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Rapid Lateral Diffusion of the Variant Surface Glycoprotein in the Coat of *Trypanosoma brucei*†

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ABSTRACT: The membrane form of the variant surface glycoprotein (mfVSG) is anchored in the plasma membrane of *Trypanosoma brucei* by a dimyristoylphosphatidylinositol residue connected via a glycan to the COOH-terminal amino acid. The glycoprotein molecules are tightly packed, forming a coat that is impenetrable to lytic serum components. Lateral diffusion of mfVSG was measured by the fluorescence recovery after photobleaching technique. mfVSG labeled on the cell surface with rhodamine-conjugated anti-VSG Fab fragments showed a diffusion coefficient of 1×10^{-10} cm^2/s at 37 °C and of 0.7×10^{-10} cm^2/s at 27 °C. About 80% of the molecules were mobile. Affinity-purified mfVSG molecules implanted into the plasma membrane of baby hamster kidney cells exhibited a similar mobility to that found in the trypanosome coat [$D = (0.4–0.7) \times 10^{-10}$ cm^2/s at 4 °C]. Phospholipid mobility in the plasma membrane of trypanosomes was characterized by a diffusion coefficient of 2.2×10^{-9} cm^2/s at 37 °C. It is concluded that mfVSG mobility in the surface coat of the parasite is rapid and comparable to that of other membrane-bound glycoproteins but slower than that of phospholipids.

African trypanosomes, exemplified by *Trypanosoma brucei*, are unicellular eucaryotic flagellates that parasitize the blood and tissues of their mammalian host. The entire cell surface is covered by a coat that consists of a single glycoprotein species, designated the variant surface glycoprotein (VSG). Every cell has the potential to express a large number of different VSG's, thereby enabling the parasite to evade the host's immune response (Borst, 1986).

About 10^7 VSG molecules are considered to be arranged at the surface of each cell in a tightly packed single layer of homodimers separated by a mean distance of about 40 Å (Cross, 1975; Auffret & Turner, 1981; Freymann et al., 1984; Jackson et al., 1985). The N-terminal two-thirds of the molecule form a domain that carries the antigenic determinants accessible to antibodies in live cells. Although the amino acid sequence of the N-terminal domain from different VSG's is not homologous, all of these proteins, nevertheless, appear to assume a similar, rodlike shape that is attributable to a bundle of four long α-helices per dimer (Freymann et al., 1984; Metcalf et al., 1987; Jähnig et al., 1987). VSG's are anchored in the membrane by a dimyristoylphosphatidylinositol residue connected via a glycan and an ethanolamine to the COOH terminus (Holder, 1983; Ferguson & Cross, 1984; Ferguson et al., 1985; Jackson & Voorheis, 1985). In this membrane

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form (mfVSG), VSG is the prototype of a class of proteins attached to the membrane by a lipid anchor (Cross, 1987).

The surface coat is impenetrable to molecules as large as concanavalin A (Cross & Johnson, 1976) or lytic serum components (Tetley et al., 1981; Ferrante & Allison, 1983). This dense packing may severely restrict the lateral diffusion of VSG molecules. On the other hand, there is clear evidence for lateral mobility in the coat. First, VSG molecules can be capped by specific antibodies (Barry, 1979; Baltz et al., 1986). Second, when a single trypanosome switches expression from one VSG to another, the new molecules intermix with the old ones over the entire surface (Esser & Schoenbechler, 1985). This result requires lateral diffusion because, after synthesis, VSG molecules are considered to reach the surface by exocytosis only at a specialized region of the plasma membrane lining the flagellar pocket, which is located at the base of the flagellum near the posterior end of the cell.

In this paper the rate of lateral diffusion of membrane-form VSG is quantitated by using the technique of fluorescence recovery after photobleaching. The results indicate that lateral diffusion of mfVSG is fast and similar in rate to typical integral membrane proteins but slower than that of phospholipids.

