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Chitosan Membrane Containing Chimeric Endolysin for Enhanced Antibacterial Activity Against Methicillin-Resistant *Staphylococcus aureus* in Wound Healing

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ABSTRACT

Background: The increasing prevalence of multidrug-resistant pathogens hinders effective wound healing. An alternative approach to treat such infection is utilizing phage endolysins which possess broad antimicrobial efficacy even against drug-resistant strains such as methicillin-resistant *S. aureus (MRSA)*. Chitosan-based structures are wildly used as a wound dressing and can be used as a drug carrier for antimicrobial therapeutics. This study develops and evaluates a chitosan-based membrane conjugated with a chimeric endolysin (CHAP-amidase) as a smart drug delivery system with the application as a wound dresser.

Methods: The coding gene sequence of the chimeric CHAP-amidase was subcloned into the pET-22(+) expression vector and expressed in the *E. coli* BL21 (DE3) strain. Affinity chromatography was applied to purify the recombinant protein and CHAP-amidase was visualized by SDS-PAGE and western blotting. The chitosan membrane was synthesized by the ionic gelation method and CHAP-amidase attachment was done by non-covalent ionic interaction. Then morphology of membrane was characterized using SEM microscopy imaging. The antibacterial efficiency of

chitosan membrane containing CHAP-amidase (CMCA) was evaluated against Staphylococcus aureus (*S. aureus)*, MRSA, *E. coli*, and Enterotoxigenic E. coli (ETEC) pathogens by MIC.

Results: CMCA has significantly reduced the cell count of *S. aureus* and MRSA by 3- and 4-folds respectively after 15 min. Moreover, the CMCA showed greater lytic efficiency against all tested pathogens compared with chitosan membrane and CHAP-amidase alone after 120 min. Although the CHAP-amidase was not effective against *E. coli* and ETEC, the effect of CMCA on both pathogen's cell counts was significant.

Conclusion: Regarding the excellent antibacterial activity of CMCA against *S. aureus* and MRSA and the ideal properties of chitosan for wound dressing application the CMCA could be applied as a potential wound dresser.

Keywords: Endolysin; CHAP-amidase; Chitosan membrane; antibacterial efficiency; MRSA

1. INTRODUCTION

Antibiotics were last long used to treat or prevent infections. Multiple antibacterial substances have been introduced following the discovery of penicillin in 1928, yet, one of the main challenges of using antibiotics is the continuous emergence of resistant pathogens and lack of effectiveness. *S. aureus*, a commensal bacterium in the human skin and mucous membranes, is a leading cause of severe skin and soft tissue infections (SSTIs) particularly for immune-compromised individuals [1]. The most common bacteria associated with wound infections are gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*, and gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* [2]. SSTIs are often difficult to cure and on top of that, the elevating incidence of methicillin-resistant *S. aureus* (MRSA) strains leads to increasing rates of treatment failure and relapse. MRSA is considered a major risk across the globe by increasing the mortality rate, prolonged stay at the hospital, and septic shock followed by infections [3]. Above all, the common treatment of *S. aureus* with vancomycin is highly limited with renal toxicity and the emergence of

resistant strains. Therefore, searching for and developing alternative treatments are of great interest.

Peptidoglycan hydrolases (PGH) especially, bacteriophage-encoded endolysins, are novel antimicrobials (also known as enzybiotics) in particular, against Gram-positive bacteria [4, 5]. In bacteriophages, these proteins are employed to degrade the peptidoglycan (PG) of a phage-infected bacterial host cell. This leads to rapid osmotic lysis and the death of the bacteria [6]. Compared to other classes of antibiotics, these proteins showed extremely valuable advantages including a narrow spectrum, which results in a lower rate of emerging resistant bacteria after continued exposure and more importantly prevents altering the natural microbiota [7, 8]. Notably, endolysins have already been used to treat staphylococcal infections in animal models, and many clinical trials are currently ongoing, including SAL200 and CF-301[7, 9]. Also, the commercially available Staphefekt SA.100, which contains a recombinant phage endolysin, is effectively used to treat chronic *S. aureus*-related dermatoses [10-12]. However, therapeutic application of endolysins is generally limited by challenges posed by the host system, like low bioavailability, short *in vivo* half-life (loss of activity, rapid clearance by the reticuloendothelial system, and antibody-mediated inactivation), and non-targeted delivery [13]. In this context, many efforts have been taking place to overcome these drawbacks by employing potential delivery systems for the encapsulation of endolysins [13]. Ionic attachment of CHAP-amidase protein may make more stability by reducing entropy in protein structure and increase bioavailability.

