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ORIGINAL PAPER

Development of an efficient amine-functionalized glass platform by additional silanization treatment with alkylsilane

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Abstract Aminosilane-treated molecular layers on glass surfaces are frequently used as functional platforms for biosensor preparation. All the amino groups present on the surface are not available in reactive forms, because surface amino groups interact with remaining unreacted surface silanol groups. Such nonspecific interactions might reduce the efficiency of chemical immobilization of biomolecules such as DNA, enzymes, antibodies, etc., in biosensor fabrication. To improve immobilization efficiency we have used additional surface silanization with alkylsilane (capping) to convert the remaining silanol groups into Si–O–Si linkages, thereby liberating the amino groups from nonspecific interaction with the silanol groups. We prepared different types of capped amine surface and evaluated the effect of capping on immobilization efficiency by investigating the fluorescence intensity of Cy3-NHS (*N*-hydroxysuccinimide) dye that reacted with amino groups. The results indicate that most of the capped amine surfaces resulted in enhanced efficiency of immobilization of Cy3-NHS compared with the untreated control amine surface. We found a trend that trialkoxysilanes had greater capping effects on immobilization efficiency than monoalkoxysilanes. It was also found that the aliphatic chain of alkylsilane, which does not participate in the capping of the silanol, had an important function in enhancing immobilization efficiency. These results would be useful for preparation of an amine-modified surface platform, with enhanced immobilization efficiency, which is essential for developing many kinds of biosensors on a silica matrix.

Keywords Aminosilane · Glass surface modification · Capping

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Introduction

Aminoalkane-substituted silanes are extensively used for chemical modification of different silica surfaces used as bonded phases in chromatography [1, 2], in trace metal analysis [3], and as coupling agents in the treatment of glass fibers [4], etc. In recent years, amine-functionalized silica surfaces have also been widely used as microarray substrates for printing and archiving of biomolecules such as nucleic acids [5, 6]. They are usually obtained by self-assembly of aminoalkoxysilanes on a silica matrix. In this process, aminosilanes react with silanol groups on the silica matrix to form the –Si–O–Si–R–NH_2 structure, leaving the amine functionality on the surface. These reactive primary amines on the surface then serve as a functional platform for a variety of technological applications.

Silanol groups on silica surface are an important factor in obtaining dense and homogenous surface layers during silanization. They act as precursors for condensation of the aminosilane with the substrate, by forming hydrogen bonds which are later transformed into covalent silyl ether linkages. It has, however, been reported that, because of steric factors, only 70% of surface silanol groups are used, even

in the most rigorous silanization reactions, so a significant number of silanol groups are left unreacted [7]. For this reason, silanol groups remaining on the surface interact with introduced amino groups, which could reduce the number of reactive primary amines available for the desired applications.

In the presence of surface silanol groups on silica surface after silanization the amino groups are not in the free form, for example $R–NH_2$ or $R–NH_3^+$(in equilibrium), but, instead, form hydrogen bonds with residual unreacted silanol groups, –Si–O–H ····· NH_2–R–, or participate in ion-pairing reactions, $–Si–O^-$ ····· NH_3^+–R– [8]. This is because silanols have acidic properties rather than those of an alcohol. Protons from surface silanols are, moreover, transferred to amino groups to form the NH_3^+forms, which are not sufficiently reactive to form covalent bonds [9–11]. Consequently, these interactions between amino groups and surface silanols, especially in microarray fabrication, might reduce the efficiency of chemical immobilization of biomolecules on the surface.

In this report we describe one possible way of reducing nonspecific interaction of amino groups with silanol groups. An additional silanization (capping) step with alkylsilanes was used to convert unreacted silanol groups on the substrate in to Si–O–Si linkages. A variety of mono and trialkoxysilanes with aliphatic chains of different length were used to prepare amine-modified surface platforms with enhanced immobilization efficiency for development of reliable biosensor tools on the silica matrices. The results obtained were compared.

