Thermo-Responsive Hydrogel Coatings on Polymer Surfaces for Controlled Cell Adhesion and Detachment

Polymere mit thermo-reversiblen Eigenschaften sind von großer Bedeutung für biomedizinische Anwendungen. Bekannte Vertreter sind Poly(N-isopropylacrylamid) (PNiPAAm) und Poly(N,N-diethylacrylamid) (PDEAAm) mit Übergangstemperaturen von 32 bis 33 °C. In der vorliegenden Arbeit wurden Copolymere dieser Materialien mit Poly(ethyleneglycol) (PEG) synthetisiert, um so die Übergangstemperatur in den physiologischen Bereich nahe 37 °C zu verschieben. Die Copolymere (PNiPAAm-g-PEG, PDEAAm-g-PEG) wurden mittels Plasmaimmobilisierung als dünne Schicht auf Zellkulturträgern präpariert. Es konnte gezeigt werden, dass derartige Träger die schonende Ablösung adhärenter Zellen durch eine kurzzeitige Absenkung der Temperatur um wenige K gestatten.

Keywords
cell culture
stimuli responsive polymers
plasma immobilization

Bearbeiter
D. Schmaljohann
J. Oswald
B. Jørgensen
M. Nitschke
D. Beyerlein
C. Werner

Förderer
Bundesministerium für Bildung und Forschung im Rahmen des Kompetenzzentrums für Materialien im Blut- und Gewebkontakt Dresden (Max-Bergmann-Zentrum für Biomaterialien Dresden)

Introduction

Polymers with a volume phase transition play an important role in biomedical application. Especially, those materials are of interest which show a thermally stimulated transition, in particular a lower critical solution temperature (LCST). Poly(*N*-isopropylacrylamide) (PNiPAAm) and poly(*N*,*N*-diethylacrylamide) (PDEAAm) are polymers that have a LCST at 32 to 33 °C. A transition in that region is desirable for biomedical applications like biosensors [1], because a small change in the temperature causes a dramatic change in the polymer properties. Surface-immobilized, thermo-responsive hydrogels are utilized for e.g. cell culture carriers to facilitate cell harvest without enzymatic treatments [2]. Furthermore, hydrogels which respond to small changes in the environmental temperature by swellling or collapsing can serve additional functions like protein entrapment. Low-pressure plasma treatment allows the immobilization of polymer films of a few nanometers thickness on polymeric substrates. This technique was applied to various combinations of substrates and coatings [3-5]. Recently, we reported the preparation of thin hydrogel coatings on PTFE-like fluorocarbon surfaces by plasma immobilization. The thermo-reversible swelling was studied on these hydrogels based on novel PNiPAAm-*g*-PEG and PDEAAm-*g*-PEG graft copolymers, which change the degree of swelling upon temperature changes between 30 and 45 °C [6, 7]. Poly(ethyleneglycol) (PEG) is used in these materials not only to adjust the LCST, but also to alter the interaction with proteins and cells. This work now focuses on the study of the response of cells towards the different environmental conditions, especially the ability to adhere cells above the LCST and to release them below the transition temperature.

Polymer Synthesis and Characterization

The graft copolymers P1 to P4 (Fig. 1) were synthesized from NiPAAm or DEAAm and poly(ethyleneglycol) monomethacrylate via free radical copolymerization. Experimental details are given elsewhere [8, 9].

Low Pressure Plasma Immobilization

Non-branched fluorocarbon films with a structure close to PTFE were prepared on silicon substrates with an oxide layer of 50 nm by plasma polymerization. The fluorocarbon surfaces were pre-treated in argon plasma for 120 s as described below to allow spin coating of thin films of the polymers P1-P4 (solution 0.5 % w/w in $CHCl_3$, 5000 rpm/s, 5000 rpm). In the next step the films were immobilized using argon plasma. The treatment was carried out in a MicroSys apparatus by Roth & Rau, Germany (microwave excitation, effective power 120 W, argon gas flow 38 sccm, pressure $8 \cdot 10^{-3}$ mbar, treatment time 8 s). Afterwards the film was rinsed with $CHCl_3$ to remove polymer residues which had not been immobilized.

Ellipsometry

The used Ellipsometer M-44 (Woollam Co. Inc., USA) is a rotating analyzer type with an detector array of 44 wave-lengths between 428 and 763 nm. Measurements took place in a home-built flow-cell [10] with 74.8° as angle of incident. For modeling a five layer system (see Fig. 2) was assumed. All polymers were described with Chauchy functions. In the swollen state, the thickness and the optical constants were fitted as one homogeneous layer.

