SUPPORTING INFORMATION

Micrometre and Nanometre Scale Patterning of Binary Polymer Brushes, Supported Lipid Bilayers and Proteins

Alexander Johnson,[1] Jeppe Madsen,[1] Paul Chapman,[1,2] Abdullah Alswieleh, [1] Omed Al-Jaf, [1] Peng Bao,[3] Claire R. Hurley,[1] Michaël L. Cartron,[4] Steven P. Armes,[1] Stephen D. Evans,[3] Jamie K. Hobbs,[3] C. Neil Hunter[4] and Graham J. Leggett[1*]

[1]*Department of Chemistry, University of Sheffield, Brook Hill, Sheffield S3 7HF, UK*

[2]*Department of Physics and Astronomy, University of Sheffield, Sheffield S3 7RH, UK*

[3]*Molecular and Nanoscale Physics Group, School of Physics and Astronomy, University of Leeds, Leeds LS2 9JT, UK*

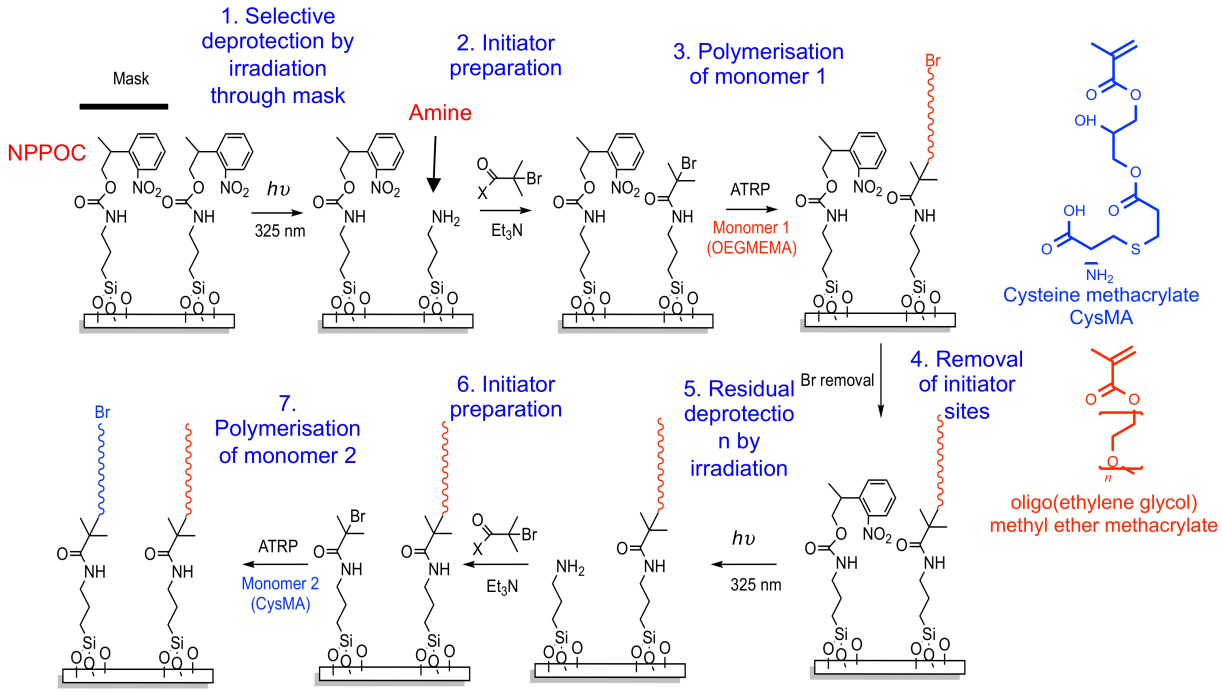
[4]*Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, UK*

OPTIMISATION OF NPPOC-APTES PATTERNING AND DERIVATISATION CHEMISTRY

Silicon oxide based surfaces react with {N-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropyl}-triethoxysilane (NPPOC-APTES) to give {N-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropyl}-functionalised surfaces (NPPOC surfaces).[1] Upon irradiation, the NPPOC group is cleaved to yield a primary amine, which is highly reactive towards electrophiles. In particular the formation of 2-bromoisobutyramides by reaction of the amine with activated 2-bromoisobutyric compounds is of interest, because these can be used as initiators for ATRP. The NPPOC surfaces are therefore convenient starting points for the preparation of binary brush structures, provided that the unmodified NPPOC groups do not react with the activated 2-bromoisobutyric compound used, i.e. that it acts as an efficient protecting group. Preliminary work has indicated that under certain experimental conditions, a reaction with NPPOC occurs and that this is detrimental to the formation of well-defined binary brushes. The nature and conditions under which this reaction proceeds are examined here. In addition, conditions are identified that ensure chemoselective reaction of deprotected NPPOC and guidelines are sketched for the use of NPPOC as a protecting group.

Synthetic strategy for using NPPOC-APTES films to prepare patterned binary brush structures

The strategy for formation of binary brush structures consisting of patterned poly(oligoethylene glycol) methyl ether methacrylate (POEGMEMA) and poly(cysteine methacrylate) (PCysMA), is shown in **Scheme 1**. The scheme is reproduced here for convenience. The NPPOC-APTES surfaces are first irradiated through a photomask to selectively convert the non-masked areas into surface-bound amines (step 1).[1] The amines are then reacted with an activated 2-bromoisobutyric acid to form the amide (step 2), followed by polymerisation of OEGMA to form a patterned POEGMEMA brush pattern (step 3). Step 4 consists of removing the initiating groups from the POEGMEMA brushes. This may be facilitated by several means, for example by nucleophilic substitution of the bromine groups with azide or by trapping of radicals in the ATRP process. In the next step, irradiation is repeated without the mask to deprotect the remaining NPPOC surface groups (step 5) and this is followed by initiator preparation from the remaining amines (step 6). In the final step, CysMA is polymerised from the initiator sites to form PCysMA brushes (step 7).



Scheme 1. *Strategy for the preparation of binary brush patterns using NPPOC protection group chemistry.*

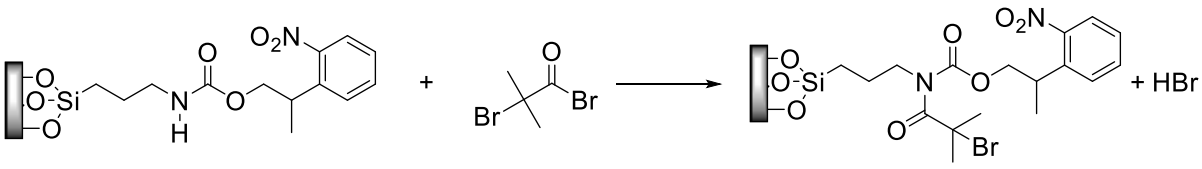
Issues

The irradiative removal of the 2-(2-nitrophenyl)propan-1-oxycarbonyl has been described both in solution[2] and on surfaces.[1] On testing the strategy proposed in **Scheme 1**, it was found that after irradiation, reaction with 2-bromoisobutyryl bromide and polymerisation of OEGMEMA (**Scheme 1**, steps 1-3), patterned POEGMEMA brushes formed, as measured by a height difference in AFM. This suggests that successful deprotection of NPPOC and acylation of the amines. However, after removal of initiator sites on POEGMEMA using sodium azide in solution, irradiation, reaction with 2-bromoisobutyryl bromide and polymerisation with CysMA (**Scheme 1** steps 4-7), TOF-SIMS showed that there was a significant amount of PCysMA *on top* of the POEGMEMA brushes. In addition, AFM indicated that the height difference between POEGMEMA and PCysMA was comparable to the height difference of the patterns before PCysMA growth. These observations suggest a combination of poor removal of initiator sites in step 4 and poor initiation efficiency from the deprotected NPPOC that had been reacted with 2-bromoisobutyryl bromide (steps 6-7). Thus, in order to optimise conditions, both the acylation step and the bromine removal step were examined in further detail.

REACTION OF THE NPPOC PROTECTING GROUP WITH 2-BROMOISOBUTYRYLATION AGENTS

Brush Growth from APTES-NPPOC treated with 2-bromoisobutyryl bromide

A literature survey revealed that acylation of carbamates with acylating reagents has been demonstrated.[2] The proposed reaction between NPPOC surfaces and 2-bromoisobutyryl bromide (BiB) to give an N-(2-bromoisobutyryl) functional NPPOC surface (BiB-NPPOC surface) is given in **Scheme 2**.



Scheme 2. *Reaction of a NPPOC surface with 2-bromoisobutyryl bromide to give a BiB-NPPOC surface.*

The 2-bromoisobutyryl functionality on the BiB-NPPOC surface can potentially act as an ATRP initiator. This was investigated by using a non-patterned BiB-NPPOC surface as a substrate for growth of PCysMA brushes and measuring the thickness using ellipsometry. The results are shown in **Figure 1**, where results from the use of an APTES-BiB initiator as polymerisation substrate are included for comparison. Brushes grown from BiB-NPPOC surface are around 10 nm thick. In contrast, brushes grown from APTES-BiB grow to a thickness of around 20 nm under the same conditions. These results indicate that the BiB-NPPOC surface does act as an ATRP initiator, which supports the proposed reaction shown in **Scheme 2**, although the initiation efficiency is significantly less than observed when using the corresponding APTES-BiB surface. The lower apparent efficiency may be due to differences in initiator structure or due to a lower surface coverage of initiator groups because of the bulkier NPPOC groups. This has not been investigated further.

