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Tracking Bulk and Interfacial Diffusion using Multiplex Coherent Anti-Stokes Raman Scattering Correlation Spectroscopy

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Abstract

Multiplex coherent anti-Stokes Raman scattering correlation spectroscopy (CARS-CS) is shown as a label-free, chemically specific approach for monitoring the molecular mobility of particles in solution and at interfaces on the millisecond time scale. The CARS spectral range afforded by broadband excitation facilitates a quantitative measurement for the number of particles in the focal volume, whereas the autocorrelation of spectral data elucidates dynamic events, such as diffusion. The measured diffusion coefficients for polymer beads ranging from 100 nm to 1.1 μm in diameter are on the order of 10^{-8} – 10^{-9} cm^2/s , in good agreement with predicted Stokes-Einstein values. Diffusion at different interfaces shows particles are fastest in bulk medium, marginally slower at the liquid/glass interface, and 1.5-2 times slower rate at the air/liquid interface. Multivariate curve resolution analysis of distinct spectral features in multiplex CARS measurement distinguishes different composition lipid vesicles in a mixture diffusing through the focal volume. The observed diffusion is consistent with results obtained from single particle tracking experiments. This work demonstrates the utility of multiplex CARS correlation spectroscopy for monitoring particle diffusion from different chemical species across diverse interfaces.

Graphical abstract

I. Introduction

The ability to track individual particles and single molecules has provided increased understanding of molecular and nanoscale transport and interactions in a wide variety of applications.¹⁻⁶ Fluorescence correlation spectroscopy (FCS) has played a key role in this

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Supporting Information. Additional Experimental Details, Figure S-1, Figure S-2, Figure S-3, Table S-1, and Table S-2 are provided.

increased understanding.⁷⁻⁸ In the 1990s, improvements in confocal microscopy allowed FCS to investigate an assortment of biomolecules at physiological concentrations.^{2, 8-10} In a confined observation volume, the correlation between the signal fluctuations from fluorophores, such as inherently fluorescent proteins or dye-labeled target molecules, was used to determine the concentration and size of particles, characterize translational and rotational diffusion coefficients, and track chemical reactions such as binding kinetics.^{8, 11-12} Advances in instrumentation and bioengineering have enabled fluorescence cross-correlation spectroscopy (FCCS) to study dynamic mechanisms in living cells by monitoring fluorescence fluctuations from chemically distinct fluorophores, thus gaining information on enzymatic cleavage, changes in molecular conformation, and protein-protein interactions in signaling.¹³⁻¹⁴

However, FCS and FCCS are limited to studies of fluorescent species. The system under investigation must be well known to choose the appropriate fluorescent indicator, which narrows the potential for scientific discovery. Fluorescent labels can be quite large moieties that may affect the molecular mobility of the targeted analyte and often require extensive sample preparation. Label-free techniques, such as Raman, circumvent the need for a fluorophore and can be employed to gain chemical specificity.¹⁵⁻¹⁶ Yet, spontaneous Raman scattering is an inherently weak process and is inadequate to detect low concentrations on relatively fast time scales. Surface enhanced Raman spectroscopy (SERS) is a well-established method that detects an increased Raman signal from molecules near a noble metal nanostructure.¹⁷ Recent SERS and surface enhanced resonance Raman spectroscopy studies were done to determine the shape and rotational diffusion of various nanoparticles,¹⁸⁻¹⁹ and characterize the diffusion of particles on a planar SERS substrate.²⁰⁻²¹ An underutilized detection method for correlation spectroscopy is coherent anti-Stokes Raman scattering (CARS). CARS detects fluctuations in the intrinsic vibrational signal of a species without the use of fluorescent dyes, metal colloids, or a metal substrate in the system and broadens the range of biomolecules that can be examined in an unperturbed environment.

The resurgence of nonlinear Raman techniques was driven by the development of solid-state laser systems and laser scanning microscopes also in the 1990s. CARS is widely used in biomedical applications for its chemical sensitivity and video-rate imaging comparable to fluorescence microscopy without the use of exogenous agents.²²⁻²⁷ In coherent anti-Stokes Raman scattering, a pump field (ω_P), Stokes field (ω_S), and probe field (ω_P ; typically $\omega_P = \omega_S$) interact with a sample in a four wave mixing process. When the pump and Stokes fields are spatially and temporally overlapped with a frequency difference, or beat frequency, resonant with a molecular vibration, $\omega_P - \omega_S = \Omega_{\text{vib}}$, coherently driving molecular oscillators at this frequency in the focal volume, a strong anti-Stokes signal is generated at $\omega_{AS} = 2\omega_P - \omega_S$. This anti-Stokes signal is nonlinearly dependent on the incident fields and quadratically dependent on analyte concentration, $I_{\text{CARS}} \propto \chi^{(3)^2} I_P^2 I_S$.^{23, 28-30} In addition, a nonresonant background is generated that shows complex interference with the resonant signal. There have been advances in the instrumentation and data analysis to extract the spontaneous Raman spectrum from the CARS signal.^{28, 30-32}

