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Metal Nano Clusters as Transducers for Bioaffinity Interactions

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Summary. Metal clusters excited by electromagnetic radiation exhibit high local field enhancement and nanoscale resonant behavior. Absorptive properties of these metal clusters bound to a surface are the basis of various new and highly promising setups to transduce biorecognitive interactions into an optical signal.

Immunogold labelling and immunogold chromatography as well as SPR transduction of metal cluster binding have been successfully commercialized. Multilayered highly resonant systems have been proposed and recently realized employing a metal mirror, a polymer distance layer, a biomolecule interaction layer, and biorecognitively bound metal nano clusters. Experiments indicate a strong influence of cluster symmetry and cluster shell on the strong reflection minima induced by the resonant behavior. Modified clusters, clearly exhibit at least one additional narrow reflection minimum in the red or infrared region and therefore far away from spherical gold colloids (<520 nm). Glass-type metal clusters synthesized by interruption of the thermal step of Au^{3+}-reduction as well as metal-dielectric shell clusters synthesized by multiple shell deposition processes enables the shift to a near-mid IR resonance.

It is possible to convert directly (without use of additional analysis steps) biorecognitive binding processes and catalytic activity of proteins by the application of surface enhanced clusters into a visible optical signal (color change of sensor surface). Disposable single step assays as well as multiuse-monitoring devices have been established employing *e.g.* lectin-sugar, antigen-antibody, protein-receptor, or DNA-DNA interactions.

Keywords. Metal nano clusters; Surface enhanced absorption; Array; Teststick.

Metallnanocluster als Transducer bioaffiner Wechselwirkungen

Zusammenfassung. Metallcluster, die durch elektromagnetische Strahlung angeregt werden, zeigen starke lokale Feldüberhöhung und resonantes Verhalten. Absorptive Eigenschaften dieser Metallcluster, welche an oder nahe einer Oberfläche gebunden sind, können als Basis neuartiger analytischer Meßsysteme dienen, welche biorekognitive Wechselwirkungen in ein optisches Signal umzuwandeln vermögen.

Immunogoldmarkierung, Immunogoldchromatographie und die SPR-Transduktion der biorekognitiven Bindung von Metallclustern an Oberflächen wurden bereits mit Erfolg kommerziell verwertet. Mehrschichtige hochresonante Systeme wurden zuerst theoretisch berechnet und in den

letzten Jahren durch einen Aufbau aus metallischer Spiegelschicht, einer nanometerdicken Polymerschicht, einer biorekognitiven Interaktionsschicht und daran gebundenen metallischen Clustern realisiert. Experimentell konnte gezeigt werden, daß die Symmetrie und der schalenförmige Aufbau der Cluster einen starken Einfluß auf die Lage und das Auftreten von resonanten Absorptionsbanden hat. Insbesondere Cluster mit glasartig-amorpher Struktur zeigen ein schmalbandiges Reflexionsminimum im Rot- bzw. IR-Bereich und liegen daher weit niederenergetischer als sphärische Goldkolloide (< 520 nm). Modifizierte Metallcluster, welche durch Unterbrechen des thermischen Reduktionsschritts aus Au^{3+}-Lösungen oder durch Metall-Dielektrikum-Schalenaufbau synthetisch zugänglich sind, zeigen die erwähnte starke Resonanz im IR-Bereich.

Der Sensoraufbau ermöglicht es, direkt (d.h. ohne zusätzliche Verfahrensschritte) biorekognitive Bindungsvorgänge und katalytisch-enzymatische Prozesse unter Anwendung der oberflächenverstärkten Clusterabsorption in ein optisches Signal (Farbe der Chipoberfläche) umzuwandeln. Sowohl einschrittige Assays in Form von Teststreifen oder Sensoren als auch kontinuierliche Meßsysteme konnten unter Nutzung von Lectin-Zucker-,Antigen-Antikörper-,Protein-Rezeptor-,Metall-Ligand- oder Nukleinsäurewechselwirkungen aufgebaut werden.

Introduction

The choice of metal clusters as signal transducers of molecular binding events is based on their 100 to 1000 times higher extinction coefficients compared to conjugated chromophores based on molar concentrations. Using cluster based assays it is possible to visualize the binding of biomolecules at a given surface by a bound layer of ligand modified metal clusters. A similar direct approach of detection is impossible with chromophores without using an additional amplification by enzymes (*e.g.* employed in enzyme linked immuno assays = ELISA).

Since the first introduction of immunogold and silver staining methods, signal transduction by metal nano clusters has experienced an enormous evolution. Various methods have been developed to synthesize clusters in solution by chemical means. Especially narrow size distribution and surface ligand modification enabled application as a transducer of various biorecognitive binding reactions. The success of cluster based visualization is based on

- electron dense particles with highly resonant electrons,
- preparation as monodisperse particles by chemical synthesis,
- efficient stabilization and coating with various biomolecules,
- direct optical visualization,
- secondary enhancement by deposition of silver, and
- signal stability (contrary to chromophores and fluorophores).

Moreover, cluster probes are not only efficient markers, but within the last years they have become the basis of new devices employing cluster resonance, cluster field enhancement, and cluster-cluster interactions.

Theory of metal clusters

Discussing optical spectroscopy of metal clusters in terms of electrodynamics, it is appropriate to apply a quasi-static regime only for clusters smaller than $\lambda/50$ (Ag: < 10–20 nm for visible light) [1, 2, 3]. Within a static regime, phase shifts in the

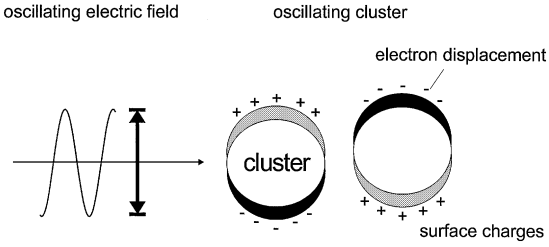

Fig. 1. Cluster excited by electromagnetic field

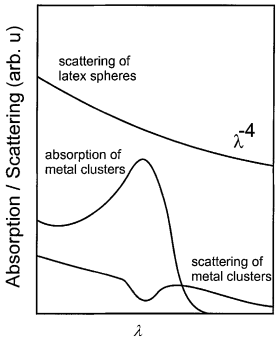

Fig. 2. Absorption and scattering of metal clusters and dielectric spheres

particle are small enough to be neglected and would simplify the excitation of the particle to an oscillating dipole (Fig. 1).

For clusters of any size or geometry the dominant features of the optical spectra are those with the strongest oscillation strength (Fig. 2). Resonance of metal clusters is dominated by collective oscillation of the electron gas within the cluster either forming plasmons or plasmon polaritons. In contrast, in an extended metal unconfined electron movement is possible, resulting in strong, unspecific reflectivity, well known as metallic lustre. Leaving apart the wide area of vibrational spectroscopy (surface enhanced *Raman* spectroscopy, SERS) and the magneto-optical *Kerr* effect, we will mainly discuss electronic excitation from visible light to IR.

In a realistic setup, the resonance of clusters is smeared out due to cluster size and shape distribution. Furthermore, the influence of matrix boundaries as well as a finite penetration of the electromagnetic wave ($\delta = \lambda/2\pi k$, *e.g.* Au about 30 nm) have to be considered. It is obvious that in particular the electron mean free path plays a key role in optical properties of metal clusters. Whereas for small particles (< 10 nm) the field attenuation is small, in larger particles only surface regions are excited by the field.

