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SYNTHESIS OF *CLICKABLE* COATING POLYMERS BY POST-POLYMERIZATION MODIFICATION: APPLICATION IN MICROARRAY TECHNOLOGY

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KEYWORDS: polymer coating, click chemistry, post-polymerization modification, microarray, oriented immobilization

Abstract

In this work we report on the post-polymerization modification (PPM) of a polymer to introduce new functionalities that enable click chemistry reactions, for microarray applications. The parent polymer, named Copoly(DMA-NAS-MAPS), is composed of *N,N*-dimethylacrylamide (DMA), a monomer that self adsorbs onto different materials through weak interactions such as hydrogen bonding or Van der Waals forces, 3-(trimethoxysilyl)propyl methacrylate (MAPS) that strengthens the stability of the coating through the formation of covalent bonds with siloxane groups on the surface to be coated, and *N*-acryloyloxysuccinimide (NAS), an active ester group, highly reactive towards nucleophiles, that enables bioprobe immobilization. This copolymer has been widely exploited to coat surfaces for microarray applications, but exhibits some limitations due to the potential hydrolysis of the active ester (NHS ester). The degradation of NHS ester hampers the use of this coatings in some situations, for example when probe immobilization cannot be accomplished through a microspotting situation, but in large volumes for example in microchannels derivatization or micro/nanoparticles functionalization.

In order to overcome the limitations of NHS esters, we have developed a family of polymers that originates from the common copolymer precursor, by reacting the active ester contained in the polymer chain with a bifunctional amine. In particular, the functional groups introduced in the polymer by PPM, enable click chemistry reactions such azide/alkyne or thiol/maleimide 'click' reactions with suitably modified biomolecules. The advantages of such reactions are quantitative yields, orthogonality of functional groups and insensitivity of the reaction to pH. The new click functionalities, inserted with quantitative yield, improve the stability of the coating, enabling the attachment of biomolecules directly from solution, avoiding spotting reduced volumes (pL) of probes.

Finally we have demonstrated the applicability of the click surfaces in a highly effective solid-phase PCR for the genotyping of KRAS G12D mutation.

1. Introduction

The immobilization of bioprobes, for the development of solid phase biosensors, demands an accurate and precise control of the chemical-physical surface properties. It is fundamental to preserve capture probe functionality and maximize its density onto the surface to increase

accuracy of data analysis as well as reliability and reproducibility. It is widely recognized that, among the different methods employed to immobilize biomolecules on the surface of biosensors, those that elevate the probes from the substrate should be preferred, as they prevent sterical limitations during the hybridization of the target to probes[1,2]. Three-dimensional (3D) coatings allow the distribution of immobilization points within their thickness and are known to produce better signal-to-noise ratios, and wider dynamic ranges through a unique combination of characteristics that include low non-specific binding and high probe loading capacity[3]. In 2004, our group has introduced one of such 3D coatings (named *Copoly(DMA-NAS-MAPS)*) realized by the combination of physi- and chemisorption of a copolymer obtained by random radical polymerization of *N,N*-dimethylacrylamide, a monomer that adheres to different types of surface by hydrogen and van der Waals interactions, a silane monomer that, through condensation with hydroxyl or silanol groups, stabilizes the polymer on the surface and a succinimide ester monomer, reactive towards nucleophile groups[4]. The characteristics that have mostly contribute to its success are: simplicity of the coating procedure, that does not require chemistry labs or skilled personnel, uniformity of the nanometric coating, which is suitable for application in optical biosensors[5], low level of non-specific adsorption of bioprobes[6], together with a high immobilization density[6].

The functional group of this copolymer is a succinimidyl ester (NHS ester), highly reactive towards nucleophiles such as amine, a group naturally present in proteins and peptides that can be also easily inserted in oligonucleotides. Covalent immobilization of biomolecules through reaction with NHS ester is widely exploited [7,8,9] as the resulting amide bond is very stable. However, the use of active esters has some drawbacks: they are quite unstable in water, especially at the alkaline pH required for reaction with aliphatic amines (pH 6-9)[10]. Under these conditions, the hydrolysis of the ester group competes with the amidization process, potentially degrading significantly the efficiency of the coupling chemistry[11] and also affecting experiment reproducibility.

Another consequence of NHS ester hydrolitic instability is that, attempts to bind biomolecules to surfaces are successfull only when the molecules to be immobilized are arrayed by microspotting. In this case, a fast evaporation of water takes place, increasing probe concentration and accelerating its reaction rate with NHS ester on the surface. On the contrary, when the probe is in solution or in a macrodrop, due to NHS ester competing hydrolysis, very low immobilization efficiency is achieved[12]. In addition, buffers that contain free amines such as tris(hydroxymethyl)aminomethane (Tris) or glycine must be avoided when using any amine-

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reactive probe. Also, ammonium contaminants, often used in protein precipitation, must be carfully removed before performing conjugation. Furthermore, NHS esters are reactive not only with primary amines, but also with several other functional groups, such as secondary amines, alcohols, phenols, and thiols, thus restricting the regioselectivity of the coupling reaction.

In order to ovecome the limitations of NHS esters, we have developed a family of polymers with different functionalities that originate from the common polymer precursor Copoly(DMA-NAS-MAPS), by post-polymerization modification (PPM) of NHS ester moieties: by reacting with a proper amine, NHS ester is selectively transformed into other functional groups. The polymers, recovered by precipitation, mantain their solubility in water and self-adsorbing properties. Advantages of PPM over direct polymerization of the respective functional monomers are manifold: first of all, not every functional group can be introduced by direct polymerization. Some of them do not tolerate the polymerization conditions due to their participation in side reactions that lead to uncontrolled polymerization or deactivation. In addition, some functional groups may simply react with the polymerizable group itself, as, for example, seen in amino- or maleimido containing acrylates [13].

In this work, the functional groups introduced on the polymer by PPM enable the so called "click chemistry" reactions including thiol-based addition, azide-alkyne cycloadditions and Diels-Alder reactions[14,15] with suitably modified biomolecules. The advantages of such reactions are: quantitative yields, controlled orthogonal and chemoselective probe immobilization, insensitivity of the reaction to pH. Moreover, click chemistry reactions offer the possibility of immobilizing biomolecules directly from solution: in a recent work, in fact, we have introduced a new approach to bind antibodies onto gold nanoparticles by means of a Cu(I)-catalyzed azide/alkyne cycloaddiction [16].

In this article we show examples of application of these new "clickable" polymers in DNA microarray.

2. Materials and methods

N,N-Dimethylacrylamide (DMA), 3-(trimethoxylsilyl)propyl methacrylate (MAPS), propargylamine, *N*-(2-aminoethyl)maleimide trifluoroacetate salt, dibenzocyclooctyne-amine, cisteamine, copper sulfate ($CuSO_4$), ascorbic acid, Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), *N*,*N*-diisopropylethylamine (DIPEA), dithiotreitol (DTT), α,α'-Azoisobutyronitrile (AIBN), anhydrous tetrahydrofuran (THF), ammonium sulphate ($(NH_4)_2SO_4$), phosphate buffered saline (PBS),

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3 ethanolamine, Geneframes were purchased from Sigma Aldrich (St. Louis, MO, USA). All solvents were used as received. *N*-acryloyloxysuccinimide and 3-azido-1-propylamine were synthesized as reported elsewhere[17] [18]. All the oligonucleotides for hybridization tests, solid-phase PCR and genotyping were synthesized by MWG-Biotech AG (Ebevsberg, Germany) with the following sequences: 5'-GCCCACCTATAAGGTAAAAGTGA-3' (COCU8; modified in 5' with the following functionalities: amine (NH_2), dibenzocyclooctyne (DBCO), thiol (SH), azide (N_3)), 5'-Cy3-TCACTTTTACCTTATAGGTGGGC-3' (COCU10), 5'-DBCO-CAGGACTGTCGT-3' (SmallDBCO), 5'-Cy3-ACGACAGTCCTG-3',(SmallCy3), 5'- GCCTGCTGAAAATGACTGAA -3' (forward) and 5'-AGAATGGTCCTGCACCAGTAA-3' (reverse). COCU10 and SmallCy3 were labeled with the fluorophore Cyanine 3 for fluorescence detection. These oligonucleotides were freeze-dried and re-suspended in DI water at a final concentration of 100 µM before use.

Untreated silicon slides 1000Å Thermal Oxide (14 X 14 mm) were supplied by SVM, Silicon Valley Microelectronics Inc. (Santa Clara, CA USA) and were pretreated using a HARRICK Plasma Cleaner, PDC-002 (Ithaca, NY, USA) connected to an oxygen line.

Contact angle measurements were aquired via the sessile drop method using a CAM 200 instrument (KSVLtd), which combines video capture and subsequent image analysis.

FT-IR spectra were recordered using a Jasco-660 spectrometer and analyzed with Spectra Manager software 1.52 (Jasco, MD, USA). Dual polarization interferometry (DPI) analyses were conducted using an Analight Bio 200 (Farfield Group, Biolin Scientific, Manchester, UK) supporting Analight Explorer software.

Spotting is perfomed using a SciFLEXARRAYER S5 (Scienion, Berlin, Germany).

Gel permeation chromatography analyses were conducted with a Jasco 880 system connected to a UV detector Jasco Uvidec-100-III mounting Schodex columns OHpak SB-G (guard column), OHpak SB-804 M HQ, OHpak SB-803 HQ, and OHpak SB-802.5 HQ (New York, NY, USA). The spectra were analysed with the ChromNAV Chromatography data system (Jasco, MD, USA).

