Polarization-dependent fluorescence of proteins bound to nanopore-confined lipid bilayers

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Lipid bilayers are essential structural component of biological membranes of all the living species: from viruses and bacteria to plants and humans. Biophysical and biochemical properties of such membranes are important for understanding physical mechanisms responsible for drug targeting. Binding events between proteins and the membrane may be ascertained by introducing fluorescence markers (chromophores) to the proteins. Here we describe a novel biosensing platform designed to enhance signals of these fluorescence markers. Nanoporous aluminum oxide membranes with and without gold (Au) surface coating have been employed for optical detection of bound conjugated streptavidin to biotinylated lipid bilayers—a model system that mimics protein docking to the membrane surface. Unexpectedly, it was found that fluorescence signals from such structures vary when pumped with E-polarized and H-polarized incident optical beams. The origin of the observed polarization-dependent effects and the implications for enhanced fluorescence detection in a biochip format are being discussed. © 2008 American Institute of Physics. [DOI: [10.1063/1.2972143](https://doi.org/10.1063/1.2972143)]

I. INTRODUCTION

Plasma membranes are essential structural elements of eukaryotic cells that define their outer surface. These membranes that are composed from lipids, proteins, and other molecules form highly selective barriers allowing cells to actively control individual biochemical environment at desirable levels. Such environment is cell specific and crucial to the cell function. The active control of intracellular concentrations is achieved by membrane proteins and, primarily, ion channels that respond to various stimuli. Yet, despite growing literature on protein microarrays (see Ref. 1 for a review), there are only a relatively few reports on membrane protein arrays and their use for ligand screening.^{2–4} Lack of microarray methods for membrane proteins, which represent the single most important class of drug targets, is considered by some as a fundamental limitation to further development of the biochip technology. Previously, it has been shown that lipid bilayers and membrane proteins can be assembled in functional conformations inside macroscopically aligned nanochannels of anodic aluminum oxide (AAO) membranes.^{5–10} Recently, we have reported on sub-wavelength hybrid metallo-dielectric structures for the enhancement of Raman spectroscopic signals^{11,12} and one might wonder whether the same applies to fluorescence signals. Others also pointed to the fluorescence enhancing properties of such platforms albeit for optical beams at normal incidence.¹³ Here we report on polarization-dependent fluorescence of a protein marker bound to biotinylated lipid bilayers that were deposited into nanochannels of commercial AAO membranes from aqueous dispersions. While commercial AAO substrates are easily available, they are known to lack highly ordered honeycomb pore packing and a narrow

distribution of pore diameters as achieved with some of our in-house substrates and prepared by a double-anodization method. Nevertheless, we have found that even these relatively poorly organized commercial AAO membranes are suitable for fluorescence detection of protein binding to lipid bilayers and exhibit unexpected polarization effects.

Our initial hypothesis was that chromophores imbedded in the pores of commercially made nanoporous substrates *would not* exhibit variations in their fluorescence signals as a function of sample orientation, owing to significant dispersion in the pores' diameter, their shape, the pore packing, and the fact that these pores are much smaller than the interrogating optical wavelengths used. For the experiments we employed zwitterionic lipid bilayers that could be incorporated into nanoporous aluminum oxide membranes with minimal perturbation to the bilayer properties.^{5–10} The bilayers were doped with 0.5–5.0 mol % of biotinylated lipids and exposed to 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF)—a conjugated streptavidin—to mimic protein docking to the membrane surface. Fluorescence signals were measured as a function of the angle between the optical polarization state of the incident beams and the orientations of macroscopically ordered AAO nanochannels.

Our hypothesis was proven wrong: the fluorescence signals varied when pumped with E-polarized (E-pol or s-polarized) and H-polarized (H-pol or p-polarized) incident optical beams. The origin of the observed polarization-dependent effects and the implications for enhanced fluorescence detection in a biochip format are being discussed.

