



Polyethylene glycol-conjugated alkylamines - A novel class of surfactants for the saturation of immunoassay solid phase surfaces

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ABSTRACT

All solid-phase immunoassay techniques depend on so-called blocking reagents to suppress the background that is caused by unwanted adhesion of assay system components to the solid support. Commonly used blocking reagents based on biological materials bear severe inherent drawbacks such as heterogeneity and cross-reactivity, while synthetic alternatives often show insufficient background prevention. In this study, polyethylene glycol-conjugated alkylamines were synthesized via a versatile building block approach and were studied as novel blocking reagent candidates in immunoassays. The newly developed substances outperformed commonly used blocking reagents in two different ELISA setups, enabling both, excellent prevention of non-specific binding and particularly high assay sensitivity. This class of surfactants therefore may contribute significantly to the field of assay technology.

1. Introduction

Immunoassays, in particular enzyme-linked immunosorbent assays (ELISA), represent a simple, yet essential tool for clinical diagnostics and for bioanalytics. One critical issue of such heterogeneous assay systems is the unwanted adhesion or non-specific binding (NSB) of reagents or sample components to the solid phase surface. NSB frequently causes false-positive detection signals, thereby compromising the assay's specificity, or high background signals which lead to a decreased assay sensitivity. This problem is commonly addressed by employing a "blocking" reagent which saturates the solid phase by adsorption and thus passivates the surface. Although blocking reagents based on biological materials (e.g. animal sera, skimmed milk, bovine serum albumin (BSA), gelatin) [1–4] work reasonably well in many applications, they have severe drawbacks such as heterogeneity, lot-to-lot variation, restrictions due to bio-hazardous risks, storage limitations and cross-reactivity [5,6]. Besides that, proteinaceous blocking reagents may inhibit important recognition processes such as streptavidin-biotin binding [7] or disturb detection and thus decrease the sensitivity of the assay [8–10]. Consequently, some attempts have been made to adjust assays to a setup obviating blocking with proteinaceous blocking reagents [11]. Synthetic molecules, e.g. detergents like Tween20 or polymers such as polyvinyl alcohol or Ficoll, have been used as alternatives for proteinaceous blocking reagents [12–15], but they often do

not prevent non-specific binding to a sufficient extent [4]. In light of this, there ought to be demand for better blocking techniques. Here we present a class of substances which perform excellently as blocking reagents in solid phase immunoassays with regard to both prevention of non-specific binding and assay sensitivity.

2. Materials and methods

2.1. Synthesis of blocking reagents

Details on materials along with an exact description of the procedures for synthesis of the blocking reagents are given in the Supporting Information.

2.2. Preparation of human serum

Human serum was prepared from freshly collected blood samples of healthy adult blood donors as approved by the Ethics Committee of the Medical University of Lübeck (revision number 06–093). Blood was allowed to clot at room temperature, then serum was collected after centrifugation and aliquots were snap-frozen in liquid nitrogen and stored at -80°C .

Abbreviations: ELISA, enzyme-linked immunosorbent assay; NSB, non-specific binding; BSA, bovine serum albumin; PEG, polyethylene glycol; OD, optical density

