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Interactions of Microcapsules with Human Polymorphonuclear Leucocytes

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Two types of microcapsules with different negative surface potentials, poly(1,4-piperazinediylterephthaloyl) microcapsules containing various concentrations of sodium polystyrene sulfonate solution and hemolysate-loaded polyamide microcapsules of different degrees of carboxylation, were prepared by using an interfacial polymerization technique, and the interactions of these microcapsules with human polymorphonulcear leucocytes (PMNs) were investigated as a function of the surface potential of the microcapsules in the absence and presence of plasma proteins. The rate of oxygen consumption by PMNs was taken as a measure of the interaction.

In the absence of plasma proteins, the rate of oxygen consumption by PMNs increased with increasing difference in surface potential between the microcapsules and PMNs. The presence of plasma proteins affected the rate of oxygen consumption, which was dependent on the protein species present and related to the surface potential of the microcapsules. This was interpreted as showing that the surface potential of the microcapsules exerts a great influence on the mode and amount of protein adsorption on the microcapsule surface.

Keywords——microcapsules; leucocyte interaction; surface potential; plasma protein; protein adsorption

Hemolysate-loaded poly(N^{α},N^{ε}-L-lysinediylterephthaloyl) microcapsules have been studied by Kondo and coworkers in the hope that they may be used in transfusion as artificial red blood cells.[1-8] These artificial red blood cells enclose mammalian hemolysate within an ultrathin synthetic polymer film which has a high permeability towards low molecular weight solutes. Their oxygen absorption curves, enzymatic activities, and rheological properties were found to be comparable to those of native erythrocytes.[1-3] It was also found that platelet adhesion is greatly affected by the surface potential of the cells and adsorption of plasma components on the cell surface.[4-7] Moreover, their interaction with fibrinogen was found to cause disintegration of the cells under certain conditions.[8]

The present paper deals with the interaction of microcapsules with human polymorphonuclear leucocytes as a part of our studies on artificial red blood cells, because the cells should experience this sort of interaction when they are injected into the blood stream. Two types of microcapsules, poly(1,4-piperazinediylterephthaloyl) microcapsules containing sodium polystyrene sulfonate solution and hemolysate-loaded carboxylated polyamide microcapsules, were prepared by using an interfacial polymerization technique and the interaction of the microcapsules with the leucocytes was studied as a function of the surface potential of the microcapsules in the absence and presence of plasma proteins.

As poly(1,4-piperazinediylterephthaloyl) microcapsules containing various concentrations of sodium polystyrene sulfonate solution have membranes of the same chemical composition and probably identical physicochemical properties (except for their surface potential, which is dependent on the concentration of the polyelectrolyte solution en-

capsulated[9]), they are expected to be a very good model for studying the effect of surface potential on the interaction of microcapsules with polymorphonulcear leucocytes. The surface potential of microcapsules can also be varied by introducing various amounts of such ionizable groups as carboxyl groups into the polymer chains constituting the microcapsule membranes. This is done by using mixtures of piperazine and L-lysine, which has a carboxyl group in the molecule, because the carboxyl group remains unreacted in the interfacial polycondensation reaction between the amino acid and any acid dichloride.[10] In this case, the composition and properties of the microcapsule membrane vary with the ratio of L-lysine to piperazine in the mixture.

Experimental

Preparation of Microcapsules——Poly(1,4-piperazinediylterephthaloyl) (PPiP) microcapsules containing various concentrations of sodium polystyrene sulfonate (PSS) solution were prepared in the following way. A 0.4 M piperazine solution (4 ml) containing 0.45 M sodium carbonate was added to 4 ml of PSS solution containing dextran in a 200 ml round-bottomed flask surrounded by ice. The alkali served to neutralize hydrogen chloride formed as the by-product in the interfacial polycondensation reaction between piperazine and terephthaloyl dichloride. The concentration of PSS solution was varied by mixing 6% (w/v) dextran solution in various volume ratios.

