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Microfluorometric technique for the determination of localized heating in organic particles

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We describe a novel microfluorometric technique, based on the temperature-dependent fluorescence emission from single dye-labeled phospholipid vesicles, for the determination of localized heating effects. An increase in sample temperature results in a red shifting of the probe fluorescence spectrum. As individually calibrated microthermometers, fluorescent liposomes exhibit a temperature sensitivity of ~0.1 °C in the vicinity of the bilayer phase transition temperature. Through modification of the bilayer components, both the sensitivity and operating temperature range of these microthermometers can be controlled. Micron spatial resolution is achieved at a signal-to-noise ratio in excess of 10^3:1. We use the above technique, for the first time, to determine localized heating effects induced by a laser beam focused to its near-diffraction limited spot size. At the laser wavelength of λ=1.064 μm, a temperature change of 1.1 °C/100mW in 10-μm-diam organic liposomes is reported. Implications for the real-time optical monitoring of temperature in biological systems are discussed.

To date, a number of probe-based[1] and optical diagnostic techniques[2–5] have been developed, including laser-induced fluorescence and Raman spectroscopy,[2–5] photothermal absorption spectroscopy,[6] and Zeeman interferometry,[7] for the determination of temperature changes and heating effects in dielectric and organic samples. These techniques are often limited by poor spatial, temporal, or thermal resolution, and are not easily adapted to biological systems for *in situ* microthermometric measurements. Herein, we report the development of a technique for the determination of localized heating and temperature effects in organically engineered liposome particles, using temperature-dependent fluorescence emission spectroscopy. The microfluorometric technique described herein is noninvasive, and offers the additional advantages of high-temperature sensitivity (0.1 °C), submicron spatial resolution, a large signal-to-noise ratio (>10^3:1), and broad applicability to a variety of dielectric and biological samples. The technique is applied, for the first time, to the measurement of temperature changes associated with the irradiation of single, micron-sized organic liposomes by an infrared (Nd:YAG) laser.

The mechanism for sensing localized temperature changes is based upon the fluorescence emission derived from an environmentally sensitive fluorophore, Laurdan (6-dodecanoyl-2-dimethylaminon-aphthalene), embedded within the organic phospholipid bilayer DPPC (dipalmitoylphosphatidyl choline) of a liposome. The temperature sensitivity of Laurdan in phospholipid (PL) vesicles, and the study of PL relaxation dynamics, was first described by Parasassi *et al.*[8,9] Liposomes are spherical organic vesicles consisting of an aqueous core and a single- or multi-lamellar organic lipid bilayer shell that resembles the real bilayer membrane of most biological cells and organisms.[10–12] Multi-lamellar vesicles (MLVs) typically range in size from 0.1 to 20 μm in diameter. Single-lamellar vesicles (SLVs) possess a single 5-nm-thick membrane bilayer, and can be fabricated to similar dimensions. When liposomes are heated, the bilayer undergoes a phase transition from a gel to liquid-crystalline state, thereby altering the membrane permeability to water. Since the Laurdan dye incorporated in the bilayer is highly sensitive to the dipolar relaxation of the surrounding medium, a large temperature-dependent Stokes shift (>40 nm) is observed as the probe microenvironment changes. Hence, small temperature changes can be detected with great sensitivity in the region of the bilayer phase transition temperature (T_t). An optical technique is therefore employed to monitor temperature variations, or to produce thermal maps, with submicron spatial resolution.

Fluorescence detection is accomplished by using a charge-coupled device (CCD) fluorescence detection system within an optical microscope geometry, as shown in Fig. 1. Multilamellar vesicles (~10 μm) are first prepared according to the method of New *et al.*, [11] impregnated with the Laurdan

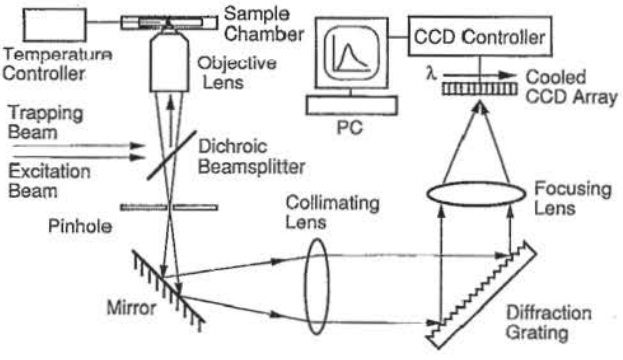

FIG. 1. Schematic diagram of a fluorescence emission microscope, in a confocal configuration, and its spectral analysis system.

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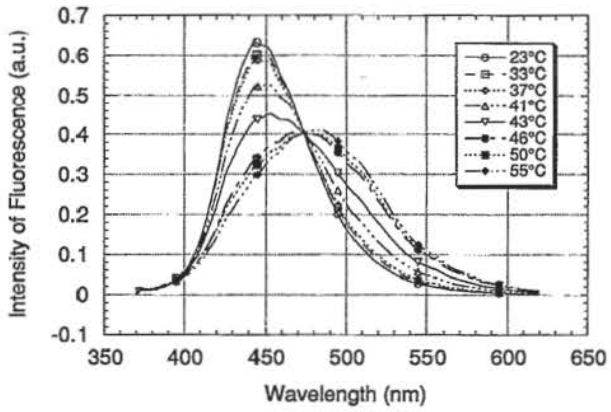

FIG. 2. Temperature-dependent emission spectra from a 10-μm-diam liposome. The emission red shifts, by more than 40 nm, above the liposome phase transition temperature (42 °C).

