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Effect of antioxidant contained in poly(ethylene glycol) on cell fusion

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The effect of antioxidants contained in poly(ethylene glycol) (PEG) on cell fusion was studied using L929 cells in the monolayer state. Hydroquinone monomethyl ether (HQME), 2-mercaptobenzimidazole (MB), butyl hydroxyanisole (BHA) and 2,6-di-(t-butyl)-4-methylphenol (BHT) were chosen from the antioxidants that have currently been used to protect commercially available PEG from oxidation. Cell culture was conducted in 40% w/w aqueous solution of PEG with a molecular weight of 3000 in the presence of different concentrations of antioxidants. BHA clearly enhanced membrane fusion of L929 cells with increasing concentration in PEG solution, whereas HQME, MB and BHT had no significant effect on cell fusion. The enhancement of cell fusion by BHA might be ascribed to balanced hydrophobicity and high water solubility in comparison with the other three antioxidants.

(Keywords: membrane fusion; poly(ethylene glycol); antioxidant)

INTRODUCTION

It has been pointed out very often that the success rate of cell fusion by poly(ethylene glycol) (PEG) greatly depends on the origin of the PEG, such as the manufacturer, the lot number and the purity, when the molecular weight and concentration of PEG are fixed[1–3]. Antioxidants are generally added to commercially available PEG to prevent PEG oxidation during storage. Therefore, it is possible that antioxidants present in the PEG product affect cell fusion. Indeed, Honda *et al.* reported that fusion of human erythrocytes by PEG was suppressed when PEG was purified by reprecipitation with ether before use. This seems to indicate that PEG itself has no or very low fusogenic activity, and needs cooperation with its contaminants such as catalyst, terminator of PEG polymerization, or antioxidant in order to induce effective membrane fusion[4]. On the other hand, Smith *et al.* reported that PEG could induce membrane fusion even after PEG purification[1]. They purified five different commercial sources of PEG with molecular weight of 6000 and studied their ability to fuse erythrocytes. Extensive fusion occurred for all five unpurified preparations of PEG, when erythrocytes were incubated with 45% w/w PEG for 15 min. Under this condition, however, the fusogenic activity of four preparations of PEG was not affected at all by purification involving reprecipitation in chloroform and ether and/or dialysis, although a decrease in the fusogenic activity was observed for one of them. They concluded that PEG itself has fusogenic activity, which is possibly enhanced by the contaminating substances. In our series of work we always employed PEG samples containing no antioxidant and found that cell fusion effectively took place if an appropriate cell culture condition was selected[5–7].

So far, there have been few reports focusing on the effect of antioxidants used for commercial-grade PEG on cell fusion, especially on what chemical property of the antioxidants affects membrane fusion. In this report, the effect of four kinds of antioxidants contained in PEG on cell fusion is studied, and the relationship between the physicochemical properties of the antioxidants and membrane fusion will be discussed.

EXPERIMENTAL

Materials

PEG with number-average molecular weight of 3000 (PEG3000) was kindly supplied by Daiichi Kogyo Seiyaku Co. Ltd, Kyoto, Japan. It was free of any additives and used without further purification. The purity of PEG was higher than 99% and contained practically no contaminants such as carbonyl compounds and peroxides.

Four kinds of antioxidant were supplied by Daiichi Kogyo Seiyaku Co. Ltd. They were all phenol derivatives with purity higher than 99% and were used without any further purification. They include hydroquinone monomethyl ether (HQME), 2-mercaptobenzimidazole (MB), butyl hydroxyanisole (BHA) (which is a mixture of 3-t-butyl-4-hydroxyanisole (94%) and 2-t-butyl-4-hydroxyanisole (6%)) and 2,6-di-(t-butyl)-4-methylphenol (BHT). Their chemical structures are shown in *Figure 1*.

