containing triblock copolymer membrane is about 4 nS higher than that of the protein-free membrane. This suggests that 27 maltoporin trimers are inserted. The same preparation was subsequently polymerized with UV light. Interestingly the conductance decreases considerably. This could reflect a closure or an expulsion of some of the channels during the crosslinking reaction, probably due to internal stress occurring in the membrane during the polymer chain reaction which may lead to a steric contraction of the hydrophilic blocks of the polymers.[3, 6] Such a steric contraction should depend sensitively on the length of the hydrophilic blocks. This remains to be clarified. This argumentation is also supported by the slight decrease in conductance observed in the protein-free membrane during the crosslinking reaction (see Figure 4). Presumably, the polymerization induces a reorganization within the films that allows small membrane defects to be healed.

The remaining maltoporin-trimers in the polymerized PMOXA-PDMS-PMOXA triblock copolymer membrane were subsequently titrated with maltooligosaccharides (maltodextrin). Figure 4 shows the conductance of the polymerized membrane during stepwise addition of 6 μL of a 10^{-1} M maltohexaose solution. Analysis of the conductance data in the usual way[15, 18] gave a binding constant of $K = 7100\,\mathrm{M}^{-1}$ between the proteins and the sugar. The sugar affinity constants for maltoporin within the polymerized triblock copolymer membrane were the same and in good agreement with previous investigations on maltoporin in conventional lipid membranes.[12, 15] Evidently, the conformation of the protein is not influenced by the surrounding membrane and its functionality is fully preserved.

The present investigations show clearly that a functional reconstitution of membrane proteins can be achieved in completely artificial ultra-thin films. This opens the possibility to benefit from the enhanced stability and diversity of such block copolymer aggregates and to incorporate membrane proteins into such a complete artificial polymer membrane. The resulting protein–polymer hybrid materials can be expected to possess great potential for applications in the area of diagnostics, sensor technology, protein crystallization, and even drug delivery.

Received: May 11, 2000
Supplemented: August 28, 2000 [Z15109]

[1] L. Zhang, A. Eisenberg, *Science* **1995**, *268*, 1728.
[2] A. S. Jenekhe, X. L. Chen, *Science* **1998**, *279*, 1903.
[3] C. Nardin, T. Hirt, J. Leukel, W. Meier, *Langmuir* **2000**, *16*, 1035.
[4] Y-Y. Won, H. Ted Davis, F. S. Bates, *Science* **1999**, *283*, 960.
[5] B. M. Disher, Y-Y. Won, D. S. Ege, J. C. M. Lee, F. S. Bates, D. E. Disher, D. A. Hammer, *Science* **1999**, *284*, 1143.
[6] C. Nardin, M. Winterhalter, W. Meier, *Langmuir*, in press.
[7] C. Wang, R. J. Stewart, J. Kopecek, *Nature* **1999**, *397*, 417.
[8] D. A. Hajduk, M. B. Kossuth, M. A. Hillmyer, F. S. Bates, *J. Phys. Chem. B* **1998**, *102*, 4269.
[9] D. D. Lasic, *Liposomes: From Physics to Applications*, Elsevier Science, Amsterdam, **1993**.
[10] T. Hirt, R. C. Baron, D. Lohman, W. Meier, WO-A 99/12059, **1999**.
[11] W. Meier, *Macromolecules* **1998**, *31*, 2212.
[12] R. Benz, K. Bauer, *Eur. J. Biochem.* **1988**, *176*, 1.
[13] P. Mueller, D. P. Rudi, H. T. Tien, W. C. Wescott, *J. Phys. Chem.* **1963**, *67*, 534.
[14] H. Nikaido, *Mol. Microbiol.* **1992**, *6*, 435.
[15] M. Winterhalter, *Colloids Surf. A* **1999**, *149*, 547.
[16] T. Schirmer, T. A. Keller, Y.-F. Wang, J. P. Rosenbusch, *Science* **1995**, *267*, 512.
[17] B. Eisenberg, *Acc. Chem. Res.* **1998**, *31*, 117.
[18] R. Benz, A. Schmid, R. E. W. Hancock, *J. Bacteriol.* **1985**, *162*, 722.
[19] N. Saint, K. L. Lou, C. Widmer, M. Luckey, T. Schirmer, J. P. Rosenbusch, *J. Biol. Chem.* **1996**, *271*, 20676.
[20] J. H. Lakey, *FEBS Lett.* **1987**, *211*, 1.
[21] Y.-F. Wang, R. Dutzler, P. J. Rizkallah, J. P. Rosenbuch, T. Schirmer, *J. Mol. Biol.* **1997**, *272*, 56.
[22] R. Dutzler, Y.-F. Wang, P. J. Rizkallah, J. P. Rosenbusch, T. Schirmer, *Structure* **1996**, *4*, 128.

