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INFLUENCE OF CALCIUM IONS ON THE SURFACE CHARGE DENSITY AND LIGHT-SCATTERING IN *PLECTONEMA BORYANUM* SPHEROPLASTS

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Summary. The effect of divalent Ca^{2+} ions on the changes of surface charge density (SCD) and light-scattering (LS) in spheroplasts of the cyanobacterium *Plectonema boryanum* was studied.

Cells were grown on 3 different culture media as follows: an iron-sufficient medium (as a "control" variant), an iron-deficient medium (as a "Fe-starved" variant) and an excess of iron supply medium (as a "20×Fe" variant). Calcium ions induced changes in surface charge density due to cell membrane alterations. Ca^{2+} binding induced a large increase in net negative SCD of "20×Fe" spheroplasts vesicles as it was shown in the electrophoretic mobility experiments. On the contrary, a significant reduction in the net negative SCD of the "control" and "Fe-starved" spheroplasts after $CaCl_2$ treatment was registered.

After calcium binding the LS of the "control" and "Fe-starved" spheroplasts vesicles were altered in a higher extent than was that of the "20×Fe" spheroplast particles.

The application of the electrokinetic and LS methods based on the biophysical characterization of the spheroplast model structure upon an iron stress applied, was discussed.

Key words: calcium ions, cyanobacterium, light-scattering, *Plectonema boryanum*, spheroplast, surface charge density

Abbreviations: EPM – electrophoretic mobility, HEPES – 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, LS – light-scattering, SCD – surface charge density (σ).

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Introduction

The microbial accumulation of heavy metals and a potential application for photosynthetic prokaryotes has been reviewed recently (Gadd, 1988; Reed and Gadd, 1990).

The influence of Ca^{2+} ions on the electron transfer and oxygen evolution in the cyanobacteria may differ from this in higher plants (Debus, 1992). Ca^{2+} requirement for cyanobacterial cells may also be different from these in plants as it is among various species of cyanobacteria (Debus, 1992). For example, thylakoid membranes from *Synechocystis* sp. PCC 6714 have been reported to require >5 mM Ca^{2+} for maximal oxygen-evolving activity (Astier et al., 1986).

Because of their indisputable application in biotechnology, *Pl. boryanum* spheroplasts were used in our study as a model system to examine the stress responses against different iron contents in the cultural medium. It is known that spheroplasts are osmosensitive wall-free vesicles whose modified peptidoglycan can no longer control their morphology (Papageorgiou, 1989).

Three variants of cyanobacterum were examined to ascertain the extent of cation binding ability to the spheroplast surfaces of "iron-starved" and of "excess of iron supply" variants compared to the "iron-sufficient" variant in a low ionic strength medium.

As most biological membranes, spheroplasts isolated *in vitro* as closed vesicles are negatively charged at neutral pH. According to Riviere et al. (1988) the value of σ of isolated membrane vesicles from *Anacystis nidulans* in normal growth medium was calculated by laser light-scattering technique to be -2.0 ($e^{-}/\mu m^2.10^{-4}$). There is no information available about SCD of spheroplast vesicles from *Plectonema boryanum*. We tried to study their electrokinetic behaviour in dependence on iron content in cultivating medium.

Compensation of the negative surface charge by screening and by neutralization by metal cations (i.e. $CaCl_2$) has been described (Papageorgiou, 1989). The role of Ca^{2+} on the electrokinetic properties and the aggregation ability of the spheroplast vesicles by light-scattering was assessed.

According to Benderliev et al. (1988), the electrokinetic approach is helpful for registration of cell damage caused by free radical attack and lipid peroxidation.

The aim of the present study was to give an information about electrokinetic behaviour of the spheroplasts in cases of iron stress.

Materials and Methods

Plectonema boryanum Gom. (*Leptolyngbya boryana* (Gomont), Anagnastidis et Komarek), strain 594, from Cyanobacterial Culture Collection, Sofia University, was grown autotrophically on the nutrition medium of Allen & Arnon (1955). It was cultivated in 200 ml vessels. Carbon source was provided by bubbling sterile 2% (vol/vol)

CO_2 in air through the cultures. Illumination was provided by luminescent lamps at light intensity of 8 000 lx, at 32 °C.

