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3 **Macromolecular Crowding Effect upon *in vitro* Enzyme Kinetics:**  
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5 **Mixed Activation-Diffusion Control of the Oxidation of NADH by**  
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7 **Pyruvate Catalyzed by Lactate Dehydrogenase**  
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**ABSTRACT**

Enzyme kinetics studies have been usually designed as dilute solution experiments, which differ substantially from *in vivo* conditions. However, cell cytosol is crowded with a high concentration of molecules having different shapes and sizes. The consequences of such crowding in enzymatic reactions remain unclear. The aim of the present study is to understand the effect of macromolecular crowding produced by Dextran of different sizes and at diverse concentrations in the well-known reaction of oxidation of NADH by pyruvate catalyzed by L-Lactate Dehydrogenase (LDH). Our results indicate that the reaction rate is determined by both the occupied volume and the relative size of Dextran obstacles in respect to the enzyme present in the reaction. Moreover, we analyzed the influence of macromolecular crowding on the Michaelis-Menten constants,  $v_{max}$  and  $K_m$ . The obtained results show that only high concentrations and large sizes of Dextran reduce both constants suggesting a mixed activation-diffusion control of this enzymatic reaction due to the Dextran crowding action. From our knowledge, this is the first experimental study that depicts mixed activation-diffusion control in an enzymatic reaction due to the effect of crowding.

*Keywords: enzyme kinetics, macromolecular crowding, LDH, Dextran, mixed activation-diffusion control.*

## INTRODUCTION

It is well known that up to a 40% of the volume of the cytosol is occupied by a wide variety of macromolecules and solutes<sup>1</sup>. For this reason, any solute moving through the intracellular environment will see its diffusion rate affected with respect to diffusion in aqueous solution<sup>2-8</sup>, either being reduced or presenting anomalous diffusion at short times.

Macromolecular crowding inside the cell, does not only affect diffusion processes, but also biochemical reaction processes by inducing the enzyme to undergo protein folding, self-association or protein binding processes<sup>9-22</sup>, which in turn alter enzymatic activity. Thus, in order to obtain more accurate rates for enzymatic reactions, it is important to perform studies of biochemical processes in nature-like microenvironments that try to mimic this effect. Indeed, there are an important number of works which have studied how enzyme kinetics is affected by crowded environments, even in vitro<sup>23-41</sup>.

The best approach for this sort of studies would certainly be using cell extracts. However, the experimental data collection and their interpretation would be challenging because cell extracts are complex media that present a high heterogeneity in their geometrical and physical properties. Therefore, the major experimental studies on crowding effect have used purified macromolecules as crowding agents. Dextran is one of them and its use is widely spread due to its lack of reactivity and high solubility in water. Moreover, its flexibility and random coil shape in solution are suitable for modelling many macromolecules present in the natural state of the cell. It is also readily available in various sizes and large quantities.

