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Short communication

Immobilisation of oligo-peptidic probes for microarray implementation: Characterisation by FTIR, Atomic Force Microscopy and 2D fluorescence☆

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This work is dedicated to the memory of Jean-Paul Chauvet, professor at Ecole Centrale de Lyon, who initiated the project.

Abstract

Proteomic microarrays show a wide range of applications for the investigation of DNA–protein, enzyme–substrate as well as protein–protein interactions. Among many challenges to build a viable "protein microarray", the surface chemistry that will allow to immobilised various proteins to retain their biological activity is of paramount importance. Here we report a chemical functionalisation method allowing immobilisation of oligo-peptides onto silica surface (porous silica, glass, thermal silicon dioxide). Substrates were first derivatised with a monofunctional silane allowing the elaboration of dense and uniform monolayers in highly reproducible way. Prior to the oligo-peptides grafting, this organic layer was functionalised with an amino-polyethyleneglycol. The coupling step of oligo-peptides onto functionalised supports is achieved through activation of the C-terminal function of the oligo-peptides. Chemical surface modifications were followed by FTIR spectroscopy, AFM measurements and fluorescence scanning microscopy. A systematic study of the oligo-peptide grafting conditions (time, concentration, solvent) was carried out to optimise this step. The oligo-peptides grafting strategy implemented in this work ensure a covalent and oriented grafting of the oligo-peptides. This orientation is ensured through the use of fully protected peptide except the terminal primary amine. The immobilized peptides will be then deprotected before biological recognition. This strategy is crucial to retain the biological activity of thousands of oligo-probes assessed on a microarray.

Keywords: Oligo-peptide immobilization; Microarray; Biotin; Streptavidin; FTIR; AFM; Fluorescence

1. Introduction

Protein microarrays are becoming widely available and more broadly applied with a market estimated to grow up to US$ 500 million in 2006 [1]. Indeed, they help identifying proteins from different cells and under various conditions allowing their use in basic research as well as biomedicine [2–4]. Therefore, they enable high throughput technology to proteomics in order to understand protein interactions [5]; catalytic specificities [6] and identify small molecules that regulate protein activities [7].

The complexity of protein structure, which may result in the loss of protein functionality after immobilization,

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explains why proteomic microarrays still need strong improvements compared to DNA chips [8].

Developing effective, rapid and non destructive immobilisation protocols and detection methodologies applicable to protein chips is a major challenge for proteomic researchers.

The most commonly used protocol of immobilisation is adsorption. This method has been widely developed in biosensor production due to its simplicity and cost-effectiveness. However, the adsorption technique relies on non-specific electrostatic and hydrophobic interactions to immobilise proteins. Surfaces with adsorbed biomolecules in solution are susceptible to instability as desorption may occur due to the reversible nature of non-covalent attachment [9]. As an alternative, covalent binding of proteins to solid surfaces is increasingly investigated to increase the protein stability, and the control of protein-binding site availability [10]. Proper surface chemistry will allow immobilised proteins of diverse types to retain their secondary and tertiary structure, and thus their biological activity. Different strategies can be considered depending on the nature of the ligands (proteins, antibody, epitope, etc., . . .). In all the cases, the aim of covalent binding is to create an oriented linkage between surface and ligands (protein, peptide, drugs, etc., . . .) in order to homogenise and optimise the biological activity of the thousands different bio-molecules immobilised on the same support.

The other key parameter to take into consideration in protein chips development is the method of detection. Conventional enzyme-linked immunosorbent assay (ELISA) and probes labelling with fluorescent dyes were first used [11]. Subsequently, other detection methods were studied and validated. For instance, Volle et al. [12], who compared different surface characterisation techniques of biochips: ellipsometry, AFM, XPS and fluorescence spectroscopy, reported that both methodologies were applicable to a large variety of biosensors.