II. EXPERIMENTS

Most of the experiments were conducted with commercial “0.02 μm ” Anodisc 13 membranes (Whatman International, Maidstone, UK) that are shaped as disks of 13 mm in

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FIG. 1. SEM image of commercial AAO after depositing streptavidin bound to biotinylated lipid bilayers. The pores are extending throughout the sample (approximately 60 μm thick).

diameter and are about 60 μm thick. Examination of these substrates with scanning electron microscopy (SEM) (Fig. 1) yielded an average pore diameter of 177 ± 20 nm on one side, whereas the average diameter for the filtration side (not shown) was 29 ± 7 nm. Each Anodisc substrate was cut into two halves, one half was flipped, and then the two halves were subjected to the same Au sputter coating from just the exposed side. The coating was sufficiently thin and did not block the pores. The other side of each half remained uncoated. Such a procedure yielded two types of AAO substrates containing a thin Au layer at either large pore or small pore (filtration) side. Hence, altogether four different nanoporous substrate samples were prepared for depositing lipid bilayers and further analysis. These less ordered AAO substrates were compared to in-house AAO platform (50 nm thick alumina on an aluminum film). The latter exhibited remarkably periodic array of nanowells.¹²

Synthetic zwitterionic phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and biotinylated 16:0 Biotinyl Cap PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl), sodium salt) were purchased from Avanti Polar Lipids (Alabaster, Alabama) in chloroform solutions and were stored in a freezer at -80°C prior to use. Multilamellar vesicles were prepared by mixing chloroform solutions of DMPC and Biotinyl Cap PE in 20:1 or in 200:1 molar ratio. Consequently, chloroform was removed by a rotary evaporator yielding a thin lipid film on the surface of a round bottom flask. Residual chloroform was removed overnight by keeping the flask open to a liquid nitrogen trap and a vacuum pump. Multilamellar vesicles were formed by adding 50 mM Hepes, pH 7.0, buffer and cycling the flask for at least ten times between liquid nitrogen at -197°C (77 K) and a water bath at 30°C . The final concentration of lipids in aqueous media was 150 mg ml^{-1} .

Streptavidin is a 53 kD tetrameric protein known for its extraordinarily strong affinity for the biotin moiety: the dissociation constant, $K_d \approx 10^{-15}$ mol/l, ranks the biotin-streptavidin complex as one of the strongest among the non-covalent interactions.¹⁴ DTAF-conjugated streptavidin¹⁵ was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and dissolved in a 50 mM Na/K phosphate buffer at pH=7 (VWR International, West Chester,

PA). DTAF-conjugated streptavidin was mixed with an excess of biotinylated lipids immediately before the experiments. Each of the four AAO surfaces described above (namely, two surfaces coated with Au and two uncoated) was exposed to aqueous lipid dispersion while the entire sample was kept at approximately 30°C to ensure that the lipid membrane is in a fluid bilayer phase. Such an exposure resulted in an immediate wetting of the nanoporous substrate, which became semitransparent. Consequently the samples were allowed to air dry before optical measurements. Previously, we have estimated that such lipid bilayer deposition results in a formation of up to four nanotubular bilayers nestled per each of the nanopore throughout the entire available surface area of the substrate assuming 100% surface coverage.¹⁶ While some of the lipids left on the outside AAO surface were washed away during the preparation, some residual lipids were observed by SEM for the air-dried samples (Fig. 1). It should be noted here that the condition of full hydration that is essential for lipids to remain assembled into a bilayered structure is likely to break down for the air-dried samples. Therefore, the lipids in such samples are expected to be somewhat disordered and not necessarily assembled into bilayers.

The deposited nanohole array was mounted on a rotational stage and excited by a 10 mW 488 nm line of an Ar ion laser, which was further focused to a spot of $50\text{ }\mu\text{m}^2$ by a 10 cm lens in a confocal configuration [Fig. 2(a)]. An optical filter in front of the spectrometer ($\lambda=495$ nm) provided cutoff of the laser line. The scattered light (fluorescence signal) was collimated and focused onto the spectrometer slit. A photomultiplier tube was used for detection. A typical fluorescence signal of biotinylated streptavidin is shown in Fig. 2(b).

