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Modulation of enzyme catalytic properties and biosensor calibration parameters with chlorides: Studies with glucose oxidase

Margarita Kagan, Kairi Kivirand, Toonika Rinken*

Institute of Chemistry, University of Tartu, Ravila 14a, Tartu, Estonia

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ABSTRACT

We studied the modulation of calibration parameters of biosensors, in which glucose oxidase was used for bio-recognition, in the presence of different chlorides by following the transient phase dynamics of oxygen concentration with an oxygen optrode. The mechanism of modulation was characterized with the changes of the glucose oxidase catalytic constant and oxygen diffusion constant. The modulation of two biosensor calibration parameters were studied: the maximum calculated signal change was amplified for about 20% in the presence of sodium and magnesium chlorides; the value of the kinetic parameter decreased along with the addition of salts and increased only at sodium chloride concentrations over 0.5 mM. Besides glucose bioassay, the amplification of calibration parameters was also studied in cascaded two-enzyme lactose biosensor, where the initial step of lactose bio-recognition, the β-galactosidase – catalyzed lactose hydrolysis, was additionally accelerated by magnesium ions.

1. Introduction

The concept of biosensors, relating the concentration of an analyte to a measurable signal of a selective bio-recognition reaction, was introduced almost 50 years ago [1]. Since then, numerous studies have attempted to interface different biological materials to determine the ability of a biosensor to recognize the molecules of a particular analyte and signal transducer [2]. The properties of a bio-recognition system determine the selectivity, sensitivity and speed of analysis of a biosensor.

In addition to natural bio-selective materials, which are available for only a limited number of natural compounds and artificial structures, such as synthetic and engineered receptors, have been developed for a more effective detection of both natural and man-made substances [3]. A convenient option for the up-down regulation of bio-recognition efficiency involves the physical and/or chemical modulation of the properties of a biologically active material. The activity of enzymes, which are the most frequent bio-recognition materials, has been modified through different factors, including a static magnetic field [4], light [5,6], pressure [7], change of pH [8,9], immobilization on an insoluble carrier [10] and chemical modification [11].

Glucose oxidase (EC 1.1.3.4) is the most widely used enzyme in biosensors, serving as the bio-recognition material in glucose biosensors that are primarily used for the detection of blood sugar. Together with β-galactosidase (EC 3.2.1.23), glucose oxidase is used in lactose cascaded biosensors, where β-galactosidase catalyzes the hydrolysis of lactose into galactose and glucose and glucose is further oxidized through glucose oxidase. This cascaded process is applied because no acceptable direct method for the detection of lactose reactions is available for application in biosensors. Lactose detection is essential for production control in the dairy industry [12] and medicine, as 70% of the world's adult population suffers from either lactose maldigestion or intolerance [13].

In addition, the catalytic properties of enzymes can be modified with the addition of salts of varying ionic strengths. However, the information concerning the effects of salt on glucose oxidase is conflicting. Liu and Cui studied the effect of sodium chloride (NaCl) on glucose oxidase activity and detected a negative impact on glucose conversion. The enzyme activity was 5 times lower at an NaCl concentration of 200 mM compared with salt-free solutions [14]. In contrast, Tongbu observed the activation of glucose oxidase at low NaCl concentrations and the inactivation of glucose oxidase at higher NaCl concentrations [15]. Moreover, sodium nitrate ($NaNO_3$) is a competitive inhibitor of glucose oxidase [16]. There are numerous data concerning the inhibiting the effects of heavy metals ions, such as Cu^{2+}, Hg^{2+} and Fe^{3+}, on glucose oxidase, which are measured using amperometric biosensors [17,18]. Furthermore, measuring the products of glucose catalytic oxidation spectrophotometrically has revealed that $HgCl_2$, Hg_2Cl_2, $CuCl_2$ and CuCl activate glucose oxidase in a nonessential manner, with activation constants of 7.76×10^{-5} M, 3.26×10^{-4} M, 1.12×10^{-4} M and 1.88×10^{-4} M, respectively. This activation might result from conformation changes in the active site, facilitating the

* Corresponding author. Tel.: +372 7375167.
E-mail address: toonika.rinken@ut.ee (T. Rinken).

