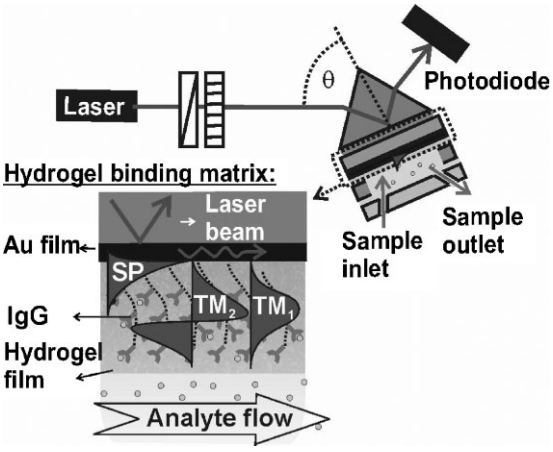
Optical Waveguide Spectroscopy for the Investigation of Protein-Functionalized Hydrogel Films[a]

Alena Aulasevich, Robert F. Roskamp, Ulrich Jonas, Bernhard Menges, Jakub Dostálek,* Wolfgang Knoll

This article reports the implementation of optical waveguide spectroscopy (OWS) for the quantitative time-resolved observation of changes in the swelling behavior and mass density of protein-functionalized hydrogel films. In the experiment, a thin film of an *N*-isopropylacrylamide (NIPAAm)-based polymer that supported optical waveguide modes is attached to a metallic sensor surface. IgG molecules are in situ immobilized in this gel by using novel coupling chemistry with a charge-attraction scheme based on a tetrafluorophenol sulfonate active ester. The anti-fouling properties of the functionalized hydrogel network and the kinetics of the affinity binding of protein molecules in the gel are investigated.



Introduction

Hydrogels are hydrophilic polymer networks that are finding an increasing number of applications in daily life goods (e.g., as superabsorbers and in contact lenses) and hold potential to spread into other important areas such as medicine and biotechnology.[1] Hydrogel materials can be modified with protein biomolecules in order to provide specific functionality such as controlled drug release,[2] defined interactions with cells,[3] or capture of target analyte molecules.[4,5] For the observation of thin hydrogel films, various optical methods have been used, which include surface plasmon resonance (SPR) and optical waveguide mode spectroscopy (OWS),[6,7] applied for the study of structural and swelling characteristics, and fluorescence correlation spectroscopy (FCS) and dynamic light scattering (PCS), for the investigation of their dynamics.[8,9] In this communication, we report the implementation of OWS for quantitative time-resolved characterization of mass changes and the swelling behavior of protein-functionalized *N*-isopropylacrylamide (NIPAAm)-based hydrogel films. This thermal responsive gel can be used for the design of advanced biosensor binding matrices. For instance, the hydrogel film can be swollen in an aqueous sample (e.g., blood sample) in order to capture a target analyte in the sample by a ligand

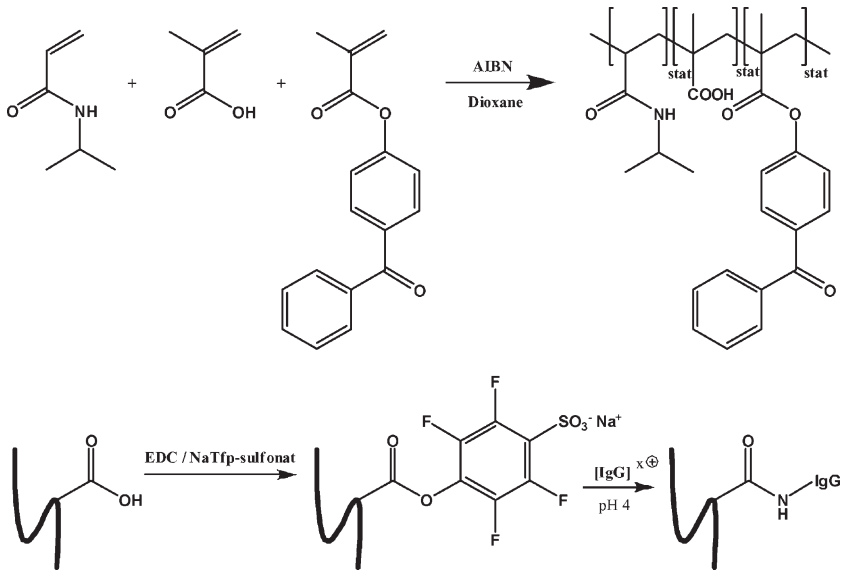
A. Aulasevich, R. F. Roskamp, U. Jonas, B. Menges, J. Dostálek
Max-Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany
Fax: +43 (0) 50 550-4399; E-mail: jakub.dostalek@arcs.ac.at
U. Jonas
FORTH/IESL, Voutes Str. 1527, 71110 Heraklion, Greece
J. Dostálek, W. Knoll
Austrian Research Centers, Tech Gate, Donau-City-Strasse 1, 1220 Vienna, Austria

