels with high consistency may not come out of the tube, while gels with low viscosity may flow too freely. The extrudability of all hydrogel formulations (F1–F5) is shown in Figure 6G, with values of $74.9 \pm 0.01\%$, $73.37 \pm 0.05\%$, $70.3 \pm 0.06\%$, $65.13 \pm 0.22\%$ and $44.17 \pm 0.11\%$, respectively. More than 70% of the contents of the gel formulations F1–F3 can be extruded, demonstrating their strong extrusion capacity, with the exception of F4 and F5, where only 60% of the contents could be extruded. Based on statistical analysis, F1 has no significant values to F2 and F3. Furthermore, F4 and F5 are significant to F1, F2 and F3. This indicates that the increased chitosan concentration affects the increasing viscosity, thereby reducing the ease of extrusion from the tube.

3.12.6 Bioadhesive test

Good bioadhesive properties can ensure a long retention time for the applied drug formulation to guarantee effective local drug concentrations and expected pharmacological responses. Bioadhesive measurements are found in Figure 6F, which shows that the higher the concentration of chitosan used, the higher the bioadhesive strength of the preparation with F1 (1682.36 ± 171.41 dyne/cm²), F2 (2276.13 ± 171.41 dyne/cm²), F3 (8906.59 ± 1484.43 dyne/cm²), F4 (11628.05 ± 1133.75 dyne/cm²), and F5 (18852.29 ± 771.33 dyne/cm²) value. The statistical analysis showed that F1 and F2, as well as F4 and F5, showed no significant value ($p > 0.05$). The increased flexibility of chitosan hydrogel of CPL MPs can improve the contact between the gel and the skin tissue, thereby encouraging the penetration of polymer chains into the tissue to form a strong bond, which leads to an increase in the strength of adhesion [61]. Thus, it also increases the effectiveness of the active substance in reaching the target. Therefore, in this test, the F3 formula was chosen as the optimum formula with a concentration of 1.5% (w/w) that can provide good pH, dispersion, viscosity, skin occlusivity and bioadhesiveness.

Table 3. Dermatokinetic parameters of hydrogel in infected *ex vivo* skin infection models formed by SA following the application of pure-CPL Hydrogel, and CPL MP Hydrogel (means $\pm$ SD, n = 3).

Condition	Formula- tion	C_{max} ($\mu\text{g}/\text{cm}^3$)	T_{max} (h)	$T_{1/2}$ (h)	AUC (h. $\mu\text{g}/\text{cm}^3$)	MRT (h)
Skin Infection model by SA	Pure CP Hydrogel	3.58 ± 0.30	6.67 ± 0.57	35.71 ± 11.99	47.51 ± 3.32	56.22 ± 16.37
	CPL MPs hydrogel	24.44 ± 0.95	6 ± 0.00	496.50 ± 749.87	417.62 ± 4.22	719.07 ± 1082.14

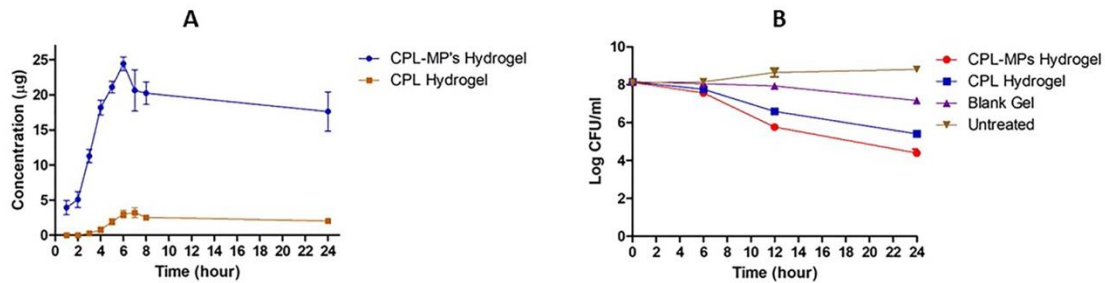


Figure 7. Dermatokinetic tests with CPL hydrogel and CPL MP hydrogel revealed total CPL (A) (means $\pm$ SD, n = 3). Bacterial viability (log CFU/mL) in an *ex vivo* skin infection wound model (*S. aureus*) following the administration of free CPL in the form of a hydrogel, MPs hydrogel and blank hydrogel (means $\pm$ SD, n = 3) (B).

3.13 *Ex vivo* dermatokinetic study

Ex vivo dermatokinetic study was performed on non-infected rat skin models. To investigate the kinetics of the release of CPL MPs formulation, dermatokinetic studies of the hydrogel are carried out after application to the skin. To confirm that the release of CPL occurs in the skin, the skin sample was vortexed with 2 mL of methanol at each time interval. We compared the dermatokinetic profile of this study with a hydrogel containing CPL without formulation in the form of MPs as a control to assess whether the release of drugs affects the state of drug particles. Our findings in Figure 7A show that at 1 h and 2 h after measurements, no CPL was found in the pure-CPL hydrogel supernatant and that free CPL permeated to the receiver compartment more rapidly in contrast to CPL MPs hydrogel after 2h. There was $2.05 \pm 0.22 \mu\text{g}$ of CPL after 24 h, which indicates that the CPL without MPs formulation showed hydrophobic properties, making it difficult to penetrate the skin membrane. On the other hand, measurements at 1 h to 24 h show a CPL found in the CPL MPs hydrogel supernatant with an optimal concentration of $24.43 \pm 0.95 \mu\text{g}$ at 6 h. This is in line with the main objective of this study, i.e., to deliver CPL MPs to the local target of the first layer of skin, where SA colonizes, infecting the skin locally.

The concentration of CPL released from the MPs form in the skin vs. time of administration is depicted as the kinetic profile of hydrogel in normal skin. Table 3 shows the dermatokinetic profile of CPL MP hydrogel and pure-CPL hydrogel after application, including C_{max} , T_{max} , $T_{1/2}$, AUC , and MRT . The results also showed that the CPL content in dermal layers obtained pure-CPL hydrogel was remarkably lower compared with that of CPL MP hydrogel. The C_{max} ($24.44 \pm 0.95 \text{ g/cm}^3$) was reached in 6.00 h, according to the kinetic profile of CPL MPs. In comparison to CPL MP hydrogel, the profile of pure-CPL hydrogel had a lower C_{max} ($3.58 \pm 0.30 \text{ g/cm}^3$ in $6.67 \pm 0.57 \text{ h}$), but the differences were not statistically significant ($p > 0.05$). Additionally, the C_{max} for both the pure-CPL hydrogel and the CPL MP hydrogel in the dermal layers was reached in the same T_{max} . The findings show that, in comparison to pure-CPL hydrogel, CPL MP hydrogel can deliver much higher concentrations of CPL to the skin. The great *ex vivo* skin bioavailability of the microparticle method is shown by the AUC value of CPL MPs hydrogel, which was found to be substantially higher ($p < 0.05$) than that of pure-CPL hydrogel. It was discovered that the MRT value of CPL MPs hydrogel was considerably different from the pure-CPL hydrogel ($p < 0.05$) in terms of retention duration in the skin. A high MRT value can be advantageous for reducing the application time of CPL MPs hydrogels in treating skin infections associated with local infection formation [62]. This reduced

application time may increase patients' acceptance of (and compliance with) this approach. Our results conclude that a responsive CPL MPs hydrogel approach can successfully deliver MPs into *ex vivo* infection models.

3.14 *Ex vivo* antibacterial activity test using infection skin model

To test the efficiency of CPL MPs hydrogel as an antibacterial agent, researchers used an *ex vivo* skin infection model. We sought to determine whether CPL MPs hydrogel could reduce bacterial bioburdens produced from SA by counting cells' log CFU/ml number. This test was carried out on CPL MPs hydrogel, pure-CPL hydrogel, blank hydrogel without CPL, and an untreated sample as a control. The test results are found in Figure 7B. In *ex vivo* skin infections, the highest antibacterial activity was achieved after applying CPL MPs hydrogels compared to pure-CPL hydrogel, as measured by the reduction of bacterial bioburden after 24 hours. A value of 8.22 ± 0.11 log CFU/ml to 8.81 ± 0.18 log CFU/ml increase in bacterial bioburden was seen in untreated samples, which indicates the validity of the control sample. Blank hydrogel without drugs showed a value of 8.16 ± 0.04 log CFU/ml to 7.11 ± 0.07 log CFU/ml decrease in bioburden properties. The antibacterial activity in the blank hydrogel was due to the ability of chitosan as a natural polymer to inhibit microorganisms, as explained in a previous study [20].

Analysis of *ex vivo* antibacterial activity showed an enhancement of 8.02 ± 0.10 log CFU/ml to 4.40 ± 0.20 log CFU/ml after 24 hours when CPL is incorporated into MPs in hydrogel form. This indicates that chitosan hydrogel may enhance the antibacterial activity of CPL MPs. Incorporating antibacterial compounds into chitosan hydrogels has been shown to boost their antibacterial activity in some experiments [20,34,63]. Furthermore, dermatokinetic experiments demonstrated the bioavailability of CPL MPs hydrogel in *ex vivo* skin, which is why this method was successful in eliminating 99.98% SA infections. Therefore, there are two primary benefits associated with the delivery of CPL-loaded MPs in chitosan hydrogel. First, this approach can control the dermatokinetic profile of CPL MPs. Second, this approach may improve the efficacy of CPL MPs hydrogels in *ex vivo* infection models of rat skin formed by SA compared to CPL without MPs hydrogel formulations. The encouraging findings of this study have validated the study's premise, demonstrating that CPL can be efficiently transported to the skin utilizing MPs, and then, once incorporated into the chitosan hydrogel formula, can exhibit its antimicrobial action to target infection. To improve the antibacterial efficacy of wound therapy, our delivery system's key advantage lies in its selective delivery

ability to locally target as well as its long retention period in areas of infection on the skin. The combination of MPs with chitosan hydrogels can selectively be a feasible alternative to existing wound infection care, as MPs are employed for topical administration of antimicrobial drugs to wounds.

4. Conclusion

Wound treatments using dermal delivery system are now receiving significant attention as potential for staving off infection. In this study, hydrogels are made from chitosan polymers in which the MPs of whey protein containing CPL are embedded inside the system. Based on the findings presented in this study, incorporating CPL in the form of MPs provides good physical characteristics and increases the drug release rate with a controlled release model. The advanced formulation in the form of hydrogel exhibits physical properties as well; in particular, it improves CPL penetration in *ex vivo* models of infections in rat skin, as indicated by the dermatokinetic profile of CPL and the reduced bioburden of large colonies of bacteria. Compared to pure-CPL hydrogel, the combination delivery method developed in this study has a higher residence duration in the skin, which may increase the effectiveness of antibacterial treatment in skin wound infections. It appears likely that hydrogels created using this technique can be utilized to heal wounds successfully. To further examine the efficacy of this technique, *in vivo* investigations on suitable animal models should be conducted.

Acknowledgement

Tri Puspita Roska expresses gratitude to Deutscher Akademischer Austausch Dienst (DAAD) for providing the scholarship. The Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia also provided funding for this study under contract number 090/E5/PG.02.00/PT/2022. The authors thank Glanbia Nutritionals, Inc, Fitchburg, USA for providing whey protein (WPI).

Conflict of interest

The authors state that they have no conflicts of interest.

References

- [1] J.F. Guest, N. Ayoub, T. McIlwraith, I. Uchegbu, A. Gerrish, D. Weidlich, K. Vowden, P. Vowden, Health economic burden that different wound types impose on the UK's National Health Service, *Int Wound J.* 14 (2017) 322–330. <https://doi.org/10.1111/iwj.12603>.
- [2] F. Gottrup, E. Peters, S. Probst, EWMA Document: Antimicrobials and Non-healing Wounds Evidence, controversies and suggestions, 2013. www.Ewma.org.
- [3] A. Brown, Acute wounds, 2018. www.nursingtimes.net.
- [4] J.E. Cefalu, K.M. Barrier, A.H. Davis, Wound Infections in Critical Care, *Crit Care Nurs Clin North Am.* 29 (2017) 81–96. <https://doi.org/10.1016/j.cnc.2016.09.009>.
- [5] J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G.M. Eccleston, Wound healing dressings and drug delivery systems: A review, *J Pharm Sci.* 97 (2008) 2892–2923. <https://doi.org/10.1002/jps.21210>.
- [6] S.G. Ingebrigtsen, N. Škalko-Basnet, A.M. Holsæter, Development and optimization of a new processing approach for manufacturing topical liposomes-in-hydrogel drug formulations by dual asymmetric centrifugation, *Drug Dev Ind Pharm.* 42 (2016) 1375–1383. <https://doi.org/10.3109/03639045.2015.1135940>.
- [7] D.A. Kuznetsova, D.R. Gabdrakhmanov, G.A. Gaynanova, L.A. Vasileva, D.M. Kuznetsov, S.S. Lukashenko, A.D. Voloshina, A.S. Sapunova, I.R. Nizameev, G. v. Sibgatullina, D. v. Samigullin, M.K. Kadirov, K.A. Petrov, L.Y. Zakharova, Novel biocompatible liposomal formulations for encapsulation of hydrophilic drugs – Chloramphenicol and cisplatin, *Colloids Surf A Physicochem Eng Asp.* 610 (2021). <https://doi.org/10.1016/j.colsurfa.2020.125673>.
- [8] S.I. Miller, Antibiotic resistance and regulation of the Gram-negative bacterial outer membrane barrier by host innate immune molecules, *MBio.* 7 (2016). <https://doi.org/10.1128/mBio.01541-16>.
- [9] A.D. Permana, Q.K. Anjani, Sartini, E. Utomo, F. Volpe-Zanutto, A.J. Paredes, Y.M. Evary, S.A. Mardikasari, Muh.R. Pratama, I.N. Tuany, R.F. Donnelly, Selective delivery of silver nanoparticles for improved treatment of biofilm skin infection using bacteria-responsive microparticles loaded into dissolving microneedles, *Materials Science and Engineering: C.* 120 (2021) 111786. <https://doi.org/10.1016/j.msec.2020.111786>.
- [10] S. Yasasvini, R.S. Anusa, B.N. VedhaHari, P.C. Prabhu, D. RamyaDevi, Topical hydrogel matrix loaded with Simvastatin microparticles for enhanced wound healing activity, *Materials Science and Engineering C.* 72 (2017) 160–167. <https://doi.org/10.1016/j.msec.2016.11.038>.

- [11] X. Delabranche, A. Berger, J. Boisramé-Helms, F. Meziani, Microparticles and infectious diseases, *Med Mal Infect.* 42 (2012) 335–343. <https://doi.org/10.1016/j.medmal.2012.05.011>.
- [12] M. Lengyel, N. Kállai-Szabó, V. Antal, A.J. Laki, I. Antal, Microparticles, microspheres, and microcapsules for advanced drug delivery, *Sci Pharm.* 87 (2019). <https://doi.org/10.3390/sci-pharm87030020>.
- [13] A. George, P.A. Shah, P.S. Shrivastav, Natural biodegradable polymers based nano-formulations for drug delivery: A review, *Int J Pharm.* 561 (2019) 244–264. <https://doi.org/10.1016/j.ijpharm.2019.03.011>.
- [14] A.R. Madureira, C.I. Pereira, A.M.P. Gomes, M.E. Pintado, F. Xavier Malcata, Bovine whey proteins – Overview on their main biological properties, *Food Research International.* 40 (2007) 1197–1211. <https://doi.org/10.1016/j.foodres.2007.07.005>.
- [15] M.A. Farooq, M. Aquib, S. Ghayas, R. Bushra, D. Haleem Khan, A. Parveen, B. Wang, Whey protein: A functional and promising material for drug delivery systems recent developments and future prospects, *Polym Adv Technol.* 30 (2019) 2183–2191. <https://doi.org/10.1002/pat.4676>.
- [16] Z. Liu, C. Liu, X. Sun, S. Zhang, Y. Yuan, D. Wang, Y. Xu, Fabrication and characterization of cold-gelation whey protein-chitosan complex hydrogels for the controlled release of curcumin, *Food Hydrocoll.* 103 (2020). <https://doi.org/10.1016/j.foodhyd.2019.105619>.
- [17] A. Aliyah, W.W. Oktaviana, K.S. Dwipayanti, A.P. Erdiana, R.N. Utami, A.D. Permana, Enhanced skin localization of doxycycline using microparticles and hydrogel: Effect of oleic acid as penetration enhancer, *Pharmaciana.* 11 (2021) 239. <https://doi.org/10.12928/pharmaciana.v11i2.21044>.
- [18] C. Xiao, R. You, Y. Fan, Y. Zhang, Tunable functional hydrogels formed from a versatile water-soluble chitosan, *Int J Biol Macromol.* 85 (2016) 386–390. <https://doi.org/10.1016/j.ijbiomac.2016.01.006>.
- [19] K. Mohan, A.R. Ganesan, T. Muralisankar, R. Jayakumar, P. Sathishkumar, V. Uthayakumar, R. Chandirasekar, N. Revathi, Recent insights into the extraction, characterization, and bioactivities of chitin and chitosan from insects, *Trends Food Sci Technol.* 105 (2020) 17–42. <https://doi.org/10.1016/j.tifs.2020.08.016>.

- [20] M.E. Abd El-Hack, M.T. El-Saadony, M.E. Shafi, N.M. Zabermawi, M. Arif, G.E. Batiha, A.F. Khafaga, Y.M. Abd El-Hakim, A.A. Al-Sagheer, Antimicrobial and antioxidant properties of chitosan and its derivatives and their applications: A review, *Int J Biol Macromol.* 164 (2020) 2726–2744. <https://doi.org/10.1016/j.ijbiomac.2020.08.153>.
- [21] A. Jain, G. Sharma, G. Ghoshal, P. Kesharwani, B. Singh, U.S. Shivhare, O.P. Katare, Lycopene loaded whey protein isolate nanoparticles: An innovative endeavor for enhanced bioavailability of lycopene and anti-cancer activity, *Int J Pharm.* 546 (2018) 97–105. <https://doi.org/10.1016/j.ijpharm.2018.04.061>.
- [22] A.D. Permana, A.J. Paredes, F. Volpe-Zanutto, Q.K. Anjani, E. Utomo, R.F. Donnelly, Dissolving microneedle-mediated dermal delivery of itraconazole nanocrystals for improved treatment of cutaneous candidiasis, *European Journal of Pharmaceutics and Biopharmaceutics.* 154 (2020) 50–61. <https://doi.org/10.1016/j.ejpb.2020.06.025>.
- [23] A.D. Permana, M. Mir, E. Utomo, R.F. Donnelly, Bacterially sensitive nanoparticle-based dissolving microneedles of doxycycline for enhanced treatment of bacterial biofilm skin infection: A proof of concept study, *Int J Pharm X.* 2 (2020) 100047. <https://doi.org/10.1016/j.ijpx.2020.100047>.
- [24] M. Mir, A.D. Permana, I.A. Tekko, H.O. McCarthy, N. Ahmed, Asim. ur. Rehman, R.F. Donnelly, Microneedle liquid injection system assisted delivery of infection responsive nanoparticles: A promising approach for enhanced site-specific delivery of carvacrol against polymicrobial biofilms-infected wounds, *Int J Pharm.* 587 (2020) 119643. <https://doi.org/10.1016/j.ijpharm.2020.119643>.
- [25] A.D. Permana, R.N. Utami, A.J. Courtenay, M.A. Manggau, R.F. Donnelly, L. Rahman, Phytosomal nanocarriers as platforms for improved delivery of natural antioxidant and photoprotective compounds in propolis: An approach for enhanced both dissolution behaviour in bio-relevant media and skin retention profiles, *J Photochem Photobiol B.* 205 (2020) 111846. <https://doi.org/10.1016/j.jphotobiol.2020.111846>.
- [26] U.C. Galgatte, A.B. Kumbhar, P.D. Chaudhari, Development of *in situ* gel for nasal delivery: design, optimization, *in vitro* and *in vivo* evaluation, *Drug Deliv.* 21 (2014) 62–73. <https://doi.org/10.3109/10717544.2013.849778>.

- [27] A.D. Permana, I.A. Tekko, M.T.C. McCrudden, Q.K. Anjani, D. Ramadon, H.O. McCarthy, R.F. Donnelly, Solid lipid nanoparticle-based dissolving microneedles: A promising intradermal lymph targeting drug delivery system with potential for enhanced treatment of lymphatic filariasis, *Journal of Controlled Release*. 316 (2019) 34–52. <https://doi.org/10.1016/j.jconrel.2019.10.004>.
- [28] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G.D.-O.C. Mateo, R. Fernández-Lafuente, J.M. Guisán, Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions, *Enzyme Microb Technol*. 39 (2006) 877–882. <https://doi.org/10.1016/j.enzmictec.2006.01.014>.
- [29] C. le Bourvellec, C.M.G.C. Renard, Interactions between Polyphenols and Macromolecules: Quantification Methods and Mechanisms, *Crit Rev Food Sci Nutr*. 52 (2012) 213–248. <https://doi.org/10.1080/10408398.2010.499808>.
- [30] C. Wang, X. Zhou, H. Wang, X. Sun, M. Guo, Interactions between β -Lactoglobulin and 3,3'-Diindolylmethane in Model System, *Molecules*. 24 (2019) 2151. <https://doi.org/10.3390/molecules24112151>.
- [31] A. Abbasi, Z. Emam-Djomeh, M.A.E. Mousavi, D. Davoodi, Stability of vitamin D3 encapsulated in nanoparticles of whey protein isolate, *Food Chem*. 143 (2014) 379–383. <https://doi.org/10.1016/j.foodchem.2013.08.018>.
- [32] Q. Liu, Y. Sun, J. Cheng, M. Guo, Development of whey protein nanoparticles as carriers to deliver soy isoflavones, *LWT*. 155 (2022) 112953. <https://doi.org/10.1016/j.lwt.2021.112953>.
- [33] A. Khan, C. Wang, X. Sun, A. Killpartrick, M. Guo, Preparation and Characterization of Whey Protein Isolate–DIM Nanoparticles, *Int J Mol Sci*. 20 (2019) 3917. <https://doi.org/10.3390/ijms20163917>.
- [34] W. Zhang, J. Chen, Y. Chen, W. Xia, Y.L. Xiong, H. Wang, Enhanced physicochemical properties of chitosan/whey protein isolate composite film by sodium laurate-modified TiO₂ nanoparticles, *Carbohydr Polym*. 138 (2016) 59–65. <https://doi.org/10.1016/j.carbpol.2015.11.031>.
- [35] B. Vardhanabhuti, M.A. Kelly, P.J. Luck, M.A. Drake, E.A. Foegeding, Roles of charge interactions on astringency of whey proteins at low pH, *J Dairy Sci*. 93 (2010) 1890–1899. <https://doi.org/10.3168/jds.2009-2780>.

- [36] I. Evdokimov, L. Alieva, V. Varlamov, V. Kurchenko, V.- Haritonov, T. Butkevich, USAGE OF CHITOSAN IN DAIRY PRODUCTS PRODUCTION, *Foods and Raw Materials*. 3 (2015) 29–39. <https://doi.org/10.12737/13117>.
- [37] M. Li, Y. Ma, M.O. Ngadi, Binding of curcumin to β -lactoglobulin and its effect on antioxidant characteristics of curcumin, *Food Chem.* 141 (2013) 1504–1511. <https://doi.org/10.1016/j.foodchem.2013.02.099>.
- [38] Y. Liu, L. Gao, J. Yi, Y. Fan, X. Wu, Y. Zhang, α -Lactalbumin and chitosan core-shell nanoparticles: resveratrol loading, protection, and antioxidant activity, *Food Funct.* 11 (2020) 1525–1536. <https://doi.org/10.1039/C9FO01998G>.
- [39] A. Jain, G. Sharma, G. Ghoshal, P. Kesharwani, B. Singh, U.S. Shivhare, O.P. Katare, Lycopene loaded whey protein isolate nanoparticles: An innovative endeavor for enhanced bioavailability of lycopene and anti-cancer activity, *Int J Pharm.* 546 (2018) 97–105. <https://doi.org/10.1016/j.ijpharm.2018.04.061>.
- [40] Y. Zhang, M. Zhao, Z. Ning, S. Yu, N. Tang, F. Zhou, Development of a Sono-Assembled, Bifunctional Soy Peptide Nanoparticle for Cellular Delivery of Hydrophobic Active Cargoes, *J Agric Food Chem.* 66 (2018) 4208–4218. <https://doi.org/10.1021/acs.jafc.7b05889>.
- [41] R.V. de B. Fernandes, S.V. Borges, D.A. Botrel, C.R. de Oliveira, Physical and chemical properties of encapsulated rosemary essential oil by spray drying using whey protein-inulin blends as carriers, *Int J Food Sci Technol.* 49 (2014) 1522–1529. <https://doi.org/10.1111/ijfs.12449>.
- [42] X. Shen, C. Zhao, J. Lu, M. Guo, Physicochemical Properties of Whey-Protein-Stabilized Astaxanthin Nanodispersion and Its Transport via a Caco-2 Monolayer, *J Agric Food Chem.* 66 (2018) 1472–1478. <https://doi.org/10.1021/acs.jafc.7b05284>.
- [43] Y. Luo, Z. Teng, Q. Wang, Development of Zein Nanoparticles Coated with Carboxymethyl Chitosan for Encapsulation and Controlled Release of Vitamin D3, *J Agric Food Chem.* 60 (2012) 836–843. <https://doi.org/10.1021/jf204194z>.
- [44] Z. Teng, Y. Luo, Q. Wang, Carboxymethyl chitosan–soy protein complex nanoparticles for the encapsulation and controlled release of vitamin D3, *Food Chem.* 141 (2013) 524–532. <https://doi.org/10.1016/j.foodchem.2013.03.043>.
- [45] A. Goyal, V. Sharma, M.K. Sihag, S.K. Tomar, S. Arora, L. Sabikhi, A.K. Singh, Development and physico-chemical characterization of microencapsulated flaxseed oil powder: A

- functional ingredient for omega-3 fortification, *Powder Technol.* 286 (2015) 527–537. <https://doi.org/10.1016/j.powtec.2015.08.050>.
- [46] C. Wang, X. Zhou, H. Wang, X. Sun, M. Guo, Interactions between β -Lactoglobulin and 3,3'-Diindolylmethane in Model System, *Molecules*. 24 (2019) 2151. <https://doi.org/10.3390/molecules24112151>.
- [47] C.A. Busatto, M.E. Taverna, M.R. Lescano, C. Zalazar, D.A. Estenoz, Preparation and Characterization of Lignin Microparticles-in-Alginate Beads for Atrazine Controlled Release, *J Polym Environ.* 27 (2019) 2831–2841. <https://doi.org/10.1007/s10924-019-01564-2>.
- [48] M.M. Agwa, S. Sabra, N.A. Atwa, H.A. Dahdooh, R.M. Lithy, H. Elmotasem, Potential of frankincense essential oil-loaded whey protein nanoparticles embedded in frankincense resin as a wound healing film based on green technology, *J Drug Deliv Sci Technol.* 71 (2022) 103291. <https://doi.org/10.1016/j.jddst.2022.103291>.
- [49] M. Schneider, F. Stracke, S. Hansen, U.F. Schaefer, Nanoparticles and their interactions with the dermal barrier, *Dermatoendocrinol.* 1 (2009) 197–206. <https://doi.org/10.4161/derm.1.4.9501>.
- [50] P.-E. Lee, W.-S. Choo, Characterization of flaxseed oil emulsions, *J Food Sci Technol.* 52 (2015) 4378–4386. <https://doi.org/10.1007/s13197-014-1495-3>.
- [51] N. Kamaly, B. Yameen, J. Wu, O.C. Farokhzad, Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release, *Chem Rev.* 116 (2016) 2602–2663. <https://doi.org/10.1021/acs.chemrev.5b00346>.
- [52] M. Tato, Y. López, M.I. Morosini, A. Moreno-Bofarull, F. Garcia-Alonso, D. Gargallo-Viola, J. Vila, R. Cantón, Characterization of variables that may influence ozenoxacin in susceptibility testing, including MIC and MBC values, *Diagn Microbiol Infect Dis.* 78 (2014) 263–267. <https://doi.org/10.1016/j.diagmicrobio.2013.11.010>.
- [53] M. Abdollahi, S. Mostafalou, Chloramphenicol, in: *Encyclopedia of Toxicology*, Elsevier, 2014: pp. 837–840. <https://doi.org/10.1016/B978-0-12-386454-3.00709-0>.
- [54] W. Yuan, Y. Feng, H. Wang, D. Yang, B. An, W. Zhang, M. Khan, J. Guo, Hemocompatible surface of electrospun nanofibrous scaffolds by ATRP modification, *Materials Science and Engineering: C.* 33 (2013) 3644–3651. <https://doi.org/10.1016/j.msec.2013.04.048>.

- [55] Ó.L. Ramos, J.C. Fernandes, S.I. Silva, M.E. Pintado, F.X. Malcata, Edible Films and Coatings from Whey Proteins: A Review on Formulation, and on Mechanical and Bioactive Properties, *Crit Rev Food Sci Nutr.* 52 (2012) 533–552. <https://doi.org/10.1080/10408398.2010.500528>.
- [56] M.M. Ibrahim, S.A. Hafez, M.M. Mahdy, Organogels, hydrogels and bigels as transdermal delivery systems for diltiazem hydrochloride, *Asian J Pharm Sci.* 8 (2013) 48–57. <https://doi.org/10.1016/j.ajps.2013.07.006>.
- [57] N. Harish, P. Prabhu, R. Charyulu, M. Gulzar, E.V.S. Subrahmanyam, Formulation and evaluation of *in situ* gels containing clotrimazole for oral candidiasis, *Indian J Pharm Sci.* 71 (2009) 421. <https://doi.org/10.4103/0250-474X.57291>.
- [58] D.P. Chattopadhyay, M.S. Inamdar, Aqueous Behaviour of Chitosan, *Int J Polym Sci.* 2010 (2010) 1–7. <https://doi.org/10.1155/2010/939536>.
- [59] L. Montenegro, L. Rapisarda, C. Ministeri, G. Puglisi, Effects of Lipids and Emulsifiers on the Physicochemical and Sensory Properties of Cosmetic Emulsions Containing Vitamin E, *Cosmetics.* 2 (2015) 35–47. <https://doi.org/10.3390/cosmetics2010035>.
- [60] D.R. Kryscio, P.M. Sathe, R. Lionberger, L. Yu, M.A. Bell, M. Jay, J.Z. Hilt, Spreadability Measurements to Assess Structural Equivalence (Q3) of Topical Formulations—A Technical Note, *AAPS PharmSciTech.* 9 (2008) 84–86. <https://doi.org/10.1208/s12249-007-9009-5>.
- [61] X. Zhang, Y. Jiang, L. Han, X. Lu, Biodegradable polymer hydrogel-based tissue adhesives: A review, *Biosurf Biotribol.* 7 (2021) 163–179. <https://doi.org/10.1049/bsb2.12016>.
- [62] A.D. Permana, R.N. Utami, P. Layadi, A. Himawan, N. Juniarti, Q.K. Anjani, E. Utomo, S.A. Mardikasari, A. Arjuna, R.F. Donnelly, Thermosensitive and mucoadhesive *in situ* ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis, *Int J Pharm.* 602 (2021) 120623. <https://doi.org/10.1016/j.ijpharm.2021.120623>.
- [63] M.O. Ilomuanya, N. v Enwuru, E. Adenokun, A. FATUNMBI, A. Adeluola, C.I. IGWILO, Title: Chitosan-based microparticle encapsulated *Acinetobacter baumannii* phage cocktail in hydrogel matrix for the management of multidrug resistant chronic wound infection, *Turk J Pharm Sci.* 0 (2021) 0–0. <https://doi.org/10.4274/tjps.galenos.2021.72547>.

