Electrophoretic Determination of *Lactococcus lactis* Modified by Zinc Ions

B. Buszewski^{1,2} · A. Król^{1,2} · P. Pomastowski² · V. Railean-Plugaru^{1,2} · Małgorzata Szultka-Młyńska^{1,2}

Received: 29 June 2018 / Revised: 3 October 2018 / Accepted: 15 November 2018 / Published online: 29 November 2018
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Abstract

The developed capillary electrophoresis (CE) method was applied to evaluate the aggregation activity of divalent metal ions (zinc) on bacterial cells. In our study, the influence of the *Lactococcus lactis* surface modification by zinc ions at different concentration (1, 3 and 10 mM, respectively) was determined. Electrophoretic results in pseudo isotachophoretic mode indicated that using a $\text{Zn}_{\text{aq}}^{2+}$ caused the uncontrolled aggregation of bacterial cells and, as a result, new signals of microbial agglomerates were observed in the electropherogram. Moreover, the observed process is strongly related with the concentration of the used $\text{Zn}_{\text{aq}}^{2+}$ solution—the number of bacterial agglomerates increased with an increasing concentration of modifier. The phenomenon of *L. lactis* uncontrolled clumping was also evaluated by application of size distribution and spectrometric in infrared range measurements (FT-IR) measurements. The obtained data indicated that the main groups involved in the bacterial cells aggregation process are deprotonated carboxyl and amid groups derived from microbial surface proteins. As a consequence, the proposed mechanism of the *L. lactis* uncontrolled clumping process is presented. In addition, data from fluorescence microscopy pointed out damage cells analyzed direct after electroanalysis. Therefore, CE may be a potential method for evaluating the aggregation activity of different types of bivalent metal ions against microbial cells.

Keywords Capillary electrophoresis · Bacterial aggregation · Mechanism · Zinc ions · *Lactococcus lactis*

Introduction

Nowadays, the use of capillary electrophoresis (CE) for the analysis, identification, and characterization of different types of microorganisms has received much limited attention. This technique has been found to provide many advantages such as high separation efficiency, short analysis time or the possibility of direct analysis of biological samples [1–4]. There

is a few examples of the CE use for the determination of bacterial pathogens [5–7], yeast cells [8] or various types of human and plant viruses [9, 10]. Although this analytical method provides many solutions in the field of the microorganisms identification, it is important to remember about its limitations and drawbacks such as uncontrolled aggregation (clumping) of bacterial cells and their adhesion to the capillary surface [5, 11]. This can also be a source of capillary clogging, if aggregation occurs to a large extent. Moreover, factors such as pH, osmolarity, or high electric fields, can also affect the analysis of bacterial cells by CE [12]. Microorganisms, according to the electrokinetic theory, are often considered as biocolloids, mainly due to the complex structure of their cell wall and formed protonation equilibria at interface, and the understanding of the electrophoretic process is more complicated for such a system. The cell wall composition is characteristic for various types of bacterial species—they have different content of proteins, phospholipids, polysaccharides or another organic components [13]. The structure of the cell wall also determines the division of bacteria into two main types—Gram(+) and Gram(−). The major component is a peptidoglycan, a biopolymer consisting of *N*-acetylmuramic

Published in Chromatographia's 50th Anniversary Commemorative Issue.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10337-018-3665-3>) contains supplementary material, which is available to authorized users.

✉ B. Buszewski
bbusz@chem.umk.pl

¹ Faculty of Chemistry, Nicolaus Copernicus University, 7 Gagarina Str., 87-100 Toruń, Poland

² Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, 7 Gagarina Str., 87-100 Toruń, Poland