[a] Supporting information for this article is available at the bottom of the article's abstract page, which can be accessed from the journal's homepage at http://www.mrc-journal.de, or from the author.

Macromol. Rapid Commun. **2009**, *30*, 872–877

DOI: 10.1002/marc.200800747

immobilized at the hydrogel polymer backbone. Upon externally triggered gel collapse (e.g., by a temperature increase[6]), excess liquid and unbound species would be expelled from the hydrogel matrix while the specifically bound analyte would be concentrated at a sensor surface and thus allow for its effective detection. Immunoglobulin G (IgG) molecules were in situ covalently coupled into the NIPAAm-based gel by using a novel charge-attraction scheme based on a tetrafluorophenol sulfonate active ester. Compared to other methods,[4,10] this approach provides stable covalent coupling of IgG molecules without exposing the protein to harsh conditions. The anti-fouling properties of the functionalized hydrogel network were investigated and its potential to serve as a binding matrix is demonstrated in an experiment in which the kinetics of the affinity binding of neutravidin (NA) molecules to the immobilized biotinylated IgG was monitored.



Scheme 1. Schematics of the NIPAAm-based polymer and the coupling chemistry used.

Experimental Part

Chemicals

Sodium acetate, acetic acid, and ethanolamine hydrochloride were purchased from Aldrich (USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was from Pierce (USA). Biotin-labeled immunoglobulin antibody (IgG-b) was obtained from Acros (Germany). Immunoglobulin antibody without biotin labels (IgG) was from Biodesign (USA). Neutravidin (NA) and newborn calf serum were purchased from Invitrogen GmbH (Germany). Phosphate buffered saline (PBS, 10^{-2} M phosphate, 137×10^{-3} M NaCl, 2.7×10^{-3} M KCl with 0.005% surfactant P-20), pH 7.4, was obtained from CalBiochem (USA) and 10^{-2} M acetate buffer (ACT), pH 4, was prepared in house. Ethanolamine was dissolved in water at 1 M concentration with the pH of the solution being adjusted to 8.5 with sodium hydroxide. Sodium *para*-tetrafluorophenol sulfonate (TFPS) was synthesized in house as described in the literature.[11]

Preparation and Modification of Hydrogel Films

The synthesis of the NIPAAm-based hydrogel composed of the terpolymer with *N*-isopropylacrylamide, methacrylic acid, and 4-methacryloyl benzophenone was performed as described elsewhere.[6] A thin hydrogel film was deposited on a gold surface modified by a benzophenone-terminated thiol layer by spin-coating from ethanol solution (4 wt.-% of the polymer) and dried overnight in vacuum at 50 °C. The polymer chains were cross-linked and anchored to the gold surface through benzophenone units by UV irradiation ($\lambda = 365$ nm, irradiation energy density of 2 J · cm^{-2}). The thickness of the dry crosslinked polymer layer was 300 nm as measured with a surface profiler. The polymer structure and the used coupling chemistry are shown in Scheme 1.