Cell Cultivation

L929 mouse fibroblasts were obtained from DSMZ, Braunschweig, Germany and cultivated in RPMI medium containing 10 % FCS and antibiotics. Cells grow as a monolayer and were passaged when they reached confluence using trypsin (0.25 % w/v in PBS, Biochrom). The temperature-controlled cell detachment was monitored with a Zeiss Cell Observer, a special controlled incubator mounted on a heated scanning microscope table. The heating of the table and the air stream was adjusted manually. The microscope was connected to a digital camera (Zeiss AxioCam Colour) in order to record the cell behaviour in the incubator during the experimental course. Images were taken every 30 s and subsequently analyzed with the software AxioVision (Zeiss).

Preparation and Characterization of the Stimuli-Responsive Coatings

The polymers used for plasma immobilization were synthesized according to the scheme in Fig. 1. The reactivity of both monomers is similar. Thus one obtains almost random copolymers, which means that the PEG side chains are randomly distributed along the polymer backbone.

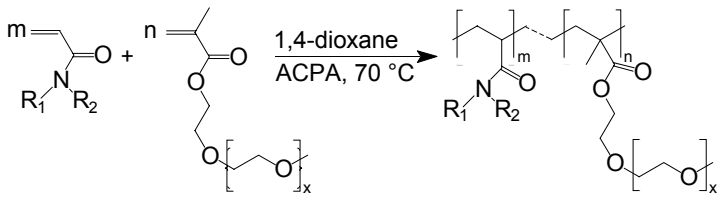

Fig. 1:
Synthetic scheme and composition of the investigated graft copolymers

P1: R_1 = H, R_2 = iPr, m = 0,987; n = 0,013; x = 44
P2: R_1 = H, R_2 = iPr, m = 0,979; n = 0,021; x = 44
P3: R_1 = Et, R_2 = Et, m = 0,994; n = 0,006; x = 44
P4: R_1 = Et, R_2 = Et, m = 0,960; n = 0,040; x = 7

These soluble polymers were used as model compounds in order to evaluate the LCST behavior and to select the material with the desired transition temperature. Afterwards, the same polymers were used for the plasma immobilization procedure. Simple spin coating and subsequent low-pressure plasma treatment yield the desired surface-immobilized polymer layer, which gives the corresponding hydrogel when immersing into water or cell culture medium. Fig. 2 depicts the layer system of the hydrogel, which was also the basis of the ellipsometry data treatment.

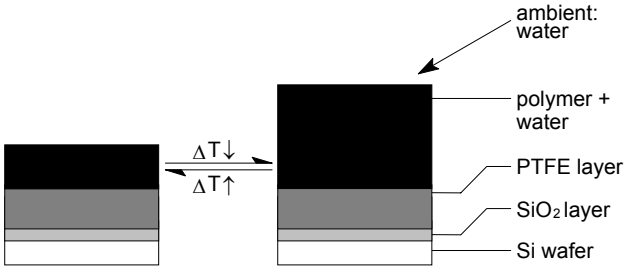

Fig. 2:
Layer system of the surface-immobilized hydrogel

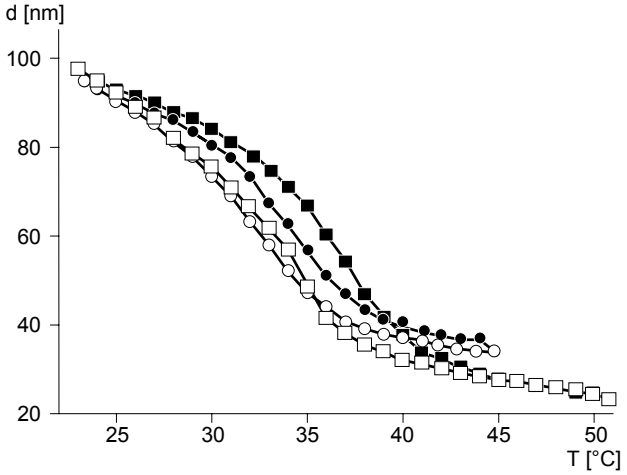

Fig. 3:
Film thickness d versus temperature T for P1 containing hydrogel in deionized water and in PBS solution studied by ellipsometry
(■ P1, deionized water, second heating, □ P1, deionized water, second cooling, ● P1, PBS solution, second heating, ○ P1, PBS solution, second colling)

