Direct observation of Brownian motion of lipids in a membrane

(colloidal gold/Nanovid microscopy/lateral diffusion/supported planar bilayer/fluorescein phosphatidylethanolamine)

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ABSTRACT Nanovid microscopy, which uses 30- to 40-nm colloidal gold probes combined with video-enhanced contrast, can be used to examine random and directed movements of individual molecules in the plasma membrane of living cells. To validate the technique in a model system, the movements of lipid molecules were followed in a supported, planar bilayer containing fluorescein-conjugated phosphatidylethanolamine (Fl-PtdEtn) labeled with 30-nm gold anti-fluorescein (anti-Fl). Multivalent gold probes were prepared by conjugating only anti-Fl to the gold. Paucivalent probes were prepared by mixing an irrelevant antibody with the anti-Fl prior to conjugation. The membrane-bound gold particles moved in random patterns that were indistinguishable from those produced by computer simulations of two-dimensional random motion. The multivalent gold probes had an average lateral diffusion coefficient (D) of 0.26×10^{-8} cm²/sec, and paucivalent probes had an average D of 0.73×10^{-8} cm²/sec. Sixteen percent of the multivalent and 50% of the paucivalent probes had values for D in excess of 0.6×10^{-8} cm²/sec, which, after allowance for stochastic variation, are consistent with the D of 1.3×10^{-8} cm²/sec measured by fluorescence recovery after photobleaching of Fl-PtdEtn in the planar bilayer. The effect of valency on diffusion suggests that the multivalent gold binds several lipids forming a disk up to 30–40 nm in diameter, resulting in reduced diffusion with respect to the paucivalent gold, which binds one or a very few lipids. Provided the valency of the gold probe is considered in the interpretation of the results, Nanovid microscopy is a valid method for analyzing the movements of single or small groups of molecules within membranes.

Nanometer-size colloidal gold probes combined with video-enhanced microscopy (nanovid microscopy) is a useful tool for studying the movements of proteins within the plasma membrane of living cells (1–7). For example, the value of nanovid microscopy already has been demonstrated for examining putative flow and transport in locomoting cells (2–7). In addition, the possibility of following the movements of individual membrane molecules has unique potential for studying the existence and size of domains in the plasma membrane (3, 4, 8). An important question for these new probes of motion is whether the attachment of the colloidal gold particle alters the diffusion characteristics of the bound membrane molecule. When compared with values obtained by fluorescence recovery after photobleaching (FRAP), the lateral diffusion coefficient is lower for gold-Con A on macrophages (4) but not for gold-anti-2A1-A on growth cones (5). In some instances, steric hindrance to the movements of the gold molecule could be produced by the glycocalyx, which can be as much as 50-nm thick on some cell types (9). Additionally, motion may be affected by the number of antigen binding sites on the gold probe and its degree of aggregation.

In this study we characterize the effect of the colloidal gold probe on Brownian motion of membrane lipid molecules by using a model membrane, the supported planar bilayer (for a review, see ref. 10). The movements of fluorescein phosphatidylethanolamine (Fl-PtdEtn) molecules within the membrane are followed with gold anti-fluorescein (anti-Fl). The random nature of the motion is demonstrated by comparing the detected patterns of movement to those produced by computer simulation of two-dimensional random motion. The effect of the number of antigen binding sites is studied by reducing the number of anti-Fl molecules bound to each gold particle. This is done by adding a competing, irrelevant antibody during probe preparation. Whether the gold label hinders lipid diffusion is evaluated by comparing the lateral diffusion coefficient D for gold-labeled lipids with D for Fl-PtdEtn obtained by FRAP.

