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Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 773–783

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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High-throughput assays on the chip based on metal nano-cluster resonance transducers

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Received 16 May 2000; received in revised form 2 October 2000; accepted 5 October 2000

Abstract

High throughput transducers using metal cluster resonance technology are based on surface-enhancement of metal cluster light absorption. These devices can be used for detection of biorecognitive binding, as well as structural changes of nucleic acids, proteins or any other polymer. The optical property for the analytical application of metal cluster films is the so-called anomalous absorption. An absorbing film of clusters positioned 10–400 nm to a mirror surface reacts in a similar way to a reflection filter. At a certain distance of the absorbing layer to the mirror the reflected electromagnetic field has the same phase at the position of the absorbing cluster as the incident fields. This feedback mechanism strongly enhances the effective cluster absorption coefficient. The system is characterised by a narrow reflection minimum whose spectral position shifts sensitively with the interlayer thickness, because a given cluster–mirror distance and wavelength defines the optimum phase. Based on this principle a set of novel tools including biochips and micro arrays is presented, which enabled us to transduce binding, as well as changes of protein-, DNA- and polymer-conformation, quantitatively into an optical signal which can be observed directly as a colour change of a sensor-chip surface. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: HTS; Protein micro pads; Protein structure; Biochip; Surface enhanced resonance; Metal clusters

1. Introduction

Whereas high throughput monitoring of molecular binding is often based on fluorescent detection, measuring of effects on protein structure caused by drug interaction or variation of the local microenvironment is achieved through expensive and complex spectroscopic devices like X-ray scattering, circular dichroism (CD) [1], optical rotation dispersion (ORD), or nuclear magnetic resonance (NMR) [2]. It is rather time consuming and costly to monitor these effects in individual proteins. Therefore, a novel technology is necessary to provide conformation data quickly, reliably, and in high throughput.

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PII: S0731-7085(00)00545-8

To monitor binding of proteins, the capture chip was set up, consisting of a metal mirror, and a resonance layer (either non-swelling polymer or inorganic glass). Proteins, such as various enzymes and serum proteins, were attached via micro dotting, adsorptive or covalent coupling as well as spin coating — followed by photo cross-linking — onto designed mirror-chip surfaces as thin films up to 200 nm. Metal-nano clusters, synthesised via chemical means or sputter coating were deposited on top of the proteins. Protein was made visible by biorecognitive interaction with coated gold clusters (12–55 nm) using a 20-fold concentrated cluster solution. For transduction of nanometric structural changes we have taken advantage of our 'Metal Island Coated Swelling Polymer Over Mirror System' (MICSPOMS) device for direct visualisation of the structural rearrangement of polymers [3–5]. In the new device we use an optical nanometric thin layer system consisting of a metal mirror, a transparent interlayer (protein crystal or gel, about 5–80 molecules thick), and a gold cluster film as a topmost layer. Due to the above mentioned spe-