After addition of 40 ml of a mixed organic solvent (chloroform–cyclohexane, 1 : 3 (v/v)) containing 15% (v/v) sorbitan trioleate to the mixed aqueous solution in the flask, the content of the flask was mechanically emulsified for 20 min to give a W/O emulsion. Without stopping the stirring, 40 ml of 0.04 M terephthaloyl dichloride solution in the mixed organic solvent was added to the emulsion, and the stirring was continued for 30 min. Then, 120 ml of cyclohexane was added to the system to stop the interfacial polycondensation reaction. The microcapsules thus obtained were separated from the organic phase by centrifugation and washed three times with cyclohexane. The separated microcapsules were completely dispersed in a small volume of 50% (v/v) polyoxyethylene sorbitan monolaurate solution and the resultant dispersion was diluted with distilled water, followed by centrifugation to separate the microcapsules. The collected microcapsules were washed ten times with distilled water and an isotonic phosphate-buffered saline containing 1 mM Ca^{2+} and 2 mM Mg^{2+} (PBS, pH 7.4), and dispersed in the same medium.

The microcapsules were then fractionated into three fractions of different capsule sizes by consecutive centrifugations at 1000, 2000, 3000 and 4000 rpm. The mean diameters of the three fractions were 2.43 ± 0.64, 1.42 ± 0.44, and 1.13 ± 0.22 μm, respectively. These fractions were the sediments in the second, third, and last centrifugations. Each of these fractions was redispersed in PBS and dialyzed against PBS for 3 d, and the concentration was adjusted to be approximately 2.4×10^{10} capsules/ml in PBS.

Sheep hemolysate-loaded carboxylated polyamide (CPA) microcapsules were prepared as follows.[2] A sheep hemolysate (1.5 ml) containing piperazine, L-lysine, and sodium carbonate was passed through a needle at a constant rate of 0.0042 ml/min by means of a microfeeder into 100 ml of a mixed organic solvent (chloroform–cyclohexane, 1 : 3 (v/v)) containing terephthaloyl dichloride, 1.5×10^{-4} M tetraethylammonium chloride, and 10% (v/v) sorbitan trioleate in a beaker surrounded by ice. The concentrations of terephthaloyl dichloride used were 0.04, 0.05, and 0.06 M. Piperazine and L-lysine were employed at 0.067 and 0.134 M and 0.4 and 0.2 M, respectively. A platinum wire (used as the cathode) was immersed in the organic solvent and an electric potential of 1000 V was applied between this wire and the needle (used as the anode) during the addition of the hemolysate. Spontaneous emulsification occurred and a shower of very fine hemolysate droplets was formed. The quaternary ammonium salt served to give electroconductivity to the organic solvent.

On the surface of each hemolysate droplet, polycondensation took place between the diamine mixture and terephthaloyl dichloride to form a carboxylated polyamide membrane. Hydrogen chloride generated in the reaction was immediately neutralized by the alkali in the aqueous phase. After stirring of the system for 30 min, 100 ml of cyclohexane was added to stop the interfacial polycondensation reaction. The microcapsules thus formed were separated by centrifugation and washed three times with cyclohexane, followed by dispersion in a small volume of 50% (v/v) aqueous polyoxyethylene sorbitan monolaurate solution under stirring with a magnetic stirrer.

The hemolysate-loaded microcapsules were fractionated into three fractions of different capsule sizes by centrifugation in a sucrose gradient, formed by layering 10 ml each of 1.0, 0.6, and 0.2 M sucrose solutions and microcapsule suspension in that order in a centrifuge tube. The tube was centrifuged at 2500 rpm for 20 min. The microcapsules gathered at the interfaces between sucrose solution of different concentrations were collected separately. Then, carbon monoxide gas was passed through suspensions of the fractionated microcapsules for 3 h to convert hemoglobin into carboxyhemoglobin, which does not react with oxygen. The microcapsule concentration in each suspension was determined turbidimetrically after the suspension had been dialyzed against PBS for 3 d.

