

Microspot surface enhanced fluorescence from sculptured thin films for control of antibody immobilization

Alina Karabchevsky^{*a}, Christian Patzig^b, Bernd Rauschenbach^b, I. Abdulhalim^a

^aDepartment of Electrooptic Engineering and the Ilse Katz Institute for NanoScale Science and Technology, Ben Gurion University of the Negev, Beer Sheva 84105, Israel

^bLeibniz-Institut für Oberflächenmodifizierung e.V., Permoserstrasse 15, 04318 Leipzig, Germany

ABSTRACT

Nano-sculptured thin films (STF) are prepared by the glancing angle deposition technique and take different forms of nano columnar structures. Varieties of STFs are investigated to find the optimum structure for biosensing based on the surface enhanced fluorescence (SEF). The highest amplification of fluorescent signal is found for Ag based STFs on fused silica giving an enhancement factor of x23, where $h=400\text{nm}$, $d=75\text{nm}$, $\alpha=23^\circ$ relative to Ag dense film using fluorescent dye Rhodamine 123. Based on this, a demonstration of monitoring of antibodies and even confirmation of successful immobilization of the receptors presented. Bound antibody to the thiol self assembly monolayer on sample surface is then quantified by means of the fluorescent signal. Upon excitation of the fluorophore by Hg source light, a CCD camera with a controlled exposure time detects the pattern of fluorescent antibody/E-coli bacteria colonies on the STF surface. A fiber optic holder attached to the microscope allowed quantitative measurement of the fluorescence spectrum on a microspot.

Keywords: Surface Enhanced Fluorescence (SEF), Sculptured Thin Films (STF), biosensing, self assembly monolayer (SAM).

1. INTRODUCTION

Fluorescence is one of the optical phenomena which can be used to detect biopathogens on surfaces¹. However fluorescent emission from analytes especially from biological assays is weak. In order to increase the fluorescent signal, attempts have been made to increase the number of dye molecules bound to the surface but this produces concentration quenching, reducing first the quantum efficiency and then the total emission. Field enhancement and therefore fluorescence can be achieved due to special morphology of the metal nano-sculptured surfaces, while the mechanism for surface enhanced processes is predominantly electromagnetic in nature. Metal nanoparticles and nanostructured metal films possess localized surface plasmon resonances (LSPRs) that imbue these materials with a number of unique and useful optical properties. A clear evidence for the role of local plasmon resonances in the excitation and emission processes has been presented². LSPRs are responsible for the size and shape³ dependent optical spectra that have led to the use of metal nanoparticles in a variety of bio-diagnostic applications^{4, 5} and plasmon modes have been implicated in the surface plasmon imaging based sensors^{6, 7}, extraordinary transmission (EOT) of light through nanoscale hole⁸ and slit⁹⁻¹¹ arrays. It was shown recently, that the reason for resonances in EOT spectra from periodic nanoslits with very thin metal $< 100\text{nm}$ is the excitation of classic surface plasmon resonance at the boarders of metal above and below the dielectric¹¹. The highly confined local electric field enhancements that accompany the excitation of LSPRs are used in a variety of near-field enhanced spectroscopy and imaging modes, from near-field scanning optical microscopy^{12, 13} to surface-enhanced Raman spectroscopy (SERS)¹⁴⁻¹⁷. Although SERS applications have motivated much of the research into surface-enhanced spectroscopy in the past decade, the widespread use of fluorescence-based sensing in biology and the importance of radiative decay near metal electrodes in organic optoelectronics^{18, 19} are two factors that have led to a great deal of new interest in the study of simple fluorescence near metal nanostructures²⁰. Although planar metal films