The approach reported herein consists of using well-defined surface chemical modifications to ensure a good uniformity and thus the best activity of immobilised ligands. Since protein activity is controlled by short amino-acid sequences (epitopes) localized at the active site of protein, we propose, besides, a strategy based on the covalent immobilisation of oligo-peptides. Such oligo-peptides will be used as probes to detect antibodies in further works. Aminosilanised porous silica, thermal silicon dioxide supports and glass slides were prepared and used as solid supports for coupling biotinylated oligo-peptides. Silanisation step was characterised by AFM measurements to estimate the morphology, and the homogeneity of the organic silane monolayer grafted at the Si/SiO_2 surface. Coupling steps were followed by FTIR spectroscopy to validate the covalent coupling. Indirect fluorescent labeling of immobilised biotinylated peptides, using Cy3-streptavidin, was accomplished to measure relative densities of peptides and to compare several immobilisation conditions. Detection was performed by fluorescence scanning. Similar strategies were successfully used by Bieri et al. [13], who immobilised G-protein-coupled receptors within a synthetic lipid bilayer membrane anchored to a self-assembled monolayer by way of streptavidin–biotin interactions, and Chapman-Smith et al. [14] who directly immobilised biotinylated proteins to supports modified with avidin.

2. Experimental

2.1. Materials

Peptide synthesis reagents, including Fmoc-Gly-Wang resin and Fmoc-Gly-OPfp, were supplied by Bachem (Bubendorf, Switzerland). Biotin O-Su was from Novabiochem (distributed by VWR, Fontenay sous Bois, France). 10-(Carbomethoxy)decyldimethylchlorosilane (CDCS) and iodotrimethylsilane (ITMS) were purchased from Roth-Sochiel (Lauterbourg, France) and used as received. Sulfuric acid 99%, hydrogen peroxide 30%, tetrahydrofurane (THF) purum grade, *N*-hydroxysuccinimide (NHS) purum grade and diisopropylcarbodiimide (DIPCDI) purum grade were obtained from Fluka (St. Quentin Fallavier, France). Polyethyleneglycol bis(3-aminopropyl) terminated (PEG) was obtained from Sigma (St Quentin Fallavier, France). Dimethylformamide (DMF), dimethylsulfoxide (DMSO) and *N*-methyl pyrrolidone (NMP) were anhydrous grade, obtained from Fluka and stored under dry gas blanket. Tween was obtained form Sigma. All other solvents and reagents were analytical grade and obtained from SDS. Water was purified using an Elga Option 4 deionised water system. Glass slides were obtained from Menzel. Silicon/silicon dioxide supports used for porous silica preparation were n-doped (phosphorus: 10^{14}–10^{15} atom/cm^3) and bearing a gold/chrome ohmic contact. They were purchased from Tronic's (Grenoble, France).

2.2. Buffers and solutions

PBS buffer (Sigma) was 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. Solution was stored at room temperature and renewed regularly. 1% Triton X-100 solution was made of 1 ml Triton X-100 diluted in 100 ml of water. Cy3-streptavidin conjugate solution was supplied from Amersham Biosciences, aliquoted in Eppendorf tubes and stored at −20 °C.

2.3. Synthesis and characterisation of oligo-peptides

Biotinylated hexa- and octaglycine were synthesized by the solid-phase method using Fmoc chemistry. Fmoc-Gly-Wang resin (140 mg; 110 μmol) was first deprotected by piperidine 20%. After washing with DMF, a cycle of coupling of Fmoc-Gly-OPfp (excess 4) was performed five or seven times in presence of HOBt. After the last deprotection, biotin-OSu was added (excess 4). The peptide was split from the resin by TFA 95%, and precipitated by ethylic ether. It

was characterised by FAB-mass spectrometry and its purity checked by HPLC (>95%).

2.4. Porous silica supports preparation

Supports of 1 cm^2 area were prepared by anodic etching of bare Si/SiO_2 wafer in HF 45%/pure ethanol solution (1:1, v/v) during 6 min at 20 mA/cm^2 under illumination (Tungsten lamp 60 W), thus forming porous silicon supports. Samples were washed with water, dried under nitrogen flow and heated at 500 °C for 2 h under synthetic air to oxidise porous silicon to porous silica. Porous thickness was 9 μm, and the size of the meso-pores was distributed between 0.5 and 1 μm [15]. Samples were then ready for silanisation.

2.5. Chemical functionalisation of porous silica, thermal Si/SiO_2 supports and glass slides

Silanisation was performed following protocols previously described [16]. Cleavage of methyl ester was carried out under mild conditions with iodotrimethylsilane [17], supports were left to react in 0.1 M ITMS solution in anhydrous dichloromethane overnight at room temperature. The generated carboxylic acid terminal groups were then activated overnight with an equimolar mixture of NHS and DIPCDI in THF, at room temperature to obtain NHS activated esters. They were then left to react with 0.1 M PEG in THF overnight at room temperature to generate an aminated surface, washed with THF, ethanol and water. The supports could then be stored at room temperature in darkness for several weeks.