Experimental

Materials

All silane reagents were purchased from Shinetsu (Osaka, Japan) and used without further purification. The silane reagents used in the study are listed in Table 1. Toluene, methanol, acetone, and distilled water were obtained from Nacalai Tesque (Osaka, Japan) and sulfuric acid and hydrogen peroxide from Wako Pure Chemicals (Osaka, Japan). Glass slides (25.4 cm×76.2 cm (±0.05 cm)) were obtained from Matsunami (Osaka, Japan). All silanization reactions were performed in stainless-steel containers purchased from AS-ONE (Osaka, Japan). The dye Cy3-NHS (*N*-hydroxysuccinimide) ester was obtained from Amersham Biosciences (Piscataway, NJ, USA).

Substrate pre-preparation

To remove all contaminants, glass slides were cleaned by sonication in acetone for 10 min, dried under vacuum for 10 min, and treated with freshly prepared Piranha solution (70% H_2SO_4, 30% H_2O_2) at 55°C for 30 min. The glass slides were cleaned with copious amounts of distilled water and dried under vacuum. Glass slides treated in this way were enriched with surface hydroxyl groups and were therefore suitable for functional silanization, as reported elsewhere [12].

Silanization

The prepared glass slides were cleaned by sonication with methanol, methanol–toluene, 1:1(*v*/*v*), and toluene, each for 10 min. Silanization was performed by immersing the glass slides in aminosilane solution (2% in toluene). During silanization containers were placed in orbital shaker at room temperature with gentle shaking (70 rpm). The glass slides were then cleaned in ultrasonic bath with toluene, methanol–toluene, 1:1(*v*/*v*), and methanol, each for 10 min. The slides were baked at 110°C for 1 h. Capping treatment was also performed by following the silanization procedure described above except that alkylsilane was used in place of aminosilane. Finally, the silanized glass slides were stored in vacuo at room temperature.

Cy3 spotting, scanning, and fluorescence detection

Cy3-NHS dye (50 pmol μL^{-1}) was spotted on the prepared glass slides with an inkjet spotter (custom built by NGK, Nagoya, Japan), with 100 μm spacing. The spotted glass

Table 1 The aminosilanes and alkylsilanes used in this work

Full name	Abbreviation	Formula	Type
3-Aminopropyltriethoxysilane	APS	$H_2N(CH_2)_3Si(OC_2H_5)_3$	Trialkoxyaminosilane
3-Aminopropyldimethylethoxysilane	APDMS	$H_2N(CH_2)_3CH_2CH_2SiOC_2H_5$	Monoalkoxyaminosilane
2-(2-Aminoethylthioethyl) triethoxysilane	AETS	$H_2NCH_2CH_2SCH_2CH_2Si(OC_2H_5)_3$	Trialkoxyaminosilane
Octadecyltriethoxysilane	ODTES	$CH_3(CH_2)_{17}Si(OC_2H_5)_3$	Trialkoxyalkylsilane
Butyltrimethoxysilane	BMS	$CH_3(CH_2)_3Si(OCH_3)_3$	Trialkoxyalkylsilane
Methoxytripropylsilane	MTPS	$(C_3H_7)_3SiOCH_3$	Monoalkoxyalkylsilane
Ethoxytrimethylsilane	ETMS	$(CH_3)_3SiOC_2H_5$	Monoalkoxyalkylsilane
Methyltrimethoxysilane	TMS	$CH_3Si(OCH_3)_3$	Trialkoxyalkylsilane

slides were baked at 80°C for 1 h. Blocking, to passivate the unspotted areas, was performed with succinic anhydride for 20 min; the slides were then rinsed thoroughly with water, soaked in distilled water (3 min) and ethanol (1 min), and dried at room temperature for 30 min. The glass slides were also treated with hybridization buffer (5×SSC (sodium chloride–sodium citrate) containing 0.5% SDS (sodium dodecyl sulfate)) with a cover slip and incubated at 42°C for 16 h. The incubated glass slides were then immersed for 5 min in 2×SSC containing 0.1% SDS. The immersed glass slides were then subjected to gentle shaking in 1×SSC and 0.1×SSC, each for 5 min. After spin drying, the slides were stored in desiccator until used for analysis. If kept for later analysis the glass slides were stored under ambient conditions in the dark. Glass slides were scanned with a DNA MicroArray Scanner (Model G2505A) from Agilent Technologies (Palo Alto, CA, USA). The fluorescent image intensities and the location of each analyte spot on the array were measured by use of mapping software (GenePixPro Ver 5.0 or 5.2; Molecular Devices, Sunnyvale, CA, USA).

