EFFECT OF THE PREPARATION METHOD ON THE DRUG LOADING OF ALGINATE-CHITOSAN MICROSPHERES

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Alginate-Chitosan microspheres obtained by polyelectrolyte complexation are pH-sensitive, bio-compatible and adhesive, and are an excellent candidate for delivery of drugs, proteins and peptides in the human body. A wide variety of methods for the production of these polymeric complexes have been provided. The water-in-oil emulsion is a complex production method, but generally enhances the control of particle size and particle size distribution of the microspheres, extremely necessary for obtaining repeatable controlled release behavior. In this work, a novel and facile water-in-oil emulsion method for the of ALG-CHI polyelectrolyte complexes is discussed. The method proposed produced ALG-CHI microspheres with improved morphology and enhanced drug loading in comparison with the aqueous medium method. The drug loading in the water-in-oil emulsion was over 40% higher than in the aqueous medium, indicating that by the new method proposed the common drug leaching during the microspheres’ preparation is avoided, being an interesting alternative to encapsulate drugs of hydrophilic nature.

Keywords: Microspheres, Alginate, Chitosan, Hydrogels, drug loading.

Introduction

Alginate-Chitosan hydrogels (ALG-CHI) have been proposed as drug delivery system in the past decade, due to their attractive combination of pH-sensitivity, bio-compatibility and adhesiveness and requires relative mild gelation conditions for the network formation [1]. A great deal of processes was developed for these hydrogels’ production in the last few years [2]. One of the limitations of these hydrogels is the drug leaching during their preparation [3] which can be reduced by controlling the reactions conditions [4-7]. In a previous work [8] several ALG-CHI formulations were statistically investigated in order to modulate and control the polyelectrolyte complexation and subsequently the hydrogel properties. It was obtained microspheres in a high yield with low particle size and reduced swelling ratio. Emulsion methods for higher drug encapsulation efficiency by low water solubility polymers have been proposed [9-11]. Gel bead system based on Calcium alginate and chitosan were successfully produced in oil-in-water emulsion for oral delivery of ally isothiocyanate [9]. Hydrogel microspheres of chitosan crosslinked with glutaraldehyde with uniform-size has been produced by a membrane emulsification technique [10], and a water-in-oil (W/O) emulsion coalescence technique was proposed for the production of CHI using vegetable oil [11]. Both methods generated microspheres with enhanced control of particle size and particle size
distribution, which are important for repeatable controlled release behavior [10-11]. Despite some different water-in-oil methods have been used to produce microspheres [12-15], they had not been utilized for the preparation of ALG-CHI microspheres to enhance the encapsulation of watersoluble drugs. In this work, a novel W/O emulsion method was designed for ALG-CHI microspheres’ preparation using two types of crosslinker and surfactant agents with a view to increase the drug loading of some model drugs. The properties of the ALG-CHI particles produced in emulsion by different methods were compared with those obtained by the aqueous method.

Experimental

Materials
Alginic acid sodium salt (~ 250 cP viscosity at 25°C, 64.4 kDa) and polyvinyl pirrolidone (PVP) was purchased from Sigma. Low molecular weight Chitosan (90% deacetylated, 6.68 kDa) was purchased from Aldrich. Calcium chloride and polyvinyl alcohol (PVA) were purchased from Synth. Genipin was purchased from Challenge Bioproducts. Fluoresceín salt were supplied by Synth, Lisinopril and Fluorescein Isothiocianate were supplied by Sigma. All reagents were analytical grades and were used as received.

Microspheres Preparation
ALG-CHI microspheres were produced in W/O emulsion, testing two types of surfactant an two types of crosslinker, totalizing four different formulations in emulsion. Also it was produced ALG-CHI microspheres in aqueous mode as described in our earlier work [8], for comparison of properties, totalizing 6 formulations, as showed on Table I.

<p>| Table I - Formulations used for production of ALG-CHI microspheres in W/O emulsion and aqueous method |</p>
<table>
<thead>
<tr>
<th>Formulations</th>
<th>Preparation Method</th>
<th>Surfactant type</th>
<th>Crosslinker type</th>
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<tbody>
<tr>
<td>1- AQ/Ca</td>
<td>Aqueous</td>
<td>CaCl₂</td>
<td></td>
</tr>
<tr>
<td>2- AQ/gen</td>
<td>Aqueous</td>
<td>Genipin</td>
<td></td>
</tr>
<tr>
<td>3- EM/PVA/Ca</td>
<td>Emulsion</td>
<td>PVA</td>
<td>CaCl₂</td>
</tr>
<tr>
<td>4- EM/PVA/Gen</td>
<td>Emulsion</td>
<td>PVA</td>
<td>Genipin</td>
</tr>
<tr>
<td>5- EM/PVP/Ca</td>
<td>Emulsion</td>
<td>PVP</td>
<td>CaCl₂</td>
</tr>
<tr>
<td>6- EM/PVP/Gen</td>
<td>Emulsion</td>
<td>PVP</td>
<td>Genipin</td>
</tr>
</tbody>
</table>