acid and *N*-acetylglucosamine with attached the peptide chain of three amino acids [14]. All compounds present in the bacterial cell wall structure strongly affects the surface charge of microorganisms. It can be explained by the presence of many functional groups undergoing the protonation process. Therefore, it is important to develop a method allowing to minimize the uncontrolled aggregation and adhesion problem. Some research groups have proposed the addition of poly(ethylene oxide) (PEO) to the buffer solution [15–17] which functioned as a focusing agent. Kłodzińska et al. [17] have identified the *E. coli* and *H. pylori* in human urine by using PEO in the CE analysis. Another approach is capillary surface modification by the chemicals such as divinylbenzene (DVB) or trimethylchlorosilane acrylamide [18, 19]—Buszewski et al. [18] have used a chemical modification for the separation of the four bacterial strains. Moreover, Yu and Li [20] have emphasized the importance of the proper bacteria samples preparation—the vortex and sonication processes seem to be crucial in the decreasing of the microorganisms aggregation level. Recently, the new approach was suggested; it is based on the modification of the bacterial surface by specific divalent metal ions such as e.g. calcium [17, 21]. Application of this method may result in the controlled aggregation of microorganisms cells. Rogowska et al. [21] have investigated the impact of the cells surface modification by Ca^{2+} ions on the control clumping of *S. cerevisiae* and on the effectiveness of electrophoretic mobility. Although several surface modifications by calcium ions have been evaluated for the separation of microorganisms [17, 21], there is a lack of papers described bacteria surface modification via another types of divalent metal ions, e.g. zinc ions (Zn^{2+}). Therefore, understanding the influence of different ions on microorganisms surfaces and their potential use for further modifications, is pivotal for analytical chemistry.

Such a problem led our research group to the main task of the experiments presented in this paper - the aim of this study was to investigate the electrophoretic behavior of lactic acid strain of Gram(+) *Lactococcus lactis* during CE analysis and to examine the influence of $\text{Zn}_{\text{aq}}^{2+}$ ions at different concentration on the bacterial cells aggregation. Furthermore, the size distribution, spectrometric in infrared range measurements and viability assay of *L. lactis* before and after CE analysis by using fluorescence microscopy was performed.

Materials and Methods

Materials

All solvents and materials were purchased from Avantor Gliwice (Poland). Ultra-pure water was obtained from Milli-Q RG system by Millipore (Millipore Intertech, Bedford, MA, USA). *Lactococcus lactis* 56 used in this study, deposited

in the Polish Collection of Microorganisms (PCM) under deposit no. B/00116 [22], were cultured in Tryptone Soya Agar (Soybean Casein Digest Medium, Oxoid, Basingstoke, United Kingdom).

Sample Preparation for CE Analysis

The required number of cells in 1 mL of the suspension was achieved by the serial dilution and the final number of *L. lactis* cells was 9×10^8 CFU/mL. Afterwards the bacteria were separated from the medium by centrifugation (20 °C, 9000 rpm, 15 min). The bacterial pellet were then suspended in the solution of zinc nitrate at 1, 3 or 10 mM concentration and incubated for 1 h at the room temperature. After the incubation, the suspension was centrifuged (20 °C, 9000 rpm, 15 min). The obtained precipitate was washed twice with distilled water to remove unbound Zn^{2+} ions and after that transferred to the outlet TB buffer (Tris and boric acid; pH = 8.0). As a control, unmodified *L. lactis* cells were used.

Capillary Electrophoresis Analysis

CE analysis were performed using PA 800 plus Beckman Coutner system, Brea, CA, USA equipped with a DAD with the use of fused silica capillary (id = 75 μm ; L_{tot} = 33.5 cm; L_{eff} = 25 cm; Composite Metal Services, Shipley, United Kingdom). Before the use, a new capillary was rinsed with 1 M NaOH, deionized water, and TB (outlet buffer) for 10 min. The bacterial samples were injected into the capillary with a pressure mode (10 psi, 8 s) and the analysis were performed at a constant voltage (20 kV) and the temperature at 23 °C. As the inlet buffer, TBH (Tris, boric acid and hydrochloric acid; pH = 7.3) were choose. The samples were detected at $\lambda = 214$ nm. Between runs, the capillaries were washed with 0.1 M NaOH, deionized water and running buffer for 2 min each.

The electrophoretic mobility of *L. lactis* during the CE assay was calculated from the equation:

$$\mu_e = \frac{L_{\text{tot}} L_{\text{eff}}}{V} \left(\frac{1}{t_m} - \frac{1}{t_{\text{EOF}}} \right), \quad (1)$$

where μ_e is an electrophoretic mobility, L_{tot} is the total length of the capillary, L_{eff} is the length to the detector, t_m is the migration time and V is the applied potential.

