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Protein resistance of dextran and dextran-PEG copolymer films

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Abstract

The protein resistance of dextran and dextran-poly(ethylene glycol) (PEG) copolymer films was examined on an organosilica particle-based assay support. Comb-branched dextran-PEG copolymer films were synthesized in a two step process using the organosilica particle as a solid synthetic support. Particles modified with increasing amounts ($0.1\text{--}1.2\text{ mg m}^{-2}$) of three molecular weights ($10\,000$, $66\,900$, $400\,000\text{ g mol}^{-1}$) of dextran were found to form relatively poor protein-resistant films compared to dextran-PEG copolymers and previously studied PEG films. The efficacy of the antifouling polymer films was found to be dependent on the grafted amount and its composition, with PEG layers being the most efficient, followed by dextran-PEG copolymers, and dextran alone being the least efficient. Immunoglobulin gamma (IgG) adsorption decreased from ~ 5 to 0.5 mg m^{-2} with increasing amounts of grafted dextran, but bovine serum albumin (BSA) adsorption increased above monolayer coverage (to $\sim 2\text{ mg m}^{-2}$) indicating ternary adsorption of the smaller protein within the dextran layer.

Keywords

ternary adsorption; tertiary adsorption; antifouling; dextran; poly(ethylene glycol) PEG; poly(ethylene oxide) PEO; polysaccharide; copolymer; polymer grafting

Introduction

A chief limitation to the performance and lifespan of materials used in biological applications are their susceptibility to biofouling, the nonspecific adsorption of biomolecules on their surface. For example, nonspecific protein adsorption on surfaces has been shown to impair industrial equipment (Norde 2003), induce inflammation and rejection of biomedical implants and nanoparticles (Dobrovolskaia and McNeil 2007; Anderson et al. 2008) and reduce the signal-to-noise ratio of molecular diagnostics (Gong and Grainger 2007; Chen et al. 2009). Currently the most effective method to reduce nonspecific adsorption is to create an antifouling (AF) steric boundary on the surface, commonly composed of a grafted polymer layer. The importance and complexity of these AF layers is demonstrated by the plethora of previous studies performed.

A number of reviews have been written on grafted polymer properties and their effect on AF efficiency (Szeleifer 1997; Currie et al. 2003; Malmsten 2003). The current consensus is that the effectiveness of these layers is dependent on polymer solubility (hydrophilicity), grafted amount, density and conformation on the surface. However, it must be noted that numerous studies have found results that contradict the conventional understanding of how these layers function. This includes the influence of terminal end group functionality (Ostuni et al.

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2001), the thickness of the AF layer (eg the effectiveness of short oligo poly(ethylene glycols)) (Prime and Whitesides 1993; Feldman et al. 1999) and ternary adsorption of proteins within the AF film itself (Bosker et al. 2005; Halperin and Kroger 2009).

The most widely studied and used AF polymer is poly(ethylene glycol) (PEG). However, a number of studies and reviews have highlighted the limitations of PEG and the AF properties of other polymer structures. One such class of AF polymers is the naturally produced polysaccharides, such as dextran. Like PEG, dextran is a hydrophilic neutral polymer. However, unlike PEG, dextran can be grafted side-on to a surface by modifying and immobilizing through the numerous hydroxyl side groups of the polysaccharide backbone (Fournier et al. 1995; Heinze et al. 2006). This gives rise to complex, train, loop and tail polymer conformations at the interface. These conformations have been shown to be more effective than physisorbed (Osterberg et al. 1993; Delgado et al. 2001) and end-on grafted (Osterberg et al. 1995) dextran layers. Up to 65-90% reduction in protein adsorption has been reported for surfaces grafted with dextran (Osterberg et al. 1995; Frazier et al. 2000; McArthur et al. 2000; Bosker et al. 2007).

The structure, large size, and grafted conformation of dextran can also give rise to increased surface loading. Hydroxyl groups not taking part in immobilization can be activated to attach moieties such as biomolecules (Heinze et al. 2006) or additional polymers (Delgado et al. 2001) as has been explored in this and previous studies. Herein, the synthesis, characterization and AF application of dextran-PEG copolymers are presented. The copolymer was assembled from 66 900 g mol⁻¹ dextran and 3 000 g mol⁻¹ amino-PEG using a two-step on-particle synthesis strategy. The AF effectiveness of the copolymer was compared to surfaces grafted with increasing amounts of dextran (of three molecular weights of 10 000, 66 900 and 400 000 g mol⁻¹) and PEG-grafted surfaces reported in a previous study (Chen et al. 2008).

