Mucoadhesive nanofibrous membrane with anti-inflammatory activity

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Received: 21 February 2018 / Revised: 18 September 2018 / Accepted: 16 November 2018 © Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract
For the higher patient compliance in periodontal disease, the drug delivery system should be released with a mucoadhesive membrane in a higher rate to minimize the sink condition in mouth. The herbal compounds have been well known as a branch of therapeutic agents, especially for inflammatory injuries. Herein, carbopol or carboxymethyl cellulose was employed to prepare a matrix system containing a therapeutic agent, namely *Ziziphus jujuba* extract. The corresponding polymer was electrospun with polyacrylonitrile, and the release process was studied in the following. Approximately, 80% of the extract was released after 60 min following the Higuchi model and the anti-inflammation reaction of the extract was confirmed after stimulating inflammation of human umbilical vein endothelial cells by lipopolysaccharide. Also, the mechanical mucoadhesion of prepared scaffold was exposed that approximately 4 N/m² was required to separate from mucoadhesive substrates. Also, Fourier transform infrared spectroscopy confirmed the presence of cyclic saccharides belonged to *Z. jujuba* extract. All results have approved that the membrane prepared with carbopol and *Z. jujuba* extract could be used as a patch for the treatment of periodontal injuries.

Keywords Mucoadhesive membrane · Carbopol · Anti-inflammatory activity · Electrospinning · Nanofibers

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Introduction

Periodontal disease is a general term characterized by the degeneration of periodontal ligaments, alveolar bone, and dental cementum and also inflammation of gums [1]. Various medications and methods have been developed to limit the destruction of periodontal tissue including the use of a guided tissue regeneration, barrier membranes and bone replacement grafts [2–4]. One of the motivations is to diminish the host response mechanisms including the usage of anti-inflammatory drugs to inhibit inflammatory destruction in periodontal disease.

Topical drug delivery is used for the treatment of localized disorders, e.g., oral and periodontal diseases and infection within the oral cavity. The main advantage of this drug delivery mode is the ability to deliver bioactive agents directly to the site, and maintenance of the suitable concentration of drug at the desired region. However, the drawback of many topical adhesive systems is a poor stability because oral cavity is located at a very wet location due to oral fluids and mucosal secretion, and it has many movements due to swallowing and chewing [5–8]. Mucoadhesion refers to the interaction of a polymeric platform with a biological substrate coated with mucus. So, the mucoadhesive systems present a possible method for the retention of drug dosage at the site of therapy. However, the potential of mucoadhesive systems is dependent on the polymer’s characteristics. Therefore, choosing a suitable polymer in the designing of the mucoadhesive systems is one of the most important issues [9, 10]. Most of these polymers are large macromolecules containing amine and carboxylic groups which can bind to the mucus. In addition, most of the mucoadhesive polymers go through hydration and swelling and then penetrate into mucus by changing the surface tension. Since in many buccal diseases fast treatment is needed, polymers with moderate adhesion, such as carbopol polymers, can be used. Carbopol or carbomer is a highly hydrophilic polymer which shows linear swelling [11] and different viscosities in different pH values [4]. The increase in pH will lead to an intensification of viscosity which will ultimately result in the formation of a gel. Water absorption of this polymer which occurs in the less adhesion time is completely noticeable compared to the other polymers. The usage of this polymer along with polar polymers in the presence of saliva and mucus can quickly cause hydrogen bonds resulting in better mucoadhesion [12]. Carbopol-polyacrylonitrile (PAN) has been introduced as a biocompatible, mucoadhesive polymer which has applications in transdermal drug delivery [13]. Electrospinning process provides to obtain a nanofibrous structure of substrates that can improve the reaction points [14]. Thus, the resultant besdless nanofibers are required to obtain high surface area-to-volume ratio for more release kinetic [15]. With a research group, the higher surface area was obtained using electrospinning compared to a substrate with no regular shape and the interactions of surface extra environment and thus, made higher positive charge [16]. Also, in our previous study, the interactions of cells with nanofibrous scaffold substrate had enhanced [17]. As a function of these reports, the electrospinning process was chosen here to produce a nanofibrous pattern of corresponding polymers.
The buccal inflammatory disease is a kind of ulcer affecting the surface of the mouth and tongue, which can even penetrate into their inner parts. Based on Iranian traditional medicine books and local healers, powders extracted from stem bark and leaves of Jujuba have been used to cure wounds including oral ones [5, 18]. Z. jujuba obtained from Rhamnaceae family is grown in the Eastern, South-eastern and Central parts of Iran [19]. Ziziphus jujuba (Z. jujuba) stem bark has been showing anti-inflammatory and antimicrobial effects to treat this disease [5, 18, 20, 21]. Secondary metabolites in the stem bark of Z. jujuba are terpenoids, tannins, and zizipho tannic acid. The most important part of these agents is the zizipho tannic acid, which makes Z. jujuba suitable for treatment of inflammatory diseases of mouth [18, 21, 22]. Other Z. jujuba constituent agents are flavonoids, such as quercetin, which is isolated from stem bark and is recognized as antioxidant agents [22].