The fluorescence signals decayed over time. This may be attributed to a competition between photobleaching¹⁷ and recovery processes. In order to further investigate such a phenomenon, successive fluorescence scans such as shown in Fig. 2(b) were taken at 5 min intervals. The peak intensity for each of the scans always stayed at $\lambda=515$ nm and is plotted in Fig. 2(c). As seen by the least squares trend curve the signal mildly decays according to a power law, $\approx 1/\sqrt{t_n}$, where t_n is the time of the n th spectrum in a sequence. The fluorescence reaches quasi-steady-state conditions after approximately 1 h. Thus, in order to ensure a constant response, all the consequent data acquisitions were made after exposing the sample to 100 mW of laser intensity for 1 h.

Biotinylated lipid bilayers alone (i.e., without DTAF-streptavidin bound) did not exhibit any measurable fluorescence signals, as expected. AAO substrates were also exposed to DTAF-streptavidin aqueous solution at 30°C following the same procedure as for aqueous lipid dispersion; namely, excess material was washed by buffer and the substrates were air dried. Figure 2(d) shows that such samples exhibit fluorescence signal similar to Fig. 2(b) albeit a bit weaker than the biotinylated lipid/streptavidin complex. This demonstrates some nonspecific binding of DTAF-streptavidin to alumina surfaces in accord with previous observations.¹⁶ The minute difference in the curve ampli-

FIG. 2. (Color online) (a) System configuration. (b) Fluorescence spectrum of DTAF-streptavidin bound to biotinylated lipid bilayers in AAO. (c) Fluorescence peak intensity as a function of time (filled diamonds) and least squares fit to $\approx 1/\sqrt{t_n}$ (solid line). (d) Fluorescence spectra of biotinylated lipid bilayers deposited into either exposed or Au coated AAO substrates before (filled diamonds) and 10 min after being introduced to the streptavidin. The samples were not exposed to the laser light prior to taking the data hence the relative large signal.

tudes is attributed to local fluctuations. The samples were not exposed to laser light prior to taking the data hence the relative large signals.

As mentioned above, we overcame the time dependent fluorescence signal issue by exposing the samples to laser radiation of 100 mW for 1 h prior to taking the data. Only then we conducted our measurements at 10 mW—a tenth of the initial laser intensity. Fluorescence signals were recorded as a function of incidence angle for two polarization states, either *H*-polarization or *E*-polarization, using a confocal arrangement with the help of a spectrometer and a photomultiplier [Figs. 3(a) and 3(b)]. Successive wavelength scans were made for each data point in order to determine the exact position of the fluorescence peak.

A. Theoretical considerations

In general, the intensity of the fluorescence peak is expected to be affected by individual hole geometry, the periodic pattern of the pores, and by surface plasmons, which are excited in the metal layer if the latter is present. A geometrical factor accounts for the orientation of the pores and macroscopic alignment (if any) of dipoles of the fluorescing chromophores with respect to the incident polarized beam. While surface plasmons may only be excited in metallic surfaces, surface waves may also exist in perforated dielectric films.

The effective area of a given pore, which is directly ex-

posed to the laser beam, is a function of the tilt angle. On the other hand, if the dipole moment of streptavidin is aligned along the pore axis, the fluorescence due to the *E*-polarization mode [*E*-pol; also denoted as *s*-pol by some; see Fig. 3(a) for definitions] is expected to be independent of the tilt angle. The excitation component, which is partially parallel to the biotin/streptavidin dipole moment, increases for the *H*-polarization (*H*-pol; also known as *p*-pol by some) as the tilt angle increases. Such geometrical effects are expected to maximize the fluorescence peak for the *H*-pol state at some tilt angle. The peak fluorescence angle for the *E*-pol state would be at normal incidence. Further details on simulations are provided in the Discussion and the Appendix sections.