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transfer of electrons from the substrate to the electron acceptor [16]. This controversial data might reflect differences in the experimental methods and parameters that were used to characterize the enzyme-catalyzed reactions.

Magnesium ions (Mg^{2+}) activate β-galactosidase, although the mechanism of this action remains elusive [19,20]. The activating effect of Mg^{2+} has been used to increase the sensitivity of lactose biosensors, and an optimal concentration of 2 mM Mg^{2+} has been suggested [21].

Different metal cations affect enzymatic activity through the modulation of the flexibility of the enzyme active site [22]. Salts also modulate the external diffusion limitation of different compounds. The molecular diffusion coefficients in water are dependent on the concentration of electrolytes, and increasing salt concentrations decrease the diffusivity of oxygen, which is a substrate in oxidase-catalyzed reactions. The oxygen diffusivity is decreased to approximately 25% in a 0.1 M NaCl solution compared with pure water [23] and is associated with an increase in the viscosity of the solution [24]. It has been suggested that the change in viscosity might also reduce the rate constants for the association and dissociation of the enzyme with substrates and/or dampen the structural fluctuations of proteins through frictional effects [25].

The aim of the present study was to characterize the modulation of the catalytic properties of glucose oxidase using different salts and to identify convenient and simple options for the amplification of glucose oxidase-based biosensor calibration parameters to accelerate the rate and improve the quality of analysis.

2. Experimental

2.1. Materials and methods

β-galactosidase (β-GAL, from *Aspergillus oryzae*, 11.2 IU/mg) and glucose oxidase (GOD, from *Aspergillus niger*, 200 IU/mg) were obtained from Sigma. All reagents used were of analytical grade.

The kinetic measurements were performed in an airtight temperature regulated (25 °C) glass cell (V = 28 ml) with constant stirring in a 0.1 M acetic buffer (pH = 5.6) (using glucose oxidase only) or a 0.1 M phosphate buffer (pH = 6.5) (using glucose oxidase and β-galactosidase). A fiber-optical oxygen sensor was plunged into the air-saturated reaction medium, containing 3 mM of glucose and a defined amount of salt, and the oxygen output signal was stabilized before initiating the reaction through the injection of 100 μl of GOD solution into the reaction cell, generating a final enzyme concentration of 1.3 IU/ml. In experiments using β-GAL, the salt-containing air-saturated 5% lactose solution was incubated with β-GAL (10.08 IU/ml) for 10 min at 25 °C (with oxygen sensor immersed), and glucose production was determined as previously described.

The change in the dissolved oxygen concentration (DOC) was recorded using an oxygen optrode constructed at the University of Tartu Institute of Physics. This sensor measures the oxygen-induced phosphorescence quenching of Pd-tetraphenylporphyrin, which is encapsulated into a thermally aged polymethyl methacrylate (PMMA) film, covering a 30 mm PMMA optical fiber with diameter of 1 mm [26]. The oxygen-sensitive film was additionally covered with a thin black silicone coating to eliminate the effect of divergent light. The optrode signal was recorded with an interval of 0.1 s; each experimental curve was recorded in 3 replicates, comprising a minimum of 2000 data points. The DOC was calculated according to the Stern–Volmer relationship:

$$\frac{I_0}{I} = 1 + K_{SV} p \quad (1)$$

where I_0 is the luminescence intensity without oxygen, I is the luminescence intensity at oxygen partial pressure, p, and K_{SV} is the Stern–Volmer coefficient, characterizing the sensitivity of the sensor material. According to Henry's law, p and DOC are directly proportional, with a value of 769.2 l atm/mol for Henry's coefficient, k_H, for dissolved oxygen at 298 K in water.

