Preparation, characterization, and in vitro antioxidant activity of pH-sensitive resveratrol microcapsule in simulated intestinal fluids

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**ABSTRACT**

Shellac resin ammonium salts (SRAS) were prepared from the natural product of shellac resin and ammonium hydroxide by amidation. Resveratrol (RES) was embedded with SRAS to improve its thermostability, antioxidant activity, and bioavailability. The absorption of RES was promoted in the intestinal tract. Meanwhile, an enteric-coated microcapsule-loaded RES was successfully established by spray drying technology. The RES microcapsule exhibited a spherical shape and perfect stability as well as outstanding sustainable release character in simulated intestinal release experiment. Moreover, SRAS/RES presented a dose-dependent manner with a much higher radical scavenging activity of DPPH\textsuperscript{•} and ABTS\textsuperscript{+} compared to the pure RES. Therefore, a kind of new pH-sensitive RES microcapsule established may act as a favorable carrier for an insoluble antioxidant ingredient in the potentially new food delivery system on functional food supplements.

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**Introduction**

With the continuous improvement of living standard, people tend to purchase green, healthy, and nutritious food. Antioxidants’ development also gradually changed from synthetic into natural which as an important part of food process. Among them, plant antioxidants such as polyphenols, tannins, vitamin, and others, due to the raw material sources, natural green, non-toxic, health, and other advantages, which has a broad application prospect in food and health-care products and other fields.\textsuperscript{[1–3]} Precisely, resveratrol (RES) is a kind of natural antioxidant with polyphenols structure and widely existed in the plant of grape, peanut, mulberry, and \textit{Polygonum cuspidatum}. It can remove the destructive free radical, prevent lipid peroxide, inhibit DNA damage, and so on.\textsuperscript{[4–6]} Studies on the application of natural RES to protect the intestinal mucosal injury, regulate intestinal function, and prevent tumors such as colon cancer have been reported.\textsuperscript{[7–10]} However, a large proportion of RES is broken down and lose activity in the process of human liver and intestinal metabolism, lead to poor absorption, and low bioavailability, which seriously limits the application of RES.\textsuperscript{[11]}

Delivery system is a preparation that transports the active component exclusively to the site where it is required and has little or no interaction with other tissues. It is widely used in medicine and food delivery. In the past decades, various types of polymer materials used in the delivery system, including synthesis polymer and macromolecular compounds extracted from natural products such as peptides, polysaccharide, protein, etc.\textsuperscript{[12–14]} Shellac resin (SR) is a natural resinous material, with easy to degradation, non-toxic, and other excellent properties, which are obtained from secretions of scale insects. It was a mixture containing terpenic resin (70–80\%), wax (6–7\%), coloring matter (4–8\%), and other impurities.\textsuperscript{[15,16]} The good mechanical and film-forming properties of SR
make it widely used in food, medicine, cosmetics, and coatings industry. Moreover, because of the acid insoluble of SR, the coating material which is used as the enteric soluble functional component has a certain pH-response in the aspect of food and medicine. Nevertheless, congenital defects such as poor water solubility and heat resistance limit the application of SR. SR containing a large amount of active groups such as hydroxyl and carboxyl group, so the disadvantages can effectively be improved by using chemical modification, then applied to the coating material of enteric drugs, the goal of controlled release of functional components will be attained.

The aim of our work was to enhance the stability, bioavailability, and antioxidant of RES by using natural SR. To this purpose, the ammonium hydroxide was used to SR modification, and then SRAS was applied to the microcapsules of RES. In particular, we investigated the thermostability, pH-sensitive performance in simulated physiological conditions and antioxidant activity of the microcapsules.

Material and methods

Chemicals and reagents

Chemicals, including Ammonium hydroxide (NH$_3$·H$_2$O), Tween80, sodium dihydrogen phosphate (NaH$_2$PO$_4$), disodium hydrogen phosphate (Na$_2$HPO$_4$), and ethyl acetate were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. SR was native to Yunnan province of China. DPPH· and ABTS$^+$· agents were brought from Shanghai Yuanye Bio-Technology Co., Ltd. RES (98%) were provided by Huayuan Hengyuan plant biochemical Co., Ltd. All other chemicals used were analytical grade, and water was double-distilled just before use.

