CHAPTER ONE

Star Polymer Surface Passivation for Single-Molecule Detection

Jürgen Groll *and* Martin Moeller

Contents

Abstract

Poly(ethylene oxide) (PEO) is known as an excellent coating material to minimize nonspecific protein adsorption. For an examination of biomolecules attached to surfaces with sensitivities down to the single-molecule level, demands on the surface additionally comprise low-intrinsic fluorescence of the coating material and a possibility to immobilize biomolecules in their functional conformation. One strategy that combines the protein-resistant properties of PEO with chemical functionality is the use of star-shaped PEOs that allow for interpolymer cross-linking. Our system consists of six-arm PEO-based star polymers functionalized with reactive isocyanate groups at the ends of the polymer chain. The isocyante groups allow intermolecular cross-linking so that high grafting densities may be achieved, which render the surfaces extremely resistant to protein adsorption. Application by spin coating offers a simple procedure for the preparation of minimally interacting surfaces. The reactive end groups may be further biofunctionalized to recognize specific biomolecules such as streptavidin or His-tagged proteins in specific geometries or as single isolated molecules. These properties, together with the advantageous chemical properties of PEO, render the surfaces ideal for immobilizing proteins with detection limits down to the single molecule level. This chapter focuses on the preparation of substrates that

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are suitable for single-molecule experiments. Besides a detailed description of surface preparation, two examples for the single-molecule detection of immobilized proteins, nucleosomes and RNase H, are presented that demonstrate the advantages of the star-polymer derived coatings over linear-grafted PEO.

1. Introduction

Poly(ethylene oxide) (PEO) is a hydrophilic uncharged polymer that has been recognized as particularly efficient for achieving protein-resistant surfaces (Harris and Zalipsky, 1997). While most studies deal with the grafting of linear PEO chains to the material surface, changes in the molecular architecture of the polymer are beneficial for higher polymer segment density on the surface and a higher density of end-group-functionalities. In this chapter, we focus on star-shaped PEO molecules and especially on their use for the preparation of biofunctional surfaces that can be used for single-molecule experiments. Layers consisting of cross-linked, end-functionalized, six-armed, PEO-based star molecules were found to be as good as or better than linear-PEO-modified surfaces at reducing protein adsorption (Groll *et al.*, 2004, 2005a). In contrast to the effort that is necessary to obtain surfaces with high grafting density by the surface grafting of linear PEO, such films can be prepared by simple spin or dip coating from aqueous solution, since chemical cross-linking ensures a high polymer segment density on the substrate (Gasteier *et al.*, 2007). Since the reactive end-groups can be used for further functionalization, particular attention is paid to the ability to generate biofunctional films by a one-step preparation method through spin-casting. The experimental procedure for substrate preparation will be discussed in detail, and examples are given for single-molecule experiments demonstrating the negligible nonspecific interaction of proteins with the surface and the unperturbed function of proteins that are immobilized on such coatings.

2. Surface Grafting of PEO and Protein Repellence

The general strategy to render surfaces inert toward proteins is to introduce a coating layer that prevents protein adsorption either thermodynamically, so that attractive surface interactions are overcompensated by repulsive interactions with the layer, or at least kinetically by creating a free energy barrier of sufficient height that cannot be overcome on relevant time scales (Halperin, 1999). An abundance of studies have shown that PEO-coated surfaces display exceptional protein resistance (Gasteier *et al.*,

2007; Harder *et al.*, 1998; Harris and Zalipsky, 1997; Kingshott *et al.*, 2002; Malmsten *et al.*, 1998; McPherson *et al.*, 1998; Sofia *et al.*, 1998; Unsworth *et al.*, 2008). On substrates such as glass or silicon, protein-repellant coatings are frequently made from long, randomly coiling linear PEO chains terminally anchored to the surface. Only in the brush regime, the grafting density of linear chains is high enough that the attached polymer chains stretch out perpendicularly to the surface, thus avoiding unfavorable monomer–monomer interactions and maintaining optimal solvation. Consequently, the grafted chains provide adequate coverage and thickness to form a very effective steric barrier against protein adsorption (Szleifer and Carignano, 2000; Yang *et al.*, 1999). Grafting density and chain length are thus the two essential experimental control parameters by which the degree of protein resistance is governed (Malmsten *et al.*, 1998; McPherson *et al.*, 1998; Unsworth *et al.*, 2006). It has recently been shown that, for molecular weights between 600 and 2000 g/mol, a grafting density of 0.5 linear OH-terminal PEO chains/nm^2 is the threshold for minimal protein adsorption (Unsworth *et al.*, 2008).

