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Optimized Crosslinking of Gelatin Microspheres with Fructose

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ABSTRACT

Crosslinking reactions are a way to minimize the solubility of gelatin in water and thus enable its application in drug delivery systems. In this paper the use of fructose as a crosslinker was studied in order to minimize the cytotoxic effect caused by petroleum derivates as glutaraldehyde. The crosslinking of gelatin with fructose was carried out by water-in-oil emulsion and microspheres were prepared without surfactant utilization. The experimental parameters were optimized by a complete 2^3 factorial design, to study the effects of pH, emulsion heating time and fructose concentration. The variance analisys results showed that pH and emulsion heating time have statistical significance to gelatin crosslink while fructose concentration has not a greater influence in this propertie. The samples' solubility declined when using high pH and long heating times, indicating greater crosslinking degree under these conditions. However thermogravimetric and amino group titration analisys revealed that gelatin microspheres prepared with pH=9, emulsion heating time of 60 min and fructose concentration of 100% (w/w) were the best experimental parameters to produce gelatin microspheres crosslinked with fructose under the conditions studied in this work.

Keywords: Gelatin, Crosslinking, Fructose, Experimental Planning

I. INTRODUCTION

Biomedical devices and systems based on polymers for controlled drug release are of great importance in many medical treatments. Many studies have been conducted in this respect, analyzing a wide range of natural and synthetic polymer materials [1–5].

However, for specific application as drug carriers, the device or system must have certain properties, in particular biocompatibility, to minimize toxicity and other adverse effects, and biodegradability, to prevent the need for surgical intervention to remove the device. Gelatin is both biocompatible and biodegradable[6–10]. For this reason, we chose it as the polymer matrix to prepare microspheres, for prospective use to anchor the anticancer drug doxorubicin.

When being placed in a physiological medium, which is predominantly aqueous, gelatin absorbs water and swells before dissolving [11,12]. This characteristic prevents its application in biomedical devices/systems. To overcome this limitation, its polymer chains must be submitted to a crosslinking process. In this study, we substituted glutaraldehyde, which is a cytotoxic crosslinking agent [13,14], with fructose, which is a biocompatible reagent. According to Cortesi, Nastruzzi and Davis [15], the crosslinking reaction between gelatin and reducing sugars can occur in various ways, with the mechanism elucidated by Hodge [16] being plausible, as shown in Figure 1.

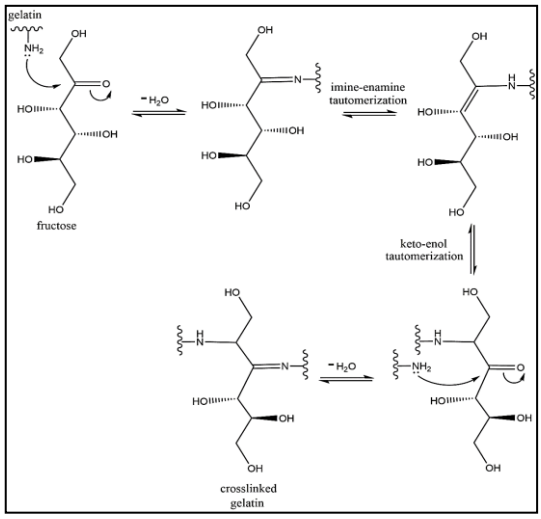

Figure 1 - Crosslinking reaction mechanism of gelatin by fructose

The crosslinking of gelatin involves nucleophilic attack of the amine groups of gelatin on the carbonyl groups of the fructose molecule. However, the reactivity of fructose in aqueous media is subject to equilibrium between its open and cyclic forms (Figure 2).

