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SHORT COMMUNICATION

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Kinetic study of cell disruption by ionic polymers with varied charge density

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Abstract The effect of charge density and hydrophobicity of the polymeric cations on cell disruption is studied thermodynamically and kinetically by using the budding yeast protoplast. It is found that cationic polymers drastically disrupt the cells above a certain concentration while nonionic and anionic ones do not. Reduced charge density of copolymers of cationic and nonionic monomers resulted in decreased cell disruption. However, it is further experimentally proved that the disruption of the cells occurs only when the polycation has a certain hydrophobicity. The stronger the hydrophobicity of the cationic polymer, the more cooperatively the cells are disrupted.

Key words Yeast protoplast · Polycation · Membrane disruption · Charge density · Hydrophobicity

Introduction

Understanding polymer-cell interactions is important for practical applications in the field of cell engineering and related technology. There have been many experimental studies on the mechanism and process of polymer-cell or polymer-vesicle interactions in terms of cell viability, membrane fluidity or fusion, and the contribution of cationic charges, as well as hydrophilic and hydrophobic balance have been emphasized [1]. For example, it is generally accepted that polymeric cations increase the membrane fluidity of the cells to give cell disruption due to binding with the negative membrane surface of the cells [2, 3]. Polymeric and monomeric surfactants are known as hemolytic compounds [4, 5], and several authors have pointed out the importance of hydrophobicity on membrane solubilization, hemolytic, and antibacterial activities [4, 6–8]. However, the polymer-cell interactions still remain unclear because of the complexity of cell membranes, and so far as authors know, few systematic studies on the thermodynamics and kinetics of cell disruption have been made.

Ionene polymers are polyelectrolytes carrying charges on the chain backbone separated by alkylene groups and they show antibacterial activity [9]. And previously, we have reported the cell (yeast protoplast) binding of cationic ionenes varying the charge density and hydrophobicity [10]. It was found that the ionenes with a lower charge density but separated with a longer hydrophobic segment disrupt the cells more effectively than those with a higher charge density. However, the kinetics of cell disruption was not much discussed.

In this paper we report the results of the systematic study of effect of charge density and hydrophobicity of polycations on the kinetics of yeast protoplast disruption. Yeast protoplast was used because the protoplast is not expected to have any specific affinities with polymers. We found that polycations with hydrophobicity induce cell disruption, while nonionic or anionic polymers do not. Increased charge density resulted in cell disruption at lower concentration. Ionene polymers with low hydrophobicity but high charge density did not induce cell disruption, suggesting that hydrophobicity is the major factor in cell disruption although cationic charges are required to interact with negatively charged cell. It was also found that hydrophobic polycations such as 12,12-ionene show high velocity of cell disruption due to cooperative interaction with cells.

Experimental section

Materials

Yeast was purchased from Nihon Seifun Co. (Tokyo, Japan). Dried yeast extract D-3 was from Nihon Seiyaku Co. Ltd. (Tokyo, Japan) and sucrose and sorbitol were from Junsei Chemical Co. (Tokyo, Japan). Kitalase was obtained from Wako Pure Chemicals (Tokyo, Japan). *N*,*N*-Dimethylformamide (DMF) was obtained from Wako Pure Chemicals and purified by conventional methods. Poly(ethylene glycol) (PEG, Mw. 20,000) was obtained from Wako Pure Chemicals and purified by dialysis in water. Chitosan was purchased from Funakoshi Co. Ltd., used as received. 1,4-Dibromobutane, sodium styrene sulfonate (NaSS), and 2-acrylamido-2-methylpropane sulfonic acid (AMPS) were purchased from Wako Pure Chemical Industries, Ltd., 1,x-dibromoalkane (x = 6, 8, 10, or 12), *N*,*N*,*N*′,*N*′-tetramethyl-1,3-diaminopropane, acrylamide (AAm), 1,3-dibromopropane, and *N*,*N*,*N*′,*N*′-tetramethyl-1,6-diaminohexane from Kanto Chem. Co., Ltd., were used as received. Potassium persulfate from Kanto Chem. Co. Ltd. was recrystallized from water. Quaternized dimethylaminopropyl-acrylamide (DMAPAA-Q) (Kojin Co. Ltd.) was used as received. *N*,*N*,*N*′,*N*′-Tetramethyl-1,12-diaminododecane was synthesized according to the Leuckart's reaction [11].