To apply electromagnetic theory, the positive charges are assumed to be immobile, and the negative charges are allowed to move being excited by the external field. Metals able to resonate in the field are free electron metals based on the fact that most of the electronic properties are caused by conduction electrons. All free electron metals have completely filled valence bands and partially filled conduction bands. For free electron metals, the optical response is based on the

conduction electron band, whereas other metals exhibit various interband transitions. Noble metals exhibit both free electron and interband transition behavior depending on the frequency range. For theoretical studies, alkali metals proved to be very useful, but due to their instability in ambient aqueous environment, silver, gold, aluminum, and magnesium are advantageous for analytical purposes. The response of a metal to an electromagnetic field is described by the dielectric function $\varepsilon(\omega)$ of *Drude-Lorentz-Sommerfeld* [1]. The internal field of a metal cluster can be calculated by adding the boundary conditions of the sphere surface: $E_{\mathrm{cl}} = E_{\mathrm{o}} \cdot 3\varepsilon_{\mathrm{m}}/(\varepsilon + 2\varepsilon_{\mathrm{m}})$ with ε_{m} as dielectric constant of the embedding medium. In a general case this can be transformed into the classical static electric polarizibility of a spherical cluster of radius R: $\alpha_{\mathrm{cl}} = 4\pi\varepsilon_{\mathrm{o}}R^3 \cdot (\varepsilon - \varepsilon_{\mathrm{m}})/(\varepsilon + 2\varepsilon_{\mathrm{m}})$. This formula is well suitable for small metal clusters because clusters feel a time dependent but spatially constant phase of the electromagnetic wave. Whereas ε_{m} is taken as a constant, ε has to be replaced by $\varepsilon(\omega)$ and extended to a complex dielectric function $(\varepsilon = \varepsilon_1 + \mathrm{i} \cdot \varepsilon_2)$. Thus, for small ε_2 a resonance occurs when $\varepsilon_1 = -2\varepsilon_{\mathrm{m}}$; its resonance frequency at $\varepsilon_{\mathrm{m}} = 1$ is $\omega = \sqrt{(e^2/(m_{\mathrm{e}}4\pi\varepsilon_{\mathrm{o}}R^3))}$. The dipolar absorption of a metal cluster can be expressed by its extinction cross section: $\sigma(\omega) = 9V_{\mathrm{o}} \cdot \varepsilon_{\mathrm{m}}^{3/2} \cdot \omega/c \cdot \varepsilon_2(\omega)/((\varepsilon_1(\omega) + 2\varepsilon_{\mathrm{m}})^2 + \varepsilon_2(\omega)^2)$.

Sometimes ω is denoted as a surface plasmon frequency; nevertheless, it is a resonance (and polarization) of a particle. Summing up, the conduction electrons in clusters act like an oscillator system, whereas those in a bulk metal act as a relaxator system with no excitation at the *Drude* eigenfrequency ω_{p}.

The discussion above holds as a useful estimate for sufficiently small particles. Realistic coupling of the electron gas with the ion core can be described by refinement procedures, *e.g.* introducing an effective optical mass instead of the electron mass, and was first introduced by *Mie*, *Gans*, and *Happel*. The theory is based on a solution of *Maxwell*'s equations with appropriate boundary conditions expanding the setup to multipol excitation. *Mie* divided the problem into two parts: the electromagnetic one and that based on material properties: *Mie* omitted a theoretical treatment of material behaviour by introducing a phenomenological dielectric function $\varepsilon(\omega, R)$. This function $\varepsilon(\omega, R)$ includes an average of all electronic and atomic functions of the cluster as dipolar, quadrupolar, and higher electric and magnetic modes of absorption and scattering. The success of this theory is based on extracting data from experiments correlating ε_1 with $\Delta\lambda_{\max}$, changes of peak height with ε_2, and changes in band width with changes in refractive index. Without going to much into detail it should be mentioned that steep $\varepsilon_1(\omega)$ induces narrow resonance peaks, whereas flat $\varepsilon_1(\omega)$ results in broad band absorption.

Metal clusters of noble metals

For alkali metals the dipolar surface plasmon is very pronounced, since damping is small in the visible spectral region. In contrast, noble metals (mainly Ag, Au, and Cu; see Fig. 2) show significant deviation from free electron behavior due to interband transitions from, *e.g.*, 4d to 5sp for Ag. These transitions exhibit a strong influence on the spectra in the visible region. The plasmon energy limit shifts from

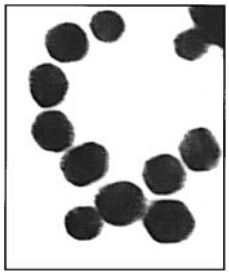

Fig. 3. Electron microscopy of a 14 nm Au cluster with high crystallinity

the free electron value (above 9 eV) to less than 4 eV. Thus, this resonance cannot be regarded as a free electron resonance; it resembles a cooperative effect based on conduction and 4d electrons. Whereas silver is the less pronounced member of the group of noble metals cited above, in copper the plasmon energy limit shifts from more than 9 eV to about 2 eV; therefore, no resonance can be obtained by embedding it in media with ε_m values around 1. It is thus obvious that only Ag shows a well developed resonance near 400 nm, whereas Au displays a peak around 520 with a shoulder at higher energy. Resonance of Pt clusters is damped in the visible region of the spectrum, but is useful in the near IR for *e.g. Raman* enhancement. Other metals like Ti, Ta, Th, *etc*. exhibit plasmons combined with various elementary excitation states of the metal. Dielectric spheres of *e.g.* MgO exhibit weak *Mie* resonance based on phonon polaritons at μm sized particles in the IR range.

A new route to the synthesis of colloidal Au clusters with diameters between 30 and 100 nm has been described by *Natan et al*. [4]. On the basis of surface catalyzed reduction of Au^{3+} by NH_2OH, the approach grows existing nano particles into larger particles of a size determined solely by the initial particle diameter and the amount of Au^{3+} added. The resulting clusters exhibit improved monodispersity relative to those prepared in one step by the reduction of Au^{3+} by citrate. Moreover, surface confined colloidal Au clusters can also be enlarged by this method, providing an attractive route to colloidal Au monolayers with variable inter particle spacing at fixed particle coverage.

Metal clusters at phase boundaries

Whereas a simple discussion of $\varepsilon(\omega)$ depicts the setup of single spherical clusters in an isotropic material, it has to be extended for anisotropic phase boundary systems [1]. Clusters on surfaces are influenced by the adjacent medium. In a very simple case, ε_m can be replaced by an averaged dielectric ε of the surrounding media giving reasonable results.

Cluster-cluster interaction

Effective medium theory can only be employed reasonably well as long as no significant coagulation of the clusters takes place. The origin of the two resonance peaks observed (see Fig. 4) can be deduced from experiments, clearly showing that the low frequency peak is reduced with increasing *p*-polarisation in contrast to the

high-energy peak which is nearly insensitive to a change of polarisation of the exciting field. This behaviour can be understood if the low-frequency peak is caused by longitudinal mode excitation, whereas the high-frequency peaks corresponds to the transverse mode of the system.

The stability of a cluster system against coagulation and further on coalescence is due to repulsive electrostatic-ionic interactions of the clusters. This interaction, sometimes combined with added polymers blocking cluster-cluster contact, prevents a close approach of clusters induced by thermal motion and migration.

Conjugates prepared by coupling of antibodies on colloidal gold particles have been used in a homogeneous sol particle immunoassay (SPIA) for various proteins (*e.g.* HCG fertility test) [5–7]. The technique is based on sol particle coagulation resulting in colour reduction. Optimal results were obtained using buffered conjugates prepared from 50 nm particles. Addition of polyethylene glycol (PEG) 6000 to the conjugates increased the agglutination rate considerably. The optimal PEG concentration of the conjugate depends on the desired incubation time and measuring range. The influence of temperature on the agglutination is small in the temperature range between 4 and 45°C. Higher conjugate concentrations result in steeper dose-response curves. The dose-response curves for *e.g.* HCG dissolved in buffer or in urine are almost identical, and the reproducibility in biological fluids is satisfactory. Homogeneous SPIAs have a high practicability, are easy to automatize, and provide an interesting new tool for the measurement of a variety of analytes. The moderate sensitivity of the assay may be considered as a disadvantage.

Based on the appearance of the second low frequency peak (Fig. 4), a new analytical device has been developed by *Mirkin et al*. [8–10]. The method is based on colorimetric detection of polynucleotides based on gold nanoparticles modified by mercapto-alkyl coated oligonucleotides. Introduction of a hybridizing oligonucleotide into a solution containing the modified particles results in the formation of a polymeric network of clusters. This coagulation of clusters can be directly monitored as a red to purple shift of the solution. Hybridization can be facilitated by freezing and thawing of the solution. Transfer of the coagulated

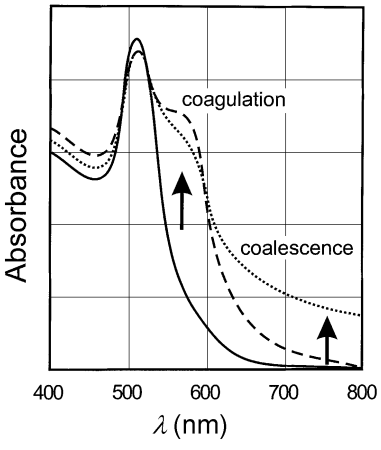

Fig. 4. Spectral changes induced by cluster coagulation and coalescence

cluster network to a porous silica surface results in a shift of the signal to a clearly visible blue spot. The system can detect femtomolar concentrations of low molecular weight oligonucleotides. A modification of the procedure enables the detection of single base-pair mismatches for application in medical diagnosis.