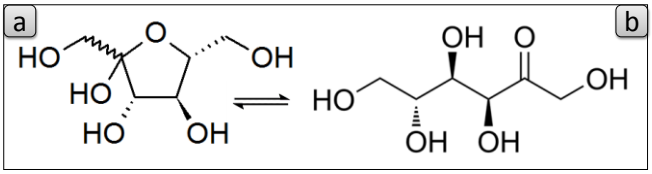

Figure 2 – Fructose cyclic (a) and open (b) forms

When the sugar is in cyclic form, the carbonyl group in the molecule is not available for reaction. Therefore, for the crosslinking reaction to occur, this equilibrium must be shifted to favor breaking the peptide bond and increasing access to the carbonyl. In light of this fact, we performed experiments to determine which parameters best improve the crosslinking reaction of the gelatin chains by fructose.

Experiments can involve investigation of a large number of factors or variables that influence the results. Thus, careful planning is necessary to design suitable experiments and decrease the number of repetitions, to reduce the time and cost and increase the quality (reliability) of the results. One way to do this is by simultaneously analyzing several variables.

Many factors can influence the reactivity and/or reaction kinetics of materials. Among these factors are: (1) By the Le Châtelier's principle, increased concentration of reagents promotes shifting the equilibrium, increasing the concentration of products. Therefore, the effects of rising concentration of the crosslinker should be the first factor analyzed. (2) According to the study of Abete, Gado, Arcangelis, Serughetti and Djabourov [17], the behavior of a drug release system can be extremely sensitive to changes in pH. They concluded that the reaction kinetics of crosslinkers with low reactivity depends on the formation of single crosslinker-gelatin bonds and that the speed of this reaction step declines with decreasing pH. (3) The reaction time can be very important in determining the number of crosslinks formed by the molecules. In light of the possible influence of these factors, we designed experiments to evaluate the influence of concentration of the crosslinker, pH of the reaction medium and reaction time on the formation of crosslinks in microspheres based on gelatin and fructose.

II. METHODS AND MATERIAL

A. Preparation of the gelatin microspheres

The influence of the factors of interest was analyzed through a complete 2^3 factorial design involving variation of the pH of the reaction medium, emulsion heating time and fructose concentration. All the experiments were conducted in triplicate in random order.

To obtain the microspheres, we used an adaptation of the method described by Souza [18]. Ten milliliters of a gelatin solution 10% (w/v) containing the crosslinker was preheated to 60 °C under magnetic stirring. Then, it was dropwise added to a three-necked flask with capacity of 250 mL containing 40 mL of corn oil preheated to 80 °C under mechanical stirring at 500 rpm. The emulsion obtained was kept under heating for a while and then cooled in an ice bath for 30 minutes. After this, 50 mL of chilled acetone was added to complete the dehydration and solidification of the microspheres. The stirring and cooling were maintained for one more hour, and the beads formed were purified by filtration and washed with chilled acetone. Finally, they were dried in a desiccator at room temperature.

B. Dissolution tests

Samples were formed containing about 0.4 g of gelatin microspheres. Each of these samples was transferred to a 50-ml round-bottom beaker. The volume in the beaker was completed with purified water (Milli-Q) and the beaker was then placed in a water bath at 37 °C for one hour. After heating, the sample was cooled to room temperature and filtered through a polyethersulfone membrane (45 µm mesh). The filtrate was reserved for subsequent analysis of concentration of proteins. This procedure was repeated for all the samples.

C. Measurement of the protein concentration by the Biuret method

A 0.2-mL aliquot of the filtrate obtained in the dissolution test was placed in a test tube to react with 5.0

mL of Biuret reagent for 30 minutes in the dark. Then the absorbances of the solutions obtained were detected in a spectrophotometer (Biospectro SP-22) at 550 nm. The concentration of gelatine released into the solution was calculated using the equation of a straight line obtained by the calibration curve constructed from patterns of known concentration. Then, the percent dissolution was calculated according to equation 1:

$$Dissolution\ \% = \frac{C_f}{C_i}\ x\ 100 \quad (1)$$

Where C_f = gelatin concentration released to solution at time t and C_i = initial concentration of gelatin.