Materials and methods

3-Aminopropyl trimethoxysilane (APS), triethylamine (TEA), O-(2-aminoethyl) polyethylene glycol 3 000 MW (amino-PEG), dextran from *Leuconostoc mesenteroides* (10 000, 66 900, 400 000 MW), 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride), FITC conjugated bovine serum albumin (BSA, 68 kDa) and bovine immunoglobulin gamma (IgG, 150 kDa) were purchased from Sigma-Aldrich. 3-Mercaptopropyl trimethoxysilane (MPS, 95%) was purchased from Lancaster, UK.

Particle synthesis, dextran activation, surface grafting and characterization

Organosilica particles (~4.60 µm) were synthesized from MPS and then amine-modified by reacting with APS and TEA in ethanol for 2 h under constant agitation (Miller et al. 2005; Corrie et al. 2008). Dextran hydroxyl groups (1:9 or 1:30) were activated with equal molar amounts of tresyl chloride and TEA in anhydrous dimethylsulfoxide (DMSO) for 2 h under an argon atmosphere. Dextran was purified from DMSO twice by precipitation with anhydrous dichloromethane (DCM) and the solvent removed by rotary evaporation.

Increasing concentrations (0-50% w/v) of 1:9 or 1:30 tresyl-activated dextran were grafted on 50 mg of particles overnight in anhydrous DMSO with 5 µl of TEA. Washing the particles three times in water removed any ungrafted dextran and regenerated the hydroxyl groups. Dextran-PEG comb copolymers were synthesized on-particle by first grafting on dextran. These coated particles were then resuspended in anhydrous DMSO and 25 µl of tresyl chloride and TEA were added under an argon atmosphere and left to react for 2 h. The particles were then washed three times in anhydrous DMSO and then 250 µl of a 100 mg ml⁻¹ amino-PEG in DMSO solution were added, along with 5 µl of TEA, and left to react

overnight. Unreacted amino-PEG was removed by washing three times in DMSO followed by a 50:50 ethanol water solution. The amount of grafted dextran and dextran-PEG copolymer on the particles was determined from the C1S XPS peak using a Kratos Axis ULTRA X-ray photoelectron spectrophotometer. Spectra were composed of three sweeps with a 20 eV pass energy at 0.1 eV increments.

Protein antifouling on dextran and dextran-PEG copolymer modified particles

Flow cytometric analysis of protein adsorption was conducted on a DakoCytomation Mo Flo™. The AF characteristics of dextran and dextran-PEG copolymer-modified surfaces were investigated by incubating 100 000 particles with 50 µl of FITC labelled BSA or bovine IgG suspended in a standard PBS solution (pH 7.4, 137 mM NaCl, 10 mM phosphate buffer, 3 mM KCl) at 100 µg ml⁻¹ for 2 h, under constant agitation at room temperature. Particles were washed once with and then resuspended in 100 µl of PBS for flow cytometric analysis. An average of 1000 individual particle data points were collected for each sample and analysed using Summit V4.1 software. Protein adsorption measurement values and standard deviations (SDs) were calculated from the mean and SD of the particle fluorescence histogram, respectively, *via* the calibration method outlined in Kozak et al. (2008). Briefly, this methodology is based on a simplified version of the theory originally outlined by Chatelier and Ashcroft (1987) and has been independently validated by solution depletion measurements for a range of different proteins, including BSA and IgG. Greater detail of this methodology is provided in the Supplementary Information.

Results and discussion

Dextran activation and surface grafting

Hydroxyl groups along the dextran polymer were activated with tresyl chloride at a 1:9 or 1:30 ratio, corresponding to 1:3 or 1:10 tresyl-activated sites per saccharide monomer. These tresyl-activated groups form covalent alkyl bonds when reacted with a nucleophilic, eg amino-, sulfhydryl, or hydroxyl- containing ligand. In this way, dextran-PEG copolymers were synthesized by first grafting the dextran to the amine groups on the particle followed by reactivation and modification with a 3 000 M_w amino-PEG, as shown in Figure 1. Any tresyl-activated groups not reacted with either the particle surface or amino-PEG react with water to re-form hydroxyl groups.

