

## Enhancement of Viral Fusion by Nonadsorbing Polymers

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**ABSTRACT** Nonadsorbing polymers such as dextran and poly(ethylene glycol) enhance binding as well as extents of fusion of influenza virus with erythrocytes. Kinetics and extent of viral membrane fusion were measured using an assay based on lipid mixing of a fluorescent dye. The effects of nonadsorbing polymers were in the concentration range from 0 to 10 wt%, far below the concentration required to overcome hydration repulsion forces. The enhancing effects were dependent on the molecular weight of nonadsorbing polymer, and only occurred at molecular weight > 1500; this links the phenomena we observe to the so-called "excluded volume effect" of nonadsorbing polymers. The time delay between triggering and the onset of influenza virus fusion was significantly reduced in the presence of nonadsorbing polymers. High molecular weight poly(ethylene glycol) also induced fusion of vesicular stomatitis virus with intact erythrocytes, which do not serve as target of vesicular stomatitis virus fusion in the absence of the polymer. The forces between membranes which determine rate-limiting processes in viral fusion and how they are affected by nonadsorbing polymers are discussed.

### INTRODUCTION

Influenza virus (orthomyxoviruses) enters host cells via receptor-mediated endocytosis. Acidification of the endosome leads to fusion between viral and endosomal membrane, thereby releasing nucleocapsid material to the cytosol (1). Both attachment of viral envelope to cell surface receptors and fusion with the endosomal membrane are mediated by the influenza virus spike protein, the hemagglutinin (HA)<sup>1</sup> (2). The HA undergoes an irreversible conformational change at low pH leading to the exposure of a hydrophobic segment of the HA2 subunit and promotion of the fusion reaction (3, 4). The plasma membrane of human erythrocytes has been widely used as a model target membrane to investigate fusion between influenza virus and biological membranes. In this system, the fusion process is regulated by the pH of the suspension medium (5, 6). Fusion of bound influenza virus with erythrocyte membranes is preceded by a pH-dependent lag time, reflecting a series of activation steps, the molecular details of which, are unclear at present (6-13).

Recently, we have proposed that the duration of the delay time of influenza virus fusion with red blood cells (RBCs) may also depend on the hydrophobicity of both fusing membranes. This proposal was based on two findings: (i) the

dependence of the delay time on the surface density of HA capable of exposing the hydrophobic sequences of the HA ectodomain (6), (ii) dependence of the delay time on the lipid composition of the external leaflet of the erythrocyte membrane, but no apparent dependence on headgroup composition (6, 14). The generality of this proposal for fusion of enveloped viruses with target membranes is suggested by our study of low pH-induced fusion of vesicular stomatitis virus (VSV, rhabdovirus) with erythrocyte membranes. Erythrocyte membranes only became susceptible to fusion with VSV upon increasing the hydrophobicity of their outer leaflet (15).

It is not yet clear which activation step(s) of enveloped virus fusion might be affected by the membrane surface hydrophobicity. One reasonable assumption is that the increase of the hydrophobicity translates into a decreased repulsion between membranes forcing them into closer apposition appropriate for fusion. In this study we investigate how nonadsorbing polymers such as poly(ethylene glycol) and dextran affect *acid-triggered* fusion of influenza virus (A/PR 8/34) and VSV with intact human red blood cells. Exclusion of high molecular weight nonadsorbing water soluble polymers from macromolecular surfaces can generate a large osmotic pressure in aqueous solution which has been shown to dehydrate membranes and promote aggregation of phospholipid vesicles (16-20) and cells (21). We use a fluorescence dequenching assay to monitor viral fusion with the erythrocyte membrane, based on the relief of self-quenching of the "lipid-like" probe octadecylrhodamine (R18) initially incorporated into virus membranes (22). The particular advantage of using acid-triggered viral fusion models in these studies is that the fusion event is dominated by virus bound to the membrane at the time of mixing. Thus, we can neglect consideration of diffusion limited virus-cell association in our analysis. In addition, virus fusion-mediated dequenching of

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<sup>1</sup> Abbreviations used: HA, hemagglutinin; PBS, phosphate-buffered saline; R18, octadecylrhodamine B chloride; PEG, poly(ethylene glycol); VSV, vesicular stomatitis virus; FDQ, fluorescence dequenching; RBC, red blood cell.