* rudenko@bgu.ac.il; phone +972-(0)8-6428599 ; fax +972-(0)8-6479494

generally quench the emission from nearby fluorophores^{21, 22}, the effects of metal nanostructures are more complicated. Depending on the details of the system under investigation, fluorescence quenching²³⁻²⁵, enhancement^{1, 3, 26, 27}, or both²⁸ have been reported in experimental studies of fluorescent dyes and quantum dots near nanostructured metals. Sculptured thin films (STFs)^{29, 30} are nanostructured materials with unidirectionally varying properties that can be designed and realized in a controllable manner using variants of physical vapor deposition techniques. The growth mechanism is based on self-organized nucleation during deposition and subsequent highly directional growth due to atomic shadowing mechanism. A particle flux, incident to the substrate at an angle β (usually $\beta \geq 80^\circ$, as measured to the substrate normal), enables preparation of columnar thin films under glancing angle deposition (GLAD)³⁰. The ability to instantaneously change the growth direction of their columnar morphology, through simple variations in the direction of the incident vapor flux, leads to a wide spectrum of columnar forms such as spiral, screws, vertical columns, chevrons etc. By immobilizing fluorescent antibody to the thiol self assembly monolayer on surfaces we show in this study the applications of the STF as a sensor for control of antibodies immobilization.

2. EXPERIMENTAL

STFs were prepared using the glancing angle deposition technique (GLAD)³¹. STFs of different materials, such as Si, Ag, Au, and Cu on different substrates, were prepared by methods including sputter deposition and e-beam evaporation. The deposition angle was adjusted to $\alpha = 85^\circ$ (deposition angle α as measured with respect to the substrate normal as it is demonstrated in Figure 1) to form nanostructures of manifold shapes (spirals, screws, vertical columns)³⁰ using appropriate substrate rotation scheme. Subsequently, dense films of each material were also deposited, with the deposition angle $\alpha = 0^\circ$, as reference samples. Pre-patterned substrates, comprising of monolayers of SiO_2 nanospheres prepared a self-assembly³² method, and Au dot honeycomb-like array gained by evaporating Au through the voids of such self-assembled nanospheres³³, were used to form periodic nanostructure arrays. A schematic illustration for the GLAD setup is demonstrated in Figure 1.

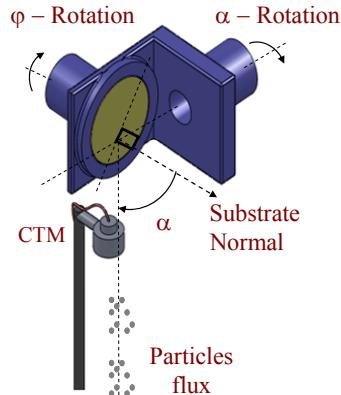


Figure 1. Schematic of the GLAD setup. One stepper motor controls deposition angle α , and a second stepper motor is used for substrate rotation φ about the substrate normal. CTM abbreviates crystal-thickness monitor.

For fluorescence measurements, first the samples were spin coated at 4000 RPM with a fluorescent dye Rhodamine 123 diluted in methanol at 0.6%wt. The thickness of the spun dye layer was estimated by atomic force microscope measurements to be approximately 50nm. Care was taken in selecting the spinning conditions to obtain uniform dye films and similar thicknesses both on the sample and its reference. After comparison between the structures prepared for the study using fluorescence dye, fluorescent receptor Anti-Rabbit IgG (whole molecule) - FITC catalog number from Sigma Aldrich: F7512 was immobilized on the structure which gave highest fluorescent signal using Rhodamine 123 fluorescent dye compared to the dense film of the same material (Figure 2). We immersed our samples in long chain thiol 11-murcaptonoic acid for 24h to prevent silver from oxidation and mainly to attach the receptor to the surfaces. Due to the chemical basis of the functionalization of gold/silver surfaces a self assembled monolayer (SAM) of thiol was used to cover the surface. In this study we show the existence of the receptor in SEF images on nano-STFs versus the flat silver substrates. An enhancement in the fluorescence signal is observed due to the localized surface plasmon resonance (LSPR) effect in nanorods.