III. RESULTS

The peak of the fluorescence signal as a function of the tilt angle θ for the *H*-polarized incident beam is shown in Fig. 3. Clearly seen is a quasisymmetric curve for all the four AAO surfaces examined, with minima appearing at normal incidence. The experiment was repeated one month later with similar results [Fig. 3(c)]. When the sample was azimuthally rotated by 90° and assessed again with the *H*-polarized incident beam [Fig. 3(d)], the response was basically flat for all of the surfaces except for the uncoated (exposed) AAO sample (2), which has the largest average pore diameter.

FIG. 3. (Color online) (a) Experimental configuration with marked *H*- and *E*-polarization modes. (b) Fluorescence peak intensity for *H*-polarized incident beam. Normal incidence position is at $\theta = 43^\circ$. (c) Repeated experiment for exposed (uncoated) AAO substrate (1) after one month: the curve has the same extinction ratio $(I_{\max} - I_{\min})/I_{\min}$, albeit with an overall intensity value differences. The inset indicates out-of-plane tilt. (d) The sample was rotated azimuthally (in-plane rotation) by 90° and the experiments were repeated.

Figure 4 exhibits fluorescence signal as a function of the tilt angle for the *E*-pol incident beam. In contrast to the *H*-pol incident beam results, a quasiflat response was observed for all of the four surfaces: a shallow dip whose position varied from sample to sample was sometimes observed (Fig. 4). The experiment was repeated one month later with basically similar results (not shown). When the sample was azimuthally rotated by 90° and assessed with *E*-pol incident beam, the response was yet again, quasiflat.

When a polarized light impinges on a sample at normal incidence its polarization state (the direction of electric field

oscillations) is parallel to the substrate surface (not to be confused with the surface of the pores, which extend throughout the substrate). This holds true regardless of the *H*- or *E*-polarized mode used. We have monitored the peak intensity of the fluorescence signal as we rotated the sample azimuthally [in-plane rotations as indicated by the inset of Fig. 5(a)] about its normal to the AAO surface [Figs. 5(a) and 5(b)]. As can be seen from the figure, the signal intensity oscillates with two periods: 240° and 120° . Such oscillations imply an effect of crystallography of the hole array on the fluorescence signal. The radial plot indicates asymmetry in

FIG. 4. (Color online) (a) Fluorescence peak intensity for *E*-polarized incident beam. Normal incidence position is at $\theta = 43^\circ$ with the exception of $\theta = 40^\circ$ for the exposed sample (1). (b) The samples were azimuthally rotated by 90° and the tilt experiments were repeated. Normal incidence position is at $\theta = 60^\circ$ with the exception of $\theta = 43^\circ$ for the exposed samples.

FIG. 5. (Color online) (a) Fluorescence peak intensity as a function of azimuthal angle (in-plane rotations as indicated by the inset) for the exposed (uncoated) sample (2) at normal incidence. (b) Radial plot: the asymmetry in the curve is likely due to sample inhomogeneities. (c) Fluorescence as a function of azimuthal angle for Au-coated sample (2) at normal incidence. The purple curve is a shifted sinusoidal curve indicating a threefold symmetry. (d) Repeated measurements demonstrate acceptable reproducibility for the radial plot.

the curve which is likely due to inhomogeneities within the sample as it is rotated about its normal axis. Nevertheless, the fluctuations in the curve upon rotating the sample by 360° are less than 10%. Notably, the observed angular period seems to be multiples of 60° but do not follow the C_6 symmetry exactly. We speculate that the main reason for this is local scatterings, which are due to the inhomogeneity of the hole array. Such scatterings are reduced somewhat when the surface of the sample is coated with a thin layer of Au [Figs. 5(c) and 5(d)] probably because the electric field better confines to the sample surface.

