CONTENT *Optical methods are used to study the interaction of molecules in fields ranging from genomics to biophysics. For example, high-throughput DNA and protein microarrays are now standard tools in biology. Furthermore, highly sensitive state-of-the art optical biosensors based on surface plasmon resonance (SPR) are a mature and widely used technique for the analysis of label-free molecules in real time. However, to reach ultimate single-molecule sensitivity in combination with label-free, high-throughput approaches in real-time, new methods are required to boost current detection limits. The following article describes a novel, emerging technique for the label-free analysis of biomolecules using optical resonances in microsphere sensors. This new method enables with an unprecedented sensitivity the quantitative analysis of such molecular properties as molecular weight, binding kinetics, affinity, and conformation. It is suitable for high-throughput approaches. This combination of features renders micro-optical resonators promising tools for a wide range of applications in the life sciences.*

Taking detection to the limit

Label-free, high-sensitivity detection of biomolecules using optical resonance

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• Recognition and specific interactions between proteins and other macromolecules within the cell are the fundamental prerequisite for biological functions. They are, for example, required to transmit and amplify chemical and electrical signals, for the assembly of larger molecular machines, e.g. the ribosome, and are sequentially combined in metabolic and signalling pathways. The modular structure of amino acids and nucleic acids enables the cell to generate a wide variety of molecules with very different binding specificities and thus with a large variety of possible interactions. On an evolutionary timescale, biomolecular function is the result of the natural selection of interactions which have become heritable parts of the genetic and proteomic circuits of the cell.

One recent interdisciplinary effort of genomics and proteomics aims to elucidate the complete network of biomolecular interactions in the cell. This research requires methods and techniques that examine molecular interactions with high sensitivity and high throughput. Most of the established high-throughput techniques use light to detect molecules interacting on a surface with immobilized binding partners. Examples of this include DNA[1] and protein-based[2] microarrays. In these approaches, DNA or protein molecules must be tagged with strongly fluorescent dyes to be detectable. A laser can then rapidly scan for labelled molecules spotted at specific locations on a glass support. A charge-coupled device (CCD) camera records the fluorescent image of the microarray surface and a computer analyses the image pixel by pixel. This technique makes it possible to analyse 1.3 million unique oligonucleotides on an array with an 11-µm »spot« size (www.affymetrix. com). However, the necessity to label the molecules prevents real-time detection. The sample must be processed, which takes time and skill, and can change the relative levels of the molecules originally present. For example, total mRNA must be extracted by a phenol-chloroform procedure from a cell suspension, reversely transcribed into cDNA and labelled before being analysed on a microarray.

The direct detection of the dynamics and interactions of untagged – and in many approaches – single molecules is therefore one of the ultimate goals for a wide variety of analyses.

Label-free, high-sensitivity detection of biomolecular interactions

• To reach this goal, highly sensitive optical approaches were developed. These enable analyses of molecular interactions in real-time, and tagging the molecules of interest with a label is unnecessary[3]. Using these approaches, biomolecular interactions can be examined in greater detail: kinetic rate constants, affinity, binding position, and thermodynamic parameters are measurable for e.g. proteins or oligonucleotides in solution binding to a ligand immobilized on a surface. The state-of-the art technique to determine these parameters uses optically excited surface plasmon resonance (SPR) for detection[4-6]. Surface plasmon resonances are oscillations of conduction electrons collectively excited in a thin gold or silver film coated on a prism surface[7,8]. The SPR method is a mature biophysical technique; ever since the commercial