Spectroscopy of Optical Waveguide Modes

A hydrogel film that is attached to a reflecting metallic surface and swollen in a liquid can support guided light waves if its refractive index n_h and the thickness d_h are sufficiently large. The propagation constant β of these guided light waves can be determined from the dispersion relation:

$$\tan(\kappa d_h) = \frac{\gamma_b n_h^2/\kappa n_b^2 + \gamma_m n_h^2/\kappa n_m^2}{1 - (\gamma_b n_h^2/\kappa n_b^2)(\gamma_m n_h^2/\kappa n_m^2)}, \quad (1)$$

where n_m is the refractive index of the metal, n_b is the refractive index of the liquid, $k_0 = 2\pi/\lambda$ is the light propagation constant in vacuum, and $\kappa^2 = (k_0^2 n_h^2 - \beta^2)$, $\gamma^2{}_m = \beta^2 - k_0^2 n^2{}_m$, and $\gamma^2{}_b = \beta^2 - k_0^2 n^2{}_b$ are the transverse propagation constants in the hydrogel film, the metal, and the liquid, respectively.

In order to excite the hydrogel guided waves (HWs), we used an optical setup utilizing an attenuated total reflection method (ATR) with a Kretschmann configuration as described before.[6] Briefly, a transverse magnetic (TM) polarized light beam emitted from a He-Ne laser ($\lambda = 633$ nm) was coupled to a LaSFN9 glass prism with a sensor chip optically matched to its base. The sensor chip consisted of a LaSFN9 glass slide coated by 2 nm of chromium and 50 nm of gold layers with the hydrogel film on the top. As shown in Figure 1, a flow-cell with a volume of ≈10 μL was attached to the sensor chip surface and liquid samples were flowed with the flow rate of 1 mL · min^{-1}. This assembly was mounted on a rotation stage to control the angle of incidence of the light beam. By using this setup, the coupling to HWs and surface plasmons (SPs) propagating along the metal surface occurs when the excitation laser beam and a guided wave are phase-

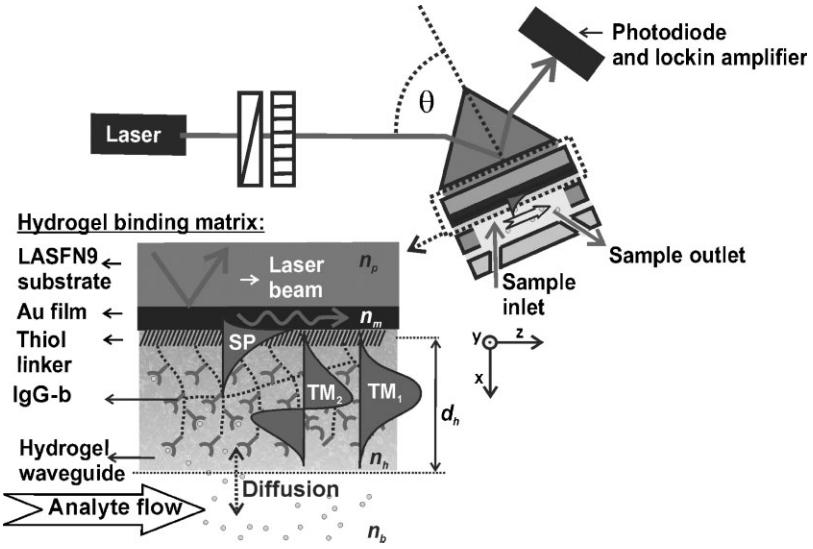


Figure 1. Optical setup for the excitation and interrogation of guided waves within a thin hydrogel film.

matched along the metal surface:

$$k_0 n_p \sin(\theta) = \mathrm{Re}\{\beta\}, \tag{2}$$

where n_p is the refractive index of the prism, θ is the coupling angle, and Re{β} states the real part of a complex propagation constant β. As Figure 2(A) shows, the coupling is manifested as a series of resonant dips in the angular reflectivity spectrum centered at the angles for which the condition (2) holds.