Star-shaped PEO molecules (star PEO) have a central core region from which the PEO arms extend. Due to this constraint, their density is higher than that of a linear chain, which offers the opportunity to produce PEO surfaces with higher grafting density (Douglas *et al.*, 1990; Sofia *et al.*, 1998). Moreover, the ends of the arms are preferentially located near the periphery due to the steric constraints in the interior of the star (Irvine *et al.*, 1996). Therefore, the probability is increased for end-functionalized groups to bind to the surface. Star PEO systems thus appear an attractive choice to confer protein resistance to surfaces. Indeed, star PEO with 70 arms and a molecular weight of 5200 g/mol per arm have been reported to pack closely on the surface and to efficiently reduce protein adsorption, although the efficiency is reduced for small proteins such as cytochrome *c* (Sofia *et al.*, 1998). In another study, star PEO with 24 arms and a molecular weight of 9700 g/mol per arm as well as star PEO with 72 arms and a molecular weight of 4500 g/mol per arm were surface grafted (Irvine *et al.*, 1998). Atomic force microscopy and reflectivity measurements show that the hydrated star molecules are overlapping, and since the star segments are depleted near the substrate, the authors explain the residual adsorption by the diffusion of small proteins such as the 12,000 g/mol cytochrome *c* through the low PEO density seams between molecules and subsequent surface adsorption. As a result, surfaces obtained by grafting the smaller star PEO molecules prevent protein adsorption better since their packing on the surface is denser. By contrast, due to the spherical shape of the high-molecular-weight PEO stars, gaps remain between the stars that appear sufficiently large for small proteins to reach the surface and adsorb to it.

From these protein-adsorption properties of surfaces obtained by the grafting of PEO stars with different sizes, two strategies become clear and these have to be followed in order to improve star PEO-derived surface

coatings. First, both the number and molecular weight of the arms have to be reduced. Smaller stars seem better for the modification of surfaces since their packing is denser on the surface. In addition, a more flexible core than the poly-divinylbenzene core used in the aforementioned studies would make the stars more flexible and, in combination with a smaller number of arms, less spherical. A second approach is to functionalize the stars with reactive groups at the ends of the arms that enable intermolecular cross-linking of the PEO stars. Covalently cross-linked stars on the surface will result in a more homogeneous lateral PEO density profile and higher surface coverage. Moreover, cross-linking would allow for a more variable control of the layer thickness and the preparation of layers that are thicker than monolayers, since the end-groups are not restricted to lateral reactions. A further advantage concerns the functionalization of stars with molecules that react with the end-groups. The spatial distribution of such molecules in the layer can be controlled on the nanoscale by attaching them to the stars before or after surface grafting (Maheshwari *et al.*, 2000). In this way, either a random distribution or a nanoclustered ligand pattern can be achieved.

3. The NCO–sP(EO-*stat*-PO) System

We followed both strategies mentioned in the last paragraph for the development of our six-arm, star-shaped molecules with terminal reactive functional groups. The backbone consists of a statistical copolymer of ethylene oxide and propylene oxide in a ratio of 4:1; each arm has a molecular mass of 2000 g/mol with low polydispersity and is attached to a sorbitol core (sP(EO-*stat*-PO)). The arms of the star molecules are initially terminated with OH and can be functionalized with various reagents to yield molecules with different reactivities. This chapter will concentrate on isocyanate (NCO–) terminated star molecules (NCO–sP(EO-*stat*-PO)) that are obtained through functionalization with isophorone diisocyanate (IPDI; Goetz *et al.*, 2002). As IPDI is chemically attached to the star molecules without a catalyst, the primary, less reactive aliphatic isocyanate groups remain as functional groups at the ends of the arms, so that the NCO–sP (EO-*stat*-PO) molecules can be dissolved in water and coatings can be applied from aqueous solutions. When the NCO–sP(EO-*stat*-PO) material is dissolved in water, hydrolysis of the isocyanate groups lead to the formation of carbaminic acid which, at neutral pH, instantly decarboxylates to form amine groups. These amines react with unreacted isocyanate groups to form urea bridges between the NCO–sP(EO-*stat*-PO) molecules. Since the kinetics of amine addition to isocyanate is much faster than hydrolysis (Caraculacu and Coseri, 2001), urea bridge formation occurs preferentially until steric restrictions significantly lower the reaction probability.

The aqueous NCO–sP(EO-*stat*-PO) solution can be used for coating surfaces either by simple dip-, spin-, or spray-coating. However, due to the ongoing hydrolysis and aminolysis of isocyanate groups, a time window of maximum 20 min after the addition of water to NCO–sP(EO-*stat*-PO) should not be exceeded. After coating, the system requires at least 12 h for completion of the cross-linking reaction within the layer. During this time, all isocyanate groups hydrolyze and then either react with other isocyanate groups to form urea bridges or remain as free amino groups that can be further functionalized (Groll *et al.*, 2005a,b,c). Figure 1.1 shows the chemical reactions that occur during film formation and presents a model of the resulting surface coatings.

One particular advantage of the NCO–sP(EO-*stat*-PO) system is the change in reactivity from isocyanate groups, which are reactive toward nucleophilic groups, such as alcohols, amines, and thiols, to amine groups during the layer preparation and curing of the coating. Addition of water-soluble compounds that bear nucleophilic groups to the aqueous NCO–sP (EO-*stat*-PO) solution before coating thus results in covalent attachment of these molecules to the coating. It is important to emphasize that, after