2.1 Synthesis of Copoly Azide, Copoly Alkyne, Copoly Maleimide, Copoly Thiol, Copoly DBCO and surface coating

2.1.1 Synthesis and post modification

All the copolymers have been obtained by post-modification reactions of the parent polymer Copoly(DMA-NAS-MAPS) (**Figure 1a**), constituted of DMA (97% mole percent), NAS (2% mole percent) and 3-(trimethoxysilyl)propyl methacrylate (MAPS, 1% mole percent). A schematic

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representation of the polymers obtained by post-polymerization modification is shown in **Figure 1**. In particular, we have reacted the succinimidyl copolymer with 3-azido-1-propylamine (**Figure 1b**), propargylamine (**Figure 1c**), dibenzocyclooctyne amine (**Figure 1d**), *N*-(2-aminoethyl)maleimide trifluoroacetate salt (**Figure 1e**), and cisteamine (**Figure 1f**).

The parent polymer was synthesized by free radical polymerization as reported elsewhere[19]. The total concentration of the monomer feed in the solvent was 20% w/v. Briefly, after degassing anhydrous THF with helium, DMA, NAS and MAPS were added to the reaction flask so that the total monomer feed was 20% w/v. The reaction mixture was heated to 65°C for two hours in presence of α, α′-azoisobutyronitrile (AIBN). The crude material was cooled to room temperature and diluted 1:1 with dry THF; the solution was then precipitated in petroluem ether (10 times the volume of the reaction mixture) to eliminate unreacted monomers. The polymer was collected by filtration as a white powder and dried under vacuum at room temperature. To introduce the new functionalities, a 20% w/v solution of the copolymer was prepared by dissolving it in dry THF and a 2.5 molar excess respect to the moles of NAS of the proper amine was added to the crude material, assuming that the concentration of NAS along the polymer chain is 20 mM. The post-modification reaction with *N*-(2-aminoethyl)maleimide trifluoroacetate salt required also the addition of *N,N*-diisopropylethylamine (DIPEA), while the reaction with cisteamine necessitated the addition of dithiotreitol (DTT) (both 2.5 molar excess respect to the moles of NAS). The mixture was stirred for 5 h at room temperature and then diluted 1:1 with anhydrous THF. The polymers were precipitated in petroleum ether (10 times the volume of the reaction mixture), filtered on a bucner funnel and dried under vacuum at room temperature. To further purify the obtained powder, the polymers were dissolved again in anhydrous THF to a final concentration of 10% w/v and re-precipited in petroleum ether. The powder was finally filtered and dried again under vacuum at room temperature.

2.1.2 Surface coating

Silicon oxide slides were pretreated with oxygen plasma for 10 min: the oxygen pressure was set to 1.2 bar with a power of 29.6W. Each copolymer was dissolved in DI water to a final concentration of 2% w/V and then diluted 1:1 with a solution of ammonium sulfate 1.6 M. The slides were immersed into each solution for 30 minutes, then rinsed with DI water, dried with a nitrogen stream and finally cured under vacuum at 80°C for 15 minutes.

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2.2 Goniometry

Contact angle measurements were aquired via the sessile drop method using a CAM 200 instrument (KSVLtd), which combines video capture and image analysis.

The solvent utilized in the analysis was deionized water, whose purity was confirmed by correlating the measured surface tension based on the pendant drop shape to the literature values for pure water (72 mN/m at 25 °C).

2.3 GPC analysis

The molecular weigth of each copolymer was characterized using Gel Permeation Chromatography (GPC), connected to a UV detector. The GPC setup is composed of three polyhydroxymethacrylate aqueous GPC columns connected in series with a decreasing exclusion limit and thermostatted at 40°C: OHpak SB-G (guard column), OHpak SB-804 M HQ, OHpak SB-803 HQ and OHpak SB-802.5 HQ. The molecular weight is extrapolated from a calibration curve of polyacrylamide standards with molecular weights ranging from 22KDa to 400 KDa.

Dry copolymers samples were dissolved using the GPC mobile phase (100 mM NaCl, 50 mM NaH_2PO_4, titrated to pH 3.5 using H_3PO_4, 10%v/v acetonitrile) to a concentration of 0.25 mg/mL and the samples were run three times through the GPC system to test for reproducibility. Each run injected 20 µL of sample and the flow rate through the system was set at 0.35 mL/min.

2.4 FT-IR spectra analyses

FT-IR spectra were recordered using a Jasco-660 spectrometer and analyzed with Spectra Manager software 1.52 (Jasco, MD, USA). The samples were mixed with KBr and compressed to obtain a tablet. Then 32 scans were recorded over the range 4000–400 cm^{-1} at a resolution of 4 cm^{-1} at intervals of 1 cm^{-1}.

All the spectra have been analyzed after subtracting the spectrum of a blank KBr pellet.

2.5 NMR characterization

^{13}C spectra were acquired at 500 Bruker DMX and 600 Bruker DRX spectrometers equipped with 5mm TXI probe with z gradient at room temperature (300K) to characterize Copoly Azide and Copoly DBCO polymers. About 30 mg of polymer were dissolved in in DMSO-d^6 solvent. Spectra were calibrated on DMSO solvent signal at 40.45 ppm.

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2.6 DPI measurements

To measure the coating thickness, a silicon oxynitride AnaChipT^{M} slide was pretreated with oxygen plasma, then inserted into the fluidic compartment of Analight Bio 200 and kept at 20°C under PBS flow. A solution of Copoly Azide (prepared as described in paragraph 2.1.2) was slowly injected into the chip channels at a flow rate of 6 µL/min for 15 minutes. The flow was interrupted for 30 minutes, to allow the adsoprtion of the polymer onto the surface. Subsequently, PBS was injected into the channel at a flow rate of 5 µl/min for 16 hours, to rinse the excess of polymer.

A standard calibration procedure was conducted before each experiment, using 80 % (w/v) ethanol and MQ water solutions. The collected data were analyzed using Analight Explorer software.

2.7 Functional test of microarray slides and quantification of of immobilized probe density

2.7.1 Spotting

The oligonucleotide 5'-GCCCACCTATAAGGTAAAAGTGA-3' (COCU8), with the appropriate modification (NH_2, DBCO, N_3, SH) was dissolved in a 150 mM sodium phosphate buffer solution at pH 8.5 with a final concentration of 10 µM in the presence of various additives. The spotting of azido-modified oligonucleotides on Copoly Alkyne coated slides required the addition of $CuSO_4$/Ascorbic Acid/THPTA (6.525 mM, 0.1mM, 0.4 mM, respectively) to the spotting solution to perform the click reaction. The oligonucleotides were deposited onto the surface using a non-contact microarray spotter (SCENION sci-FLEXARRAYER S5) using a 80 µm nozzle. The spot volume, temperature and humidity were precisely controlled to 400 pL, 22°C and 65%, respectively. Immediately after spotting, all the chips were stored overnight in a sealed chamber, saturated with sodium chloride (40 g/100 mL H_2O). After incubation, only the silicon chips coated with Copoly(DMA-NAS-MAPS) were treated with an ethanolamine blocking solution (50mM in 0.1 M TRIS/HCl buffer pH 9), pre-heated and kept at 50°C for 15 minutes. The slides coated with the click-modified copolymers did not need this step. Consequently, all the chips were rinsed with DI water and immersed in a solution containing 4X SSC (600 mM sodium chloride, 60 mM sodium citrate, pH 7.0) and 0.1% SDS, pre-heated at 50°C, and kept at this temperature for 15 minutes, before rinsing with DI water and drying with a nitrogen flow.

For the quantification of the mass of spotted oligonucleotide, a silicon slide with a 500 nm silicon oxide layer, coated with Copoly Azide was spotted using several concentrations (1-50 µM) of DBCO-modified COCU8 (as reported above) and analysed with the *Interferometric Reflectance*

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Imaging Sensors (IRIS) platform[20] to evaluate the mass of the immobilized probe. Images of the printed probes were acquired with Zoyray Acquire Software (Zoyray, Boston, MA, USA) and quantified providing a value of spot quality and bound mass density according to previous protocols.

2.7.2 Hybridization

The printed chips were incubated with a complementary oligonucleotide target, 5'-Cy3-TCACTTTTACCTTATAGGTGGGC-3' (COCU10) tagged with Cyanine 3 for fluorescence detection. COCU10 was diluted to a final concentration of 1 µM in an aqueous solution containing 2X SSC, 0.1% SDS and 0.2 mg/ml of BSA; 15 µl of this solution were layered on the hybridization area, and covered with a coverslip. The reaction was performed in a humid chamber at 65°C for 2 hours. Finally, the chips were washed in a 4X SSC solution at room temperature (to remove the coverslip) and then any unbounded oligonucleotide was removed using two successive rinses (5 minutes each) with a 2X SSC/0.1% SDS solution, pre-warmed at hybridization temperature (65°C). Other two washes with 0.2X SSC and 0.1X SSC, carried out both at room temperature for 1 min, were then performed and finally, the slides were dried using the nitrogen stream.

Fluorescent images of each chip were obtained using the a confocal laser scanner (ScanArray Lite, Perkin Elmer) with the laser power set at 55% and the photomultiplier tube gain (PMT) at 55% and analyzed using ScanArray Express software.