Preparation of PEG solutions containing antioxidant

PEG solutions containing different concentrations of antioxidant were prepared as follows. An antioxidant was melt-mixed at 60°C with PEG3000 to have the

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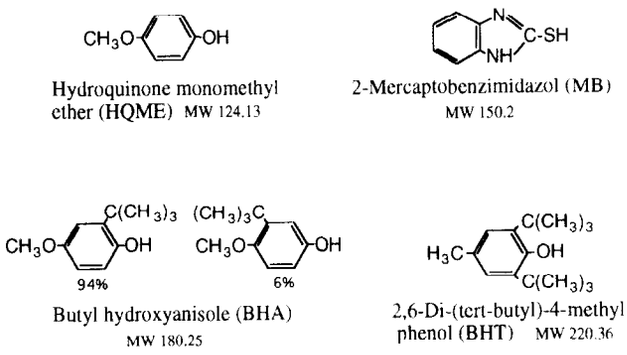

Figure 1 Chemical structures of the antioxidants used in this study

concentration of 2% w/w and a given amount of the melt mixture was mixed with a known weight of pure PEG, dissolved in Eagle's minimum essential medium (EMEM) without serum to have the PEG concentration of 40% w/w and the given concentration of antioxidant, and then used for fusion experiments.

Cell culture and fusion

The cell used in this study is L929, which is an established mouse fibroblast cell line. Cells were cultured with EMEM in a 10 cm diameter tissue culture dish at 37°C and 5% CO_2 with moisture. EMEM was used supplemented with 0.25% w/v sodium hydrogencarbonate, 0.03% w/v L-glutamine and 10% v/v fetal bovine serum (FBS). Semiconfluent cells in the logarithmic growth state were rinsed with EMEM without serum, trypsinized with 0.25% w/v trypsin and 0.02% w/v EDTA-2Na (disodium salt of ethylenediaminetetracetic acid) in PBS(−) (phosphate-buffered saline) for 5 min at 37°C. The isolated cells were transferred to a 50 ml centrifuge tube supplemented with the same volume of EMEM to inhibit the trypsin activity. After centrifugation at 1000 rev min^{-1} for 5 min, cells were resuspended in EMEM. The cell density was measured with a haemocytometer to prepare a cell suspension with a density of 1.5×10^5 cells/ml. For cell fusion experiments, accurately 2 ml of suspension was gently stirred for 10 min and put in a 24-well multidish culture plate (Corning, NY) drop by drop under gentle stirring. Cells were allowed to settle for 2 h in that position and then incubated at 37°C for 24 h. In the course of incubation, cells underwent sufficient attachment to the plate and spreading on it. The culture medium was aspirated out and 0.2 ml of 40% w/w PEG solution containing various concentrations of antioxidant was added and incubated for 1 min at room temperature. Then, cells were rinsed three times with 2 ml of EMEM without serum to remove the PEG solution and 1 ml of new EMEM was added. After further incubation at 37°C for 24 h, cells were fixed with 2.5% v/v glutaraldehyde at 4°C for 3 h and stained with Mayer's haematoxylin. Finally, optical microphotographs were taken so as to contain randomly about 500 cells per field and the degree of fusion (fusion index) was evaluated as a function of cell nuclei using the following equation:

$$\text{fusion index (\%)} = \frac{\text{total number of nuclei in fused cells}}{\text{total number of nuclei in all cells}} \times 100$$

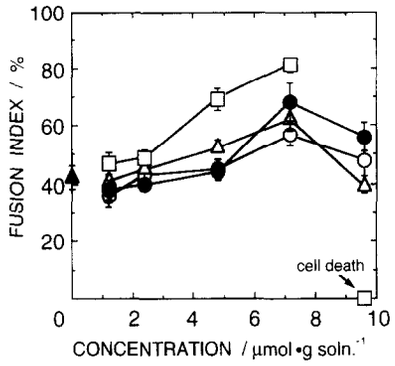

Figure 2 Effect of antioxidants added to 40% w/w PEG3000 on the fusion of L929 cells: (○) HQME, (△) MB, (□) BHA, (●) BHT, and (▲) without antioxidant. In the case of BHT, the experiment was carried out in heterogeneous system. Error bars show the standard error ($n = 6$)

In this study, two pieces of photographs were taken for every well and three wells were used for each sample to obtain the average fusion index.

Cytotoxicity test of antioxidants

The cytotoxicity test of antioxidants was carried out with L929 cells. They were first seeded in 24-well culture plate, similar to the fusion experiment. Two grams of 2% w/w melt of an antioxidant in PEG3000 kept at 60°C was added to 25 ml of EMEM without serum. The culture medium containing various concentrations of antioxidant was prepared by diluting this solution with EMEM. In this experiment, PEG was used only to enhance the solubility of antioxidants in the medium and no fusion took place at such a low PEG concentration as used in this experiment. Cells were treated with the antioxidant solution for 1 min at room temperature, rinsed three times with EMEM, and then further incubated with 1 ml of new medium for 2 h at 37°C. After trypsinization, the cells that were still alive and negatively stained with erythrosine were counted with a haemocytometer to estimate the cell viability, which was defined as the ratio of the number of living cells in antioxidant-treated cells to that in untreated ones.