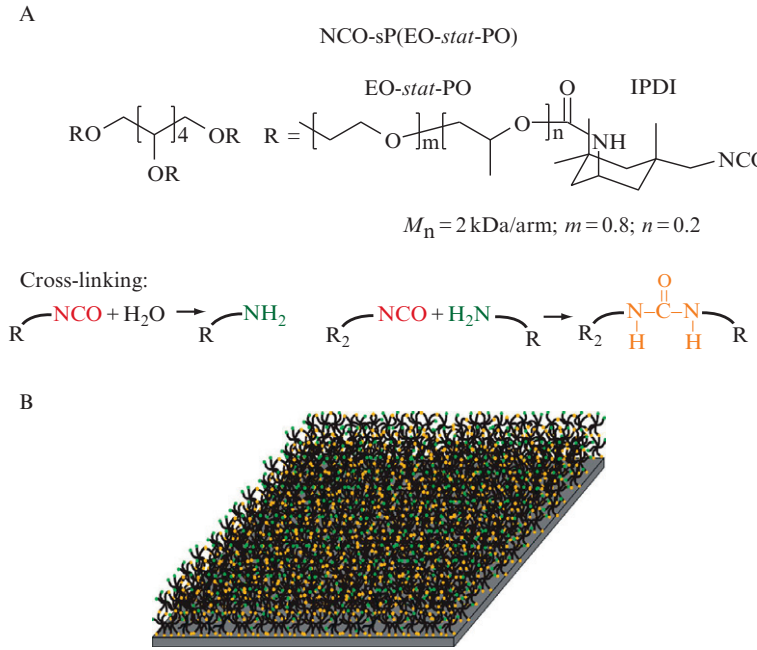

Figure 1.1 Surface coating with star polymers. (A) Schematic of the chemical composition of the NCO–sP(EO-*stat*-PO) system and the cross-linking reaction induced by water. (B) Schematic of a cross-linked sP(EO-*stat*-PO) surface coating where all isocyanate groups are hydrolyzed or aminolyzed resulting in urea bridges between the stars (yellow) and free amino groups (green). Figure partially reprinted with permission from Heyes *et al.* (2007), copyright Royal Society of Chemistry.

complete hydrolysis of the isocyanate groups, the compounds that are covalently bound to the layers are embedded in a coating that inhibits nonspecific interactions with proteins and cells, so that the immobilized molecules can interact specifically. This feature is achieved in a one-step layer preparation without the use of further chemical blocking-agents. The remainder of this chapter will focus on the preparation of substrates for single-molecule experiments according to this layer preparation method; a broader overview of different strategies for functionalization and application of the NCO–sP(EO-*stat*-PO) system for cell culture and biomaterials has recently been given elsewhere (Gasteier *et al.*, 2007).

4. Preparation of sP(EO-*stat*-PO)-Coated Substrates for Single-Molecule Experiments

Fluorescence is among the most sensitive analytical tools available and has been extensively applied to study biomolecules. In order to achieve single-molecule detection on surfaces, it is of utmost importance that the substrates have extremely low background fluorescence. Consider that a standard wide field fluorescent microscope has a point spread function approximately half the wavelength of the emitted light, which is ~300 nm for visible fluorophores. The fluorescent label has a diameter of ~1 nm. Therefore, in order to observe a single molecule, the number of emitted photons from the fluorophore covering approximately a $1 \times 1\ nm^2$ area must be significantly higher than the integrated background fluorescence over the entire $300 \times 300\ nm^2$ area. This stringent requirement necessitates that all preparation steps are carried out either in a clean room, or under a laminar flow hood with an air-filtration system. Glass slides, chemicals, solvents, and especially, the aminofunctional material used to functionalize the substrate and the PEO polymers, must be of the highest purity possible. The substrates are prepared according to the following steps:

- Ultrasonication in water for 2 min.
- Ultrasonication in isopropanol for 2 min.
- Cleaning and activation in a plasma oven or by UV/ozone treatment.

We have used different plasma machines, for example, the TePla 100-E system with 100 W at a process gas pressure of 0.5 mbar, with either oxygen or filtered air as flow gas, and process times between 10 and 15 min. For UV/ozone treatment, we have used a home-made device using a 40 W UV lamp (main emission 185 nm; UV-Technik Speziallampen GmbH) in an oxygen stream of 350 ml/min with a sample distance of 5 mm to the lamp for 10–15 min. Both processes result in clean substrates that were suitable for single-molecule experiments.

To covalently bind NCO–sP(EO-*stat*-PO) to the substrate, the surface must be functionalized with isocyanate-reactive groups, such as alcohols, amines, or thiols, as anchor points. Since the NCO–sP(EO-*stat*-PO) molecules cross-link on the substrates, the system tolerates a much lower number of functional groups on the surface than other systems that use classical grafting techniques. Furthermore, the cross-linking also results in a certain stability of the film against desorption from the surface in aqueous solution even without covalent attachment. For contact times with aqueous solutions of less than 10 h, films are sufficiently stable and may simply be coated on cleaned glass substrates. If covalent attachment is necessary, amino functionalization may be achieved by a variety of methods, for example, electrostatic adhesion of polyamines such as poly-L-lysine, chemical vapor deposition of 4-amino [2,2′]-paracyclophanes, or aminosilanization. We have used aminosilanization according to the following protocol:

- Cleaning and activation of the substrates as described earlier.
- Transfer of the substrates into a glove box under nitrogen atmosphere.
- Immersion into a solution of 3 ml *N*-[3-(trimethoxysilyl)propyl] ethylenediamine in 50 ml dry toluene for 2 h. Aminopropylsilane (APTES) may be used alternatively.
- Repeated rinsing of the substrates with dry toluene.
- Storage of the substrates in dry toluene until further use.