Preparation of yeast protoplast

Yeast (2 mg) was cultivated in a medium (10 g of sucrose and 1 g of yeast extract in 200 ml distilled water) at 30 °C for 10 h. The cultivated cells were centrifuged (3000 r.p.m., 5 min, 5 °C) and suspended into 100 ml of kitalase buffered solution (5 mg/ml; 0.9 mol/l sorbitol/20 mmol/l phosphate buffer at pH 6.0). The enzymatic treatment was carried out at 37 °C for 2 h. The protoplasts were obtained with an average diameter of 5.6 μm, which was confirmed by microscope. The cell was washed with 0.9 mol/l sorbitol buffered solution (pH 6.0) twice by centrifugation (1000 r.p.m., 10 min, 5 °C).

Preparation of polymers

x, y-Ionene (x = 3, 6, or 12; y = 3, 4, 6, 8, 10, or 12) bromide polymers were synthesized through the successive Menschutkin reaction of *N*,*N*,*N*′,N′-tetramethyl-1,x-diaminoalkane and 1,y-dibromoalkane in DMF [12]. PNaSS, PAMPS, PAAm, PDMAPAA-Q, and their copolymers were obtained by radical polymerization using potassium persulfate as an initiator. Synthesis and polymerization of poly(acryloyloxydodecylpyridinium bromide) (PADPB) were made according to the literature [13]. Chemical structure of the polymers are shown in Fig. 1.

Measurement

Determination of cell disruption. Disruption of protoplast was determined by measuring the absorbance change at 500 nm, since the absorbance is proportional to the cell concentration. The calibration was made with the absorbance against the cell concentration counted by Thoma's hemocytometer. Cell suspensions (10^7 cell/ml) were mixed with equivalent volume of polymer solutions and the absorbance change was measured with time. The microscope observation was also carried to confirm the shape change of cells. All the experiments were carried in 0.9 mol/l sorbitol/phosphate buffer (pH 6.0).

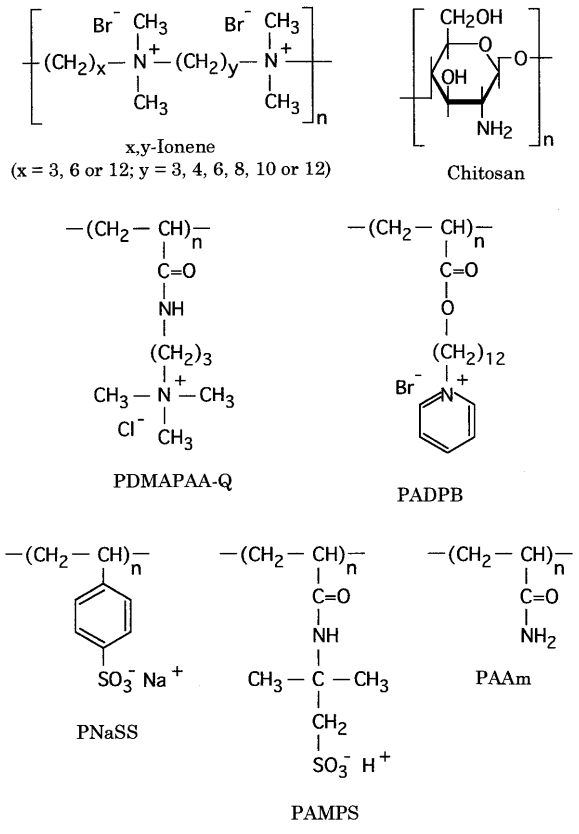

Fig. 1 Molecular structures of polymers used in this work

Results and discussion

Cell viability after prolonged interaction

A series of cationic, nonionic, and anionic polymer solutions (unit concentration of the polymers: 2.4×10^{-5} mol/l) summarized in Fig. 1 were added to the protoplast suspension to assay the effect of polymers on the cell viability with time. Fig. 2 shows time profiles of cell viability after addition of polymer solution. We have already confirmed experimentally that fragmentation of the cell (diameter: 5.6 μm) occurs by addition of these polymers and that the time profile of the cell viability can be followed by change with absorbance at 500 nm of the suspension [10]. Therefore, cell viability was quantitatively evaluated by calibrating changes in absorbance with hemocytometric observation under the microscope. As shown in Fig. 2, anionic polymers (PAMPS and PNaSS) and nonionic polymers (PAAm and PEG) induce no effect on the cell viability at all, while the cationic polymers such as PADPB, chitosan, PDMAPAA-Q, and 12,12-ioenene show strong effect and disrupt more than 70% of the cells in 30 min. The experimental fact that the presence of

0.6 mol/l of KCl totally prevents such cell disruption occurring by PDMAPAA-Q suggests that the cell disruption is mainly induced via electrostatic interactions between the negatively charged cell and the cationic polymers. Another interesting feature observed here is that the initial velocity of cell disruption strongly depends on the species of the polycation. This is discussed in the following section.

