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SHORT COMMUNICATION

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Effects of charge density and hydrophobicity of ionene polymer on cell binding and viability

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Abstract The binding of cationic ionenes onto budding protoplast was investigated and the results were associated with the cell viability. There are critical numbers of carbon atoms to induce effective cell disruption and cell binding. The longer the alkyl chain of the ionene, the lower the concentration at which cell disruption occurs. The ionenes with increased charge density undergo effective binding, while almost 2 orders of magnitude higher concentration are required for effective cell disruption. These results were associated with the cooperativity of the binding process, which induces local stress and solubilization of the lipid membrane.

Key words Yeast protoplast · Membrane disruption · Cooperativity · Ionene polymer

Introduction

Although many experimental studies have been done, the mechanism and the process of cell–polymer interactions still remain unclear despite the biological and medical importance of the problem. A part of the reason apparently originates from the heterogeneity and the complexity of the cell membrane. The cell consists of a lipid bilayer, membrane proteins and a sugar moiety attached to it and they form hydrophobic/hydrophilic surfaces with both positive and negative charges. Besides, the lipids and the membrane proteins undergo dynamic motion, due to lateral diffusion and rotational diffusion. Nevertheless, it is generally accepted that the interaction of concentrated polymeric cations increases the membrane fluidity and the membrane disruption due to binding with the negative surface charge of the cell [1, 2]. While anionic polymers with hydrophobic branches solubilize lipids [3, 4], some neutral polymers, such as poly(ethylene glycol) and dextran, bring about cell aggregation, which is nowadays used as a cell fusion technique [1, 5].

It was reported that ionene polymers have antimicrobial effects [6]; however, systematical work on the effects of charge density and hydrophobicity on the interactions with cells have not been reported as far as the authors know. In this article, we report a quantitative study of cell (yeast protoplast) binding of cationic ionene polymers by changing the charge density and hydrophobicity associated with the cell viability. Yeast protoplast was used as the cell type because protoplast is not expected to have any specific affinities with polymers and because of the ease of mass production. It was found that the ionenes with a higher charge density did not disrupt the cells at all despite their increased binding, while those with a lower charge density, but with a longer hydrophobic segment, destroyed the cell substantially although the amount of bound polymer was much lower. The mechanism is briefly discussed in terms of cooperativity and the binding process.

Materials and methods

Materials

Yeast was purchased from Nihon Seifun Co. (Tokyo, Japan). Dried yeast extract D-3 was from Nihon Seiyaku Co. (Tokyo, Japan) and sucrose and sorbitol were from Junsei Chemical Co. (Tokyo, Japan). Kitalase was obtained from Wako Pure Chemicals (Tokyo, Japan). Polycations carrying charges on the chain back-

bone, *x,y*-ionene (x = 3, 6, 12; y = 3, 4, 6, 12) and *x,X*-ionene (x = 3, 6, 12; X = xylylene) bromide polymers, were synthesized through the successive Menschutkin reaction according to methods in the literature [7, 8]. The chemical structures of the ionenes are shown in Fig. 1.

Preparation of yeast protoplast

Yeast (2 mg) was cultivated in a medium (10 g sucrose and 1 g yeast extract in 200 ml distilled water) at 30 °C for 10 h. The cultivated cells were centrifuged (3000 rpm, 5 min, 5 °C) and suspended in 100 ml kitalase buffered solution (5 g/l; 0.9 M sorbitol/20 mM phosphate buffer at pH 6.0). The enzymatic treatment was carried at 37 °C for 2 h. The protoplasts obtained had an average diameter of about 5 μm, which was confirmed by microscopy. The cell was washed twice with 0.9 M sorbitol buffered solution (pH 6.0) by centrifugation (1000 rpm, 10 min, 5 °C).

Measurements

Determination of cell disruption

Cell suspensions (2×10^7 cell/ml) were mixed with equivalent volumes of polymer solutions. After 30 min, the cell viability was determined by counting the protoplast concentration using a Thoma hemocytometer. Microscope observation was also carried out to confirm the shape change of the cells. All the experiments were carried out in 0.9 M sorbitol/phosphate buffer (pH 6.0), unless otherwise noted. It was confirmed that the viability change caused by added polymer was reproducible (errors less than 10%).