Isolation of Polymorphonuclear Leucocytes from Blood——Polymorphonuclear leucocytes (PMNs) were isolated from fresh human blood by the dextran-Ficoll method.[11] Fresh human blood was centrifuged for 10 min after the

addition of an anticoagulant to separate platelet-rich plasma, and an erythrocyte precipitate containing leucocytes was obtained. The precipitate was placed in a polycarbonate beaker, to which was added an equal volume of 3% (v/v) dextran solution in saline. The mixture was stirred well and then left to stand. After 30 min, 20 ml of the leucocyte suspension was gently poured on top of an equal volume of Ficoll–Hypaque in each of six polycarbonate centrifuge tubes and the tubes were centrifuged for 30 min. As the pellets of PMNs obtained were contaminated with a small amount of erythrocytes, a 0.83% (w/v) aqueous ammonium chloride solution was added to hemolyze the erythrocytes. The PMNs were dispersed well in PBS, and the dispersion was allowed to stand for 15 min, then centrifuged for 10 min to separate the cells. The separated cells were washed three times with PBS and redispersed in the same medium. The number of cells in the dispersion was counted with a Coulter counter and the cell concentration was adjusted to be approximately 1.0×10^7 cells/ml by the addition of PBS.

In some cases, the PMN dispersion was further centrifuged and the cell pellets were dispersed in an equal volume of a plasma protein solution. The plasma protein solutions used were human fresh plasma with heparin added (20 units/ml blood), human serum preincubated at 37 °C for 30 min, human inactivated serum preincubated at 56 °C for 30 min, 1% (w/v) human serum albumin (HSA) (Sigma, crystallized and lyophilized, essentially globulin-free A-8763) in PBS, and 1% (w/v) human γ-globulin (HγG) (Sigma, γ-globulins human Cohn fraction II) in PBS.

Determination of Rate of Oxygen Consumption——It is accepted that when the surface membranes of PMNs are stimulated an increased oxygen consumption is observed in the presence or absence of phagocytosis. Hence, if the rate of oxygen consumption is measured with a suitable oxygen electrode apparatus (Yellow Springs Co., U.S.A.) it can provide an index of the interaction between PMNs and microcapsules.

Two ml of the PMN suspension was placed in a measuring chamber of the apparatus at 37±0.1 °C. The cell suspension was then stirred with a magnetic stirrer for 5 min. At the end of this period, 100 μl of a microcapsule suspension was added to the cell suspension, the chamber was quickly and firmly closed, and the oxygen consumption was recorded on a chart.

The analysis of the rate of oxygen consumption was carried out as follows using the equation

$$r = \frac{io}{cp}$$

where r is the rate of oxygen consumption (Δ nmol $O_2/10^7$ cells/min),[12] p is the PMN concentration (1×10^7 cells/ml), c is the chart value for the medium in which the cells are suspended (divisions), and i is the slope of the tangent of the curve on the chart (Δ divisions/min). The value of r calculated using the maximum inclination was called the maximum rate of oxygen consumption, while that calculated using the inclination determined 2 min after microcapsule addition was named the initial rate of oxygen consumption.

Measurement of Electrophoretic Mobilities of Microcapsules and PMNs——Electrophoretic mobilities of the microcapsules and the PMNs were determined with a microelectrophoresis apparatus (Sugiura Lab., Inc., Tokyo). Mobility was obtained by measuring the time needed for a microcapsule or a cell to travel a certain distance. At least 30 particles were timed in each direction to eliminate the polarization effect of the electrodes.

The electrophoretic mobility, u, of a particle was calculated by use of the following equation

$$u = \frac{dks}{it}$$

where d is the distance covered by the particle, k is the electric conductance of the dispersion medium, s is the cross-sectional area of the measuring cell, i is the electric current in the cell, and t is the time required for the particle to cover the distance d.