2.8 Coating stability

To evaluate the coating stability, silicon slides coated with Copoly(DMA-NAS-MAPS) and Copoly Azide were immersed in a 300 mM sodium fosfate, pH 8.5 solution for 4 hours, immediately after the coating step. Then, the slides were washed with DI water, dried with a nitrogen stream and under vacuum. Finally they were spotted using the appropriate modified (NH_2 or DBCO) COCU8 (as described in section 2.5.1) at a concentration of 10 µM, incubated with 1 µM of Cy3-COCU10 (as described in section 2.5.2) and analysed using confocal laser scanner to evaluate fluorescence intensity. Similarly, two slides that did not undergo the accelerated aging process were used as a comparison.

2.9 Binding in non spotting condition

Two glass slides were coated with Copoly(DMA-NAS-MAPS) and Copoly Azide (as reported in section 2.1.2) and a multiwell cell culture system plate was bound by gentle finger pressure to

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form 8 wells on each slide. Four wells on Copoly Azide coated slides were incubated with 20 µL of a solution of a double stranded SmallDBCO (obtained by pre-incubating SmallDBCO and its complementary strand, $SmallCy_3$, in 2X SSC 0.1% SDS 0.2 mg/mL BSA buffer for 2 hours at 33°C) at a final concentration of 1 and 10 µM. Similarly, Copoly(DMA-NAS-MAPS) slides were incubated with the amino-modified COCU8, pre-incubated with $COCU10Cy_3$ at 65°C for 2 hours (2X SSC 0.1% SDS 0.2 mg/mL BSA buffer). To confirm the specificity of the immobilization, 20 µL of a non-modified oligonucleotide (COCU8NNT), pre-incubated with its complementary strand COCU10 Cy_3, were deposited on the remaining 4 wells of each slide, at a final concentration of 1 and 10 µM. The incubation was conducted in a humid chamber for 2 h at room temperature, then they were rinsed using two consecutive washes (5 minutes each) with a 2X SSC/0.1% SDS solution, (pre-warmed at the hybritization temperature). Other two washes with 0.2X SSC and 0.1X SSC, both carried out at room temperature for 1 min, were then performed and finally, the slides were dried using the nitrogen stream and analysed using a confocal laser scanner.

2.10 Solid phase PCR on silicon slides coated with Copoly Azide and KRAS G12D genotyping

Exon 2 of the KRAS gene was amplified directly on the slides using 5’- GCCTGCTGAAAATGACTGAA -3’ (forward) and 5’- AGAATGGTCCTGCACCAGTAA-3’ (reverse) as primer set, generating a 167 bp fragment. The reverse spotted primer, modified with DBCO group at 5’ terminus, was diluted in the printing buffer (sodium phosphate 150mM, pH 8.5, 0.01% Triton X100) at a final concentration of 20 M and deposited on coated silicon slides using a piezoelectric spotter, as previously reported (see paragraph 2.7.1). In particular, 3 different subarrays were printed, in order to perform different amplification reactions using distinct DNA control template (wild-type, G12D heterozygous, G12D mutant homozygous). As described in paragraph 2.7.1, after spotting, all the chips were stored overnight in a sealed humid chamber. The following day, the slides were simply rinsed in a 4X SSC, 0,1% SDS solution for 15 minutes, then dipped in water and dried with a nitrogen stream. The PCR was performed and processed as previously reported [21]. For the genotyping, in order to denature the bound double-stranded amplicons, the silicon slides were immersed in 0.1 M NaOH for 5 min, then rinsed with water and dried. Reporter sequences and stabilizers are described by Galbiati S. and collaborators [22]. Firstly, 0.6 µL of the stabilizer oligonucleotide were mixed with 59.4 µL of hybridization buffer (2X SSC, 0.1% SDS, 0.2 mg/mL BSA) up to 1 µM final concentration and then 15 µL of this hybridization solution were deposited onto the 3 subarrays and covered with 3 cover slips. The silicon slides were incubated using the

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3 Thermomixer Comfort (Eppendorf) hybridization chamber at 20°C for 30 min. The slides were then washed in a 4X SSC buffer at room temperature (to remove the cover slips) and then quickly (30 s) in a low-salt buffer (0.2X SSC). Afterward, to detect of G12D KRAS mutation, the reporter for the wild-type and the mutated sequences and their corresponding universal oligonucleotides labelled with Cy3 and Cy5 respectively, were mixed together in equimolar concentrations (1 µM) and added to the hybridization buffer (2X SSC, 0.1% SDS, 0.2 mg/mL BSA). The chips were incubated in the Thermomixer Comfort (Eppendorf) hybridization chamber at 37° C for 1 h. Eventually, the silicon slides were washed with a series of solutions as described in section 2.7.2 and scanned as previously described [22].

3. Results and discussion

Our group has devoted considerable efforts in the development of tailored polymeric coatings for biosensing, and in particular, the copolymer introduced in 2004[4], has proven to be an ideal candidate for probe immobilization, thanks to the presence of *N*-acryloyloxysuccinimide (NAS), an active ester group, highly reactive towards several nucleophiles, in particular amine, which are naturally present in biomolecules like proteins or peptides. Despite its widespread employment, active ester coupling chemistry exhibits some disadvantages, and in addition, it lacks of orientation control when a substrate that contains several amines, for example an antibody or a peptide, must be immobilized. Potentially, a correct orientation of a bioprobe onto the surface, achieved, for example, through the so called "click-chemistry" reactions, would improve dramatically the efficiency of target capture, thus enhancing the overall analytical performance of the assay.

Considering the high reactivity of NHS ester towards nucleophiles, we propose here a simple method to insert along the polymer chain different "click" groups through post-polymerization reactions (see **Figure 1**). The widely exploited Copoly(DMA-NAS-MAPS) becomes a scaffold to produce several polymeric coatings for oriented immobilization of bioprobes by means of click chemistry reactions. Post-polymerization modifications allow the introduction of functionalities, which would otherwise be incompatible with the radical polymerization process. For istance, Zilio and collaborators have recently introduced a similar copolymer bearing alkyne groups for the immobilization of azido-modified glycans in microarray format, exploiting the copper-catalyzed azide/alkyne cycloaddition (CuAAC)[24]. In that case, the polymer was obtained through a process that included protection of the triple bond before polymerization, followed by deprotection,

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3 dialysis and liophilization. Here we propose a simpler and versatile method of functionalization based on the quantitative reaction of different amines, bearing “click” groups, with NHS ester in mild, organic conditions. The modified polymers are easily purified and collected by precipitation. The coating is then obtained, as for Copoly(DMA-NAS-MAPS), by simply immersing the slides into a diluted aqueous solution of the copolymer, and it is particularly stable to solvents[19] and high temperatures[21].

3.1 Polymer and coating characterization

3.1.1 Goniometry

We first demonstrated that the introduction of new functionalities along the polymer chain does not affect the adsorption of the coating onto the surface, nor its hydrophilicity. The water contact angle values reported in **Table 1** confirm that each of the post-modified polymers forms a coating of hydrophilicity similar to that of the parent polymer Copoly(DMA-NAS-MAPS). Only Copoly Thiol and Copoly DBCO exhibit a moderate increase in hydrophobicity, probably because of the formation of disulfur bonds in case of the Copoly Thiol and because of the presence of benzene rings, in case of Copoly DBCO. The formation of disulfide bridges does not prevent the immobilization of thiol probes onto the surface because of the easy cleavage operated by a simple thiol-disulfide exchange.

Table 1 Water contact angle measured on surfaces coated with Copoly(DMA-NAS-MAPS) and with the modified polymers

Polymer	Water Contact Angle
Copoly(DMA-NAS-MAPS)	32.96 ± 1.00
Copoly Thiol	51.50 ± 1.37
Copoly DBCO	40.44 ± 2.09
Copoly Azide	26.16 ± 1.95
Copoly Maleimide	24.83 ± 1.26
Copoly Alkyne	25.97 ± 2.19

3.1.2 FT-IR and NMR analyses

In order to verify the efficacy of the postpolymerization reactions, all the modified polymers were analyzed by FTIR and ^{13}C-NMR.

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As shown in **Figure 2**, each FTIR spectrum shows typical signals of the introduced functional group. For example, in **Figure 2a** the typical signal of triple bonds stretching is evident at 2090 cm^{-1}, while **Figure 2b** evidences typical signals of the azide groups at and 2100 cm^{-1}; the carbonyl vibration of imide group (1776 cm^{-1}) is highlighted in the maleimide modified copolymer (**Figure 2c**), and similarly, **Figure 2d** and **e** show typical signals of thiol (2512 cm^{-1}) and DBCO (1700 cm^{-1} and 754 cm^{-1}). Furthermore, the quantitative conversion of NHS ester into the new functionalities is demonstrated by the disappearance of the signal at 1740 cm^{-1} (**Figure 2f**) that corresponds to the stretching of NHS ester carbonyl present in Copoly(DMA-NAS-MAPS).

The NMR spectra of the modified copolymers were compared with that of the parent polymer to confirm the insertion of the azido and DBCO moieties. **Figure 3** highlights the spectral regions showing the main differences between the copolymers. The most indicative signals are those belonging to the different carbonyls, between 160 and 180 ppm (**Figure 3a**). In this region, a new signal appears at 173.8 ppm (**Figure 3a**, red frame) in both modified polymers and it is attributed to the new amido group formed between the amine of the substituents and the backbone. In the region between 120 and 135 ppm the DBCO aromatic signals are clearly detected (**Figure 3b**, blue frame), whereas between 48-50 and 39 ppm the azido signals are visible (**Figure 3c**, green frames).