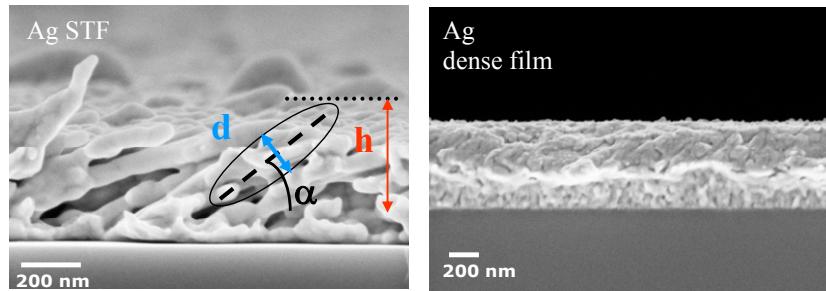


Figure 2. SEM micrographs of the side views of Ag STF (left) structure with $h \approx 400$ nm , $d \approx 75$ nm, $\alpha \approx 23^\circ$ compared to the dense silver film (right) with $\alpha \approx 85^\circ\text{-}90^\circ$ both structures are on fused silica substrate.

Fluorescence measurements were performed using an Olympus fluorescence upright microscope BX51 with an Hg arc lamp as excitation light source having three fluorescence filter cubes with the following excitation-emission wavelengths and dichroic filter (DF) wavelengths: $(\lambda_{ex}, \lambda_{em}, \lambda_{DF}) = f$. The green Hg line at 546nm was used for excitation in all of the experiments and the emission was detected using the red filter at 590nm (Figure 3).

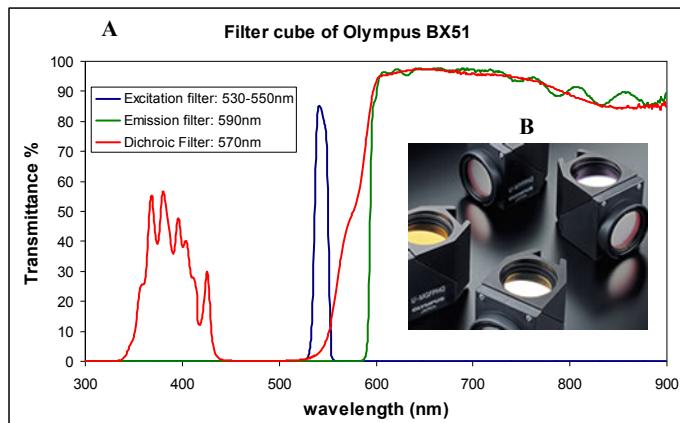


Figure 3. A Transmittance vs. wavelength of filters used in the study, B Filter cubes.

The detection of the image/signal obtained in the intermediate image plane were performed: 1) using a high sensitivity cooled CCD camera with a controlled exposure time, the acquired images were then analyzed using Matlab and the average intensity was compared between the sample and its reference; and 2) the measurements were also performed using fluorescence spectrometer. Due to integration of the microscope with spectrometer the fluorescence emission spectrum was grabbed simultaneously with the image grab. Optical fiber holder was designed (Figure 4) to fit in place of the standard microscopic eyepiece with possible XY and focus adjustments. The distal end of the fiber is connected to the spectrometer and the strongest signal is obtained when the fiber is placed in the plane of the primary image of the microscope. The microspot diameter D_{spot} , being inspected is determined by the microscope primary magnification M and the diameter of the fiber D_{fiber} ($D_{spot} = D_{fiber}/M$). Hence in our case using a 1mm diameter fiber and x50 magnification the micropot diameter is 20 μ m. Enhancement factor was calculated as a relation between fluorescent signal $F(\lambda)_{STF}$ from STF vs. fluorescent signal from dense film $F(\lambda)_{ref}$:

$$\text{Enhancement Factor} = \frac{F(\lambda)_{STF}}{F(\lambda)_{ref}} \quad (1)$$

Fluorescent signals $F(\lambda)$ were achieved by reducing the background: $F(\lambda) = F(\lambda) - \min\{ F(\lambda) \}$. Other enhancement factors were calculated according to the fluorescence images.

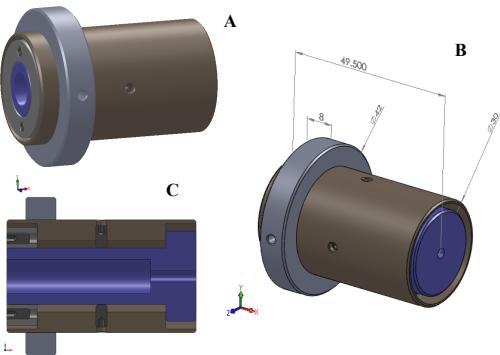


Figure 4. Design of the Fiber optic housing: A side view, B back view and C intersection.