Data Evaluation

From the dispersion relation in Equation (1) it follows that propagation constants β of the HWs and SPs are a function of optical properties of the hydrogel film, which, consequently, affects the resonant coupling to these modes. Therefore, the measured angular reflectivity spectra were fitted by using a transfer matrix-based model in order to determine these optical properties. Further, we assumed the dependence of the refractive index $n(x)$ on the distance from the gold sensor surface as:

$$n(x) = n_b + (n_h - n_b)H(d_h - x), \tag{3}$$

where x is the axis perpendicular to the sensor surface ($x = 0$ is located at the interface between the gold and hydrogel) and H is the Heaviside step function. The parameters of the layer structure used for the excitation of two HWs (noted as TM_1 and TM_2) and SP are stated in Table 1 and the profile of their electric intensity field is shown in Figure 2(C). By fitting the angular positions of TM_1 and TM_2 resonances measured with the accuracy of 10 mdeg, the thickness and the refractive index of the gel film was determined with the error of $\delta d_h \approx 20$ nm and $\delta n_h \approx 10^{-4}$, respectively. In a further analysis, we assumed that the refractive index changes with the concentration of proteins and the NIPAAm polymer as $\partial n_h/\partial c = 0.2\ \mu L \cdot mg^{-1}$. The surface mass density Γ of the hydrogel film including immobilized protein was calculated as:

$$\Gamma = (n_h - n_b) \times d_h \times \frac{\partial c}{\partial n_h} \tag{4}$$

Results and Discussion

Swelling of the Gel

Firstly, the hydrogel film was swollen in ACT buffer for 45 min after which a stable optical response was observed. By fitting the measured angular reflectivity spectrum (I) presented in Figure 2(A), we determined the thickness and the refractive index of the hydrogel film as $d_h = 2.5\ \mu m$ and $n_h = 1.3467$, respectively. These parameters correspond to the surface mass density of the hydrogel film of $\Gamma = 171\ ng \cdot mm^{-2}$. The stability of the swollen gel was tested and no significant changes in its optical properties were observed after several hours incubation in ACT buffer and after subsequent flow of ACT and PBS buffers with a pH of 4 and 7.4, respectively.

Modification with IgG

The carboxylic groups in the swollen gel were activated by 90 min incubation in a solution with TFPS and EDC dissolved in water at a concentration of 21 and 75 $mg \cdot mL^{-1}$, respectively (see Supporting Information for other tested active esters). Afterwards, the surface was rinsed with ACT buffer for 3 min and the solution with IgG-b (concentration of 100 $\mu g \cdot mL^{-1}$) was pumped through the cell for 75 min. The IgG-b molecules were dissolved in ACT buffer at pH 4, which is below their isoelectric point ($I_p \approx 5.5$). Therefore, the positive charge of these molecules allowed for their strong Coulomb attraction into the hydrogel network that exhibited a net negative excess charge as a result of the sulfonic moieties. After the uptake of IgG-b molecules and their reaction with the activated carboxylic groups, the gel was rinsed with the ACT buffer for 10 min, incubated in ethanolamine for 10 min in order to block the unreacted active ester sites, and washed with ACT buffer for 15 min.

As seen in Figure 2(A), the binding of IgG-b into the gel is manifested as a shift of the resonant dips associated with the excitation of TM_1 and TM_2 modes. The fitting of spectrum II measured after the uptake of IgG-b molecules revealed that the loading of the gel with IgG-b induced a