3.1.3 GPC analysis

Molecular weigths (Mw) and polydispersity index of the polymers, obtained by GPC analyses are reported in **Table 2**. The values of both Mw and polydispersity are in line with the conditions of the synthesis, a free-radical polymerization performed in organic solvent. As expected, Mw values are very similar for all the samples, considering they all originate from a communal parent polymer. Slight differences of Mw can be ascribed to possible rearrangement and entanglement of the polymer chains in aqueous solution, due to different solubility of the newly introduced functionalities (for example, the benzene rings of DBCO are more hydrophobic than the azide).

Table 2 Molecular weigth (Mw) and polydispersity measured by GPC of the parent polymer and of the modified ones.

	Mw (g/mol)	Polydispersity
Copoly(DMA-NAS-MAPS)	1.03×10^4	3.12
Copoly Thiol	1.03×10^4	6.63
Copoly Azide	1.21×10^4	4.27

Copoly Maleimide	1.40×10^4	3.55
Copoly DBCO	4.83×10^3	2.83
Copoly Alkyne	1.25×10^4	3.31

3.1.4 Dual Polarization interferometry analysis

Dual polarization interferometry (DPI) measurements were performed to evaluate the thickness, density and mass of the coating layer. DPI technique assesses optical phase changes in an evanescent dual polarization interferometer, as comprehensively presented in reference [25]. Briefly, DPI provides information on the molecular dimension, packing and surface loading[26] of biomolecules immobilized onto a surface. A DPI chip was coated by flowing a Copoly Azide solution into the two channels, after an oxygen plasma activation step; then mass, thickness and density of the obtained polymeric film were calculated and reported in **Table 3**. The data are average of measurements collected after flowing PBS for 16 hours, demonstrating the high stability of the adsorbed polymeric film.

Table 3 Mass, thickness and density measured by Dual Polarization Interferometry of the Copoly Azide coating

	Thickness (nm)	Mass (ng/mm^2)	Density (g/cm^3)
Copoly Azide	7.83 ± 1.02	1.40 ± 0.34	0.18 ± 0.05

3.2 Functional test and quantification of immobilized probe density

To further demonstrate the successful modification of the parent polymer, we performed a functional test, spotting onto silicon/silicon oxide slides (coated by the polymers of **Figure 1**), oligonucleotides modified with a functionality specifically reactive with the one on the coating: for example, on the azide bearing coating, a DBCO-modified oligonucleotide was immobilized, while a thiol-modified oligo was spotted on the coating exposing maleimide functional groups (**Figure 4a**; see on Supporting Information the protocol for deprotection of thiolated oligonucleotides). A SiO_2 layer of 100 nm was used to exploit the constructive optical interference for enhanced fluorescence detection[27]. **Figure 4b** depicts the spotting scheme: in the central part of the array there is the functional oligonucleotide, while in two external frames there are two negative controls: an oligonucleotide modified in 5' position with an amine group, and an unmodfied

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oligonucleotide. A typical fluorescence image obtained after hybridization of the immobilized probes with their complementary oligonucleotides is shown in **Figure 4b**, while **Figure 4c** shows histograms of avarage fluorescence signal intensity of the immobilized spots on each surface. Complementary click groups consent to immobilize properly bioprobes with very high specificity, and possibly, the detection is facilitated by the presence of a stiff heterocycle (for example the maleimide ring or the triazole deriving from the reaction between the azide and the alkyne group) that orients the double strand complex on the surface. In fact, overall, the fluorescence signal, is slightly improved respect to the signal obtained onto the control surface coated with Copoly(DMA-NAS-MAPS).

As shown in **Figure 4b**, the immobilization is highly specific: no fluorescence signals were detected in the external frame where the control oligonucleotides were immobilized: in this case, no reaction occured between the surface and the probe, in fact the corresponding fluorescence signals were not discernible from the background. This is true for all reactions but for maleimide and thiol: it is well known that maleimide and thiols reacts with amine, therefore in this case a fluorescence signal, although minimal, was detected (see **Figure S1** in Supporting information). However, the intensity is negligible respect to that obtained through the specific click reaction. In fact, the addition of amine to the double bond of maleimide or the reaction between thiols and amine are slower than click reactions and are more sensitive to operational conditions, such as solvents, pH and temperature. Conversely, the absence of non-specific signals of the control oligonucleotides on the other coatings demonstrates that the postpolymerization reaction had a quantitative yield.

In a second experiment, we have evaluated the immobilization efficiency by measuring the mass of the probe grafted per surface unit using a label free sensing platform called *Interferometric Reflectance Imaging Sensor* (IRIS). Briefly, this platform is a label-free microarray sensing technique that utilizes a Si/SiO_2 biochip for high throughput, multiplexed detection. The IRIS detection principle is based on the quantification of the shifts in the spectral reflectance signature to evaluate the addition of mass onto the surface using four discrete wavelengths (455, 518, 598, 635 nm) and measuring the distinctive reflection intensities by means of a CCD camera[20].

Figure 5 shows the density of the spotted oligo (expressed as number of oligonucleotide molecules/mm^2) onto a slide coated with Copoly Azide. Copoly Azide was used in this experiment (and the following ones) as DBCO-modified oligonucleotide reacts by click chemistry in the absence of catalysts and, unlike thiolated oligonucleotides this couple of reactants does not need

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a deprotection step. In particular, different concentrations of a DBCO-modified oligonucleotide were spotted on a Copoly Azide coated slides. As shown in **Figure 5**, the probe density increased with the concentration in the spotting solution, reaching a plateau around 40 µM. The number of immobilized probe is in the order of 1,5 x 10^{11} molecules/mm^2 which corresponds to the number of molecules immobilized on the parent polymer (data not shown) and in general on 3D surfaces[28]. This result also confirms the efficiency of the bulky post-modification reaction to introduce new functionalities.

3.3 Coating stability

As already mentioned, one of the drawbacks of Copoly(DMA-NAS-MAPS) is the hydrolysis of the active ester which reduces the number of functional groups available for probe immobilization and, moreover, generates negative charges, thus affecting the overall assay performance. Functional groups such as azide or triple bonds are far more stable than NHS ester as they do not undergo the same degradation process, hence offering consistent surface characteristics over time and application conditions. To demonstrate the stability of the azide versus NHS ester, we have accelerated the rate of NHS ester hydrolysis by immersing two slides, one coated with Copoly(DMA-NAS-MAPS) and one with Copoly Azide in 300 mM sodium fosfate solution at pH 8.5 for 4 hours; the slides were then spotted using the appropriate modified (NH_2 or DBCO) oligonucleotide and hybridized with the complementary strand, labeled with Cy_3 for fluorescence detection. **Figure 6** shows the avarage spot fluorescence intensity recorded with a confocal laser scanner at the end of the assay: it is clear that on Copoly(DMA-NAS-MAPS) slides, the immobilization of amino-modified oligo is hampered by the reduction of functional groups on the surface due to hydrolysis; on the contrary azide groups do not suffer for this degradation process and the fluorescence intensity after the aging treatment is comparable to the signal obtained on the fresh coating. The difference in fluorescence intensity of Copoly Azide is negligible, and most likely conncected to the statistical chip-to-chip variability.

Overall, this behaviour suggests that the azide coating is more stable to hydrolysis, thus coated slides could be prepared far in advance before use and their performance would remain constant.

3.5 Binding in non spotting conditions

The volume of the drop delivered onto the surface and its evaporation rate are key factors in the immobilization process. In fact, when the conjugation takes place in large volumes as in microliter

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drops, the hydrolysis rate of NHS ester is quite fast and prevents probe immobilization. On the contrary, when the probe is spotted in pL volumes, the conjugation reaction takes place in peculiar conditions: the fast water evaporation causes a drammatic increase in probe concentration that facilitates binding to the surface[12]. This volume related issue is crucial in DNA immobilization, where there is just one group (for example amine in 5' position) which can selectively react with the functionality on the surfaces, whereas it is less important for other bioprobes. Proteins, for instance, are less sensitive to hydrolisis of the anchoring point as they are more prone to non-specific binding by weak, non-covalent, interactions. The use of a functional group that is not degraded by hydrolysis (such as the azide) is very useful in those situations in which spotting is not possible, for instance in the bio-functionalization of microchannels, nanoparticles, microspheres, in applications such as flow cytometry, surface plasmon resonance and next generation sequencing[12]. Here we demonstrate the attachment of DBCO-modified oligonucleotide onto a Copoly Azide coated surface without using a spotter, but simply contacting an aqueous solution of the probe with the surface. In particular, we have created 8 different areas on coated surfaces and deposited a macrodrop of 20 µL of probe in each well (**Figure 7**). As expected, based on our previous experience and as reported by Sobek and collaborators[12], on Copoly(DMA-NAS-MAPS) coating, the yield of the reaction between amino-modified oligo and NHS ester is very low in these conditions (spot of 20 µL incubated in a high humidity chamber) resulting in an extremely low probe density .

On the contrary, the binding to the surface was very efficient on Copoly Azide coated slides, because the click reaction is fast and there is no competing hydrolysis. **Figure 7** demonstrates also that the binding onto the Copoly Azide is mediated by the formation of a triazole between the azide groups on the surface and the DBCO on the oligonucleotide strand, because there is no fluorescence signal were a non-modified oligonucleotides was deposited. In fact, on the remaining four areas where two different concentrations of a non-modified oligonucleotide (pre incubated with its complementary fluorescently labeled strand) were immobilized only a minor fluorescence signal was detected demonstrating that there is no conjugation of the probe to the coated surface.

