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Anchoring of Fluorophores to Plasma-chemically Modified Polymer Surfaces and the Effect of Cucurbit[6]uril on Dye Emission

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Abstract Polypropylene supports were functionalized by plasma-deposition of polymeric allylamine layers. The surface amino groups generated were wet-chemically reacted with xanthene dyes resulting in fluorescent polymer films. The effect of polymer-attachment of the dyes on their emission features was studied fluorometrically and different methods were tested to improve the fluorescence properties of the films. Modification with cucurbit[6]uril (CB6) yields a moderately enhanced fluorescence as well as an improved photostability. The observed effect is most likely due to CB6-induced rigidization of the linker molecules which seems to reduce fluorescence quenching dye–dye and fluorophore–surface interactions.

Keywords Fluorescence · Cucurbituril · Surface modification · Plasma functionalization · Polymer surface · Xanthene dyes

Introduction

Miniaturization processes in science and technology have reached dimensions where the tailoring of surface properties is a prerequisite for successful applications in many fields such as electronics, medicine, and bioanalysis.

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Polymeric materials are used in many of these areas because of their advantageous mechanical and chemical features, comparably low costs, and suitability for fabrication in automated processing. Several methods employing physical and chemical strategies were introduced to application-specifically tune the surface properties of polymeric materials. Straightforward and popular tools are plasma-chemical treatment techniques for coating, finishing, or etching thereby increasing the degree of surface tailoring [1–4]. Plasma-chemically generated reactive groups are often used as anchoring sites for molecules with distinct functional properties such as sensor or biomolecules. Synthetic concepts for the reaction of molecules with surfaces require also reliable methods to characterize and quantify surface functionalities and grafted molecules. Despite the enormous potential of inexpensive fluorescence techniques, there exist only few papers describing the use of fluorophore labeling techniques for the characterization of surface groups and quantification purposes [5–8]. Straightforward and sensitive fluorescence measurements for the monitoring of reactive groups at surfaces are complicated by several factors such as non-specific adsorption of unreacted fluorescent labels, penetration of dye molecules into the polymer, inhomogeneous dye distribution, and quenching of fluorescence by dye–environment or dye–dye interactions [9–11]. To minimize dye–surface interactions, we studied the effect of the macrocyclic host cucurbit[6]uril (CB6; cf. Fig. 1) on the fluorescence properties and the photostability of surface-attached fluorophores. Cucurbiturils are frequently used for e.g. molecular recognition [12, 13], selective adsorption of molecules [14, 15] and sensoric applications [16]. Moreover, similarly as host structures like cyclodextrines [17, 18], also cucurbiturils can have a beneficial influence on the fluorescence intensity of incorporated fluorophores [19–
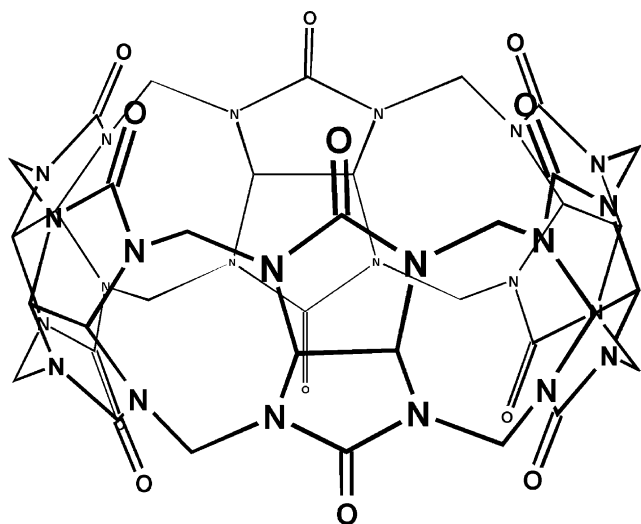
Fig. 1 Structure of CB6

21] or on the environmental dependence of their spectroscopic properties [22, 23].

In this work, we report on the spectroscopic properties of plasma-chemically amino-functionalized polypropylene (PP) surfaces, labeled with fluorescein and rhodamine dyes. These fluorophores were chosen as they are strongly fluorescent in polar protic solvents like water, yet are only moderately sensitive to the polarity of their microenvironment. In addition, they are prone to concentration-dependent, fluorescence-quenching aggregation. This renders these polymer-linked fluorophores ideal candidates for the spectrofluorometrical study of confinement effects caused by a modification with the macrocyclic host CB6 [22, 23].