Surface enhanced metal cluster systems

The optical property necessary for the analytical application of metal island films is the so-called anomalous absorption. An absorbing thin film positioned at a defined distance to a metal mirror represents a special kind of reflection interference filter. At a certain distance of the absorbing layer to the mirror the electromagnetic field reflected by the mirror has the same phase at the position of the absorbing layer as the incident fields; by this feedback mechanism the effective absorption coefficient of the absorbing layer is strongly enhanced. As for a given interlayer distance the optimum phase is only given for a defined wavelength, the system is characterized by a spectroscopically narrow reflection minimum whose spectral position shifts sensitively with the interlayer thickness (Figs. 5, 6)

The optical characteristics of the sensor can be modelled by either the stratified medium theory [11] or the CPS theory developed by *Chance*, *Prock*, and *Silbey*. These theories focus either on optical thin films or on a polarizable particle in vicinity to a metal surface. The stratified medium theory can be applied for the calculation of any kind of optical thin films. It is based on the solution of *Maxwell*'s equations under the boundary restrictions represented by the interfaces between the different materials forming the multilayer system. Application of the stratified medium theory is based on the knowledge of the complex optical constants in all

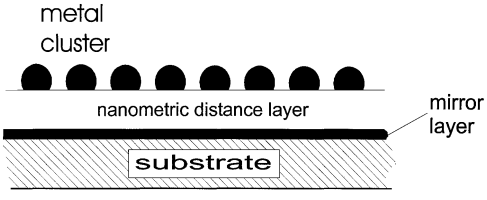

Fig. 5. Setup of enhanced cluster absorption

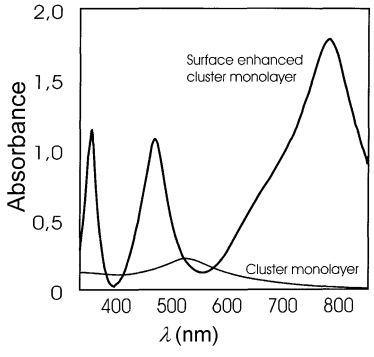

Fig. 6. Comparison of a simple and enhanced cluster monolayer

three layers (metal mirror, interlayer (transparent at a nanometric scale), and island film). Whereas optical constants of possible mirror metals (Ag, Au, Al) can be found in the corresponding literature, the optical constants of island films depend strongly on preparation parameters and must be evaluated experimentally. Appropriate methods for the determination of thin film optical constants are based on at least two independent measurements of the island film's reflection and transmission. From calculations of optimal conditions it can be concluded that at a mean mass thickness of 5 nm a maximum of the signal can be obtained. Dependent on the number of linker-bound clusters, the reflection varies over several orders of magnitude. At optimized enhancement conditions even single clusters close to the surface are visible as color changes upon irradiation with white light.

The optical reflection of a mirror-cluster system has been calculated as a function of interlayer thickness and wavelength for a gold mirror, an interlayer of any transparent material, and a 5 nm mass thickness gold island film. The reflectance r is defined as $r = \log(I_r/I_{in})$ where I_r and I_{in} are the reflected and the incident intensities, respectively.

As can be seen by a 2D plot based on stratified medium theory [11], there are several regimes of reflectivity change. According to the special swelling properties of the polymer used and to the desired dynamic range of which the system should operate, the optimum wavelength and layer thickness can be found. A horizontal section of the 3D plot represents the spectral dependence of the reflectivity for a certain interlayer thickness. It can be seen that the spectral reflectivity minimum strongly depends on the interlayer thickness, and thus a thickness change will be visually observable upon white-light illumination by the altered colour impression of the reflected light.

Nonspherical and core shell metal clusters

Experiments indicated a strong influence of cluster symmetry and cluster shell, observable as an increase in absorbance at $\lambda > 600$ nm. Based on the theory of *Mie* [1], several extensions have been developed dealing with nonspherical cluster shape and core shell particles. Based on the quasi-static approach, various geometric forms have been treated (*e.g.* oblates, ellipsoids, cubes, and cylinders). The axial ratio a/b as a main parameter is suitable for calculations extending the polarization to L_i ($i = a,\ b,\ c,\ \Sigma L_i = 1$; for spheroids: $L_a \neq L_b = L_c$). To treat samples with arbitrarily oriented ellipsoids, the signal has to be averaged over all orientations (Figs. 7, 8).

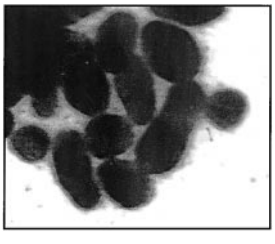

Fig. 7. Electron microscopy of slightly asymmetric, non-crystalline 14 nm Au clusters

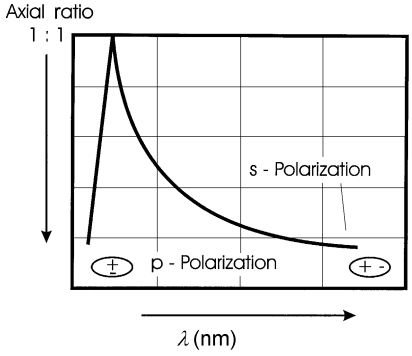

Fig. 8. Metal cluster with varying axial ratios near a metal surface

Clusters of single size and eccentricity exhibit a splitting of the peak to three more or less distinct peaks of about equal magnitude; clusters of continuous distribution of axial ratios correspond to a flat and broad absorptive region due to averaging.

Clusters composed by more than one element can be treated by combining the functions of pure metal particles. In contrast to homogenous clusters, broadband and multipeak spectra are observed. For very thick layers, spectra converge to the spectrum of the unmodified metal cluster. Since already very thin surface layers exhibit pronounced effects in the visible region, even small adlayers of sulfides or oxides lead to a significant deterioration of the spectra. This effect is well known in surface plasmon resonance of chemically reactive Ag films. Especially glass-type variation of the metal shell or core can significantly modify long-wave absorption of metal-cluster films.

Ordered arrays of metal columns deposited on or etched out of a substrate surface exhibit similar or even enhanced effects to those cited above. Especially high local fields enable efficient signal generation with *Raman*-type devices.

Immunogold labelling and chromatography

Based on well known gold-colloid labelling, various colloidal gold-based semi-quantitative manual immunoassay methods for the detection of antibodies or antigens had been developed and are available at the market as teststick-like devices [12, 13]. The colloidal gold particles are coated with an organic thiol reagent which in turn is attached to the antibody by covalent [S–*Me*] bonds. Appropriate antibodies or antigens for a sandwich type immunoassay are immobilized on the cluster surface and on simple chromatography paper. The paper strips are developed with an appropriate immunogold reagent in a plastic tube in the presence of urine (Pregnancy or Ovulation test) or serum (Rubella test). The mixture migrates up the strips towards the test band. A purple band develops which indicates the presence of the corresponding antigen. About 50 mIU of hCG in urine can be detected in 5 minutes or less, and antibody to Rubella virus at an HAI titer equivalent of 8U or above in serum can be detected in 10 minutes or less. These test kits are simple, rapid, and require no instruments to perform. A disadvantage

may by the moderate sensitivity of the assay and the lack of two-dimensional multianalyte capabilities.

SPR transduction of metal cluster binding

SPR excitation is observed when photons are coupled to a metal/dielectric interface inducing a resonant charge density oscillation at that surface. The wave is a perturbation of the electron plasma or, more precisely, a surface plasmon polariton extending up to 500 nm into the medium. The wave exhibits an exponential electromagnetic decay of the field perpendicular to the interface. Figures 9 and 10 illustrate a planar monochromatic wave incident upon a planar interface of two media.