Direct Observation of the Lower Critical Solution Temperature of Surface-Attached Thermo-Responsive Hydrogels by Surface Plasmon Resonance**

Erik Wischerhoff,* Thomas Zacher, André Laschewsky,* and El Djouhar Rekaï

Affinity biosensing is an important method for the investigation of biochemical binding processes: it allows the monitoring of biomolecular interaction in real time and therefore enables the user to deduce kinetic constants from experimental data.[1] One of the most popular transduction principles in affinity biosensing is surface plasmon resonance (SPR).[2] The excitation of surface plasmons in thin metal films—usually gold—by totally internal reflected light can be observed as a minimum in the intensity of the reflected light at a certain angle of incidence Θ_{SPR}, the resonance angle. Changes of the refractive index in close proximity to the gold surface lead to a shift in the resonance angle. SPR offers the advantage of interaction measurements in real time and does not require labeled analytes.[3]

An important prerequisite for the success of affinity biosensing is a chemically tailored sensor interface. Typically, hydrogels are employed as a biocompatible matrix to prohibit the denaturation and to maintain the unique functions of biomolecules which are covalently immobilized at sensor

[*] Dr. E. Wischerhoff,[+] Dipl.-Phys. T. Zacher[+]
BioTuL AG
Gollierstrasse 70, 80339 München (Germany)
E-mail: e.wischerhoff@pharm.uu.nl
Prof. Dr. A. Laschewsky, Dr. El D. Rekaï
Department of Chemistry
Université catholique de Louvain
Place L. Pasteur 1, 1348 Louvain-la-Neuve (Belgium)
Fax: (+32) 10-472836
E-mail: laschewsky@cico.ucl.ac.be
[+] New address:
Department of Pharmaceutical Proteomics
Universiteit Utrecht
Sorbonnelaan 16, 3508 TB Utrecht (The Netherlands)
Fax: (+31) 30-251-8219

[**] The work was supported by the European Commission (research grants CEE BIO-CT97-962372 and ERBFMBCT 982915).

  1433-7851/00/3924-4602 $ 17.50+.50/0

interfaces.[4] Frequently, polysaccharides have been chosen for such hydrogels.[5] Although they fulfil this task in many respects satisfactorily, synthetic hydrophilic polymers are attractive alternatives as they offer some advantages. First, they may be more resistant to harsh conditions (strong acids, fouling etc.). Second, they provide the opportunity to create polymers with new physical properties by tailoring their structure. An impressive example for the potential provided by tailored hydrogels are so-called thermo-responsive or thermo-sensitive hydrogels.[6]

The behavior of thermo-responsive polymers in aqueous media often contradicts intuition because they generally exhibit a lower critical solution temperature (LCST). Upon heating, when the temperature increases beyond a certain value commonly referred to as the cloud point, they precipitate from solution. Thermo-responsive hydrogels should undergo an analogous transition even under the particular conditions existing in confined space at a surface.[7] Such a behavior is of special interest for applications at sensor surfaces because it allows one to switch their properties, or to introduce new functionalities. Some applications already utilize the phase transition on a solid surface.[8] However, such a transition has not yet been directly observed at an interface up to now. Herein, we report the direct observation of the LCST of a new thermo-responsive hydrogel linked to a gold surface by using surface plasmon resonance.