Experimental

Materials

PP films (thickness of 100 μm, Goodfellow, U.K.) were ultrasonically cleaned in diethyl ether (15 min). Allylamine (>99%) was purchased from Merck (Germany) and distilled prior to use. All the solvents (tetrahydrofuran (THF), ethanol) were dried and distilled before use. The derivatization reagent pentafluorobenzaldehyde (>98%, Sigma-Aldrich, USA) was employed as received. Glutaraldehyde (GA) solution (assay ~25% in water) was purchased from Fluka (Switzerland). A 5% solution was used for spacer coupling. 1,6-diaminohexane (DAH, purity >99%) was obtained from Sigma-Aldrich and used as received. The fluorescent labels fluorescein 5 (6)-isothiocyanate (FITC), and the rhodamines 5(6)-carboxy-X-rhodamine-N-succinimidyl ester (ROX) and 5(6)-carboxytetramethylrhodamine-N-succinimidyl ester (TAMRA), that should reveal a purity>95% according to the manu-

facturer, were purchased from Fluka (Switzerland) and were employed without further purification. Cucurbit[6] uril (CB6) was synthesized and purified as described [24].

Methods

Plasma-chemical generation of amino functions at PP

PP was functionalized by depositing a 50 nm-allylamine plasma polymer layer (PP-ppAAm). The deposition process was carried out in a cylindrical plasma reactor of 50 dm^3 volume at 50 W. The reactor was equipped with a pulsed radio-frequency generator (rf 13.56 MHz), with a linear rf electrode (5.35 cm^2), a mass flow controller for gases, a liquiflow controller (Bronkhorst High Tech, The Netherlands) for liquids, a heatable gas distributor inside of the reactor, and a turbomolecular pump. In the pulsed mode, the polymer layers were deposited with a duty cycle d=0.1 or d=0.5, where d represents the ratio between active (t_{on}) and nonactive (t_{off}) plasma periods ($d = t_{on}/(t_{on} + t_{off})$). The deposition rate was determined with a quartz microbalance (FTM5, Film Thickness Monitor, BOC-Edwards, (U.K)).

Surface analysis

The surface of modified PP was studied by XPS. The XPS data were acquired with a SAGE 150 Spectrometer (Specs, Germany) using non-monochromatized Mg K$_\alpha$ radiation (12.5 kV and 250 W) at a pressure of ca. 10^{-7} Pa. XPS spectra were obtained in the constant analyzer energy (CAE) mode at a 90° take-off angle. Peak analysis was performed using the peak fit routine from Specs. Prior to XPS analysis, the NH$_2$ groups were derivatized with the XPS tag pentafluorobenzaldehyde [25]. In this case, the measured concentration of fluorine atoms is directly related to the number of surface amino functions. Measurements of water contact angles performed with an Automatic Contact Angle System G2 (Kruess, Germany) provide a measure for the hydrophilicity of the polymer surface.

Fluorescence spectroscopy

Emission spectra of fluorophore-labeled polymer films were measured between two quartz windows in 30°/60° front-face geometry. The excitation polarizer of the spectrofluorometer (Spectronics Instruments 8100) was set to 90° and the emission polarizer to 54.7°, respectively. Prior to each fluorescence measurement, the fluorophore-labeled films were washed with ethanol to remove adsorbed dye molecules. For each series of measurements, blank or so-called reference samples were prepared by reaction of the unmodified polymer film with the respective fluorescent label using the same procedure as employed to covalently

Fig. 2 Structures of the fluorescent probes FITC, TAMRA, and ROX

FITC   TAMRA   ROX
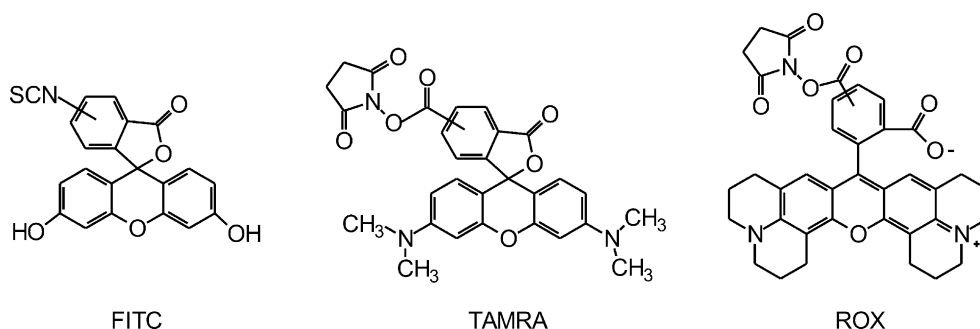
attach fluorophores to the surface-functionalized films. Unless otherwise stated, all the fluorescence spectra presented were corrected for the wavelength- and polarization-dependent spectral response of the detection system.