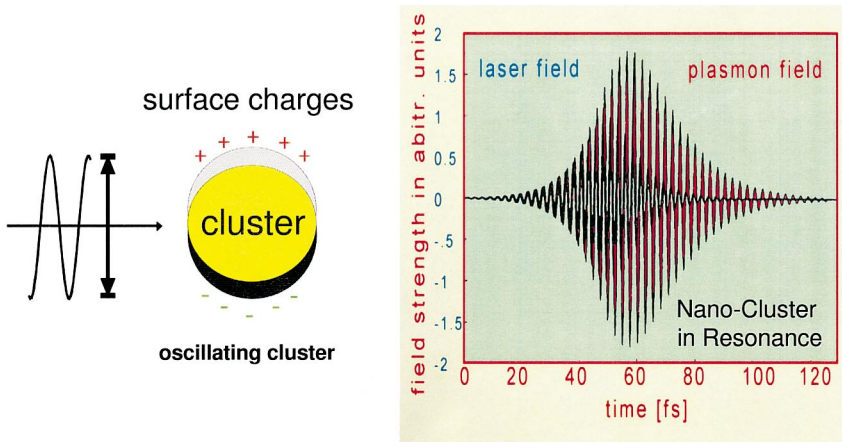


Fig. 1

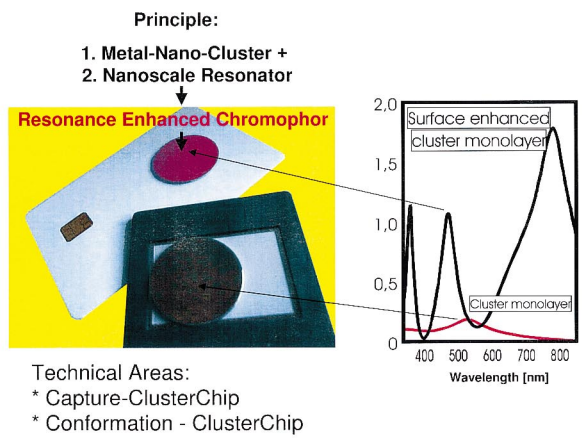


Fig. 2

Fig. 1. Nano cluster in resonance, fs-timing of nanocluster resonance based on laser-double-pulse experimental data. (fs: femtoseconds).

Fig. 2. Visual appearance of surface enhanced cluster coated chips. Spectrum of enhanced and non-enhanced cluster-layers of identical cluster-density on chip.

cial optical behaviour this system (Fig. 1 and Fig. 2) shows a characteristic spectral reflection minimum, being strongly dependent on the thickness of the transparent interlayer. The properties of this nanometric set-up have first been shown with photocrosslinked polyvinylpyrrolidone (PVP). With that polymer immobilised as a thin film (resembling a protein backbone) structural changes transduced as swelling and shrinking have been monitored for the first time dependent on different ionic and chaotropic species [6–8]. Using ultra-thin protein layers (only a few molecules thick) enabled us to prove that, for example, there is an direct correlation between a real structural expansion or contraction of proteins and the chaotropic or cosmotropic character of effector molecules. Upon incubation with effector molecules these nano-resonant systems changed their optical resonance properties resulting in micro-arrays of coloured dots representing the different stages of protein folding or biorecognitive interactions.

The response of the novel sensor chip was measured either via a reflectometric scanner or spectroscopically in the visible and IR range of the spectrum.

2. Materials and methods

2.1. Chip design

In principle, any solid surface with sufficient optical flatness can be used as the support, e.g. polyethylene terephthalate (PET) sheets metallized with titanium or aluminium (Goodfellow), silicon chips, object glass slides or reflective aluminium foils.

To obtain a maximum of compatibility with micro-arrayers we mainly used glass slides (Fig. 3) or slide sized chips metallized via sputter coating. To set-up an appropriate resonance interlayer it is vital to deposit a polymer film by spin coating at, for example. 5000 rpm for 40 s or an inorganic glass via reactive sputter coating. For low resolution–low prize applications highly polished aluminium chips (diameter: 12 mm, thickness: 0.5 mm from AMAG, Ranshofen, Austria) served both as support and mirror layer in a polymer-based-chip design. The most critical step for chip stability was the deposition of stable but still swelling protein layers, which had to be performed with exact thickness and uniformity. This was done via dipping, dotting or by spin coating the protein. Ultrathin layers of various proteins including: bovine serum albumin (BSA: fraction V, Sigma), glucose oxidase (GOD: Boehringer Mannheim) and urease (Sigma), were applied. Typically, a nanoscale film of protein was coated onto the surface by spin coating a solution of the protein (5%) at 4000 rpm for 30 s.

Most proteins, such as BSA were immobilised via two different photochemical methods

1. A 1–5% (w/v) solution of the protein in distilled H_2O was either dotted with an arrayer (dot size 100–125 μm) or spin-coated directly onto the chip surface. Crosslinking was done be exposing the chip to the UV-light of a Stratagene DNA-crosslinker at 25 mJ for 5 min. At the wavelength employed (about 300 nm) aromatic amino acids (Tyr, Trp, Phe) and thiols on the outside of the protein seemed to be activated and thus couple to other proteins as well as to the surface of the support. Finally, either a gold cluster film (max. mass thickness 3–5 nm) was deposited by sputter coating (at a vacuum of 0.1 mbar Argon, Varian turbo molecular pump) onto the protein surface using a sputter coater or clusters were coated from aqueous solution via chemisorption.
2. Other proteins such as GOD and urease were applied via spin-coating and reactive photocrosslinking. Stock solutions containing 1–5% (w/v) protein and 3% (w/v) 4,4′-diazidostilbene-2,2′disulfonic acid disodiumsalt tetrahydrate (DIAS) solutions in distilled water were mixed 10:1 (v/v). The solution containing protein and crosslinker was spin-coated on the chip and activated with a monolayer of an adhesion promotor, such as 1-(3-aminopropyl)-methyl-diethoxysilane (Fluka). Silanisation of aluminium-coated chips was done via gas phase silanisation using amino-silane in a vacuum desiccator overnight. The

chips were baked at 100°C for 30 min to crosslink the amino-silane. To remove unbound amino-silane the chips were washed with methanol and dried. Crosslinking of the spin-