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the fluorescence probe can be well separated from dequenching by nonspecific processes, e.g., lipid transfer, at neutral pH.

## EXPERIMENTAL PROCEDURES

### Materials

Octadecylrhodamine B chloride (R18) was purchased from Molecular Probes (Junction City, OR). Poly(ethylene glycol) of different average molecular weights were from Sigma (St. Louis, MO: PEG 8000, PEG 200) or Fluka AG (Buchs: PEG 1540, PEG 20000) and dextran of a molecular weight of 9400 from Sigma. Fresh blood from healthy donors was obtained from the National Institutes of Health Blood Bank. Purified VSV (Indiana) and influenza virus A/PR 8/34 was provided by J. Brown (University of Virginia) and A. Loyter (Tel Aviv), respectively.

### Red blood cell preparation

RBCs were washed three times in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% bovine serum albumin.

### Labeling of virus

4  $\mu$ l of 1 mg/ml R18 in ethanol was added with rapid vortexing to 1 ml of A/PR 8/34 (1 mg of influenza virus protein/ml). After incubation for 15 min at room temperature (in the dark) virus was washed with 10 volumes of ice-cold PBS on a sucrose cushion (70% sucrose) using a LM8-ultracentrifuge (rotor Ti-70, 30,000 rpm, 30 min, 4°C; Beckman Instruments, Inc., Palo Alto, CA) and resuspended to a concentration of 1 mg/ml. Labeling of VSV was performed as described for influenza with the modification that 9  $\mu$ l of R18 stock solution was added to 1 mg of VSV protein.

### Virus binding to cells

0.1 mg of R18 labeled A/PR 8/34 (or 0.03 mg of R18 labeled VSV) was incubated for 45 min on ice with 1 ml of RBC suspension containing  $10^8$  cells/ml as determined with a Coulter Multisizer (Coulter Instruments Inc., Hialeah, FL). The suspension was then washed in 10–15 volumes of ice-cold PBS and resuspended to a cell concentration of  $10^9$  cells/ml.

### Fusion analysis

Fluorescence dequenching of R18-labeled virus attached to RBCs was measured using a Model 8000 spectrofluorometer (SLM-Aminco; Urbana, IL). Usually, fluorescence data were recorded with 1-s time resolution (integration rate, 0.9 s). To assess the delay of the onset of fusion at 30°C, a 0.2-s time resolution (integration rate, 0.1 s) was chosen. 20  $\mu$ l of virus-RBC suspension was transferred in a disposable plastic cuvette containing 2 ml of prewarmed (37°C) PBS, pH 7.4, and the desired concentration of poly(ethylene glycol) or dextran. The suspension was stirred continuously with a 2  $\times$  8-mm Teflon-coated magnetic stir bar. After 1 min the pH was lowered by injecting appropriate amounts of 0.25 M citric acid. Usually, the fluorescence was monitored for 380 s ( $\lambda_{ex}$  = 560 nm,  $\lambda_{em}$  = 590 nm, cut-off filter 570 nm), after which Triton X-100 (0.05% final concentration) was added to obtain maximum R18 fluorescence  $F(\infty)$ . The percentage of fluorescence dequenching FDQ was calculated as described previously (23):

$$\% \text{FDQ} = 100 \times [F(t) - F(0)]/[F(\infty) - F(0)] \quad (1)$$

with  $F(0)$  and  $F(t)$  corresponding to the fluorescence intensity of the virus before starting fusion and the fluorescence intensity at a given time  $t$ , respectively.