Materials and Methods

Preparation of Fab Fragments. VSG of variant MITat 1.2 was purified as described by Cross (1984). Anti-VSG antibodies from the serum of an immunized rabbit were isolated by affinity chromatography on a VSG–Sepharose column. Bound antibodies were eluted with 0.2 M glycin, pH 2.5, and immediately neutralized by the addition of 1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0. After dialysis overnight against 0.1 Tris-HCl and 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, the purified anti-VSG antibodies were incubated with papain (10 μg/mg of antibody) for 60 min at 37 °C in the presence of 1 mM dithiothreitol (DTT). Papain was activated before use for 15 min at 37 °C in 0.1 M Tris-HCl, 2 mM EDTA, and 1 mM DTT, pH 8.0. The reaction was terminated by the addition of iodoacetamide (final concentration 20 mM). After 15 min at 0 °C, the solution was dialyzed overnight against PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). Fc and Fab fragments were separated on a protein A column and concentrated by using an Amicon PM10 membrane.

Conjugation of Antibodies or Derived Fab Fragments with Rhodamine. Conjugation with rhodamine was performed in 0.25 M carbonate buffer, pH 9.5. Seven milligrams of affinity-purified antibody or derived Fab fragments in 1 mL of buffer was mixed with 50 μg of rhodamine isothiocyanate (10 mg/mL in dimethyl sulfoxide) and kept at room temperature for 2 h. Labeled protein and free dye were subsequently separated by gel filtration on a Sephadex G-25 column.

Immunofluorescence Staining of Trypanosomes. MITat 1.2 trypanosomes (4×10^7) from the blood of an infected mouse were washed once in PBS and incubated with rhodamine-labeled anti-VSG antibody or anti-VSG Fab fragments in 200 μL of PBS for 60 min on ice. Thereafter, the cells were washed twice in PBS and incubated for 30–60 min at 0 °C in the presence of 20 μM pentalenolactone [kindly provided by Dr. M. Duszenko, Tübingen; cf. Duszenko and Mecke (1986)]. To prevent floating of the immobilized trypanosomes, the cells were embedded in 1% low-melting agarose, spread on a glass cover slip, and mounted in a circular plexiglas chamber containing 400 μL of PBS. The chamber was sealed on both faces with molten paraffin and transferred to the stage of the photobleaching microscope.

Incorporation of mfVSG into the Plasma Membrane of Baby Hamster Kidney (BHK) Cells. mfVSG was purified by affinity chromatography as described by Bülow and Overath (1985), but octyl glucoside was used at 10 mg/mL instead of decyl poly(oxyethylene) ether. The neutralized eluant of the affinity column was dialyzed and diluted 6-fold with PBS to a final concentration of 1 μM mfVSG and 5 mM octyl glucoside, 5 times below the critical micellar concentration. BHK cells were grown on cover slips as described previously (Gruenberg & Howell, 1986). The cover slips were washed twice with ice-cold PBS, incubated for 30 min at 0 °C with the diluted mfVSG/octyl glucoside solution, and then rinsed 5 times at 5-min intervals with ice-cold PBS containing 0.1% bovine serum albumin. This protocol is currently used to insert water-insoluble lipid molecules into the plasma membrane of tissue culture cells. The cell-associated detergent molecules are back exchanged during the washings (data not shown). The cells were then incubated with rhodamine-labeled anti-VSG Fab fragments for 30 min on ice and washed twice with PBS. The cover slips were either mounted in a circular Plexiglass chamber as mentioned above for photobleaching studies or fixed with 4% paraformaldehyde and embedded in moviol for conventional fluorescence microscopy.

Labeling of Trypanosomes with N-Rhodamine Phosphatidylethanolamine. MITat 1.2 trypanosomes (2×10^7) from the blood of an infected mouse were washed once in PBS and incubated in 200 μL of incomplete Iscove's medium (Flow Laboratories, Meckenheim, FRG) containing 5 mg/mL bovine serum albumin. To remove part of the surface coat, 10 μg of trypsin was added, and the suspension was then incubated at 37 °C for 5 min. After the addition of 1 mL of cold medium containing 10% inactivated fetal bovine serum, the cells were washed twice in PBS + 1 g/L glucose and incubated for 30 min at 0 °C in the presence of 1 μM *N*-rhodamine phosphatidylethanolamine and 5 mM octyl glucoside. To remove octyl glucoside, the labeled cells were washed 5 times at 5-min intervals with PBS + 1 g/L glucose and 0.1% BSA. For fluorescence photobleaching, the cells were immobilized and embedded in agarose as described above.