In recent years, the effects of crowding on enzyme catalysis have been explored

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3 by different works, excellently depicted by Zhou et al<sup>8</sup> and Noris and Malys<sup>34</sup>. Most of  
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5 them indicate that under Michaelis-Menten conditions in crowded media, excluded  
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7 volume is the major player in modulating enzymatic behavior. Moreover, gathering all  
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9 the contributions published so far, some general trends are encountered. For instance, a  
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11 slight reduction in the apparent substrate-binding affinity constant,  $K_m$  is usually  
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13 reported, despite of the characteristics of the crowding agent<sup>9, 24, 26-32</sup>. In contrast, in  
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15 crowded media experiments  $k_{cat}$  can increase in some cases<sup>24-25, 30-33</sup>, or it can also  
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17 decrease<sup>9, 25, 27-28</sup>.  
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22 Previously, we reported how the kinetic behavior of two enzymatic reactions are  
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24 influenced by crowding. Our first study, the hydrolysis of N-succinyl-L-phenyl-Ala-p-  
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26 nitroanilide catalyzed by alpha-chymotrypsin<sup>37</sup>, showed that the total volume excluded  
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28 by Dextran, but not its size, is the property that makes significant changes on the  
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30 reaction rates. We obtained a  $v_{max}$  decrease and an  $K_m$  increase when the concentration  
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32 of Dextran in solution is heightened. The slower diffusion of the alpha-chymotrypsin in  
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34 presence of Dextran was responsible of the rise of  $K_m$ . The obtained diminish in  $v_{max}$   
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36 could only be explained by a mixed inhibition by product, enhanced by crowding. In  
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38 our second study, the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)  
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40 (ABTS) by Hydrogen Peroxidase ( $H_2O_2$ ) catalyzed by Horseradish Peroxidase (HRP)<sup>41</sup>,  
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42 results revealed that the reaction rate was also significantly influenced by the excluded  
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44 volume effect, but it was independent of the Dextran size. Both  $v_{max}$  and  $K_m$  decay  
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46 when increasing the concentration of the crowder in solution, suggesting an activation  
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48 control. In other words, the catalytic constant ( $k_{cat}$ ) brings a significant contribution as  
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50 a result of the environmental surroundings influence. This contribution may be the  
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52 consequence of one or the additive effect of the following aspects: the rise of the ratio of  
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54 the activity coefficients between the enzyme and the complex formed by enzyme and  
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3 substrate as a consequence of a crowded microenvironment; the boost of water chemical  
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5 activity favored by dense solution; and the conformational change of the enzyme active  
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7 site induced by crowding.  
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11 Despite the range of experimental conditions covered by previous studies, in our  
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13 opinion, more information and experiments in crowded media are required to elucidate  
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15 the key factors that govern the enzyme kinetics in these conditions. Therefore, in this  
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17 paper we study another enzymatic system in order to obtain more information about  
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19 how enzymatic reactions could be affected by macromolecular crowding.  
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23 Here, we study how the crowding of Dextran at several concentrations and sizes  
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25 affects the catalytic reaction of LDH (oxidation of NADH by Pyruvate). We chose this  
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27 enzymatic reaction as a model process for several reasons. First, it is a well-known  
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29 reaction in which small substrates and products lead to the occurrence of the reaction  
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31 without a significant variation in excluded volume. Second, there are no interactions  
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33 between the enzyme and the Dextran used as crowding agents. And third, the LDH size  
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35 ( $M_w = 140$  kDa) is intermediate between those of the selected Dextran (from 50 to 410  
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37 kDa).  
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41 Therefore, here we illustrate how the reaction catalyzed by Lactate  
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43 Dehydrogenase is strongly influenced by different amounts of neutral polymers of  
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45 different dimensions, mimicking the known intracellular crowding levels. Specifically,  
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47 we study the dependence of  $v_{max}$  and  $K_m$  parameters on the total volume of the solution  
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49 excluded by the crowder and the individual volume occupied by each of the polymeric  
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51 coils.  
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## 54 55 **MATERIALS AND METHODS** 56 57 58 59 60

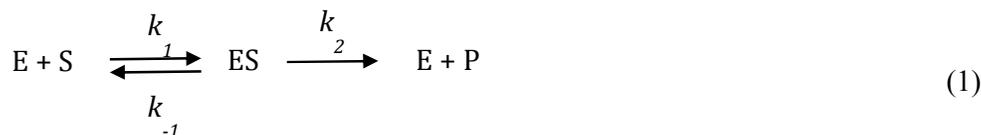
### Chemicals

Rabbit muscle L-Lactate Dehydrogenase (E.C. 1.1.1.27) ( $140 \text{ U mg}^{-1}$ ), received as a purified and lyophilized powder, sodium pyruvate and  $\beta$ -NADH were acquired from Sigma-Aldrich Chemical (Milwaukee, WI, USA). Four Dextrans (Fluka) of 410, 275, 150 and 50 molecular weight were used without further purification. All the chemicals were of analytical or spectroscopic reagent grade.