MATERIALS AND METHODS

Colloidal Gold Probes. Mouse anti-Fl (a monoclonal IgG, designated 2-3-6, supplied by E. Voss, University of Illinois, Urbana) was conjugated to 30-nm colloidal gold (Amersham) essentially as directed ("AuroBeads Colloidal Gold for Macromolecule Labelling", Janssen). Briefly, the antibody was dialyzed against 2 mM borate buffer (pH 9) for 1 hr at 4°C and then centrifuged. The colloidal gold was adjusted to pH 8.9 with 0.2 M K₂CO₃ and 0.2 M H₃PO₄, the protein was added, and the mixture was mixed with a Vortex for 2 min and incubated for 8 min at room temperature. The gold probe was stabilized with 1% bovine serum albumin (BSA) and 0.05% 20M Carbowax. After three washes in 0.05% Carbowax/1% BSA/20 mM Tris/150 mM NaCl, pH 8.2, glycerol was added to 50% (vol/vol). The gold probe was stored at –20°C. Immediately before use an aliquot of the gold probe was washed three times with 1% BSA in Ca²⁺/Mg²⁺-free phosphate-buffered saline (BSA/PBS). For paucivalent probes, the anti-Fl was mixed 1:20 with purified monoclonal rat anti-mouse IgM, μ -chain specific (Zymed Laboratories), after dialysis and centrifugation.

Planar Membranes. Liposomes, composed of egg phosphatidylcholine, cholesterol (Avanti Polar Lipids), and Fl-PtdEtn (Molecular Probes) in a molar ratio of 500:100:1, were prepared by evaporating the solvent from the lipids under N₂, adding PBS, mixing with a Vortex, and then sonicating under N₂ for 1–2 hr until the solution was almost clear. After centrifugation (15,000 $\times g$ for 15 min), the liposomes were stored under N₂ at 4°C for up to 1 wk. Aliquots were centrifuged before use.

The day before use, microscope slides and no. 1, 22-mm² coverslips were sonicated in 10% hot Micro detergent (International Products, Trenton, NJ) for 30 min, rinsed extensively in tap H₂O followed by doubly distilled H₂O and

FIG. 1. Video images of multivalent colloidal gold anti-FI moving on a supported planar membrane containing FI-PtdEtn. The images have been processed to enhance detection as described. Single gold particles are small black spots. Larger spots are aggregates. The images are 165 msec apart. The displacement of the gold particles can best be seen by comparing the position of moving particles (arrowheads) with immobile particles (x). All of the gold particles shown are attached to the membrane. Particles that were free in solution came into focus only for brief moments. (Bar = 1 μ m.)

air-dried in a dust-free place. Chambers were assembled by mounting a coverslip on a slide with two strips of double-stick tape (Scotch no. 410). The liposomes were added, and the chamber was placed in a dark, moist container for 30 min at room temperature. The newly formed planar membrane was rinsed by flowing 2 ml of PBS in 200- μ l aliquots through the chamber. BSA/PBS was added 10–20 min prior to adding the gold anti-FI. After a 15-min incubation, the unbound gold was rinsed off with three 200- μ l aliquots of BSA/PBS. The chamber was sealed with VALAP (1:1:2 vasoline/lanolin/paraffin).

Video Microscopy and Image Analysis. Planar membranes were examined on a Zeiss Axiovert 10 microscope using bright-field optics: $\times 100$, 1.3 n.a. oil-immersion objective and 1.4 n.a. oil-immersion condenser. The images were projected with a $\times 4$ adapter to a Hamamatsu C2400 video camera, Newicon tube (Photonic Microscopy, Oak Brook, IL). The Hamamatsu camera was selected because the background noise was low enough to allow detection of the rapidly moving gold particles without image-averaging. After background subtraction and contrast enhancement in real time by an Image-1 image processor (Universal Imaging, West Chester, PA), the images were recorded at video rate in sequences of 200 to a Panasonic TQ-2028F optical disk recorder. Image-1 was also used for image retrieval, processing, and analysis. Processing involved removal of shading, enhancing the contrast of the gold particles with respect to the background, and removal of fine-grained noise by smoothing. After processing, the centroids of the gold particles in each image were determined by using the Object Measurement Mode function of Image-1. A program written in C language was used to determine the tracks of each particle by locating the nearest centroid in succeeding images. The accuracy of the resulting tracks was verified by playing back the images and matching the pattern traced by individual gold particles with that produced by the tracking program.