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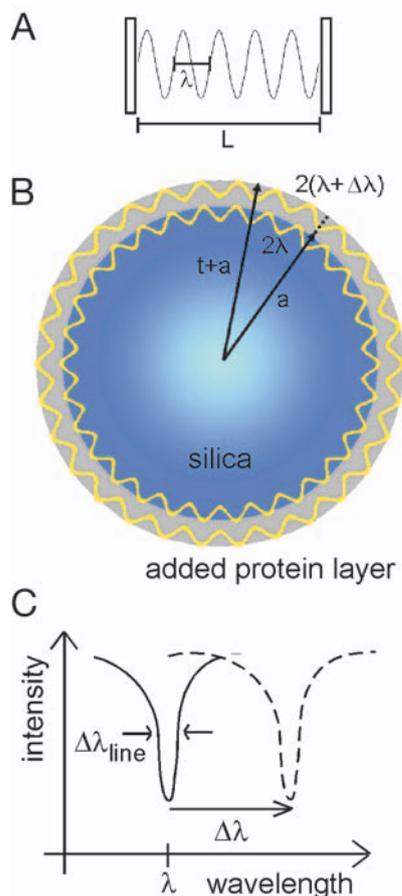
FIG. 1: (A) Optical resonance in a Fabry-Perot cavity. (B) Optical microsphere resonance (WGM): light confined by total internal reflection orbits inside a micron-sized silica glass sphere (blue) with specific resonant wavelength λ. Principle for detecting molecular adsorbates: a change Δλ of the resonant wavelength λ is anticipated due to the addition of a protein layer (grey) of thickness t on the microsphere of radius a. (C) Corresponding spectroscopic wavelength shift Δλ of the resonant line of finite width Δλ_line.

introduction of SPR sensors in the 1990s, they belong to the standard instruments in research and industry (www.biacore.com). SPR sensors are used for different biological molecules, e.g. proteins, oligonucleotides, oligosaccharides, lipids, small molecules, viral particles, cells as well as in different experimental contexts e.g. proteomics, genomics, drug discovery, and membrane studies[9]. One advantage of this method is its high sensitivity, enabling it to detect molecules at low concentrations, to monitor binding of small molecules, to measure weak binding constants, to analyse the kinetic off-rates of ex-

* since f = s / λ, s being the speed of sound

tremely strong interactions, and to detect conformational changes[10-12].

The smallest detectable molecular weight using SPR sensors is ~180 Da[13]. In terms of mass loading, this sensitivity corresponds to a detection limit of ~ 10 pg/mm^2[14], or about one hundred million bovine serum albumin molecules (molecular weight: ~ 66 kDa) bound to a sensing area of 1 mm^2. This level of sensitivity for a real-time, label-free optical biosensor system is still unmatched.

While there is theoretically no limit to their application, the development of SPR biosensors reached its practical sensitivity limit before single molecules could be studied[15]. In the following article, I shall therefore introduce a novel optical method for the analysis of biomolecules – the so-called whispering gallery mode (WGM). The applications of the WGM sensor are as wide as those of SPR sensors. However, we are confident that its ultimate sensitivity is much higher, possibly on the single-molecule level, since it uses optical resonance for detection. In addition, and unlike to currently used label-free detection methods, it is suitable to high-throughput analyses, enables real-time measurements and detailed quantitative analysis of molecular binding events.

Optical resonances

· WGM sensors derive their high sensitivity from the use of a resonance phenomenon. Resonators are well known in all scientific disciplines, one common example being the violin string. The violin string is in resonance (upon which we can hear a particular tone) when an equal number of oscillations N of wavelength λ fit one resonator roundtrip. Here this is twice the length L of the string: N × λ = 2 L. If the violinist now decreases the length L of the string with his/her finger, the resonance will accommodate to the shorter length of the string and will occur at a reduced (shorter) wavelength λ. As a result, we hear a tone of a higher frequency f*.

In optics, an example analogous to a violin string is a resonant cavity formed by two opposed reflecting

surfaces (*Figure 1A*). In analogy, the oscillating violin string can now be imagined as an electromagnetic wave of wavelength λ confined to a total length L between the two mirrors. Such Fabry-Perot cavities are essential components in lasers. Below, I will introduce a special optical resonator where light is reflected along the inside of a glass sphere only fractions of a millimetre in size, i.e. usually 100-500 μm in diameter[16]. We use the resonance phenomenon in such microspheres to amplify signals, thereby enabling high sensitivity and possibly single-molecule detection.