The fluorometer, that was equipped with a 450 W Xenon lamp (Ushio UXL 451O) and double monochromators (excitation, emission), was also used for photostability studies.

Anchoring of fluorescence dyes to allylamine-modified PP surfaces

Allylamine was polymerized both in the continuous wave and in the pulsed plasma regime. The amount of NH_2 groups in the poly(allylamine) layers was calculated from XPS data to 12–16 NH_2/100 C [25]. The surface amino groups of the modified PP samples were labeled either directly or via the spacer molecules GA and diaminohexane (DAH) with FITC or with *N*-succinimidyl ester derivatives of the xanthene fluorophores depicted in Fig. 2 (see Schemes 1 and 2).
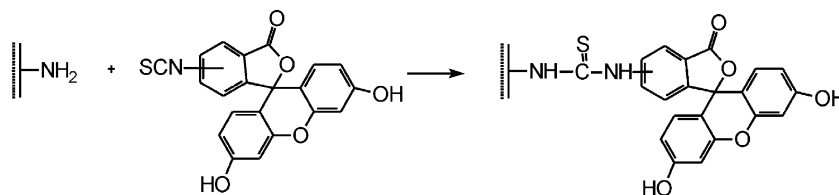
Direct attachment of FITC to surface amino functions at PP

To attach FITC labels directly to surface amino functions, the NH_2-modified films were immersed into an ethanolic solution of the fluorophore immediately after the plasma deposition process (see Scheme 1). This procedure minimizes undefined incorporation of oxygen into the deposited layers, known as post-plasma auto-oxidation [27, 28].

Insertion of spacer molecules between PP surface and functional group

To study the effect of surface–fluorophore interactions on dye emission, spacer-linked amino groups were generated

by the following process: the PP films were pretreated with an argon plasma for 5 s at 100 W and 20 Pa followed by deposition of a 50 nm plasma-polymerized allylamine layer at 50 W (cw). After removing the plasma-modified samples from the reactor, they were immediately immersed into a 5% aqueous GA solution and shaken for 2 h. In a subsequent step, the resulting CHO-modified surfaces were treated for 24 h with 1,6-diaminohexane (DAH) solution in water. This procedure generates spacer-linked amino functions. The resulting "surface-separated" NH_2-groups were reacted directly, or after modification with CB6, with different fluorophores as shown in Scheme 2. The reactions with the dyes were carried out in THF solution (2 h at room temperature for FITC and 3 h for ROX and TAMRA).

Modification of the amino-functionalized spacer by CB6

To improve the emission features of surface-attached fluorophores the hexamethylene spacer-modified PP surfaces were derivatized with CB6 as supposed by Buschmann et al. [26]. Spacer-linked amino-modified films were treated with a 50% solution of CB6 in formic acid for 5–25 min resulting in rotaxane-like structures (cf. Scheme 2). Covalent attachment of fluorophores was carried out after repeated washing steps with water and $NaHCO_3$ solution (0.025 M; pH 7–8) as recently described [25].

Results and discussion

Fluorophores directly attached to plasma-chemically modified PP

To optimize the experimental parameters for the plasma-chemical surface modification with polymeric allylamine

Scheme 1 Reaction of amino-functionalized PP (PP-NH_2) to PP-FITC accompanied by formation of a thiourea bond

