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Issues Associated with using Fluorescent Microspheres to Evaluate the Barrier Integrity of Natural Rubber Latex Films

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It has been reported that 100 nm – 110 nm fluorescent microspheres can pass through more natural rubber (NR) latex condoms than can the 27 nm virus ΦX174 as found by other investigators. Are there properties of fluorescent polystyrene microspheres that could be responsible for these disparate results? This study demonstrated that the microspheres adsorbed to a brand of condom made of NR latex as well as a highly-adsorptive virus, PRD1 (ΦX174 did not adsorb). The study also found that free fluorescent dye was not released when the microspheres were in close contact with the NR latex. These findings argue against microsphere properties being responsible for the disparate results. In addition, an increase in the fluorescence of buffer after contact with NR latex condom was found, in the absence of microspheres. This increase may require extended contact. Thus, this study found an artifact that could be misconstrued as evidence of fluorescent microspheres passing through NR latex barriers.

Natural rubber (NR) latex is a very important material used to make products that serve as physical barriers to disease transmission. For example, most condoms and medical gloves are made of NR. The AIDS epidemic had led to heightened concern about barrier integrity, and thus a number of tests have been developed to assess barrier effectiveness. As the standard water leak tests used to assure quality can at best only detect 3 μm diameter holes in condoms[1] and 25 μm holes in gloves[2] and because human viruses are very much smaller (0.025 μm – 0.20 μm)[3,4], tests have been designed to specifically determine whether these latex products are effective barriers to virus passage.

Both viruses and fluorescent polystyrene microspheres have been used as challenge probes to test the barrier integrity of latex rubber[4–19]. Microspheres theoretically have the capacity to serve as a surrogate for viruses, since they can be manufactured to be approximately the same size[13]. A fluorescent dye embedded in the polystyrene matrix allows the presence and concentration of the

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microspheres to be quantified with a spectrofluorometer. Microspheres have the advantage that quantitative measurements can be obtained faster than with viruses. On the other hand, tests utilising viral probes can readily be designed to be many times more sensitive.

Questions regarding the validity of condom testing with these probes arose when, using the same test protocol but different test probes, different groups obtained significantly different results. Using bacteriophage ΦX174 (27 nm diameter) as the viral probe, Lytle *et al.* found that 3 of 60[14] and 12 of 470[17] latex condoms allowed passage of virus. In contrast, using essentially the same protocol but with 110 nm (size of HIV, the AIDS virus) fluorescent microspheres instead of virus particles, Carey *et al.*[15] concluded that 29 of 89 latex condoms allowed particles to pass. In addition, Roland *et al.*[18] reported passage by diffusion of 100 nm particles through two different 8 cm^2 pieces of latex condom, as well as much lower passage of 1000 nm particles through two other pieces.

Are there properties of the viruses or microspheres that could be responsible for these apparently disparate results? One possible explanation is that the virus adsorbs to the rubber, thereby yielding negative results when a hole is present in the condom, while the microsphere adsorbs less well, if at all. A second possible explanation is that the fluorescent dye may separate from the microsphere and pass through pores that are too small for either the virus or the microsphere.

The purpose of this study was to investigate whether the properties of the microspheres support either possible explanation of the disparate results. The ability of the microspheres to adsorb to NR was determined by a method developed to compare the adsorption of different viruses to NR[20]. Microspheres of two sizes were used as a means of verifying the role of particle diffusion in adsorption. The possible release of dye from the microspheres before or after contact with the rubber was determined by measuring the amount of fluorescent signal from free dye in samples from the adsorption experiment. In addition, a simultaneous test of passage by diffusion through latex condoms by 100 nm microspheres and 27 nm bacteriophage ΦX174 was conducted.

MATERIALS AND METHODS

Microspheres

The fluorescent polystyrene microspheres (Polysciences, Inc., Warrington, PA) contained fluorescein dye embedded in each microsphere. The microspheres had diameters of 48 nm [standard deviation (S.D.), 5] or 100 nm (S.D., 3), provided by the manufacturer. For experiments, the microspheres were suspended in Dulbecco's phosphate-buffered saline without Ca^{++} or Mg^{++} (DPBS; Gibco, Inc., BRL, Gaithersburg, MD).