Fig. 3 shows time profiles of cell viability on addition of 12,12-ionene at various concentrations. One can see that increase in the ionene concentration results in increased velocity of cell disruption and decreased cell viability at the final static state. Drastic change in cell viability occurs in a small concentration range, around a certain concentration (1.8×10^{-5} mol/l).

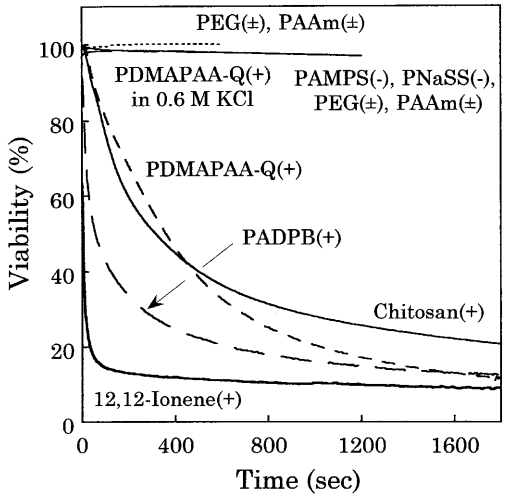

Fig. 2 Time profiles of cell viability on addition of various polymers. Charged unit concentration: 2.4×10^{-4} mol/l

Cell viability 30 min after adding various polymers is plotted against charge concentration of the polymers in Fig. 4. Nonionic and anionic polymers do not induce cell disruption in the concentration range studied. Polycations such as 12,12-ionene, PDMAPAA-Q, chitosan, and PADPB drastically disrupt cells at a certain concentration about 10^{-5} mol/l, suggesting that the disruption occurs cooperatively. It was found that the concentration of disruption by these polymers are almost the same, despite the difference in chemical structure, suggesting positive charges concentration are important for cell disruption.

In order to examine further the effects of charge density on the cell disruption, copolymers of DMAPAA-Q and AAm with various molar ratios F (F is the molar ratio of DMAPAA-Q to the total monomer content in feed) were used. Note that AAm is used since it is a hydrophilic nonionic monomer and its polymer is inert to the cell. Cell viability 30 min after polymer addition is plotted as a function of charge concentration of the copolymer (Fig. 5). The cell viability V decreases drastically at a certain concentration, the same as the homopolymers in Fig. 4, but the critical concentration value to induce the disruption of the cell increases with the decrease in F.

Since the cell viability shows a sigmoidal curve with the polymer charge concentration, the following equation with two parameters should be held phenomenologically:

$$V = \frac{100 \cdot C^{*\sqrt{u}}}{C^{\sqrt{u}} + C^{*\sqrt{u}}} \tag{1}$$

where C is the charged unit concentration of the polymer, u is the cooperativity parameter of the disruption, and C^* is the concentration at which cell

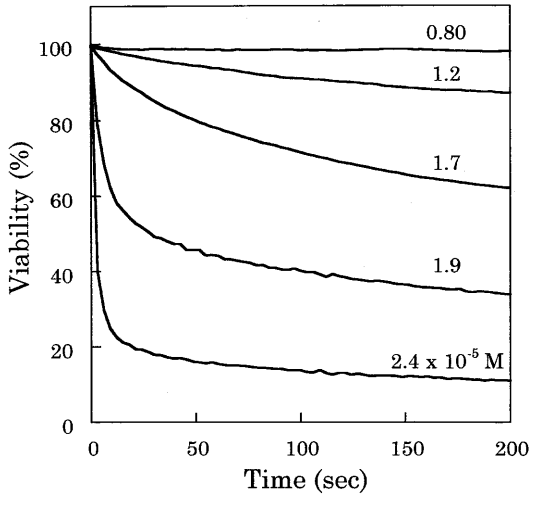

Fig. 3 Time profiles of cell viability on addition of 12,12-ionene at various concentrations