Fringe Pattern Photobleaching. Theory and practice of the technique have been described (Davoust et al., 1982, 1988). The conditions used to perform the experiments on living cells have already been described (Pollerberg et al., 1986). Briefly, the fluorescently labeled trypanosomes mounted in a chamber were transferred to the photobleaching microscope (Zeiss IM 35), and individual cells were illuminated by two coherent laser beams that create a fringe pattern as shown in Figure 1. During the bleaching pulse of 100 ms (1500 W cm^{-2}, $\lambda = 514$ nm), between 10 and 20% of the total rhodamine-labeled Fab fragments present on the illuminated fringes (see Figure 1) were irreversibly bleached. This low level of bleaching was used to minimize photodamage of the cells. After pattern photobleaching, the decay of the periodic fluorescence concentration profile was monitored continuously by an interrogation fringe pattern (less than 1 W cm^{-2}) that scans back and forth (3 kHz) (Davoust et al., 1982, 1988). The resulting modulated emission of fluorescence (MEF) was detected by using a photomultiplier coupled to a lock-in amplifier and stored after digitalization with a time resolution of 10–60 ms. Only the periodic distribution of bleached versus nonbleached fluorophores contributes to the signal. The amplitude of MEF, which is a measure of the periodic contrast created by the fringe pattern photobleaching, was normalized with respect to its peak value (100%). The MEF decayed as a function

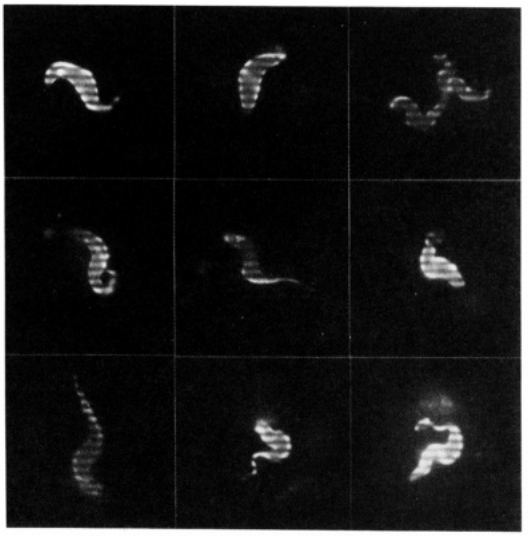

FIGURE 1: *T. brucei* under fringe pattern illumination. Trypanosomes were labeled with rhodamine-coupled Fab fragments against the variant surface glycoprotein. After transfer to the temperature-controlled stage of the fringe pattern photobleaching microscope, the cells were illuminated with two attenuated coherent laser beams that cross in the plane of focus, creating a set of vertical planar fringes (interfringe distance 1.55 μm). The fluorescence micrographs show representative cells having different orientations with respect to the fringe pattern illumination profile (magnification 916-fold). In the subsequent photobleaching experiments the fringe pattern was used to bleach irreversibly the enlighted fluorophores. In the case of rapid surface diffusion, the intensity of fluorescence intensity in the non-bleached stripes vanishes, and the surface of the cells becomes again uniformly labeled.

of time because of the randomization of fluorochrome-labeled anti-mfVSG antibodies. Per cover slip, 10–35 individual photobleaching measurements could be performed within 1 h.

Results

Measurement of VSG lateral diffusion in trypanosomes by the photobleaching technique required motionless cells. It was found that immobilization without detectably damaging cell morphology could be achieved by incubation with the irreversible inhibitor of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, pentalenolactone (Duszenko & Mecke, 1986), for 30–60 min at 0 °C. This treatment depletes the ATP level of the cells, which is solely maintained by glycolysis. Therefore, cells were first labeled with affinity-purified rhodamine-conjugated anti-VSG antibodies or similarly tagged monovalent anti-VSG Fab fragments and then treated with pentalenolactone. After the cells were embedded in low-melting agarose photobleaching by the fringe pattern technique was performed as previously described (Davoust et al., 1982, 1988; Pollerberg et al., 1986).