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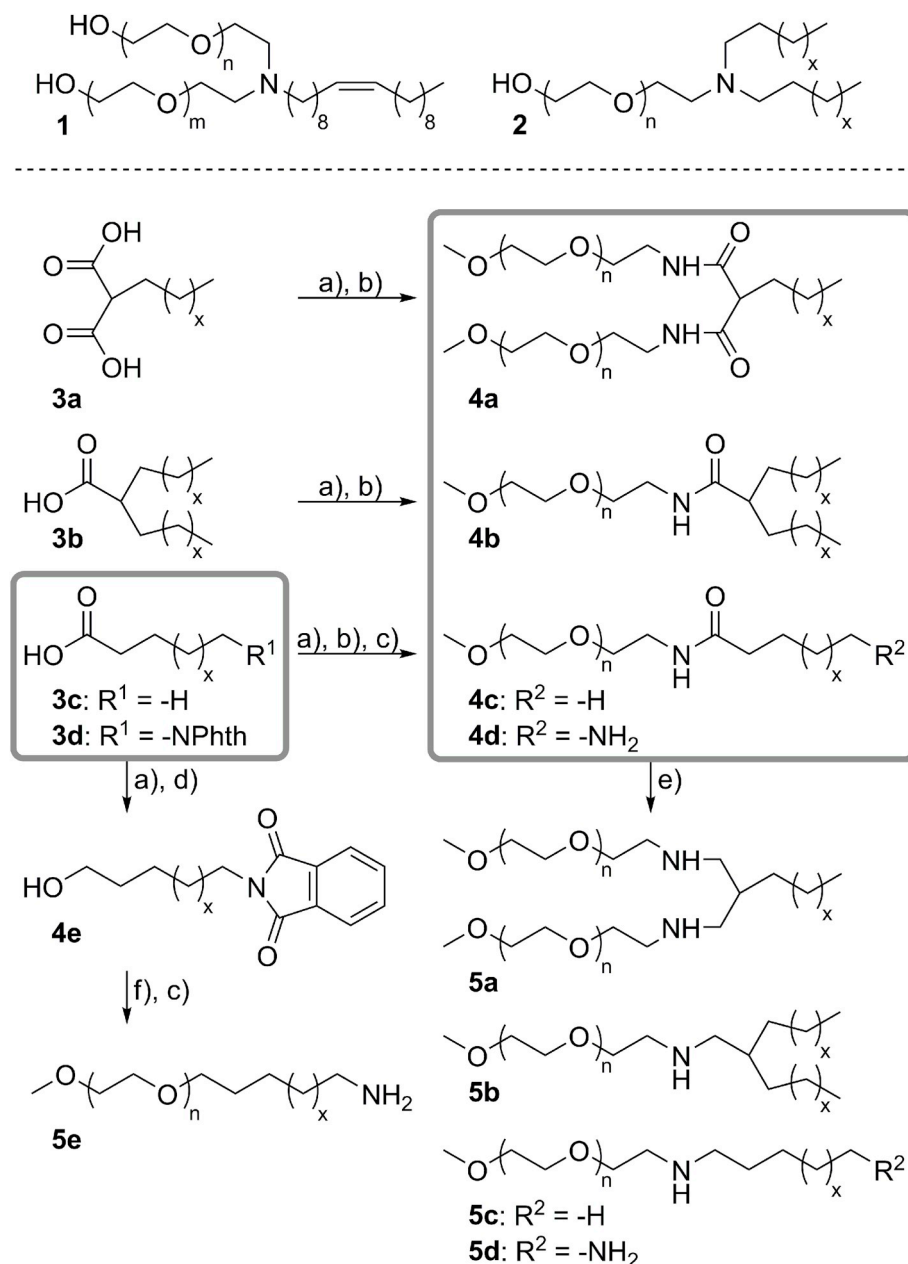
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Scheme 1. Synthesis and molecular structures of PEG-conjugated alkylamines tested as blocking reagents. Top: Commercially available cationic surfactants OA200 (1) and DKA 300 (2). (n + m)_{average} = ~20 for 1, n_{average} = ~30 and x_{average} = ~11 for 2. Bottom: reaction scheme for synthesis of cationic surfactants. n_{average} = ~11 or ~44, x = 10 or 18. a) SOCl₂; b) Me-PEG-NH₂, Et₃N, Toluene; c) H₂NNH₂·H₂O, EtOH (N/A for 3c); d) Li(t-BuO)₃AlH, THF; e) BH₃·THF, THF; f) Tf₂O, Me-PEG-OH, NaH, DME.

2.3. ELISA experiments

Details on buffers and on the different blocking reagents are given in the Supporting Information.

In all ELISA experiments, the following blocking reagent formulations were used: BSA, fish gelatin, casein, casein hydrolysate: each 1% (w/v) in Dulbecco's phosphate-buffered saline (D-PBS); skimmed milk: 5% (w/v) in D-PBS; Tween20: 0.5% (v/v) in D-PBS; AquaBlock: ready to use solution according to manufacturer; Rotiblock: 1:10 diluted in H₂O according to manufacturer; all novel cationic surfactants: each 1% (w/v) in D-PBS, except for substances 1 (0.5% (w/v)) and 5b(550,20) (0.2% (w/v)).