### Binding assay

The influence of polymer on the amount of virus released from RBC was measured at 37°C, pH 7.4. 25  $\mu$ l of influenza-RBC suspension ( $10^8$  cells/ml) was transferred to a disposable plastic cuvette containing 2.5 ml of prewarmed buffer together with the specified concentration of the polymer (PEG or dextran). After 5 min of continuous stirring, 1 ml of the suspension was diluted 1:1 with PBS, and the fluorescence intensity obtained in the presence of 0.05% Triton X-100 was taken as a measure of the total amount of virus present  $F(\text{tot})$  (attached and released). The amount of released virus was ascertained from the fluorescence intensity of the supernatant  $F(\text{sn})$  after centrifugation of the suspension at low speed (Beckman Microfuge 11, 3 min) and 1:1 dilution of the supernatant PBS (Triton X-100, 0.05% final concentration). From  $F(\text{tot})$  and  $F(\text{sn})$  the percentage of released virus with respect to the total amount of virus present was obtained.

### Hemolysis assay

After removal of unbound virus, 20  $\mu$ l of the A/PR 8/34-RBC suspension ( $10^8$  cells/ml) was transferred to 180  $\mu$ l of PBS, pH 7.4, with the desired concentration of PEG 8000 (0–10 wt%). After 5 min of incubation 1.8 ml of prewarmed (37°C) buffer (150 mM NaCl, 20 mM sodium acetate buffer, pH 5.0) containing PEG 8000 was added. Following an incubation at 37°C for 5 min the suspension was centrifuged, and 0.2 ml of the supernatant was diluted in 1 ml of 0.5%  $\text{NH}_4\text{OH}$  and the absorbance was measured at 540 nm. The reference (100% hemolysis) was determined by mixing of 0.2 ml of the suspension in 1 ml of 0.5%  $\text{NH}_4\text{OH}$ .

## RESULTS

### The influence of PEG 8000 and Dextran 9400 on fusion of influenza virus with erythrocytes

Fig. 1 shows the influence of Dextran 9400 on the time course of R18 dequenching due to fusion of prebound, labeled influenza virions with RBC at pH 4.9, 37°C. Clearly the extent of fusion was enhanced in the presence of the polymer.

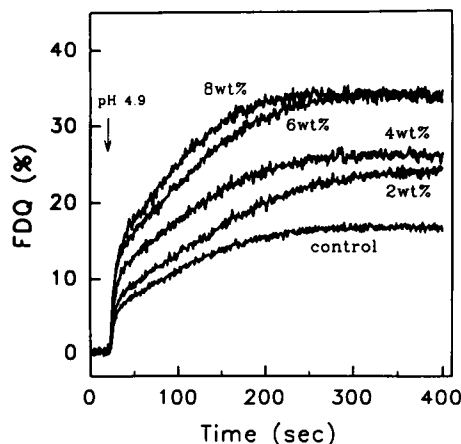


FIGURE 1 Influence of Dextran 9400 on fusion of A/PR 8/34 with RBC at pH 4.9, 37°C. R18 A/PR 8/34 was bound to RBC, washed, and incubated in PBS of different polymer concentration (marked on the curve), pH 7.4, at 37°C. The pH in the medium was changed as indicated by the arrow. %FDQ was calculated according to Eq. 1. Kinetics of FDQ were measured with a time resolution of 1 s.

The kinetics of low pH-triggered influenza virus fusion in the presence of PEG 8000 was similar to that observed with the Dextran 9400 (data not shown). In Fig. 2 the dependence of extent of influenza fusion on concentration of both polymers is given. No significant differences between PEG 8000 and Dextran 9400 were observed.

The fusion extent reached its maximum value at polymer concentrations  $\geq 6$  wt%. The initial rate of fluorescence dequenching normalized to the final fusion extent was almost independent of the presence of polymers.

The pH dependence of influenza virus fusion with erythrocyte membranes was shifted to higher pH in the presence of PEG 8000 (8 wt%) (see Fig. 3). The maximal fusion extent in the presence of PEG was obtained at higher pH compared to the control (no polymer). A plateau phase was found where fusion extent was independent of pH (pH 5.2–4.9). Comparable results were found with Dextran 9400 (Fig. 2). The similar values obtained for the extent of these two plateaus (Figs. 1 and 2) suggest to us that the maximum degree of fusion that our system can achieve, has been reached.

