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Determination of the Association Constant of 6-thiopurine and Chitosan Grafted β-Cyclodextrin

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Abstract

Mono-oxidation of β-cyclodextrin was performed along with its incorporation into chitosan by a direct and efficient reductive coupling reaction. The inclusion ability of the obtained product was studied spectrophotometrically, using 6-thiopurine as guest molecule. In addition a new procedure for the determination of association constants in polymer-incorporated cyclodextrins is proposed and tested. Experimental results demonstrate an increase in the inclusion ability of β-cyclodextrin after its incorporation into polymer chains.

Introduction

Cyclodextrins are a family of cyclic oligosaccharides with the outstanding ability of forming inclusion complexes with a large variety of organic and inorganic guests. The most important cyclodextrins are those composed by six, seven and eight glucose units linked by α-(1–4) bonds and are named α, β and γ-cyclodextrins, respectively. Of the three types, β-cyclodextrin has the most widespread use [1–3]. One of the most remarkable applications of cyclodextrins is their use as drug carriers in controlled release systems. As drug carriers, cyclodextrins allow the solubilization, stabilization and transport of hydrophobic drugs together with several pharmacological benefits such as the reduction of unwanted side effects [4–5].

Polymeric drug delivery devices have many advantages over conventional systems, because they allow a release of a bioactive substance over longer periods of time with a constant level in the plasma [6, 7]. Of all the potentially useful polymers in drug delivery systems, naturally occurring polysaccharides appear as very attractive alternatives due to their low cost, high biodegradability and biocompatibility [7–8]. One of the most promising polymers in this sense is chitosan, a bioactive polysaccharide prepared by the N-deacetylation of chitin, a natural polysaccharide present in shells of crustaceans such as crabs and shrimps. Moreover, chitosan allows specific chemical modifications since it has primary amine groups at the C-2 position of its monomeric units. These reactive sites enable the grafting of a large variety of properly functionalized molecules [8–10].

Grafting cyclodextrin molecules into chitosan-reactive sites may lead to a molecular carrier that possess the cumulative effects of inclusion, size specificity and transport properties of cyclodextrins as well as the controlled release ability of the polymeric matrix [11].

In order to avoid crosslinking of polymer chains, cyclodextrin needs to be carefully mono-functionalized before its incorporation into chitosan. The synthesis of chitosan-grafted cyclodextrins has been reported previously, but no comparison between free- and grafted-cyclodextrin inclusion ability has yet been performed [11]. Furthermore, the determination of association constants in grafted cyclodextrins has not been reported for most of this kind of system [12–14].

At the present time, available methods provide only qualitative descriptions of the inclusion phenomena in polymeric systems. These are often inaccurate and even unreliable due to the fact that their results are extremely sensitive to small changes in concentration measurements. Another problem in the determination of association constants in polymeric systems arises from the fact that almost always the polymer's molecular weight remains unknown [15].

This work describes the synthesis of chitosan-grafted β-cyclodextrin by a simple reductive coupling reaction, followed by a spectrophotometric study of its inclusion performance by using 6-thiopurine as a guest model. 6-thiopurine, a poorly soluble and unstable drug, is efficient in the treatment of acute leukemia and in inflammatory bowel disease [16, 17].

The previous mono-functionalization of β-cyclodextrin was performed by using IBX (1-hidroxy-1,2-benziodoxol-3(1H)-one 1-oxide) as oxidant agent in dimethylsulfoxide [18, 19]. Furthermore, a new method is proposed for the determination of association constants in polymer-linked cyclodextrins.

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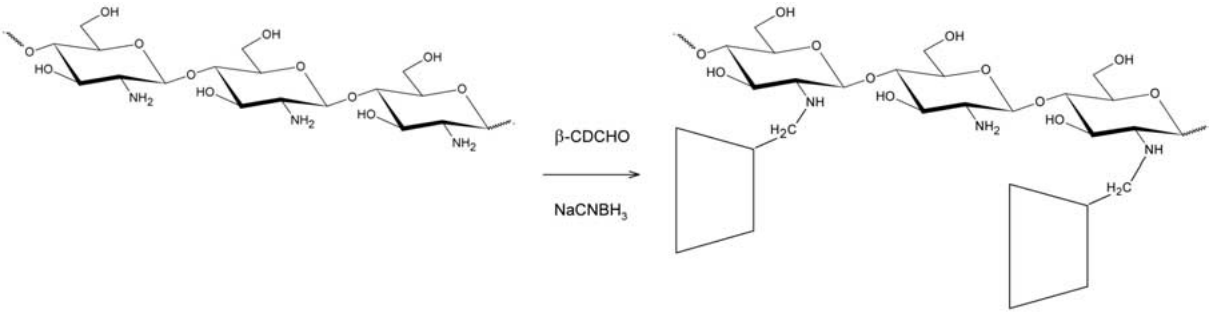