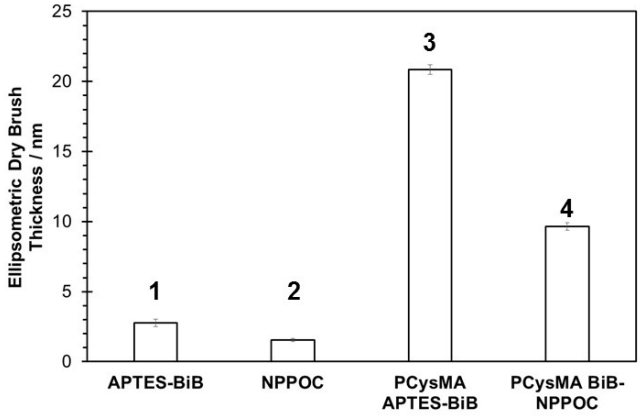
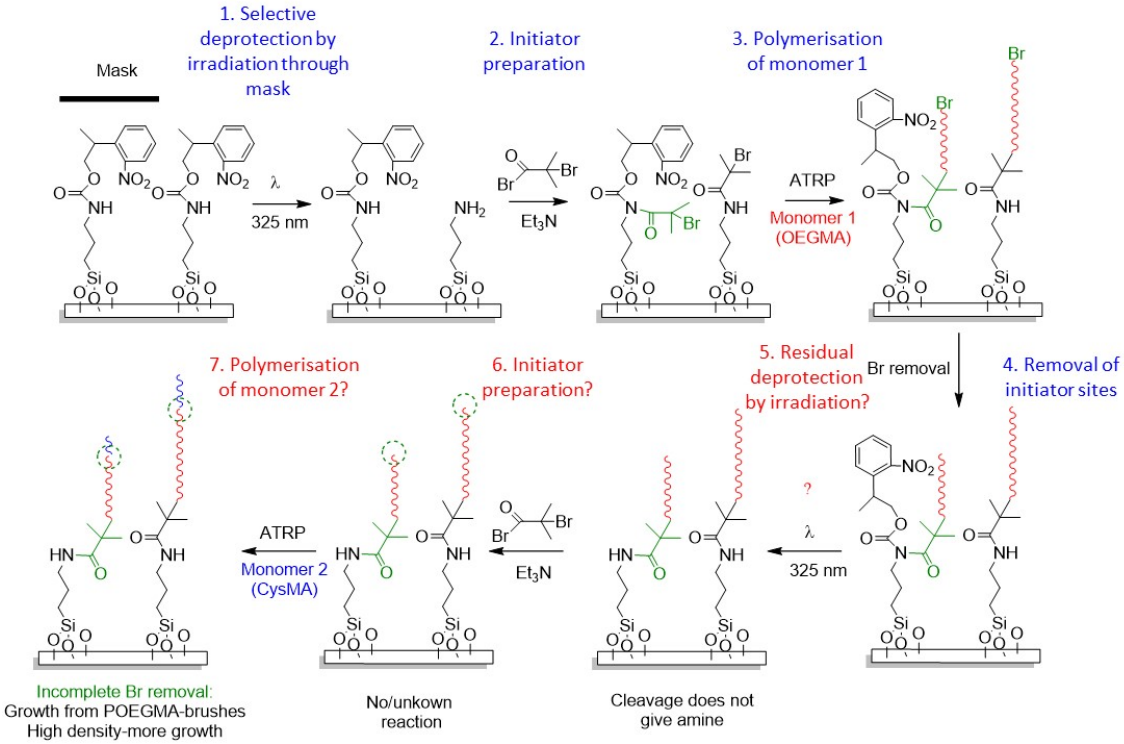


Figure 1. *Growth of PCysMA brushes measured by ellipsometry on functional silicon surfaces. (1) APTES-BiB initiator thickness. (2) NPPOC surface, non-modified. (3) PCysMA brushes grown from APTES-BiB (4) PCysMA brushes grown from BiB-NPPOC surface Conditions: [CysMA] = 0.88 mol/g in H_2O, [CysMA]:[CuBr]:[CuBr2]:[bpy] = 100:1:0.5:3. 30 °C, 2 h.*

Consequences of BiB-NPPOC formation on brush patterns

The reaction between NPPOC and BiB on surfaces and the polymer brush formation from the product may potentially be disruptive to the formation of binary brush structures, as summarised in **Scheme 3**: after patterning (step 1), BiB reacts both with amines and with NPPOC (step 2). Both of these adducts are capable of acting as ATRP initiators as demonstrated in **Figure 1**. Brushes grown from amine functionalised with BiB are thicker than those grown from the NPPOC adduct. Therefore, a pattern with thicker POEGMEMA brushes in the irradiated areas should emerge. If only the height difference is considered, this pattern would be difficult to distinguish from a pattern without POEGMEMA brushes in the non-irradiated areas. However, removal of bromine will remove initiating groups from the entire surface (step 4). Subsequent irradiation (step 5) may still cleave off the NPPOC protecting group, but the result is now an *amide* rather than an *amine*. The resulting amide is

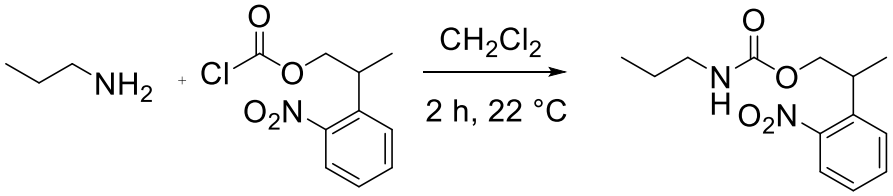
partly shielded by the POEGMEMA brushes and the reactivity towards BiB (step 6) is unknown but lower than for easily accessible amines. Therefore there is expected to be few, if any initiating groups in this area, which is why growth of PCysMA brushes (step 7) is limited. Thus, PCysMA growth is expected to mainly be due to incomplete removal of bromine in step 4. Complete removal of bromine will not lead to the formation of binary brushes.



Scheme 3. *Possible consequences of reaction between BiB-NPPOC formation on binary brush pattern formation.*

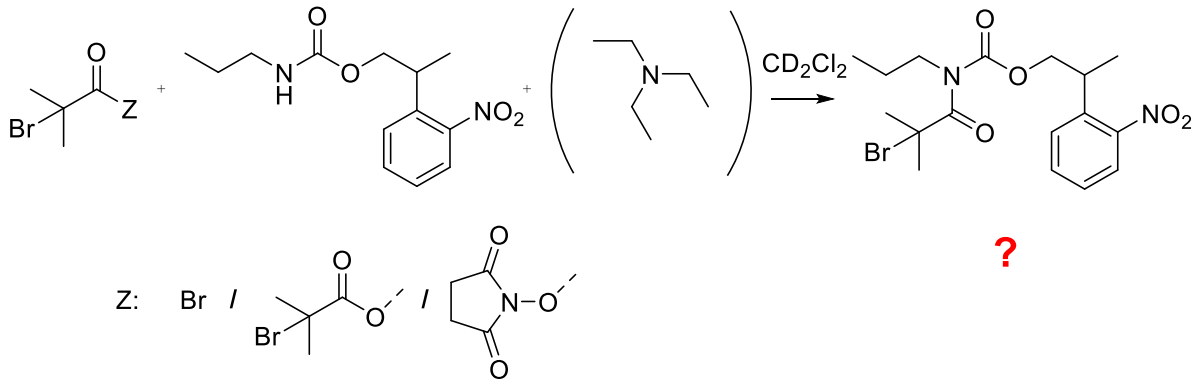
Model reactions of N-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropane with 2-bromoisobutyryl bromide: Confirmation of surface reaction by solution chemistry

Although the brush growth from surface-bound NPPOC and 2-bromoisobutyryl bromide indicated the reaction proposed in **Scheme 2**, the employment of common surface characterisation techniques such as XPS and TOF-SIMS did not allow for complete confirmation of the proposed structure. In order to confirm this, a method for testing the reaction in solution was developed. In solution, methods for structural determination such as NMR can easily be applied and, in addition, the interpretation of mass spectra for small molecules is often simpler than the interpretation of mass spectra from surfaces. Furthermore, experimental conditions in solution are relatively easy to survey, which allows for rapid optimisation to find conditions under which the protection group chemistry works.



Scheme 4. *Preparation of NPPOC-AP: NPPOC-APTES analogue without triethoxysilane.*

Triethoxysilanes are relatively prone to reaction with a range of reagents, including water and carboxylic acids[3] and it is in this role they are used for attachment to silicon oxide surfaces. However, once attached, the resulting Si-O bonds are a lot less reactive and function as an inactive linker. Therefore an analogue without triethoxysilane, N-[2-(2-nitrophenyl)propan-1-oxycarbonyl]-3-aminopropane (NPPOC-AP) was prepared for work in solution in order to avoid any irrelevant side-reactions with the triethoxysilane. The synthetic route to this is shown in scheme 4.



Scheme 5. *Model reaction between NPPOC-AP and acylating reagent in solution. Typical reaction conditions: [NPPOC-AP] ~0.04 M. BiB:(Et3N):NPPOC-AP = 2:(2):1, 22 °C, 24 h.*

The reaction between NPPOC-AP and 2-bromoisobutyryl bromide (see **Scheme 5**, *Z* = Br) was tested in NMR tubes both with and without triethylamine in CD_2Cl_2. The resulting assigned ^{1}H NMR spectrum for the experiment without triethylamine are reproduced in **Figure 2** B along with the assigned ^{1}H NMR spectrum of the unmodified NPPOC-AP (see **Figure 2** A).