The mobilities of the microcapsules coated with plasma proteins were determined in the same manner. Coating of the microcapsules with plasma proteins was done as follows. The microcapsules were dispersed in one of the following: human fresh plasma, human serum, human inactivated serum, 1% HSA in PBS and HγG in PBS. The dispersions were incubated with shaking at 37 °C over night. The protein-coated microcapsules were then separated by centrifugation, washed three times with PBS, and redispersed in the same medium.

Results and Discussion

Choice of PMN and Microcapsule Concentrations

The concentration of PMN relative to that of microcapsules is important in relation to the interaction.[13,14] Figure 1 shows the rate of oxygen consumption in the interaction of PPiP microcapsules containing water alone with PMNs as a function of the PMN concentration when the microcapsule concentration is 2.4×10^{10} capsules/ml.

The rate of oxygen consumption increased linearly with increasing PMN concentration up to a value of 1.3×10^7 cells/ml, beyond which the rate showed a tendency to level off. This

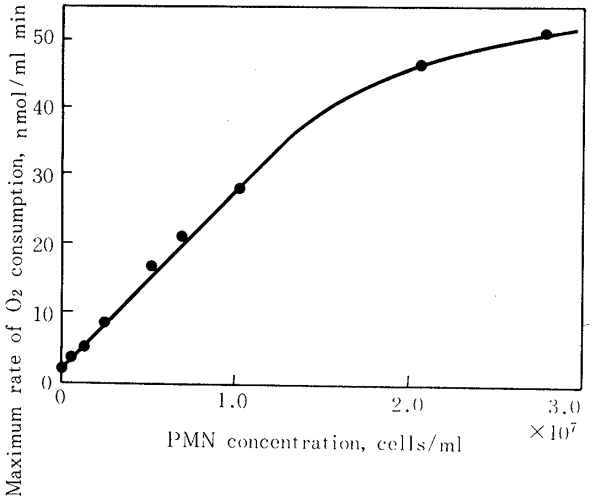

Fig. 1. Effect of PMN Concentration on the Rate of Oxygen Consumption by the Cells in Their Interaction with PPiP Microcapsules

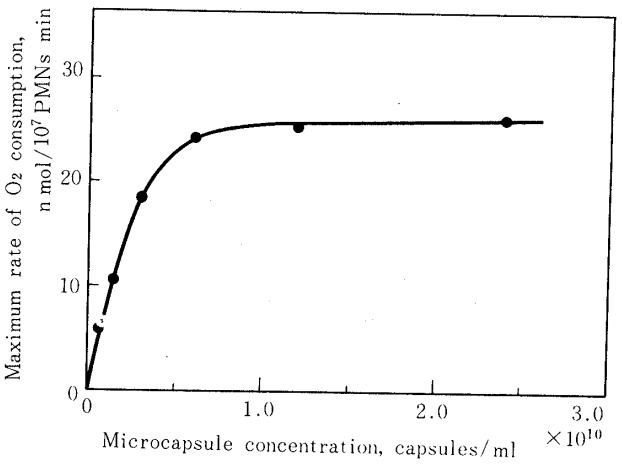

Fig. 2. Effect of Microcapsule Concentration on the Rate of Oxygen Consumption by PMNs in Their Interaction with Microcapsules

TABLE I. Effect of Microcapsule Size on the Rate of Oxygen Consumption

Microcapsule size (μm)	Initial rate of oxygen consumption (nmol/10^7 cells/min)	Maximum rate of oxygen consumption (nmol/10^7 cells/min)
1.13±0.22	25.0±2.3	25.5±3.4
1.42±0.44	24.8±2.1	26.0±2.1
2.43±0.64	23.2±1.5	24.3±0.4

may be either due to a lowering of stimulation caused by aggregation of the cells at high cell concentrations or due to a decrease in contact frequency brought about by low ratios of the number of microcapsules to that of cells in this range of cell concentration. As it was convenient to operate the oxygen electrode at as high an oxygen consumption as possible, the PMN concentration was fixed at 1.0×10^7 cells/ml in this work.