Binding of polymer onto the cell

The binding experiment was made by measuring the free *x,X*-ionene (x = 3, 6, 12) concentration to determine the amount of bound polymer. The polymer concentration was determined by measuring the absorbance at 267 nm and was expressed in moles per litre with respect to the charges of the polymer. The Equilibrating time was 1 h. Incubation of the protoplast with the polymer caused a high blank at 267 nm, which should interfere with the detection of free polymer. Thus, glutaraldehyde (GA) fixation of the cell was made, which enabled all the experiments to be carried out with negligible blank. The protoplast suspension (1 ml) was mixed with 5 ml GA/1.2 M sorbitol buffered solution (4%, pH 4.9), and the solution was incubated at 5 °C for 1 h. The cells were washed with distilled water four times by centrifugation (3000 rpm, 5 min, 5 °C), then suspended in the same buffer solution as used for the disruption measurement. For the binding measurement, the cell concentration was 4×10^7 cell/ml.

Results and discussion

In order to clarify the roles of the charge density and the hydrophobicity of ionenes on the cell disruption, a series of ionenes containing ammonium positive charges separated with alkyl chains (*x,y*-ionene; x = 3, 6, 12, y = 3, 4, 6, 12) or a xylylene group (*x*,X-ionene; x = 3, 6, 12, X = xylylene) were synthesized (Fig. 1). The cell viability was determined using a Thoma hemocytometer 30 min after introducing the polymer solution to the cell suspension. As shown in Fig. 2, no disruption occurred at all when ionenes with increased charge density but short alkyl groups (3,3-, 6,4-, 6,6-) were added in the concentration range 10^{-6}–10^{-4} M, though they caused the cell to deform into a tear-drop shape. On the other hand, substantial cell disruption occurred when ionenes with less charge density but more hydrophobic segments