Scheme 2 Reaction of plasma-polymerized allylamine layers with GA (1), 1,6-diaminohexane (2), and fluorophores (*Fl*) without (3) or in presence of CB6 (4). *Fl* denotes the fluorophores FITC, TAMRA, or ROX
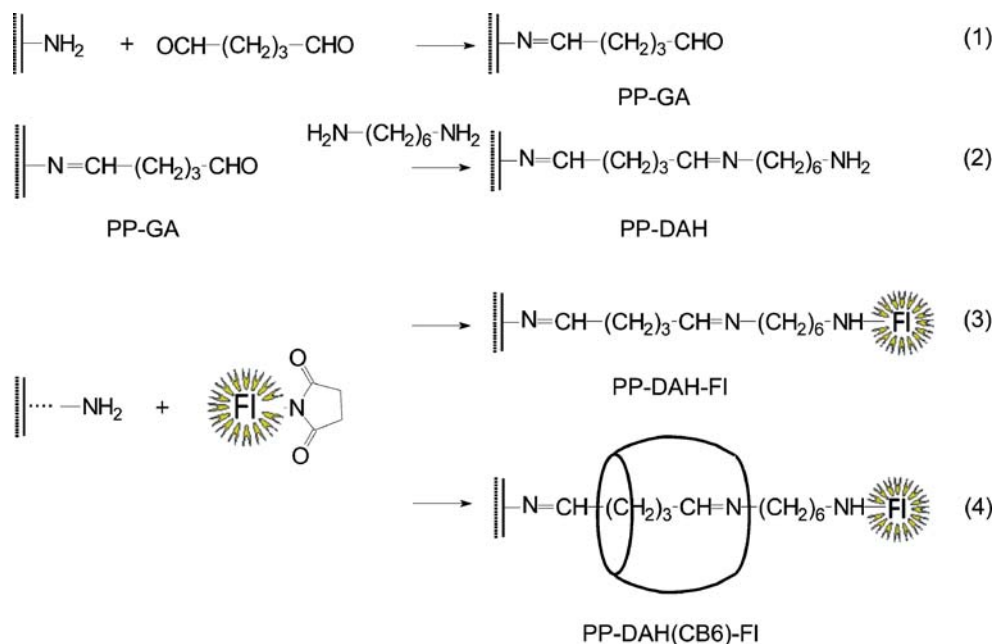
layers, the effect of different plasma conditions on the number of generated surface amino groups was investigated by XPS and fluorescence spectroscopy. In Fig. 3, the XPS results are exemplary shown for allylamine layers polymerized at a duty cycle of 0.5. The XPS data are also related to the fluorescence intensities displayed by the corresponding FITC-labeled PP films. The amino-sensitive FITC fluorophore was chosen here because its sulfur (S) atom can be simultaneously exploited as XPS tag, thereby elegantly circumventing further derivatization steps. Interestingly, neither th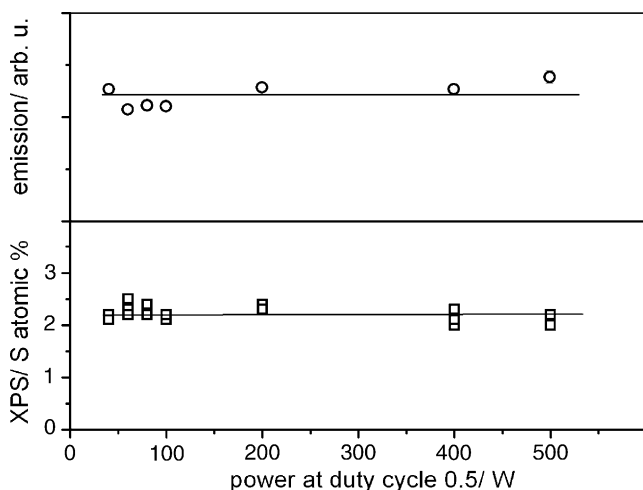e very weak fluorescence intensity (upper part), nor the sulfur concentration (lower part) of the FITC-labeled PP surface show a significant dependence on the plasma power applied.

Similar results were obtained for measurements of the plasma-chemically deposited polymer layers at a duty cycle of 0.1 or in the cw mode [25].
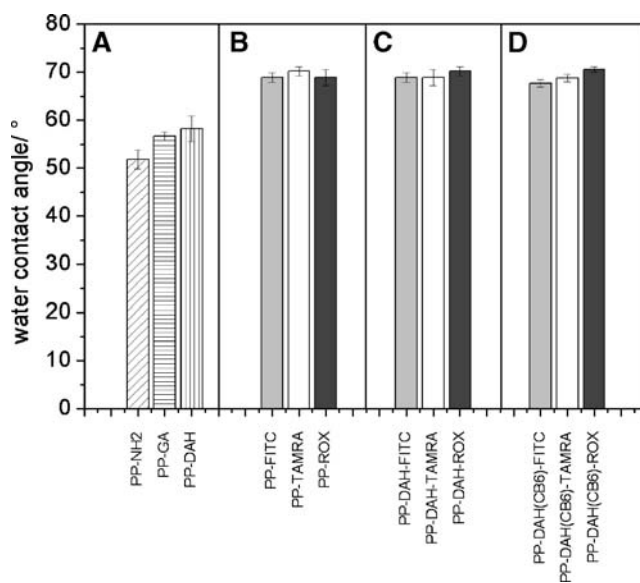

Fig. 3 Fluorescence intensity (*open circles*) and S concentration (*open squares*) obtained for FITC-labeled allylamine layers deposited on PP by pulsed plasma

Fig. 4 Water contact angles of fluorophore-labeled PP films (FITC *light gray bars*, TAMRA *white bars*, ROX *dark gray bars*) directly attached to the surface (**B**), spacer-linked (**C**), and spacer-linked and modified with CB6 (**D**). Contact angles of non-fluorescent amino-functionalized reference films (PP-NH₂) and films containing different spacer-linked functional groups are shown for comparison (**A**)