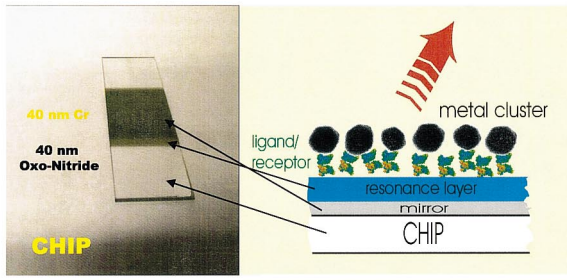


Fig. 3

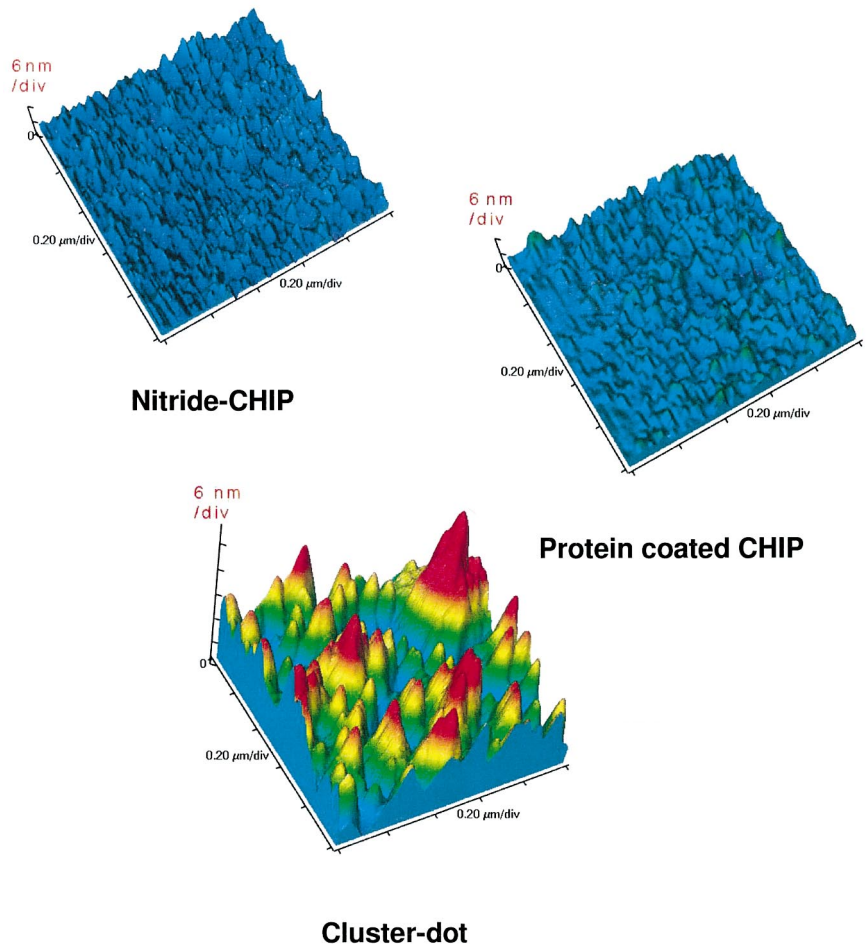
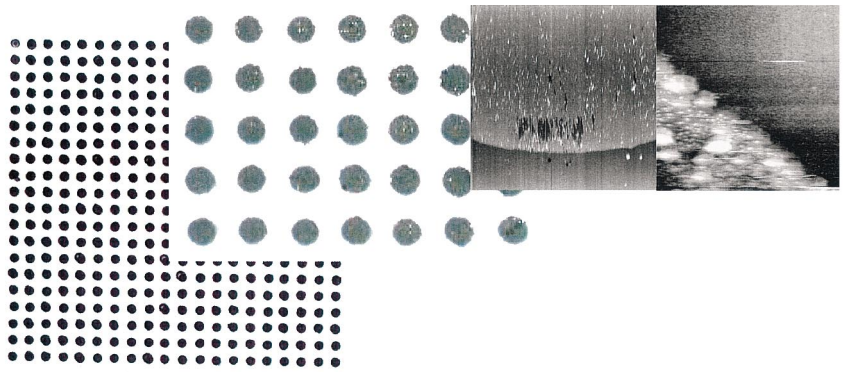