Preparation of SRAS

After crushing and screening, SR (20.00 g) was added to 0.1 mol/L NH$_3$·H$_2$O aqueous solution to prepare the SRAS at the temperature of 40°C with continuous stirring. Standing and then cooling after reaction over, take the supernatant liquor to freeze drying. Then, SRAS was obtained.

Preparation of SRAS/RES

SRAS was first dissolved in water at the temperature of 50°C. Then, RES is added to the solution, and a small amount of Tween80 is added dropwise. The prepared solution is stirred for 1 h using a magnetic stirrer. The RES concentration of 0%, 5%, and 10% solutions were prepared, respectively. Then, microcapsules were prepared according to the methodology of Carvalho et al. with modifications. Spray drying was performed in a B-290 laboratory scale spray dryer (BUCHI, Switzerland) with a nozzle atomization system with a 0.7 mm diameter nozzle. The emulsion was pumped into the primary chamber through a peristaltic pump at 25°C using a magnetic stirrer. Feed flow rate was 6 mL/min, and the drying airflow rate was 50 m$^3$/h. Experimental tests were carried out with an inlet air drying temperature of 140°C and outlet air temperature of 65°C. The production by spray drying was performed in duplicate.

Determination of moisture, encapsulation efficiency, and loading capacity

The moisture of fresh microcapsules was determined by free-drying and weighed. Encapsulation efficiency and loading capacity were measured according to the method reported by Wang et al. with a slight modification. In brief, 20 mg sample was washed using ethyl acetate for three times and filtered. The retention liquor was added to the sample. Based on the equation for the RES standard curve, the retention liquor was collected to determine the free RES amount by measuring the absorbance at 305 nm. The standard curve of RES was determined by measuring the absorbance
(305 nm) of a known concentration of RES using a TU-19 UV/VIS spectrophotometer (Beijing Purkinje General Instrument Co, Ltd.). Moisture, EE, and LC were calculated by using the following equations:

\[
Moisture (\%) = \frac{W_0 - W_1}{W_0} \times 100
\]  

(1)

\[
EE (\%) = \frac{M_0 - M_1}{M_0} \times 100
\]

(2)

\[
LC(\%) = \frac{M_0 - M_1}{M} \times 100
\]

(3)

where \(W_0\) and \(W_1\) were the weight of microcapsule before and after free-drying, respectively. \(M_0\) and \(M_1\) were the mass of initial addition RES and un-embedding RES, \(M\) was the mass of microcapsule.

**Morphology of SRAS/RES**

Morphologies of the SRAS/RES microcapsule were observed using a field-emission scanning electron microscopy (SU8010, Hitachi, Japan). All of the samples were sputter-coated with gold just before observation and observed under a working voltage of 10 kV.

**Solid-state study**

The sample was tested by using Fourier-transform infrared spectroscopy (FT-IR) and Differential scanning calorimetry-thermogravimetric (DSC) analyses. FT-IR of the samples were recorded using an Alpha (Bruker Ltd., USA) at a resolution of 2 cm\(^{-1}\) with 24 scans over the wave-number range of 400–4000 cm\(^{-1}\). All FT-IR experiments were performed in triplicate. DSC analysis (Mettler-Toledo, Switzerland) was performed under a nitrogen atmosphere at a flow rate of 50 mL/min. Samples were accurately weighed and sealed in 40 \(\mu\)L aluminum pans, and heated at temperature from 30°C to 400°C under the purging of nitrogen gas with heating rate of 10°C/min.