CARS correlation spectroscopy (CARS-CS) utilizing single-frequency excitation was initially reported independently by Xie et al. and Zumbusch et al. in 2002;³³⁻³⁴ however, little has been done since to expand on CARS-CS. Most recently, inverse CARS-based correlation spectroscopy monitored changes in signal from surrounding bulk water as particles diffused through the focal volume.³⁵ Alternatively, nonlinear correlation spectroscopy (NLCS), specifically second harmonic generation (SHG), was used to track molecular aggregates at a liquid interface.³⁶⁻³⁷ Although NLCS is able to detect nanoparticles in a complex biological medium, the methods used to date lack the chemical specificity obtained from measuring the spectrum. SHG is also limited to observing non-centrosymmetric molecules, useful at interfaces, but difficult for bulk measurements.^{36, 38-40} In this report, we demonstrate the capabilities of multiplex coherent anti-Stokes Raman scattering correlation spectroscopy for monitoring the molecular mobility of particles at the air/liquid interface, the liquid/solid interface, and in bulk solution.

II. Experimental Section

Coherent anti-Stokes Raman Detection

The CARS microscope consists of an electronically synchronized (Coherent SynchroLock AP, Model 2.0b) picosecond pulsed beam (Coherent Mira Ti:sapphire Model 900-P, 2 ps, 76 MHz) with a broadband femtosecond pulsed beam (Coherent Mira Ti:sapphire Model 900-F, 100 fs, 76 MHz). Both beams are linearly polarized and collinearly combined into an upright microscope equipped with a motorized nosepiece (Scientifica, SliceScope Pro) for focus control. An x-y-z piezo stage (Mad City Labs Inc., Nano-LP200, Nano-Drive) is used for point-by-point imaging. Three different objectives were used to excite the different samples: two air objectives (Olympus MD Plan 0.9 NA and Olympus Plan N 0.65 NA) and an oil immersion objective (Olympus U Plan FLN 1.3 NA). For all correlation spectroscopy experiments, the CARS signal was collected in the forward-direction using a Nikon objective (Nikon LPlan 0.70 NA), then focused with an objective (Olympus Plan N 0.25 NA) into an optical fiber and dispersed by a spectrograph onto a CCD camera (Andor iDus). Imaging experiments were performed to determine the optical resolution of the microscope with the 0.9 NA air objective and 1.3 NA oil immersion objective, which were collected in the epi-direction through the excitation objective and focused by an objective onto the fiber optic into the spectrograph and CCD camera.

For broadband excitation of approximately 300 cm^{-1} region, the narrow band pump and broadband Stokes beams were centered at $\lambda_P=720$ nm and $\lambda_S=909$ nm to excite the aliphatic CH_2 and CH_3 region between -2800 to -3000 cm^{-1} , and tuned to $\lambda_P=785$ nm and $\lambda_S=850$ nm to excite the aromatic ring stretches region near -1000 cm^{-1} . The pump and Stokes laser powers ranged between 20-30 mW and 10-20 mW, respectively. Kinetic series of polymer bead samples were collected at 10, 25, and 50 ms acquisitions resulting in a cycle time of 25.32, 40.31, and 65.31 ms for each spectrum. The length of the series varied from 500 to 3000 spectra with a duration of 20 seconds to 2 minutes. A total of 913 kinetic series of polymer bead samples were collected and data analysis was performed in Matlab (Mathworks) and Igor Pro (Wavemetrics).

For supercontinuum excitation over a 1600 cm^{-1} range, the broadband Stokes beam centered at $\lambda_S=800\text{ nm}$ was coupled into a 12-cm photonic crystal fiber (NKT Photonics, Femto White 800) to generate a femtosecond pulse spanning 400 nm to 1100 nm. The supercontinuum filtered wavelengths of $\lambda_{SC}=785\text{--}945\text{ nm}$ and pump beam centered at $\lambda_P=720\text{ nm}$ gave rise to CARS signal from -1800 to -3400 cm^{-1} . The spectral range is limited by the grating and size of the CCD camera chip. The pump beam and supercontinuum laser powers were 30-40 mW and 4-5 mW, respectively. Kinetic series of lipid vesicle samples were collected at 50 ms acquisitions for 5000 spectra. A total of 112 kinetic series were recorded.

Sample Preparation

Polymer bead samples were prepared by diluting 100 nm, 300 nm, and $1.10\text{ }\mu\text{m}$ diameter polystyrene bead solutions (PSB, Sigma-Aldrich, 10% solid in aqueous suspension) with ultrapure water ($18.2\text{ M}\Omega$, Barnsted Nanopure). Diluted samples were sonicated for 20 minutes to prevent any bead aggregation prior to each experiment. A $100\text{ }\mu\text{L}$ drop of 0.01% wt solution was placed in a polydimethylsiloxane (PDMS, Ellsworth Adhesives) well with a height of 0.8 mm and diameter of 20 mm adhered to a glass slide. An air objective was used for measurements observed at the air/solution and glass/solution interfaces and in bulk. A coverslip was placed on the PDMS well when using the oil immersion objective to excite particles at the glass/solution interface. Data for all kinetic series were collected in the forward direction.

