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Roadmap

Roadmap on biosensing and photonics with advanced nano-optical methods

Enzo Di Fabrizio[1,12], Sebastian Schlücker[2], Jérôme Wenger[3], Raju Regmi[3], Hervé Rigneault[3], Giuseppe Calafiore[4], Melanie West[4], Stefano Cabrini[4], Monika Fleischer[5], Niek F van Hulst[6,7], Maria F Garcia-Parajo[6,7], Annemarie Pucci[8], Dan Cojoc[9], Charlotte A E Hauser[10] and Ming Ni[11]

[1] Physical Sciences and Engineering Division, King Abdullah University of Science and Technology, Thuwal, 23955-6900, Saudi Arabia
[2] Physical Chemistry I, Faculty of Chemistry and Center for Nanointegration Duisburg-Essen (CENIDE), University of Duisburg-Essen, Universitätsstr. 5, Essen 45141, Germany
[3] CNRS, Aix-Marseille Université, Centrale Marseille, Institut Fresnel, UMR 7249, 13013 Marseille, France
[4] The Molecular Foundry, Lawrence Berkeley National Laboratory, One Cyclotron Road, Berkeley, CA 94702, USA
[5] Institute for Applied Physics and Center LISA$^+$, Eberhard Karls University of Tübingen, Auf der Morgenstelle 10, 72076, Tübingen, Germany
[6] ICFO-Institut de Ciencies Fotoniques, The Barcelona Institute of Science and Technology, Mediterranean Technology Park, 08860 Castelldefels (Barcelona), Spain
[7] ICREA-Institució Catalana de Recerca i Estudis Avançats, 08010 Barcelona, Spain
[8] Kirchhoff Institute for Physics, University of Heidelberg, Im Neuenheimer Feld 227, 69120 Heidelberg, Germany
[9] CNR-IOM Institute of Materials, Area Science Park—Basovizza, S.S. 14 km 163.5, Trieste 34149, Italy
[10] Division of Biological & Environmental Science & Engineering, Laboratory for Nanomedicine, King Abdullah University of Science and Technology, 4700 KAUST, Thuwal 23955-6900, Saudi Arabia
[11] Institute of Bioengineering and Nanotechnology, A*Star, 31 Biopolis Way, The Nanos, #04-01, Singapore 138669

E-mail: enzo.difabrizio@kaust.edu.sa

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Abstract

This roadmap, through the contributions of ten groups worldwide, contains different techniques, methods and materials devoted to sensing in nanomedicine. Optics is used in different ways in the detection schemes. Raman, fluorescence and infrared spectroscopies, plasmonics, second harmonic generation and optical tweezers are all used in applications from single molecule detection (both in highly diluted and in highly concentrated solutions) to single cell manipulation. In general, each optical scheme, through device miniaturization and electromagnetic field localization, exploits an intrinsic optical enhancement mechanism in order to increase the sensitivity and selectivity of the device with respect to the complex molecular construct. The materials used for detection include nanoparticles and nanostructures fabricated with different 2D and 3D lithographic methods. It is shown that sensitivity to a single molecule is already accessible whether the system under study is a single cell or a multitude of cells in a molecular mixture. Throughout the roadmap there is an attempt to foresee and to suggest future directions in this interdisciplinary field.

[12] Guest editor of the roadmap.

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Keywords: biophotonics, biosensing, nanomedicine, nanophotonics, plasmonics

(Some figures may appear in colour only in the online journal)

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1. Foreword

Enzo Di Fabrizio

King Abdullah University of Science and Technology

Introduction

Why should we write a roadmap on biosensing and photonics in these fast and changing times?

Firstly, let us state that in this roadmap there is no pretension to be exhaustive on the complete subject matter for the self-evident reason that it would be an impossible task. We were purposely agile with respect to completeness. Our aim is to give readers, especially young scientists, modular ideas that can be reclaimed for new and different tasks.

Optics and spectroscopy, along with nano manipulation, are the main themes of the roadmap. These disciplines started in earnest almost together and have progressed continuously over the centuries. What is new now is that experimenters mix, in unprejudiced and skilled ways, far-field, near-field and guided optics, with a general task to control the electromagnetism on a scale accessible to a few molecules or cells. To do this, miniaturization plays a key role. Today, when talking about nano-optics, nanofabrication is always involved. This can happen through the use of lithography or through colloidal synthesis, but commonly the sensitive material is a nanostructure where nano-optics is active.

Not long ago the most sophisticated optical system accessible to biology and medicine was the confocal microscope. Today, it is very common to meet biologists and physicians trained in advanced spectroscopy and optical manipulation techniques. The groups led by the authors of this roadmap have contributed substantially over the years to creating this new situation in modern laboratories.

What is expected in the near future is that new tools will become available on the market that provide researchers who are not the developers of these new methods with the possibility to access these new methodologies and extend them to their own research fields.

Going back to the question posed at beginning, the main reason to write a roadmap in this field is because it is useful. It is an effort to clarify the new possibilities at hand and the future developments and applications in biosensing and photonics.

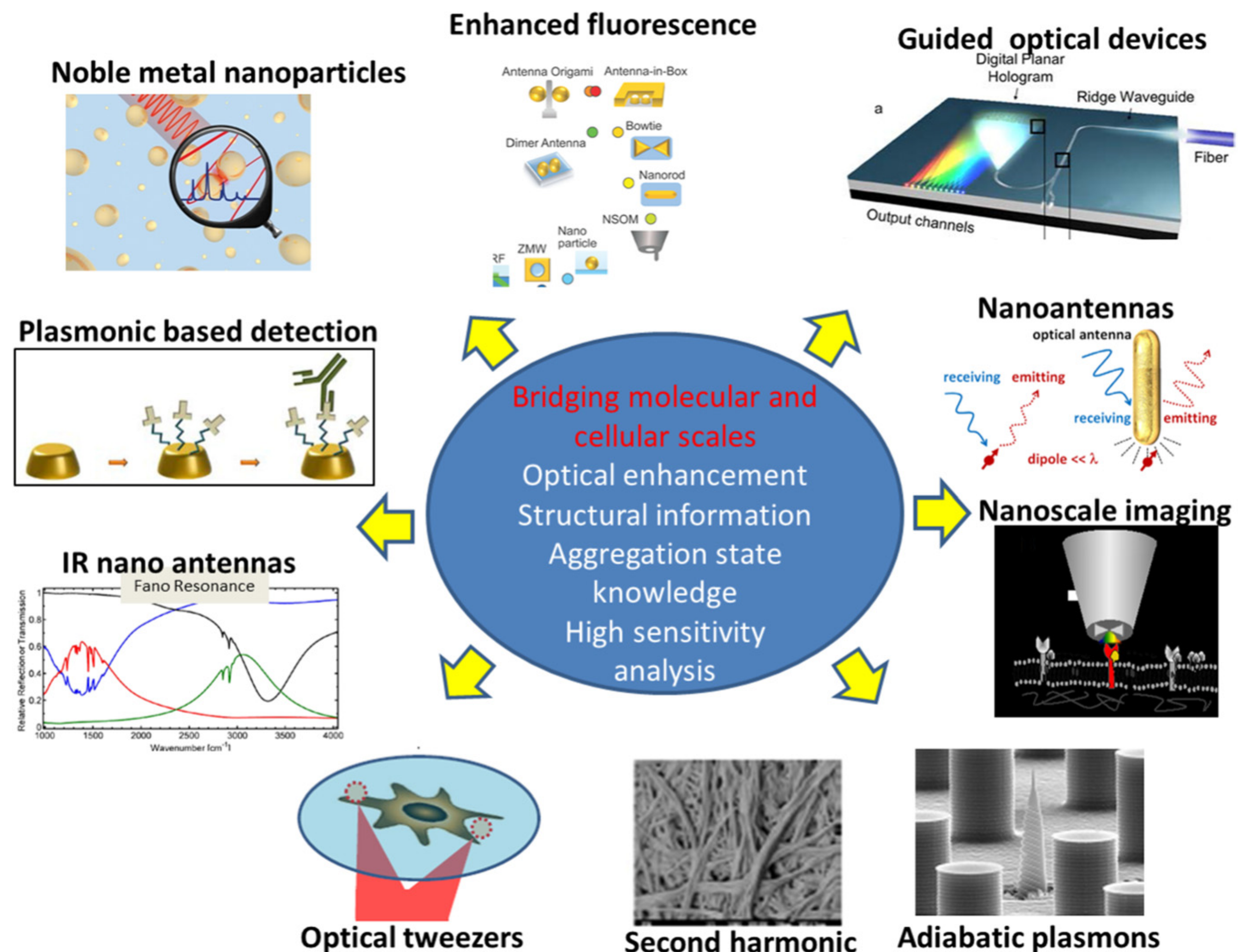

Figure 1. Illustration of how the themes covered within this roadmap tie together. Images are described in the individual sections.

What is important in reading the roadmap is to see the connections between different approaches and how they can be used to reach a higher level of information on the system under study. To this end, the schematic illustration in figure 1 shows where the subjects covered by this roadmap are explicitly connected.

The knowledge of optics in the matter is bridging the macro world with the molecular world. The knowledge of phenomena on this wide length scale will probably contribute to the comprehension of the mystery of how local phenomena due to a few molecules contribute to collective phenomena acting at the macro scale.

Concluding remarks

In a fast changing landscape we are aware that a roadmap is just an effort, for ourselves and for others, to offer a clear direction to a research field. In our opinion this effort is worthwhile because we can compare in a lapsed time manner what was correct, what was wrong and what was an illusion. We will see in future years where these methods and related scientific questions will take us. Above all we are certain that what we will do in our day-to-day work is accountable and this verifiability will guide us, and whoever else is interested, to make the next leap.

We hope that this initiative will be useful for a wide community and will be inspiring for young researchers who approach this field.

Acknowledgments

I thank all the contributors for their prompt responses to and enthusiasm for the invitation from the publisher, Jarlath McKenna, and myself; thanks too to the referees, who with their suggestions improved the content of this work.

2. Optical spectroscopy and chemistry with noble metal nanoparticles

Sebastian Schlücker

University of Duisburg-Essen

Status

Noble metal nanoparticles exhibit fascinating optical and chemical properties. Our ancestors used them for aesthetic reasons long before the rise of nanoscience and nanotechnology. One ancient example is the famous Roman Lycurgus cup containing colloidal gold. In the beautiful medieval windows in various European cathedrals, the bright and impressive colours are due to the inclusion of noble metal colloids into glass. The physics of the absorption and scattering of light from small particles is nowadays well understood. German physicist Gustav Mie pioneered the theoretical description in 1908, including the treatment of the optical properties of colloidal gold.

The free electrons of the metal nanoparticles can be forced to perform collective oscillations upon resonant excitation by light at the plasma frequency. The resulting localized dipolar surface plasmon resonance (LSPR) shown in figure 2 can decay via two different channels. In the radiative decay channel, the oscillating Hertzian dipole leads to the emission of radiation at the excitation frequency. The particle acts as an optical antenna (or nanoantenna) and elastic/resonant Rayleigh scattering is observed. In addition, Raman scattering from molecules on or near the metal surface is enhanced by several orders of magnitude in a process known as surface-enhanced Raman scattering (SERS). In the non-radiative decay channel, electron–hole pairs are generated via intra- or inter-band excitation above the Fermi level. Highly energetic, so-called hot electrons and holes can be used for reduction and oxidation chemistry of molecules on or near the metal surface. A limitation is the fast recombination of these charge carriers. Due to electron–phonon coupling, the temperature in the nanoparticle finally rises. This photothermal heating can be exploited, for example, in targeted tumor therapy.

Chemists have developed numerous elegant strategies for the synthesis of noble metal nanoparticles with control over important parameters such as size and shape. Various characterization techniques such as electron microscopy and optical spectroscopy are important to understand the correlation between size and shape (i.e. structure) on the one hand and optical/LSPR properties on the other hand. Experiments on single particles are particularly useful for identifying structure–activity (LSPR) relations.

Applications of noble metal colloids include, among other areas, LSPR biosensing, labeling and imaging, catalysis and surface-enhanced spectroscopies. Although much is known about the physics and chemistry of noble metal colloids, several challenges remain to be addressed in the 21st century.