NR Latex Condoms

Trojan-enz (Carter-Wallace, Inc., New York, NY) non-lubricated NR latex condoms with receptacle tips were used. Condoms of the same lot were used in earlier experiments investigating the adsorption of various viruses[20,21]. They did not contain silica (Carter-Wallace, Inc., private communication).

These condoms were used as representative of those NR latex condoms that adsorb some viruses[20]. In this laboratory, three of six NR latex products from different manufacturers adsorbed PRD 1 bacteriophage: 2 of 3 brands of condoms, and 1 of 3 brands of gloves (unpublished data).

Fluorescence Measurements

The microspheres in suspension were quantified by measuring the fluorescence with an SLM Aminco SPF-500 Spectrofluorometer (SLM Instruments, Inc., Urbana, IL). An excitation frequency of 440 nm was used with fluorescence measured at 488 nm. The fluorescence was linear with microsphere concentration over the ranges investigated (conversion factors: 0.05 and 0.7 fluorescence units per 10^8 microspheres per ml for 50 nm and 100 nm microspheres, respectively). A low level of signal was obtained with the buffer alone and was usually subtracted from the fluorescence measurements; the exceptions are discussed below. The minimum detectable concentration of 100 nm microspheres was about 3×10^6 particles per ml.

Adsorption Experiments

The basic method compared the concentrations of microspheres in a suspending buffer before and after passage through a narrow adsorption channel lined with NR latex condom, previously described in detail[20,21]. Briefly, the testing apparatus consisted of two flat polycarbonate blocks covered with one condom each and clamped together with paper spaces to define a channel whose length, width and height were, respectively, 15.5 cm, 1 cm, and 50 μm – 90 μm (determined by the spacer thickness and the tightness of the clamps). The microsphere suspension was placed in the reservoir at the top and allowed to flow down the narrow channel under gravity so that the microspheres had the opportunity to contact, and perhaps adsorb to, the rubber surfaces. The microspheres were used at initial concentrations of 3.6×10^{11} or 4.5×10^{10} particles per ml for the 50 nm and 100 nm microspheres, respectively, to provide similar levels of fluorescence.

The ratio of the microsphere concentration after passage to that before passage was defined as the transmitted fraction[20], *i.e.*, the fraction of microspheres that apparently passed down the rubber-lined channel without being adsorbed. For example, a value of 0.70 indicated that 70% of the microspheres were not adsorbed (30% were adsorbed). The fraction adsorbed was expected to increase with time spent in the channel, because there would be more opportunity to diffuse to the rubber surface where adsorption could occur[20]. After adsorption, the virus PRD 1 could be completely recovered[21].

The flow rate of the microsphere suspension was controlled by the clamp tightness and the angle of the apparatus from the vertical[21]. Individual drops were collected in petri dishes. The timing between the drops and the volume of the drops allowed calculation of the flow rate. Aliquots of selected drops and of the control suspension in the reservoir were diluted 100-fold before being measured for fluorescence in the spectrofluorometer.

In order to determine whether the clamp tightness and angle of the channel were sufficient to provide the desired flow rate, an initial trial with buffer alone (*i.e.*, without microspheres) was used to determine the flow rate. This had the additional advantage of removing loose particles from the surface of the rubber that were found to interfere with accurate and reproducible fluorescence measurements of microspheres (data not shown).

Measurement of Free Dye

To determine whether the fluorescent dye was released from the microspheres upon exposure to the NR latex film, we separated free dye, if present, from a microsphere suspension by ultrafiltration through a Millipore™ Ultrafree 0.5 ml Centrifugal Filter

(100 K NMWL), which removes particles above 100 000 molecular weight (approximately 7 nm diameter). This was done with the original suspension and with pooled drops collected during an adsorption experiment. The filtrates were diluted 1:10 into buffer before measuring the fluorescence. Comparison of the fluorescence of the filtrate with that of the mocrosphere suspensions (before or after exposure to the latex) allowed determination of the extent of free-dye release.