For each particle, the tracking program also computed the mean square displacement (MSD) for every time interval by using the formula (11):

$$\text{MSD}(t) = \langle (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2 \rangle, \quad [1]$$

where (x_{i+n}, y_{i+n}) describes the particle position following a time interval, t , given by $n \times$ video frame time (0.033 sec) after starting at position (x_i, y_i) . i ranges from 1 to $N - n$, where N is the total number of particle positions recorded, and n takes on values 1, 2, 3 . . . $N - 1$.

For a two dimensional random walk,

$$\text{MSD} = \langle r^2 \rangle = 4Dt, \quad [2]$$

where D is the lateral diffusion coefficient and t is the time interval (11). By using this equation, D was computed from the linear portion of the MSD versus time plot.

The number of immobile gold particles was determined by overlaying the first and last images in a sequence in red and green, respectively. The number of red spots were counted as the total number of particles, and overlapping red and green spots were counted as immobile. The particles defined in this way as immobile had $D \leq 4 \times 10^{-10}$ cm²/sec.

Computer Simulations. Computer simulations of two-dimensional Brownian motion were produced based on microscopic theory of diffusion (12, 13). The simulated random walk of a particle consisted of a series of jumps with each jump consisting of 100 steps. Since a step was 0.33 msec, a jump corresponds to the frame interval used to record images (video rate). The length of each step (r) was constant during the course of the walk and was determined by $(4Dt)^{1/2}$, where t is the time duration of the step (0.33 msec) and D is the chosen lateral diffusion coefficient. The direction of each step was chosen randomly and independently from the previous step.

FRAP. By using the same equipment and procedure as previously described (14, 15), a small spot (1.1- μ m radius) of FI-PtdEtn was bleached in the planar membrane with a $\times 40$, 1.3 n.a. oil-immersion objective. The bleach was for 10 msec when using a laser power of 300 mW at 515 nm. For monitoring recovery, the bleaching beam was attenuated 10,000-fold, and fluorescence was measured with a photon counter (Thorn EMI

FIG. 2. (A–C) Patterns of random motion of gold anti-FI attached to a planar membrane. (D) The MSD (μ m²) plots for each of the tracks shown. D for each of the gold particles was 1.1×10^{-8} cm²/sec (A), 1.0×10^{-8} cm²/sec (B), and 1.3×10^{-8} cm²/sec (C). For this figure and all following figures, the time interval is defined by $t = n \times$ video frame time.

GenCom, Fairfield, NJ) coupled to a multichannel analyzer (Nucleus, Oak Ridge, TN). For isotropic lateral diffusion, $D = W_s^2 \gamma / 4\tau_{1/2}$, where W_s is the e^{-2} radius of the Gaussian profile of the laser beam, γ depends on the strength of photobleaching, and $\tau_{1/2}$ is the half time for full recovery. The FRAP experiments were done at room temperature.

Controls. The specificity of the colloidal gold probe was tested in three ways. (i) The gold anti-FI could be blocked from the planar membrane by competition with fluorescein-conjugated goat anti-rabbit antibody. (ii) The gold anti-FI bound to cells labeled with FI-PtdEtn but not to cells that had not been labeled with FI-PtdEtn. (iii) When gold anti-pgp-1 (a membrane glycoprotein) was added to planar membranes, there was no binding.

To verify that our video microscopy system could indeed detect individual gold particles, cells cultured on gold locator grids were labeled with either gold anti-pgp-1 or gold anti-FI, images were recorded with the video system, and then the cells were fixed and processed as whole mounts for examination by transmission electron microscopy. The gold particles in electron micrographs of the cells matched those in the video images, and many were single gold particles (see below).