Fig. 4

Fig. 3. Set-up of the sensor with (1) chip with reflecting layer (preferentially electron-conducting metal or cluster layer), (2) resonance layer (3) biointeraction layer and a (4) topmost layer of metal nanoclusters.

Fig. 4. Contact-mode atomic force microscopy study of uncoated chip surface, protein coated chip surface and cluster-coated visual dot.



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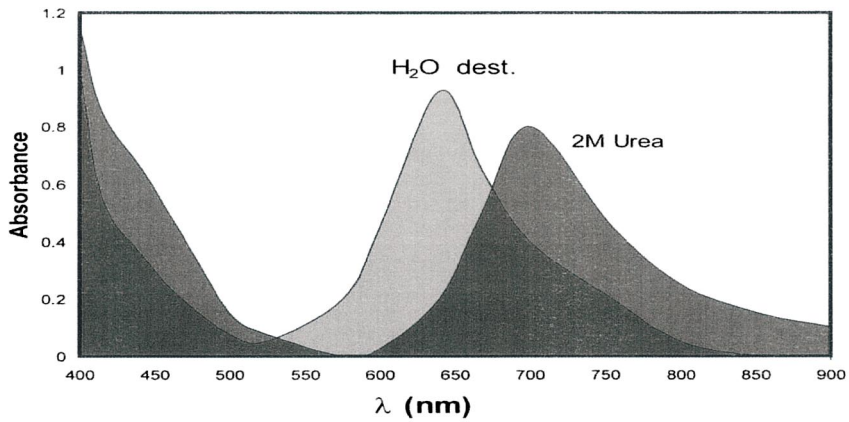

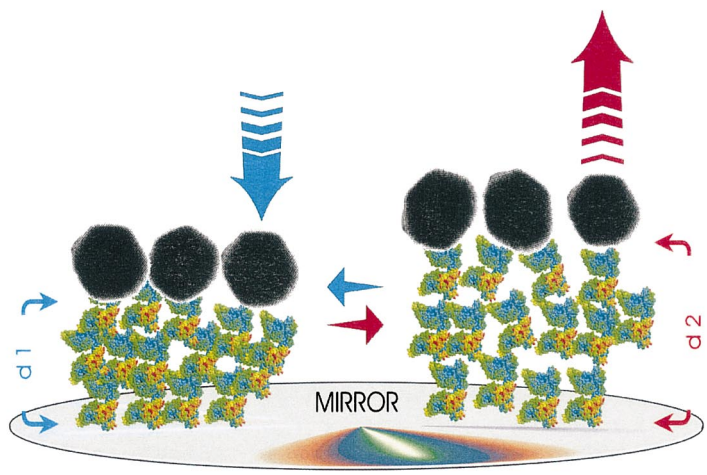

Fig. 6

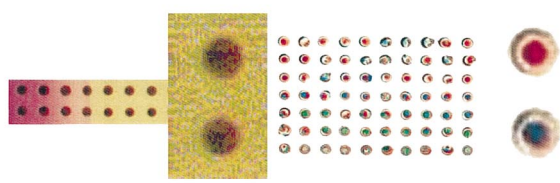

Fig. 7

spin-coated film was done by irradiation of the chips with UV-light for 30 s (350 nm, 60 W). A DNA-crosslinker is not recommended because its emission near 300 nm causes DIAS decomposition and leads to negligible crosslinking.

The gold cluster film was deposited by covalent coupling or adsorptive binding of colloids synthesised in solution via tannic acid or citric acid methods prepared according to the method described by Frens [9]. Chemically synthesised clusters of 9–40 nm were concentrated (20-fold) via centrifugation and resuspension in H_2O and deposited by adsorption to the protein surface (1–3 min). Sensors were rinsed with H_2O to prove chip-stability.

Clusters were deposited on a protein film via sputter coating under a high vacuum process.