Macromol. Rapid Commun. **2009**, *30*, 872–877

Macromolecular Journals
DOI: 10.1002/marc.200800747

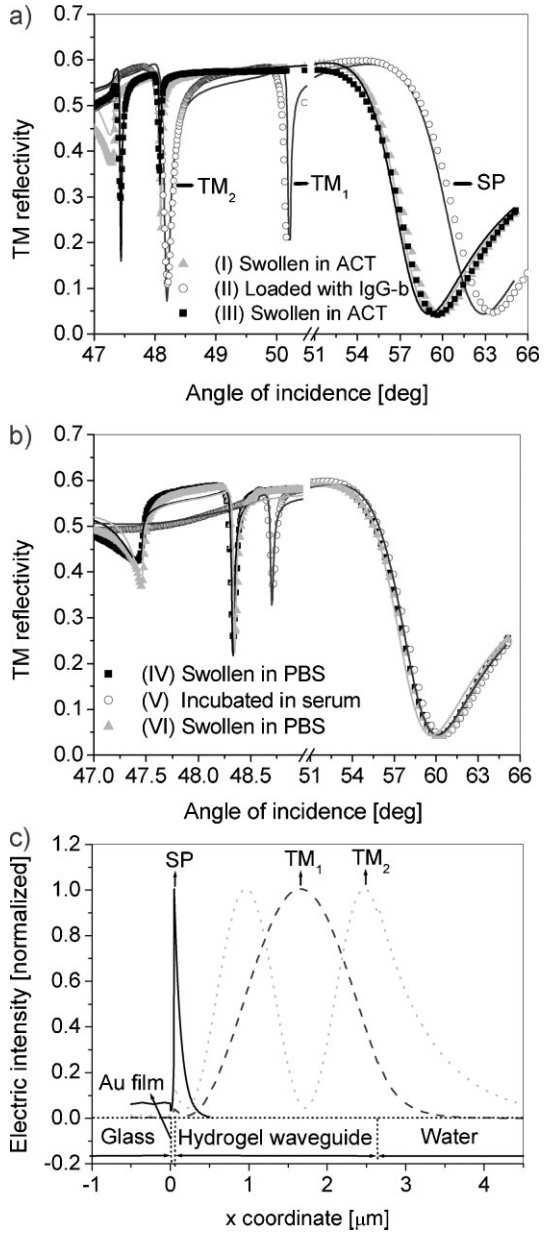


Figure 2. A) Reflectivity spectra measured for the hydrogel film swollen in ACT buffer (squares), loaded with IgG molecules (circles), and after the incubation in ethanolamine (triangles). B) Reflectivity spectra for the gel swollen in PBS (squares), upon the incubation on blood plasma (circles), and after the washout with PBS (triangles). Corresponding fits are shown as lines. C) Simulations of the profile of the electric intensity field of SP and HW waves (layer parameters stated in Table 1).

decrease in its thickness to $d_h = 1.94$ μm and an increase in its surface mass density to $\Gamma = 407$ ng · mm^{-2}. The thickness decrease was probably a result of the screening of the negatively charged gel moieties by the positively charged IgG-b, and the 2.4 fold increase in the surface mass density was caused by the uptake of IgG-b molecules. After

Table 1. Optical parameters of the layer structure used for the excitation of hydrogel waveguide modes.

Material	Refractive index *n*, thickness *d*
Glass substrate	$n_p = 1.845$
Chromium layer	$n = 3.14 + 3.32i$, $d = 2$ nm
Gold layer	$n_m = 0.34 + 3.51i$, $d_m = 45$ nm
Linker layer	$n = 1.5$, $d = 1.37$ nm
Hydrogel	$n_h = 1.3467$, $d_h = 2.50$ μm
Liquid buffer	$n_b = 1.3330$

the incubation in ethanolamine, the resonant dips were shifted to lower angles as the loosely bound IgG-b molecules were rinsed out of the gel (see spectrum III). This led to a decrease in the gel surface mass density to 190 ng · mm^{-2} and an increase of the thickness to $d_h = 3.2$ μm. The thickness increase can be attributed to electrostatic repulsion within the gel between bound IgG-b molecules that are positively charged at pH 4 (note that the negatively charged carboxylic groups without IgG substituents were converted into neutral hydroxyl groups through their reaction with EDC-TFPS and ethanolamine). The surface mass density of the covalently immobilized IgG-b molecules of $\Delta\Gamma = 19$ ng · mm^{-2} was determined as the difference between the surface mass density Γ before the gel activation and after the incubation in ethanolamine. The changes in the swelling and the surface mass density of the hydrogel are summarized in Table 2. The thickness and mass density of the gel and immobilized IgG-b was determined for a set of three identical samples as $d_h = 2.32 \pm 0.28$ μm, $\Gamma = 168 \pm 11$ ng · mm^{-2}, and $\Delta\Gamma = 31 \pm 10$ ng · mm^{-2}, respectively (± denotes the standard deviation).

After modifying the gel with IgG-b antibodies, the hydrogel was swollen in PBS at pH 7.4. As spectrum IV in Figure 2B shows, after the swelling in PBS the refractive index increased to $n_h = 1.35$ and the thickness decreased to $d_h = 2.38$ μm, which can be attributed to reduced repulsion force within the gel because of a higher ionic strength of PBS.