ELISA setup I was specifically designed to evaluate the capacity of "blocking" substances to prevent non-specific binding. For this, polystyrene microtiter plates (Corning 96-well half area, high-bind) were

filled with 150 µl/cavity of the respective blocking solution and left at room temperature (RT) for 7 h. After washing the plates 4x with Dulbecco's phosphate-buffered saline (D-PBS), 45 µl/cavity human serum in blocking solution (20% (v/v)) or blocking solution alone was added and the plates were incubated over night at 4 °C. The plates were washed (4x, D-PBS), incubated for 3 h at RT with biotin-labeled anti-human IgG antibody (Southern Biotech, Birmingham, AL, USA) (45 µl; 0.25 µg/mL in blocking solution) and washed again (6x, D-PBS with 0.05% (w/v) Tween20 (PBST)). Streptavidin-horseradish peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA) (45 µl; 1 µg/mL in blocking solution) was added and the plates were incubated for 90 min at RT. After washing the plates (6x, PBST), a freshly prepared tetramethyl benzidine/H₂O₂ solution [16] was added and allowed to develop color in the dark for 10 min at RT. The reaction was stopped by adding 1 M H₂SO₄, and the optical density (OD) at 450 nm was

measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

ELISA setup II was used to determine the influence of the blocking reagent on assay sensitivity. For this, microtiter plates were coated with a recombinant protein fragment (murine prion protein amino acid 90–231 (PrP90-231) in Lite-PBS) (45 µl/cavity; 50 ng/mL) or with buffer alone as a negative control over night at 4 °C. After washing (3x, PBST), the plates were blocked with the respective blocking solution (150 µl/cavity) for 7 h at RT, washed again (4x, PBST) and incubated over night at 4 °C with monoclonal anti-PrP antibody 6H4 (Prionics, Schlieren, CH) (75 µl/cavity; 0.25 ng/mL in blocking solution containing additional 0.1% (v/v) Tween20). The plates were subjected to another series of washes (4x, PBST), HRP-labeled anti-mouse IgG antibody (Southern Biotech, Birmingham, AL, USA) (45 µl/cavity; 1:2000 diluted in blocking solution) was added and the plates were incubated for 90 min at RT. After washing the plates (6x, PBST), color was developed and OD was measured as described above.

The ELISA procedures I and II were carried out independently in duplicate for substances **4d**, **5a**, **5b**, **5c**, **5d** and **5e** and in quadruplicate for commercially obtained blocking reagents as well as for surfactants **1** (OA200) and **2** (DKA300).

3. Results and discussion

3.1. Generation of PEG-alkylamine-based blocking reagents

Solid phases commonly used in microplate immunoassays are mainly made of hydrophobic materials – for the most part polystyrene – which promote adherence of substances mainly via hydrophobic interactions. Special high-bind polystyrene plates also display negatively charged (carboxylate) residues and allow binding via ionic interactions [17]. In line with this, it has been found that certain cation-rich peptides can adhere very strongly to high-bind polystyrene microtiter plates [18]. Expanding further from this observation we wanted to investigate the potential of polyethylene glycol (PEG)-conjugated alkylamines to saturate microplate surfaces and reduce non-specific binding: PEG-conjugated alkylamines combine a biorepulsive moiety - PEG is well known to act biorepulsively and has long been used for protein-resistant surface modifications [19–21] - and a hydrophobic adsorptive part additionally equipped with cationic groups - as can be found in polystyrene-adhering peptides [18]. We first selected two commercially available surfactants which exhibit the desired structural characteristics (OA200, polyoxyethylene oleylamine ether; DKA300, polyoxyethylene dicocosamine ether) (Scheme 1, structures **1** and **2**) to test them for any potential “blocking” capability. Furthermore, a series of structurally related PEG-conjugated alkylamines was synthesized in a

versatile building block approach. Synthesis protocols were based on coupling substituted malonic- or carboxylic acid derivatives with methoxy PEG amines and subsequent reduction, or on an ether synthesis of alcohols with methoxy PEGs. Optionally present phthalimido-protected primary amino functions were deprotected after coupling and utilized to investigate the effects of terminal amine groups (Scheme 1, see Supporting Information for details on synthesis).

For a simplified presentation, all compounds with the same core structure but different PEG- or alkyl chain length are symbolized in the following by a unique combination of numbers in parentheses which indicate the molecular mass of the PEG moiety and the alkyl chain length, separated by a comma. E.g. **5c**(550, 20) represents the PEG-alkylamine **5c** in Scheme 1, with an average molecular mass of the PEG chain of approximately 550 g/mol and an alkyl chain length of 20 carbon atoms.

