Sains Malaysiana 53(9)(2024): 3183-3195
http://doi.org/10.17576/jsm-2024-5309-22

Thermosensitive Hydrogel Containing Chinese Herbal Compound Extract and Hyaluronic Acid-Chitosan Quaternary Ammonium Salt Microspheres for Topical Application: Preparation and Characterization

(Hidrogel Termosensitif Mengandungi Ekstrak Sebatian Herba Cina dan Asid Hialuronik-Kitosan Mikrosfera Garam Amonium Kuaterner untuk Penggunaan Topikal: Penyediaan dan Pencirian)

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Received: 21 April 2024/Accepted: 5 August 2024

ABSTRACT

Allergic contact dermatitis (ACD) is a highly prevalent allergic inflammatory dermatosis with persistent severe pruritus. Zhi-Yang-Fang (ZYF), a topical Chinese herbal compound used in clinical practice for treating ACD, has certain drawbacks such as inconvenience in use and its impact on aesthetics. This research aimed to overcome these shortcomings by developing an F127/F68-based thermosensitive hydrogel containing ZYF aqueous extract and hyaluronic acid-chitosan quaternary ammonium salt (HA-HTCC) microspheres. The 19 major components of ZYF aqueous extract were preliminarily identified using ultra-high-performance liquid chromatography coupled to Quadrupole-Exactive Orbitrap mass spectrometry (UPLC-Q Exactive Orbitrap-MS) analysis. HA-HTCC microspheres and HA-HTCC microspheres containing ZYF aqueous extract were prepared using the physical cross-linking method, and the success of the preparation was confirmed through a series of characterisations. The concentration of F127 and F68 was screened. The proportions of each substance in the final hydrogel formulation were determined, prepared using the cold method, and characterized using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and a rotational rheometer. These technologies verified the successful preparation, temperature sensitivity, and stability of the hydrogel. The hydrogel developed may be considered a promising approach for the management of ACD.

Keywords: Allergic contact dermatitis; Chinese herbal compound extract; microspheres; thermosensitive hydrogel; topical application

ABSTRAK

Dermatitis hubungan alahan (ACD) adalah dermatosis keradangan alahan yang sangat lazim dengan pruritus teruk yang berterusan. Zhi-Yang-Fang (ZYF), sebatian herba Cina topikal yang digunakan dalam amalan klinikal untuk merawat ACD, mempunyai kelemahan tertentu seperti kesulitan yang digunakan dan kesannya terhadap estetika. Penyelidikan ini bertujuan untuk mengatasi kekurangan ini dengan membangunkan hidrogel termosensitif berasaskan F127/F68 yang mengandungi ekstrak akueus ZYF dan mikrosfera garam amonium asid hyaluronik-kitosan (HA-HTCC). Sembilan belas komponen utama ekstrak akueus ZYF dikenal pasti awal menggunakan kromatografi cecair berprestasi ultra tinggi ditambah dengan analisis spektrometri jisim Orbitrap Quadrupole-Exactive (UPLC-Q Exactive Orbitrap-MS). Mikrosfera HA-HTCC dan mikrosfera HA-HTCC yang mengandungi ekstrak akueus ZYF disediakan menggunakan kaedah penghubung silang fizikal dan kejayaan penyediaan telah disahkan melalui satu siri pencirian. Kepekatan F127 dan F68 telah ditayangkan. Perkadaran setiap bahan dalam formulasi hidrogel akhir ditentukan, disediakan menggunakan kaedah sejuk dan dicirikan menggunakan imbasan mikroskop elektron (SEM), spektroskopi

inframerah transformasi Fourier (FTIR) dan rheometer putaran. Teknologi ini mengesahkan penyediaan yang berjaya, kepekaan suhu dan kestabilan hidrogel. Hidrogel yang dibangunkan boleh dianggap sebagai pendekatan yang berpotensi untuk pengurusan ACD.

Kata kunci: Dermatitis hubungan alahan; ekstrak sebatian herba Cina; hidrogel termosensitif; mikrosfera; penggunaan topikal

INTRODUCTION

Allergic contact dermatitis (ACD) causes a localized inflammatory response in the skin upon re-exposure to the same antigen, classified as a type IV hypersensitivity reaction (Johansen et al. 2022; Kaplan, Igyarto & Gaspari 2012; Vocanson et al. 2009). ACD affects up to 20% of the general population (Alinaghi et al. 2019). Upon re-exposure to the semi-antigen, effector T-cells in the dermis become active, leading to inflammation characterized by symptoms like redness, rash, itching, dry skin, swelling, and blisters (Olusegun & Martincigh 2021). Research indicates multiple adverse impacts of ACD (Hong, Koo & Koo 2008; Kalboussi et al. 2019; Mossing et al. 2022). ACD can appear on any part of the body, but hand dermatitis is most common due to frequent exposure to allergens, affecting the patient's quality of life (Kalboussi et al. 2019; Karlberg et al. 2008). ACD tends to negatively affect the patient's social relationships, psychological state, and professional career (Kadyk et al. 2003).

Presently, various medical treatments for ACD exist, including topical glucocorticoids (Ludriksone et al. 2021), Janus kinase inhibitors (Fukuyama et al. 2015; Ludriksone et al. 2021), phototherapy (Ludriksone et al. 2021; Mørk & Austad 1983; Sjövall & Christensen 1986), calcineurin inhibitors (Luger & Paul 2007), and immunosuppressants (Ludriksone et al. 2021), with glucocorticoids remaining the preferred option for medical professionals. Because of the persistent nature of the illness, healthcare professionals and patients should exercise caution when contemplating the administration of glucocorticoids due to their potential for adverse effects (Li & Li 2021). Therefore, there is a need to develop a drug that is safe and effective in refractory ACD with few side effects to fulfil the medical need. Currently, the means of treating ACD are limited and prevention is the mainstay, Traditional Chinese Medicine (TCM) has great potential in treating ACD due to its effective and safe features (Wang et al. 2021).

In China, TCM boasts an extensive usage history, marked by its distinct therapeutic effectiveness and minimal adverse effects (Zhang, Wang & Zheng 2000). This study focused on developing a pharmaceutical formulation of Zhi-Yang-Fang (ZYF), a Chinese herbal compound, to treat allergic contact dermatitis in clinical settings. Despite the variety of Chinese medicinal treatments for ACD, the availability of external medications is limited, and ZYF, a clinical topical lotion, shares common flaws such as its archaic dosage, impractical application, low bioavailability, ease of removal, and aesthetic impact. There's a critical medical necessity to explore the use of highly efficient and user-friendly topical forms of TCM in treating ACD.