Multilamellar vesicles (MLV) of varying sizes were prepared by rehydrating 10 mg of dry 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, Inc.) in 1 mL of ultrapure water, resulting in a 1% wt. lipid suspension. The lipid suspension was subjected to 5 cycles of heating/cooling, repeatedly heated above the phase transition point, 41°C , vortexed, and cooled on ice. Similarly, 10 mg of dissolved 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Inc.) was dried and rehydrated in a similar manner to form a 1% wt. solution of multilamellar vesicles. Diluted 0.05% wt. DOPC and 0.02% wt. DPPC were independently sized using a Zetasizer Nano Series (Malvern) in a backscattering configuration. Individual sizing runs consisted of 13 measurements with an attenuation factor of 7. The MLV sizes were averaged from 3 different runs of each sample. Aliquots from each 1% wt. MLV solution were combined and diluted to form a 0.2% wt. DOPC and 0.2% wt. DPPC mixture. Multiplex CARS-CS measurements were taken of the individual lipid vesicle solutions and mixture using an oil immersion objective with a coverslip on a PDMS well.

Single Particle Tracking

Single particle tracking (SPT) experiments were performed on $1.10\text{ }\mu\text{m}$ diameter polystyrene bead samples prepared under similar conditions. An Olympus BX51 microscope with a wide-field air objective (Olympus LM Plan FLN 0.5 NA) was used for focusing the excitation light onto the solution and collecting the darkfield scattered light, which was directed onto a CMOS camera (Hamamatsu C11440). Darkfield time-lapse videos were recorded for 2 minutes each with a cycle time of 50 ms acquisition per frame, resulting in a total of 2399 frames per video. The z-axis of the sample stage was adjusted to focus at the

air/solution interface, into the bulk, and at the glass/solution interface at the bottom of the PDMS well. A total of 140 single particle trajectories from 10 different videos were calculated using Nikon NIS Elements Advanced Research software.

III. Results

Multiplex CARS correlation spectroscopy has the ability to detect the fluctuations of intrinsic vibrational signal of particles diffusing through the excitation volume. Figure 1 displays the recorded CARS signal of 1.10 μm polystyrene beads (PSB) in solution undergoing Brownian motion in a PDMS well. In Figure 1A, the excitation volume is focused at the glass/solution interface and 2000 consecutive CARS spectra are collected with a 50 ms acquisition time. The multiplex CARS spectra in Figure 1B clearly shows the fluctuating vibrational signal of the -1000 cm^{-1} aromatic ring stretch from the PSB with a consistent nonresonant background attributed to the glass interface. Alternatively, Figure 1C and 1D depict a kinetic series measured in the bulk solution, in which the fluctuating signal arises from the -2850 cm^{-1} band of PSB with minimal nonresonant background. The emitted CARS signal is directionally dependent on the size and shape of the analyte due to the summation of coherently induced dipoles, and scales quadratically to analyte

concentration, $I_{\text{CARS}} \propto \chi^{(3)2} I_p^2 I_s$.^{23, 28} When the relative diameter of a spherical analyte is $d > 0.3\lambda_p$, the CARS signal radiates predominately in the forward direction.⁴¹ For this reason, kinetic series of 1.10 μm PSB and 300 nm PSB samples were collected in the forward-direction. Although 100 nm PSB had a diameter much smaller in comparison to the pump wavelength, $d < 0.3\lambda_p$, there was sufficient CARS intensity generated and collected in the forward direction in order to maintain the continuity of the data analysis for all experiments.

Extensive work has been done to describe CARS intensity autocorrelation functions for CARS correlation spectroscopy.³³⁻³⁴ To gain a better understanding of the multiplex CARS signal in Figure 1, we implemented the forward-detected CARS-CS autocorrelation function as $G(t)$ in Equation 1 to characterize freely diffusing species in a three-dimensional (3D) trajectory.³³ Equation 1 correlates the signal intensity at a given time, $I(t)$, to a signal intensity at some time delay τ , represented by $I(t+\tau)$.

$$G(t) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2} = \frac{1}{[N]} \left(\frac{1}{1 + \frac{t}{\tau_D}} \right) \left(\frac{1}{\sqrt{1 + \frac{\left(\frac{r_0}{\sqrt{2}}\right)^2 t}{\left(\frac{z_0}{\sqrt{2}}\right)^2 \tau_D}}} \right) \quad \text{Eq. 1}$$

Analogous to fluorescence correlation spectroscopy analysis, the first term defines the inverse of the average number of particles in the focal volume, $N = c \cdot V_{\text{eff}}$. The second term in Eq. 1 details the hyperbolic decay of the autocorrelation function corresponding to the Gaussian profile of the excitation beam,⁹ and the third term relates the decay time, τ_D , to the effective excitation volume and optical resolution of the instrument.¹⁰ The coherent properties of CARS are accounted for in the aspect ratio of its lateral and axial dimensions,

r_0 and z_0 , which represents the cylindrical shape of the excitation volume. In order to acquire a precise autocorrelation function, the lateral resolution of our CARS microscope was determined by imaging 300 nm diameter polystyrene beads (PSB) dried on a glass slide, see Supporting Information Figure S-1. By plotting the intensity profile of the bead and fitting the full width at half maximum (FWHM), the effective focal radius, r_0 , was obtained by Eq. 2.