Fourier Transform Infrared Spectroscopy Analysis

The determination of active functional groups present on the *L. lactis* surface before and after $\text{Zn}_{\text{aq}}^{2+}$ modification were performed using FT-IR method (Direct Detect spectrophotometer, Merck Millipore, Germany). 2 μL of sample was

pressed into a card and dried. All IR spectra were recorded at room temperature in the range of $\nu = 1350\text{--}1850\text{ cm}^{-1}$.

***L. lactis* Cells Size Determination**

The size of non-modified and modified *Lactococcus lactis* cells was determined using the Mastersizer 3000 (Malvern Panalytical, Malvern, United Kingdom).

Fluorescence Microscopy Analysis

Determination of *Lactococcus lactis* cells viability before and after the CE analysis was performed by using fluorescence microscopy approach according to the [22]. During the CE analysis, fractions of bacterial cells not and modified with zinc ions were collected. Obtained bacterial samples were then stained with acridine orange ($0.12\text{ }\mu\text{g/mL}$) and ethidium bromide ($0.4\text{ }\mu\text{g/mL}$) and analyzed using a Zeiss Axio Observer.D1 (Zeiss, Oberkochen, Germany) fluorescence microscope with the set of filters (43 He and 38). Recorded images were analyzed with Axio Vision 4.8. software.

Results and Discussion

In this study, Gram(+) *Lactococcus lactis* modified with the different concentration of zinc ions (1, 3 and 10 mM) were examined in an applied voltage of 20 kV. Bacterial strain without any surface modification was tested as a control sample (Fig. 1a). The electromigration time of the probiotic strain modified by the zinc nitrate solution at concentration of 1, 3 and 10 mM was 2.026 (RSD = 0.784%), 2.064 (RSD = 1.287%) and 2.083 (RSD = 0.512%) min respectively. Application of electrophoretic buffers (TB and TBH; isotachophoretic mode of CE) and without the surface modification by $\text{Zn}_{\text{aq}}^{2+}$ allowed focusing the zone at the electromigration time of 1.670 min (RSD = 1.794%). It is in a good correlation with the results obtained by Pomastowski et al. [23], who have observed the peak of *Lactococcus lactis* ATCC 11454 at migration time of about 2 min. Such a short electromigration time can be explained by the higher zeta potential of Gram(+) strain in comparison with Gram(−) bacterial cells in used buffer system [23]. All electrophoretic analysis were in the range of 0.1–3% RSD (Table S1). Moreover, the value of *L. lactis* electrophoretic mobility ($\mu\text{, cm}^2/\text{Vs}$) non- and modified with zinc ions were decreasing with the increasing CE duration time (Table S1).

Adhesion to the capillary surface and uncontrolled clumping of bacterial cells is a significant drawback of capillary electrophoresis and many researcher groups have tried to develop an efficient method to eliminate this problem. One of the popular approach is bacterial surface modification by

specific divalent metal ions such as e.g. calcium, which is believed to be one of the most important factors enabling aggregation of bacterial cells [24, 25]. Rogowska et al. [21] have examined the impact of the cells surface modification by Ca^{2+} ions on the control of the yeast cells analysis by capillary electrophoresis. The results pointed out the crucial impact of the microorganism surface modification on its electrophoretic mobility. During the CE analysis of *Saccharomyces cerevisiae* modified by 5 mM Ca^{2+} and with the increasing value of medium pH, they observed the increase in the electromigration time of modified yeast cells and decrease in the electrophoretic mobility. In addition, the surface modification resulted in the sharpening of the peaks and the reduction in the number of aggregates of *S. cerevisiae*. It was correlated with the function of calcium ions in the controlled clumping of yeast cells by the binding surface-carboxyl groups or lectin-like mechanisms of flocculation in which Ca^{2+} enables the lectins to acquire a suitable active conformation and thus interact with carbohydrate residues of α -mannans present on the cell surface [24]. Furthermore, the study of Rogowska et al. [21] have shown that pH value of culture medium is a significant factor since its changes the ionization of the functional residues present on the cell surface, mainly the carboxyl group, thus modifying the availability of Ca^{2+} and allow the collecting of *S. cerevisiae* zone in CE. The same observation have described Pomastowski et al. [23] who have noted that the change in the electrophoretic mobility of different types of bacteria cells and the reduction of repulsive forces by Ca^{2+} surface modification resulted in signal amplification. However, described results indicated the promising use of calcium ions in the controlled clumping of microorganisms [21, 23], the role of other physiologically important bivalent metals has been largely neglected. Accordingly, the main aim of our study was to investigate the impact of *L. lactis* surface modification with $\text{Zn}_{\text{aq}}^{2+}$ at their electrophoretic mobility and aggregation process. The most widely used thermodynamic model for the description of the aggregation phenomenon is DLVO (Derjaguin, Landau, Verwey, Overbeek) theory. Bacterial adhesions mediated by interplay between Lifshitz–van der Waals forces and electrostatic interactions originating from the overlap of electrical double layers and the solid surface. This theory has been extended to acid–base interactions [24]. It is in a good correlation with the chemical properties of the zinc element and may allow to described the possible mechanism of the *Lactococcus lactis* uncontrolled clumping mechanism.