Initial swelling studies have been carried out in deionized water (Fig. 3), similar to the LCST determination of the soluble polymer with UV/Vis turbidity measurement. The comparison between LCST of the soluble polymer with the transition temperature of the immobilized hydrogel yield a decrease by 2 to 4 K in the transition temperature due to the immobilization step, which is accompanied by a broadening of the observed transition. The treatment with phosphate buffer saline (PBS) solution or RPMI + 10 % FCS cell culture medium, respectively, caused a further downshift in the transition temperature by 2 to 3 K (Fig. 3). Finally, all applied immobilized hydrogels have a transition temperature between 32 and 37 °C in PBS with a hysteresis of 2 to 3 K. Thus, the hydrogels are in the collapsed state at 37 °C. Yet, the presence of the PEG makes the hydrogels hydrophilic enough that they maintain certain hydrogel properties. A degree of swelling DS = $[d_{swollen}]:[d_{dry}]$ = 2 to 3 is observable at T = 37 °C, whereas at T = 25 °C DS is 6 to 7. Even though the difference in degree of swellling as well as surface polarity is modest as compared to the corresponding hydrogel prepared from PNiPAAm or PDEAAm homopolymer, we believe that the addition of PEG supports the cell release behavior.

Cell Cultivation and Detachment via Thermal Stimulus

L929 mouse fibroblasts were cultivated on hydrogel P1 for several days in RPMI medium. The cells adhere well, spread and proliferate on the surface at 37 °C, which showed that the applied hydrogels are suitable as cell cultivation carriers. The gels were confirmed to be non-toxic and did not show impaired cell adhesion due to their PEG contents.
In a second experiment the cell detachment was studied upon a thermal stimulus. After cultivation over night, cells were placed at 37 °C into the Zeiss Cell Observer and the temperature was reduced at a rate of ca. 0.1 $K \cdot min^{-1}$ to follow the response of the cell towards the changes in film thickness and surface polarity. Already 1 to 2 K below 37 °C, the cells round up. And after decreasing the temperature by 3 K to 34 °C, complete cell detachment was observed. Fig. 4 shows micrographs of the cells up to the point, when the cells finally go off the substrate.

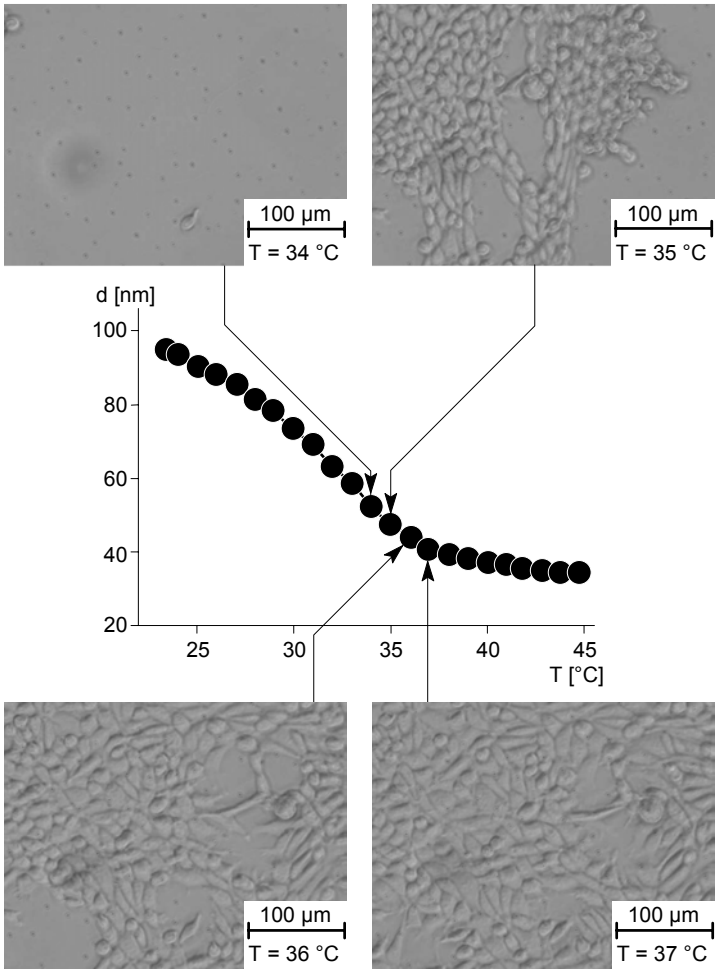

Fig. 4:
Micrographs of mouse fibroblasts during cell detachment at decreasing temperature and correlation to the film thickness d of the P1 hydrogel
(○ P1, PBS solution, second cooling)

Fig. 4 also indicates how the changes in cells are correlated to the changes of the hydrogel film. Long before the swelling is completed, the cells already detach from the surface. We attribute the immediate response of the cells towards the temperature to the substantial amount of PEG in the hydrogel. Upon swelling, the PEG gains further mobility, which allows it to diffuse to the surface. The cell detachment occurred within 20 min. Thus, we prepared a fast responding hydrogel, which again can be attributed to the presence of the hydrophilic PEG.