Anti-Fouling Properties

The non-specific sorption of blood plasma to the IgG-modified NIPAAm-based film was investigated. Blood plasma (100%) was circulated through the flow-cell for 40 min followed by rinsing with PBS buffer for 130 min. Fitting of the reflectivity spectrum V measured upon the incubation in plasma showed an increase in the gel refractive index to $n_h = 1.3535$, which indicates that plasma had diffused inside the gel, see Figure 2(B). After

Table 2. Characteristics of the hydrogel film upon its modification with protein molecules.

Hydrogel	n_b	d_h	n_h	Γ
		μm		ng · mm^{-2}
(I) Swollen in ACT	1.3330	2.50	1.3467	171
(II) Loaded with IgG-b	1.3330	1.94	1.3757	407
(III) After passivation	1.3330	3.20	1.3449	190
(IV) Swollen in PBS	1.3347	2.38	1.3500	182
(V) Incubated in plasma	1.3480	2.20	1.3535	–
(VI) Swollen in PBS	1.3347	2.44	1.3500	187
(VII) After NA binding	1.3347	2.53	1.3507	203

the rinsing step, the surface mass density of non-specifically bound plasma compounds was determined to be $\Delta\Gamma = 5$ ng · mm^{-2} as the difference in the surface mass density before the injection of plasma (spectrum IV) and after the rinsing (spectrum VI).

Affinity Binding

The functionalized hydrogel can also be used as a matrix for affinity binding studies. Figure 3(A) shows the time evolution of the angular position of the TM_1 resonant dip upon the binding of NA to the hydrogel film with immobilized biotinylated IgG-b molecules. Firstly, PBS was flowed across the gel for 5 min in order to establish a stable baseline. Afterwards, a solution with NA dissolved in PBS at a concentration of 100 μg · mL^{-1} was flowed for 50 min followed by rinsing with PBS. As seen in Figure 3(A), the binding of NA into the gel network with biotin moieties was observed as a gradual increase of the coupling angle, which reached its saturation after approximately 40 min. In the control experiment, we observed no binding of NA in the gel that was modified by IgG molecules without biotin tags. Diffusion characteristics and association and dissociation binding constants of the reaction can be determined by fitting the measured kinetics with an appropriate model.[12] The fitted NA association rate (≈10^3 M^{-1} · s^{-1}) is two orders of magnitude lower than that for the interaction between (strept)avidin derivatives and biotin (≈10^5 M^{-1} · s^{-1} [13]), which indicates that the binding in the gel was strongly diffusion controlled.

The analysis of angular spectra (VI–VII) in Figure 3(B) reveals the increase in the surface mass density as a result of the capture of NA to biotin moieties of 16 ng · mm^{-2} (the mass density of immobilized IgG-b was 11 ng · mm^{-2}). Assuming the molecular weight of IgG (MW = 150 kDa) is 2.5-fold higher than that of NA (MW = 60 kDa), the average number of NA molecules captured by an IgG-b molecule was determined. This ratio was measured in triplicate as 4.6 ± 1.1 (standard deviation), which is within the expected range (3–6 biotin tags are anchored to an individual IgG molecule), and indicates that the coupling of IgG-b molecules to flexible polymer chains in a swollen hydrogel network allows for efficient binding of the affinity partner molecules.

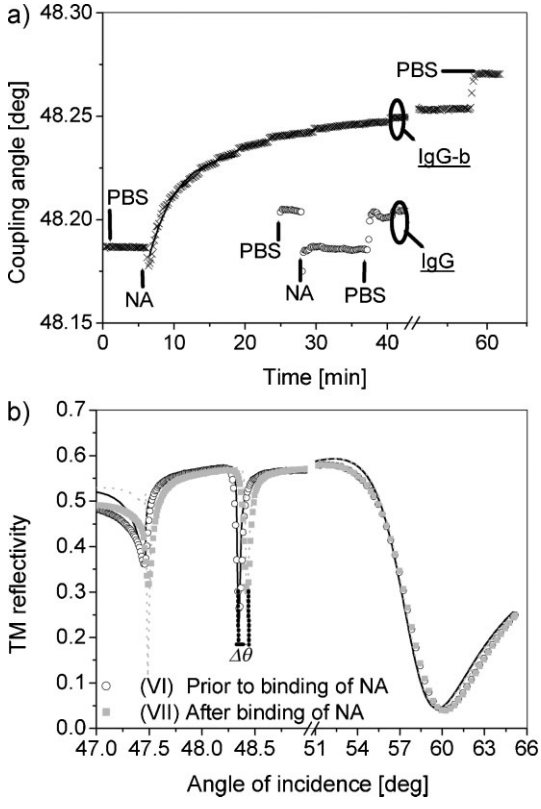


Figure 3. A) Time evolution of the TM_1 coupling angle showing the binding of NA to a gel modified with IgG-b and IgG. B) Measured reflectivity spectra before (circles) and after (squares) the affinity binding of NA with corresponding fits (lines).

