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Analysis of individual lipoproteins and liposomes

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ABSTRACT

We describe the application of single molecule detection (SMD) technologies for the analysis of natural (serum lipoproteins) and synthetic (liposomes) transport systems. The need for advanced analytical procedures of these complex and important systems is presented with the specific enhancements afforded by SMD with flowing sample streams. In contrast to bulk measurements which yield only average values, measurement of individual species allows creation of population histograms from heterogeneous samples. The data are acquired in minutes and the analysis requires relatively small sample quantities. Preliminary data are presented from the analysis of low density lipoprotein, and multilamellar and unilamellar vesicles.

2. INTRODUCTION

Over the past seven years several groups have developed the sensitivity necessary to detect and identify single fluorophores in aqueous systems.¹⁻¹⁰ Several review articles on this subject have been published recently.¹¹ The ability to detect and analyze samples at the single molecule level represents the ultimate in chemical analysis and is achieved by the combination of a sensitive optical detection apparatus and by reducing the background due to fluorescent impurities, Raman scattering, and scattering at interfaces. This background reduction is accomplished primarily by decreasing the probe volume defined by the intersection of the illuminating light path and the detector field of view. We use hydrodynamic focusing,¹² a focused laser beam, and a high resolution microscope objective to obtain a probe volume of approximately 1 pL. Other workers have used confocal techniques to detect single molecules in femtoliter probe volumes which produce a minimum of interfering signal. However, efficient detection requires that each analyte flow through the center of the detection region, which becomes increasingly difficult as the probe volume is decreased since many analytes will skirt the edge of the detection region or be missed entirely. Consequently, a balance must be reached between an extremely small detection region and a region large enough to ensure the analytes will pass through it. With careful attention to details to ensure all of the analyte molecules pass through the center of the detection volume, detection efficiencies greater than 90 % have been attained.^{10, 13c} Detection of analytes in flow with picoliter probe volumes offers the advantage of efficient, rapid analysis combined with single molecule sensitivity.

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Ultrasensitive fluorescence detection in flow is based on the technique of flow cytometry (or flow microfluorimetry) which has existed for three decades.¹² Flow cytometry has enabled major advances in biomedical science by providing rapid, quantitative, and sensitive multiparameter measurements of individual particles (cells). Because each particle is analyzed individually, histograms can be produced which reveal not only population averaged properties but also information on the heterogeneity of the population. Thus, analysis of individual particles in flow provides information which cannot be obtained by bulk phase analysis. Conventional flow cytometers are capable of making several measurements simultaneously on individual particles (*i.e.* multicolor fluorescence and small angle and orthogonal light scatter), making it possible to identify different cell types in a heterogeneous suspension on the basis of combinations of morphology, surface molecules, metabolic state, or a variety of other physiologically important criteria. Combined with an analysis rate of hundreds to thousands of particles per second and the ability to physically sort particles of interest, these features have made flow cytometry an indispensable tool in immunology, cancer biology, and other fields of biomedical research. One limitation of conventional flow cytometry is the inability to measure small (less than 0.5 μm) or dim (less than several hundred fluorescent molecules) particles. A great variety of important biological particles, molecules, and molecular assemblies fall into these categories. It is our long term goal to develop ultrasensitive flow cytometric approaches to access this domain of biological entities.

Over the past several years we have developed applications of SMD for the analysis of a variety of biological species. Sequencing DNA by detecting single fluorescently labeled nucleotides as they are sequentially cleaved in flow is one current area of intense investigation for application of this technique.¹³ Others are DNA Fragment sizing¹⁴ and DNA fingerprinting. This application is based on the detection of intercalating fluorophores which bind stoichiometricly (with respect to DNA base pairs) to individual DNA fragments.¹⁴ In this paper we present additional biomedical applications of this SMD technology to the analysis of lipoproteins and liposomes.