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:



MINISTRY OF EDUCATION CULTURE OF INDONESIA
HASANUDDIN UNIVERSITY
FACULTY OF PHARMACY

Alamat Jalan Perintis kemerdekaan Km.10, Makassar 90245
Telepon (0411) 588556, Faksimili (0411) 590663
Laman: farmasi@unhas.ac.id

The Editor

Journal of Biomaterials Advance

October 14, 2022

Dear Sir/Madam,

I wish you to re-consider our manuscript entitled **“Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy”** for publication in ***Journal of Biomaterials Advance***.

We have made some changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved. We have addressed each of the reviewers' comments in the response to the reviewer file. Importantly, we have made a great effort to improve the English and the discussion parts of our revised manuscript.

We believe that these findings will be of interest to scientists working on the development of dermal delivery system for skin infection treatment using natural biopolymer. This manuscript has not been previously published in any language anywhere and that it is not under simultaneous consideration by another journal. We appreciate your attention. We hope you will now consider publishing our research in ***Journal of Biomaterials Advance*** and look forward to hearing from you in due course.

Yours Sincerely,

Andi Dian Permana (on behalf of all authors)
Faculty of Pharmacy
Hasanuddin University
Indonesia
Email: andi.dian.permana@farmasi.unhas.ac.id

Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Tri Puspita Roska¹, Sartini¹, Mukarram Mudjahid¹, Ardiyah Nurul Fitri Marzaman¹, Nana Novriana Payung Datu¹, Andi Dian Permana^{1*}

1. Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia

***Corresponding author:**

Andi Dian Permana

Faculty of Pharmacy, Hasanuddin University, Indonesia

Email: andi.dian.permana@farmasi.unhas.ac.id

Abstract

Skin wounds have been reported to increase the number of microbial colonies susceptible to infection. Treatments using oral antibiotics have been limited due to their toxicity and hydrophobic characteristics. In this study, we developed a formulation of chloramphenicol microparticles (CPL MPs), which was modified into chitosan hydrogel to increase treatment efficiency in targeting infections and creating an optimal environment to support the healing process. CPL MPs were prepared by a cross-linker stabilized method using whey protein (WPI) biopolymer, and the CPL MPs hydrogel was designed using chitosan biopolymer. Based on the result, CPL-loaded MPs showed desired physical and encapsulation characteristics. In the *in vitro* study, drug release of CPL MPs in simulated wound fluid represented approximately $99.40 \pm 7.01\%$ of the system after 24 h. The antibacterial activity of CPL-loaded MPs formulation (MIC value 12.5 $\mu\text{g/ml}$, MBC 25 $\mu\text{g/ml}$) was effective as MIC concentration increased. Furthermore, the formulation of CPL MPs into hydrogel showed a better dermatokinetic profile compared to hydrogel with pure CPL. Interestingly, the antibacterial activity of the *ex vivo* infection model showed that *Staphylococcus aureus* activity decreased by up to 99.98% after 24 h administration of CPL MPs hydrogel when compared to pure-CPL hydrogel and blank hydrogel. These studies have confirmed that incorporating CPL MPs into hydrogel can provide a promising approach to skin infection treatment.

Keywords: Chloramphenicol; Hydrogel; Microparticle; Skin Wounds; Whey Protein.

1. Introduction

A skin wound is a condition in which the skin has impaired integrity, anatomical structure and tissue function, and may be caused by chronic disease, burns or postoperative trauma. Skin wounds are a healthcare problem that is estimated to occur in about 4–5% of the adult population [1]. Injuries that compromise the skin's integrity allow microorganisms to colonize the area. This can lead to infection issues that can extend a patient's treatment time and necessitate the administration of more medication. The most common aerobic microorganisms causing wound infections include *S. aureus*, *S. pyogenes*, *P. aeruginosa*, MRSA, *S. epidermidis*, *Enterococcus faecalis*, coliforms, and *Acinetobacter baumannii* [2]. Wound infections can cause bacteremia, sepsis, and organ dysfunction, so an effective method is needed to prevent wound infections, one of which is the administration of appropriate topical antibiotics [3].

One antimicrobial with a broad spectrum of action is chloramphenicol (CPL), which is used for skin infections and can maintain its activity against bacterial resistance, with a bacteriostatic mechanism specifically inhibiting bacterial protein synthesis [4]. However, the effectivity of oral route of CPL is still limited due to its toxicity [5], and the physicochemical properties of hydrophobicity, make it difficult to penetrate the skin and inhibit drug transport to the target of infection [6]. This limitation can be overcome via the dermal route, which facilitates the loading of hydrophobic drugs with direct action activity to the target of infection.

Drug delivery systems targeting local infections could be developed by designing drugs into microparticles [7]. Microparticles (MPs) are widely preferred because they responsively release drugs, increase the effectiveness of the drug on the target, and provide local effects on the skin. In addition, drug-containing MPs offer high drug-loading flexibility and good physical and chemical stability [8]. MPs have also been reported to be designed for local administration with desired effects, including wound care. The MPs approach has also been widely developed for the treatment of infectious diseases to increase efficiency at selectively targeting infections through specific stimulus responses [9].

The drug substance can be incorporated into MPs through various methods such as ionotropic gelation, single or double emulsification, polymerization, and stabilization with cross-linkers [10]. MPs prepared by a cross-linker method using natural biopolymers as drug carriers offer optimal

75 drug loading. Hydrophobic drug encapsulation using natural biopolymers was chosen because of
76 its biodegradability, biocompatible, and suitability for skin delivery system formulation [11]. Whey
77 protein is a high-quality protein from cheese processing or cow's milk and is primarily consisted of
78 major proteins including β -lactoglobulin, α -lactalbumin, immunoglobulin, and lactoferrin [12]. A
79 study has shown that the binding of whey protein biopolymers with hydrophobic drugs can increase
80 drug solubility and dissolution [13]. Liu et al. used whey protein for curcumin encapsulation by
81 employing the spray-drying method to increase curcumin solubility and bioavailability [14]. This
82 can be chosen as a strategy to increase the effectiveness of drugs in treating skin infections. How-
83 ever, there are limitations in using whey protein in formulations because it tends to have a weak
84 protein bond, is easily damaged by heating, and may be susceptible to degradation. Therefore, hy-
85 drogel formulation is one of the preparations to overcome this problem.

86 Hydrogels are polymer networks with hydrophilic properties, which are needed in wound treatment
87 because they have excellent biocompatibility and provide an optimal environment for wound heal-
88 ing. Hydrogels can be applied in various physical forms, such as nanoparticles, MPs, and films [15].
89 Chitosan, the natural polymers in the manufacture of hydrogels, contain many hydroxyl and amine
90 groups typically used as functional groups to react with cross-linking agents, which stabilize the
91 protein structure [16]. In the treatment of wound infections, there are several reported benefits of
92 using chitosan as a biopolymer, including good antibacterial activity [17], anti-inflammatory and
93 hemostatic properties, enhanced skin regeneration; superior biocompatibility and biodegradability;
94 good water absorption and skin occlusivity properties; and due to the presence of amino and hy-
95 droxyl groups in the molecular chain, many biological activities can be improved by grafting on
96 additional groups and chemical elements [17,18].

97 For the potential enhancement of wound infection therapy, in this study, we provide a novel com-
98 bination delivery method of chitosan hydrogel and chloramphenicol microparticles (CPL MPs).
99 CPL MPs was formed and optimized by solvent evaporation and stabilized using a cross-linker.
100 Additionally, tests were done on drug loading, entrapment effectiveness, zeta potential, particle size,
101 polydispersity index, and antibacterial activity. The fabrication capacity to deliver CPL intrader-
102 mally was further tested in an *ex vivo* dermatokinetic study on healthy rat skin. Lastly, the penetra-
103 tion and antibacterial activity of the formulation were evaluated in an *ex vivo* skin infection model.

The major findings of this study have the potential to provide new insights for solving problems in the treatment of skin wound infections.

2. Materials and Methods

2.1 Materials

Whey protein (WPI) containing 80% was gifted by Glanbia Nutritionals, Inc, Fitchburg, USA. Chloramphenicol, Chitosan (MW 161.156 and deacetylation degree $\geq 75\%$), glutaraldehyde, 2-alpha naphthol, tryptone soya agar (TSA) medium, nutrient broth (NB) medium, phosphate-buffered saline (PBS), and sodium hydroxide were purchased from Sigma–Aldrich Pte Ltd., Singapore, Singapore. The reagents utilized in this investigation were all analytical grade.

2.2 Preparation of CPL MPs

CPL MPs were prepared according to a previous study (Jain et al., 2018), with slight modifications. WPI (with concentration of 2.5%, 5%, 10%, 15% and 20% (w/v)) was dispersed in deionized water, and the protein solution's pH was adjusted to 8 using NaOH (1 M). The solution was swirled for one hour with a magnetic stirrer before being refrigerated overnight (4°C) to fully hydrate the biopolymer. The solution was centrifuged at 1000 rpm for 5 minutes, and the supernatant was separated for further use. CPL (with a concentration of 12.5, 25, 50, 100, and 200 mg/mL) was dissolved in 5 mL ethanol and added to 20 mL of protein solution dropwise while continuously stirring at a temperature of 40°C. Immediately after dissolution using methanol, 2 mL of 5% (v/v) glutaraldehyde was added to facilitate cross-linking of the MPs. The detailed concentration of formulation is depicted in Table 1. The cross-linking procedure was carried out for 24 hours at 40°C with agitation; this also caused any residual organic solvent to evaporate. The obtained dispersion was thoroughly washed with deionized water three times. The mixtures were then freeze-dried.

2.3 Characterization of CPL MPs

2.3.1 Evaluation of particle size, zeta potential and polydispersity index (PDI)

The particle size, polydispersity index (PDI) and zeta potential of formulation were determined using the size analyzer (Malvern Instruments, Malvern, UK). The MPs were first dispersed with deionized water (0.01% v/v), and 1 mL of each sample was used for the analysis process. All samples were analyzed at 25°C and a scattering angle of 90°.

2.3.2 Measurement of entrapment efficiency (EE) and drug loading (DL) percentages

Microparticle EE was measured using the indirect method. CPL MPs suspension was centrifuged at 1000 rpm for 20 minutes to separate the supernatant. The supernatant was then analyzed using a UV-Vis spectrophotometer at a wavelength of 488.7 nm after being derivatized with 2- alpha naphthol (0.01 M) and sodium bicarbonate (0.01 M). A derivatization procedure was performed on CPL to produce peaks that did not overlap with WPI and showed accurate measurement data. The reduced CPL MPs solution was created by dissolving the CPL MPs in methanol, then reacting it with zinc powder and HCl (12 M) while stirring it for 5 minutes in bath water at a temperature of 50° C. The reduced solution was filtered into a flask and diluted with deionized water. In a volumetric flask containing the CPL MPs solution, 1 ml of 2-alpha naphthol and 0.5 mL of sodium bicarbonate (0.1 M) were added to complete the reduced process. To create an orange-red result, the solution was heated at 70° C in bath water for 10 min. The amount of CPL was calculated according to the standard curve equation $Y = 0.0769x - 0.0097$. The encapsulation efficiency of CPL MPs (EE) % is the actual amount of the CPL found in MPs divided by the total amount of the CPL added during the preparation. Drug loading of CPL (DL) % is the mass of the CPL-MPs in the dried powder divided by the total mass of the MPs recovered from the drying process. The calculation of % EE and DL follows the equations below [20]:

$$\% \text{ Entrapment Efficiency (EE)} = \frac{\text{Total drug amount} - \text{unencapsulated drug amount}}{\text{total drug amount}} \times 100\% \quad (1)$$

$$\% \text{ Drug Loading (DL)} = \frac{\text{Mass of encapsulated drug}}{\text{total mass of microparticle}} \times 100\% \quad (2)$$

2.3.3 Powder X-ray diffraction (PXRD) analysis

The crystallinity pattern of the sample was analyzed with a powder X-ray diffraction (Rigaku Corporation, Kent, England) instrument using 1 gram of sample in a very high-resolution Cu-K α radiation diffraction system. The CPL MPs was placed in an aluminium holder at a voltage of 40 kV, current of 30 mA and scanned 2 θ angle range of 10 to 90°.

2.3.4 Differential scanning calorimetry (DSC) analysis

The materials were thermally examined using a DSC 2920 TA instrument (Surrey, UK). The detection parameters for this measurement were a 5 mg powder sample, a temperature range of 25° to 300°C, a heating rate of 10°C/min, and a nitrogen cleaning rate of 50 mL/min.

2.3.5 Fourier-transform infrared spectroscopy (FT-IR) analysis

Chemical interaction between chemical components in CPL MPs in WPI polymer was analyzed by FT-IR spectrophotometer instrument (Accutrac FT/IR-4100™ Series, Perkin Elmer, USA) in the wavenumbers range of 4000–400 cm⁻¹.

2.3.6 Scanning electron microscope analysis

A scanning electron microscope (TM3030 microscope, Hitachi, Krefeld, Germany) was used to examine the morphologies of CPL MPs. CPL MPs were freeze-dried under vacuum by lyophilizing. The CPL MPs were directly attached to the carbon tape and observed with the following operating conditions: accelerating voltage 15 kV, probe current 45 nA, and counting time 60 s.

2.3.7 In vitro release studies of CPL MPs

In vitro drug release of CPL MPs and CPL using simulated wound fluid. Simulated wound fluid was prepared using 0.36 g of sodium hydrogen carbonate, 5.84 g of sodium chloride, 0.28 g of calcium chloride, 30.30 g of potassium chloride, and 33.00 g of bovine albumin in 1000 mL of deionized water [21]. Two Erlenmeyers contained 50 mL of simulated wound fluid for each formulation. CPL MPs equivalent to 50 mg of CPL were added into simulated wound fluid with 5 mL of samples taken at predetermined time intervals (0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 24 h) and replaced with the same volume of new release media. The test was carried out at a temperature of 37°C and a speed of 100 rpm. The CPL released was further determined using a UV-Vis spectrophotometer. The amount of CPL in simulated wound fluid was calculated according to the standard curve equation $Y = 0.0368x + 0.011$ with $R^2 = 0.9996$ and the drug release percentage was calculated using the equation below[22] :

$$\% \text{ Drug released} = \frac{\text{Amount of CPL release}}{\text{Amount of CPL tested (50 mg)}} \times 100\% \quad (3)$$

2.3.8 Mathematical modelling of the kinetics of drug release

The principal mathematical models of drug release were tested, along with the relevant equations and parameters, using the Excel® (Microsoft Corporation, Redmond, USA) Add-In-DDSolver to model the systems' drug release data. The adjustment of the theoretical models to the experimental data was compared using the adjusted coefficient of determination (adjusted- R^2). The kinetics model follows the equations below [23]:

Zero-order:

$$Q_t = Q_0 + K_0 t \quad (4)$$

First-order:

$$\ln Q_t = \ln Q_0 + K_1 t \quad (5)$$

Higuchi:

$$Q_t = K_H \sqrt{t} \quad (6)$$

Hixson-Crowell:

$$Q_0^{1/3} - Q_t^{1/3} = K_s t \quad (7)$$

Korsmeyer-Peppas:

$$Q_t = K_t n \quad (8)$$

where Q_0 is the preliminary value of Q_t , t is the time, n is the exponent of diffusion release, K_0 , K_1 , K_H , K_t , and K_s are the coefficients of release according to the relevant kinetic model, and Q_t (%) is the percentage of CPL released at time t .

2.4 In vitro antibacterial activity of CPL MPs

2.4.1 Preparation of bacterial cultures

The bacterial strain used was *Staphylococcus aureus* (ATCC®25923), which was stored at 4°C and sub-cultured in fresh media. Bacterial culture was prepared by growing bacteria on TSB media with a temperature of 37°C. Test bacteria are made equivalent to 0.5 mc Farland equivalent to 2×10^8 CFU / mL.

2.4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluation

The MIC and MBC from CPL and CPL MPs were determined using microtiter dilution techniques in accordance with the Clinical and Laboratory Standards Institute protocol [24]. CPL and CPL MPs solution with a concentration of 1000 µg/mL was made in a 10 mL volumetric flask. The

sample was entered into 96-well plates with a concentration of 100 µg/mL. Next, 100 µL of bacterial suspension was added to the wells, then homogenized using micropipettes. TSB with a concentration of 100 µL medium was added to the wells. Wells containing only pure CPL and TSB media alone were used as the positive and negative control, respectively. All filled wells were closed and incubated at 37°C for 24 hours. To determine MBC value, 20 µL from 96-well plates with selected MIC was cultured on a Petri dish containing TSA medium and incubated at 37°C for 24 h. After that, the number of bacteria in the Petri dish colony was then determined. MBC was defined as the lowest concentration that eliminates 99.9% of bacterial growth.

2.4.3 Time–kill assay of CPL MPs

Time–kill assay of CPL and CPL MPs against *Staphylococcus aureus* (SA) was determined. CPL, blank MPs, and CPL MPs were added to the bacterial solution in the same quantities as MIC, 2 x MIC, and 4 x MIC, resulting in 2×10^5 CFU/mL of bacteria. After that, bacterial cultures were cultured at 37°C. 20µL aliquot from the culture was collected at specified time intervals and inoculated aseptically into a Petri dish containing TSA medium. The Petri dish was then incubated at 37°C for 24 hours. The procedure was performed with 3 replications and created a log CFU/mL curve against time. CPL, blank MPs, and CPL MPs were added to the bacterial solution in the same quantities as MIC, 2 x MIC, and 4 x MIC, resulting in 2×10^5 CFU/mL of bacteria.

2.4.4 Evaluation of hemolytic activity

By incubating the sample with red blood cells (RBC) and quantifying the percentage of hemoglobin produced from lysed erythrocytes, the overall hemolysis of the formulation was examined. *In vitro* hemolytic assays were carried out in accordance with a previously described method to determine the hemolytic activity of CPL, CPL MPs and CPL MPs hydrogel [25]. First, EDTA tubes containing freshly drawn rat blood were centrifuged for 10 minutes at 3000 rpm to separate the RBCs. After scraping off the plasma layer at the top of the tube, the remaining cells were further washed twice with PBS and diluted to 1:10, v/v. We mixed 100 µL of the prepared cell suspension with 900 µL of the sample containing CPL, CPL MPs and CPL MPs hydrogel (serially dissolved in PBS) at concentrations ranging from 5 to 500 mg/L. After 60 minutes of incubation at 37°C, the mixture was centrifuged at 3000 rpm for 10 minutes. Free hemoglobin was estimated using UV-visible spectroscopy measurements by analysing the supernatant taken at wavelength 540 nm. Minimal (negative) and maximum (positive) hemolytic controls were performed using phosphate-buffered

saline and distilled water, respectively. Blood sample hemolysis was analyzed by visually inspecting for colour changes in the serum and plasma. For each concentration, the experiments were repeated three times. In the following equation, we included the hemolysis percentage calculation:

$$\text{Hemolysis (\%)}: \frac{\text{test sample} - \text{negative control} \times 100}{\text{positive control} - \text{negative control}} \quad (9)$$

2.5 Formulation of chitosan hydrogel from CPL MPs

Chitosan was dissolved in 2% acetic acid solution at 60°C to produce the CPL MPs hydrogel. The chitosan solution was combined with glycerin (10% w/w) and DMDM hydantoin (0.01% w/w) while being stirred with a magnetic stirrer. CPL MPs (1% w/w) were then added to the mixture. The foaming components of the preparation were eliminated using a sonicator for 30 minutes at a temperature of 50°C, thereby adjusting the hydrogel pH to 5 with the addition of NaOH 8.3 M. As a control, a hydrogel containing CPL was created.

2.6 Characterization of CPL MPs hydrogel

2.6.1 pH measurement

A pH meter was used to determine the pH of CPL MPs hydrogel by inserting the electrode into the gel and recording the result after two minutes. All measurements are carried out 3 times to obtain the average value and standard deviation.

2.6.2 Evaluation of viscosity and rheology

With the use of a Brookfield viscometer with a seven-speed spindle running at 50 rpm, the viscosity of the CPL hydrogel was measured. The rheology of the CPL hydrogel was also determined using the same seven-speed spindle tool. Measurements are carried out at speeds of 5, 10, 20, 50, and 100 rpm.

2.6.3 Spreadability

Two sets of standard-sized glass slides were taken in this study. The spreadability of the hydrogel was evaluated by applying 0.5 g of the gel to a circle that had been previously marked on a glass plate with a diameter of 2 cm and then again using a second glass plate. After 1 minute, the diameter of the hydrogel was measured. The spreadability measurement was carried out with loads of 500 g.

2.6.4 Skin occlusivity examination

The previous technique was used to test *in vitro* skin occlusivity [26]. The 100 mL beaker was filled with 50 mL of deionized water, which was covered carefully with Whatman filter paper. Whatman filter paper with a size of 2.5 mm was applied with 250 mg of hydrogel. As a control, filter paper without CPL MPs hydrogel was used. All samples were stored at a temperature of 32°C, and the weight of Whatman paper was calculated at 0 h, 6 h, 24 h and 48 h. Occlusivity (O) was calculated through Eq:

$$\text{Occlusivity percentage (O)} = \frac{\text{mass of control} - \text{mass of sample}}{\text{mass of control}} \times 100\% \quad (10)$$

2.6.5 Extrudability percentage

About 10 g of hydrogel were sealed inside a collapsible aluminum tube, the end of which was crimped, and a clamp was used to keep the tube from unwinding. The gel was released after the cap was removed, and the quantity of gel that was extruded was measured. The amount of gel that was forced out of the extruder was determined as follows:

$$\% \text{ Extrudability} = \frac{\text{total mass of extruded hydrogel}}{\text{Total mass of hydrogel}} \times 100\% \quad (11)$$

2.6.6 Bioadhesivity test

Part of the rat skin tissue was cut and immediately secured with the mucosa side out into each glass vial via rubber bands and aluminum caps. Phosphate-buffered saline (PBS) was applied to vials containing rat skin tissue for 10 minutes. The test was carried out by gluing the skin onto the upper and lower vial bases, after which the hydrogel preparation was placed between the rat skin on the scale's left arm. The hydrogel was moistened with PBS and then attached to the skin. The vial's height was then altered to accommodate the application of hydrogel between the mucosal tissues of both vials. Once two vials were linked, the weights were held at that level. The detachment stress (dyne/cm²) between two vials was calculated using the adhesive forces presented below [27]

$$\text{Bioadhesive strength} = \frac{m \cdot g}{A} \quad (12)$$

where m is the weight required to remove the gel from the skin, g is the acceleration due to gravity (980 cm/s²), and A is surface area of the exposed mucosa (cm²).

2.6.7 *Ex vivo dermatokinetic study*

Dermatokinetic assays using Franz diffusion cells were carried out following the previous method [28]. Rat skins were cut and soaked in PBS pH 7.4 for 30 minutes before being attached to Franz diffusion cells. The medium used in this study was simulated wound fluid, which was arranged as a receiver compartment. The receiver compartment was heated to $37\pm1^{\circ}\text{C}$ and agitated at 600 rpm. Parafilm® was used to close off the donor area. A 1 g sample of CPL MPs hydrogel was put into the skin using a syringe needle for 30 seconds, and a 5 g cylindrical weight was placed on top of the skin to keep the formula in place during the experiment.

The samples were taken at intervals of 1, 2, 3, 4, 5, 6, 8, and 24 hours. After removing the hydrogel, the skin was cleansed with deionized water to remove any residue particles. The biopsy incision was 5 mm in diameter and was made in the skin. Next, the skin was transferred to a 1.5 mL Eppendorf® tube and heated in a water bath at 60°C for 2–3 minutes. The epidermal layer was separated from the dermis layer using forceps. The extraction process of the drug on the skin layer was carried out by adding 1 mL of methanol separately, and each mixture was homogenized for 10 minutes. The sample was then centrifuged at 3000 rpm for 15 minutes. Supernatants were collected and then measured using a UV-Vis spectrophotometer and following procedure section 2.3.2. C_{max} , T_{max} and AUC_{0-24} were determined using PK Solver software (China Pharmaceutical University, Nanjing, China).

2.6.8 *Ex vivo antibacterial activity test using infection skin model*

2.6.8.1 *Ex vivo test preparation on rat skin infection model*

The skin was sterilized by immersing it in 70% ethanol for one hour. The skin was sensitized to laminar airflow (LAF) for 20 minutes prior to the experiment. Furthermore, the sterilized skin was administered an intradermal injection of 50 μL of BSA solution, which was equal to 0.5 mg Farland. The skin piece was next positioned aseptically on a Petri plate filled with TSA media. The skin was then aseptically transferred to a new TSA Petri dish, and the Petri dish was incubated at 37°C for 7 days.

2.6.8.2 *Antibacterial activity test of ex vivo hydrogel preparations on skin infection model*

The antibacterial activity of the *ex vivo* skin infection model was carried out using diffusion Franz diffusion cells [29]. In this study, rat skin of average thickness was used, and a skin infection model

was placed on diffusion cells. Before the study, a dilution of CPL MPs hydrogel and CPL hydrogel was prepared by dissolving in DMSO. The skin was collected and 1.5 mL of sterile water was added to a 1.5 ml Eppendorf tube after hydrogel treatments had been left on for 6, 12, and 24 hours, respectively. Next, the mixture was homogenized and TSA medium was added to a Petri dish with 20 mL of homogenous samples. The sample with the medium was incubated for 24 hours at 37°C. Additionally, the same method was used to apply both blank hydrogel and CPL hydrogel to infected skin as a control. We determined the log CFU/mL of the samples.

2.7 Analytical statistic

All data obtained for this study were reported as means of the standard deviation (SD) and computed using Microsoft Excel® 2016. (Microsoft Corporation, Redmond, USA). The data were analyzed using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA) and the SPSS version with a significance value of $p = 0.05$.

3 Result & Discussion

3.1 Particle size, zeta potential and polydispersity index (PDI) of CPL MPs

All ingredients used in the preparation of CPL MPs have been reported to be non-toxic and safe for clinical purposes [30]. As a cross-linker agent, CPL MPs were prepared by solvent evaporation and stabilized by glutaraldehyde. Moreover, glutaraldehyde has also been reported to introduce intermolecular cross-linking in proteins or to modify adsorbed proteins on amino-glutaraldehyde bonds [31]. Glutaraldehyde-mediated cross-linking of whey proteins was formed by aggregates of whey protein by whey protein intermolecular amide bonds (β -lactoglobulin (BLG) bands) and glutaraldehyde as follows the reaction in Figure 1. This approach successfully fabricated MPs in this study by designing formulations with a variation of CPL, WPI concentration and WPI:CPL ratio. The MPs' physical characteristics were then evaluated in Table 1 and Figure 2A.

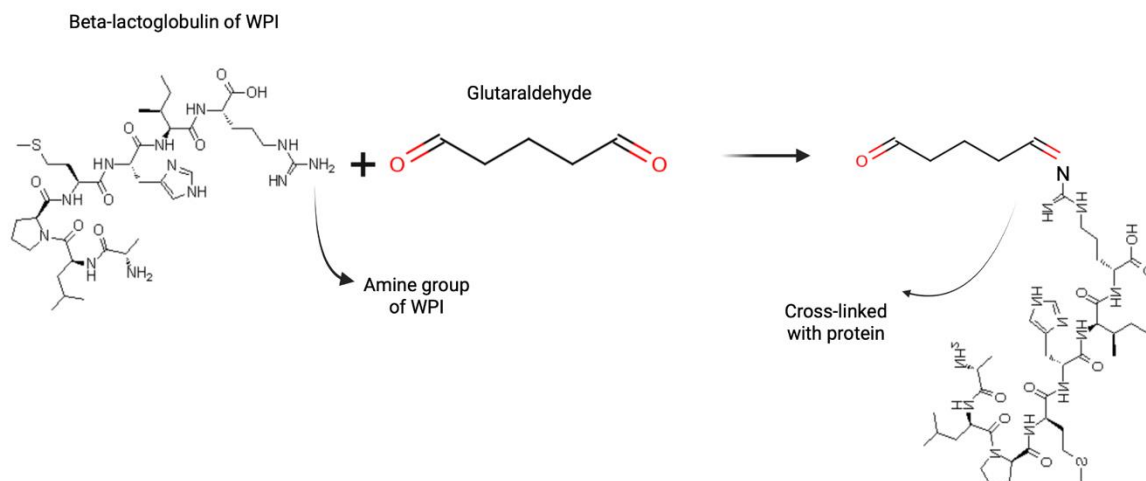


Figure 1. The cross-linked reaction of Amine group of WPI and glutaraldehyde

The results show that particle sizes of CPL MPs were produced in the range of $0.56 \pm 0.13 \mu\text{m}$ to $35.71 \pm 5.23 \mu\text{m}$. Conversely, samples with variation in CPL concentration show significant particle size variation between formulations. Moreover, the formulation with a variation of WPI concentration also shows significant particle size variation between formulations ($p < 0.05$) with an increase of $3.62 \mu\text{m}$. It concludes that the concentration of WPI can produce a more compact protein structure so that the higher the protein concentration, the more it will be able to increase particle size [32]. The same trend also occurs in variations in the ratio of WPI and CPL (1:1, 2:1, 8:1, 16:1), of which a 1:1 ratio was found to yield significantly larger particle size compared to the rest ($p < 0.05$). This increase may be due to increased hydrophobic interactions between encapsulated and absorbed CPL in the main component of the protein isolate β -lactoglobulin with an increase in WPI concentration. Solution at pH 8.0 allows ligands to enter the hydrophobic nucleus and bind to many hydrophobic molecules, causing an increase in particle size [33]. These findings also showed that the CPL/WPI microparticle size was dependent on the ratio of WPI: CPL in the system (core: coating ratio), and that this ratio could be raised by increasing the WPI content in the formulations [34].

Table 1. Details of the different formulation characteristics utilized to create CPL MPs, including their PDI, zeta potential, and particle size (means $\pm$ SD, n = 3).