A glass microsphere is able to confine light on a circular path due to multiple total internal reflections along the inside of the sphere surface (*Figure 1B*). These special optical resonances, the WGMs, occur when the light waves, having completed one roundtrip along the equator of the sphere, begin to overlap, i.e. interfere constructively. As a result, an integer number N of wavelength λ fits the circumference of the sphere with a given radius a: N × λ = 2 π a.

The number of roundtrips of the light is limited due to the loss of photons. Losses are mainly caused by absorption or scattering. However, losses in silica glass are minimal and the sphere forms a very good light container. In fact, glass microspheres are the ultimate optical resonators: light can be confined up to ~ 3 μs[17]. Considering the speed of light (~ 10^8 m/s), photons can orbit a sphere up to 300,000 times, corresponding to a path-length of ~ 300 metres travelled inside the sphere. Importantly, the circulating light can be used for the detection of molecules. The resonance phenomenon leads to an amplification of the signal (and its changes) which is ideally strong enough to detect a single molecule.

Resonant biodetection

· In first experiments, we showed that the resonant microsphere can be used as a molecular sensor to detect the binding of a molecule from solution to a ligand fixed to the sphere. The binding event is detected from a

change of the microsphere resonance wavelength. This so-called frequency-domain detection principle can be intuitively understood from a geometrical argument for a resonant light wave circulating the inside of a sphere (*Figure 1B*). The resonance is characterized by a certain number of wavelength N (here 30) that fit the circumference (radius a) of the sphere's equator. Assuming that material such as protein adsorbs to the surface of the sphere to a final thickness t, the wavelength λ at which resonance occurs then increases by Δλ due to the increased circumference of the sphere: $\Delta\lambda/\lambda = t/a$[18]. Note that the binding of proteins is detected *not* from a change of intensity of the light, but from the change of its resonant wavelength. Since the microsphere can be connected to a simple

optical fibre, its resonant wavelength can be read out instantaneously, thus enabling analyses of biomolecules in real-time. However, before going into greater technical detail, I will discuss the ultimate sensitivity of the WGM sensors.

Optical losses set the detection limit for the resonance shift. The damping of light intensity by absorption and scattering limits the number of roundtrips for a given resonance and thus also the associated width $\Delta\lambda_{line}$ of the resonant line (*Figure 1C*). In a loss-less cavity, the number of oscillations would be infinite and a sharp resonant line would be observed at the resonant wavelength λ. In reality, any oscillator experiences losses which limit the number of oscillations to a finite number and the resonance to a certain line width

$\Delta\lambda_{line}$. We can observe a resonance shift Δλ induced by binding of molecules only if the magnitude of the shift is larger or of the order of the line width $\Delta\lambda_{line}$.

To compare different resonators, we and others defined a quality factor as $Q = \lambda/\Delta\lambda_{line}$. Q's of ~ 10^7 are measured for microspheres simply fabricated by melting the tip of a standard telecommunications optical fibre (a 125-μm-diameter, solid silica glass fibre) in a hot, butane-nitrous oxide flame. Surface tension forms a sphere on a stem structure, fractions of one millimetre in diameter. The stem does not interfere with the resonance localized at the equator of the sphere. Ultimate Q's of ~ 10^{10} have been reported[17] when the adsorption of atmospheric water to the nascent sphere was avoided. In an aqueous environment, i.e. in most biological applications, Q can be reduced by the vibrational light absorption of H_2O molecules. However, even a »modest« Q of 10^7 enables detection of a resonance shift which corresponds to an adsorbed layer of thickness* of 10 picometre or 0.1 Ångstrom – a tenth of the diameter of an atom! Proteins are several nanometres in size and detection of a monolayer is thus an »easy« task for a WGM sensor.