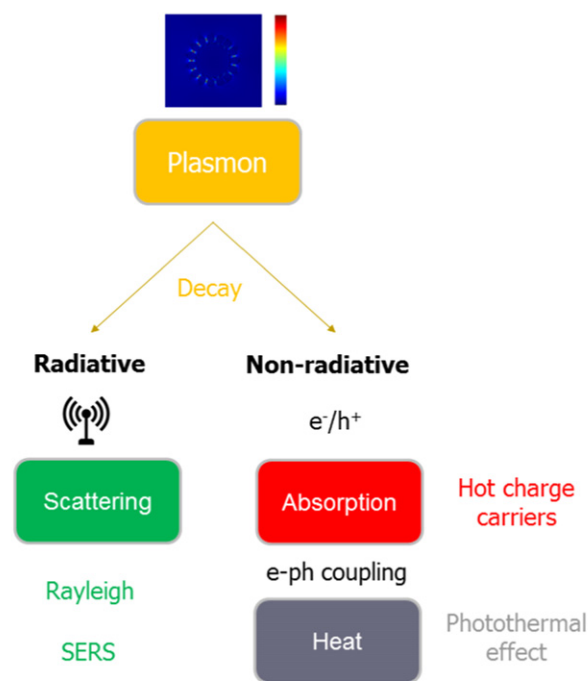

Figure 2. Schematic decay of a localized dipolar surface plasmon resonance.

Current and future challenges

A selection of grand challenges is necessarily subjective and biased by the ignorance and limited expertise of the author. Nevertheless, this section tries to address this issue below by highlighting three 'hot' research topics on noble metal nanoparticles from the perspective of physics and chemistry: (i) quantum plasmonics [1–3], (ii) single molecule SERS, and (iii) chemistry with hot electrons/holes [4–6].

(i): In most cases, classical physics is sufficient to reliably predict the optical properties of noble metal colloids, including parameters such as plasmon peak/LSPR position and local field enhancements. However, under certain circumstances a description by classical physics is not sufficient. For instance, in coupled plasmonic nanostructures such as dimers of nanoparticles with gaps <1 nm, quantum mechanical phenomena such as tunneling occur. Therefore, the incorporation of quantum mechanics is required. However, a full quantum mechanical treatment of, for example, a dimer of 50 nm gold nanoparticles is computationally extremely demanding and far from being feasible at the moment. On the experimental side, controlling the gap size experimentally between particles with sub-nanometer, i.e. Ångstrøm resolution, is challenging as well. A different attractive option is to control the 'communication' between the two particles by using rationally designed molecular bridges with well-defined physico-chemical properties including electric conductivity.

(ii): Hot spots, i.e. highly localized extreme field enhancements occurring in the gap between two particles or at tips, are required for single molecule SERS. Nanofabrication techniques provide access to many (nearly identical) plasmonic nanostructures with control of the position of hot spots. For the observation of single molecule SERS, the

corresponding molecule must travel through this hot spot. The most conclusive demonstration of single molecule SERS is currently the isotopologue approach, in which isotopic substitution allows spectral discrimination between 'chemically identical' molecules with the same surface affinity and scattering cross section. Although significant advances in the field of single molecule SERS have been made, this technique is far from being a routine tool in molecular vibrational spectroscopy.

(iii): Many reactions in chemistry involve the transfer of electrons from an electron donor to an electron acceptor. Such redox reactions typically involve molecules as oxidation and reducing agents. Plasmonic nanostructures are an attractive addition to this concept since hot electrons for reduction and hot holes for oxidation chemistry can be exploited. The option to control the injection of electrons into specific orbitals of a molecule on the metal surface, for instance, paves the way to highly selective reaction routes and mild reaction conditions. The potential to complement and expand current chemical synthesis routes is immense. However, a detailed fundamental understanding of the underlying processes is required to fully exploit this potential.

Advances in science and technology to meet challenges

A number of advancements are required to address the challenges facing the three research topics identified above.

(i): Since a full quantum mechanical description of complex plasmonic nanostructures is computationally extremely costly, intelligent strategies to combine and bridge classical and quantum mechanics are required. Experimentally, both innovative top-down and bottom-up approaches are required to control very short distances between two or more particles. Rationally designed linker molecules, typically involving organic synthesis, are one attractive alternative for achieving this.

(ii): Observing continuous rather than sporadic single molecule SERS signals is quite challenging. Specifically, this requires the molecule to be selectively placed into the hot spot. Innovative routes for achieving this are required. Using additional molecular linkers or bridges to immobilize the single molecule of interest or exploiting the photophysical properties of the hot spot itself may be opportunities towards permanent single molecule SERS.

(iii): The recombination of charge carriers generated upon LSPR excitation is a severe problem since hot electrons and hot holes are necessary for reduction and oxidation chemistry of molecules on the metal surface. Recovering holes and transforming them back by using additional external chemical agents with a high surface affinity may be one approach with general applicability [6]. Sophisticated experimental techniques in the space, time and frequency domain, ideally applied to single plasmonic nanostructures, are required to resolve the underlying mechanisms. Further, a close interaction between experimentalists and theoreticians is highly desired for analysis, modeling, and also the prediction of chemical reactivity and reaction mechanisms.

Concluding remarks

Optical spectroscopy and chemistry with noble metal nanoparticles requires expertise from various scientific disciplines, ranging from physics, chemical synthesis and physical chemistry to material science. Single research groups typically have expertise in a particular field. However, efforts to solve the above mentioned challenges will greatly benefit from international collaborations across disciplines, e.g. between groups with skills in solid-state physics, plasmonics, surface science, space-, time- and frequency-resolved spectroscopy, inorganic, organic, physical and theoretical chemistry.

Acknowledgments

I thank the members of the nanobiophotonics lab at the UDE in Essen, Germany, for many stimulating discussions and the German Science Foundation (DFG) for financial support. The image in section 1 is reproduced with acknowledgment from [7].

3. Plasmonic-enhanced fluorescence detection of single molecules at high concentrations

Jérôme Wenger, Raju Regmi, Hervé Rigneault

CNRS, Aix-Marseille Université

Status

Single molecule detection techniques have revolutionized biological sciences by enabling two major applications: super-resolution optical microscopy with nanometer accuracy and single molecule DNA sequencing. These breakthroughs offer a powerful new vision to observe how single molecules work and interact in a physiological environment with the long-term goal of elucidating the mechanisms that drive the function of living cells.

Although enormous progress has been made in the optical detection of single molecules over the last two decades, efficiently detecting a single molecule remains a major challenge. The main limiting factor is the fundamental phenomenon of diffraction of light. Owing to the size mismatch between a single molecule (below 5 nm) and the wavelength of light (around 500 nm), efficient interaction between the propagating light field and the molecular emitter is not possible using diffraction-limited optics. The direct consequences are low fluorescence signals and large statistical noise.

Moreover, optical microscopes generate detection volumes on the order of 0.5 fL which impose nanomolar concentrations of the fluorescent species in order to isolate a single molecule in the detection volume. However, this condition of low molecular density does not meet the requirements of a large majority of enzymes and proteins which call for concentrations in the micro to millimolar range so as to reach relevant reaction kinetics and biochemical stability (figure 3). To observe a large class of enzymes and proteins at physiologically relevant conditions with single molecule resolution, the optical detection volume must be reduced by more than three orders of magnitude as compared to confocal microscopy [8].

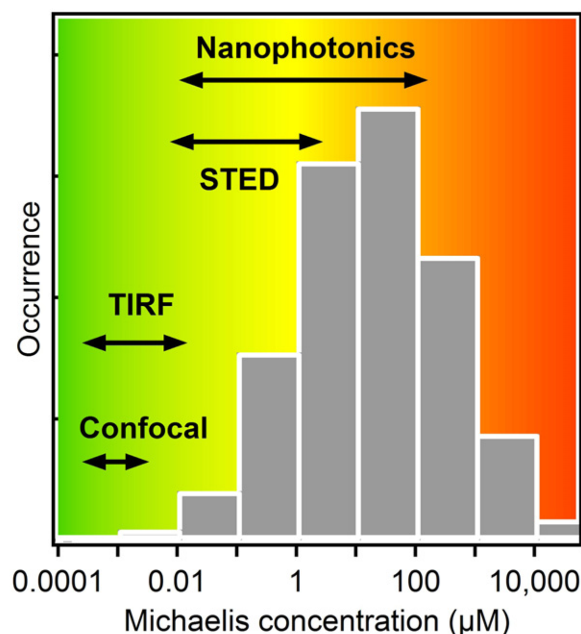

Figure 3. Histogram of Michaelis constant (the substrate concentration such that the reaction rate is half the maximum achievable rate) for 118 000 enzymes taken from the Brenda database (http://www.brenda-enzymes.org/). The arrows indicate the concentration regime reached by different techniques (TIRF = total internal reflection fluorescence microscopy, STED = stimulated emission depletion microscopy).

Current and future challenges

Several challenges must be met to breach the diffraction limit and monitor single molecules at high physiological concentrations [9]. First, light must be confined on the nanometer scale, far below the optical wavelength size. This can be reached by exploiting the unique optical properties of metallic nanostructures that support local surface plasmon modes, and has led to the concept of optical antenna. Like their radiofrequency counterparts, optical antennas convert propagating radiation into localized energy and enable efficient interaction between light and single quantum emitters. While metals provide nanoscale localization of intense light fields, metal losses limit the performance of plasmonic antennas via the phenomenon of non-radiative energy transfer from the molecule to the metal. This fluorescence quenching critically depends on the molecular distance and orientation to the metal as well as the fluorescence emission spectra position respective to the antenna mode resonances. Therefore, a balance must be found to maximize the net fluorescence enhancement.

A specific requirement for the detection of fluorescent molecules in solution at high concentration is the rejection of the background signal from molecules a few tens of nanometers away from the plasmonic antenna, yet still in the confocal detection volume. At the targeted concentrations of several micromolar, the number of non-enhanced molecules in the confocal volume can reach several thousand, and their fluorescence emission can severely outshine the signal from the single molecule enhanced by the plasmonic antenna.

While a specific design must be found for the plasmonic antennas and their integration into a microscope apparatus, the nanofabrication itself is another great challenge. Classical top-down techniques like electron-beam lithography or focused ion beam milling made decisive contributions to the development of nanophotonics, yet they are both expensive techniques to run and slow in throughput. Bottom-up self-assembly or stencil nanolithography offer alternative strategies to overcome these limitations.

Lastly, the selective immobilization of a target molecule in conjunction with the plasmonic antenna is another major challenge. This shall enable the specific observation of single molecule activity with enhanced sensitivity at physiological concentrations.

Advances in science and technology to meet challenges

The scientific field was pioneered in 2003 by the groups of Harold Craighead and Watt Webb by using a single nanometric aperture milled in an opaque metallic film to confine light below the diffraction limit [10]. As the aperture diameter is significantly reduced below half of the optical wavelength, the light does not propagate though the aperture and evanescently decays within a few tens of nanometers inside the nanoaperture, which has thus been called a zero-mode waveguide (ZMW). The nanoaperture provides a detection volume in the attoliter range enabling real-time single molecule DNA sequencing [11]. However, the fluorescence enhancement remains weak due to the lack of intense field localization (figure 4).

Plasmonic nanoantennas with sharp tips can sustain high local electromagnetic intensities thanks to the combination of the lightning rod effect, local surface plasmon resonance and coupling between nanoelements. For instance, a single crystalline gold nanorod [12] or bowtie nanoantenna [13] were shown to provide fluorescence enhancement factors above a thousandfold.

Specific strategies are being developed to overcome the background from the thousands of molecules in the diffraction-limited confocal volume. Current approaches involve using low quantum yield emitters and/or adding a metal cladding layer. The design termed 'antenna-in-box' combines a gap-antenna inside a nanoaperture [14], enabling single molecule operation at concentrations above 20 μM.

As an alternative strategy to top-down lithography techniques, DNA origami is a powerful method to achieve exquisite nanofabrication control for both the self-assembly of nanoparticles into complex antenna designs and localizing the desired target molecule in the antenna hot spot [15]. Advances in top-down lithography were also made to realize high-throughput fabrication of large-scale arrays of nanoantennas [16]. The approach relies on blurring-free stencil lithography patterning by dry etching through nanostencils to achieve reproducible and uniform control of nanoantenna structures.

Near-field scanning optical microscopy combined with plasmonic antennas brings another dimension for true nanoscale imaging with a spatial resolution close to the size of a single molecule. Using fluorescence localization techniques, two fluorescent molecules separated by only 2 nm could be separately visualized [17].

Concluding remarks

The recent achievements of plasmonics and nanophotonics make it possible to overcome the diffraction limit and confine the light towards dimensions similar to the size of a single molecule. These promising new techniques pave the way to bring single molecule analysis into a new dimension of molecular concentration reaching physiological conditions close to the native environment of living cells. A huge number of proteins and enzymes which until now were only monitored using ensemble measurements can now be investigated with single molecule resolution to reveal sample heterogeneity, subpopulations and dynamic disorder. Applying plasmonics and nanophotonics to single molecule detection at high concentrations holds great promise of revealing new insights into biological functions and dynamics.