Unlike PEG, which only has terminal hydroxyl groups for activation, dextran has three hydroxyl groups per saccharide monomer that can be tresyl-activated. This gives rise to complex polymer grafting conformations such as ‘trains’ and ‘loops’ on the particle surface. These are expected to change as a function of the degree of tresyl activation and grafting concentration, as shown in Figure 1. For example, polymer trains are expected to dominate the surface as the degree of polymer activation (number of possible anchoring points) is increased and the grafting concentration is much less than that required for surface saturation. Increasing the grafting concentration above and beyond surface saturation levels and reducing the degree of activation should cause dextran to go from train, to loop, to end-on ‘tail’ conformations. In contrast, grafted PEG can only attach end-on to the surface.

Grafted amounts of dextran between 0.1 and 1.1 mg m⁻² were achieved by increasing the grafting concentration from 0.1 to 500 mg ml⁻¹ (0.01 - 50 % w/v), as shown in Figure 2. Each grafting curve is composed of a minimum of two independent sample preparations and measurements. The grafted amount for each point was calculated by multiplying the dry layer thickness of the dextran layer by its crystalline density ~1.032 g cm⁻³ (Park et al. 1998). Dry layer thicknesses were calculated from the increase in the ratio of the dextran C-O ether peak at 285.5 eV to the particle peak at 284 eV of the XPS C1S spectra

(Supplementary Information), following the theory outlined in Sofia et al. (1998) and used in a previous study of grafted PEG layers (Chen et al. 2008). As expected, increasing the grafted amount of dextran also showed a decrease in silica, sulfur and nitrogen elemental ratios in the XPS wide spectra. Although not used in this study, the changes in the elemental ratios also could have been used to calculate the dextran layer thickness.

Grafting was observed to be independent of the degree of tresyl-activation and behaved like a high affinity Type I isotherm. All of the grafting conditions, degree of activation and dextran MW, exhibited a transition point at 10 mg ml^{-1} going from high grafting efficiency to a maximum-grafted-amount plateau. Interestingly, the 10 000 and 400 000 MW polymers (smallest and largest studied) gave rise to a similar maximum grafted amount of $\sim 1.1 \text{ mg m}^{-2}$, which was three times higher than the grafted amount from the 66 900 MW dextran (0.4 mg m^{-2}). This difference in grafted amount maxima was also observed for the two tresyl-activation ratios studied. Typically, the plateau maximum is expected to increase with polymer MW (Fleer et al. 1993), similar to the findings of Piehler et al. (1999) for side-on grafted dextran. However, because polymer grafting is highly dependent on the grafting conditions, such as solvency, a number of studies have observed contradictory findings; eg grafting to be independent of MW (Sofia et al. 1998) or even contrary to the expected trend (Chen et al. 2008). The reason for the lack of a consistent grafting trend observed in this study is, therefore, unknown. However, this anomaly only impacted the grafted amount range of the 66 900 MW dextran that could be used for AF studies.

Particle dextran-PEG copolymer synthesis

Comb-branched dextran-PEG copolymer structures were synthesized on the particle using the $66\,900 \text{ g mol}^{-1}$ dextran as a backbone. Dextran activated 1:10 was grafted on the particles at 10, 100 and 300 mg ml^{-1} giving rise to three particle sets with grafted amounts of 0.25, 0.44 and 0.44 mg m^{-2} , respectively (Table 1). These particles were then reactivated with tresyl chloride and reacted with a 3 000 MW amino-PEG to form comb-branched dextran-PEG copolymers on the particles. The amount of PEG incorporated in the copolymer was calculated from the additional increase in the XPS 286 eV C-O peak compared to the 284 eV C-C peak. Interestingly, up to 3.4 mg m^{-2} of PEG was incorporated, which is double the maximum amount of PEG grafted on the particles (1.6 mg m^{-2}) in a previous study (Chen et al. 2008). This is believed to be due to the 3-D structure and the mobility of the grafted dextran, which gives rise to conformational changes that enable a greater amount of PEG to be attached.

Protein adsorption: antifouling efficacy of dextran and dextran-PEG films

The protein resistance of dextran and dextran-PEG copolymer films were examined against the two most common serum proteins, albumin and immunoglobulin gamma. These proteins have been associated with the initial fouling of medical implants and molecular diagnostics and represent two proteins of varying size and charge (Malmsten 2003). BSA is a smaller ($14 \times 4 \times 4 \text{ nm}$), negatively charged protein (Zhang et al. 1998) compared to the larger ($8.5 \times 14.5 \times 4 \text{ nm}$) and commonly neutral to negatively charged IgG properties at pH 7 (Silverton et al. 1977).