$$r_0 = \frac{\text{fwhm}_{r(\text{avg})}}{\sqrt{\ln 2}} \quad \text{Eq. 2}$$

Similarly, CARS spectra were collected at a fixed region on a thin polystyrene film spin coated on a glass slide while changing the z-axis of the stage, which produced a depth profile to determine the axial resolution, z_0 , in Eq. 3:

$$z_0 = \frac{\text{fwhm}_{z(\text{avg})}}{\sqrt{\ln 2}} \quad \text{Eq. 3}$$

As a result, the effective focal volume, V_{eff} , for forward-detected CARS-CS was calculated by Eq. 4:³³

$$V_{\text{eff}} = \pi^{\frac{3}{2}} \left(\frac{r_0}{\sqrt{2}} \right)^2 \left(\frac{z_0}{\sqrt{2}} \right) \quad \text{Eq. 4}$$

The optical resolution for each objective is summarized in Supporting Information Table S-1, which are the coefficients, r_0 , z_0 , and V_{eff} , used in the autocorrelation fit in Eq. 1 for each corresponding kinetic series.

To account for the resonant and nonresonant contributions observed in a multiplex CARS measurement, the data was analyzed as follows. First, the individual CARS spectra were examined in each kinetic series. Figure 2 displays representative data analysis for 0.01 wt% 100 nm, 300 nm, and 1.10 μm diameter polystyrene beads diffusing at the glass/solution interface. In Figure 2, the data shown were collected under identical experimental conditions (0.9 NA air objective, 50 ms acquisition time per spectrum). Figure 2A and 2B show multiplex CARS spectra of the aliphatic CH_2 region for 100 nm PSB, and the aromatic ring stretch for 1.10 μm PSB, respectively. At the glass/solution interface, a significant nonresonant background was observed, evidently arising from the glass surface and surrounding medium. The minimum CARS spectrum containing the nonresonant background without the presence of a vibrational signal from a polystyrene bead was used as a reference to normalize, by division, all the collected CARS spectra within the same kinetic series. Nonresonant background was either not observed or insignificant in the CARS spectra measured at the air/solution interface and in bulk solution such that the kinetic series was analyzed without normalizing by a reference. To examine the correlation between the

signals observed in the multiplex measurements in Figure 1B and Figure 2B, a Pearson's r cross-correlation analysis was performed as described in Eq. 5.⁴²

$$r = \frac{\sum_m \sum_n (A_{mn} - \bar{A})(B_{mn} - \bar{B})}{\sqrt{\sum_m \sum_n (A_{mn} - \bar{A})^2} \sqrt{\sum_m \sum_n (B_{mn} - \bar{B})^2}} \quad \text{Eq. 5}$$

In this case, A and B are the values of two individual intensity profiles in a given kinetic series, and $\bar{A}$ and $\bar{B}$ represent the mean intensities of those profiles. The cross-correlation coefficient, r , can distinguish between CARS intensity profiles bearing a statistically significant correlation versus uncorrelated noise. We compared the intensity profiles of the CARS resonant signal at -1000 cm^{-1} and the nonresonant shoulder at -975 cm^{-1} , which resulted in an average cross-correlation coefficient of $r > 0.92$. Additionally, we compared the weak resonant vibrational band at -1035 cm^{-1} , also an aromatic ring stretch, to the strong resonant signal at -1000 cm^{-1} and obtained an average Pearson's value of $r > 0.80$. The strong correlation between the intensity profiles of the nonresonant signal and two vibrational bands at -1035 cm^{-1} and -1000 cm^{-1} indicate that the fluctuations in signal arise from the same particles moving through the focal volume.

The intensity profile associated with vibrational resonances in each kinetic series was determined for the three different particle sizes as shown in Figure 2C. For CARS signal observed at the aliphatic CH_2 region, a Gaussian function was fit to the vibrational band centered at -2850 cm^{-1} to obtain a FWHM of 10 cm^{-1} . To remove any mechanical baseline drift, the trapezoid method was used to integrate the CARS signal over the FWHM of the CH_2 stretch from -2845 to -2855 cm^{-1} and a spectral baseline region from -3300 to -3310 cm^{-1} , such that the difference results in the fluctuating intensity due to changes in the number density of molecules diffusing through the effective excitation volume. Likewise, the aromatic ring band at -1000 cm^{-1} was integrated over -995 to -1005 cm^{-1} with a corresponding spectral baseline region from -1400 to -1410 cm^{-1} to produce a similar intensity profile. Noise originating from the timing jitter of the electronically mode-locked lasers was minimized by implementing a threshold of the lower confidence interval,

$LCI = \bar{x} - 2\sigma_x$, of the mean integrated intensity and standard deviation within a kinetic series.