Table 1 Elemental composition of PP surfaces with plasma-polymerized allylamine layers reacted with spacer-linked amino functions, before and after modification with CB6 (reaction time 25 min), and the fluorophores FITC, ROX, and TAMRA

System	C/ atomic %	N/ atomic %	O/ atomic %	S/ atomic %
PP-NH$_2$	73.6±0.2	24.0±0.2	2.4±0.1	–
PP-GA	77.2±0.6	10.2±0.3	12.7±0.4	–
PP-DAH	71.1±0.9	10.1±0.6	18.8±0.3	–
PP-DAH(CB6)	68.1±0.7	14.9±0.3	17.0±0.9	–
PP-DAH-FITC	75.9±0.7	11.3±0.8	11.8±0.2	1.0±0.1
PP-DAH(CB6)-FITC	73.9±0.7	12.4±1.0	12.7±0.4	1.0±0.2
PP-DAH-ROX	78.3±0.4	10.9±0.4	10.8±0.2	–
PP-DAH(CB6)-ROX	75.9±0.7	12.3±0.6	11.8±0.1	–
PP-DAH-TAMRA	75.0±0.7	12.6±0.5	12.4±0.2	–
PP-DAH(CB6)-TAMRA	75.9±0.6	11.7±0.2	12.4±0.5	–

Insertion of spacer molecules and modification with CB6

Contact angle measurements

Measurements of the contact angle were performed in order to gain further insight into the effect of plasma-chemical modification and surface labeling. The contact angle geometry provides a sensitive tool to probe the surface energy of organic thin films on solid supports. Figure 4 summarizes the contact angles obtained for different polymer surfaces like spacer-linked functional groups before and after reaction with various fluorophores. The contact angles of plasma-functionalized supports (PP-NH$_2$), surfaces with spacer-linked aldehyde functions (PP-GA) and amino functionalities (PP-DAH) are in the range of 50° and 60° (Fig. 4A). After reaction with the fluorophores, the water contact angles increase from *ca.* 58° to *ca.* 70° (see Fig. 4B and C). Interestingly, the modification with CB6 has no significant influence on the surface energy of the films as follows from the corresponding very small change in water contact angles, see Fig. 4D. These results confirm the theoretical model [26] of threading CB6 onto flexible -NH-(CH$_2$)$_6$-NH$_2$ spacer chains, whereas the terminal groups remain uncaged. The easily accessible amino functions can be exploited for subsequent chemical reactions, in this case, for the labeling with fluorophores. For all the fluorophores investigated, the water contact angle increases after dye attachment as well as after derivatization with CB6, thereby reflecting an increased hydrophobicity of the modified surfaces.

Surface composition by XPS

Determination of the chemical composition of the surface demonstrates the successful functionalization and CB6-modification of the polymer. The elemental composition of
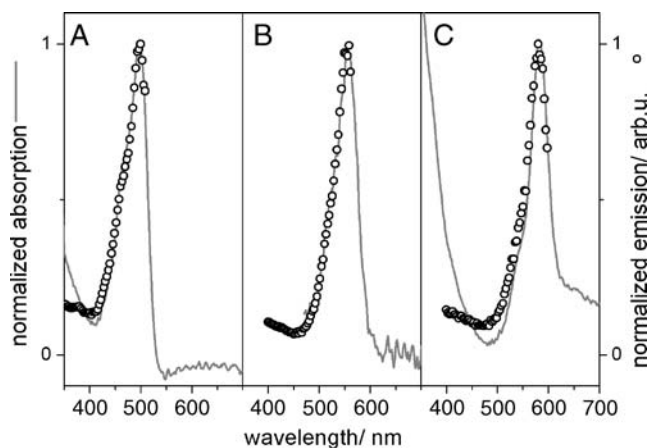

Fig. 5 Normalized absorption spectra (*solid lines*), roughly corrected for the scattering background, and fluorescence excitation spectra (*symbols*) of the fluorophores FITC (**A**), TAMRA (**B**), and ROX (**C**), attached via CB6-modified spacers to PP surfaces
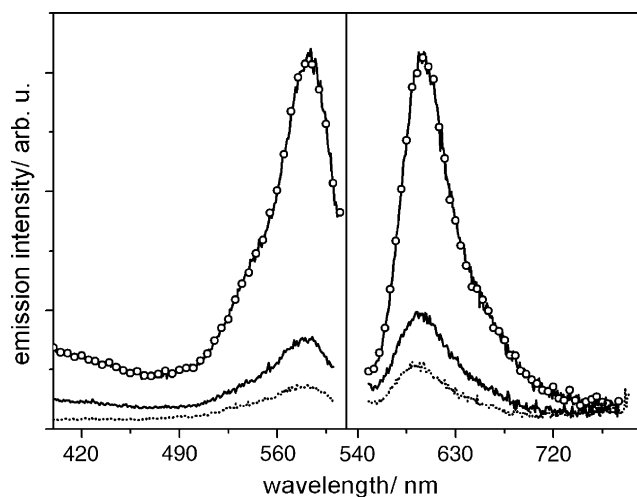