3.6 Solid phase PCR on silicon slides coated with Copoly Azide

As an example of the usefulness of the azido-modified coating, "on-chip" PCR was demonstrated in a clinically relevant application. The oncogene KRAS belongs to the signaling pathway of several different molecules. In colorectal cancer, missense mutations causing gain-of-function are often

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found in somatic cells. The identification of KRAS and BRAF mutations is clinically relevant because they distinguish patients not responding to anti-EGFR treatments. In 2013 Galbiati and co-workers developed a highly sensitive assay for the detection of common KRAS and BRAF oncogenic mutations, based on the microarray technique[22]. However, this approach shows some drawbacks such as the need of a spotter for the immobilization of the PCR products and several purification steps. Thanks to the great thermal stability of Copoly(DMA-NAS-MAPS), Damin and collaborators have improved this system by immobilizing the oligonucleotide primers directly onto the surface, thus introducing a solid-phase polymerase chain reaction (SP-PCR)[21]. Similarly, to assess the stability of the azide polymer coating, we performed SP-PCR and the genotyping of one of the G12D: the most common mutations in the codon 12 of KRAS gene.

As described in section 2.10, after the immobilization of the DBCO-modified reverse primer on the silicon slides, coated with Copoly Azide, the chips underwent the optimized SP-PCR (40 cycles, 1.9mM $MgCl_2$, 1:4 ratio reverse: forward liquid primers, 100 ng DNA template, 50°C annealing temperature). On a single silicon slide, the wild-type control sample, heterozygous and homozygous mutant DNA templates were amplified simultaneously. Afterward, the silicon slides were hybridized with the dual-colors protocol described by Galbiati et al.[22], following the denaturation of the resulting bound PCRs. Briefly, the ssPCRs bound to the surface were hybridized with a solution containing the wild-type and mutant reporters, each reporters is prolonged by a tail complementary to Cy3 and Cy5 labeled universal oligonucleotides that anneals to wild-type and mutant reporters respectively. Genotyping of G12D mutation is illustrated in **Figure 8**, that demonstrates that the peculiar characteristics of the coating such as thermal stability and tridimentionality, consents the execution of a very efficient solid-phase PCR. The presence of alternative functionalities does not interfere with the performance of the experiment, as the results are similar to the amplification and genotyping obtained with the parent polymer Copoly(DMA-NAS-MAPS)[21]. Furthermore, the genotyping of the G12D mutation was successfully achieved by applying the dual-color fluorescent hybridization to the amplicon. The solid-phase PCR combined with the genotyping of the KRAS mutation shown in this study, is used to demonstrate the potential applications of these substrates in the context of a microarray assay.

4. Conclusions

In this work we have exploited the reactivity of Copoly(DMA-NAS-MAPS), a polymer widely used to functionalize microarray slides, to generate several polymer derivatives that form coatings with

different functional groups. In particular, by a simple post modification reaction with a bifunctional amine, NAS yielded functionalities that enable click chemistry reactions. The new moieties not only allow a specific and oriented immobilization of bioprobes onto the surface, but also overcome the disadvantages connected to the hydrolysis of active ester, extending the shelf-life of the coatings that are not sensitive to water or moisture. Furthermore, the improved stability and the advantages of click reactions, enable the attachment of biomolecules directly from solution, avoiding spotting reduced volumes (pL) of probes. This is particular relevant in those applications in which spotting is not possible such as microchannel functionalization of derivatization of nanoparticles.

The polymers produced by post-polymerization reactions were extensively characterized by FTIR while the coatings, obtained by dip and rinse approach of silicon/silicon oxide slides in an aqueous solutions of these polymers, were characterized by Goniometry, Dual Polarization Interferometry, IRIS and by functional tests aimed at demonstrating the advantages provided by the new surfaces in microarray technology. In particular, the stability of one of such coatings containing azido groups, was demonstrated in a solid-phase PCR experiment for the genotyping of KRAS G12D mutation.

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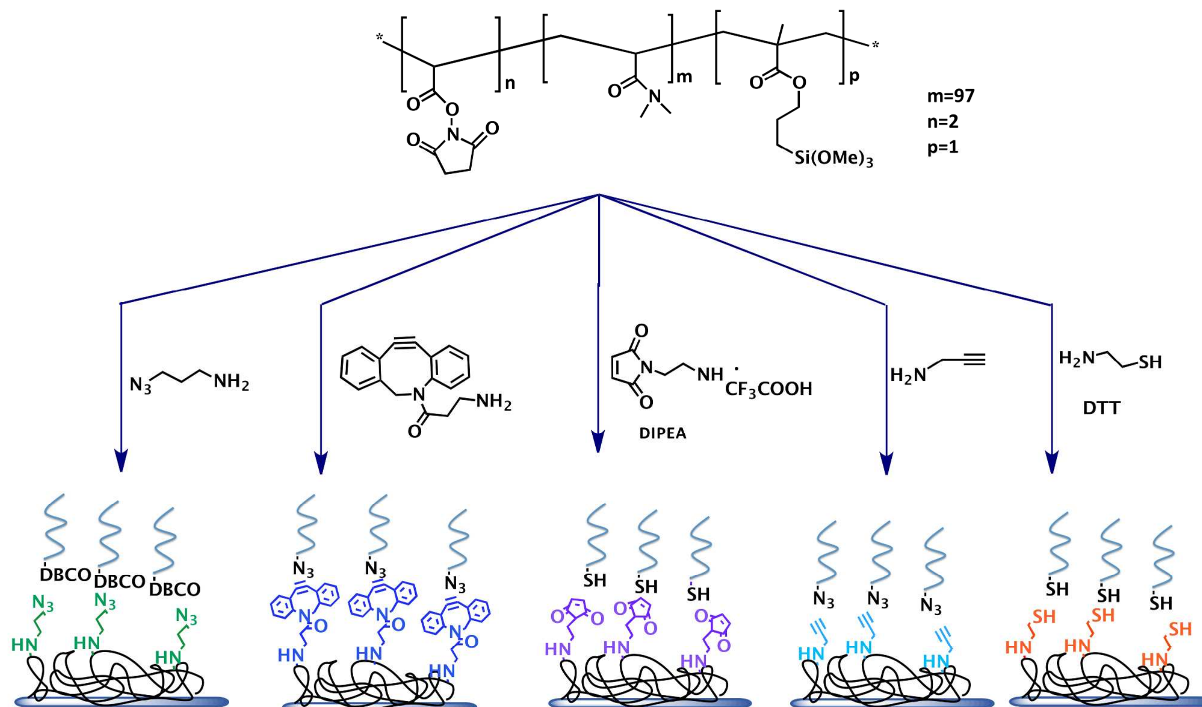

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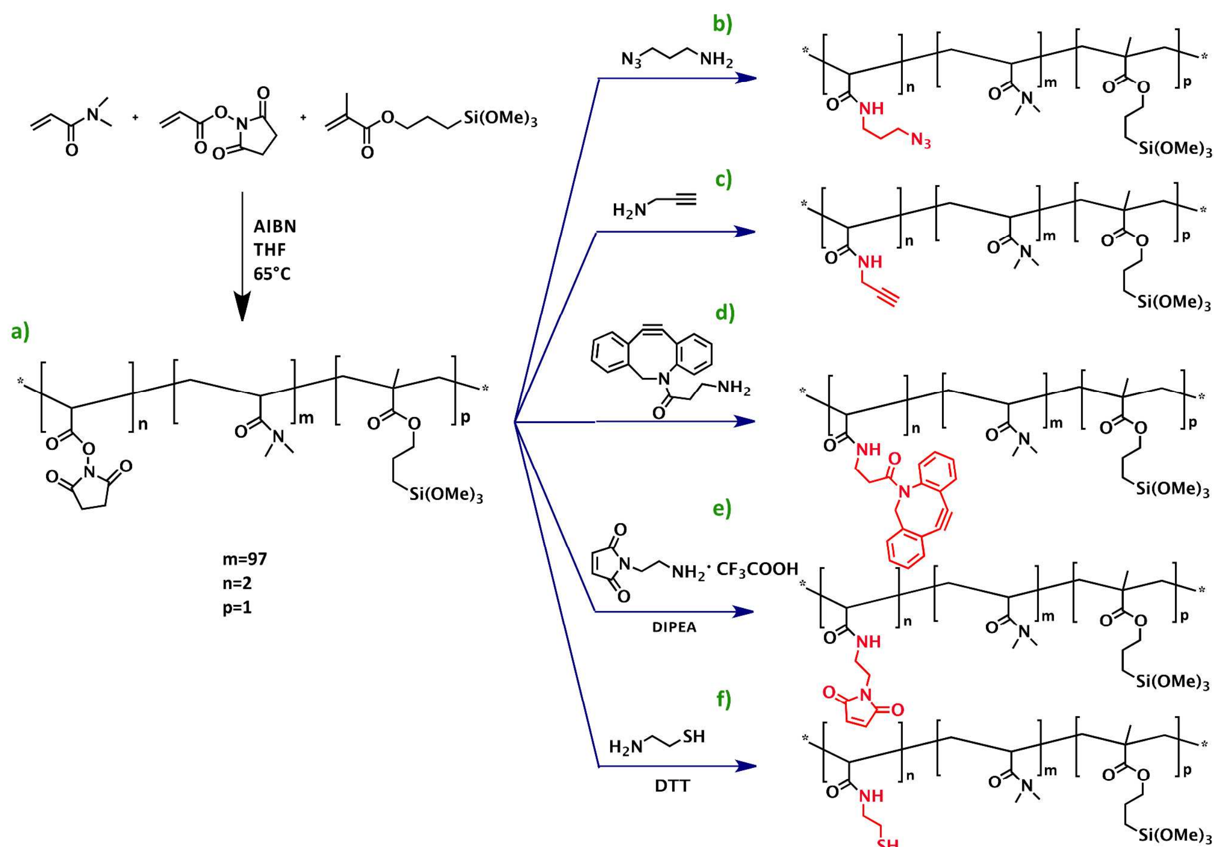

Figure 1 Scheme of the synthesis of Copoly(DMA-NAS-MAPS), the precursor of Copoly Azide, Copoly Alkyne, Copoly DBCO, Copoly Maleimide, Copoly Thiol, obtained by post-polymerization reactions with a proper amine.