Formulation code	WPI in water (%)	CPL in ethanol (mg)	Ratio WPL:CPL	Particle size (μ m)	PDI	Zeta potential (mV)
MP1	5	12.5	4:1	1.33 $\pm$ 0.09	0.127 $\pm$ 0.005	-28.27 $\pm$ 0.81
MP2	5	25	4:1	1.40 $\pm$ 0.14	0.129 $\pm$ 0.005	-29.25 $\pm$ 1.01
MP3	5	50	4:1	1.44 $\pm$ 0.05	0.13 $\pm$ 0.003	-29.44 $\pm$ 0.67
MP4	5	100	4:1	1.45 $\pm$ 0.11	0.14 $\pm$ 0.007	-29.14 $\pm$ 1.06
MP5	5	200	4:1	1.47 $\pm$ 0.10	0.13 $\pm$ 0.010	-29.21 $\pm$ 1.65
WP1	2.5	50	4:1	0.56 $\pm$ 0.13	0.13 $\pm$ 0.004	-24.67 $\pm$ 0.71
WP2	10	50	4:1	2.92 $\pm$ 0.20	0.13 $\pm$ 0.005	-31.95 $\pm$ 0.87
WP3	15	50	4:1	7.65 $\pm$ 0.99	0.14 $\pm$ 0.005	-34.23 $\pm$ 1.14
WP4	20	50	4:1	11.42 $\pm$ 1.07	0.14 $\pm$ 0.009	-35.17 $\pm$ 0.84
CP1	10	50	1:1	134.62 $\pm$ 10.29	0.63 $\pm$ 0.029	-27.73 $\pm$ 1.43
CP2	10	50	2:1	33.03 $\pm$ 4.08	0.40 $\pm$ 0.006	-28.36 $\pm$ 0.91
CP3	10	50	8:1	1.29 $\pm$ 0.05	0.13 $\pm$ 0.007	-32.32 $\pm$ 2.00
CP4	10	50	16:1	1.29 $\pm$ 0.07	0.12 $\pm$ 0.009	-32.56 $\pm$ 2.04

PDI was carried out to characterize the molecular weight of a polymer. Based on the results (Figure 2B), samples with a variation of CPL WPI concentrations show no significant results between formulations ($p > 0.05$). While the different outcomes are noticeably seen in the ratio of WPI:CPL, the higher solution of WPI in formulation shows decreased PDI value. The PDI value indicates the same trend as the particle size. PDI smaller than 0.5 is produced when the mass of WPI in WPI:CPL is within the range of 2:1 to 16:1, indicating high particle aggregation with a broad particle size distribution [30].

Measurement of zeta potential can predict the colloidal solution's stability, indicating the repulsive force between MPs. The zeta potential graphic of the CPL MPs is shown in Figure 2C. CPL MPs have a relatively high zeta potential (i.e., close to around -30 mV), indicating good stability of MPs exhibited in this study. Furthermore, increasing the concentration of CPL in the formulation has no significant effect ($p > 0.05$) on the zeta potential of MPs, indicating that the significant amount of drugs trapped in the core has no effect on the surface area of β -lactoglobulin. All formulation have

extremely negative zeta potentials, which improve the physical stability of many systems by increasing the repulsive force between MPs [35]. In addition, The negative zeta potential found in this investigation may be attributable to the structure of the whey protein opening during heating at the microparticle preparation, which reveals more polar groups [36]. Furthermore, β -Lg of WPI has an isoelectric point in the range of 4.9–5.4. The second significant constituent of whey protein is α -La, with an isoelectric point of 4.6. When pH value increases above the isoelectric point, the protein produces a negative charge, which facilitates negative zeta potential. Additionally, the isoelectric point of β -Lg is in the 4.9–5.4 range. Whey protein's second important component (α -La) has an isoelectric point of 4.6. Proteins create a negative charge when the pH level rises above the isoelectric point, which supports the negative zeta potential [37,38]

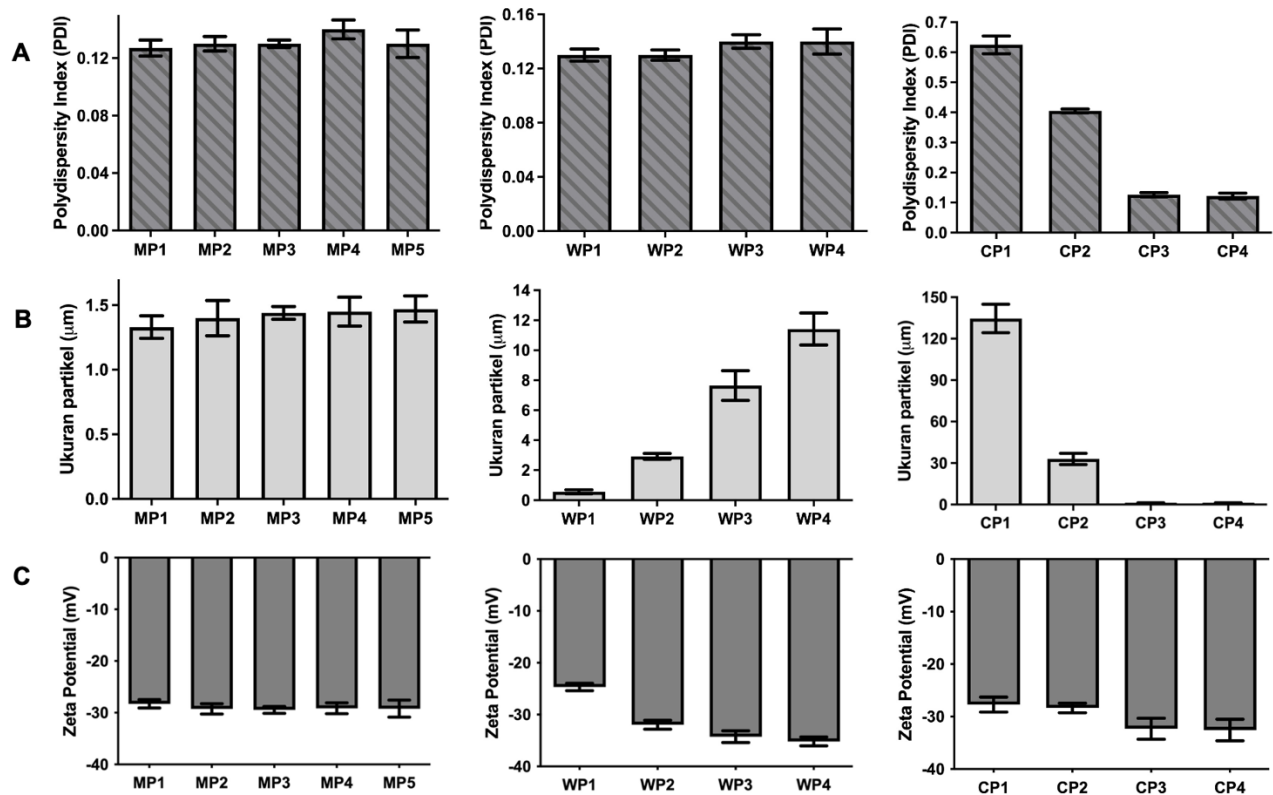


Figure 2. Particle size (A), Polydispersity Index (B) and Zeta Potential (C) of CPL MPs prepared with Whey Protein (WPI) (means $\pm$ SD, n = 3).

3.2 EE and DL percentages

Entrapment efficiency (%EE) and drug loading (%DL) of CPL MPs are shown in Figure 3A. CPL MPs have a higher EE value ($68.78 \pm 1.40\%$) when the mass of WPI is in the range of 10–20%, but

there is no noticeable difference between WP2, WP3 and WP4 ($p > 0.05$). The same trend occurred in the WPI: CPL ratio (8:1 and 6:1), experiencing an increase in EE ($55.67 \pm 3.55\%$ and $45.98 \pm 1.47\%$). This value decreased significantly to $13.50 \pm 2.67\%$ when the WPI:CPL ratio was 1:1. WPI has an amphiphilic structure that can self-assemble into different forms, thereby increasing drug encapsulation. However, this ability can be reduced if DL has been maximized. According to Li et al., the increased surface hydrophobicity of WPI aggregates can explain their higher CPL loading capacity [39]. Li et al. investigated whether the enhanced contact between hydrophobic groups during the chemical cross-linking procedure was causing the surface hydrophobicity of WPI to rise. They found that the more active hydrophobic patches among the generated WPI aggregates for binding to CPL accounted for their increased %EE and %DL.

Drug loading of CPL MPs increased significantly from $3.40 \pm 1.68\%$ to $20.43 \pm 2.15\%$ (Figure 3B). Similar results have also been reported in utilizing α -lactalbumin-based nanoparticles to encapsulate resveratrol, in which the DL value increased significantly as drug content was increased, indicating a higher microparticle loading capacity with the addition of drugs [40]. Moreover, methanol as the solvent to dissolve CPL at higher concentrations modifies the protein solution's dielectric constant and induces the transition into aggregates [41]. Consequently, The total WPI aggregate formed depends on CPL concentration and the amount of solvent in the formulation. The DL of WPI aggregates produced in this preparation was much higher than what Zhang et al. reported for protein based-nanoparticles formulation ($12.69 \mu\text{g}/\text{mg}$ nanoparticles) [42].

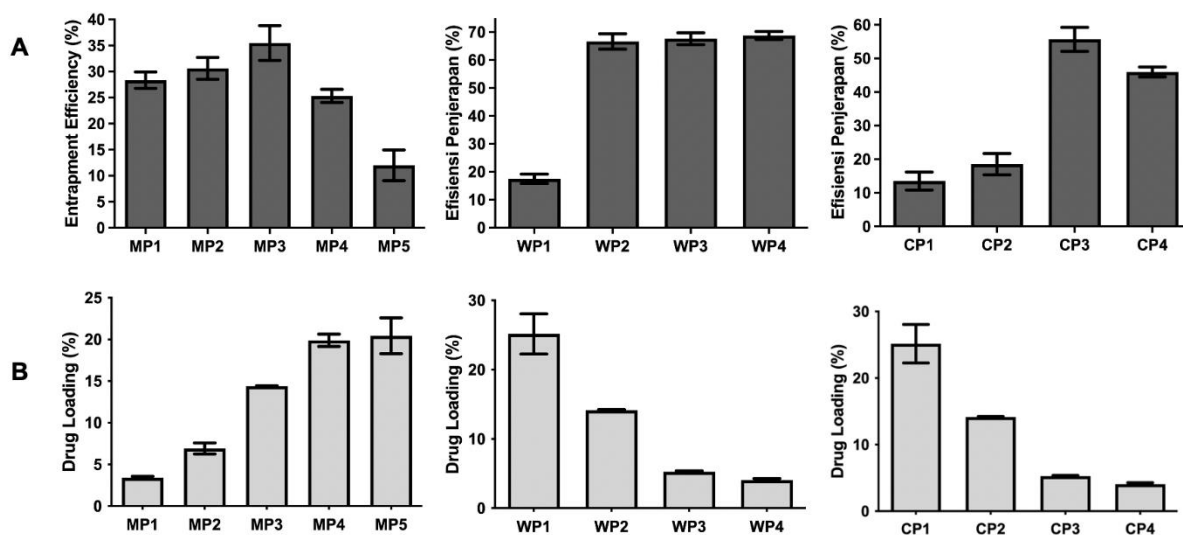


Figure 3. EE (A) and DL of (B) diagram of CPL MPs with a variation of CPL, WPI and ratio of CPI: WPI concentration (means $\pm$ SD, n = 3).

3.3 Powder X-ray diffraction (PXRD)

X-ray diffraction (PXRD) is essential to identify the physical state of the drug and the effect of homogenization on the crystalline structure of active substances. XRD patterns of the pure WPI, pure CPL, physical mixture (1.5:1), and CPL MPs have been compared in Figure 4C. The sharp peaks are associated with the crystalline fraction, whereas the amorphous part is represented as broad peaks [43]. It can be observed that firm crystalline peaks are observed in CPL and physical mixture diffraction. There are two sharp dominant peaks at $2\theta = 31.82^\circ$ and $2\theta = 26.1^\circ$ in CPL and $2\theta = 30.64^\circ$ and $2\theta = 25.98^\circ$ in the physical mixture (1.5:1). Two broad peaks were also observed at around 21.5° in CPL-loaded MPs and 21.46° in WPI. The same pattern was observed previously for WPI studies [43]. However, the crystalline peaks almost disappeared in the XRD pattern of CPL MPs and WPI, indicating the disruption of the crystalline form of CPL after encapsulation and entrapment of CPL into WPI-based MPs.

3.4 Differential scanning calorimetry (DSC)

To assess the physicochemical interactions between the formulation chemicals and to ascertain whether crystallinity was preserved during the encapsulation procedure, a thermal analysis of the DSC was carried out. Thermograms of pure CPL, pure WPI, physical mixture and CPL-loaded MPs are presented in Figure 4B. The DSC curve of pure WPI exhibits a broad endothermic peak between 40 and 120°C , centred at 85°C . The primary whey protein components (β -lactoglobulins and α -lactalbumin) undergo heat-induced transitions, which cause this distinctive endotherm. Similar characteristics were also seen in the physical mixture WPI:CPL (1.5:1) and in prior literature [44]. The endothermic point remained sharp at 159°C , representing CPL's melting point and crystallinity (pure CPL and physical mixture). The endothermic point at 159°C was not investigated in the pure WPI and CPL-loaded MPs. However, in CPL MPs, an endothermic peak was observed only in pure WPI, showing that CPL was distributed molecularly in the polymer matrix and hence properly encapsulated in WPI MPs. Endothermic peaks and melting points were identified following the encapsulation of DIM with protein nanoparticles, providing strong evidence of nanoencapsulation [45].

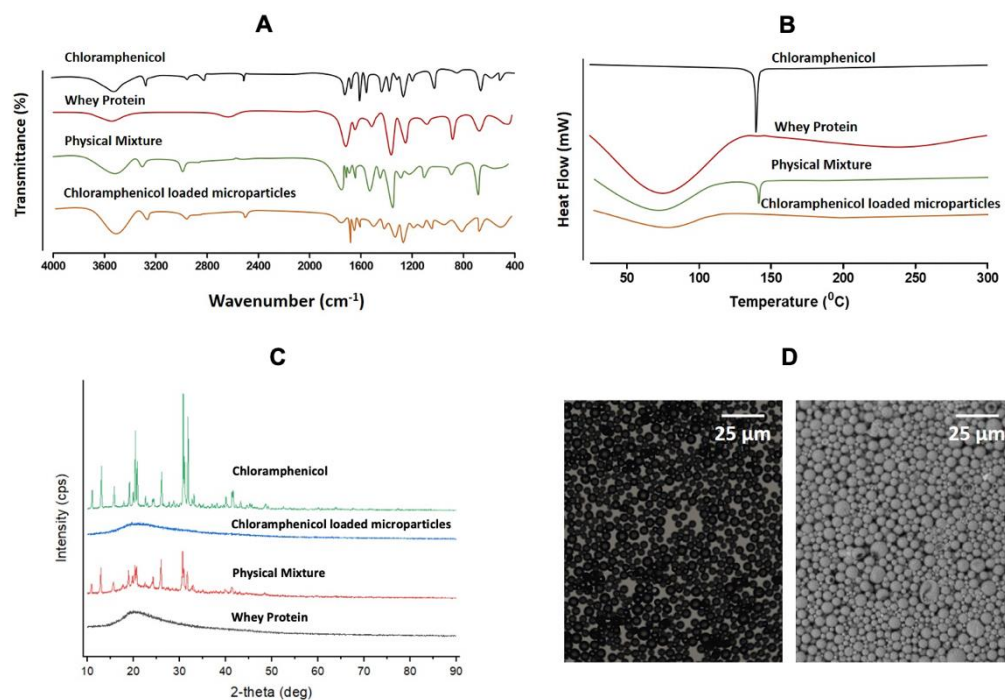


Figure 4. FT-IR Spectra of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (A). DSC thermogram of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (B). X-ray diffractogram of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (C). SEM images of CPL-loaded MPs (D).

3.5 Fourier-transform infrared spectroscopy (FT-IR) analysis

FT-IR analysis was used to determine the functional groups and interactions between pure WPI, pure CPL, physical mixture, and CPL-loaded MPs. The results are presented in Figure 4A. Major interesting peaks are observed in 3200–3600 cm^{-1} , indicating the presence of O–H and N–H bonds. The broad peak at around 2800–2500 cm^{-1} is also associated with O–H stretching vibration. O–H stretching was remarkably shifted after CPL was encapsulated in WPI MPs due to the interaction of CPL and WPI. These changes suggested that hydrogen bonding was formed between β -lactoglobulin and CPL. In the infrared spectrum, major interesting peaks are observed in 3200–3600 cm^{-1} , indicating the presence of O–H and N–H bonds [34]. The presence of strong peaks appeared on the CPL spectrum at 3493 cm^{-1} , which is associated with O–H stretching vibration; spectrum 3218 cm^{-1} is associated with N–H stretching vibration bond; spectrum 2978 cm^{-1} is associated with aromatic C–H stretching vibration, spectrum 1668 cm^{-1} with C=O stretching vibrations; and spectrum 1561 cm^{-1} is associated with NO_2 stretching peak [46]. The entire spectrum was detected in CPL encapsulated into MPs, indicating that encapsulation had been successfully performed on the system.

Amides I, II, and III are regarded as typical WPI spectra, indicating the presence of WPI's primary components, β -lactoglobulin and α -lactalbumin. The amide I band mainly confirms the presence of C=O stretch vibration and N–H bending. The middle absorption band II shown correlates with the structural combination group of C–N stretch with N–H deformation, while the amide III bands are associated with C–O bending vibration [47]. Amide I in the spectrum of 1745 cm^{-1} , amide II at 1451 cm^{-1} , and amide III at 1241 cm^{-1} are present in CPL-loaded MPs and physical mixture. The C=O stretching vibration carbonyl groups are primarily responsible for the Amide I observed in the encapsulated CPL at $1600\text{--}1700\text{ cm}^{-1}$. The binding of CPL to β -lactoglobulin was demonstrated by comparing the WPI spectrum with the encapsulated CPL. Wang et al. reported similar outcomes [48]. The spectrum of CPL-loaded MPs shows most of the characteristic peaks identical to the spectrum of WPI and CPL, which indicates the encapsulation procedure was successfully carried out. The change in intensity indicates the hydrophobic interaction of CPL and lactoglobulin complexes. These findings align with DIM-based polymerized whey protein nanoparticles [35].

3.6 SEM analysis

Morphology of CPL-loaded microparticle formulation was observed using SEM. As shown in Figure 4D, the CPL MPs show the spherical shape of the particles. The size of CPL MPs found in this experiment was similar to that observed in the DLS analysis ($\sim 25\text{ }\mu\text{m}$). Particle size is closely related to drug penetration into target tissues; Figure 3D shows that CAP MPs meet the particle size criteria to penetrate the stratum corneum lipid layer through the lipidic intercellular route or aqueous pores, which can penetrate to a depth of $> 2\text{ mm}$ efficiently into hair follicles. Another study also used the solvent evaporation method and prepared MPs, resulting in particle sizes of $6\text{--}25\text{ }\mu\text{m}$ [49]. Similar studies have also been described by previous studies [20], in which nanoparticle formulation of frankincense essential oil-loaded whey with a particle size of $321 \pm 22.42\text{ nm}$ showed a good healing effect for skin wounds. This capability can be supported if the particle size is within the range of $10\text{--}100\text{ nm}$ based on confirmation by studies on human, porcine or murine skin [50]. Moreover, the permeability of MPs into the skin is influenced by the shape of the particles. A spherical shape has an excellent ability to penetrate the skin because it is symmetrical in all three dimensions; thus, it can be located deep in the epidermis and dermis layers.

3.7 In vitro microparticle drug release of CPL MPs

The drug release behaviour of CPL compared to CPL MPs formulation is presented in Figure 5A. To mimic the physiological condition of the skin, *in vitro* drug release was prepared using simulated wound fluid. In this study, encapsulation of the drug into microparticle form improved the drug release profile. The drug release of CPL MPs was examined as the time in which the drugs needed to be completely soluble and migrate from the initial form as in the polymeric system to the simulated wound fluid until attained maximum drug accumulation. The experiment showed that $99.40 \pm 7.01\%$ CPL was released from the microparticle formulation after 24 hours. Meanwhile, the amount of drug release from pure CPL was around $95.43 \pm 2.41\%$ after 6 h and then CPL reached maximum release. For simulated wound fluid, the estimated drug release during the first hour was $4.76 \pm 0.16\%$. After that, approximately $10.31 \pm 0.23\%$, $24.81 \pm 1.03\%$, $42.04 \pm 4.21\%$ and $62.39 \pm 1.68\%$ of CPL were released from MPs within 2 h, 4 h, 6 h, and 8h, respectively. By comparing both CPL and CPL MPs, a quick release lasting up to only 6 hours was observed in pure CPL, while that in CPL MP's needed 24 hours to completely release, showing the contribution of particle encapsulate and the sustained released drug was achieved. The CPL MPs formulations showed sustained release patterns up to 24 h. This behaviour is related to an enlarged CPL surface area, leading to an increased drug release, with a controlled release profile [51]. Following that, a quick release lasting up to 24 hours was observed; weakly bound CPL that was released and attached to the surface of the microparticle may have caused the drug release. The solubility of WPI in simulated wound fluid can be attributed to the release profile of WPL from MPs, which predicts prolonged release by causing erosion of the microparticle matrix.

3.8 Mathematical modeling of the kinetics of drug release

The experimental drug release was employed in multiple kinetic models to further understand the process of CPL release from MPs. The most acceptable mathematical model is chosen based on the correlation coefficient value. Based on Table 2, It was concluded that the best results of CPL MPs were observed with R2 value of 0.91877. According to the results, the release behaviour of the CPL-loaded MPs formulation followed the Hixson–Crowell kinetic model. The drug release from these spherical sustained-release dosage forms depends on matrix drug diffusion and polymeric matrix erosion during polymer degradation [52]. The regular area of a collection of particles was found by Hixson and Crowell to be proportional to the volume cube root. The Hixson-Crowell cube root law describes particle release from a system where the surface area and diameter of the particles change [22]. In this work, the applicability of these equations was tested. Hixson and Crowell recognized

that the rate of dissolution depends on the surface contacting with the applied solvent. Increased surface area leads to faster dissolution [21]. Because of the differences in surface area and diameter, this model corresponds well to drug release from MPs.

Table 2. Kinetic modeling of pure-CPL and CPL-loaded MPs (means $\pm$ SD, n = 3).

Formulation	R and K value of Kinetic model										
	Zero-order		First-order		Higuchi		Krosmeyer-Peppas			Hixson-Crowell	
	R	K	R	K	R	K	R	K	n	R	K
Pure CPL	0.9814	0.015	0.7495	0.000	0.7623	0.029	0.9658	0.007	0.001	0.91877	0.000
CPL MPs	0.8481	0.005	0.6279	0.000	0.8677	0.018	0.2097	0.006	0.001	0.9642	0.000

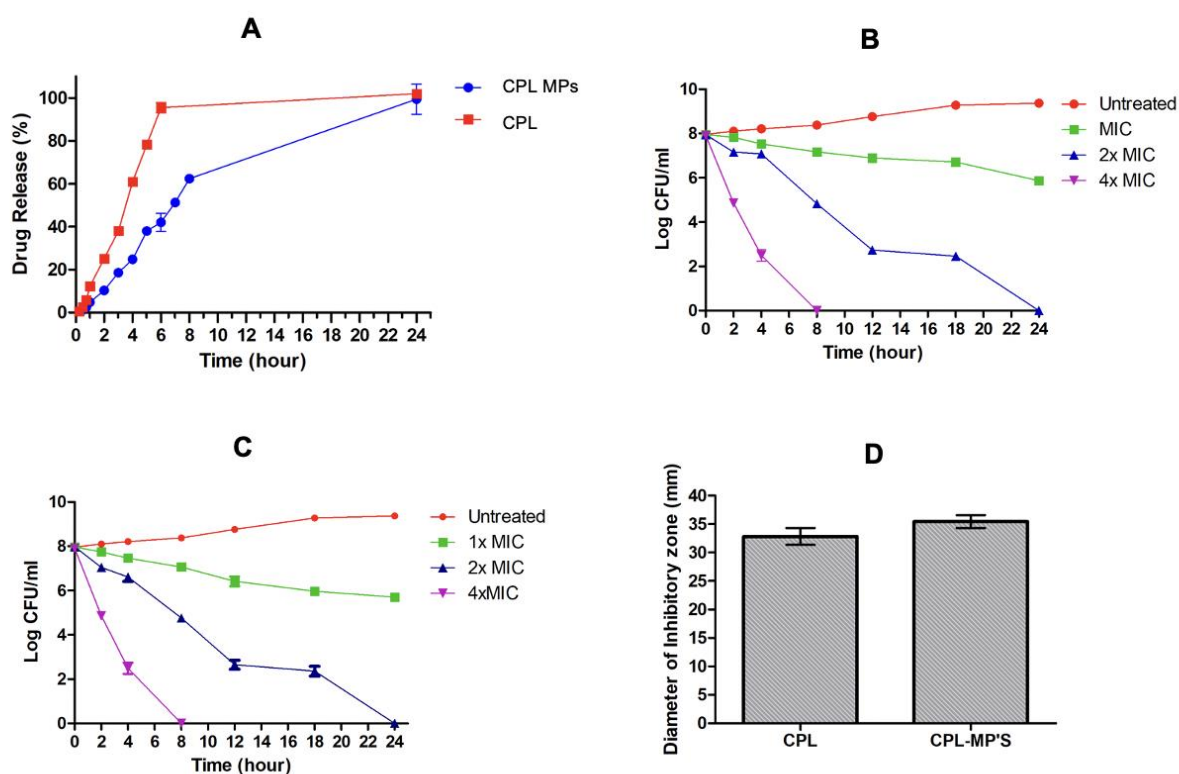


Figure 5. *In vitro* release profiles of CPL-loaded MPs in comparison with the pure CPL (A). Time-kill assay of CPL-loaded MPs (B) and pure CPL (C) against *Staphylococcus aureus* (means $\pm$ SD, n = 3). Diameter of inhibitory zone of CPL-loaded MPs against *Staphylococcus aureus* (means $\pm$ SD, n = 3) (D).

3.9 Determination of MIC and MBC

This work compared the bactericidal properties of pure CPL and CPL MPs. The MIC and MBC values of CPL MPs and pure CPL are 12.5 μ g/mL and 25 μ g/mL, respectively. The result shows

that the MIC value at the lowest concentration indicates strong antibacterial activity. Next, the MBC value is determined. The MBC value was greater than the MIC value, indicating that a larger concentration of CPL was required to kill bacterial cultures. Moreover, MIC values of samples were lower than MBC values. The ratio of MBC to MIC is < 4 . A ratio of 4 indicates bactericidal activity, and a ratio > 4 shows bacteriostatic activity [53]. Accordingly, a higher concentration of CPL MPs was required to kill the SA. Our study also suggests that CPL should be in solution form or MPs formulation to exhibit bactericidal activity.

The results showed that the inhibition zones of pure CPL and CPL MPs were 35.45 ± 1.14 mm and 32.82 ± 1.48 mm, respectively (Figure 6C). This might be because the substance disperses in the media, improving its capacity to penetrate the SA cell membrane. Previous research has shown that CPL prevents bacterial ribosomes from producing peptidyl transferase. The 50S ribosomal subunit's 23S rRNA contains residues A2451 and A2452, which this activity preferentially binds to [54]. CPL's ability to successfully permeate SA cell membranes may have contributed to its strong antibacterial action against SA when used in solution form. Notably, our control study using DMSO alone did not show antibacterial activity. Moreover, the formulation in the form of MPs showed higher antibacterial activity compared with CPL solutions. This may be due to the increased water solubility of CPL once in the MPs, enhancing CAP's capacity to efficiently permeate SA cell membranes.

3.10 Time–kill assay

The time–kill assay was performed to examine the time required for the CPL to completely kill the SA. Time–kill curves of free CPL and CPL MPs in DMSO versus SA are depicted in Figure 5B–C. The viable SA colony-forming unit increased by about 9.37 ± 0.05 log CFU/ml after 24 hours of cultivation in the control (untreated) group. With MIC values, CPL solutions and CPL MPs were unable to kill 99.99% of SA cultures after 24 hours. When 2x MIC values were observed, no feasible SA cultures were found after 24 hours in the case of either CPL solutions or CAP MPs. Furthermore, the time needed to eliminate 100% of CA cultures was decreased to 8 hours after incubation with 4x MIC values of both CPL and CPL MPs solutions. The results achieved in this work indicate that the killing rate of CPL in the microparticle-based formulation is based on how much CPL is present in the bacterial culture medium.

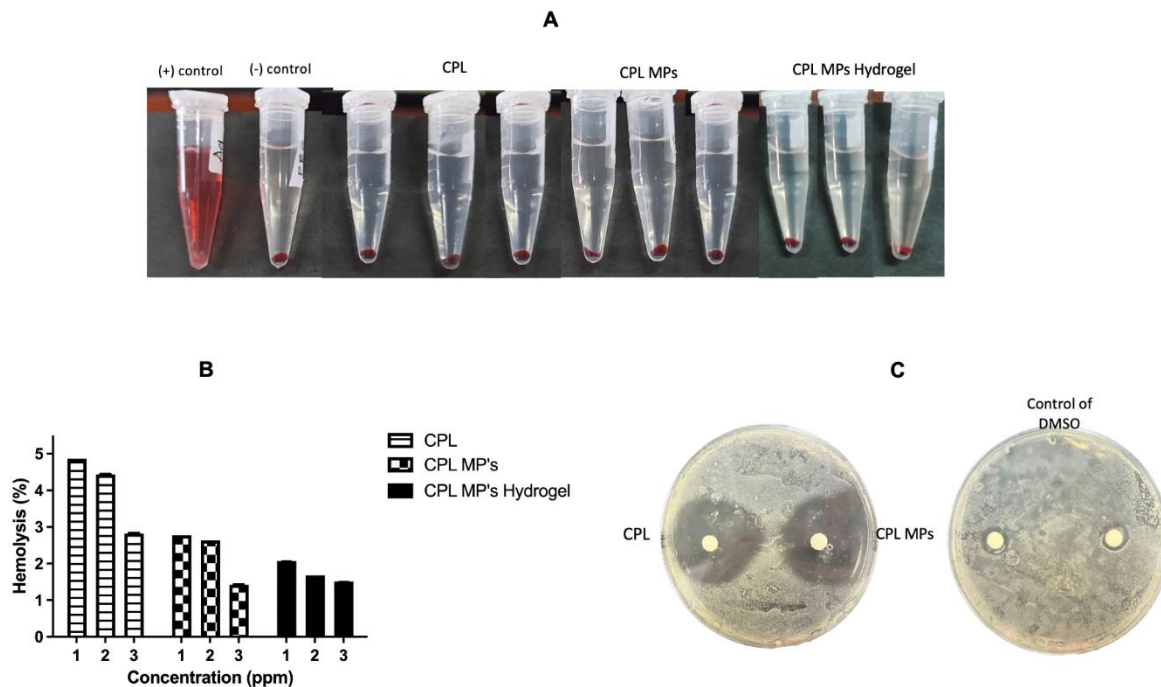


Figure 6. Hemolysis result of CPL Mps hydrogel, CPL-loaded MPs and pure CPL with concentrations of 500, 50 and 5 $\mu\text{g/mL}$ (Aquadest and PBS as positive and negative controls, respectively) (A). Hemolysis percentage of CPL-loaded MPs and pure CPL (B). Inhibitory zone of CPL-loaded MPs against *Staphylococcus aureus* compared with control (means $\pm$ SD, n = 3) (C).