Developing a system for topical application with extracts of Chinese herbal compounds might be viewed as an innovative method for treating ACD. The production of this drug delivery system also presents a chance to be utilized in crafting topical drug formulations for various other illnesses.

Microgel is a polymer with a mesh structure and particle size between 0.1 and 100 μm. It has a small size, high drug capacity, environmental responsiveness, and biocompatibility, making it ideal for drug release systems (Kittel, Kuehne & De Laporte 2022). The microgels in the study were created by combining chitosan quaternary ammonium salt (HTCC) and hyaluronic acid (HA) through electrostatic adsorption, forming physically crosslinked structures. These physical microgels are simpler to make and more eco-friendly than chemically crosslinked ones.

Due to their abundant water content, supple texture, versatility, and compatibility with living organisms (Li & Mooney 2016), hydrogels are experiencing a surge in popularity. Thermo-responsive hydrogels are liquid at low temperatures and become viscous at higher temperatures (at body temperature), making them ideal for drug delivery systems (Luo et al. 2023). The temperature-sensitive properties of hydrogels may help to increase contact with tissues, making the drug more adherent to the affected area and less likely to be dislodged and maintaining the therapeutic concentration of the drug for a prolonged period (Fan et al. 2022). All these offer possibilities for optimizing the ACD treatment.

Poloxamer is a safe, water-soluble copolymer commonly used for its temperature-sensitive properties. It is easily accessible, made of PEO/PPO, and comes in a wide range of molecular weights (Russo & Villa 2019). Due to their thermoreversible gelation process, Poloxamer is now the main material used for making biocompatible temperature-sensitive hydrogels, with Poloxamer 188 (F68) and Poloxamer 407 (F127) being the most commonly used and FDA-approved for drug formulations (Urbán-Morlán et al. 2008). Poloxamer is

increasingly used in gel systems for various drug delivery methods, allowing for controlled drug release in specific areas like the skin, eyes, and rectum (Russo & Villa 2019; Zarrintaj et al. 2020).

The objective of this research was to develop an F127/F68-based thermosensitive hydrogel containing ZYF aqueous extract and hyaluronic acid-chitosan quaternary ammonium salt (HA-HTCC) microspheres. The ZYF aqueous extract was mixed with HA-HTCC microspheres and incorporated into a hydrogel matrix consisting of F127/F68. Firstly, the major constituents of ZYF aqueous extract were characterized using ultra-high-performance liquid chromatography coupled to Quadrupole-Exactive Orbitrap mass spectrometry (UPLC-Q Exactive Orbitrap-MS). HA-HTCC microspheres were prepared and characterized. Subsequently, the ZYF aqueous extract was incorporated into the HA-HTCC microspheres and characterized. Additionally, the concentrations of F127 and F68 were screened and optimized to ensure gelation at human skin temperature. Finally, a novel thermosensitive hydrogel containing ZYF aqueous extract and HA-HTCC microspheres was prepared, and its characterization was conducted by scanning electron microscopy (SEM), rotational rheometer, and Fourier transform infrared spectroscopy (FTIR). To summarize, this study presents a newly developed temperature-sensitive hydrogel that incorporates ZYF aqueous extract and HA-HTCC microspheres. This innovative formulation offers a potential therapeutic approach for managing allergic contact dermatitis and holds considerable prospects for future clinical utilization.

MATERIALS AND METHODS

MATERIALS

F127 and F68 were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Hyaluronic acid was provided by Bloomage Biotechnology Corporation Limited (Jinan, China). Chitosan quaternary ammonium salt was purchased from Shanghai Macklin Biochemical Technology Co., Ltd (Shanghai, China). Acetonitrile hvperarade for LC-MS LiChrosolv was provided by Merck KGaA (Darmstadt, Germany).

The root of *Stellaria dichotoma* L.var. *lanceolata* Bge. (YCH), the ripe fruit of *Prunus mume* (Sieb.) Sieb. et Zucc. (WM), the ripe fruit of *Schisandra chinensis* (Turcz.) Baill. (WWZ), the root of *Saposhnikovia divaricata* (Turcz.) Schischk. (FF), the root and rhizome of *Cynanchum paniculatum* (Bge.) Kitag. (XCQ), the ripe fruit of *Cnidium monnieri* (L.) Cuss. (SCZ), the ripe fruit of *Kochia scoparia* (L.) Schrad. (DFZ), the fruit with involucre of *Xanthium sibiricum* Patr. (CEZ), the root bark or near-root bark of *Pseudolarix amabilis* (Nelson) Rehd. (TJP), the root of *Sophora flavescens* Ait. (KS), the root bark of *Dictamnus dasycarpus* Turcz. (BXP), the root of *Arnebia euchroma* (Royle) Johnst. (ZC), the cortical shell of *Cryptotympanapustulata* Fabricius (CY), and Borneolum Syntheticum (BP). were purchased from LBX pharmacy (Guangzhou, China) and were authenticated by Professor Hong Nie (College of Pharmacy, Jinan University, Guangzhou, China).

PREPARATION OF ZYF AQUEOUS EXTRACT

150 g of TCM compound (YCH, WM, WWZ, FF, XCQ, SCZ, DFZ, CEZ, TJP, KS, BXP, ZC, CY, BP, with a ratio of 1:1:1:1:1:1:1:1:1:3:1:1:1:0.1) was soaked in 500 mL of water in a decocting container for 1 h. It was then boiled at 100 °C, simmered at 70 °C for 20 min, and filtered through four layers of gauze. The filtrate was collected and the residue was kept in the container. Added 400 mL of water to the decocting container, boiled again to 100 °C, then simmered at 70 °C for 20 min, filtered with four layers of gauze, combined the filtered solution, centrifuged (7000 rpm, 10 min), took the supernatant, used a rotary evaporator (60 °C, 100 rpm) to concentrate to 1.0 g/mL (calculated by raw drug amount), stored at -20 °C.