2.2. Theoretical considerations and calculations

The reduction in the DOC over time was characterized using 2 independent parameters, the total signal change parameter (A) and the kinetic parameter (B), calculated from the biosensor transient signal using a modified dynamic biosensor model. The model considers the ping-pong mechanism of enzyme kinetics, substrate diffusion and system inertia [27]:

$$\frac{C_{O_2}(t)}{C_{O_2}(0)} = A\exp(-Bt) + (1-A) - 2A\sum_{n=1}^{\infty}(-1)^n \frac{(\tau_s H^2)}{B-\tau_s}\left[\exp(-Bt)\exp-\left(-n^2\frac{t}{\tau_s}\right)\right] + (1-A), \quad (2)$$

where $C_{O_2}(t)$ is the oxygen concentration at time t and $C_{O_2}(0)$ is the oxygen concentration at the start of the reaction; τ_s is the time constant of system inertia and the reaction parameters A and B are dependent on the substrate concentration, expressed as:

$$A = \frac{k^*_{cat}[E]_{total} c_S^{bulk}}{k_{dif}^{O_2} K_S K_{O_2} + (k^*_{cat}[E]_{total} + k_{diff}^{O_2} K_{O_2}) c_S^{bulk}}, \quad (3)$$

$$B = \frac{(k^*_{cat}[E]_{total}/K_{O_2}) c_S^{bulk}}{K_S + c_S^{bulk}} + k_{diff}^{O_2} \quad (4)$$

In Eqs. (3) and (4), k^*_{cat} denotes the catalytic constant of the reaction, $[E]_{total}$ is the overall enzyme concentration, $k_{diff}^{O_2}$ is the apparent oxygen diffusion coefficient, K_S is the formation of enzyme-substrate complex, K_{O_2} characterizes the binding of oxygen to the active enzyme, and C_S^{bulk} denotes the initial substrate concentration.

The signal parameters A and B are used for the calibration of biosensors, and the modulation of these parameters reduces the impact of experimental noise, thereby improving the quality of the results, expanding the biosensor working range and raising the sensitivity of the biosensor.

To determine the values of parameters A and B, only k^*_{cat} and $k_{diff}^{O_2}$ are dependent on the concentration of the electrolytes in solution (if $[E]_{total}$ and C_S^{bulk} are kept constant). Considering Eqs. (3) and (4), parameters A and B can be expressed as the functions of two variables: the catalytic coefficient k^*_{cat} and diffusion coefficient $k_{diff}^{O_2}$:

$$A = \frac{const_1 k^*_{cat}}{const_2 k_{diff}^{O_2} + const_1 k^*_{cat}}, \quad (5)$$

$$B = \frac{const_1}{const_2} k^*_{cat} + k_{diff}^{O_2}, \quad (6)$$

where

$$const_1 = [E]_{total} C_S^{bulk}, \quad (7)$$

$$const_2 = K_{O_2}(K_S + C_S^{bulk}) \quad (8)$$

In the applied system, the total glucose oxidase concentration, $[E]_{total}$, was 1.0×10^{-5} M and the glucose concentration, C_S^{bulk}, was 3.0×10^{-3} M. The value of K_{O_2} for oxygen as a substrate was 2.0×10^{-4} M [28,29], and the K_S for glucose was 2.2×10^{-3} M [27]; thus, the values for $const_1$ and $const_2$ for our system, according to Eqs. (7) and (8), were 3.0×10^{-8} and 1.3×10^{-6}, respectively. Although $const_1$ and $const_2$ characterize a particular system, these values do not influence the relative values of k^*_{cat} and $k_{diff}^{O_2}$ in solutions of different salts.

3. Results and discussion

First, we determined the values of signal parameters A and B to determine the GOD in a catalyzed reaction using the biosensor model (Eq. (2)) in a salt-free glucose solution and at different concentrations of different electrolytes (Fig. 1). The standard deviations of the calculated biosensor signal parameters ranged from 0.002 to 0.009 for parameter A and 0.02×10^{-4} to 7.4×10^{-4} for parameter B. The modulation of the output signal parameters depends on the nature of the parameter itself and the nature and concentration of the added electrolyte. The experimentally determined value of the maximum signal change parameter A, which is the steady state signal of the reaction, is the most commonly used reaction indicator in biosensor studies, although side processes and the correct determination of the biosensor response time notably influenced this parameter [30].