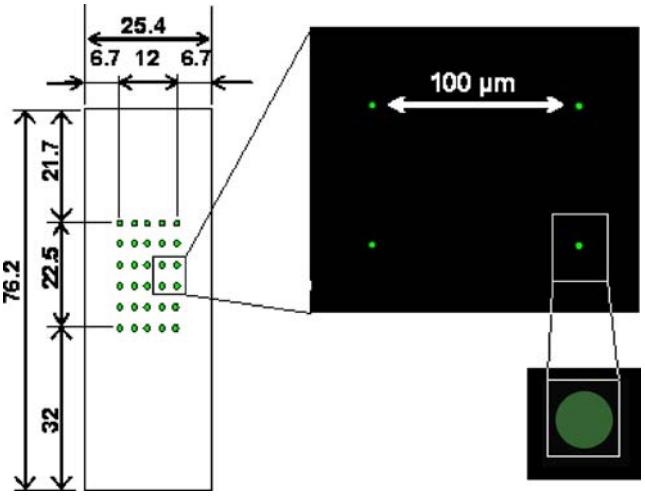


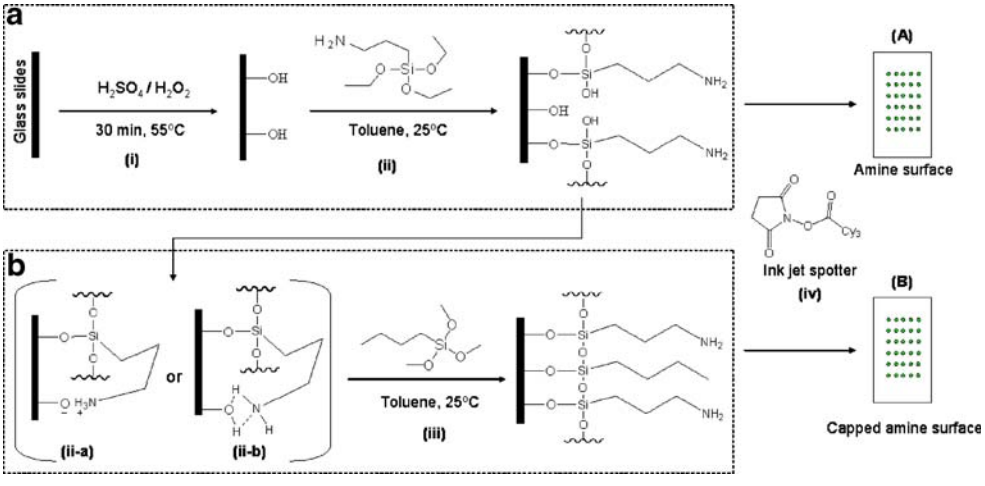
Fig. 1 Five by six spots of fluorescent labels on amine-functionalized glass slides. Cy3-NHS dye (50 pmol μL^{-1}) was spotted on the prepared glass slides with an inkjet spotter (custom built by NGK, Nagoya, Japan), with 100 μm spacing. The inkjet-deposited spots had good uniformity in terms of size and shape. The unit of length is cm