Pl. boryanum was cultivated intensively at three different iron concentrations in the nutrition medium: 9.4 mg/l (control variant); 20 times higher quantity of iron (188 mg/l) and in iron-free growth medium. After 48-hours of cultivation the algal cells were harvested at the end of the exponential phase of growth by filtration and were washed twice with distilled water. For the spheroplast preparation the cells were suspended in 50 ml of 0.5 M sucrose, 2% BSA, 30 mM potassium phosphate buffer (pH 7.0), and 1 mg/ml lysozyme (Ivanova, 1987) and then incubated in the dark for 4 hours at 37 °C on the "Elpan" water bath shaker, type 357. The lysozyme treatment was stopped by centrifugation for 10 min at 5 000×g at 4 °C. Sedimented spheroplasts were washed twofold by a buffer containing 0.5 M sucrose, 30 mM potassium phosphate buffer (pH 7.0) and 2% BSA. The final chlorophyll *a* concentration of the spheroplast suspension was approximately 6 µg chl/ml. The preparation was stored on ice in the dark until used (within 2 h).

To 20 ml of buffer containing 25 mM HEPES/KOH, 10 mM NaCl (pH 7.50), 200 µl of the spheroplast suspension were added.

EPM studies were performed using an OPTON Cytopherometer (Doltchinkova et al., 1993). The observation light (with intensity of 13 $\mu E/m^2.s$) was filtered by a green (545 nm) interference filter through a 16 V/15 W lamp. To obtain the photoinduced effect the observation light with intensity of 920 $\mu E/m^2.s$ was achieved by removing the green filter. After a minute of illumination of the spheroplast suspension the green filter was reinserted and the EPM of the sample was measured.

For a negatively-charged surface bathing in a mixed solution containing monovalent (NaCl) and divalent ($CaCl_2$) salts, the surface charge density, σ, was determined according to Chow et al. (1991).

The aggregation of the spheroplasts was measured by light-scattering at 480 nm as a function of cation concentration using the Specol 10 Spectrophotometer (Carl Zeiss Yena). The scattering level at an angle of 90° represented the degree of aggregation.

Data were averaged of triplicate measurements.

Results

Effects on the EPM of three types spheroplasts were examined over a low concentration range of Ca^{2+}.

Ionic binding and shielding of surface charges caused a decrease in "control" spheroplasts EPM, as it was seen when the $CaCl_2$ concentration was increased (Fig. 1).

Addition of 0.5 mM $CaCl_2$ to the "control" spheroplast suspension reduced the EPM from -2.40×10^{-8} $m^2/V.s$ (without $CaCl_2$) to -1.24×10^{-8} $m^2/V.s$. The electrostatic effect at 0.5 mM $CaCl_2$ concentrations was decreasded to 51% (see legend below