The light strikes the interface at an defined angle. Hence, any coating of the surface of the metal with an overlayer of varying ε will give rise to a change of the mode properties of the resonant system. This change induces a shift in the resonant angle up to a few degrees. Based on this principle, various devices are commercially available which directly (without any label) transduce biochemical binding into a variation of resonant angle.

Although a direct transduction is very useful for basic interaction studies, overall sensitivity is low. Thus, based on the large difference of ε_{H_2O} and ε_{metal} a significant increase in assay sensitivity has been obtained adding a metal nano cluster label to the assay [14]. Disadvantages of this setup are the high costs and the limited multianalyte capabilities.

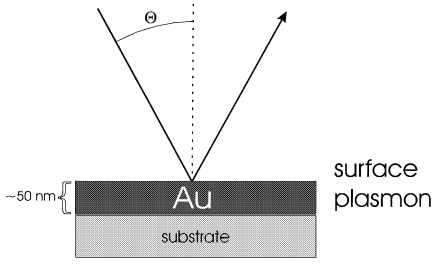

Fig. 9. Setup of surface plasmon resonance

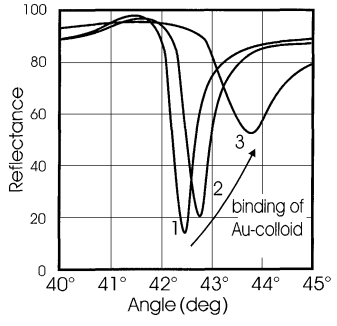

Fig. 10. Shift of resonant angle induced by cluster binding to the chip surface

Metal cluster setup

The metal cluster setup technology is based on surface enhanced metal cluster systems by means of which nucleic acids, enzymes, or binding proteins can be detected in a simple and reproducible way. The analytes induce binding or dissociation of metallic clusters which have been or will be bound at a defined distance to a reflecting, preferentially electron conducting, substrate surface (Fig. 11). The binding or dissociation is transduced into a clearly detectable optical signal through resonant enhancement of clusters interacting with their mirror dipoles.

The setup aims at reducing technical measurement restrictions through a novel one step test procedure [15–17]. Rapid and sensitive test kits for clinical and laboratory use can be set up based on this technology. Possible fields of application include *e.g.* the diagnosis of urinary tract infections, screening of allergens, detection and quantification of pathogenic bacteria, glucose, C-reactive protein, *etc*.

A displacement sensor consists of a metal layer on a substrate surface and an inert distance layer, deposited *e.g.* by spin coating or chemical vapor deposition, on top of which individual linker molecules with clusters are coupled. The diameter of the clusters is preferentially chosen to be smaller than 40 nm. If the analyte interacts with the linker, it induces either changes in the packing density of the cluster layer on a molecular scale or changes in the spatial arrangement of bound clusters, both leading to characteristic changes of the optical appearance of the sensor surface. The color of the surface is altered due to the catalytic activity of an analyte or the addition of an enzymatically or biorecognitively active component. The small size of the metal clusters or colloids (see above) serves to suppress multipol peaks in the spectrum and to enhance diffusion.

Given an optimized setup, the spectral position is highly dependent on the spatial arrangement, especially the distance of the cluster layer to an electron conducting surface and the local variation of ε_m. Moreover, the sensor setup can transduce changes in the extent of surface coverage with bound clusters into a clearly visible optical signal either by a strong change in absorption at a defined wavelength or a spectral shift of an absorption maximum.

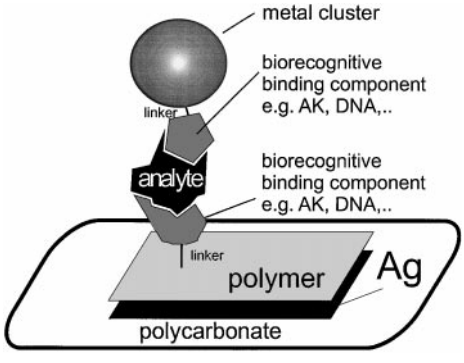

Fig. 11. Setup of enhanced cluster absorption biosensor

To estimate the ultimate sensitivity of the chip, clusters of a diameter of 25 nm are arranged in a two dimensional lattice of 100 nm at an optical resolution of 1/10 mm, inducing a change of 10% of the signal equal to 2×10^5 molecules. This sensitivity has been proved with an antigen-antibody setup detecting *E. coli* proteins in the femtomolar range. The application of catalytically active analytes or labels, *e.g.* urease, increases the sensitivity significantly and allows single molecule detection. Moreover, *Aussenegg* and *Leitner* were able to monitor the resonance of single metal clusters bound to a surface employing an AFM with near-field fiber optics.

In general, clusters (preferentially silver or gold cluster) can be bound by means of any biochemical linkers at a defined distance to a mirror. A detectable signal will result if these linkers are either formed or cut by biochemical recognition or by catalysis or if their spatial arrangement is altered. According to the development for a displacement sensor *e.g.* oligonucleotides are applied as linkers which can then be cut by specific restriction enzymes from microorganisms. Many pathogenic microorganisms express specific restriction endonucleases and can therefore be detected by means of the new sensor without expensive instrumentation.

Due to their particle structure, cluster or colloid films do not form a barrier of diffusion to gases or fluids. The analyte concentration can be measured with high sensitivity *via* visual inspection of the sensor surface. In order to reduce unspecific background absorption of the sample, measurement can be carried out at two angles of observation. Whereas the absorption of chromophores is independent of the angle of observation, the specific signal strongly shifts dependent on the angle. Simple subtraction of both signals thus eliminates the background resulting from matrix effects. The intensity of the absorption band is directly proportional to the number of interacting clusters. Any reduction of the number of clusters by chemically induced cut-off results in a lowering of the absorption of the system and-at a high surface coverage-in a spectral shift due to a change in cluster-cluster interactions.

Metal cluster biosensors

Surface enhanced absorption is defined as an increased optical absorption of *e.g.* cluster layers in nanometric vicinity to a metal surface. Total enhancement in absorption of spherical metal colloids is at least 8-fold, relative adsorption enhancement more than 100-fold and independent of the way of application of sputtered clusters but depending on the attachment of colloids under given conditions (Fig. 12; see also Fig. 6).

Up to a wavelength of 600 nm, the optical properties of sensor chips covered with clusters are equivalent to chips covered with sputtered metal clusters. At any wavelength substantially above 600 nm, sputtered cluster layers show optical properties dramatically different from those of crystalline colloids attached to the polymer surface *via* affinity interactions; this results from the difference in shape as well as from varying degrees of crystalline nano-structure (Fig. 13).

In the course of gold colloid synthesis based on the procedure of *Frens* [23], remarkable changes in the color of the reaction mixture occur. Upon adding the

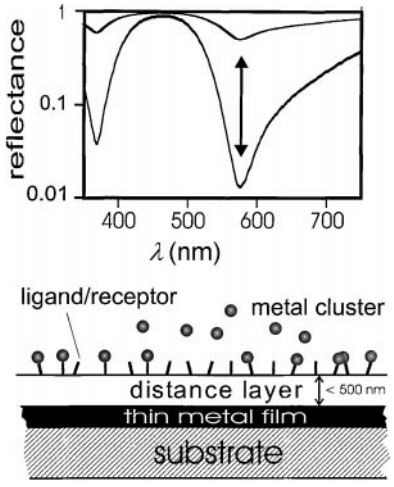

Fig. 12. Spectral response of an enhanced cluster absorption biosensor

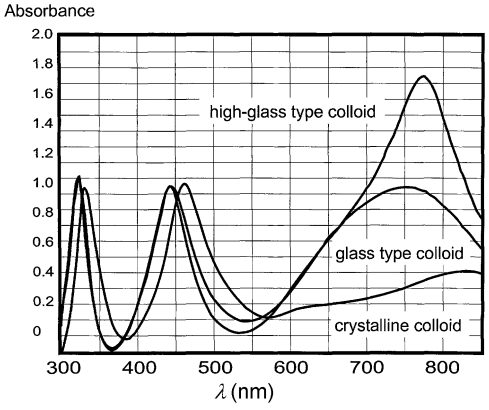

Fig. 13. Spectral response of an enhanced cluster absorption using Au clusters of varying crystallinity

reducing agent to a boiling yellow gold solution, the color changes within minutes from black to dark blue, violet, and finally shifts suddenly to red. By comparing the absorption spectra of the reaction mixture as a function of time during the synthesis, one finds that the characteristic sharp absorption peak of nano-crystalline colloidal gold develops only slowly. Earlier stages of the synthesis absorb light also above 600 nm; these can be isolated by a rapid cool-down or addition of metal ions blocking crystallization of the gold cluster (Fig. 14).