Based on the CARS intensity profiles, autocorrelation curves were generated in Matlab.⁴³ The experimental autocorrelation curves were fit with the forward-detected CARS-CS autocorrelation function, Eq. 1, to obtain dynamic information of the freely diffusing particles. The results are illustrated in Figure 2D for 100 nm, 300 nm, and $1.10 \mu\text{m}$ PSB samples, which exhibit the expected trend of slower decay times, τ_D , for larger particles.

The diffusion coefficients for the polystyrene beads freely circulating in solution were calculated with Eq. 6:³³

$$D = \frac{\left(\frac{r_0}{\sqrt{2}}\right)^2}{4\tau_D} \quad \text{Eq. 6}$$

using the τ_D determined from the fit to Eq. 1 and focal radius of each experiment. The mean radius of the three polystyrene bead solutions were measured by dynamic light scattering and used to calculate the expected Stokes-Einstein diffusion coefficients for comparison with our experimental values.¹⁷ The Stokes-Einstein relation in Eq. 7:

$$D = \frac{k_B T}{6\pi\eta r} \quad \text{Eq. 7}$$

utilizes the Boltzmann constant (k_B), temperature (T), viscosity of the sample medium (η), and particle radius (r) to describe the diffusion coefficient for a particle undergoing Brownian motion through the excitation volume. Spectra were acquired at 10, 25, and 50 ms to distinguish the temporal resolution of the multiplex CARS measurement and CCD camera relative to experimental parameters, such as laser power, excitation objective, and analyte size and concentration. The mean diffusion coefficients and standard deviation of the mean for the three particle sizes at the glass/solution interface are depicted in Figure 3 and summarized in Table 1. Diffusion coefficients for 300 nm and 1.10 μm PSB are mean values from multiple kinetic series collected at 10, 25, and 50 ms acquisitions, such that the varying cycle times attained similar τ_D for the given bead size. However, the mean diffusion coefficient for 100 nm PSB is the combination of kinetic series acquired at 25 and 50 ms per spectrum. The 100 nm PSB did not generate an appreciable signal with 10 ms acquisitions due to the low concentration and small size of the particles.

In addition to single particle diffusion, various anomalies, such as clustered diffusion was also detected. For example, Figure 4A and 4B display the normalized maximum CARS spectra and intensity profiles for a single 1.10 μm PSB and randomly oriented aggregate. From the autocorrelation fit in Figure 4C, the concentration coefficient, $[c]$, can ascertain the number of beads in the cluster. In this case, $c_{\text{aggregate}} = 3.65 \times 10^{12}$ and $c_{\text{single}} = 1.18 \times 10^{12}$, such that the ratio of this parameter results in a cluster of approximately 3 polystyrene beads. This agrees with the maximum peak area in Figure 4A and 4B, which is 9.3 times higher in the aggregate kinetic series in comparison to the single particle kinetic series.

We employed multiplex CARS-CS to monitor diffusion dynamics of PSB samples at two different interfaces and in the bulk medium illustrated in the sample diagram in Supporting Information Figure S-2. For measurements at the air/solution interface, the effective excitation volume was positioned on the surface of the polystyrene bead solution within the PDMS well. For bulk solution measurements, the effective excitation volume was focused approximately 100 μm below the solution surface. Alternatively, glass/solution interfacial measurements were done at the bottom of the PDMS well on the surface of the glass slide, or on the top of the PDMS well at the surface of the glass coverslip. The diffusion coefficients for 300 nm and 1.10 μm polystyrene beads at the air/solution, glass/solution

interfaces and in the bulk medium are listed in Supporting Information Table S-2 and presented in Figure 5A and 5B, respectively. The observed diffusion coefficients at the air/solution interface were slow relative to the Stokes-Einstein prediction, while the bulk and glass/solution interfaces are within error of the predicted value.

To validate the diffusion behavior observed by CARS-CS, control experiments using wide-field single particle tracking experiments were performed. Single particle trajectories of 1.10 μm polystyrene beads were calculated from darkfield time-lapse videos. Mean square displacement analysis determined the diffusion coefficient, D , of individual particles in solution by Eq. 8:⁴⁴

$$D = \frac{\langle r^2 \rangle}{6t} \quad \text{Eq. 8}$$

where r is the displacement and t is the time interval describing the particle movement between each frame. In this case, the mean square displacement is an average of every 10 frames in a trajectory and the time interval is 50 ms cycle time per SPT image. All calculated diffusion coefficients at the three sample configurations are depicted as histograms in Figure 5D-F. Two distinct subpopulations of slow and fast moving particles are observed at the air/solution and glass/solution interfaces, whereas only one population of fast diffusion is seen in bulk solution. A Gaussian distribution was fit to each subpopulation to obtain the mean diffusion coefficients for both slow and fast trajectories. The weighted mean diffusion coefficient for each histogram is plotted in Figure 5C and compared to the diffusion coefficients obtained by multiplex CARS-CS and Stokes-Einstein prediction.