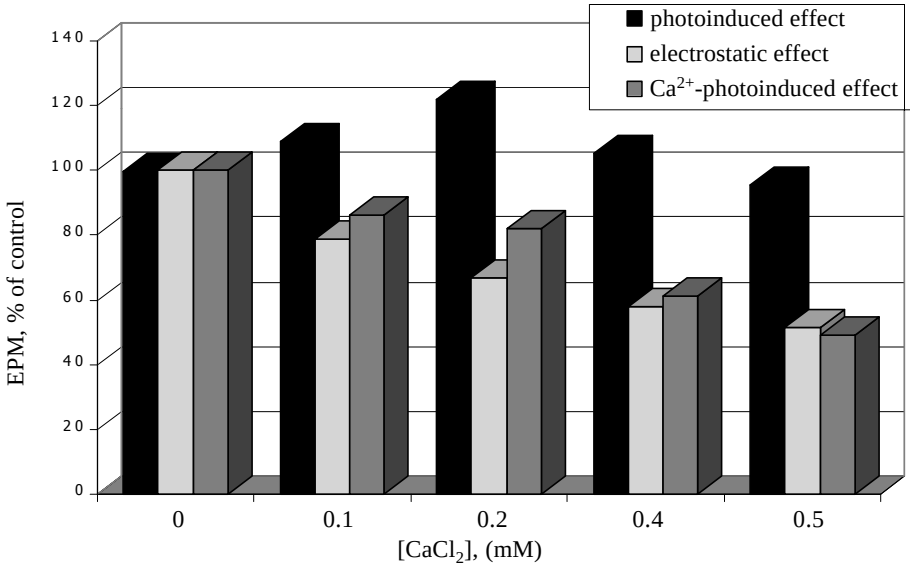

Fig. 1. Effects of Ca^{2+} ($CaCl_2$) on the EPM changes in spheroplasts of *Plectonema boryanum*, cultivated on iron-sufficient nutrition medium. Suspending medium consisted 25 mM HEPES (KOH) pH 7.5, 10 mM NaCl ($I = 0.01 mol/dm^3$). The electrostatic effect was calculated as ratio of EPM with $CaCl_2$ normalized EPM in the absence of calcium ions ($100\% = -2.42 \times 10^{-8} m^2/V.s$, $n = 20 \div 27$, $p < 0.01$). The effect of Ca^{2+} and photoinduction was measured after 1 min preillumination at 25 °C and chlorophyll *a* concentration of 6 mg/ml in the same buffer medium ($100\% = -2.41 \times 10^{-8} m^2/V.s$, $n = 21 \div 28$, $p < 0.05$). The photoinduced effect was given relatively to the EPM without light treatment.

Fig. 1). A similar trend to decrease in the negative surface charge caused by cation binding after the illumination was observed (Fig. 1, Ca^{2+}-photoinduced effect). A significant enhancement of photoinduced EPM effect with 21.9% at the "control" spheroplasts in the presence of 0.2 mM $CaCl_2$ in the suspension medium was registered (Fig. 1). The higher level of the photoinduced EPM effect of spheroplasts as compared to the untreated particles with different concentrations of $CaCl_2$ was explained as being indicative of a light-stimulated generation of fixed negative surface charges on their surfaces.

As it was shown in our previous study (Doltchinkova et al., 1995), after photoinduction the negative SCD was increased by 30% for "iron-starved" spheroplasts in comparison with the "iron-sufficient" particles. The $CaCl_2$ treatment was tested on the EPM of the vesicles as follows: "control" spheroplasts at $100 \div 500$ µM concentration range, "Fe-starved" spheroplasts at $25 \div 200$ µM concentration range and "20×Fe" spheroplasts at $1 \div 10$ µM. The higher Ca^{2+} concentrations needed to neutralize the surface charges led to a creation of large spheroplast aggregate complexes and impeded the EPM measurement.

We investigated the electrostatic effects of Ca^{2+} on the above mentioned spheroplast variants as it was shown by the percentage changes in EPM caused by calcium

