Preparation of Rosemary Collagen Chitosan Blend Membrane Using Response Surface Methodology (RSM)

Ming He, Yuhan Zhai, Yuqing Zhang, Shuo Xu, Shaoxuan Yu, Haifang Xiao and Yuanda Song

Colin Ratledge Center for Microbial Lipids, School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, China

Abstract: The present research provided a formula for the optimization of rosemary/collagen/chitosan blend membrane. Rosemary Extract (RE) was used as additive and membrane was prepared using tensile strength, elongation at break and elongation length as the indexes. The results of single factor experiment showed that the addition ratio of collagen to chitosan, additive content, glycerol content and drying temperature all affected the mechanical properties and thickness of the blend membrane, among which the addition ratio of collagen to chitosan and glycerol content had a great influence. Box-Behnken Design (BBD) of Response Surface Methodology (RSM) was used to optimize the process and the parameters were obtained as follows: The ratio of collagen to chitosan (39:61, v/v), the addition of RE (0.6 mg/mL) and the glycerol content (30.2%). The mechanical properties and thickness of the optimized formula were verified, which proved the feasibility of the scheme and the broad application prospect of rosemary/collagen/chitosan blend membrane in meat packaging industry. The optimal data of tensile strength, elongation at break, elongation length and thickness were 2789.65 g, 49.92%, 117.55 and 0.143 mm, respectively. In the future, the research of blend membrane in food industry will more meet the diversified and high-performance market demand.

Keywords: Blend Membrane, Mechanical Properties, Response Surface Methodology

Introduction

With the improvement of people's requirements on food quality and shelf life as well as the enhancement of people’s awareness of environmental protection, the edible packaging made of natural biological materials has become a research hotspot in the field of food packaging (Petkoska et al., 2021). Compared with the traditional polymer packaging materials, the edible film is more environmentally safe and easier to degrade. In some aspects, it has the potential to replace the traditional polymer packaging materials (Dou et al., 2018).

Collagen is a triple-helical protein capable of self-assembly into the fibrillar structure, which exhibits tensile strength and excellent ability to support cell adhesion (Irawan et al., 2021). The manufacturing cost of artificial collagen casing is low (only half of the cost of natural casing) and the preparation process is simple. Previous study has found that the physical properties of pure collagen membrane are far from meeting the requirements of casing (Chu et al., 2017). After extraction and in vitro recombination of collagen in natural materials, the hierarchical structure of collagen is changed and its mechanical properties are significantly reduced (Xiao et al., 2021). Unoptimized collagen membrane is prone to poor mechanical strength, adhesion, caking and other problems. Blending with another natural polymer is an effective method to optimize the mechanical properties of collagen membrane (Li et al., 2019). To overcome these limitations, biological materials like chitosan can be incorporated with collagen (Gurumurthy et al. 2018). Chitosan, the most abundant biopolymer second to cellulose, consists of β-(1-4)-2- acetalamiño-α-glucose and β-(1-4)-2- amino-α-glucose units (Sun et al., 2017). Chitosan has been found to be nontoxic, biodegradable, biofunctional, biocompatible in addition to possess antimicrobial characteristics (Vedula et al., 2021). In addition, chitosan is an excellent component of edible film due to its film-forming capacity and valuable mechanical properties for forming transparent films, which can fulfill various packaging
needs (Singh et al., 2015). Untreated collagen presents a fast biodegradation rate and low mechanical strength. However, addition of chitosan interacting with collagen nanofiber enhances fiber network and increases the pore size of scaffold (in the freeze-dried form), resulting in a significant improvement in mechanical strength (Oliveira et al., 2019).

In addition, natural active ingredients can be added in edible membrane to improve its antioxidant and antibacterial capacities. For instance, prepared chitosan/β-cyclodextrin/tannic acid biocomposite membrane, the oxidation resistance of the membrane was obtained successfully (Ulu et al., 2021). Li et al. (2021) found that bletilla striata polysaccharide can improve antibacterial activity from fabricating bletilla striata polysaccharide/chitosan membranes. Rosemary Extract (RE) are widely used as additives in the food (e.g., flavorings, antioxidant and antimicrobial agent) (Moczewska et al., 2020). RE from the Lamiaceae family are a source of bioactive ingredients including phenolic acids, flavonoids, diterpenoids and triterpenes (De Paiva et al., 2021). The main polyphenolic compound found in rosemary is rosmarinic acid, which exhibits high antioxidant and antimicrobial properties. The addition of RE to the chitosan films had positive effect on lipid oxidation and microbial contamination of the fresh pork sausages (Jancikova et al., 2019). Natural active ingredients slow down the automatic oxidation of meat and can also affect the physical and mechanical properties of the film. Elongation at break of all films supplemented with RE was reduced by higher 60% compared with the control group (Piñeros et al., 2017). Yan et al. (2013) also found that tensile strength and elongation at break of starch-alginate composite membrane decreased after adding RE. Natural phenolic compounds have been successfully used in active packaging. Through increasing the content of phenolic compounds and activities of anti-free radicals, the interaction between macromolecules and active groups have been increased and the physical properties such as the barrier property of the film have been greatly improved (Lei et al., 2019).