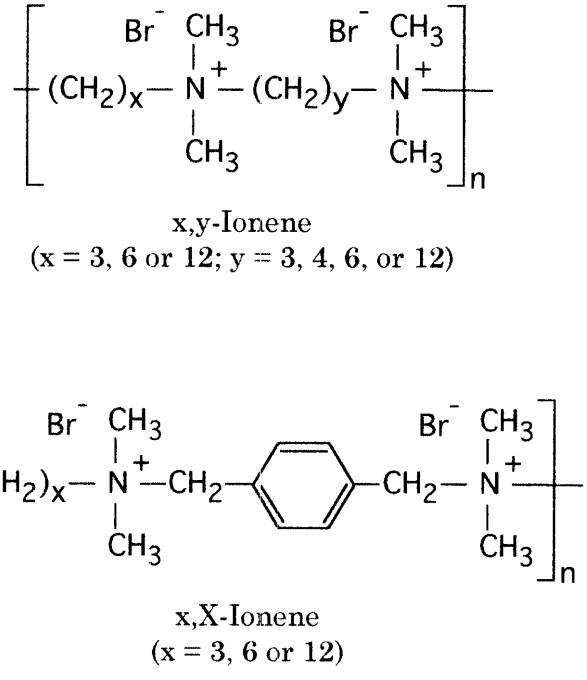

Fig. 1 Molecular structures of the polymers used in this work

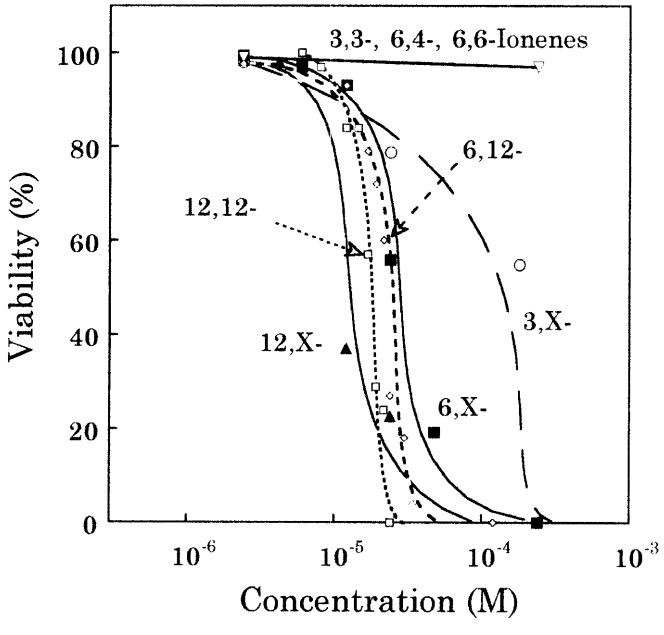

Fig. 2 $1/c^*$ as a function of $x + y$ or $x + X$ of ionenes, where X = 8

were added, especially when the concentration exceeded a certain critical value (c^*). The 6,12- and 12,12-ionenes drastically disrupted the cell at $c^* = 2.3 \times 10^{-5}$ and 1.7×10^{-5} M, respectively. The c^* of the x,y-ionenes strongly depended on the alkyl chain length and the longer x and y, the lower c^* as shown in Fig. 2. The 12,X-ionene induced substantial cell disruption at very low concentrations, such as 1.1×10^{-5} M, while the 6,X- and 3,X-ionenes disrupted the cell at higher concentrations of 3×10^{-5} and 2×10^{-4} M, respectively, indicating that the hydrophobicity of the polymers plays a crucial role in cell disruption rather than the charge density.

If $1/c^*$, which represents the reciprocal of the concentration at which 50% of the cell is disrupted, is plotted against the number of carbons separating the ammonium cations, one obtains the plot in Fig. 3. As clearly indicated in Fig. 3, a minimum number of carbons on the main chain of the ionene is required to induce an effective cell disruption and the values are $x + y = 12$ for the x,y-ionenes and $x + X = 10$ for the x,X-ionenes, respectively.

In order to associate the viability with the amount of cell-bound ionenes, the binding isotherms of the x,X-ionenes onto the protoplast were determined at 25 °C. Incubation of the protoplast in the presence of the ionenes caused cell disruption; however, the cell disruption caused substantial numbers of cellular compounds including proteins to come out and made it difficult to carry out the spectrophotometric assay at 267 nm; thus, GA fixation of the cell was carried out. This GA treatment successfully prevented the cellular compounds from effusing out of the cell due to the high osmotic pressure. The effects of GA fixation on the cell surface properties have been reported previously [10]. It was confirmed by thin layer chromatography that the lipid bilayer remained on the cell surface, even after the GA fixation. We measured the ζ potential of the GA-treated protoplasts and found that the number of negative charges decreased with an increase in GA concentration.

Binding isotherms of x,X-ionenes onto GA-treated protoplast at 25 °C are shown in Fig. 4. An increase in the alkyl size of the ionenes increased the minimum concentration at which the binding started. This result indicated that electrostatic interaction plays a major role in binding since the ionenes with higher charge density showed higher amounts of binding. The absolute amount of bound polymer was evaluated by comparing with amount of lipid at the cell surface. It is reported that there are approximately 5×10^6 lipid molecules in a 1-μm^2 area of the lipid bilayer (8×10^{-18} mol/μm^2) [11]. Since the surface area of the yeast protoplast can be calculated as 78 μm^2 as the average diameter is 5 μm, there should be 4×10^8 lipid molecules or 7×10^{-16} mol/cell, which coincided well with the amount of bound ionene at the initial stage of binding in Fig. 4. In other words, the x,X-ionenes can roughly undergo one-to-one stoichiometric binding with each lipid molecule in the initial step, while they continue to bind with an increase in concentration and adsorb nonstoichiometrically or penetrate inside the cell.

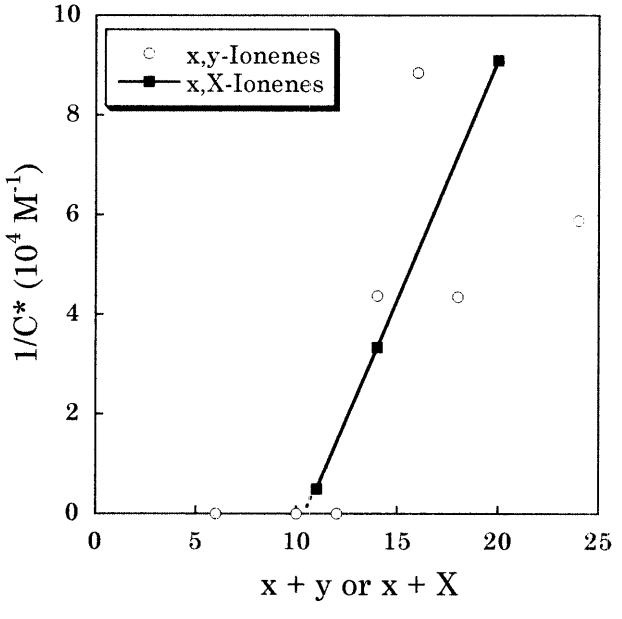

Fig. 3 Cell viability after 30 min as a function of the x,X-ionene polymer concentration (no glutaraldehyde, *GA*, treatment)