Herein, a mucoadhesive membrane was prepared by electrospinning technique using carbopol polymer and PAN as the main component of the membrane and the extract derived from Z. jujuba constituent as an anti-inflammatory property. The corresponding scaffold has been evaluated by SEM, FTIR and water contact angle assays. The mechanical mucoadhesion test was studied beside to kinetic mechanism of extract release for 1 h. Antimicrobial influence of Z. jujuba extract and also anti-inflammatory response were discussed in the following. Finally, the relevant results determined the required properties of corresponding scaffolds as an inflammatory membrane and also mucoadhesive buccal patch for periodontal disease.

Methods

Preparation of the extract

After Z. jujuba was obtained, fresh parts were cleaned and dried at 40 °C prior to powdering by electric mills. Then, 96% ethanol was employed to extract by Soxhlet apparatus from the pulverized stem bark with a yield percent of 11.46% w/w. The resultant extract was stored at 2–4 °C and partitioned by ethanol before any further application [23, 24].

Preparation of electrospinning solution

The solutions were prepared for the electrospinning process by dissolving carbopol (Sigma-Aldrich, 0.05 gr) with polyacrylonitrile (PAN) (Sigma-Aldrich, 0.18 gr) in 4 ml DMF. In the following, the extract, namely Z. jujuba (0.2 gr), was added and vigorously stirred under magnetic stirring for 5 h to get a homogenous solution. The electrospinning process was performed by 18 kV of positive voltage–power, and the produced nanofibers were collected on the aluminum foil of rotating collector with a speed of 240 rpm. The feed rate and distance were set at 0.1 ml/h and 18 cm, respectively.
Nanofiber examinations

Morphology of the electrospun mat of carbopol-PAN-extract was studied using a scanning electron microscope (SEM, Philips XL30, Netherlands). The nanofibrous samples were coated with gold using a sputter coater, and the mean diameter of nanofibers was exposed by the software of Microstructure Measurement and reported as a mean ± standard error. Also, the obtained composite scaffold was studied using Invert microscope (Motic, AE31) before and after swelling in phosphate buffer serum (PBS, Gibco).

FTIR assessments

Fourier transform infrared spectroscopy (FTIR) was employed for the assessment of functional groups of the nanofibrous carbopol-PAN-extract mat. The related spectra were recorded with a BOMEM spectrometer (BM-102) equipped with a DTGS detector from 500 to 4000 cm⁻¹ at the ambient temperature.

Water contact angle measurement

The surface wettability of scaffold with and without *Z. jujuba* extract was studied by measuring the contact angle with water molecules. The samples of corresponding scaffolds were mounted on a stage of a G10 Kruss contact angle goniometer. Then, the analysis was performed using the sessile drop method with a G10 Kruss contact angle goniometer and the angles were reported after 10 s.

In vitro drug release studies

In vitro extract release evaluation of carbopol-PAN-extract nanofibers was carried out based on US Pharmacopeia at 37 °C and 100 rpm in a tank of artificial saliva [25]. The corresponding assay was done after a nanofibrous mat with the thickness of 30 µm, and the surface area of 0.25 cm² was placed at the bottom of a beaker in 5 ml of artificial saliva. The assay was done for 60 min, with 10-min time intervals, at pH 6.9 and thermally controlled at 37 °C. 1 ml of solution vessel was replaced with artificial saliva every 10 min and analyzed using polyphenolic compounds release assay which will be described in the following section [26]. These investigations were conducted in triplicate, and the phenolic compound release value was plotted against time.