Base on the above, chitosan and RE were used in collagen blend membrane in this study. Single factor experimental design and Response Surface Methodology (RSM) was employed to optimize the preparation process of the blend membrane. The objective of this study was to evaluate the effect of ratio of collagen and chitosan, addition of RE and glycerol content on the mechanical properties and thickness of blend membrane, with the aim of optimizing the formulation for food packaging. Our results in this study will provide a basis for optimizing the formulation of blend film for food packaging industry.

Materials

Collagen was purchased from Shandong Zhongtian Biotechnology Co., Ltd (Zibo, China). Water soluble RE was obtained from Zhengzhou Wanbo Chemical Products Co., Ltd (Zhengzhou, China). Chitosan, glycerol and acetic acid were purchased from Sinopac Chemical Reagent Co., Ltd (Beijing, China). All of the reagents used in the experiment were analytically pure.

Methods

Preparation of Blend Membrane

Chitosan was dissolved in 1% (v/v) acetic acid firstly, then collagen and chitosan solutions (6 g: 200 mL m/v) were both prepared using distilled water. RE was added to cowhide collagen solution for mixing. Then the RE-collagen mixture was blended with chitosan solution using digital magnetic stirrers. Meanwhile glycerol was added to the mixture as plasticizer quantitatively. Film forming solution (30 mL) was accurately measured and cast in 10×10 cm polystyrene culture dishes. After removing bubbles on the surface, the film was dried for 16 h.

Single Factor Experimental Design of Blend Membrane

Single factor design was used to analyze the influences of various variables on mechanical properties and thickness of the blend membrane. Ratios of collagen to chitosan were set as 20:80, 30:70, 40:60, 50:50 and 60:40 (v/v), respectively. The count of water-soluble RE addition was set as 0.2-1.0 mg/mL. Glycerol content (percentage of total collagen and chitosan content) was set at 20%-40% and drying temperature was set at 30-50°C.

Determination of Mechanical Properties

Each prepared blend film was cut into six 6 cm×1 cm strip. Tensile strength, elongation at break and elongation length were measured by texture analyze (lotus scienceco, Ltd), respectively. The thin film strip was clamped between the gripping clamp probe A/MTG, then stretched at 1 mm/s crosshead capacity, with the initial distance of 40 mm and the tensile speed of 0.5 mm/s.

Determination of the Thickness

Film thickness was measured using the electronic digital display helical thickness gauge (Aipu Metrology Instrument Co., Ltd) at three random sites 20 mm from the edge.
Box-Behnken Design (Bbd) for the Blend Membrane

Base on the single-factor test results, the factors and levels affecting the mechanical properties and thickness of RE/chitosan/collagen blend membrane were determined. A three-level-three-factor, BBD was adopted to further optimize the membrane preparation process. Ratios of collagen to chitosan \((X_1)\) (30:70-50:50, v/v), addition of RE \((X_2)\) (0.4-0.8 mg/mL), glycerol content \((X_3)\) (25-35%) on the dependent variables of the films were used in the current study. The stated variables included tensile strength \((Y_1/\text{g})\), elongation at break \((Y_2/\%)\), elongation length \((Y_3/\text{mm})\) and thickness \((Y_4/\text{mm})\).

The design matrix and corresponding results of blend membrane process optimization were listed in Table 1. The process flow diagram of blend membrane was shown in Fig. 1. Multiple regression analysis methods were used to process the experimental data (Saberi et al., 2016). The relationship between response of film forming performance and test variables can be expressed by the following second-order polynomial equation:

\[
Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_i X_i^2 + \sum_{i<j}^{3} \beta_{ij} X_i X_j
\]

where, \(Y\) is the predicted response; \(\beta_0, \beta_i, \beta_i, \) and \(\beta_{ij}\) are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. \(X_i\) and \(X_j\) are the independent variables.

Statistical Analysis

Origin was selected to process and plot the test data of single factor test. Design-expert 8.0.6 was used for data analysis and variance analysis of RSM. \(p\) values less than 0.05 were considered to be statistically significant. All data were measured three times and averaged.

Results and Discussion

Effect of Mass Ratio of Collagen to Chitosan on Mechanical Properties and Thickness of Blend Membrane