The value of the calculated maximum signal change parameter A, increased 1.2 times from its initial value of 0.82 in salt-free solutions to a maximum value of 1.00 in 0.5 M NaCl solution (Fig. 1(A1)); the kinetic parameter B, in the same conditions was reduced 3-fold (Fig. 1(B1)).

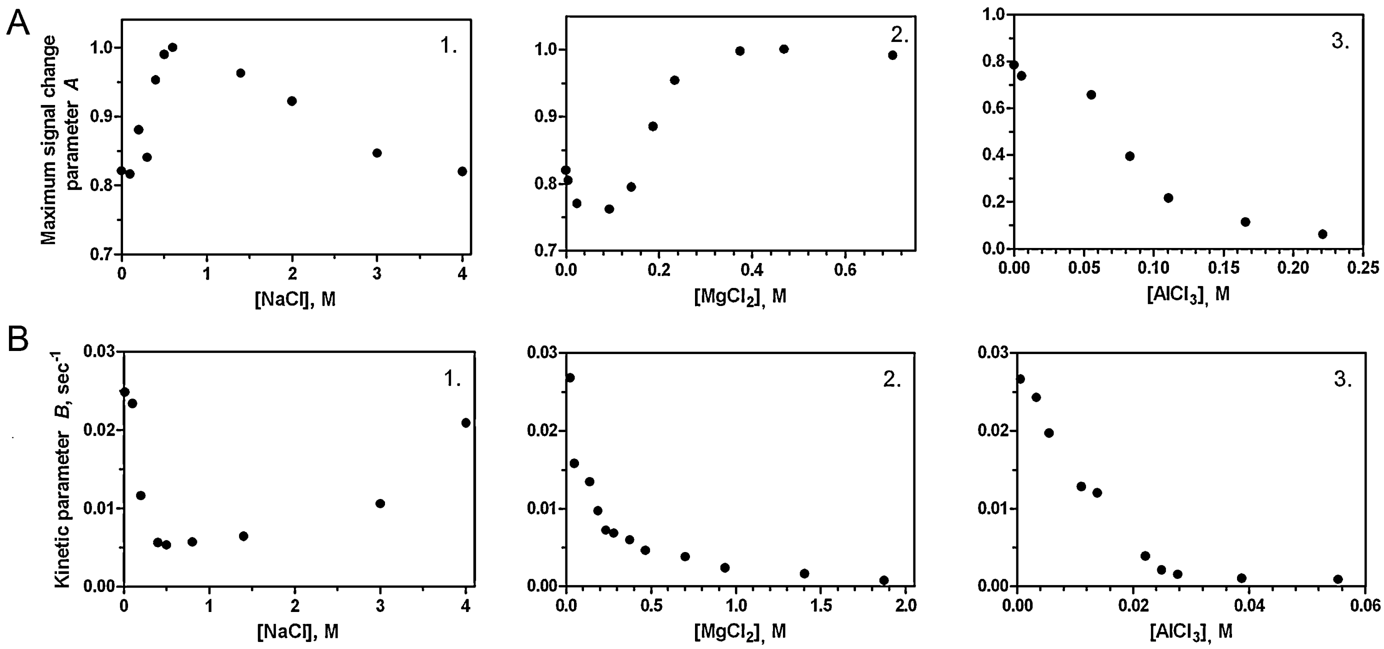

Fig. 1. The dependence of biosensor signal parameters on the concentrations of different chlorides. (A) Maximum signal change parameter *A*; (B) kinetic parameter *B*. All measurements were carried out in air-saturated 0.1 M acetic buffer (pH = 5.60) at 25 °C; $c_{glucose}$ = 3 mM, $c_{glucose\ oxidase}$ = 1.3 IU/ml.