ANALYSIS USING UPLC-Q EXACTIVE ORBITRAP-MS

The chemical components present in ZYF aqueous extract were isolated and characterized by a High-Resolution Orbitrap Mass Spectrometer (Thermo Scientific, Q Exactive Plus, Germany). The ACQUITY UPLC HSS T3 Column (2.1×100 mm, 1.8 μm, Waters, Milford, MA, USA) was used for separations with water as eluent A and acetonitrile as eluent B. The elution process took 51 min at 30 °C. The flow rate was 0.3 mL/min with an injected volume of 2 μL. The separation was conducted using the following mobile phase conditions: (i) 0-23 min, 1.00-36.28% B, (ii) 23-27 min, 36.28-44.12% B, (iii) 27-31 min, 44.12-59.80% B, (iv) 31-36 min, 59.80-79.40% B, (v) 36-41 min 79.40-99.00% B, and (vi) 41-51 min 99% B. MS^n analysis was performed in positive and negative ion modes. The mass spectrometer scanned from 100 to 1500 m/z at a sheath gas pressure of 206.84 kPa and an auxiliary gas flow of 11.0 L/min. The spray voltage was 3.5 kV for both positive and negative ion modes, and the capillary temperature was 325 °C. MS^1 had a resolution of 70,000 FWHM, while MS^2 had a resolution of 17,500 FWHM. Data was collected using Thermo Xcalibur 4.1. The structures of ZYF-related compounds were qualitatively analyzed using data from the Traditional Chinese Medicine Systems Pharmacology Database and relevant literature.

PREPARATION AND CHARACTERIZATION OF HA-HTCC MICROSPHERES

PREPARATION

Hyaluronic acid (HA) and chitosan quaternary ammonium salt (HTCC) were each formulated into a 50 mL aqueous solution at a concentration of 1.0 mg/mL, and then the formulated aqueous HTCC solution was injected slowly and homogeneously into the aqueous hyaluronic acid solution, followed by agitation for 3 h at 900 rpm. After mixing, the resulting solution was centrifuged (7000 rpm, 10 min) to obtain the supernatant, which was the HA-HTCC microsphere solution, and then freeze-dried to obtain lyophilised HA-HTCC microspheres.

CHARACTERIZATION OF TRANSMISSION ELECTRON MICROSCOPY (TEM)

The HA-HTCC microsphere solution was diluted 10 times with ultrapure water, treated with ultrasound, coated onto a copper mesh, and dried before being observed under a TEM (JEOL, JEM-1400Flash, Japan).

PARTICLE SIZE ANALYSIS

The HA-HTCC microsphere solution was diluted 10 times with ultrapure water, treated with ultrasound, and measured by a Nanoparticle size analyzer (Malvern Panalytical, NANO ZS, UK).

FOURIER-TRANSFORM INFRARED SPECTROMETRY (FTIR) CHARACTERIZATION

Samples of HA, HTCC, and lyophilized HA-HTCC microspheres weighing 2 mg each were analyzed for transmittance using an FTIR spectrometer (PerkinElmer, Spectrum Two, USA) with the wavelength from 400 to 4000 cm^{-1}.

PREPARATION AND CHARACTERIZATION OF HA-HTCC MICROSPHERES CONTAINING ZYF AQUEOUS EXTRACT

PREPARATION

The ZYF aqueous extract and lyophilised HA-HTCC microspheres were dissolved in ultrapure water at 900 rpm for 24 h, resulting in final concentrations of 0.2%(w/w) and 1.0%(w/w), respectively. The volume of the final sample is 10 mL.

TRANSMISSION ELECTRON MICROSCOPY (TEM) CHARACTERIZATION

The HA-HTCC microspheres with ZYF extract solution were diluted 10 times with ultrapure water, treated with ultrasound, coated onto a copper mesh, and dried before being observed under a TEM (JEOL, JEM-1400Flash, Japan).

PARTICLE SIZE ANALYSIS

The HA-HTCC microspheres with ZYF extract solution were diluted 10 times with ultrapure water, treated with ultrasound, and measured by a nanoparticle size analyzer (Malvern Panalytical, NANO ZS, UK).

FOURIER-TRANSFORM INFRARED SPECTROMETRY (FTIR) CHARACTERIZATION

Samples of lyophilized ZYF extract, lyophilized HA-HTCC microspheres, and lyophilized HA-HTCC microspheres with ZYF extract weighing 2 mg each were analyzed for transmittance using an FTIR spectrometer (PerkinElmer, Spectrum Two, USA) with the wavelength from 400 to 4000 cm^{-1}.

SCREENING OF F127 DOSAGE AND DETERMINATION OF PHASE TRANSITION TEMPERATURE AND GEL FORMATION TIME

PREPARATION

F127 solutions were made by mixing F127 with ultrapure water and refrigerating at 4 °C for 24 h. Different concentrations (18-24%w/w) were prepared (10 mL per sample) following the 'Cold Method' (Schmolka 1972), and samples were photographed at 4 °C and 37 °C.

DETERMINATION OF PHASE TRANSITION TEMPERATURE

A 10 mL sample was taken from the refrigerator at 4 °C, transferred to a bottle, and allowed to warm up to room temperature (25 °C) for 10 min. It was then heated in a water bath from 25 °C to 50 °C at a rate of 1 °C per min, with the bottle tilted at a 45-degree angle for every 1 °C increase. The solution flow and water temperature were monitored. The phase change temperature was determined by measuring when the solution solidified into a gel and did not pour within 10 s when the vial was inverted. Three measurements were taken for each sample and the average and standard deviation were calculated.

DETERMINATION OF GEL TIME

The gel time was determined by warming a 10 mL sample from 4 °C to 25 °C, then placing it in a 37 °C water bath and observing its flow every 30 s. Gel formation was confirmed when the solution turned into a gel and remained still for 10 s in the inverted bottle. The time from placing the bottle in the water bath to gel formation was recorded and measured three times for each sample to calculate the average and standard deviation.

PREPARATION OF F127 SOLUTION CONTAINING ZYF AQUEOUS EXTRACT AND HA-HTCC MICROSPHERES AND DETERMINATION OF ITS PHASE TRANSITION TEMPERATURE AND GEL TIME

Samples (10 mL per sample) were created by mixing ultrapure water with three concentrations of F127, stirring for 24 h at 4 °C and 900 rpm. Phase transition temperature and gel time were measured using the methods outlined above. The three sample compositions were: a) lyophilized ZYF aqueous extract, lyophilized HA-HTCC microspheres, and F127 at a concentration of 0.2%, 1.0%, and 19% respectively (w/w), b) lyophilized ZYF aqueous extract, lyophilized HA-HTCC microspheres, and F127 at a concentration of 0.2%, 1.0%, and 20% respectively (w/w), and c) lyophilized ZYF aqueous extract, lyophilized HA-HTCC microspheres, and F127 at a concentration of 0.2%, 1.0%, and 21% respectively (w/w).