We investigated the effect of ratio of collagen to chitosan on mechanical properties of blend membrane. It can be seen from Fig. 2, as the proportion of collagen increasing, tensile strength, elongation at break, elongation length and thickness of blend membrane tended to increase at first and then decrease and peaked at 40:60 \((\text{v/v})\). The peak values of tensile strength, elongation at break, elongation length and thickness of the membrane were 2674.119 g, 18.746%, 41.658 mm and 0.134 mm, respectively. Results in Fig. 2 showed that the addition of chitosan to the blend film led to an increase in tensile strength by approximately 50%. Chitosan contains more polar groups. Under the same humidity, with the increase of the amount of chitosan, moisture absorption rate of blend membrane increases (Glampedaki et al. 2011). The plasticizing effect of water improves motility of the molecular chain segment of blend membrane and then enhances elongation at break of composite membrane (Lewandowska et al., 2016). As the content of chitosan increases, the degree of collagen cross-linking increases substantially, which reduces fluidity of molecular chain macroscopically showing that elongation at break decreases (Chen et al., 2014). As a consequence, with the increasing of the chitosan content, the elongation at break of the membrane firstly increased and then decreased, which was consistent with our research results. In addition, compared with previous studies on fibrin/chitosan based composite membrane added with rosemary extract, the RE/chitosan/collagen blend membrane has better elongation at break (Du et al., 2021). Tensile strength of blend membrane was inversely proportional to the proportion of chitosan, possibly because augment of the chitosan content cannot form.

Fig. 1: The process flow diagram of rosemary/collagen/chitosan blend membrane with high properties
sufficient intermolecular force between collagen and chitosan (Yi et al., 2019). The thickness of the blend membrane depends on its composition and processing parameters. Where higher concentrations of biopolymer in the formulation leads to an increase in solids in the polymer matrix, thus the blend membrane becomes thicker evidently (Batista et al., 2019).

Effect of Re on Mechanical Properties and Thickness of Blend Membrane

Adding RE to food products could slow down or prevent the oxidation reactions of processed meat and fishery products (EFSA, 2008). As can be seen in Fig. 3, with the increase of RE addition, the elongation at break and elongation length of blend membrane decreased significantly. With the addition of RE within the set concentration range, the tensile strength and thickness first increased and then decreased and reached the maximum value at 0.6 mg/mL. The possible reason for this phenomenon is that the polyphenols in RE cross-linking with chitosan and then reduce the tensile properties of blend membrane. Previous study found that the chitosan-based film, became stronger and less stretchable due to the enhancement of cross-linking agent after added with thyme extract. (Talón et al., 2017).

Effect of Glycerol on Mechanical Properties and Thickness of Blend Membrane

Glycerol can be used as plasticizer to improve the processing performance of edible collagen membrane. Previous study reported that due to the addition of plasticizer, the elongation at break of composite film could be improved significantly, thus optimized the problem for easy to break (Chen et al., 2014). In the present study, glycerol was employed in preparing blend membrane. As illustrated in Fig. 4, tensile strength, elongation at break, elongation length and thickness of blend membrane all increased with the increase of glycerol content. When the content of glycerol exceeded 30%, thickness of blend membrane had no obvious changes and elongation length of blend membrane began to decrease. The probable explanation might be that glycerol could soft the rigid structure of blend membrane, increase the free volume of system and fluidity of molecular chain by weakening the macromolecular or intramolecular interaction (Ghasemliou et al., 2011).

Effect of Drying Temperature on Mechanical Properties and Thickness of Blend Membrane

Higher heat treatment temperature is conducive to improve strength on the interaction force of collagen and chitosan molecules gradually, sequentially the formed membrane structure tends to be compact (Su et al., 2021). The softness and elongation at break of the composite film reach ideal condition but viscosity of the film increased when drying temperature is close to 50°C (Chen et al., 2014). Therefore, it was necessary to choose appropriate drying temperature in this research. As can be seen in Fig. 5, tensile strength, elongation at break and elongation length of blend membrane were relatively high, when drying temperature was 30°C, which was consistent with the research results of on the influence of drying temperature on mechanical properties of collagen-chitosan blend membrane (Wang et al., 2020). Therefore, drying temperature was set as 30°C in this study.

Optimization the Preparation of Blend Membrane by RSM

Models Fitting and Statistical Analysis

The influences of the variables (ratio of collagen to chitosan, addition of RE and glycerol content) on tensile strength, elongation at break, elongation length and thickness of blend membrane were investigated through 3³ factorial experimental design of RSM. The experimental design and observation of BBD dependent variable data were summarized in Table 2. ANOVA was used to judge whether the quadratic model was significant or not. BBD for model fitting was shown in Table 3. Through multiple regression analysis of experimental data, four second-order polynomial mathematical models were obtained. These mathematical models were used to demonstrate the relationship between independent variables and the mechanical properties and thickness of RE/chitosan/collagen blend membrane. The final equations obtained according to the coding factor were as follows:

\[
Y_1 = 49.54 - 4.33X_1 + 0.13X_2 + 3.42X_3
\]
\[
+ 1.77X_1X_2 + 4.91X_1X_3 - 2.4X_2X_3
\]
\[-15.42X_1^2 - 10.38X_2^2 - 6.46X_3^2
\]
\[Y_2 = 117.19 - 5.79X_1 + 0.99X_2 + 6.28X_3
\]
\[-3.92X_1X_2 - 4.20X_1X_3 - 0.61X_2X_3
\]
\[-44.41X_1^2 - 31.12X_2^2 - 25.89X_3^2
\]
\[Y_3 = 0.15 - 4.75 \times 10^{-3}X_1 + 1.5 \times 10^{-3}X_2
\]
\[+ 5 \times 10^{-4}X_3 - 2.75 \times 10^{-3}X_1X_2 - 3.25 \times 10^{-3}X_1X_3
\]
\[+ 5.75 \times 10^{-3}X_2X_3 - 0.042X_1^2 - 0.013X_2^2 - 0.013X_3^2
\]