A. Simulations

Simulations of the peak fluorescence signal as a function of the tilt angle are presented in Fig. 6. The qualitative model assumes a macroscopic alignment of the streptavidin dipole with respect to the beam. Such alignment should scale the signal as $\sim |\sin \theta|^2$, where θ is the tilt angle with respect to the surface normal. The effective pore area with respect to the incoming pump laser beam is changing: a circular pore

FIG. 6. (Color online) Simulations: normalized fluorescence signal for H -pol (red line) and E -pol (blue dash line) modes. The molecules are assumed to be aligned along the pore axis but the position below AAO surface is insignificant.

with a radius r for a normal incidence beam changes effectively to an ellipse with an area of $A = \pi r^2 \cos \theta$ when the sample is tilted by an angle of θ . Thus, the effective area scales as $\cos \theta$. The detection efficiency also scales as $\cos \theta$ since the acceptance angles for the optical system as well as the spectrometer opening are fixed.

Along the discussion in the Appendix section, there are two major requirements for fluorescence signal enhancement: (i) an efficient coupling to a surface mode and (ii) a phase match condition with an appropriate crystallographic plane of holes (air rods). The first requirement dictates a proper tilt and azimuthal (in-plane rotation) angles such that the light is coupled by the first order of diffraction to a surface mode. If we treat the hole array as a grating with a given pitch, the efficiency of this coupling process may be approximated by $\sin^2(\Delta\eta/2)$, where $\Delta\eta$ is the phase difference between the grating and the propagating beam. The second requirement is related to a Bragg scattering between this surface mode and the array of holes when the beam propagates inside the AAO substrate. Such scattering is expected to be affected by only the azimuthal or in-plane rotation angle.

In the model we assumed a phase match condition [hence the $\sin^2(\Delta\eta/2)$ term is unity]. We included a reflection coefficient for the incident beam between the AAO and air. We also included an effective reflection coefficient between the oxide and its related air rods. All of those coefficients are dependent on the incident angle θ . The simulation results shown in Fig. 6 demonstrate that while the model succeeded in qualitatively following the trend in the fluorescence response for the H -polarized modes, it failed to account for the relatively flat experimental results for the E -polarized incident beams.

IV. DISCUSSION

We set to investigate effects of polarized incident beams on the fluorescence signal of DTAF-conjugated streptavidin, which was bound to biotinylated lipid bilayers. The latter were imbedded in nanopores of commercial anodized aluminum oxide platforms. The streptavidin was labeled with fluorescein, a chromophore marker. Our initial hypothesis was that the fluorescence signals from these chromophores will not be substantially affected by the orientation of the substrate, nor its pores, when pumped with various polarized incident beams. We varied the sample orientation with respect to the pumping optical beam in two ways: (i) by tilt angle θ —this was to test the effects of local chromophore orientation with respect to the polarized incident beam—and (ii) by in-plane rotations, or, azimuthal angle ϕ at normal incidence beam—this was to study the effect of the crystallographic hole-array orientation with respect to the beam's linear polarization state. Our hypothesis was proven wrong: unexpectedly, the experimental data demonstrated variations in the fluorescence signal both upon tilting the sample with respect to the beam direction and with respect to the azimuthal (in-plane) sample rotations.

In general, the larger signal variability was achieved with the H -polarization mode for a certain crystallographic sample orientation. The data for the E -polarized incident

beam also exhibited some signal fluctuations as a function of tilt angle, yet, its extinction ratio $(I_{\max} - I_{\min})/I_{\min}$ was much smaller than the corresponding value for the H -polarized mode. In addition, no correlation between the position of the signal minima (whenever present) and approximate crystallographic orientation of the hole array could be established. We, therefore, hypothesize that the observed polarization effects may be attributed to the formation of selective light intensity distribution within the holes of the AAO substrates.¹²