Among the recently proposed materials, polysaccharides, a versatile class of biopolymers, are of great interest and hold a central position due to their unique nature [14]. In particular, chitosan, the second most abundant polysaccharide in nature, is typically obtained by deacetylation of chitin which is the main constituent of the exoskeleton of shells in crabs, lobsters, and shrimps [15]. Chitosan exhibits multiple appealing characteristics such as excellent biocompatibility, antimicrobial activity, biodegradability, nontoxicity, and excellent hemostatic properties [16]. Hence, it has been employed in varying applications such as wound dressing, tissue engineering, health-care food supplements, drug delivery systems, etc. [17, 18]. An ideal wound dressing material must prevent infection, balance moisture, and should possess good permeability for the exchange of gases [19]. Thereafter, infection control and speedy wound healing are most required

and are the main challenges for wound healing experts. By combination of Chitosan polymer and CHAP-amidase the antibacterial activity can be induced against both Gram positive and negative bacteria.

In a previous study CHAP-amidase, a chimeric protein variety of endolysin was introduced. CHAP-amidase showed an excellent lytic effect on methicillin-resistant *S. aureus* (MRSA) [20]. In the present study, we designed, synthesized a chitosan membrane and encapsulate the CHAP-amidase to achieve a novel drug delivery system. The aim is evaluating the antibacterial property of a CMCA against gram negative and positive bacteria in compare with Chitosan membrane and CHAP-amidase solely.

2. MATERIALS AND METHODS

2.1.Materials

All the chemicals were obtained from either Sigma Aldrich (St. Louis, Missouri, USA) or Merck (Kenilworth, New Jersey, USA) with appropriate grades. All of the solvents were provided by Merck. Cell lines were purchased from Pasteur Institute, Tehran, Iran. Cell culture medium and FBS were provided by Gibco (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.2. Bacterial strains, plasmid, and media

E. coli BL21 (DE3) (BD Biosciences, San Jose, CA) strains were used as host strains. Plasmid pQE-80L (Qiagen, Hilden, Germany) was used as the cloning and expression vector containing the ampicillin resistance gene. The *E. coli* strains were grown at 37 ºC in Luria–Bertani (LB) medium in the presence of 100 µg/ml ampicillin and LB plates prepared and solidified with 2% agar.

2.3. Expression and purification of recombinant CHAP-amidase

Expression of recombinant was performed using *E. coli* BL21 (DE3) strain transformed with the pET-22b –CHAP-amidase plasmid. Starter cultures were prepared by growing a single colony of recombinant *E. coli* cells overnight at 37 ºC in flasks containing LB broth supplement with 1% glucose, 0.5% glycerol, and 100 µg/ml ampicillin. *E. coli* BL21 (DE3)/ pET-22b–CHAP-amidase expressed with 1 mM IPTG at 37 ºC with orbital shaking at 180 rpm for 4 h. *E. coli* BL21 (DE3)/

pET-22b was used as the negative control. All experiments were carried out in triplicates. The cells were harvested by centrifugation (8500 ×g for 5 min) and resuspended in 1/20 volume of PBS buffer pH 7.4. The cell disruption was conducted by sonication (60 kW) for 15 min in 30 S intervals for a 20 ml sample (Bandelin electronic GmbH & Co., Germany); the cell debris was removed by centrifugation at 8500 ×g, 10 min, 4 °C (Sigma 2-16PK, UK), and the supernatant was kept at -20 °C for further analysis.

The supernatant was then loaded onto Ni-NTA agarose (Qiagen, Hilden, Germany) affinity column chromatography (20 x 10 mm) and eluted with 300 mM of imidazole in the PBS. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze fractions under the standard protocol [25]. The purified CHAP-amidase sample was dialyzed against PBS with the buffer being replaced three times during 24 hours and further concentrated with polyethylene glycol. The protein concentration was determined by Bradford assay using BSA as standard [26].

2.4. Western blotting

15 ng of total protein samples were dissolved in SDS sample buffer, containing 5% β-mercaptoethanol and loaded onto a 10% polyacrylamide gel, electrophoresed and transferred onto nitrocellulose membrane using a semi-dry transfer system, utilizing a mini trans-blot transfer cell and pre-cooled transfer buffer at 4 °C, 100 V, for 2 h. CHAP-amidase was identified using the anti-his tag antibody (Sigma-Aldrich, product No. A7058).

2.5. Chitosan membrane synthesis

Chitosan hydrogel was synthesized by ionic gelation method using sodium tripolyphosphate (TPP) as cross-linking agent (22). Briefly, 1% of chitosan solution prepared in 20 ml of 1% acetic acid solution under gentle string for 1 h. The pH value was adjusted to 7.4–8.4 using 20 wt.% aqueous sodium hydroxide solution and furthered the cloudy white particles of chitosan polymer was appeared then centrifuge at 5000 rpm for 20 min. The precipitation was intermix with 10 ml ultrapure water. Then the appropriate amount of endolysin solution (0.5 mg/ml) was added to the chitosan mixture and cast gently into 3 Cm diameter Petri dishes. Then dried at 45 °C for 24 h.