3. RESULTS AND DISCUSSION

Figure 5A shows fluorescence spectra taken from microspot with diameter of $20\mu\text{m}$ while Ag-nanorod STF and Ag dense film on fused silica were spin coated at 4000 RPM with Rhodamine 123 diluted in methanol at 0.6%wt. The pattern of Ag-nanorod STF and Ag dense film on fused silica were shown on SEM micrographs on Figure 2. To apply STFs as biosensors we immobilized fluorescent receptor Anti-Rabbit IgG on the Ag STF and Ag dens films to demonstrate the STF as a biosensor. Figure 5B shows an experimental results of two fluorescence images, one for a Ag STF ($h=400$ nm) deposited by e-beam evaporation on fused silica. The STF consists of rod-like nanostructures with rod diameter $d \approx 75$ nm, inclined at an angle $\alpha \approx 23^\circ$ with respect to the substrate plane. The left image corresponds to the continuous Ag film used as a reference, which has thickness of approximately 415 nm (deposited on fused silica).

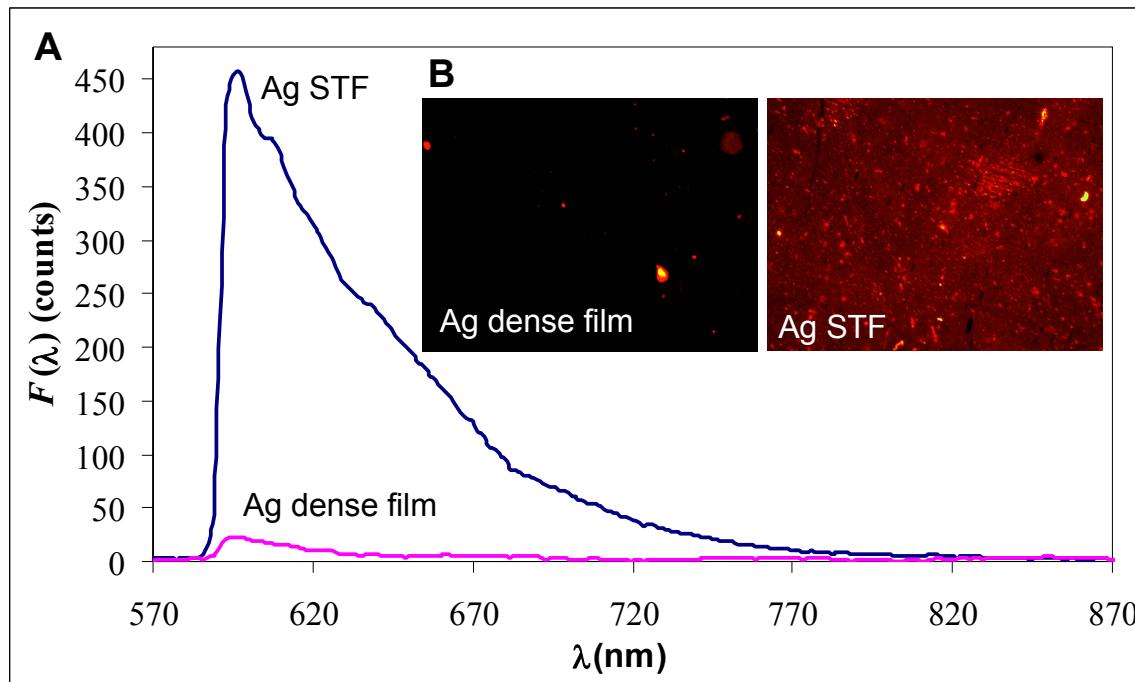


Figure 5. A: Fluorescence spectra taken from microspot with diameter of $20\mu\text{m}$ while Ag-nanorod STF and Ag dense film on fused silica were spin coated by Rhodamine 123. B: Fluorescence images from: dense Ag film and Ag-nanorod STF on fused silica. Both slides were immersed in diluted 1/100 (antibody to PBS) Anti-Rabbit IgG (whole molecule).