Zinc is one of the most essential microelements and is crucial for the proper function of a large number of macromolecules and enzymes as far it participates in bone mineralization, healing wounds and is responsible for the proper functioning of the immune system or insulin secretion [25]. In 1956, G. Schwarzenbach and Ahrland Chatt and Davis

Fig. 1 Electropherogram of non-modified *L. lactis* (**a**); modified *L. lactis* by 1 mM (**b**), 3 mM (**c**) and 10 mM (**d**) $\text{Zn}(\text{NO}_3)_2$. Conditions: inlet buffer: TBH (pH=7.3), outlet buffer: TB (pH=8), suspensive

buffer: TB (pH=8); $I=100\ \mu\text{A}$, $U=20\ \text{kV}$, $t=23\ ^\circ\text{C}$, $\lambda=214\ \text{nm}$, $L=33.5\ \text{cm}$, $L_{\text{eff}}=25\ \text{cm}$, $\varphi=100\ \mu\text{m}$, injection: 10 psi, $t=8\ \text{s}$

[26, 27] classified the metal ions into two classes called *a* and *b* types. Those two research groups have explained that the class *a* of metals prefer to bind with class *a* of donors and accordingly class *b* of metals prefer to interact with class *b* of donors. It was also proved that such an interaction shall lead to the formation of a more stable compound. Metal cations (Me^{2+}) with the completely filled d-electron shells such as e.g. zinc ions (Zn^{2+}), belongs to the second group in which the interactions with the ligand are predominantly covalent. Zinc has also an exceptional coordination chemistry - in the acidic aqueous solution zinc forms the colourless stable aqua complexes, $[\text{Zn}(\text{H}_2\text{O})_6]^{2+}$ which are acids according to the Brønsted–Lowry theory [28]. Furthermore, it may exchange their water molecules when binding to other ligands [29]. The interaction between zinc and ligands (water or organic anions) is highly connected to its nature but is not widely described in the literature. The polarization of water

molecules by the central zinc ion causes the dissociation of a proton and as a result, an aqua-hydroxo complex is formed. Further, the deprotonation process of zinc aqua complexes leads to the formation of species with different charges - they are crucial for interaction with proteins and other groups of ligands [29–30].

Despite its pivotal properties, zinc ions may play an important role in the adhesion and biofilm formation by microorganisms. Biofilms are the most common type of bacterial growth in nature and consist of the microbial cells adhesion within the extracellular polymeric substances (EPS), a composition of extracellular polysaccharides and proteins [31]. However, in contrast to calcium chemistry, the zinc ions are d-electrons elements, possess Lewis acid properties, caused the spontaneous interaction with active Lewis base such as amino groups components of bacterial cell walls [32]. Moreover, the ability to form biofilms and