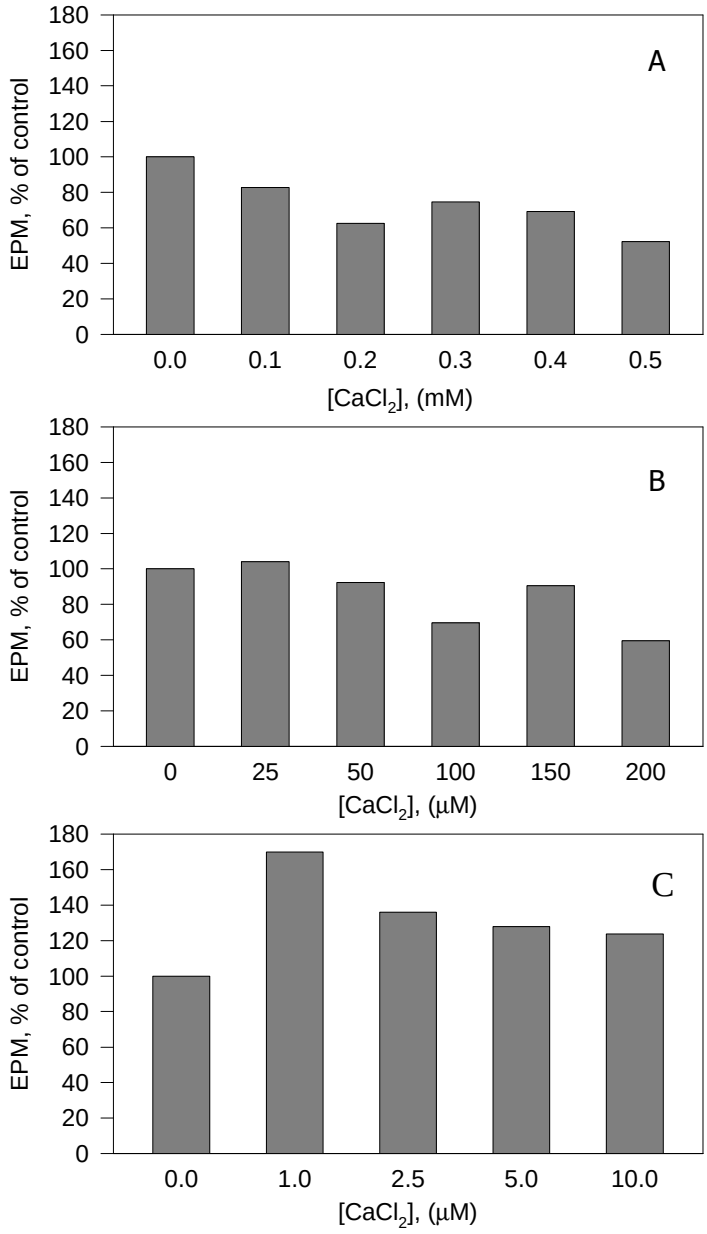

Fig. 2. Effects of $CaCl_2$ concentration on EPM of *Plectonema boryanum* spheroplasts. Suspending medium was the same as in Fig.1. **A**: spheroplasts from cells grown on iron-sufficient medium (100% = -2.37×10^{-8} m^2/V.s, n = 20÷24, $p<0.05$); **B**: spheroplasts from cells grown on iron-deficient medium (100% = -1.97×10^{-8} m^2/V.s, n = 15÷20, $p<0.05$); **C**: spheroplasts from cells grown on 20-fold increased content of iron supply medium (100% = -1.60×10^{-8} m^2/V.s, n = 15÷20, $p<0.05$).

binding (Fig. 2). We obtained similar EPM decrease after $CaCl_2$ treatment of both "control" and "Fe-starved" vesicles (Fig. 2A, B). There was a noticeable reduction of 17% in the EPM of "iron-starved" spheroplasts (-1.97×10^{-8} m^2/V.s) compared to the EPM of "iron-sufficient" ones both without $CaCl_2$ in the suspending medium (-2.37×10^{-8} m^2/V.s). Evidently, the EPM changes observed were not connected with a significant structural deterioration of the plasma membrane.

In case "20×Fe" spheroplast preparations were suspended in a low ionic strength HEPES buffer, addition of 1 μM calcium cations induced a significant enhancement of EPM (Fig.2, C), from the level of -1.60×10^{-8} m^2/V.s to -2.72×10^{-8} m^2/V.s. At higher concentrations (2.5–10 μM) $CaCl_2$ the increase in EPMs was lower than that for 1 μM $CaCl_2$ EPM values. The net negative charges on plasma membrane were probably due to the maximum quantities of lipids and carbohydrates reached in the cyanobacterial cells at iron sublethal concentrations in suspension medium (Chaneva et al., 1995).

Mobility measurements allowed us to monitor the reorientation of the spheroplasts, their "linear aggregates" and polarized ruffle structures, as in the case of the "20×Fe" spheroplasts (σ = -0.0024 C/m^2). Fig. 2 A and B illustrates that Ca^{2+} ions were adsorbed more tightly to spheroplast vesicles at the concentrations we had examined: The electrostatic effect was reduced for all $CaCl_2$ concentrations for the "Fe-starved" spheroplast structures, except for 25 μM $CaCl_2$, where no EPM effect was detected.