Magnesium chloride ($MgCl_2$) caused a 1.3-fold essential increase of the maximum signal change parameter *A* in a salt concentration, ranging from 0.1 to 0.4 M. At lower concentrations, the value of parameter *A* was slightly reduced, and at concentrations greater than 0.4 M, parameter *A* maintained its maximum value (Fig. 1(A2)). Kinetic parameter *B* showed a gradual decrease (Fig. 1(B2)) but not as quickly as observed in the presence of NaCl. At $MgCl_2$ concentrations greater than 1 M, the glucose oxidation reaction was slow; thus, although the maximum value for the signal change parameter was observed (meaning that at the reaction steady state DOC equals zero), the experimental determination and interpretation of steady state was problematic.

Both studied reaction parameters were reduced in the presence of aluminum chloride ($AlCl_3$). Reaction kinetic parameter *B* reached its minimum at lower $AlCl_3$ concentrations compared with the maximum signal change parameter *A* (Fig. 1(A3) and (B3)), indicating the complete inactivation of GOD.

Considering the calculated constants, $const_1$ and $const_2$ (Eqs. (7) and (8)), and the reaction parameters, *A* and *B* (Eqs. (3) and (4)), the values of the catalytic coefficient, k^*_{cat}, and the diffusion coefficient, $k^{O_2}_{diff}$, at different salt concentrations were identified (Fig. 2). k^*_{cat} is the apparent catalytic constant of the oxidoreductases, which catalyze a two-stage ping-pong mechanism for oxidation. The value of k^*_{cat} was $0.94 \pm 0.01\ s^{-1}$ when no electrolytes were added to the reaction medium. This value included the characteristics of a particular measuring system and served only as a basic landmark to characterize the effects of salt. The k^*_{cat} value for *A. niger* GOD toward glucose in water has been reported to lie between 0.3 and 337 s^{-1} [28,31], demonstrating a variability of 1000 times, which suggests the essential dependence of the k^*_{cat} value on the characteristics of the method used for measuring.

The addition of NaCl or $MgCl_2$ rapidly reduced the k^*_{cat} value in an exponential manner (with a "half-life" of 0.127 M) and increased the salt concentration to 0.5 M. The observed inhibiting effect was not dependent on the charge of the metal ion or the ionic strength of the solution, suggesting a nonspecific interaction with the enzyme activity center. The inhibiting effect of NaCl and $MgCl_2$ was different at concentrations over 0.5 M. In solutions containing Mg^{2+} ions, the exponential decrease of the apparent catalytic coefficient continued until total inhibition of the enzyme at a Mg^{2+} concentration of 1.9 M. In addition, the inhibiting effect of Na^+ ions was noticeably reduced at Na^+ concentrations over 0.5 M (Fig. 2).

Comparing the dynamics of k^*_{cat} values with the dependence of the calculated reaction parameters on the concentrations of $MgCl_2$ revealed that at higher salt concentrations (above 0.5 M), the inactivation of the enzyme influences the biosensor signal parameters.

Aluminum chloride ($AlCl_3$) inhibited the enzyme at low concentrations (5 mM), and at higher salt concentrations over 0.1 M, the GOD catalytic activity was completely inhibited.

The other variable factor, the oxygen diffusion coefficient, $k^{O_2}_{diff}$, depends on the viscosity of the solution and decreases with increasing electrolyte concentration [23,32]. In pure water, the value of $k^{O_2}_{diff}$ is $1.9 \times 10^{-5}\ cm^2/s$ [33]; in the present system, the value of this coefficient for the Teflon film-covered oxygen optrode (0.5 mm film thickness) in solutions containing no