Diffusion of Viruses and Microspheres through NR Condoms

NR condoms were challenged with a suspension of 100 nm fluorescent microspheres (4.6×10^{11} particles per ml) and ΦX174 (10^8 pfu/ml) to compare their passages. Eight ml of the mixed suspension were placed in each condom, which was in turn submerged into 50 ml of collection buffer in a 100-ml glass media bottle. Approximately 2/3 of the condom surface was submerged and covered by the challenge suspension on the inside and the collection buffer on the outside, yielding no hydrostatic pressure but allowing diffusion through most of the condom. Aliquots of the collection buffer were assayed subsequently to determine if viruses or microspheres had passed through the barrier. To investigate the likelihood that microspheres that passed through the condom could be detected, the collection buffer for one condom with no challenge microspheres inside was 'spiked' with a 10^4-fold dilution of the challenge suspension and aliquots assayed later.

The fact that at least 9×10^7 microspheres must have passed into the collection buffer to be detectable by their fluorescence, this test could detect passage of 2×10^{-4} mL of challenge suspension or more. Similarly, passage of 25 viruses or 2.5×10^{-7} mL of challenge suspension was the detection limit with ΦX174. Thus, the test of the condoms with the virus was 10^3-fold more sensitive than with the microspheres for detecting passage through the barrier.

RESULTS AND DISSUSSION

Microsphere Adsorption to NR Latex Film

The passage of the microspheres down the latex film-covered adsorption channel was determined for a number of different flow rates. The residence time, *i.e.*, the time spent traversing the length of the channel, was then calculated using the flow rate and the trough angle[21]. The results are shown in *Figure 1*. The transmission of the microspheres decreased, indicating adsorption to the rubber surface in the channel. The decreased transmission with increasing residence time fit exponential kinetics for each size of microsphere, indicating that the microsphere adsorption to the NR film was likely to be a first-order process[21].

The transmission of the microspheres also depended on microsphere size, with the smaller ones displaying more adsorption. This was expected because smaller particles diffuse faster across the channel to the rubber film than the large ones (the diffusivities of the particles are inversely proportional to their radii[20]), providing more opportunity to interact with the film during particle passage down the adsorption channel. When the residence time was normalised by the diffusion time, *i.e.*, to diffuse from the centre of the channel to the rubber surface[20], for the different-sized microspheres (*Figure 2*), the transmission data essentially overlap, indicating that the primary difference in the adsorption of the different-sized microspheres resulted from different diffusivities.

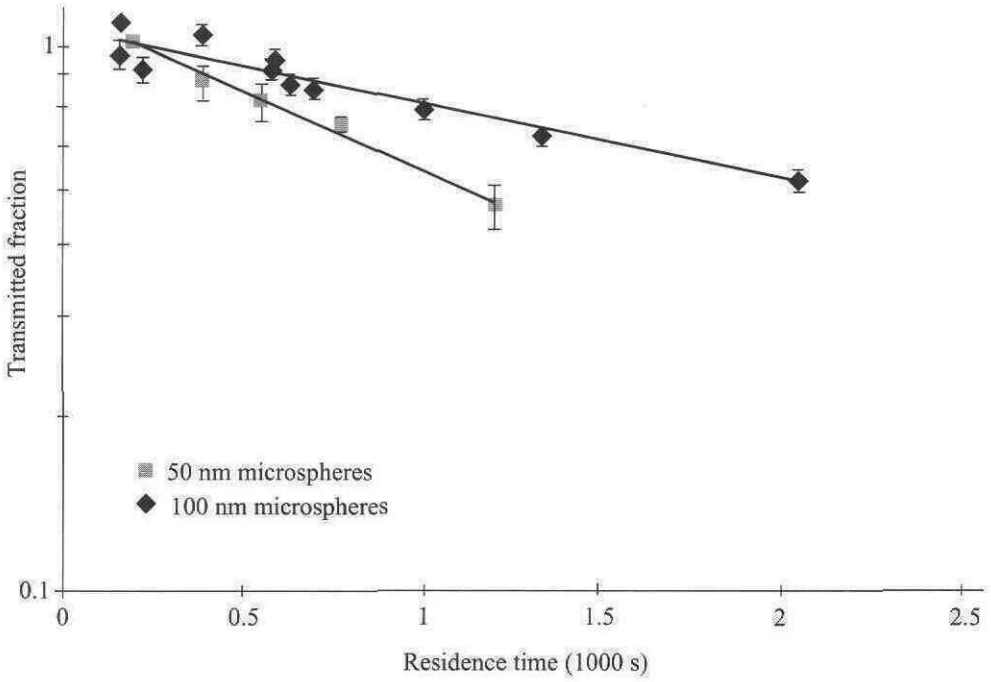

Figure 1. Transmitted fraction of different-sized microspheres as a function of residence time in the NR latex film channel. The lines represent the best-fit exponential regression lines for each microsphere size. Error bars represent standard errors of the means of three measurements.