Before reaction with 2-bromoisobutyryl bromide, signals assigned to the single urethane proton (k,k') can be detected. These show two isomers due to the restricted rotation about the C-N bond.[4] This is reflected in the two signals with similar relative intensities assigned to the methylene protons neighbouring the urethane nitrogen (h,h') which reflect the different interaction with the urethane proton. After reaction with 2-bromoisobutyryl bromide, the signals assigned to the urethane proton has disappeared and in addition, the methylene protons (h) now only constitute one triplet due to coupling with i. This suggests substitution on the urethane nitrogen. In addition, the aromatic pattern is essentially unchanged, which excludes any aromatic substitution. Around 2.0 ppm a signal from the methyl groups of the isobutyryl imide (l) has appeared slightly upfield from the methyl groups of excess 2-bromoisobutyryl bromide. All other signals from the NPPOC-AP can be identified in the spectrum and none of these exhibit any significant shift. Finally, the TOF ES+ signal shows a dominant peak at m/z = 437.2, which corresponds to the molecular mass plus the mass of sodium of BiB-NPPOC-AP. In addition, the peaks integrate as expected from the structure of BiB-NPPOC-AP. Combined, these results suggest that substitution of BiB on the urethane nitrogen of NPPOC-AP is an efficient high-yielding reaction. This supports the hypothesis that BiB-NPPOC forms on NPPOC functional surfaces. The reaction was also found to proceed in the presence of triethylamine with comparable efficiency (not shown, but see below).

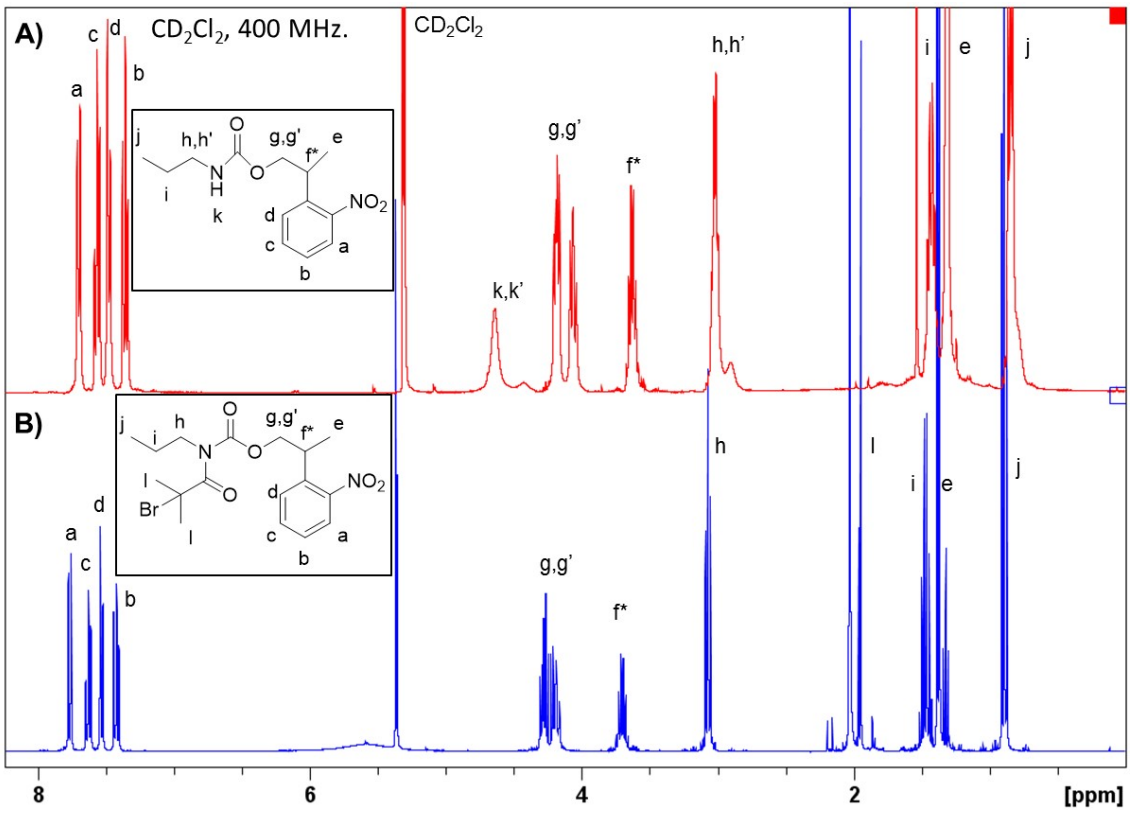


Figure 2. *Assigned ^{1}H NMR spectra of A) NPPOC-AP and B) 0.04 M NPPOC-AP exposed to 2 equivalent BiB in CD_2Cl_2 after 24 h at 22 °C.*

Exploiting a model reaction in solution to examine reaction of NPPOC-AP with other amino-reactive 2-bromoisobutyrate acylation agents.

The model reaction in solution presents an efficient tool in finding conditions where acylation of amines proceed but where NPPOC-AP does not react as it is easy to test other acylating reagents and experimental conditions.

Commercially available alternatives to BiB as acylating reagent are 2-bromoisobutyric anhydride (BiAnh) and 2-bromoisobutanoic acid N-hydroxysuccinimide ester (BiNHS). These react efficiently with amines but are generally less active than the acid bromide; this is why their reaction towards NPPOC-AP was tested in solution. The ^{1}H NMR spectrum of the reaction between NPPOC-AP with two equivalent 2-bromoisobutyric anhydride and four equivalent triethylamine is shown in **Figure 3** A. After 120 h at room temperature, signals from the urethane proton (k,k') are still visible. In addition, the aromatic pattern remains unchanged and while the methylene protons neighbour to the urethane nitrogen are mostly hidden under signals assigned to triethylamine at around 3 ppm, the pattern and position of the signals assigned to methylene protons neighbouring the urethane *oxygen*, g,g' at 4.0-4.3 ppm resembles those in NPPOC-AP (see **Figure 3** B) rather than those in AP-BiB-NPPOC (see **Figure 2** B). This supports that the NPPOC-AP does not react with the anhydride. In addition, it was found that the NPPOC-AP did not react with 2-bromoisobutanoic acid N-hydroxysuccinimide ester either, but that both reagents reacted with 1-aminopropane as expected (not shown).

Figure 3 A shows formation of several methacrylic signals at 5.9-6.4 ppm (I, I'). These are probably due to triethylamine base induced elimination of the bromine from 2-bromoisobutyric anhydride and of adducts with triethylamine (which explains the multiple

peaks). This side reaction is undesired on a surface as it may potentially reduce the number of initiating sites. It can be suppressed by reducing the amount of base present (not shown).

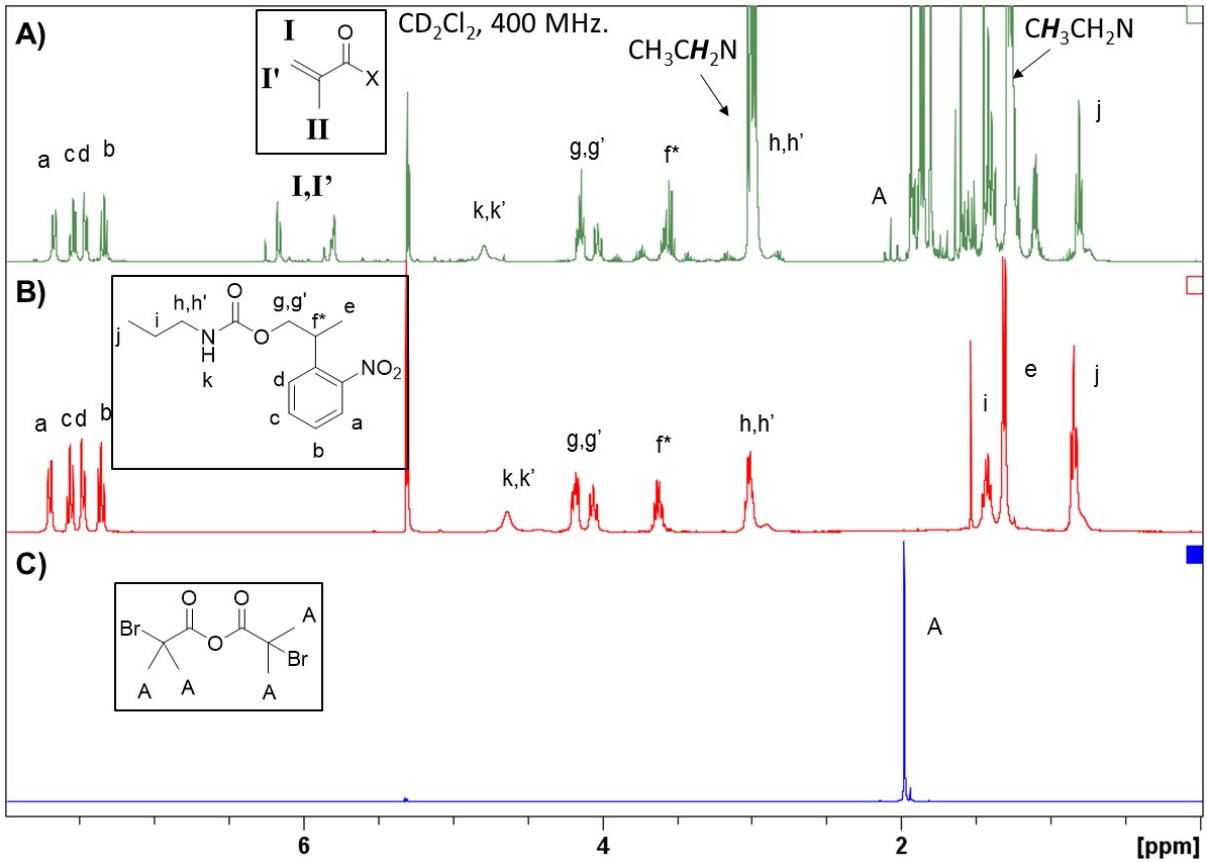
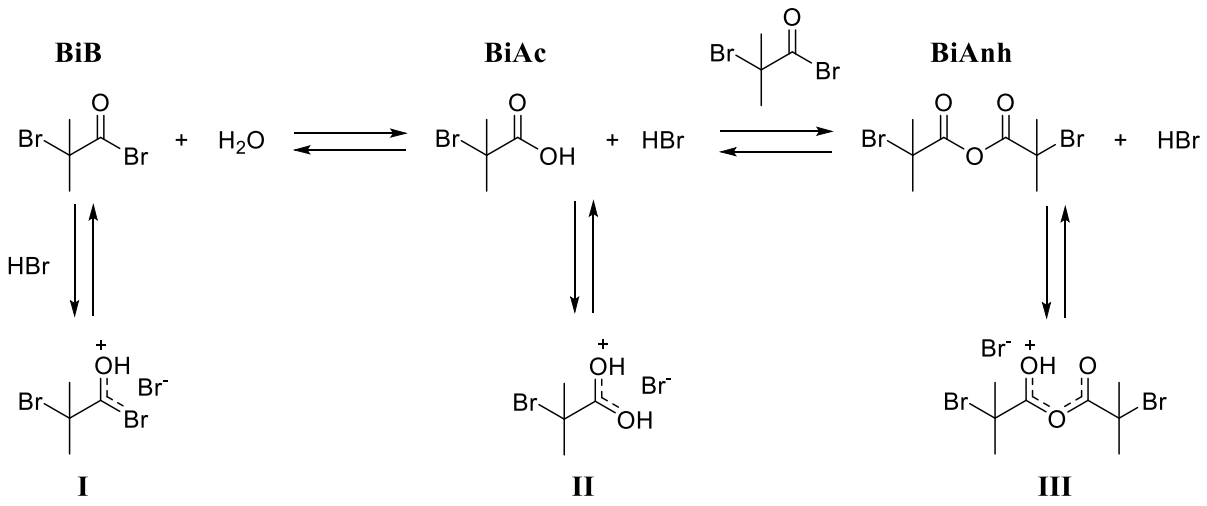