For fluorescent dye Rhodamine 123 on the STF (Figure 5A) the enhancement factor was only 20 while with receptor molecules immobilized on the STF (Figure 5B) the enhancement factor is more than 32. In contrast to SERS, maximal enhancement of fluorescence does not occur from molecules adsorbed directly on the metal surface. Emission from fluorescent dyes adsorbed directly on the metal surfaces is quenched due to rapid nonradiative energy transfer to the metal. The ratio of the fluorescence intensity observed from molecules near a roughened silver surface to that from molecules adsorbed on a reference glass substrate can be described by the product of two terms:

$$Y = |L(\lambda_{exc})|^2 Z(\lambda_{flu}) \quad (2)$$

The term $|L(\lambda_{exc})|^2$ is the enhancement of local electromagnetic field intensity near the STFs at the excitation wavelength λ_{exc} . It is representative of the ability of the metal to concentrate the electromagnetic energy when the excitation wavelength is coincident with the surface plasmon resonance of the metal. The second factor $Z(\lambda_{flu})$ describes the relative radiative yield of the excited molecules on the two substrates. The radiative decay rate of the coupled system consisting of the metal particles and adsorbed molecules is proportional to the square of the local field enhancement factor $|L(\lambda_{exc})|^2$ at the fluorescence excitation wavelength λ_{exc} ³⁴. Because of the coupling between the molecular dipole and the metal, the latter can radiate a photon before the excitation is dissipated by nonradiative pathways within the fluorescent molecule. This effect increases the relative radiative yield because the factor Z can be as large as $1/Q_0$, where Q_0 is the fluorescence quantum yield of the molecules³⁵. Obviously, the factor Z does not provide significant enhancement for fluorophores with high quantum yields. A maximum enhancement of $1/Q_0$ can be obtained only when the fluorophore is at an optimum distance from the metal surface, and the value of Z decreases rapidly as the distance is decreased below this value. The quenching is a result of a very strong nonradiative energy transfer from fluorophore molecules to the metal substrates. The radiationless transfer is a short-range effect relative to the enhancement of the local electromagnetic field. As a consequence, a maximum of fluorescence intensity is predicted for dye molecules spaced at some optimal distance from the metal surface. Maximal fluorescence amplification is observed only from molecules at certain distances from the surface³⁶ while here, receptor molecules were immobilized on functionalized films with thiol layer of 2-4nm.

4. CONCLUSIONS

Surface Enhanced Fluorescence from metallic nano Sculptured Thin Films (STFs) is investigated for biosensing applications. Fluorescent signals were captured by camera. Emission spectra of the samples were collected using fluorescence spectroscopy which enables to investigate the effects of amplification of the fluorescent signal from STFs and dens films in more quantitative fashion. For this purpose, we designed and manufactured a fiber housing which is a compact adjustable mechanical module that connects to the microscope in place of its eyepiece. This enabled collection of the light from the primary image plane of the Olympus BX51 light microscope guiding it to the spectrometer. Highest fluorescent enhancement factor of ~20 was obtained from the silver nanocolumns deposited by 23° with respect to the fused silica substrate. By using this nanostructure we have demonstrated that the STF can serve as a thin film platform for biosensing. The combination of column like pattern of the sensing element and fluorescence detection schemes which is fluorescence microscopy with the resonant coupling of surface plasmons as the excitation ‘light source’ at a metal/dielectric interface, i.e. of the sensor surface in contact with the analyte media, allows monitoring of antibodies and even confirmation of successful immobilization of the receptors on the silver surfaces.

Acknowledgement:

This work is supported by the ministry of science under "Tashtiot program" and the Graduate school 'BuildMoNa (University Leipzig, Germany) funded within the German Excellence Initiative of the Deutsche Forschungsgemeinschaft (DFG). Special thanks to Mr. Lev Tsapovsky M. Sc. student from Biotechnology Department, Ben-Gurion University of the Negev for the preparation of the antibody.

REFERENCES

- [1] I. Abdulhalim, A. Karabchevsky, C. Patzig, B. Rauschenbach, B. Fuhrmann, E. Eltzov, R. Marks, J. Xu, F. Zhang and A. Lakhtakia, "Surface - enhanced fluorescence from metal sculptured thin films with application to biosensing in water," Applied Physics Letters 94, 063106 (2009).