Aqueous solution of ALG (1% w/v) were prepared and diluted to a final concentration of 0.2% (w/v) using distilled water. CHI solution (1% w/v) were dissolved in a pH 3 acetic acid solution, and further diluted to (0.2% w/v) with distilled water. The ALG-CHI-Ca⁺² microspheres were prepared by placing the solution of CHI 0.2% and ALG 0.2% in separated tubes and adding the 2mM CaCl₂ solution into the tube with CHI solution and homogenized. The surfactant powder was added in each tube in a 1.5% w/v and homogenized in an ultrasonic bath for 25 min. Both tubes
were carefully added to a vessel containing mineral oil in a volume ratio 6/1 mineral oil/aqueous phase. The mixture was vigorously sonicated with an ultrasonic probe for 3 min producing a stable emulsion, and then replaced in the ultrasonic bath for additional 20 min. The emulsion was centrifuged at 3500 rpm for 30 min for aqueous and oil-phase separation, and then the aqueous-phase was again centrifuged and the solid obtained was lyophilized. The preparation of the microspheres ALG-CHI-genipin was similar, with the difference of adding genipin solution (0.1% w/w) in the tube containing ALG. For the preparation of doped microspheres, the models drugs Fluorescein or Lisinopril labeled with FITC were added in the tube containing the CHI solution and homogenized. Figure I show a schematic representation of the components in all steps of the ALG-CHI nanoparticles preparation by the water-in-oil method.

Microspheres characterization

Hydrogel samples were dried and then sputter-coated with gold for Scanning Electron Microscope (SEM) characterization in a Jeol JSM 5800 microscope, using an acceleration voltage of 5 kV. The morphology was investigated through Fluorescence Optical microscope (OM) (Leitz Wetzlar). The encapsulation efficiency (EE) was evaluated for fluorescein as model drug and for lisinopril labeled with Fluorescein Isothiocianate (FITC) through absorbance of in an UV spectrometer at 489 and at 499 nm, respectively.

Results and Discussion

In a previous study [16], it was seen that CHI based microspheres prepared at aqueous medium showed low encapsulation efficiency of hydrophilic drugs. This could be related to the drug highly hydrophilic character, which present stronger interaction between drug-solvent (water) than the electrostatic interactions between drug-microspheres. In this work, the production of ALG-CHI microspheres with controlled morphology was studied on aqueous medium and also in water-in-oil emulsion method, focusing in differences in the morphology, drug distribution pattern and encapsulation efficiency.
**Morphology**

The morphology of the ALG-CHI microspheres was observed through SEM, and is showed in Figure II. The ALG-CHI hydrogel particles showed spherical shape and the average particle size and particles size distribution varied according to the preparation method, if in aqueous medium or W/O emulsion, and to the crosslinker type, if with genipin or CaCl$_2$. In general, there was an increase in the regularity of the particles shape when genipin was used as crosslinker agent, as revealed by the SEM micrographs Fig. II (a), (c) and (e). ALG-CHI particles produced in W/O emulsion with PVA using both crosslinker agents presented a more spherical shape and regular structure, revealed by the SEM micrographs Fig. II (c) and (d).

![SEM micrograph images of ALG-CHI hydrogel particles obtained using CaCl$_2$ as crosslinker: by aqueous method with CaCl$_2$ (a) and with genipin (b), and by W/O emulsion method using PVA with genipin (c) and with CaCl$_2$ (d) and using PVP with Genipin (e) and with CaCl$_2$ (f).](image)

Figure III shows OM micrographs of ALG-CHI microspheres obtained by both aqueous and emulsion method. Microspheres produced through the former method were more irregular in shape and there was a tendency to form agglomerates (Fig. III (a) and (b)). On the other hand, microspheres prepared through the emulsion method presented uniform size with self-avoided particles (Fig. III (c) and (d)), being potential more adequate for drug delivery.

Table II shows the average particle size of ALG-CHI microspheres. The average particle size of the ALG-CHI microspheres corresponds to the diameters average measured in fifty particles from each batch. It can be seen from the average diameter values that the particles size varied according to the preparation method and composition used. The hydrogel particles produced by the W/O emulsion method were smaller having narrower particles size distribution than those produced by the aqueous
method. Some data on emulsion methods usually report particles having broad size range [15] but through the method proposed here the particles size distribution is narrower than those produced in aqueous medium.

![Figure III: MO micrograph images of ALG-CHI hydrogel particles obtained in aqueous medium with genipin (a) and with CaCl2 (b), and by W/O emulsion method using PVA with genipin (c) and PVP with CaCl2 (d).](image)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Encapsulation Efficiency (%)</th>
<th>Particle size (µm)</th>
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<tbody>
<tr>
<td></td>
<td>Fluorescein</td>
<td>Lisinopril</td>
</tr>
<tr>
<td>1- AQ/Ca</td>
<td>38.4</td>
<td>45.2</td>
</tr>
<tr>
<td>2- AQ/gen</td>
<td>49.8</td>
<td>59.7</td>
</tr>
<tr>
<td>3- EM/PVA/Ca</td>
<td>83.2</td>
<td>80.0</td>
</tr>
<tr>
<td>4- EM/PVA/Gen</td>
<td>77.2</td>
<td>85.5</td>
</tr>
<tr>
<td>5- EM/PVP/Ca</td>
<td>78.4</td>
<td>73.6</td>
</tr>
<tr>
<td>6- EM/PVP/Gen</td>
<td>28.7</td>
<td>36.5</td>
</tr>
</tbody>
</table>