The specific balance of hydrophilic and hydrophobic groups is a characteristic feature for many thermo-responsive polymers exhibiting a LCST in aqueous solution.[9] To control the thermal transition temperature, we have modified water-soluble precursor polymers by partial acylation.[10] Compared to the widely applied approach of employing copolymerization, this strategy facilitates the making of chemically homogeneous, random copolymers, thus enabling a sharp transition in priciple. The precursor polymer chosen for this work was poly-*N*-[tris(hydroxymethyl)methyl]acrylamide (P-THMA; see inset in Figure 1) that is known for biomedical applications.[11] Partially acetylated P-THMA derivatives exhibit a LCST in aqueous solution,[10] the observed cloud points depending on the concentration. P-THMA bearing 40 mol% of acetoxy groups covers the physiologically interesting temperature range of 20 to 45 °C (Figure 1).

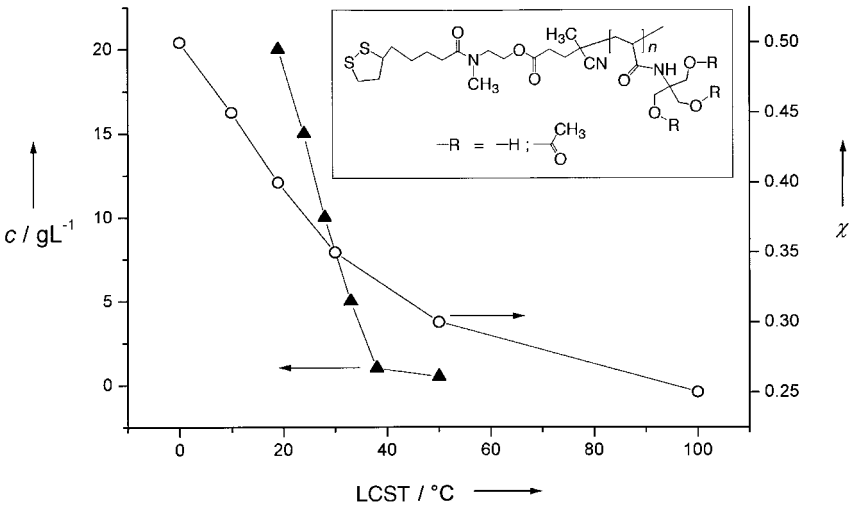

Figure 1. Dependence of the LCST of aqueous solutions of acetylated P-THMA on the molar fraction of acetylation χ (concentration 20 g L^{-1}; open circles) as well as on the polymer concentration c (degree of acetylation 40%; filled triangles). The lines only serve as a guide to the eye and have no physical meaning. The inset shows the structure of P-THMA with the azo initiator fragment which bears disulfide moieties.

However, it is not evident how polymers that are thermo-responsive in solution will behave when grafted to a surface. For measuring the temperature-dependent conformational state of the grafted polymers, we have used a SPR-prototype setup (BioTul AG, Munich, Germany). Because the evanescent field of the surface plasmons decays exponentially from the surface, the collapse of the hydrogel when passing the cloud point will change the local average refractive index (Figure 2). Consequently, it should be possible to track the collapse by monitoring the angle shift in the intensity minimum.

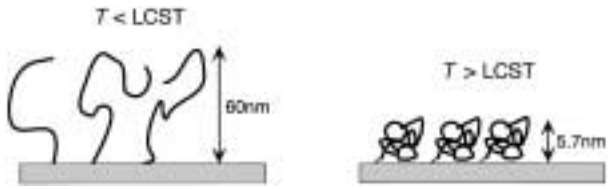

Figure 2. Schematic representation of the thermo-responsive effect of acetylated P-THMA attached to a gold surface as determined and quantified with SPR. The phase transition occurs at LCST $\approx$ 26 °C.

The SPR setup used employs a diode laser ($\lambda = 784$nm) as light source and an angular resolution of 0.005° is available. The monochromatic light is directed through a cylindrical prism to a gold-coated glass slide (BK7 glass, $n = 1.511$) coupled through immersion oil to the glass prism. The intensity of the reflected light was measured by a photodiode as a function of the angle of incidence.