3.11 Hemolysis test

The cytotoxicity of chemicals on RBCs can be quickly assessed using the hemolysis test. These RBCs will burst open entirely in the presence of water, releasing their hemoglobin content. In a similar vein, changes in osmotic pressure on the surface of RBCs caused by exposure to external elements can cause significant lysis. When RBCs are damaged or lysed, they emit adenosine diphosphate, which stimulates platelet assembly on the foreign body surface, speeds up the coagulation and thrombotic cascade pathways, and disrupts the body's natural ability to heal the wound [55]. Therefore, a good wound dressing membrane should not compromise the coagulation pathway or the integrity of the circulating RBC. By spectrophotometrically lysing RBC at 540 nm, the hemolytic potential of CPL MPs and CPL MPs hydrogel was calculated. As shown in Figures. 6A–B, different concentrations of CPL MPs (5–500 $\mu\text{g/mL}$) showed hemolysis percentages in the range of 1.42–2.76% and CPL MPs hydrogel in the range of 1.51–2.07%, whereas pure CPL showed a higher hemolysis percentage of 2.82–4.86%. According to the standard, materials exhibiting hemolysis

greater than 5% are thought to be hemolytic, those between 2 to 5% are thought to be slightly hemolytic, and those less than 2% are thought to be non-hemolytic [20]. From the results above, it can be concluded that the fabrication in the form of CPL-loaded MPs is compatible with RBCs because it consists of natural polymer.

3.12 Characteristics of hydrogel

3.12.1 pH measurement

Because the application of CPL MPs hydrogel should not irritate the skin, the pH of the formulation is very important. The pH of the hydrogel surface for each formula is in the range of 5.45 ± 0.04 to 5.55 ± 0.01 , as shown in Figure 7B, which is very close to normal skin pH of 5 to 6 [56], indicating that the formulation will be tolerated by the skin and will not interfere with the wound healing process.

3.12.2 Viscosity and rheological evaluation

Viscosity evaluation in this study used a Brookfield viscometer with spindle 7. The viscosity of CPL MPs hydrogel preparation can be seen in Figure 7E, which shows that the viscosity of preparations obtained in this study for F1 to F5, respectively, were 2266.66 ± 230.94 cPs, 3200 ± 173.20 cPs, 6900 ± 173.21 cPs, 9433.33 ± 152.75 cPs, and 23066.66 ± 832.67 cPs. Then, the viscosity test results from statistical analysis data showed that F1, F2 and F3 with respective chitosan concentrations of 1% (w/w), 1.25% (w/w) and 1.5% (w/w) have no significantly different values ($p > 0.05$). Meanwhile, the F4–F5 formulation has a significantly different value ($p < 0.05$) from F1. The good viscosity range in gels is in the field of 2000–4000 cPs, and other studies state that the viscosity of gels can reach up to 16000 cPs; therefore, F1, F2 and F3 are within the required viscosity range [57,58]. More importantly, chitosan has an intrinsic viscosity of 1.2×10^5 to 3.2×10^5 Da with molecular weight (MW) in the range of 3.2 to 6.52 dL/g [59], in which the amount of chitosan concentration in the formulation can increase the value of hydrogel viscosity. Based on the results of rheological measurements (Figure 7G), all sample flow behaviors exhibited non-Newtonian or pseudoplastic behavior ($n < 1$). Pseudoplastic flow is a viscosity flow that continues to decrease as the rate of shear increases. Thus, it can be estimated that an Increase in spreadability will occur at higher shear speeds or after dilution with liquids [57].

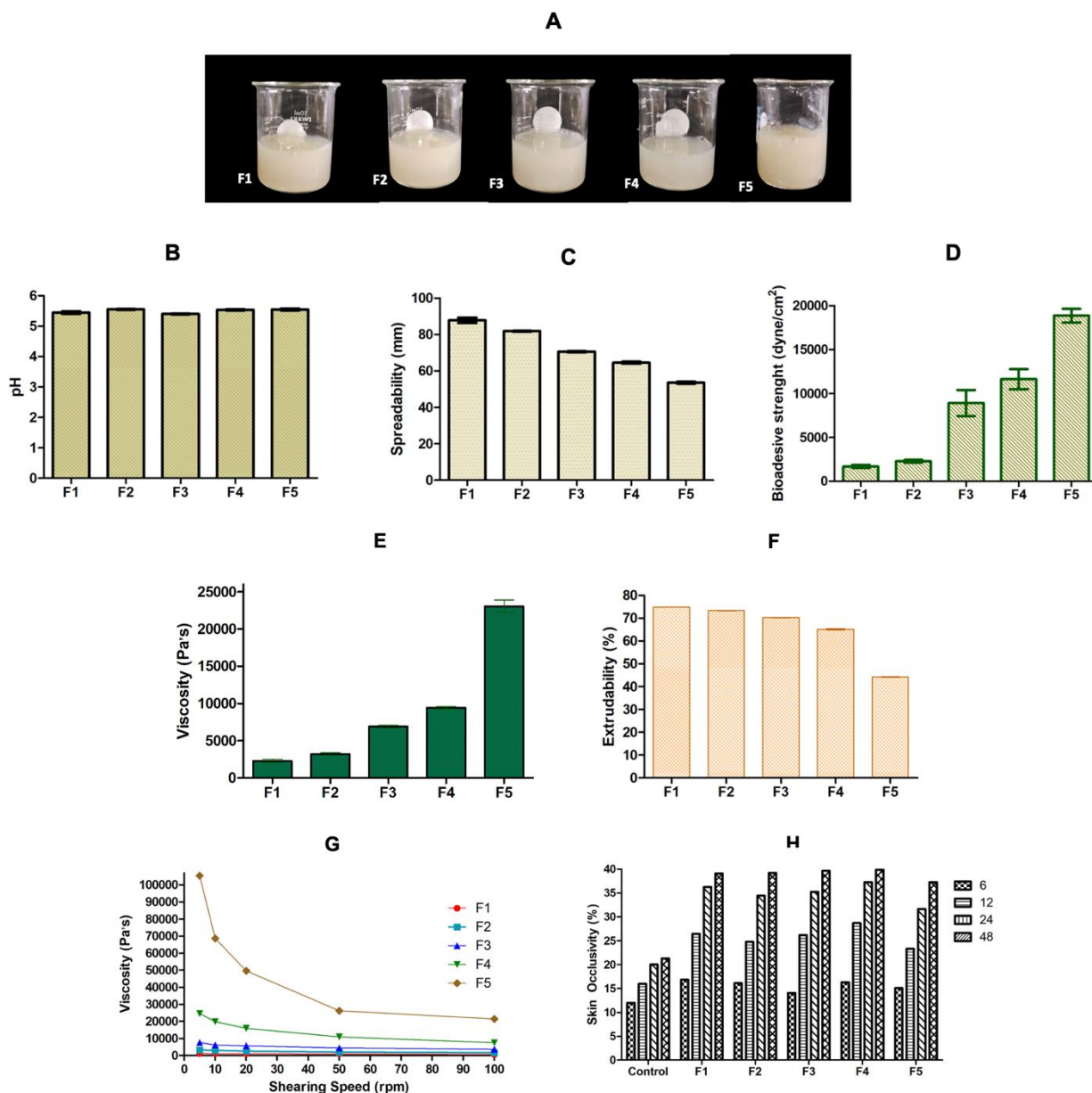


Figure 7. Formulation of CPL MPs Hydrogel with variation concentration of chitosan (F1: 1%; F2: 1,25%; F3: 1,5%; F4: 1,75%; F5: 2%) (A). pH (B), Spreadability (C), Bioadhesive strength (D), Viscosity (E), Extrudability (F), Rheology (G) and Skin occlusivity (H) of CPL MPs hydrogel (means $\pm$ SD, n = 3).

3.12.3 Spreadability

Poor spreadability can cause inhomogeneous distribution of particles, which can in turn affect the amount of dose applied as well as the efficiency of the active ingredients penetrating the skin [60]. The spreadability of hydrogel preparation was determined by the hydrogel's ability to spread on the

skin's surface. Good spreadability can ensure optimal distribution of hydrogel when applied to the skin. The spread test results of CPL MPs Hydrogel preparations reveal a value between 53.52 ± 0.50 mm to 87.84 ± 1.45 mm (Figure 6C), which indicates that CPL MPs hydrogel has good spreadability. The results of the ANOVA analysis show that the spreadability of CPL MPs hydrogel is not significant between all formulations ($p > 0.05$), with the exception of F4 and F5 to F1. Spreadability is inversely proportional to viscosity [61]. Good spreadability of the preparation is in the range of 50–70 mm; therefore F3, F4 and F5 are qualifying criteria, and F3 is the most optimal formulation. The results of the measurement in Figure 7C show that the greater the concentration of chitosan, the smaller the spreadability of the hydrogel.

3.12.4 Skin occlusivity test

The capacity of the formulation to preserve skin moisture properties was predicted using an *in vitro* model of skin occlusivity. Over 48 hours, the percentage of occlusion factors (Figure 7H) was evaluated for each formulation (F1–F5), and a filter paper without formulation was utilized as a reference. The occlusion factor values of all hydrogel formulas showed a significant difference ($p < 0.05$) compared to the control, and chitosan represented a good occlusion factor. This indicates that incorporating CPL MPs into the hydrogel can increase the occlusive value. Due to the lowering of the corneocyte slits, higher occlusion may improve skin moisture for 48 hours, allowing drugs to penetrate to deeper layers of the skin [26]. According to statistical analysis, there was no difference between any CPL MPs hydrogel formulation's occlusive value ($p > 0.05$).

3.12.5 Extrudability percentage

Hydrogel extrusion from the tube is essential during application. A sufficient consistency is necessary to remove the gel from the tube because hydrogels with high consistency may not come out of the tube, while gels with low viscosity may flow too freely. The extrudability of all hydrogel formulations (F1–F5) is shown in Figure 7F, with values of $74.9 \pm 0.01\%$, $73.37 \pm 0.05\%$, $70.3 \pm 0.06\%$, $65.13 \pm 0.22\%$ and $44.17 \pm 0.11\%$, respectively. More than 70% of the contents of the gel formulations F1–F3 can be extruded, demonstrating their strong extrusion capacity, with the exception of F4 and F5, where only 60% of the contents could be extruded. Based on statistical analysis, F1 has no significant values to F2 and F3. Furthermore, F4 and F5 are significant to F1, F2 and F3. This indicates that the increased chitosan concentration affects the increasing viscosity, thereby reducing the ease of extrusion from the tube.

3.12.6 Bioadhesive test

Good bioadhesive properties can ensure a long retention time for the applied drug formulation to guarantee effective local drug concentrations and expected pharmacological responses. Bioadhesive measurements are found in Figure 7D, which shows that the higher the concentration of chitosan used, the higher the bioadhesive strength of the preparation with F1 (1682.36 ± 171.41 dyne/cm²), F2 (2276.13 ± 171.41 dyne/cm²), F3 (8906.59 ± 1484.43 dyne/cm²), F4 (11628.05 ± 1133.75 dyne/cm²), and F5 (18852.29 ± 771.33 dyne/cm²) value. The statistical analysis showed that F1 and F2, as well as F4 and F5, showed no significant value ($p > 0.05$). The increased flexibility of chitosan hydrogel of CPL MPs can improve the contact between the gel and the skin tissue, thereby encouraging the penetration of polymer chains into the tissue to form a strong bond, which leads to an increase in the strength of adhesion [62]. Thus, it also increases the effectiveness of the active substance in reaching the target. Therefore, in this test, the F3 formula was chosen as the optimum formula with a concentration of 1.5% (w/w) that can provide good pH, dispersion, viscosity, skin occlusivity and bioadhesiveness.

Table 3. Dermatokinetic parameters of hydrogel in infected *ex vivo* skin infection models formed by SA following the application of pure-CPL Hydrogel, and CPL MPs Hydrogel (means $\pm$ SD, n = 3).

Condition	Formula- tion	C _{max} ($\mu\text{g}/\text{cm}^3$)	T _{max} (h)	T _½ (h)	AUC (h. $\mu\text{g}/\text{cm}^3$)	MRT (h)
Skin Infection model by SA	Pure CP Hydrogel	3.58 $\pm$ 0.30	6.67 $\pm$ 0.57	35.71 $\pm$ 11.99	47.51 $\pm$ 3.32	56.22 $\pm$ 16.37
	CPL MPs hydrogel	24.44 $\pm$ 0.95	6 $\pm$ 0.00	496.50 $\pm$ 749.87	417.62 $\pm$ 4.22	719.07 $\pm$ 1082.14

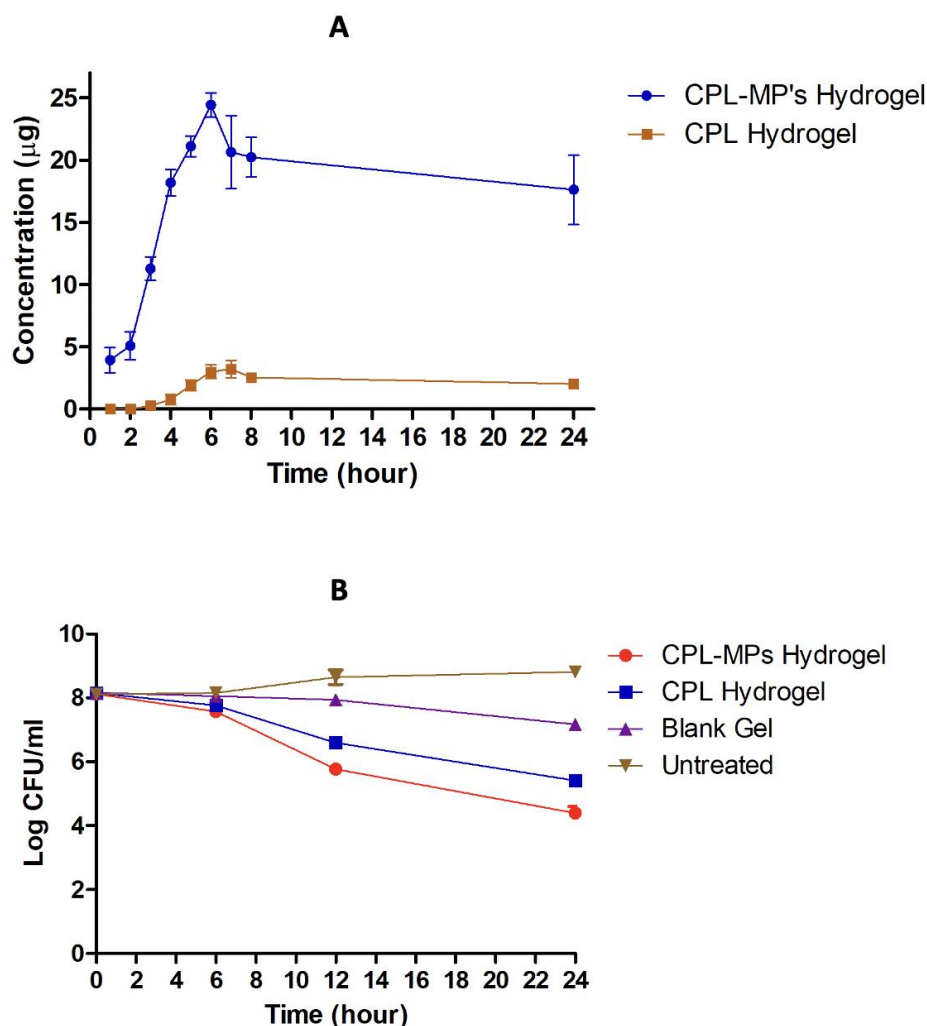


Figure 8. Dermatokinetic tests with CPL hydrogel and CPL MPs hydrogel revealed total CPL (A) (means $\pm$ SD, n = 3). Bacterial viability (log CFU/mL) in an *ex vivo* skin infection wound model (*S. aureus*) following the administration of free CPL in the form of a hydrogel, MPs hydrogel and blank hydrogel (means $\pm$ SD, n = 3) (B).

3.13 *Ex vivo* dermatokinetic study

Ex vivo dermatokinetic study was performed on non-infected rat skin models. To investigate the kinetics of the release of CPL MPs formulation, dermatokinetic studies of the hydrogel are carried out after application to the skin. To confirm that the release of CPL occurs in the skin, the skin sample was vortexed with 2 mL of methanol at each time interval. We compared the dermatokinetic profile of this study with a hydrogel containing CPL without formulation in the form of MPs as a control to assess whether the release of drugs affects the state of drug particles. Our findings in Figure 8A show that at 1 h and 2 h after measurements, no CPL was found in the pure-CPL hydrogel

supernatant and that free CPL permeated to the receiver compartment more rapidly in contrast to CPL MPs hydrogel after 2h. There was 2.05 ± 0.22 μg of CPL after 24 h, which indicates that the CPL without MPs formulation showed hydrophobic properties, making it difficult to penetrate the skin membrane. On the other hand, measurements at 1 h to 24 h show a CPL found in the CPL MPs hydrogel supernatant with an optimal concentration of 24.43 ± 0.95 μg at 6 h. This is in line with the main objective of this study, i.e., to deliver CPL MPs to the local target of the first layer of skin, where SA colonizes, infecting the skin locally.

The concentration of CPL released from the MPs form in the skin vs. time of administration is depicted as the kinetic profile of hydrogel in normal skin. Table 3 shows the dermatokinetic profile of CPL MPs hydrogel and pure-CPL hydrogel after application, including C_{max} , T_{max} , $T_{1/2}$, AUC , and MRT . The results also showed that the CPL content in dermal layers obtained pure-CPL hydrogel was remarkably lower compared with that of CPL MPs hydrogel. The C_{max} (24.44 ± 0.95 g/cm^3) was reached in 6.00 h, according to the kinetic profile of CPL MPs. In comparison to CPL MPs hydrogel, the profile of pure-CPL hydrogel had a lower C_{max} (3.58 ± 0.30 g/cm^3 in 6.67 ± 0.57 h), but the differences were not statistically significant ($p > 0.05$). Additionally, the C_{max} for both the pure-CPL hydrogel and the CPL MPs hydrogel in the dermal layers was reached in the same T_{max} . The findings show that, in comparison to pure-CPL hydrogel, CPL MPs hydrogel can deliver much higher concentrations of CPL to the skin. The great *ex vivo* skin bioavailability of the microparticle method is shown by the AUC value of CPL MPs hydrogel, which was found to be substantially higher ($p < 0.05$) than that of pure-CPL hydrogel. It was discovered that the MRT value of CPL MPs hydrogel was considerably different from the pure-CPL hydrogel ($p < 0.05$) in terms of retention duration in the skin. A high MRT value can be advantageous for reducing the application time of CPL MPs hydrogels in treating skin infections associated with local infection formation [29]. This reduced application time may increase patients' acceptance of (and compliance with) this approach. Our results conclude that a responsive CPL MPs hydrogel approach can successfully deliver MPs into *ex vivo* infection models.

3.14 *Ex vivo* antibacterial activity test using infection skin model

To test the efficiency of CPL MPs hydrogel as an antibacterial agent, researchers used an *ex vivo* skin infection model. We sought to determine whether CPL MPs hydrogel could reduce bacterial bioburdens produced from SA by counting cells' log CFU/ml number. This test was carried out on

CPL MPs hydrogel, pure-CPL hydrogel, blank hydrogel without CPL, and an untreated sample as a control. The test results are found in Figure 8B. In *ex vivo* skin infections, the highest antibacterial activity was achieved after applying CPL MPs hydrogels compared to pure-CPL hydrogel, as measured by the reduction of bacterial bioburden after 24 hours. A value of 8.22 ± 0.11 log CFU/ml to 8.81 ± 0.18 log CFU/ml increase in bacterial bioburden was seen in untreated samples, which indicates the validity of the control sample. Blank hydrogel without drugs showed a value of 8.16 ± 0.04 log CFU/ml to 7.11 ± 0.07 log CFU/ml decrease in bioburden properties. The antibacterial activity in the blank hydrogel was due to the ability of chitosan as a natural polymer to inhibit microorganisms, as explained in a previous study [18].

Analysis of *ex vivo* antibacterial activity showed an enhancement of 8.02 ± 0.10 log CFU/ml to 4.40 ± 0.20 log CFU/ml after 24 hours when CPL is incorporated into MPs in hydrogel form. This indicates that chitosan hydrogel may enhance the antibacterial activity of CPL MPs. Incorporating antibacterial compounds into chitosan hydrogels has been shown to boost their antibacterial activity in some experiments [18,36,63]. Furthermore, dermatokinetic experiments demonstrated the bioavailability of CPL MPs hydrogel in *ex vivo* skin, which is why this method was successful in eliminating 99.98% SA infections. Therefore, there are two primary benefits associated with the delivery of CPL-loaded MPs in chitosan hydrogel. First, this approach can control the dermatokinetic profile of CPL MPs. Second, this approach may improve the efficacy of CPL MPs hydrogels in *ex vivo* infection models of rat skin formed by SA compared to CPL without MPs hydrogel formulations. The encouraging findings of this study have validated the study's premise, demonstrating that CPL can be efficiently transported to the skin utilizing MPs, and then, once incorporated into the chitosan hydrogel formula, can exhibit its antimicrobial action to target infection. To improve the antibacterial efficacy of wound therapy, our delivery system's key advantage lies in its selective delivery ability to locally target as well as its long retention period in areas of infection on the skin. The combination of MPs with chitosan hydrogels can selectively be a feasible alternative to existing wound infection care, as MPs are employed for the topical administration of antimicrobial drugs to wounds.

4. Conclusion

Wound treatments using dermal delivery system are now receiving significant attention as potential for staving off infection. In this study, hydrogels are made from chitosan polymers in which the

MPs of whey protein containing CPL are embedded inside the system. CPL-loaded MPs showed desired physical and encapsulation characteristics with particle sizes in the range of $0.56 \pm 0.13 \mu\text{m}$ to $35.71 \pm 5.23 \mu\text{m}$ and EE value of $68.78 \pm 1.40\%$. The drug release behavior of CPL from CPL MPs formulation showed a value of $99.40 \pm 7.01\%$ after 24 hours, and then the cytotoxicity of MPs chemicals on RBCs can be quickly assessed using the hemolysis test. Essentially, analysis of *ex vivo* antibacterial activity showed an enhancement of 99.98% after 24 hours when CPL was incorporated into MPs in hydrogel form. Compared to pure-CPL hydrogel, the combination delivery method developed in this study has a higher residence duration in the skin, which may increase the effectiveness of antibacterial treatment in skin wound infections. It appears likely that hydrogels created using this technique can be utilized to heal wounds successfully. To further examine the efficacy of this technique, *in vivo* investigations on suitable animal models should be conducted.

Acknowledgement

Tri Puspita Roska expresses gratitude to Deutscher Akademischer Austausch Dienst (DAAD) for providing the scholarship. The Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia also provided funding for this study under contract number 090/E5/PG.02.00/PT/2022. The authors thank Glanbia Nutritionals, Inc, Fitchburg, USA for providing whey protein (WPI).

Conflict of interest

The authors state that they have no conflicts of interest.

References

- [1] J.F. Guest, N. Ayoub, T. McIlwraith, I. Uchegbu, A. Gerrish, D. Weidlich, K. Vowden, P. Vowden, Health economic burden that different wound types impose on the UK's National Health Service, *Int Wound J.* 14 (2017) 322–330. <https://doi.org/10.1111/iwj.12603>.
- [2] J.E. Cefalu, K.M. Barrier, A.H. Davis, Wound Infections in Critical Care, *Crit Care Nurs Clin North Am.* 29 (2017) 81–96. <https://doi.org/10.1016/j.cnc.2016.09.009>.
- [3] J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G.M. Eccleston, Wound healing dressings and drug delivery systems: A review, *J Pharm Sci.* 97 (2008) 2892–2923. <https://doi.org/10.1002/jps.21210>.

- 767 [4] S.G. Ingebrigtsen, N. Škalko-Basnet, A.M. Holsæter, Development and optimization of a
768 new processing approach for manufacturing topical liposomes-in-hydrogel drug formula-
769 tions by dual asymmetric centrifugation, *Drug Dev Ind Pharm.* 42 (2016) 1375–1383.
770 <https://doi.org/10.3109/03639045.2015.1135940>.
- 771 [5] D.A. Kuznetsova, D.R. Gabdrakhmanov, G.A. Gaynanova, L.A. Vasileva, D.M. Kuznetsov,
772 S.S. Lukashenko, A.D. Voloshina, A.S. Sapunova, I.R. Nizameev, G. v. Sibgatullina, D. v.
773 Samigullin, M.K. Kadirov, K.A. Petrov, L.Y. Zakharova, Novel biocompatible liposomal
774 formulations for encapsulation of hydrophilic drugs – Chloramphenicol and cisplatin, *Col-
775 loids Surf A Physicochem Eng Asp.* 610 (2021).
776 <https://doi.org/10.1016/j.colsurfa.2020.125673>.
- 777 [6] S.I. Miller, Antibiotic resistance and regulation of the Gram-negative bacterial outer mem-
778 brane barrier by host innate immune molecules, *MBio.* 7 (2016).
779 <https://doi.org/10.1128/mBio.01541-16>.
- 780 [7] A.D. Permana, Q.K. Anjani, Sartini, E. Utomo, F. Volpe-Zanutto, A.J. Paredes, Y.M. Evary,
781 S.A. Mardikasari, Muh.R. Pratama, I.N. Tuany, R.F. Donnelly, Selective delivery of silver
782 nanoparticles for improved treatment of biofilm skin infection using bacteria-responsive mi-
783 croparticles loaded into dissolving microneedles, *Mat Science & Eng C.* 120 (2021) 111786.
784 <https://doi.org/10.1016/j.msec.2020.111786>.
- 785 [8] S. Yasasvini, R.S. Anusa, B.N. VedhaHari, P.C. Prabhu, D. RamyaDevi, Topical hydrogel
786 matrix loaded with Simvastatin microparticles for enhanced wound healing activity, *Mat Sci-
787 ence & Eng C.* 72 (2017) 160–167. <https://doi.org/10.1016/j.msec.2016.11.038>.
- 788 [9] X. Delabranche, A. Berger, J. Boisramé-Helms, F. Meziani, Microparticles and infectious
789 diseases, *Med Mal Infect.* 42 (2012) 335–343. [https://doi.org/10.1016/j.med-](https://doi.org/10.1016/j.med-mal.2012.05.011)
790 [mal.2012.05.011](https://doi.org/10.1016/j.med-mal.2012.05.011).
- 791 [10] M. Lengyel, N. Kállai-Szabó, V. Antal, A.J. Laki, I. Antal, Microparticles, microspheres, and
792 microcapsules for advanced drug delivery, *Sci Pharm.* 87 (2019). [https://doi.org/10.3390/sci-](https://doi.org/10.3390/sci-pharm87030020)
793 [pharm87030020](https://doi.org/10.3390/sci-pharm87030020).
- 794 [11] A. George, P.A. Shah, P.S. Shrivastav, Natural biodegradable polymers based nano-formu-
795 lations for drug delivery: A review, *Int J Pharm.* 561 (2019) 244–264.
796 <https://doi.org/10.1016/j.ijpharm.2019.03.011>.
- 797 [12] A.R. Madureira, C.I. Pereira, A.M.P. Gomes, M.E. Pintado, F. Xavier Malcata, Bovine whey
798 proteins – Overview on their main biological properties, *Food Research Intern.* 40 (2007)
799 1197–1211. <https://doi.org/10.1016/j.foodres.2007.07.005>.
- 800 [13] M.A. Farooq, M. Aquib, S. Ghayas, R. Bushra, D. Haleem Khan, A. Parveen, B. Wang,
801 Whey protein: A functional and promising material for drug delivery systems recent devel-
802 opments and future prospects, *Polym Adv Technol.* 30 (2019) 2183–2191.
803 <https://doi.org/10.1002/pat.4676>.

- 804 [14] Z. Liu, C. Liu, X. Sun, S. Zhang, Y. Yuan, D. Wang, Y. Xu, Fabrication and characterization
805 of cold-gelation whey protein-chitosan complex hydrogels for the controlled release of cur-
806 cumin, *Food Hydrocoll.* 103 (2020). <https://doi.org/10.1016/j.foodhyd.2019.105619>.
- 807 [15] A. Aliyah, W.W. Oktaviana, K.S. Dwipayanti, A.P. Erdiana, R.N. Utami, A.D. Permana,
808 Enhanced skin localization of doxycycline using microparticles and hydrogel: Effect of oleic
809 acid as penetration enhancer, *Pharmaciana.* 11 (2021) 239. <https://doi.org/10.12928/pharmacia.v11i2.21044>.
810
- 811 [16] C. Xiao, R. You, Y. Fan, Y. Zhang, Tunable functional hydrogels formed from a versatile
812 water-soluble chitosan, *Int J Biol Macromol.* 85 (2016) 386–390.
813 <https://doi.org/10.1016/j.ijbiomac.2016.01.006>.
- 814 [17] K. Mohan, A.R. Ganesan, T. Muralisankar, R. Jayakumar, P. Sathishkumar, V. Uthaya-
815 kumar, R. Chandirasekar, N. Revathi, Recent insights into the extraction, characterization,
816 and bioactivities of chitin and chitosan from insects, *Trends Food Sci Technol.* 105 (2020)
817 17–42. <https://doi.org/10.1016/j.tifs.2020.08.016>.
- 818 [18] M.E. Abd El-Hack, M.T. El-Saadony, M.E. Shafi, N.M. Zabermawi, M. Arif, G.E. Batiha,
819 A.F. Khafaga, Y.M. Abd El-Hakim, A.A. Al-Sagheer, Antimicrobial and antioxidant prop-
820 erties of chitosan and its derivatives and their applications: A review, *Int J Biol Macromol.*
821 164 (2020) 2726–2744. <https://doi.org/10.1016/j.ijbiomac.2020.08.153>.
- 822 [19] A. Jain, G. Sharma, G. Ghoshal, P. Kesharwani, B. Singh, U.S. Shivhare, O.P. Katore, Ly-
823 copen loaded whey protein isolate nanoparticles: An innovative endeavor for enhanced bi-
824 oavailability of lycopene and anti-cancer activity, *Int J Pharm.* 546 (2018) 97–105.
825 <https://doi.org/10.1016/j.ijpharm.2018.04.061>.
- 826 [20] M.M. Agwa, S. Sabra, N.A. Atwa, H.A. Dahdooh, R.M. Lithy, H. Elmotasem, Potential of
827 frankincense essential oil-loaded whey protein nanoparticles embedded in frankincense resin
828 as a wound healing film based on green technology, *J Drug Deliv Sci Technol.* 71 (2022)
829 103291. <https://doi.org/10.1016/j.jddst.2022.103291>.
- 830 [21] C. Bradford, R. Freeman, S.L. Percival, In Vitro Study of Sustained Antimicrobial Activity
831 of a New Silver Alginate Dressing, *J Am Col Certif Wound Spec.* 1 (2009) 117–120.
832 <https://doi.org/10.1016/j.jcws.2009.09.001>.
- 833 [22] B.B.K. Reddy, K.E.V. Nagoji, S. Sahoo, Preparation and in vitro & in vivo evaluation
834 of cephalexin matrix tablets, *Braz J Pharm Sci.* 54 (2018). <https://doi.org/10.1590/s2175-97902018000317277>.
835
- 836 [23] A.D. Permana, A.J. Paredes, F. Volpe-Zanutto, Q.K. Anjani, E. Utomo, R.F. Donnelly, Dis-
837 solving microneedle-mediated dermal delivery of itraconazole nanocrystals for improved
838 treatment of cutaneous candidiasis, *Euro J Pharm and Biopharm.* 154 (2020) 50–61.
839 <https://doi.org/10.1016/j.ejpb.2020.06.025>.