No fluorescence increase in the presence of polymers was observed with A/PR 8/34 at pH 7.4, 37°C, or with virus that was inactivated by preincubation at pH 5.0, 37°C. This indicates that the fluorescence dequenching in the presence of polymers is related to viral fusion and not to nonspecific effects.

In studies with HA-expressing cells it was shown that low pH-induced membrane fusion is preceded by a delay time which depends on the temperature (7, 8). With intact influenza virus, time delays between triggering and the onset of fusion in the range of 1–10 s have been observed at temperatures above 30°C (Ref. 6, see also Ref. 9). In agreement with previous results on the pH dependence of fusion (6) we show that a longer delay time correlates with a reduced extent of virus fusion. In Fig. 4 the influence of PEG on the delay

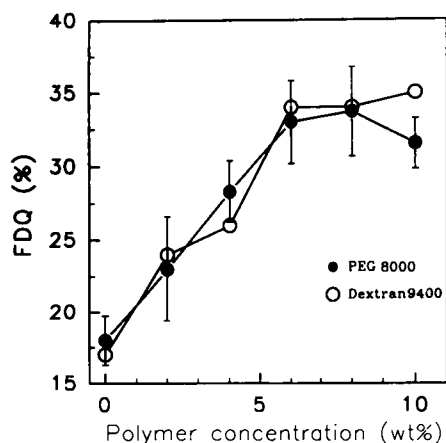


FIGURE 2 Comparison of the influence of PEG 8000 (●) and Dextran 9400 (○) on the fusion of A/PR 8/34 with RBC at pH 4.9, 37°C. The extent of fluorescence dequenching was measured 380 s after lowering the pH as described in the legend to Fig. 1. Standard deviation is given for PEG values. Data represent three independent measurements.

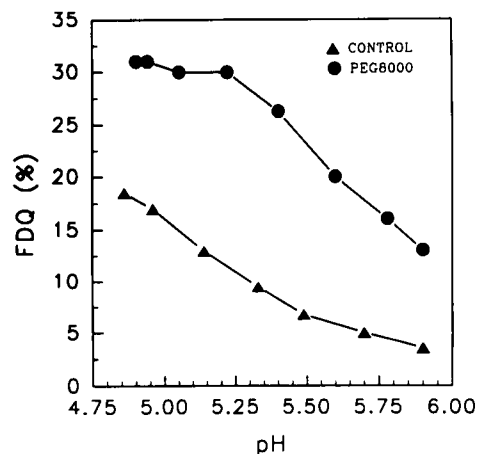


FIGURE 3 Effect of PEG 8000 on the pH dependence of fusion of A/PR 8/34 with RBC. A/PR 8/34-RBC complexes were incubated either in the absence (control, ▲) or presence of 8wt% PEG (●) at pH 7.4, 37°C before lowering pH (cf. Fig. 1). The extent of fluorescence dequenching was measured 380 s after lowering the pH as described in the legend to Fig. 1. A typical experiment is presented. The standard deviation is in the order of 10% of the average.

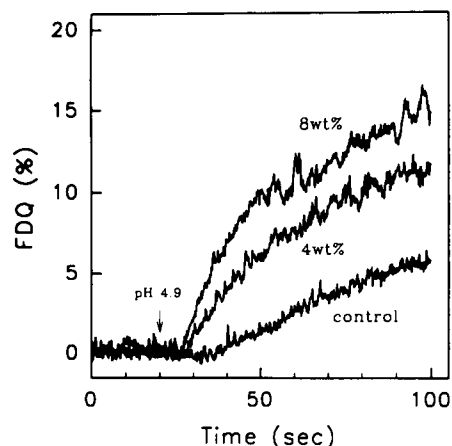


FIGURE 4 Influence of PEG 8000 on the delay time of the onset of fusion after lowering the pH. These experiments were done at 30°C to resolve the lag phase. The pH was shifted to 4.9 as indicated by the arrow (experimental details see legend to Fig. 1). Each curve represents the average of four single kinetics which were measured with a time resolution of 0.2 s. PEG concentrations are marked on the curves.

before the onset of fusion at 30°C is shown. In the absence of PEG the delay was about 18 s at pH 4.9.