The rate of oxygen consumption also changed with the number of microcapsules added to the PMN suspension. Figure 2 shows the rate of oxygen consumption in the interaction of PPiP microcapsules containing water with PMNs as a function of the microcapsule concentration.

The rate of oxygen consumption rises first and then levels off with increase in the microcapsule concentration. Consequently, the microcapsule concentration was adjusted to be approximately 2.4×10^{10} capsules/ml in all cases in the present work to make the oxygen consumption as high as possible.

Effect of Particle Size on the Interaction

It is possible that particle size affects the interaction, and small particles may be phagocytosed more easily than large ones.[15,16] As any microcapsule sample has a size distribution, it would be necessary to take this fact into consideration in the analysis of oxygen consumption data unless the effect of capsule size is small. Fortunately, the rates of oxygen consumption for the three fractions of PPiP microcapsules were found to be identical, as shown in Table I.

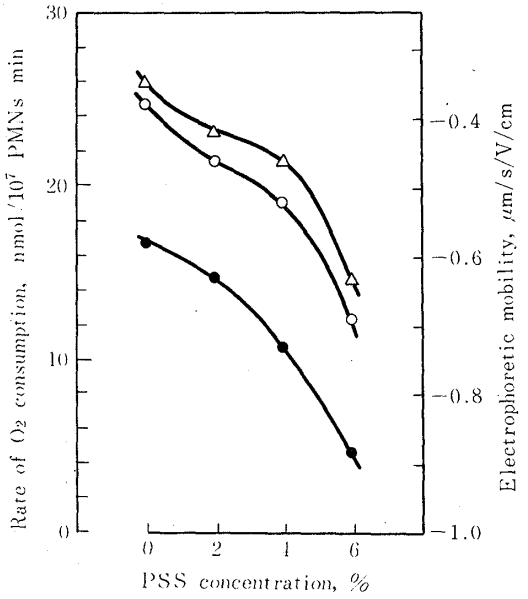

Fig. 3. Effect of Surface Potential on the Rate of Oxygen Consumption by PMNs in Their Interaction with PSS-Loaded PPiP Microcapsules

Initial oxygen consumption rate (○), maximum oxygen consumption rate (△), and electrophoretic mobility (●).

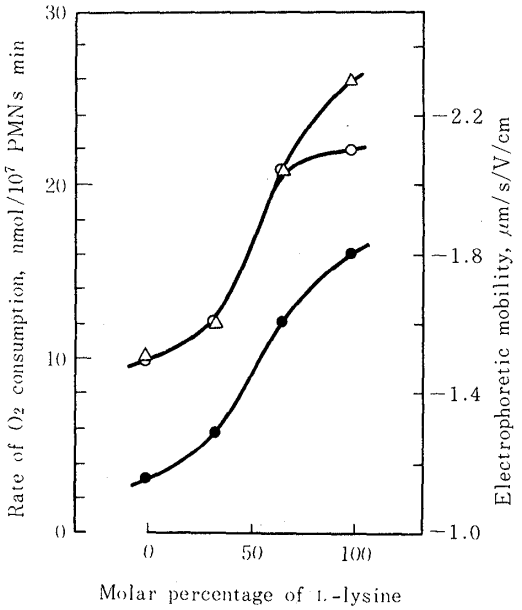

Fig. 4. Effect of Surface Potential on the Rate of Oxygen Consumption by PMNs in Their Interaction with Hemolysate-Loaded CPA Microcapsules

Initial oxygen consumption rate (○), maximum oxygen consumption rate (△), and electrophoretic mobility (●).

Effect of Surface Potential on the Interaction

Figures 3 and 4 show the effect of surface potential, which parallels the concentration of encapsulated PSS or the mole percent of L-lysine in the diamine mixture used in the microcapsule preparation, on the rate of oxygen consumption in the interaction with human PMNs of PPiP microcapsules containing PSS solution and hemolysate-loaded CPA microcapsules, respectively. In both figures, electrophoretic mobility, a measure of surface potential, is given as a function of the concentration of encapsulated PSS or the mole percent of L-lysine in the diamine mixture, instead of the potential itself.