Results and discussion

Preparation of functional amino group-modified glass slides

Amine functionalization of the glass surfaces was performed by chemical treatment with aminosilanes such as 3-aminopropyltriethoxysilane (APS), 3-aminopropyldimethylethoxysilane (APDMS), and 2-(2-aminoethylthioethyl) triethoxysilane (AETS). The procedure is illustrated schematically in Scheme 1a. First, glass slides were cleaned and hydroxylated (step (i) of Scheme 1a). Glass slides with the OH-enriched surface were then immersed in aminosilane solution (2% in toluene) and incubated at room temperature for different reaction times (step (ii) of Scheme 1a). After baking at 110°C for 1 h, amine-functionalized glass slides were obtained. To evaluate the efficiency of chemical immobilization of biomolecules on the amine surface, a fluorescent label (Cy3-NHS dye), which can form stable amide bonds with amino groups, was spotted by using an inkjet spotter (step (iv) of Scheme 1). Five by six fluorescent spots were deposited on each glass slide. As shown in Fig. 1, the size and shape uniformity of the inkjet-deposited spots was good. Fluorescent data from over 90 spots (three glass slides) were measured and the statistical data obtained were used to evaluate the efficiency of chemical immobilization on the modified surface.

Capping of silanol groups remaining on aminosilane-modified surface was performed as shown in Scheme 1B. It has previously been reported that primary amino groups interact strongly with the remaining surface silanols [9–11]. As illustrated in steps (ii-a) and (ii-b) of Scheme 1b,



Scheme 1 Preparation of an amine-functionalized surface by use of 3-aminopropyltriethoxysilane (APS) (**a**) and additional treatment with butyltrimethoxysilane (BMS) for capping of the amine-functionalized surface (**b**): (*i*) activation of glass slides with Piranha solution for 30 (min at 55°C. (*ii*) aminosilanization of glass slides with APS-toluene at 25°C. (*iii*) capping of APS-treated glass slides with BMS-toluene at 25°C. (*iv*) spotting of fluorescent label (Cy3-NHS dye) on the amine-glass slides by use of an inkjet spotter

interactions such as hydrogen bonding, –Si–O–H ····· NH_2–R–, or ion-pair formation, –Si–O^- ····· NH_3^+–R– are expected. Such interactions could reduce amine functionality on the surface and, therefore, the efficiency of chemical immobilization of biomolecules. Although the post-deposition curing process liberates some of the amino groups from these nonspecific interactions, it was known that some amino groups still do not remain sticking outward but are bent toward the solid surface [12]. To reduce these nonspecific interactions and increase amine functionality, we performed an additional silanization step with alkylsilane, in which unreacted surface silanols are converted to Si–O–Si linkages. These conversions might liberate the amino groups from the nonspecific interactions so they can participate in chemical immobilization of biomolecules. First, aminosilane-treated glass slides were prepared as a basic platform, as presented in Scheme 1a. Several different alkylsilane solutions (2% in toluene) were then used to cap the silanol groups on the surface (step (iii) of Scheme 1b). After capping, the fluorescent label (Cy3-NHS dye) was spotted on the capped glass slides, as described above (step (iv) of Scheme 1). By investigating the fluorescence intensity, the efficiency of chemical immobilization on the amine surface (without capping) and the capped amine surface were compared, and the effect of the capping on the efficiency of immobilization was estimated.

In the preliminary experiment, three different aminosilanes, APS, APDMS, and AETS, were compared to determine their ability to mediate the amine functionality on the glass slides. APS treatment was found to result in abundant formation of functional amino groups on the silica surface; the efficiency of treatment with APDMS and AETS was lower than that of APS treatment (approx. 40–50% that of APS treatment), although they also effectively mediated formation of an amine-functionalized surface. Consequently, in the next investigation to evaluate the effect of capping on chemical immobilization, the APS-modified glass surface was adopted as the basic platform.