It decreases in the presence of PEG 8000 to about 8 and 6 s at polymer concentration of 4 and 8 wt%, respectively.

#### Influence of different molecular weights of PEG on fusion with A/PR 8/34

Fig. 5 shows the dependence of the extent of influenza virus fusion on molecular weight of PEG. With PEG of molecular weight  $\leq 1540$  the fusion of influenza with RBC was not

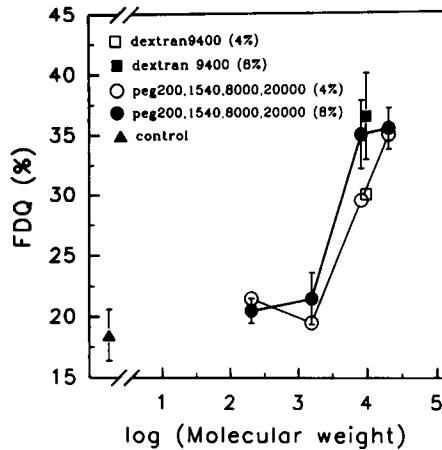


FIGURE 5 Influence of the molecular weight of the polymer on the fusion of A/PR 8/34 with RBC. PEG 200, 1540, 8000, 20000 (○, ●) and Dextran 9400 (□, ■) were used. A/PR 8/34-RBC complexes were incubated either in the absence (control, ▲) or presence of 4 wt% (open symbols) or 8 wt% polymer (filled symbols), respectively, at pH 7.4, 37°C before lowering pH to 4.9 (cf. Fig. 1). The extent of fusion (FDQ) was measured 380 s after lowering the pH. Standard deviation is given for control and values of 8 wt% polymer of different molecular weight. Data represent three independent measurements.

affected. However, beyond that molecular weight delay times were reduced and the subsequent extents were enhanced.

Similar molecular weight dependence of the enhancement of fusion was observed at pH 5.4 (data not shown).

### Influence of polymers on the stability of virus binding to RBC

The fusion extent may be affected by dissociation of virus from the target membrane during preincubation at pH 7.4 and/or after triggering the fusion process at (low pH). Therefore, we have investigated the influence of polymers on the stability of virus binding to RBC membranes. To avoid interference with fusion these experiments were done at pH 7.4. About 20–30% of virus was released from the RBC into the supernatant after incubation at 37°C and pH 7.4 for 5 min (control, absence of polymers). In Fig. 6 the relative amount of virus released from RBC after incubation at 37°C, pH 7.4, for 5 min is shown relative to the control as a function of polymer concentration for different polymers.

Dissociation of virus from RBC was inhibited in the presence of polymers of high molecular weights (PEG 8000, PEG 20000, and Dextran 9400), and at concentrations  $\geq 5$  wt% there was virtually no release. The PEG 1540 also had an inhibitory effect at higher polymer concentrations, but the PEG 200 did not prevent release of virus from RBC.

The extent of low pH-induced fusion was independent of the period (1–5 min) of preincubation at 37°C, pH 7.4, indicating that the spontaneous release of virus does not cause a decrease of the number of virions fused. This implies that the attachment of those viruses which will fuse with target are quite stable and are not released in the time course of the