### Oxidation of NADH

The oxidation of NADH was made at  $25^\circ\text{C}$  in Imidazole-Acetic Acid buffer (30 mM, pH = 7.5, containing 60 mM of  $\text{CH}_3\text{COOK}$  and 30 mM of  $\text{MgCl}_2$ ). Each sample contains the same concentration of  $8.2 \cdot 10^{-13} \text{ M}$  of LDH and  $1.17 \cdot 10^{-4} \text{ M}$  of NADH. Michaelis-Menten plots were obtained by measuring initial velocity of the reaction at different pyruvate concentrations, in a range between  $7.1 \cdot 10^{-5}$  and  $5.4 \cdot 10^{-4} \text{ M}$ . This process was first done without the addition of crowding agent. After the incorporation and homogenization of the enzyme into a sample, which contains NADH and pyruvate, the reaction starts. Subsequently, we dissolved into the same sample mixture different Dextran concentrations (25, 50 and 100 mg/mL) for each Dextran size mentioned above: 410, 275, 150 and 50 kDa, which will be referred as D410, D275, D150 and D50, respectively. The reaction progress and the data analysis was described in detail in Pastor et al.<sup>37</sup>. Briefly, in this case we follow the reaction by the absorbance change that occurs as NADH is oxidized into  $\text{NAD}^+$ , which no longer absorbs at 320 nm. Initial reaction velocity,  $v_0$ , was obtained by linear fitting of the initial data points in the absorbance-time plot. Blank solution containing substrate and Dextran in the same concentrations than the sample was measured in each case.

The oxidation of NADH by Pyruvate catalyzed by LDH can be treated as a single substrate reaction and its kinetic study can be performed using the scheme proposed by Henri<sup>42</sup>:



where  $k_1$ ,  $k_{-1}$ , and  $k_2$  are rate constants. This system can be described using Michaelis-Menten equation<sup>42</sup>:

$$v_0 = \frac{v_{\max}[S]_0}{K_m + [S]_0} \quad (2)$$

where  $v_{\max}$  is the maximum velocity and  $K_m$  is the Michaelis-Menten constant. With the combination of (1) and (2) some definitions can be stated:  $K_m$  can be defined as  $K_m = (k_{-1} + k_2)/k_1$ , and for  $v_{\max}$ ,  $v_{\max} = k_{\text{cat}}[E]_T$ , and  $k_{\text{cat}} = k_2$ .

Since Michaelis and Menten studies, it has been assumed that enzyme activity can be studied applying equation (2), obtaining data to fit that equation by performing measurements of the initial rate of product formation at different substrate concentrations.

## RESULTS AND DISCUSSION

For a suitable evaluation of the influence exerted by macromolecular crowding on the kinetic parameters, one must consider at least two important features of the study. First, the reactions under investigation should be processes whose kinetic behavior has an established and generally accepted interpretation. In our case, the