The fluorescence micrographs in Figure 1 show representative cells illuminated by the fringe pattern in different orientations. The staining was uniform over the entire cell surface; no aggregation or patching of the tagged mfVSG could be detected. In subsequent experiments the pattern was used to bleach the fluorophores in the illuminated stripes and then to measure the decay in contrast to the periodic fluorescence intensity profile as a function of time. No decrease in fluorescence intensity between the beginning and end of the bleach was observed, suggesting that on the time scale of these experiments the antibodies did not dissociate.

The randomization of the immunolabeled VSG on the surface of trypanosomes is illustrated in Figure 2. At 37 °C mfVSG labeled with rhodamine-conjugated anti-VSG Fab

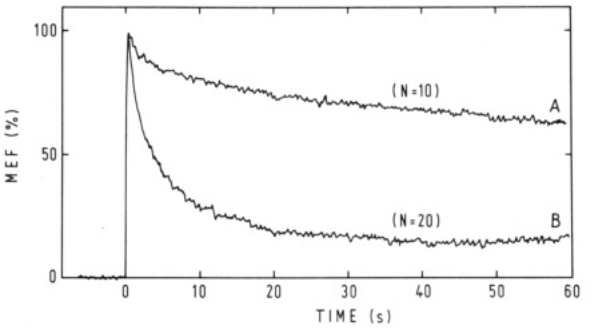

FIGURE 2: Lateral diffusion of the membrane-form variant surface glycoprotein on the surface of *T. brucei*. The fluorescence-labeled trypanosomes were illuminated at 37 °C with a fringe pattern as shown in Figure 1. At time 0, 10–20% of the fluorophores of a single cell was bleached in less than 100 ms with an intense fringe pattern. Thereafter, the amplitude of the periodic fluorescence concentration profile was measured as a function of time by using a scanning fringe pattern. Represented here is the modulated emission of fluorescence (MEF) measured before and after pattern bleaching of trypanosomes labeled with rhodamine-coupled bivalent anti-VSG antibodies (curve A) or rhodamine-coupled univalent anti-VSG Fab fragments (curve B). The interfringe distance was 1.47 and 1.55 μm, respectively, and N is the number of measurements averaged for obtaining the curves. The standard deviation of the collection of individual curves did not exceed 3% of the initial value of the signal.

Table I: Comparison of Surface Mobilities of Membrane-Form Variant Surface Glycoprotein or Phosphatidylethanolamine in *T. brucei* or BHK Cells[a]

interfringe distance (μm)	mobile fraction (%)	diffusion coeff ($cm^2/s \times 10^{10}$)	N	temp (°C)
Rhodamine-Labeled Anti-VSG Fab Fragments Attached to Trypanosomes				
1.55	82 ± 2	1.04 ± 0.02	20	37
1.55	79 ± 2	0.74 ± 0.02	15	27
2.67	81 ± 6	0.94 ± 0.07	4	27
1.57	81 ± 2	0.42 ± 0.05	4	4
Rhodamine-Labeled Anti-VSG Antibodies Attached to Trypanosomes				
1.47	33 ± 3	0.24 ± 0.02	10	37
N-Rhodamine Phosphatidylethanolamine Inserted into Trypanosomes				
2.45	92 ± 2	22.0 ± 0.2	10	37
1.57	93 ± 2	6.7 ± 0.05	10	4
Rhodamine-Labeled Anti-VSG Fab Fragments Attached to mfVSG Inserted into BHK Cells				
1.47	56 ± 3	0.66 ± 0.02	10	4

[a] The traces of lateral diffusion (cf. Figure 2) were decomposed into the sum of exponential components plus a base line by using a program based on the cubic spline model developed by Vogel (1986). The analysis showed that the data can be adequately simulated by a single-exponential model that gives an apparent lateral diffusion coefficient very close to the average of the coefficients obtained by a multiexponential model. The lateral diffusion coefficient D is related to the single-exponential time constant τ by $D = l^2/4\pi^2\tau$, where l is the interfringe distance. The mobile fraction is equal to the fractional amplitude of the exponential decay. The results are expressed as the mean plus or minus the standard deviation of the mean. N represents the number of individual measurements.