Figure 3. *^{1}H NMR spectra of A) 0.04 M NPPOC-AP with two equivalent 2-bromoisobutyric anhydride and four equivalent triethylamine reacted for 24 h at 22 °C B) NPPOC-AP C) 2-bromoisobutyric anhydride.*

Examining the effect of water on the reaction between NPPOC-AP and 2-bromoisobutyryl bromide



Scheme 6. *Addition of water to 2-bromoisobutyryl bromide*

In all experiments 2-bromoisobutyryl bromide was used as received, and all experiments described above were carried out using a bottle fresh from the manufacturer. Therefore, the purity was assumed to be in accordance to the manufacturer's information i.e. around 98 %. However, given the high reactivity of acid halides towards nucleophiles like water, reaction with atmospheric water is likely to occur upon exposure to the atmosphere if no special precautions are taken to transfer the reagent dry. This may lead to reduced reactivity over time. The reaction with sub-stoichiometric amounts of water is expected to proceed as described in **Scheme 6**: water can hydrolyse the 2-bromoisobutyryl bromide (BiB) to 2-bromoisobutyric acid (BiAc), which will liberate hydrogen bromide. The BiAc may then react with another molecule of BiB to give the 2-bromoisobutyric anhydride (BiAnh). Notably, the liberation of HBr in the process may lead to protonation of BiB, BiAc or BiAnh to give species **I, II, III**. These are expected to be less reactive towards nucleophiles than their non-protonated counterparts. One water molecule may effectively reduce the reactivity of up to four BiB molecules, therefore. Release of HBr may also reduce the reactivity of the electrophile by protonation.

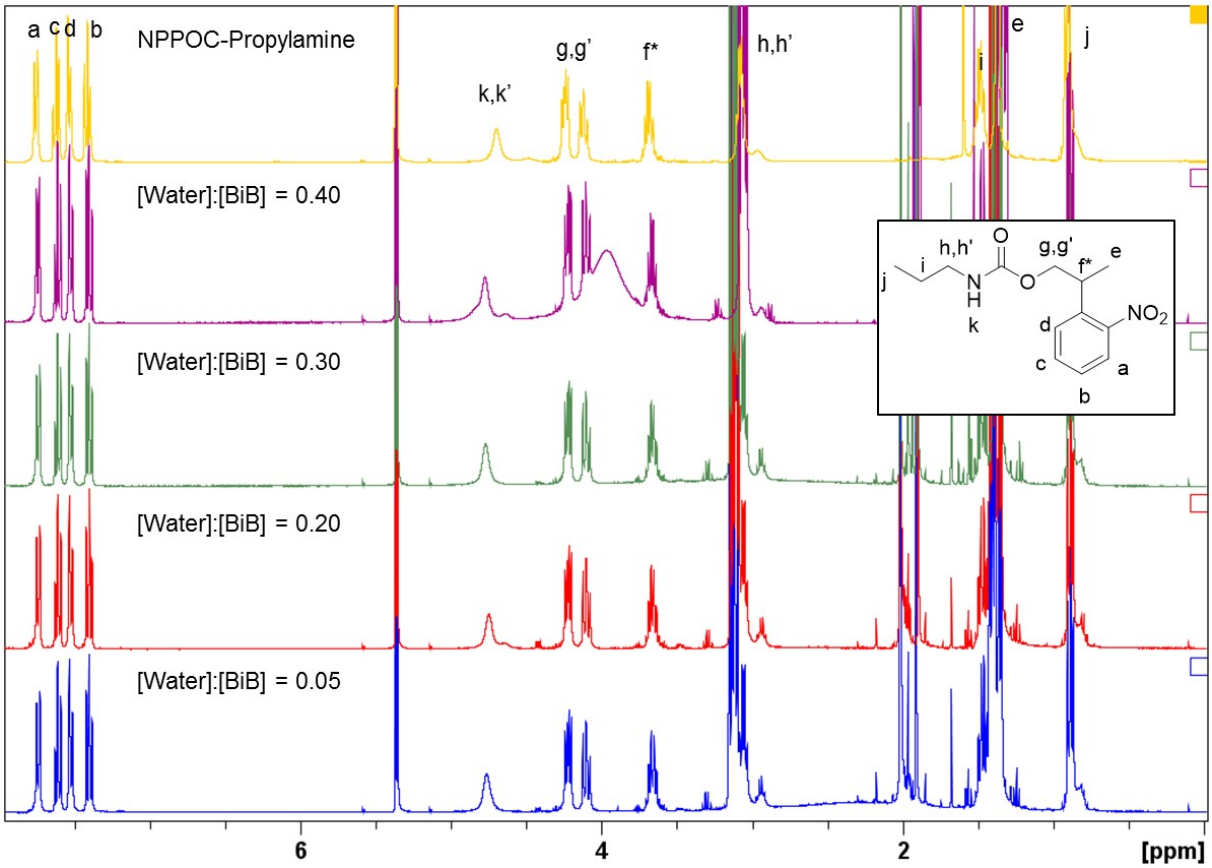


Figure 4. *^{1}H NMR spectra of the mixture of NPPOC and 2-bromoisobutyryl bromide when substoichiometric amounts of water are added to the latter. Procedure: Water and 2-bromoisobutyryl bromide is mixed separately for 5 min at room temperature. The mixture is then added to a solution of triethylamine and NPPOC in CD_2Cl_2 in an NMR tube. Conditions: [NPPOC]:[Et_3N]:[BiB] = 0.5:0.95:1. [BiB] = 0.1 M. The reaction mixture was left for 6 h at 22 °C before recording the NMR spectrum.*

The effect of sub-stoichiometric amounts of water on the reaction between BiB and NPPOC-AP was tested by pre-mixing BiB and water in molar ratios of water to BiB from 0.05 to 0.40 prior to addition to a pre-made mixture of NPPOC-AP and triethylamine in deuterated CD_2Cl_2. The resulting ^{1}H NMR spectra are shown in **Figure 4**, where they are

compared with the NPPOC-AP starting material. The peaks assigned to NPPOC-AP, in particular the urethane N-H proton (k, k') are still present and in addition, no shifts of peaks assigned to other protons (in particular g,g' and f*) are observed. This suggests that substitution on the urethane nitrogen has been suppressed. It is somewhat surprising that as little as 0.05 equivalent water leads to apparently complete suppression of the reaction based on the discussion above (where it was expected that at least 0.25 equivalent was necessary), and also that the reaction is suppressed in the presence of triethylamine. This suggests that the reaction may be more complicated than previously thought. Currently we have no explanation for this discrepancy. However, it should be emphasised that the BiB was used as received and handled in air, this is why the actual water content may be somewhat higher than the added amount.

Regardless, it is useful that the reaction of BiB with NPPOC can be suppressed completely by the addition of relatively small amounts of water. The question remains whether BiB with added water still reacts with more reactive nucleophiles such as amines. This was examined by testing the reaction between 1-aminopropane and BiB with 0.4 equivalent water (see **Figure 5**). If the reaction proceeds, the expected product is the N-propyl-2-bromoisobutyramide. As **Figure 5** shows, signals due to the amine completely disappears and are replaced with signals assigned to the amide. In particular, the signal assigned to the methylene group neighbour to the nitrogen in the amine at 2.6 ppm (A) disappears and a new signal at 3.2 ppm (a) occurs. This corresponds to the control experiment, where the amide was prepared using the anhydride. The methylene and methyl groups further down the propyl chain, B and C also shifts to lower field upon reaction (to b and c), which further supports amide formation.