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3 reaction between NADH and pyruvate catalyzed by LDH is a well-known process  
4 following a Michaelis-Menten mechanism and it can easily be monitored by UV-  
5 spectroscopy. Second, the crowding induced effect should be accounted only by the  
6 changes incurred by the crowding polymers with regard to its dimensions and  
7 concentration. Besides, as stated above, the selected reaction is accompanied by an  
8 insignificant variation of excluded volume during the progression of the reaction, owing  
9 to the small size of NADH, pyruvate,  $\text{NAD}^+$  and L-lactate.  
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19 We studied how the selected system presents a different kinetic response than in  
20 dilute solution when different concentrations and sizes of Dextran are added to the  
21 reaction media. An initial linear decrease followed by a plateau is observed when  
22 measuring the absorbance of NADH at 320 nm in all the studied cases (results not  
23 shown). As explained in Material and Methods, initial velocity ( $v_0$ ) values were  
24 acquired by the fitting of the linear part of the absorbance-time plot for each sample.  
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33 Figure 1 shows  $v_0$  values as a function of the substrate concentration. Each data  
34 point is obtained from the mean between 3 to 5 samples with the same reaction  
35 conditions in terms of substrates, enzyme and Dextran concentrations. Each figure, A to  
36 D, represents a given Dextran size, from the smallest to the biggest Dextran. Dextran  
37 concentration in mg/mL and excluded volume are directly proportional. A significant  
38 dependence of  $v_0$  on both the excluded volume and the obstacle dimension is clearly  
39 found. Figure 2 reveals that reaction rate decreases as a function of Dextran size when  
40 high Dextran concentrations ( $> 50$  mg/mL) are used. In this case, the process behavior  
41 depends not only on the excluded volume but also on the dimension of the obstructive  
42 particles present in the reaction media.  
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3 This result is consistent with the results of Homchaudhuri et al.<sup>29</sup>. These authors  
4 stated that alkaline phosphatase makes the hydrolysis of p-nitrophenyl phosphate with a  
5 velocity which depends on the crowder size for a given excluded volume conditions.  
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7 They showed that for samples of Dextran of about 20% (w/w) the reaction rates  
8 decrease with the increase of Dextran size: a decrease of about 2, 5 and 7-fold was  
9 observed for Dextran of 15-70, 200 and 500 kDa, respectively. However, it is in  
10 contrast with the results showed in our previous works<sup>37, 41</sup> and with the results of  
11 Minton et al.<sup>2, 8-12</sup>. In all these works, only excluded volume but not obstacle size was  
12 presented as a major factor influencing enzymatic reactions occurring in reaction media  
13 crowded by macromolecules.  
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26 The major difference between the studies is the relative dimension of the protein  
27 in respect to the obstacle. It has to be acknowledged that the size of alkaline  
28 phosphatase (105 kDa) is comparable with that of LDH (140 kDa). As such, the rate of  
29 the reactions they catalyze has similar behavior in crowded media unlike the rate of the  
30 other investigated processes catalyzed by much smaller proteins: alpha-chymotrypsin  
31 (25 kDa) and HRP (42 kDa). As revealed by Homchaudhuri et al.<sup>29</sup>, occasioned by the  
32 big size of the protein, large crowding agents make enzyme and substrates to come  
33 across less often. However, this effect is partially offset when using small obstacles and  
34 the decrease is less significant, due to a certain caging effect that causes an enhanced  
35 enzymatic activity. Our results underline the fact that the degree of crowding effect  
36 relies both on the size and concentration of the obstructive particles. In spite of that, the  
37 relative size of the enzyme in respect to the crowder seems to exert a significant  
38 influence when larger molecules are present in the system.  
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3 The values of Michaelis Menten constants,  $K_m$  and  $v_{max}$ , were estimated by  
4 fitting Eq. 2 to each data set in Figure 1, in order to get information about how the  
5 excluded volume could affect the oxidation of NADH by LDH in crowded media.  
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10 Figures 3 and 4 and Table 1 show how  $K_m$  and  $v_{max}$  are dependent on the amount of  
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12 volume excluded by macromolecular inert obstacles. In fact, we can perceive that only  
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14 high concentrations and large sizes of Dextran affect both  $v_{max}$  and  $K_m$  values, whereas  
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16 for smaller sizes of Dextran, the enzymatic system behaves in a similar way to our  
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18 previous studies with  $\alpha$ -Chymotrypsin<sup>37</sup>.  
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22 Firstly, examining  $v_{max}$  values we see (Figure 3 and Table 1) that they diminish  
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24 with the increase of size and concentration of Dextran. In the Figure it is shown that the  
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26 value of  $v_{max}$  in presence of D50, which size is lower than LDH, decreases slightly with  
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28 respect to the value in diluted solution, but it is independent of the concentration of  
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30 D50. Moreover, the presence of low concentrations (25 and 50 mg/mL) of D150 and  
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32 D410, whose sizes are, respectively, equal and two times the LDH size, affects the value  
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34 of  $v_{max}$  in the same sense, i.e. its value decays slightly with respect to the value in  
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36 diluted solution, but it is independent of the concentration. However, high concentration  
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38 of both Dextrans, D150 and D275, decreases hugely the value of  $v_{max}$ . In the same  
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40 direction but even enhanced, the presence of a high concentration of D410, which size is  
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42 three times the LDH size, causes biggest decreases of  $v_{max}$ .  
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47 As stated previously,  $v_{max}$  is defined as  $v_{max} = k_{cat}[E]_T$ , with  $k_{cat} = k_2$ .  
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49 Therefore, its decrease with high concentration or large size of Dextrans could be due to  
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51 two main reasons: the variation of the effective enzyme concentration or the deviation  
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53 of the catalytic rate constant ( $k_{cat}$ ). On the one hand, the excluded volume in crowded  
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55 media is responsible for an increase of the effective enzyme concentration<sup>2, 8-12</sup>. In fact,  
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3 an increase of  $v_{\max}$  in crowding situations is usually explained as a result of a raise in  
4 the effective enzyme concentration<sup>24, 31, 33</sup>. On the other hand, when a decrease in  $v_{\max}$   
5 is found in crowding situations, it is normally explained by the reduction of  $k_{\text{cat}}$  as a  
6 result of conformational changes of the catalytic centre of the protein derived from  
7 surrounding variations induced by the crowded media<sup>27-28, 30, 33, 44</sup>. In our case, it is  
8 reasonable to assume that  $k_{\text{cat}}$  would be unaffected by the presence of Dextran. Since  
9 the catalytic site is protected from the bulk solution, Dextran can not denaturize or  
10 somehow alter the inner cavities of the protein in contrast with other molecules<sup>22, 23-24,</sup>  
11 <sup>27-28, 30, 33, 43-44</sup>. In fact, in this particular case we should also consider the comparable  
12 size between LDH and the obstacles to understand the decrease of  $v_{\max}$  in crowded  
13 media. In presence of large obstacles, the comparable size between both, LDH and  
14 Dextran, causes a reduction of the encounters between enzyme and substrates. On the  
15 contrary, a minor decrease in the  $v_{\max}$  value is found in presence of small obstacles  
16 because the crowding effect is partially compensated by the improvement of enzymatic  
17 activity that a certain cage effect can induce. In our opinion, only this situation could  
18 explain our results.