The synthesized surfactants **4d**, **5a**, **5b**, **5c**, **5d** and **5e**, as well as the two commercially available surfactants **1** and **2** (Scheme 1) were tested as blocking reagents in two different ELISA setups and compared to commonly used blocking reagents.

3.2. Prevention of non-specific binding of serum components

Determination of antibody titers in human serum is one of the most important applications of immunoassays. Thus, it is a key requirement for a blocking reagent to be able to prevent non-specific binding of antibodies present in human serum. ELISA of serum samples suffers particularly from “background” problems due to the hydrophobic binding of serum components to polystyrene plates [22–24]. We therefore used human serum in our first ELISA setup as a reference material: we determined the degree of non-specific binding caused by serum-borne human IgG antibodies and evaluated its prevention by different blocking agents. The following blocking reagents were tested in this setup: the surfactants OA200 (**1**), DKA300 (**2**) and the PEG-conjugated alkylamines **4d**, **5a**, **5b**, **5c**, **5d** and **5e**, the protein-based blockers BSA, fish gelatin, casein, casein hydrolysate, skimmed milk and AquaBlock, the detergent Tween20, and the synthetic polymer-based Rotiblock. Polystyrene microtiter plates were first blocked with the respective blocking solutions to be tested and then incubated with human serum. Non-specifically bound IgG was detected with biotin-labeled anti-human IgG antibody/horseradish-peroxidase-labeled streptavidin and quantitated by measuring the optical density (OD) after addition of a colorigenic substrate. In this setup, high read-out values evidence high binding of serum IgG to the plate, low values indicate efficient blocking (Fig. 1). Most of the blocking reagents tested provide a good prevention of NSB under these experimental conditions. This holds true for commercially available reagents as well as for our

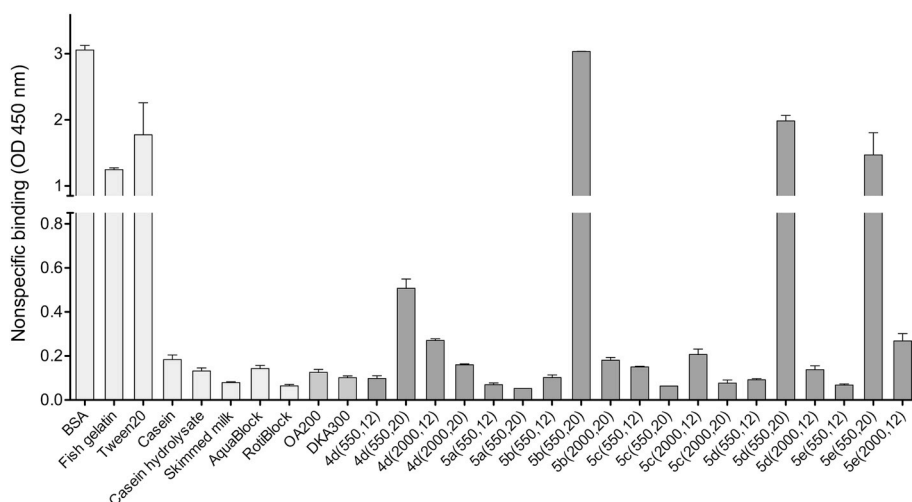


Fig. 1. Prevention of non-specific binding by different blocking reagents. Adsorption of IgG from human serum to polystyrene microtiter plates after blocking with various blocking reagents was measured in an ELISA set-up. High OD read-outs indicate high non-specific binding, low values are indicative of good blocking capabilities. Commercially available “standard” blocking reagents (light bars) are compared to surfactants **1** and **2** (medium bars) and to newly synthesized PEG-alkylamines (dark bars).

newly synthesized ones. Yet some reagents perform poorly: although BSA is a widely used reference reagent [6], it shows one of the worst blocking efficiencies next to gelatin, Tween20 and few of the novel blocking reagents, as indicated by an OD > 0.5 after incubation of the blocked microplate with 20% human serum. In case of the novel blocking reagent candidates exhibiting poor performance, the high NSB always coincides with a “short” PEG chain in combination with a “long” alkyl chain (4d(550/20), 5b(550/20), 5d(550/20), 5e(550/20)). It is conceivable that the long hydrophobic chains can be particularly prone to interactions with (lipophilic) components from the serum which in turn may facilitate dissociation of the blocking molecule from the plate surface - a similar phenomenon as seen when detergent blockers are dissociated from the plate surface by hydrophobic analyte components or when - vice versa - detergents detach lipid antigens from ELISA plate surfaces [25,26]. As a result, the blocking reagent would be exchanged with serum proteins, thus leading to the undesired high background in this setup. The adverse effect of the long alkyl chain can obviously be counteracted by a longer hydrophilic PEG chain, or by the presence of two PEG-chains instead of one - compare bad performance of 5b(550,20) with good performance of 5b(2000,20) or of 5a(550,20).