Enhancement of immobilization efficiency by capping with alkylsilane

A variety of alkylsilanes with different numbers of alkoxy groups and different types of non-alkoxy chain were used to cap of amine-functionalized glass slides and Cy3 spotting was the performed. The fluorescence intensity of Cy3 immobilized on each glass slide sample was measured once a month for up to 3 months. The fluorescence intensity of the APS-modified surface observed immediately after spotting (month zero) was used as the standard value for each set of alkylsilane treatments. As shown in Fig. 2, it was observed that fluorescence intensity measured on most of the capped amine surfaces was greater than that measured on a noncapped surface (APS-modified surface), indicating that capping of the remaining free silanols enhanced the efficiency of immobilization on the solid surface. These increased Cy3 fluorescence intensities were maintained almost constant for 3 months. In contrast, no Cy3 signal was observed in a control test in which glass slides treated with same alkylsilanes, but without the aminosilane treatment, were subjected to the similar fluorescence intensity analysis (data not shown). These results suggest there was no nonspecific adsorption of the Cy3-NHS dye by alkylsilane or glass slides and that the increased intensity observed on the capped amine surface therefore resulted from fluorescent labels covalently bound with the reactive amino groups present on the surface.

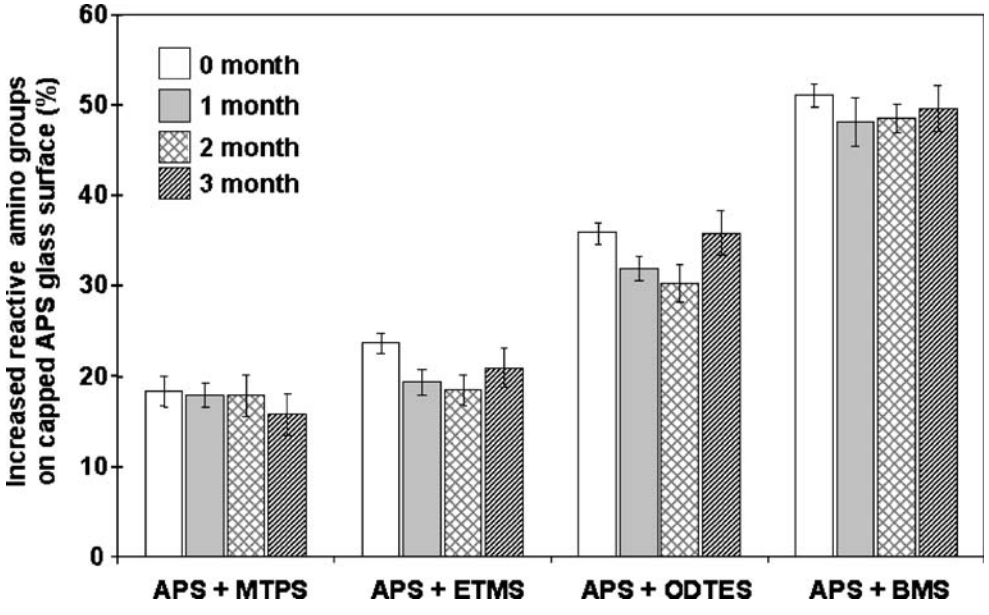


Fig. 2 Comparison of Cy3-NHS dye immobilization efficiency on APS glass slides capped with different alkylsilanes: octadecyltriethoxysilane (*OTDES*), butyltrimethoxysilane (*BMS*), methoxytripropylsilane (*MTPS*), and ethoxytrimethylsilane (*ETMS*). The fluorescence intensity of the APS-modified surface immediately after spotting was used as the standard value for each set of alkylsilane treatments. The *bars* and *error-bars* are average values and standard deviations, respectively, of fluorescence intensity, and were calculated by measurement of 90 spots for each set of experiments