D. Thermogravimetric analysis

The thermal stability of the microspheres was analyzed with a TA Instruments Q50 V6.4 Build 193 analyzer. About 10 mg of each sample was placed in an aluminum capsule and heated under a nitrogen atmosphere with flow of 100 mL/min in the temperature range from 10 °C to 500 °C, at a heating rate of 20 °C/min.

E. Titration of the free NH_2 groups

In this procedure, about 50 mg of microspheres was immersed in 20 mL of deionized water and left to rest for 30 minutes to allow them to swell. Then the NH_2 groups were protonated with HCl 0.2 M to pH 1.5 with the addition of 0.1 mL increments and resting time of 30 seconds after each addition. For each sample, the number of combined protons was calculated according to Equation 2.

$$Combined\ H^+ = \frac{C_1}{g\,(1-10^{(pH_w - pH_g)})} \quad (2)$$

Where: C_1 is the concentration of hydrochloric acid, g is the sample mass, pHw is the pH of the deionized water, and pHg is the pH of the sample suspension.

F. Morphology of the microspheres

The morphology of the gelatin microspheres was investigated with a scanning electron microscope (SEM). The samples were coated with a fine layer of gold to increase their conductivity and protect them against localized heating. The images were captured using secondary electron detectors and electron acceleration voltage of 10kV.

III. RESULTS AND DISCUSSION

Gelatin microspheres must be crosslinked for biomedical applications. Without this process, the high solubility of the polymer in aqueous media prevents its use. In most published studies on gelatin microspheres, researchers have used analysis of terminal amino groups to determine the material's crosslinking degree. Normally trinitrobenzenesulfonic acid (TNBS) is used for this purpose, but this substance is hard to obtain (it is not even produced in Brazil, and its importation is slow and expensive because of its explosive nature). This situation required using a different technique to determine the crosslinking efficiency of the microspheres, namely the percent of gelatin dissolution in the aqueous medium. Theoretically, the higher the gelatin percentage found dissolved, the lower the crosslinking degree of the sample. The gelatin dissolution was quantified by calorimetry of the Biuret reagent. The results of these analyses are reported in Table 1. These data were treated statistically and submitted to analysis of variance (ANOVA).

The analysis of variance produced a p-value for lack of fit greater than 0.05, meaning a suitable model at 95% confidence. Besides this, the correlation coefficient was 0.94, indiating the model explains approximately 94% of the solubiltiy variance of the samples. Therefore, the model can be used for prediction.

Table 1 – Samples composition and dissolution tests results

Sample	pH	Time (min)	Fructose (% w/w)*	Dissolution (%)
1	5	10	40	75.5
2	9	10	40	83.7
3	5	60	40	51.8
4	9	60	40	32.1
5	5	10	100	67.6
6	9	10	100	47.4
7	5	60	100	65.9
8	9	60	100	35.1
Center point	7	35	70	54.9
Center point	7	35	70	52.3
Center point	7	35	70	48.0

*relative to the gelatin

With respect to the factors studied, the effect generated by each one can be visualized in the Pareto chart (Figure 3). In this chart, the size of the bars is proportional to the estimated values of the prinicpal effects. The vertical line corresponds to the 95% confidence interval, so an effect that exceeds this line can be considered significant. It can be seen that the variables pH and heating time, along with the second-order interaction between heating time and fructose concentration, had significant effects on the percentage of gelatin dissolved in the aqueous medium. It can also be observed that both heating time and pH had negative effects on solubility, i.e., the higher the pH and longer the heating time, the lower the solubility of the samples was.