The lack of fit test and analysis of variances were used to evaluate the fitted model and suitability of true response surface. The coefficient of determination \(R^2\) of the model was 1.00, signified that 100% of experimental data can be predictably fitted with model data for tensile strength. The predicted residual sum of square (PRESS) for the model is an indication of how well the predictive model matches each point in the design (Saberi et al., 2016). According to analysis of the model, the high-level F value (15.66) and low-grade p value (<0.01) implied that the model was a sky-high statistically significance.
According to the ANOVA in Table 3, p value of the model was lower than 0.0001, indicated that the regression model was at an extremely significant level, the regression equation could accurately reflect the relationship between tensile strength and various factors in the test conditions. The lack of fit item was not significant as its p value was 0.1967, indicated that the actual test and regression model were well fitted. In this model, $R_{adj}^2 (0.9542)$ indicated that the variation of tensile strength mainly was distributed among three design factors. The multivariate correlation coefficient $R^2 (0.9799)$ fully indicated that there was an admirable fitting degree between the actual and predicted results.

The determination coefficient ($R^2$) of elongation at break model (Table 3) was estimated to be 0.9575, which further indicated that there was a commendable correlation between the predicted value and experimental value. Lack of fit value and p value of the model were calculated to be 0.2587 and 0.0005, respectively, which in turn revealed that the mathematical model was satisfactory for prediction of elongation at break of blend membrane.

The RSM mathematical model of elongation length was calculated and the results showed that elongation length p value was lower than 0.001. The determination coefficient ($R^2$) of elongation length model (Table 3) was estimated to be 0.97, which in turn revealed that the mathematical model was satisfactory for prediction of elongation length of blend membrane.

Fig. 2: (a) Effect of ratio of collagen to chitosan on mechanical properties of blend membrane; (b) Effect of ratio of collagen to chitosan on thickness of blend membrane

Fig. 3: (a) Effect of RE content on mechanical properties of blend membrane; (b) Effect of RE supplemental addition on thickness of blend membrane
Fitting the model for thickness showed in Table 3 that $R^2$ value of the models was 0.94. The p value for lack of fit, F value and p value of the model for thickness were 0.28, 12.6 and less than 0.01, respectively. Experimental results verified the reliability of the model in predicting thickness of blend membrane.

Analysis of Response Surface Plot and Contour Plot

Response surface analysis and contour variation represented the interaction between various factors and the degree of influence of different factors on tensile strength, elongation at break, elongation length and thickness. The contour shape showed the strength of interaction between various factors. The circle meant that two factors were not significant, while the ellipse meant that they were relatively significant. The steepness of the 3D graph on the response surface indicated the influence of various factors on the experimental indicators.

The Effect of Independent Variables on Mechanical Properties

Tensile strength of blend membrane in response surface test ranged from 1174.14 to 2868.83 g. As shown in Table 4, $X_1$, $X_1X_3$, $X_1^2$, $X_2^2$ and $X_3^2$ significantly affected the tensile strength of the blend membrane, indicated that the ratio of collagen to chitosan ($X_1$) was a more significant variable than addition of RE ($X_2$) and glycerol content ($X_3$). As can be seen from Fig. 6, when glycerol content was fixed at 0 level, tensile strength increased first and then decreased with ratio of collagen to chitosan ($X_1$). Moreover, the highest tensile strength values were obtained at 38.8:61.2 (v/v) of ratio of collagen to chitosan and 29.8% of glycerol content, respectively. In Fig. 6B, the more...
obvious the ellipse shape of the contour line was, indicated that the interaction between X₁ and X₃ was obvious.

In RSM, elongation at break of blend membrane ranged from 15.55 to 52.68%. As shown in Table 4, coefficients of X₁, X₃ and X₁X₃ were significant based on a 95% confidence level in affect elongation at break of blend membrane, showed that two variables had a significant influence on elongation at break and X₁ and X₃ interacted significantly. Moreover, there was no significant interaction between X₁X₂ and X₂X₃. In order to investigate the effects of the independent variables and interactions between them on elongation at break, response surface plots and contour plots displayed in Fig. 7 were used. The results showed that the ratio of collagen to chitosan and glycerol content had a significant influence on elongation at break of the blend membrane, meanwhile there was a significant interaction between them.

Elongation length of blend membrane varied from 33.44-123.36 mm in RSM experiments (Table 2). The quadratic effects of ratio of collagen to chitosan (X₁²), addition of RE (X₂²) and glycerol content (X₃²) on elongation length were significant (p<0.01), however the interaction effects were non-significant. Fig. 8C showed the influence of addition of RE and glycerol content on elongation length. When the ratio was fixed at 0 level, elongation length of both increased continuously and then decreased. According to plasticizer impacted elongation length due to glycerol to reduce the interactions among polymer chains, thus decreased film resistance and increased flexibility (Teixeira et al., 2021).