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40 Secondly, we analyzed the  $K_m$  behavior. As it is shown in Figure 4 and Table 1,  
41 only a high concentration of large Dextran, 100 mg/mL of D150, D275 or D410, decays  
42 the  $K_m$  value. As was explained in Materials and Methods section, the Michaelis-  
43 Menten constant is defined by  $K_m = (k_{-1} + k_2)/k_1$  following the general chemical  
44 equation for enzymatic processes presented in Eq. 1. In a situation of diffusion control,  
45 an increase in  $K_m$  value can be expected since the bimolecular constant ( $k_1$ ) will  
46 decrease with the crowding<sup>45-47</sup>. In the literature, there are few works that report this  
47 situation<sup>21, 37, 45-47</sup>. In these reported cases, a high diffusion resistance in the sample is  
48 responsible of a  $K_m$  increase with Dextran concentration. However, most of the  
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3 enzymatic reaction studies in crowding situations showed a slight decrease in  $K_m$   
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5 compared to that of the dilute solution. These studies excluded a diffusion control of the  
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7 enzymatic process and the modification of  $k_1$  by the presence of crowding agents (e.g.  
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9 reference 31). A variation in  $k_{cat}$  with crowding is usually found in the cases where a  
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11  $K_m$  decrease is reported. The  $K_m$  constant is sensitive to the sample composition since it  
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13 depends on the substrate activity coefficient. Therefore, a decrease in  $K_m$  with crowding  
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15 is usually attributed to several factors. On the one hand, due to non-ideal conditions of  
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17 the crowded solution, chemical activity of the substrate is modified. On the other hand,  
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19 there is an increase of the activity coefficients relation between the free enzyme and the  
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21 complex formed by enzyme and substrate<sup>27-28,30</sup>. Moreover, substrate binding event is  
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23 depending on water activity, because both the substrate and the active site must be  
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25 dehydrated for the binding to occur. Therefore,  $k_2$  value may be affected. In our  
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27 opinion, as it was explained in detail in our previous work<sup>37</sup>, both hindered diffusion  
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29 and alterations in the active site must be considered to better understand the results. In  
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31 fact, the behavior showed in this study by  $K_m$  at high concentration of large Dextran is  
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33 in agreement with these previous studies. In this case, at high concentration of large  
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35 Dextran, the decrease of both constants,  $v_{max}$  and  $K_m$ , are in agreement with a decrease  
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37 in the  $k_2$  value, which depends on the amount of encounters between enzyme and  
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39 substrates. The existence of a high quantity of large Dextran reduces the number of  
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41 these encounters and consequently the constant value.  
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49 In addition, if we compute the  $v_{max}/K_m$  ratio (Table 1), we observe a decrease in  
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51 its value at high concentration of large Dextran, which could indicate some possible  
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53 effect of inhibition. Excess-substrate inhibition of rabbit muscle LDH is well  
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55 documented for the case of pyruvate<sup>48-51</sup>. However, we observe a slight and constant  
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3 decrease of the value of the  $v_{\max}/K_m$  ratio with respect to the case of diluted solution  
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5 until the excluded volume achieves some limit value. This behavior suggests that the  
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7 possible substrate inhibition reported in the earlier literature does not explain the  
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9 obtained values. Recent simulation and experimental studies reported on diffusion  
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11 processes of enzymes in crowded media<sup>47, 52-55</sup> show that the effective diffusion  
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13 coefficient is higher in dilute samples than in crowding situations. A decrease in the  
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15 diffusion coefficient may imply a decrease in the bimolecular constant ( $k_1$ ) with  
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17 crowding<sup>45-46</sup>. In a situation where the enzymatic reaction depends on the diffusion, the  
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19  $K_m$  value should increase because of the decrease of  $k_1$ . However we find that  $K_m$   
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21 remains constant until high concentration of large Dextran. On the other hand, our  
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23 results show that  $k_2$  decreases with crowding, which is in agreement with an activation  
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25 control. Thus, a mixed activation-diffusion control could explain the rough decreases of  
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27 the experimental fitted values of  $v_{\max}$  and  $K_m$  for high Dextran sizes and concentrations  
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29 In particular, the decrease  $v_{\max}$  is produced by the decrease of  $k_2$  with size and  
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31 concentration of Dextran. The quasi-constant value of  $K_m$  can be seen as a result of the  
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33 combined effect of the decreasing of the diffusion-controlled constant  $k_1$ , with size and  
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35 concentration of Dextran and the decreasing of  $k_2$ . This concordance breaks for high  
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37 concentration of large Dextran when  $K_m$  is not constant anymore.  
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45 In conclusion, our results reveal, on the one hand, that the initial velocity of the  
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47 reaction depends on both the size and amount of Dextran present in the media. In  
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49 addition, and in contrast with previous reported works, we have found that the enzyme  
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51 relative size respect to the crowding molecules represents another important factor  
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53 influencing the velocity of the reactions occurring in crowded media. When enzymes  
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55 are small the reaction rate mainly relies on the excluded volume of the solution.  
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3 However, for large enzymes (in our case LDH) the reaction rate is also influenced by  
4 the size of obstacles present in the reaction environment. On the other hand, we  
5 obtained a decrease in both Michaelis-Menten constants,  $v_{\max}$  and  $K_m$ , with the amount  
6 of crowding agent in the sample, but this decrease is only significant in the case of  
7 samples with high concentrations of large Dextran. The decrease in  $v_{\max}$  also depends  
8 on the size of Dextran present in the media. This result is attributable to the activation  
9 control of the enzymatic process, but it must be taken into account the fact that as a  
10 result of the relative large size of LDH, large obstacles reduce the encounters among  
11 enzymes and substrates. However, an activation control of the LDH reaction is against  
12 the decay found in  $K_m$  only in cases with high concentration of large Dextran. This  
13 decrease can be explained by a mixed activation-diffusion control of this enzymatic  
14 process in crowding media produced by Dextran. Only a mixed control can explain the  
15 behavior found in both Michaelis-Menten constants, and probably it is due to the  
16 relative large size of the LDH and the difference between the enzyme and the crowding  
17 agent size. In our opinion, the enzyme relative size with respect to the crowding agent  
18 represents a significant factor to be considered in enzymatic reaction studies carried out  
19 in macromolecular crowded media.  
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Table 1