Figure 1. Synthesis of chitosan-linked β-cyclodextrin.

Experimental

Cyclodextrin was purchased from Calbiochem. The chitosan used has an average viscosimetric molecular weight of 80,000 with an acetylation degree of 20% (moles).

^{1}H NMR experiments were performed using a Bruker AC250P spectrometer in DMSO-d_6 as solvent. Absorbance measures were carried out in a Shimadzu UV-vis spectrophotometer model UV-1603.

Synthesis of β-cyclodextrin mono aldehyde

To a solution of 1.0 g of cyclodextrin in 20 mL of DMSO, 0.5 g of IBX (1-hidroxy-1,2-benziodoxol-3(1H)-one 1-oxide) was added. The resulting solution was stirred at 40 °C in a water bath for 1.5 hours. The product was precipitated by saturating the solution with acetone. It was then filtered, washed with acetone, and dried under a vacuum. In order to attain the best conversion to monoaldehyde, the reaction time, temperature, and cyclodextrin to IBX ratio were determined by previous assays [18, 19]. ^{1}H RMN (DMSO-d_6, TMS) δ (ppm): 9.7 (1H, CHO), 5.7–5.8 (14H, C2-OH and C3-OH of glucopyranose units), 4.9–5.0 (7H, C1-H of glucopyranose units), 4.4–4.5 (6H, C6-OH). ^{13}C RMN (DMSO-d_6, TMS) δ (ppm): 195 (1C, CHO), 102 (C-1), 87 (C hydrate), 81 (C-4), 74 (C-3), 73 (C-5), 72 (C-2), 60 (C-6). IR ν (cm^{-1}): 3380 (broad band, O–H), 2928 (C–H).

Synthesis of chitosan grafted β-cyclodextrin

To a solution of 0.25 g of chitosan in 200 mL of pH 4.7 buffer (acetic acid and sodium acetate 0.2 mol L^{-1}), 0.8 g of β-cyclodextrin monoaldehyde in 50 mL of water were slowly added. After one hour of stirring, 0.52 g of $NaBH_3CN$ was added at once and stirring was continued for 48 hours more (Figure 1). Diluted ammonia was added until the solution was alkaline, and the product was then precipitated by saturation with ethanol, while maintaining vigorous stirring. The white solid was filtered, washed with ethanol and dried at room temperature under a vacuum.

Characterization of chitosan-grafted β-cyclodextrin

The product was characterized by elemental analysis and differential thermogravimetric analysis (DTGA). DTGA of the cyclodextrin-chitosan polymer shows a single decomposition peak in agreement with the chemical linking of

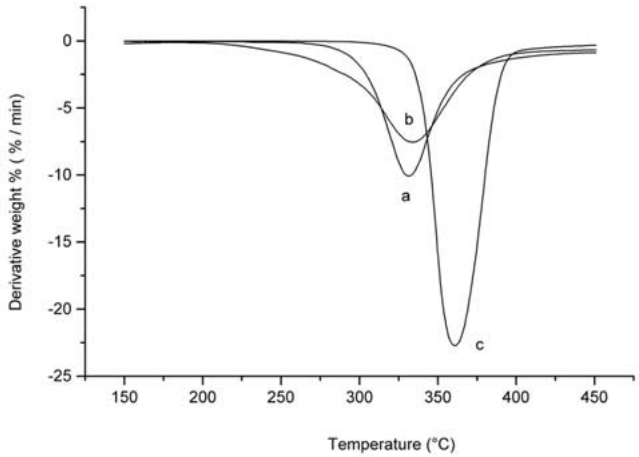

Figure 2. Differential thermal analysis of (a) pure chitosan, (b) chitosan linked β-cyclodextrin and (c) β-cyclodextrin.