**The Effect of Independent Variables on Thickness**

In RSM, the maximum and minimum thickness of blend membrane were 0.079 and 0.152 mm respectively (Table 2). The contour plots of Fig. 9A and 9B were similar, indicated that X₂, X₃, X₁X₂ and X₁X₃ had no significant elongation or interaction on the blend film. According to previous correlated report, the thickness of optimized film did not differ (p>0.05) compared to control, which was consistent with the results of our study (Batista et al., 2019).

**Verification of Predictive Model**

The desirability function was used for simultaneous optimization of the multiple responses (Saberi et al., 2016). The ratio of collagen to chitosan, the addition of RE and glycerol content were 38.97:61.03 (v/v), 0.6 mg/mL and 30.21%, respectively. Tensile strength, elongation at break, elongation length and thickness were 2789.65 g, 49.92%, 117.55 and 0.143 mm, respectively. In order to verify the feasibility of the response surface optimization results, verification tests were carried out. The optimal conditions were determined as follows: Ratio of collagen to chitosan was 39:61 (v/v), addition of RE was 0.6 mg/mL and glycerol mass fraction was 30.2%. It can see from Table 5, there was slightly different between the predicted values and experimental values, indicated that it was feasible and of practical significance to optimize the process of the blend membrane by RSM.

**Table 1: Factors and coding value of response surface experimental design**

| Levels   | -1  | 0   | 1   |
|----------|-----|-----|-----|
| Ratio of collagen to chitosan (X₁) /v/v | 30: | 70  | 50: |
| Addition of RE (X₂) mg/mL              | 0.4 | 0.6 | 0.8 |
| Glycerol content (X₃) %                | 25  | 30  | 35  |

**Table 2: Box-behnken experimental design and results for mechanical properties and thickness**

| Runs | X₁  | X₂  | X₃  | Y₁ /g | Y₂ /% | Y₃ /mm | Y₄ /mm |
|------|-----|-----|-----|-------|-------|--------|--------|
| 1    | -1  | 0   | 1   | 1174.14 | 29.93 | 68.72  | 0.093  |
| 2    | 0   | 0   | 0   | 2868.83 | 50.18 | 110.96 | 0.144  |
| 3    | 0   | 1   | -1  | 1557.72 | 34.79 | 59.54  | 0.104  |
| 4    | 0   | 0   | 0   | 2994.91 | 52.68 | 112.08 | 0.147  |
| 5    | 1   | 0   | -1  | 1242.15 | 15.55 | 33.44  | 0.096  |
| 6    | -1  | 0   | -1  | 1882.82 | 31.31 | 34.02  | 0.097  |
| 7    | 0   | 0   | 0   | 2858.09 | 49.34 | 122.32 | 0.135  |
| 8    | 0   | 0   | 0   | 2776.01 | 44.43 | 117.18 | 0.152  |
| 9    | 1   | 0   | 0   | 1317.73 | 33.82 | 51.35  | 0.079  |
| 10   | 0   | 1   | 1   | 1307.16 | 35.21 | 57.13  | 0.128  |
| 11   | -1  | -1  | 0   | 2042.03 | 33.23 | 41.63  | 0.093  |
| 12   | -1  | 1   | 0   | 2113.44 | 25.62 | 55.84  | 0.102  |
| 13   | 1   | -1  | 0   | 1372.85 | 18.32 | 34.04  | 0.087  |
| 14   | 0   | -1  | -1  | 1189.02 | 25.39 | 61.99  | 0.125  |
| 15   | 0   | 0   | 0   | 2792.71 | 51.06 | 123.36 | 0.135  |
| 16   | 1   | 1   | 0   | 1589.53 | 17.79 | 35.09  | 0.085  |
| 17   | 0   | -1  | 1   | 1295.2  | 35.40 | 62.01  | 0.126  |

X₁ = Ratio of collagen to chitosan (v/v), X₂ = Addition of RE (mg/mL), X₃ = Glycerol content (%), Y₁ = Tensile strength (g), Y₂ = Elongation at break (%), Y₃ = Elongation length (mm), Y₄ = Thickness (mm)
Table 3: Analysis of variance for determination of model fitting

| Sources of variation | Y₁ | Y₂ | Y₃ | Y₄ |
|----------------------|----|----|----|----|
| F ratio of model     | 38.01 | 17.51 | 23.37 | 12.640 |
| p of model F         | <0.0001<sup>a</sup> | 0.0005<sup>a</sup> | 0.0002<sup>a</sup> | 0.0015<sup>a</sup> |
| PRESS                | 683700 | 2169.24 | 17571.47 | 0.01 |
| Lack of fit          | 0.1967 | 0.2587 | 0.0846 | 0.2803 |
| R²                   | 0.9799 | 0.9575 | 0.9678 | 0.9420 |
| Adjusted R²          | 0.9542 | 0.9028 | 0.9264 | 0.8675 |