Alternatively, the commercial reagent Vectabond™ from Vector Labs may be used according to the detailed protocol provided by the supplier. While this method can be performed without the need of a glove box, it is important to use a new, unopened bottle of Vectabond each time, as it does not stay clean for long after it has been opened.

Spin-coating of aqueous NCO–sP(EO-*stat*-PO) solutions provides precise control of the layer thickness via rotation speed and prepolymer concentration and results in homogeneous layers (Groll *et al.*, 2005b). NCO–sP(EO-*stat*-PO) is preweighed in portions of typically 50 mg in nitrogen atmosphere and provided in airtight glass vials. For spin-coating, anhydrous THF is added to the vial to predissolve the prepolymer, so that upon the addition of water, a homogeneous mixture is formed immediately. Typically, THF is added to result in solutions with a concentration of 10–20 mg/ml NCO–sP(EO-*stat*-PO). Then, water is added until the aqueous solution is diluted to typically 1 mg/ml NCO–sP(EO-*stat*-PO). After gentle shaking to ensure homogeneous mixing, the solution is left to react for exactly 5 min. After this time, the solution is slightly opaque. In the meantime, the aminosilanized glass coverslip is placed on the spin coater. The solution is drawn up into a syringe, and a 0.02-μm membrane filter (Whatman Anotop 10) is attached to the syringe Luer connection. The solution is pressed through the filter directly onto the amino functional coverslip placed on the spin coater. The whole coverslip is homogeneously

covered with solution (~0.5 ml is needed per 24 × 24 mm coverslip). It is important not to touch the coverslip with the syringe as this may cause problems with the homogeneity of the spin-coating. The spin coater is turned on and the substrate rotated at 2500 rpm for 45 s. After that, the coverslip is placed into a glass petri dish that has been cleaned either by plasma- or UV/ozone treatment. The petri dish is covered, sealed with parafilm, and stored at ambient conditions over night for cross-linking of the surface. Figure 1.2 presents a flow diagram for the layer preparation

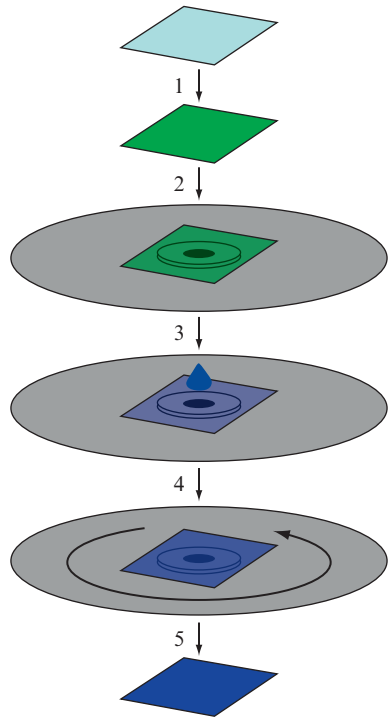

Figure 1.2 Flowchart of the coating procedure for generation of NCO–sP(EO-*stat*-PO)-coated slides. Glass microscopy slides are cleaned by ultrasonication, activated either by oxygen plasma or UV/ozone treatment and, if long-term stability of the films on the substrates in aqueous conditions is required, subsequently aminofunctionalized (1). These substrates are fixed on a spin coater (2), ideally in dust-free or (not of) dust-reduced conditions, and dropwise covered with an aqueous solution of NCO–sP (EO-*stat*-PO) 5 min after dissolution of the star-shaped prepolymers in a typical concentration of 1 mg/ml. The solution is dropped onto the substrate from a syringe and filtered through 0.02 μm syringe filters (3). When the substrate is completely covered by the solution, spin-casting is initiated (4) with 2500 rpm and left rotating for 45 s. Afterward, the samples are carefully taken off the spin-coater using a teflon tweezer (5) and stored for 24 h in clean glass petri dishes under ambient conditions (ideally dust free or dust reduced) for cross-linking of the coating.

procedure. As hydrolysis and subsequent cross-linking between the star-molecules continuously proceed in solution, only a limited number of maximally five substrates can be produced from each solution. The remaining aqueous NCO–sP(EO-*stat*-PO) solution has to be discarded. It was shown using scanning confocal fluorescence microscopy with single-molecule detection sensitivity that the background fluorescence of cross-linked sP(EO-*stat*-PO) surfaces carefully prepared according to this protocol is low enough to detect the fluorescence of single molecules (Groll *et al.*, 2004). The nonfouling (passivation) properties of sP(EO-*stat*-PO) coatings were compared to surfaces modified with linear methoxy-terminated PEO chains and BSA (Groll *et al.*, 2004; Koopmans *et al.*, 2008). In both studies, the sP(EO-*stat*-PO) films exhibited superior nonfouling properties (Fig. 1.3).

For binding of proteins to such cross-linked sP(EO-*stat*-PO) films, ligands for specific immobilization have to be covalently linked to the polymer. One often-used coupling-system of proteins to surfaces relies on the complex formation between biotin and streptavidin. Streptavidin recognizes biotin with high specificity and affinity, binding with a K_d of $\sim 10^{-15}$ *M*. Streptavidin is tetravalent toward biotin and is able to subsequently bind a biotinylated protein of interest. Biotinylated surfaces may be prepared by the addition of biocytin, a biotin derivative that contains a free amine group, to the aqueous NCO–sP(EO-*stat*-PO) solution prior to spin coating. Experimentally, only one variation of the layer preparation protocol above is necessary. Instead of adding pure water to the NCO–sP(EO-*stat*-PO) material dissolved in THF, an aqueous solution of biocytin is added. During the 5- min interval between mixing and spin-casting, the amine group of biocytin reacts with the isocyanate groups of the polymer. After spin-casting, the covalently linked biotin moieties are homogeneously distributed in the polymer film. The amount of biocytin that is dissolved in water determines the degree of layer functionality. Typically, 2 mg of biocytin are used per 50 mg NCO–sP(EO-*stat*-PO) prepolymer.