- [2] S. Kuhn, U. Hakanson, L. Rogobete and V. Sandoghdar, "Enhancement of Single-Molecule Fluorescence Using a Gold Nanoparticle as an Optical Nanoantenna," *Physical Review Letters* 97, 017402 (2006).
- [3] K. M. Mayer and J. H. Hafner, "Localized Surface Plasmon Resonance Sensors," *Chemical Reviews* 111, 3828-3857 (2011).
- [4] A. J. Haes and R. P. Van Duyne, "A unified view of propagating and localized surface plasmon resonance biosensors," *Analytical and Bioanalytical Chemistry (Formerly: Analysis)* 379, 920 (2004).
- [5] L. Joseph, "Plasmonics in biology and plasmon - controlled fluorescence," *Plasmonics* 1, 5 - 33 (2006).
- [6] A. Karabchevsky, S. Karabchevsky and I. Abdulhalim, "Fast surface plasmon resonance imaging sensor using Radon transform," *Sensors and Actuators B: Chemical* 155, 361-365 (2011).
- [7] A. Karabchevsky, S. Karabchevsky and I. Abdulhalim, "Nanoprecision algorithm for surface plasmon resonance determination from images with low contrast for improved sensor resolution," *Journal of Nanophoton* 5, 051813 (2011).
- [8] T. W. Ebbesen, H. J. Lezec, H. F. Ghaemi, T. Thio and P. A. Wolff, "Extraordinary optical transmission through sub - wavelength hole arrays," *Letters to Nature* 391, 667 (1998).
- [9] A. Karabchevsky, O. Krasnykov, M. Auslender, B .Hadad, A. Goldner and I. Abdulhalim, "Theoretical and Experimental Investigation of Enhanced Transmission Through Periodic Metal Nanoslits for Sensing in Water Environment," *Plasmonics* 4, 281 - 292 (2009).
- [10] A. Karabchevsky, O. Krasnykov, I. Abdulhalim ,B. Hadad, A. Goldner, M. Auslender and S. Hava, "Metal grating on a substrate nanostructure for sensor applications," *Photonics and Nanostructures - Fundamentals and Applications* 7, 170-175 (2009).
- [11] A. Karabchevsky, M. Auslender and I. Abdulhalim, "Dual Surface Plasmon Excitation with Thin Metallic Nanoslits," *Journal of Nanophoton* (2011) [DOI: 10.1117/1.3609276]
- [12] C. Neacsu, G. Steudle and M. Raschke, "Plasmonic light scattering from nanoscopic metal tips," *Applied Physics B: Lasers and Optics* 80, 295-300 (2005).
- [13] E. J. Sanchez, L. Novotny and X. S. Xie, "Near-Field Fluorescence Microscopy Based on Two-Photon Excitation with Metal Tips," *Physical Review Letters* 82, 4014 (1999).
- [14] S. Nie and S. R. Emory, "Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering," *Science* 275, 1102-1106 (1997).
- [15] A. M. Michaels, M. Nirmal and L. E. Brus, "Surface Enhanced Raman Spectroscopy of Individual Rhodamine 6G Molecules on Large Ag Nanocrystals," *Journal of the American Chemical Society* 121, 9932-9939 (1999).
- [16] K. Kneipp, "Surface - enhanced Raman scattering and biophysics," *Journal of Physics : Condensed Matter* 14, R597 (2002).
- [17] C. L. Haynes and R. P. Van Duyne, "Plasmon-Sampled Surface-Enhanced Raman Excitation Spectroscopyâ , " *The Journal of Physical Chemistry B* 107, 7426-7433 (2003).
- [18] J. Vuckovic, M. Loncar and A. Scherer, "Surface plasmon enhanced light-emitting diode," *Quantum Electronics, IEEE Journal of* 36, 1131-1144 (2000).
- [19] P. A. Hobson, S. Wedge, J. A. E. Wasey ,I. Sage and W. L. Barnes, "Surface Plasmon Mediated Emission from Organic Light-Emitting Diodes," *Advanced Materials* 14, 1393-1396 (2002).
- [20] J. R. Lakowicz, "Radiative Decay Engineering: Biophysical and Biomedical Applications," *Analytical Biochemistry* (2001) 1-24 ,298
- [21] R. R. Chance, A. Prock and R. Silbey, *Molecular Fluorescence and Energy Transfer Near Interfaces*, Advances in Chemical Physics, John Wiley & Sons, Inc., NJ, 1-65, (2007).
- [22] W. L. Barnes, "Fluorescence near interfaces :The role of photonic mode density," *Journal of Modern Optics* 45, 661 - 699 (1998).
- [23] E. Dulkeith, A. C. Morteani, T. Niedereichholz, T. A. Klar, J. Feldmann, S. A. Levi, F. C. J. M. van Veggel, D. N. Reinhoudt, M. Moller and D. I. Gittins, "Fluorescence Quenching of Dye Molecules near Gold Nanoparticles: Radiative and Nonradiative Effects," *Physical Review Letters* 89, 203002 (2002).
- [24] E. Dulkeith, M. Ringler, T. A. Klar, J. Feldmann, A. Munoz Javier and W. J. Parak, "Gold Nanoparticles Quench Fluorescence by Phase Induced Radiative Rate Suppression," *Nano Letters* 5, 585-589 (2005).
- [25] F. Cannone, G. Chirico, A. R. Bizzarri and S. Cannistraro, "Quenching and Blinking of Fluorescence of a Single Dye Molecule Bound to Gold Nanoparticles," *The Journal of Physical Chemistry B* 110, 16491-16498 (2006).
- [26] I. Abdulhalim, A. Karabchevsky, C. Patzig, B. Rauschenbach and B. Fuhrmann, "Comparative study of enhanced fluorescence from nano sculptured thin films," *SPIE, Nanostructured Thin Films* 7041, 70410G-7 (2008).
- [27] V .Giannini, A. I. Fernandez-Dominguez, S. C. Heck and S. A. Maier, "Plasmonic Nanoantennas: Fundamentals and Their Use in Controlling the Radiative Properties of Nanoemitters," *Chemical Reviews* 111, 3888-3912 (2011).