In the interaction of PPiP microcapsules with PMNs, both the initial and maximum rates of oxygen consumption decreased with increasing concentration of encapsulated PSS or rising negative surface potential. In contrast, the rates increased as the mole percent of L-lysine increased when hemolysate-loaded CPA microcapsules stimulated PMNs. It was evident, however, that in both cases the rate of oxygen consumption, either initial or maximum, increases with increase in the difference of surface potential between the microcapsules and the PMNs, whose mobility value was $-1.0\,\mu$m/s/V/cm.

According to the theory of heterocoagulation based on the DLVO theory of colloid stability,[17] the electrostatic interaction between two colloidal particles with surface potentials of the same sign but different magnitudes gives rise to an attractive force between the particles at small interparticle distances as a result of overlapping of electric double layers around them, and this attractive force starts acting at larger interparticle distances as the difference in the magnitude of surface potential between the particles becomes large, while a repulsive force operates at large interparticle distances. Although great care should be taken in applying this theory to the interaction between PMNs and microcapsules, the theory might still be useful, at least in the early stages of the interaction where microcapsules and PMNs approach each other. Those microcapsules which have surface potentials much higher or lower than that of PMNs will adhere easily to the surface of PMNs due to the attractive force operating between

them, while those with surface potentials equal or very close to that of the leucocytes will be less adhesive. As adhesion of microcapsules to the surface of PMNs will be a prerequisite for stimulation of the cells, it seems reasonable to assume that the more microcapsules adhere to the surface of PMNs, the more the rate of oxygen consumption rises.

It was reported that polystyrene latex particles are readily phagocytosed by PMNs, accompanied with an increased respiration, when they adhere to the cells.[18] Similarly, microcapsules are quite likely to be phagocytosed by PMNs because there are only minor differences in size and surface characteristics between the two types of fine particles. However, it was not possible to obtain direct evidence of phagocytosis of microcapsules by PMNs in the present work because of experimental difficulty.

Effects of Plasma Proteins on the Interaction

When microcapsules come into contact with blood they must adsorb some components of plasma before they interact with PMNs. In view of this, the effects of plasma proteins on the interaction of microcapsules with PMNs are very important. The effects of plasma proteins on the interaction of PSS-loaded PPiP microcapsules with PMNs are shown in Fig. 5, where the maximum rate of oxygen consumption is plotted against the electrophoretic mobility of the bare microcapsules.

Oxygen consumption in the interaction was enhanced by the presence of human fresh plasma or human serum, while HγG and HSA suppressed oxygen consumption by the leucocytes. Human inactivated serum had no appreciable effect on the interaction. Similar results were obtained when the initial rate of oxygen consumption was adopted as the index of the interaction, though the data are not shown here.

Plasma, serum, inactivate serum, serum γ-globulin, and serum albumin all diminished the

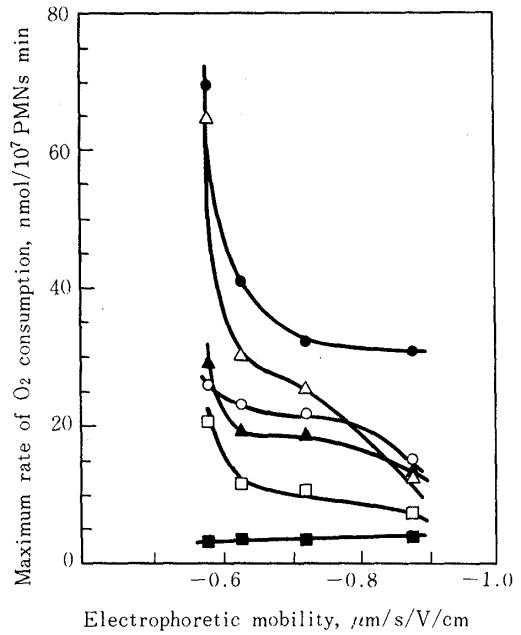

Fig. 5. Effects of Plasma Proteins on the Maximum Rate of Oxygen Consumption by PMNs in Their Interaction with PSS-Loaded PPiP Microcapsules Having Different Surface Potentials

Human plasma (●), human serum (△), human inactive serum (▲), HγG (□), HSA (■), and buffer (○).