In the experiments, pure P-THMA and partially acetylated P-THMA were chemisorbed on gold through their terminal disulfide groups and then the coated substrates were immersed in water which resided in a flow chamber. Water heated stepwise to certain temperatures was injected into the flow chamber and SPR measurements were carried out simultaneously (Figure 3). Several single measurements were averaged to filter the noise.

A polynomial fit to the data points of simple P-THMA (Figure 3, open circles) serves as a reference curve and reflects the temperature dependence of the refractive index of water in accordance with the data listed by Schiebener et al.[12] The average slope of the curve amounts to -0.011 deg K^{-1} which equals a temperature-dependent change of the refractive index of water of -1.0×10^{-4} K^{-1}.

The analogous measurements carried out on modified P-THMA bearing 40% of acetylated hydroxy groups are shown in Figure 3 (filled circles). Apart from the

  1433-7851/00/3924-4603 $ 17.50+.50/0

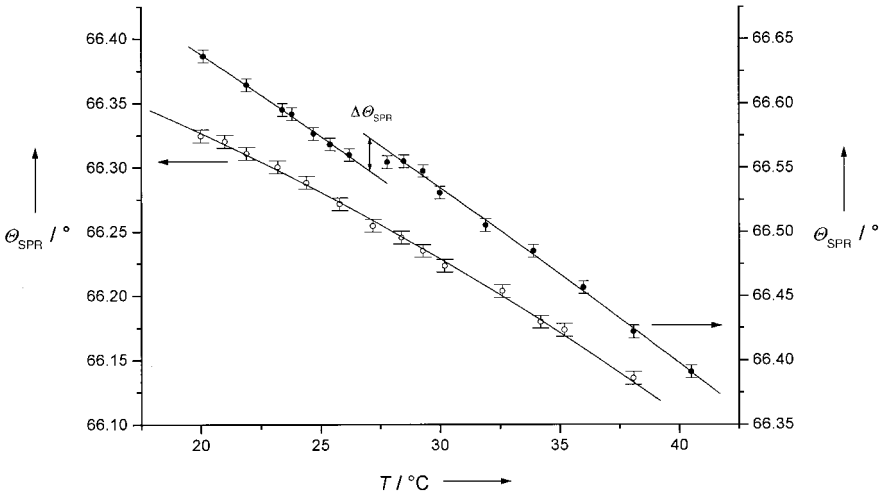

Figure 3. Open circles: $\Theta_{SPR}(T)$ of non-stimuli-responsive P-THMA. The curve reflects the evolution of the refractive index of water with temperature and serves as a reference. Filled circles: $\Theta_{SPR}(T)$ of acetylated P-THMA (degree of acetylation 40%). The collapse of the hydrogel at $T =$ LCST leads to a significant shift $\Delta\Theta_{SPR}$, indicating the phase transition as visualized in Figure 2.

overall decrease of the resonance angle with temperature, there is a distinct change in the slope between about 26 °C and 28.5 °C, leading to an offset of $\Delta\Theta_{SPR} = 0.026°$. This indicates the phase transition of the acetylated P-THMA triggered by the increasing temperature. Calculations based on applying Fresnel equations[13] to a model layer system assuming homogeneous layers reveal that the measured values of $\Theta_{SPR}(T)$ and the observed shift of $\Delta\Theta_{SPR} = 0.026°$ correspond to a swollen hydrogel film which is approximately 60 nm thick at $T <$ LCST, and which collapses to a 5.7 nm thick film when the temperature exceeds the LCST. For the collapsed hydrogel a refractive index of 1.478 was assumed, for the swollen hydrogel a mixed refractive index of 1.344 calculated according to Garnet.[14] The width ΔT of the phase transition can be estimated to amount to $\Delta T \approx 2.5$ °C and is believed to be caused by the polydispersity of the compound. (A fully acetylated sample from the same batch was analyzed by gel-permeation chromatography. The resulting number for the apparent polydispersity was 8.)