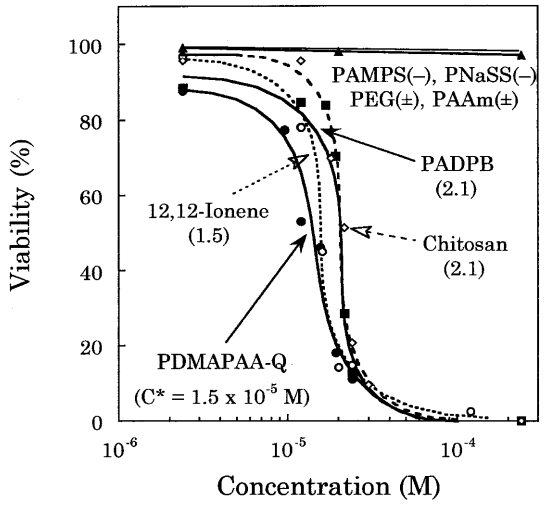

Fig. 4 Cell viability after 30 min as a function of unit concentration of various polymers. C^* is the concentration at 50% viability

viability is 50%. Thus $1/C^*$ indicates critical numbers of charges to induce effective cell disruption. $1/C^*$ and u of various polycations are summarized in Table 1. If $1/C^*$ is plotted as a function of F, Fig. 5 (inset) is obtained which shows that increase in F results in increased $1/C^*$, demonstrating that high charge density favors the disruption of the cells.

One can see the effect of hydrophobicity on the cooperativity of the disruption by comparing PDMAPAA-Q and PADPB, a hydrophobic analog of PDMAPAA-Q. As shown in Fig. 4 and Table 1, PADPB shows higher cooperativity than PDMAPAA-Q. We have previously reported that ionenes with increased hydrophobic nature induce the cell disruption at lower concentration [10]. It is indicated that hydrophobicity of the polymer plays an important role in cell disruption.

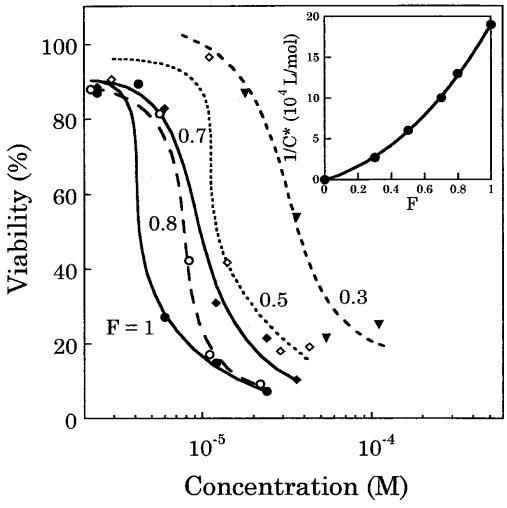

Fig. 5 Cell viability after 30 min as a function of charged unit concentration of P(DMAPAA-Q-*co*-AAm). F is the molar ratio of DMAPAA-Q to total amount of monomer in feed. Inset: $1/C^*$ as a function of F

Table 1 Thermodynamic parameters of cell viability change on addition of various polycations as determined by Eq. (1)

Polycations	$1/C^*$ (10^4 l/mol)	Cooperativity parameter u
P(DMAPAA-Q-*co*-AAm)		
F = 0	< 0.1	
0.3	2.7	4.0
0.5	6.0	7.3
0.7	10	4.0
0.8	13	18
1	19	72
x,y-ionene		
3,3-	< 0.1	
6,4-	< 0.1	
6,6-	< 0.1	
6,8-	4.4	1.7
6,10-	8.8	9.0
6,12-	4.5	36
12,12-	5.7	53
PADPB	4.8	260
chitosan	4.7	53

In order to investigate the effect of hydrophobicity on cell disruption, a series of viability experiment was made using ionenes with different x and y (Fig. 6). The ionene polymers are categorized into two groups in terms of cell disruption. The first group is composed of the ionenes with $y \geqq 8$, which bring about drastic and extensive cell disruption at the concentration of 10^{-5} mol/l or higher. Ionene polymers with shorter alkyl chain segments, such as 3,3-, 6,4- or 6,6-ionenes, are categorized into the second group, which do not disrupt the cells despite the high cationic charge density. They only turn the cells to a tear-drop shape. This result demonstrates that hydrophobicity as high as that of alkyl chains longer than at least C8 is required for ionenes to induce effective cell disruption. These facts suggest that, in addition to electrostatic interactions, the hydrophobicity of the polymer plays an important role in the effective cell disruption. Although the species of cells and additives are different, this experimental result coincides with the conclusion in the references that hemolytic activity or membrane solubilization of surfactants is proportional to the alkyl chain length [4, 5].