aggregates is characteristic of some lactic acid bacteria (LABs) such as e.g. *Lactobacillus rhamnosus* or *Lactobacillus plantarum* [33]. The formation of the specific microbial biofilm is affected by many factors, including the properties of the bacterial surface, medium pH and composition or temperature. Another crucial factor can be addition of the metal ions to the nutrient or surface modification by Me^{2+} . Work of the Formosa-Dague [34] have shown that zinc ions strongly increases the bacterial cell wall rigidity and activates the adhesive function of *Staphylococcus aureus* surface protein (SasG). They have tested the influence of the 1 mM zinc chloride (ZnCl_2) on the biophysical properties of the *S. aureus* surface. After addition of the zinc ions, cell aggregates at 5–10 μm in size were observed in bacteria with the SasG protein expressing. Those results were confirmed by data collected from the AFM study which pointed out that addition of Zn^{2+} greatly changed the adhesive properties of microbial surface and caused formation of bacterial agglomerates. Brown et al. [35] also investigated the influence of the zinc on the biofilms formation and their results shown that with the Zn^{2+} concentrations increase, the aggregation of bacteria rise too. The described effect was found to be zinc-specific, because another types of ions (copper and manganese) did not affect biofilm formation. Outcomes of our study show that after the *L. lactis* surface modification by $\text{Zn}_{\text{aq}}^{2+}$ ions, the formation of bacterial aggregates with different size and surface charge occurred; it was observed by an increasing number of signals in the electropherograms (Fig. 1b–d). The number of bacterial agglomerates rises with an increasing concentration of modifier. In the case of 1 and 3 mM modification, the number of CE zones occurred at 18, whereas using of 10 mM zinc nitrate resulted in the observation of 23 signals. What is more, the study of the size of modified *Lactococcus lactis* cells indicates that the zinc ions have a significant impact on the size distribution of the examined cells in comparison with the control sample (Fig. 3). It corresponds to the predicted size of unmodified *L. lactis*—from 0.5 to 1.5 μm [36]. The observed differences in the intensity, number and time of some signals, can be a consequence of the various sorption and interaction of zinc at different concentration level with active functional group of e.g. bacterial proteins. Our previous work [37] have shown that addition of 3 mM zinc nitrate to the *Lactobacillus paracasei* LB3 cell suspension have resulted in the intracellular formation of zinc oxide nanocomposites which could also form aggregates and be observed as a multiplied signals on the CE electropherogram. According to their data from FT-IR analysis, the main groups involved in the ZnO biosynthesis are carboxyl and amid groups derived from bacterial proteins. It is strongly related with results obtained in our study—Fig. 2 shows the FT-IR spectra of *L. lactis* unmodified and modified by $\text{Zn}_{\text{aq}}^{2+}$ ions. It indicated that the main groups involved in the bacterial cells aggregation

process are deprotonated carboxyl groups (spectral bands at $\nu = 1530\text{--}1560\text{ cm}^{-1}$) (2) which can derive from aminoacids of bacterial proteins as well as peptidoglycan of their cell wall [38, 39]. The spectra band at $\nu = 1610\text{--}1670\text{ cm}^{-1}$ (3) corresponds with amide groups of bacterial proteins. Another characteristic band appears at $\nu = 1440\text{ cm}^{-1}$ (1) may derives from the stretching vibration of C–N and N–H bond from surface microbial proteins. Furthermore, as a result of the modification, the increase of the signal at $\nu = 1720\text{ cm}^{-1}$ (4) intensity can be observed. It is related with the stretching vibration of carbonyl groups (C=O). The obtained data prove that the examined bacterial strain exhibits the ability of the sorption of divalent metal cations such as zinc ions. Additionally, it can be observed that the most significant changes occur in the spectrum of the *Lactococcus lactis* modified by 10 mM zinc nitrate. It is in a good correlation with the knowledge about interactions between zinc and functional groups presented on the biocolloids surface such as bacterial cells. Pomastowski et al. [39] have also performed the FT-IR analysis and proved that carboxyl groups of amino acids residues (e.g. asparagine and glutamine) are mainly involved in the described phenomena. The proposed mechanism of the uncontrolled clumping of *Lactococcus lactis* caused by the $\text{Zn}_{\text{aq}}^{2+}$ ions is shown in the Fig. 4.

Capillary electrophoresis has already been useful in the identification of pathogens [5–7], yeast cells [8] or various types of viruses [9, 10]. A CE approach has also been applied for the direct detect of the Hela cells apoptosis [40].