To demonstrate the chemical specificity of CARS-CS, we further measured the diffusion of individual DPPC and DOPC multilamellar vesicles in a mixture. The structures of these lipids are depicted in Figure 6A, in which DPPC and DOPC differ in length by only two carbon atoms with an unsaturated C=C bond. In Figure 6B, the CARS spectra of DPPC and DOPC vesicles have vibrational modes at -2850 cm^{-1} and -2930 cm^{-1} corresponding to CH_2 in the acyl chain and CH_3 in the lipid head group, respectively, with similar nonresonant background from the spectral structure of the supercontinuum excitation source. The observed peak at -3015 cm^{-1} is indicative of the CH vibrational stretch on the C=C bond in DOPC, which is not present in the DPPC spectrum. A multivariate curve resolution (MCR) model composed of a data set containing 60 CARS spectra of DOPC, DPPC, and supercontinuum background signal generated 3 components displayed in Figure 6C. Component 1 (15.04%) is representative of the CH_2 and CH_3 stretches in DPPC, whereas Component 2 (12.46%) has overlapping spectral features and contains the additional CH stretch attributed to DOPC. Component 3 (72.50%) contains the largest percent variance captured by the model, and is attributed to the supercontinuum background evident in every spectrum.

CARS-CS kinetic series of a mixture with equal concentrations of DPPC and DOPC vesicles is depicted in Figure 7A. The MCR model is capable of distinguishing which multiplex CARS spectra contain vibrational signals of DPPC and DOPC by one vibrational peak

indicated in Figure 7B. Even though both MLVs give rise to the -2850 cm^{-1} signal in the intensity profile in Figure 7C, the contributions of each DPPC and DOPC can be separated by the MCR scores of Component 1 and Component 2, respectively, as shown in Figure 7D. The autocorrelation curves calculated from these MCR scores describe the individual diffusion dynamics of DPPC and DOPC vesicles in the mixture in Figure 7E. Control experiments were performed using dynamic light scattering (DLS). The diffusion coefficient for DOPC ($D_{\text{DOPC}}=4.56\times 10^{-9}\text{ cm}^2/\text{s}$) has a mobility consistent with the DOPC vesicle sizes of approximately $1.61\pm 1.35\text{ }\mu\text{m}$ in diameter, measured by DLS and displayed in Supporting Information Figure S-3. The decay time observed for DPPC is slower, as expected, due to the polydispersity of the lipid solution, as measured by DLS, with multilamellar vesicles ranging from $1.2 - 5.5\text{ }\mu\text{m}$ in diameter.

IV. Discussion

Here we have demonstrated the value of obtaining a multiplex CARS spectrum for monitoring the diffusion of chemically distinct particles at different interfaces. Previous CARS-CS studies using an avalanche photodiode detector and hardware correlator established single-frequency CARS correlation spectroscopy as a valid method to determine the sizes of different particles and viscosities of various sample media.³³⁻³⁴ Similar to reports of single frequency CARS-CS,³³⁻³⁴ this multiplex CARS-CS approach is able to accurately measure the diffusion of particles. The results in Table 1 and Figure 3 verify the agreement between our results and that expected from the Stokes-Einstein prediction. The relative error for each PSB size is 10%, or less, which appears to arise from the heterogeneity of sizes within the polystyrene bead samples. In correlation analysis, diffusion coefficients within 10% error for a single freely diffusing species in solution is an acceptable comparison to Stoke-Einstein values.⁴⁵ However, by collecting a multiplex CARS spectrum, such as Figure 1 and Figure 7, there is the potential to obtain more chemical information from the monitored analyte and its surrounding environment. It may also be possible to monitor competition between different particles for interfacial sites. The direct measurement of spectral changes in the multiplex CARS signal can provide insight into the changes of chemical composition and molecular interactions between multiple species interacting within a given system.

The observed signals provide a quantitative measurement of the different particles in the observation area. In contrast to SERS, which has a strong surface selection that complicates quantification, CARS signals are proportional to the number of analyte molecules interrogated. By integrating over the FWHM of the vibrational band of interest and applying a spectral baseline correction and lower confidence bound threshold, the observed CARS

intensity profiles are quantitatively consistent with the expected change, $I_{\text{CARS}} \propto \chi^{(3)^2} I_{\text{p}}^2 I_{\text{s}}$, in the number of particles diffusing through the focal volume. The concentration coefficient attained from the autocorrelation function can further distinguish between single particle and aggregate diffusion and quantify the number of particles within a given cluster shown in Figure 4. The maximum CARS intensity of the aggregate scaled quadratically to the estimated concentration of particles in the excitation volume.

Numerous studies have been conducted towards understanding the diffusion dynamics and chemical interactions at the air/liquid and liquid/solid interfaces.^{37, 46-47} Here, we utilize multiplex CARS correlation spectroscopy to monitor particle diffusion at the air/solution, glass/solution interface and in bulk solution. For both multiplex CARS-CS and SPT measurements, the polystyrene beads diffuse fastest in the bulk medium, which is supported by the Stokes-Einstein relation of free Brownian motion unhindered by any interfacial boundary. In Figure 5, we expected the diffusion coefficients at the glass/solution interface to be marginally slower due to the increased drag force and particle interactions with the glass surface, resulting in restricted diffusion.⁴⁸ However, shown in Figure 5D-5F, single particle tracking is able to distinguish different modes of motion in two subpopulations of slow diffusion in the presence of obstacles, such as the solid interface, and fast pure diffusion, comparable to that bulk diffusion. This indicates that the multiplex CARS-CS measurement is biased toward faster moving particles at the liquid/solid interface.