Haemolysis of erythrocyte by antioxidants

Chloroform solution (2% w/w) was prepared from antioxidants except for MB, which was dissolved in methanol, and a given amount of this solution was placed in a vial. After thorough evaporation of the solvent, a given amount of PBS(−) was added to prepare the antioxidant PBS(−) solution with different concentrations. Sheep erythrocytes were suspended in PBS(−) at the concentration of 5×10^8 cells/ml and 1 ml of antioxidant solution was added to 1 ml of erythrocyte suspension, followed by incubation for 1 h at 37°C under gentle shaking. The mixture was centrifuged at 3000 rev min^{-1} for 5 min and 1 ml of supernatant was diluted at 3 ml with PBS(−). The percentage haemolysis of erythrocytes was estimated as the ratio of absorbance in the supernatant at 541 nm to that in the positive control treated with 0.1% w/v sodium dodecyl sulfate (SDS).

Determination of hydrophobicity of antioxidants

Hydrophobicity of antioxidants was evaluated by two methods as follows.

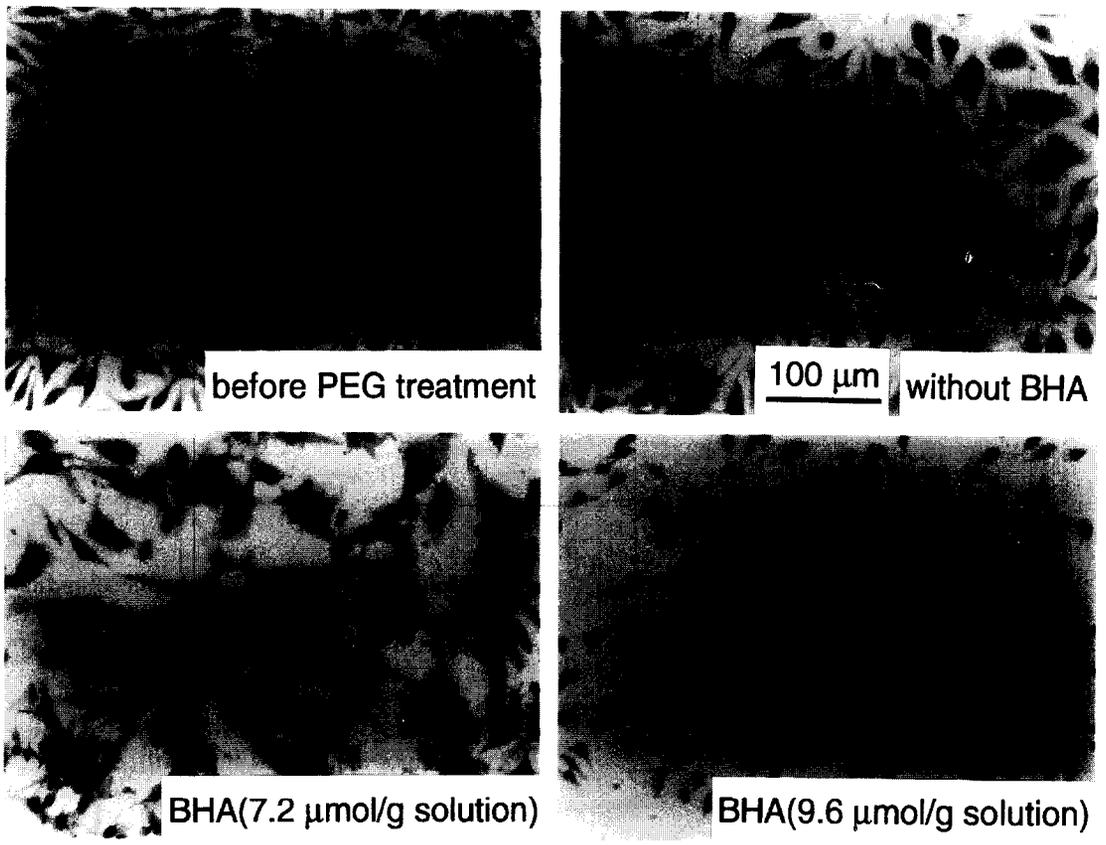

Figure 3 Optical microphotographs of L929 cells treated with 40% w/w PEG3000 for 1 min in the presence of different concentrations of BHA. Cells were stained with haematoxylin after fixation