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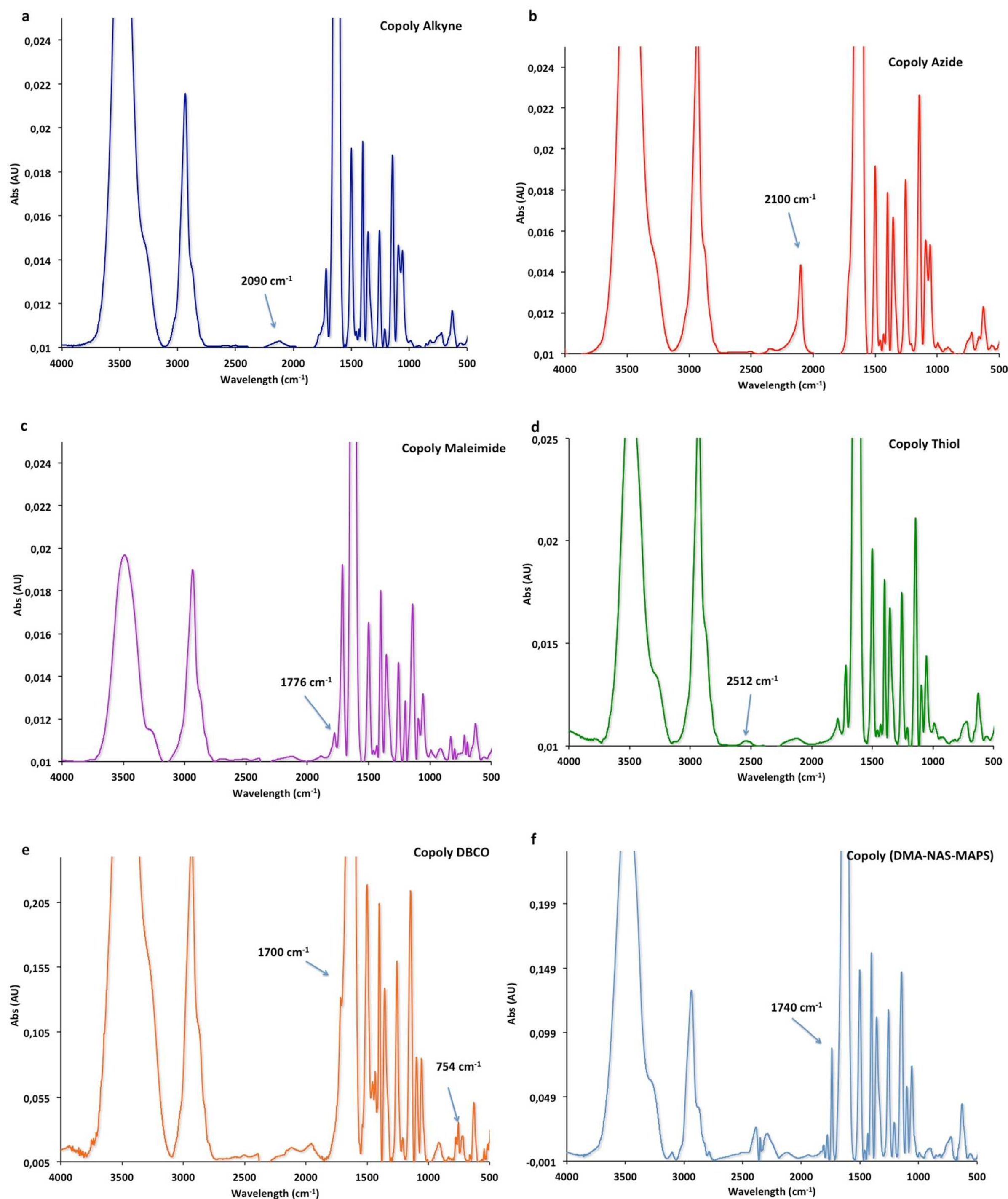

Figure 2 FTIR spectra of Copoly Alkyne, Copoly Azide, Copoly Maleimide, Copoly Thiol, Copoly DBCO and Copoly(DMA-NAS-MAPS), registered using a Jasco 660 spectrophotometer. A total of 32 scans were recordered over the range 4000-400 cm^{-1} at a resolution of 4 cm^{-1} at intervals of 1 cm^{-1}. The spectra are magnified to highlight the signals typical of each introduced group.

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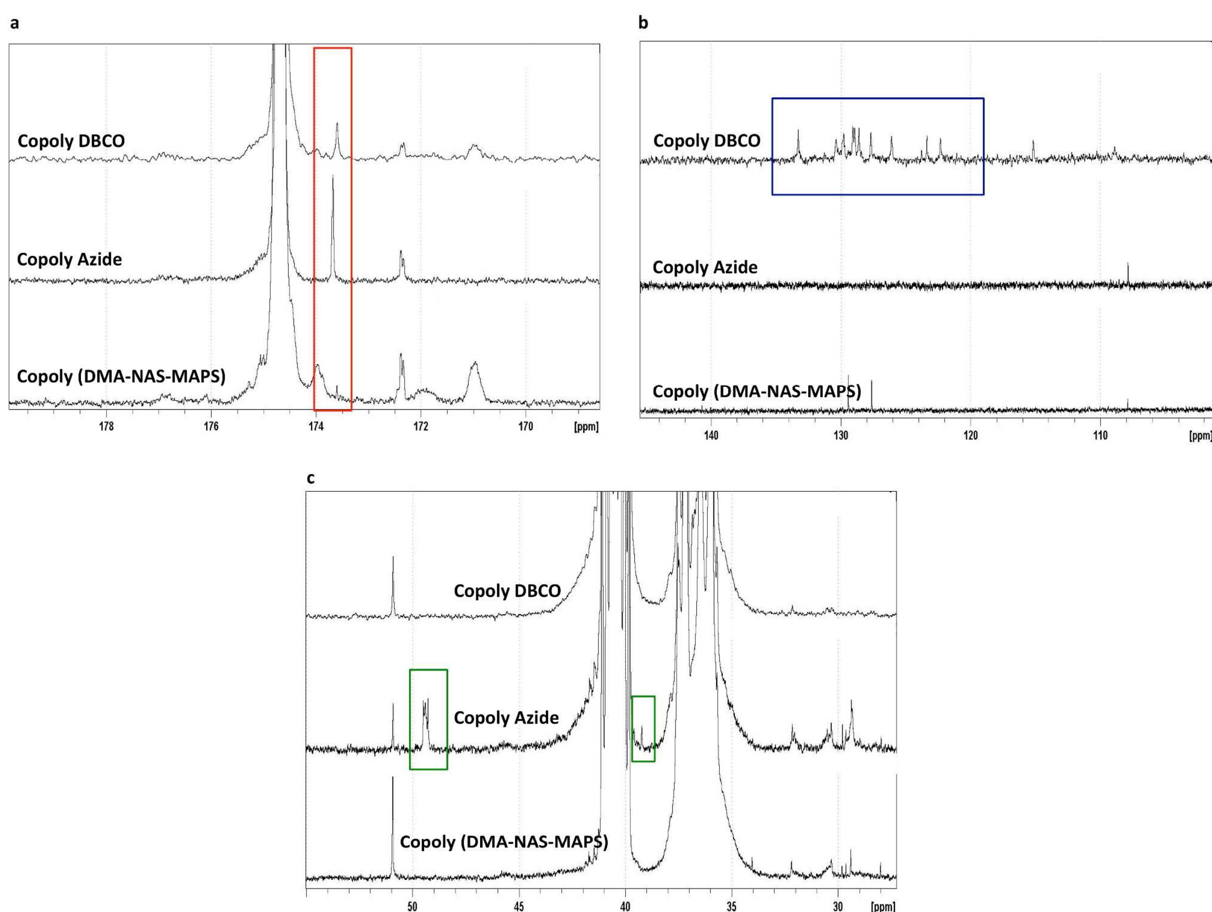

Figure 3 ^{13}C-NMR spectra of Copoly(DMA-NAS-MAPS) Copoly Azide and Copoly DBCO registered in DMSO-d^6 using a 500 Bruker DMX and 600 Bruker DRX spectrometers equipped with 5mm TXI probe with z gradient at 300K a) comparison of the three spectra to highlight the formation of a peak in the modified polymers, attributed to the formation of a new amide bond b) DBCO aromatic signals are clearly detected in the blue frame, while are not present in the other copolymers c) signals typical of azido group are highlighted in the green frames