If such glass-type non-crystalline colloids are applied in a surface enhanced absorption setup, resonance enhancement at $\lambda > 600$ nm is significant. This enables to shift the desired absorption peak all over the visible spectrum to IR and therefore to wavelengths favorable for technical reasons of measurement, as *e.g.* concerning matrix absorption or light source.

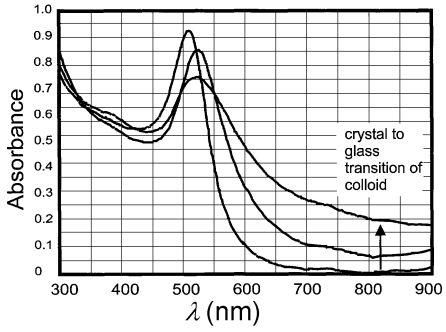

Fig. 14. Spectra of Au clusters in aqueous solution with varying crystallinity

The following scheme gives a typical example for the stepwise construction of a sensor chip, including the bio-affinity binding events of gold colloids to the distance layer.

1. The chemically inert distance layer is treated with DC or RF oxygen plasma.
2. Oxygen plasma treatment modifies the surface of the polymer distance, generating OH, CO, and COOH groups.
3. Horse radish peroxidase (HRP) is attached to the distance layer.
4. A solution of ConA is added. Since HRP presents multiple glycosylation and mannosylation sites, ConA binds to the HRP coated surface *via* specific lectin-sugar interactions.
5. To perform the assay, a solution is added containing gold colloids onto which horse radish peroxidase (HRP) has been bound; the coated colloids bind to the free sugar recognition sites of surface bound ConA tetramers.

The binding in the last step can be monitored in real time. Such direct and sensitive kinetic monitoring enables to transduce a number of molecular binding events into a macroscopic optical signal visible to the eye.

In the following, a typical setup applying surface enhanced absorption is described. A glucose sensor employing competitive binding of HRP coated colloids and varying amounts of glucose was set up according to the above procedure. The sensor surface is covered with ConA. Upon incubation with a mixture of HRP coated colloids and a test solution containing sugar, colloids will compete for the binding sites at the sensor surface. As a result, the sensor color will depend on the concentration of sugar in the test solution since sugar molecules interfere with the HRP coated colloid-lectin binding. The lower the concentration of sugar in the solution, the more HRP coated colloids will be able to bind to the ConA coated surface. Unbound colloids may be washed off from the sensor. Figure 15 shows the kinetics of the binding of HRP coated colloids as well as of the binding of colloids in the presence of an increasing amount of mannose acting as a competitor of colloid binding to the reactive sensor surface.

The binding kinetics of the bioaffinity interactions are strongly influenced by the number of sugar residues per colloid bound to the surface. Therefore, variation of surface concentrations of sugar residues will strongly influence the overall

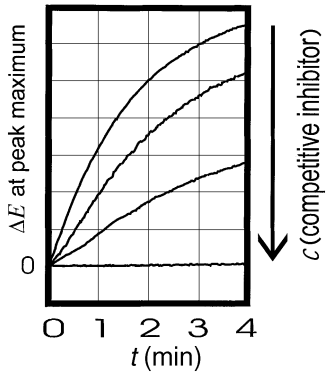

Fig. 15. Kinetics of the binding of Au cluster to the chip surface *via* biorecognitive interactions

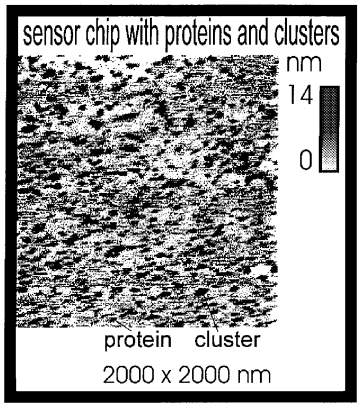

Fig. 16. AFM scan of a biosensor chip surface with immobilized proteins and attached 14 nm Au clusters

number of colloids bound to the solid support. The choice of an appropriate lectin is a way to influence the speed and equilibrium of cluster binding.

To visualize the absolute extent of surface coverage with colloids, scanning techniques based on STM and AFM have been employed. Figure 16 shows about 10^3 ConA molecules covering about 5 percent of the surface of which about 1 to 10 percent have bound a colloid, whereas the polymer surface is smooth at a nanoscale level. Activation of the sensor surface by plasma chemistry enables surface modification with good homogeneity.

Any conventional ELISA can be adapted to the new detection protocol transducing various antigen-antibody interactions. *E.g.*, *anti-E. coli* (IgG) has been used employing 14 nm gold colloids. *E. coli* proteins were adsorbed to the polymer distance layer. Upon incubation of *E. coli* covered sensor surfaces, the *anti-E. coli* coated colloids bound to the sensor surface and the sensor developed a color visible to the eye. The detection limit of *E. coli* protein was in range of fmol/mm^2. To study interactions of small haptens with antibodies, the sensor chip was covered with a polymer distance layer modified with 2,4-dinitrophenyl labeled BSA, a well-

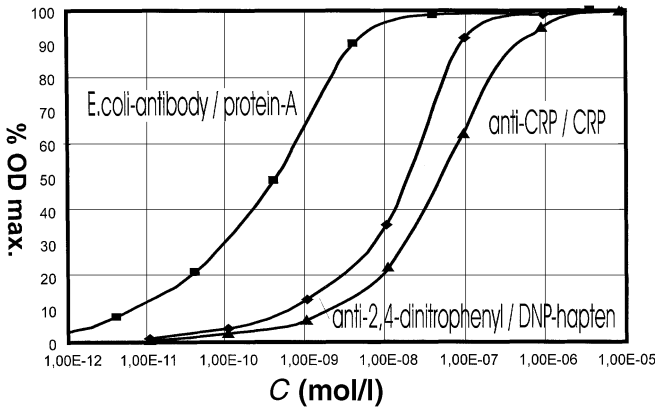

Fig. 17. Reflectance *vs.* concentration graphs of an enhanced cluster absorption biosensor using antibodies, proteins, and allergens as analytes

known allergen. Incubating the chip with varying dilutions of *anti*-DNP sera, the antibodies were detected with recombinant protein G coated clusters (Fig. 17) [16].

One significant advantage of the surface enhanced optical sensors is the ultra-high density which can be achieved using printing techniques on surfaces similar to a CD-ROM or DVD (digital versatile disk). Areas of 1×1 μm easily can be read out with rather cheap and simple devices. Due to the nonporous ultraflat surface array based sensor, chips are under construction using photolithography and inkjet printing. Using these techniques, hundreds to millions of different drugs, ligands, proteins, or oligonucleotides can be immobilized in a well defined array on a chip. In addition to the direct techniques described above, surface enhanced optical sensors can also transduce the action of an enzymatic label, *e.g.* by application of a *pH* or ion sensitive distance layer undergoing reversible volume changes upon a *pH* shift. The setup can be as follows: Receptors to be assayed for the binding of pharmaceuticals are immobilized to the sensor. Urease is coupled to an antidrug antibody as a label. Binding to receptors on the sensor surface occurs in a narrow compartment. Upon incubation with urea, the enzyme will produce ammonium and carbonate which will lead to local *pH* and ion concentration changes which are reversibly transduced into a macroscopic optical signal. This setup offers highest spatial resolution and access to direct use of ELISA protocols.

A direct kinetic monitoring of biomolecular interactions has been achieved by transforming a molecular binding event into an optical signal visible to the eye [15]. The optical sensor enables to setup one step assays because detection does not necessitate any extra incubation step. Surface enhanced absorption transduces changes in surface coverage of sub-monomolecular cluster layers.