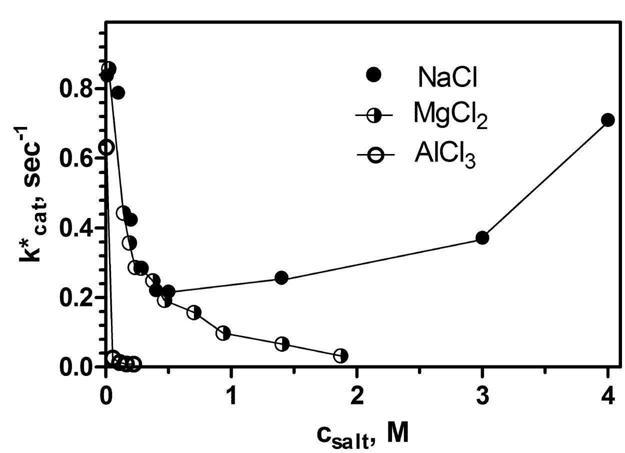

Fig. 2. The dependence of the glucose oxidase catalytic coefficient k^*_{cat} on the concentration of the added chlorides. All measurements were carried out in air-saturated 0.1 M acetic buffer (pH = 5.60) at 25 °C; $c_{glucose}$ = 3 mM, $c_{glucose\ oxidase}$ = 1.3 IU/ml.

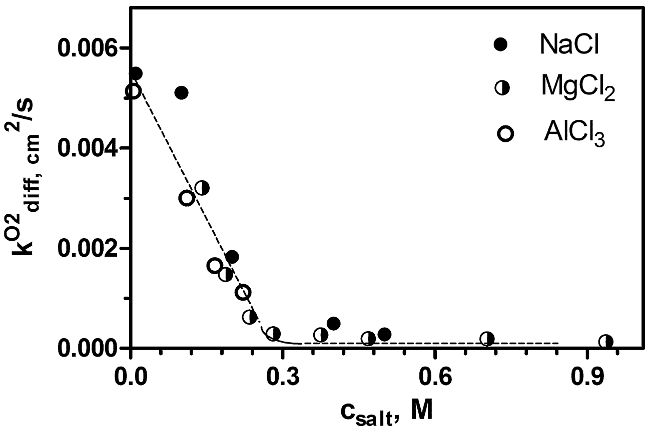

Fig. 3. The dependence of the oxygen diffusion coefficient $k_{diff}^{O_2}$ on the concentration of the added chlorides. All measurements were carried out in air-saturated 0.1 M acetic buffer (pH = 5.60) at 25 °C; $c_{glucose}$ = 3 mM, $c_{glucose\ oxidase}$ = 1.3 IU/ml.

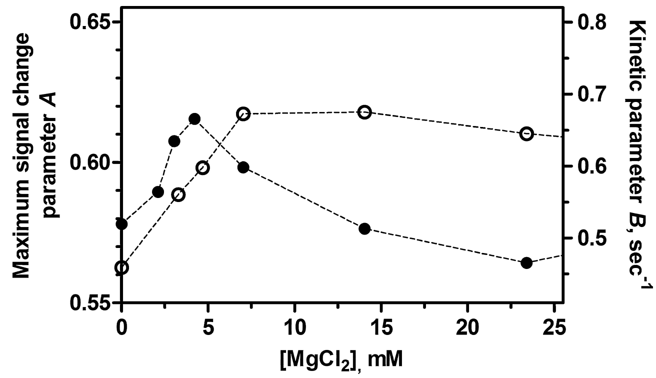

Fig. 4. Lactose biosensor signal parameters at different $MgCl_2$ concentrations (● maximum signal change parameter *A*; ○ kinetic parameter *B*). All measurements were carried out in air-saturated 0.1 M phosphate buffer (pH = 6.50) at 25 °C; $c_{lactose}$ = 0.14 M, $c_{\beta-galactosidase}$ = 10.08 IU/ml, $c_{glucose\ oxidase}$ = 1.3 IU/ml.