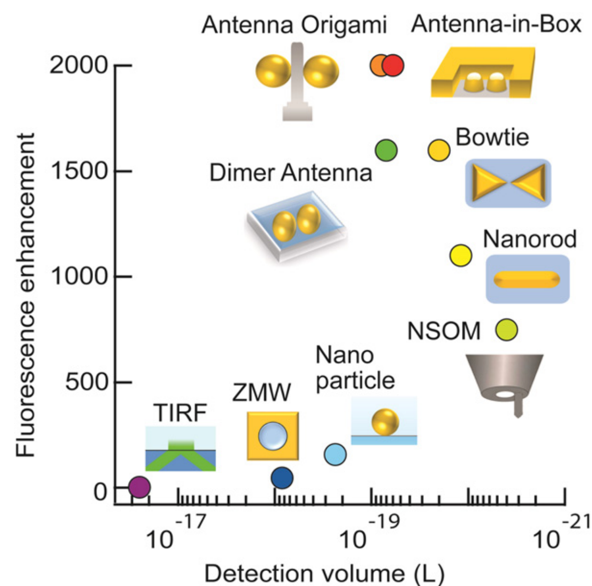

Figure 4. Comparison between different approaches to confining light and enhancing fluorescence. The fluorescence enhancement factors are estimated for an emitter with 2% quantum yield.

Acknowledgments

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4. Nanophotonic devices for biosensing

Giuseppe Calafiore, Melanie West, Stefano Cabrini

Lawrence Berkeley National Laboratory

Status

Current biomedical tools for sensing and diagnostics in laboratories consist of microplates—such as nucleic acid microarrays, or enzyme-linked immunosorbent assays (ELISA)—that simultaneously probe multiple samples for their responses to chemicals, living organisms or antibodies. Color changes in labels associated with compounds on the plates can signal the presence of particular proteins, drugs, or gene sequences. While such systems offer high multiplexing capabilities, they suffer from a slow response and long processing time. Moreover, these systems are often bulky and require laboratory equipment to function.

Conversely, miniaturized biosensing devices and high-throughput screening technologies could offer a new opportunity to realize the dream of personalized medicine. The inherent advantage of nano-biosensors is that the analyzed volume is much smaller compared to traditional detection tools, while maintaining a high sensitivity and excellent limit of detection (LOD) [18]. Unlike conventional techniques, biosensors can also be portable, more user-friendly, disposable, and fabricated at a low cost. A new class of biosensors is enabled by nanophotonics, which allows us to control the interaction between light and matter at the nanometer scale. Nanophotonic devices for sensing mainly exploit two different working principles to obtain a transduction signal: change in the refractive index (Δn), and confinement and enhancement of the electromagnetic field below the diffraction limit [18]. Based on the former principle, interferometric and resonant cavity based devices are coated with an antibody and a binding event with the target analyte causes a local change in the refractive index. As an example, Young and Mach–Zehnder interferometers [19] can yield LODs down to 10^{-7}, but require a long interaction length in order to aggregate a detectable Δn. Resonating photonic structures exploit their high quality factor to overcome such a limitation, with detection limits down to 10 pM, which rivals those of conventional ELISA [20]. Such sensors are based on microsphere, microring, bottleneck, Fabry–Perot, microcoil, photonic crystal, and whispering gallery modes resonators. Another class of nanophotonic biosensors exploits surface plasmon resonances (SPRs) to allow sensing [21]. Approaches like angular, spectral and local SPR can reach a LOD on the order of 100–1000 pg cm^{-2} [18], but still suffer from large interaction lengths that prevent spatial resolution. In recent years, near-field nanoprobes have provided evidence of unprecedented capabilities in terms of spatial resolution, while preserving a high sensitivity, down to single molecule detection [22]. All these devices promise great advancements in the field of nanomedicine, but present some challenges.

Current and future challenges

Nanophotonic devices, especially near-field plasmonic probes [23, 24], have made enormous progress in laboratories, but are very challenging to mass-produce due to their complex three-dimensional (3D) shape. Moreover, sub-10 nm patterns in plasmonic nanostructures can be fabricated only by timely and costly lithography techniques, i.e. electron-beam lithography. For this purpose, a promising technique with sub-8 nm resolution and high throughput is nanoimprint lithography (NIL) [25]. Although simpler lithography techniques to fabricate plasmonic devices exist—e.g. nanosphere lithography—they cannot support complicated layouts that characterize the next generation of nano-biosensors. NIL is potentially inexpensive and offers the flexibility to handle the fabrication of both photonic and fluidic structures at the same time.

Another key challenge that must be addressed to bring research grade biosensors to the clinical market is the on-chip integration of auxiliary components, such as spectrometers, micro/nanofluidic channels, nano-mechanical devices, microcontrollers, and a read-out system. On-chip spectrometers provide a solution for integrating optical functionality with multiplexing capabilities for pathogen detection [26]. By means of different biomarkers or labels, fluorescence spectral analysis can identify the analyte composition and gain a quantitative reading of different pathogens on the same platform (figure 5). Near-field Raman spectroscopy is another powerful multiplex detection technique that, unlike the previous approach, allows for actual label-free detection by reading the spectral fingerprint of analyte molecules. However, there are several challenges associated with the development of a hand-held near-field Raman analysis device: nanometer alignment between the near-field probe and target molecule, complex on-chip integration, high-resolution lithography, and analyte immobilization. The combination of a plasmonic probe and a nanofluidic channel can effectively address these constraints and allow for real-time, single molecule detection, and DNA sequencing (figure 6) [25]. Unlike most label-free optical biosensors, this approach uses a self-aligned configuration where target molecules are delivered to the sensing area, which is a complicated task and major drawback for point-of-care applications. Such a device can be fabricated in parallel by NIL, where multiple nanochannels and nanoantennas of varying dimensions can be fabricated in one step.

Advances in science and technology to meet challenges

NIL has already proved its great potential for addressing the challenges of future nanophotonic biosensors. It has demonstrated the possible integration of microfluidics and plasmonics in a single, low-cost fabrication step, and is shown to be powerful with the fabrication of complex photonic devices. Nevertheless, NIL as a mere lithography tool cannot address all of the technological challenges of photonic biosensors. Primarily, the need to add bio-recognition agents—i.e. antibodies—and localized surface functionalization cannot be

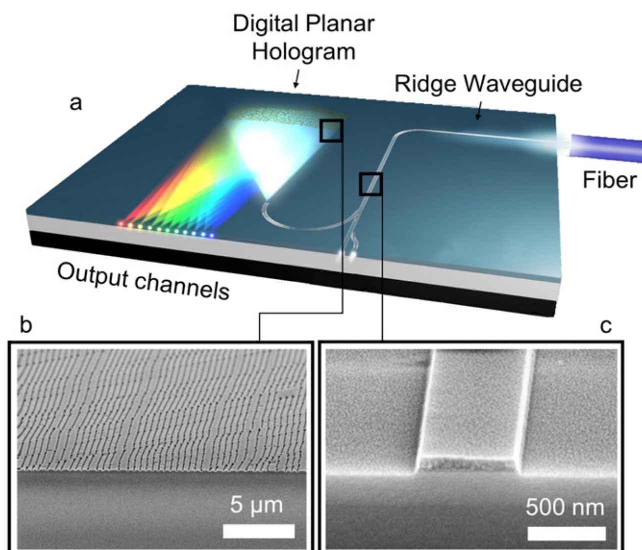

Figure 5. (a) Scheme of the on-chip spectrometer that integrates a digital planar hologram (DPH) together with an optical circuitry. (b) SEM micrograph of the DPH (feature size below 100 nm). (c) SEM micrograph of the ridge waveguide.

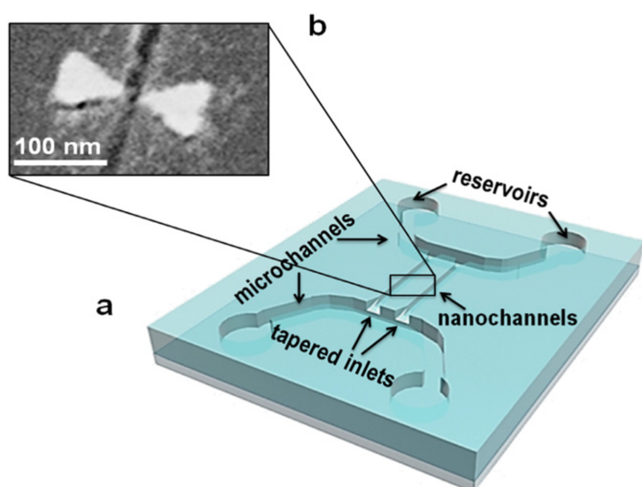

Figure 6. (a) Scheme of the biosensor device with a fluidic system fabricated by NIL. (b) SEM image of a fluidic nanochannel and plasmonic nanoantenna, where target molecules are delivered to the sensing area.

performed directly by NIL. Just as the successful manufacturing of a photonic biosensor relies on the comprehensive integration of different functionalities, the fabrication techniques must compatibly interface with each other in a cost-effective manner. An interesting route towards this goal would be the combination of NIL—as a lithography means—with molecular imprinting—a technique used in chemistry to create template-shaped cavities for molecular recognition. This would allow the inexpensive patterning of biological agents in compatibility with the lithography requirements of the photonic components. The multidisciplinary nature of the project also calls for fabrication techniques that can make use of functional materials. Such materials embed specific functions required by the device. For instance, the imprint of a high-refractive index material could allow a single-step fabrication of the photonic components in the sensor [27], while NIL of a conductive polymer could facilitate the integration of contacts and other simple electrical components.

Further advancements in the capabilities of high-resolution 3D patterning are also necessary. Current solutions for 3D patterning mainly consist of two-photon lithography and layer-by-layer fabrication. Although progress in this field is coming at a very fast pace, the former technique cannot cope with the high-resolution requirements of plasmonics, while the latter is costly and time consuming. 3D patterning technologies with nanometer scale resolution are highly desirable as they are expected to trigger a fast development in the field of nanophotonic biosensors.

Concluding remarks

The underlying physical properties of nanophotonics are seamlessly integrated with a wide range of chemical surface modification techniques, nanofluidics, and biological interfacing schemes to yield hand-held biosensors with superior sensitivity and detection limits. Plasmonic devices operate label-free and can reach sub-10 nm spatial resolution, enabling the detection of new biomolecular interactions at the single molecule level. Although the potential of these technologies to benefit point-of-care diagnostics has been demonstrated for decades, the majority of lab-on-a-chip systems still lack the required cost-effectiveness to make a large impact on the medical diagnostic industry. To maximize the benefits provided by biosensors, multiple functions should be integrated onto the same stand-alone, user-friendly platform, which can efficiently handle a wider range of clinical samples.

From the technological standpoint, this can be achieved by means of fabrication techniques like NIL, which can meet the required resolution, throughput, and cost-effectiveness. The use of functional materials in NIL for direct patterning and further advancements in 3D patterning can speed up the time-frame for the widespread use of nanophotonic biosensors in clinics and fields.

Acknowledgments

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5. Plasmonics and detection

Monika Fleischer

Eberhard Karls Universität Tübingen

Status

Sensitive detection plays a major and increasing role in fields such as medicine, chemistry, biology, environmental or health monitoring, security, and more. Various detection schemes are aiming towards ever improved detection limits, specificity, multiplexing, signal levels, and signal-to-noise ratio.

With the advent of plasmonics a new tool became available for label-free detection [28–31]. Plasmons denote collective oscillations of the free electron density that are located at metal–dielectric interfaces (known as surface plasmons, or SPs) or plasmonic nanostructures (known as localized surface plasmons, or LSPs; see also Roadmap sections 2 and 6). SPs and LSPs can be excited by electromagnetic waves and are associated with enhanced electric near-fields, which are well adapted for molecule detection and real-time probing of kinetic binding processes. The surface may be functionalized with a recognition structure (figure 7).

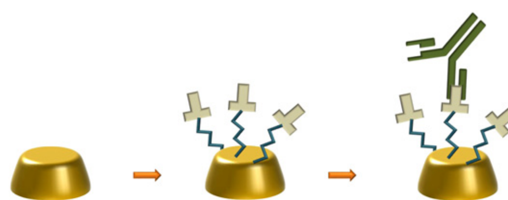

Figure 7. Antibody detection via specific binding to plasmonic nanostructures (courtesy of A Horrer).