SCREENING OF THE DOSAGE OF F68 IN ZYF THERMOSENSITIVE HYDROGEL

A 21% concentration of F127 was chosen for the next stage of development, with F68 added to control the system's phase transition temperature. Samples (10 mL per sample) with different F68 concentrations were made using ultrapure water and stirred for 24 h at 4 °C and 900 rpm. Phase transition temperature and gel time were then determined for the three sample compositions. a) F68, lyophilized ZYF aqueous extract, lyophilized HA-HTCC microspheres, and F127 at a concentration of 0.5%, 0.2%, 1.0%, and 21% respectively (w/w), b) F68, lyophilized ZYF aqueous extract, lyophilized HA-HTCC microspheres, and F127 at a concentration of 1.0%, 0.2%, 1.0%, and 21% respectively (w/w), and c) F68, lyophilized ZYF aqueous extract, lyophilized HA-HTCC microspheres, and F127 at a concentration of 1.5%, 0.2%, 1.0%, and 21% respectively (w/w).

PREPARATION AND CHARACTERIZATION OF ZYF THERMOSENSITIVE HYDROGEL

PREPARATION

A mixture of lyophilized ZYF aqueous extract, lyophilized HA-HTCC microspheres, F127, F68, and ultrapure water was thoroughly mixed for 24 h at 4 °C and 900 rpm. The formulation contained 0.5% F68, 0.2% lyophilized ZYF aqueous extract, 1.0% lyophilized HA-HTCC microspheres, and 21% F127 (w/w). The sample (10 mL) was observed at 4 °C and 32 °C and photographed on a white background.

RHEOLOGICAL CHARACTERIZATION

The samples were tested using a rotational rheometer (TA Instruments, DHR-2, USA). The linear viscoelastic zone was identified by adding a 0.5 mL sample, controlling the temperature at 32 °C, maintaining a frequency of 1 Hz, and using a logarithmic scale for scanning parameters to ensure equal acquisition points in each order of magnitude. The strain range was 0.001% to 100%, with 5 points per decade.

A study measured changes in G' and G" with temperature by adding a 0.5 mL sample and testing at 20-37 °C with a 20-mm parallel plate and 0.1% strain at 1 Hz frequency. The temperature-viscosity curve was created by adding 0.5 mL of the sample to a 20 mm parallel plate and increasing the temperature by 1 °C per minute. Viscosity measurements were taken every 10 s between 20 and 37 °C to plot the curve. The frequency scan was done with a 0.5 mL sample at 32 °C, using a strain of 0.1% within the linear viscoelastic range. A logarithmic scale was used to ensure uniformity in the number of points across orders of magnitude. The scan ranged from 100-0.1 rad/s, with 5 points acquired per order of magnitude.

FOURIER-TRANSFORM INFRARED SPECTROMETRY (FTIR) CHARACTERIZATION

Samples of lyophilized HA-HTCC microspheres with ZYF extract, F127, F68, and lyophilized ZYF thermosensitive hydrogel weighing 2 mg each were analyzed for transmittance using an FTIR spectrometer (PerkinElmer, Spectrum Two, USA) with the wavelength from 400 to 4000 cm^{-1}.

SCANNING ELECTRON MICROSCOPE (SEM) CHARACTERIZATION

The hydrogel samples were frozen, dried, cut, and analyzed using a scanning electron microscope (Hitachi, FlexSEM1000, Japan).

STATISTICAL ANALYSIS

Numerical data are presented as the mean value ± standard deviation.

RESULTS AND DISCUSSION

ANALYSIS OF THE MAJOR CONSTITUENTS OF ZHI-YANG-FANG (ZYF) AQUEOUS EXTRACT

The ZYF aqueous extract was analyzed using UPLC-Q-Exactive Orbitrap-MS to identify and characterize its chemical components. The compounds were successfully separated under optimized UPLC and MS conditions

shown in Figure 1(a) and 1(b). Nineteen compounds were identified (Table 1) by comparing retention time and molecular ion peaks with literature and TCMSP data (https://old.tcmsp-e.com/tcmsp.php).

CHARACTERIZATION OF HA-HTCC MICROSPHERES

TEM analysis confirmed the uniform size and spherical shape of microspheres (Figure 2(a) and 2(b)). FTIR spectrum of HA-HTCC microspheres (Figure 2(c)) showed characteristic peaks of HA and HTCC without new peaks, indicating physical cross-linking and unchanged chemical properties. The infrared spectrograms of HA and HTCC were consistent with previous studies, confirming the accuracy of the determination method (Hu & Wang 2016; Karami et al. 2021; Tang et al. 2016). The measurement of particle size revealed an average size of 546.6± 31.5 nm (Figure 2(d)).

CHARACTERIZATION OF HA-HTCC MICROSPHERES CONTAINING ZYF AQUEOUS EXTRACT

HA-HTCC microspheres with ZYF extract were confirmed to be spherical, uniform in size, and non-adhesive in TEM analysis (Figure 3(a)). The FTIR spectrum (Figure 3(b)) showed that the infrared spectra of HA-HTCC microspheres containing ZYF aqueous extract exhibited the characteristic peaks of both ZYF aqueous extract and HA-HTCC microspheres, without the emergence of novel characteristic peaks. This observation suggested the absence of any new chemical reactions during the preparation process. Analysis of particle size indicated an average size of 1933.0±215.3 nm, which exceeded the average particle size of HA-HTCC microspheres (Figure 3(c)). Consequently, these findings confirmed the successful preparation of HA-HTCC microspheres containing ZYF aqueous extract.

OPTIMIZATION AND PREPARATION OF ZYF THERMOSENSITIVE HYDROGEL

To achieve the optimal gel formation, a range of F127 solution concentrations were examined and visually documented at both 4 and 37 °C. Additionally, the phase transition temperatures of each F127 solution group and the gelation time at 37 °C were determined. It was observed that F127 solutions with concentrations ranging from 18% to 24% (w/w) exhibited desirable fluidity and appeared transparent at 4 °C. Furthermore, all F127 solution groups demonstrated the ability to undergo phase transition and form gels at 37 °C (Figure 4(a)). When the concentration of F127 increased, the phase transition temperature and gelation time decreased (Figure 4(b) and 4(c)). To achieve phase transition temperatures comparable to that of human skin, F127 concentrations of 19, 20, and 21% (w/w) were selected for further development.