Then 1%TPP was dissolved in ultrapure water. The chitosan membrane was immersed in TPP solution for 1 h, rinsed with water.

2.6. Chitosan membrane characteristics

The morphological characteristics of the hydrogels were studied utilizing a high-resolution Scanning electron microscope (SEM, Tecnai G20, FEI, Netherland). The functional group of the chitosan hydrogels was also investigated using Fourier-transform infrared spectroscopy (FTIR) analysis. To evaluate the ability of the polymer for maintaining proper moisture and at the same time absorbing the fluid secreted from the wound, the water absorption properties were performed. Accordingly, the chitosan membrane was cut into square shapes, weighed (w_1), immersed in PBS, and incubated at 37 °C, for 1 h. Next, the specimens were weighed again (w_2). The weight gain value (W_g) was calculated using the following equation:

$$W_g = (w_2 - w_1)/w_1 \times 100$$

2.7. Broth Microdilution Assay and MIC Estimation

Ninety-six well plates containing 200 µL per well of Muller-Hinton broth were inoculated 10% with the 0.5 McFarland suspension of tested pathogens followed by addition of chitosan membrane, CMCA, and CHAP-amidase in concentrations of 20 µg/mL and incubated at 37 °C for 15, 60 and 120 min. The concentration and time points were selected according to H. Haddad Kashani etc. [20]. Turbidity was recorded at 600 nm. Antibacterial susceptibility tests were performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucas t.org).

2.8. Statistics

All experiments were performed in triplicate. The data is presented as mean ± SD. the graphs were drawn using graph pad PRISM v.6. Unpaired t-test and One-Way ANOVA (Tukey posttest) were used to compare the relative expression levels of genes. $P < 0.05$ was considered as the level of statistical significance.

3. RESULTS

3.1. Protein characterization

The recombinant CHAP-amidase was expressed in *E.coli* BL21 and purified by Ni-NTA affinity chromatography (Figure 1a). The molecular weight of the recombinant protein was estimated to be 29.5 ± 5 Da at SDS-PAGE and the heterologous protein band was confirmed by western blotting and visualized using anti-His Tag antibody (Figure 1b).