**Drug release of SRAS/RES**

To evaluate the release profiles of RES, a fully calibrated dissolution apparatus using the paddle method were performed in 600 mL phosphate buffers with the pH of 1.0, 6.8, and 7.4, respectively.\[^{[24]}\] Briefly, samples contain 100 mg RES were placed to dialysis tube (MD3500, Viskase, USA), clamped and sank at the bottom of the vessel at the beginning of the experiment. The temperature was maintained at 37°C ± 0.5°C, and vessel speed was kept at 100 rpm. 1 mL of supernatant was collected and replenished with 1 mL of fresh PBS, and the accumulated amount of released factor in solution was quantified by a TU-19 UV/VIS spectrophotometer. Accumulated release percentage of the drug was determined as follows:

\[
Q = \sum_{t=0}^{t} \frac{M_t}{M_0} \times 100
\]

(4)

where \(M_t\) was the weight of growth factor released at time \(t\), and \(M_0\) was the total amount mass of the RES in the microcapsule theoretically. All experiments were tested in triplicate.

**Determination of antioxidant activity on SRAS/RES**

DPPH radical scavenging assay and ABTS\(^+\) scavenging assay were used to evaluate the antioxidant activity of the SRAS/RES. Precisely, radical scavenging activity of the SRAS/RES samples against the DPPH- was done using a UV/VIS spectrophotometer in accordance with the method reported by
Musa et al. with minor modification.\textsuperscript{[25]} Briefly, 0.5 mL of each tested solution (RES concentration were 200, 400, 600, 800, 1000 \( \mu \)g/mL, respectively) was blended with 5.0 mL of 79 mg/L alcoholic solution of DPPH radical. The mixture was shaken energetically and kept in the dark for 1 h at 310.15 K. The absorbance of supernatant was recorded at 517 nm. The percentage of DPPH radical scavenging activity was calculated as follows:

\[
DPPH \cdot (\%) = \frac{A_s - A_c}{A_b - A_c} \times 100\% \hspace{1cm} (5)
\]

where \( A_c \), \( A_b \), and \( A_s \) were the absorbance of control, blank, and blended solutions at 517 nm, respectively. Radical cation scavenging ability of the SRAS/RES samples against the ABTS\(^+\) was assayed in accordance with Intarasirisawat et al. method with slight modification.\textsuperscript{[26]} ABTS radical cation was obtained by the mixture of 3.84 g/L ABTS\(^+\) aqueous solution with 1.34 g/L potassium persulfate aqueous solution at a ratio of 1:1 (v/v). The mixed solution was retained for 12 h in the dark at room temperature. Prior to using, necessary absorption 0.7 ± 0.02 at 734 nm was obtained by diluting the mixed solution with PBS solution. The samples (0.5 mL) with different concentrations of RES (40, 60, 80, 100, and 120 \( \mu \)g/mL, respectively) were blended with 10 mL of ABTS radical cation and then all mixtures were left for 1 h at 310.15 K in dark. The absorbance was tested at 734 nm.

\[
ABTS\cdot^+ (\%) = \frac{A_s - A_c}{A_b - A_c} \times 100\% \hspace{1cm} (6)
\]

where \( A_c \), \( A_b \), and \( A_s \) were the absorbance of control, blank, and blended solutions at 734 nm, respectively.

**Statistical analysis**

Each experiment was performed three times, and the results are given as the mean ± standard deviation (SD). All data were collected and converted to graphs using Origin 8.0 software.

**Results and discussion**

**Physical parameters**

Table 1 shows that the encapsulation efficiency of RES was preferable by using SRAS. The encapsulation efficiency was significantly reduced after increase in the proportion of RES. The main reason is the limited embedding ability of the wall material on the core material. When the amount of the core material increases significantly, the concentration of the wall material decreases, and the encapsulation efficiency of the core material (RES) decreases significantly.\textsuperscript{[27,28]}