SEM images of these commercial AAO substrates revealed an imperfect pore homogeneity and packing. Nevertheless, polarization-dependent signal along certain crystallographic directions alludes to selective coupling between the fluorescence mode, which propagate in the AAO sample and the radiative modes (those modes are scattered back, propagate in free space, and detected by the spectrometer/detector system). Moreover, the clearer signal for Au-coated surfaces implies the role of surface plasmons in the coupling process.¹⁸ While qualitative simulations using a simplified model reproduced the main data trends for the H -polarized incident beams, the model failed to account for the fluorescence results obtained with the E -polarized incident beam. We attribute this to (i) a poor pore ordering and heterogeneity of pore diameters in the commercial AAO membranes and (ii) oversimplifications of our initial model. These considerations justify further efforts toward data modeling and call for additional experiments with nanoporous platforms with better defined hole-array platforms.

The fluorescence signal exhibited a rather mild decay over time when exposed to prolonged irradiation of the laser light. Such decay behavior could be well fitted by a power law, $\approx 1/\sqrt{t_n}$ [solid line in Fig. 2(c)]. In general, if the lifetime of the fluorescing molecule is relatively long, a steady state may be reached. For DTAF-streptavidin the half-lifetime value is about 16–21 sec depending on the solvent.¹⁷ On the other hand, if there is a feedback mechanism (such as anisotropy from scattering by the array of holes), it is expected to impact the signal decay of such long-lived fluorescing molecules. Therefore, we may attribute the time dependent fluorescence signal to the additional scatterings by the porous platform besides the inherent photobleaching effect. Platforms built upon arrays with homogeneous and periodically packed nanopores ought to accentuate such behavior even further.

V. CONCLUSIONS

An analytical methodology has been introduced to utilize nanoporous aluminum oxide substrates for enhanced fluorescence detection of protein bound to lipid bilayers. Specifically, it was found that a model protein-lipid bilayer complex, formed upon noncovalent binding of DTAF-conjugated streptavidin to biotinylated lipid bilayers and deposited into a nanoporous array in anodized aluminum oxide films, exhibited different fluorescence characteristics for E -polarized and H -polarized incident optical beams. Variations in the fluorescence signals in the presence of a thin coating of gold on the sample surface were also observed.

FIG. 7. (Color online) Unit vectors and ideal arrangement of holes.

The origin of the observed polarization-dependent effects could be explained by a simplified model which assumed a macroscopic alignment of the fluorescing dipole with respect to the oscillating pumping electric field. Understanding the mechanisms of such behavior may assist us in designing better platforms for biochemical detection and further development of this analytical tool.

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APPENDIX: OPTIMAL FLUORESCENCE CONDITIONS WITH AN ARRAY OF HOLES

The AAO slab may be viewed as a particular case of photonic crystal with hexagonal array of air rods.¹⁹ There are several limitations to this analogy: (1) the pore packing in commercial AAO is far from ideal and variations in the hole diameter are large; thus, such hole array is only quasiperiodic; (2) the array pitch is much smaller than the propagating wavelength; (3) the AAO substrates employed are very thick (on the order of 60 μm) with respect to the propagating wavelengths; and (4) the chromophores may be (partially) aligned within each nanohole, thus, complicating the picture. Nevertheless, one could gain some insights by examining an ideal two dimensional (2D) array of holes.

Let us consider an ideal 2D hexagonal case (Fig. 7) and start by identifying two lattice vectors, $a_1 = \hat{x}a$ and $a_2 = \hat{x}a/2 + \hat{y}\sqrt{3}a/2$, where a is the distance between the nearest neighbor holes. The reciprocal lattice vectors are $G_1 = \hat{x}2\pi/a - \hat{y}2\pi/a\sqrt{3}$, and $G_2 = \hat{y}4\pi/a\sqrt{3}$. Light is coupled to the periodic array of holes by momentum conservation. For example, if the wave has a propagation component along the y -direction, then

$$k_y + q_2 G_2 = \beta_y, \quad (\text{A1})$$

where $k_y = k_0 \sin(\theta) \cos(\phi)$ to account for the tilt θ and the azimuthal ϕ angles; q_2 is integer (both negative and positive numbers are allowed).