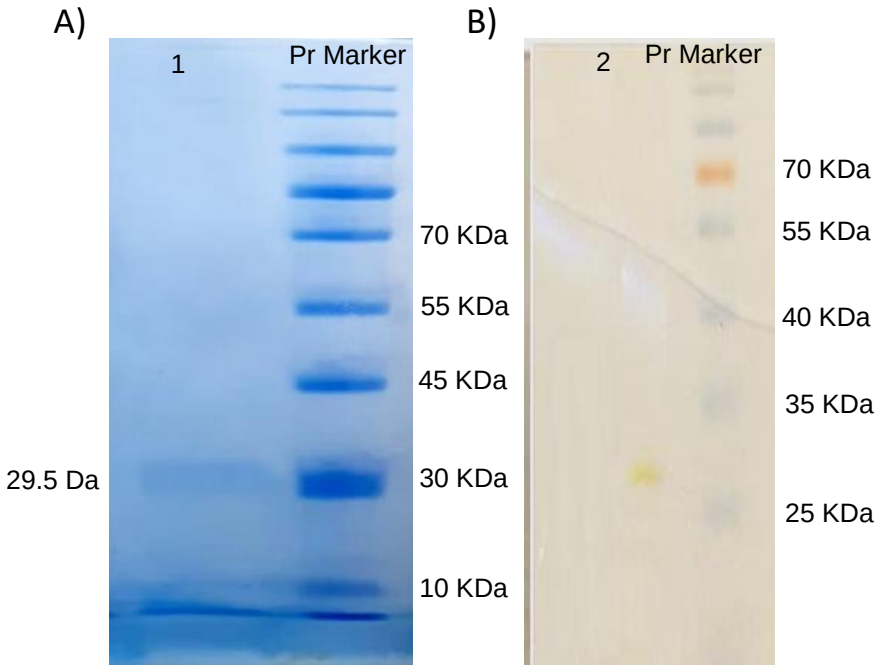


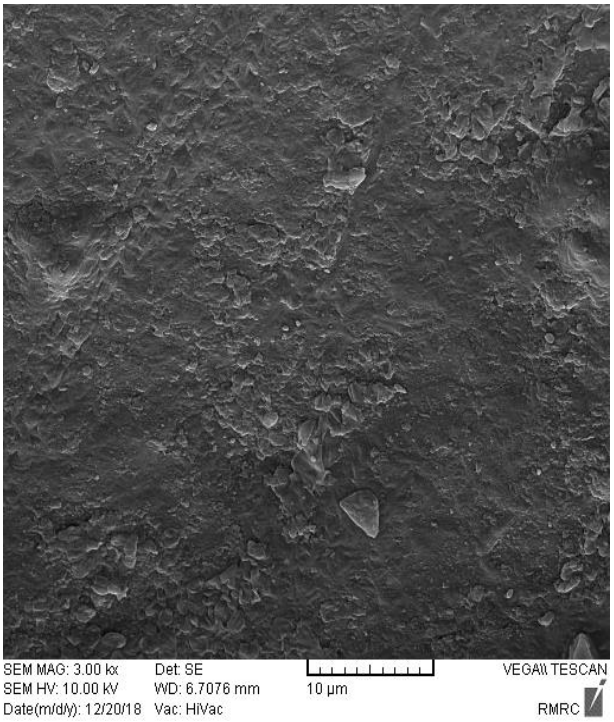
Figure 1. Expression and purification of recombinant protein in *E.coli* BL21. (a) SDS-PAGE: lane 1 purified protein and proteins marker. (b) Western blotting, lane 1 recombinant protein visualized using anti-His Tag antibody and proteins marker.

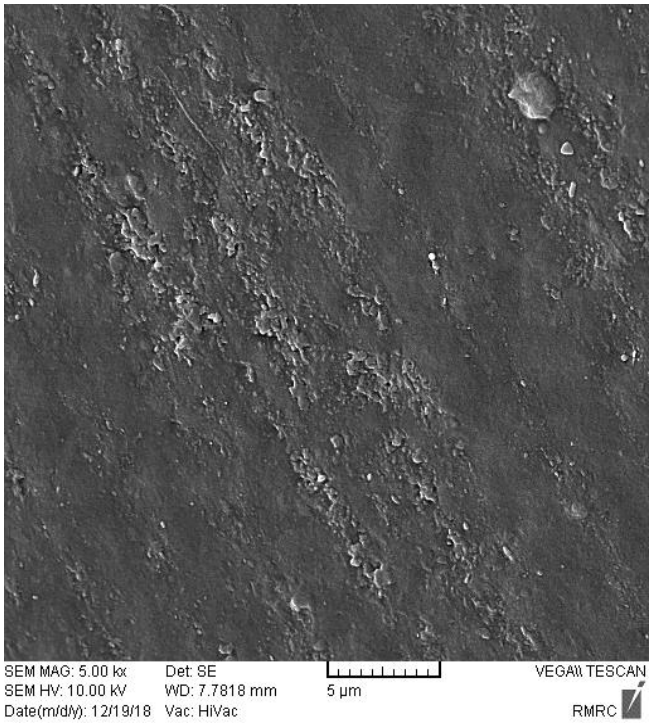
3.2. Chitosan membrane characterization

The morphology of CMCA was analyzed by Scanning Electron Microscopy (SEM) images. As illustrated from SEM images (Fig. 2), the Chitosan membrane is, semispherical shaped and well homogenous. As illustrated in Figure 2b, the sharp peaks in the pure chitosan, represented the tensile vibration of OH and CH bonds at 3444, and 2875.9 $cm^{-1,}$ respectively. also, the flexural vibration of NH at 1599 cm^{-1}, C=O, and The C-O-C at 896.83-1157.33 cm^{-1} are related to the polysaccharide skeleton. The moderate peak at 3439.19 cm^{-1} sowed the vibration of the OH groups

and the decrease in hydrogen bonds in cross-linked chitosan nanoparticles is due to the cross-linking of chitosan with sodium triphosphate (see Figure 2c) [21]. The absorption region at 2854.74 to 3007 cm^{-1} is related to the binding of TPP to chitosan and the two peaks at 1413.87 and 1541.18 cm^{-1} belong to the flexural vibration of NH_4^+ groups within the polymer. These peaks represent the ionic bond between the positive charge in the amine groups and the negative ions of triphosphate, which cause the interaction between the phosphate group and the chitosan amine group. the transfer of 2875.96 cm^{-1} bands from the C-H group related to chitosan to 2922 cm^{-1} chitosan nanoparticles improves the converting of the chitosan into nanoparticles [22]. The water absorption capacity of the chitosan membrane and CMCA were analyzed. The precentral of water in the total mass of wet membrane for chitosan membrane and CMCA was 96.9 % and 91.5 % respectively.