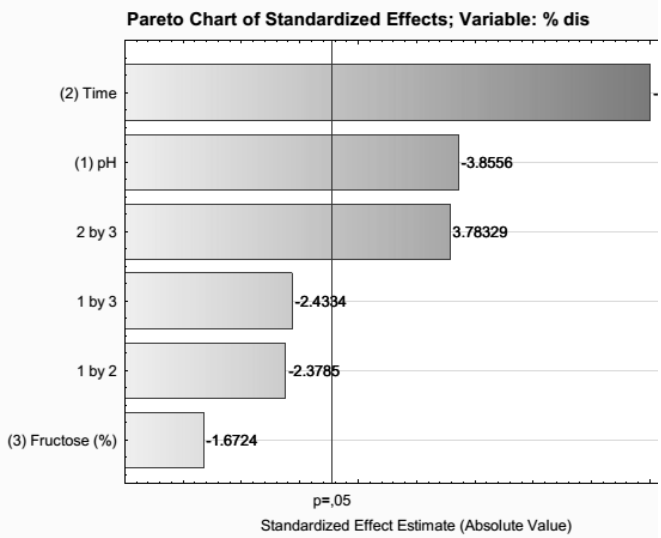

Figure 3 - Pareto chart

With respect to the effect of the second-order interaction between concentration and crosslinker, the Pareto chart indicates a positive effect. However, to better assess this interaction, it is necessary to analyze the contour graphs shown in Figure 4.

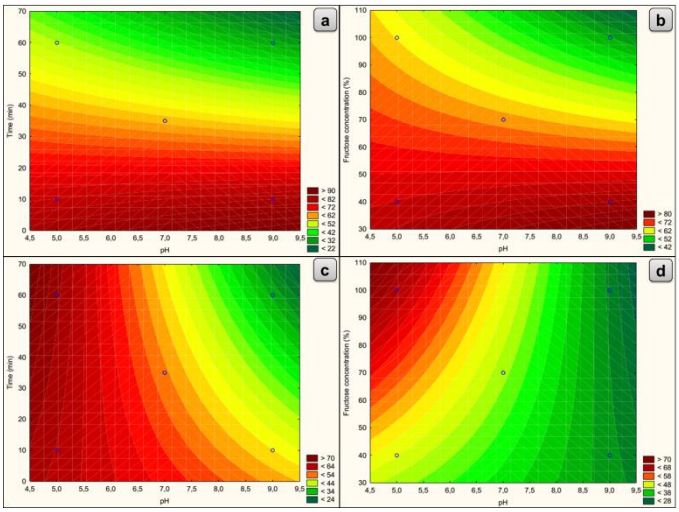

Figure 4 - Contour graphs: (a) 40% sugar concentration; (b) time of 10 minutes; (c) 100% sugar concentration and (d) time of 60 minutes.

The surfaces presented indicate that when the concentration of the crosslinker was fixed at the lowest level (40%)(Figure 4a), solubility rates below 42% were obtained when the heating time was near 60 minutes and the pH varied from 7.5 to 9.0. However, the experimental conditions leading to samples with solubility below 32% were only obtained under conditions indicated in a small region of the graphs. Analysis of the response region where the fructose concentration was set at 100%(Figure 4c), the pH range became narrower while it was possible to diminish the heating time necessary to obtain samples with solubility below 44%. With respect to the surface obtained with heating time of 10 minutes (Figure 4b), it can be seen that the lowest solubility values were only attained when the concentration of the crosslinker was high and the pH was also high. On the other hand, with a heating time of 60 minutes (Figure 4d), it can be seen from the graph area with lower solubility that the concentration of the crosslinker was not relevant in a broad pH range.

In light of the profiles presented by the contour surfaces, it can be said that since the objective is to minimize the dissolution of the gelatin in aqueous media, the best pH is near 9 and the optimal heating time is 60 minutes. Regarding the crosslinker concentration, it is not a significant factor under these experimental conditions. For this reason, from this point on we performed FTIR and thermogravimetric analyses, measured loss of NH_2 groups and examined the microspheres' morphology only with samples 4 and 8, which were prepared with

pH 9 and heating time of 60 minutes. These samples differ only in crosslinker concentration (40% in sample 4 and 100% in sample 8).