fragments was highly mobile [trace B, $D = (1.04 \pm 0.02 \times 10^{-10}$ cm^2/s]. The mobile fraction amounted to 82 ± 2% of all fluorescent molecules. The diffusion coefficient decreased by about 20% or 60% when the temperature was lowered to 27 or 4 °C, respectively, while the percentage of the mobile fraction remained the same (Table I). These results demonstrate that mfVSG in the surface coat of trypanosomes is highly mobile. Furthermore, the lateral diffusion coefficients measured with two different interfringe distance scales (1.55 and 2.67 μm) were very similar, indicating a Brownian type

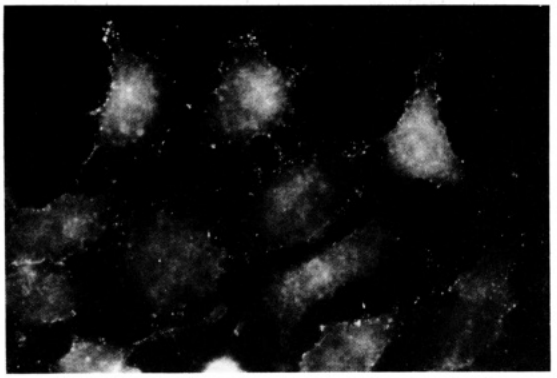

FIGURE 3: Insertion of mfVSG into the plasma membrane of BHK cells. The affinity-purified mfVSG was inserted into the surface of BHK cells in the presence of 5 mM octyl glucoside at 0 °C as described under Materials and Methods. The labeled cells were then reacted with the rhodamine-coupled anti-mfVSG Fab fragments, fixed, and photographed with rhodamine optics in a conventional fluorescence microscope. Anti-mfVSG gave no background in control BHK cells. The magnification of the fluorescence micrograph is 400-fold.

of motion of mfVSG on the surface of trypanosomes (Table I). In a given preparation, the standard deviation of the lateral diffusion coefficient and of the mobile fraction was very small. Experiments performed using a different batch of trypanosomes also gave very similar results [$D = (1.24 \pm 0.04) \times 10^{-10}$ cm^2/s; mobile fraction = 82 ± 3%], indicating a small intrinsic variability of the diffusion coefficient but no variation in the mobile fraction. Cross-linking of the mfVSG molecules by treatment with a bivalent antibody decreased the rate of lateral diffusion 5-fold, and only 33 ± 3% of all labeled molecules remained mobile (Figure 2, trace A, and Table I).

Next, the measurement of the lateral diffusion of phospholipids was attempted. Incubation of bloodstream forms at 0 °C in the presence of *N*-rhodamine phosphatidylethanolamine and octyl glucoside at a concentration (5 mM) below its critical micellar concentration (25 mM) did not lead to labeling of the cells. This protocol is currently used to label the plasma membrane of BHK cells and to study the rate of endocytosis of fluorescent phospholipid analogues (data not shown). In trypanosomes, the surface coat appeared to prohibit the insertion of this low molecular weight amphipathic compound into the plasma membrane. However, if the N-terminal domain of most VSG molecules was first removed by treatment with trypsin, the cell membrane could be labeled with the fluorescent lipid. Photobleaching experiments showed that the inserted phospholipids were essentially all highly mobile [$D = (2.2 \pm 0.2) \times 10^{-9}$ cm^2/s; mobile fraction = 92 ± 2%]. A reduction in temperature from 37 to 4 °C reduced the rate by a factor of 3.

Finally, it was of interest to investigate how mfVSG would behave in the plasma membrane of a different eucaryotic cell. Affinity-purified mfVSG was inserted into the BHK cell plasma membrane by incubating the cells in the presence of 1 μM mfVSG in 5 mM octyl glucoside at 0 °C. The cell-associated detergent molecules were then removed by several washings with a solution containing 1 mg/mL bovine serum albumin as described for the insertion of fluorescent phospholipids. The mfVSG present at the surface of BHK cells (Figure 3) exhibited a rate of lateral diffusion at 4 °C very similar to that in trypanosomes [$D = (0.66 \pm 0.02) \times 10^{-10}$ versus $(0.42 \pm 0.05) \times 10^{-10}$ cm^2/s; cf. Table I]. The reduction of the mobile fraction to 56 ± 3% compared to 82 ± 2% for the trypanosomes is probably due to a fraction of aggregated molecules attached rather than inserted into the plasma membrane of BHK cells (cf. spots in Figure 3). The experiments were performed at 4 °C in order to rule out the possibility of internalization of the mfVSG Fab complex.