2.6. Atomic Force Microscopy characterisation

A topographical investigation of the silane surface was performed by Atomic Force Microscopy (AFM). We used a stand alone SMENA (NT-MDT) microscope operating in air using the amplitude modulation mode, spring constants of 5–14 $N\,m^{-1}$ driven near their resonant frequency of 150–300 kHz. Nominal tip curvatures were less than 10 nm.

2.7. Oligo-peptide immobilisation

After solubilisation of the biomolecules, carboxylic acid terminal moieties of oligo-peptides were activated with NHS/DIPCDI overnight at 22 °C (peptide:NHS:DIPCDI molar ratio of 1:2:2). 0.5 μl of NHS activated peptide solutions were then deposited onto functionalised glass slides, and left to react under saturated solvent vapours at 22 °C. After the reaction, each slide was (1) rinsed with deionised water, (2) washed in 40 ml of 1% Triton X-100 aqueous solution at room temperature 15 min under ultrasounds, (3) rewashed in 40 ml of water, 5 min under ultrasounds, and (4) stored in water until use.

2.8. IR characterisation

Fourier Transform IR spectroscopy was performed on porous silica supports using an ATR universal Perkin-Elmer spectrophotometer. Spectrophotometer was purged with anhydrous nitrogen for 15 min to remove traces of water vapour. Sixteen scans were recorded for each sample, with a wave number resolution of 4 cm^{-1}.

2.9. Labelling of biotinylated peptides immobilised onto glass slides with Cy3-streptavidin conjugates

An aliquot of Cy3-streptavidine conjugate solution was left to warm at room temperature, then diluted 1:100 with water. Ten microliters of diluted solution were deposited per peptide spot, and left to incubate at 22 °C in darkness and water saturated atmosphere for 5 h. Slides were then thoroughly rinsed with water, and dried under gaseous nitrogen flow.

2.10. Fluorescence scanning

Slides were analysed using a fluorescence scanner developed at LEOM (for more details see [18]). The laser was filtered (514 nm) and focused on the surface sample with the objective of microscope. Fluorescent light emission was collected through the same objective, filtered (560 nm) and directed to a photomultiplier.

2.11. Fluorescence image analysis

Fluorescence scans were analysed using a homemade software named “Target”, and dedicated to monochromatic fluorescence image analysis. Relevant fluorescence signals were extracted from each spot using a semi-automatic “4 sigma” image analysis segmentation algorithm. This algorithm was used to select pixels considered as significantly fluorescent, and discard pixels corresponding to background fluorescence. The average pixel fluorescence intensities is computed for each spot. Results correspond to the average of several repetitions (minimum 3)

3. Results and discussion

Primary amine was chosen as surface chemical group for either peptide immobilisation or peptide direct synthesis. Immobilisation of peptides can be performed by reaction of their C-terminal end with amine-derivatised surface. Several carboxylic acid activation methods are available to yield reactive intermediates. In our work NHS ester activated proved to be the most efficient for our application and present results will only deal with this activation method. Primary amine derivatized surface is also of interest for direct chemical synthesis of peptides on solid support, using Fmoc strategy [19]. This approach on our functionalised surfaces will be presented in further papers.

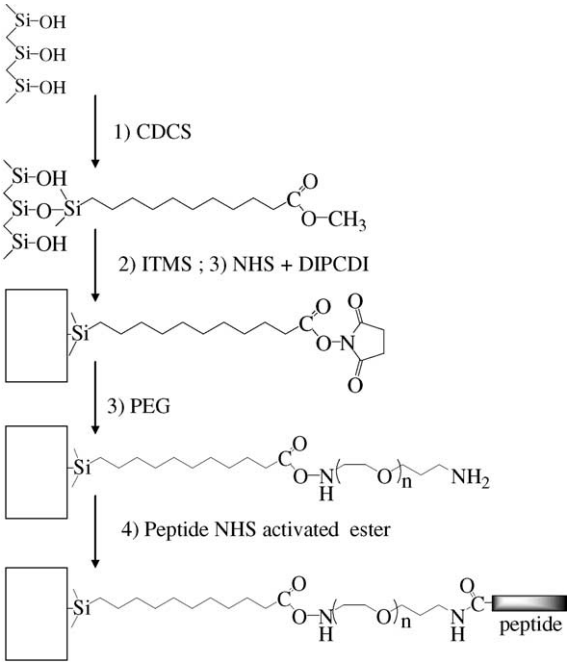

Fig. 1. Steps of immobilisation of peptides.