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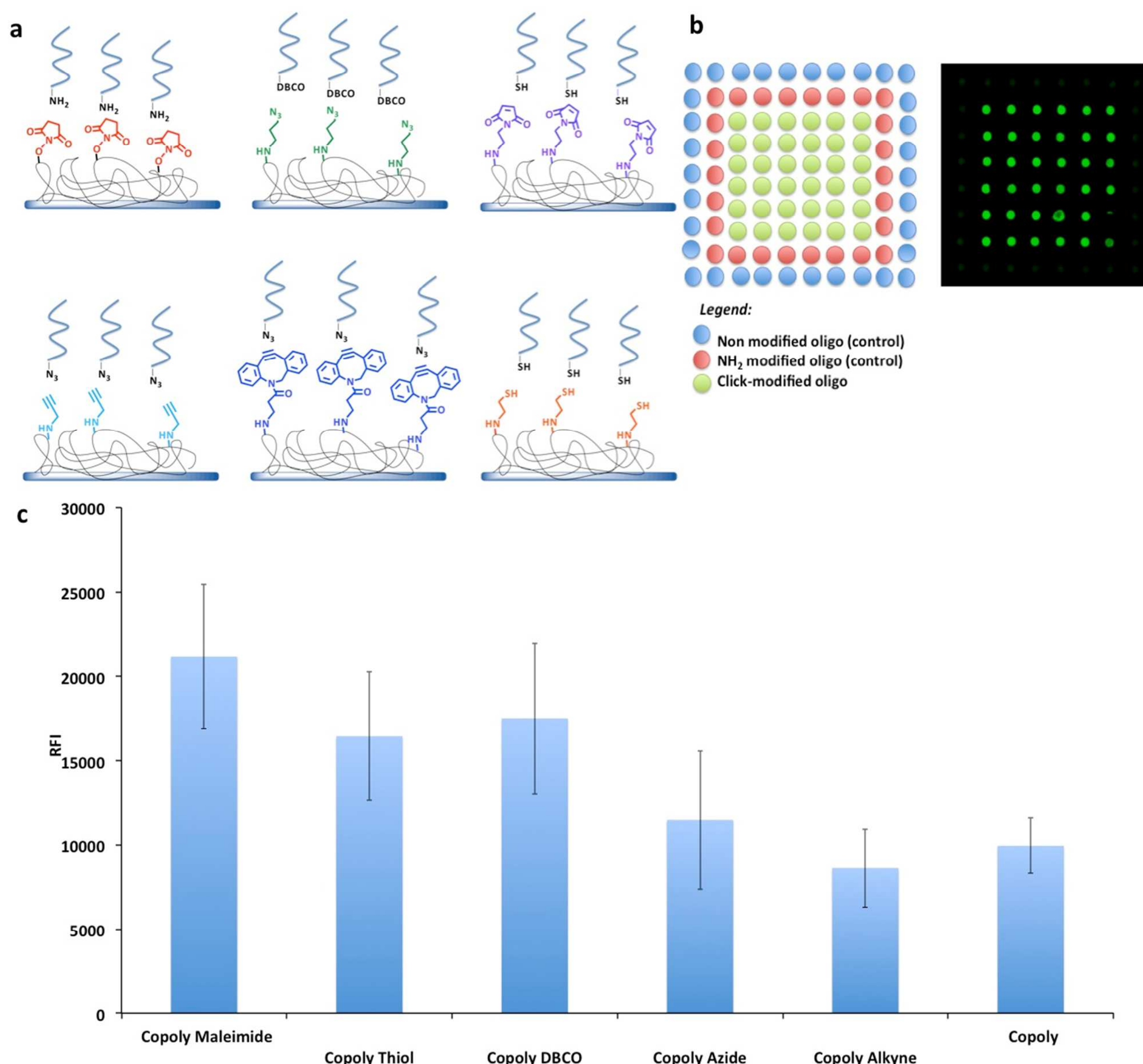

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Figure 4 a) Schematic representation of the oligonucleotides immobilized on the surfaces, coated with the *click copolymers*. In particular, each oligonucleotide has a modification in 5' position compatible with the functional group of the coating, in order to be immobilized throught a click chemistry reaction; b) spotting scheme and a typical fluorescence image obtained after hybridization of the immobilized probes with the complementary Cy3 labeled oligonucleotide; c) histograms of avarage fluorescence signal intensity of the immobilized spots on each surface. Values are the average of the spots of each array constituted of 6 x 6 spots; error bars are the standard deviations of the fluorescence intensity of each array. The arrays were scanned using 55% laser power and 55% fo the photomultiplier tube gain (PMT).

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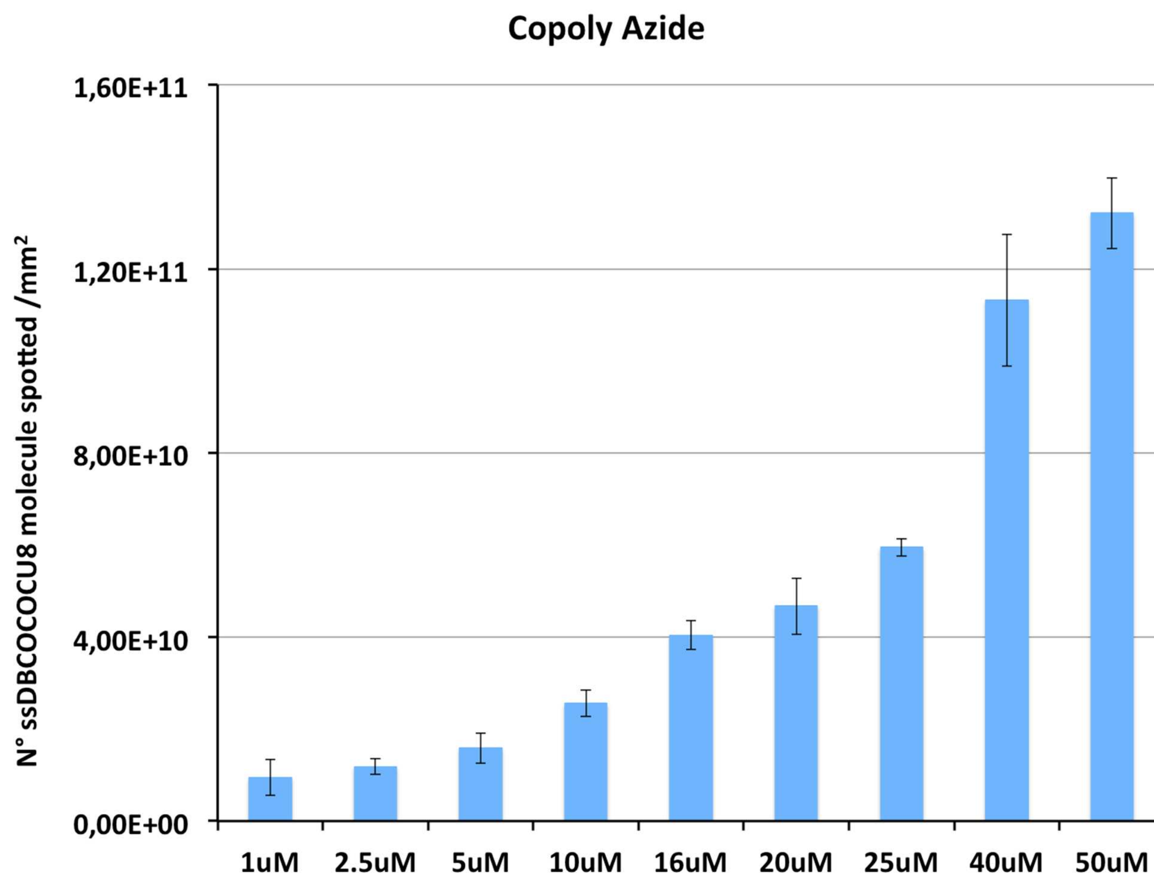

Figure 5 Histogram of the density of the spotted oligo (expressed as number of oligonucleotide molecules/mm^2) onto a slide coated with Copoly Azide. An oligonucleotide modified in 5' position with a DBCO groups was immobilised at increasing concentrations onto a Copoly Azide coated slide. After an overnight incubation in a humid chamber, the slide was washed and analysed using the *IRIS* platform. The values are average of the spots of two subarrays constituted of 6 spots each; error bars are the standard deviations of the fluorescence intensity of each subarray.