(a)





(b)

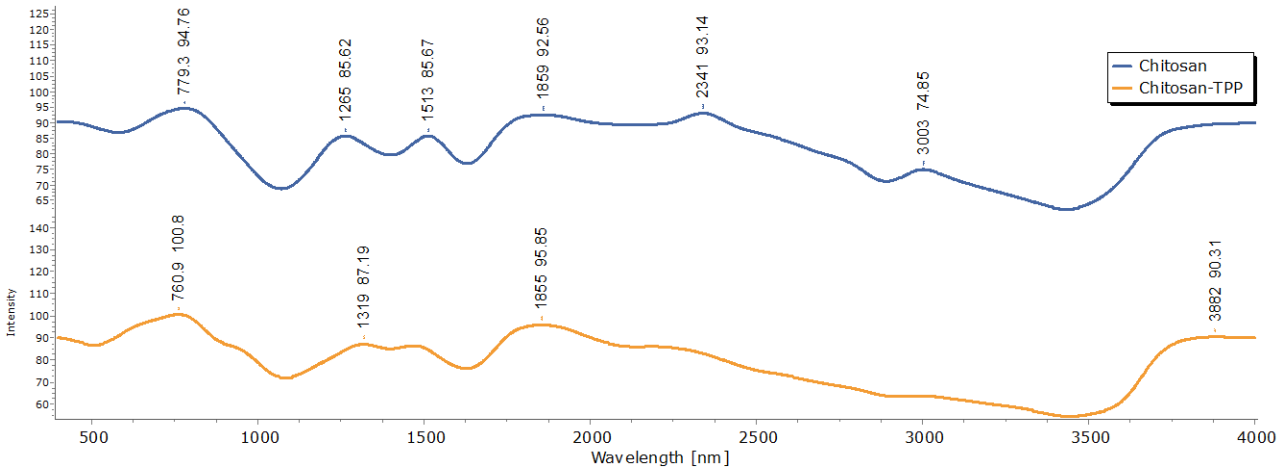


Figure 2. a) The SEM images synthetic CMCA. The SEM images showed the semispherical particles overlying the membrane. b) the FTIR diagram of chitosan nanoparticles. The spectrum showed the binding of triphosphate to chitosan and NH bending between the chitosan amine group and triphosphate moieties.

The antibacterial activity of CMCA was investigated on four pathogens, *E. coli*, ETEC, *S. aureus*, and MRSA as described in the method section. As illustrated in figure 3, concentration of CMCA, including 20 µg/mL, can reduce MRSA titer with a ΔOD 600 of 0.076 during 120 min incubation at 37◦C ($P < 0.05$) (figure 3a). The same was observed for *S. aurous* titer (figure 3b). Moreover, CMCA inhibits the growth of *E. coli* and ETEC as well (figure 4). The ETEC titer decreased with a ΔOD 600 of 0.18 after 120 min incubation at the concentration of 20 µg/mL (figure 4a). CHAP-amidase inhibits all pathogen's growth (except for ETEC) in a time-dependent manner starting after 15 min of incubation and reaching the maximum at the end of the experiment (120 min). Nevertheless, the chitosan membrane only reduced pathogens titer after 120 min of incubation. *S. aureus* was the most susceptible among all to chitosan membrane (ΔOD 600 of 0.14). Interestingly, both CHAP-amidase and CMCA were most effective on all of the pathogens at the concentration of 20 µ/mL at all three-time points. These observations indicate that the chitosan membrane showed weaker lytic activity, even though, the inhibitory effect of CMCA on pathogens growth is higher than CHAP-amidase alone with 1.5 fold, 1.16 fold, 1.5 fold at 20 µg/mL for *E. coli*, *S.*

aureus and MRSA respectively. Importantly, CMCA reduced ETEC titer with a ΔOD 600 of 0.18 at 20 µg/mL after120 min of incubation where CHAP-amidase showed no effect on ETEC (figure 4a).