**Encapsulation Efficiency**

Using 2% of loading of fluorescein or lisinopril, the encapsulation efficiency was determined using a calibration curve in a UV-visible spectrometer. The calibration curves for Fluorescein and for Lisinofril labeled with FITC are on Figure IV and are given respectively by the Equations 1 and 2:

\[ y = 0.035 x - 0.004 \quad R^2 = 0.998 \] (1)
\[ y = 0.0204 x -0.0715 \quad R^2 = 0.992 \quad (2) \]

Where: \( y \) corresponds to the absorbance of the sample in aqueous solution, \( x \) is the drug concentration in the sample.

Each one of the curves presented excellent linearity for low and high drug concentration, with a correlation factor \( R^2 > 0.99 \). Table II shows the encapsulation efficiency values for each system produced calculated using the equations given in Figure IV. Comparing the EE values for each formulation is seen that particles produced in aqueous medium presented relatively low EE in comparison with the majority of the EE values of the microspheres obtained by W/O emulsion method, which successfully entrapped over 70% of lisinopril. The EE of Lisinopril-FTIC was higher or equivalent than Fluorescein, probably due to the lower hydrophilic character which may cause lower drug leaching. When genipin was used as crosslinker also the EE was higher independently of the preparation method for both drug models. For lisinopril, there was an increase of 30% in the encapsulation efficiency, changing from 45 to around 60%, when genipin was used in the hydrogel preparation through the aqueous method. As previously reported [16], controlled release rates of indomethacin from ALG-CHI particles were achieved by increasing the genipin content in the hydrogel due to a higher crosslinking density. In fact, the genipin content can be manipulated in order to control the chitosan crosslink density [17]. Also, when PVA was used as a surfactant agent it was obtained the best result. As already reported by Reis et al [18], PVA has the ability to form hydrogen bonds with CHI and these intermolecular interactions could provide an entangled network with improved encapsulation efficiency in comparison with PVP. The formulation prepared with PVP/gen showed the lowest encapsulation efficiency of all, suggesting that maybe there is a negative interaction between genipin and PVP. On the other hand, when CaCl\(_2\) was used as crosslinking agent the surfactant type did not have strong influence in the efficiency of the Lisinopril encapsulation.
Figure V shows the lisinopril-FITC encapsulation and distribution within the particles investigated by fluorescence Optical microscopy (FOM). It is evidenced in the lisinopril-FITC-ALG-CHI microspheres micrographs that the lisinopril-FITC distribution pattern varied according to the preparation method and crosslinker type. In aqueous medium, the drug dispersion was favored by replacing CaCl$_2$ with genipin (Fig. V (a) and (b), respectively) also corroborated by the EE values. Fig. V (c) and (d) show a homogeneous dispersion of the drug inside of the ALG-CHI microspheres when produced through the W/O emulsion method. Particularly, the surfactant seems affect the encapsulation efficiency of ALG-CHI particles crosslinked with genipin. The reported mechanism for genipin-chitosan crosslinking reaction is a nucleophilic attack of chitosan amino groups in the dihydropiran ring of genipin [17]. A high loss of hydrogen ions from protonated amino groups to the carboxylic groups favors the nucleophilicity of amino groups, which in turn may increase the CHI crosslinking density [17]. In the case of the microspheres here reported, the residual carboxylic groups (25%) in the PVA molecules favor the chitosan deprotonation, facilitating the amino nucleophilic attack, increasing the crosslinking density, and naturally the encapsulation efficiency (85% for R5 batch hydrogel). On the other hand, in the case of the ionic crosslinker (calcium chloride), PVP and PVA were a good surfactant for the W/O system and the EE average was around 75%.

In summary, ALG-CHI microspheres produced in W/O emulsion with PVA as surfactant presented highly spherical particles with narrower size distribution than conventional aqueous method. In addition, those particles formed self-avoided domains, avoiding aggregation. Moreover, these particles presented higher encapsulation efficiency reaching values up to 80% no matter which crosslinker type was used.
Conclusion

It was tested different formulations for production of ALG-CHI microspheres in order to increase the encapsulation efficiency and optimize the morphology. Regarding to the crosslinker type, genipin increased significantly the EE of microspheres produced in aqueous medium. It was observed that the emulsion method in general was more efficient to encapsulate lisinopril than the aqueous method. In addition to this, microspheres produced in emulsion using PVA as surfactant presented highly spherical particles with narrower size distribution than conventional aqueous method, and those particles formed self-avoided domains, avoiding aggregation. Moreover, these particles presented higher encapsulation efficiency reaching values up to 80% no matter which crosslinker type was used, indicating that the emulsion method using PVA as emulsifier is a promising route to encapsulate hydrophilic drugs.

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References