Optical characteristics of a MICSPOMS type device

The Metal Island Coated Swelling Polymer Over Mirror System (MICSPOMS) is an optical thin layer system consisting of a metal mirror, a transparent interlayer (in this case represented by the active analyte induced swelling polymer), and a metal cluster (island) films as the topmost layer (Fig. 18) [18–22]. Due to the special

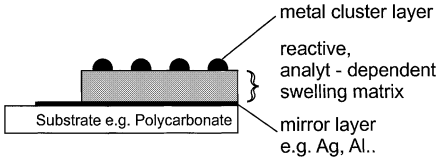

Fig. 18. Setup of a MICPOMS sensor device

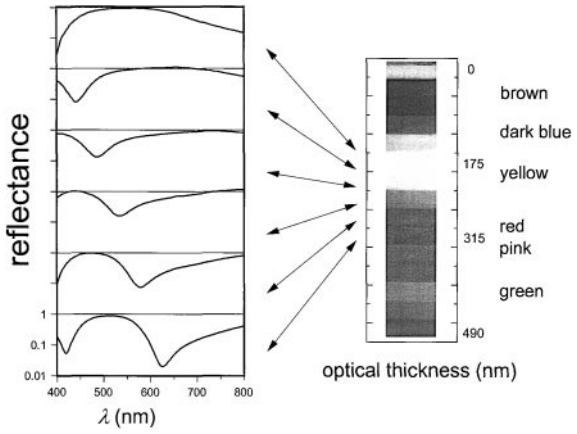

Fig. 19. Spectral response of a MICPOMS sensor device

optical behaviour of an island film and due to the special layer design, this system shows the characteristic spectral reflection behaviour cited above, being strongly dependent on the thickness of the transparent interlayer (Fig. 19).

Polyvinylpyrrolidone crosslinked with sulfonated bisazidostilbenes exhibits ion dependent shrinking and swelling which can be observed by using this polymer as interlayer polymer in a MICSPOMS device. The response of the sensor depends on type, charge, and concentration of the ion. It is fully reversible and exhibits a fast volume change due to the direct exposure of the very thin swelling polymer layer to the analyte. As a chemical sensor, the MICSPOMS system was used to monitor different ions, *pH*, organic solvents, and polyphenols.

As the main result it could be shown that due to their related structure the effect of chaotropic agents on polyvinylpyrrolidone polymers and on proteins is very similar. Chaotropic agents do not only interact with the surface of a polymer, but also increase the free volume of the polymer chains. Moreover, the experiments pointed out that the device can directly and dynamically monitor volume changes of polymer layers of one or several molecules thickness. The crosslinking of polyvinvlpyrrolidone (*PVP*) with *DIAS* (4,4′-diazidostilbene-2,2′-disulfonic acid disodium salt tetrahydrate) is shown in Fig. 20.

The visual impression obtained by observation of the reflected light upon diffuse white light illumination of MICSPOMS in the interlayer optical thickness range from 0 to 490 nm is shown in Fig. 19 (optical thickness = thickness × refractive index). This photograph shows that there is sufficient colour contrast for visually distinguishing thickness changes of a few nanometers. Swelling of the

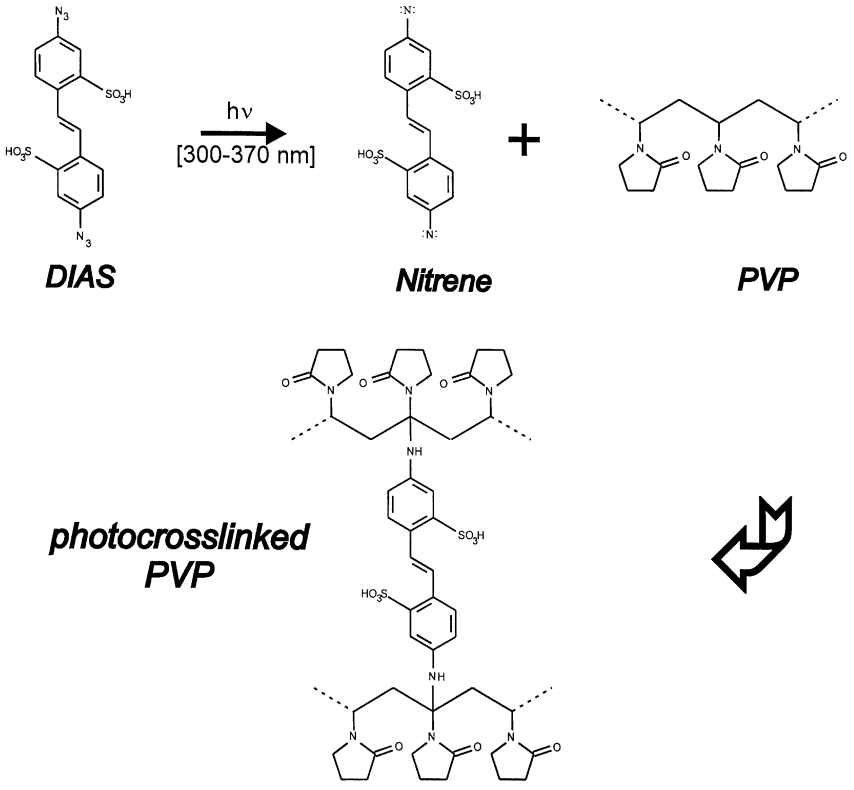

Fig. 20. Photochemical crosslinking of polyvinylpyrrolidone with reactive azides

interlayer polymer is equivalent to increasing the optical interlayer thickness. The volume response of a MICSPOMS sensor induced by different inorganic ions is fully reversible. Thus, the spectra obtained upon variation of ions in the polymer layer can be compared with calculated spectra and calibrated in effective thickness. The increase in absorbance (which corresponds to a decrease in reflectance) at a certain wavelength is an appropriate measure for the interlayer thicknes change. For small changes, a linear relation between absorbance and thickness change can be assumed. A typical absorbance *vs*. time graph is given in Fig. 21.

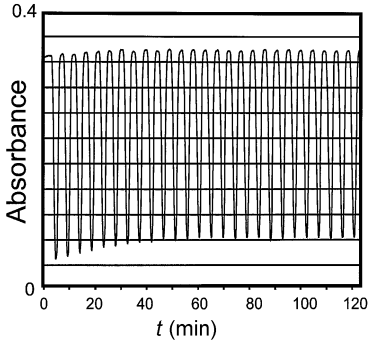

Fig. 21. Reversible response of a MICPOMS device to changing analyte concentrations

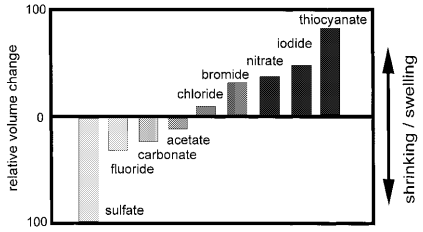

Fig. 22. Response of a MICPOMS device to various ions of the *Hofmeister* series

In most cases, a distinct reversible colour change could be observed when the concentration of an ion in a buffered solution was changed. Ion variations were established using buffer systems similar to human blood. Variations of chloride induced within the pathological concentration range were transduced after exclusion of large proteins from the polymer. No or only minor changes were observed near *pH* 7. The shrinking of the polymer layer induced by different ions is not restricted to a neutral *pH* and was also observed at higher and lower *pH* values. As can be seen from Fig. 22, different salts induce either shrinking or swelling of the polymer.

Regarding a wide concentration range, a sigmoid logarithmic calibration curve of the sensor shrinking response was obtained using monovalent cations and anions. Bivalent cations induce a more complex behaviour of the polymer gel. The calibration curve for Ca^{++} is an overlay of the sigmoid logarithmic shrinking behaviour with a swelling effect at high ionic concentrations. Due to the direct exposure of the active swelling polymer layer to the analyte, an immediate response (within seconds) to changes in the ionic environment can be observed.

A comparative study of the ion effect using a crosslinked polyvinylpyrrolidone polymer which is solubilized in aqueous solutions by the interaction of its amide structure with the solvent demonstrated a fundamental correlation of the polymer swelling properties with the *Hofmeister* series of chaotropic agents [20]. The ion effects are in good accordance with the molecular theory of chaotropic agents. Thus, it could be shown that the main effect of chaotropic agents in amid polymers and in proteins is very similar due their similar structure. As the main result from these experiments the following conclusions are to be considered: PVP is a model system for the solvent interaction of protein chains, chaotropic agents do not only interact with the surface of a polymer but increase the free volume of polymer chains, and the device can directly and dynamically monitor volume changes of polymer layers of only one to some molecules thickness.