Polyphenolic compounds release assay

Gallic procedure was used to evaluate the total phenolic content of released extracts from carbopol-PAN-extract nanofibers based on the protocol described previously [27]. Briefly, the phenolic compound (1 ml) was mixed with diluted Folin–Ciocalteu reagent (5 ml), before the addition of sodium bicarbonate solution (7.5 w/v). The absorbance at 765 nm was detected using a UV spectrophotometer (Pharmacia
Biotech, Germany) after 30 min, and Gallic acid was used as a standard sample. The total phenolic content is expressed as mg of gallic acid equivalents (GAE) per g of dry extract using the below standard curve equation [18]:

\[
Y = 0.0074X - 0.0756 \quad \text{and} \quad R^2 = 0.9858
\]

**Mechanical mucoadhesion test**

Universal Testing Machine (Instron, Hounsfield H50KS, England) was utilized to simulate the in vitro adhesion strength of prepared scaffold to the wound area. The related measurement was taken in terms of the force needed to pull out the nanofibrous scaffold from a gelatin gel solution (30% wt/wt). The obtained electrospun membrane was fixed in the upper jaw of the machine and wetted using 0.1 ml of water and pulled out from the gel layer. The upper jaw was advanced at 180 mm/s of velocity until the electrospun scaffold was separated from the gel. The ultimate detected stress was the adhesive bond strength between the scaffold and gel layer [28].

**Microbial assay of *Z. jujuba* extract**

*Porphyromonas gingivalis* and *Fusobacterium nucleatum* microbial samples were prepared from Baqiyatallah University of Medical Sciences, Tehran, Iran. Bacterial strains of corresponding microbial samples were isolated according to Slots’ rapid identification method [29]. The susceptibilities to *Z. jujuba* extract (50 µg/ml) and antibiotic disks of penicillin G (Pen), tetracycline (Tet), ciprofloxacin (Cip), erythromycin (Ery) and clindamycin (Clin) were studied using the disk diffusion susceptibility test (DDS test: Kirby–Bauer’s modified method for anaerobic bacteria). An inoculum of $10^5$ CFU was applied with a Steers replicator (Craft Machine Inc., Woodline, PA), and the plates were incubated in an anaerobic jar with gas pack A for 48 h at 37 °C.

**The inflammatory response to *Z. jujuba* extract**

To investigate the anti-inflammatory effect of *Z. jujuba* extract, permeability assay was used as a standard method according to our previous report [30] and exposed as a schematic view in Fig. 1. Human umbilical vein endothelial cells (HUVEC) were purchased from the cell bank of School of Advanced Technologies in Medicine (Shahid Beheshti University of Medical Sciences, Tehran, Iran) and cultured on the transwells of 24-well plate insert with 3.0 µm pore size and 6.5 mm diameter (with cell density of $1 \times 10^5$ cells per well under 250 µl basal media (DMEM supplemented with 10% FBS) and 750 µl fresh basal media were also added to the bottom chambers. After 48 h monolayer of HUVEC, the cells will be ready to perform a permeability assay. First, it is needed to transfer the upper chambers to the side empty wells without media. Four groups were designed as follows: (1) lipopolysaccharide (LPS, 10 ng/mL for 3 h) as an inflammatory agent, (2) LPS for 3 h and then extract (50 µg/ml) for 1 h, (3) extract as a pretreatment for 1 h and then LPS for 3 h,
(4) only extract for 1 h. After the removal of media and reagents from upper chambers and washing with PBS, 250 μl phenolsulfonphthalein (phenol red) (Bioreagent, Sigma-Aldrich) (0.67 mg/mL diluted in DMEM containing 4% FBS) was added to upper chambers and incubated at 37 °C, then placed on wells with bottom basal media. After 30 min, bottom media were removed and read by a microplate reader at 650 nm. Culture media were used as a blank.

Statistical analysis of results

The difference between groups was analyzed using SigmaPlot software 12.0. (Sysstat Software Inc., UK). Two-tailed Student’s t test was recruited to show the differences between data means of applied groups. A P value of less than or equal to 0.05 was considered statistically significant, and the corresponding data were shown as a mean ± standard error.