In order to perform single-molecule measurements on such substrates, first streptavidin has to be bound to the biotin, followed by binding of the biotinylated and fluorescently labeled protein to the streptavidin. All these steps should be performed in aqueous solution, so that sequential flushing of solutions has to be enabled. This can be achieved by constructing substrate "sandwiches" following the procedure schematically shown in Fig. 1.4. One 32 × 24 mm coverlip and one 20 × 20 mm coverslip are required for each sandwich. At least one of the substrates has to be coated with a biotinylated cross-linked sP(EO-*stat*-PO) film, whereas the second one may be coated with a nonfunctionalized sP(EO-*stat*-PO) layer. The sandwiches are prepared by taking the large coverslip from the petri dish and placing two pieces of double-sided tape on the upside to leave a 2–3 mm channel in the center. Then, the small coverslip is placed on top of the tape. Care should be taken to ensure that the polymer-coated upside now faces

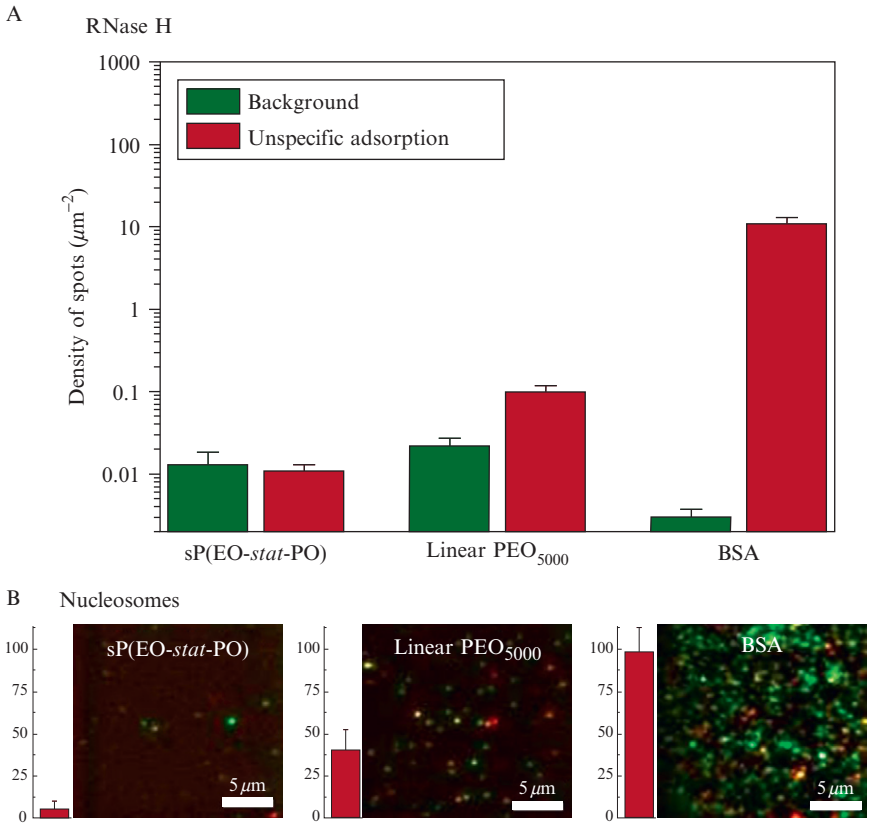

Figure 1.3 Quality of surface passivation. Nonspecific protein adsorption on cross-linked sP(EO-*stat*-PO) films is compared to that on grafted linear PEO_{5000} and physisorbed BSA using single molecules of Alexa Fluor 546 labeled RNase H (A, density of spots) and Cy3-ATTO647N labeled nucleosomes (B, fluorescence images and normalized fluorescence signal intensities). Significantly higher levels of nonspecific adsorption were observed on BSA than on PEO surfaces. sP(EO-*stat*-PO) surfaces showed negligible nonspecific adsorption. Figure partially reprinted with permission from Groll *et al.* (2004), copyright American Chemical Society and Koopmans *et al.* (2008), copyright Wiley-VCH Verlag GmbH& Co. KGaA.

down into the channel. The tape is cut along the edges of the coverslip and the sandwich is placed in a clean, sealed glass petri dish to transport to the microscope. For the experiments, the streptavidin solution (typically 20 μg/ml) and biotinylated/labeled protein (typically 200 p*M*) are flushed through the channel by adding a droplet of solution to one open side of the channel and waiting for 10 min each time before the removal of the droplet and addition of the next one.