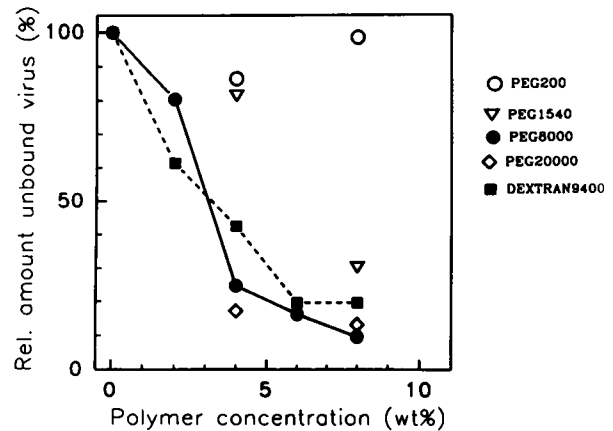


FIGURE 6 Effect of the Dextran 9400 and PEG of different molecular weight on the extent of A/PR 8/34 binding to RBC at pH 7.4, 37°C. A/PR 8/34-RBC complexes were incubated either in the absence (control) or presence of the polymer, respectively. After 5-min incubation at pH 7.4, 37°C, the amount of virus released from RBC was measured as described under Experimental Procedures. The virus released from RBC in the absence of any polymer (control) was set to 100%. Usually, the absolute amount of virus released for the control was about 20–30% of the total virus. Dextran 9400 (■); PEG 200 (○); PEG 1540 (▽); PEG 8000 (●); PEG 20000 (◇).

experiment. The dramatic effect of PEG cannot be ascribed to a proportionate increase in the pool of bound virus.

### Influence of PEG 8000 on A/PR 8/34-induced hemolysis of RBC

Fusion of influenza virus with erythrocytes is often accompanied by swelling and, subsequently, the release of hemoglobin (hemolysis) (24). We have examined the influence of PEG 8000 on the virus-induced hemolysis at pH 5.0, 37°C. Fig. 7 shows that PEG 8000 significantly reduced the hemolysis which is directly opposite to its effect on fusion of A/PR 8/34 with RBC (Fig. 2).

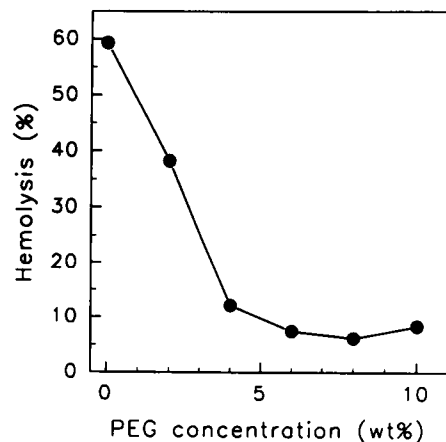


FIGURE 7 Low pH-induced hemolysis of RBC by A/PR 8/34 as a function of the concentration of PEG 8000. Hemolysis was measured 5 min after lowering the pH to 5.0, 37°C, as described under Experimental Procedures.

This observation can be rationalized by the fact that colloid-osmotic swelling and subsequent hemolysis is reduced by the presence of high molecular weight substances in the extracellular medium which cannot permeate the membrane. It has been shown that, due to the high osmotic pressure, a shrinkage of RBC occurs in suspension media containing PEG of high molecular weight over a similar concentration range (25). Since virus-cell fusion was enhanced, our results indicate that swelling is not a necessary condition for fusion, consistent with our previous observations (26, 27).

### Effect of PEG 8000 on fusion of VSV with RBC

The influence of PEG (molecular weight 8000) on the fusion of Sendai virus with erythrocyte membrane which is triggered by the viral fusion protein F at *neutral pH* (28) is different from that on fusion with *acid-triggered* influenza virus reported in this study. Although the initial rate of the fusion was enhanced in the presence of PEG, delays of the onset of fusion and extents of Sendai virus fusion were not affected by the polymer.

We wondered whether the effects of nonadsorbing polymers upon influenza fusion might be representative of a general phenomenon associated with low pH triggered viral fusion. We chose the well characterized VSV-erythrocyte system as a model to corroborate this idea (10, 15). It has been previously shown that fusion of VSV with human erythrocyte membranes occurs only after modifying the lipid composition of the outer RBC leaflet by preparing lipid-symmetric ghosts (15, 29). Fig. 8 shows low pH-triggered fusion of VSV with intact RBC in the presence of 8 wt% PEG 8000. No such fusion was seen in the absence of PEG, or in the presence of low molecular weight PEG. The extent of fusion was lower than that of low pH-induced fusion of VSV with lipid-symmetric ghosts (10, 15).