- 840 [24] A.D. Permana, M. Mir, E. Utomo, R.F. Donnelly, Bacterially sensitive nanoparticle-based
841 dissolving microneedles of doxycycline for enhanced treatment of bacterial biofilm skin in-
842 fection: A proof of concept study, *Int J Pharm* X. 2 (2020) 100047.
843 <https://doi.org/10.1016/j.ijpx.2020.100047>.
- 844 [25] M. Mir, A.D. Permana, I.A. Tekko, H.O. McCarthy, N. Ahmed, Asim. ur. Rehman, R.F.
845 Donnelly, Microneedle liquid injection system assisted delivery of infection responsive na-
846 noparticles: A promising approach for enhanced site-specific delivery of carvacrol against
847 polymicrobial biofilms-infected wounds, *Int J Pharm.* 587 (2020) 119643.
848 <https://doi.org/10.1016/j.ijpharm.2020.119643>.
- 849 [26] A.D. Permana, R.N. Utami, A.J. Courtenay, M.A. Manggau, R.F. Donnelly, L. Rahman,
850 Phytosomal nanocarriers as platforms for improved delivery of natural antioxidant and pho-
851 toprotective compounds in propolis: An approach for enhanced both dissolution behaviour
852 in biorelevant media and skin retention profiles, *J Photochem Photobiol B.* 205 (2020)
853 111846. <https://doi.org/10.1016/j.jphotobiol.2020.111846>.
- 854 [27] U.C. Galgatte, A.B. Kumbhar, P.D. Chaudhari, Development of *in situ* gel for nasal delivery:
855 design, optimization, *in vitro* and *in vivo* evaluation, *Drug Deliv.* 21 (2014) 62–73.
856 <https://doi.org/10.3109/10717544.2013.849778>.
- 857 [28] A.D. Permana, I.A. Tekko, M.T.C. McCrudden, Q.K. Anjani, D. Ramadan, H.O. McCarthy,
858 R.F. Donnelly, Solid lipid nanoparticle-based dissolving microneedles: A promising intra-
859 dermal lymph targeting drug delivery system with potential for enhanced treatment of lym-
860 phatic filariasis, *J of Control Release.* 316 (2019) 34–52. <https://doi.org/10.1016/j.jconrel.2019.10.004>.
- 862 [29] A.D. Permana, R.N. Utami, P. Layadi, A. Himawan, N. Juniarti, Q.K. Anjani, E. Utomo,
863 S.A. Mardikasari, A. Arjuna, R.F. Donnelly, Thermosensitive and mucoadhesive *in situ* oc-
864 ular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the
865 treatment of fungal keratitis, *Int J Pharm.* 602 (2021) 120623.
866 <https://doi.org/10.1016/j.ijpharm.2021.120623>.
- 867 [30] Q. Liu, Y. Sun, J. Cheng, M. Guo, Development of whey protein nanoparticles as carriers to
868 deliver soy isoflavones, *LWT.* 155 (2022) 112953.
869 <https://doi.org/10.1016/j.lwt.2021.112953>.
- 870 [31] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G.D.-O.C. Mateo, R. Fer-
871 nández-Lafuente, J.M. Guisán, Different mechanisms of protein immobilization on glutaral-
872 dehyde activated supports: Effect of support activation and immobilization conditions, *En-
873 zyme Microb Technol.* 39 (2006) 877–882. <https://doi.org/10.1016/j.enzmictec.2006.01.014>.
- 874 [32] C. le Bourvellec, C.M.G.C. Renard, Interactions between Polyphenols and Macromolecules:
875 Quantification Methods and Mechanisms, *Crit Rev Food Sci Nutr.* 52 (2012) 213–248.
876 <https://doi.org/10.1080/10408398.2010.499808>.

- 877 [33] C. Wang, X. Zhou, H. Wang, X. Sun, M. Guo, Interactions between β -Lactoglobulin and
878 3,3'-Diindolylmethane in Model System, *Molecules*. 24 (2019) 2151.
879 <https://doi.org/10.3390/molecules24112151>.
- 880 [34] A. Abbasi, Z. Emam-Djomeh, M.A.E. Mousavi, D. Davoodi, Stability of vitamin D3 encapsulated in nanoparticles of whey protein isolate, *Food Chem.* 143 (2014) 379–383.
881 <https://doi.org/10.1016/j.foodchem.2013.08.018>.
- 883 [35] A. Khan, C. Wang, X. Sun, A. Killpartrick, M. Guo, Preparation and Characterization of
884 Whey Protein Isolate–DIM Nanoparticles, *Int J Mol Sci.* 20 (2019) 3917.
885 <https://doi.org/10.3390/ijms20163917>.
- 886 [36] W. Zhang, J. Chen, Y. Chen, W. Xia, Y.L. Xiong, H. Wang, Enhanced physicochemical
887 properties of chitosan/whey protein isolate composite film by sodium laurate-modified TiO₂
888 nanoparticles, *Carbohydr Polym.* 138 (2016) 59–65. <https://doi.org/10.1016/j.carbpol.2015.11.031>.
- 890 [37] B. Vardhanabhuti, M.A. Kelly, P.J. Luck, M.A. Drake, E.A. Foegeding, Roles of charge interactions on astringency of whey proteins at low pH, *J Dairy Sci.* 93 (2010) 1890–1899.
891 <https://doi.org/10.3168/jds.2009-2780>.
- 893 [38] I. Evdokimov, L. Alieva, V. Varlamov, V. Kurchenko, V.- Haritonov, T. Butkevich, USAGE
894 OF CHITOSAN IN DAIRY PRODUCTS PRODUCTION, *Food and Raw Mat.* 3 (2015) 29–
895 39. <https://doi.org/10.12737/13117>.
- 896 [39] M. Li, Y. Ma, M.O. Ngadi, Binding of curcumin to β -lactoglobulin and its effect on antioxidant characteristics of curcumin, *Food Chem.* 141 (2013) 1504–1511.
897 <https://doi.org/10.1016/j.foodchem.2013.02.099>.
- 899 [40] Y. Liu, L. Gao, J. Yi, Y. Fan, X. Wu, Y. Zhang, α -Lactalbumin and chitosan core-shell
900 nanoparticles: resveratrol loading, protection, and antioxidant activity, *Food Funct.* 11 (2020)
901 1525–1536. <https://doi.org/10.1039/C9FO01998G>.
- 902 [41] A. Jain, G. Sharma, G. Ghoshal, P. Kesharwani, B. Singh, U.S. Shivhare, O.P. Katore, Lycopene loaded whey protein isolate nanoparticles: An innovative endeavor for enhanced bioavailability of lycopene and anti-cancer activity, *Int J Pharm.* 546 (2018) 97–105.
903 <https://doi.org/10.1016/j.ijpharm.2018.04.061>.
- 906 [42] Y. Zhang, M. Zhao, Z. Ning, S. Yu, N. Tang, F. Zhou, Development of a Sono-Assembled, Bifunctional Soy Peptide Nanoparticle for Cellular Delivery of Hydrophobic Active Cargoes, *J Agric Food Chem.* 66 (2018) 4208–4218. <https://doi.org/10.1021/acs.jafc.7b05889>.
- 909 [43] R.V. de B. Fernandes, S.V. Borges, D.A. Botrel, C.R. de Oliveira, Physical and chemical properties of encapsulated rosemary essential oil by spray drying using whey protein-inulin blends as carriers, *Int J Food Sci Technol.* 49 (2014) 1522–1529.
910 <https://doi.org/10.1111/ijfs.12449>.

- 913 [44] X. Shen, C. Zhao, J. Lu, M. Guo, Physicochemical Properties of Whey-Protein-Stabilized
914 Astaxanthin Nanodispersion and Its Transport via a Caco-2 Monolayer, *J Agric Food Chem.*
915 66 (2018) 1472–1478. <https://doi.org/10.1021/acs.jafc.7b05284>.
- 916 [45] Y. Luo, Z. Teng, Q. Wang, Development of Zein Nanoparticles Coated with Carboxymethyl
917 Chitosan for Encapsulation and Controlled Release of Vitamin D3, *J Agric Food Chem.* 60
918 (2012) 836–843. <https://doi.org/10.1021/jf204194z>.
- 919 [46] Z. Teng, Y. Luo, Q. Wang, Carboxymethyl chitosan–soy protein complex nanoparticles for
920 the encapsulation and controlled release of vitamin D3, *Food Chem.* 141 (2013) 524–532.
921 <https://doi.org/10.1016/j.foodchem.2013.03.043>.
- 922 [47] A. Goyal, V. Sharma, M.K. Sihag, S.K. Tomar, S. Arora, L. Sabikhi, A.K. Singh, Develop-
923 ment and physico-chemical characterization of microencapsulated flaxseed oil powder: A
924 functional ingredient for omega-3 fortification, *Powder Technol.* 286 (2015) 527–537.
925 <https://doi.org/10.1016/j.powtec.2015.08.050>.
- 926 [48] C. Wang, X. Zhou, H. Wang, X. Sun, M. Guo, Interactions between β -Lactoglobulin and
927 3,3'-Diindolylmethane in Model System, *Molecules.* 24 (2019) 2151.
928 <https://doi.org/10.3390/molecules24112151>.
- 929 [49] C.A. Busatto, M.E. Taverna, M.R. Lescano, C. Zalazar, D.A. Estenoz, Preparation and Char-
930 acterization of Lignin Microparticles-in-Alginate Beads for Atrazine Controlled Release, *J*
931 *Polym Environ.* 27 (2019) 2831–2841. <https://doi.org/10.1007/s10924-019-01564-2>.
- 932 [50] M. Schneider, F. Stracke, S. Hansen, U.F. Schaefer, Nanoparticles and their interactions with
933 the dermal barrier, *Dermatoendocrinol.* 1 (2009) 197–206.
934 <https://doi.org/10.4161/derm.1.4.9501>.
- 935 [51] P.-E. Lee, W.-S. Choo, Characterization of flaxseed oil emulsions, *J Food Sci Technol.* 52
936 (2015) 4378–4386. <https://doi.org/10.1007/s13197-014-1495-3>.
- 937 [52] N. Kamaly, B. Yameen, J. Wu, O.C. Farokhzad, Degradable Controlled-Release Polymers
938 and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release, *Chem Rev.* 116
939 (2016) 2602–2663. <https://doi.org/10.1021/acs.chemrev.5b00346>.
- 940 [53] M. Tato, Y. López, M.I. Morosini, A. Moreno-Bofarull, F. Garcia-Alonso, D. Gargallo-Vi-
941 ola, J. Vila, R. Cantón, Characterization of variables that may influence ozenoxacin in sus-
942 ceptibility testing, including MIC and MBC values, *Diagn Microbiol Infect Dis.* 78 (2014)
943 263–267. <https://doi.org/10.1016/j.diagmicrobio.2013.11.010>.
- 944 [54] M. Abdollahi, S. Mostafalou, Chloramphenicol, in: *Encyclopedia of Toxicology*, Elsevier,
945 2014: pp. 837–840. <https://doi.org/10.1016/B978-0-12-386454-3.00709-0>.
- 946 [55] W. Yuan, Y. Feng, H. Wang, D. Yang, B. An, W. Zhang, M. Khan, J. Guo, Hemocompatible
947 surface of electrospun nanofibrous scaffolds by ATRP modification, *Materials Sci & Eng C.*
948 33 (2013) 3644–3651. <https://doi.org/10.1016/j.msec.2013.04.048>.

- 949 [56] Ó.L. Ramos, J.C. Fernandes, S.I. Silva, M.E. Pintado, F.X. Malcata, Edible Films and Coat-
 950 ings from Whey Proteins: A Review on Formulation, and on Mechanical and Bioactive Prop-
 951 erties, Crit Rev Food Sci Nutr. 52 (2012) 533–552.
 952 <https://doi.org/10.1080/10408398.2010.500528>.
- 953 [57] M.M. Ibrahim, S.A. Hafez, M.M. Mahdy, Organogels, hydrogels and bigels as transdermal
 954 delivery systems for diltiazem hydrochloride, Asian J Pharm Sci. 8 (2013) 48–57.
 955 <https://doi.org/10.1016/j.ajps.2013.07.006>.
- 956 [58] N. Harish, P. Prabhu, R. Charyulu, M. Gulzar, E.V.S. Subrahmanyam, Formulation and eval-
 957 uation of *in situ* gels containing clotrimazole for oral candidiasis, Indian J Pharm Sci. 71
 958 (2009) 421. <https://doi.org/10.4103/0250-474X.57291>.
- 959 [59] D.P. Chattopadhyay, M.S. Inamdar, Aqueous Behaviour of Chitosan, Int J Polym Sci. 2010
 960 (2010) 1–7. <https://doi.org/10.1155/2010/939536>.
- 961 [60] L. Montenegro, L. Rapisarda, C. Ministeri, G. Puglisi, Effects of Lipids and Emulsifiers on
 962 the Physicochemical and Sensory Properties of Cosmetic Emulsions Containing Vitamin E,
 963 Cosmetics. 2 (2015) 35–47. <https://doi.org/10.3390/cosmetics2010035>.
- 964 [61] D.R. Kryscio, P.M. Sathe, R. Lionberger, L. Yu, M.A. Bell, M. Jay, J.Z. Hilt, Spreadability
 965 Measurements to Assess Structural Equivalence (Q3) of Topical Formulations—A Technical
 966 Note, AAPS PharmSciTech. 9 (2008) 84–86. <https://doi.org/10.1208/s12249-007-9009-5>.
- 967 [62] X. Zhang, Y. Jiang, L. Han, X. Lu, Biodegradable polymer hydrogel- based tissue adhesives:
 968 A review, Biosurf Biotribol. 7 (2021) 163–179. <https://doi.org/10.1049/bsb2.12016>.
- 969 [63] M.O. Ilomuanya, N. v Enwuru, E. Adenokun, A. FATUNMBI, A. Adeluola, C.I. IGWILO,
 970 Title: Chitosan-based microparticle encapsulated Acinetobacter baumannii phage cocktail in
 971 hydrogel matrix for the management of multidrug resistant chronic wound infection, Turk J
 972 Pharm Sci. 0 (2021) 0–0. <https://doi.org/10.4274/tjps.galenos.2021.72547>.

973

Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Tri Puspita Roska¹, Sartini¹, Mukarram Mudjahid¹, Ardiyah Nurul Fitri Marzaman¹, Nana Novriana Payung Datu¹, Andi Dian Permana^{1*}

1. Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia

***Corresponding author:**

Andi Dian Permana

Faculty of Pharmacy, Hasanuddin University, Indonesia

Email: andi.dian.permana@farmasi.unhas.ac.id

Abstract

Skin wounds have been reported to increase the number of microbial colonies susceptible to infection. Treatments using oral antibiotics have been limited due to their toxicity and hydrophobic characteristics. In this study, we developed a formulation of chloramphenicol microparticles (CPL MPs), which was modified into chitosan hydrogel to increase treatment efficiency in targeting infections and creating an optimal environment to support the healing process. CPL MPs were prepared by a cross-linker stabilized method using whey protein (WPI) biopolymer, and the CPL MPs hydrogel was designed using chitosan biopolymer. Based on the result, CPL-loaded MPs showed desired physical and encapsulation characteristics. In the *in vitro* study, drug release of CPL MPs in simulated wound fluid represented approximately $99.40 \pm 7.01\%$ of the system after 24 h. The antibacterial activity of CPL-loaded MPs formulation (MIC value 12.5 $\mu\text{g/ml}$, MBC 25 $\mu\text{g/ml}$) was effective as MIC concentration increased. Furthermore, the formulation of CPL MPs into hydrogel showed a better dermatokinetic profile compared to hydrogel with pure CPL. Interestingly, the antibacterial activity of the *ex vivo* infection model showed that *Staphylococcus aureus* activity decreased by up to 99.98% after 24 h administration of CPL MPs hydrogel when compared to pure-CPL hydrogel and blank hydrogel. These studies have confirmed that incorporating CPL MPs into hydrogel can provide a promising approach to skin infection treatment.

Keywords: Chloramphenicol; Hydrogel; Microparticle; Skin Wounds; Whey Protein.

1. Introduction

A skin wound is a condition in which the skin has impaired integrity, anatomical structure and tissue function, and may be caused by chronic disease, burns or postoperative trauma. Skin wounds are a healthcare problem that is estimated to occur in about 4–5% of the adult population [1]. Injuries that compromise the skin's integrity allow microorganisms to colonize the area. This can lead to infection issues that can extend a patient's treatment time and necessitate the administration of more medication. The most common aerobic microorganisms causing wound infections include *S. aureus*, *S. pyogenes*, *P. aeruginosa*, MRSA, *S. epidermidis*, *Enterococcus faecalis*, coliforms, and *Acinetobacter baumannii* [2]. Wound infections can cause bacteremia, sepsis, and organ dysfunction, so an effective method is needed to prevent wound infections, one of which is the administration of appropriate topical antibiotics [3].

One antimicrobial with a broad spectrum of action is chloramphenicol (CPL), which is used for skin infections and can maintain its activity against bacterial resistance, with a bacteriostatic mechanism specifically inhibiting bacterial protein synthesis [4]. However, the effectivity of oral route of CPL is still limited due to its toxicity [5], and the physicochemical properties of hydrophobicity, make it difficult to penetrate the skin and inhibit drug transport to the target of infection [6]. This limitation can be overcome via the dermal route, which facilitates the loading of hydrophobic drugs with direct action activity to the target of infection.

Drug delivery systems targeting local infections could be developed by designing drugs into microparticles [7]. Microparticles (MPs) are widely preferred because they responsively release drugs, increase the effectiveness of the drug on the target, and provide local effects on the skin. In addition, drug-containing MPs offer high drug-loading flexibility and good physical and chemical stability [8]. MPs have also been reported to be designed for local administration with desired effects, including wound care. The MPs approach has also been widely developed for the treatment of infectious diseases to increase efficiency at selectively targeting infections through specific stimulus responses [9].

The drug substance can be incorporated into MPs through various methods such as ionotropic gelation, single or double emulsification, polymerization, and stabilization with cross-linkers [10]. MPs prepared by a cross-linker method using natural biopolymers as drug carriers offer optimal

drug loading. Hydrophobic drug encapsulation using natural biopolymers was chosen because of its biodegradability, biocompatible, and suitability for skin delivery system formulation [11]. Whey protein is a high-quality protein from cheese processing or cow's milk and is primarily consisted of major proteins including β -lactoglobulin, α -lactalbumin, immunoglobulin, and lactoferrin [12]. A study has shown that the binding of whey protein biopolymers with hydrophobic drugs can increase drug solubility and dissolution [13]. Liu et al. used whey protein for curcumin encapsulation by employing the spray-drying method to increase curcumin solubility and bioavailability [14]. This can be chosen as a strategy to increase the effectiveness of drugs in treating skin infections. However, there are limitations in using whey protein in formulations because it tends to have a weak protein bond, is easily damaged by heating, and may be susceptible to degradation. Therefore, hydrogel formulation is one of the preparations to overcome this problem.

Hydrogels are polymer networks with hydrophilic properties, which are needed in wound treatment because they have excellent biocompatibility and provide an optimal environment for wound healing. Hydrogels can be applied in various physical forms, such as nanoparticles, MPs, and films [15]. Chitosan, the natural polymers in the manufacture of hydrogels, contain many hydroxyl and amine groups typically used as functional groups to react with cross-linking agents, which stabilize the protein structure [16]. In the treatment of wound infections, there are several reported benefits of using chitosan as a biopolymer, including good antibacterial activity [17], anti-inflammatory and hemostatic properties, enhanced skin regeneration; superior biocompatibility and biodegradability; good water absorption and skin occlusivity properties; and due to the presence of amino and hydroxyl groups in the molecular chain, many biological activities can be improved by grafting on additional groups and chemical elements [17,18].

For the potential enhancement of wound infection therapy, in this study, we provide a novel combination delivery method of chitosan hydrogel and chloramphenicol microparticles (CPL MPs). CPL MPs was formed and optimized by solvent evaporation and stabilized using a cross-linker. Additionally, tests were done on drug loading, entrapment effectiveness, zeta potential, particle size, polydispersity index, and antibacterial activity. The fabrication capacity to deliver CPL intradermally was further tested in an *ex vivo* dermatokinetic study on healthy rat skin. Lastly, the penetration and antibacterial activity of the formulation were evaluated in an *ex vivo* skin infection model.

The major findings of this study have the potential to provide new insights for solving problems in the treatment of skin wound infections.

2. Materials and Methods

2.1 Materials

Whey protein (WPI) containing 80% was gifted by Glanbia Nutritionals, Inc, Fitchburg, USA. Chloramphenicol, Chitosan (MW 161.156 and deacetylation degree $\geq 75\%$), glutaraldehyde, 2-alpha naphthol, tryptone soya agar (TSA) medium, nutrient broth (NB) medium, phosphate-buffered saline (PBS), and sodium hydroxide were purchased from Sigma–Aldrich Pte Ltd., Singapore, Singapore. The reagents utilized in this investigation were all analytical grade.

2.2 Preparation of CPL MPs

CPL MPs were prepared according to a previous study (Jain et al., 2018), with slight modifications. WPI (with concentration of 2.5%, 5%, 10%, 15% and 20% (w/v)) was dispersed in deionized water, and the protein solution's pH was adjusted to 8 using NaOH (1 M). The solution was swirled for one hour with a magnetic stirrer before being refrigerated overnight (4°C) to fully hydrate the biopolymer. The solution was centrifuged at 1000 rpm for 5 minutes, and the supernatant was separated for further use. CPL (with a concentration of 12.5, 25, 50, 100, and 200 mg/mL) was dissolved in 5 mL ethanol and added to 20 mL of protein solution dropwise while continuously stirring at a temperature of 40°C. Immediately after dissolution using methanol, 2 mL of 5% (v/v) glutaraldehyde was added to facilitate cross-linking of the MPs. The detailed concentration of formulation is depicted in Table 1. The cross-linking procedure was carried out for 24 hours at 40°C with agitation; this also caused any residual organic solvent to evaporate. The obtained dispersion was thoroughly washed with deionized water three times. The mixtures were then freeze-dried.

2.3 Characterization of CPL MPs

2.3.1 Evaluation of particle size, zeta potential and polydispersity index (PDI)

The particle size, polydispersity index (PDI) and zeta potential of formulation were determined using the size analyzer (Malvern Instruments, Malvern, UK). The MPs were first dispersed with deionized water (0.01% v/v), and 1 mL of each sample was used for the analysis process. All samples were analyzed at 25°C and a scattering angle of 90°.

2.3.2 Measurement of entrapment efficiency (EE) and drug loading (DL) percentages

Microparticle EE was measured using the indirect method. CPL MPs suspension was centrifuged at 1000 rpm for 20 minutes to separate the supernatant. The supernatant was then analyzed using a UV-Vis spectrophotometer at a wavelength of 488.7 nm after being derivatized with 2- alpha naphthol (0.01 M) and sodium bicarbonate (0.01 M). A derivatization procedure was performed on CPL to produce peaks that did not overlap with WPI and showed accurate measurement data. The reduced CPL MPs solution was created by dissolving the CPL MPs in methanol, then reacting it with zinc powder and HCl (12 M) while stirring it for 5 minutes in bath water at a temperature of 50° C. The reduced solution was filtered into a flask and diluted with deionized water. In a volumetric flask containing the CPL MPs solution, 1 ml of 2-alpha naphthol and 0.5 mL of sodium bicarbonate (0.1 M) were added to complete the reduced process. To create an orange-red result, the solution was heated at 70° C in bath water for 10 min. The amount of CPL was calculated according to the standard curve equation $Y = 0.0769x - 0.0097$. The encapsulation efficiency of CPL MPs (EE) % is the actual amount of the CPL found in MPs divided by the total amount of the CPL added during the preparation. Drug loading of CPL (DL) % is the mass of the CPL-MPs in the dried powder divided by the total mass of the MPs recovered from the drying process. The calculation of % EE and DL follows the equations below [20]:

$$\% \text{ Entrapment Efficiency (EE)} = \frac{\text{Total drug amount} - \text{unencapsulated drug amount}}{\text{total drug amount}} \times 100\% \quad (1)$$

$$\% \text{ Drug Loading (DL)} = \frac{\text{Mass of encapsulated drug}}{\text{total mass of microparticle}} \times 100\% \quad (2)$$

2.3.3 Powder X-ray diffraction (PXRD) analysis

The crystallinity pattern of the sample was analyzed with a powder X-ray diffraction (Rigaku Corporation, Kent, England) instrument using 1 gram of sample in a very high-resolution Cu-K α radiation diffraction system. The CPL MPs was placed in an aluminium holder at a voltage of 40 kV, current of 30 mA and scanned 2 θ angle range of 10 to 90°.

2.3.4 Differential scanning calorimetry (DSC) analysis

The materials were thermally examined using a DSC 2920 TA instrument (Surrey, UK). The detection parameters for this measurement were a 5 mg powder sample, a temperature range of 25° to 300°C, a heating rate of 10°C/min, and a nitrogen cleaning rate of 50 mL/min.

2.3.5 *Fourier-transform infrared spectroscopy (FT-IR) analysis*

Chemical interaction between chemical components in CPL MPs in WPI polymer was analyzed by FT-IR spectrophotometer instrument (Accutrac FT/IR-4100™ Series, Perkin Elmer, USA) in the wavenumbers range of 4000–400 cm⁻¹.

2.3.6 *Scanning electron microscope analysis*

A scanning electron microscope (TM3030 microscope, Hitachi, Krefeld, Germany) was used to examine the morphologies of CPL MPs. CPL MPs were freeze-dried under vacuum by lyophilizing. The CPL MPs were directly attached to the carbon tape and observed with the following operating conditions: accelerating voltage 15 kV, probe current 45 nA, and counting time 60 s.

2.3.7 *In vitro release studies of CPL MPs*

In vitro drug release of CPL MPs and CPL using simulated wound fluid. Simulated wound fluid was prepared using 0.36 g of sodium hydrogen carbonate, 5.84 g of sodium chloride, 0.28 g of calcium chloride, 30.30 g of potassium chloride, and 33.00 g of bovine albumin in 1000 mL of deionized water [21]. Two Erlenmeyers contained 50 mL of simulated wound fluid for each formulation. CPL MPs equivalent to 50 mg of CPL were added into simulated wound fluid with 5 mL of samples taken at predetermined time intervals (0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 24 h) and replaced with the same volume of new release media. The test was carried out at a temperature of 37°C and a speed of 100 rpm. The CPL released was further determined using a UV-Vis spectrophotometer. The amount of CPL in simulated wound fluid was calculated according to the standard curve equation $Y = 0.0368x + 0.011$ with $R^2 = 0.9996$ and the drug release percentage was calculated using the equation below[22] :

$$\% \text{ Drug released} = \frac{\text{Amount of CPL release}}{\text{Amount of CPL tested (50 mg)}} \times 100\% \quad (3)$$

2.3.8 *Mathematical modelling of the kinetics of drug release*

The principal mathematical models of drug release were tested, along with the relevant equations and parameters, using the Excel® (Microsoft Corporation, Redmond, USA) Add-In- DDSolver to model the systems' drug release data. The adjustment of the theoretical models to the experimental data was compared using the adjusted coefficient of determination (adjusted- R^2). The kinetics model follows the equations below [23]:

Zero-order:

$$Q_t = Q_0 + K_0 t \quad (4)$$

First-order:

$$\ln Q_t = \ln Q_0 + K_1 t \quad (5)$$

Higuchi:

$$Q_t = K_H \sqrt{t} \quad (6)$$

Hixson-Crowell:

$$Q_0^{1/3} - Q_t^{1/3} = K_s t \quad (7)$$

Korsmeyer-Peppas:

$$Q_t = K_t n \quad (8)$$

where Q_0 is the preliminary value of Q_t , t is the time, n is the exponent of diffusion release, K_0 , K_1 , K_H , K_t , and K_s are the coefficients of release according to the relevant kinetic model, and Q_t (%) is the percentage of CPL released at time t .

2.4 In vitro antibacterial activity of CPL MPs

2.4.1 Preparation of bacterial cultures

The bacterial strain used was *Staphylococcus aureus* (ATCC®25923), which was stored at 4°C and sub-cultured in fresh media. Bacterial culture was prepared by growing bacteria on TSB media with a temperature of 37°C. Test bacteria are made equivalent to 0.5 mc Farland equivalent to 2×10^8 CFU / mL.

2.4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluation

The MIC and MBC from CPL and CPL MPs were determined using microtiter dilution techniques in accordance with the Clinical and Laboratory Standards Institute protocol [24]. CPL and CPL MPs solution with a concentration of 1000 µg/mL was made in a 10 mL volumetric flask. The

sample was entered into 96-well plates with a concentration of 100 µg/mL. Next, 100 µL of bacterial suspension was added to the wells, then homogenized using micropipettes. TSB with a concentration of 100 µL medium was added to the wells. Wells containing only pure CPL and TSB media alone were used as the positive and negative control, respectively. All filled wells were closed and incubated at 37°C for 24 hours. To determine MBC value, 20 µL from 96-well plates with selected MIC was cultured on a Petri dish containing TSA medium and incubated at 37°C for 24 h. After that, the number of bacteria in the Petri dish colony was then determined. MBC was defined as the lowest concentration that eliminates 99.9% of bacterial growth.