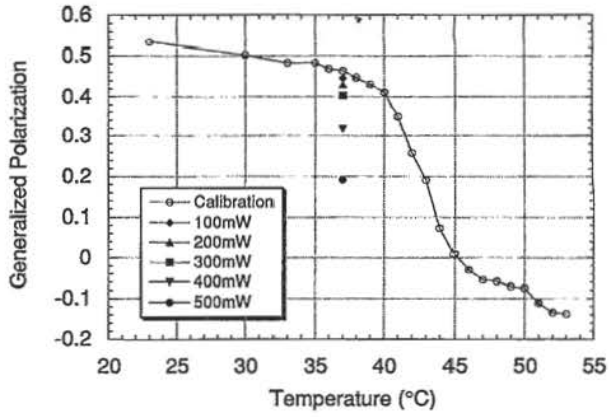

FIG. 3. Dependence of generalized polarization (GP) on sample temperature, as determined from the fluorescence emission curves. $\Delta(GP)/\Delta T$ is equal to 0.092/°C at 42 °C for the pure DPPC bilayer. The change in GP as a function of incident laser power is also shown. The initial setpoint temperature of the DPPC liposome, prior to laser exposure, was 37 °C.

dye,[8,9] and then suspended in a phosphate buffered saline (PBS) solution within a custom-built chamber. A 365-nm UV excitation beam derived from a mercury arc lamp is then focused by a microscope objective lens onto the liposome sample. The fluorescence emission is collected by the same objective lens, and passed through an adjustable pinhole located at the image plane of the microscope objective. The light is then dispersed by a 300 g/mm grating and focused onto an electrically cooled CCD camera. For sample sizes in the 2–15 μm size range, the detected fluorescence signal-to-noise ratio (S/N) is greater than $\sim 10^3$:1. Optical spectra are recorded and analyzed with a personal computer. Here, the combination of an objective lens and a pinhole creates a confocal microscope geometry that permits the measurement of fluorescence spectra only from localized, micron-sized regions of the sample. It can also facilitate the mapping of temperature variations across the sample, via pinhole translation. The combined use of the liposome with the designated spectral analysis system therefore facilitates a high sensitivity, microthermometric measurement. For calibration purposes, a thermocouple was placed at the center of the sample chamber in order to monitor the background solvent solution temperature. This temperature is electrically controlled by a heating coil that is embedded within the chamber. However, due to the possible existence of temperature gradients inside the chamber, the location of the liposome to be calibrated was chosen to be very close (20–100 μm) to the tip of the thermocouple in order to minimize calibration errors.

The results of measuring fluorescence spectra from UV-excited Laurdan-doped DPPC liposomes immersed in a water-based PBS solution at different temperatures are shown in Fig. 2. All spectra have been corrected for instrument response. When the solution temperature is set at 23 °C, for example, a temperature which is well below the liposome phase transition temperature T_t of 42 °C, the entire liposome membrane exists in a pure gel phase. Here, the fluorescence emission exhibits a peak at 440 nm, and has a full width half-maximum (FWHM) of ~60 nm. A nearly identical emission spectrum is also obtained at 33 °C, which is still below T_t. As the temperature is increased from 33° to 43°, the peak of the fluorescence emission red shifts. This shift directly corresponds to the phase transition occurring within the sample membrane region. At 55 °C, which is well above T_t, the phospholipid membrane has converted into a pure liquid crystalline state, and displays its peak fluorescence at 485 nm. For temperatures in the vicinity of T_t, phase coexistence and partial conversion between the gel and liquid-crystalline phases result in a fluorescence spectrum that represents an admixture from the two different phases, and produces a spectral shift ratio that is a maximum.

To quantify the spectral shift with temperature, the "generalized polarization" (GP) was calculated.[8] GP, an intensity contrast ratio, is defined as the ratio $(I_G - I_L)/(I_G + I_L)$, where I_G and I_L are the fluorescence intensities measured at the maximum emission wavelengths of Laurdan in the gel and liquid-crystalline phases, respectively. In Fig. 3, the GP ratio is plotted over a temperature range of nearly 30 °C. For temperatures below T_t (~22–39 °C), the GP of pure DPPC changes very slowly while the lipid bilayer is in its gel state, with the slope of the curve, $\Delta(GP)/\Delta T$, equal to ~0.005/°C at 30 °C. A similar behavior is observed at temperatures above ~45 °C, while the bilayer is in its pure liquid-crystalline state. In comparison, during phase transition, $\Delta(GP)/\Delta T$ reaches its maximum value. For example, at $T = 42$ °C, corresponding to T_t, $\Delta(GP)/\Delta T = 0.092$/°C is obtained, a value which is nearly 18 times larger than the GP slope at 30 °C. The ability to measure GP changes as small as 0.01, as shown in Fig. 3, means that ΔT variations as small as 0.11 °C at T_t can be resolved. We note that the greatest temperature sensitivity and the largest GP slope occurs near T_t. Hence, by choosing different phospholipids in either pure or mixed form, it is possible to shift T_t and alter the GP slope, thereby "engineering" the temperature range and sensitivity of the liposome sensor and improving the stability of the phase states. For example, GP data for a liposome bilayer consisting of the phospholipids DPPC and DMPC (di-myristoyl phosphocholine), mixed in a 1:1 ratio,