The fluorescence dequenching is attributed to fusion of VSV and not to nonspecific effects because: (i) no fluores-

cence dequenching was observed in the presence of PEG at pH 7.4; (ii) no dequenching was observed at pH 5.6 (without or with PEG) after inactivation of VSV (10 min at 56°C) (30) prior to binding.

### DISCUSSION

Many barriers or repulsive forces exist between biological membranes, e.g., steric, electrostatic, deformation, hydration, which prevent spontaneous fusion (31). Catalysis of membrane fusion involves interactions designed to decrease the activation energy necessary to overcome those barriers. We will argue that our observations indicate that the application of a moderate, osmotically derived, force decreases the delay time (following application of a low pH trigger) and concomitantly enhances the final extent of influenza virus fusion with RBC membranes. How can the force we apply achieve this?

### Generation of osmotic forces

It has been shown that PEG is able to aggregate proteins, lipoproteins, lipid vesicles, and cells (16, 32, 33). This ability strongly depends on the molecular weight of the polymer up to 6000, beyond which only a weak dependence is observed. This can be accounted for by the excluded volume effect of nonadsorbing polymers which is now well documented (17–19, 34). Interaction of PEG with polar surfaces is disfavored (33, 35). This is due to repulsive forces between the hydrated polymer and the hydrated membrane surface (hydration force) combined with its entropic (steric) exclusion from the surface depending on the molecular weight of the polymer (36). Electrophoretic measurements have confirmed that a layer depleted of high molecular weight nonadsorbing polymers exists at the surface of erythrocyte membranes (37–38). When two membrane surfaces become apposed this interaction is stabilized by an osmotic force deriving from a gradient in the PEG concentration toward the bulk solution. Although the absolute osmotic pressure of a bulk solution of PEG is almost independent of molecular weight (19), one observes a molecular weight dependence because of the differential exclusion of different molecular weight polymers from a membrane surface (17–19, 39). That we see such a molecular weight dependence on the parameters we measure (e.g., Fig. 4) is a good indication that the excluded volume effect is the basis of the influence of PEG on the system. Larger molecules should be excluded more effectively from the gap between virus and erythrocyte membranes because of hydration repulsion. The failure of low molecular weight PEG to enhance the fusion of both A/PR 8/34 and VSV is due to the fact that it is not excluded from the hydrated gap between the apposed membranes.<sup>2</sup> The inability of low molecular weight PEG (PEG 200 and 1540) to affect fusion

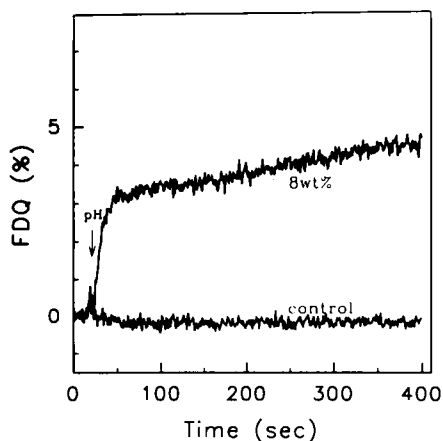


FIGURE 8 Fusion of VSV with RBC at pH 5.6. R18 VSV was bound to RBC, washed, and incubated in PBS in the absence or presence of 8 wt% PEG 8000 at pH 7.4, 37°C. The pH in the medium was changed as indicated by the arrow. %FDQ was calculated according to Eq. 1.

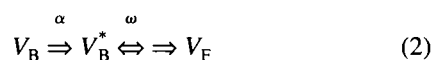
<sup>2</sup> The enhanced binding of influenza virus to RBC in the presence of PEG 1540 does not necessarily contradict its inability to support viral fusion, as seen for higher molecular weight polymers. The molecular weight given

indicates that the polymer does not enhance influenza fusion with RBC by a direct interaction with fusing membranes.

### Virus binding, delay, and extent of fusion

We show that high molecular weight polymers of PEG stabilize binding of influenza to erythrocyte membranes. However, this relatively small increase in virion attachment (21%; 8 wt% PEG 8000; compared to the control 0 wt% PEG) at the time of triggering fusion cannot account for the dramatic increase in the extent of fusion we observe (87%). Thus, the ratio of fused virus to bound virus was increased by about 50% at 8 wt% PEG 8000 compared to the control. Moreover, PEG 1540 enhanced binding of A/PR 8/34 to RBC (17%, 8 wt% PEG 1540) but did not increase fusion.