β-cyclodextrin and chitosan. In addition, there is no evidence of the existence of free cyclodextrin in the product as is shown in Figure 2. Elemental analysis shows a significant reduction of nitrogen content when comparing the obtained product (2.5%) with pure chitosan (8.3%). The nitrogen content of the product corresponds to a substitution degree higher than 70%, showing that almost all free amino groups in chitosan were grafted with β-cyclodextrin monoaldehyde. The product is fairly soluble in both neutral and acidic media, in contrast with chitosan that is soluble only in acidic media.

Inclusion assays

Measurement solutions were prepared at a fixed concentration of the guest (35 μmol L^{-1}) and at a concentration of the polymeric host ranging from 0.02 to 0.12 g/L. In all cases, distilled water was used as solvent. UV-vis spectra were recorded after 3 hours of stirring at 25 °C at a wavelength of 314 nm.

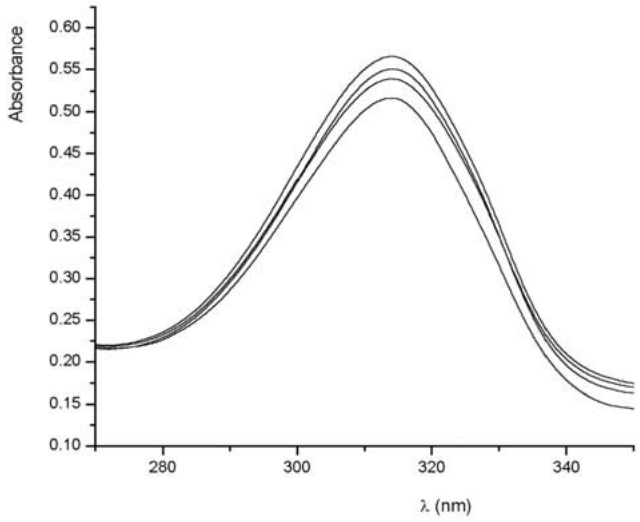

Figure 3. Absorption spectral changes of 6-thiopurine upon the addition of β-cyclodextrin-chitosan polymer. 6-Thiopurine concentration is 35 μmol L^{-1} and the polymer concentration range was 0.02–0.10 g/L from the up to down curves.

Results

Inclusion performance of chitosan-linked β-cyclodextrin

The inclusion performance of the product with 6-thiopurine as a guest model is shown in Figure 3. As the polymer concentration increases, larger absorbance variations are observed and the polymer addition induces a hypochromic effect on the absorption band.

The absorbance variations observed are a consequence of 6-thiopurine inclusion in the cyclodextrin sites available in the polymer. The existence of other polymer-guest interactions different from inclusion was discarded by previous tests that showed no absorbance changes when pure chitosan solutions were put in contact with 6-thiopurine solutions. Therefore, the polymer has one type of interaction sites, which is composed only by cyclodextrin cavities.

Even though inclusion occurs only in cyclodextrin, chitosan polymer chains affect inclusion kinetics considerably by increasing the equilibrium time from a few minutes in native cyclodextrin to more than three hours in the cyclodextrin-chitosan polymer.

The determination of the association constant between 6-thiopurine and grafted β-cyclodextrin is based on the absorbance variations observed during inclusion assays. Two procedures were employed: the first is a classical procedure used in the determination of association constants in polymeric receptors [15, 20] and the second method is an extension of the work of Yang *et al.* [21] in the field of polymeric systems. This new method is proposed and employed for the determination of association constant in cyclodextrin-chitosan polymers, but it can be applied on different kinds of polymeric systems.

In the determination of association constants, both methods consider cyclodextrin cavities as equivalent sites where inclusion takes place [15]. The guest-polymer equilibrium is established between cyclodextrin sites (S) and the guest (G), and is characterized by an association constant K.

$$\begin{aligned} S + G &\rightleftharpoons SG \\ K &= \frac{[SG]}{[S][G]}, \end{aligned} \tag{1}$$

where SG represents an inclusion complex formed between S and G.

In order to determine K, the equilibrium concentrations of all involved species need to be known. These concentrations can be easily obtained from the molar fraction of the complexed guest, X, which can be measured by several methods although the spectrophotometric technique is one of the simplest and widely used [22–23].