Prominent examples of plasmonic detection

SP resonance detection. In *SP resonance detection*, a thin metal film on a prism is illuminated in total internal reflection [28, 29]. The intensity of the reflected beam is measured under variation of, for instance, the angle (or the wavelength, intensity, or phase [31]). A sharp minimum is observed at the angle where the evanescent wave couples to the SP when their wave vectors match. Upon molecules binding to the surface, the effective local refractive index changes. The resulting shift in the angle can be detected with high precision (figure 8(a)).

LSP resonance shift sensing. In *LSP resonance shift sensing*, the plasmon resonances of individual nanostructures are spectrally detected [31, 32]. When an analyte attaches to the surface from liquid or air, a noticeable concentration-dependent shift in the resonance wavelength is observed due to a change in the local refractive index (figure 8(b)). The shift can be detected with high precision.

Surface enhanced Raman sensing. In *surface enhanced Raman sensing* (SERS), the Raman intensity from molecules at nanostructured plasmonic surfaces can be increased by many orders of magnitude through the enhanced electric near-field, allowing for chemically specific detection (figure 8(c)) [33].

By advancing plasmonic detection, contaminant traces can be more easily detected, smaller test volumes suffice for health and safety applications, and detectors can be miniaturized for field use or point-of-care [34].

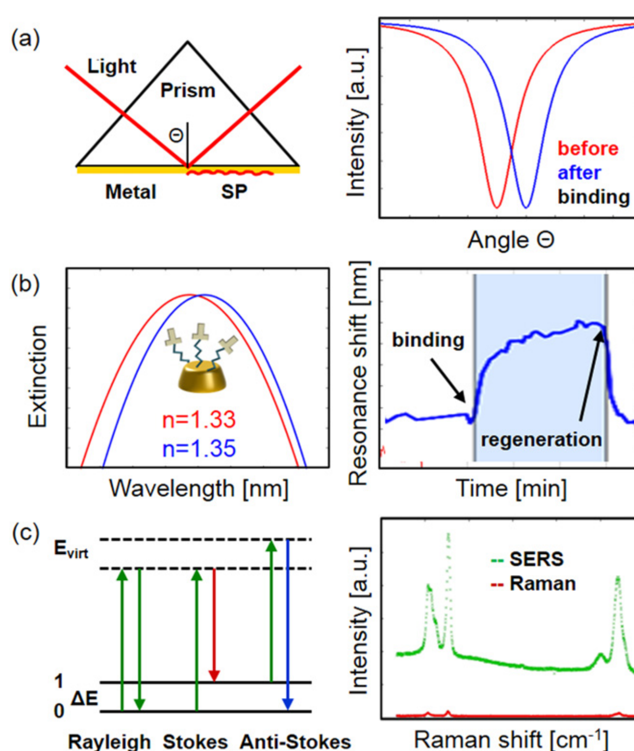

Figure 8. Plasmonic detection schemes: (a) SP resonance, (b) LSP resonance, (c) SERS (courtesy of A Horrer and C Schäfer, M Fleischer group).

Current and future challenges

SP resonance sensors are well established for characterizing and quantifying biomolecular interactions. Key performance factors are their sensitivity, resolution, and limit of detection, where limits of ng ml^{-1} down to pg ml^{-1} can routinely be reached [29]. For further development, additional affordable bio-specific surfaces with strong suppression of non-specific binding are required. Since the detection signal depends on the modification of the local dielectric function and does not carry any chemical information, non-specific binding can lead to strong artefacts. Complex analyte solutions need to be pre-treated to avoid contamination of the microfluidic environment. As further challenges, multiplexing for parallel detection of different analytes, high throughput and detection speed, and miniaturization are targeted.

Here the advantages of LSP sensing and SERS come into play. Nanostructured surfaces can be implemented in lab-on-chip environments for small hand-held devices. Individual nanostructures may be functionalized with specific

recognition structures to allow for densely packed multiplexing. The wave vector matching condition for the excitation of SPs is relaxed for LSPs, which allows for excitation by simple illumination.

In LSP detectors, a major challenge lies in increasing the sensitivity and limit of detection, ideally down to single molecule sensing. For this purpose the figures of merit need to be optimized, requiring sharp resonances and large shifts per refractive index unit.

In SERS, the homogeneity of the signal enhancement across the surface is a main concern. SERS depends minutely on nanoscale variations of the topography and local near-field, beyond the level of precision currently accessible with nanofabrication. While SERS has the great advantage of providing chemical information, quantification of the analyte is extremely challenging.

Both LSP resonance sensing and SERS suffer from the kinetics of analyte binding. For low concentrations, the probability that a molecule attaches to the active areas of the nanostructures is extremely low. Therefore techniques for targeted analyte delivery are required (cf section 10).

From a technical point of view, challenges furthermore include cost-effective parallel fabrication processes (cf sections 4 and 7) and fast and automated read-out. From a fundamental point of view, an ever improving understanding of the underlying Raman and energy transfer processes as well as of the metal–organic interface is pursued. Improved modelling could pave the way to simultaneous multi-resonance sensing at different length scales.

Advances in science and technology to meet challenges

LSP sensing including SERS depends sensitively on the morphology of the nanostructures. Nano-plasmonic detection was only made possible through the rapid development of nanotechnology. Still, important limitations remain. The accuracy of most lithographic techniques is limited to ~10 nm. Single digit lithography, atomic layer deposition, advances in colloidal synthesis or size-selected cluster preparation as well as low-defect passivation and functionalization layers need to be further pursued. Transfer into cost-effective, robust, reproducible and scalable fabrication techniques is mandatory. In many cases plasmonic detection still requires highly trained operators and elaborate post-processing. Here extensive databases of Raman spectra together with advanced system integration and fast, simple, reliable read-out and data processing are important features to bring nanoplasmonic detection from the laboratory to the general market place.

Various recent publications demonstrate interesting paths towards advanced nanostructure design for high figures of merit in LSP resonance sensing, e.g. using hole arrays [35] or Fano resonances, core–shell structures or collective modes to achieve narrow full-widths-at-half-maximum, as well as harnessing hybrid multi-component structures. Miniaturization for hand-held devices and parallelization in advanced microfluidic schemes that enable multiple analyte mixing ratios and detection sites are under development for medical applications [36]. Superhydrophobic nanostructured surfaces with designated detection sites as well as force gradients are being used for targeted delivery of analyte at ultra-low concentrations [34]. Reference nanostructures are being included to correct for drifts and artefacts in real time. Such promising approaches need to be further advanced and optimized, also using simulations for parameter space studies. Successful approaches need to be scaled up using micro- and nanofabrication to meet the aforementioned challenges. Advances in science require a still deeper understanding of light−matter interaction, intensity enhancement, local orientation and dielectric tensors of molecules, etc.

At the same time new developments keep appearing that may lead to paradigm shifts in the existing approaches, such as the introduction of different materials (e.g. highly doped oxides) that offer the advantages of fewer losses, better tunability and CMOS compatibility compared to the typically used noble metals. New basic concepts are being proposed for plasmonic detection, such as combining plasmonic apertures with optical trapping, exploiting nonlinear effects (see section 11), or moving towards active plasmonic sensing [34]. Such advances cannot always be predicted, but may open entirely new roads for plasmonic detection.

Concluding remarks

Plasmonics has been a fast-growing research area for the last few decades. Sensing has been identified as one of the main application areas for plasmonic structures [37]. Optical detection offers the advantages of non-invasiveness, easy access, and fast signal transfer as well as mature excitation and read-out schemes. Using plasmonics brings the added value of high surface sensitivity and strong electric near-field enhancement for improved detection limits, as well as label-free detection. Although impressive progress has been made in plasmonic detection, with the exception of SP resonance sensing the techniques are still mostly confined to research environments. With some additional efforts in theoretical description, high-precision and parallel nanofabrication, data processing and surface chemistry however, plasmonics-based detection offers high potential for widely distributed miniaturized sensing devices with ultra-high sensitivity and chemical specificity.

Acknowledgments

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6. Optical antennas for localized and enhanced interaction with single-photon emitters

Niek F van Hulst

The Institute of Photonic Sciences, ICFO, and Catalan Institution for Research and Advanced Studies, ICREA

Status

Optical antennas offer unprecedented possibilities to control and enhance the interaction of light with nanoscale matter: emission and absorption can be controlled by near-field coupling to a properly designed antenna mode. Optical antennas have been exploited to improve single molecule detection, to brighten single-photon sources and to achieve true nanometric resolution microscopy [38, 39]. Ultimately optical antennas do provide complete control over the local optical field to engineer electromagnetic parameters like phase, dispersion, fs-response, polarization state, direction of radiation and even optical angular momentum, which are all the subject of active research.

The local near-field coupling is crucial in improving the interaction of an object with the optical radiation of an optical antenna. The 'object', a molecule, quantum dot, protein or color center, both receives and emits light through the antenna modes. Metallic nanoparticles are especially suited as optical antennas because they support confined plasmon modes that respond strongly to light: the electronic transitions of quantum emitters can be controlled, the excitation and emission rates enhanced, the spectral dependence shaped, and the angular emission directed.

The modes of optical antennas are analytically described by the Mie solutions for ellipsoids, while nanorod antennas are usually described in terms of resonators or Fabry–Perot cavities. If the wave vector along the rod antenna is known, the relation between length and spectral resonance can be determined [40, 41]. However, the functionality of an antenna is not just given by its resonance length or wavelength, rather the local near-field interaction of the antenna modes with the quantum emitter is crucial. Strongest local field effects are generally found at the fundamental dipole mode (half-lambda-mode) or in nanogaps between antennas. Considering nanorods as one-dimensional cavities, analytical models have been derived for the interaction of dipolar transitions with radiation through nanorod optical antenna modes, which accurately describe the super-radiant, sub-radiant, and dark modes, and all the emission characteristics: the radiative decay rate, quantum efficiency, and angular emission [42].

Current and future challenges

A free emitter in vacuum experiences a certain mode density which dictates its spontaneous decay rates. In proximity to an antenna the mode density can be strongly increased by proximity of the metal interface and further enhanced by local concentration of field lines and resonance with a plasmonic mode. Figure 9 depicts a nanorod acting as receiving and emitting antenna for a dipolar quantum emitter close to the rod end. Field lines come together at the rod end, creating a hot spot, a position with high mode density. For optimal interaction the dipole emitter should have its transition dipole moment along the local field lines. The angular emitted power increases by tuning the antenna length. Figure 9 shows the transition rate as a function of the antenna length. The strength of the coupling reaches a maximum rate of 100–1000 times the vacuum rate at the dipolar resonance length of the nanorod. The rate enhancement occurs both for emission and reception. An incident plane wave, at the resonance frequency, will equally be enhanced at the hot spot position of the dipole, i.e. acting as a nanofocusing point. The challenge now is to create experimentally the conditions as sketched in figure 9, for which both fabrication of the nanoantenna and positioning of the dipole emitter are critical.

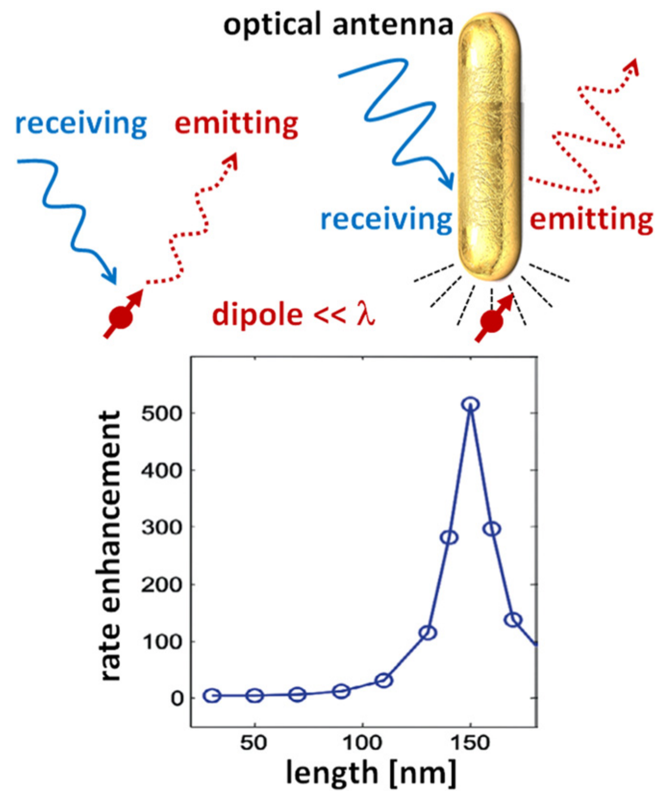

Figure 9. Optical antennas for enhanced and localized interaction. Both excitation and radiative rates of a single dipole oscillator are enhanced at high field density points of an optical antenna (top). By tuning the length of a rod antenna to its dipole resonance the rates can be enhanced by 2–3 orders of magnitude (bottom) [43].