3.1. Functionalisation of solid supports

Steps allowing the immobilisation of peptides are described in Fig. 1. (1) Silanisation of the support with CDCS; (2) capping of non reacted Si-OH, and hydrolysis of methyl esters with iodotrimethylsilane; (3) activation of COOH groups by formation of reactive NHS esters; (4) grafting of an aminated polyethyleneglycol linker; (5) grafting of oligo-peptides after the formation of NHS ester at its C-terminal end. Currently microarrays are made on conventional glass slide supports. However, the glass slides are unsuitable for surface characterisations. In order to validate each steps of the support functionalisation, various silica supports were used.

Porous silica is a large specific surface area support [15] suitable for FTIR surface analysis spectroscopy. Thermal silicon dioxide are well-defined supports characterised by a low root mean square roughness (RMS) which allows accurate Atomic Force Microscopy analysis. The surface of porous silica and silicon dioxide substrates bear surface silanol groups. The organosilanes grafting on "silica" surface involves the reaction with these surface hydroxyl groups. The cross-analyses obtained by FTIR spectroscopy on porous silica and AFM measurements on silicon dioxide support are assumed to be well-correlated to the glass slide functionalisation.

Functionalisation of silica surface is currently done by silane coupling agent. These organic layers can be achieved in several ways. In order to obtain reproducible and uniform silane monolayers, we have investigated the surface silanisation with monofunctional silane. These molecules prevent problems of polymerisation which can appear with more reactive trifunctional silane.

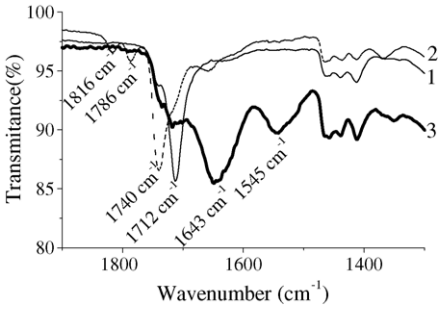

Fig. 2. IR characterisation of grafting of PEG linker onto silanised porous silica. (1) Thin line: starting surface = carboxylic acid function; (2) dashed line: NHS ester activated; (3) bold line: PEG linker coupled to COOH through amide bond.

Fig. 2 shows the evolution of infra-red characteristics spectra of the solid supports along functionalisation steps. After silanisation and ester-deprotection with ITMS, the surface exhibits terminal carboxylic acid groups characterised by a band at 1712 cm^{-1}. Activation with NHS (spectrum 2) shifts the band towards 1740 cm^{-1} together with the appearance of two bands at 1786 and 1816 cm^{-1} corresponding to NHS ester [20]. Grafting of PEG linker via amide bond is shown by the decrease of NHS ester band and the appearance of characteristic amide bands (1545 and 1643 cm^{-1}).

Chemical surface functionalisation was followed by IR-spectroscopy. AFM measurements were also performed to characterise the topological surface homogeneity. Silicon dioxide supports were analysed by Atomic Force Microscopy both before and after the silanisation step, to ensure complete coverage of their surfaces. Before silanisation, AFM probes (silicon nitrate tips) led to a strong interaction with the oxidized silicon surface and the glass slides, which made the capture of images very difficult (tip sticked to the surface). After the silane layer deposition, AFM measurements were facilitated by the change of the tip-surface interaction. This is the first sign of the presence of silane. Fig. 3a shows that molecules organize themselves in small islands of 50 nm × 30 nm size and 0.4 nm high.

The length of the silane molecule is approximately 1.5 nm, however this height was not measured with the AFM images. This indicates that molecules are lying flat on the surface. At a large scale, the grafting layer presents a smooth and flat surface with a root mean square (RMS) roughness of 0.25 and 0.86 nm on oxidized silicon and glass slides, respectively (Fig. 3b). These values correspond exactly to the values measured on bare supports. This result indicates that silane layer covers the substrate homogeneously.