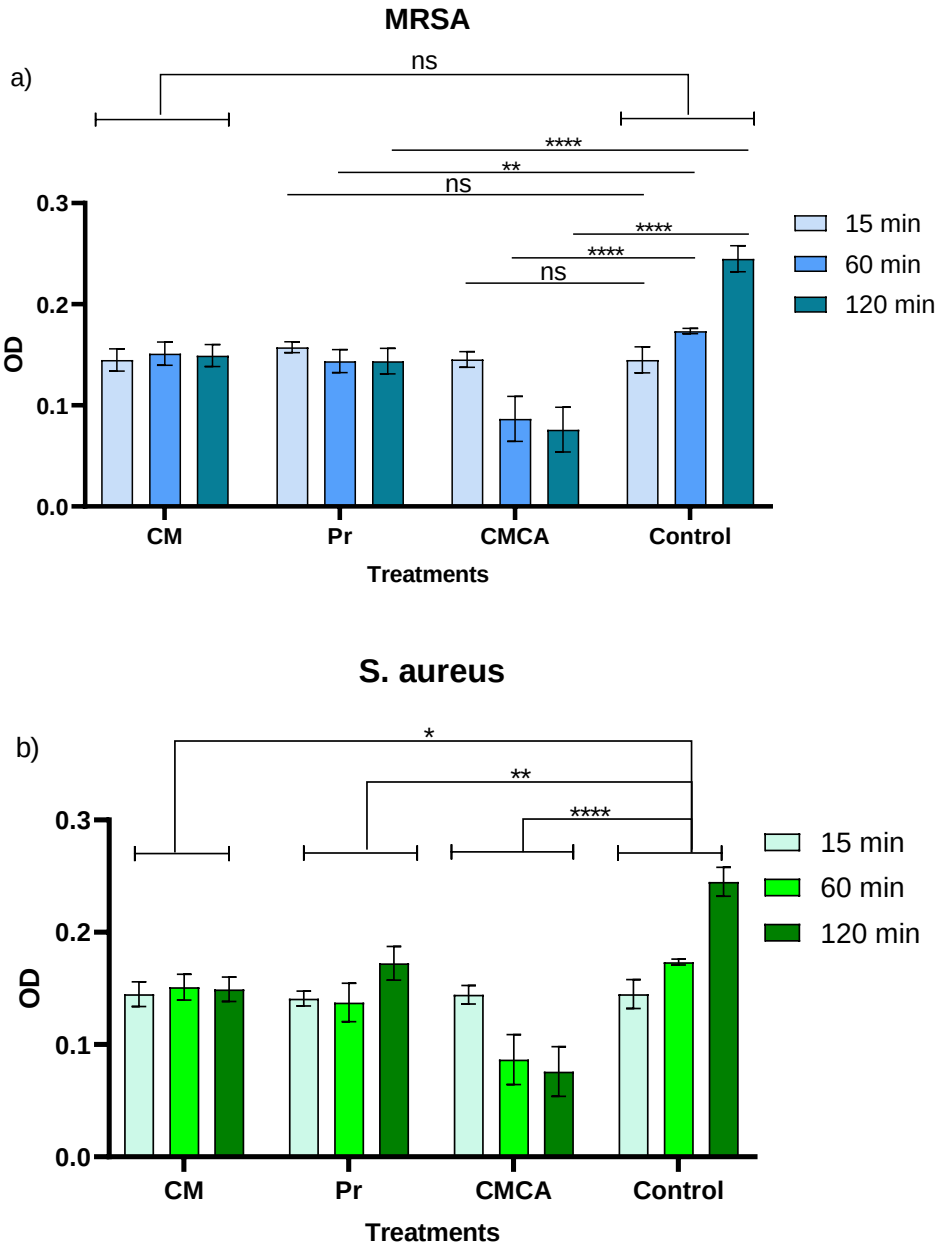


Figure 3. The lytic effect of chitosan membrane conjugated CHAP-amidase (CMCA), chitosan membrane (CM), and CHAP-amidase (Pr) on (a) methicillin-resistant *S. aureus* (MRSA) and (b) *S. aureus*. The mean ± STD of three experiments is shown.