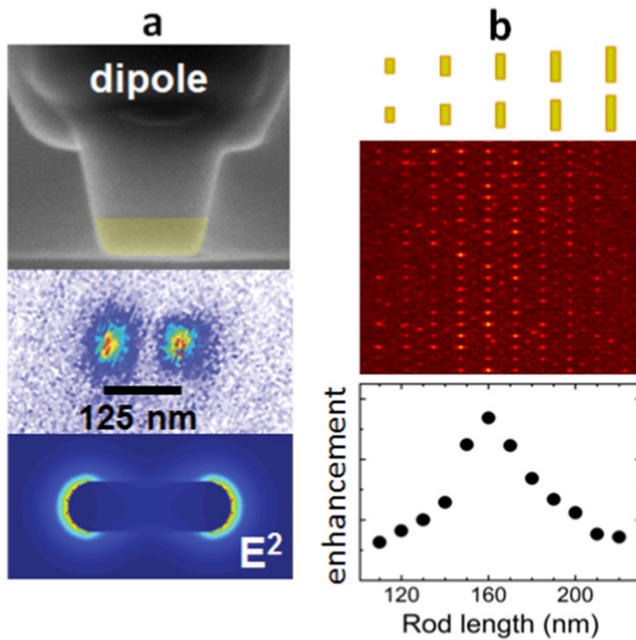

Figure 10. (a) local antenna field: a dipole antenna (top) scanning over a single molecule reveals its localized hot spots (middle, 30 nm FWHM), in agreement with simulations (bottom) [44]. (b) Resonance enhancement: an array of rods with increasing length reveals its dipole length in resonance with the fluorescence of molecules on top [45].

Advances in science and technology to meet challenges

For strong antenna enhancement several conditions need to be met.

Principally, the quantum emitters need to be positioned in the hot spot of the antenna mode for optimal coupling. Such nanoscale control can be achieved by fabricating the resonant antenna at the vertex of a scanning tip (figure 10(a)). Next, scanning the nanoantenna in close proximity to single fluorescent molecules the local plasmonic mode is revealed with nanometer molecular resolution. In fact one maps the x-, y-, and z-field components of the dipole antenna [44]. Figure 10 displays the mode map for a z-oriented molecule, which is invisible in conventional confocal imaging. In imaging mode such an antenna probe reveals single molecules with a 30 nm FWHM response function, making the scanning resonant antenna an ideal tool for extreme resolution bioimaging.

Obviously, for optimized enhancement the antenna needs to be resonant with the excitation and/or emission wavelength of the quantum emitter. Varying the length of a nanorod is a basic approach to tune the resonance. Figure 10(b) shows a schematic picture of an antenna array with increasing length. Such an array is easily fabricated by e-beam lithography and subsequent lift-off. After spin coating the array with fluorescent molecules the confocal fluorescence image reveals the dipolar longitudinal mode, resonant with the emission and excitation bands of the molecules. The boosting of fluorescence is firstly due to excitation enhancement, while for emitters with low quantum efficiency also emission enhancement plays a role [45]. Only a small fraction of the molecules is located in the antenna hot spots; the fluorescence of these strongly enhanced molecules is effectively reduced by a higher number of unenhanced molecules.

Concluding remarks

Clearly optical antennas are ideal for both enhanced detection and extreme resolution microscopy. The main challenges are the nanofabrication, the control of resonance and the nanoscale localization of the molecules to be probed.

Acknowledgments

I thank the members of my Molecular NanoPhotonics team at ICFO. This research was funded by ERC Advanced Grant 247330-NanoAntennas, EU-project NanoVista (FP7-ICT-2011-7, 288263), Plan Nacional projects FIS2009-08203 and FIS2012-35527, CONSOLIDER CSD2007-046 NanoLight, Fundació CELLEX (Barcelona), Marie-Curie International Fellowship COFUND and the ICFOnest program.

7. Nanophotonic approaches for nanoscale imaging and nanospectroscopy in living cells

Maria F Garcia-Parajo

ICFO-The Institute of Photonic Sciences and ICREA- Institucio Catalana de Recerca i Estudis Avancats

Status

Understanding how the surface machinery of living cells works is crucial to distinguish healthy from pathological cell responses. This requires the visualization of individual molecular interactions as they dynamically occur in living cell surfaces. Although modern molecular biology has made enormous progress in identifying a full repertoire of proteins, lipids and other molecular components both inside and at the cell surface, monitoring how molecules interact in space and time remains challenging. The difficulty arises because of the high concentration levels of membrane molecular components, their dynamic behavior and their reduced interaction space (figure 11). Indeed, the lateral distribution of molecules on the cell surface occurs at the nanometer scale [46–48], a size regime not accessible by standard optical microscopy as it suffers from diffraction.

In recent years, the development of far-field optical techniques able to surpass the diffraction limit of light is advancing our understanding of the cell surface organization at the nanometer scale. These 'super-resolution' techniques make use of specific photophysical properties of fluorescence probes in conjunction with tailored methods of diffraction-limited illumination to reach optical resolutions or localization accuracies on the order of tens of nanometers. Moreover, dual color imaging on fixed cells with localization accuracies below 10 nm have been recently reported. Unfortunately, increasing the localization accuracy beyond these values using far-field optics has proven to be technically challenging due to experimental inaccuracies in registering multi-color single molecule localization events. Importantly, extending these approaches to living cells is far from trivial since current methods are very slow compared to the dynamic nature of the cell membrane.

Current and future challenges

The pressing need to visualize molecular dynamic events at the nanoscale in living cells has led in recent years to the development of novel techniques that do not rely on imaging but rather on the recording of fluorescence from diffusing molecules as they traverse the illumination region. For instance, stimulated emission depletion microscopy (STED) has been combined with fluorescence correlation spectroscopy (FCS) to reduce the illumination area to ~20 nm [49]. However, STED-FCS suffers from drawbacks that require further technological development, including the high power density of the STED depletion beam (10–100 MW cm^{-2}), which compromises cell viability, and the difficulty of extending the technique to simultaneous multi-color excitation.

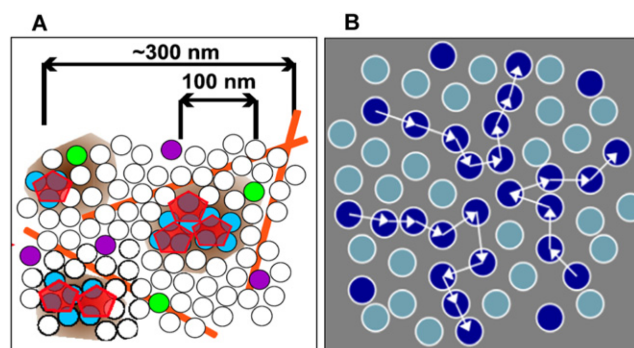

Figure 11. Graphical representation of the cell membrane. (A) Lipids (white circles) and different proteins (green, purple and cyan circles) organize in small heterogeneous compartments smaller than 100 nm in size (gray shadow regions) that are further compartmentalized by the actin cytoskeleton in regions varying from 100 to 300 nm (red lines). (B) This organization is dynamic and molecular components diffuse differently on the cell membrane (illustrated by three different trajectories of dark blue diffusing species).

As such, several technological challenges must be overcome to reach the final goal of mapping the full dynamic landscape of living cell membranes.

First: observation volumes should be confined at the nanometer scale, matching the spatial scales of the molecular organization on cell membranes;

Second: multi-color imaging should become available so that multiple components can be simultaneously mapped with sub-nanometric accuracy;

Third: multi-color approaches must be extended to simultaneous recording of dynamic interactions between different molecular components;

Fourth: methods should become scalable to account for the inherent heterogeneity of living cells.

Nanophotonic approaches have recently emerged as attractive tools to investigate the nanoscale dynamics of living cell membranes, potentially overcoming most of the barriers associated with diffraction-limited and super-resolution methods. In particular, metallic nanostructures known as photonic antennas hold great potential for biology as they both confine and amplify optical fields at the nanometer scale (figure 12) [50, 51]. Moreover, they can be designed for broadband operation allowing for multi-color light confinement in the visible regime [17]. While the unique physical properties of these devices have been extensively studied with first applications to different fields of science, their extension to biology is still in its infancy. Current and future challenges remain on the nanofabrication side, such as the compatibility with live cell studies and the complex nature of the near-field components emanating from these nanostructures, which complicates the reliable analysis of fluorescence imaging and dynamic recording in living cells.

Advances in science and technology to meet challenges

The nanofabrication challenge

The investigation of biological processes in living cells demands cost-efficient methods able to fabricate large-scale antenna arrays while maintaining nanoscale control of their geometries.

Amongst the many approaches explored so far, stencil lithography (SL) stands out in terms of design flexibility and reliable geometrical control at the nanoscale over large scales. We recently reported on a novel blurring-free SL-based patterning technique that exploits localized reactive ion etching to fabricate large arrays of nanoaperture-based antennas [16]. Using this approach we fabricated chip arrays containing over 400 000 antennas with features controlled down to 20 nm. We further showed their applicability by recording the diffusion of individual lipids on living cell membranes in regions of 20 nm in size [16]. Combining these antennas with developments in array detectors should in the future allow the parallel recording of thousands of molecules at the same time.

Bringing photonic antennas to living cells

A limitation of 2D antenna arrays relates to the need for cell membranes to adhere to the substrate, causing membrane curvature effects and/or non-specific adhesion of the membrane close to the nanostructure edges. To overcome these limitations, current efforts are focused on planarization strategies that simultaneously maintain substrate biocompatibility.

A different approach relies on the use of self-standing antennas carved at the end of tapered optical fibers and integrated into a scanning optical microscope (figure 12(B)). This approach has several advantages. *First*, there is no physical interaction between the antenna and the living cell membrane, removing the potential artifacts associated with 2D array approaches. *Second*, the experimental configuration enables both nanoscale imaging in a multi-color fashion [17] and recording of fluorescence from diffusing molecules on nanoscale excitation volumes. Indeed, we have recently shown that monopole antennas fabricated on bowtie nanoaperture antennas provide multi-color single molecule detection with true optical resolution of 20 nm and sub-nanometer localization accuracy [17].

Extracting biological information from antennas

The rich information contained in the 3D near-field components emanating from antennas together with their complex geometries add an additional level of complexity to the study of biological processes in living cells. As a result, extracting reliable biological information from the process under study might be cumbersome and lead to artifactual conclusions. As the field evolves, a great effort should be invested in the development of suitable analytical tools that can discriminate the effect of different excitation profiles from real biological information.

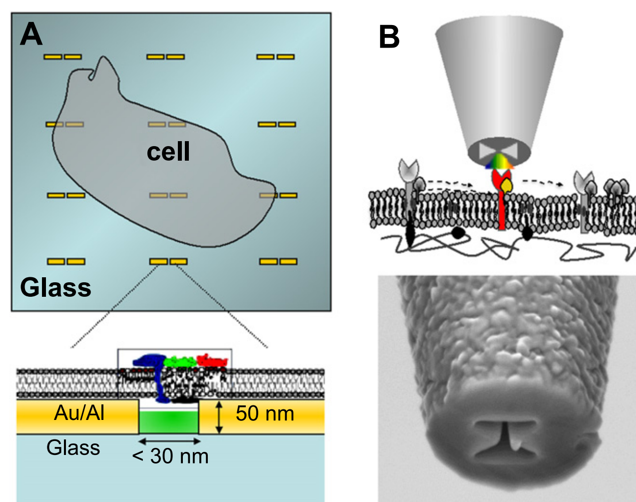

Figure 12. Different photonic antenna configurations for live cell membrane research. (A) 2D antenna arrays provide multiple hot spots of illumination to investigate the dynamics of individual membrane components. (B) Photonic antennas carved at the end of Al-coated tapered optical fibers can be used for nanoscale imaging (in scanning mode) or for dynamic measurements (maintaining the antenna stationary above the cell surface). The SEM image below shows a monopole antenna fabricated on a bowtie nanoaperture antenna. The monopole tip is 20 nm in size.

Concluding remarks

Recent advancements in the field of nanophotonics have led to the development of photonic antenna devices that overcome the diffraction limit of light and allow the confinement and amplification of light in ultra-small volumes. These devices have already been applied to nanoscale imaging of intact cell membranes [52] and single molecule detection at ultra-high sample concentrations both in *in vitro* conditions [14] (see also section 3) and on living cells [16]. While these exciting results convincingly demonstrate the potential of these nanostructures, they also show important technological challenges that need improvement before their routine application in life sciences. Driven by their enormous potential, we expect that current technological obstacles will soon be overcome and that biology-compatible antenna geometries will be readily available to the biological community in the coming years.