Studies have shown polymer diffusion at the air/solution interface under different solution conditions.⁴⁹⁻⁵¹ For both 300 nm and 1.10 μm PSB in Figure 5A-B, the rate of diffusion is slowest at the air/solution interface. Our results are agreeable, in which the measured diffusion coefficients are relatively 1.5 to 2 times slower at the air/solution interface in comparison to bulk measurements as a result of air pressure, surface tension, and potential capillary forces inhibiting the mobility of particles at that interface. Alternatively, SPT results observe a predominant subpopulation of hindered diffusion, similar to that of multiplex CARS-CS. The single particle tracking measurements again identified two subpopulations of particle movement; however, the CARS-CS result was more consistent with the slower moving, interfacial population.

The bias observed in the CARS-CS, toward the solution species at the solid/liquid interface and the interfacial species at the air/liquid interface, suggests that the finer spatial resolution associated with the focal volume in CARS imaging selects for the first interface. Refraction effects are known to alter focusing parameters and may erode the sensitivity of CARS into sub-interfacial regions.⁵²

Multiplex CARS-CS also sacrifices peak power to gain more chemical information. The CARS intensity is driven by the quadratic dependence of the pump power, such that the linear dependence of the Stokes beam does not diminish the signal to noise when using a weaker supercontinuum excitation source. In Figure 6 and Figure 7, the micron-size MLVs generate resonant vibrational signal discernible from the broader nonresonant background from the sample medium and supercontinuum. Without preprocessing the CARS spectra, a multivariate curve resolution model can discriminate between two similar lipids in a mixture. The unsaturated DOPC chain has a CH bond on the C=C bond, giving rise to a vibrational signal near -3015 cm^{-1} that is not observed in the saturated DPPC spectrum in Figure 7B. Based on this spectral information, MCR analysis can elucidate the dynamic contributions of DOPC and DPPC diffusing through the focal volume as demonstrated in Figure 7C-7E.

In regards to the temporal resolution of a CCD camera compared to a conventional avalanche photodiode, it is difficult to observe sub-millisecond events due to the limited

acquisition rate per spectrum. In Figure 5A and 5B, the relative error for the mean diffusion coefficients for all three sample configurations is approximately twice as large for the 300 nm PSB in comparison to the 1.10 μm . This is a result of the CARS signal dependency on the molecular susceptibility of the analyte, such that dilute concentrations and smaller sized particles present challenges. With our given setup, analysis of PSB smaller than 100 nm in water is not possible; however, other small particles with larger susceptibilities could still be detected. Importantly, multiplex CARS correlation spectroscopy can be an advantageous method to discern chemical interactions for slower diffusing mechanisms such as molecular transport through cellular membranes.⁵³ There is a growing interest to investigate the nano/bio interface to understand the diffusion dynamics, and chemical and biological effects of nanoparticles in cells.^{46, 54-56} Inherently, CARS is a four-wave mixing processes that is sensitive to the large susceptibility of metal nanoparticles,⁵⁷⁻⁶⁰ and may prove to be a valuable tool for observing molecular interactions of functionalized nanoparticles in biological systems.

V. Conclusions

Multiplex CARS-CS has sufficient signal to noise to identify and monitor the diffusion of particles in solution, and at multiple interfaces, on the millisecond time scale. Multiplex CARS-CS also has the ability to obtain particle sizing and three dimensional diffusion parameters for simple systems, analogous to dynamic light scattering, fluorescence correlation spectroscopy, and nonlinear correlation spectroscopy measurements; however, it also retrieves chemically specific information in a label-free, unperturbed manner. The trade-off between spectral density for chemical information is still sufficient to monitor the diffusive motion of a 100 nm nonresonant polymer particle in aqueous solution. Our results indicate that this analysis becomes signal to noise limited for fast moving, and low signal, events that require sub-millisecond temporal resolution. Preliminary work on multilamellar vesicles performed with a supercontinuum excitation source as the Stokes beam to generate a broader spectral range provides chemical information that suggests straight-forward cross-correlation analysis. This will allow for the examination of multiple vibrational bands from different interacting analytes and its surroundings within a kinetic series by CARS cross-correlation spectroscopy, which has yet to be done. The increased spatial resolution of a nonlinear focus suggests that multiplex CARS-CS has utility for investigating particle diffusion in a wide variety of environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Examples of 1.10 μm PSB diffusing through the focal volume at (A, B) the glass/solution interface and in (C, D) bulk solution. (A) The CARS heat map represents a kinetic series containing 2000 spectra collected at 50 ms acquisitions over 130.62 s. (B) The CARS spectra from the highlighted section in (A) depicts 40 spectra over 2.61 s with vibrational signal at -1000 cm^{-1} and -1035 cm^{-1} and broadband nonresonant background. (C) The CARS heat map of 1000 spectra recorded at 50 ms has corresponding (D) CARS spectra from the highlighted section in (C) with fluctuating signal at -2850 cm^{-1} from the polystyrene beads.