Real-time analysis of protein-protein interactions

· The binding of a single molecule to the surface of the glass sphere is enough to shift the wavelength at which resonance occurs. Thus, the analysis of the wavelength shift over time reveals the binding kinetics of molecules. To demonstrate the potential of a microsphere-based biomolecule detector, we developed and tested a simple table-top set-up (*Figure 2A*)[19]. In the set-up, a ~ 300-μm-diameter glass microsphere fabricated by melting a standard optical silica fibre is positioned in a liquid-filled chamber. The microsphere is placed in contact with an optical fibre, which is connected on one end to a tuneable laser, and on the opposite
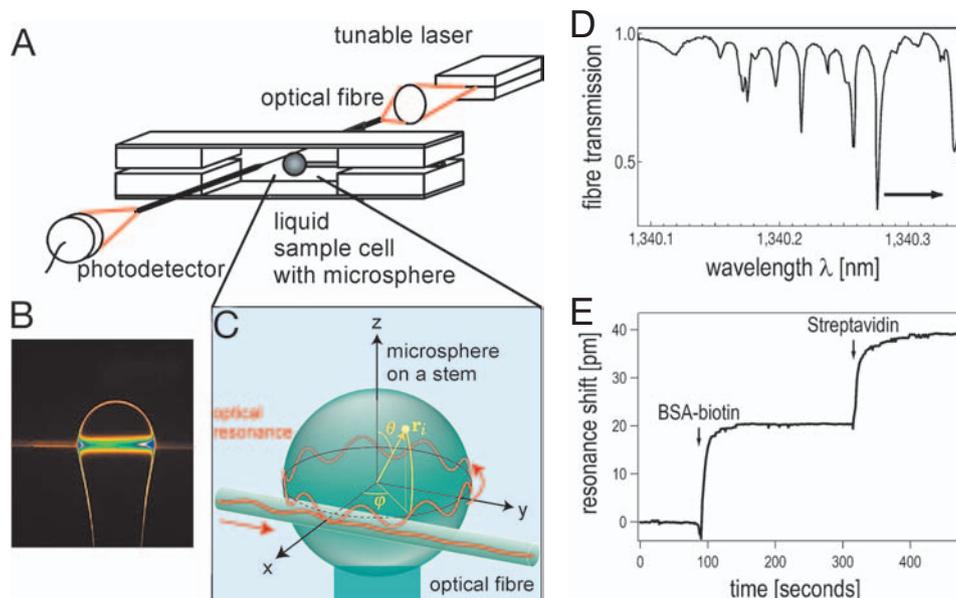
FIG. 2: (A) Experimental set-up to detect optical resonance. Optical resonances in a microsphere on a stem are excited and measured via an optical fibre. (B) Actual image of light circulating inside a microsphere at resonance. An optical fibre running horizontally through the image is used to deliver the light to and, at resonant wavelength, into the sphere. (C) Illustration of a resonance excited inside a microsphere connected to the optical fibre. Light circulating within the sphere is depicted as a red wave (D) The wavelength of the infrared laser is continuously changed to scan for resonances. Whenever the output wavelength of the laser matches the resonant wavelength of the sphere, the light travelling down the fibre passes from the fibre into the sphere. A drop in the fibre transmission is then recorded by the photodiode connected to the other end of the fibre. A complete scan of the laser repetitively reveals the same resonant lines in the transmission spectrum recorded every 10 ms. A resonant wavelength is identified from the minimum of a resonant line. Molecules binding to the sphere surface will shift all resonant lines by the same Δλ , as indicated by the arrow. (E) The plot shows a time trace of one resonant wavelength which monitors the binding of proteins to the sphere surface. First, biotinylated BSA is injected in the liquid sample cell from where it adsorbs to the sphere surface. Specific protein detection is demonstrated from a second wavelength shift after injection of streptavidin.