**Morphology of SRAS/RES delivery system**

In order to confirm the morphological characteristic of the RES-loaded microcapsules, SEM was used as shown in Figure 1. The results showed the morphology of pure RES (a) demonstrated a slender crystal structure. The 0% SRAS/RES microcapsule (b) had no core material of RES showed

| No. | Proportion | Moisture/% | EE/% | LC/% |
|-----|------------|------------|------|------|
| 1   | SRAS(2g)/RES(0g)/Tween80(0.5g) | 8.13 ± 0.12 | –    | –    |
| 2   | SRAS(2g)/RES(0.132g)/Tween80(0.5g) | 6.11 ± 0.09 | 95.40 ± 0.17 | 4.77 ± 0.01 |
| 3   | SRAS(2g) RES(0.278g)Tween80(0.5g) | 3.41 ± 0.13 | 82.70 ± 0.13 | 8.27 ± 0.02 |
adhesion together, and with the increase of the amount of RES (c and d), microcapsule sphere structure gradually forming, surface seemed more smoothly with smaller particle size and uniform distribution, indicating a preferable embedding effect.

Solid-state study

FT-IR was conducted to investigate the change of the functional group and the compatibility between the active ingredients and the wall material in the microcapsule. The FT-IR spectra of SR, SRAS, the microcapsules, and RES were showed in Figure 2. Except for RES, all spectra displayed a broadband at 3000~3600 cm$^{-1}$ indicating the overlap of O–H and N–H stretches in the same region. Compared to SR, the FT-IR spectra of 3150 cm$^{-1}$ has a strong enhancement in this region, and a new spectra of 1559 cm$^{-1}$ was appeared which represented N–H stretches and R–COO$^-$, respectively. The changes of the spectra of 1380 cm$^{-1}$ due to the formation of amido bond through ammonolysis by lactone of SR. All the results showed that the amino-group was successfully bonded with the carboxyl group of SR to form amido bond. After embedding RES, the FT-IR spectra of microcapsule showed obvious characteristic absorption peak of RES, especially around the spectra of 1600~1450 cm$^{-1}$ and 900~500 cm$^{-1}$ indicating that RES was part of the formation of the microcapsules.

DSC analysis was performed to investigate the thermal stability and physical state of RES in the microcapsules. As Figure 3 shows, the thermograms of all substances showed a slight endothermic peak around 50–100°C which were associated with the loss of water. The pure RES showed an endothermic sharp peak at 266.98°C with a specific enthalpy of transition (DH) 241.2 J/g. This corresponds to the melting of RES, which is in accordance with Kobierski et al. But, it was not found for the microcapsules. Therefore, it was proved that the formation of microcapsules could be achieved by the application of the natural polymers for the encapsulation of RES.
In vitro release study

The studies of the in vitro release of RES loaded microcapsules were performed under simulated gastrointestinal tract on food products. Thus, it was studied the cumulative release of RES from obtained microcapsules in phosphate buffers at a pH of 1.0, 6.8, and 7.4, respectively. In microspheres/microcapsules, the Peppas equation (Equation 7) was usually used to explain the type of RES release mechanisms.

\[
Q = k \cdot t^n
\]  

(7)

where \( Q \) is the accumulative release percentage of RES released at time \( t \); \( k \) is a rate constant related to structural and geometric characteristics of the material; \( n \) is the release exponent that indicates the mechanism of the RES release. (\( n \leq 0.43 \) indicates the dominant release mechanism is the Fickian diffusion; \( 0.43 \leq n < 0.85 \) indicates non Fickian or anomalous transport; \( n \geq 0.85 \) corresponds to zero order release kinetics)
As Figure 4 shows, the release profiles of RES almost the same with different pH conditions. The release rate of RES was rapid in front of 10 h and no any pH-sensitive behavior. However, the microcapsules exhibited a controlled and sustained release profile of RES. More precisely, during the first 2 h at pH 1.0, only a small percentage of RES was released into the PBS solution. There can be considered as the RES dissolution on the surface of microcapsules, and the RES of inner was difficult to transport into solution through the wall. But in simulated intestinal juice, especially at the pH of 6.8, RES can quickly be released from the microcapsules, so as to achieve the aim of colon-targeting release.

The fitting results by Peppas equation of the release rate showed all of these depend simultaneously by the swelling and the diffusion processes specific to a non Fickian mechanism (Table 2). The release kinetics of RES from microcapsules at pH of 6.8 were best described by the Peppas equation (0.990 and 0.982, respectively).