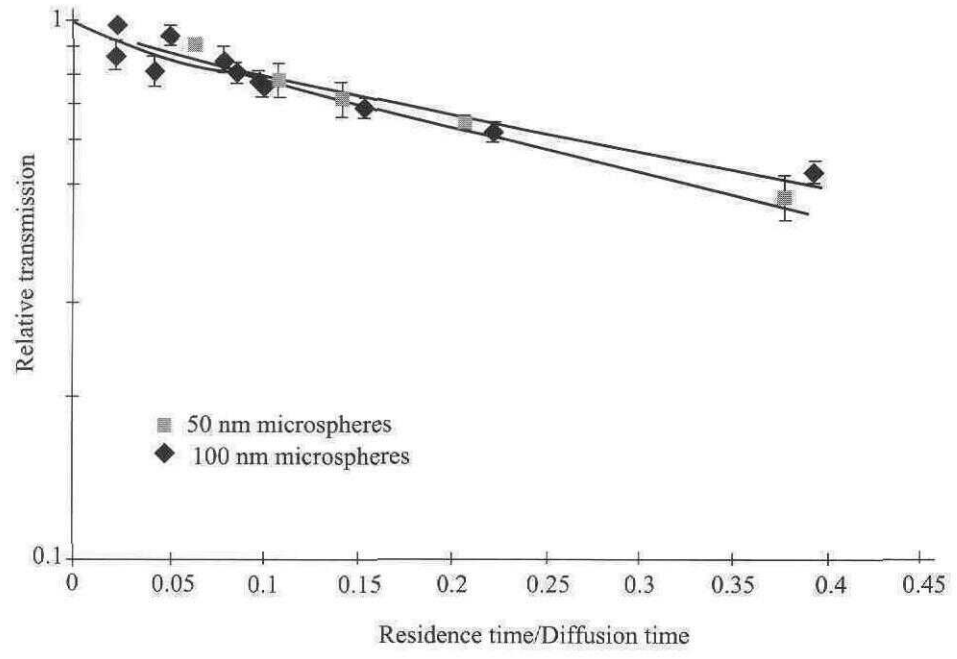

Figure 2. Relative transmission of different-sized microspheres through a NR latex film channel as a function of normalised residence time (residence time/time for the microspheres to diffuse from the centre of the channel to the rubber wall). Perfect adsorption is represented by the lower curve, PRD 1 adsorption by the middle curve, and ΦX174 adsorption by the upper, horizontal line.

Further, when these data are compared with the transmission curves for 'perfect adsorption' (*i.e.*, when the probability of adsorption is 1.0 for any particle approaching within one particle diameter of the channel wall[20]) (*Figure 2*), it is clear that the polystyrene microspheres were also highly-adsorptive. Comparison with the adsorption of a highly-adsorptive virus, PRD 1[20], (*Figure 2*) indicates that the microspheres were highly adsorptive relative to viruses, as well.

Lack of Free Dye

Microsphere suspensions were examined for the presence of free dye before and after contact with NR latex film by comparing samples collected after traversing the adsorption channel with the original suspension. Samples from the buffer-only trial at the beginning of the experiment were also compared. The results are presented in *Table 1*. The fluorescence of the samples containing microspheres displayed the expected drop after traversing the adsorption channel, indicating microsphere adsorption. The filtrates, which would contain the free dye, if any, but not microspheres or other particles above 100 000 molecular weight, displayed no fluorescence above that of the buffer alone. Thus, there was no free dye (less than 1 part in 1000) in the original suspension nor in the suspension after contact with the latex film in the adsorption trough.

Lack of Passage of Microspheres or Virus through Condoms

The simultaneous challenge of the condom barrier with microspheres and virus could provide the critical evidence regarding the utility of either probe for such tests. However, the data in *Table 2* show that no viruses were found in the collection buffers, and the fluorescences of the collection buffers were no higher than that of a control test with no microspheres in the condom. Thus, there was no evidence of passage by either particle and no way to differentiate their abilities to pass through any of the four condoms, even after 48 h or 72 h. On the other hand, this evidence is consistent with latex film being an effective barrier.