* $t = a/Q = 10$ picometre

end to a photodetector. Resonance in the sphere was excited by laser light transmitted via the optical fibre[20]. The tuneable telecommunication laser operated at a nominal wavelength of 1.3 μm and the laser output wavelength was repetitively and continuously changed to probe for resonances.

Whenever the wavelength of the light sent into the fibre matches a resonant wavelength of the sphere, the light will almost completely pass from the fibre into the sphere where it circulates on its resonant orbit (*Figure 2B, C*). Since light with the resonant wavelength is »trapped« inside the sphere, it no longer reaches the photodetector at the end of the fibre and the detector records a drop in light intensity for this particular wavelength (*Figure 2D*). The microsphere was located in a sample cell filled with phosphate-buffered solution to allow for biological measurements. Its Q was measured as 2×10^6, and was thus high enough to resolve the binding of proteins to the microsphere.

Following the successful first proof-of-principle experiments, we modified the set-up to demonstrate binding of the negatively charged protein bovine serum albumin (BSA) as a model. We coated the glass sphere with an aminosilane layer, thereby introducing positively charged amino groups on the surface[21]. Upon injection of the solution containing BSA into the liquid-filled chamber, we observed a shift of the resonance wavelength due to BSA adsorption to the surface of the sphere by electrostatic interactions (*Figure 2E*). Each resonance shifted by the same $\Delta\lambda$, and, from the trace of one resonance wavelength, we calculated that the BSA-saturated surface shifts the resonance by 21 pm. With our simple formula ($\Delta\lambda$ /λ = t/a) we estimated the thickness t of the BSA layer to be ~ 3.2 nm, the thickness of a monolayer shown with crystallographic experiments[22]. The Langmuir-like isotherm also confirmed that there is only one layer of surface-adsorbed BSA molecules. Furthermore, from the adsorption isotherm[19] we could determine the

affinity constant of BSA for binding to aminosilane (~ 1.5 nM).

Our next step was to examine whether we could specifically detect binding of the biomolecules. To this end, the BSA molecules immobilized on the surface of the sphere carried biotin groups (BSA-biotin). In a second injection, we added streptavidin to our sample cell since it is known to selectively bind to biotin. From the magnitude of the resonance shift (*Figure 2E*) with respect to the molecular weight of each protein, we estimated the binding stoichiometry as ~ 1:1 – a value anticipated for a complete second protein monolayer composed of streptavidin. The results thus demonstrated that our set-up indeed facilitates the monitoring of specific protein binding.

The applications of the WGM biosensor are boundless. Molecular interactions of any biological material can be recorded and analysed in real-time. From the binding kinetics, it is possible to determine on and off rates. From equilibrium measurements at different temperatures and concentrations, thermodynamic parameters such as binding entropies and enthalpies can be determined. The silica material of the microsphere is a well known substrate. All classes of biomolecules can be covalently linked to its silanol (Si-OH) groups[23]. Moreover, for the detecton of small molecular weight compounds, such as oligonucleotides, it is desirable to increase the number of binding sites per surface area. This can be relatively easily achieved using our approach of adsorbing hydrogels such as biotinylated dextran to the surface of the microsphere before coating it with the molecules of interest, e.g. the oligonucleotides[24].

Towards a label-free DNA microsphere array

• Since each microsphere in a sensor array can carry a different biomolecule, WGM sensors are amenable to high-throughput detection required in areas such as genomics and proteomics. To this end, several microspheres can be connected to the same

optical fibre for parallel measurements of their resonant wavelengths. We used this approach to develop a microsphere-based DNA array for the detection of single nucleotide polymorphisms (SNP)[24]. For this analysis, we coupled two microspheres to the same optical fibre (*Figure 3*). The two spheres differed slightly in size due to the manufacturing process. Their resonances thus appeared at different wavelengths and each could be unambiguously identified. Each sphere was coated with a different 27-mer oligonucleotide: biotinylated oligonucleotides were immobilized to a biotinylated dextran hydrogel matrix adsorbed to the sphere by crosslinking with streptavidin. The complementary strands were then sequentially injected into the liquid-filled chamber.