In this study, increasing the amounts of grafted dextran from 0.1 and 1.1 mg m^{-2} had very different effects on BSA and IgG adsorption, as shown in Figure 3. Overall, the AF effectiveness of dextran layers was independent of MW, in accordance with previous findings on grafted dextran (Osterberg et al. 1995) and PEG (Chen et al. 2008) AF layers. Increasing the grafted amount of dextran only reduced the adsorption of the larger IgG protein. In contrast, BSA adsorption was found to be greater than that expected for

monolayer coverage on dextran layers with low and high grafted amounts, below 0.6 and above 1 mg m⁻² of dextran, respectively.

The efficacy of an AF polymer film is dependent upon the formation of a dense polymer layer on the surface. For end-on grafted polymers, increasing the grafting density begins to force neighboring polymers to overlap and form a polymer brush. The point at which polymer overlap occurs can be estimated from the radius of gyration (R_g) or Flory radius of the polymer (Fleer et al. 1993). In this study the transition point was expected to occur between 0.4 – 0.6 mg m⁻², as the R_g of the 10 000, 66 900 and 400 000 MW dextrans are ~ 3, 8 and 20 nm, respectively (Ioan et al. 2000). However, as this transition point is modeled for end-on grafted polymers, it is presented here only as a guide and is not expected to accurately represent the overlap point for polymers such as dextran that are grafted in loops and trains on a surface.

At grafted amounts < 0.4 mg m⁻², the adsorption of BSA and IgG was very high and broad. This is believed to be due to the minimal polymer overlap expected to exist at these low grafting conditions, below the predicted transition point of all the polymers used. BSA and IgG adsorption (2 and 4.2 mg m⁻², respectively) were higher than would be expected for a randomly orientated packed monolayer on the bare particle surface (Kozak et al. 2008) or other similar hydrophobic surfaces (Malmsten 1994). This is thought to be a result of the loosely packed dextran interface allowing proteins to penetrate between the polymer coils to adsorb both on the surface and within the dextran layer, eg ternary adsorption. As would be expected, this was more pronounced for the smaller BSA. Similar greater-than-monolayer ternary adsorption of BSA has been observed on dextran-modified PDMS (Farrell et al. 2010) and on PEO-modified surfaces (Bosker et al. 2005; Halperin and Kroger 2009).

Grafted amounts of dextran > 0.6 mg m⁻² gave rise to a clear decrease in the adsorption of the larger IgG protein. IgG adsorption was reduced by ~90%, from 4.2 to 0.4 mg m⁻², at the highest grafted amount of dextran (1.2 mg m⁻²). This was similar to the findings of Frazier et al. (2000) who observed an 82% decrease in IgG adsorption on dextran that was side-on grafted at 1.8 mg m⁻². Interestingly, an almost linear decrease in IgG adsorption with increasing amount of grafted dextran was observed. This was similar to the trend observed by Fournier et al. (1995) for BSA adsorption on grafted dextran. In this study, BSA readily adsorbed on and within the dextran layer, reaching a maximum adsorbed amount of 4 mg m⁻², which is double the amount of a monolayer, on 1.2 mg m⁻² of dextran.

Increasing the number of possible anchoring sites by tresyl-activation qualitatively improved the AF properties of the dextran layer. Delgado et al. (2001) reported a similar, yet more pronounced, trend by increasing the number of surface-anchoring groups *via* the degree of hydrophobic substitution of dextran. The improved AF was believed to be due to increased polymer density close to the interface. However, they also observed an increase in BSA adsorption at high hydrophobic substitution (> 1:4 glucose monomers) due to the hydrophobic group orientation toward the solution, facilitating protein interactions. Tresyl-activated dextran holds the advantage that groups not reacted with the surface will re-form native hydroxyl groups. This is expected to eliminate any contribution that modifying the polymer for immobilization may have on protein adsorption.

Results from a previous study on protein adsorption as a function of PEG grafting density (Chen et al. 2008) were compared to the dextran results of this study. The AF efficacy of dextran layers was found to be worse than PEG layers of similar grafted amounts. Other studies have also observed that dextran is not as effective an AF polymer as PEG at the same grafting density (Osterberg et al. 1995; Bosker et al. 2007). The improved AF properties of

PEG are often attributed to its more flexible nature and larger excluded volume in water (Carignano and Szleifer 2000).