The thermal transition on the gold surface occurs at $T \approx$ 26 °C which would correspond to a concentration of about 15 g L^{-1} in aqueous solutions. However, the dimensions of the collapsed and swollen hydrogel film as determined by the SPR measurements imply a local concentration of the polymer in the chemisorbed swollen hydrogel film of about 120 g L^{-1}, assuming a density of 1.2 g cm^{-3} for the polymer. Two factors may explain this difference: first, the temperatures measured may be systematically slightly too high due to heat flow effects, but, more important, it is very likely the steric restrictions for the polymer chains at the interface have a significant influence on the phase transition,[7] shifting the transition temperature to higher values for polymer chains in confined space. An analogous increase of transition temperatures was reported, for example, for surface-attached brushes of liquid crystalline polymers.[15]

In conclusion, controlled chemical modification allows one to induce and to tune the LCST of well-known water-soluble polymers, as in the case of acetylated P-THMA. The chemisorption of such polymers leads to thermo-responsive hydrogels on gold surfaces. We have directly evidenced the thermal transition by SPR and quantified the thermo-responsive effect by comparison of the measurements with calculated values. Owing to their straightforward structure together with a marked thermo-responsiveness, the presented hydrogels have the potential for interesting biochemical applications, for example control of binding processes at sensor surfaces by variation of the temperature.

Received: May 18, 2000
Revised: September 20, 2000 [Z15143]

[1] M. Fivash, E. M. Towler, R. J. Fisher, *Curr. Opin. Biotechnol.* **1998**, *9*, 97–101.
[2] B. Liedberg, K. Johansen in *Methods in Biotechnology, Vol. 7* (Eds.: K. R. Rogers, A. Mulchandani), Humana, Totowa, **1998**, pp. 31–53.
[3] A. Szabo, L. Stolz, R. Granzow, *Curr. Opin. Struct. Biol.* **1995**, *5*, 699–705.
[4] S. Löfås, B. Johnsson, *J. Chem. Soc. Chem. Commun.* **1990**, 1526–1528.
[5] a) J. H. Elam, H. Nygren, M. Stenberg, *J. Biomed. Mater. Res.* **1984**, *18*, 953–959; b) J. Piehler, A. Brecht, K. E. Geckeler, G. Gauglitz, *Biosens. Bioelectron.* **1996**, *11*, 579–590.
[6] N. A. Peppas, R. Langer, *Science* **1994**, *263*, 1715.
[7] P. W. Zhu, D. H. Napper, *Macromol. Chem. Phys.* **1999**, *200*, 698–705.
[8] a) N. Yamada, T. Okano, H. Sakai, F. Karikusa, Y. Sawasaki, Y. Sakurai, *Macromol. Chem. Rapid Commun.* **1990**, *11*, 571–576; b) T. Oya, T. Enoki, A. Grosberg, S. Masamune, T. Sakiyama, Y. Takeoka, K. Tanaka, G. Wang, Y. Yilmaz, M. S. Feld, R. Dasari, T. Tanaka, *Science* **1999**, *286*, 1543–1545; c) K. Uchida, K. Sakai, O. H. Kwon, E. Ito, T. Aoyagi, A. Kikuchi, M. Yamato, T. Okano, *Macromol. Chem. Rapid Commun.* **2000**, *21*, 169–173.
[9] L. D. Taylor, L. D. Cerankowski, *J. Polym. Sci. Polym. Chem. Ed.* **1975**, *13*, 2551–2570.
[10] A. Laschewsky, E. D. Rekaï, E. Wischerhoff, *Polym. Prepr. Am. Chem. Soc. Div. Polym. Chem.* **1999**, *40*, 189–190.
[11] P. Barthélemy, J. C. Maurizis, J. M. Lacombe, B. Pucci, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1559–1562.
[12] P. Schiebener, J. Straub, J. M. H. Levelt Sengers, J. S. Gallagher, *J. Phys. Chem. Ref. Data* **1990**, *19*, 677–715.
[13] K. S. Johnston, S. R. Karlsen, C. C. Jung, S. S. Yee, *Mater. Chem. Phys.* **1995**, *42*, 242–246.
[14] M. Garnet, *Philos. Trans. R. Soc. A* **1904**, *203*, 385–420.
[15] B. Peng, D. Johannsmann, J. Rühe, *Macromolecules* **1999**, *32*, 6759–6766.

  1433-7851/00/3924-4604 $ 17.50+.50/0