As shown in *figure 3*, the local hybridization events on each sphere could be followed independently and in real-time – without requiring a label as in conventional DNA microarrays. Moreover, instead of a simple all-or-nothing response of most gene chips, WGM sensors enable us to extract more information from the binding kinetics such as the concentration of the analytes. If two spheres were coated with oligonucleotides differing only in one base, a complementary strand could be examined for the base present at this position (e.g. A or T, G or C). Thus, four spheres are required to sequence one nucleotide. N × 4 spheres would be necessary to sequence an N-mer. A real-time, differential measurement, with one sphere acting as a reference, eliminates background noise due to e.g. temperature fluctuations and unspecific binding. A SNP can thus be detected with a high signal-to-noise ratio[24], demonstrating the wide applicability of the method.

Mass production of such devices is possible, since both microcavities and optical waveguides can be fabricated on silicon chips using photolithographic techniques[25,26]. Such chips could allow the integration of a microcavity DNA array with »spot« sizes of ~ 10 μm.
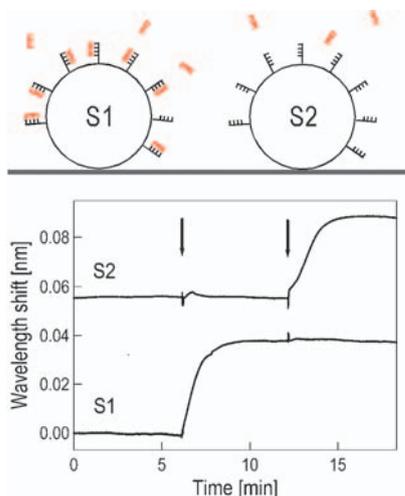
FIG. 3: Towards a DNA microsphere array: Two spheres are connected to the same optical fibre. Each sphere S1 and S2 is coated with a different oligonucleotide. Specific hybridization after sequential injection of the complementary strands can be observed in real time and with high sensitivity.

$$\frac{\Delta\lambda}{\lambda} = \frac{\alpha_{ex}\sigma}{(n_s^2 - n_m^2)a}$$
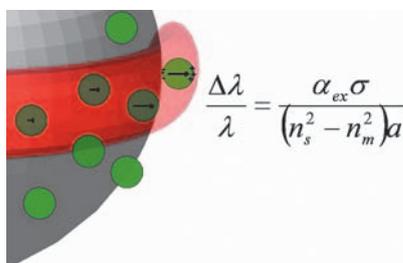
FIG. 4: Quantitative theory for the wavelength shift (refer to text). The optical resonance inside the sphere is associated with an evanescent field (red) extending about one wavelength (~ 1 μm) from the surface into the sample medium. The evanescent field induces polarization of bound molecules which causes the shift of the resonant wavelength.

Polarizability of molecules and stochastic sensing

• The technique also enables us to obtain quantitative information on molecules and their interactions, since the contribution from each single molecule to the overall observed wavelength shift, and in particular the magnitude of the shift, can be discerned theoretically. The magnitude of the wavelength shift, in turn, can be examined by the quantitative first-order perturbation theory[18,19,27,28].

To successfully perform this detailed analysis, we must modify our intuitive picture of the microsphere resonance. The light orbiting within the sphere is associated with an evanescent field, extending about one wavelength from the surface of the sphere (*Figure 4*). The evanescent field is an extension of the electromagnetic wave circulating the sphere, and as such will induce polarization of bound molecules. The energy required by a resonance to polarize the molecules bound within the evanescent field directly above its orbit compared to its total energy content is a measure of the resonant wavelength shift.