3. INSTRUMENTATION

The instrument used to collect the presented data is shown in Figure 1 and is similar to previous experimental designs.^{5,14c} A brief description of the experimental setup is given below. Excitation was accomplished with an Ar⁺ laser operating at 488.0 or 514.5 nm. The output power was attenuated with a polarizer/half wave plate assembly and focused to a 20 μm ($1/e^2$ diameter) horizontally polarized circular spot at the center of a 250 x 250 μm^2 square bore sheath flow cuvette (NSG Precision Cell, Inc.). Fluorescence was collected at 90° with a 40x, 0.85 NA microscope objective (Nikon Fluor) and spatially filtered with a 400 μm slit located at the image plane of the microscope objective. The probe volume thus defined was approximately 3 pl. Light passing through the slit was spectrally filtered with a 30 nm bandpass filter centered at 535 or 575 nm. The filtered light was refocused on the 200 x 200 μm^2 active area of a silicon photon counting module (SPCM-AQ or SPCM-PQ, EG&G Optoelectronics Canada) using a 10x microscope objective.

The sheath fluid, ultra pure water (Millipore water system), was delivered to the flow cell using gravity feed. The volumetric sheath flow rate (~ 10 $\mu\text{L}/\text{min}$.) was adjusted to give transit times through the probe volume of about 1 ms, as determined by the autocorrelation function.^{14c} Samples dissolved in TE (Tris-EDTA, pH 8) or PBS (phosphate buffered saline) at concentrations from 1×10^{-8} to 1×10^{-12} M were forced through a capillary (O.D. 90 μm , I.D. 20 μm , Polymicro Technologies) via a pressure differential. Sample molecules, eluted from the tapered capillary tip into the center of the flow cell, were then hydrodynamically focused by the sheath fluid. The focused laser beam intersected the sample stream approximately 100 μm downstream of the capillary tip. The sample concentration was adjusted to minimize coincidences.

Photoelectron pulses (TTL) from the photodiode were passed directly to a multichannel scalar (MCS) PC card (Oxford Instruments), which integrates and stores pulses in successive time bins (typically 50 μs). An Interactive Data Language (Research Systems, Inc.) program was used to analyze the data. Photon bursts were smoothed (Lee filtering algorithm) and sifted from the data by setting a threshold just above the background level. A series of consecutive points rising above this threshold was defined as a burst. After sifting, the burst areas were integrated and histogrammed. A second instrument configuration employed