<sup>a</sup>Significant difference with p<0.05

Table 4: Analysis of variance for the experimental results

| Indicators | df | Y₁ F value | p value | Y₂ F value | p value | Y₃ F value | p value | Y₄ F value | p value |
|------------|----|------------|---------|------------|---------|------------|---------|------------|---------|
| X₁         | 1  | 17.87      | 0.0039<sup>a</sup> | 10.88      | 0.0132<sup>a</sup> | 3.21      | 0.1165 | 2.05      | 0.1951 |
| X₂         | 1  | 2.80       | 0.1383   | 0.01       | 0.9216   | 0.09      | 0.7679 | 0.20      | 0.6647 |
| X₃         | 1  | 3.0929     | 6.78     | 0.0352<sup>a</sup> | 3.77      | 0.0931   | 0.02      | 0.8844   |
| X₁X₂       | 1  | 0.26       | 0.6232   | 0.91      | 0.3718   | 0.52      | 0.4948 | 0.34      | 0.576  |
| X₁X₃       | 1  | 7.69       | 0.0275<sup>a</sup> | 7.01      | 0.033<sup>a</sup> | 0.84      | 0.3889 | 0.48      | 0.5106 |
| X₂X₃       | 1  | 1.59       | 0.2474   | 1.67      | 0.2372   | 0.02      | 0.8982 | 1.50      | 0.2598 |
| X₁<sup>2</sup> | 1  | 45.72      | 0.0003<sup>a</sup> | 72.75     | <0.0001<sup>a</sup> | 99.38     | <0.0001<sup>a</sup> | 85.54     | <0.0001<sup>a</sup> |
| X₂<sup>2</sup> | 1  | 59.81      | 0.0001<sup>a</sup> | 32.94     | 0.0007<sup>a</sup> | 48.81     | 0.0002<sup>a</sup> | 7.81      | 0.0267<sup>a</sup> |
| X₃<sup>2</sup> | 1  | 173.70     | <0.0001<sup>a</sup> | 12.78     | 0.009<sup>a</sup> | 33.78     | 0.0007<sup>a</sup> | 8.43      | 0.0228<sup>a</sup> |

<sup>a</sup>Significant difference with p<0.05, df = Degree of freedom
Fig. 6: Response surface plots (3D) and contour plots of tensile strength as a function of significant interaction between factors. (A) Ratio of collagen to chitosan and addition of RE; (B) Ratio of collagen to chitosan and glycerol content; (C) Addition of RE and glycerol content.

Fig. 7: Response surface plots (3D) and contour plots of elongation at break as a function of significant interaction between factors. (A) Ratio of collagen to chitosan and addition of RE; (B) Ratio of collagen to chitosan and glycerol content; (C) Addition of RE and glycerol content.
Fig. 8: Response surface plots (3D) and contour plots of elongation length as a function of significant interaction between factors. (A) Ratio of collagen to chitosan and addition of RE; (B) Ratio of collagen to chitosan and glycerol content; (C) Addition of RE and glycerol content
Table 5: Predicted and experimental response values at optimum

| Response | Predicted value | Experimental value |
|----------|----------------|--------------------|
| Y₁ (g)   | 2789.65        | 2716.59±3.8706     |
| Y₂ (%)   | 49.92          | 50.42±0.0854       |
| Y₃ (mm)  | 117.55         | 119.24±0.7280      |
| Y₄ (mm)  | 0.143          | 0.134±0.0004       |

Conclusion

The optimal formulation of chitosan-based blend membrane was successfully developed from cowhide collagen and RE. The findings showed that ratio of collagen to chitosan and glycerol content significantly affected tensile strength, elongation at break, elongation length of the blend membrane. The optimum formulation of RE/chitosan/collagen blend membrane was found to be ratio of collagen to chitosan of 38.97:61.03 (v/v), addition of RE of 0.6 mg/mL and glycerol content of 30.21%, respectively. Measurement under optimal conditions of tensile strength, elongation at break, elongation length and thickness were 2789.65 g, 49.92%, 117.55 mm and 0.143 mm, respectively. This study manifested that cowhide collagen, RE and chitosan could be used to make the blend membrane having choiceness packaging properties. The resulting blend membrane possessed a potential application value in the meat industry.

Acknowledgement

This work was supported by Shandong Provincial Natural Science Foundation, China (No. ZR2014CQ002 and No. ZR2019BC104), the National Natural Science Foundation of China (No.31972851) and Taishan Industry Leading Talent Project (No. LJNY201606). Therefore, we are grateful for the funding and support of this research.