TABLE 1. FLUORESCENCE OF SAMPLES AFTER CONTACT WITH NR LATEX FILM

Samples	With microspheres	Buffer only
Before exposure to latex film		
Microspheres + free dye[a]	2.91 ± 0.05[c]	0.024 ± 0.004
Free dye[b]	0.012 ± 0.005	
After exposure to latex film		
Microspheres + free dye[a]	1.49 ± 0.10	0.015 ± 0.004
Free dye[b]	0.025 ± 0.006	

[a] Samples before centrifugation (possibly containing microspheres and free dye) were diluted 100-fold before fluorescence measurements.

[b] Samples after centrifugation (possibly containing free dye, but not microspheres) were diluted 10-fold before fluorescence measurements.

[c] Mean value of fluorescence from 2–6 adsorption experiments, with standard error of the mean.

TABLE 2. LACK OF PASSAGE OF 100 NM MICROSPHERES OR VIRUS THROUGH LATEX CONDOMS

Sample	Microspheres[a] (Fluorescence units)	ΦX174 (pfu/ml)
Original challenge (after 10^4-fold dilution)		
1 h	0.265	$1.15 (\pm 0.01) \times 10^8$
3 h	0.265	
24 h	0.262	
48 h	0.261	$1.06 (\pm 0.02) \times 10^8$
72 h		$0.99 (\pm 0.01) \times 10^8$
Inside condom (after 10^4-fold dilution)		
1 h	0.230	$0.86 (\pm 0.06) \times 10^8$
3 h	0.189	
24 h	0.119	$0.96 (\pm 0.02) \times 10^8$
48 h	0.126	$1.15 (\pm 0.14) \times 10^8$
72 h		$1.03 (\pm 0.06) \times 10^8$
In collection buffer[b]		
1 h	0.037 ± 0.001	<25[c]
3 h	0.037 ± 0.002	<23
24 h	0.063 ± 0.001	<21
48 h	0.042 ± 0.001	<19
Collection buffer (w/o microspheres)		
0 h	0.022	
21 h	0.062	
45 h	0.041	
Spiked collection buffer		
1 h	0.249	
3 h	0.210	
24 h	0.160	
48 h	0.109	

[a] Fluorescence intensity after 10^4-fold dilution of original challenge and challenge inside condom, other samples were measured with no dilution.

[b] n = 4

[c] No virus detected; calculated detection limit presented.

There are two particularly interesting observations that can be made from these data. First, the concentration of microspheres decreased in the challenge inside the condom and in the 'spike' outside the condom, consistent with adsorption of the microspheres to latex film. Second, the fluorescence of the collection buffer increased with time above the level of buffer alone (0.02 units), with or without microspheres inside the condom. This

latter result is similar to that found in other experiments where buffer or distilled water was used to rinse the inside of condoms. An increase in fluorescence was found after low-speed centrifugation to remove powder and, at a lower level, after ultrafiltration (as above) to remove particles above 100 000 molecular weight (data not shown).

CONCLUSIONS

The results of this study clearly demonstrated that the virus-size polystyrene microspheres adsorbed to latex film (in buffer), at a rate comparable to highly-adsorptive viruses and much more than the virus ΦX174[20]. For virus adsorption to be responsible for the disparate results described earlier in the introduction, adsorption by ΦX174 would have to be greater than that by the microspheres. Thus, particle adsorption does not explain the disparate results. Furthermore, free dye was not released when the microspheres were in close contact with the film. Thus, the properties of these microspheres were not responsible for the disparate results obtained when viruses or microspheres were used to evaluate barrier integrity of latex films.

The reason(s) for the disparity lies elsewhere. For example, an increase in the fluorescence of buffer was found after extended contact with NR latex film, in the absence of microspheres. This increase was not apparent in buffer samples taken from the adsorption experiment after much shorter duration contact and after the film had been previously rinsed. Thus, apparent evidence of fluorescent microspheres passing through rubber barriers could originate as an artifact that would vary depending partly on previous exposure of the rubber film to other fluids. It is not known whether this explanation would be sufficient to explain the disparate results previously reported[15,18].

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