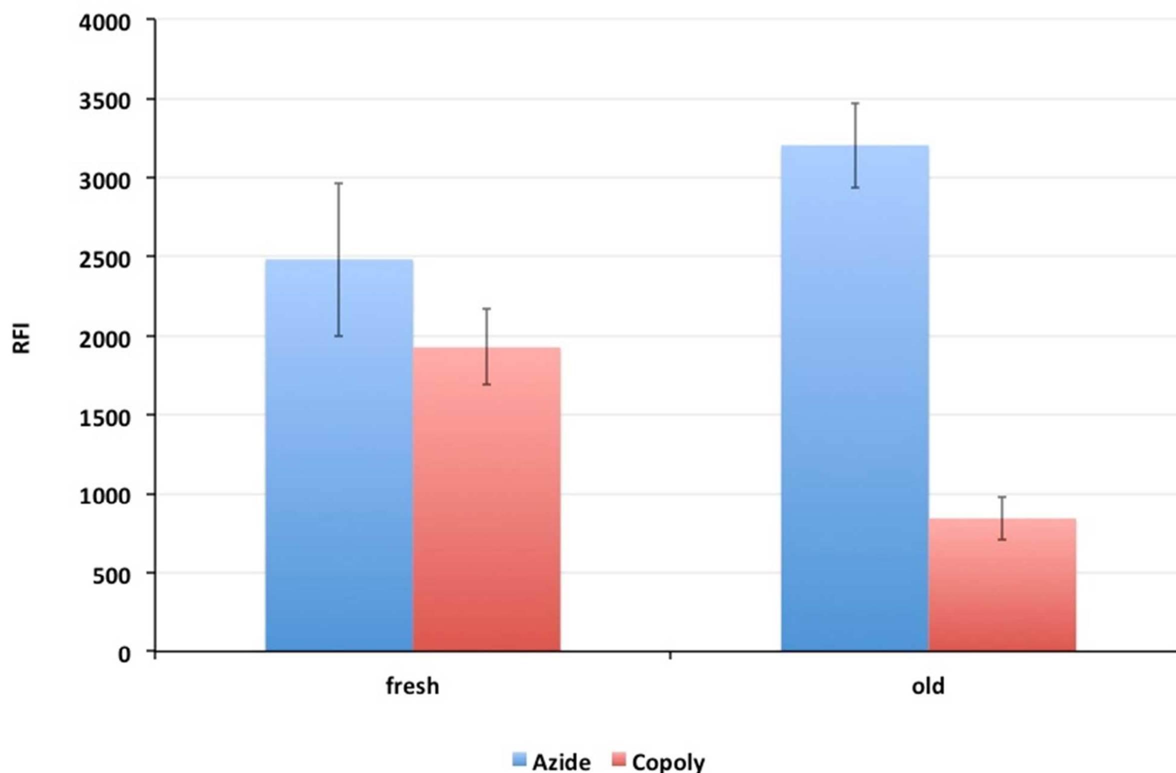

Figure 6 Fluorescence intensity signals obtained on slides coated with Copoly Azide and Copoly(DMA-NAS-MAPS) before and after the alkaline treatment to accelerate hydrolysis degradation. On Copoly(DMA-NAS-MAPS) slides, the immobilization of amino-modified oligo is hampered by the reduction NHS ester due to hydrolysis; on the contrary azide groups do not suffer for this degradation process and the fluorescence intensity after the aging treatment is comparable to the signal obtained on the fresh coating. Signals are the average of the spots of each array constituted of 6 x 6 spots; error bars are the standard deviations of the fluorescence intensity of each array. The arrays were scanned using 55% laser power and 55% fo the photomultiplier tube gain (PMT).

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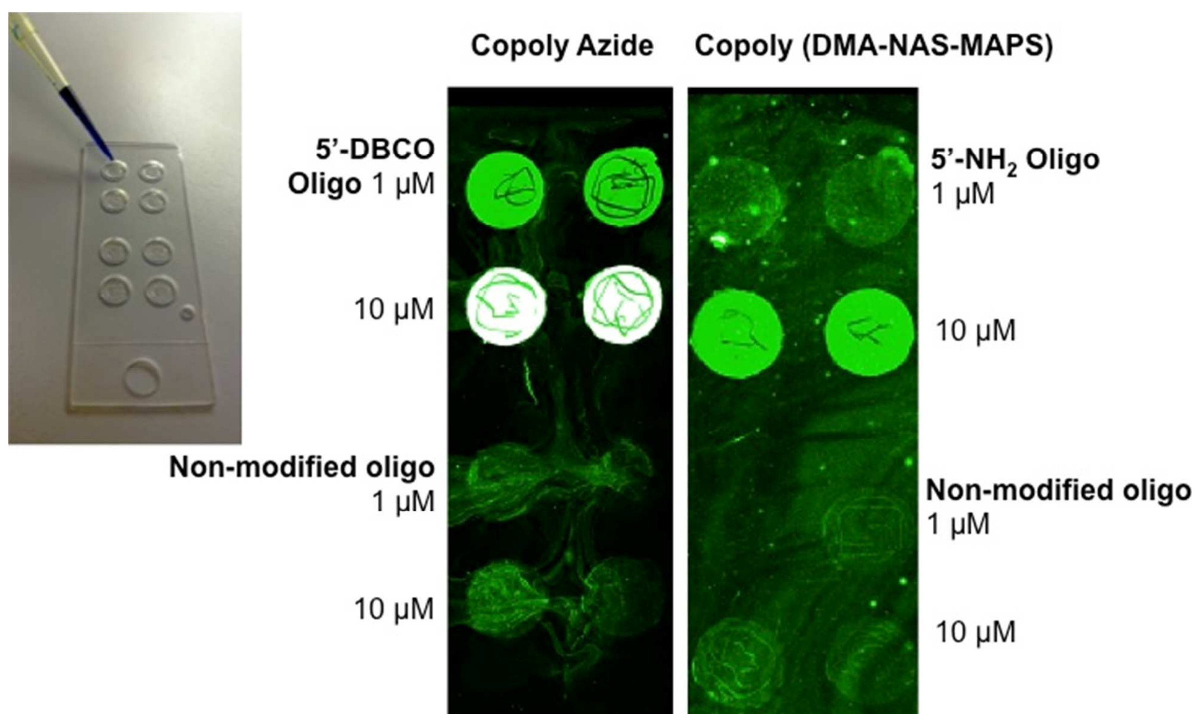

Figure 7 Image of the silicon frame used to obtain 8 wells of slides coated with Copoly Azide and Copoly(DMA-NAS-MAPS). Four wells on Copoly Azide were incubated with 20 µL of a Cy3 labeled double stranded oligonucleotide modified with a DBCO group in 5' position; similarly, four wells on Copoly(DMA-NAS-MAPS) were incubated with 20 µL of a Cy3 labeled double stranded oligonucleotide modified with an amine in 5' position. The fast hydrolysis rate of NHS ester on Copoly(DMA-NAS-MAPS) hampers the immobilization of amino probes when using large volumes, but the stability of click groups, such as the azide, facilitates the biomolecules conjugation to the surface

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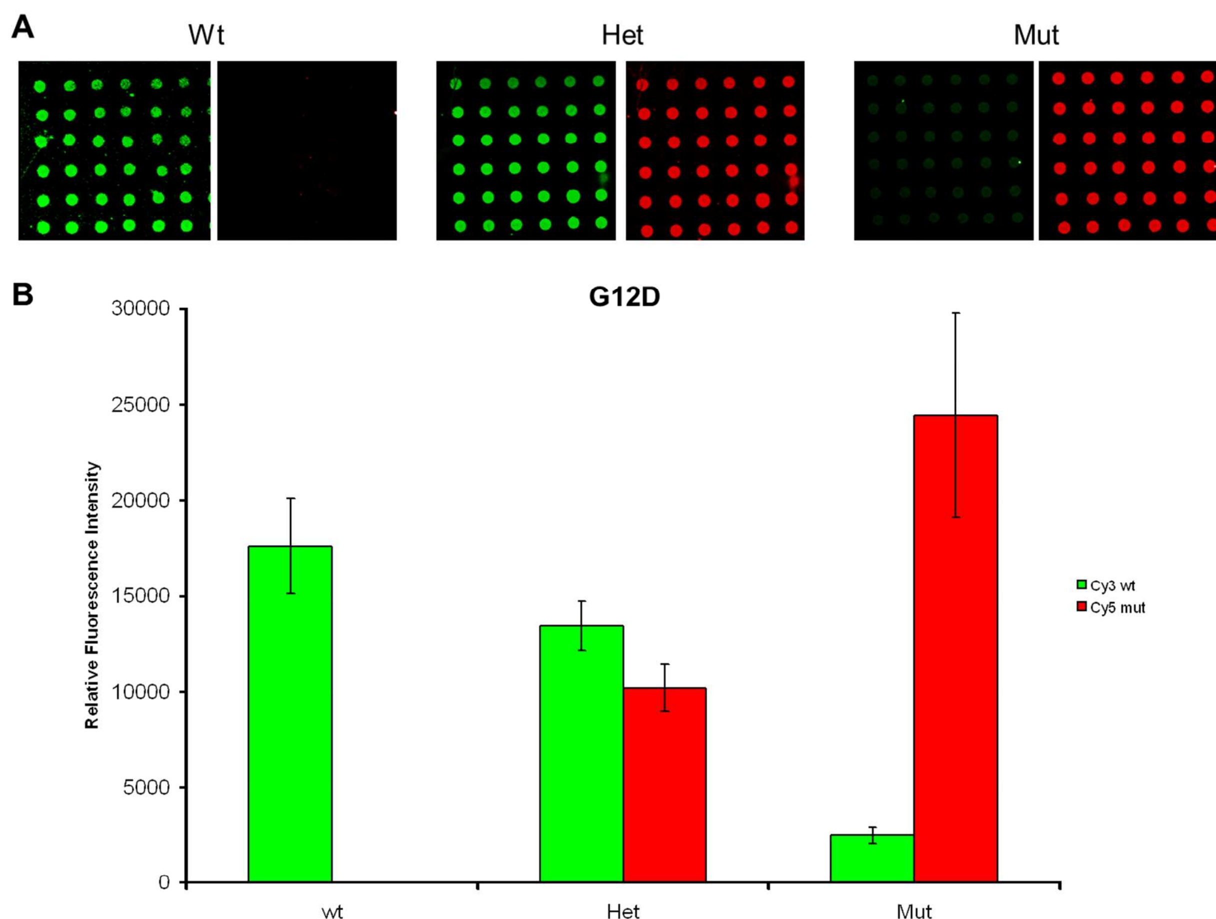

Figure 8 Microarray images for the genotyping of the G12D KRAS mutation; a) fluorescence image of the Cy3 fluorescence (green) corresponding to the wild-type allele and Cy5 fluorescence (red) corresponding to the mutated allele; b) normalized relative fluorescence intensity after hybridization of known control samples with the reporters complementary to the G12D variation. Bars are the average of the intensity of the 36 replicates of each sample. The error bars are the standard deviations of the fluorescence intensity of each sample. Wt, wild-type control sample; Het, heterozygous control sample; Mut, homozygous mutated control sample.