Fig. 2 FT-IR spectra for non-modified *L. lactis* (a) and modified *L. lactis* by 1 mM (b), 3 mM (c) and 10 mM (d) $\text{Zn}(\text{NO}_3)_2$, ν (cm^{-1}): 1: 1420–1480, 2: 1530–1560, 3: 1610–1670, 4: 1710–1740

Fig. 3 Size distribution of non- and modified *L. lactis* cells by $\text{Zn}_{\text{aq}}^{2+}$

Due to the promising use of capillary electrophoresis to determine cell viability, it is necessary to investigate and describe its effect on the microorganisms lifespan first. Ebersole and McCormick [41] have performed the separation of the *E. faecalis*, *S. pyogenes*, *S. agalactiae*, *S. pneumoniae* and *S. aureus* by CZE. The data from their study have indicated that more than 90% of the bacterial cells remained viable after separation. Szumski et al. [42] have executed the assessment of the viability of *S. aureus* and *E. coli* cells during capillary electrophoretic process. They have tested two different applied voltage (20 kV and 30 kV) and collected bacterial zones after CE were cultivated on the Broth Agar. In comparison to the control sample (without electric treatment, 0 kV), only 3.4% and 0.7% of the *E. coli* cells survived the applied voltage treatment of 20 kV and 30 kV, respectively. *S. aureus* strains were found to be more resistant to the electric field—results of the experiment have shown that about 35% and 20% *S. aureus* cells survived voltage of 20 kV and 30 kV, respectively. Szumski and coworkers [42] have emphasized such a significant differences is related to the different cell wall composition of treated strains. The bigger peptidoglycan layer of Gram(+) (*S. aureus*) cell wall make them more tough and resistant to capillary electrophoresis conditions. Data from the described papers [40, 41] are in a good correlation with those received in our study—in the fluorescence microscopy assay the amount of total cells before CE were compared to the control after the CE

analysis (Fig. 5). After staining with an acridine orange and ethidium bromide, the live cells exhibit green fluorescence and dead one are visible as a red. Both of used dyes are able to intercalate or bind with the nucleic acid (DNA/RNA) present in organisms. This kind of interaction is the result of the electrostatic interactions of dyes molecules between the nucleic acid base pairs. The results of the fluorescent microscopy analysis indicated that capillary electrophoresis do not cause the death of microbial cells but only their damage (yellow colour on the Fig. 5). It can be explained by the fact if *Lactococcus lactis* used in our study is the Gram(+) strain with the thick peptidoglycan layer in its bacterial cell wall. Application of the fluorescence microscopy confirmed also that the modification of the bacterial cell surface with 1, 3 and 10 mM zinc ions causes their uncontrolled aggregation (Fig. 5b, c). This result is in good correlation with the data presented on the electropherograms (Fig. 1b, c, d). The results obtained during the experiment also indicate that zinc ions with the highest concentration (10 mM) caused the higher aggregation of the *Lactococcus lactis*.

Conclusions

In this work, CE was used to evaluate the effect of zinc ions at different concentration level (1, 3 and 10 mM) on the *Lactococcus lactis* cells aggregation. This study confirms

Fig. 4 The proposed mechanism of the *L. lactis* uncontrolled clumping

the occurrence the formation of bacterial cells agglomerates under the specific surface modification. According to the FT-IR data, the main groups involved in those process are carboxyl and amid groups which may derive from surface bacterial proteins. The results of the fluorescence microscopy showed that *L. lactis* cells stay alive after surface modification and during capillary electrophoresis. In comparison with previous work focused on the microbial

surface modification by calcium ions, it can be concluded that Zn_{aq}^{2+} is not sufficient for the *Lactococcus lactis* controlled clumping in CE approach. However, the described work shed new light on several important issues, including an interpretation of probiotic bacteria aggregation process and understanding the influence of zinc ions on microorganisms surfaces and their potential use for further modifications, which seems to be crucial for separation science field.

Fig. 5 *Lactococcus lactis* non-modified (**a**) and modified by 1 mM (**b**), 3 mM (**c**) and 10 mM (**d**) of $\text{Zn}(\text{NO}_3)_2$ after the capillary electrophoresis

Acknowledgements This work was supported by the Opus 11 No. 2016/21/B/ST4/02130 (2017–2020) from the National Science Centre, Poland. Moreover, we would like to thank Dr Tomasz Kowalkowski for technical assistance in size distribution analysis.

Compliance with ethical standards

Conflict of interest The authors declared that they have not conflicts of interest.

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