In the particular case of spectrophotometric determinations, X is related to absorbance changes due to inclusion complexation ΔA by the following expression:

$$X = \frac{\Delta A}{G_0 \Delta\epsilon b}, \tag{2}$$

where G_0, $\Delta\epsilon$ and b represent the initial guest concentration, the difference of molar absorption coefficient between free and complexed guest and the optical path length of Lambert–Beer's law, respectively.

The classical method

The first method is a classical procedure employed in the determination of association constants in polymeric receptors, such as proteins [15, 20]. By considering individual equilibrium between n equivalent inclusion sites per mole of polymer and guest, the following lineal relationship can be obtained:

$$\frac{G_{\text{comp}}}{G_{\text{free}} L} = \frac{nK}{M} - \frac{G_{\text{comp}}}{L} K, \tag{3}$$

where G_{comp} and G_{free} are the molar concentrations of complexed and free guest respectively, n is the number of inclusion sites per mole of polymer, M is the molecular weight of the polymer, L is its concentration in g/L, and K is the association constant in mol L^{-1}. According to this expression, K can be determined from the slope of the straight line

$$\frac{G_{\text{comp}}}{G_{\text{free}} L} \text{ vs. } \frac{G_{\text{comp}}}{L}.$$

Equilibrium concentrations for each species can be calculated from absorbance measurements with Equation (2). It is important to note that in order to apply this method, $\Delta\epsilon$ (the difference of molar absorption coefficient between the free and complexed guest) must be previously determined. This requirement is restrictive and prohibits the application of the method in most cases where the total guest inclusion is not reached under experimental conditions, even though large concentrations of host molecule are used. More over, although $\Delta\epsilon$ is known, the determination of association constants by this method is highly affected by experimental error, and consequently inaccurate results are often obtained.

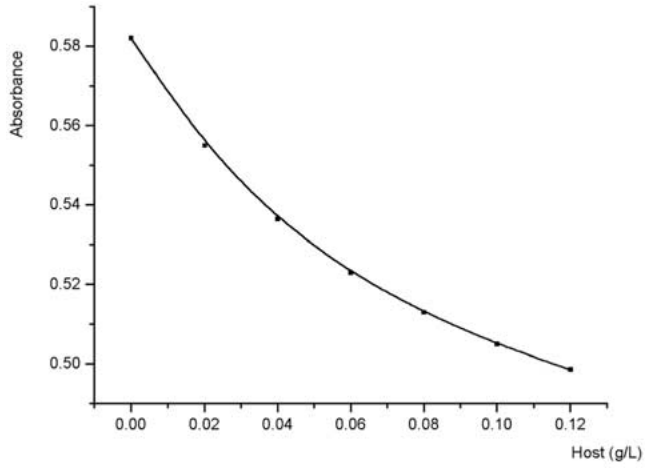

Figure 4. Absorbance changes due to inclusion complexation of 6-thiopurine in chitosan grafted β-cyclodextrin. Measures were performed at 314 nm in aqueous solution.

In this work the total inclusion of the guest was not attained as it is showed in the Figure 4, which show the absorbance changes versus the concentration of the polymer. The curve monotonically decreases with the addition of polymer and no plateau is reached since the solubility of the polymer precluded the use of higher concentrations. Thus, an estimated value of $\Delta\epsilon$ was employed to test method performance in the determination of association constants. The estimated value of $\Delta\epsilon$ (4700 cm^{-1} M^{-1}) was chosen from previous inclusion assays with 6-thiopurine and native β-cyclodextrin. Thus an association constant of $K \approx 2 \times 10^3$ mol L^{-1} was obtained. However, the calculated association constant was largely affected ($\sim$200%) by introducing little variations (1%) in ΔA data. Since small changes in ΔA, and hence small changes in the concentrations of the involved species produce extremely high changes in slope, the measurement of the association constant by this method is inaccurate and unreliable.

The new method

The second procedure is a new method based on the work of Yang *et al*. [21], who proposed a non-approximate procedure for the determination of association constants in cyclodextrins. This method offers some advantages compared to traditional procedures, such as obtaining more accurate results with less experimental data. By using a similar procedure to the one employed by Yang *et al.* [21], a new method is proposed for the determination of association constants in polymeric systems, where guest-polymer interactions take place in cyclodextrin cavities. In contrast to traditional procedures, the new method does not require the determination of the molecular weight of the polymer nor does it set any constraint on the relative concentrations of host and guest.