Finally, the concentration of calcium cation required to produce charge neutralization, decreased (Fig. 2 A, B).

In case the net negative surface charge of the spheroplasts was shielded by the addition of cations, the plasma membranes coalescence and a change in light-scattering properties would be expected in these spheroplast suspensions. We measured LS at an angle of 90° to obtain an indication of aggregation in the various spheroplast preparations (Fig. 3). Calcium-influenced aggregation of the three types of spheroplasts was investigated in dependence of the different concentrations of $CaCl_2$. Thus, in the presence of 0.1 mM $CaCl_2$ an increase in the degree of aggregation was observed as shown by an increase in LS up to 120% (Fig. 3A) resulting from a maximal Ca^{2+} binding to the negatively exposed residues on the spheroplast surfaces. Absence of changes in the LS of calcium-treated spheroplasts was found at the concentration range of 0.2–0.5 mM $CaCl_2$ in suspension.

Vesicle aggregation experiments were conducted for "Fe-starved" spheroplasts as a function of Ca^{2+} as well (Fig. 3B). Only at 100 μM Ca^{2+} the degree of aggregation decreased and a LS minimal value (83.5%) was obtained.

In contrast, the "20×Fe" vesicles displayed little or no aggregation in the presence of Ca^{2+}. The results were shown in Fig. 3C and demonstrated that "20×Fe" spheroplasts were induced to aggregate to a higher extent with 5 μM Ca^{2+}, indicating that calcium ions were adsorbed to the spheroplast membrane surfaces at concentrations of 5 and 10 μM Ca^{2+}.