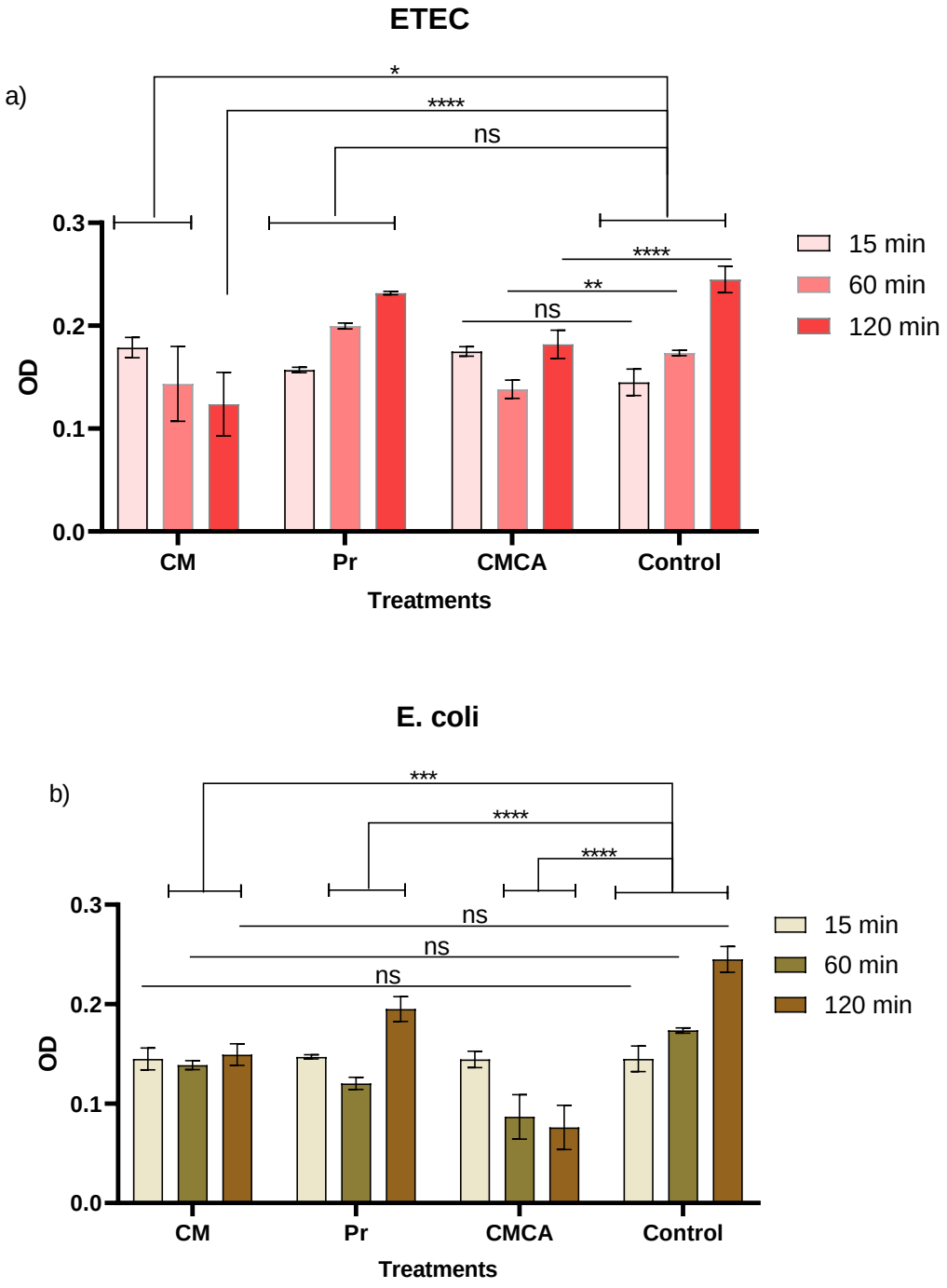


Figure 4. The lytic effect of chitosan membrane conjugated CHAP-amidase (CMCA), chitosan membrane (CM), and CHAP-amidase (Pr) on (a) enterotoxigenic *E. coli* (ETEC) and (b) *E. coli.* The mean ± STD of three experiments are shown

4. DISCUSSION

In the present study, we designed, synthesized, and evaluate the lytic activity of a novel chitosan membrane encapsulated a chimeric protein, comprised of CHAP and amidase domains of LysK endolysin. The synthesized hydrogel exhibited a homogenous polymeric structure with semispherical chitosan particles. Also, the high-water absorption capacity of the chitosan hydrogels helped to maintain the moisture and absorbed extra fluids of the wound. The modular structure of endolysin is feasible for the designing of novel tailored endolysins by domain deletion and shuffling [23]. In this context, many notable efforts have been aimed to develop phage-derived lytic proteins effective on *S. aureus* [9]. Many of these proteins, including CHAPK, LysH5, CHAP-SH3b, ClyH, or ClyF, have been reported to inhibit or eliminate biofilms [9, 24]. The production of the CHAP-amidase has been previously described by Kashani et al. The CHAP-amidase reduced the MRSA cell count by 3.2 log and showed a strong synergic effect with vancomycin by 8-fold reduction in the MIC of vancomycin [20]. Similarly, our data showed that CHAP-amidase displayed lytic activity against *S. aureus* and MRSA whereas no significant effect was observed against *E. coli* and ETEC. As mentioned before, instability and short *in vivo* lifetime of endolysins are the major drawbacks that limit their application. Using biopolymers to encapsulate and deliver endolysins is an interesting approach to overcome such issues. Chitosan-based structures hold great interest for smart drug delivery applications because of their unique characterizations. The anti-bacterial properties of chitosan biopolymers are well stabilized in the literature [25-30]. Generally, for biotherapeutics applications, chitosan-based formulations are preferable approaches over other types of delivery systems. Chitosan-based delivery systems elevated the bioavailability of therapeutic agents as well as safe removal from the host system. A few mechanisms of action of chitosan antibacterial activity are described in the literature, which is disrupting the cell membrane/cell wall, interacting with genomic DNA, chelating of nutrients, and formatting a dense polymer film on the cell surface [31]. Although many reports are available in the literature on bacteriophage encapsulation systems, few are described the endolysins encapsulation system [32]. A truncated variant of LysK endolysin domain (CHAPK) and lysostaphin were encapsulated in thermally triggered Poly (N-isopropyl acrylamide) (PNIPAM) nanoparticles [33]. Portilla et al. described the encapsulation of LysRODI endolysin in pH-sensitive liposomes with a significant impact on the cell count of *S. aureus* at pH 5 [34]. Here we