In the *pH* range of 5–8 no distinct change in the protonation of quaternized amines (for PVP copolymers), no change in the protonation of sulfonic groups of the crosslinking agent, and no protonation of the amide nitrogen occurs. For a given buffer, a significant variation of the reflectivity maximum as a function of *pH* was observed at high and low *pH* values. Thus, between *pH* 5 and *pH* 8 ion effects can well be separated from *pH* effects. The volume response of the sensor induced by different ions is fully reversible over more than 500 cycles.

Applications

Enhanced metal cluster absorption enables the transduction of molecular binding events into visually detectable signals. The focus of the development was to provide an optical biochip which allows detection of analytes such as DNA/RNA, proteins (allergens, restriction enzymes, antibodies), cell metabolites, and even living cells. The setup allows to replace conventional binding assays (like ELISA), overcoming the various technological limits as there are multiple incubation steps, harmful reagents, and spatial resolution. Three major areas of product development are under investigation:

1. *Diagnostics*:
   Various setups for C-reactive proteins, *E. coli* proteins, blood glucose, allergens, and various antibodies have been realized. *E.g.*, in a commercial allergen screening sensor the surface will be covered with a well defined pattern of different allergens upon reaction with serum and subsequent detection with anti-IgE coated colloids; spots will develop when the patients blood contained antibodies against the particular antigen.
2. *Drug screening*:
   A. High spatial resolution, combinatorial chemistry on nano particles (mix + split), and analyte diffusion restricted to defined areas can be achieved, also in combination with photo patterning of the sensor surface. The signal can be read out on conventional microplate readers, scanners, CD-ROM-based devices, or by means of video microscopy.
   B. Microstructuring of the sensor surface as well as application of living cells allows to visualize the metabolism of whole cells attached to the sensor chip. In order to exploit the high spatial resolution of the chip, cells are bound to an array of various adhesion sites. The cell restricts diffusion of excreted products after being attached to the chip surface, forming a narrow nanoscale compartment beneath (Fig. 23).
3. *Environmental monitoring*:
   Online monitoring of analytes such as atrazine-and pentachlorophenol-like substances and xeno-estrogenes are further goals of research.

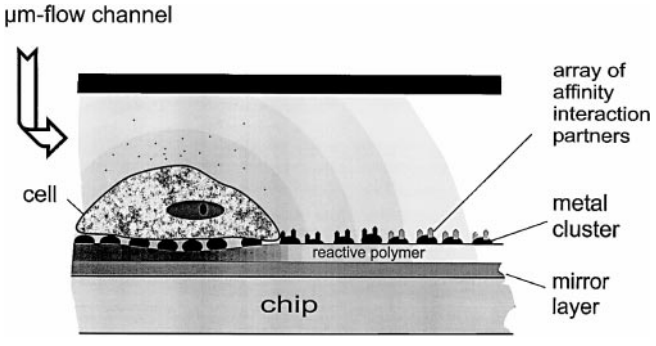

Fig. 23. Setup of enhanced cluster absorption with attached living cell forming a small subcellular compartment

All devices described above take advantage of a combination of conventional binding assays with the new visually detectable label. Ultra-high spatial resolution further speeds up detection, simplifies handling, and increases cost efficiency of drug screening devices.

Experimental Peculiarities

Substrates

Glass slides (Assistent-Germany, $12 \times 12 \times 0.8$ mm), polycarbonate sheets (Goodfellow-UK) hydrophilized by oxygen plasma etching, or any other sufficiently flat and adhesive surfaces can be used as substrates.

Deposition of mirror layers

Silver, gold, or gold-palladium is deposited by DC sputter coating. Sputtering has to be optimized for the optical quality of the metal film (highest possible reflection) as well as adhesion of the metal film on the support and applicability of the metal film for spinning of varying polymer dilutions. Attachment of silver on glass and of gold on polycarbonate is good, adhesion of gold-palladium on glass is rather poor. Typically, sputtering was carried out in an sputter coater for 60 seconds at 0.1 mbar Argon and 40 mA DC.

Aluminated polyethylene terephthalate can be applied instead of the above given substrates coated with Ag or Au. Moreover, metal foils or sheets can be used provided that their surface is sufficiently smooth on a nanometric scale.

Chemical stability of the mirror is excellent for gold, whereas Ag darkens quickly and Al is etched in aqueous environment if the solvent can penetrate the protecting distance layer.

Spin coating of polymer interlayers

Adhesion of the polymer film on the metal or mirror surface is critical for chip stability. A nanoscale film of hexyl polymethacrylate (with an average molecular weight of 400.000; Aldrich) is applied by spin coating at 4000 rpm using varying dilutions of the polymer in AZ 1500 Photothinner (Hoechst) and *n*-decane (Sigma). The appropriate solvent for the polymer should be chosen depending on the molecular coating of the metal film.

Etching of polymer layers by oxygen plasma

The polymer surface is etched with oxygen plasma for 10 sec at 45 mA at an oxygen pressure of 0.1 mbar. Strong hydrophilicity is observed by exposure to oxygen plasma.

Chemical activation of polymer surface by alkaline hydrolysis of ester bonds

As an alternative way to create functional COOH groups at the polymer surface, ester bonds of the polymer can be broken. Hydrolysis is achieved by incubation

with 2–4 *N* KOH. To limit polymer damage by total ester hydrolysis, the reaction time should be less than 10 minutes. No substantial deterioration of film stability is observed; however, a slight film hydrophilization occurs.

Sputtercoating of cluster layers

Deposition is carried out by DC sputtering similar to the metal film underneath but reducing coating time to about 10–50 seconds (Figs. 24, 25)

Attachment of ligands to the (bio)recognitive layer

For application in a glucose sensor setup, HRP was attached either covalently onto carbodiimide activated surfaces or bound *via* non covalent absorption to a plasma etched polymer surface. Alternatively to the HRP attachment, 4-aminophenyl-α-*D*-glucopyranoside can be bound *via* an amide linkage to the plasma treated surface. Coupling of 4-aminophenyl-α-*D*-glucopyranoside to carbodiimide activated sur-

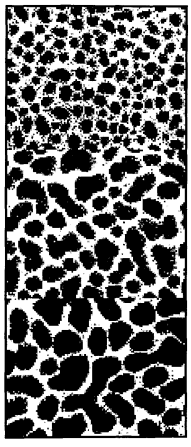

Fig. 24. Electron microscopy of evaporated Au islands with increasing evaporation time (mean diameter about 20 nm)

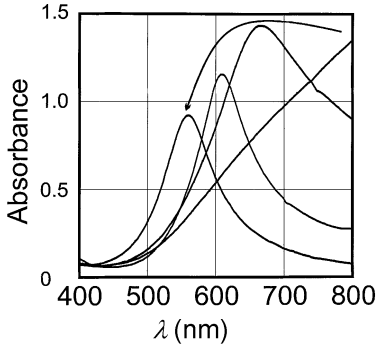

Fig. 25. Spectral change of a sensor device with increasing evaporative coating time (graphs 1–4: 20–50 s)

faces should be carried out at $pH = 5.0$. For most applications, simple adsorption was used to bind the ligand layer to the polymer surface adapting know-how from the ELISA technology [15].

Synthesis of metal clusters

Various types of colloids can be synthesized by chemical reduction of noble metal salts. Gold colloids varying in size from 9 to 50 nm have been prepared according to the sodium citrate method of *Frens* [23]. Alternatively, gold colloids have been prepared following the method of *Slot* [24]. Silver colloids have been prepared according to the citrate-tannine method (*Garbowski*, [25]). Gold cluster synthesis provided colloids with a very narrow size distribution. Comparing the analytical properties of nanoscale gold and silver colloids, gold proved to be more stable under assay conditions.

Coating of colloid surfaces

Gold colloids are coated with HRP in 2- to 3-fold excess for 12 h. Excess of HRP has to be removed by centrifugation. Coating can take place with reduced amounts of HRP or any other protein if in addition to the functional protein in a second step an unreactive protein is applied for the stabilization of colloids. In a similar way, other proteins can be attached to the colloid surface considering their isoelectric points.