(A) Partition coefficient in water/octanol. Ten millilitres of 0.02 mg ml^{-1} antioxidant solution in 1-octanol was mixed with exactly 200 ml of water and gently stirred for 2 h at 25°C. From the decrease of the antioxidant concentration in the 1-octanol phase estimated by pre-measured calibration curves in 1-octanol, the partition coefficient of antioxidant in the water/1-octanol system was determined.

(B) Measurement with hydrophobic probe. First, 0.05 ml of an aqueous solution of *N-p*-tolyl-2-naphthylamine-6-sulfonic acid (TNS) (0.02 mg ml^{-1}) was added to 4 g of dioxane contained 72 μmol of antioxidant mixed with 6 g of water. The fluorescence intensity of TNS was measured by scanning the emission peak at 420 nm with excitation wavelength fixed at 365 nm, as described in a previous work[7].

RESULTS AND DISCUSSION

The effect of four different antioxidants on the membrane fusion of L929 cells in 40% w/w aqueous PEG3000 solution is given in *Figure 2*. The antioxidant concentration in the PEG solution was changed from 1.2 to 9.6 μmol/g of solution. In the case of BHT, the cell culture medium was heterogeneous because of its low solubility in water. For comparison, the fusion index observed for the PEG solution without antioxidants is also shown as a function of 'antioxidant concentration' in *Figure 2*, simply because cell fusion was also carried out without antioxidant in parallel to a series of cell fusion studies with PEG solutions containing different concentrations of antioxidants. As can be seen from *Figure 2*, only BHA could enhance membrane fusion with an increase in the concentration among the antioxidants used in this study. No statistically significant effect was observed for other antioxidants. Most L929 cells were killed when the BHA concentration increased to 9.6 μmol g^{-1}. Optical microphotographs of L929 cells treated with PEG containing different concentrations of BHA are given in *Figure 3*. Apparently, cell morphology as well as membrane fusion were greatly influenced by the presence of PEG, but much more remarkably by the addition of BHA. In addition, no membrane fusion occurred when cells were treated with antioxidants in the absence of PEG (data not shown).

It is likely that enhanced cell fusion by very low concentrations of BHA is related to the degree of induced membrane damage, because the addition of 9.6 μmol g^{-1} BHA very severely destroyed the cell membrane, as shown in *Figure 3*. To study the viability of L929 cells when treated with PEG solution containing an antioxidant, the killed cells were counted after 1 min incubation. The result is given in *Table 1*. As can be seen, only BHA showed cytotoxicity to L929 cells, at least in the concentration range investigated. To confirm further the cytotoxic effect of the antioxidant, haemolysis of sheep erythrocytes was examined after their treatment with antioxidants. *Table 2* shows the result. Again, only BHA destroyed the erythrocyte membrane, suggesting a strong interaction of BHA with the cell membrane at high BHA concentrations, whereas other antioxidants

Table 1 Cytotoxicity of antioxidants to L929 cells treated for 1 min at 37°C, quoted as cell viability (%)[a]

	Concentration of antioxidants ($\mu g\,ml^{-1}$)						
	25	50	100	200	400	800	1600
HQME	100	100	100	100	100	100	100
MB	100	100	100	100	100	100	100
BHA	100	100	100	58 ± 6	53 ± 6[b]	0[b]	0[b]
BHT	100	100	100	100[b]	100[b]	100[b]	100[b]

[a] Data = average ± SE·($n = 3$)
[b] Solution of antioxidants was heterogeneous

Table 2 Haemolysis (%)[a] of sheep erythrocytes by antioxidants

	Concentration of antioxidants ($\mu mol\,g^{-1}$ of solution)				
	1.2	2.4	4.8	7.2	9.6
None	0	0	0	0	0
HQME	0	0	0	0	0
MB	0	0	0	0	0
BHA	0	0	0	64	53[b]
BHT	0	0	0	0	0