Additionally, we investigated the impact of HA-HTCC microspheres containing ZYF aqueous extract on the gelation time and phase transition temperature of F127. The addition of HA-HTCC microspheres containing ZYF aqueous extract increased the phase transition temperature and gelation time of the system (Figure 5(a) and 5(b)). To be able to obtain a phase transition temperature close to the temperature of human skin (32 °C), we chose 21% (w/w) as the concentration of F127 for subsequent development.

The inclusion of F68 into the system was undertaken for adjustment. The study centred on analyzing the influence of different levels of F68 (0.5%, 1.0%, and 1.5% w/w) on both the temperature at which phase transition occurred and the duration it took for the system to form a gel. The results indicated a positive relationship between the F68 concentration and both the system's phase transition temperature and gel time, as shown in Figure 5(c) and 5(d). Ultimately, a concentration of 0.5% w/w F68 was determined as the optimal choice for formulation development. These results indicated successful screening of F127 and F68 concentrations for formulation development.

CHARACTERIZATION OF ZYF THERMOSENSITIVE HYDROGEL

The appearance of the prepared ZYF thermosensitive hydrogel was observed and subjected to a series of characterizations. The hydrogel exhibited favourable flowability at a temperature of 4 °C, possessed an orange-yellow hue, and did not exhibit any precipitation (Figure 6(a) and 6(b)). Moreover, it demonstrated the ability to undergo gelation at a temperature of 32 °C. The FTIR spectrum (Figure 6(c)) showed that the infrared spectra of ZYF thermosensitive hydrogel exhibited the characteristic peaks of F127, F68, and HA-HTCC microspheres containing ZYF aqueous extract, without the emergence of novel characteristic peaks. This observation suggested the absence of any new chemical reactions during the preparation process. The infrared spectral features of F127 and F68 were in agreement with previous studies, validating the accuracy of the determination method (Mohamed et al. 2017; Silva et al. 2018).

To further elucidate the microstructure, the freeze-dried samples of ZYF thermosensitive hydrogel and blank hydrogel were subjected to observation using SEM. The SEM analysis of the cross sections demonstrated that both the blank hydrogel and the ZYF thermosensitive hydrogel (Figure 6(d)-6(g)) exhibited a characteristic 3D network structure (Sharun et al. 2024), consistent with the structure of this type of hydrogel demonstrated in

previous related studies (Chatterjee et al. 2019; Liang et al. 2023; Suhail et al. 2023). 3D network structure facilitates drug encapsulation (Li et al. 2023). Importantly, upon closer examination, microspheres embedded within the hydrogel were observed in the ZYF thermosensitive hydrogel compared to the blank hydrogel, providing additional evidence for the formation of a composite hydrogel system.

Hydrogel rheological properties were assessed, and the results from strain scanning (Figure 6(h)) indicated that within the strain range of 0.001-0.1%, the energy storage modulus (G') and loss modulus (G") of the hydrogel exhibited stability, suggesting the structural stability of the hydrogel during this period. Nevertheless, once the strain exceeded 0.1%, both the energy loss modulus and storage modulus underwent alterations, underscoring the significance of restricting the hydrogel's linear viscoelastic region to a strain of 0.1%. G' and G" of hydrogel were determined as a function of temperature (Figure 6(i)). The G' and G" started to increase after 25.9 °C, and G' began to be steadily larger than the loss modulus G", indicating the beginning of gelation, with a period of a sharp increase in the middle, and G' steadily exceeded G" when the temperature reached 32.1 °C and entered into a plateau period, indicating that the formed hydrogel system had typical viscoelastic behaviour and stability (Jose et al. 2024).

The findings from the frequency scanning analysis (Figure 6(j)) indicated that the energy storage modulus (G') exhibited stability within the frequency range (100-0.1 rad/s), thus demonstrating its sustained stability following gel formation. The temperature-viscosity curve was measured (Figure 6(k)). Within the temperature range of 20-25 °C, the viscosity exhibited minimal variation. However, beyond 25 °C, a notable increase in viscosity occurred, indicating a solution state to a gel state. Subsequently, a plateau was gradually attained after reaching 30 °C, accompanied by a deceleration in the rate of viscosity change, signifying the progressive stabilization of the gel state. The rheological characterization results exhibited rheological properties consistent with those reported in prior studies on F127 and F68 (Liang et al. 2023; More et al. 2022), thereby affirming the feasibility and accuracy of the employed research methodology. Consequently, these findings confirmed the successful preparation of ZYF thermosensitive hydrogel.

TABLE 1. Identification of constituents in Zhi-Yang-Fang (ZYF) aqueous extract

Peak No.	t_R (min)	Compound	Formula	Scanning mode	Measured value (m/z)
1	5.20	Eciphin	$C_{10}H_{15}NO$	Positive ion mode	166.0861
2	6.68	Cytisine	$C_{11}H_{14}N_2O$	Positive ion mode	191.1176
3	8.25	N-Methylcytisine	$C_{12}H_{16}N_2O$	Positive ion mode	205.1332
4	8.92	Baptifoline	$C_{15}H_{20}N_2O_2$	Positive ion mode	261.1595
5	11.04	Matrine	$C_{15}H_{24}N_2O$	Positive ion mode	249.1954
6	11.45	Oxymatrine	$C_{15}H_{24}N_2O_2$	Positive ion mode	265.1904
7	14.42	2'-Methoxykurarinone	$C_{27}H_{32}O_6$	Positive ion mode	453.3421
8	16.30	Cimifugin	$C_{16}H_{18}O_6$	Positive ion mode	307.1167
9	17.42	1,3,5-tri-o-caffeoylquinic acid	$C_{34}H_{30}O_{15}$	Positive ion mode	679.5096
10	19.75	Diosmetin	$C_{16}H_{12}O_6$	Positive ion mode	301.1039
11	20.65	Cnidimol F	$C_{15}H_{14}O_6$	Positive ion mode	291.1220
12	24.74	Arenarine B	$C_{14}H_{14}N_2O_2$	Positive ion mode	243.1011
13	31.10	Obacunone	$C_{26}H_{30}O_7$	Positive ion mode	455.2030
14	42.19	Kadsulignan B	$C_{25}H_{30}O_9$	Positive ion mode	475.3248
15	3.26	Palmitic acid	$C_{16}H_{32}O_2$	Negative ion mode	255.0495
16	12.97	Lineolone	$C_{21}H_{32}O_5$	Negative ion mode	363.0832
17	29.21	Rugosal	$C_{15}H_{22}O_4$	Negative ion mode	265.1466
18	36.99	Deoxyharringtonine	$C_{28}H_{37}NO_8$	Negative ion mode	514.3220
19	44.99	Maackiain	$C_{16}H_{12}O_5$	Negative ion mode	283.2628