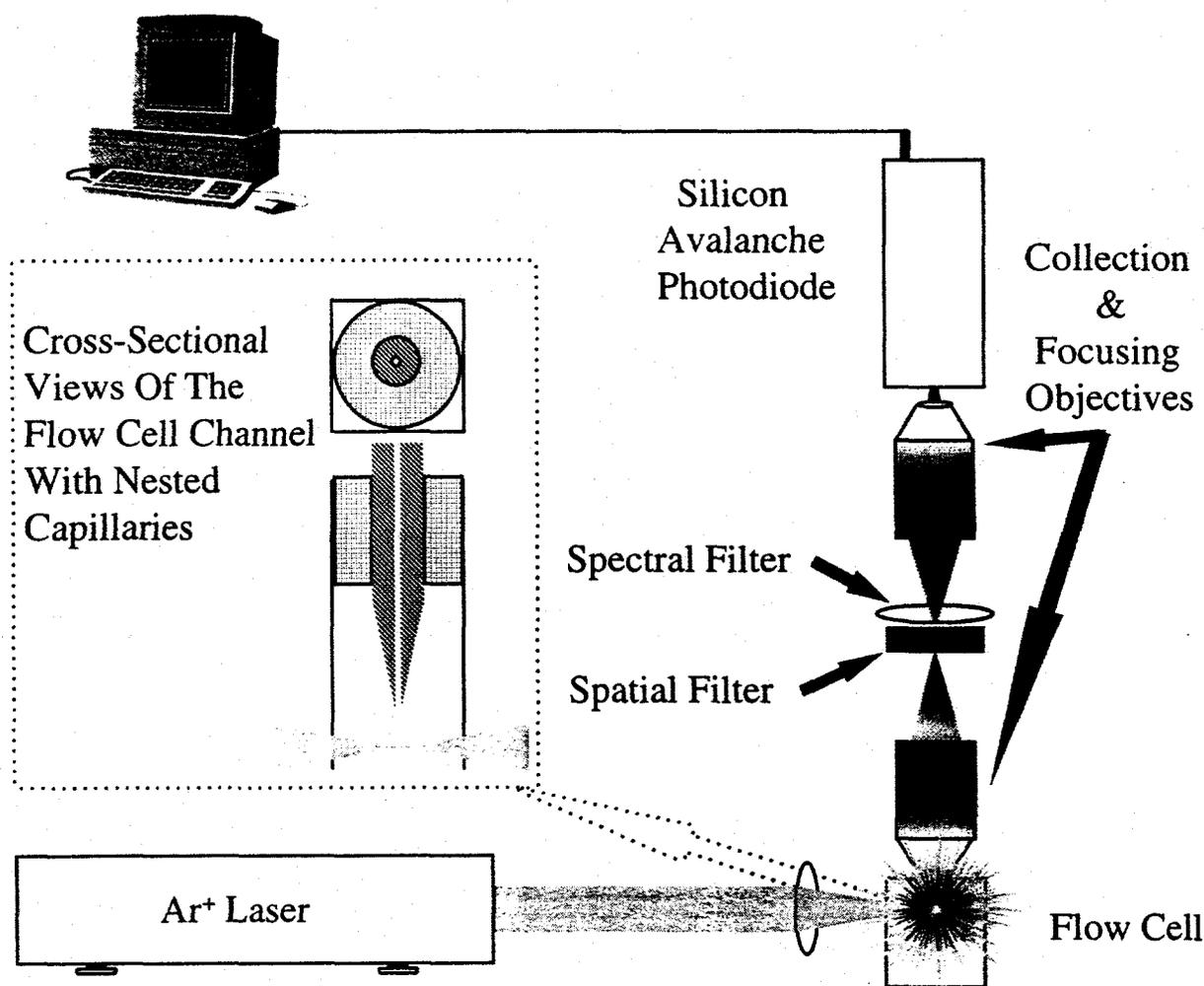


Figure 1: Single molecule detection apparatus consisting of four primary components: laser source (continuous or mode locked Ar⁺ laser), flow cell, detection optics, and detector (active or passively quenched avalanche diode with output passed to a multichannel scalar or to specialized electronics for temporal gating). The conical shaped capillary tip (inset) is formed by burning a small section (~ 0.5 mm) of the polyimide coating off of commercially available stock (O.D. 90 μm , I.D. 20 μm , Polymicro Technologies). This section of bare quartz is acid etched (hydrofluoric acid) under slight tension until the capillary breaks. The overhanging polyimide coating is removed and the capillary is nested in a larger capillary (O.D. 240 μm , I.D. 100 μm) for positioning and inserted into the square bore flow cell (250 x 250 μm^2). The tapered contour, formed by this process, allows for smooth sheath flow to the tip where samples are eluted.

a mode locked Ar⁺ laser to discriminate temporally, fluorescence from prompt scatter. This was accomplished by collecting photoelectrons in a fixed time window, delayed with respect to each laser excitation pulse.⁵

4. SAMPLE PREPARATION

Multilamellar vesicles (MLVs) were prepared with 1% (m/m) DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine-perchlorate, Molecular Probes, Inc. Cat # D-282) and egg PC (phosphatidylcholine, Sigma Chemical Company). Small unilamellar vesicles (SUVs) were prepared with ~10% (m/m) head group-labeled phospholipid molecules, NBD-PE (1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (7-nitro-2-1,3-benzoxadiazol-4-yl), Avanti Polar-Lipids Inc., Cat. # 810145), and egg PC. Lipids and fluorescent probes in chloroform were mixed and solvent evaporated. Dried lipids were hydrated in TE forming MLVs. SUVs were formed from MLVs by extrusion (~30 passes) through two polycarbonate filters (Avestin, Inc.). All vesicle preparations were made to a 1 mM lipid concentration and diluted in TE for delivery without further modification or chromatographic separation. Calcein (Molecular Probes, Inc. Cat. # C-481) loaded vesicles were prepared by hydration of dried lipids with 1×10^{-3} to 1×10^{-5} M calcein in TE and were extruded as described above. Fluorescence from untrapped dye was significantly reduced by dilution and quenching with Co²⁺ (CoCl₂).¹⁵

Low density lipoprotein (LDL) from human plasma, fluorescently labeled with DiI, was purchased from Molecular Probes Inc. (DiI AcLDL, cat # L-3484). LDL was diluted in TE and analyzed without modification.