$1/C^*$ and u of ionenes are also summarized in Table 1. $1/C^*$ is independent of $x + y$ among the ionenes in the first group, suggesting that C^* is a function of both charge density and hydrophobicity. However, u monotonously increases with increase in $x + y$, suggesting that cooperativity of the cell disruption is determined by hydrophobicity. This is in agreement with our previous result that ionene binds

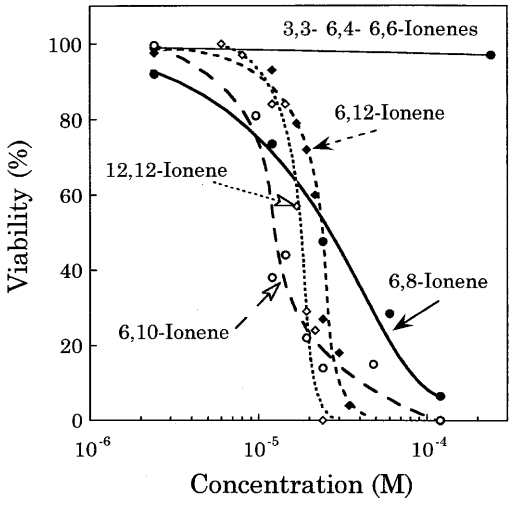

Fig. 6 Cell viability after 30 min as a function of cation concentration of various ionene polymers

onto the cell cooperatively, and the cooperativity increases with increase in hydrophobicity [10].

It is generally accepted that polycations bind with cell membrane mainly by electrostatic attractive force, meaning that increased charge density of the polymers increases the interaction [1, 2]. It is also known that increased hydrophobicity of the polyelectrolytes favors the membrane binding [1]. Our results strongly suggest that the electrostatic interaction is important to bind with cells although it is not sufficient to induce cell disruption. The strong hydrophobicity plays a crucial role in disrupting the cells as a whole, presumably due to preferentially binding to the cell membrane.

Kinetics of protoplast disruption

As shown in Fig. 2, the initial velocity of cell disruption depends on the species of polycations and, despite the cell viability, reaches almost the same value at the static state. The initial velocity of cell disruption after addition of various polycations is calculated from the slope of the time profile at the very initial stage and the logarithmic plot of the charge concentration of the polymers and the velocity is shown in Fig. 7. Fig. 7 shows that the velocity of the cell disruption by these ionenes increased in a power law with increase in the concentration. 12,12-Ionene and PADPB, which have strong hydrophobic moieties, show surprisingly higher velocity than other polycations, for example, 1 or 2 orders of magnitude higher values than those of chitosan and PDMAPAA-Q at 2.4×10^{-5} mol/l. Though it is difficult to make a comparison among these polycations because of the difference in chemical structure, this result suggests that hydrophobicity enhances the velocity of cell disruption.

In order to confirm the effect of hydrophobicity on the velocity of cell disruption, the initial velocity of cell disruption after addition of various ionenes as a function of charge concentration was measured and shown in Fig. 8. It was found that the ionenes have higher absolute value of the disruption velocity than the other polycations in Fig. 7, confirming the effect of hydrophobicity.

As shown in Figs. 7 and 8, the velocity of the cell disruption v (%/s) follows a power law with the concentration C (M), therefore we have

$$v = k \cdot C^{\alpha} \tag{2}$$

where factor k is equivalent to v at $C = 1$, and determines the potency of the polymer on the value of v. Index α, which is determined from the slope of the fitting line in Fig. 7, represents the number of charges to induce cell disruption in cooperation. Here one can see that increasing alkyl size of the ionenes results in increased k and α. If the values of log k and α of the ionenes are