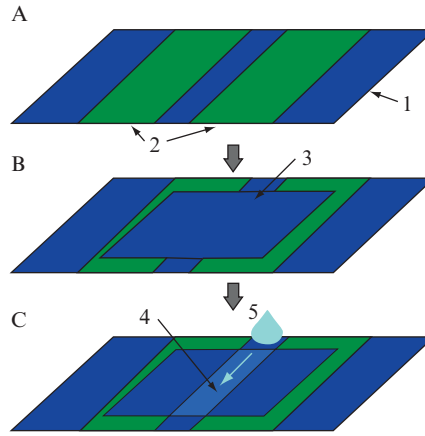

Figure 1.4 Schematic of substrate preparation for single-molecule experiments. For optimal results, preparation should be carried out under clean room conditions. The substrate-sandwiches are prepared by taking the large coverslip (32 mm × 24 mm; (1)) from the petri dish, and placing two pieces of double-sided tape (2) on the upside to leave a 2–3 mm channel in the center (A). Then, the small coverslip (20 × 20 mm; (3)) is placed on top of the tape with the coated side facing down toward the big coverslip (B). The tape is cut along the edges of the coverslip, and the sample can be placed in a clean, sealed glass petri dish to transport to the microscope. The channel between the two coated coverslips (4) allows for repeated flushing with solutions by adding a droplet at the edge of the small glass slide (5) and waiting for 10 min.

5. Analysis of Protein Structure and Function on NCO–sP(EO-*stat*-PO) Surfaces

To take advantage of the applications of surface-immobilized proteins, for example, as biosensors, it is necessary to achieve:

(i) high preference of specific adsorption over nonspecific adsorption, and
(ii) binding of the protein in its native, functional structure.

Ideally, the protein should completely refold into its native form if it is temporarily exposed to denaturing conditions. Maintenance of the folded state of the protein and its ability to refold after denaturation can be addressed using single-molecule fluorescence (or Förster) resonance energy transfer (FRET). More specifically, the labeling of proteins with two spectrally different fluorophores enables experiments in which the fluorophore with the higher excitation energy, the donor, is selectively excited. The energy is then nonradiatively transferred via dipole–dipole coupling to the lower energy fluorophore, the acceptor (Förster, 1948; Stryer and Haugland, 1967). Since the efficiency of this process is proportional to

R^{-6}, where R is the distance between the two chromophores, structural information of the protein may be inferred from the FRET efficiency. In order to study single-molecule FRET of surface-immobilized molecules, scanning confocal microscopy or total internal reflection microscopy may be used to limit background fluorescence.

A schematic representation on how FRET is used to infer the structural details of immobilized biomolecules is presented in Fig. 1.5. The surface is coated with a protein-resistant material, such as linear PEO or cross-linked sP(EO-*stat*-PO), which contains a bioactive group such as biotin (represented as a red antenna). In order to compare the cross-linked sP (EO-*stat*-PO) with other surface preparation techniques, linear-PEO-coated surfaces were prepared by grafting linear PEO (MW = 5000 Da) with amine-reactive NHS end-groups from aqueous solution, of which a small fraction (~1%) was also functionalized with biotin. Cross-linked sP (EO-*stat*-PO) surfaces were prepared as described earlier in a convenient, single-step layer preparation. Subsequent addition of streptavidin to these surfaces and then of biotinylated, FRET-labeled protein as described earlier allows specific immobilization of the protein.

Maintenance of a functional protein structure can be followed through the FRET efficiency. Upon structural changes and unfolding of the peptide chain, the distance between the chromophores increases and the efficiency of energy transfer decreases so that an increased fluorescence of the donor dye can be detected. By this method, the binding specificity of biotinylated and FRET-labeled nucleosomes to three different surfaces and especially, the structural integrity of the surface-bound nucleosomes, were measured (Koopmans *et al.*, 2008). On biotinylated BSA, binding specificity was only 2%, and only 28% of the immobilized nucleosomes retained structural integrity. On linear PEO_{5000}, the values increased to 60% and 53%, respectively. Cross-linked sP(EO-*stat*-PO) films, by contrast, resulted in 90% specificity of binding, and 78% of the immobilized nucleosomes were bound in their intact structure. Spontaneous unwrapping of nucleosomal DNA, so called nucleosome breathing, was measured on the surface-immobilized nucleosomes and the dynamics of the process compared to that in solution. The lifetimes of both the closed and the open states are approximately fivefold faster on cross-linked sP(EO-*stat*-PO) films than the dynamics observed on the linear-PEO-coated surface (1.5 s closed state, 120 ms open state), and they perfectly agree with the breathing kinetics of nucleosomes in solution (Li *et al.*, 2005). Thus, nucleosomes can be specifically immobilized on sP(EO-*stat*-PO) coatings while maintaining their structural integrity and their dynamic nature.