5. SERUM LIPOPROTEINS

Here we present the detection and diagnostics of individual serum lipoproteins by high sensitivity flow cytometry. Quantification is important because of the many diseases associated with lipoprotein transport and metabolism. Most notable is coronary artery or heart disease which is associated with elevated levels of specific serum lipoprotein fractions and remains the leading cause of death in the United States. Risk factors include abnormal levels of low density lipoprotein (LDL) and lipoprotein(a). Many diseases have been correlated with lipoprotein disorders involving the various fractions of serum lipoproteins (Table 1). Therefore, it is desirable to have a technique that characterizes this class of particles completely. Currently, ultracentrifugation is the only technique offering a complete description. However, ultracentrifugation is slow, labor intensive, requires large quantities of blood, and is expensive. The expense comes from the fact that a single machine can be tied up continuously for periods of one week or more for a single patient. Additionally, unique and important subclasses of lipoproteins may be too near in density for discrimination by centrifugation.

	Particle Size (nm)	Surface Area (Centroid) (nm ²)	Molecular Weight (amu)	Density (g/ml)
VLDL	30 to 80	9.5×10^3	10 to 20,000,000	0.93 to 1.006
IDL	25 to 35	2.8×10^3	5 to 10,000,000	1.006 to 1.019
LDL	18 to 25	1.4×10^3	2,300,000	1.019 to 1.063
HDL ₂	9 to 12	3.5×10^2	360,000	1.063 to 1.125
HDL ₃	5 to 9	1×10^2	175,000	1.125 to 1.210

Table 1.¹⁶ Very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL).

There are several major areas in basic and clinical research where the analysis of diseases associated with serum lipoprotein disorders are limited by the cost of existing technology and the lengthy time of analysis. These include but are not limited to: 1) clinical analysis of the complete lipoprotein distribution in human patients, 2) analysis of the time dependence of lipoprotein distributions in patients who have eaten a fatty meal, changed their diet, or taken medications which alter their lipid metabolism,

3) the analysis of lipoprotein metabolism in animal models or human subjects for the purpose of drug development by pharmaceutical companies, and 4) the evaluation of lipid metabolism in genetically altered animal models of heart disease. Enhanced analytical capabilities will greatly benefit these areas of study.

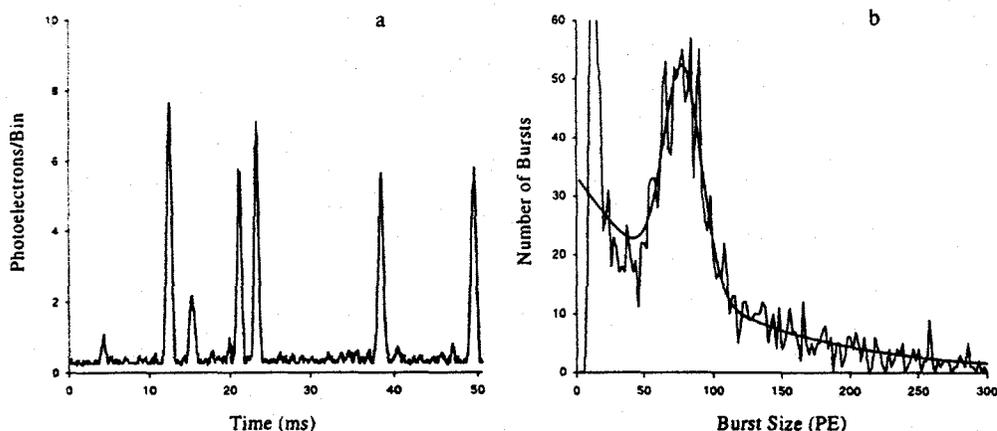


Figure 2: Single molecule detection (time gated detection) of DiI labeled LDL from human serum. a) A segment of smoothed data exhibiting photon bursts from LDL-DiI complexes. b) Burst size distribution obtained from 60 seconds of raw data. The fit is from the sum of a decaying exponential (representing background) and a Gaussian ($A_1 \cdot \exp(-B \cdot x) + A_2 \cdot \exp(-((x-m)/\sigma)^2/2)$, where A_1 and A_2 are pre-exponential amplitudes, B is a constant, x is the burst size, m is the mean, and σ is the standard deviation.) The mean burst size obtained from this fit is 78.2 ± 1.1 photoelectrons and the standard deviation is 13.4 ± 1.2 .