Results and discussion

Fibers morphology examinations

The obtained electrospun scaffolds were examined using SEM and shown in Fig. 2a, b. The electrospun carbopol-PAN presented a range of fiber diameter.
from 1714 to 2018 nm, and carbopol-PAN-extract was 600–900 nm which was in agreement with higher conductivity of solution after addition of extract [31] in Table 1. As observed in SEM figures, there are distances between fiber-to-fiber contacts providing the desired space for water diffusion and facilitated the release of extract compound. Also, it was clear the uniform structure of nanofibers could provide a high surface area-to-volume ratio helping to greater release value of extract compared to substrates without nanofibrous structure [17, 32]. Figure 2c shows the macroscopic morphology of the prepared composite scaffold with 30 µm in thickness that could facilitate its employment as a mucoadhesive membrane. Also, the nanofiber reactions before (Fig. 2d) and after (Fig. 2e) swelling in PBS were studied using inverted optical microscopy. It seems that the fibers diameter was the same between different conditions, while the swelled nanofibers after incubation in PBS loose the compact structure of fibers. Thus, the space

<table>
<thead>
<tr>
<th>Polymer solution</th>
<th>Temperature (°C)</th>
<th>Conductivity value (µs/cm)</th>
<th>Fiber diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbopol-PAN</td>
<td>26.8</td>
<td>34.85</td>
<td>1866 ± 152</td>
</tr>
<tr>
<td>Carbopol-PAN-extract</td>
<td>25.8</td>
<td>219.4</td>
<td>750 ± 150</td>
</tr>
<tr>
<td>DMF</td>
<td>24</td>
<td>2.1</td>
<td>–</td>
</tr>
</tbody>
</table>
between fibers became larger facilitating release process due to solution transport into fibers network [33].

**FTIR assessments**

The related peak to carbopol was intensively exposed at 1730 cm⁻¹ which is certified to C=O stretching [34] and a band at 2934 cm⁻¹ related to C–H stretching moved to 2875 cm⁻¹. The corresponding peak appeared with nanofibrous carbopol-PAN-extract. The extract compound of *Z. jujuba* belongs to plant polyphenols which were exposed the band between 1036 and 1105 cm⁻¹ representative of C–O–C stretching of cyclic saccharides in *Z. jujuba* extract with the composite type of the corresponding scaffold [11] (Fig. 3). Moreover, there is a broad region at 3000–3500 cm⁻¹ related to OH groups with the extract and composite structure of membrane that is representative of water molecules existence [35].

**Water contact angle measurements**

The both scaffold types inclusive pristine and composite types were studied to compare the impact of *Z. jujuba* extract to surface hydrophilicity. Figure 4 shows the related angle number to the scaffolds types. It is observed that the angle was reduced after addition of the extract from 24° with pristine type to composite type with 36°. The difference was statistically acceptable and confirmed the positive role of the extract to increase the surface wettability. As before reported, the polyphenols are classified as hydrophilic nature than lipophilic due to their phenolic compound [36]. Thus, the presence of derived polyphenolic molecules from the extract caused the

![Fig. 3 FTIR spectroscopy results of electrospun scaffolds types and Z. jujuba extract](image-url)
lower water contact guaranteeing the higher swelling compared to pristine type and also facilitating the release process in the following.

**Ziziphus jujuba extract release assay**

During the electrospinning process, contents with higher conductivity tend to move to the outer layers of electrospinning fibers [37]. The polymeric composite containing *Z. jujuba* extract has a higher conductivity compared to the pure polymeric solution of carbopol-PAN, so the produced fibers have an increasing gradient of *Z. jujuba* from the core to the surface. Additionally, during the first 20 min, scaffolds have shown a burst release of the encapsulated drug (Fig. 5). In this manner, more than 45% of *Z. jujuba*, which was loaded into the scaffold, had been released by the diffusion after 20 min. This will also result in empty spaces in polymeric chains and pores on the surface of fibril structure that could improve the release process. The release amount from 20 min till 40 min obeyed the Peppas equation of \( Y = 21.4X^{0.26} \) and \( R^2 = 0.96 \). The obtained value of resulted exponent \( (n) \) as 0.26 approved the Fickian diffusion in the region [38]; however, after 40 min the model had deviated from the Peppas model. After 40 min, an accelerated release behavior was observed, resulting in up to 24% of total loaded drug to be released. This is attributed to the diffusion of artificial saliva into polymeric structure and finally polymeric swelling matrix and more distances between fibers. Thus, as well as larger surface area has been formed, the escalated diffusion of drug content in out of scaffolds occurs more [39]. Ultimately, after 60 min of release, more than 80% of the encapsulated drug had been released from the scaffold. Due to the positive slope of obtained release curve as 1.17 with \( R^2 = 0.91 \), zero-order kinetic was developed. The corresponding kinetic type approved the extract release was dependent on time meaning with more incubation time, the released fraction will increase. There was a deviation from first-order mechanism for this release profile because of the lower solubility kinetic of corresponding polymers [40] in spite of hydrophilic nature. On the other hand, the kinetic release was fitted in Higuchi model with \( R^2 \) and \( K_H \) of, respectively, 0.98 and 0.12. Higuchi model has been reported for the systems with lower solubility confirming the associated mechanism as controlled release type [41] opposite to 45%
fractional of release immediately in less than 20 min. While as a function of scaffold degradation and also sink conditions, Fickian phenomena again occurred [42].