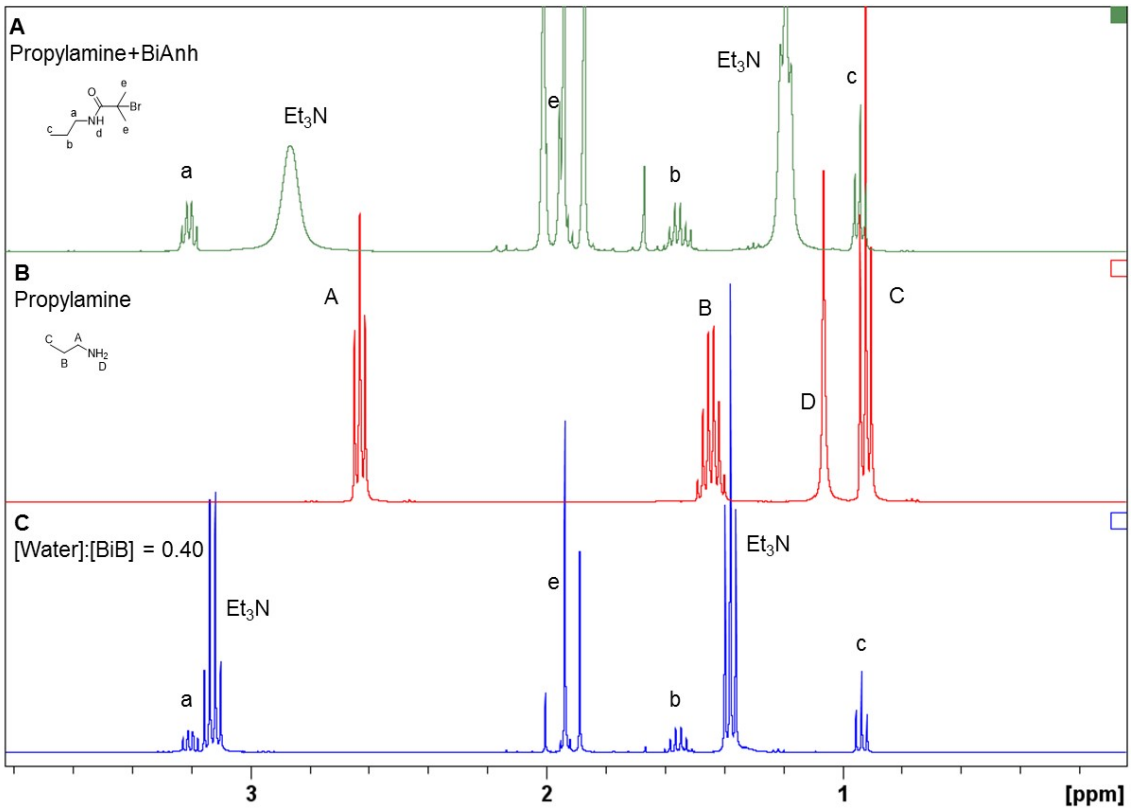
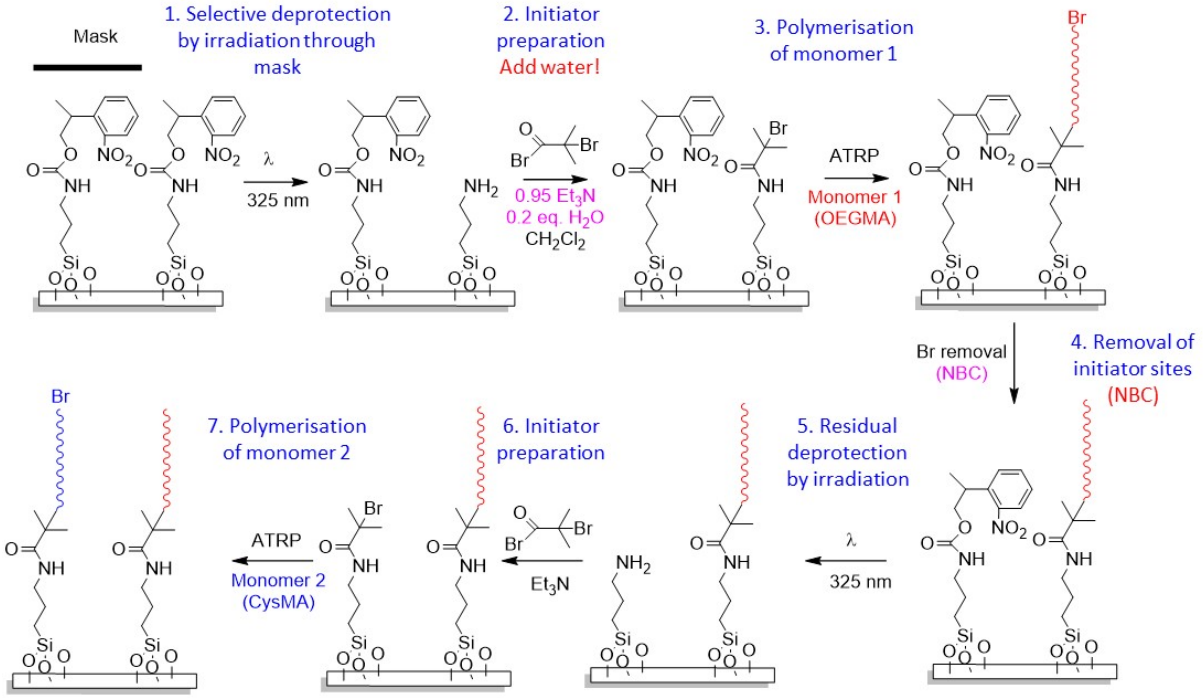


Figure 5. *^{1}H NMR spectra of the reaction of propylamine (B) with (A) 2-bromoisobutyric acid anhydride [Propylamine]:[Et_3N]:[BiAnh] = 1:2:2.7, [BiAnh] = 0.33 M and with (C) 2-bromoisobutyryl bromide pre-mixed with 0.4 equivalent water. [Propylamine]:[Et_3N]:[BiB] = 0.5:0.95:1, [BiB] = 0.1 M.*

These results suggest that the simplest route to selectively functionalise amines in the presence of NPPOC (such as after photo-patterning) is by using 2-bromoisobutyryl bromide to which a substoichiometric amount of water is added.

Formation of binary brush patterns using 2-bromoisobutyryl bromide with added water to selectively functionalise photodeprotected amines

The above results allow a strategy for the preparation of binary brush patterns using NPPOC to be devised. This is summarised in **Scheme 7**. The main differences to the original strategy (**Scheme 1**) is the addition of 0.2 equivalent water in step 2 to protect NPPOC from substitution. In addition, a spin trap (Nile Blue 2-(methacryloyloxy)ethyl carbamate, NBC)[5] has been used for termination/bromine removal instead of sodium azide as we found the former to be efficient and at the same time allow fluorescent labelling of the chains. This is consistent with the work presented elsewhere,[6] in which Nile Blue dyes were found to act as spin trap. A separate manuscript is under preparation on the use for halide removal in surface ATRP.



***Scheme** 7. Modified strategy for the preparation of binary brush patterns using NPPOC protection group chemistry with added water in step 2. Note that bromine is removed using NBC as a spin trap in situ.*

The resulting patterned surfaces were characterised by TOF-SIMS and by AFM (see **Figure 6**). TOF-SIMS show that HS^- ions specific to PCysMA are located in the expected pattern (see **Figure 6** A), which confirms the specific growth of PCysMA in these areas. In addition, $C_2H_3O^-$ which are more common to POEGMEMA (but can be an ion fragment of PCysMA) show higher intensity around the PCysMA brushes. In addition, the AFM height image in **Figure 6** C shows a height difference of around 14 nm between the 2 brushes (as opposed to around 20 nm before PCysMA growth, not shown), which suggest a PCysMA thickness of around 6 nm. Furthermore, **Figure 6** D shows a distinct phase difference between the two brushes, which supports that the two areas interact differently with the AFM tip.