As an approach into this area we have focused on detecting serum lipoproteins whose surfaces have been fluorescently labeled, by the addition of an amphipathic dye, such as DiI. Upon partitioning into the lipoprotein surface, DiI's fluorescence increases. Because lipoprotein fractions exhibit distinct sizes (Table 1), the surface area available for labeling will vary as a function of diameter. It is thus, in principle, possible to detect and quantify the amount of each lipoprotein present in a serum sample based on particle size. This approach depends on the resolution, as reflected in the experimentally measured standard deviation, which will have contributions from instrumentation, sample, and sample labeling. In the limit of small numbers of photons detected, the standard deviation has a square root dependence on the signal mean. This means that the resolution increases as the signal increases. Larger signals may be obtained by an increase in the number of dyes per particle. However, an increase in the number of fluorescent labels on a finite surface area can cause a decrease in overall fluorescence due to dye-dye fluorescence quenching via Förster energy transfer. The intra-label distance at which fifty percent fluorescence quenching is expected is termed R_0 and for DiI in a membrane this radius is 6.8 nm.¹⁷ For a probe restricted to a two dimensional surface, this radius corresponds to a circular area of 145 nm² per probe molecule. Consequently, a spherical LDL with a surface area of 1400 nm² would exhibit 50% quenching at ~10 probe molecules per lipoprotein. However, at low surface densities the standard deviation will have a significant contribution from labeling statistics.

Molecular Probes reports an average of 36 DiI molecules per LDL. At this labeling density considerable fluorescence quenching is expected. Sixty seconds of data were collected for analysis. The first 50 ms of smoothed data are shown in Figure 2a. The transit time through the focused laser beam (11 mW) was adjusted to 2 ms. Approximately 1200 LDL photon bursts were collected in one minute and analyzed to generate the histogram shown in Figure 2b, yielding a peak size of 78.2 ± 1.1 photoelectrons

with a standard deviation of 13.4 ± 1.2 . This standard deviation is reasonable considering labeling and signal statistics.

To estimate our ability to resolve heterogeneous mixtures of surface labeled lipoproteins ranging from high to very low density, we simulated the expected burst sizes based upon their median surface areas (Table 1). The surface area median for each fraction was scaled linearly according to the number of photoelectrons measured for LDL (78.2 photoelectrons). Standard deviations were based on that measured for LDL and scaled according to the square root of the surface area medians. We assume that both the statistics of labeling and instrumental contributions to the standard deviation are proportional to the square root of the mean. The simulation (solid curve, Figure 3) exhibits resolvable peaks for HDL, LDL, IDL, and VLDL, given equimolar concentrations. The simulated histogram is significantly different (cross-hair curve, Figure 3) when the pre-exponential intensity factors are weighted according to the relative lipoprotein concentrations expected for a normal fasting male (HDL₃ = 80%, HDL₂ = 9%, LDL = 10%, IDL = 0.3%, VLDL = 0.7%).¹⁶ The simulation will be altered by changes in sub-fraction particle size range and by reduction of surface lipid available for staining due to protein at the surface. These calculations indicate that these lipoprotein classes can be discriminated on the basis of a fluorescent surface label alone.

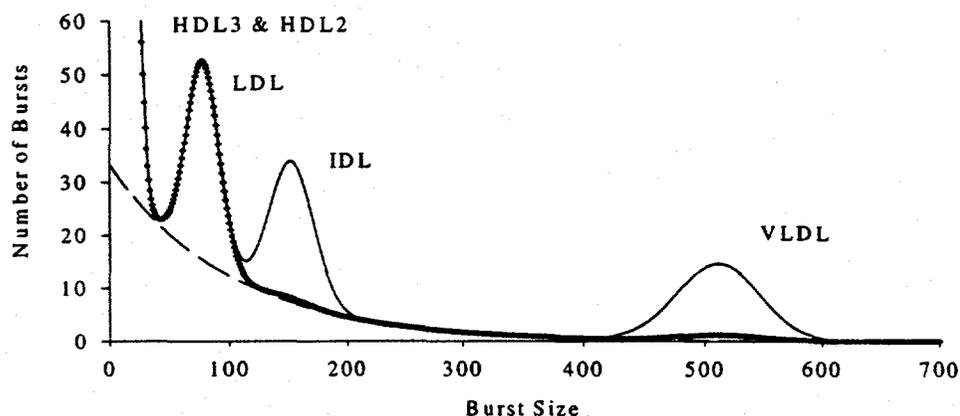


Figure 3: Simulated burst size distributions of surface labeled serum lipoprotein. Simulations are generated from the sum of 5 Gaussians and an exponential. The exponential and LDL Gaussian parameters are taken from the fit to data shown in Fig. 2. Parameters for HDL₂, HDL₃, IDL, and VLDL are scaled according to their surface areas relative to LDL. The solid curve is generated assuming equimolar concentrations for each lipoprotein class. The crosshair curve accounts for lipoprotein concentrations relative to LDL for a fasting male.¹⁶ The dashed curve represents the exponentially decaying background.