RESULTS AND DISCUSSION

The gold anti-FI attached to FI-PtdEtn moved on the planar membrane in random walks (Figs. 1 and 2A–C). In the sample images shown in Fig. 1, individual gold particles (arrowheads) can be seen to move relative to adjacent stationary particles (x). One can also see that there is considerable variation in the distance moved by individual particles. The diverse patterns formed by their tracks are similar to those produced by computer simulation of two-dimensional random motion (Fig. 3A–C). In both the gold and the simulated tracks, examples were found of “running and tumbling” (Figs. 2A and 3A) (16), of space filling (Figs. 2B and 3B), and of looping where the endpoint is near the start point (Figs. 2C and 3C). Since the patterns of Brownian motion are fractal (17), producing similar patterns even when data are collected over longer times, a random pattern such as running and tumbling could be mistaken for flow. Thus, there is a definite need for statistical methods to discriminate between random motion and flow or corraling when studying cells (4, 5, 7).

The theoretical MSD plot for random motion is a straight line (2, 4, 11). The experimental MSD plots are linear for only short time intervals for both the individual gold tracks (Fig. 2D) and the simulated tracks (Fig. 3D). The nonlinearity at the longer time intervals is not due to measurement errors because such errors are random and thus do not affect the MSD plots (26). However, with the longer time intervals, there is greater stochastic variation in the net displacements of the particles from their original positions. For instance, compare the start (S) and end (E) positions of Fig. 2A with C and Fig. 3A with C. This stochastic variation is not averaged out because there are fewer square displacements

Table 1. Summary of D data computed from MSD plots of individual gold and simulated tracks

Track type	$D \times 10^8$, cm ² /sec		Linear portion of MSD plot			N*
	Mean $\pm$ SD	Range	Extent, sec	R^2		
MV†	0.33 $\pm$ 0.29	0.04–1.3	1.7 $\pm$ 1.18	0.972 $\pm$ 0.050		74
PV	0.76 $\pm$ 0.46	0.07–2.1	1.6 $\pm$ 0.74	0.990 $\pm$ 0.008		19
PVG	0.70 $\pm$ 0.44	0.26–1.7	1.5 $\pm$ 0.62	0.985 $\pm$ 0.012		13
SIM1	0.42 $\pm$ 0.13	0.22–0.59	2.2 $\pm$ 0.97	0.989 $\pm$ 0.007		10
SIM2	1.17 $\pm$ 0.33	0.59–1.7	1.0 $\pm$ 0.50	0.995 $\pm$ 0.006		10
SIM2	1.34 $\pm$ 0.41	0.59–2.1	1.1 $\pm$ 0.43	0.995 $\pm$ 0.006		30

D was computed from the slope of the linear part of the MSD-versus-time curve that extended from the origin to a time interval unique to each track. The average ($\pm$ SD) of that time interval is given under “Extent.” The average ($\pm$ SD) regression coefficient, R^2 , is given to demonstrate how well a straight line fits these portions of the MSD plots.

*N, number of tracks.

†MV, multivalent gold; PV, paucivalent gold; PVG, paucivalent gold with 30% glycerol; SIM1, computer simulations of Brownian motion with $D = 0.4 \times 10^{-8}$ cm²/sec; SIM2, simulations with $D = 1.3 \times 10^{-8}$ cm²/sec.

to average at the longer time intervals (Fig. 4A *Inset*). Despite the increased stochastic variation, the lines appear smooth because the data points are not independent of each other (apart from the first time interval, the MSD are computed from overlapping displacements; see Eq. 1). Since the plots were linear near the origin, D could be calculated for each trajectory (Table 1). This approach proved to be accurate because the mean D s computed for the simulations from the individual MSD plots are almost the same as the D s used to generate the trajectories (see Table 1).

The above observations are further supported by averaging the MSDs for several tracks. The resulting MSD plots are linear for greater distances (Fig. 4). All tracks for the mobile gold particles ($D > 4 \times 10^{-10}$ cm²/sec) were averaged together because the distribution of D appeared continuous with no obvious break above the immobile fraction (Fig. 5). The linear portion of the combined plot for the multivalent gold tracks (Fig. 4A) and for the paucivalent gold tracks (Fig. 4B) yields diffusion coefficients of 2.6×10^{-9} cm²/sec and 7.3×10^{-9} cm²/sec, respectively (Table 2). These values are only slightly different than the mean D computed from the individual plots (compare with Table 1). D for the combined plot of the simulated tracks (Fig. 4C) is 4.1×10^{-9} cm²/sec, almost the same as the value used to generate the tracks, 4.0×10^{-9} cm²/sec.