Fig. 6 Fluorescence excitation (*left*) and emission spectra (*right*) of ROX attached to PP surfaces directly, via a spacer (*solid lines*), and CB6-modified (*open symbols*). Spectra of an unmodified reference film (*dotted lines*) are shown for comparison to reveal fluorescence contributions from adsorbed dye molecules
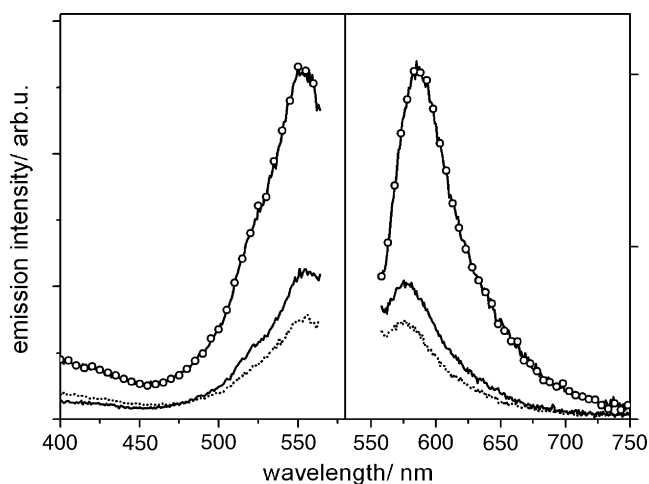
Fig. 7 Fluorescence excitation (*left*) and emission spectra (*right*) of TAMRA attached to PP surfaces directly, via a spacer (*solid lines*) and CB6-modified (*open symbols*). Spectra of an unmodified reference film (*dotted lines*) are shown for comparison to reveal fluorescence contributions from adsorbed dye molecules

the investigated systems are summarized in Table 1. For comparison, data obtained for non-modified surfaces with spacer-linked and FITC-labeled amino groups are also included.

The observed oxygen content of allylamine layers deposited onto PP and the data obtained for GA-modified films are consistent with other reports describing enhanced oxygen percentages due to postplasma oxidation processes [27, 28]. Interestingly, the PP-DAH films generated by reaction of the allylamine layers with GA and DAH in aqueous solution show an unexpectedly high oxygen content. In agreement with studies on the effect of

autoclaving on plasma-polymerized allylamine films that reveal a similarly strong increase in oxygen content [28], we attributed the high oxygen percentage for PP-DAH to a reaction of the allylamine layer with water during the synthesis in aqueous solution. The CB6-modified film (PP-DAH(CB6)) displays the expected significantly higher N content, as compared to films without CB6 (PP-DAH). The increase in N content is due to threaded, nitrogen-rich CB6 and reflects the covering of the surface with -NH-$(CH_2)_6$-NH_2(CB6)-chains. Subsequent attachment of fluorophores barely affects the chemical composition of the surface.

Fluorescence spectroscopic characterization

To investigate the effect of spacer insertion, as well as CB6-modification on the optical properties of the fluorophore-labeled polymers, detailed spectroscopic studies have been performed. The absorption and fluorescence excitation and emission spectra of FITC and the rhodamine dyes ROX and TAMRA covalently linked to the PP surfaces are summarized in Figs. 5 to 7 and in Table 2 for direct dye attachment, attachment via a spacer, and subsequently CB6-modified systems, respectively. In most cases, precise absorption measurements with the fluorophore-labeled PP surfaces failed due to strong and non-uniform scattering processes (see e.g. Fig. 5). Therefore, the Stokes shift $\Delta\tilde{v}_{abs-em}$ was obtained from fluorescence excitation spectra.

All the spectra of the PP-attached dyes show fluorescence excitation bands which resemble their absorption spectra (see Fig. 5), and a Stokes-shifted emission band of mirror image shape. This is exemplary shown in Figs. 6 and 7 for the rhodamine labels. Both the shape and the maxima

Table 2 Spectroscopic data of plasma-chemically modified and fluorophore-labeled polymer systems