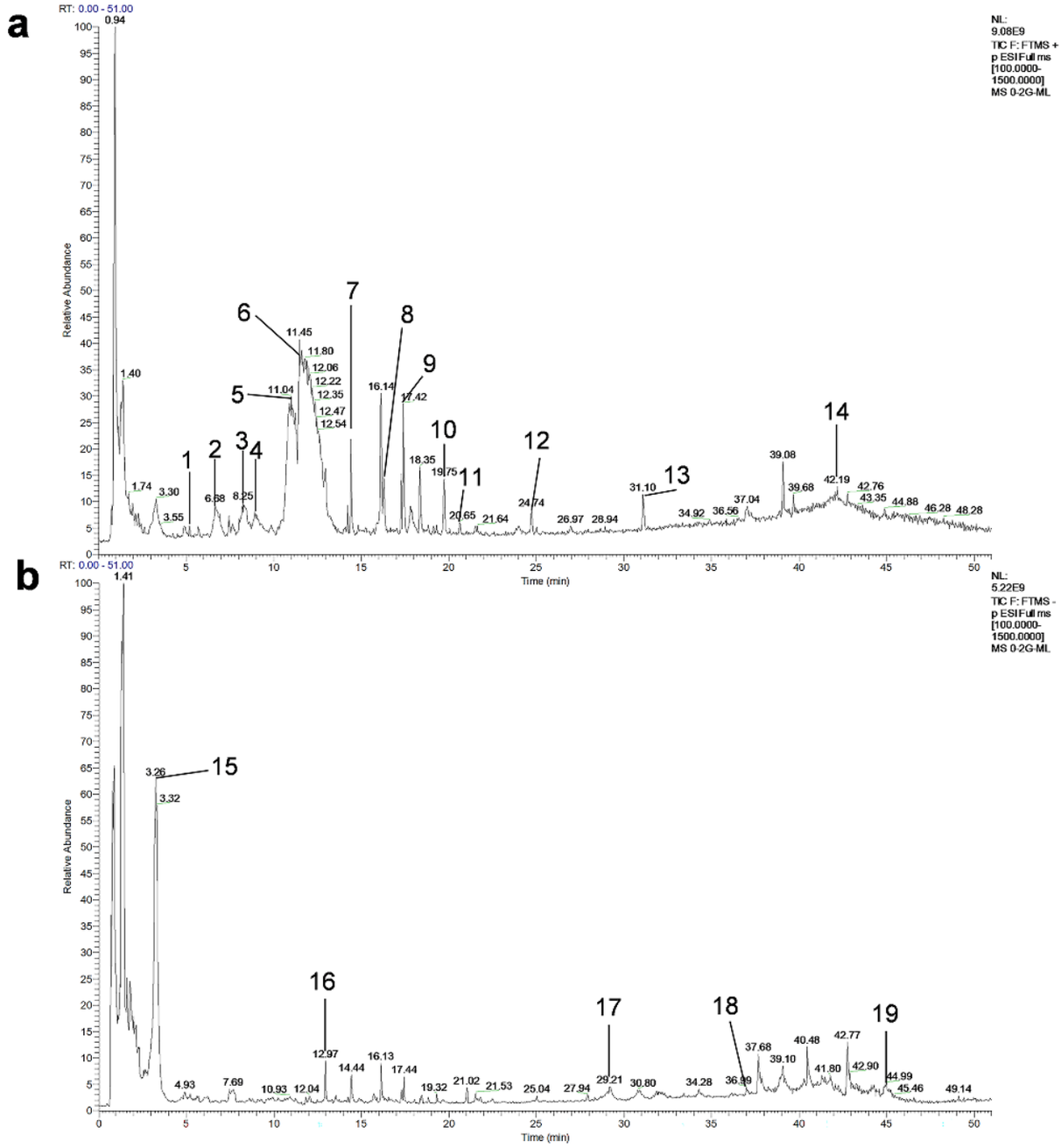

FIGURE 1. Total ion chromatogram of Zhi-Yang-Fang (ZYF) aqueous extract.
(a) Positive and (b) Negative ion modes

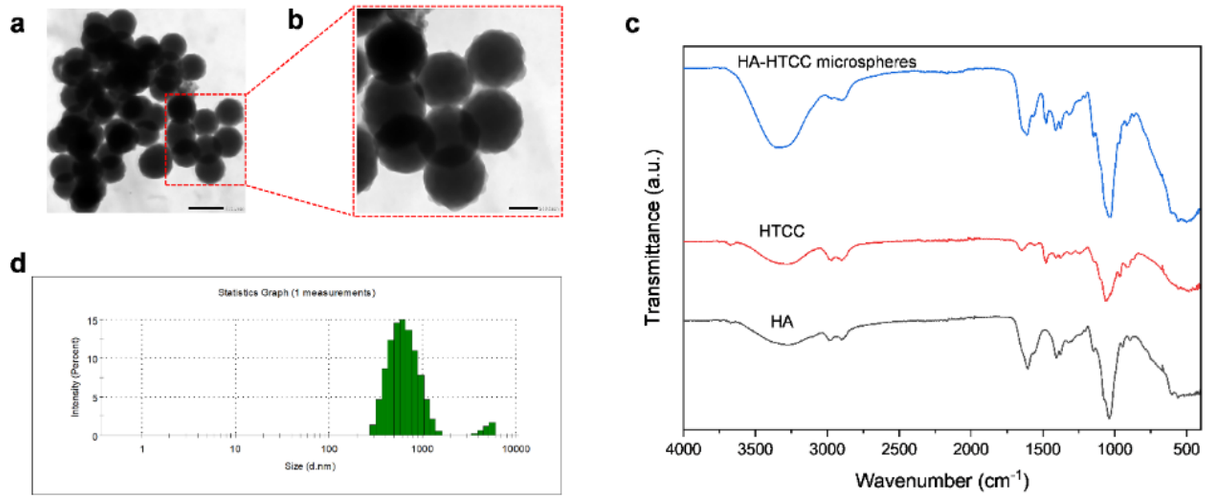

FIGURE 2. Characterization of hyaluronic acid-chitosan quaternary ammonium salt (HA-HTCC) microspheres. Transmission electron microscopy (TEM) micrographs. Scale bar: 500 nm (a) and 250 nm (b); (c) Fourier transform infrared spectroscopy (FTIR) spectrum; (d) Particle size distribution