Macromol. Rapid Commun. **2009**, *30*, 872–877

DOI: 10.1002/marc.200800747

Conclusion

We implementated the optical waveguide modes spectroscopy (OWS) for the time-resolved quantitative studies of protein-functionalized hydrogel films. We showed that this method allows for the observation of changes in mass distribution in a gel film through the simultaneous measurement of its thickness and refractive index. OWS was applied for the investigation of the coupling of protein molecules to a NIPAAm-based hydrogel film, which was achieved by using a novel charge-attraction scheme that exploited tetrafluorophenol-sulfonate active ester chemistry. We demonstrate that the gel can be used as a binding matrix into which protein molecules can diffuse and affinity bind to the immobilized catcher molecules. In addition, the investigation of the non-specific binding of blood plasma to the gel film reveals its good anti-fouling properties, which makes it, for instance, a good candidate for future applications in affinity biosensors.

Acknowledgements: The authors thank *Natalie Horn* for help with SPR spectrometer measurements. Partial support for this work was provided by the *Deutsche Forschungsgemeinschaft* (KN 224/18-1, Schwerpunktprogramm "Intelligente Hydrogele", SPP 1259) and by the *European Commission* in the Communities 6th Framework Programme, Project TRACEBACK (FOOD-CT-036300).

Received: December 1, 2008; Revised: February 3, 2009; Accepted: February 6, 2009; DOI: 10.1002/marc.200800747

Keywords: hydrogels; proteins; optical sensor; optical waveguide; surface plasmon resonance

[1] N. A. Peppas, J. Z. Hilt, A. Khademhosseini, R. Langer, *Adv. Mater.* **2006**, *18*, 1345.
[2] J. S. McGonigle, G. Tae, P. S. Stayton, A. S. Hoffman, M. Scatena, *J. Biomater. Sci., Polym. Ed.* **2008**, *19*, 1021.
[3] J. A. Rowley, G. Madlambayan, D. J. Mooney, *Biomaterials* **1999**, *20*, 45.
[4] T. Miyata, N. Asami, T. Uragami, *Nature* **1999**, *399*, 766.
[5] W. Knoll, A. Kasry, F. Yu, Y. Wang, A. Brunsen, J. Dostalek, *J. Nonlinear Optic. Phys. Mater.* **2008**, *17*, 121.
[6] P. W. Beines, I. Klosterkamp, B. Menges, U. Jonas, W. Knoll, *Langmuir* **2007**, *23*, 2231.
[7] J. Dostalek, R. F. Roskamp, W. Knoll, *Sens. Actuators B* **2008**, in press (doi 10.1016/j.snb.2008.07.002).
[8] M. Gianneli, P. W. Beines, R. F. Roskamp, K. Koynov, G. Fytas, W. Knoll, *J. Phys. Chem. C* **2007**, *111*, 13205.
[9] M. Gianneli, R. F. Roskamp, U. Jonas, B. Loppinet, G. Fytas, W. Knoll, *Soft Matter* **2008**, *4*, 1443.
[10] K. Derwinska, L. A. Gheber, U. Sauer, L. Schorn, C. Preininger, *Langmuir* **2007**, *23*, 10551.
[11] K. R. Gee, E. A. Archer, H. C. Kang, *Tetrahedron Lett.* **1999**, *40*, 1471.
[12] D. A. Edwards, B. Goldstein, D. S. Cohen, *J. Math. Biol.* **1999**, *39*, 533.
[13] M. H. Qureshi, J. C. Yeung, S. C. Wu, S. L. Wong, *J. Biol. Chem.* **2001**, *276*, 46422.