3.3. Influence of blocking reagent on assay sensitivity

Good reduction of NSB is a necessary yet not sufficient requirement for effective blocking. If a blocking reagent interferes with the detection process, for instance by covering or replacing the coating material, the signal will be false-negative and the sensitivity of the assay is decreased. This aspect was investigated in a second ELISA system, evaluating the same set of blocking reagents as above. In this setup, prion protein (PrP) which is known to be a very adhesive material and is thus supposedly hard to replace [27] was chosen as model analyte. Polystyrene microtiter plates were coated with a recombinant PrP fragment (PrP90-231) [28] or left uncoated to determine background “noise”, and were then blocked with the respective blocking solutions. PrP detection was carried out with a monoclonal anti-PrP antibody and a horseradish peroxidase-labeled secondary antibody and the OD was measured after visualization with tetramethylbenzidine. The OD read-out of PrP-coated wells was set in relation to that of background control wells in order to obtain the signal-to-noise ratio (S/N) as well as the signal-minus-noise value (S - N) as measures for the sensitivity of the assay (Fig. 2). In this setup, the signal strength correlates with assay sensitivity, e.g. blocking reagents interfering with the antigen-antibody interaction produce lower signals and vice versa. The readout obtained for the background control, on the other hand, exhibits the degree of non-specific binding which could not be prevented by the blocking reagent. In this ELISA setup, an elevated signal of the background control was only seen for BSA (OD > 0.5), all other reagents were preventing non-specific

binding quite efficiently (OD = 0.04–0.11).

When comparing assay sensitivity, the commercially available “standard” blocking reagents show a poor to mediocre performance. Especially those reagents which had demonstrated very good results in prevention of non-specific binding in the first ELISA setup, such as RotiBlock or AquaBlock, can scarcely produce signals above background in the “sensitivity” ELISA setup (S/N = ~1.0), casein and milk yield readouts of only 0.1–0.2 OD above background signals. Tween20 exhibited at least a moderate S/N of ~8, with absolute readout values of ~0.5 OD. In terms of sensitivity, the performance of our novel surfactant-based blocking solutions is clearly better than that of the “standard” blocking reagents, with the top candidates reaching S/N ratios of up to ~60 and absolute readout values up to > 3 OD.

3.4. Mechanistic advantages of novel blocking reagents

To come up with a possible explanation for the superior performance of the novel surfactants with respect to both, blocking capacity and assay sensitivity we took a closer look at two variables, namely the blocking mechanism and the blocking reagent size. Proteinaceous blocking reagents “passivate” the surface of ELISA plates against unwanted binding by adsorbing themselves to “empty” regions of the plate - i.e. surface areas which are not occupied by e.g. coating antigen. Adsorption varies between different proteins [29] and is usually based on hydrophobic interactions between blocking reagent and plate surface, but it can also include ionic interactions especially in case of the high-bind plates carrying carboxylate residues at their surface [17]. A side-effect of this “hydrophobic adsorption principle” is the potential of multilayer formation of the blocking proteins [29–31]. In addition, surface-bound protein-based blocking reagents can sometimes interact with sample or analyte proteins [32]. This unwanted interference is especially problematic in lectin- or glycoprotein-involving assays, since many of the typical proteinaceous blocking reagents contain carbohydrate structures [9,33].

Detergent-based blocking reagents, such as Tween20, can also adsorb to hydrophobic plate surfaces [14], yet this adsorptive blocking is often inefficient and non-permanent, and the blocking detergent can easily be washed off the plate surface or exchange with proteins from added solutions [32,34]. In addition, however, detergents usually exercise their blocking power by interacting with added proteins in solution, thereby minimizing any hydrophobic binding of those proteins to the microtiter plate. Therefore, they are usually used not only in the blocking step, but also added to all buffers used in subsequent washing and incubation steps [32,35], although they may be detrimental in some assays where the antigens are phospholipids or glycolipids [25,26].