2.2. Experimental

A change in cluster–mirror distance can be visualised directly with a photometer or a Canon (9-micron resolution) optical scanner. Spectroscopic measurements were carried out on a Hitachi spectrophotometer U-2000 over the range 400 and 900 nm. With dots more than 1 mm in diameter a clear visual impression of protein binding or conformation changes is obtained by just looking at the chip surface.

All measurements were done in buffered solutions mostly employing physiological conditions. The chips were placed in a flow through cell and solutions with different effectors (up to 5 ml/min) were passed through this cell. Structural changes of the protein nanolayers (dependent on denaturing agents, pH and ions) were measured, employing phosphate and acetate buffers. Solvent dependent effects were measured in water to avoid precipitation of buffer ions at high solvent concentrations. We either measured the shift of the peak maxima or we chose a defined wavelength at the rising or falling side of the peak measuring increase or decrease of reflectance (which is more sensitive). These data characterised expansion or shrinking of the protein. To obtain a nearly linear range, the dynamic signal was chosen not to exceed 0.5 OD. Most signals were normalised for 0.5 OD = 100% of swelling and shrinking of the protein nanolayer.

Binding assays (Fig. 4 and Fig. 5) were carried out using biotin-labelled-BSA and avidin, digoxigenin coupled proteins and digoxin antibodies (Roche), small haptens (dinitrophenyl) as well as protein-type allergens and the appropriate antibodies or other related biorecognitive systems (e.g. CRP, protein-A/antibody,…).

3. Results

3.1. Sensor set-up

Ultra-flat glass and polymer substrates coated with a metal mirror (deposited via sputter coating) were used as sensor support. Various metals were tested and mirrors of very divergent quality were obtained. Depending on process control and sputter rates mirror formation is obtained within 60s (Au) to 6 min (Al). Use of aluminium as a mirror turned out to be quite unfavourable due to low productivity. Significantly higher sputter rates were obtained with platinum, but due to the expensive material and the low adhesion this metal is not the first choice. Sandwiched aluminium–platinum mirrors exhibit a good mirror quality with moderate costs, but production is nevertheless rather time consuming. Mirror qualities comparable to aluminium, but at lower production costs, were obtained with chromium. This mirror even turned out to be superior in reflection properties and a significantly deeper colour can be obtained in the surface enhanced mode (Fig. 3).

Fig. 5. 400 dot cut-out of a 1600 dot chip, 35 dot optical magnification, AFM-scan of the border region of a micro-dot and zoom-in to the border of the dot clearly showing proteins and clusters.

Fig. 6. Chip design and response of a BSA-biosensor device to the denaturing agent urea.

Fig. 7. Micro-dotted protein-gel-pads photocrosslinked with bis-nitrenes.

Table 1
Polymers with their solvents used for resonance layer production of the capture chip. The table also gives the concentration of the spincoated polymers

Polymer	Mol. weight (g/mol)	Solvent(s) applied	Concentration for optimal spin films (%)
Poly-styrene	280 000	toluene	2–7
Poly-hexylmethacrylate	320 000	decane, octane, AZ 1500	2–19
Poly-(4-tert-butylstyrene)	300 000	hexane, decane, AZ 1500	3–10
Poly-styrene bromated	480 000	cyclohexanone	5–10
Poly-methylmethacrylate	350 000	acetone	2–8

Fig. 6. Chip design and response of a BSA-biosensor device to the denaturing agent urea.
Fig. 7. Micro-dotted protein-gel-pads photocrosslinked with bis-nitrenes.

3.2. Mechanical, chemical and thermal stability of the mirror

For high throughput use in bioapplications stability in aqueous buffer at 20–90° is vital. As a first step incubation in water, phosphate buffer, ELISA-coating buffer (carbonate pH 9.0), ethanol, isopropanol as well as acid wash at pH 2.0 were tested. To test for adhesion under mechanical stress the standard peel off tape test was applied. Thermal stability was tested up to 300°C in dry air.

Aluminium is stable in an oxidising environment, adhesion was satisfying, but long-term as well as thermal stability is limited due to chemical reactivity. Platinum is chemically most stable, but due to very low surface adhesion peels off under chemical and mechanical stress, heating tends to stabilise the platinum layer. Use of aluminium as an adhesion layer stabilises the platinum film. Chromium is stable under all conditions tested, superior in the peel off test and thermally stable within the temperature range.