System	Absorption λ_{max}/nm	Excitation[a] λ_{max}/nm	Emission λ_{fl}/nm	Stokes shift[b] $\Delta\tilde{v}_{abs-em}\big/cm^{-1}$
FITC	492[c]		518	1,020
PP-FITC		499	527	1,065
PP-DAH-FITC		494	523	1,120
PP-DAH(CB6)-FITC	498	495	523	1,080
TAMRA	543[d]		576	1,055
TAMRA	554[c]		584	927
PP-DAH-TAMRA		554	577	720
PP-DAH(CB6)-TAMRA	556	556	586	920
ROX	575[e]		600	725
PP-DAH-ROX		581	601	570
PP-DAH(CB6)-ROX	581	583	601	520

Fluorescence spectra were recorded upon excitation at 488 nm (FITC), and 543 nm (TAMRA and ROX)
[a] Maxima of the excitation spectra
[b] Stokes shift defined as $\Delta\tilde{v}_{(abs-em)} = \tilde{v}_{abs} - \tilde{v}_{em}$, where $\tilde{v}_{abs}$ is here the wave number of the maximum of the fluorescence excitation band and $\tilde{v}_{em}$ is the wave number of the high energy emission band.
[c] Absorption in 0.1 M phosphate pH 8 [29]
[d] Absorption in methanol from [29]
[e] Absorption in 0.1 M phosphate pH 7 [29]

of the excitation and emission bands of the polymer-linked dyes resemble the well-investigated spectral features of xanthene dyes in solution [29]. The small spectral shifts and slightly reduced Stokes shifts of the attached rhodamines compared to the non-attached dyes suggest reduced geometrical and solvent relaxation as expected for more restricted fluorophores at polymer surfaces or in a confined environment [22, 30, 31]. Interestingly, CB6-modification of the amino-terminated spacer-chains results in a considerable increase in the emission intensity of the fluorophore-labeled films (see e.g. Figs. 6 and 7). Even when possible variations in the absorption of the investigated dyes were taken into account, still a slight increase in the absorption-corrected fluorescence efficiency (defined as fluorescence intensity I_{fl} divided by the absorbance A at the excitation wavelength) could be achieved (see Figs. 8 and 9). The measured fluorescence intensities remained unchanged after a reaction time with CB6 of 5 min. This finding coincides with previously reported results from surface sensitive characterization techniques. The chemical surface composition as determined by XPS and water contact angles of similar films was found to remain unchanged for reaction times >5 min as well [29]. The corresponding fluorescence enhancement by a factor of 1.4 for FITC and by factors up to 7.5 for the rhodamine-labeled films is illustrated in Fig. 9.

The enhanced emission of the CB6-modified films points to an interaction with the macrocyclic host molecules. CB6 threading by the spacer molecules at the PP surface most likely leads to a rigidization of the flexible spacer chain and can thereby prevent fluorophore–fluorophore interactions that can result in fluorescence quenching. In addition, it can also circumvent a potentially unfavorable close contact between the dyes and the film surface [26].

Fig. 9 Fluorescence enhancement caused by CB6-modification of fluorophore-labeled systems (*dark gray*). The values for the CB6-modified systems represent the means of fluorescence efficiencies (including their standard deviation), measured after different reaction times with CB6, as exemplary shown in Fig. 8

Photostability studies

Besides the often reported fluorescence enhancement by cucurbituril [17–19], supramolecular interactions between the macrocyclic host molecules and dyes can result not only in favorably altered spectroscopic features due to the suppression of undesired aggregation and supramolecular stabilization, yet these interactions can also affect the photochemical properties of such systems. The latter can lead to a significant decrease in the quantum yield of photodecomposition [19, 22, 23]. In addition to an intense emission, an improved photostability of surfaces-linked dyes
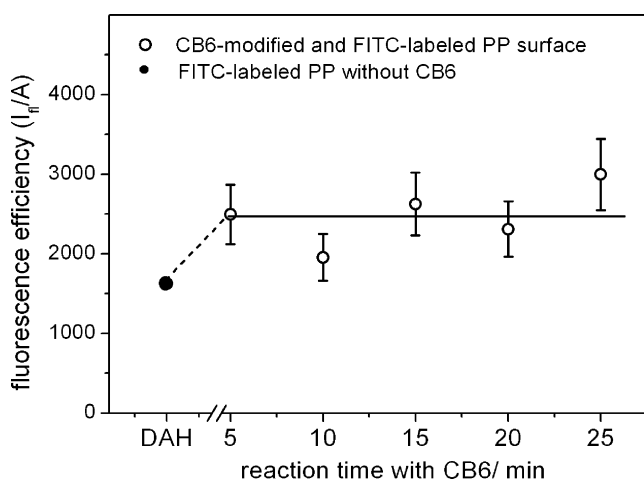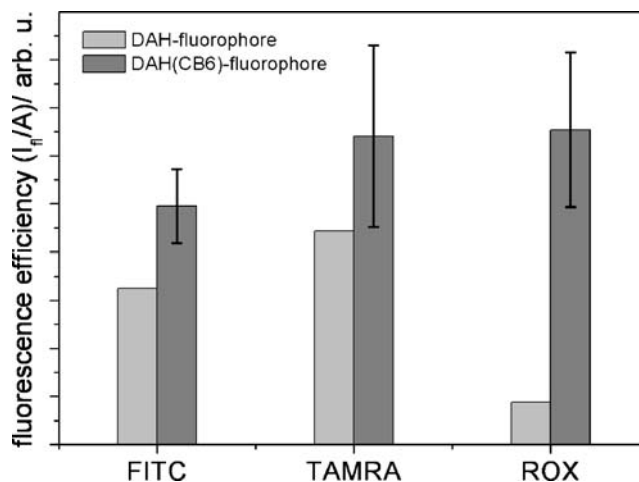