	Concentration (mg/mL)	$v_{\max}$ (mM/s)	$K_m$ (mM)	$v_{\max}/K_m$ (s <sup>-1</sup> )	$r^2$
<b>Diluted Solution</b>	-	0.81 ± 0.01	0.47 ± 0.01	1.7 ± 0.1	0.9825
<b>D50</b>	25	0.63 ± 0.02	0.45 ± 0.01	1.4 ± 0.1	0.9893
	50	0.57 ± 0.02	0.46 ± 0.01	1.2 ± 0.1	0.9758
	100	0.54 ± 0.05	0.46 ± 0.01	1.2 ± 0.1	0.9733
<b>D150</b>	25	0.53 ± 0.03	0.43 ± 0.01	1.2 ± 0.1	0.9879
	50	0.53 ± 0.03	0.43 ± 0.01	1.2 ± 0.1	0.9820
	100	0.21 ± 0.01	0.34 ± 0.01	0.6 ± 0.1	0.9918
<b>D275</b>	25	0.58 ± 0.03	0.41 ± 0.01	1.4 ± 0.1	0.9865
	50	0.59 ± 0.02	0.41 ± 0.01	1.4 ± 0.1	0.9846
	100	0.21 ± 0.01	0.35 ± 0.01	0.6 ± 0.1	0.9965
<b>D410</b>	25	0.59 ± 0.02	0.43 ± 0.01	1.4 ± 0.1	0.9925
	50	0.39 ± 0.03	0.43 ± 0.01	0.9 ± 0.1	0.9855
	100	0.11 ± 0.07	0.37 ± 0.01	0.3 ± 0.1	0.9970