Acknowledgments

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8. Linear nanoantennas for surface-enhanced infrared absorption

Annemarie Pucci

Heidelberg University

Status

Infrared (IR) spectroscopy of vibrational excitations is a well known and powerful analytical tool. It allows the chemical analysis of molecules in an almost non-destructive process. Both high spectral resolution and fast measurement can be achieved with modern instrumentation that also provides a huge dynamic range of at least four orders of magnitude in photometric sensitivity. Modern spectrometers allow very fast measurements and, for example, reasonable monolayer spectra can be acquired today in less than a second. In such kinds of measurements the focal spot amounts to ca. 1 cm^2, which means for a monolayer that ca. 10^{15} molecules contribute to the measured signal. In a sensing application in which a much lower number of molecules may be present, IR spectroscopy is hampered by the very tiny vibrational absorption cross section that is much smaller than the squared IR wavelength. The sensitivity for such situation can be strongly improved by electromagnetic field enhancement at the sites of the molecules. Since the last decades of the 20th century, IR vibrational signal enhancement (by up to three orders of magnitude) of molecules on metal nano-films has been studied, and is known as surface-enhanced IR absorption (SEIRA) [53]. Such systems of adsorbates on metal-particle aggregates can be described by effective media theories if the particles are very small regarding the wavelength [53, 54]. The mutual interaction of the nanoparticles in the aggregates broadens and extends the resonance spectrum down to the IR region. Recently, the considerably higher SEIRA (by about six orders of magnitude) from molecules on micron-long metal nanowires with their plasmonic resonances at the vibrational ones was detected and identified as a Fano-type effect with the plasmonic excitation spectrum as the background ([55, 56] and see figure 13). Many experiments with various plasmonic objects that are resonant in the IR confirmed the huge vibrational signal enhancement (see for example the references given in [56]). It was also shown that there is an optimum distance range for signal enhancement [58] and that directly at the surface quantum effects may decrease the vibrational signal [59]. Several application-related SEIRA devices with resonant metal nanostructures have been demonstrated already, for example [60].

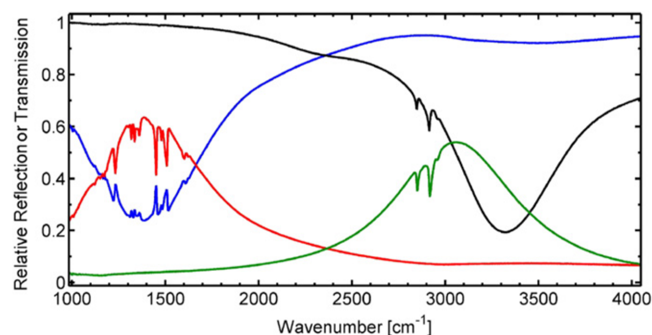

Figure 13. Typical SEIRA spectra measured as relative reflectance (broad plasmonic resonances pointing upwards, red and green lines) and as relative transmittance (resonance pointing downwards, blue and black lines). The narrow features on the plasmonic background are Fano-type lines resulting from vibrations of molecules on the gold nanorods. The plasmonic object for the higher wavenumber resonance is covered by a monolayer of octadecanethiol (ODT, green and black curve). The nanorod array with the lower wavenumber resonance is covered by a 5 nm thick layer of 4,40-bis (N-carbazolyl)-1,10-biphenyl (CBP, red and blue curve). The CBP layer has shifted the plasmonic resonance of the bare rods (not shown) by about 140 cm^{-1}. As a reference for the reflectance, a gold mirror was used, but for the transmittance, a sample area without nanorods was used [57].

Current and future challenges

For SEIRA as a Fano-type effect the strength of the coupling between the molecular oscillator and the plasmonic excitation is essential. For a given molecule, the coupling strength is governed by the near-field at the molecular position, which represents the main challenge in order to get maximum SEIRA signals. First of all, the maximum near-field strength for any plasmonic particle is achieved at the plasmonic resonance. For linear nanoantennas in the infrared, this resonance expressed in vacuum wavelengths of light is linearly proportional to the length of the rod and thus can be tuned via changing this length, for example [61]. Since the plasmonic resonance is broadened by intrinsic and radiation damping, it has to be taken into account that the near-field resonance is red shifted from the far-field extinction maximum [56]. Also related to the damping, the near-field resonance maximum decreases remarkably if the intrinsic damping is strongly increased by bad metal quality. A further important issue is the right site for the molecules. Because the plasmonic nanorods show a resonant near-field confinement at the apex, the molecules at the apex give huge SEIRA signals. For the immobilization of biomolecules (for example, marker molecules), a controlled functionalization of the rod's apexes is necessary, which represents an open problem currently (see section 5). The controlled functionalization needs a well-defined metal surface. For reasons of chemical stability, mostly gold is used for the plasmonic structures, but in typical production processes up to now, no control of the apex structure on the atomic level has been possible. Even more problematic is the use of adhesion layers in the top-down production. Atoms from these layers may diffuse onto the gold surface and thus strongly increase the amount of surface impurities that may disturb the self-organization of an ordered functionalization monolayer. There are more aspects that should be considered. For the plasmonic system, the transverse distances (or lattice constants) of the nanorod array should support constructive interferences at the plasmonic resonance [62]. For the quantitative analysis of the SEIRA

measurement, careful referencing is important. The baseline should be described accurately [56]. Otherwise the Fano-type signals from the molecules appear modified in an uncontrolled manner.

Advances in science and technology to meet challenges

With the correct description of the plasmonic background the Fano-type line-shape can be quantitatively analyzed (see figure 14), and so the correct vibrational oscillator data can be numerically derived [56], which is important for molecular identification and for monitoring molecular changes. First studies on biomolecular detection and modification by means of SEIRA with antenna arrays implemented into microfluidic sensors have been shown already, for example [60]. It is important to note that the vibrational as well as the plasmonic damping [63] are crucial for the plasmonic coupling strength and thus the SEIRA signal size. A recent study clarifies that for optimum SEIRA signals the intrinsic and the radiation scattering of the plasmonic nanoantenna should be similar [64]. Currently, a lot of efforts are put into simplified production methods (for example, interference lithography) of large area plasmonic arrays and into exploiting other plasmonic materials like graphene [65, 66] and doped semiconductors.

Concluding remarks

SEIRA with resonant plasmonic objects enables huge vibrational signal enhancement of molecules that are located in the near-field of the plasmonic object. The enhancement factor can reach 1 000 000 or even more if the strongest plasmonic near-field resonance is tuned to the vibrational frequency and if the molecule is located in the enhanced near-field. High resonant near-field enhancement can be achieved with simply shaped linear nanorods for which the resonant near-field confinement concerns the apexes. Therefore, the preparation of a well-defined metallic surface, especially at the apexes, is important, in addition to the best tuning of the plasmonic resonance.

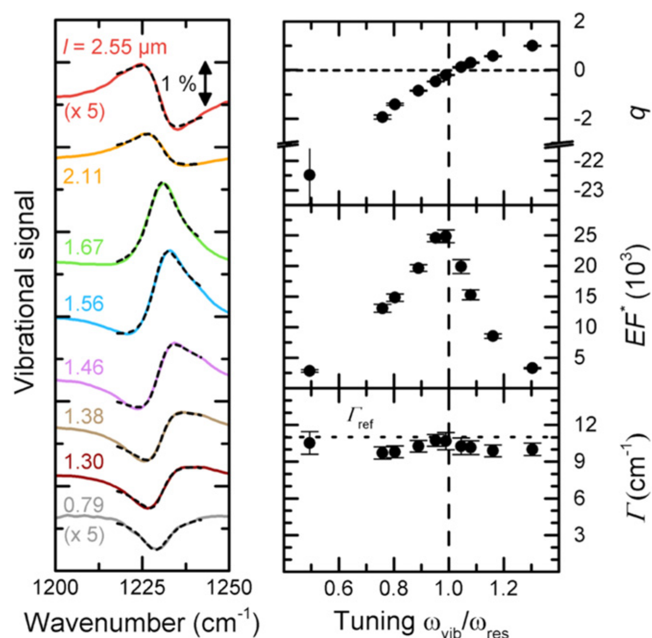

Figure 14. Left: Baseline corrected relative transmittance spectra showing enhanced vibrational signals (SEIRA) of the carbazole C–H deformation mode (1230 cm^{-1}) of about 5 nm CBP covering arrays of various nanoantenna lengths (given in microns, see numbers next to the spectra) and thus different tuning ratios of the vibrational resonance to the plasmonic one. Depending on the tuning ratio, different line shapes are found which can be fitted to a Fano model (dashed lines) [56]. The spectra are shifted vertically. Right: Fano-fit results for the various tunings. The asymmetry parameter q (upper right panel) and the SEIRA signal enhancement factor EF^* (middle panel, right) strongly depend on the tuning. For all tunings, the vibrational line width Γ (lower panel, right) equals the value for a layer without a plasmonic substrate, which indicates that the physisorbed molecules are not changed by the plasmonic coupling. In Fano's theory, maximum coupling corresponds to $q \rightarrow 0$ whereas $q \rightarrow \infty$ means vanishing coupling. The figure is reprinted with permission from [56]. Copyright 2015 RSC.

Acknowledgments

The author acknowledges the author of [57] for permission to reuse figure 3.18 as figure 13. Permission to reuse figure 5 from reference [56] as figure 14 is also gratefully acknowledged.

9. Structured light and optical tweezers for cell biology

Dan Cojoc

CNR-IOM, Trieste

Status

Optical tweezers technology has been continuously developed to control and probe matter at length scales ranging from nanometers to tens of micrometers. Structuring the laser beam in optical tweezers has opened new opportunities such as multiple particle trapping and manipulation in 3D arrays, or transfer of the angular momentum of helical beams to particles [67].

Light shaping is usually achieved by controlling the amplitude and/or the phase of the trapping beam through diffractive optics elements (DOEs) implemented on a spatial light modulator (SLM) [68]. The main advantages of using DOEs/SLMs are: to generate static/dynamic 3D discrete/continuous intensity patterns, and to convert Gauss beams to complex beams such as Laguerre-Gauss or Bessel. Structured light and optical tweezers setups can be easily integrated with other optical microscopy techniques, making them very practical for lab-on-chip systems to enable optical manipulation, actuation and sensing [69].

Although the applications of structured light combined with optical tweezers are powerful and hence have led to many discoveries in the field of physics, it has become very important to explore their wider interest for the biomedical sciences. The ability of optical tweezers to access nanometer-length distances and piconewton forces makes them one of the most important tools for biological studies at the single molecule level [70].

Force probing and local mechanical stimulation of living cells is another interesting application of optical tweezers for cell biomechanics and mechano-transduction. Microbeads can be organized in a cage-like geometry to apply controllable forces to a cell at multiple sites simultaneously (figure 15). This technique has been combined with living cell microscopy showing that mechanical constraints can be applied on the dorsal surface of a cell whilst performing its fluorescence optical sectioning [71].

Silica or polystyrene beads are mostly used in optical tweezers. Nevertheless, micro- and nano particles with various shapes and of different materials can be optically trapped and manipulated as well. Thus, by using an appropriate infrared laser, to avoid cell damage, living cells and subcellular organelles can be manipulated. Structured light and optical tweezers (SLOT) have been applied to pattern heterotypic living cells into arbitrary 3D geometries with very high spatial resolution [72].

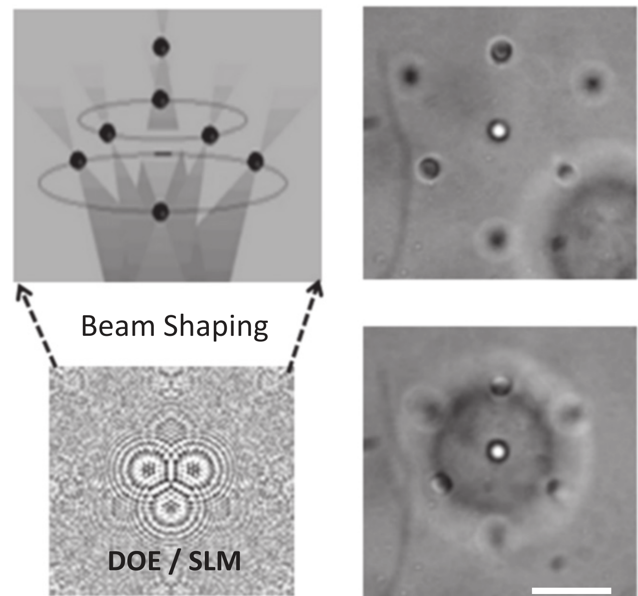

Figure 15. Beam shaping of the trapping laser by DOE displayed on SLM makes it possible to build a dynamic ‘cage’ (left) which is applied on the top of a cell. The images on the right show two instances of a seven bead cage on a HeLa cell (adapted from [77]). Scale bar 15 μm.