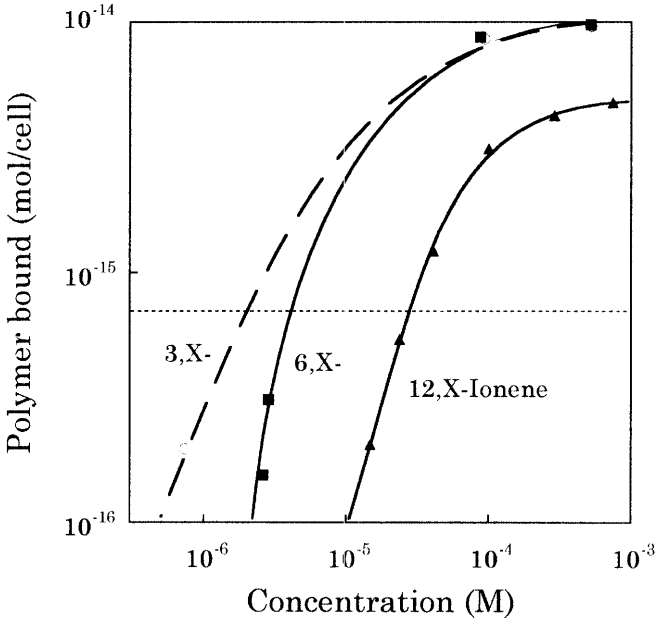

Fig. 4 Binding isotherms of x,X-ionene polymers onto GA-treated protoplast at 25 °C. The *dashed line* shows the number of negative charges derived from the lipid membrane of the cell (mol/cell)

It would be difficult to directly compare the results of the disruption experiment with that of binding on the chemically modified protoplasts; however, one could assume that the cell disruption does not occur simply due to the amount of ionene bound on the cell but also due to the nature of the binding. If the amount of ionene bound with the cell (Fig. 4) is compared with the cell viability in Fig. 2, one can notice that the binding of the 3,*X*-ionene started at such a low concentration as 10^{-6} M, while the effective cell disruption occurred only at a concentration of 10^{-4} M, at which 10^{-14} mol/cell 3, X-ionene had completed binding. On the other hand, the 12,*X*-ionene started binding at a higher concentration of 1×10^{-5} M, at which substantial disruption was already completed. This result suggested that the hydrophobic ionene is able to disrupt the cells effectively by stoichiometric binding, while ionenes with a higher charge density can bind effectively with the cell through the strong electrostatic interaction. It was found that the amount of bound ionene with a high charge density for the effective cell disruption was much higher than that of hydrophobic ionene. The reason might be associated with an enhanced local solubilization of the lipid membrane due to hydrophobic interaction. It was reported that the hemolytic activity of surfactant was proportional to the alkyl size of the surfactant but that the electrostatic interaction was less important [9].

The binding curves in Fig. 4 not only show the presence of a critical concentration at which the binding starts, but also demonstrate its cooperative nature because the slope of the curve is high. The cooperativity is, as is well established, the process which facilitates binding at a site adjacent to an already occupied site; as a result it leads to a long sequence without branches or voids. In previous articles, we have reported that the hydrophobic interaction plays a dominant role for the cooperative binding process of surfactant onto the oppositely charged polyelectrolyte and its gels [8, 12, 13]. The continuous sequences of bound molecules induce the strong local stress and a conformational change of the complexes. The same is true for the binding with the cell. This cooperative binding of ionene induces a strong stress on a certain area of the cell membrane and changes its fluidity and solubilization to finally result in cell destruction. The presence of c^*, the minimum concentration to induce the effective destruction in Fig. 2, coincides well with this consideration.

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