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3 FIGURE 1.  $v_0$  versus substrate concentration plot for the oxidation of NADH in  
4 presence of pyruvate catalyzed by LDH in Dextran crowded media with different  
5 Dextran sizes: (A)  $M_w = 50$  kDa; (B)  $M_w = 150$  kDa; (C)  $M_w = 275$  kDa and (D)  $M_w =$   
6 410 kDa. In each figure, the curves corresponding to four Dextran concentrations are  
7 plotted: 0 mg/mL (solid square), 25 mg/mL (open circle), 50 mg/mL (solid up-triangle)  
8 and 100 mg/mL (open down-triangle).  
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17 FIGURE 2. Example of  $v_0$  variation with Dextran size and concentration for the  
18 oxidation of NADH in presence of pyruvate catalyzed by LDH for three fixed substrate  
19 concentrations: 0.22 mM (A), 0.34 mM (B) and 0.40 mM (C). In each figure, three  
20 Dextran concentrations are plotted: 25 mg/mL (open circle), 50 mg/mL (solid up-  
21 triangle) and 100 mg/mL (open down-triangle).  
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29 FIGURE 3. Plot of the variation of  $v_{max}$  values with Dextran size and concentration for  
30 the oxidation of NADH in presence of pyruvate catalyzed by LDH. The four Dextran  
31 sizes used are plotted: D50 (solid square), D150 (open circle), D275 (solid up-triangle)  
32 and D410 (open down-triangle).  
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39 FIGURE 4. Plot of the variation of  $K_m$  values with Dextran size and concentration for  
40 the oxidation of NADH in presence of pyruvate catalyzed by LDH. The four Dextran  
41 sizes used are plotted: D50 (solid square), D150 (open circle), D275 (solid up-triangle)  
42 and D410 (open down-triangle).  
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Figure 1