2.4.3 Time–kill assay of CPL MPs

Time–kill assay of CPL and CPL MPs against *Staphylococcus aureus* (SA) was determined. CPL, blank MPs, and CPL MPs were added to the bacterial solution in the same quantities as MIC, 2 x MIC, and 4 x MIC, resulting in 2×10^5 CFU/mL of bacteria. After that, bacterial cultures were cultured at 37°C. 20µL aliquot from the culture was collected at specified time intervals and inoculated aseptically into a Petri dish containing TSA medium. The Petri dish was then incubated at 37°C for 24 hours. The procedure was performed with 3 replications and created a log CFU/mL curve against time. CPL, blank MPs, and CPL MPs were added to the bacterial solution in the same quantities as MIC, 2 x MIC, and 4 x MIC, resulting in 2×10^5 CFU/mL of bacteria.

2.4.4 Evaluation of hemolytic activity

By incubating the sample with red blood cells (RBC) and quantifying the percentage of hemoglobin produced from lysed erythrocytes, the overall hemolysis of the formulation was examined. *In vitro* hemolytic assays were carried out in accordance with a previously described method to determine the hemolytic activity of CPL, CPL MPs and CPL MPs hydrogel [25]. First, EDTA tubes containing freshly drawn rat blood were centrifuged for 10 minutes at 3000 rpm to separate the RBCs. After scraping off the plasma layer at the top of the tube, the remaining cells were further washed twice with PBS and diluted to 1:10, v/v. We mixed 100 µL of the prepared cell suspension with 900 µL of the sample containing CPL, CPL MPs and CPL MPs hydrogel (serially dissolved in PBS) at concentrations ranging from 5 to 500 mg/L. After 60 minutes of incubation at 37°C, the mixture was centrifuged at 3000 rpm for 10 minutes. Free hemoglobin was estimated using UV-visible spectroscopy measurements by analysing the supernatant taken at wavelength 540 nm. Minimal (negative) and maximum (positive) hemolytic controls were performed using phosphate-buffered

saline and distilled water, respectively. Blood sample hemolysis was analyzed by visually inspecting for colour changes in the serum and plasma. For each concentration, the experiments were repeated three times. In the following equation, we included the hemolysis percentage calculation:

$$\text{Hemolysis (\%)}: \frac{\text{test sample} - \text{negative control} \times 100}{\text{positive control} - \text{negative control}} \quad (9)$$

2.5 Formulation of chitosan hydrogel from CPL MPs

Chitosan was dissolved in 2% acetic acid solution at 60°C to produce the CPL MPs hydrogel. The chitosan solution was combined with glycerin (10% w/w) and DMDM hydantoin (0.01% w/w) while being stirred with a magnetic stirrer. CPL MPs (1% w/w) were then added to the mixture. The foaming components of the preparation were eliminated using a sonicator for 30 minutes at a temperature of 50°C, thereby adjusting the hydrogel pH to 5 with the addition of NaOH 8.3 M. As a control, a hydrogel containing CPL was created.

2.6 Characterization of CPL MPs hydrogel

2.6.1 pH measurement

A pH meter was used to determine the pH of CPL MPs hydrogel by inserting the electrode into the gel and recording the result after two minutes. All measurements are carried out 3 times to obtain the average value and standard deviation.

2.6.2 Evaluation of viscosity and rheology

With the use of a Brookfield viscometer with a seven-speed spindle running at 50 rpm, the viscosity of the CPL hydrogel was measured. The rheology of the CPL hydrogel was also determined using the same seven-speed spindle tool. Measurements are carried out at speeds of 5, 10, 20, 50, and 100 rpm.

2.6.3 Spreadability

Two sets of standard-sized glass slides were taken in this study. The spreadability of the hydrogel was evaluated by applying 0.5 g of the gel to a circle that had been previously marked on a glass plate with a diameter of 2 cm and then again using a second glass plate. After 1 minute, the diameter of the hydrogel was measured. The spreadability measurement was carried out with loads of 500 g.

2.6.4 Skin occlusivity examination

The previous technique was used to test *in vitro* skin occlusivity [26]. The 100 mL beaker was filled with 50 mL of deionized water, which was covered carefully with Whatman filter paper. Whatman filter paper with a size of 2.5 mm was applied with 250 mg of hydrogel. As a control, filter paper without CPL MPs hydrogel was used. All samples were stored at a temperature of 32°C, and the weight of Whatman paper was calculated at 0 h, 6 h, 24 h and 48 h. Occlusivity (O) was calculated through Eq:

$$\text{Occlusivity percentage (O)} = \frac{\text{mass of control} - \text{mass of sample}}{\text{mass of control}} \times 100\% \quad (10)$$

2.6.5 Extrudability percentage

About 10 g of hydrogel were sealed inside a collapsible aluminum tube, the end of which was crimped, and a clamp was used to keep the tube from unwinding. The gel was released after the cap was removed, and the quantity of gel that was extruded was measured. The amount of gel that was forced out of the extruder was determined as follows:

$$\% \text{ Extrudability} = \frac{\text{total mass of extruded hydrogel}}{\text{Total mass of hydrogel}} \times 100\% \quad (11)$$

2.6.6 Bioadhesivity test

Part of the rat skin tissue was cut and immediately secured with the mucosa side out into each glass vial via rubber bands and aluminum caps. Phosphate-buffered saline (PBS) was applied to vials containing rat skin tissue for 10 minutes. The test was carried out by gluing the skin onto the upper and lower vial bases, after which the hydrogel preparation was placed between the rat skin on the scale's left arm. The hydrogel was moistened with PBS and then attached to the skin. The vial's height was then altered to accommodate the application of hydrogel between the mucosal tissues of both vials. Once two vials were linked, the weights were held at that level. The detachment stress (dyne/cm²) between two vials was calculated using the adhesive forces presented below [27]

$$\text{Bioadhesive strength} = \frac{m \cdot g}{A} \quad (12)$$

where m is the weight required to remove the gel from the skin, g is the acceleration due to gravity (980 cm/s²), and A is surface area of the exposed mucosa (cm²).

2.6.7 *Ex vivo dermatokinetic study*

Dermatokinetic assays using Franz diffusion cells were carried out following the previous method [28]. Rat skins were cut and soaked in PBS pH 7.4 for 30 minutes before being attached to Franz diffusion cells. The medium used in this study was simulated wound fluid, which was arranged as a receiver compartment. The receiver compartment was heated to $37\pm1^{\circ}\text{C}$ and agitated at 600 rpm. Parafilm® was used to close off the donor area. A 1 g sample of CPL MPs hydrogel was put into the skin using a syringe needle for 30 seconds, and a 5 g cylindrical weight was placed on top of the skin to keep the formula in place during the experiment.

The samples were taken at intervals of 1, 2, 3, 4, 5, 6, 8, and 24 hours. After removing the hydrogel, the skin was cleansed with deionized water to remove any residue particles. The biopsy incision was 5 mm in diameter and was made in the skin. Next, the skin was transferred to a 1.5 mL Eppendorf® tube and heated in a water bath at 60°C for 2–3 minutes. The epidermal layer was separated from the dermis layer using forceps. The extraction process of the drug on the skin layer was carried out by adding 1 mL of methanol separately, and each mixture was homogenized for 10 minutes. The sample was then centrifuged at 3000 rpm for 15 minutes. Supernatants were collected and then measured using a UV-Vis spectrophotometer and following procedure section 2.3.2. C_{max} , T_{max} and AUC_{0-24} were determined using PK Solver software (China Pharmaceutical University, Nanjing, China).

2.6.8 *Ex vivo antibacterial activity test using infection skin model*

2.6.8.1 *Ex vivo test preparation on rat skin infection model*

The skin was sterilized by immersing it in 70% ethanol for one hour. The skin was sensitized to laminar airflow (LAF) for 20 minutes prior to the experiment. Furthermore, the sterilized skin was administered an intradermal injection of 50 μL of BSA solution, which was equal to 0.5 mc Farland. The skin piece was next positioned aseptically on a Petri plate filled with TSA media. The skin was then aseptically transferred to a new TSA Petri dish, and the Petri dish was incubated at 37°C for 7 days.

2.6.8.2 *Antibacterial activity test of ex vivo hydrogel preparations on skin infection model*

The antibacterial activity of the *ex vivo* skin infection model was carried out using diffusion Franz diffusion cells [29]. In this study, rat skin of average thickness was used, and a skin infection model

was placed on diffusion cells. Before the study, a dilution of CPL MPs hydrogel and CPL hydrogel was prepared by dissolving in DMSO. The skin was collected and 1.5 mL of sterile water was added to a 1.5 ml Eppendorf tube after hydrogel treatments had been left on for 6, 12, and 24 hours, respectively. Next, the mixture was homogenized and TSA medium was added to a Petri dish with 20 mL of homogenous samples. The sample with the medium was incubated for 24 hours at 37°C. Additionally, the same method was used to apply both blank hydrogel and CPL hydrogel to infected skin as a control. We determined the log CFU/mL of the samples.

2.7 Analytical statistic

All data obtained for this study were reported as means of the standard deviation (SD) and computed using Microsoft Excel® 2016. (Microsoft Corporation, Redmond, USA). The data were analyzed using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA) and the SPSS version with a significance value of $p = 0.05$.

3 Result & Discussion

3.1 Particle size, zeta potential and polydispersity index (PDI) of CPL MPs

All ingredients used in the preparation of CPL MPs have been reported to be non-toxic and safe for clinical purposes [30]. As a cross-linker agent, CPL MPs were prepared by solvent evaporation and stabilized by glutaraldehyde. Moreover, glutaraldehyde has also been reported to introduce intermolecular cross-linking in proteins or to modify adsorbed proteins on amino-glutaraldehyde bonds [31]. Glutaraldehyde-mediated cross-linking of whey proteins was formed by aggregates of whey protein by whey protein intermolecular amide bonds (β -lactoglobulin (BLG) bands) and glutaraldehyde as follows the reaction in Figure 1. This approach successfully fabricated MPs in this study by designing formulations with a variation of CPL, WPI concentration and WPI:CPL ratio. The MPs' physical characteristics were then evaluated in Table 1 and Figure 2A.

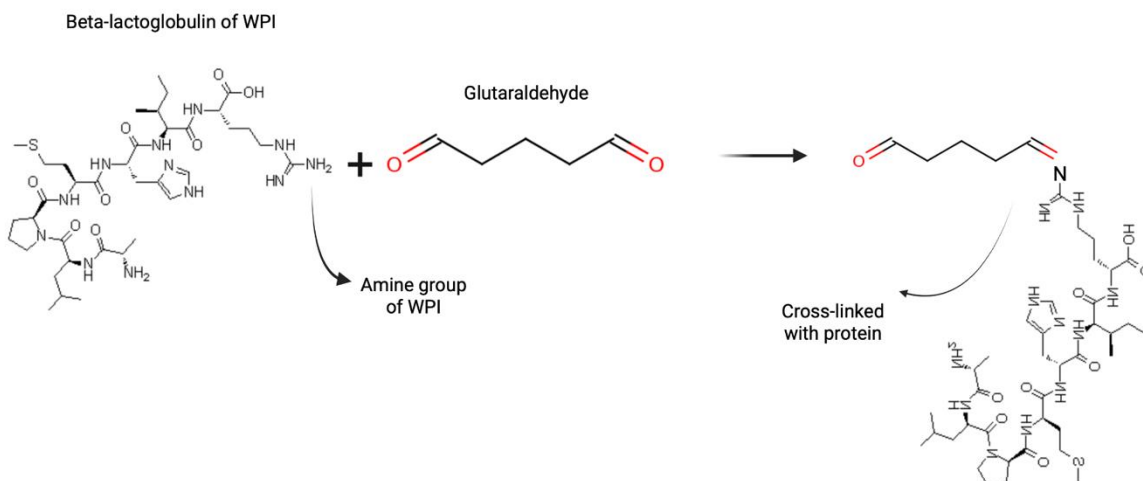


Figure 1. The cross-linked reaction of Amine group of WPI and glutaraldehyde

The results show that particle sizes of CPL MPs were produced in the range of $0.56 \pm 0.13 \mu\text{m}$ to $35.71 \pm 5.23 \mu\text{m}$. Conversely, samples with variation in CPL concentration show significant particle size variation between formulations. Moreover, the formulation with a variation of WPI concentration also shows significant particle size variation between formulations ($p < 0.05$) with an increase of $3.62 \mu\text{m}$. It concludes that the concentration of WPI can produce a more compact protein structure so that the higher the protein concentration, the more it will be able to increase particle size [32]. The same trend also occurs in variations in the ratio of WPI and CPL (1:1, 2:1, 8:1, 16:1), of which a 1:1 ratio was found to yield significantly larger particle size compared to the rest ($p < 0.05$). This increase may be due to increased hydrophobic interactions between encapsulated and absorbed CPL in the main component of the protein isolate β -lactoglobulin with an increase in WPI concentration. Solution at pH 8.0 allows ligands to enter the hydrophobic nucleus and bind to many hydrophobic molecules, causing an increase in particle size [33]. These findings also showed that the CPL/WPI microparticle size was dependent on the ratio of WPI: CPL in the system (core: coating ratio), and that this ratio could be raised by increasing the WPI content in the formulations [34].

Table 1. Details of the different formulation characteristics utilized to create CPL MPs, including their PDI, zeta potential, and particle size (means $\pm$ SD, n = 3).

Formulation code	WPI in water (%)	CPL in ethanol (mg)	Ratio WPL:CPL	Particle size (μ m)	PDI	Zeta potential (mV)
MP1	5	12.5	4:1	1.33 $\pm$ 0.09	0.127 $\pm$ 0.005	-28.27 $\pm$ 0.81
MP2	5	25	4:1	1.40 $\pm$ 0.14	0.129 $\pm$ 0.005	-29.25 $\pm$ 1.01
MP3	5	50	4:1	1.44 $\pm$ 0.05	0.13 $\pm$ 0.003	-29.44 $\pm$ 0.67
MP4	5	100	4:1	1.45 $\pm$ 0.11	0.14 $\pm$ 0.007	-29.14 $\pm$ 1.06
MP5	5	200	4:1	1.47 $\pm$ 0.10	0.13 $\pm$ 0.010	-29.21 $\pm$ 1.65
WP1	2.5	50	4:1	0.56 $\pm$ 0.13	0.13 $\pm$ 0.004	-24.67 $\pm$ 0.71
WP2	10	50	4:1	2.92 $\pm$ 0.20	0.13 $\pm$ 0.005	-31.95 $\pm$ 0.87
WP3	15	50	4:1	7.65 $\pm$ 0.99	0.14 $\pm$ 0.005	-34.23 $\pm$ 1.14
WP4	20	50	4:1	11.42 $\pm$ 1.07	0.14 $\pm$ 0.009	-35.17 $\pm$ 0.84
CP1	10	50	1:1	134.62 $\pm$ 10.29	0.63 $\pm$ 0.029	-27.73 $\pm$ 1.43
CP2	10	50	2:1	33.03 $\pm$ 4.08	0.40 $\pm$ 0.006	-28.36 $\pm$ 0.91
CP3	10	50	8:1	1.29 $\pm$ 0.05	0.13 $\pm$ 0.007	-32.32 $\pm$ 2.00
CP4	10	50	16:1	1.29 $\pm$ 0.07	0.12 $\pm$ 0.009	-32.56 $\pm$ 2.04

PDI was carried out to characterize the molecular weight of a polymer. Based on the results (Figure 2B), samples with a variation of CPL WPI concentrations show no significant results between formulations ($p > 0.05$). While the different outcomes are noticeably seen in the ratio of WPI:CPL, the higher solution of WPI in formulation shows decreased PDI value. The PDI value indicates the same trend as the particle size. PDI smaller than 0.5 is produced when the mass of WPI in WPI:CPL is within the range of 2:1 to 16:1, indicating high particle aggregation with a broad particle size distribution [30].

Measurement of zeta potential can predict the colloidal solution's stability, indicating the repulsive force between MPs. The zeta potential graphic of the CPL MPs is shown in Figure 2C. CPL MPs have a relatively high zeta potential (i.e., close to around -30 mV), indicating good stability of MPs exhibited in this study. Furthermore, increasing the concentration of CPL in the formulation has no significant effect ($p > 0.05$) on the zeta potential of MPs, indicating that the significant amount of drugs trapped in the core has no effect on the surface area of β -lactoglobulin. All formulation have

extremely negative zeta potentials, which improve the physical stability of many systems by increasing the repulsive force between MPs [35]. In addition, The negative zeta potential found in this investigation may be attributable to the structure of the whey protein opening during heating at the microparticle preparation, which reveals more polar groups [36]. Furthermore, β -Lg of WPI has an isoelectric point in the range of 4.9–5.4. The second significant constituent of whey protein is α -La, with an isoelectric point of 4.6. When pH value increases above the isoelectric point, the protein produces a negative charge, which facilitates negative zeta potential. Additionally, the isoelectric point of β -Lg is in the 4.9–5.4 range. Whey protein's second important component (α -La) has an isoelectric point of 4.6. Proteins create a negative charge when the pH level rises above the isoelectric point, which supports the negative zeta potential [37,38]

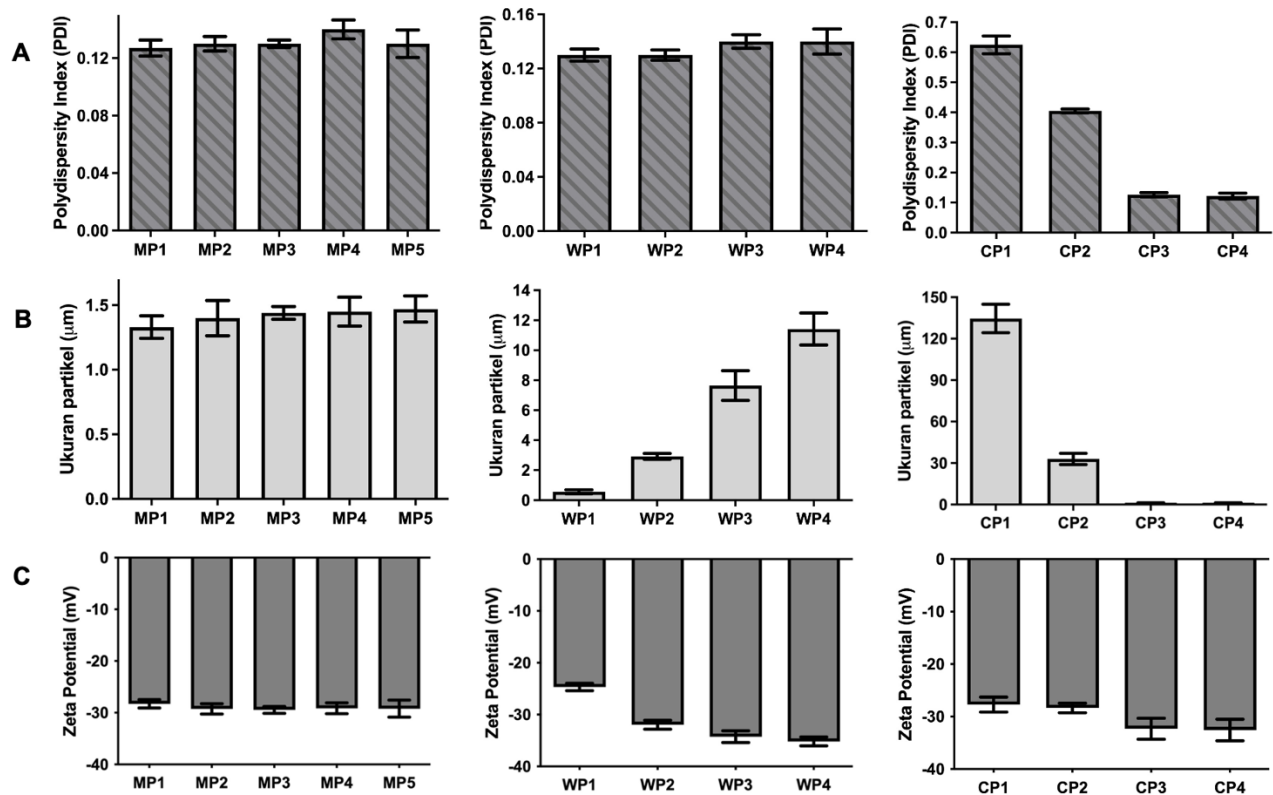


Figure 2. Particle size (A), Polydispersity Index (B) and Zeta Potential (C) of CPL MPs prepared with Whey Protein (WPI) (means $\pm$ SD, n = 3).

3.2 EE and DL percentages

Entrapment efficiency (%EE) and drug loading (%DL) of CPL MPs are shown in Figure 3A. CPL MPs have a higher EE value ($68.78 \pm 1.40\%$) when the mass of WPI is in the range of 10–20%, but

there is no noticeable difference between WP2, WP3 and WP4 ($p > 0.05$). The same trend occurred in the WPI: CPL ratio (8:1 and 6:1), experiencing an increase in EE ($55.67 \pm 3.55\%$ and $45.98 \pm 1.47\%$). This value decreased significantly to $13.50 \pm 2.67\%$ when the WPI:CPL ratio was 1:1. WPI has an amphiphilic structure that can self-assemble into different forms, thereby increasing drug encapsulation. However, this ability can be reduced if DL has been maximized. According to Li et al., the increased surface hydrophobicity of WPI aggregates can explain their higher CPL loading capacity [39]. Li et al. investigated whether the enhanced contact between hydrophobic groups during the chemical cross-linking procedure was causing the surface hydrophobicity of WPI to rise. They found that the more active hydrophobic patches among the generated WPI aggregates for binding to CPL accounted for their increased %EE and %DL.

Drug loading of CPL MPs increased significantly from $3.40 \pm 1.68\%$ to $20.43 \pm 2.15\%$ (Figure 3B). Similar results have also been reported in utilizing α -lactalbumin-based nanoparticles to encapsulate resveratrol, in which the DL value increased significantly as drug content was increased, indicating a higher microparticle loading capacity with the addition of drugs [40]. Moreover, methanol as the solvent to dissolve CPL at higher concentrations modifies the protein solution's dielectric constant and induces the transition into aggregates [41]. Consequently, The total WPI aggregate formed depends on CPL concentration and the amount of solvent in the formulation. The DL of WPI aggregates produced in this preparation was much higher than what Zhang et al. reported for protein based-nanoparticles formulation ($12.69 \mu\text{g}/\text{mg}$ nanoparticles) [42].

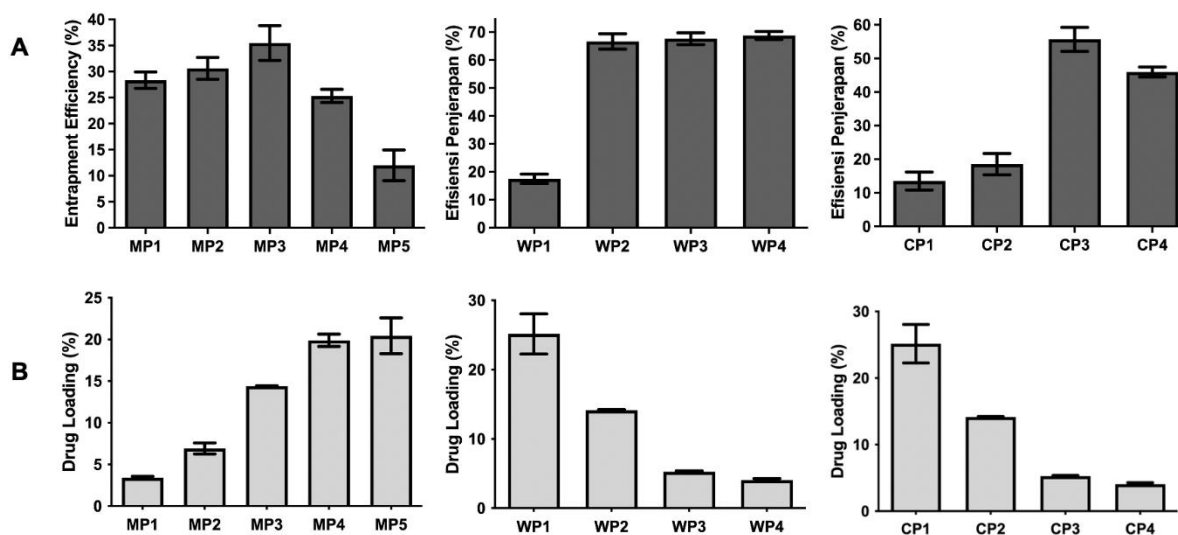


Figure 3. EE (A) and DL of (B) diagram of CPL MPs with a variation of CPL, WPI and ratio of CPI: WPI concentration (means $\pm$ SD, n = 3).

3.3 Powder X-ray diffraction (PXRD)

X-ray diffraction (PXRD) is essential to identify the physical state of the drug and the effect of homogenization on the crystalline structure of active substances. XRD patterns of the pure WPI, pure CPL, physical mixture (1.5:1), and CPL MPs have been compared in Figure 4C. The sharp peaks are associated with the crystalline fraction, whereas the amorphous part is represented as broad peaks [43]. It can be observed that firm crystalline peaks are observed in CPL and physical mixture diffraction. There are two sharp dominant peaks at $2\theta = 31.82^\circ$ and $2\theta = 26.1^\circ$ in CPL and $2\theta = 30.64^\circ$ and $2\theta = 25.98^\circ$ in the physical mixture (1.5:1). Two broad peaks were also observed at around 21.5° in CPL-loaded MPs and 21.46° in WPI. The same pattern was observed previously for WPI studies [43]. However, the crystalline peaks almost disappeared in the XRD pattern of CPL MPs and WPI, indicating the disruption of the crystalline form of CPL after encapsulation and entrapment of CPL into WPI-based MPs.

3.4 Differential scanning calorimetry (DSC)

To assess the physicochemical interactions between the formulation chemicals and to ascertain whether crystallinity was preserved during the encapsulation procedure, a thermal analysis of the DSC was carried out. Thermograms of pure CPL, pure WPI, physical mixture and CPL-loaded MPs are presented in Figure 4B. The DSC curve of pure WPI exhibits a broad endothermic peak between 40 and 120°C , centred at 85°C . The primary whey protein components (β -lactoglobulins and α -lactalbumin) undergo heat-induced transitions, which cause this distinctive endotherm. Similar characteristics were also seen in the physical mixture WPI:CPL (1.5:1) and in prior literature [44]. The endothermic point remained sharp at 159°C , representing CPL's melting point and crystallinity (pure CPL and physical mixture). The endothermic point at 159°C was not investigated in the pure WPI and CPL-loaded MPs. However, in CPL MPs, an endothermic peak was observed only in pure WPI, showing that CPL was distributed molecularly in the polymer matrix and hence properly encapsulated in WPI MPs. Endothermic peaks and melting points were identified following the encapsulation of DIM with protein nanoparticles, providing strong evidence of nanoencapsulation [45].

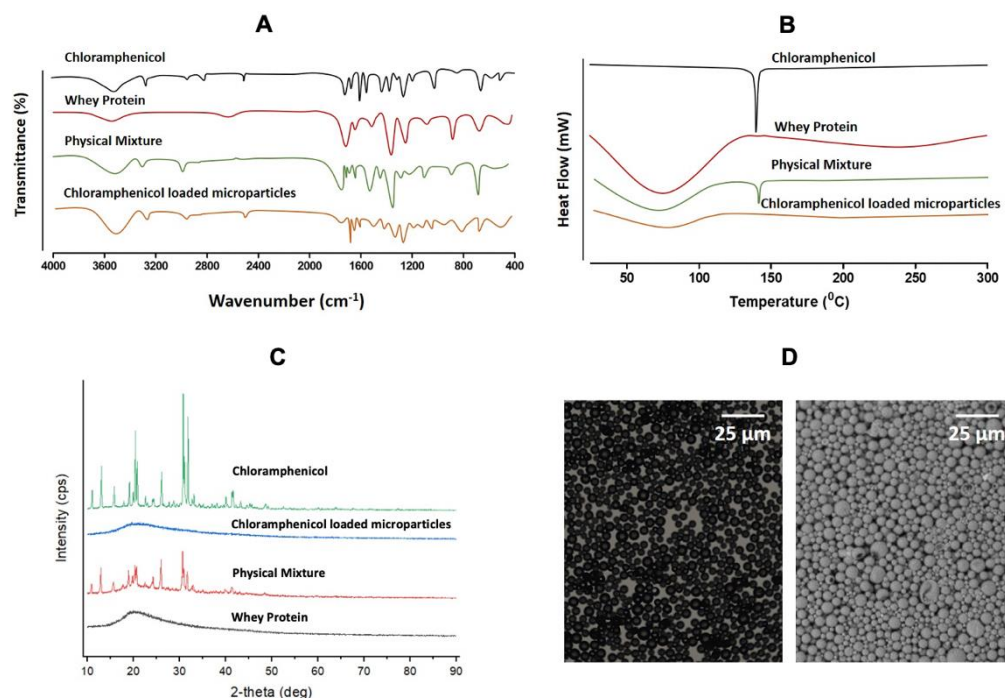


Figure 4. FT-IR Spectra of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (A). DSC thermogram of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (B). X-ray diffractogram of CPL, WPI, Physical Mixture (1.5:1) and CPL MPs (C). SEM images of CPL-loaded MPs (D).

3.5 Fourier-transform infrared spectroscopy (FT-IR) analysis

FT-IR analysis was used to determine the functional groups and interactions between pure WPI, pure CPL, physical mixture, and CPL-loaded MPs. The results are presented in Figure 4A. Major interesting peaks are observed in 3200–3600 cm^{-1} , indicating the presence of O–H and N–H bonds. The broad peak at around 2800–2500 cm^{-1} is also associated with O–H stretching vibration. O–H stretching was remarkably shifted after CPL was encapsulated in WPI MPs due to the interaction of CPL and WPI. These changes suggested that hydrogen bonding was formed between β -lactoglobulin and CPL. In the infrared spectrum, major interesting peaks are observed in 3200–3600 cm^{-1} , indicating the presence of O–H and N–H bonds [34]. The presence of strong peaks appeared on the CPL spectrum at 3493 cm^{-1} , which is associated with O–H stretching vibration; spectrum 3218 cm^{-1} is associated with N–H stretching vibration bond; spectrum 2978 cm^{-1} is associated with aromatic C–H stretching vibration, spectrum 1668 cm^{-1} with C=O stretching vibrations; and spectrum 1561 cm^{-1} is associated with NO_2 stretching peak [46]. The entire spectrum was detected in CPL encapsulated into MPs, indicating that encapsulation had been successfully performed on the system.

Amides I, II, and III are regarded as typical WPI spectra, indicating the presence of WPI's primary components, β -lactoglobulin and α -lactalbumin. The amide I band mainly confirms the presence of C=O stretch vibration and N–H bending. The middle absorption band II shown correlates with the structural combination group of C–N stretch with N–H deformation, while the amide III bands are associated with C–O bending vibration [47]. Amide I in the spectrum of 1745 cm^{-1} , amide II at 1451 cm^{-1} , and amide III at 1241 cm^{-1} are present in CPL-loaded MPs and physical mixture. The C=O stretching vibration carbonyl groups are primarily responsible for the Amide I observed in the encapsulated CPL at $1600\text{--}1700\text{ cm}^{-1}$. The binding of CPL to β -lactoglobulin was demonstrated by comparing the WPI spectrum with the encapsulated CPL. Wang et al. reported similar outcomes [48]. The spectrum of CPL-loaded MPs shows most of the characteristic peaks identical to the spectrum of WPI and CPL, which indicates the encapsulation procedure was successfully carried out. The change in intensity indicates the hydrophobic interaction of CPL and lactoglobulin complexes. These findings align with DIM-based polymerized whey protein nanoparticles [35].