We believe that the enhancement of extent derives from the polymer-induced decrease in the delay time preceding fusion (Fig. 4). In our system the recorded fusion is dominated by virus bound to the membranes at the time of triggering fusion so that virus-cell association kinetics need not figure in the following discussion. In previous papers we have proposed the following scenario (8, 10), shown schematically as:



where  $V_B$ ,  $V_B^*$ , and  $V_F$  are, respectively, populations of bound, bound-activated, and fused virus. Process  $\alpha$  is a pH-dependent triggering event, whereas process  $\omega$  represents a complex series of steps, some of which may be reversible. From any point along this pathway one can invoke an inactivation process which competes with the forward reaction. We have previously fitted the pH dependence of VSV fusion to a model which incorporates these steps, although, owing to the complexity of the event, it is impossible to achieve a unique solution (10). The relevant point for our purposes here is that, by increasing the forward rate from a state which can also be inactivated, high molecular weight polymers can increase fusion yield. Support for this interpretation comes from the observed reduction in delay time with 4 and 8 wt% PEG 8000 (Fig. 4). If the pH-triggered conformational change of viral protein is rate-limiting, then PEG may reduce the threshold number of activated proteins/virion required to induce fusion. However, we have also observed decreased delay times in the presence of high molecular weight PEG for the X31 strain of influenza (Clague et al., unpublished results) for which it has been established that the conformational change is not rate-limiting (9, 40)

We are challenged to propose how this enhancement of rate may occur. The magnitude of the forces applied may give some clues. The osmotic pressure of an aqueous solution of PEG 8000 is about  $4 \times 10^4$  Pa and  $1.5 \times 10^5$  Pa for a

concentration of 4 and 8 wt%, respectively (19). This is tiny in comparison to that shown to be required to directly overcome the hydration repulsion force between two bilayers (19, 20, 34). It is interesting to note that the repulsive pressure of cell membranes resulting from the steric compression of the extracellular part of membrane proteins and the accompanied difference of the chemical potential of water between intermembrane space and the bulk phase is in the order of  $10^5$  Pa (41–43). Our data would be consistent with the idea that fusion rates are enhanced by reducing the intermembrane distance imposed by the hydrated extracellular glycoprotein matrix and facilitating the interaction of hydrophobic parts of HA with the target membrane. Another possibility is that the polymer alters the intermembrane water structure (44), and thus acts to increase the hydrophobicity of the membrane surfaces. Ohki and Arnold (1990) (45) have measured a reduction of about 25% in the surface dielectric constant of lipid vesicles in the presence of 10 wt% of high molecular weight PEG (molecular weight 6000). This would correlate with our previous observations of decreased delay times in response to factors which increase the hydrophobicity of surfaces destined to fuse, e.g., increased density of HA in fusion active conformation (6) and exposure of polyunsaturated lipids at the erythrocyte surface (6, 15, 17).<sup>3</sup> By designing experiments to discriminate between these proposals, factors which determine the rate of low pH-induced virus fusion will be elucidated.

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<sup>3</sup> From preliminary experiments we deduced from the blue shift of the emission spectrum of the polarity-sensitive fluorophore PATMAN (6-palmitoyl-2-[[[2-trimethylammonium]ethyl]methylaminonaphthalen chloride) that the surface dielectric constant of the outer leaflet of erythrocyte membranes is lowered by about 10% when exposing polyunsaturated lipids by disruption of the well known transverse lipid asymmetry of RBC (Schroth and Herrmann, unpublished results). A similar fluorescence technique has been used by Ohki and Arnold (1990) (45) to measure the surface dielectric constant of lipid vesicles.

corresponds to an average value. Therefore, polymers with a molecular weight < 1540 are present which should be able to enter the gap between the virus and erythrocyte membrane.

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