Author’s Contributions

Ming He: Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.
Haifang Xiao: Contributed to the study design, the interpretation of the results and manuscript preparation.
Yuhan Zhai, Yuqing zhang and Shuo Xu: Participated in part of the experimental design.
Shaoxuan Yu: Ameliorated the manuscript.
Yuanda Song: Contributed to the guidance of experimental design and ameliorated the manuscript.
Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

References

Batista, J. T. S., Araújo, C. S., Joele, M. P., Júnior, J. S., & Lourenço, L. F. H. (2019). Study of the effect of the chitosan use on the properties of biodegradable films of myofibrillar proteins of fish residues using response surface methodology. Food Packaging and Shelf Life, 20, 100306. doi.org/10.1016/j.fpsl.2019.100306

Chen, D. J., Zhao, L., Yuan, M. L., Su, W., Liu, H., & Chen, L. L. (2014). Mechanical properties of collagen-chitosan composite membrane, Food Science, 35, 112-118.

Chu, C., Deng, J., Man, Y., & Qu, Y. (2017). Evaluation of nanohydroxyapatite (nano-HA) coated epigallocatechin-3-gallate (EGCG) cross-linked collagen membranes. Materials Science and Engineering: C, 78, 258-264. doi.org/10.1016/j.msec.2017.04.069

De Paiva, G. B., Trindade, M. A., Romero, J. T., & da Silva-Barreto, A. C. (2021). Antioxidant effect of acerola fruit powder, rosemary and licorice extract in caiman meat nuggets containing mechanically separated caiman meat. Meat Science, 173, 108406. doi.org/10.1016/j.meatsci.2020.108406

Dou, L., Li, B., Zhang, K., Chu, X., & Hou, H. (2018). Physical properties and antioxidant activity of gelatin-sodium alginate edible films with tea polyphenols. International Journal of Biological Macromolecules, 118, 1377-1383. doi.org/10.1016/j.ijbiomac.2018.06.121

Du, H., Liu, C., Unsalan, O., Altunayar-Unsalan, C., Xiong, S., Manyande, A., & Chen, H. (2021). Development and characterization of fish myofibrillar protein/chitosan/rosemary extract composite edible films and the improvement of lipid oxidation stability during the grass carp fillets storage. International Journal of Biological Macromolecules, 184, 463-475. doi.org/10.1016/j.ijbiomac.2021.06.121

EFSA. (2008). Use of rosemary extracts as a food additive-Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food. European Food Safety Authority. EFSA Journal, 6(6), 721. doi.org/10.2903/j.efsa.2008.721

Ghasemlou, M., Khodaiyan, F., & Oromiehie, A. (2011). Physical, mechanical, barrier and thermal properties of polyol-plasticized biodegradable edible film made from kefiran. Carbohydrate Polymers, 84(1), 477-483. doi.org/10.1016/j.carbpol.2010.12.010

Glampedaki, P., Jocic, D., & Warmoeskerken, M. M. (2011). Moisture absorption capacity of polyamide 6, 6 fabrics surface functionalised by chitosan-based hydrogel finishes. Progress in Organic Coatings, 72(3), 562-571. doi.org/10.1016/j.porgcoat.2011.06.019

Gurumurthy, B., Griggs, J. A., & Janorkar, A. V. (2018). Optimization of collagen- elastin-like polypeptide composite tissue engineering scaffolds using response surface methodology. Journal of the Mechanical Behavior of Biomedical Materials, 84, 116-125. doi.org/10.1016/j.jmbbm.2018.04.019

Irawan, V., Kajiwara, D., Nakagawa, Y., & Ikoma, T. (2021). Fabrication of mechanically robust bilayer membranes of hydroxyapatite/collagen composites. Materials Letters, 291, 129514. doi.org/10.1016/j.matlet.2021.129514

Jancikova, S., Jamroz, E., Kulawik, P., Tkaczewska, J., & Dordevic, D. (2019). Furcellaran/gelatin hydrolysate/rosemary extract composite films as active and intelligent packaging materials. International Journal of Biological Macromolecules, 131, 19-28. doi.org/10.1016/j.ijbiomac.2019.03.050

Lei, Y., Wu, H., Jiao, C., Jiang, Y., Liu, R., Xiao, D., & Li, S. (2019). Investigation of the structural and physical properties, antioxidant and antimicrobial activity of pectin- konjac glucomannan composite edible films incorporated with tea polyphenol. Food Hydrocolloids, 94, 128-135. doi.org/10.1016/j.foodhyd.2019.03.011

Lewandowska, K., Sionkowska, A., Grabska, S., Kaczmarek, B., & Michalska, M. (2016). The miscibility of collagen/hyaluronic acid/chitosan blends investigated in dilute solutions and solids. Journal of Molecular Liquids, 220, 726-730. doi.org/10.1016/j.molliq.2016.05.009

Li, M., Han, M., Sun, Y., Hua, Y., Chen, G., & Zhang, L. (2019). Oligoarginine mediated collagen/chitosan gel composite for cutaneous wound healing. International journal of biological macromolecules, 122, 1120-1127. doi.org/10.1016/j.ijbiomac.2018.09.061