Mechanistically, our novel blocking surfactants combine favorable

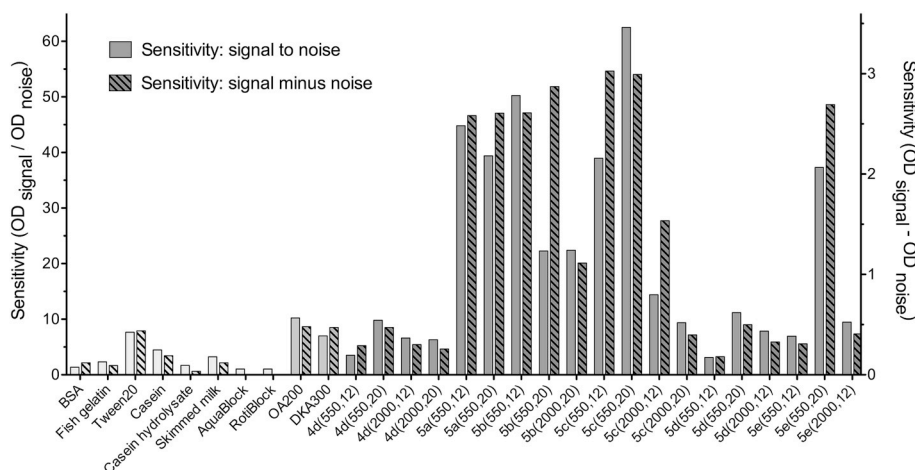


Fig. 2. Influence of blocking reagent on assay sensitivity. A prion protein fragment adsorbed onto polystyrene microtiter plates was detected by standard ELISA procedure after blocking with various blocking reagents. The assay sensitivity is given as signal-to-background quotient (filled bars) and as signal-minus-background difference (hatched bars). Commercially available “standard” blocking reagents (light bars) are compared to surfactants 1 and 2 (medium bars) and to newly synthesized PEG-alkylamines (dark bars).