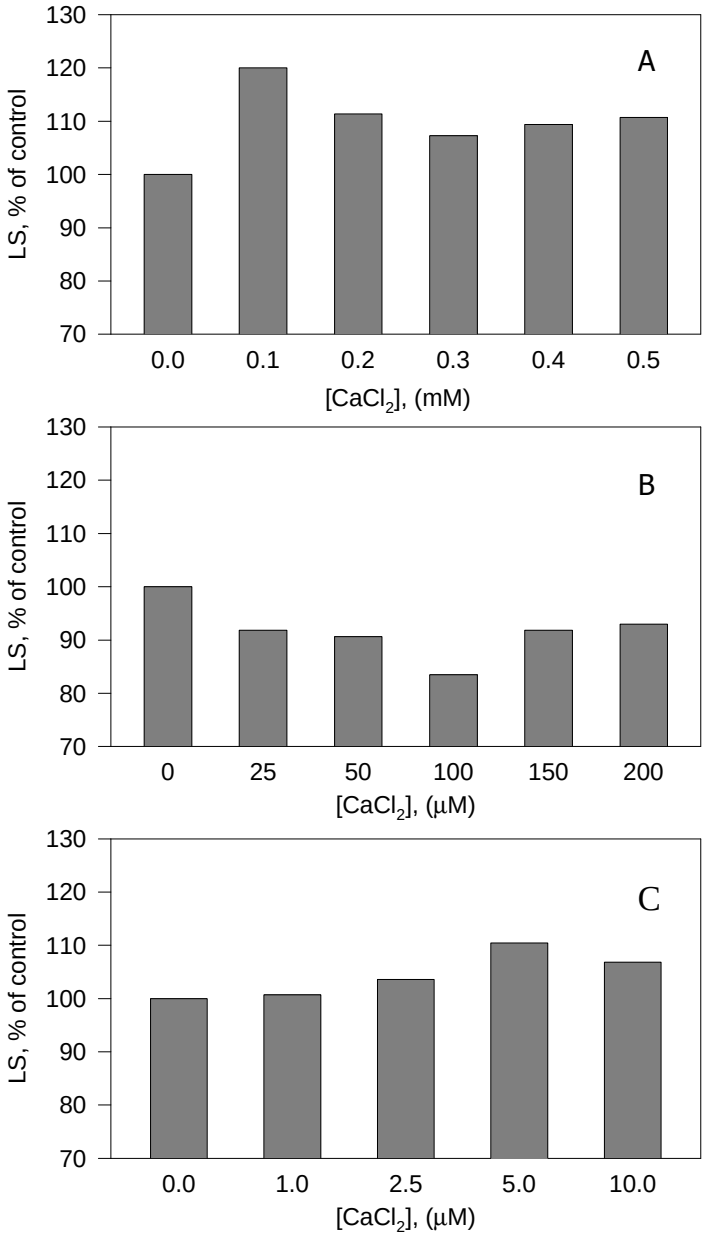

Fig. 3. LS dependencies of *Plectonema boryanum* spheroplasts in the presence of $CaCl_2$ and suspending media as shown in Fig. 1. **A**: spheroplasts from cells grown on iron-sufficient medium (100% = 149 arbitrary units); **B**: spheroplasts grown on iron-deficient medium (100% = 85 arbitrary units); **C**: spheroplasts grown on 20-fold increased content of iron supply medium (100% = 139 arbitrary units).

The results shown in Fig. 2 and 3 also indicated that the extent of these changes in LS values depended on spheroplast variant studied (i.e. indirectly on iron content in growth culture medium). The EPM of "20×Fe" spheroplast vesicles was strongly altered due to most probably the electrostatic interactions of calcium cations to the negatively surface charge exposed groups on the spheroplast membrane in comparison to the EPM of "iron-sufficient" particles.

For "Fe-supplied" variant the concentration of 0.1 mM $CaCl_2$ caused a maximal increase of LS value (Fig. 3A). Contrary, in "Fe-starved" spheroplasts at the same calcium concentration the maximal decrease of the LS effect was registered (Fig. 3B).

Discussion

Experimental data showed that the surface charge density affects the binding of calcium cations to spheroplasts significantly.

A significant reduction in the net negative SCD of "control" (from -0.0040 C/m^2 without $CaCl_2$ to -0.0023 C/m^2 in the presence of 0.5 mM $CaCl_2$) and "iron-starved" spheroplasts (from -0.0033 C/m^2 without $CaCl_2$ to -0.0020 C/m^2 in the presence of 0.2 mM $CaCl_2$ in suspension) was observed.

An increase of the photoinduced electrokinetic potential of "iron-sufficient" spheroplasts was confirmed (Fig.1).

Alterations on the apparent change of charge density upon illumination could come from redistribution of ions and most probably by changes of the local dielectric constant and density in the interface and ionic double layer.

The results of cation-induced EPM change showed the "20×Fe" spheroplasts bear a smaller amount of charges on the surface than "iron-deficient" and "iron-sufficient" ones.

Surface charge densities were affected by calcium treatment and this parameter was very sensitive to iron content in the external medium of cultivation. Electrokinetic measurements indicated that the observed modifications of spheroplast membrane composition and SCD did not significantly affect the degree of aggregation in the presence of Ca^{2+} cations. Obviously, after calcium treatment a characteristic conformation in the spheroplast membrane was not altered.

It could be supposed that the sublethal iron stress was accompanied by destabilization of the membrane structure (i.e., the surfaces became more negative as observed by an increase in the EPM) in the case of "20×Fe" spheroplasts. Even the highest micromolar concentrations of $CaCl_2$ did not neutralize effectively the negative surface charges on the plasma surfaces. Accumulation of negatively charged products of peroxidation could not be excluded.

Application of electrokinetic and LS methods was based on the characterization of the biophysical properties of the spheroplast model system in response of the iron stress applied.

For a more quantitative treatment of SCD of spheroplast particles, either a spherical or ellipsoidal a Poisson's equation and Boltzmann's law in the Gouy-Chapman treatment of the electric double layer could be applied as shown in the article of Chow et al. (1991).

Vesicles formed from a given variant of *Plectonema boryanum* cells adsorbed Ca^{2+} equally well (Fig. 2). $CaCl_2$ was not sufficient to produce a marked aggregation of spheroplast vesicles.

The results of the present study demonstrate some electrokinetic properties of spheroplast particles, including the effect of calcium ions, and provide some useful information for better understanding of the biological functions of Ca^{2+} – spheroplast membrane interactions.

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