described an endolysin delivery system comprising of chitosan membrane-anchored with CHAP-amidase. Our findings showed that the CMCA showed greater lytic activity against all tested pathogens compared with chitosan membrane and CHAP-amidase alone. Moreover, the CMCA influenced the *E. coli* and ETEC cell counts after 20 mins, where CHAP amidase did not exhibit any lytic activity. Similarly, Gondil et al reported an increase in the antibacterial effect of Cpl-1 endolysin encapsulated in chitosan nanoparticles in both *in vitro* and *in vivo* conditions. They developed A mucoadhesive chitosan nanoparticles containing a full-length Cpl-1 endolysin with anti-streptococcal activity [7, 35]. Kaur et al. also reported a chitosan-alginate-based endolysin delivery system for efficient delivery of LysMR-5, an anti-staphylococcal endolysin in vitro [36]. At the end, the significant reduction in pathogen titers demonstrated in vitro positions CMCA as a compelling candidate for wound dressing applications. However, the dynamic environment of a real wound (e.g., presence of exudate, serum proteins, varying pH, and host immune components) could influence CHAP-amidase stability and chitosan membrane performance. Therefore, subsequent investigation in preclinical wound models is essential to confirm CMCA's antibacterial efficacy and wound-healing promotion under conditions mimicking clinical use, particularly for challenging MRSA-infected wounds.

5. CONCLUSION

This study demonstrates that the chitosan-based CHAP-amidase (CMCA) formulation exhibits superior antibacterial activity against Staphylococcus aureus and methicillin-resistant S. aureus (MRSA) compared to chitosan membrane or CHAP-amidase alone [13]. Chitosan’s wound-healing properties extend beyond its antibacterial effects, promoting rapid re-epithelialization, granulation tissue formation, and fibroblast proliferation [32, 33]. Its biocompatibility creates a moist wound environment conducive to cell migration, while its hemostatic activity forms a tight seal, reducing bleeding and exudate [34]. The incorporation of CHAP-amidase enhances these properties by selectively lysing pathogenic bacteria, minimizing infection-related inflammation, and fostering a cleaner wound bed for tissue regeneration [24]. Chitosan enhances CHAP-amidase’s antibacterial efficacy through multiple mechanisms. Its positively charged structure disrupts bacterial cell membranes, increasing susceptibility to CHAP-amidase’s enzymatic lysis [18]. Additionally, chitosan’s matrix stabilizes CHAP-amidase, ensuring sustained activity,

while its mucoadhesive properties localize the enzyme to infected sites, enhancing biofilm disruption. Compared to existing wound dressings, CMCA offers distinct clinical advantages. Silver-based dressings, such as Aquacel Ag, provide broad-spectrum antibacterial activity but risk cytotoxicity and resistance [37]. Hydrocolloid dressings maintain moisture but lack active regenerative support. CMCA's biodegradable nature and dual antibacterial-regenerative properties reduce dressing changes and improve patient comfort, positioning it as a promising alternative for infected wounds. Despite these findings, limitations include the study's in vitro focus, which may not fully reflect complex wound environments, and its evaluation against limited bacterial strains. The lack of long-term biocompatibility data further constrains clinical insights. Future research should prioritize in vivo studies in animal models, particularly for chronic wounds like diabetic ulcers, to validate CMCA's efficacy. Challenges include ensuring CMCA stability in physiological conditions, scaling up production, and navigating regulatory hurdles for clinical translation. These investigations will clarify CMCA's potential as an advanced wound dressing.

6. Declarations

- Consent to Participate:

The authors declare that they have consent to participate

- Consent to Publish:

The authors declare that they have consent to publish

- Authors' Contributions:

Sahar Sarbandi: Study concept and design, Acquisition of data, Drafting of the manuscript.

Hossein Fahimi: Critical revision of the manuscript for important intellectual content.

Hamed Hadad Kashani: technical and material support.

Sepideh Khaleghi: Statistical analysis, Administrative, technical and material support, Study supervision, Analysis, and interpretation of data.

- Funding: There is no funding/support for this project. All of the materials were provided personally. - Competing Interests: The authors declare no competing interests.

- Availability of data and materials: The data that support the findings of this study are available on request from the corresponding author

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