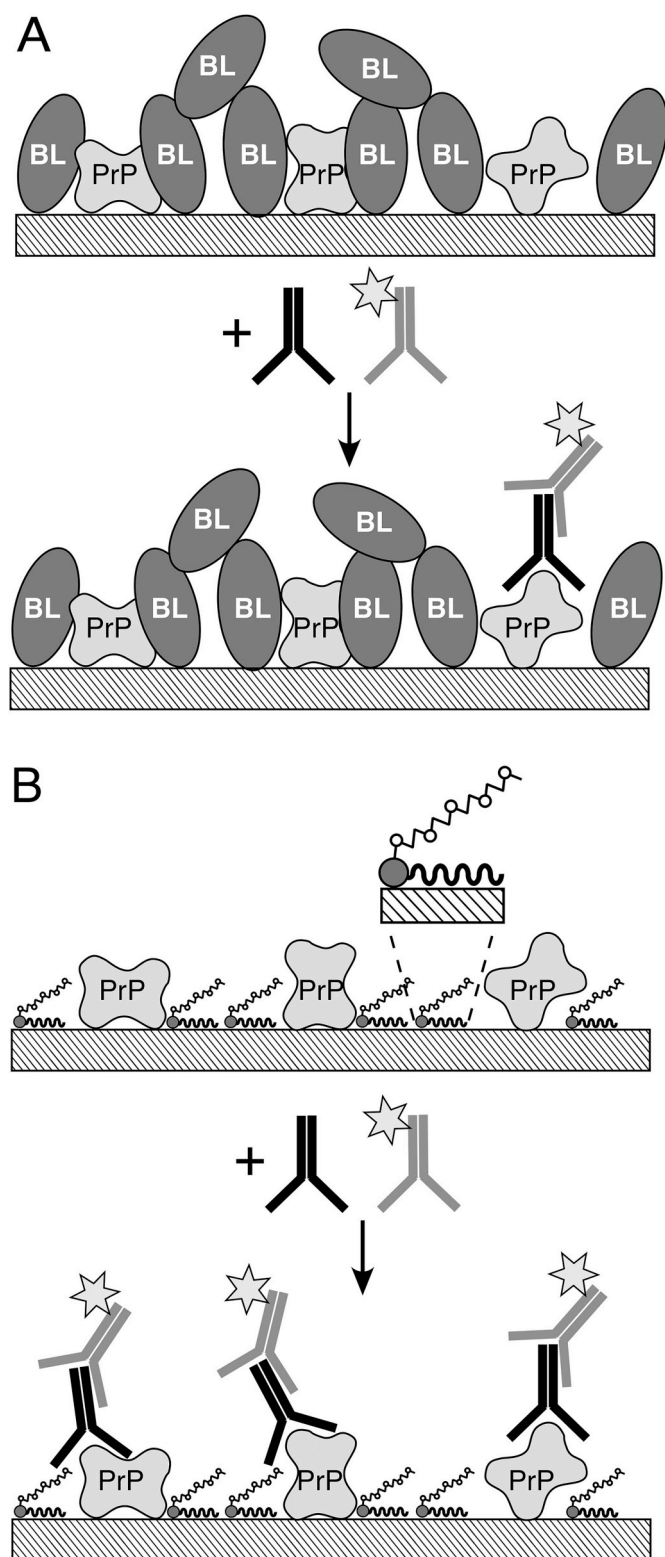


Fig. 3. Superior performance of PEG-alkylamines as blocking reagents. **A:** Conventional blocking reagents (BL) – high molecular mass polymer- or protein-based – may mask microplate-bound analyte (e.g. prion protein fragment, PrP) by steric hindrance and/or multilayer formation. Access to and detection of analyte by specific antibodies is reduced. **B:** PEG-alkylamines are considerably smaller than proteins and do not form multilayers on hydrophobic surfaces. All microplate-bound analyte is freely accessible to detection reagents.

properties of both, protein-based reagents and detergents. They adsorb to the plate surface not only by strong hydrophobic interaction but also via their positive charge which, in addition to binding to negative surface groups of high-bind ELISA wells, may enhance attachment to the polystyrene matrix via cation- π -interactions [36]. On top of that, the special feature of our novel blocking reagents is the presence of the PEG-chains which, after attachment of the hydrophobic part/positively charged alkylamine group, will presumably arrange themselves on the plate to stick away from the surface plane, thereby rendering this plate area hydrophilic (Fig. 3). This surface hydrophilicity by itself decreases background noise and will also minimize interactions of the plate-bound blocking reagent with proteins from the analyte solution [37]. On the other hand, with our novel blocking reagents being surfactants, they can presumably also loosely attach to proteins in analyte/detection solutions and prevent any non-specific interactions of those components. In this, they are similar to detergents like Tween20, and we would hence recommend to analogously use the surfactants in all the respective incubation steps.

As for the size, the commercially available blocking reagents which are either proteins (casein, fish gelatin, BSA) or at least protein-based (casein hydrolysate, skimmed milk, AquaBlock) are, by default, of at least similar size and possibly even larger than the analyte and the detection reagents. The polymer PVP, used as a blocking reagent e.g. in RotiBlock, typically has an average molecular mass of at least 40 kDa [38] which is close to the size of many proteins. Thus, even if those surface-passivating blocking reagents just build a uniform monolayer on the plate, a steric effect of adsorbed blocking reagents on adjacent coating reagents is very likely. Moreover, as blocking reagents are used in a huge excess, a protein-protein based secondary adsorption [29] and hence a formation of blocking reagent multilayers is quite possible. Blocking reagent molecules may thus cover analytes and prevent successful detection. In contrast, the usually employed detergents, but also our novel surfactants exhibit a size of < 3 kDa. They are far smaller than the typical analytes and detection molecules and, due to their amphipathic structure, not prone to forming multilayers on solid surfaces. These reagents should therefore not disturb analyte binding or interfere with the detection reaction, even if they have formed a dense and impenetrable layer on the plate surface (Fig. 3).

4. Conclusions

We present here a novel class of blocking reagents which are structurally based on cationic surfactants. A versatile building block synthesis was developed which allowed the generation of a variety of structurally related, PEG-conjugated alkylamines. Testing in two standardized ELISA setups led to the identification of several candidates which outperform the selection of commercial blocking reagents, both in prevention of non-specific binding to solid phase surface as well as by providing excellent assay sensitivity.

Declaration of competing interest

The authors are inventors on the patent “Novel synthetic blocking reagents”, EP 2 261 662 and US 8,501,199, assigned to the Research Center Borstel.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2020.120741>.

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