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Improved method to evaluate the ability of compounds to destabilize the cellular plasma membrane

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ABSTRACT

In the paper, we present an improved method for evaluation of a compound ability to destabilize erythrocyte plasma membrane. The proposed method is based on the continuous monitoring of the light scattered by erythrocytes exposed to osmotic pressure differences. The kinetics of hemolysis depends on the plasma membrane mechanics and the extent of the osmotic stress. Generally, the osmotic pressure difference of approximately 150 mOsm is taken for measurements, as a result of the equal volume mixing with the physiological salt solutions. In this approach the hemolytic process completion is not established which may result in poor quality and reproducibility of the experimental data. In consequence, inaccurate parameters of the kinetic are determined due to the low quality fitting to the, widely used, single exponential model. In the paper we propose a new experimental protocol allowing to determine the extended set of parameters for kinetics of hemolysis. Namely, the method of the minimal osmotic pressure difference determination is proposed which ensures the completeness of the hemolytic process. This step allows improving the quality and exactness of the calculated parameters. The developed methodology was tested on two qualitatively different, biologically relevant, experiments; evaluation of the peptide effect on the plasma membrane properties and differentiating between human and rabbit erythrocytes.

1. Introduction

Biological activity of a chemical compound depends on two factors; the capability to affect molecular identity, therefore influencing cell functioning, and the ability to penetrate the plasma membrane barrier (Agoram et al., 2007a,b; Aranda et al., 2005; Suwalsky et al., 2007a). Whereas the biological potency can be designed and evaluated using molecular dynamic simulations and biochemical methods, the permeability is still difficult to predict and/or determine experimentally using a currently available tests (Mager, 2006; Mager and Illes, 2006) (Fouchecourt et al., 2001; McGinnity and Riley, 2001; McGinnity and Maguire, 2001; Roberts, 2003; Tam et al., 2010; Velický et al., 2010). There are no clear criteria for chemicals, which may be used as indicator of the compounds capacity to penetrate the biological membranes. In order to overcome those difficulties a semi-empirical relations and simplified experimental models have been developed (Alper, 2002; Tam et al., 2010). The process of passive diffusion into the cell interior is preceded by an initial association of a compound with the plasma membrane. The compound association with the membrane will likely affect its properties including stability, integrity and mechanical properties (Balimane et al., 2000; Balimane and Sinko, 2000). This capability of a compound to destabilize the plasma membrane of erythrocytes is frequently used as an indicator of its toxicity and/or biological activity (Didelon et al., 2000; Fujii et al., 1984; Kitagawa et al., 2004; Mineo and Hara, 2005; Miszta et al., 2005; Parpart et al., 1947a,b; Suwalsky et al., 2007a,b). The erythrocyte has been selected for this test as a model cell because it lacks the intracellular membranes, which may interfere with the measured quantities. The extent of the membrane destabilization is routinely measured at the presence or absence of the osmotic pressure difference (Parpart et al., 1947b; Pazdzior et al., 2003; Wojewodzka et al., 2005). In the static hemolytic test, certain amounts of erythrocytes are incubated with various concentrations of the investigated compound. At a specific, arbitrary selected, time point cell suspensions are centrifuged and the amount of released hemoglobin in supernatants is measured. The compound concentration at which 50% cell erythrocytes are lyzed is used as a measure of its toxic potency (Balimane et al., 2000; Best et al., 2002; DalNegro and Cristofori, 1996; Lenormand et al., 2001; Suwalsky et al., 2002, 2009b; Van den Bos et al., 2003). However, properties of the erythrocyte plasma membrane might be altered at compound concentrations much lower than determined in the test. To account for that, two other parameters have been

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devised, so called fluidity and mechanical strength (Lenormand et al., 2001; Soares and Maghelly, 1999). The first parameter is measured with membrane incorporated molecular probes reporting on the membrane local properties. The main limitation of the approach lays in the difficulty of converting local effects into the global membrane properties (Best et al., 2002). The plasma membrane mechanical susceptibility seems to be more reliable measure of a global membrane property. In order to determine the plasma membrane fragility cells are exposed to the hydrostatic or osmotic pressure difference. The cell shape alteration caused by hydrostatic pressure difference is measured with the micromanipulation devices (Lenormand et al., 2001) which is technically demanding and very laborious due to the large number of experiments required by the statistics. The cell exposure to the osmotic pressure difference, on the other hand, is performed simultaneously on a large population of cells. The progress of cell hemolysis is monitored with scattered light using stopped-flow device (Pazdzior et al., 2003). The proper selection of the experimental conditions allows excluding the potential interferences from metabolic processes and/or alteration of the intracellular structuring (Grzybek et al., 2006; Malheiros et al., 2004; Pazdzior et al., 2003; Suwalsky et al., 2007a,c, 2009a,b). In the method equal volumes of cell suspension and pure water are rapidly mixed and the following changes of the mixture optical properties are monitored as a function of time. The experiment allows evaluating additional parameters, as compared to the statics hemolysis method (Miszta et al., 2005; Wojewodzka et al., 2005). In order to parameterize the membrane with fitting curve extracted numbers a biophysical model is needed. The model is based on the assumption of independent and complete lysis of cell population, which allows the exponential function approximation. These conditions need to be ensured by properly selected experimental settings, which may vary from sample to sample. In the paper we propose the experimental protocol, which defines additional, critical parameter, minimum osmotic pressure difference, which justifies experimental data fitting with a single exponential function. This has been done by progressive elevation of the water flux through the membrane so the lysis process is completed. Technically this can be done by changing the osmotic pressure difference across the cell plasma membrane achieved by changing the ratio of mixed volumes in the stopped-flow instrument. The purpose of the new procedure is to obtain kinetics, which can be fitted with a single exponential function, and the final level of hemolysis corresponds to the situation when all cells are ruptured. In such a case parameters of exponential fit can be well assigned to sample properties, i.e. initial transmittance corresponds to static hemolysis prior to osmotic stress application, whereas the time constant reflects the combination of membrane mechanical resilience and the water permeability (Wojewodzka et al., 2005).