3.3. Resonance layer

Depending on the mode of sensor application either non-swelling resonance layers of hydrophobic polymers — inorganic glass (for a capture chip type device) or a reactive gel resonance-interlayer of crosslinked-proteins or DNA — are applied on top of the chip.

Whereas for the active matrix approach the sub-nanometric spatial sensitivity of the resonance setup is of primary importance, for the capture chip the absolute signal is vital. Comparing signals from a non-resonant capture assay (using colloid particles) with a resonant set up an enhancement of 7–12-times at the resonance frequency and more than 100-times off resonance is obtained.

3.4. Capture chip resonance layer

The resonance layer is a nanometric thin film of a polymer or an inorganic glass based on for example, a metal oxide. Optimal resonance is achieved within 10–400 nm of cluster–mirror distance. As characteristic for all micro- and especially nano-technologies, working in a clean room environment is preferable, because dust decreases the quality of all the resulting products. This is of particular importance for the application of the resonance interlayer. The most convenient method of film production is via polymer spin coating. As is well known from semiconductor industries, a polymer is dissolved in a solvent in such a concentration that the resulting solution shows viscosity considerably higher than the solvent alone. Then the polymer solution is spread over the chip and the chip is brought into rotation of up to 5000 rpm. During the increase of rotational speed a major part of the polymer is removed from the surface of the chip. If the indices of hydrophilicity of the surface and the polymer-solution match, some of the polymer solution will coat on the surface and the remaining solvent will evaporate from the solution. Thus, a well-defined film of the polymer remains attached to the surface. We used the polymers given in Table 1 to set

up the resonance layer and coated them on chip via spincoating as described above.

Similar to polymer interlayers, metal oxide as well as metal nitride layers proved to be useful. Reactive sputter coating as well as oxidation based thermal curing was applied. Various nitrides and oxides of aluminium, chromium, tin and indium tim oxide (ITO) were coated up to 200 nm optical thickness. Chromium and aluminium nitrides resulted in unsatisfying resonance signals whereas aluminium oxide, tin oxide and nitride as well as ITO gave strong resonance (Fig. 3). Moreover, it is quite beneficial for microdotting to use a surface coating, which is hard and chemically stable.

The interlayer thickness was designed to obtain the desired colour starting from gold, brown, violet, blue, light blue, yellow, orange, red and green.

3.5. Active matrix chip resonance layer

Similar to a polymer a protein is dissolved in a suitable solvent. However, since the protein usually has to perform volume changes, but should not dissolve, it is necessary to cross-link the protein to form a thinfilm gel pad (Fig. 6). For building a sensor array various proteins representing a spectrum of properties were chosen to be applied on the chip, including BSA as a carrier protein in blood, GOD from *Aspergillus niger* (used for the enzymatic determination of glucose in aqueous solution) and urease from jack bean catalyzing the reaction from urea to ammonia and carbon dioxide.

To coat the total chip surface with a homogeneous protein gel layer spin coating of proteins proved to be the best immobilisation method. Covalent layer-by-layer immobilisation had distinct disadvantages in reproducibility and in the degree of crosslinking. An ordered protein film with a high degree of crosslinking turned out to have less flexibility of chain folding and thus less response. To obtain high throughput screening (HTS) protein conformation chips protein-gel-pads have to be deposited via a micro-arrayer. Similar to spin coating a crosslinking procedure within the deposited protein dot leads to the formation of a small (0–250 micron) gel pad (Fig. 7). Contrary to microdotting for binding assays the concentration of the protein solution microdotted as 1 nl microdots is reasonably high at up to about 5%. Crosslinking is achieved in a similar way to full-chip layers.

3.6. Cluster coating and structural integrity

As topmost layer, a gold cluster film is coated either via sputter coating or via adsorptive deposition of large metal clusters in an aqueous solution. Deposition of a gold cluster film via sputter coating onto the protein surface may lead to the denaturation of the protein, though renaturation is favoured because the protein has been crosslinked in the native state. Using covalent or adsorptive coupling of gold clusters from aqueous solutions to the protein surface under milder conditions, enabled us to retain full protein activity.

To prove the structural integrity of the gel pad protein molecules we chose urease, a sensitive enzyme catalysing the reaction from urea to ammonia and carbon dioxide. The protein was coated and cross-linked using the photo-sensitive cross-linker DIAS. As topmost layer a gold cluster film was deposited by adsorbing chemically synthesised gold clusters. Sensors were rinsed with H_2O and tested for enzymatic activity using urea (0–500 μM) in an appropriate buffer, 1 mM, pH 7.2 (Fig. 10).