electrolytes was $(6.29 \pm 0.80) \times 10^{-7}$ cm^2/s (Fig. 3), which was consistent with the values of the diffusion coefficients for Teflon films at $D_{36.8\,°C} = 2.83 \times 10^{-7}$ cm^2/s (5 mm thick films) [34] or $D_{25\,°C} = 1.52 \times 10^{-7}$ cm^2/s (1.5 mm films) [35]. A linear decrease in $k_{diff}^{O_2}$ was observed with salt concentrations of up to 0.3 M; at higher salt concentrations, the value of $k_{diff}^{O_2}$ approached zero (Fig. 3). The low values of $k_{diff}^{O_2}$ stabilized the biosensor maximum signal change parameter at a defined maximum of 1 (Eqs. (3) and (5)) for $MgCl_2$ (Fig. 1(A2)); however, for NaCl, this biosensor signal parameter was gradually reduced after reaching a maximum value (Fig. 1(A1)), which was consistent with an increase in the catalytic coefficient k_{cat}^* (Fig. 2). For $AlCl_3$, both variables $k_{diff}^{O_2}$ and k_{cat}^* decreased with an increasing salt concentration, and the biosensor signal parameters gradually declined until no reaction was detected. There was no clear dependence of $k_{diff}^{O_2}$ on the ionic strength (data not shown).

For the practical applications of biosensors, the slope and linear range of the calibration curves, based on biosensor signal parameters, determine the quality of the results. The amplification of the measured values of these parameters increased the accuracy and quality of the analysis. For glucose bioassays, the modulation of the GOD-based biosensor signal parameters with NaCl and $MgCl_2$ increased the calculated maximum signal change parameters and the slope of the calibration curve by nearly 20% compared with the initial value.

In addition, the enhanced detection of glucose is also essential in cascaded lactose biosensors, which integrate two consecutive catalytic processes: (1) the hydrolysis of lactose into galactose and glucose, catalyzed through β-GAL; and (2) the consecutive oxidation of the produced glucose, catalyzed through GOD [36]. These biosensors are commonly not applicable for quick analyses due to the relatively low speed of lactose hydrolysis [37]. However, it might be possible to detect smaller quantities of the produced glucose and obtain faster results when the sensitivity of the glucose measurements is increased.

Sodium chloride causes a similar modulation of the maximum signal change parameter *A* in lactose cascaded biosensors, such as the glucose biosensor, and, as expected, this parameter increased by 20% compared with the initial value. The value of kinetic parameter *B* also increased and reached a steady state at a NaCl concentration of 0.5 M, potentially reflecting the continuous generation of glucose through lactose hydrolysis, thereby maintaining constant glucose levels in solution. In addition, the inactivation rate of the enzyme β-GAL is increased in aqueous NaCl solutions [38]. $MgCl_2$ increased both parameter *A* and *B* values, as this salt also activates β-GAL [19,20]. Parameters *A* and *B* were increased by 1.2 and 1.5 times, respectively, at $MgCl_2$ concentrations below 5 mM (Fig. 4). The addition of $MgCl_2$ to a final concentration of 5 mM was optimal to achieve the highest amplification of the lactose biosensor calibration parameters and could be used for the acceleration of lactose analyses. The amplification of the signal parameters is vital for processes conducted at low temperatures, such as milk preservation, where lactose hydrolysis and glucose production are extremely slow.

4. Conclusions

The modulation of biosensor bio-recognition reactions and the biosensor calibration parameters, calculated from the transient phase of the biosensor output signal, were performed in the presence of different chlorides. Sodium chloride and magnesium chloride increased the biosensor maximum signal change parameter by 20% at concentrations below 0.5 M. In addition, the reaction kinetic parameter decreased, but this decrease was diminished at sodium chloride concentrations over 0.5 M. Changes in the reaction catalytic coefficient, k_{cat}^*, and the oxygen diffusion coefficient, $k_{diff}^{O_2}$, at different electrolyte concentrations caused the modulation of the reaction parameters. The modulation of the reaction parameters with magnesium chloride accelerated the lactose measurements, using bi-enzyme cascaded biosensors. The amplification was highest at a magnesium chloride concentration of 5 mM.

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