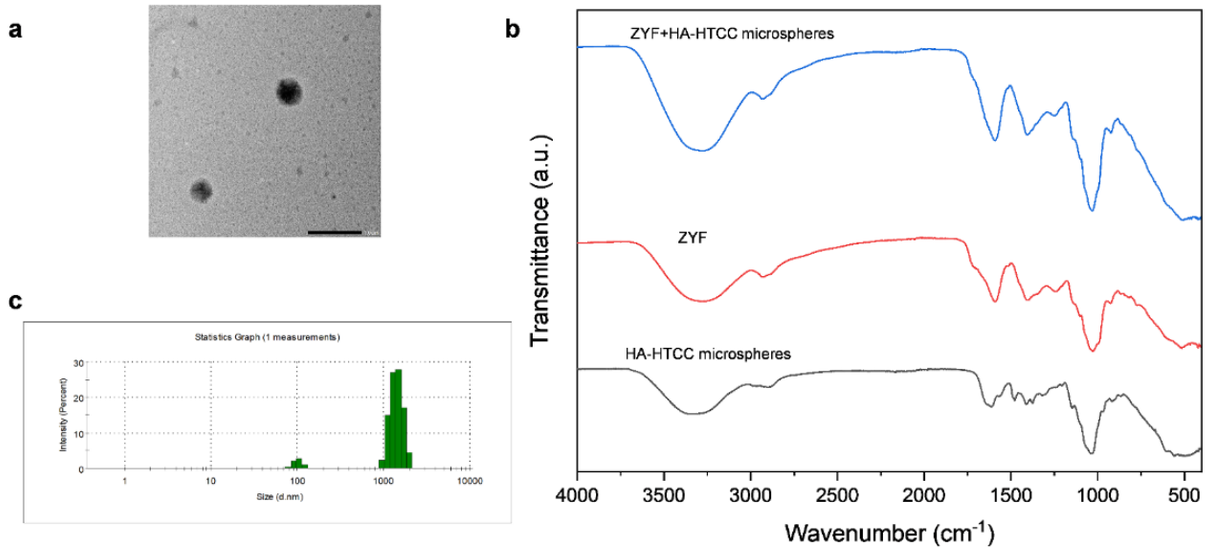

FIGURE 3. Characterization of HA-HTCC microspheres containing ZYF aqueous extract. (a) TEM micrograph. Scale bar: 1 μm; (b) FTIR spectrum; (c) Particle size distribution

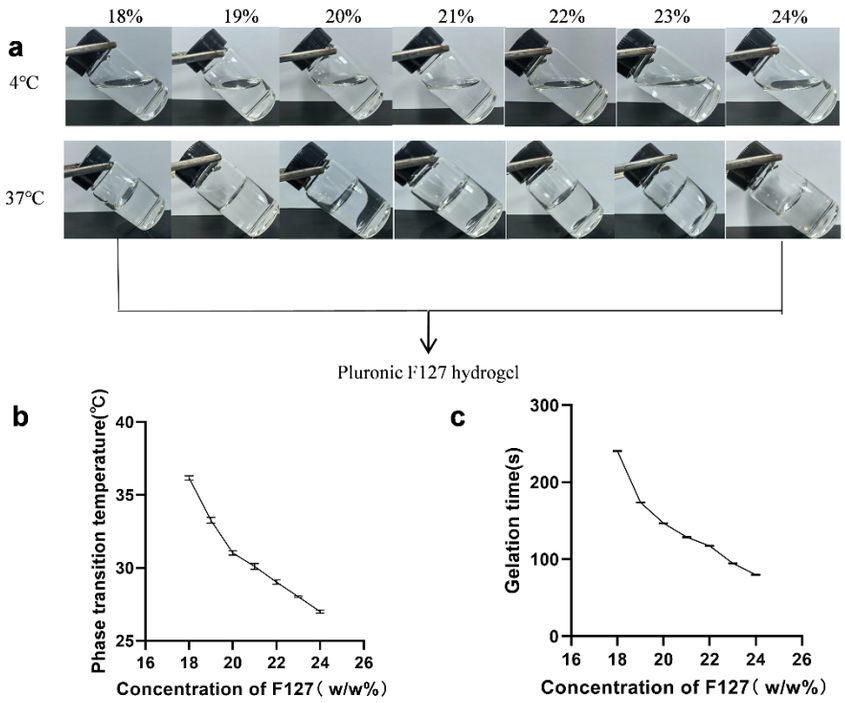

FIGURE 4. Thermosensitive properties of blank F127 hydrogel. (a) Gel resolution of different concentrations (18%, 19%, 20%, 21%, 22%, 23% and 24% w/w) at 4 °C and gel formation of F127 at 37 °C; (b) Phase transition temperatures of different concentrations of F127 (18%, 19%, 20%, 21%, 22%, 23% and 24% w/w); (c) Gelation time of different concentrations of F127 (18%, 19%, 20%, 21%, 22%, 23% and 24% w/w)