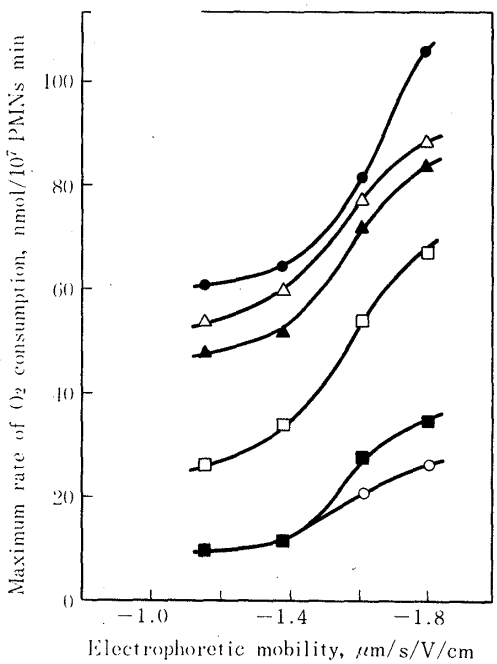

Fig. 6. Effects of Plasma Proteins on the Maximum Rate of Oxygen Consumption by PMNs in Their Interaction with Hemolysate-Loaded CPA Microcapsules Having Different Surface Potentials

Human plasma (●), human serum (△), human inactive serum (▲), HγG (□), HSA (■), and buffer (○).

difference in electrophoretic mobility with bare PSS-loaded PPiP microcapsules. The values of mobility for the protein-treated microcapsules were lower than those for the bare microcapsules, but were dependent on the individual plasma proteins used. This strongly suggests that considerable amounts of plasma proteins are adsorbed on the microcapsule surface and may promote or suppress microcapsule adhesion to PMNs. The mode and amount of adsorption for each protein would be dependent on the surface potential of bare PPiP microcapsules.

It is accepted that opsonin (the general name given to those substances which enhance stimulation of PMNs, leading to phagocytosis) includes IgG, complement α_1-globulin, β-globulin, α_2-glycoprotein. As these opsonin fractions are present in plasma and serum, some of them are presumably responsible for the observed enhancement of respiration. The difference between plasma and serum in enhancing respiration of PMNs can be ascribed to the presence of fibrinogen in the former and/or of heparin added to the former as an anticoagulant. Heating at 56 °C for 30 min causes complement to lose its activity. Hence, complement plays a part in the enhancement of the interaction of PSS-loaded PPiP microcapsules by plasma and serum.

Figure 6 shows the effects of plasma proteins on the interaction of hemolysate-loaded CPA microcapsules; the maximum rate of oxygen consumption in the presence of plasma proteins is given as a function of the electrophoretic mobility of bare CPA microcapsules.

In contrast to the case of PSS-loaded microcapsules, with the exception of serum albumin, all plasma proteins enhanced respiration of PMNs in the interaction of the cells with hemolysate-loaded CPA microcapsules. The electrophoretic mobilities of CPA microcapsules were reduced by the presence of those plasma proteins which enhanced respiration, indicating adsorption of these proteins on the microcapsule surface. HSA was found electrophoretically not to be adsorbed on the microcapsules, and it had no enhancing effect on oxygen consumption by PMNs. These data clearly suggest the existence of a relationship between the interaction and protein adsorption.

In conclusion, it is indicated that the surface potential of bare microcapsules directly affects their interaction with PMNs in the absence of plasma proteins, while it has only an indirect effect on the interaction in the presence of plasma proteins because the adsorption of these proteins on microcapsules takes place prior to their contact with PMNs.

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