The variability in the D s computed for individual gold particles complicated their comparison with D for the FI-PtdEtn in the planar membranes obtained by FRAP. Thus, we used computer simulations to evaluate how much variation in D is contributed by the stochastic nature of Brownian motion. Since the D obtained for FI-PtdEtn in the planar membranes by FRAP is 1.3×10^{-8} cm²/sec (Table 2), computer simulations of Brownian motion were generated by

FIG. 3. (A–C) Patterns of random motion simulated by computer for $D = 4 \times 10^{-9}$ cm²/sec as described. (D) MSD (μm^2) plots for each of the tracks shown. D for each of the simulated tracks was 0.5×10^{-8} cm²/sec (A), 0.5×10^{-8} cm²/sec (B), and 0.3×10^{-8} cm²/sec (C).

FIG. 4. Averaged MSD (μm^2) plots for 74 multivalent gold particles (A), 19 paucivalent gold particles (B), and 10 simulated tracks (C). The MSD for each time interval was averaged for all tracks with $D > 4 \times 10^{-10} \text{ cm}^2/\text{sec}$. (Inset in A) Number of displacements that were squared and averaged to give the MSD for each respective time interval in A. The solid lines are regression lines computed over the linear region of the curve: 0–3 sec (A), 0–1 sec (B), and 0–4 sec (C).

using a $D = 1.3 \times 10^{-8} \text{ cm}^2/\text{sec}$. When the simulations were analyzed in the same manner as for the gold particles and for a comparable number of frames, it was found that, for individual tracks, D ranged from 0.6 to $2.1 \times 10^{-8} \text{ cm}^2/\text{sec}$ (Table 1). Thus, those particles with $D \geq 0.6 \times 10^{-8} \text{ cm}^2/\text{sec}$ can be considered as having a D consistent with the FRAP value. With this line of reasoning, 16% of the multivalent gold and $\approx 50\%$ of the paucivalent gold particles can be assumed to exhibit similar lateral mobility as the unlabeled lipid analog.

Several physical reasons could contribute to the lower values of D obtained for the remaining gold particles. One reason is that multivalent gold particles may bind one or more lipid analogs. The small fraction of the multivalent particles with values of D consistent with those measured by FRAP may represent gold particles bound to only one or two lipids. For the multivalent gold particles that have a lower D , one can envision each particle binding to several lipids within the bilayer and thus creating a large disk as illustrated in Fig. 6A.

Table 2. Diffusion of FI-PtdEtn in a planar membrane: A comparison of FRAP and colloidal gold

	$D \times 10^8$, cm^2/sec	Particles with $D >$ 0.6×10^{-8} cm^2/sec , %	% mobile	N^*
FRAP	1.33 ± 0.33	—	70 ± 13	28
Multivalent gold	0.26	16	70	74
Paucivalent gold	0.73	53	84	19
PVG†	0.63	46	89	13

*For FRAP, N = number of spots bleached; for gold, N = number of mobile particles.

†PVG is paucivalent gold with 30% glycerol.

In the extreme case that this disk is essentially impermeable to other lipids in the bilayer, the Saffman–Delbruck model (19), adapted to a single monolayer of the bilayer (20), would predict that D for the 40-nm disk would be roughly half the D for a single lipid. However, the disk is most probably “free draining” or partially so, meaning that the lipids of the bilayer can permeate the disk to some extent. When such “solvent” permeation occurs, the diffusion coefficient of the disk will not be as low as if the disk were solid (21, 22). That “free draining” is plausible is shown by the fact that even when antibodies bound to lipids in a planar membrane are at a sufficient concentration to be immobilized, the bilayer lipids have nearly the same diffusion coefficient as when antibodies are not present (23, 24). A partially permeable ($\sigma = 2$; see ref. 21) disk of the size depicted in Fig. 6A would have its diffusion coefficient reduced by a factor of 1.5 relative to that for a single lipid molecule.