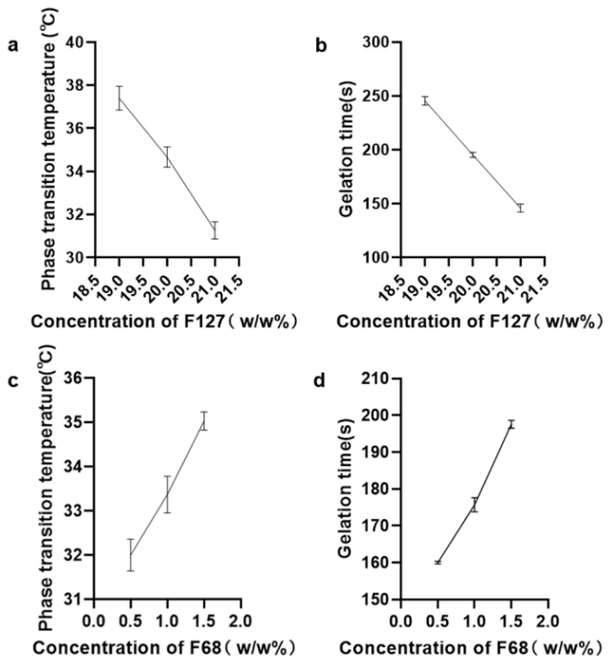

FIGURE 5. Effect of HA-HTCC microspheres containing ZYF aqueous extract on the F127. (a) Phase transition temperature; (b) Gelation time; Effect of F68 on 0.2%(w/w) ZYF aqueous extract/1.0%(w/w) HA-HTCC microspheres/21%(w/w) F127 system. (c) Phase transition temperature; (d) Gelation time

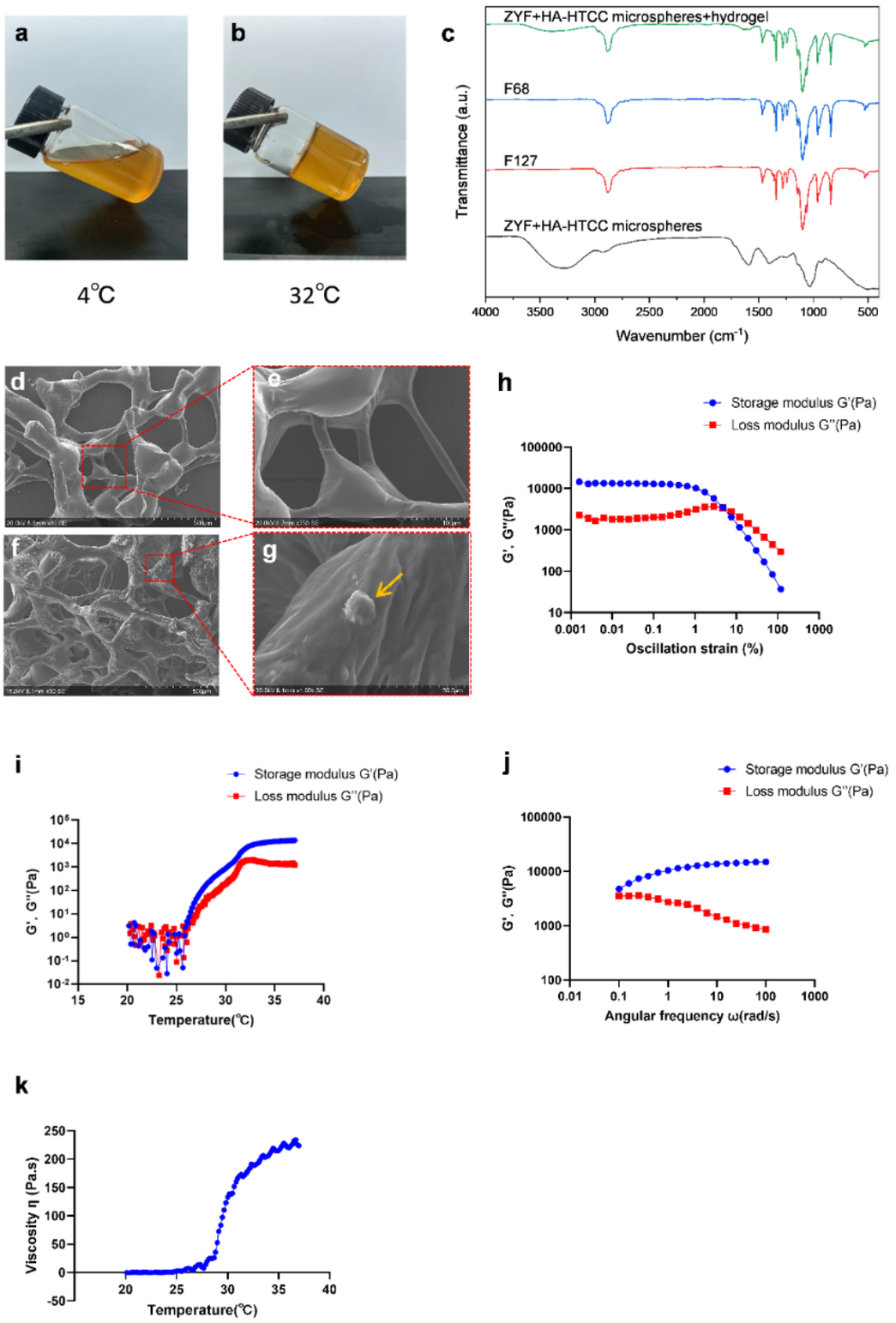

FIGURE 6. Characterization of ZYF thermosensitive hydrogel. (a) The appearance of ZYF thermosensitive hydrogel at 4 °C; (b) The appearance of ZYF thermosensitive hydrogel at 32 °C; (c) FTIR spectrum; (d) Scanning electron microscopy (SEM) micrograph of blank hydrogel. Scale bar: 500 μm; (e) SEM micrograph of blank hydrogel. Scale bar: 100 μm; (f) SEM micrograph of ZYF thermosensitive hydrogel. Scale bar: 500 μm; (g) SEM micrograph of ZYF thermosensitive hydrogel. Scale bar: 100 μm (the arrow showed the microsphere); (h-k) Rheological characterization

CONCLUSIONS

In summary, the present study analyzed and identified the 19 main components in the aqueous extract of Chinese herbal compound (ZYF), successfully prepared HA-HTCC microspheres and HA-HTCC microspheres containing the aqueous extract of ZYF, screened the concentrations of F127 and F68, and ultimately succeeded in the development of a thermosensitive hydrogel containing ZYF aqueous extract and HA-HTCC microspheres, which possessed thermal sensitivity and stability. This study offers a potential solution for managing ACD with promising clinical implications.

ACKNOWLEDGEMENTS

The authors acknowledge the support and facilities provided by the College of Pharmacy and College of Chemistry and Materials Science, Jinan University, in carrying out the present research work. This research was supported by the project of the National Natural Science Foundation of China to H.N., grant number (No. 81373993).

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