All these results indicated that additional alkylsilanization effectively increases chemical immobilization of Cy3 dye on the surface. The alkoxy group of the alkylsilane used for treatment was expected to participate in siloxane linkage formation with the surface silanol groups, liberating the adsorbed amino groups from silanol groups [13]. It was found the trialkoxysilanes were more effective than monoalkoxysilanes at increasing the number of reactive primary amines on the surface [13]. Compared with the control amine surface (APS-modified surface without capping), use of octadecyltriethoxysilane (OTDES) and butyltrimethoxysilane (BMS) to cap the amine surfaces resulted in 35% and 51% increases in fluorescence intensity, respectively, whereas use of methoxytripropylsilane (MTPS) and ethoxytrimethylsilane (ETMS) resulted in 18% and 23% increases in fluorescence intensity, respectively. This is because trialkoxysilanes (OTDES, BMS) have more than one alkoxy group for formation of siloxane linkages with surface silanols whereas monoalkoxysilanes (MTPS and ETMS) have only one alkoxy group. Among trialkoxysilane treatments, BMS capping resulted in the largest increase in the number of amino groups available for the immobilization reaction on the APS-modified surface.

Characterization of the effect of alkylsilane capping on amino group reactivity

The capping effect of BMS-treatment on amine-functionalized glass slides was investigated in terms of its relationship with APS-treatment times. For this work we prepared two sets of surfaces, one used as a control references and the other as samples. The set of control references were glass slides with different APS-layers prepared by controlling the APS reaction times (1, 3, 5, and 7 h). The sample set was prepared by capping each APS-modified glass slide with BMS (3 h treatment). Cy3-NHS dye was spotted on each surface and the fluorescence intensity was measured. As shown in Fig. 3, it was observed that the fluorescence intensity for each amine surface capped with BMS was greater than that for its control reference without capping. An especially significant increase (enhancement of fluorescence intensity 51%) was observed for glass slides prepared by treatment with APS for 3 h. It was assumed that for glass slides prepared by use of longer APS-reaction times (5 and 7 h), BMS may have less chance to react with the surface silanol groups, probably because the glass surface was already enveloped with dense layers of APS [14]. Compared with the 5 and 7 h results for the control amine surface, the fluorescence intensity was increased by 19% and 21.9%, respectively. In contrast, for the sample with 1 h APS-reaction time, BMS-capping resulted in a 47.3% increase in fluorescence intensity compared with the 1 h reaction control. This glass slide may not be sufficiently coated with APS molecular layers, however, so the total amount of functional amino groups was less. Treatment with APS for 3 h was therefore observed to furnish a proper aminosilane-modified layer for introducing the efficient capping effect of BMS treatment.

Next, the efficiency of chemical immobilization of Cy3 on the surface was investigated as a function of reaction time in the BMS-capping treatment. Amine surfaces prepared by treatment with APS for 3 h were further treated for different times (1, 3, 5, and 7 h) with BMS solution (2% in toluene). As shown by APS+BMS in