Li, Y., Ma, Z., Yang, X., Gao, Y., Ren, Y., Li, Q., ... & Zeng, R. (2021). Investigation into the physical properties, antioxidant and antibacterial activity of Bletilla striata polysaccharide/chitosan membranes. International Journal of Biological Macromolecules, 182, 311-320. doi.org/10.1016/j.ijbiomac.2021.04.037

Moczewska, M., Karp, S., Horbanczuk, O. K., Hanula, M., Wyrwisz, J., & Kurek, M. A. (2020). Effect of rosemary extract addition on oxidative stability and quality of hemp seed oil. Food and Bioproducts Processing, 124, 33-47. doi.org/10.1016/j.fbp.2020.08.002

Oliveira, P. N., Montembault, A., Sudre, G., Alcouffe, P., Marcon, L., Gehan, H., ... & David, L. (2019). Self-crosslinked fibrous collagen/chitosan blends: Processing, properties evaluation and monitoring of degradation by bi-fluorescence imaging. International Journal of Biological Macromolecules, 131, 353-367. doi.org/10.1016/j.ijbiomac.2019.02.134
Petkoska, A. T., Daniloski, D., D'Cunha, N. M., Naumovski, N., & Broach, A. T. (2021). Edible packaging: Sustainable solutions and novel trends in food packaging. Food Research International, 140, 109981. doi.org/10.1016/j.foodres.2020.109981

Piñeros-Hernandez, D., Medina-Jaramillo, C., López-Córdoba, A., & Goyanes, S. (2017). Edible cassava starch films carrying rosemary antioxidant extracts for potential use as active food packaging. Food hydrocolloids, 63, 488-495. doi.org/10.1016/j.foodhyd.2016.09.034

Saberi, B., Thakur, R., Vuong, Q. V., Chockchaisawasdee, S., Golding, J. B., Scarlett, C. J., & Stathopoulos, C. E. (2016). Optimization of physical and optical properties of biodegradable edible films based on pea starch and guar gum. Industrial Crops and Products, 86, 342-352. doi.org/10.1016/j.indcro.2016.04.015

Singh, T. P., Chatli, M. K., & Sahoo, J. (2015). Development of chitosan based edible films: Process optimization using response surface methodology. Journal of Food Science and Technology, 52(5), 2530-2543. doi.org/10.1007/s13197-014-1318-6

Su, H., Fujiwara, T. anderson, K. M., Karydis, A., Ghadri, M. N., & Bumgardner, J. D. (2021). A comparison of two types of electrospun chitosan membranes and a collagen membrane in vivo. Dental Materials, 37(1), 60-70. doi.org/10.1016/j.dental.2020.10.011

Sun, L., Sun, J., Chen, L., Niu, P., Yang, X., & Guo, Y. (2017). Preparation and characterization of chitosan film incorporated with thinned young apple polyphenols as an active packaging material. Carbohydrate polymers, 163, 81-91. doi.org/10.1016/j.carbpol.2017.01.016

Teixeira, S. C., Silva, R. R. A., de Oliveira, T. V., Stringhera, P. C., Pinto, M. R. M. R., & Soares, N. D. F. F. (2021). Glycerol and triethyl citrate plasticizer effects on molecular, thermal, mechanical and barrier properties of cellulose acetate films. Food Bioscience, 101202. doi.org/10.1016/j.foodbio.2021.101202

Ulu, A., Birhanli, E., & Ateş, B. (2021). Tunable and tough porous chitosan/β-cyclodextrin/tannic acid biocomposite membrane with mechanic, antioxidant and antimicrobial properties. International Journal of Biological Macromolecules, 188, 696-707. doi.org/10.1016/j.ijbiomac.2021.08.068

Vedula, S. S., & Yadav, G. D. (2021). Chitosan-Based Membranes Preparation and Applications: Challenges and Opportunities. Journal of the Indian Chemical Society, 100017. doi.org/10.1016/j.jics.2021.100017

Wang, F., & Lan, Z. G. (2020). Preparation and property determination of porcine skin collagen polypeptide membrane, Strait Pharmaceutical Journal, 32, 14-15.

Xiao, J., Ma, Y., Wang, W., Zhang, K., Tian, X., Zhao, K., … & Guo, Y. (2021). Incorporation of gelatin improves toughness of collagen films with a homo-hierarchical structure. Food Chemistry, 345, 128802. doi.org/10.1016/j.foodchem.2020.128802

Yan, Q. Q., Zhang, J. L., Dong, H. Z., Hou, H. X. & Guo, P. (2013). Properties and antimicrobial activities of starch-sodium alginate composite films incorporated with sodium dehydroacetate or rosemary extract. Journal of Applied Polymer Science, 127, 1951-1958. doi.org/10.1002/app.37570

Yi, F. R., Jing, J. Z., Dao, D. P., Yang, Y. S. & Jian, L. S. (2019). Physicochemical properties of goose skin collagen/chitosan composite membrane and its potential for food preservation. Shipin Kexue/Food Science, 40(1), 263-269. doi.org/10.7506/spkxl002-6630-2017.1028.333