- [28] P. Anger, P. Bharadwaj and L. Novotny, "Enhancement and Quenching of Single-Molecule Fluorescence," Physical Review Letters 96, 113002 (2006).
- [29] A. Lakhtakia and M. Russel, *Sculptured thin films: nanoengineered morphology and optics*, SPIE, (2005).
- [30] K. Robbie, M. J. Brett and A. Lakhtakia, "First thin film realization of a helicoidal bianisotropic medium," Journal of Vacuum Science & Technology A: Vacuum, Surfaces, and Films 13, 2991 - 2993 (2009).
- [31] R. Messier and A. Lakhtakia, "Sculptured thin films — II. Experiments and applications," Materials Research Innovations 2, 217-222 (1999).
- [32] C. Patzig, B. Fuhrmann, H. S. Leipner and B. Rauschenbach, "Silicon Nanocolumns on Nanosphere Lithography Templatized Substrates: Effects of Sphere Size and Substrate Temperature," Journal of Nanoscience and Nanotechnology 9, 1985-1991 (2009).
- [33] B. Fuhrmann, H. S. Leipner, H.-R. Hache, L. Schubert, P. Werner and U. Gasele, "Ordered Arrays of Silicon Nanowires Produced by Nanosphere Lithography and Molecular Beam Epitaxy," Nano Letters.(2005) 2524-2527 ,5
- [34] A. Wokaun and E. Henry, *Surface-Enhanced Electromagnetic Processes*, Solid State Physics, Academic Press, 223-294, (1984).
- [35] J. Kummerlen, A. Leitner, H. Brunner, F. R. Ausseneegg and A. Wokaun, "Enhanced dye fluorescence over silver island films: analysis of the distance dependence," Molecular Physics: An International Journal at the Interface Between Chemistry and Physics 80, 1031 - 1046 (1993).
- [36] K. Sokolov, G. Chumanov and T. M. Cotton, "Enhancement of Molecular Fluorescence near the Surface of Colloidal Metal Films," Analytical Chemistry 70, 3898-3905 (1998).