The thermogravimetric analysis allowed determining two important properties of the samples: degradation starting temperature (T_{onset}) and percentage of residue after the analysis. When evaluating the crosslinking of a material by thermogravimetric results, the expectation is that material with a greater number of cross-linkages (higher crosslinking degree) will require a higher temperature to start degrading because of the greater heat energy needed to overcome the stronger resistance afforded by the greater interconnection of the molecules. For the same reason, a larger amount of residue should be observed at the end of the analysis.

The degradation starting temperature (T_{onset}) to gelatin, sample 4 and sample 8 were 289 °C, 293°C and 294°C, respectively. Considering that the analyzer has a measurement error of ± 2 °C, it can be seen that both samples 4 and 8 showed a slight increase in the degradation onset temperature in comparison with the pure gelatin, but no significant difference in this property between each other.

With respect to the residual percentage, the values for gelatin, sample 4 and sample 8 were 25.58%, 28.55% and 29.87%, respectively. One more time, the values for samples 4 and 8 were very close and did not differ significantly. Nevertheless, when comparing these values individually versus the residue percentage of the pure gelatin, sample 4 did not differ statistically with the gelatin while sample 8 presented a statistically higher percentage than did the gelatin. These data indicate that sample 8 was more crosslinked than sample 4.

Analysis of the mechanism suggested by Hodge [16] (Figure 1) shows that the formation of crosslinks between the gelatin chains occurs through the NH_2 groups present in the gelatin's protein structure. Therefore, the quantity of this functional group that still remains in the sample is inversely proportional to the degree of crosslinking. To provide stronger support for the observations presented so far, we conducted analyses to determine the loss of the NH_2 groups with heating. The results showed that sample 4 lost 49.21% of its original amino groups while sample 8 lost 84.95%. These results allow drawing a clear distinction between the samples regarding crosslinking. The sample with the higher fructose percentage during synthesis formed a greater number of polymer-crosslinker connections, thus indicating this sample has the optimal experimental parameters for preparation of gelatin microspheres crosslinked with fructose.

Figure 5 shows the SEM micrographs of sample 8. Were formed spherical particles, with diameters in the range of 30 to 150 µm. It can be seen that the microspheres have rough surfaces, due to the presence of pores. This roughness was caused by the absence of a surfactant while preparing the emulsion. We decided not to utilize reagents, because even in small quantities they can increase the cytotoxicity of the device or system as a whole. The presence of pores in the microspheres increases their surface area and makes the polymer's functional groups more exposed to subsequent reactions. Consequently, increase the possibility of their interaction and reaction with the drug to be carried.

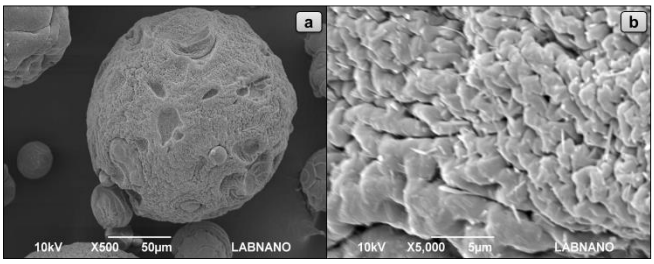

Figure 5 - Micrographs of sample 8, at magnification of 500X (a) and 5000X (b)

IV.CONCLUSION

The results obtained show that pH and heating time were the parameters that influenced the solubility of the samples. The contour graphs enabled observing that the solubility was minimized when using the highest pH and longest heating time, irrespective of the concentration of the crosslinker. The subsequent thermogravimetric analyses and measurement of the NH_2 groups lost indicated different degrees of crosslinking and allowed differentiating those two samples, suggesting that sample 8, prepared with pH=9, emulsion heating time of 60 min and fructose concentration of 100% (w/w) are the best experimental parameters to produce gelatin microspheres crosslinked with fructose under the conditions studied in this work.

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