Current and future challenges

Since SLOT can be implemented on almost all optical microscopy platforms it becomes natural to combine manipulation with high-resolution imaging and spectroscopy to reveal new mechanisms in cell biology. Although it is known that the mechanical environment influences many cell functions, it remains largely mysterious how mechanical stimuli are converted into biochemical signals by the cell. For instance, the Src protein is known to regulate the integrin-cytoskeleton interaction, which is essential for the transduction of mechanical stimuli. Src activation waves have been documented under local mechanical stimulation of cells by optical tweezers, using fluorescent resonance energy transfer (FRET) and developing a specific genetically encoded Src reporter [73]. Coupling optical manipulation with super-resolution imaging techniques such as STED or PALM is still challenging due to technical problems.

Translating quantitative single molecule force probing experiments from *in vitro* to *in vivo* in single cell experiments represents another fascinating challenge. Applying and measuring piconewton forces at different extra- and intra-cellular sites requires smaller probes and better control of them. Considerable progress has been accomplished in this realm, measuring for instance the mechanical properties of individual proteins central to cellular adhesion and even the action of molecular motors in living cells. Intra-cellular quantitative experiments at the single molecule level remain, however, challenging to implement [74].

Mechanical stimuli represent, however, only a part of the possible stimuli for a cell from its environment. The biochemical cues, usually released by other cells, are preponderent. Since SLOT allows manipulation of particles of a wide range of materials and shapes, it becomes a powerful tool to manipulate individual or multiple vectors carrying active molecules for stimulation of the cells at specific sites (figure 16). Functionalized micro/nano-beads or quantum dots (e.g. with carboxilic groups) and linking kits to attach proteins on their surface are

commercially available, making it possible to work with any type of protein. The characterization of these vectors, in terms of the density and functionality of the deposited proteins, still remains challenging. Moreover, the proteins cannot detach from the surface, limiting the field of applications. Therefore synthesis of more versatile vectors is required.

Advances in science and technology to meet challenges

Considerable progress has been made in developing new dyes for super-resolution imaging, which lowers the power of the laser and allows imaging of living cells with resolution better than 50 nm in real time. Manipulation by SLOT could thus be implemented in such platforms as a first step of the experiment. The development of new FRET probes is also moving fast, overtaking problems such as cell-toxicity or transfection efficiency.

Force probing of individual molecules inside the living cell requires a handle. The typical handles are polystyrene, metallic and magnetic beads with a variety of sizes and coatings. One challenge is to get the handle inside the cell without influencing the cell function. For some cells, this can be achieved by endocytosis. Otherwise the membrane of the cell should be penetrated mechanically or by laser ablation to inject the handles inside the cell [74].

For local cell stimulation some other vectors can be used beside coated beads. Liposomes, which are phospholipidic vesicles 100–200 nm in size, are intensively investigated for drug delivery in medicine. Micrometric vesicles, which are easy to monitor by optical phase microscopy, encapsulating active molecules of any type, are very good vectors. The vesicles can be trapped and positioned near the cell of interest and the molecules released by membrane photolysis, using a short UV laser pulse (figure 16). Knowing the initial concentration of molecules, the encapsulation efficiency can be approximated and in some cases measured [78], allowing one to estimate the number of active molecules inside a vesicle of a certain size. The precision of this estimation depends on those of the encapsulation and of the vesicle size measurement. Interesting to note is that a vesicle of one micrometer diameter filled with a solution at one nanomolar concentration contains one molecule, on average. The effect of the neuronal cues semaphorin-3A and netrin-1 on the growth cones was thus investigated, reducing the number of active molecules [75].

The above described technique implies that the molecules are released in a burst, followed by free diffusion. Another way to create spatial and temporal gradients of active molecules intercepting a cell was demonstrated in an interesting experiment with neutrophil cells. Chemo-attractant or repellent molecules were encapsulated in biodegradable polymer beads and optically manipulated for continuous release close to the cell to induce cell motility [76].

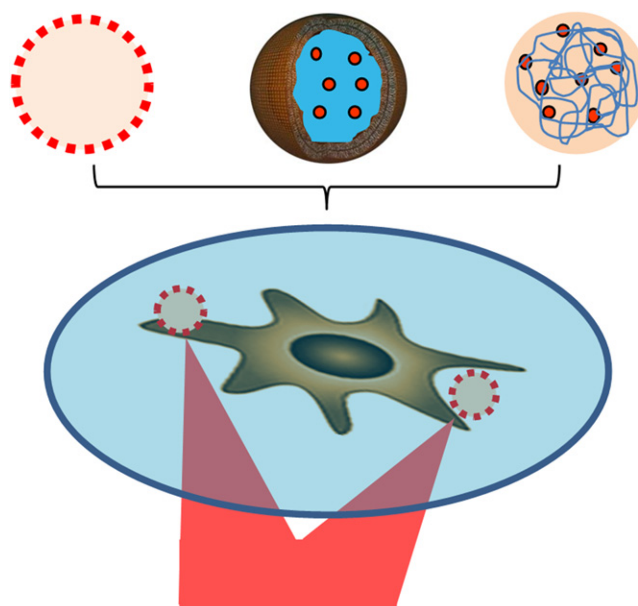

Figure 16. Focused cell stimulation at multiple sites by optically manipulated vectors. The active molecules can be charged in and carried by different type of vectors—coated micro-beads or quantum dots (upper left), filled lipid vesicles (middle), or biodegradable polymers (right).

Concluding remarks

SLOT represents a powerful tool for multiple particle manipulation and beam shaping, which opens up new opportunities in cell biology studies. Force probing at multiple sites of the cell and high-resolution imaging and spectroscopy can be integrated to increase the potential of this technique. Multi-disciplinary efforts are requested to develop new probes for live cell imaging conjugated to cell manipulation. The use of new beams for probing matter will probably contribute to this. The use of micro/nano-vectors seems very powerful, hence the characterization and development of such vectors is highly in demand.

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10. Plasmonics and scanning probes based on adiabatic compression

Enzo Di Fabrizio

King Abdullah University of Science and Technology, KAUST

Status

Sensing at a molecular level means to 'localize in space the presence of the molecule, its orientation, its chemical composition, its physical state, its structure and its biological function, taking into account the specific environment around the molecule or the molecular aggregate. The environment can range from a solution to a solid/liquid interface or to a flexible surface of a cell membrane.' The knowledge of this information, at a molecular level, defines the state of the molecule in its own environment.

Is it possible to know the state of the molecule under generic environmental conditions? Or, in other words, is there a tool that allows, exploiting some kind of interactions, quantitative measurements in order to obtain knowledge of the molecular state?

The general answer to these questions is negative but, in the near future it is conceivable to approach the problem of sensing in biology, physics, medicine, chemistry in a way that, the knowledge on the state of the molecule in its specific environment, can be reached.

We distinguish two different conditions where the molecular aggregate is considered:

1. Localized regions in space where the molecules are bound (physical or chemical binding).
2. Solutions where the molecules are dissolved at different levels of concentration.

In case (1), sensing at the level of a single molecule or few molecules, from an instrumentation point of view, means: (a) localizing a probe in a region where the molecule of interest is sitting, and (b) generating a localized interaction with few molecules even in a surrounding complex environment.

In case (2), the molecules are free to move in space and their detection relies on finding a way to provoke an encounter of the molecule with a sensing surface. Very often, the encounter is regulated by the diffusion of the molecules that, in other words, means that the diffusion dictates the encounter probability. In other cases the encounter can be governed by microfluidics or electromagnetic fields, or more general driving forces (chemical, thermodynamics etc).

Current and future challenges

What are the scientific questions that we want to address with this approach?

Our task, through molecular sensing, is to find new tools and methodologies to:

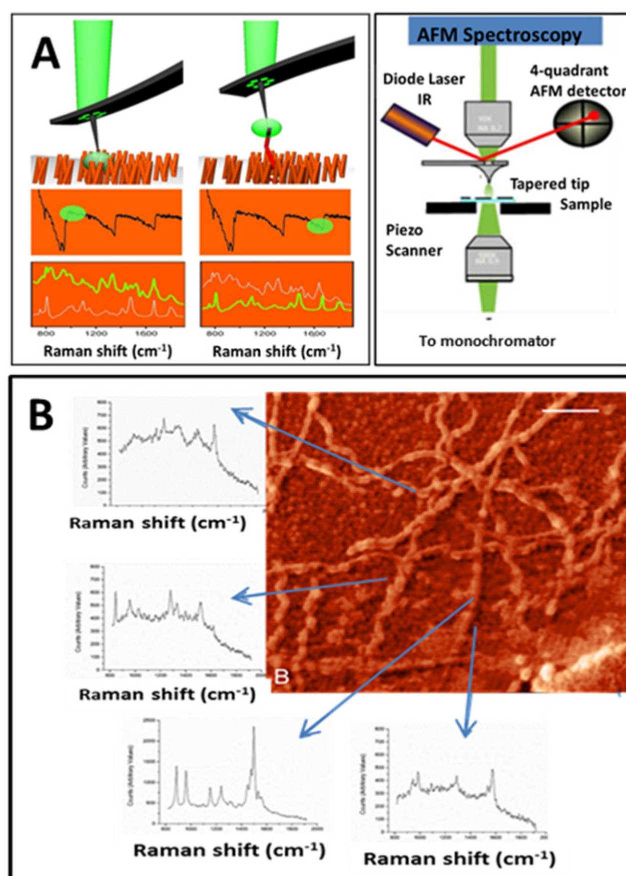

Figure 17. (A) Schematic showing Raman measurement and pulling of molecules (left), and its optical system (right). (B) Raman in different locations of an amyloid fibril sample. The bar is 50 nm.

A. Discover the structure and the function of biomolecules in living conditions, when studied in life sciences, and additionally in soft matter.
B. Detect few molecules from complex mixtures even in highly diluted conditions.

In case (A), one experimental system to be considered a priority in life sciences is the study of membrane proteins on living cells. In case of material science, the full characterization of nanostructured surfaces/thin film interfaces is also an experimental system of strong interest, especially out of vacuum conditions. This is also of interest in catalysis.

In case (B), there are different interesting experimental systems to consider, such as the complex solution in serum with some species at very low concentration, such as in early disease detection, or in environmental science, the search for dangerous pollutants in water, in air or in soils. Another important aspect of molecular sensing is that the interrogation of few molecules, even in a complex environment, will allow the direct comparison of experimental data to simulation through the latest most advanced approach of molecular dynamics (MD). In fact the length and temporal scales involved in the experiments are compatible with the latest MD algorithms. This consideration is relevant especially for

molecular, structural and functional problems where MD can help to elucidate the role played by the internal degree of freedom of a macromolecule in defining its structure and function.

Advances in science and technology to meet challenges

The concrete know-how necessary to face this research program is related to the ability to design and realize new tools that, in their function, include imaging, chemical and physical mapping, transport and optical measurements. In other words, this approach aims at designing new instrumental architectures, new materials and new methods in order to face molecular sensing as defined above.

Challenge #1: scanning probe and spectroscopy measurement by combining atomic force microscopy (AFM) and Raman spectroscopy

In this challenge [79–81, 22], AFM and Raman spectroscopy will be combined together with the aim of setting up a new 'fishing' technique. The technique consists, see figure 17, of doing force spectra while measuring the Raman signal of a molecule anchored at the tip, generated by the surface plasmon polariton created by adiabatic compression [81] that focuses at the cone apex. In figure 17(A), left side, the measurement strategy is schematically reported. When the molecules under study are proteins, the AFM force pulling induces a molecular elongation that can be viewed as a mechanical unfolding. With a force clamp strategy, it will be possible to measure the Raman signal as a function of the pulling force. AFM, molecule fishing and force clamp will be implemented both in traditional force distance mode (soft cantilevers) and by developing the low amplitude frequency modulation mode. The latter is expected to improve sensitivity and position accuracy by adding sub-nanometer modulation and by minimizing uncontrolled static bending due to tip sample interaction caused by the electromagnetic field at the tip. The combination of the two spectra, AFM and Raman, will increase the structural and functional information on the protein itself. The data collected in this experiment will be compared with MD simulations in order to reconstruct the molecular structure and compare it eventually with that of the crystal.