Okano et al. also reported that the incorporation of ionic groups or PEG accelerated the cell detachment of NiPAAm containing hydrogels [11].
Nevertheless, our results are in contrast to those reported by Okano, where they state that incorporation of 0.5 wt.-% of PEG dramatically decreases the cell adhesion. The hydrogels discussed in this paper contain about 19 wt.-% PEG and they show at 37 °C good cell adhesion behavior even without any precoating with proteins of the extracellular matrix. However, Okano et al. used bovine aortic endothelial cells in culture experiments which may behave different as compared with the L929 mouse fibroblasts applied in our study. The cell number on the PEG-containing hydrogel films was found to be roughly similar as on the polystyrene (PS) culture dish control. This unusual behavior at a relatively high PEG content can possibly be attributed to the graft architecture of the precursor polymer, which exhibits a strong interaction of the ethylenglycol units with the NiPAAm units above the transition temperature and in a less pronounced fashion also with the DEAAm units above T_{tr} [8, 9].
The micrographs in Fig. 5 compare the behavior of the cells on two substrates (P1 hydrogel and PTFE-like polymer as control) at the two temperatures (above and below T_{tr} of P1 hydrogel). During the polymer immobilization, one part of the glass slide with the PTFE-like layer (cf. Fig. 2) was covered in order to avoid plasma exposure. When rinsing the glass slide with chloroform after plasma treatment, the non-crosslinked polymer hydrogel was removed completely from this covered area of the sample. This region on the glass slide was taken as control in Fig. 5.

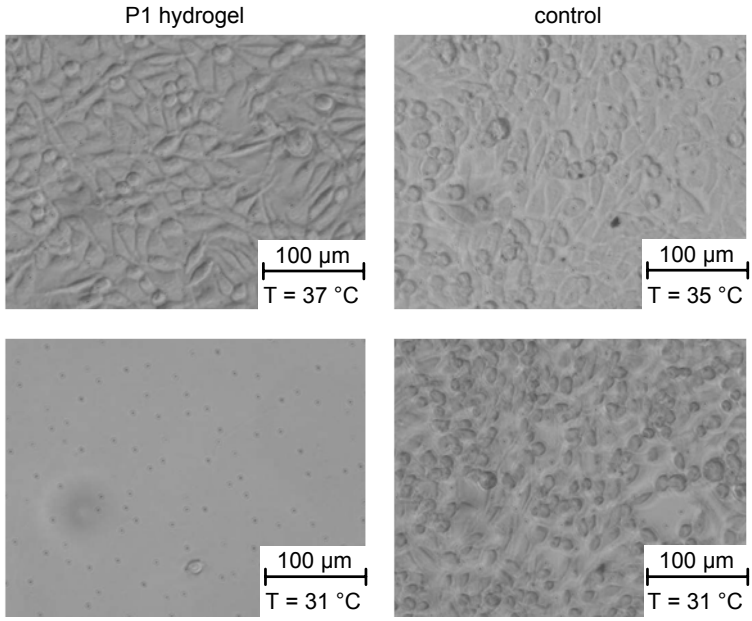

Fig. 5:
Micrographs of mouse fibroblast on P1 hydrogel (left) and PTFE-like control (right), and at 37 °C (top) and 31 °C (bottom), respectively

The fibroblasts continue to adhere well on the uncoated fluoropolymer even upon reducing the temperature to 31°C while they are efficiently removed from the thermo-responsive hydrogel at this temperature. After temperature reduction to 31 °C, the cells on the control show light shrinking in shape, but this completely reversed after going back 37 °C.

Conclusion

The stimuli-responsive surface-immobilized hydrogels based on PNiPAAm-*g*-PEG or PDEAAm-g-PEG, respectively, can be utilized as fast responding hydrogels, which allow to detach cells from the substrate without enzymatic treatment. The relatively large content of PEG (15 to 20 wt.-%) is attributed to support this fast detachment, which is caused by the collapse of the thermo-responsive polymer. Furthermore, the PEG allows to maintain certain hydrogel properties even above the transition temperature.

References

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