Figure 2.

Example of data analysis performed for 100 nm (blue), 300 nm (green), and 1.10 μm (red) diameter polystyrene beads. (A) The normalized CARS spectra are shown for the integrated signal at -2850 cm^{-1} from a 100 nm PSB kinetic series and (B) at -1000 cm^{-1} from a 1.10 μm PSB kinetic series. Experimental parameters are: Pump=30 mW, Stokes=15 mW, 50 ms acquisitions at the glass/solution interface. (C) Plots of integrated intensity over time can be characterized by (D) autocorrelation curves (dotted lines) and fits (solid lines) for all three particle sizes.

Figure 3.

Plot of mean diffusion coefficients for 100 nm, 300 nm, and 1.10 μm diameter PSB at the glass/solution interface and theoretical Stokes-Einstein diffusion coefficients (red line) are shown. Error bars represent mean standard deviation for each PSB radius and diffusion coefficient with a relative error of 7%, 10%, and 4%, respectively, compared to Stokes-Einstein prediction

Figure 4.

(A) The maximum CARS spectra at $t=98.6181$ s for single $1.10\ \mu\text{m}$ PSB and at $t=74.3228$ s for a randomly oriented aggregate are shown from (B) intensity profiles representative of single particle (black) and aggregate (red) diffusion. (C) The autocorrelation curve (dotted line) and fit (solid line) for each kinetic series illustrates the slower decay time for clustered particles.

Figure 5.

Plots of mean diffusion coefficients for (A) 300 nm and (B) 1.10 μm diameter polystyrene beads observed at the air/solution and glass/solution interfaces and in bulk solution. (C) This plot compares the mean diffusion coefficients measured via multiplex CARS-CS (black square) and single particle tracking analysis. The red line indicates the Stokes-Einstein predicted diffusion coefficient for 1.10 μm PSB. For the multiplex CARS-CS values, the error bars represent the mean standard deviation for each diffusion coefficient. The individual error markers for the particle tracking values represent the mean standard deviation for the slow (downward triangle) and the fast (upward triangle) diffusion, and the error bars represent the mean standard deviation from all measurements in each histogram in (D, E, F). Histograms of measured diffusion coefficients are shown for the two interfaces and bulk solution via mean square displacement analysis of single particle trajectories of 1.10 μm polystyrene beads. (D) The air/solution ($n_s=456$, $D_s=3.6\pm0.7\times10^{-9}$, $n_f=142$, $D_f=5.0\pm0.4\times10^{-9}$) and (F) glass/solution histograms ($n_s=1932$, $D_s=2.8\pm0.8\times10^{-9}$, $n_f=401$, $D_f=4.8\pm0.7\times10^{-9}$) have two Gaussian peak fits to discriminate subpopulations of slow (s) and fast (f) moving particles, whereas only one population of fast diffusion is observed in the (E) bulk histogram ($n_f=365$, $D_f=4.9\pm1.2\times10^{-9}$).

Figure 6.

(A) The structural formula for the saturated DPPC and unsaturated DOPC are shown. (B) The CARS spectra of pure DPPC and pure DOPC have vibrational stretches at -2850 cm^{-1} and -2930 cm^{-1} corresponding to CH_2 in the acyl chain and CH_3 in the lipid head group, respectively, with similar nonresonant background from the supercontinuum. The isolated region centered at -3015 cm^{-1} is indicative of the CH vibrational band on the unsaturated $\text{C}=\text{C}$ bond observed in DOPC, which is not present in DPPC. (C) Multivariate curve resolution analysis of a data set containing CARS spectra of pure DOPC, pure DPPC, and background references composed a model with 3 components accounting for 100% cumulative variance.

Figure 7.

(A) CARS-CS kinetic series of a mixture of DOPC and DPPC were collected at 50 ms acquisitions for 5000 spectra. (B) CARS spectra of DOPC (red) at $t=30.630$ s and DPPC (blue) at $t=33.504$ s were ascertained from the MCR model in (D). (C) The intensity profile of -2850 cm^{-1} vibrational band was separated into contributions of DOPC and DPPC represented in (D) MCR scores of Comp. 1 (blue) and Comp. 2 (red). (E) The autocorrelation curves (dotted line) and fits (solid line) describe the diffusion of DOPC ($\tau_{D,DOPC}=0.008$ s) and DPPC ($\tau_{D,DPPC}=0.372$ s) vesicles through the excitation volume.

Table 1

Mean diffusion coefficients for 100 nm, 300 nm, and 1.10 μm diameter PSB single particle diffusion with theoretical Stokes-Einstein relation.

Mean PSB Radius (nm)	Mean Diffusion Coefficient (cm^2/s)	Stokes-Einstein (cm^2/s)
53.34 ± 5.59	$3.38 \pm 0.54 \times 10^{-8}$	3.65×10^{-8}
218.97 ± 21.09	$7.98 \pm 1.43 \times 10^{-9}$	8.91×10^{-9}
433.14 ± 77.41	$4.23 \pm 0.91 \times 10^{-9}$	4.40×10^{-9}