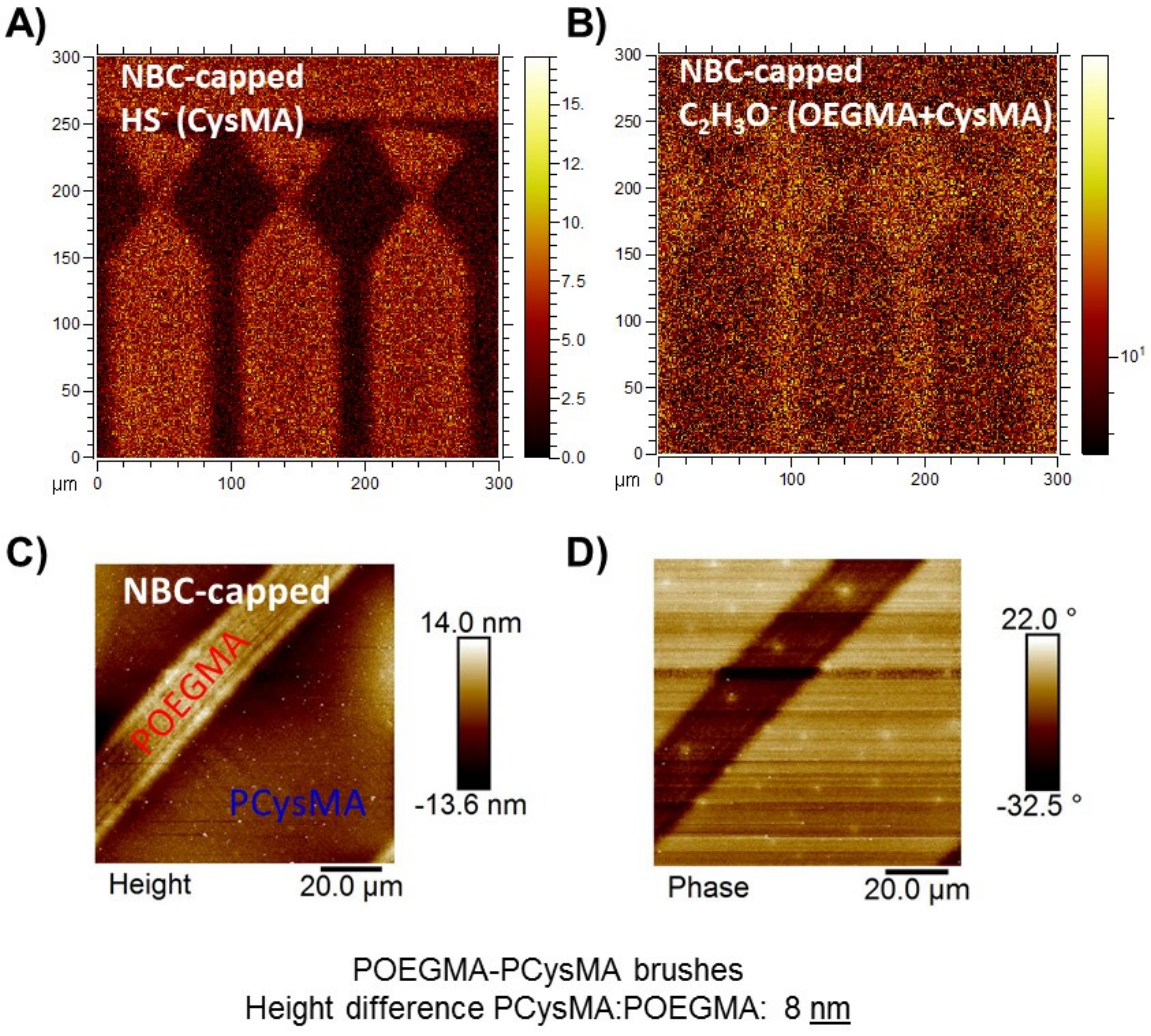


Figure 6. *Characterisation of binary PCysMA-POEGMEMA brush patterns. A) TOF-SIMS of HS^- ions specific to PCysMA on the patterned surface. B) TOF-SIMS of $C_2H_3O^-$ ions (more prevalent in POEGMEMA brushes) on the patterned surface. C) AFM height image of the patterned surface D) AFM phase image of the patterned surface.*

EXPERIMENTAL DETAILS

Materials

2-Ethylnitrobenzene (98% GC), hydrochloric acid (>30%), paraformaldehyde (>95%), 3-(triethoxysilyl)propyl isocyanate (95%), benzyltrimethylammonium hydroxide (Triton B) (40 wt. % in methanol), dimethylphenyl phosphine (DMPP) (99.0%), L-cysteine (97%), deuterium oxide (99.9%), 3-(acryloyloxy)-2-hydroxypropyl methacrylate (99%), triethylamine (>99%), oligo(ethylene glycol) methyl ether methacrylate (OEGMEMA; M_n ~ 475), 2,2'-bipyridyl (>99%), copper(I) bromide (99%), copper(II) bromide (99%), 2-bromoisobutyryl bromide (98 %, BIBB), triethylamine (99 %) and potassium dihydrogen phosphate (>99%) were supplied by Sigma-Aldrich (Poole, UK). Magnesium sulfate, dichloromethane (HPLC grade), petroleum ether (40-60), ethyl acetate (HPLC grade) and diethyl ether (anhydrous grade) were supplied by Fisher Scientific (Loughborough, UK). Silicon wafers (test grade, boron doped, <100>, 380 µm thick) were supplied by Pi-KEM (Peterborough, UK). Electron microscope grids (600 and 2000 Mesh Cu), were supplied by

Agar Scientific (Stansted, UK). 2-(Methacryloyloxy)ethyl phosphorylcholine (MPC, >99%) was purchased from Lipidure (White Plain, USA).

{*N*-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropyl}-triethoxysilane (NPPOC-APTES) was synthesised according to our previously reported methodology.[1] Cysteine methacrylate (CysMA) was synthesised as described by Alswieleh et al.[7] 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). 2-(2-Nitrophenyl)propyl chloroformate (95 %), 1-aminopropane (≥99%), 2-bromoisobutyryl bromide (98%), 2-bromoisobutyric anhydride (95 %), 2-bromoisobutyric acid N-hydroxysuccinimide ester (98%), dichloromethane (HPLC grade), Copper(I) bromide (Cu(I)Br, ≥98%), copper(II) bromide (Cu(II)Br, 99.999%), 2,2′-bipyridyl (bpy, ≥99%), N,N-dimethylformamide (DMF, HPLC grade) were purchased from Sigma-Aldrich (Poole, UK). Sulfuric acid (s.g. 1.83, >95%), hydrogen peroxide (30% v/v), ammonia solution (s.g. 0.88, 35%), Magnesium sulfate (dry), triethylamine (extra pure grade) was obtained from Fisher Scientific (Loughborough, UK).

Dichloromethane-d2 was purchased from Cambridge Isotope Laboratories

Nile Blue 2-(methacryloyloxy)ethyl carbamate (NBC) was prepared as described previously.[10]

Silicon wafers (reclaimed, p-type, ⟨100⟩) were purchased from Compart Technology (Peterborough, UK).

Silicon wafers were cleaned by immersion in piranha solution, which is a mixture of 30% hydrogen peroxide and 70% sulfuric acid, for 20-30 min, before being rinsed seven times in deionised water (caution: piranha solution is a strong oxidising agent and can explode on contact with organic material). The silicon wafers were further cleaned by immersion in a 'RCA solution' (a 5:1:1 mixture of H_2O: NH_4OH: H_2O_2) at 80 ºC for 20 min, before being rinsed seven times with deionised water. The resulting wafers were dried under a stream of nitrogen and placed in an oven at 120 ºC overnight.

Film Preparation

Clean silicon wafers were immersed in a 1 mM solution of NPPOC-APTES in toluene for 48 h. After the NPPOC-APTES film had been formed, they were rinsed in toluene, a 1:1 mixture of toluene and ethanol, and ethanol, before being dried under a stream of nitrogen and annealed in a vacuum oven at 120 ºC for 30 min.

Photolithography

Photolithography was carried out using two light sources: a He-Cd Kimmon IL3202R-D laser emitting at 325 nm and a Coherent Innova 300 C frequency-doubled argon ion laser emitting at 244 nm. Micrometre-scale grid patterns were fabricated by exposing planar substrates to 244 nm light through a mask consisting of a 600 or 2000 mesh copper grid held in place by a quartz disk. UV doses of either 1.52 or 3.92 J cm^{-2} were used to photodeprotect the NPPOC-APTES films using either the 244 nm or 325 nm laser, respectively. Interference lithography was conducted using a Lloyd's mirror two-beam interferometer, consisting of a sample and mirror set at an angle 2θ relative to each other. The interferogram consisted of alternating lines of dark and bright contrast with a period of $\lambda/2\sin\theta$. In this case, substrates were

exposed to a dose of 2.7 J cm^{-2} at 244 nm. The angle between the mirror and the sample in the interferometer was adjusted to determine the period of the resulting pattern.

Surface Functionalization

After photopatterning the NPPOC-APTES films they were functionalized in one of two ways. To perform surface-initiated polymerization directly, using the NPPOC group as a positive tone resist, substrates were patterned by exposure at 244 nm, washed with ethanol and immersed in a solution containing 0.4 M 2-bromoisobutyryl bromide (BIBB) and 0.4 M triethylamine in dichloromethane for 30 min. They were then rinsed with dichloromethane and ethanol, and dried under a stream of nitrogen. To prepare binary brush patterns, samples were initially treated in the same way. After growth of POEGMEMA brushes from the exposed regions by ATRP (see below), they were end-capped by reaction with sodium azide (see below) and then subjected to large-area UV illumination at 325 nm to remove all remaining photocleavable protecting groups. The exposed amine groups were derivatized by reaction with BIBB, and ATRP was conducted for a second time to produce the PCysMA brush.

Negative tone single brush patterns were also fabricated. Following exposure through a mask, NPPOC-APTES films were immersed in a 20 mM solution of trifluoroacetic anhydride (TFAA) in anhydrous THF containing 40 mM triethylamine. After 3 h the substrates were removed, washed with THF and dried under a stream of nitrogen. These substrates could then be exposed a second time at 325 nm to deprotect the remaining NPPOC-APTES, thus enabling functionalization of these regions by reaction with BIBB.

Atom Transfer Radical Polymerisation (ATRP)

Wafers functionalized with ATRP initiator were placed into carousel tubes, sealed, degassed and placed under nitrogen. In a round-bottom flask, water (10 mL) and methanol (10 mL) were added to OEGMEMA (20 mL) and the solution degassed by purging with nitrogen for 30 min. To this monomer solution, copper(I) bromide (0.37 g) and 2,2′-bipyridyl (0.81 g) were added and the solution was degassed for a further 5 min and sonicated. 1−2 mL of the monomer−catalyst solution was added to the carousel tubes and surface ATRP was conducted for 30 min at 20 °C. The wafers were then removed from the reaction solution, sonicated in water, rinsed with ethanol, and blown dry with nitrogen. For the POEGMEMA brushes that required capping, the final sonication step was omitted and the samples were rinsed in methanol and acetone, before being placed in clean carousel tubes under nitrogen. To form PMPC brushes, a similar protocol was used except that 12 g of the MPC monomer was dissolved in 10 mL of water and 10 mL of methanol and, following degassing of this solution, 2,2′-bipyridyl (317 mg), copper(I) bromide (96.9 mg) and copper(II) bromide (75.7 mg) were added. To form PCysMA brushes, CysMA monomer (5.0 g) was dissolved in water (12 mL) and, after degassing, 2,2′-bipyridyl (234 mg), copper(I) bromide (71.4 mg) and copper(II) bromide (55.6 mg) were added.