Application of colloids

HRP coated colloids are added to the ConA coated surface; under appropriate conditions ($pH = 6.0$, Mn^{++}, Mg^{++}, Ca^{++} all 1 m*M*), binding of the colloids to ConA occurs. The binding of ConA is monitored in real time by observation of a developing absorption peak in a setup with 125 μm aqueous top layer. Due to the fact that any compound penetrating the polymer film will potentially react with the metal mirror layer and alter its optical and adhesive properties, care has to be taken to obtain distance layers without pinholes or cracks.

Setup on Al coated foils

Onto PET metallized with Al (resistance about 3.2 Ω/cm), a 6% solution of polyhexyl metacrylate is applied by spin coating. The surface of the thin film is functionalized under oxygen plasma, and glycosylated proteins are bound *via* adsorption or EDC coupling. Multivalent ConA is added and binds to the hexoses of the exposed surface. Gold colloids with 14 nm in diameter, coated with glycosylated protein (*e.g.* peroxidase), are added and bind to ConA at the surface. Aggregation of clusters or unspecific binding is prevented by addition of 0.1% Tween 20.

Nanometric particles from polystyrene as distance layer

The use of nanometric particles as distance layer provides a way to achieve a constant distance between clusters and metal surface, even if the metal surface is

not smooth at a nanometric scale. This improves reproducibility over large and curved surfaces. The metal surface is incubated with a solution of 2% cystamine in ethanol for 30 minutes, whereby a self assembled monolayer with free amino groups is formed. Carboxylated beads (with a diameter of 50 nm) are coupled to these amino groups *via* a two step EDC coupling protocol. Such beads can be coupled with the same EDC coupling protocol onto aminosilane coated steel or aluminum of arbitrary shape. When coupled to densely functionalized surfaces, nanometric spherical particles with a narrow size distribution form a two-dimensional ordered pattern. On top of the beads, oligonucleotides with a functional amino linker can be coupled to the carboxylated microspheres according to an EDC coupling protocol. In order to ensure that only highly reactive terminal amino groups react with the carboxylated beads, the reactivity of EDC is reduced by the addition of imidazole.

Electrophoretic movement of clusters

In addition to a setup similar to that described above, 2 electrodes are attached to the sensor chip to allow microelectrophoresis. One electrode is used both as distance layer and an electrode, *e.g.* by a setup depositing indium-tin oxide by reactive gas sputtering (In/Sn target, oxygen, DC sputter system) or coating with an ion conducting polymer. Applying of an electrophoretic potential induces a movement of charged clusters either increasing local concentration and speeding up the assay or removing unbound clusters. In a similar setup, nanomagnetic particles, *e.g.* metal or metal oxide, can be moved by (electro)magnetic forces (Fig. 26).

Chemical technology of the swelling polymer layer

Any solid surface with sufficient optical flatness can be used as support, *e.g.* standard sodium silicate glasses with a dimension of $75 \times 25 \times 1$ mm. The supports are carefully cleaned by sonication in a detergent and washing in ultrapure water for 24 h. Immediately before the first deposition step the supports are treated in a plasma cleaner (N_2 plasma) in order to remove any organic contaminations. Deposition of the mirror layer is performed by evaporation or sputtering of about 200 nm of gold.

The most critical step is the deposition of the swelling polymer which has to be performed with sufficient thickness precision and uniformity. This can be done

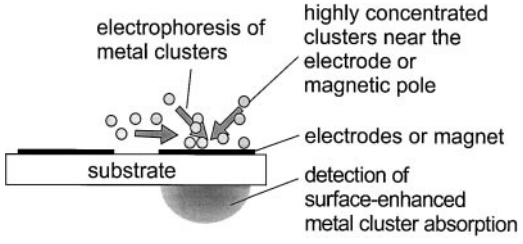

Fig. 26. Enhanced cluster biosensor using electrophoresis and magnetic particles to speed up the assay, suppress unspecific background, and actively move clusters on the chip

either by spinning (3000–5000 rpm) or dipping methods. Using a dip coating method, the formation of crystals from the crosslinker has to be avoided by lowering the crosslinker concentration. The swelling polymer is synthesized according to the following procedure: stock solutions containing 2.5% (w/v) *PVP* (polyvinylpyrrolidone, MW = 360.000) or 5% (w/v) *MA-PVP* (polyvinylpyrrolidone-dimethylaminoethyl methacrylate, quaternized) in distilled water and 0.75% (w/v) *DIAS* (4,4′-diazidostilbene-2,2′-disulfonic acid disodiumsalt tetrahydrate) in distilled water are mixed (3:2 v/v). The sensor support is spin- or dip-coated and dried. Crosslinking is done by exposing the chip to the light of a UV 50 W Hg medium pressure lamp for 5 seconds.

Finally, a metal cluster film is deposited by slow thermal evaporation of gold onto the polymer surface. The mechanism of island growing strongly depends on the mobility of single metal atoms on the support surface. For *PVP*, this criterion is widely fulfilled. If necessary, the metal cluster film can be replaced by covalent coupling of colloids to the polymer surface.

Acknowledgements

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References

[1] Kreibig U, Vollmer M (1995) In: Optical Properties of Metal Clusters. Springer, Heidelberg
[2] Dörfler HD (1994) In: Grenzflächen und Kolloidchemie. VCH, Weinheim
[3] Brezesinski G, Mögel HJ (1993) In: Grenzflächen und Kolloide. Spektrum, Heidelberg Berlin Oxford
[4] Brown KR, Natan MJ (1998) Langmuir **14**: 726
[5] Leuvering JH, Goverde BC, Thal PJ, Schuurs AH (1983) J Immunol Methods **60**: 9
[6] Leuvering JH, Thal PJ, Van der Waart M, Schuurs AH (1981) J Immunol Methods **45**: 183
[7] Leuvering JH, Thal PJ, Van der Waart M, Schuurs AH (1980) J Immunoassay **1**: 77
[8] Elghanian R, Storhoff JJ, Mucic RC, Letsinger RL, Mirkin CA (1997) Science **277**: 1078
[9] Mirkin CA, Letsinger RL, Mucic RC, Storhoff JJ (1996) Nature **382**: 607
[10] Storhoff JJ, Elghanian R, Mucic RC, Mirkin CA, Letsinger RL, (1998) J Am Chem Soc **120**: 1959
[11] Leitner A, Zhao Z, Brunner H, Aussenegg FR, Wokaun A (1993) Appl Optics **32**: 102
[12] Chakraborty UR, Black N, Brooks HG Jr, Campbell C, Gluck K, Harmon F, Hollenbeck L, Lawler S, Levison S, Mochnal D (1990) Ann Bio Clin Paris **48**: 403
[13] Xu-Z (1992) Chung Hua I Hsueh Tsa Chih Taipei **72**(4): 216
[14] Natan MJ (1998) IBC-Conference on Biosensor Technology. Boston
[15] Schalkhammer T, Leitner A, Aussenegg FR, Bauer G, Pittner F (1998) SPIE **3253**: 12
[16] Bauer G, Pittner F, Schalkhammer T (1998) Mikrochimica Acta (in press)
[17] Schalkhammer T, Pittner F, Bauer G (1997) PCT-patent application PCT/AT98/00101
[18] Schalkhammer T, Lobmaier Ch, Pittner F, Leitner A, Brunner H, Aussenegg FR (1995) Sensors and Actuators B **24**: 166–172
[19] Aussenegg FR, Brunner H, Leitner A, Lobmaier Ch, Schalkhammer T, Pittner F (1995) Sensors and Actuators B **29**: 204
[20] Schalkhammer T, Lobmaier C, Pittner F, Leitner A, Brunner H, Aussenegg FR (1995) Mikrochimica Acta **121**: 259

[21] Schalkhammer T, Lobmaier Ch, Pittner F, Leitner A, Brunner H, Aussenegg FR (1995) SPIE **2508**: 102
[22] Aussenegg FR, Brunner H, Leitner A, Pittner F, Schalkhammer T (1997) US-patent 5,611,998
[23] Frens G (1973) Nature (London) Phys Sci **241**: 20
[24] Slot JW, Geuze HJL (1985) Eur-J-Cell-Biol **38** (1): 87
[25] Garbowski (1954) In: A Short Textbook of Colloid Chemistry. Pergamon, Oxford

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