Although not effective as a protein-resistant surface on its own, the large molecular weight, numerous side groups for attachment and loop-and-train confirmation of dextran does increase the potential functional group loading of the surface (Piehler et al. 1999). This enables increased anchoring of other molecules, such as proteins, for higher capture probe loading, or polymers for new high-density, low-fouling surface layers. The amount of PEG grafted on dextran modified particles was found to be 125 to 200 % greater than the highest amount of PEG (1.6mg m^{-2}) grafted directly to the particle surface in the previous study (Chen et al. 2008). These copolymers were found to form extremely effective AF layers preventing ~ 95% of both BSA and IgG adsorption (Figure 4). This corresponded to 0.13 and 0.2mgm^{-2} of BSA and IgG adsorption, respectively, on the 30Dex_{OP}-PEG sample.

This indicates that on-particle copolymerisation can fill voids left by loops and trains of dextran molecules to prevent both surface and ternary protein adsorption. Although improved, the AF effectiveness of the copolymer was still not better than the best PEG layer from the previous study (Chen et al. 2008). However, the copolymer is believed to hold promise for providing a more biologically stable environment with increased biomolecule probe loading compared to end-on grafted polymer layers.

Conclusions

Side-on grafted dextran was found to form relatively poor AF surfaces compared to polymer films composed of PEG or dextran-PEG copolymers. This indicates that the properties of the PEG polymer (flexibility, hydrophilicity) or generated PEG layer (end-on grafting, polymer segment density profile from the surface) are more protein-resistant than dextran polymer layers. Increasing the grafted amount of dextran reduced the adsorption of IgG protein by up to 90%. In contrast, BSA (smaller than IgG) exhibited ternary adsorption amid the polymer layer, illustrated by adsorbed amounts greater than monolayer coverage (2mg m^{-2}). The MW of dextran had no effect on protein resistance, but the number of hydroxyl side groups activated with tresyl chloride for surface anchoring appeared to marginally reduce protein adsorption, in agreement with previous findings. Incorporating low-fouling PEG polymer into the dextran AF layer through the creation of dextran-PEG copolymers was found to significantly reduce protein adsorption of both IgG and BSA by > 95%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of dextran and dextran-PEG copolymer layer formation. 1) Covalent grafting of tresyl-activated dextran with increasing concentration on particle surface in train, loops and tail confirmations; 2) reactivation of grafted dextran-coated particles with tresyl chloride followed by reaction with amino-PEG to form dextran-PEG copolymers on particles.

Figure 2.

Amount of dextran grafted on particles as a function of MW 10 000 (▲, △), 66 900 (■, □) and 400 000 (●, ○) g mol^{-1} and degree of tresyl activation 1:3 (filled symbols) and 1:10 (open symbols). All data sets represent a minimum of two independent sample preparations.

Figure 3.

BSA (a) and IgG (b) adsorption on dextran modified particles of 10 000 ($\circ$, $\bullet$), 66 900 ($\square$, $\blacksquare$) and 400 000 g mol^{-1} ($\diamond$, $\blacklozenge$) that were 1:3 (open symbols) or 1:10 (closed symbols) tresyl-activated during polymer grafting. Error bars represent the SD of the individual measurements. The shaded area represents the distribution of data points observed for IgG adsorption on particles grafted with linear PEG molecules between 2 000 and 20 000 MW conducted in a previous study (Chen et al. 2008).

Figure 4.

Antifouling effectiveness of 66 900 g mol⁻¹ dextran (hashed bars) grafted at increasing concentration on particle surface compared to dextran-PEG copolymer (cross hashed bars) in preventing BSA (white) and IgG (grey) adsorption. Adsorbed amounts are normalized by the amount measured for monolayer coverage on blank particles (Γ_m), being 2 ± 0.36 and 4.2 ± 0.86 mg m⁻² for BSA and IgG adsorption, respectively. Errors bars represent the SD of the individual measurements.

Table 1

Dextran-PEG copolymer grafted amount and copolymer composition.

Dextran grafting concentration (mg ml ⁻¹)	Amount of grafted dextran (mg m ⁻²)	Amount of grafted PEG (mg m ⁻²)	Total amount of grafted polymer (mg m ⁻²)
1	0.25	2.04	2.29
100	0.44	3.39	3.83
300	0.44	2.12	2.56