The mature technology envisioned for comprehensive lipoprotein analysis will employ multi-parameter detection of fluorescence signals. The integrated burst intensity from partitioned lipophilic dyes (such as DiI) will define size classes of lipoproteins as described above, (analogous to an ultracentrifugation analysis which defines classes based on density). Fluorescence from labeled antibodies against specific apolipoproteins could be used to identify subclasses.

The efficient detection of single particles by this technique requires extremely small samples and makes possible the analysis of multiple serum samples from small animals used for models in lipoprotein research. The 1200 LDL bursts (representing one minute of data collection) used to generate the histogram in Figure 2b would be contained in just 12 nL of male human serum.¹⁶ Speed and sensitivity are advantages of this technique for the analysis of serum lipoprotein.

6. LIPOSOMES

Self assembling vesicles or liposomes represent another system well suited for flow analysis. Their study complements the lipoprotein analysis in several ways. With respect to size, the liposomes we have studied overlap with the larger lipoprotein classes and, more importantly, their amphipathic surfaces closely resemble those of lipoproteins. Liposomes are also important for artificial transport systems as carriers of drugs, vaccines, etc. Their broad applicability arises, in part, from the exquisite control afforded in their makeup which allows preparation of timed release vesicles and addition of specific molecules to their surfaces. The latter property holds promise for creating vesicles capable of targeting specific organs or tissues for drug delivery. Liposomes are also an important system for the study of cells and interactions which take place at cell surfaces. These artificial systems can serve as models for the study of biologically important phenomena such as ligand-receptor binding, signal transduction and membrane fusion.

Research in these areas is facilitated by quantitative liposome characterization. The information desired from their preparation overlaps with that mentioned for lipoprotein and includes quantitation of vesicle diameter and volume, as well as the number of adsorbed, attached, or entrapped molecules. Their similarity to lipoprotein allows for analysis by methods similar to those described above. Single event detection of vesicles extruded through 200 and 400 nm polycarbonate filters was recently reported by Fuller and Sweedler.¹⁸

MLVs, fluorescently labeled with DiI, were diluted to approximately 1×10^{-10} M and injected into a capillary for analysis. Figure 4 shows the histogram produced from approximately 1 minute of data. This histogram shows a distribution of vesicles resolved from the background. The shape of the distribution is indicative of the sample population and should be characteristic of the method of preparation. The steep drop off towards small burst size is indicative of liposomes whose radius is becoming too small to accommodate the phospholipid molecules. The long trailing edge toward larger bursts may have contributions from both large MLVs and simultaneous detection of two vesicles.

SUVs, fluorescently labeled with NBD, were diluted to approximately 1×10^{-13} M and delivered to the flow cell for analysis. Their analysis was accomplished on a different instrument^{14c} with a relatively large probe volume (50 pL), a long transit time (~7 ms), and without the use of an efficient avalanche photodiode. For this reason this measurement can not be compared directly to the MLV data. Fluorescence bursts from these SUVs are histogrammed and shown in Figure 5. This histogram has a symmetric, Gaussian distribution. These vesicles, formed by extrusion through polycarbonate filters, are expected to be symmetrically centered around an average size related to the pore size (50 nm). Thus the average size of 461 photoelectrons represents the fluorescence from 1000 NBD labels per vesicle (estimated assuming a 50 nm diameter and average lipid surface area of 0.7nm^2 .) In contrast, a 50 nm vesicle would, on average, encapsulate only 40 dye molecules, assuming a 1 mM dye concentration. Bursts from calcein encapsulated MLVs and SUVs¹⁵ (extruded through 50, 100, 200 and 400 nm polycarbonate filters) have been discriminated from background in our laboratory (not shown). However, histograms generated from this burst data exhibit a peak in the burst size distribution at zero due to an elevated background signal level from free calcein.