The new method considers the polymer as a finite collection of inclusion sites where interaction occurs. Considering that n inclusion sites exist per mole of polymer, it can be shown that the following expression is valid:

$$\begin{aligned}(nP_1\Delta A_2 \; - \; nP_2\Delta A_1)G_0b^2\Delta\epsilon^2 \\ + \; (nP_1 - nP_2)(\Delta A_2\Delta A_1)b\Delta\epsilon \\ + \; (\Delta A_2 - \Delta A_1)(\Delta A_2\Delta A_1) = 0 \qquad (4)\end{aligned}$$

where the sub indices 1 and 2 represent two single inclusion experiments, in which the initial guest molar concentration G_0 is kept constant. P_1 and P_2 correspond to the respective polymer molar concentrations used in the experiments, and X_1 and X_2 are the complexed molar fractions of the guest in each case. b represents the optical path length of the Lambert–Beer's law and $\Delta\epsilon$ is the difference between the molar absorption coefficients of the free and the complexed guest.

By solving this quadratic equation, $\Delta\epsilon$ can be numerically determined and then the concentrations of all the involved species will be calculated as well. However, in Equation (4), the terms np_i remain undetermined since the molecular weight of the polymer is unknown. In order to solve this problem it is necessary to consider that, in dilute solutions or by using low molecular weight polymers, all cyclodextrin sites are able to form inclusion complexes. If this approximation is taken into account, then a quantitative measurement of the number of available inclusion sites can be given by the substitution degree of the polymer θ. In the case of grafted chitosan, the substitution degree can be determined experimentally by several techniques, such as potentiometric titration and elemental analysis.

On the other hand, M – the polymer molecular weight – can be related with $\overline{m}$, the average molecular weight of the monomeric units of the polymer. If N is the total average number of monomeric units per mole of polymer and θ is its substitution degree, then it can be shown that

$$M = N\overline{m} \qquad (5)$$

and

$$\theta = \frac{n}{N}. \qquad (6)$$

Now, considering that L_i represents the polymer concentration in g/L, the polymer molar concentration P_i can be expressed as:

$$P_i = \frac{L_i}{M}. \qquad (7)$$

Finally, it can be demonstrated that

$$nP_i = \frac{\theta}{\overline{m}}L_i. \qquad (8)$$

By substituting the terms nP_i with their corresponding values, Equation (4) can be solved, and hence $\Delta\epsilon$ can be determined. With the calculated value of $\Delta\epsilon$, X_i can be also obtained, and finally, K can be determined by the following equation:

$$K = \frac{X_i}{[nP_i - X_iG_0](1 - X_i)}. \qquad (9)$$

In the particular case of the complexation of 6-thiopurine and chitosan-grafted β-cyclodextrin, this new method gives an association constant of 1700 $\pm$ 300 M^{-1}. In this case,

the result is affected by differences smaller than 20% when introducing 1% variations of ΔA in the input data.

An increase in the association constant is observed when comparing this result with the association constant between 6-thiopurine and native β-cyclodextrin (250 ± 30 M^{-1}) . After its incorporation into chitosan chains, β-cyclodextrin undergoes an improvement of its inclusion ability with 6-thiopurine as the guest molecule. The larger association constants of polymeric cyclodextrin compared with native β-cyclodextrin can be related to the dynamic aspect of the equilibrium, since it has been shown that the β-cyclodextrin polymer system has smaller dissociation rate constants for the inclusion process than the native β-cyclodextrin [24].

Concluding remarks

β-Cyclodextrin monoaldehyde can be easily grafted into chitosan chains by a simple reductive coupling reaction, using sodium cyanoborohydride as reductive agent. After its incorporation into chitosan chains, β-cyclodextrin retains its inclusion ability and, in the particular case of 6-thiopurine, an increase is observed in the association constant when comparing native β-cyclodextrin with polymer grafted β-cyclodextrin.

Additionally, a new method is proposed for the determination of association constants in cyclodextrin-polymer systems. This new procedure has provided more accurate and reliable results than traditional procedures and also has the advantage of requiring minimal data points in order to be applied.

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