A simple formula can be derived which relates the resonance shift $\Delta\lambda$ to the surface density (σ) of bound molecules and their excess polarizability (α_{ex}) (*Figure 4*, with n_s and n_m being the refractive index of the sphere and the surrounding medium, respectively, λ the nominal wavelength of the laser, and a the sphere radius). The excess polarizability α_{ex} is defined as the polarizability of the molecule in excess compared to an equal amount of water. The units for polarizability are Ångstrom cubed (Å^3) and the magnitude is dependent on the size and class of the biomolecule. As a rule of thumb, the excess polarizability is approximately equal to the volume of a protein or DNA molecule, e.g. α_{ex} of BSA is $(38 \text{ Å})^3$, compared to the radius of gyration for BSA of ~ 30 Å. The polarizability is therefore a proportional measure for the molecular weight. If unknown, the polarizability can be calculated from measurements of the refractive index of a protein or DNA solution[29].

This quantitative first-order perturbation theory is a valuable tool for detailed analysis of biomolecular interactions. For sphere-, or sensor-bound molecules of known polarizability, the shift signal provides a direct measure of their surface density. For example, a shift of 21 pm in BSA adsorption measurements corresponds to a surface density of 1.7×10^{12} molecules/cm^2. This density is in agreement with a BSA monolayer which covers 63% of the surface[24]. Depending on the aminosilanization method used to coat the sphere[30], surface coverage of up to 90% was achieved.

By way of comparison, the hydrogel matrix-assisted immobilization of oligonucleotides produced ~ 3.6×10^{13} cm^{-2} binding sites for the complementary oligonucleotides, with an accessibility for hybridization of ~ 78%[24]. In other words it had a much higher surface density than that measured for a single protein layer.

From the above theory, we can calculate how much molecular mass is loaded per surface area. With our current prototype set-up, a minimal mass loading of ~ 1 pg/mm^2 is detectable[30], which is already one magnitude more sensitive than commercially available SPR sensors (~ 10 pg/mm^2, or about 10 response units)[14].

Outlook

• Biomolecular analysis with micro-optical, i.e WGM, resonances is one of the most sensitive, label-free techniques now available to the biologist and biophysicist.

While experiments with prototypes have demonstrated the large potential of this new technique, there is still plenty of room for improvement. For example, implementation of microfluidic sample delivery to the microsphere would eliminate the noise caused by molecular diffusion. The detection of a single virus particle or bacterium should then be possible. Using a microsphere cavity made of sapphire and thus of ultimate Q, single-molecule detection seems theoretically possible[27]. This would enable stochastic sensing of molecular interactions in solution without the necessity to label the molecules. Such ultimate sensors would allow unprecedented insight in the non-equilibrium dynamics of molecular recognition, conformational changes, and biomolecular function: each binding and unbinding event of a molecule could be observed in real-time. Such information is usually masked by averages from bulk measurements obtained with conventional techniques.

The technique already constitutes a more sensitive tool for biodetection and analysis than commercial SPR instruments. It also has several other advantages, some of which have not

yet been explored. For example, each microsphere resonance is polarized – either orthogonally (transverse electric) or parallel (transverse magnetic) to the microsphere surface. The analysis of molecular interactions with two orthogonally polarized modes can be used to study surface morphology, orientation, and conformational changes of biomolecules[30].

Furthermore, micro-optical resonances can be excited simultaneously at different wavelengths. From measurements taken at two wavelengths, it is possible to determine the dielectric constant and thickness of an adsorbed layer in one measurement and monitor its change in real time[31]. This approach can be extended to several wavelengths, thus enabling us to determine the profile of a molecular mass distribution close to the sensor surface[28]. Such measurements could be used in biology to analyse the refractive index profile of virus particles, cells, and bacteria; measure their size and weight and thus identify the species, possibly more quickly than with any current method. It may be possible to monitor such parameters for single cells and learn about the nature of e.g. cellular development, viral infection, or bacteriophage lysis. Our prototype has demonstrated great potential in pilot experiments and opens up a multitude of applications in all areas of biology. Further development is under way to reach the aim of single-molecule sensitivity in an aqueous solution.

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