In the case of paucivalent gold particles, the number of lipids bound per gold particle will be considerably lower, and a substantial fraction of particles may bind only one lipid (Fig. 6B). Moreover, when multiple bonds occur, they will be fewer in number and thus sufficiently distant to allow the disk to be free-draining. This is consistent with the shift in distribution of paucivalent D values toward the D measured by FRAP (Table 2; Fig. 5).

Another possible reason for the reduced D for some of the particles is that they could occasionally clump together. Large clumps of four or more gold particles are easily detected as aggregates by video microscopy, and clumps of three gold particles usually appear as aggregates. However, electron microscopy is required to distinguish between one and two particles. Fortunately, as judged by electron microscopy of gold on cells, $\approx 90\%$ of the gold particles that were not in large clumps were single. For the planar membranes, particles were counted as aggregates if they appeared noticeably darker than the majority of the particles in the image. Of those judged to be aggregates, $\approx 50\%$ were stationary. The remaining aggregates accounted for $\approx 15\%$ of the total mobile gold particles. For the mobile aggregates, the average D ($0.19 \times 10^{-8} \text{ cm}^2/\text{sec}$ (multivalent) and $0.48 \times 10^{-8} \text{ cm}^2/\text{sec}$

FIG. 5. Distribution of D for individual gold particles moving on a planar membrane. (A) Multivalent gold. (B) Paucivalent gold. (C) Paucivalent gold in the presence of 30% glycerol. The numbers on the abscissa indicate the upper limit of each class. Arrows indicate the class where the D obtained by FRAP would be.

FIG. 6. Model demonstrating how a multivalent gold particle (A) could bind a disk of lipid analogs 40 nm in diameter and how a paucivalent gold particle would bind one lipid analog (B). Since the antibody molecule is expected to make multiple contacts with the gold particle (18), we presume that, in some instances, only one arm of the divalent antibody is free to bind to its epitope. We have not assessed the number of relevant antibodies bound to each paucivalent gold particle; however, the ratio of relevant to irrelevant antibody was such that the vast majority of gold particles did not attach to the membrane yielding sparse labeling. This is in contrast to the multivalent gold where dense labeling was obtained with a solution less concentrated by approximately a factor of 100. (C) A possible mechanism by which a multivalent gold particle could deform the membrane.

(paucivalent) was lower than the overall average (Table 1), but many putative single gold particles had values for D lower than those found for the aggregates (Fig. 5).

Even though the gold particle in the adjacent aqueous phase is in a medium much less viscous than the membrane, one may wonder if the mass and/or size of the gold particle could act to slow the movement of the bound lipid. To test this, the viscosity of the medium was increased by adding glycerol to 30%, yielding a viscosity of 2.16 centipoise (25). The glycerol had no effect on the average D nor on the percentage of particles with a value for $D > 1 \times 10^{-8}$ cm²/sec (Table 2; Fig. 5). However, the glycerol may have reduced the affinity of the anti-F1 for fluorescein, as this was the only condition where gold particles were observed to detach from the membrane.

It is possible that some of the multivalent gold particles had perturbed the membrane causing a reduction in lateral mobility. Since the gold is coated with antibody, it also could become coated with lipid, resulting in deformation of the bilayer (Fig. 6C). Membrane vesicles of such low diameter (40 nm) are known to exist; thus, this curvature of the bilayer is possible. However, if such curvature of the bilayer were induced, the gold particle would probably be immobilized. This may account for the higher immobile fraction observed with the multivalent gold (Table 2).

CONCLUSION

The Brownian motion of lipids in a membrane can be observed, tracked, and measured by using colloidal gold probes. The number of lipids bound to the gold affects the measured D . This has led us to propose that, on the average, a multivalent gold particle binds to several lipids creating a partially permeable disk moving through the membrane, whereas a paucivalent gold particle is attached to only one or two lipids. We anticipate that nanovid microscopy will find increasing use in the study of both membrane dynamics and domain structure. However, the valency of the gold should

be considered when interpreting the results from such experiments.

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