Discussion

On the time scale of the division cycle of a trypanosome (generation time ≥7 h) the rate of lateral diffusion of mfVSG in the surface coat is rapid. Although the mfVSG molecules are tightly packed, thereby forming an efficient barrier, they nevertheless change place at a high frequency by Brownian motion. Extrapolating these findings to the size of the trypanosomes indicates that lateral diffusion will ensure the complete randomization of VSG molecules at the cell surface in about 40 min. Indeed, Esser and Schoenbechler (1985) found that trypanosomes in the process of antigenic variation have a mixed coat of two different VSGs. The lateral diffusion will ensure the homogeneous packing of VSG, thereby counteracting possible defects induced, for example, by cell motility. The phospholipid lateral diffusion coefficient in trypanosomes [$(22.0 \pm 0.2) \times 10^{-10}$ cm^2/s] is within the range ($10^{-9} \leq D \leq 5 \times 10^{-8}$ cm^2/s) generally observed for lipid probes in biomembranes (McCloskey & Poo, 1986). Because mfVSG is anchored in the phospholipid bilayer by a phosphatidylinositol residue, it might be expected that its mobility is similar to that of a typical phospholipid. This appears not to be the case. Although the diffusion coefficient of 1×10^{-10} cm^2/s observed for mfVSG at 37 °C is within the range ($0.5 \times 10^{-10} \leq D \leq 6 \times 10^{-10}$ cm^2/s) observed for other plasma membrane glycoproteins (Peters, 1981; McCloskey & Poo, 1986), it is clearly slower than that of the Thy-1 antigen on thymocytes and fibroblasts [$(2–4) \times 10^{-9}$ cm^2/s; Ishihara et al., 1987] or the decay accelerating factor in HeLa cells (1.6×10^{-9} cm^2/s; Thomas et al., 1987). Both these proteins are anchored in the membrane via phosphatidylinositol (Low & Kincade, 1985; Tse et al., 1985; Davitz et al., 1986). The diffusion coefficient of mfVSG is also slower than that of frog rhodopsin, a multiple membrane spanning protein of 40 kDa in rod outer segments disk membranes, which has a diffusion coefficient of 4×10^{-9} cm^2/s (Poo & Cone, 1974). This latter value is very close to theoretical predictions for integral membrane proteins [for a review, see McCloskey and Poo (1984)].

The difference in mobility of phospholipid-anchored proteins cannot be attributed to interactions with the cytoskeleton because the fatty acyl chains do not cross the bilayer, nor can it be explained by an interaction with extracellular matrix components because they are absent from the surface of the trypanosomes. Also, this difference in mobility cannot be explained by a difference in size because the complex consisting of an mfVSG homodimer and two Fab fragments has a molecular weight similar to that of the Thy-1 IgG complex (Ishihara et al., 1987). Moreover, differences in size do not strongly influence the rate of lateral diffusion because antibodies bound to lipid haptens diffuse in model membrane systems as rapidly as the lipids themselves (Smith et al., 1979). Finally, the comparatively slow rate of lateral diffusion of mfVSG is not characteristic for the surface coat of the parasite because a similar mobility was observed after integration into the surface of BHK cells ($D = 0.7 \times 10^{-10}$ cm^2/s). There remains the possibility that the lateral mobility of glycoproteins is differentially modulated by rather unspecific interactions between their polypeptide and/or carbohydrate domains clustered at the surface of cell membranes. In the case of VSG such putative interactions could improve the protective properties of the coat.

Up to 82% of the mfVSG forming a protective coat on the surface of trypanosomes was found to diffuse laterally in spite of the close packing of proteins. On the other hand, only 45%

of the Thy-1 molecules in C3H cells was found to be mobile (Ishihara et al., 1987), and the mobile fraction of the decay accelerating factor was much less than 1 (Thomas et al., 1987). Possibly interactions between different membrane components result in trapping of a fraction of the molecules in relatively immobile domains. The high fraction of mobile mfVSG molecules is likely a reflection of the homogeneous properties of the surface coat.

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