Ultimately, it is desirable to relate the amount of fluorescence (photoelectrons) to the size of the vesicle. However, unlike analysis of DNA fragments or distinct lipoprotein fractions, where quantitation can be related to a known marker, measurement of unknowns without internal standards requires absolute calibration in which uncertainties arising from photobleaching and self quenching must be addressed.¹⁸

7. CONCLUSIONS

We have demonstrated analysis of single lipoproteins and liposomes in flowing aqueous sample streams. While single molecule detection in aqueous flow was accomplished more than seven years ago, detection in the presence of biological media and other biologically induced complications make this area of research especially challenging. The speed and sensitivity provided by this technology will allow novel applications in biological and analytical research and will compliment existing analytical methods.

Experiments are planned to analyze distinct fractions of serum lipoproteins such as HDL and IDL. The well-defined size range of these particles may allow partial analysis of these fractions based on surface area alone. Additionally, the narrow distribution observed for LDL particles may allow for its use as a calibration standard for liposomes or other particles. Further applications of this SMD technology,

including rare event detection of ligand/receptor pairs, DNA hybridization experiments, and protein characterization, are under investigation in our laboratory.

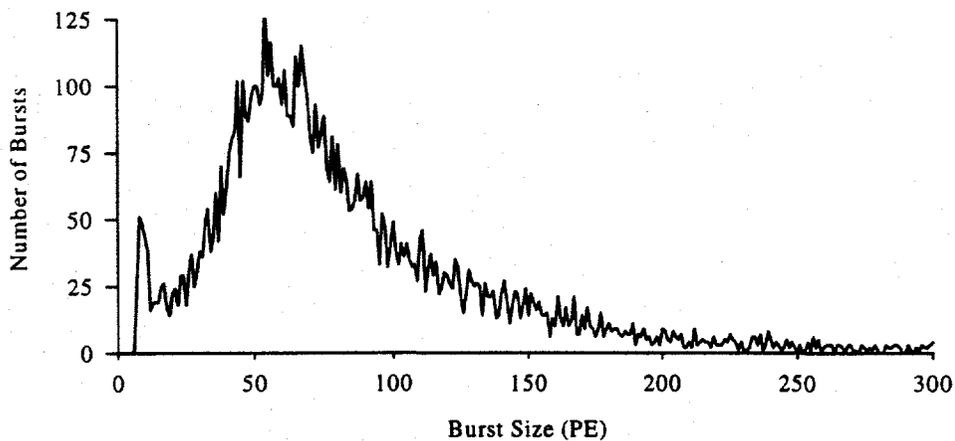


Figure 4: Burst size distribution of multilamellar vesicles composed of egg PC (99%) and DiI (1%).

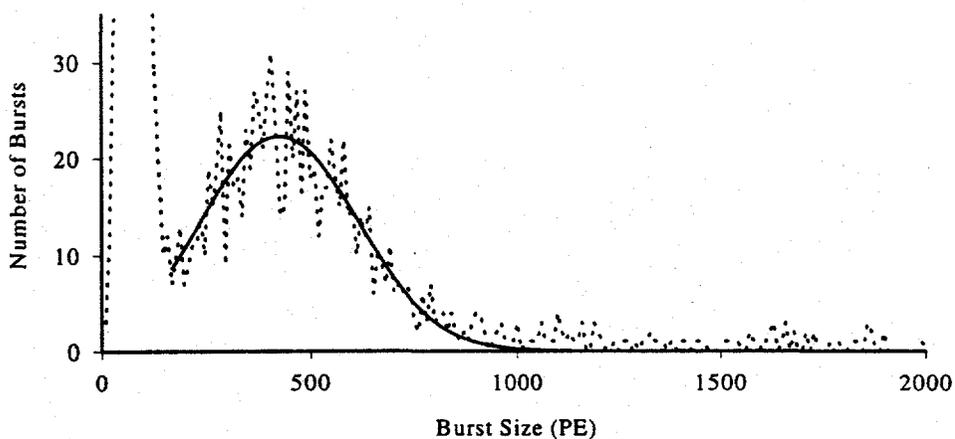


Figure 5: Burst size distribution of small unilamellar vesicles composed of egg PC (90%) and NBD-PE(10%). The SUVs were formed by extruding MLVs through 50 nm polycarbonate filters. The fit (as described in Fig. 2) yields a mean of 461 photoelectrons and a standard deviation of 185.

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