Fig. 8 Fluorescence enhancement caused by modification of PP-DAH-FITC with CB6. The *error bars* correspond to the uncertainty of fluorescence measurements on polymer surfaces, previously determined to *ca*. 15% [11]. The *solid line* represents the mean of the fluorescence efficiency. The *dashed line* is a guide for the eye only
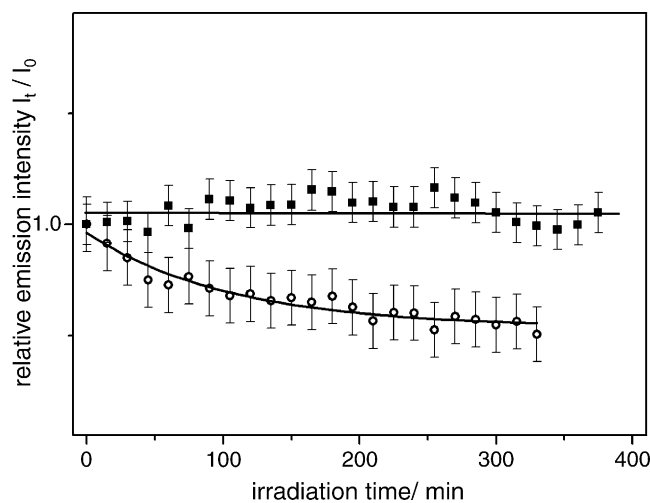

Fig. 10 Temporal evolution of the fluorescence intensity of a CB6-modified PP-film labeled with ROX (*solid squares*) compared to a non-CB6-modified system (*open circles*). The polymer films were illuminated at 530 nm within the absorption band of the fluorophore with the light source of the spectrofluorometer

is highly desirable for many (bio)analytical applications, that are based on the attachment of fluorescent sensor or biomolecules to surface functionalities. This is especially important for single molecule spectroscopic techniques and confocal laser scanning microscopy, for which the chromophore stability is one of the key parameters. To examine the photobleaching behavior of fluorophore-labeled and CB6-modified PP films in comparison to their non-CB6-modified counterparts, we performed photostability tests with a spectrofluorometer. The dependence of the fluorescence intensity on illumination time is displayed in Fig. 10 exemplary for ROX-labeled films.

As illustrated in Fig. 10, the CB6-film shows almost no loss in fluorescence intensity over a period of several hours, whereas the fluorescence of the unmodified control substantially decreases. Since the size of the ROX molecule prevents the perfect protection of the fluorophore from the environment by complete immersion in the cavity of CB6 (see also emission data in Table 2) [22], the improved photochemical stability is probably caused by a combination of different effects. The supposed rigidization of the spacer molecules by CB6 [26] can suppress the formation of dye dimers and aggregates [22]. In addition, modification with CB6 can prevent unspecific adsorption of the rhodamine molecules at the film surface [22], which has been reported to result in fluorescence quenching of xanthene dyes in solution [32].

Conclusions

The fluorometric characterization of surface functionalities on e.g. plasma-chemically modified polymer supports relies on reactive dyes that yield strongly fluorescent surface-attached species. As a strategy to enhance the fluorescence and photostability of such surface-linked dye molecules, we successfully exploited CB6 threading. This has been demonstrated by spectroscopic and stability studies with the three conventional xanthene dyes fluorescein, ROX, and TAMRA bound to amino functionalities of plasma-chemically functionalized PP surfaces either directly or via alkyl spacers. For all these dyes, that are prone to the formation of nonfluorescent aggregates and to fluorescence quenching interactions with the polymer surface, modification with CB6 resulted in an enhanced fluorescence and an improved photochemical stability. We ascribe these favorable effects to reduced fluorophore–fluorophore and fluorophore–surface interactions by CB6-induced rigidization of the spacer molecules.

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