**Mucoadhesion activity**

The adhesion strength values of electrospun mats of carbopol-PAN and carbopol-PAN- *Z. jujuba* extract were measured. The required force for the peak detachment of nanofibrous membrane of carbopol-PAN was 4.02 N/m² and for composite type was 3.48 N/m². The values statistically approved the insignificant relation between pristine and composite types of scaffolds. In our knowledge, the presence of *Z. jujuba* extract did not change the mechanical property of prepared mucoadhesive mat. Compared to other studies, the obtained values were higher than other formulated tablets for the treatment of periodontal disease from cellulose, polyacrylic and metronidazole. The resultant values were between 0.36 and 1.28 N which were lower than that obtained in this study [43]. The measured values of another study, which had resulted for bilayered tablets with a strong adhesion to gum, were ranging from 0.85 to 1.58 N [44] which was also lower than the mechanical adhesion which was provided with composite type of carbopol-PAN in this study.

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Fig. 5 Release curve of *Z. jujuba* from composite type of nanofibrous scaffold
Microbial assessment

The microbial assay was studied against microorganism related to periodontal diseases. In contrast to positive result with antibiotic disks of penicillin G (Pen), tetracycline (Tet), ciprofloxacin (Cip), erythromycin (Ery) and clindamycin (Clin), the test sample containing *Z. jujuba* extract showed an insignificant response that confirmed no antimicrobial activity with the composite electrospun membrane.

Anti-inflammatory activity

The HUVEC cells were inclusive 4 groups: (1) incubation with LPS (control +), (2) incubation with LPS and then with extract in the following, (3) treated with extract and then incubation with LPS and (4) treated with extract (control –), and the results are gathered in Fig. 6. Endothelial cells after stimulation with bacterial products as LPS produce various factors as cytokines [45]. The resultant inflammatory conditions by cytokines induce vascular permeability [46] despite the effects on tight junction proteins. The previous study approved that some plant compounds as polyphenol have inhibitory effects on inflammatory agents [47]. Also, other researches approved the anti-inflammation behavior of herbal extracts as *Areca catechu* L. [48], *Securidaca longepedunculata* root barks [49] and green tea [50]. The corresponding activity was related into phenol compounds which could prevent the activation of inflammatory pathway. The results confirmed the significant role of extract treatment to prevent inflammatory response compared to the cell group which was added solely to LPS. Moreover, the inflammatory condition of LPS was reduced

![Fig. 6](image-url) The obtained results of anti-inflammatory activity assay for corresponding groups: LPS; incubation of cells with LPS only, extract/LPS; the pretreated cells with extract and then incubation with LPS, LPS/extract; the incubation with LPS and then treatment with the extract, extract: the cells were only treated with the extract
considerably after applying the extract. There was a significant relationship between the obtained value of the inflammatory process of cells which were treated by LPS (1.34) and the sample as a negative control (0.287).

**Conclusion**

This study was aimed to produce a mat with mucosal adhesion beside anti-inflammatory activity and the highest rate of release to improve patient compliance. The corresponding membrane was prepared by electrospinning method to obtain more surface area-to-volume ratios. The lower fiber diameter was a representative of higher conductivity with carbopol-PAN-Z. *jujuba* solution after the employment of the corresponding extract. In contrast to negative result with microbial examination against *Porphyromonas gingivalis* and *Fusobacterium nucleatum* bacteria, the anti-inflammatory function was significantly improved after the addition of *Z. jujuba* extract. Also, the kinetic release mechanism was Higuchi model that considerably related to the diffusion process of extract from porous matrix of nanofibrous carbopol-PAN-Z. *jujuba* scaffold. Also, the release profile was completely in accordance with zero-order kinetic and also Fickian diffusion due to sink condition and also erosion of the employed polymers. Oppositely, first-order kinetic was not established as a result of lower solubility of the corresponding polymers in artificial saliva. Thus, this study suggests a new engineered membrane for periodontal diseases and guarantees the 80% of fractional release over 60 min in a controlled release manner leading into more patient compliance.

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