Challenge #2: Mixtures of highly diluted solution

In this challenge [82–89], the detection of a few molecules in a highly diluted solution is of paramount interest in fields including biomedicine, safety and eco-pollution in relation to rare and dangerous chemicals. Nanosensors based on plasmonics are promising devices in this regard, in that they combine the features of high sensitivity, label-free detection and miniaturization. However, plasmonic-based nanosensors, in common with general sensors with sensitive areas on the scale of nanometers, cannot be used directly to detect molecules dissolved in femto- or attomolar solutions. In other words, they are diffusion limited and their detection times become impractical at such concentrations. We demonstrated, by combining super-hydrophobic artificial surfaces and nanoplasmonic structures, that few molecules can be localized and detected even at attomolar (10^{-18} mol l^{-1}) concentration. One of these devices is reported in figure 18. Moreover, the detection can be combined with fluorescence and Raman spectroscopy, such that the chemical signature of the molecules can be clearly determined. In conclusion, the combination of super-hydrophobic surfaces and nanoplasmonics allows one to overcome the limit dictated by diffusion when sensors approach a nanoscale length.

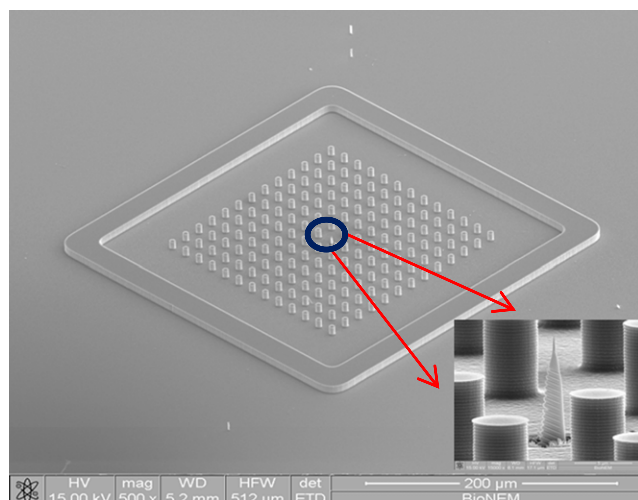

Figure 18. Superhydrophobic device with a plasmonic tip at the center for single molecule detection from a highly diluted solution; see [82].

Concluding remarks

Nano fabrication and spectroscopies, including scanning probes, can be combined to define new categories of devices that can show both sensitivity down to attomolar concentration and spatial resolution down to a few nanometers. The vibrational spectroscopies give the additional structural information for helping the determination of the structure and the function of macromolecules. The ultimate advantage of this approach is that these methods are fully compatible with physiological conditions and would be crucial for bridging diagnosis with pathology studies.

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11. Self-assembling ultrashort peptide nanoparticles: smart biomaterial as second harmonic probes for bioimaging and diagnostic applications

Charlotte A E Hauser[1] *and Ming Ni*[2]

[1]King Abdullah University of Science and Technology, KAUST
[2]Institute of Bioengineering and Nanotechnology, A*Star

Status

Second-harmonic generation (SHG) microscopy is a powerful nonlinear optical (NLO) imaging technique that has emerged in recent years to visualize structures or functions of cells, tissues and organisms [90]. It has the advantages of being label-free, having inherent three-dimensional (3D) resolution, near-infrared (NIR) excitation for superior optical penetration and low photo-damage, and being capable of providing quantitative information, which makes it an attractive tool for high-resolution imaging [90–92]. It has been used to diagnose a wide range of diseases including cancer, liver fibrosis and skin damage [93]. It has also been used in tissue engineering and regenerative medicine [94] to envision three-dimensional biomaterial scaffolds and extracellular matrices secreted by cells [95]. It provides many significant advantages over fluorescence imaging techniques for its deeper optical penetration, lower photo-damage and longer observation time [90, 93]. In all these cases, endogenous probes have been used that are already available in the body. Exogenous probes have the advantage that they can basically be provided at any time and at any spot, which gives an enormous flexibility towards application. Unlike plenty of fluorescent probes, such as dyes available for fluorescence imaging, there are only a few exogenous second harmonic (SH) probes available. These are mainly inorganic nanocrystals, including barium titanate (BaTiO3), quantum dots (QDs) and zinc oxide (ZnO) [96]. However, inorganic nanomaterials have long been challenged for their health and environmental issues, which limit their clinical applications. Under these circumstances, organic probes with low cytotoxicity will offer interesting alternatives.

Current and future challenges

Up to now, nearly no organic exogenous SH probes as nanomaterials for bioimaging have been reported. Therefore, non-immunogenic organic probes with strong SHG signals and no toxicity would overcome existing application hurdles. Excitation wavelength tunability is one of the advantages of SHG imaging. This enables us to freely tune the excitation wavelength to best match the optical properties of the sample. Biological samples, such as cells and tissues, are known to have auto-fluorescence. With the excitation wavelength tunability, we can avoid auto-fluorescence. In addition, we can further increase penetration depth in tissues by simply increasing the excitation wavelength.

Advances in science and technology to meet challenges

Peptide dimer diphenylalanine (FF) was the first reported peptide that showed nonlinear optical properties. A class of ultrashort linear peptides has been rationally designed with a

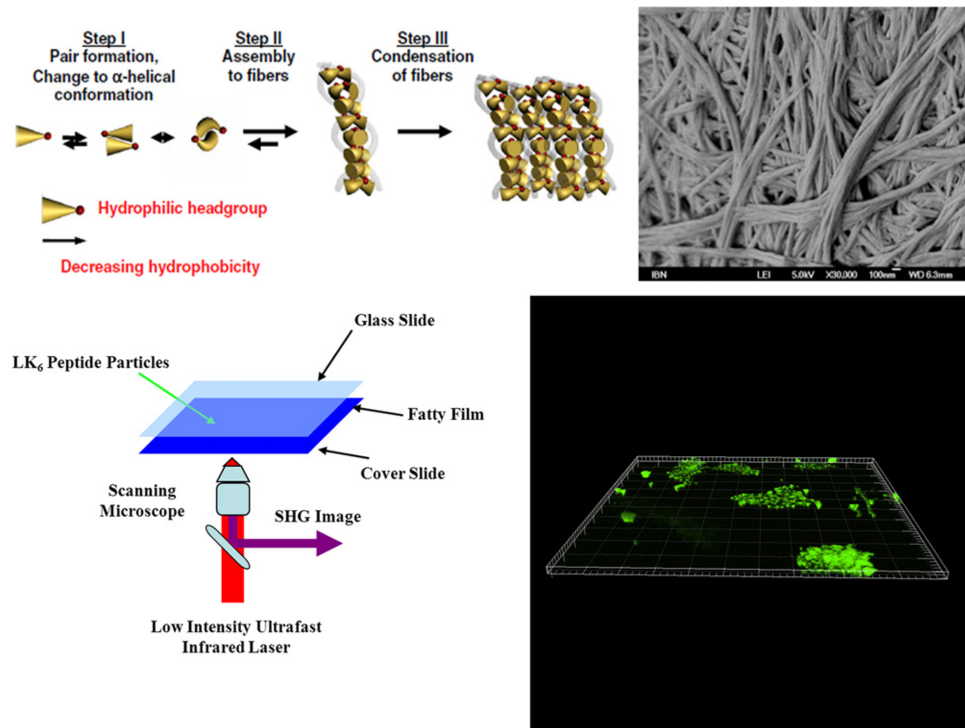

Figure 19. Proposed mechanism of ultrashort peptide self-assembly and nanofiber formation (upper panel, left). Electron micrograph of nanofibrillar networks formed by a peptide trimer (upper panel, right), reprinted from [97]. Schematic illustration of an experimental set-up showing peptide nanoparticles with fat tissue from a rat, sandwiched between glass slides (lower panel, left). 3D image of the peptide nanoparticles as a SHG z-stack (lower panel, right).

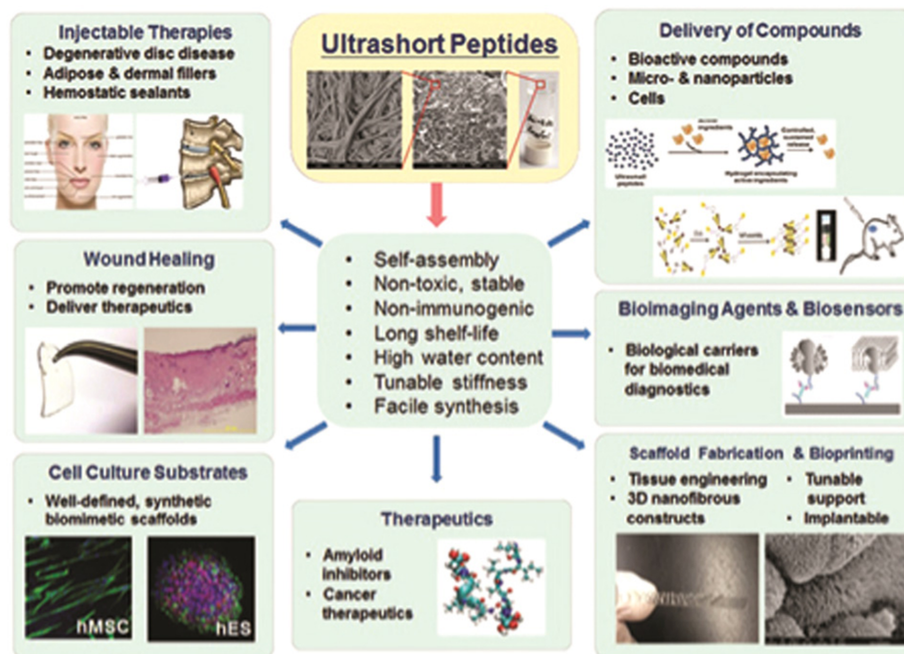

Figure 20. Ultrashort self-assembling peptides as a platform technology for diverse applications.

characteristic motif consisting of just 3–6 aliphatic amino acids. Each peptide sequence comprises aliphatic amino acid residues leucine, isoleucine, valine, alanine, and/or glycine arranged in the order of decreasing hydrophobicity toward the C-terminus (the hydrophobicity gradient is illustrated as a cone in figure 19). The designed peptide sequences are terminated at the C-terminus by a polar amino acid residue such as aspartic acid, glutamic acid, serine, or lysine. This type of arrangement favors a parallel-antiparallel stacking as we could demonstrate by molecular dynamics simulation. All peptides have an innate tendency to self-assemble into helical fibers giving rise to fibrous scaffolds and vesicle-like nanoparticles, also made from fibers (figure 19, upper panel). The fiber nanotopography and the supramolecular networks show physico-chemical properties comparable or superior to those of collagen and other ECM-like materials, so that the biomimetic material can be considered a smart biomaterial. We observed that this peptide-based biomaterial can be directly visualized by SHG microscopy. It shows excitation wavelength tenability and similar second harmonic signal intensity to endogenous collagen I. For these SHG studies a hexamer peptide LK6 was designed and synthesized in our laboratory. As a member of the above mentioned class of ultrashort peptides, this hexamer peptide can easily self-assemble into hydrogels through hydrophobic interactions, ionic interactions, hydrogen bonding and van der Waals forces. It contains a non-polar aliphatic tail and a polar head. Since it is ideal that SH probes should have defined shape and size, peptide nanoparticles were prepared by hydrodynamic focusing and solvent evaporation. The particle size ranged from submicron to 10 microns, and the particle shape was irregular. Currently, we are manipulating preparation parameters to achieve better control over the size and shape of the peptide particles. Based on our results we conclude that this purely synthetic, peptide-based material holds the potential to be used as a future SH probe for bioimaging applications. We have further explored the peptide scaffolds as smart biomaterials for biomedical applications, for regenerative medicine and tissue engineering, but also in combination with therapeutics for drug delivery, biosensing and topical applications (figure 20).

Concluding remarks

In the search for next-generation bioimaging markers, peptide nanoparticles with nonlinear optical characteristics are interesting candidates. Peptides are ideally suited for biomedical applications, since they resemble natural proteins, show chemical versatility, functionalization potential, intrinsic biocompatibility and biodegradability. Besides these favorable features that go along with the use of peptides, peptide nanoparticles offer alternative bioimaging opportunities that are currently not available with the existing solutions considering inorganic nanocrystals, quantum dots and fluorescent dyes as nanoprobes. To our knowledge, our ultrashort peptide-based biomaterial is one of the first aliphatic peptides that can be directly visualized by SHG microscopy.

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