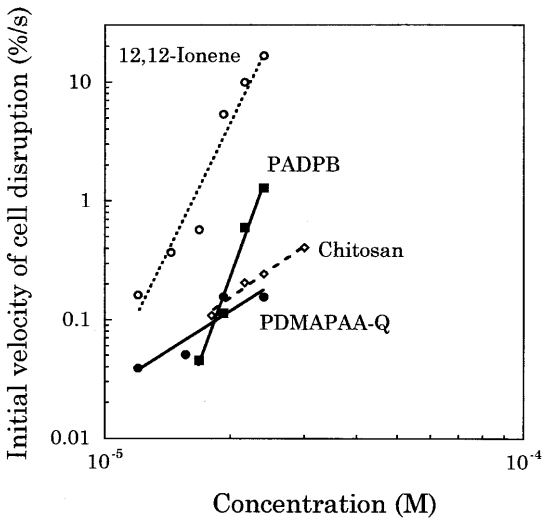

Fig. 7 The initial velocity of cell disruption as a function of cation concentration of various polymers

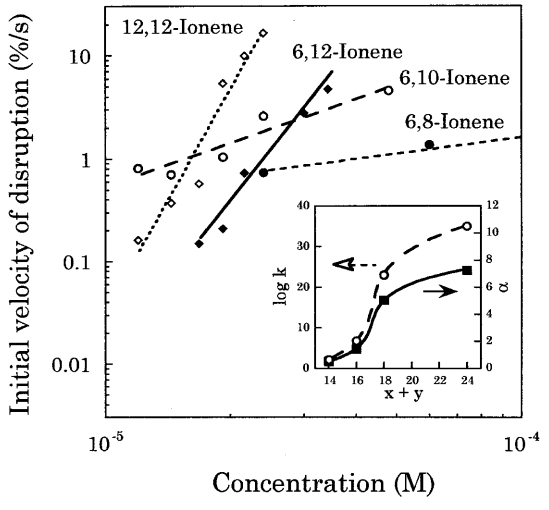

Fig. 8 The initial velocity v of cell disruption as a function of cation concentration C of various ionene polymers. v follows a power law with C, $v = k \cdot C^{\alpha}$. *Inset*: log k and α as a function of $x + y$ of ionenes

plotted against $x + y$, we obtain Fig. 8 (inset), in which log k and α increase with increase in $x + y$.

The values of log k and α of various polycations are summarized in Table 2. Chitosan, PDMAPAA-Q, and P(DMAPAA-Q-*co*-AAm) show lower log k and α than the hydrophobic polymers such as PADPB and 12,12-ionene; however, charge density dependence of log k and α are not observed. These results indicate that hydrophobicity of the polycation enhances the velocity of cell disruption; however, charge density is not a major factor in determining the velocity.

The meanings of the value of k and α are not clearly explained at the present stage. Large α suggests that the cell disruption occurs consequentially and crucially only

Table 2 Kinetic parameters of cell disruption on addition of various polycations as determined by Eq. (2)

Polycations	log k	α
P(DMAPAA-Q-*co*-AAm)		
F = 0		
0.3	13	1.3
0.5	8.0	2.2
0.7	6.1	1.4
0.8	9.7	1.9
1	5.4	3.1
x,y-ionene		
3,3-		
6,4-		
6,6-		
6,8-	2.2	0.51
6,10-	6.8	1.4
6,12-	23	5.1
12,12-	35	7.3
PADPB	46	9.9
chitosan	11	2.5

by the presence of α numbers of cationic polymers. It is demonstrated previously that hydrophobic ionene polymers cooperatively bind to cells [10]. Cooperative binding means localized increase in the bound polymer concentration at the surface of the cell; thus, it presumably facilitates the disruption by solubilizing membrane and making pores on the cells.

Conclusions

Interactions of yeast protoplast and polymers consisted of electrostatic and hydrophobic interactions. Nonionic or anionic polymers such as PAAm, PEG, PAMPS, or PNaSS did not induce cell disruption, while cationic polymers such as PDMAPAA-Q, chitosan, and PADPB drastically decreased cell viability. Ionene polymers with short alkyl chains did not induce cell disruption because of low hydrophobicity. On the other hand, ionene polymers with a longer alkyl chain than C8 showed cooperative cell disruption, due to the hydrophobic interaction of the polymers. Ionene polymers with C12, especially, showed a high velocity of cell disruption increasing with concentration in a power law, indicating cooperative interaction due to hydrophobicity. Copolymers of cationic DMAPAA-Q and nonionic AAm of various charge densities were made and the effect of charge density on the cell disruption was studied. Increased charge density resulted in increased amount of cell disruption. These results demonstrate that cationic charges were required to bind with negatively charged cells, and hydrophobicity was crucial to induce cell disruption.

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