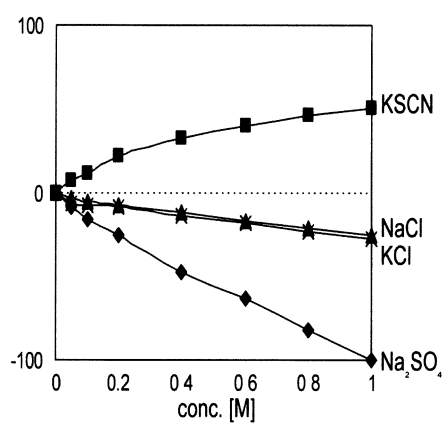

Fig. 8. Response of a BSA device to various ionic species of the Hofmeister series.

Whereas effects of ions on protein structure are in the mM range, ammonia (locally produced by the enzyme) results in structural effects up to 1000 times larger than the ionic effects. This demonstrates that the activity of the enzyme was retained during the immobilisation process. These sensors retain their full signal and sensitivity for weeks and can be stored for prolonged periods at 4°C. Studies with various other proteins including a set of active enzymes are in progress.

3.7. Chaotropic agents

High concentrations of urea (8–10M) and guanidinium chloride (GdmCl, 6M) are common chaotropic reagents and thus used to denature proteins. Their mode of action is based on surface activity and water structure. Their denaturation abilities result from a combination of two effects: (1) These reagents reduce the hydrophobic interactions that play an important role in stabilising the protein tertiary structure, and (2) they interact directly with the surface of the protein molecule [10].

GdmCl has a stronger denaturation effect on most of the proteins compared to urea (0.5–4 M in 200 mM phosphate buffer, pH 7). Contrary to that, reversibility of the conformational changes was much better for urea than for GdmCl. At low concentrations GdmCl first induces a small but distinct shrinking of the protein and at higher concentrations the destabilising effect is monitored as an increased volume of the protein. The effect can be visualised even without any photometer as a strong colourshift of the chip.

3.8. Ions

Protein shrinking and swelling was directly coherent with the Hofmeister lyotropic series of chaotropic agents [7,8], using, for example, BSA photo-crosslinked by UV (Bio-Rad DNA-crosslinker, 300 nm). As expected, effects of cosmotropic agents are stabilising while those of chaotropic agents are denaturing. Destabilising ions bind extensively to the surface of proteins, thus reducing the number of water molecules around the protein.

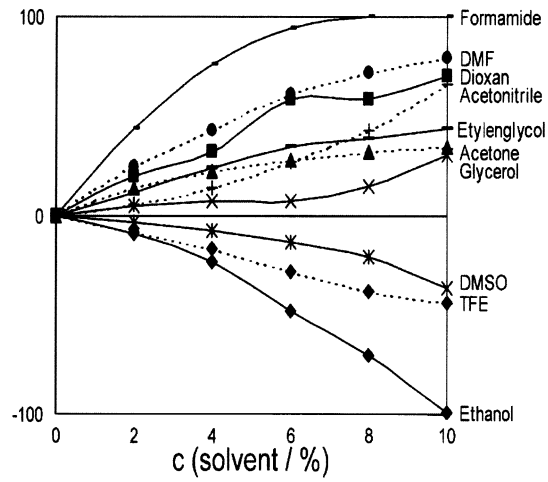

Fig. 9. Response of a BSA device to organic solvents. Stabilising and destabilising effects of organic solvents (1–10% in water).

Different monovalent salts exhibit different effects mainly caused by their anions (Fig. 8). Salts of sodium and potassium (50 mM) induced either shrinking or swelling of the protein layer at neutral pH in 100 mM phosphate buffer. Variations in ion concentration were monitored using a buffer system resembling physiological conditions. Distinct, and in most cases reversible, sensor signals were observed when a concentration jump of an ion (50 mM–1 M) in a buffered solution (200 mM phosphate, pH 7) was done.