In order to study the interaction of specifically bound proteins with the NCO–sP(EO-*stat*-PO) surface and ask whether immobilized proteins refold into their functional structure after temporary denaturation, biotinylated RNase H was labeled with Alexa Fluor 546 and Alexa Fluor 647 and

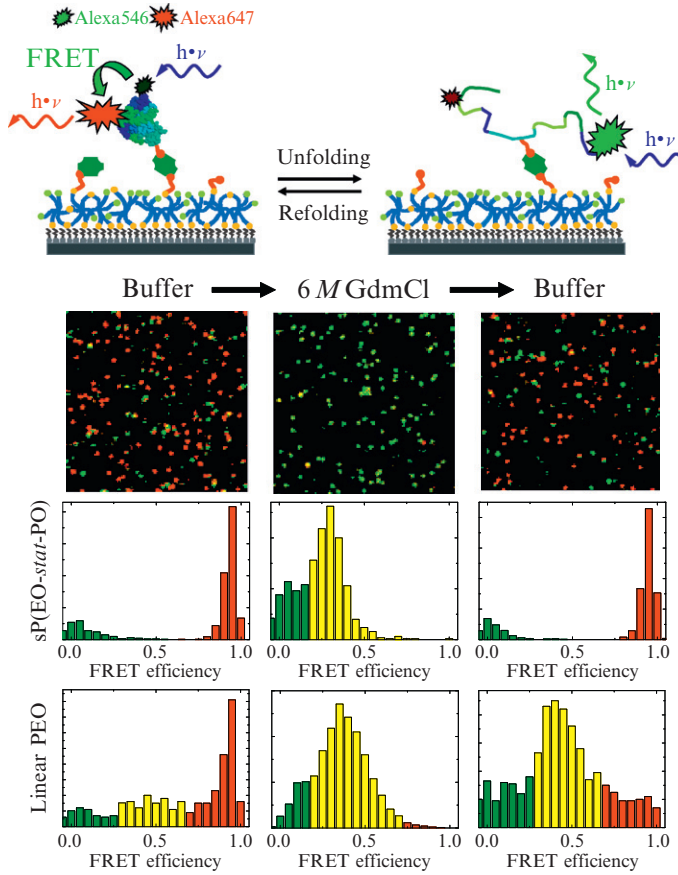

Figure 1.5 Schematic illustration of the FRET technique used to monitor structural information of immobilized proteins. In the folded state, the dye molecules, which are placed at specific sites on the protein, are close together. Upon excitation of the donor dye (green), a high transfer efficiency of the energy to the acceptor dye (red) occurs. If the protein is unfolded, the distance between the dyes increases and consequently, the energy transfer efficiency decreases. The distance dependence of the FRET efficiency can be used to infer details of the protein structure. The pictures show example scanning confocal fluorescence microscopy images of RNase H proteins immobilized on NCO–sP(EO-*stat*-PO)-coated surfaces and the resulting single-molecule FRET histograms of RNase H immobilized on NCO–sP(EO-*stat*-PO) and linear-PEO_{5000}-coated surfaces under buffer conditions, 6 *M* guanidinium chloride (GDmCl) and then subsequently reimmersed in buffer. The green fraction centered at zero FRET efficiency represents molecules that have no acceptor molecule due to incomplete labeling or dye photobleaching. The red fraction at high (∼0.9)-FRET efficiency are molecules in their folded state. The yellow fraction at low-to-intermediate FRET efficiency (∼0.4) represents molecules in their unfolded state. Figure partially reprinted with permission from Groll *et al.* (2004), copyright American Chemical Society.

bound to the biotinylated NCO–sP(EO-*stat*-PO) layers via streptavidin. The FRET dye pair was chosen and placed at sites on the protein so that a high-FRET efficiency is observed as long as the protein maintains its folded state. If the protein loses its compact-folded structure, the average distance between the dyes increases and the FRET efficiency is reduced. RNase H was labeled with Alexa Fluor 546 and Alexa Fluor 647 (Molecular Probes, Eugene, OR, $R_0 = 66$ Å in buffer) at amino acids 3 and 135, respectively (Kuzmenkina *et al.*, 2006). As a result, when unfolding of the protein chain is induced, the distance between the dyes increases and the donor fluorescence becomes detectable. If the peptide chain is able to refold to its initial conformation, the energy is again transferred to the acceptor dye and the fluorescence of this chromophore dominates. Exemplary scanning confocal fluorescence microscopy images of single, specifically immobilized, FRET-labeled RNase H on cross-linked sP(EO-*stat*-PO) surfaces are shown in Fig. 1.5, together with histograms of the calculated FRET efficiency of many single molecules on both linear PEO and cross-linked sP (EO-*stat*-PO) surfaces. The histograms in Fig. 1.5 are colored, based on their FRET efficiency ranges, to indicate proteins that are folded (red) or unfolded (yellow) or contain no acceptor dye and thus, cannot be structurally interrogated (green). For RNase H immobilized on linear PEO surfaces under buffer conditions, it is evident that there is a large distribution of FRET efficiencies from molecule to molecule, indicating that the proteins adopt a wide range of structures (many of them in a low-to-intermediate (unfolded) FRET state), while on cross-linked sP (EO-*stat*-PO) surfaces, practically all molecules that contain both dyes are in a high-FRET state, indicating that the folded state of RNase H is maintained for most molecules (Amirgoulova *et al.*, 2004; Groll *et al.* 2004).