2. Materials and methods

2.1. Preparation of the erythrocyte suspension

Fresh blood samples of healthy individuals were obtained from the blood bank (RCKiK in Wrocław), whereas animal blood samples were a generous gift from the Wroclaw University of Environmental and Life Sciences. Fresh blood samples were washed with the isotonic phosphate buffered saline with glucose (8.9 mM) at 2400 rpm for 4 min at room temperature. The hematocrit was determined by measuring the relative erythrocyte packed cell volume expressed in % of the total volume after centrifugation (centrifuge MLW TH21). The final hematocrit of the erythrocyte stock solution was 1%, from which appropriate working cell suspensions were prepared.

2.2. Stopped-flow measurements and data analysis

The kinetic of osmotically induced erythrocyte lysis was measured by means of stopped-flow technique, where the transmittance as a function of time was recorded. The instrument (BioLogic, Grenoble France) was equipped with the xenon-mercury lamp, the monochromator of the 0.5 nm slit and the quartz flow cuvette of 1 cm optical path. The erythrocyte suspension (300 mOsm) was mixed with distilled water in different ratios providing the desired osmotic pressure across the cell membrane. The transmittance of light was recorded at 610 nm with respect to the pure water, for which 100% of transparency was fixed. The initial erythrocyte suspension became more transparent as hemolysis progressed, as illustrated in Fig. 1A. Typically the curves were collected for 60 s with the probing rate of 10 ms. Since the decrease of light intensity passing through the sample may be a result of both, absorption and scattering, additional kinetics were recorded at lower wavelengths corresponded to the absorption of hemoglobin in order to exclude the effect of absorption. Regardless the light wavelength the calculated parameters were statistically indistinguishable. The sample absorption results exclusively from the presence of hemoglobin, the concentration of which remains constant during the experiment. Therefore, the amount of absorbed light is also constant and does not contribute to the time dependant processes. Light scattering, on the other hand, depends on the number and shape of cells in the sample, hence can be used as an indicator of a progress of the hemolysis (Wojewodzka et al., 2005).

It has been assumed that the cells population is uniform with respect to the membrane properties so the experimental curves can be fitted with a single exponential function as following:

$$T(t) = T_{final} + A\ \exp\left(\frac{-t}{\tau}\right) \tag{1}$$

where T_{final} indicates the final hemolysis, τ is the time constant and A is an amplitude. Consequently, the sum: $T_{final} + A$ stands for an initial transmittance, and can be correlated with a static hemolysis. All parameters derived from the fitted curves are represented as an average value with the standard deviation of at least five measurements, which were much smaller than inter-sample variation. The statistical significance of the difference between samples was evaluated with the Fisher test. Error bars in all figures indicate the data variation collected for at least five different blood samples. The transmittance curves were fitted with the least squares method to the model represented by Eq. (1). Assuming that the erythrocyte plasma membrane breaks at a certain surface area expansion (at about 4%, Gennis, 1989), what limits the maximal cell volume change:

$$\frac{\Delta V_{\max}}{V_0} = \kappa \tag{2}$$

where κ is a constant, $\Delta V_{\max}$ and V_0 are the maximal volume change and the initial erythrocyte volume, respectively. The volume change (ΔV) is a result of water flux through the plasma membrane having a constant surface area (S). Assuming that the change of the intracellular concentration is negligible until cell membrane breaks we can write:

$$\Delta V = JSt \tag{3}$$

$$J = \frac{P}{S}\Delta\Pi \tag{4}$$

$$t \approx \frac{\kappa V_0}{\Delta\Pi P} \tag{5}$$

where J is a water flux, S erythrocyte surface area, P plasma membrane permeability to water, $\Delta\Pi$ – osmotic pressure difference and t is time. We can correlate the time t, at which the statistical single