Suppose that the surface component of the wave inside the AAO sample $\beta = \beta(x, y)$ (bold letters are used for vector notations) is scattered from the same periodic structure. This

may happen because the AAO is made of densely packed holes with inter hole spacing which is much smaller than the propagating wavelength. This is also happening because of the abrupt nature of the hole in the AAO slab, invoking higher orders and suborders of diffraction. Some directions within the crystal of holes will strongly diffract the beam when the following phase match condition is fulfilled:

$$|\beta - qG| = \beta. \quad (\text{A2})$$

Again, q is an integer. One can envision that such Bragg diffraction creates a standing wave within the hole array. These considerations are true for the pump as well as for the fluorescing wavelengths.

Optimal fluorescence conditions occur when the mode propagating in the AAO is a surface mode, i.e., $\beta \sim k_0 n_{\text{eff}}$. Here n_{eff} is the effective index of refraction of AAO including the holes [the effective index of refraction is $(n_{\text{eff}})^2 = f(n_{\text{air}})^2 + (1-f)(n_{\text{oxide}})^2$, f —the fill factor—50% for holes, $n_{\text{air}}=1$; $n_{\text{oxide}}=1.77$]. It is obvious that when $\theta=0$ (normal incidence), the propagation constant inside the AAO layer has a degeneracy of two counterpropagating beams, each fulfilling the condition $\beta = q|G|$. This means that there will be a propagation component traveling parallel to the AAO surface. For 2D hexagonal hole array, with $q_1 G_1$ and $q_2 G_2$ and q_i integer (with both negative and positive values; the idea may be extended though, to subharmonics, such that, $q_i = \pm 1/2, \pm 1/3, \dots$ as well²⁰), the optimized wavelength, which fulfills this requirement, may be approximated as

$$\lambda_{\text{opt}} = \frac{a \cdot n_{\text{eff}}}{\sqrt{\left(\frac{4}{3}\right) \cdot (q_1^2 - q_1 q_2 + q_2^2)}}. \quad (\text{A3})$$

All together, at normal incidence there would be fourfold degeneracy: two beams oscillating in parallel and two beams in perpendicular to the AAO surface. Equation (A3) predicts transverse resonance effect at $\lambda \sim 545$ nm for $a \sim 225$ nm, $q_1=1/2$, $q_2=0$, and $n_{\text{eff}} \sim 1.4$. This is not quite optimized with the pump or, the fluorescence wavelengths, however, we note that the resonance may be fairly wide due to local scattering—up to 20% of the central wavelength.

When a wave incident on the surface of an AAO sample, the polarized beam is diffracted and refracted throughout the oxide medium and within the holes. As mentioned above, at normal incidence, the linear polarized wave is made of counterpropagating beams. Let us consider the mode, which propagates parallel to the slab surface.²¹ If the photonic crystal is made perfect, then there exists a bandgap for some frequencies. Namely, the propagation of these frequencies is frustrated for all crystallographic directions (or throughout the Brillion zone and its subharmonics). If the arrangement of holes is far from ideal and we operate at frequencies outside the bandgap, the reflections from the hole array in some directions within the x - y plane will be stronger than the others and will depend on the crystallographic symmetry of the holes. Thus, for normally incident beam, the fluorescing signal will depend on the in-plane rotation angle with respect to the linearly polarized state.²² The symmetry of the hole array will dictate the number of cycles upon full rotation. For hexagonal array of holes there would be symmetry of 60° or its

multiple. While commercial AAO substrates lack such high degree of symmetry, they may retain some partial hexagonal ordering. Therefore, at normal incidence, one might expect that such symmetry appear in the rotational data.

To summarize, the efficiency of the coupling between the hole array and the polarization state of the pump beam depends on the coherence of the scattering process. A proper tilt angle ensures coupling to a surface mode and in-plane rotations provides for the proper phase match conditions.

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