Polymer Brush Capping Procedure

ATRP is a controlled radical polymerization method. Thus it is necessary to prevent surface polymerization from regions where the first brush has been grown before a second brush can be grown from different regions of the same substrate. This was achieved as described previously[8] by using sodium azide to convert each terminal bromine atom into an azide group. Dry DMF (20 mL) was degassed for 20 min and sodium azide (0.26 g) was added to

make up a 0.20 M solution. Wafers were placed into carousel tubes under nitrogen and the sodium azide solution then added. After heating overnight at 60 °C, the wafers were removed, rinsed with water and ethanol, and dried under a stream of nitrogen.

Protein Adsorption

To form protein patterns, patterned wafers were immersed in a 20 μg mL^{-1} solution of green fluorescent protein (GFP) in phosphate buffered saline solution (PBS) and incubated overnight at 20 °C. The resulting wafers were then rinsed with PBS solution and deionised water before being dried under a stream of nitrogen.

Surface Analysis

Advancing sessile drop contact angles were measured using a Rame-Hart model 100-00 goniometer (Netcong, NJ).

X-ray photoelectron spectroscopy (XPS) was carried out using a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) with a monochromatic Al Kα X-ray source operating at 150 W with an emission current of 8 mA and a background pressure in the analysis chamber of 10^{-8} to 10^{-10} mbar. XPS data were processed using CasaXPS software (http://www.casaxps.com).

Atomic force microscopy (AFM) studies were conducted using a Digital Instruments Nanoscope V Multimode Atomic Force Microscope (Veeco, Santa Barbara, USA) with a 'J' scanner (0 - 125 μm). Samples were imaged in tapping mode using silicon probes with nominal spring constants ranging from 20 to 80 Nm^{-1}.

Static SIMS experiments were performed using a TOF-SIMS IV instrument (Ion-ToF GmbH, Münster, Germany) equipped with a bismuth ion gun and a single-stage reflectron time-of-flight analyser. High mass-resolution images were obtained by using high-current bunched mode, with Bi_3^{++} as the primary source and a target current of *ca.* 0.1 pA. All data were analysed with SurfaceLab 6 software (Ion-Tof).

Confocal fluorescence microscopy images of protein micro- and nano-patterns were recorded using a LSM 510 meta laser scanning confocal microscope (Carl Zeiss, Welwyn Garden City, UK). Samples were placed between two microscope glass slides and a drop of Citifluor was added as an antifade reagent (glycerol-PBS solution, AF1) (Citifluor Ltd., London, UK) was placed between the sample surface and the top glass slide. A 40x magnification oil dipping lens with a numerical aperture of 1.30 was used for imaging. A drop of immersion oil (Immersol 518 F, Zeiss) was placed between the slide and the lens. An Ar laser operating at 488 nm was used to excite NeutrAvidin and the resulting yellow-green fluorescence was recorded at wavelengths above 515 nm. All fluorescence images were analysed using Zeiss LSM image browser software.

Measurements of Diffusion in Supported Lipid Bilayers

1.0 mg of a ternary lipid mixture (DOPC: DOTAP: NBD-DOPE = 74:25:1) was dissolved in 1:1 chloroform/methanol at a concentration of 5 mg mL^{-1}. This lipid mixture was dried under a flow of nitrogen for 1 h and then rehydrated using 1 mL of phosphate buffer (a 10 mM mixture of sodium dihydrogen phosphate and disodium hydrogen phosphate in deionised water, pH 7.1). The solution (1.0 mg mL^{-1}) was vortex-mixed for 1 min (Vortex Genie2, Jencons Ltd, UK) to create multilamellar liposomes as a cloudy suspension. This solution

was tip-sonicated (Branson Sonifer 750, Branson Ultrasonics Corp, Danbury, CT) at 4 °C for 30 min to obtain a near-transparent dispersions of unilamellar liposomes of ~ 25 nm diameter. This dispersion was centrifuged (Heraeius Fresco 17, Thermo Fisher Scientific, Loughborough, UK) for 1 min at 14 500 *g*, after which the supernatant was retained. The suspension was diluted with phosphate buffer to 0.5 mg mL^{-1} prior to use and stored at 4 °C in the dark for no longer than five days.

Bilayer formation was conducted using a custom-built flow cell. The samples were first soaked in buffer solution for 10 min, followed by injection of an aqueous liposome dispersion (0.5 mg mL^{-1}), incubation for 1 h at 50 ^{o}C, and finally rinsing with degassed deionised water at a flow rate of 2.6 mL min^{-1}.

Fluorescence recovery after photobleaching (FRAP) studies were performed using an epifluorescence microscope (E600 Nikon, USA). The sample was illuminated and bleached using a high-pressure mercury arc lamp. The bleached spot size had a diameter of 28 μm. FRAP images were recorded using a Zyla sCMOS CCD (Andor Technology Ltd, Belfast, UK) until complete fluorescence recovery was observed. The Axelrod method[9] was used to calculate the diffusion coefficient and the mobile fraction in each case.

Synthesis of N-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropane, NPPOC-AP

1-aminopropane (0.222 g, 37.6 mmol) was dissolved in dichloromethane (10 mL). The mixture was placed on ice. After 10 min, 2-(2-Nitrophenyl)propyl chloroformate (0.497 g, 20.4 mmol) was added dropwise using a syringe and needle. The reaction mixture was removed from the ice bath and left for 2.5 h. Then, the reaction mixture was washed with water (3 x 10 mL), saturated sodium hydrogen carbonate (10 mL) and saturated sodium chloride (10 mL). After drying over magnesium sulfate, the organic phase was filtered and the solvent was evaporated. Yield: 0.418 g corresponding to 76 % based on 2-(2-Nitrophenyl)propyl chloroformate.

The assigned ^{1}H NMR spectrum is given in **Figure 2** A)

ESI-MS, m/z $(M+Na)^{+}$ 289

Reaction between NPPOC-AP and 2-bromoisobutyryl bromide

In an NMR tube was weighed off N-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropane (0.010 g, 37 mmol). To this was added 1 mL dichloromethane-d2, triethylamine (0.010 mL, 75 mmol) and 2-bromoisobutyryl bromide (0.0093 mL, 75 mmol) and the sample was submitted to NMR after 24 h at 22 °C. Experiments without triethylamine were conducted by omitting the addition of triethylamine.

The assigned ^{1}H NMR spectrum is given in **Figure 2** B)

ESI-MS, m/z $(M+Na)^{+}$ 437

Reaction between NPPOC-AP and 2-bromoisobutyric anhydride

These were conducted as described above by substituting 2-bromoisobutyryl bromide with 2-bromoisobutyric anhydride.

Reaction between NPPOC-AP and 2-bromoisobutyric acid N-hydroxysuccinimide ester

These were conducted as described above by substituting 2-bromoisobutyryl bromide with 2-bromoisobutyric acid N-hydroxysuccinimide ester.

Reaction between 1-aminopropane and 2-bromoisobutyryl bromide

To an NMR tube was added 1-aminopropane (0.01 mL, 122 mmol), dichloromethane-d2 (1 mL), triethylamine (0.034 mL, 244 mmol) and 2-bromoisobutyryl bromide (0.030 mL, 243 mmol). The sample was submitted to NMR after 24 h at at 22 °C.

Reaction between 1-aminopropane and 2-bromoisobutyric anhydride

These were conducted as described above by substituting 2-bromoisobutyryl bromide with 2-bromoisobutyric anhydride.

Reaction between 1-aminopropane and 2-bromoisobutyracid N-hydroxysuccinimide ester

These were conducted as described above by substituting 2-bromoisobutyryl bromide with 2-bromoisobutyric acid N-hydroxysuccinimide ester.

Reaction between NPPOC-AP or 1-aminopropane and 2-bromoisobutyryl bromide with added water

The influence of water was tested by pre-mixing 2-bromoisobutyryl bromide and water prior to addition to the NPPOC or 1-aminopropane. In a typical procedure, 2-bromoisobutyryl bromide (0.5000 mL, 40.5 mmol) and water (0.0036 mL, 0.20 mmol, 5.0 %) was allowed to react in a vial at 22 °C for 2 min. Then the 2-bromoisobutyryl:water mixture (0.01 mL, approximately 0.8 mmol 2-bromoisobutyryl bromide) was added to a mixture of a pre-made stock solution (1.000 mL, 42.4 mmol NPPOC-AP) of N-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropane (0.0508 mg, 0.191 mmol) in deuterated dichloromethane (3.600 mL) and triethylamine (0.0108 mL, 0.775 mmol). The reaction mixture was left for 6 h at 22 °C before the ^{1}H NMR spectrum was recorded.

The reaction with 1-aminopropane was examined by substituting 1-aminopropane for NPPOC-AP.

Surface amination was conducted using a literature protocol:[10] The clean wafers were placed in a glass rack in a vacuum desiccator. In a separate vial 0.1 mL APTES was added and this was placed open in the desiccator. The desiccator was evacuated to a pressure of less than 1 mbar and left for 30 min at room temperature. Then the desiccator was opened and the wafers were placed in an oven at 110 °C for 30 min. This procedure was found to give amine-functional surfaces with an R_a roughness value of around 0.10 nm and a sessile drop contact angle of 45-50 °.