3.6 SEM analysis

Morphology of CPL-loaded microparticle formulation was observed using SEM. As shown in Figure 4D, the CPL MPs show the spherical shape of the particles. The size of CPL MPs found in this experiment was similar to that observed in the DLS analysis ($\sim 25\text{ }\mu\text{m}$). Particle size is closely related to drug penetration into target tissues; Figure 3D shows that CAP MPs meet the particle size criteria to penetrate the stratum corneum lipid layer through the lipidic intercellular route or aqueous pores, which can penetrate to a depth of $> 2\text{ mm}$ efficiently into hair follicles. Another study also used the solvent evaporation method and prepared MPs, resulting in particle sizes of $6\text{--}25\text{ }\mu\text{m}$ [49]. Similar studies have also been described by previous studies [20], in which nanoparticle formulation of frankincense essential oil-loaded whey with a particle size of $321 \pm 22.42\text{ nm}$ showed a good healing effect for skin wounds. This capability can be supported if the particle size is within the range of $10\text{--}100\text{ nm}$ based on confirmation by studies on human, porcine or murine skin [50]. Moreover, the permeability of MPs into the skin is influenced by the shape of the particles. A spherical shape has an excellent ability to penetrate the skin because it is symmetrical in all three dimensions; thus, it can be located deep in the epidermis and dermis layers.

3.7 In vitro microparticle drug release of CPL MPs

The drug release behaviour of CPL compared to CPL MPs formulation is presented in Figure 5A. To mimic the physiological condition of the skin, *in vitro* drug release was prepared using simulated wound fluid. In this study, encapsulation of the drug into microparticle form improved the drug release profile. The drug release of CPL MPs was examined as the time in which the drugs needed to be completely soluble and migrate from the initial form as in the polymeric system to the simulated wound fluid until attained maximum drug accumulation. The experiment showed that $99.40 \pm 7.01\%$ CPL was released from the microparticle formulation after 24 hours. Meanwhile, the amount of drug release from pure CPL was around $95.43 \pm 2.41\%$ after 6 h and then CPL reached maximum release. For simulated wound fluid, the estimated drug release during the first hour was $4.76 \pm 0.16\%$. After that, approximately $10.31 \pm 0.23\%$, $24.81 \pm 1.03\%$, $42.04 \pm 4.21\%$ and $62.39 \pm 1.68\%$ of CPL were released from MPs within 2 h, 4 h, 6 h, and 8h, respectively. By comparing both CPL and CPL MPs, a quick release lasting up to only 6 hours was observed in pure CPL, while that in CPL MP's needed 24 hours to completely release, showing the contribution of particle encapsulate and the sustained released drug was achieved. The CPL MPs formulations showed sustained release patterns up to 24 h. This behaviour is related to an enlarged CPL surface area, leading to an increased drug release, with a controlled release profile [51]. Following that, a quick release lasting up to 24 hours was observed; weakly bound CPL that was released and attached to the surface of the microparticle may have caused the drug release. The solubility of WPI in simulated wound fluid can be attributed to the release profile of WPL from MPs, which predicts prolonged release by causing erosion of the microparticle matrix.

3.8 Mathematical modeling of the kinetics of drug release

The experimental drug release was employed in multiple kinetic models to further understand the process of CPL release from MPs. The most acceptable mathematical model is chosen based on the correlation coefficient value. Based on Table 2, It was concluded that the best results of CPL MPs were observed with R2 value of 0.91877. According to the results, the release behaviour of the CPL-loaded MPs formulation followed the Hixson–Crowell kinetic model. The drug release from these spherical sustained-release dosage forms depends on matrix drug diffusion and polymeric matrix erosion during polymer degradation [52]. The regular area of a collection of particles was found by Hixson and Crowell to be proportional to the volume cube root. The Hixson-Crowell cube root law describes particle release from a system where the surface area and diameter of the particles change [22]. In this work, the applicability of these equations was tested. Hixson and Crowell recognized

that the rate of dissolution depends on the surface contacting with the applied solvent. Increased surface area leads to faster dissolution [21]. Because of the differences in surface area and diameter, this model corresponds well to drug release from MPs.

Table 2. Kinetic modeling of pure-CPL and CPL-loaded MPs (means $\pm$ SD, n = 3).

Formula- tion	R and K value of Kinetic model										
	Zero-order		First-order		Higuchi		Krosmeyer-Peppas			Hixson-Crowell	
	R	K	R	K	R	K	R	K	n	R	K
Pure CPL	0.9814	0.015	0.7495	0.000	0.7623	0.029	0.9658	0.007	0.001	0.91877	0.000
CPL MPs	0.8481	0.005	0.6279	0.000	0.8677	0.018	0.2097	0.006	0.001	0.9642	0.000

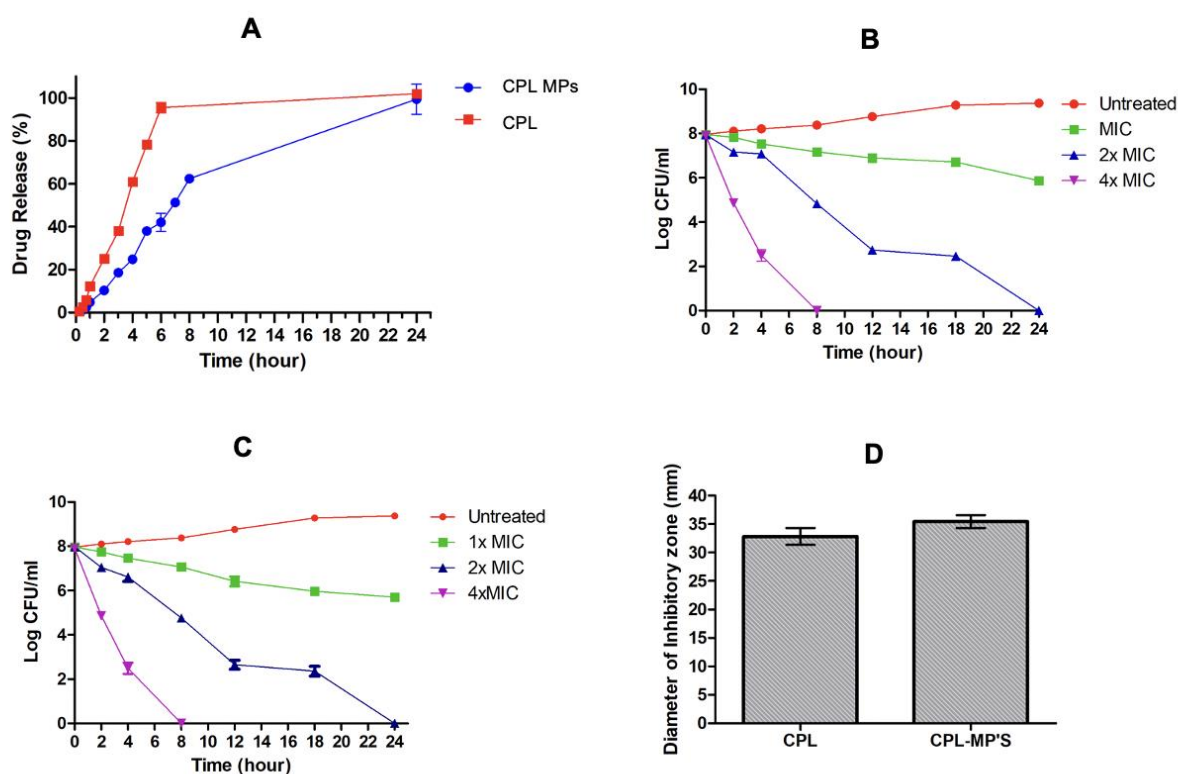


Figure 5. *In vitro* release profiles of CPL-loaded MPs in comparison with the pure CPL (A). Time-kill assay of CPL-loaded MPs (B) and pure CPL (C) against *Staphylococcus aureus* (means $\pm$ SD, n = 3). Diameter of inhibitory zone of CPL-loaded MPs against *Staphylococcus aureus* (means $\pm$ SD, n = 3) (D).

3.9 Determination of MIC and MBC

This work compared the bactericidal properties of pure CPL and CPL MPs. The MIC and MBC values of CPL MPs and pure CPL are 12.5 μ g/mL and 25 μ g/mL, respectively. The result shows

that the MIC value at the lowest concentration indicates strong antibacterial activity. Next, the MBC value is determined. The MBC value was greater than the MIC value, indicating that a larger concentration of CPL was required to kill bacterial cultures. Moreover, MIC values of samples were lower than MBC values. The ratio of MBC to MIC is < 4 . A ratio of 4 indicates bactericidal activity, and a ratio > 4 shows bacteriostatic activity [53]. Accordingly, a higher concentration of CPL MPs was required to kill the SA. Our study also suggests that CPL should be in solution form or MPs formulation to exhibit bactericidal activity.

The results showed that the inhibition zones of pure CPL and CPL MPs were 35.45 ± 1.14 mm and 32.82 ± 1.48 mm, respectively (Figure 6C). This might be because the substance disperses in the media, improving its capacity to penetrate the SA cell membrane. Previous research has shown that CPL prevents bacterial ribosomes from producing peptidyl transferase. The 50S ribosomal subunit's 23S rRNA contains residues A2451 and A2452, which this activity preferentially binds to [54]. CPL's ability to successfully permeate SA cell membranes may have contributed to its strong antibacterial action against SA when used in solution form. Notably, our control study using DMSO alone did not show antibacterial activity. Moreover, the formulation in the form of MPs showed higher antibacterial activity compared with CPL solutions. This may be due to the increased water solubility of CPL once in the MPs, enhancing CAP's capacity to efficiently permeate SA cell membranes.

3.10 Time–kill assay

The time–kill assay was performed to examine the time required for the CPL to completely kill the SA. Time–kill curves of free CPL and CPL MPs in DMSO versus SA are depicted in Figure 5B–C. The viable SA colony-forming unit increased by about 9.37 ± 0.05 log CFU/ml after 24 hours of cultivation in the control (untreated) group. With MIC values, CPL solutions and CPL MPs were unable to kill 99.99% of SA cultures after 24 hours. When 2x MIC values were observed, no feasible SA cultures were found after 24 hours in the case of either CPL solutions or CAP MPs. Furthermore, the time needed to eliminate 100% of CA cultures was decreased to 8 hours after incubation with 4x MIC values of both CPL and CPL MPs solutions. The results achieved in this work indicate that the killing rate of CPL in the microparticle-based formulation is based on how much CPL is present in the bacterial culture medium.

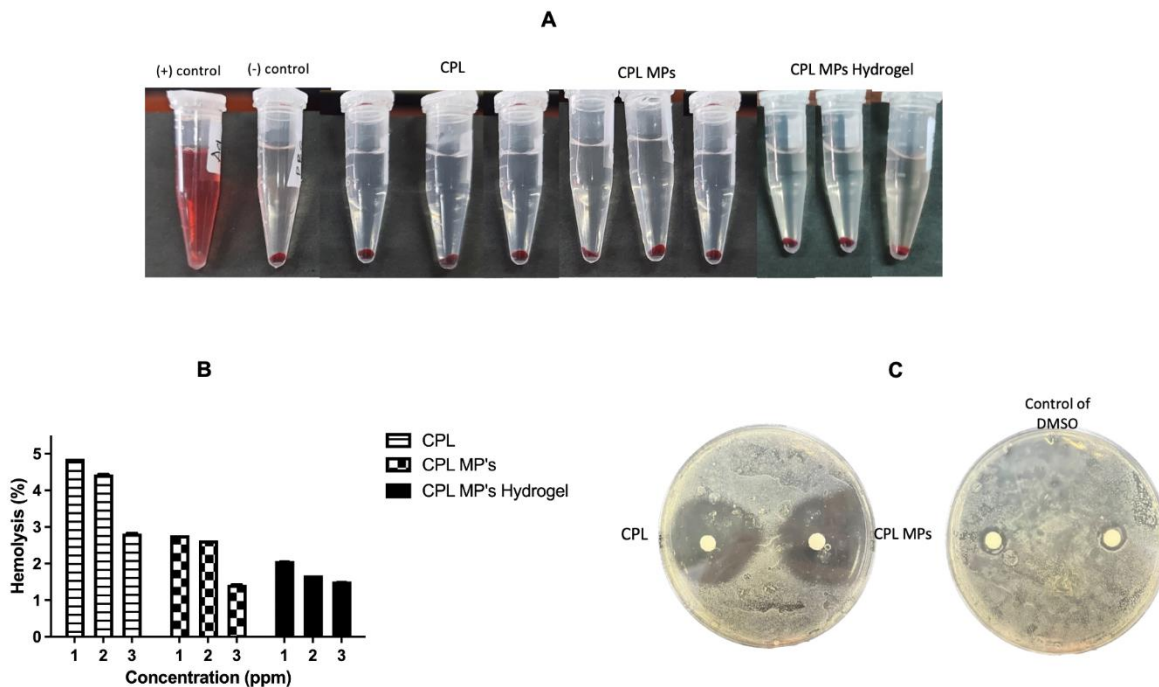


Figure 6. Hemolysis result of CPL Mps hydrogel, CPL-loaded MPs and pure CPL with concentrations of 500, 50 and 5 $\mu\text{g/mL}$ (Aquadest and PBS as positive and negative controls, respectively) (A). Hemolysis percentage of CPL-loaded MPs and pure CPL (B). Inhibitory zone of CPL-loaded MPs against *Staphylococcus aureus* compared with control (means $\pm$ SD, n = 3) (C).

3.11 Hemolysis test

The cytotoxicity of chemicals on RBCs can be quickly assessed using the hemolysis test. These RBCs will burst open entirely in the presence of water, releasing their hemoglobin content. In a similar vein, changes in osmotic pressure on the surface of RBCs caused by exposure to external elements can cause significant lysis. When RBCs are damaged or lysed, they emit adenosine diphosphate, which stimulates platelet assembly on the foreign body surface, speeds up the coagulation and thrombotic cascade pathways, and disrupts the body's natural ability to heal the wound [55]. Therefore, a good wound dressing membrane should not compromise the coagulation pathway or the integrity of the circulating RBC. By spectrophotometrically lysing RBC at 540 nm, the hemolytic potential of CPL MPs and CPL MPs hydrogel was calculated. As shown in Figures. 6A–B, different concentrations of CPL MPs (5–500 $\mu\text{g/mL}$) showed hemolysis percentages in the range of 1.42–2.76% and CPL MPs hydrogel in the range of 1.51–2.07%, whereas pure CPL showed a higher hemolysis percentage of 2.82–4.86%. According to the standard, materials exhibiting hemolysis

greater than 5% are thought to be hemolytic, those between 2 to 5% are thought to be slightly hemolytic, and those less than 2% are thought to be non-hemolytic [20]. From the results above, it can be concluded that the fabrication in the form of CPL-loaded MPs is compatible with RBCs because it consists of natural polymer.

3.12 Characteristics of hydrogel

3.12.1 pH measurement

Because the application of CPL MPs hydrogel should not irritate the skin, the pH of the formulation is very important. The pH of the hydrogel surface for each formula is in the range of 5.45 ± 0.04 to 5.55 ± 0.01 , as shown in Figure 7B, which is very close to normal skin pH of 5 to 6 [56], indicating that the formulation will be tolerated by the skin and will not interfere with the wound healing process.

3.12.2 Viscosity and rheological evaluation

Viscosity evaluation in this study used a Brookfield viscometer with spindle 7. The viscosity of CPL MPs hydrogel preparation can be seen in Figure 7E, which shows that the viscosity of preparations obtained in this study for F1 to F5, respectively, were 2266.66 ± 230.94 cPs, 3200 ± 173.20 cPs, 6900 ± 173.21 cPs, 9433.33 ± 152.75 cPs, and 23066.66 ± 832.67 cPs. Then, the viscosity test results from statistical analysis data showed that F1, F2 and F3 with respective chitosan concentrations of 1% (w/w), 1.25% (w/w) and 1.5% (w/w) have no significantly different values ($p > 0.05$). Meanwhile, the F4–F5 formulation has a significantly different value ($p < 0.05$) from F1. The good viscosity range in gels is in the field of 2000–4000 cPs, and other studies state that the viscosity of gels can reach up to 16000 cPs; therefore, F1, F2 and F3 are within the required viscosity range [57,58]. More importantly, chitosan has an intrinsic viscosity of 1.2×10^5 to 3.2×10^5 Da with molecular weight (MW) in the range of 3.2 to 6.52 dL/g [59], in which the amount of chitosan concentration in the formulation can increase the value of hydrogel viscosity. Based on the results of rheological measurements (Figure 7G), all sample flow behaviors exhibited non-Newtonian or pseudoplastic behavior ($n < 1$). Pseudoplastic flow is a viscosity flow that continues to decrease as the rate of shear increases. Thus, it can be estimated that an Increase in spreadability will occur at higher shear speeds or after dilution with liquids [57].

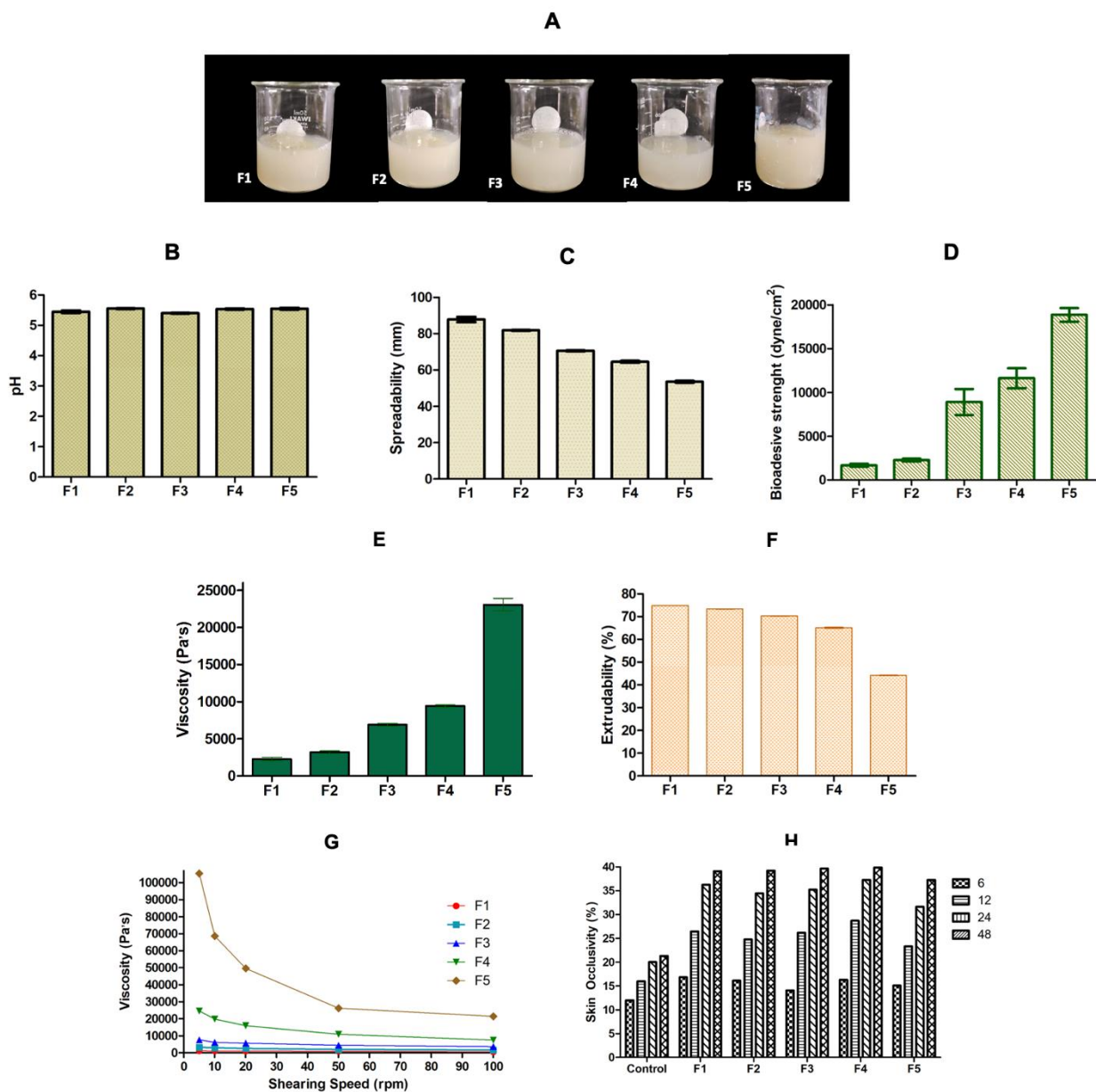


Figure 7. Formulation of CPL MPs Hydrogel with variation concentration of chitosan (F1: 1%; F2: 1,25%; F3: 1,5%; F4: 1,75%; F5: 2%) (A). pH (B), Spreadability (C), Bioadhesive strength (D), Viscosity (E), Extrudability (F), Rheology (G) and Skin occlusivity (H) of CPL MPs hydrogel (means $\pm$ SD, n = 3).

3.12.3 Spreadability

Poor spreadability can cause inhomogeneous distribution of particles, which can in turn affect the amount of dose applied as well as the efficiency of the active ingredients penetrating the skin [60]. The spreadability of hydrogel preparation was determined by the hydrogel's ability to spread on the

skin's surface. Good spreadability can ensure optimal distribution of hydrogel when applied to the skin. The spread test results of CPL MPs Hydrogel preparations reveal a value between 53.52 ± 0.50 mm to 87.84 ± 1.45 mm (Figure 6C), which indicates that CPL MPs hydrogel has good spreadability. The results of the ANOVA analysis show that the spreadability of CPL MPs hydrogel is not significant between all formulations ($p > 0.05$), with the exception of F4 and F5 to F1. Spreadability is inversely proportional to viscosity [61]. Good spreadability of the preparation is in the range of 50–70 mm; therefore F3, F4 and F5 are qualifying criteria, and F3 is the most optimal formulation. The results of the measurement in Figure 7C show that the greater the concentration of chitosan, the smaller the spreadability of the hydrogel.

3.12.4 Skin occlusivity test

The capacity of the formulation to preserve skin moisture properties was predicted using an *in vitro* model of skin occlusivity. Over 48 hours, the percentage of occlusion factors (Figure 7H) was evaluated for each formulation (F1–F5), and a filter paper without formulation was utilized as a reference. The occlusion factor values of all hydrogel formulas showed a significant difference ($p < 0.05$) compared to the control, and chitosan represented a good occlusion factor. This indicates that incorporating CPL MPs into the hydrogel can increase the occlusive value. Due to the lowering of the corneocyte slits, higher occlusion may improve skin moisture for 48 hours, allowing drugs to penetrate to deeper layers of the skin [26]. According to statistical analysis, there was no difference between any CPL MPs hydrogel formulation's occlusive value ($p > 0.05$).

3.12.5 Extrudability percentage

Hydrogel extrusion from the tube is essential during application. A sufficient consistency is necessary to remove the gel from the tube because hydrogels with high consistency may not come out of the tube, while gels with low viscosity may flow too freely. The extrudability of all hydrogel formulations (F1–F5) is shown in Figure 7F, with values of $74.9 \pm 0.01\%$, $73.37 \pm 0.05\%$, $70.3 \pm 0.06\%$, $65.13 \pm 0.22\%$ and $44.17 \pm 0.11\%$, respectively. More than 70% of the contents of the gel formulations F1–F3 can be extruded, demonstrating their strong extrusion capacity, with the exception of F4 and F5, where only 60% of the contents could be extruded. Based on statistical analysis, F1 has no significant values to F2 and F3. Furthermore, F4 and F5 are significant to F1, F2 and F3. This indicates that the increased chitosan concentration affects the increasing viscosity, thereby reducing the ease of extrusion from the tube.

3.12.6 Bioadhesive test

Good bioadhesive properties can ensure a long retention time for the applied drug formulation to guarantee effective local drug concentrations and expected pharmacological responses. Bioadhesive measurements are found in Figure 7D, which shows that the higher the concentration of chitosan used, the higher the bioadhesive strength of the preparation with F1 (1682.36 ± 171.41 dyne/cm²), F2 (2276.13 ± 171.41 dyne/cm²), F3 (8906.59 ± 1484.43 dyne/cm²), F4 (11628.05 ± 1133.75 dyne/cm²), and F5 (18852.29 ± 771.33 dyne/cm²) value. The statistical analysis showed that F1 and F2, as well as F4 and F5, showed no significant value ($p > 0.05$). The increased flexibility of chitosan hydrogel of CPL MPs can improve the contact between the gel and the skin tissue, thereby encouraging the penetration of polymer chains into the tissue to form a strong bond, which leads to an increase in the strength of adhesion [62]. Thus, it also increases the effectiveness of the active substance in reaching the target. Therefore, in this test, the F3 formula was chosen as the optimum formula with a concentration of 1.5% (w/w) that can provide good pH, dispersion, viscosity, skin occlusivity and bioadhesiveness.

Table 3. Dermatokinetic parameters of hydrogel in infected *ex vivo* skin infection models formed by SA following the application of pure-CPL Hydrogel, and CPL MPs Hydrogel (means $\pm$ SD, n = 3).

Condition	Formula- tion	C _{max} ($\mu\text{g}/\text{cm}^3$)	T _{max} (h)	T _½ (h)	AUC (h. $\mu\text{g}/\text{cm}^3$)	MRT (h)
Skin Infection model by SA	Pure CP Hydrogel	3.58 $\pm$ 0.30	6.67 $\pm$ 0.57	35.71 $\pm$ 11.99	47.51 $\pm$ 3.32	56.22 $\pm$ 16.37
	CPL MPs hydrogel	24.44 $\pm$ 0.95	6 $\pm$ 0.00	496.50 $\pm$ 749.87	417.62 $\pm$ 4.22	719.07 $\pm$ 1082.14

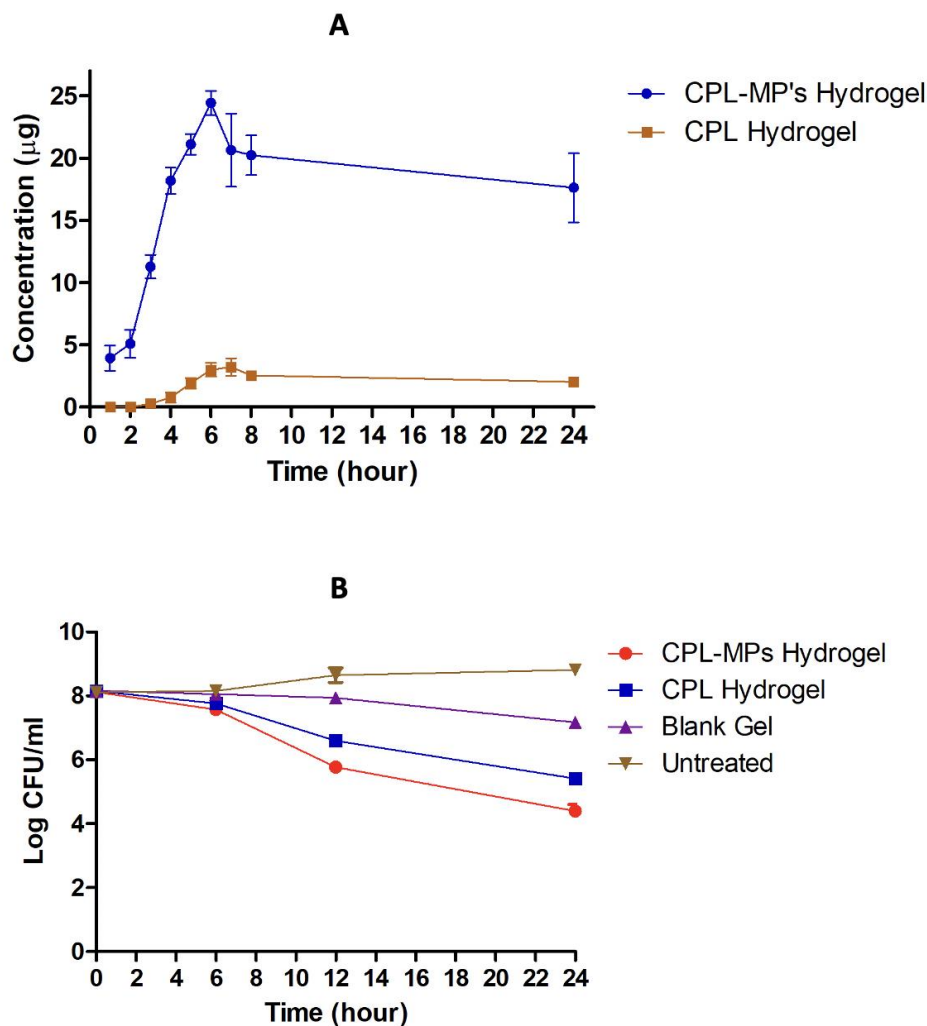


Figure 8. Dermatokinetic tests with CPL hydrogel and CPL MPs hydrogel revealed total CPL (A) (means $\pm$ SD, n = 3). Bacterial viability (log CFU/mL) in an *ex vivo* skin infection wound model (*S. aureus*) following the administration of free CPL in the form of a hydrogel, MPs hydrogel and blank hydrogel (means $\pm$ SD, n = 3) (B).

3.13 *Ex vivo* dermatokinetic study

Ex vivo dermatokinetic study was performed on non-infected rat skin models. To investigate the kinetics of the release of CPL MPs formulation, dermatokinetic studies of the hydrogel are carried out after application to the skin. To confirm that the release of CPL occurs in the skin, the skin sample was vortexed with 2 mL of methanol at each time interval. We compared the dermatokinetic profile of this study with a hydrogel containing CPL without formulation in the form of MPs as a control to assess whether the release of drugs affects the state of drug particles. Our findings in Figure 8A show that at 1 h and 2 h after measurements, no CPL was found in the pure-CPL hydrogel

supernatant and that free CPL permeated to the receiver compartment more rapidly in contrast to CPL MPs hydrogel after 2h. There was $2.05 \pm 0.22 \mu\text{g}$ of CPL after 24 h, which indicates that the CPL without MPs formulation showed hydrophobic properties, making it difficult to penetrate the skin membrane. On the other hand, measurements at 1 h to 24 h show a CPL found in the CPL MPs hydrogel supernatant with an optimal concentration of $24.43 \pm 0.95 \mu\text{g}$ at 6 h. This is in line with the main objective of this study, i.e., to deliver CPL MPs to the local target of the first layer of skin, where SA colonizes, infecting the skin locally.