As a test for reversibility of protein structure changes Na_2SO_4 (a well known stabiliser of proteins) and KSCN (a well known chaotropic agent) were used in a pulse experiment proving that changes in protein conformation are often (within a limited range of denaturation) highly reversible, even on chip surfaces. Bivalent cations induced a more complex behaviour of the proteins. The calibration curve for $CaCl_2$ or $MgCl_2 \cdot 6H_2O$ is an overlay of the sigmoid logarithmic shrinking behaviour with a swelling effect at high ionic concentration. Summing up, structural effects induced by different ions are in good accordance with the molecular theory of chaotropic agents.

3.9. Organic solvents

Water is fundamental for formation of the appropriate three-dimensional protein structure [11] as well as for protein action. In many cases protein inactivation is observed when mixtures of water and (water-miscible) organic solvents are employed [12]. To correlate volume behaviour and stability we tested several organic solvents for their stabilising (shrinking) and denaturing abilities on immobilised BSA photo cross-linked with UV-irradiation.

Some polar aprotic solvents, like dimethyl sulfoxide, stabilise the protein at low concentrations. Conversely dimethylformamide (DMF), another aprotic solvent, destabilises the protein. Additives such as ethylene glycol and glycerol are often described as stabiliser for proteins [13]. Such substances have a high content of hydroxyl groups and have the ability to form multiple hydrogen bonds and act like water. Nevertheless, we found a destabilising effect for ethylene glycol and a weaker effect for glycerol. Organic solvents such as ethanol even stabilised BSA at low concentration, although at higher concentration ($>20\%$ w/v) they destabilised the protein and caused denaturation (Fig. 9). Sometimes, mixtures of organic solvents have subtractive instead of additive effects on protein structure enabling a compensation strategy for protein stabilisation.

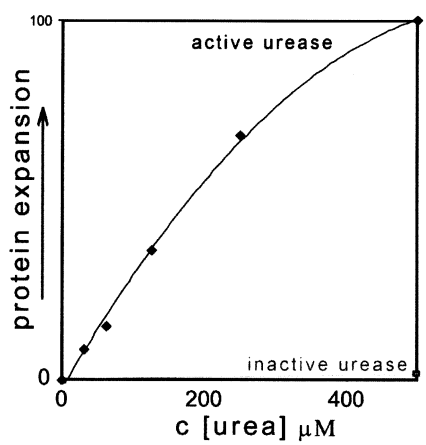

Fig. 10. Whereas effects of ions on protein structure are in the mM range, ammonia locally produced by urease from urea results in structural effects three orders of magnitude larger than the chaotropic effects.

4. Discussion

The new type of protein biochips and arrays [14] we have described here should be generally useful for characterisation of binding and conformation dynamics of proteins and protein–effector interactions. For the pharmaceutical industry (focussing on the search for specific drugs interacting with their corresponding proteins) as well as for HT-Proteomics the novel devices will enable the quantitative assessment of these important interactions by transducing binding and conformational changes.

From the technical point of view it turned out that it is vital to use chip substrates with only a few nanometric irregularities at the surface, and that working in clean air rooms increases chip quality. The chemical and mechanical stability of the novel chip surfaces enables handling even at 100°C and thus on chip protein denaturation, on chip protein and DNA digestions, on-chip-PCR and similar techniques.

It is important to consider the requirement of pure proteins with about 50 μg/ml for micro dotting on the capture chip, but up to 50 mg/ml for gel-pads on the conformation-active matrix chip. Nevertheless, even for the active matrix chip, dots as small as 125 μm need 10–50 ng of the respective protein.

In addition, these devices allow to screen for, or with, DNA, as well as polymers, including polysaccharides or synthetic polymers, that can be used as chemical sensors to monitor various analytes via structural changes, with a time resolution of less than 1 s.

Summing up, this new transducer principle enables to construct devices to measure changes of protein and DNA-structure and the activity of proteases as well as (restriction) endonucleases, to transduce conformational changes (of e.g. prions) into signals, to study structural effects of enzyme products on the structural integrity of the enzyme, to investigate refolding of disease related proteins, to study the optimal microenvironment for enzymes and the protein structure in organic sol-

vents, to optimise an enzyme-substrate reaction in non-aqueous environment and to screen for structural changes as a result of drug interactions.

Acknowledgements

We thank Professors Aussenegg and Leitner from University of Graz, Austria and Professor Sontag from University of Vienna, Austria for helpful discussions. Part of this work was covered by a grant from the Bundesministerium für Wissenschaft und Verkehr, Austria.

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