Aminated surfaces were reacted with 2-(2-Nitrophenyl)propyl chloroformate in gas phase at 150 °C at reduced pressure: The APTES functional wafer was placed in a roundbottom flask to which was added 2-(2-Nitrophenyl)propyl chloroformate (approximately 0.01 mL) separately in an amber HPLC vial without lid. The flask was then protected from light by wrapping in aluminium foil, placed in a heating mantle and connected to a vacuum line through a 3-way valve with a liquid nitrogen cooled trap. The pressure was reduced to 1 mbar and once the pressure was reached, the flask was closed using the 3-way valve and the temperature was increased to 150 °C. The reaction was left at this temperature for 12 h. While cooling to room temperature, triethylamine (0.1 mL) was added to a second round-bottom flask (25 mL), which was connected to the free arm on the 3-way valve. When the mantle temperature reached 22 °C, the flask was connected to the triethylamine and the system was left for 2 h. The wafers were then washed with ethanol, acetone and water. NPPOC wafers prepared using this route was found to be similar to wafers prepared using {N-[2-(2-Nitrophenyl)propan-1-oxycarbonyl]-3-aminopropyl}-triethoxysilane in solution (see main text).[1]

Contact angle: 82.7 ± 2.3°

Formation of binary POEGMEMA-PCysMA brush patterns using NPPOC protection group chemistry, initial procedure

First, the NPPOC functional surfaces were deprotected by irradiation through a mask using a 325 nm laser at a dose of 10 J cm^{-2}.

The patterned wafers were then covered with a solution of 2-bromoisobutyryl bromide (0.123 mL, 1.00 mmol) and triethylamine (0.132 mL, 1.00 mmol) in dichloromethane (20 mL). After 2 h, the wafers were washed with dichloromethane, ethanol and water followed by drying under a stream of nitrogen.

Patterned POEGMEMA brushes were prepared by immersing the wafers in a nitrogen-purged solution of OEGMEMA (10 g, 0.02 mol) in deionised water (10 mL) containing CuBr (0.092 g, 0.64 mmol), Cu(II)Br (0.044 g, 0.20 mmol) and bpy (0.28 g, 1.8 mmol). The brushes were allowed to grow for 2 h at 30 °C under a nitrogen atmosphere. The wafers were washed with ethanol and water and dried under nitrogen.

Next, the bromine was removed by treatment with a solution of sodium azide in DMF (0.2 M NaN_3) for 16 h at 60 °C. The wafers were washed with water and dried under nitrogen.

Residual NPPOC groups were photodeprotected by irradiation of the entire pattern without a mask using a 325 nm laser with a total dose of 10 J cm^{-2}.

The resulting amino groups were converted into initiator groups by placing the wafers in a vacuum desiccator containing a vial of 2-bromoisobutyryl bromide (approximately 0.5 mL) and triethylamine (approximately 1 mL). The pressure was reduced until triethylamine visibly boiled. Then it was closed for 12 h, followed by washing with ethanol, acetone and water and drying under a stream of nitrogen.

PCysMA brushes were grown by immersing the wafers into 1 mL of a nitrogen purged solution of CysMA (8.0 g, 0.024 mol) in water (19.71 g) containg CuBr (0.0450 g, 0.250 mmol), Cu(II)Br (0.0359 g, 0.161 mmol) and bpy (0.1148 g, 0.7350 mmol). The brushes were allowed to grow for 1 h at 30 °C under a nitrogen atmosphere.

Formation of binary POEGMEMA-PCysMA brush patterns using NPPOC protection group chemistry, modified procedure

This procedure involves adding water to the reaction of patterned photodeprotected NPPOC surfaces with 2-bromoisobutyryl bromide as well as using NBC to remove the bromine initiator.

First, the NPPOC functional surfaces were deprotected by irradiation through a mask using a 325 nm laser at a dose of 10 J cm^{-2}.

The patterned wafers were then covered with a solution of 2-bromoisobutyryl bromide (0.123 mL, 1 mmol), triethylamine (0.132 mL, 1 mmol) and water (0.0035 mL, 0.2 mmol) in dichloromethane (20 mL) to convert the photodeprotected amino-groups into initiator groups. After 2 h, the wafers were washed with dichloromethane, ethanol and water followed by drying under a stream of nitrogen.

At this stage, Residual NPPOC groups were photodeprotected by irradiation of the entire pattern without a mask using a 325 nm laser with a total dose of 10 J cm^{-2} to avoid subsequent irradiative damage of the chromophore.

Patterned POEGMEMA brushes were prepared by immersing the wafers in 1 mL of a nitrogen-purged solution of OEGMEMA (10 g, 0.02 mol) in deionised water (10 mL)

containing CuBr (0.092 g, 0.64 mmol), Cu(II)Br (0.044 g, 0.20 mmol) and bpy (0.28 g, 1.8 mmol). The brushes were allowed to grow for 2 h at 30 °C in a nitrogen atmosphere. At this stage 0.1 mL of a nitrogen-purged solution of NBC (86.4 mg, 0.17 mmol) in ethanol (10 mL) was added through syringe and the solution was left for further 12 h at 30 °C to terminate the brushes. The wafers were then washed with ethanol, acetone and water and dried under nitrogen.

The residual amino groups were converted into initiator by placing the wafers in a vacuum desiccator containing a vial of 2-bromoisobutyryl bromide (approximately 0.5 mL) and triethylamine (approximately 1 mL). The pressure was reduced until triethylamine visibly boiled. Then it was closed for 12 h, followed by washing with ethanol, acetone and water and drying under a stream of nitrogen.

PCysMA brushes were grown by immersing the wafers into 1 mL of a nitrogen purged solution of CysMA (8.0 g, 0.024 mol) in water (19.71 g) containg CuBr (0.0450 g, 0.250 mmol), Cu(II)Br (0.0359 g, 0.161 mmol) and bpy (0.1148 g, 0.7350 mmol). The brushes were allowed to grow for 1 h at 30 °C in a nitrogen atmosphere.

Instrumentation

NMR spectra were recorded using a Bruker Avance III HD 400 spectrometer. Mass spectra were obtained using a Waters LCT spectrometer.

SUMMARY

The reactivity of the NPPOC protection group towards acylation reagents was examined. Results in solution indicate that the NPPOC protection group reacts with highly reactive acylation agents such as 2-bromoisobutyryl bromide but not with less reactive acylation reagents such as 2-bromoisobutyric anhydride or 2-bromoisobutyric acid N-hydroxysuccinimide ester. Importantly, the reactivity of 2-bromoisobutyryl bromide towards the NPPOC can be reduced by addition of sub-stoichiometric amounts of water and this was exploited to device a working strategy for use of NPPOC and 2-bromoisobutyryl bromide to prepare binary brush patterns.

REFERENCES

1. Alang-Ahmad, S. A.; Wong, L. S.; ul-Haq, E.; Hobbs, J. K.; Leggett, G. J.; Micklefield, J., Micrometer- and Nanometer-Scale Photopatterning Using 2-Nitrophenylpropyloxycarbonyl-Protected Aminosiloxane Monolayers. *J. Am Chem. Soc.* **2009,** *131*, 1513–1522.
2. Wöll, D.; Smirnova, J.; Galetskaya, M.; Prykota, T.; Bühler, J.; Stengele, K.-P.; Pfleiderer, W.; Steiner, U. E., Intramolecular Sensitization of Photocleavage of the Photolabile 2-(2-Nitrophenyl)propoxycarbonyl (NPPOC) Protecting Group: Photoproducts and Photokinetics of the Release of Nucleosides. *Chem. Eur. J.* **2008,** *14*, 6490-6497.
3. Davidson, D.; Skovronek, H., The Acylation of Amides. *J. Am. Chem. Soc.* **1958,** *80*, 376-379.
4. Arkles, B., Silicon Compounds, Silicon Esters. In *Kirk-Othmer Encyclopedia of Chemical Technology*, John Wiley & Sons, Inc.: 2000.
5. Quintanilla-Licea, R.; Colunga-Valladares, J.; Caballero-Quintero, A.; Rodríguez-Padilla, C.; Tamez-Guerra, R.; Gómez-Flores, R.; Waksman, N., NMR Detection of Isomers Arising from Restricted Rotation of the C-N Amide Bond of N-Formyl-o-toluidine and N,N'-bis-Formyl-o-tolidine. *Molecules* **2002,** *7*, 662.
6. Madsen, J.; Canton, I.; Warren, N. J.; Themistou, E.; Blanazs, A.; Ustbas, B.; Tian, X.; Pearson, R.; Battaglia, G.; Lewis, A. L.; Armes, S. P., Nile Blue-Based Nanosized pH

Sensors for Simultaneous Far-Red and Near-Infrared Live Bioimaging. *J. Am. Chem. Soc.* **2013,** *135*, 14863-14870.
7. Alswieleh, A. M.; Cheng, N.; Canton, I.; Ustbas, B.; Xue, X.; Ladmiral, V.; Xia, S.; Ducker, R. E.; El Zubir, O.; Cartron, M. L.; Hunter, C. N.; Leggett, G. J.; Armes, S. P., Zwitterionic Poly(amino acid methacrylate) Brushes. *J. Am. Chem. Soc.* **2014,** *136*, 9404-9413.
8. Chapman, P.; Ducker, R. E.; Hurley, C. R.; Hobbs, J. K.; Leggett, G. J., Fabrication of Two-Component, Brush-on-Brush Topographical Microstructures by Combination of Atom-Transfer Radical Polymerization with Polymer End-Functionalization and Photopatterning. *Langmuir* **2015,** *31*, 5935-5944.
9. Axelrod, D.; Koppel, D. E.; Schlessinger, J.; Elson, E.; Webb, W. W., Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* **1976,** *16*, 1055-1069.
10. Willott, J. D.; Murdoch, T. J.; Humphreys, B. A.; Edmondson, S.; Webber, G. B.; Wanless, E. J., Critical Salt Effects in the Swelling Behavior of a Weak Polybasic Brush. *Langmuir* **2014,** *30*, 1827-1836.