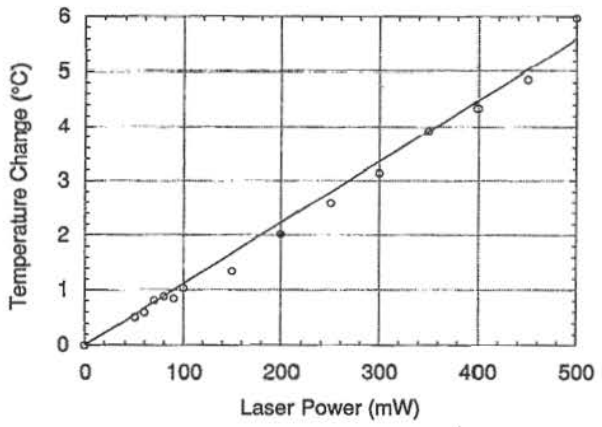

FIG. 4. Experimental data on the temperature changes induced at a given incident laser power. The straight line fit has a slope of 1.1°C/100 mW.

was found to produce a new thermal response curve having a sensitivity $\Delta(\mathrm{GP})/\Delta T = 0.06/°\mathrm{C}$ at a new T_t of 38 °C.

The spatial resolution of the temperature measurement is determined by the pinhole geometry of the detection system and the sample region from which fluorescence is collected. In the present case, a pinhole located at the image plane of the collection objective, and having a diameter ~1 mm, corresponds to a fluorescence collection area on the sample of ~10 μm^2. Hence, temperature changes and heating effects from micron-sized regions can be acquired. In principle, since liposomes can be made smaller than 0.1 μm, liposomes can be used as temperature sensors having submicron spatial resolution.

To demonstrate the feasibility of the above microfluorometric technique, it was used, for the first time, in the determination of localized heating effects in 10-μm-sized organic liposomes which were irradiated by a Nd:YAG laser operating at 1.064 μm and focused to a spot size of ~1 μm. At this wavelength, sample heating is primarily due to water absorption within the membrane region. To quantify the change in sample temperature as a result of heating by laser exposure, the liposome temperature was initially set at a value slightly below T_t, with the laser beam initially turned off. In Fig. 3, for example, this setpoint was 37 °C. As the incident laser power was increased, the fluorescence emission was continuously monitored. Fluorescence photobleaching effects were avoided by gating the incident UV excitation beam. A shift in fluorescence emission wavelength was observed, corresponding to a change in the sample GP and a new effective sample temperature. The difference between the final temperature and the initial setpoint is the actual temperature change due to heating. From Fig. 3, it can be seen that a laser power of 400 mW results in the GP change of 0.14, and a ΔT of 4.3 °C. The change in sample temperature with applied laser power is more clearly seen in Fig. 4, from which a heating rate of ~1.1 °C/100 mW is derived. Hence, exposure to higher incident laser powers results in a linear increase in sample temperature, but only for measurements made within the close proximity of T_t. Last, we note that, since the phase transition process in the liposome is a reversible one, it is possible to reduce the effects of heating and recover the initial phase state and fluorescence emission spectrum, without memory or hysteresis effects, by cooling the sample or lowering the incident laser power. Hence, for initial setpoint temperatures established above T_t, the liposome sensor can also be used to measure sample cooling rates.

As presented herein, the temperature-dependent fluorescence emission technique is ideally suited for the measurement of heating effects and temperature variations in organic particles such as liposomes. However, given the similarity between the phospholipid bilayer of the liposome and the actual membrane structure encountered in cells, it should be possible to extend the present technique to microthermometric studies of living systems, providing Laurdan, or some other environmentally sensitive dye, can be incorporated into the cell membrane. In addition, given its thermal and spatial resolution, the present technique should prove invaluable for assessing, in real time, the effects of sample exposure to highly focused laser beams, such as those encountered in optical laser traps (optical tweezers).[13,14] Last, given the high spatial resolution of this "biosensor," it should also be possible to perform metabolic imaging and thermal mapping at the submicron level.

In conclusion, we have demonstrated a new microfluorometric technique for the nondestructive measurement of heating effects in organic particles by using the temperature-sensitive fluorescence emission from Laurdan-doped liposomes studied under an optical microscope. The temperature and spatial resolutions are 0.1 °C and μm, respectively. The technique was used to determine temperature changes in micron-sized liposomes that were heated as a result of laser exposure. The potential applications for the work described herein include the quantification of thermal effects in laser-tissue interactions, cellular metabolism, and the mapping of thermal profiles within organic and biological samples.

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