Upon exposure of immobilized RNase H on either linear or cross-linked sP(EO-*stat*-PO) surfaces to high concentrations of guanidinium chloride (GdmCl), a reduction in the FRET efficiency was observed, indicating that the molecules unfold. Upon exchanging the denaturant for normal buffer conditions once again, practically all the RNase H proteins immobilized on cross-linked sP(EO-*stat*-PO) surfaces were able to refold to their compact high-FRET state. In contrast, on the linear PEO surfaces, once the protein molecules completely unfolded, practically none were able to refold to their high-FRET folded state. Moreover, on the cross-linked sP (EO-*stat*-PO) surfaces, the unfolding–refolding of RNase H molecules was found to be completely reversible over at least 50 cycles of adding and removing GdmCl (Amirgoulova *et al.*, 2004). In Fig. 1.6, the FRET state of the same single molecules before and after 50 cycles of adding and removing 6 *M* GdmCl was followed. The ability of the molecules to fold to the same high-FRET state after multiple denaturation–renaturation cycles is suggestive that the proteins are in or at least close to their native state. Further evidence stems from whether the protein is still able to function on the

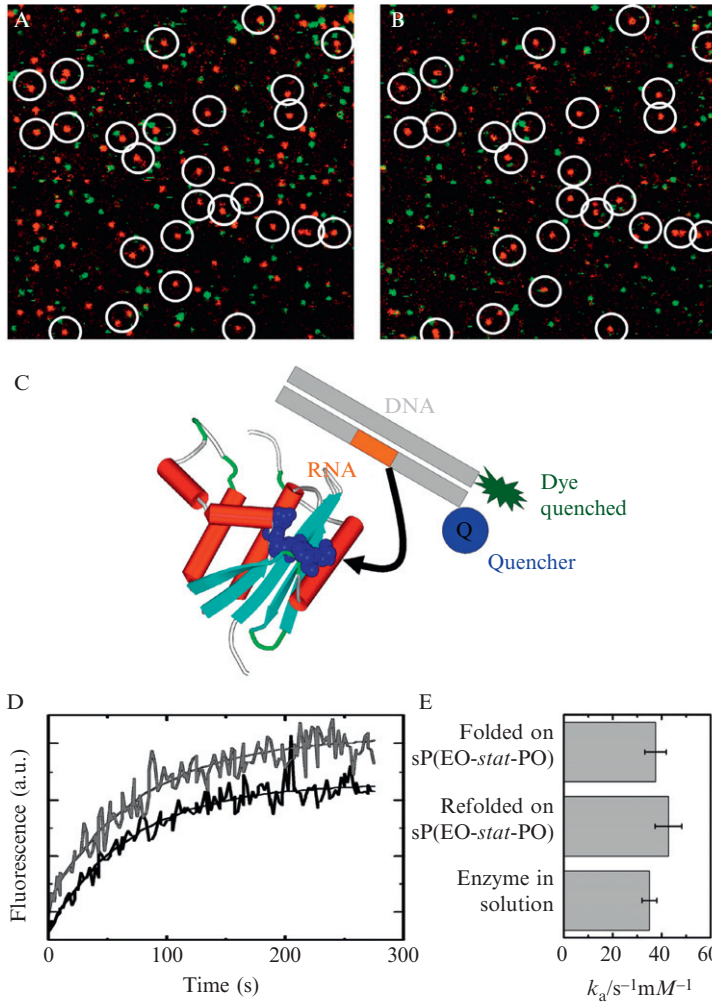

Figure 1.6 Scanning confocal fluorescence microscopy image of RNase H molecules immobilized on cross-linked sP(EO-*stat*-PO) surfaces before (A) and after (B) 50 cycles of unfolding–refolding. The same molecules that were folded before the 50 cycles were shown to completely refold after, and are highlighted in both figures. There are always a fraction of molecules that have no acceptor dye but have a donor dye. These green molecules are ignored as we cannot infer structural information from them. In the right image, some of the molecules that were visible in the left frame are no longer visible in the right frame, due to photobleaching during the scanning. Panel (C) shows a schematic representation of the enzymatic assay of RNase H immobilized on cross-linked sP(EO-*stat*-PO) surfaces. A fluorescently-quenched DNA–RNA hybrid dissociates upon RNA cleavage by the RNase H. (D) Increase in fluorescence of the dye following RNA cleavage with time upon exposure to RNase H immobilized on cross-linked sP(EO-*stat*-PO) both before and after an unfolding–refolding cycle. The refolding curve has been offset vertically for presentation purposes. (E) Calculated activity coefficients of the RNase H in solution, upon immobilization on cross-linked sP(EO-*stat*-PO), and following an unfolding–refolding cycle on the cross-linked sP(EO-*stat*-PO). Figure partially reprinted with permission from Heyes *et al.*, 2007, copyright Royal Society of Chemistry.

surface. The function of RNase H is to cleave RNA–DNA hybrids (Kanaya, 1998). An enzymatic assay for RNase H was previously developed that uses a fluorescently labeled RNA–DNA construct (Hogrefe *et al.*, 1990). The hybrid contains a fluorescent dye and a quencher, so that it does not fluoresce in its uncleaved state. Once exposed to RNase H, the RNA is cleaved and the DNA–RNA hybrid dissociates. This cleavage separates the quencher from the dye, and the dye fluoresces. By following the increase in fluorescence over time for the DNA–RNA hybrid exposed to RNase H specifically immobilized on sP(EO-*stat*-PO) surfaces, one is able to determine the activity coefficient of the enzyme. The results of this assay are shown in Fig. 1.6. Compared to the enzyme in solution, the activity coefficient of the enzyme is both unaffected by immobilization and returns to the same value after unfolding and refolding on the sP (EO-*stat*-PO) surface. More details of these FRET experiments with surface-bound RNase H are described elsewhere (Heyes *et al.*, 2007).

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