[a] Degree of haemolysis was evaluated as the ratio of absorbance at 540 nm assigned to haemoglobin to that in the positive control system treated with 0.1% w/v SDS
[b] Haemoglobin was discoloured

Table 3 Partition coefficient of antioxidants in water/1-octanol system

Antioxidant	λ_{max} (nm)[a]	C_o ($mg\,ml^{-1}$)[b]	C_w ($mg\,ml^{-1}$)[c]	C_o/C_w
HQME	294	1.186×10^{-1}	4.961×10^{-3}	23.9
MB	308	1.429×10^{-3}	3.916×10^{-5}	36.5
BHA	293	1.946×10^{-2}	4.905×10^{-5}	396.7
BHT	279	2.097×10^{-3}	1.690×10^{-5}	1240.8

[a] In pure 1-octanol
[b] Antioxidant concentration in the 1-octanol phase
[c] Antioxidant concentration in the water phase

were entirely negative in haemolysis of erythrocyte. Therefore, one can say that only BHA interacts strongly with the cell membrane among the four antioxidants, resulting in severe disturbance of the membrane structure and hence membrane fusion as reported elsewhere[8–10].

One of the methods available for estimating the interaction of foreign substances with cell membranes is to determine their partition coefficient in the membrane model system[11,12]. *Table 3* gives the partition coefficient of the four antioxidants observed for the water/1-octanol system. As can be seen, BHA showed neither the highest nor the lowest partition coefficient among the four antioxidants. The low partition coefficients of HQME and MB may indicate that these substances are not sufficiently hydrophobic to interact with the lipid bilayer of cell membrane. BHT does not have high enough solubility in water, although its hydrophilicity is very high.

BHA showed intermediate hydrophobicity and water solubility that might be optimal to interact with the lipid bilayer of a cell membrane. It follows that other chemicals that have the same extent of hydrophobicity and water solubility as BHA are thought to have a potential for membrane fusion even if they are not antioxidants. In fact, Ahkong *et al.* reported that fatty

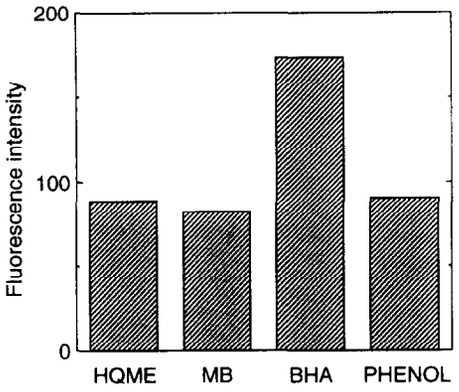

Figure 4 Dependence of the fluorescence intensity of TNS in a water and dioxane mixture (40/60 by weight) containing antioxidants of $7.2 \times 10^{-6}\,mol\,g^{-1}$. TNS concentration: 0.64×10^{-6} M

acids, esters, retinol, and α-tocopherol induced fusion of erythrocyte[13]. These substances seem to interact effectively with the erythrocyte membrane cooperatively working with PEG.

Hydrophobicity of a substance can also be estimated using TNS as a probe, because TNS is known to become fluorescent when placed in a hydrophobic environment. In a previous report, the fluorescence intensity of TNS placed in water/dioxane mixtures was shown to increase with an increase of dioxane fraction up to 0.9[7]. The fluorescence intensity of the TNS measured in a water/dioxane mixture containing antioxidants is given in *Figure 4*. Clearly, BHA showed the highest fluorescence intensity, in other words, the highest hydrophobicity among the water-soluble antioxidants used. The hydrophobicity of BHT could not be evaluated because of its poor solubility in the water/dioxane mixtures. Referring to the partition coefficient measured for the four antioxidants, BHT seems to be more hydrophobic than BHA. The reason that only BHA could promote cell fusion may be ascribed to its strong interaction with membrane components due to its sufficiently high hydrophobicity and high water solubility compared with other antioxidants. However, it should be noted that our results might be influenced by autoxidation products of the antioxidants as well as the antioxidants themselves because all experiments were carried out under aerobic conditions.

In conclusion, it may be summarized that some kinds of substance, such as BHA, added to commercial-grade PEG, can enhance cell fusion by PEG if they have well balanced hydrophobicity and water solubility to interact with cell membranes.

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