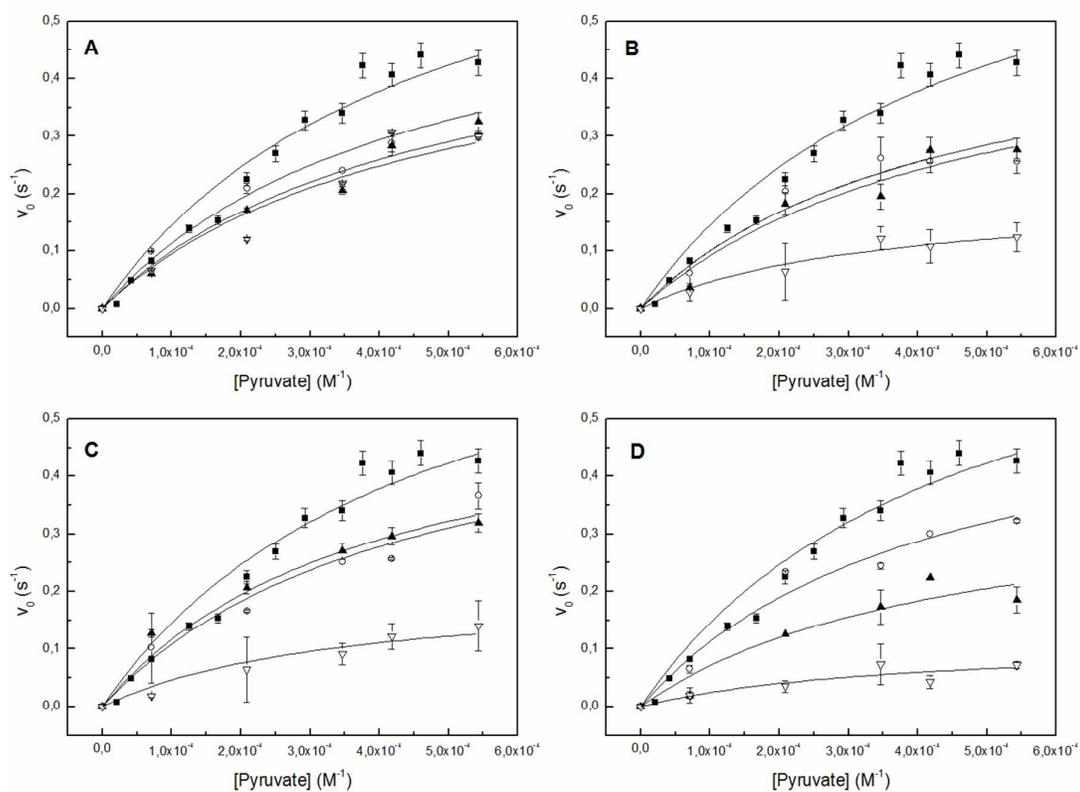


Figure 2

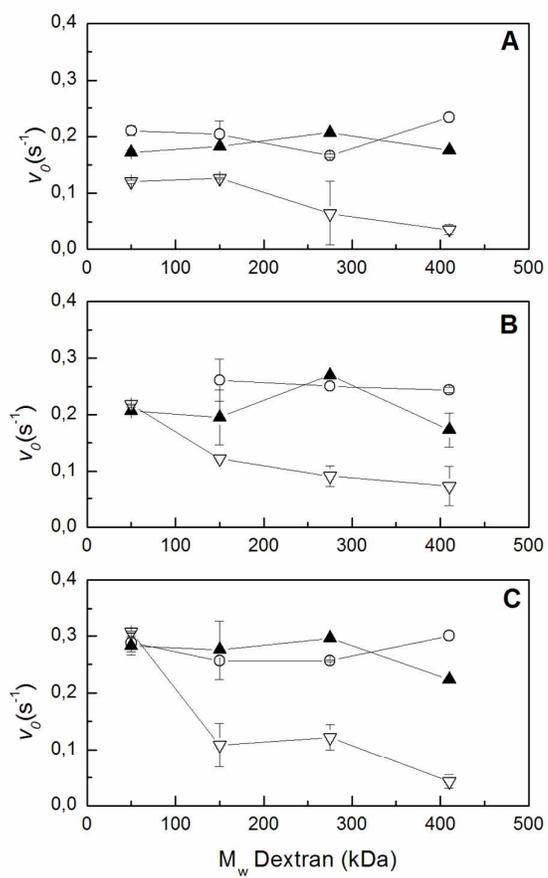


Figure 3

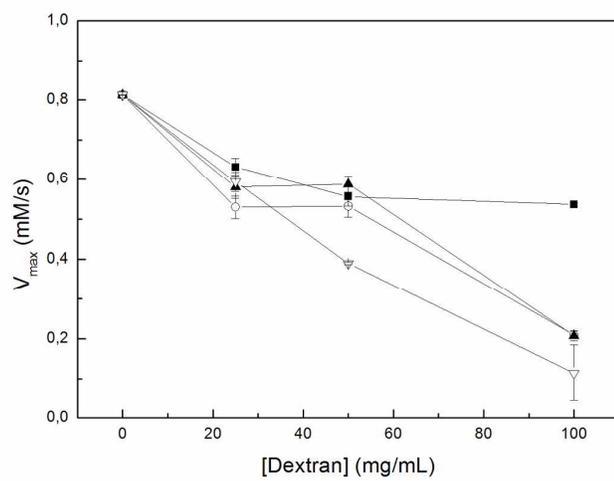
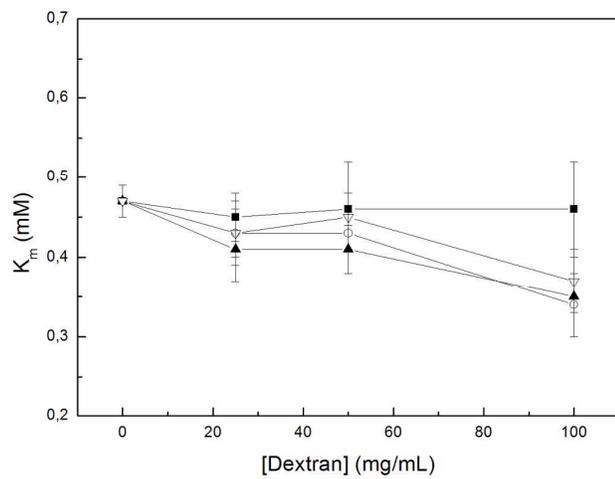


Figure 4



## TOC