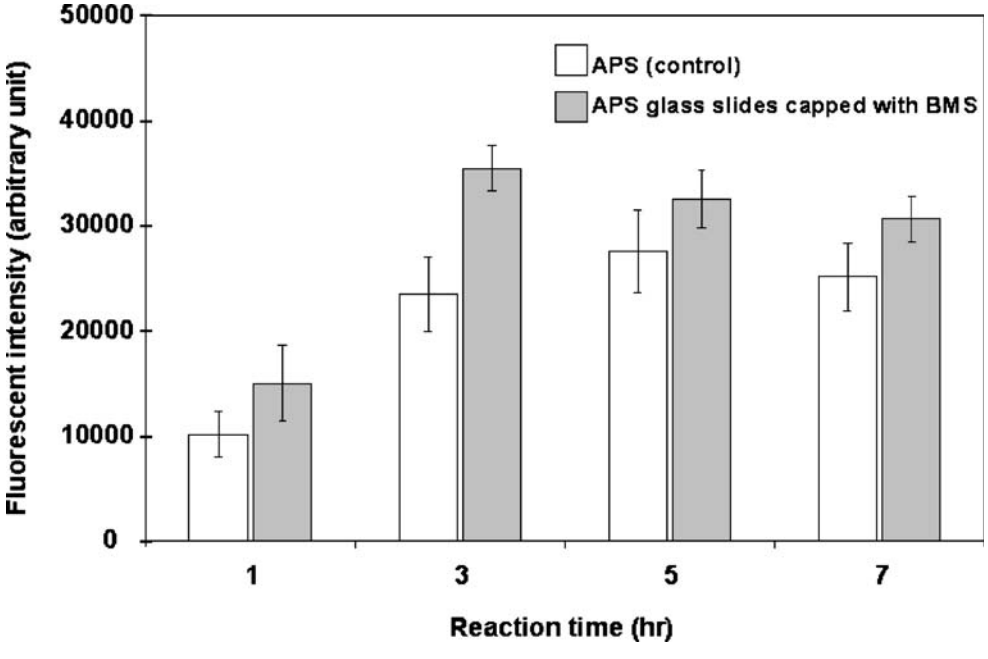


Fig. 3 Dependence on APS-treatment time of capping effect of BMS treatment on amine-functionalized glass slides. APS treatment was performed for different times (1, 3, 5, and 7 h) and each set of APS-modified glass slides was also treated with BMS for 3 h (capping treatment). The *bars* and *error-bars* are average values and standard deviations, respectively, of fluorescence intensity, and were calculated by measurement of 90 spots for each set of experiments

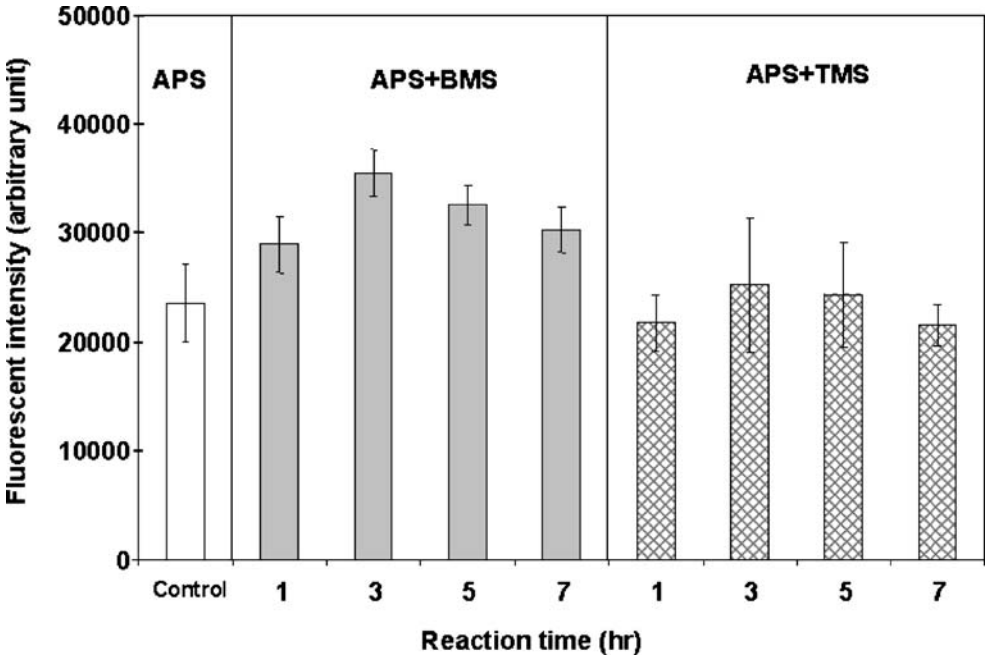


Fig. 4 Dependence on reaction time during BMS and TMS treatment in terms of the efficiency of immobilization of Cy3-NHS dye on the capped surfaces. APS treatment was performed for 3 h and each set of APS-modified glass slides was also treated with BMS or TMS (capping treatment) for 1, 3, 5, or 7 h. The *bars* and *error-bars* are average values and standard deviations, respectively, of fluorescence intensity, and were calculated by measurement of 90 spots for each set of experiments

Fig. 4, it was observed that the fluorescence intensity of Cy3 increased for BMS treatment up to 3 h. BMS-capping treatment for 1 and 3 h resulted in 22.9% and 50.8% increases in fluorescence intensity, respectively. When BMS treatment was performed for longer, however (>5 h), the effect of capping decreased. The increased fluorescence intensity of Cy3 after 5 and 7 h was 38.3% and 28.5%, respectively. It is possible that long-term treatment with trialkoxysilane induces multilayer silanization that may prevent access of Cy3-NHS to the amino groups on the surface. This implies that for effective capping, reaction time in alkylsilane treatment should be optimized.