The concentration of CPL released from the MPs form in the skin vs. time of administration is depicted as the kinetic profile of hydrogel in normal skin. Table 3 shows the dermatokinetic profile of CPL MPs hydrogel and pure-CPL hydrogel after application, including C_{max} , T_{max} , $T_{1/2}$, AUC , and MRT . The results also showed that the CPL content in dermal layers obtained pure-CPL hydrogel was remarkably lower compared with that of CPL MPs hydrogel. The C_{max} ($24.44 \pm 0.95 \text{ g/cm}^3$) was reached in 6.00 h, according to the kinetic profile of CPL MPs. In comparison to CPL MPs hydrogel, the profile of pure-CPL hydrogel had a lower C_{max} ($3.58 \pm 0.30 \text{ g/cm}^3$ in $6.67 \pm 0.57 \text{ h}$), but the differences were not statistically significant ($p > 0.05$). Additionally, the C_{max} for both the pure-CPL hydrogel and the CPL MPs hydrogel in the dermal layers was reached in the same T_{max} . The findings show that, in comparison to pure-CPL hydrogel, CPL MPs hydrogel can deliver much higher concentrations of CPL to the skin. The great *ex vivo* skin bioavailability of the microparticle method is shown by the AUC value of CPL MPs hydrogel, which was found to be substantially higher ($p < 0.05$) than that of pure-CPL hydrogel. It was discovered that the MRT value of CPL MPs hydrogel was considerably different from the pure-CPL hydrogel ($p < 0.05$) in terms of retention duration in the skin. A high MRT value can be advantageous for reducing the application time of CPL MPs hydrogels in treating skin infections associated with local infection formation [29]. This reduced application time may increase patients' acceptance of (and compliance with) this approach. Our results conclude that a responsive CPL MPs hydrogel approach can successfully deliver MPs into *ex vivo* infection models.

3.14 *Ex vivo* antibacterial activity test using infection skin model

To test the efficiency of CPL MPs hydrogel as an antibacterial agent, researchers used an *ex vivo* skin infection model. We sought to determine whether CPL MPs hydrogel could reduce bacterial bioburdens produced from SA by counting cells' log CFU/ml number. This test was carried out on

CPL MPs hydrogel, pure-CPL hydrogel, blank hydrogel without CPL, and an untreated sample as a control. The test results are found in **Figure 8B**. In *ex vivo* skin infections, the highest antibacterial activity was achieved after applying CPL MPs hydrogels compared to pure-CPL hydrogel, as measured by the reduction of bacterial bioburden after 24 hours. A value of 8.22 ± 0.11 log CFU/ml to 8.81 ± 0.18 log CFU/ml increase in bacterial bioburden was seen in untreated samples, which indicates the validity of the control sample. Blank hydrogel without drugs showed a value of 8.16 ± 0.04 log CFU/ml to 7.11 ± 0.07 log CFU/ml decrease in bioburden properties. The antibacterial activity in the blank hydrogel was due to the ability of chitosan as a natural polymer to inhibit microorganisms, as explained in a previous study [18].

Analysis of *ex vivo* antibacterial activity showed an enhancement of 8.02 ± 0.10 log CFU/ml to 4.40 ± 0.20 log CFU/ml after 24 hours when CPL is incorporated into MPs in hydrogel form. This indicates that chitosan hydrogel may enhance the antibacterial activity of CPL MPs. Incorporating antibacterial compounds into chitosan hydrogels has been shown to boost their antibacterial activity in some experiments [18,36,63]. Furthermore, dermatokinetic experiments demonstrated the bioavailability of CPL MPs hydrogel in *ex vivo* skin, which is why this method was successful in eliminating 99.98% SA infections. Therefore, there are two primary benefits associated with the delivery of CPL-loaded MPs in chitosan hydrogel. First, this approach can control the dermatokinetic profile of CPL MPs. Second, this approach may improve the efficacy of CPL MPs hydrogels in *ex vivo* infection models of rat skin formed by SA compared to CPL without MPs hydrogel formulations. The encouraging findings of this study have validated the study's premise, demonstrating that CPL can be efficiently transported to the skin utilizing MPs, and then, once incorporated into the chitosan hydrogel formula, can exhibit its antimicrobial action to target infection. To improve the antibacterial efficacy of wound therapy, our delivery system's key advantage lies in its selective delivery ability to locally target as well as its long retention period in areas of infection on the skin. The combination of MPs with chitosan hydrogels can selectively be a feasible alternative to existing wound infection care, as MPs are employed for **the topical** administration of antimicrobial drugs to wounds.

4. Conclusion

Wound treatments using dermal delivery system are now receiving significant attention as potential for staving off infection. In this study, hydrogels are made from chitosan polymers in which the

MPs of whey protein containing CPL are embedded inside the system. CPL-loaded MPs showed desired physical and encapsulation characteristics with particle sizes in the range of $0.56 \pm 0.13 \mu\text{m}$ to $35.71 \pm 5.23 \mu\text{m}$ and EE value of $68.78 \pm 1.40\%$. The drug release behavior of CPL from CPL MPs formulation showed a value of $99.40 \pm 7.01\%$ after 24 hours, and then the cytotoxicity of MPs chemicals on RBCs can be quickly assessed using the hemolysis test. Essentially, analysis of *ex vivo* antibacterial activity showed an enhancement of 99.98% after 24 hours when CPL was incorporated into MPs in hydrogel form. Compared to pure-CPL hydrogel, the combination delivery method developed in this study has a higher residence duration in the skin, which may increase the effectiveness of antibacterial treatment in skin wound infections. It appears likely that hydrogels created using this technique can be utilized to heal wounds successfully. To further examine the efficacy of this technique, *in vivo* investigations on suitable animal models should be conducted.

Acknowledgement

Tri Puspita Roska expresses gratitude to Deutscher Akademischer Austausch Dienst (DAAD) for providing the scholarship. The Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia also provided funding for this study under contract number 090/E5/PG.02.00/PT/2022. The authors thank Glanbia Nutritionals, Inc, Fitchburg, USA for providing whey protein (WPI).

Conflict of interest

The authors state that they have no conflicts of interest.

References

- [1] J.F. Guest, N. Ayoub, T. McIlwraith, I. Uchegbu, A. Gerrish, D. Weidlich, K. Vowden, P. Vowden, Health economic burden that different wound types impose on the UK's National Health Service, *Int Wound J*. 14 (2017) 322–330. <https://doi.org/10.1111/iwj.12603>.
- [2] J.E. Cefalu, K.M. Barrier, A.H. Davis, Wound Infections in Critical Care, *Crit Care Nurs Clin North Am*. 29 (2017) 81–96. <https://doi.org/10.1016/j.cnc.2016.09.009>.
- [3] J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G.M. Eccleston, Wound healing dressings and drug delivery systems: A review, *J Pharm Sci*. 97 (2008) 2892–2923. <https://doi.org/10.1002/jps.21210>.

- 767 [4] S.G. Ingebrigtsen, N. Škalko-Basnet, A.M. Holsæter, Development and optimization of a
768 new processing approach for manufacturing topical liposomes-in-hydrogel drug formula-
769 tions by dual asymmetric centrifugation, *Drug Dev Ind Pharm*. 42 (2016) 1375–1383.
770 <https://doi.org/10.3109/03639045.2015.1135940>.
- 771 [5] D.A. Kuznetsova, D.R. Gabdrakhmanov, G.A. Gaynanova, L.A. Vasileva, D.M. Kuz-
772 netsov, S.S. Lukashenko, A.D. Voloshina, A.S. Sapunova, I.R. Nizameev, G. v. Sibgatul-
773 lina, D. v. Samigullin, M.K. Kadirov, K.A. Petrov, L.Y. Zakharova, Novel biocompatible
774 liposomal formulations for encapsulation of hydrophilic drugs – Chloramphenicol and cis-
775 platin, *Colloids Surf A Physicochem Eng Asp*. 610 (2021).
776 <https://doi.org/10.1016/j.colsurfa.2020.125673>.
- 777 [6] S.I. Miller, Antibiotic resistance and regulation of the Gram-negative bacterial outer mem-
778 brane barrier by host innate immune molecules, *MBio*. 7 (2016).
779 <https://doi.org/10.1128/mBio.01541-16>.
- 780 [7] A.D. Permana, Q.K. Anjani, Sartini, E. Utomo, F. Volpe-Zanutto, A.J. Paredes, Y.M.
781 Evary, S.A. Mardikasari, Muh.R. Pratama, I.N. Tuany, R.F. Donnelly, Selective delivery of
782 silver nanoparticles for improved treatment of biofilm skin infection using bacteria-respon-
783 sive microparticles loaded into dissolving microneedles, *Materials Science and Engineer-
784 ing: C*. 120 (2021) 111786. <https://doi.org/10.1016/j.msec.2020.111786>.
- 785 [8] S. Yasasvini, R.S. Anusa, B.N. VedhaHari, P.C. Prabhu, D. RamyaDevi, Topical hydrogel
786 matrix loaded with Simvastatin microparticles for enhanced wound healing activity, *Mat
787 Science & Eng C*. 72 (2017) 160–167. <https://doi.org/10.1016/j.msec.2016.11.038>.
- 788 [9] X. Delabranche, A. Berger, J. Boisramé-Helms, F. Meziani, Microparticles and infectious
789 diseases, *Med Mal Infect*. 42 (2012) 335–343. [https://doi.org/10.1016/j.med-](https://doi.org/10.1016/j.med-mal.2012.05.011)
790 [mal.2012.05.011](https://doi.org/10.1016/j.med-mal.2012.05.011).
- 791 [10] M. Lengyel, N. Kállai-Szabó, V. Antal, A.J. Laki, I. Antal, Microparticles, microspheres,
792 and microcapsules for advanced drug delivery, *Sci Pharm*. 87 (2019).
793 <https://doi.org/10.3390/scipharm87030020>.
- 794 [11] A. George, P.A. Shah, P.S. Shrivastav, Natural biodegradable polymers based nano-formu-
795 lations for drug delivery: A review, *Int J Pharm*. 561 (2019) 244–264.
796 <https://doi.org/10.1016/j.ijpharm.2019.03.011>.
- 797 [12] A.R. Madureira, C.I. Pereira, A.M.P. Gomes, M.E. Pintado, F. Xavier Malcata, Bovine
798 whey proteins – Overview on their main biological properties, *Food Research Intern*. 40
799 (2007) 1197–1211. <https://doi.org/10.1016/j.foodres.2007.07.005>.
- 800 [13] M.A. Farooq, M. Aquib, S. Ghayas, R. Bushra, D. Haleem Khan, A. Parveen, B. Wang,
801 Whey protein: A functional and promising material for drug delivery systems recent devel-
802 opments and future prospects, *Polym Adv Technol*. 30 (2019) 2183–2191.
803 <https://doi.org/10.1002/pat.4676>.

- 804 [14] Z. Liu, C. Liu, X. Sun, S. Zhang, Y. Yuan, D. Wang, Y. Xu, Fabrication and characteriza-
805 tion of cold-gelation whey protein-chitosan complex hydrogels for the controlled release of
806 curcumin, *Food Hydrocoll.* 103 (2020). <https://doi.org/10.1016/j.foodhyd.2019.105619>.
- 807 [15] A. Aliyah, W.W. Oktaviana, K.S. Dwipayanti, A.P. Erdiana, R.N. Utami, A.D. Permana,
808 Enhanced skin localization of doxycycline using microparticles and hydrogel: Effect of
809 oleic acid as penetration enhancer, *Pharmaciana.* 11 (2021) 239.
810 <https://doi.org/10.12928/pharmaciana.v11i2.21044>.
- 811 [16] C. Xiao, R. You, Y. Fan, Y. Zhang, Tunable functional hydrogels formed from a versatile
812 water-soluble chitosan, *Int J Biol Macromol.* 85 (2016) 386–390.
813 <https://doi.org/10.1016/j.ijbiomac.2016.01.006>.
- 814 [17] K. Mohan, A.R. Ganesan, T. Muralisankar, R. Jayakumar, P. Sathishkumar, V. Uthaya-
815 kumar, R. Chandirasekar, N. Revathi, Recent insights into the extraction, characterization,
816 and bioactivities of chitin and chitosan from insects, *Trends Food Sci Technol.* 105 (2020)
817 17–42. <https://doi.org/10.1016/j.tifs.2020.08.016>.
- 818 [18] M.E. Abd El-Hack, M.T. El-Saadony, M.E. Shafi, N.M. Zabermawi, M. Arif, G.E. Batiha,
819 A.F. Khafaga, Y.M. Abd El-Hakim, A.A. Al-Sagheer, Antimicrobial and antioxidant prop-
820 erties of chitosan and its derivatives and their applications: A review, *Int J Biol Macromol.*
821 164 (2020) 2726–2744. <https://doi.org/10.1016/j.ijbiomac.2020.08.153>.
- 822 [19] A. Jain, G. Sharma, G. Ghoshal, P. Kesharwani, B. Singh, U.S. Shivhare, O.P. Katare, Ly-
823 copene loaded whey protein isolate nanoparticles: An innovative endeavor for enhanced bi-
824 oavailability of lycopene and anti-cancer activity, *Int J Pharm.* 546 (2018) 97–105.
825 <https://doi.org/10.1016/j.ijpharm.2018.04.061>.
- 826 [20] M.M. Agwa, S. Sabra, N.A. Atwa, H.A. Dahdooh, R.M. Lithy, H. Elmotasem, Potential of
827 frankincense essential oil-loaded whey protein nanoparticles embedded in frankincense
828 resin as a wound healing film based on green technology, *J Drug Deliv Sci Technol.* 71
829 (2022) 103291. <https://doi.org/10.1016/j.jddst.2022.103291>.
- 830 [21] C. Bradford, R. Freeman, S.L. Percival, In Vitro Study of Sustained Antimicrobial Activity
831 of a New Silver Alginate Dressing, *J Am Col Certif Wound Spec.* 1 (2009) 117–120.
832 <https://doi.org/10.1016/j.jcws.2009.09.001>.
- 833 [22] B.B.K. Reddy, K.E.V. Nagoji, S. Sahoo, Preparation and in vitro & in vivo evaluation
834 of cephalixin matrix tablets, *Braz J Pharm Sci.* 54 (2018). <https://doi.org/10.1590/s2175-97902018000317277>.
- 836 [23] A.D. Permana, A.J. Paredes, F. Volpe-Zanutto, Q.K. Anjani, E. Utomo, R.F. Donnelly,
837 Dissolving microneedle-mediated dermal delivery of itraconazole nanocrystals for im-
838 proved treatment of cutaneous candidiasis, *Euro J Pharm and Biopharm.* 154 (2020) 50–61.
839 <https://doi.org/10.1016/j.ejpb.2020.06.025>.

- 840 [24] A.D. Permana, M. Mir, E. Utomo, R.F. Donnelly, Bacterially sensitive nanoparticle-based
841 dissolving microneedles of doxycycline for enhanced treatment of bacterial biofilm skin in-
842 fection: A proof of concept study, *Int J Pharm X*. 2 (2020) 100047.
843 <https://doi.org/10.1016/j.ijpx.2020.100047>.
- 844 [25] M. Mir, A.D. Permana, I.A. Tekko, H.O. McCarthy, N. Ahmed, Asim. ur. Rehman, R.F.
845 Donnelly, Microneedle liquid injection system assisted delivery of infection responsive na-
846 noparticles: A promising approach for enhanced site-specific delivery of carvacrol against
847 polymicrobial biofilms-infected wounds, *Int J Pharm*. 587 (2020) 119643.
848 <https://doi.org/10.1016/j.ijpharm.2020.119643>.
- 849 [26] A.D. Permana, R.N. Utami, A.J. Courtenay, M.A. Manggau, R.F. Donnelly, L. Rahman,
850 Phytosomal nanocarriers as platforms for improved delivery of natural antioxidant and pho-
851 toprotective compounds in propolis: An approach for enhanced both dissolution behaviour
852 in biorelevant media and skin retention profiles, *J Photochem Photobiol B*. 205 (2020)
853 111846. <https://doi.org/10.1016/j.jphotobiol.2020.111846>.
- 854 [27] U.C. Galgatte, A.B. Kumbhar, P.D. Chaudhari, Development of *in situ* gel for nasal deliv-
855 ery: design, optimization, *in vitro* and *in vivo* evaluation, *Drug Deliv*. 21 (2014) 62–73.
856 <https://doi.org/10.3109/10717544.2013.849778>.
- 857 [28] A.D. Permana, I.A. Tekko, M.T.C. McCrudden, Q.K. Anjani, D. Ramadan, H.O. McCar-
858 thy, R.F. Donnelly, Solid lipid nanoparticle-based dissolving microneedles: A promising
859 intradermal lymph targeting drug delivery system with potential for enhanced treatment of
860 lymphatic filariasis, *Journal of Controlled Release*. 316 (2019) 34–52.
861 <https://doi.org/10.1016/j.jconrel.2019.10.004>.
- 862 [29] A.D. Permana, R.N. Utami, P. Layadi, A. Himawan, N. Juniarti, Q.K. Anjani, E. Utomo,
863 S.A. Mardikasari, A. Arjuna, R.F. Donnelly, Thermosensitive and mucoadhesive *in situ* oc-
864 ular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the
865 treatment of fungal keratitis, *Int J Pharm*. 602 (2021) 120623.
866 <https://doi.org/10.1016/j.ijpharm.2021.120623>.
- 867 [30] Q. Liu, Y. Sun, J. Cheng, M. Guo, Development of whey protein nanoparticles as carriers
868 to deliver soy isoflavones, *LWT*. 155 (2022) 112953.
869 <https://doi.org/10.1016/j.lwt.2021.112953>.
- 870 [31] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G.D.-O.C. Mateo, R. Fer-
871 nández-Lafuente, J.M. Guisán, Different mechanisms of protein immobilization on glutar-
872 aldehyde activated supports: Effect of support activation and immobilization conditions,
873 *Enzyme Microb Technol*. 39 (2006) 877–882.
874 <https://doi.org/10.1016/j.enzmictec.2006.01.014>.
- 875 [32] C. le Bourvellec, C.M.G.C. Renard, Interactions between Polyphenols and Macromole-
876 cules: Quantification Methods and Mechanisms, *Crit Rev Food Sci Nutr*. 52 (2012) 213–
877 248. <https://doi.org/10.1080/10408398.2010.499808>.

- 878 [33] C. Wang, X. Zhou, H. Wang, X. Sun, M. Guo, Interactions between β -Lactoglobulin and
879 3,3'-Diindolylmethane in Model System, *Molecules*. 24 (2019) 2151.
880 <https://doi.org/10.3390/molecules24112151>.
- 881 [34] A. Abbasi, Z. Emam-Djomeh, M.A.E. Mousavi, D. Davoodi, Stability of vitamin D3 en-
882 capsulated in nanoparticles of whey protein isolate, *Food Chem.* 143 (2014) 379–383.
883 <https://doi.org/10.1016/j.foodchem.2013.08.018>.
- 884 [35] A. Khan, C. Wang, X. Sun, A. Killpartrick, M. Guo, Preparation and Characterization of
885 Whey Protein Isolate–DIM Nanoparticles, *Int J Mol Sci*. 20 (2019) 3917.
886 <https://doi.org/10.3390/ijms20163917>.
- 887 [36] W. Zhang, J. Chen, Y. Chen, W. Xia, Y.L. Xiong, H. Wang, Enhanced physicochemical
888 properties of chitosan/whey protein isolate composite film by sodium laurate-modified
889 TiO₂ nanoparticles, *Carbohydr Polym.* 138 (2016) 59–65. <https://doi.org/10.1016/j.carbpol.2015.11.031>.
- 891 [37] B. Vardhanabhuti, M.A. Kelly, P.J. Luck, M.A. Drake, E.A. Foegeding, Roles of charge
892 interactions on astringency of whey proteins at low pH, *J Dairy Sci*. 93 (2010) 1890–1899.
893 <https://doi.org/10.3168/jds.2009-2780>.
- 894 [38] I. Evdokimov, L. Alieva, V. Varlamov, V. Kurchenko, V.- Haritonov, T. Butkevich, US-
895 AGE OF CHITOSAN IN DAIRY PRODUCTS PRODUCTION, *Food and Raw Mat.* 3
896 (2015) 29–39. <https://doi.org/10.12737/13117>.
- 897 [39] M. Li, Y. Ma, M.O. Ngadi, Binding of curcumin to β -lactoglobulin and its effect on antiox-
898 idant characteristics of curcumin, *Food Chem.* 141 (2013) 1504–1511.
899 <https://doi.org/10.1016/j.foodchem.2013.02.099>.
- 900 [40] Y. Liu, L. Gao, J. Yi, Y. Fan, X. Wu, Y. Zhang, α -Lactalbumin and chitosan core–shell na-
901 noparticles: resveratrol loading, protection, and antioxidant activity, *Food Funct.* 11 (2020)
902 1525–1536. <https://doi.org/10.1039/C9FO01998G>.
- 903 [41] A. Jain, G. Sharma, G. Ghoshal, P. Kesharwani, B. Singh, U.S. Shivhare, O.P. Katore, Ly-
904 copene loaded whey protein isolate nanoparticles: An innovative endeavor for enhanced bi-
905 oavailability of lycopene and anti-cancer activity, *Int J Pharm.* 546 (2018) 97–105.
906 <https://doi.org/10.1016/j.ijpharm.2018.04.061>.
- 907 [42] Y. Zhang, M. Zhao, Z. Ning, S. Yu, N. Tang, F. Zhou, Development of a Sono-Assembled,
908 Bifunctional Soy Peptide Nanoparticle for Cellular Delivery of Hydrophobic Active Car-
909 goes, *J Agric Food Chem.* 66 (2018) 4208–4218. <https://doi.org/10.1021/acs.jafc.7b05889>.
- 910 [43] R.V. de B. Fernandes, S.V. Borges, D.A. Botrel, C.R. de Oliveira, Physical and chemical
911 properties of encapsulated rosemary essential oil by spray drying using whey protein-inulin
912 blends as carriers, *Int J Food Sci Technol.* 49 (2014) 1522–1529.
913 <https://doi.org/10.1111/ijfs.12449>.

- 914 [44] X. Shen, C. Zhao, J. Lu, M. Guo, Physicochemical Properties of Whey-Protein-Stabilized
915 Astaxanthin Nanodispersion and Its Transport via a Caco-2 Monolayer, *J Agric Food*
916 *Chem*. 66 (2018) 1472–1478. <https://doi.org/10.1021/acs.jafc.7b05284>.
- 917 [45] Y. Luo, Z. Teng, Q. Wang, Development of Zein Nanoparticles Coated with Carboxyme-
918 thyl Chitosan for Encapsulation and Controlled Release of Vitamin D3, *J Agric Food*
919 *Chem*. 60 (2012) 836–843. <https://doi.org/10.1021/jf204194z>.
- 920 [46] Z. Teng, Y. Luo, Q. Wang, Carboxymethyl chitosan–soy protein complex nanoparticles for
921 the encapsulation and controlled release of vitamin D3, *Food Chem*. 141 (2013) 524–532.
922 <https://doi.org/10.1016/j.foodchem.2013.03.043>.
- 923 [47] A. Goyal, V. Sharma, M.K. Sihag, S.K. Tomar, S. Arora, L. Sabikhi, A.K. Singh, Develop-
924 ment and physico-chemical characterization of microencapsulated flaxseed oil powder: A
925 functional ingredient for omega-3 fortification, *Powder Technol*. 286 (2015) 527–537.
926 <https://doi.org/10.1016/j.powtec.2015.08.050>.
- 927 [48] C. Wang, X. Zhou, H. Wang, X. Sun, M. Guo, Interactions between β -Lactoglobulin and
928 3,3'-Diindolylmethane in Model System, *Molecules*. 24 (2019) 2151.
929 <https://doi.org/10.3390/molecules24112151>.
- 930 [49] C.A. Busatto, M.E. Taverna, M.R. Lescano, C. Zalazar, D.A. Estenoz, Preparation and
931 Characterization of Lignin Microparticles-in-Alginate Beads for Atrazine Controlled Re-
932 lease, *J Polym Environ*. 27 (2019) 2831–2841. [https://doi.org/10.1007/s10924-019-01564-](https://doi.org/10.1007/s10924-019-01564-2)
933 2.
- 934 [50] M. Schneider, F. Stracke, S. Hansen, U.F. Schaefer, Nanoparticles and their interactions
935 with the dermal barrier, *Dermatoendocrinol*. 1 (2009) 197–206.
936 <https://doi.org/10.4161/derm.1.4.9501>.
- 937 [51] P.-E. Lee, W.-S. Choo, Characterization of flaxseed oil emulsions, *J Food Sci Technol*. 52
938 (2015) 4378–4386. <https://doi.org/10.1007/s13197-014-1495-3>.
- 939 [52] N. Kamaly, B. Yameen, J. Wu, O.C. Farokhzad, Degradable Controlled-Release Polymers
940 and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release, *Chem Rev*. 116
941 (2016) 2602–2663. <https://doi.org/10.1021/acs.chemrev.5b00346>.
- 942 [53] M. Tato, Y. López, M.I. Morosini, A. Moreno-Bofarull, F. Garcia-Alonso, D. Gargallo-Vi-
943 ola, J. Vila, R. Cantón, Characterization of variables that may influence ozenoxacin in sus-
944 ceptibility testing, including MIC and MBC values, *Diagn Microbiol Infect Dis*. 78 (2014)
945 263–267. <https://doi.org/10.1016/j.diagmicrobio.2013.11.010>.
- 946 [54] M. Abdollahi, S. Mostafalou, Chloramphenicol, in: Encyclopedia of Toxicology, *Elsevier*,
947 2014: pp. 837–840. <https://doi.org/10.1016/B978-0-12-386454-3.00709-0>.
- 948 [55] W. Yuan, Y. Feng, H. Wang, D. Yang, B. An, W. Zhang, M. Khan, J. Guo, Hemocompati-
949 ble surface of electrospun nanofibrous scaffolds by ATRP modification, *Materials Sci &*
950 *Eng C*. 33 (2013) 3644–3651. <https://doi.org/10.1016/j.msec.2013.04.048>.

- [56] Ó.L. Ramos, J.C. Fernandes, S.I. Silva, M.E. Pintado, F.X. Malcata, Edible Films and Coatings from Whey Proteins: A Review on Formulation, and on Mechanical and Bioactive Properties, *Crit Rev Food Sci Nutr*. 52 (2012) 533–552. <https://doi.org/10.1080/10408398.2010.500528>.
- [57] M.M. Ibrahim, S.A. Hafez, M.M. Mahdy, Organogels, hydrogels and bigels as transdermal delivery systems for diltiazem hydrochloride, *Asian J Pharm Sci*. 8 (2013) 48–57. <https://doi.org/10.1016/j.ajps.2013.07.006>.
- [58] N. Harish, P. Prabhu, R. Charyulu, M. Gulzar, E.V.S. Subrahmanyam, Formulation and evaluation of *in situ* gels containing clotrimazole for oral candidiasis, *Indian J Pharm Sci*. 71 (2009) 421. <https://doi.org/10.4103/0250-474X.57291>.
- [59] D.P. Chattopadhyay, M.S. Inamdar, Aqueous Behaviour of Chitosan, *Int J Polym Sci*. 2010 (2010) 1–7. <https://doi.org/10.1155/2010/939536>.
- [60] L. Montenegro, L. Rapisarda, C. Ministeri, G. Puglisi, Effects of Lipids and Emulsifiers on the Physicochemical and Sensory Properties of Cosmetic Emulsions Containing Vitamin E, *Cosmetics*. 2 (2015) 35–47. <https://doi.org/10.3390/cosmetics2010035>.
- [61] D.R. Kryscio, P.M. Sathe, R. Lionberger, L. Yu, M.A. Bell, M. Jay, J.Z. Hilt, Spreadability Measurements to Assess Structural Equivalence (Q3) of Topical Formulations—A Technical Note, *AAPS PharmSciTech*. 9 (2008) 84–86. <https://doi.org/10.1208/s12249-007-9009-5>.
- [62] X. Zhang, Y. Jiang, L. Han, X. Lu, Biodegradable polymer hydrogel-based tissue adhesives: A review, *Biosurf Biotribol*. 7 (2021) 163–179. <https://doi.org/10.1049/bsb2.12016>.
- [63] M.O. Ilomuanya, N. v Enwuru, E. Adenokun, A. FATUNMBI, A. Adeluola, C.I. IGWILO, Title: Chitosan-based microparticle encapsulated *Acinetobacter baumannii* phage cocktail in hydrogel matrix for the management of multidrug resistant chronic wound infection, *Turk J Pharm Sci*. 0 (2021) 0–0. <https://doi.org/10.4274/tjps.galenos.2021.72547>.

BUKTI
ACCEPTED

Decision on submission to Biomaterials Advances

1 message

Materials Science & Engineering C <em@editorialmanager.com>
Reply-To: Materials Science & Engineering C <support@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Wed, Oct 26, 2022 at 5:03 PM

Manuscript Number: BIOADV-D-22-02327R1

Development of Chloramphenicol Wound Dressing Protein-Based Microparticles in Chitosan Hydrogel System for Improved Effectiveness of Dermal Wound Therapy

Dear Dr. Permana,

Thank you for submitting your manuscript to Biomaterials Advances.

I am pleased to inform you that your manuscript has been accepted for publication.

My comments, and any reviewer comments, are below.

Your accepted manuscript will now be transferred to our production department. We will create a proof which you will be asked to check, and you will also be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

We appreciate you submitting your manuscript to Biomaterials Advances and hope you will consider us again for future submissions.

We encourage authors of original research papers to share the research objects – including raw data, methods, protocols, software, hardware and other outputs – associated with their paper. More information on how our open access Research Elements journals can help you do this is available at https://www.elsevier.com/authors/tools-and-resources/research-elements-journals?dgcid=ec_em_research_elements_email.

Kind regards,
Changyou Gao
Associate Editor

Biomaterials Advances

Editor and Reviewer comments:

Reviewer #1: The authors responded to all my comments and in this reviewer's opinion the paper is now acceptable.

More information and support

FAQ: When and how will I receive the proofs of my article?

https://service.elsevier.com/app/answers/detail/a_id/6007/p/10592/supporthub/publishing/related/

You will find information relevant for you as an author on Elsevier's Author Hub: <https://www.elsevier.com/authors>

FAQ: How can I reset a forgotten password?

https://service.elsevier.com/app/answers/detail/a_id/28452/supporthub/publishing/

For further assistance, please visit our customer service site: <https://service.elsevier.com/app/home/supporthub/publishing/>

Here you can search for solutions on a range of topics, find answers to frequently asked questions, and learn more about Editorial Manager via interactive tutorials. You can also talk 24/7 to our customer support team by phone and 24/7 by live

chat and email

#AU_BIOADV#

To ensure this email reaches the intended recipient, please do not delete the above code.

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: <https://www.editorialmanager.com/bioadv/login.asp?a=r>). Please contact the publication office if you have any questions.