**Figure 4.** Cumulative release profiles of RES, 5% and 10% SRAS/RES at 37°C: (a) pH = 1; (b) pH = 6.8; (c) pH = 7.4.

| Sample       | pH = 1          | pH = 6.8         | pH = 7.4         |
|--------------|-----------------|------------------|------------------|
| SRAS/RES(5%) | Q = 4.184<sup>0.594</sup>, R² = 0.974 | Q = 8.655<sup>0.724</sup>, R² = 0.990 | Q = 10.04<sup>0.608</sup>, R² = 0.976 |
| SRAS/RES(10%)| Q = 4.377<sup>0.78</sup>, R² = 0.978 | Q = 9.87<sup>0.726</sup>, R² = 0.982 | Q = 11.19<sup>0.606</sup>, R² = 0.966 |
**Antioxidant activity**

There are several methods to evaluate radical scavenging activity, among which, the DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS\(^+\) [2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate)] are widely used in measuring the antioxidant capability with stable and efficiency.

As Figure 5 shows, the reduced percentage of DPPH and ABTS\(^+\) radical were all related to the concentration of the different formulations. A dose-dependent manner was discovered for all formulations. However, it was interesting that the microcapsules have a higher ability of reducing free radicals under a low concentration compared with the same concentration of RES. The EC\(_{50}\) value (concentration required to obtain a 50% antioxidant effect) was calculated of each sample against free radicals as shown in Table 3.\(^{[31]}\) It can be seen that the EC\(_{50}\) value of DPPH and ABTS free radicals were decreased after embedding resveratrol in accord with Pan et al.\(^{[32]}\) This may be due to the large surface area of the RES on the microcapsule at the reaction medium. In conclusion, after encapsulated by SR ammonium salt, the antioxidant activities of RES were remained, which has good scavenging ability for DPPH and ABTS\(^+\) free radicals.

![Graph showing scavenging effects of antioxidant formulations on the DPPH and ABTS+ radicals.](image-url)

**Figure 5.** Scavenging effects of antioxidant formulations on the DPPH- and ABTS\(^+\).
Therefore, the new resveratrol carrier manifested the hopeful properties for the new effective intestinal formulation, which could be used into the functional delivery system and regulate the balance of intestinal ecological with taking the lower amount of antioxidants and the high radical scavenging activity. The natural polymers of SR used in coating material have been reported before. In this study, the amino group grafted into SR to regard as wall material, and resveratrol microcapsule were successfully prepared to improve the stability and bioavailability of resveratrol, at the same time improve the antioxidant activity. This new delivery system can act as a favorable carrier for insoluble antioxidants in the potential use of new food delivery system on functional food supplements.

### Conclusion

The microcapsule (SRAS/RES) was successfully prepared to enhance the stability, bioavailability, and the radical scavenging activity of RES and make the best use of food delivery system on functional food supplements. The ammonium salts of SR were successfully synthesized and used to fabricate the microcapsule. The microcapsule showed perfect stability and outstanding sustained release character in vitro intestinal release test. Moreover, compared to the pure RES, the microcapsule showed a dose-dependent manner with a higher radical scavenging activity of DPPH· and ABTS⁺, which can be used widely to the further food or nutrient applied research.

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### Table 3. EC₅₀ value of antioxidant formulations on the DPPH- and ABTS⁺.

|          | RES       | SRAS/RES(5%)       | SRAS/RES(10%)       |
|----------|-----------|--------------------|---------------------|
| DPPH·    | y = 1.009x-2.091, R² = 0.968 | y = 0.701x-1.25, R² = 0.912 | y = 0.942x-1.894, R² = 0.955 |
| EC₅₀     | 369.7 μg/mL | 313.64 μg/mL       | 347.86 μg/mL        |
| ABTS⁺    | y = 0.977x-1.065, R² = 0.985 | y = 1.022x-1.1, R² = 0.983 | y = 0.859x-0.796, R² = 0.992 |
| EC₅₀     | 39.98 μg/mL | 36.78 μg/mL        | 32.44 μg/mL         |
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