In addition, the role of the aliphatic chain of BMS in the effect of capping treatment was elucidated by comparison with trimethoxymethylsilane (TMS) as a reference. Similarly to BMS-treatment, APS-treated glass slides were modified with TMS for different times. Because TMS has three alkoxy groups similar to BMS, TMS treatment was expected to result in a performance similar to that of BMS in increasing chemical immobilization efficiency. As shown in APS+TMS of Fig. 4, TMS capping had a similar effect to BMS capping, although no significant increase in fluorescence intensity was observed (the increase was below 8%, at most). In fact, although trialkoxysilane could provide more than one alkoxy group for siloxane linkage formation with surface silanols, with trialkoxysilane treatment there is also a large chance of leaving one silanol group in the molecular layer because two alkoxy groups usually participate in the siloxane linkage reaction. It is probable that the remaining alkoxy group could reduce the amino group reactivity of the surface by adsorbing amino groups again [15]. Nevertheless, as mentioned above, BMS treatment was effective at increasing immobilization efficiency. The good capping effect of BMS is probably because of the presence of a longer aliphatic chain. That is, the aliphatic chains of BMS result in a surface of sufficient hydrophobicity to mediate more efficient liberation of functional amino groups from the silanol groups in the molecular layers. The aliphatic chains of BMS may, moreover, also provide steric hindrance against re-access of reactive amino groups to silanols. Considering that the performance of TMS with its shorter aliphatic chain (one carbon atom only) is not similar to that of BMS, the aliphatic chain of the alkylsilane, although it does not participate in capping of the silanol, might also play an important role for increasing the availability of amino groups on the surface.

Conclusion

To increase the efficiency of chemical immobilization of biomolecules on aminosilane-treated glass surfaces, we performed additional surface modification of the silanol groups with alkylsilanes (capping) because we expected that capping could liberate reactive amino groups that interact with residual surface silanol groups. We also investigated the effects of capping in detail by using a variety of alkylsilanes with different numbers of alkoxy groups and different types of non-alkoxy chain. Comparison of the fluorescence intensity of spotted Cy3 showed that immobilization efficiency was enhanced for most

amine surfaces with capping compared with the control amine surface without capping. For example, for an APS-treated surface capped with BMS the amount of Cy3 dye immobilized was 51% greater than for the control without the BMS capping. It was also determined that trialkoxysilanes (OTDES, BMS) were more effective capping reagents, i.e. resulted in immobilization of more Cy3 dye, than monoalkoxysilanes (MTPS and ETMS). Excessively long aminosilanization and capping-treatment are, however, likely to lead to formation of multilayers on the surface that may reduce the accessibility of the primary amines for covalent binding. Comparison of the effect of BMS and TMS on chemical immobilization efficiency indicated that the aliphatic chain of the alkylsilane has an important effect in the enhancement of immobilization efficiency.

Recently, as substrate-based biosensors have made much progress in biotechnology, more functionalized and stabilized surfaces have been required. For the purpose, different surface-modification approaches, for example self-assembled monolayers of thiol on gold [16], zirconium phosphonate layers on silica [17], adsorption coating of functional copolymers on glass surfaces [18], and scale-controlled dendron layers [19], have been developed and used to fabricate more selective and sensitive biosensors. In this paper we report an effective method for preparing an amine surface with increased immobilization efficiency by additional silanization (capping) of residual silanols. Together with other recently developed surface modifications, our procedure for capping amine surfaces will become a practical surface-treatment method useful for efficient immobilization of several biomolecules, for example DNA, proteins, enzymes, antibodies, etc.

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