3.2. Covalent coupling of oligo-peptides onto amino-derivatised supports

The final oligo-peptides coupling step was characterised by FTIR spectroscopy on porous silica supports. Fig. 4 shows spectra before and after coupling. Spectrum "before cou-

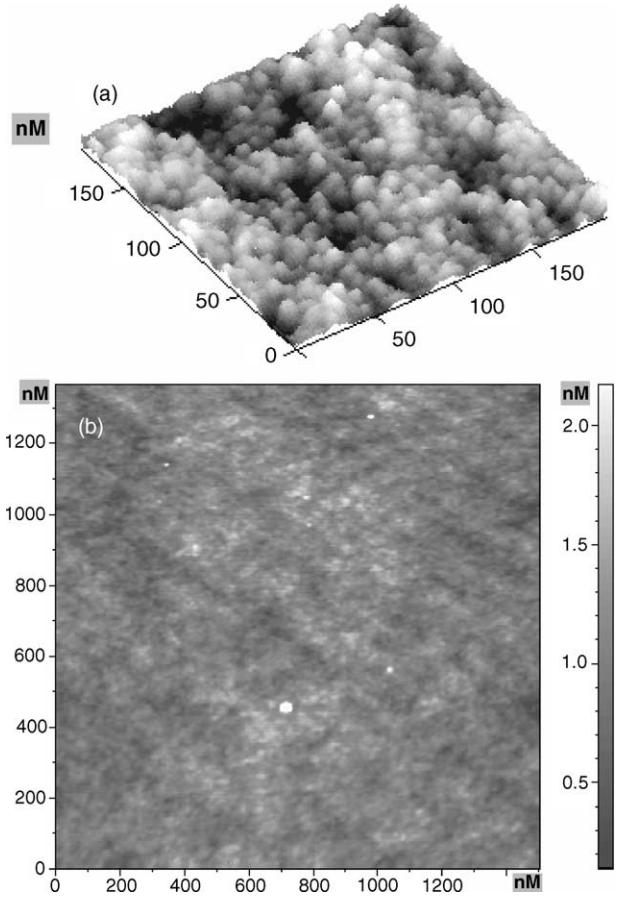

Fig. 3. AFM image of the silane layer deposited on oxidized silicon. (a) At high resolution (dimensions: 200 nm × 200 nm) molecules cover the substrate and lead to a very low roughness: $\Delta z = 1.4$ nm. (b) At large scale, 1400 nm × 1450 nm, the layer appears homogeneous with a $\Delta z = 2.3$ nm.

pling" corresponds to amino-derivatised porous silica support characterised by the amide I and amide II bands (respectively at 1656 and 1548 cm^{-1}). "After coupling" of peptide, there is a meaningful increase of the amide bands, indicating creation of new amide bonds. This result is explained meanly by the covalent binding of peptide onto PEG linker via amide bonds as the oligoglycine peptide model used bear

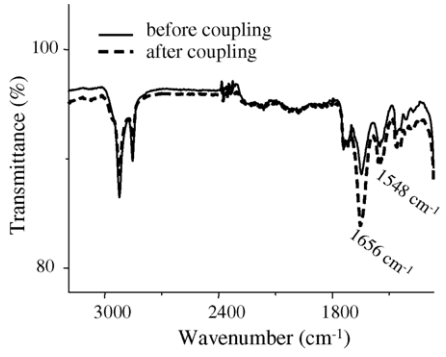

Fig. 4. IR characterisation of peptide coupling onto silane + PEG. (1) Solid line: solid support before peptide coupling; (2) dashed line: support after peptide coupling.

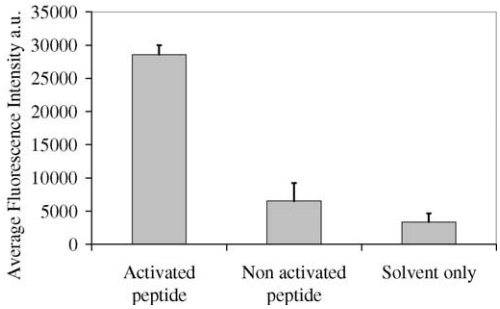

Fig. 5. Validation of indirect labelling using Cy3-streptavidin conjugate onto glass slides.

only one carboxylic acid located at C-terminal end. Therefore, FTIR measurements validate the oligo-peptide surface grafting strategy in order to obtain a well-controlled and an oriented covalent coupling of peptides onto aminated surface via amide bonds.

3.3. Fluorescence characterisation of biotinylated peptides immobilised onto glass slides

The immobilisation of oligo-peptide was then investigated on conventional glass slides using the protocol of functionnalisation developed previously on porous silicon and silicon dioxide. Since IR microscopy is difficult to perform efficiently on glass slides, characterisation of oligo-peptides grafting was implemented using indirect peptides labelling protocol. A biotinylated model peptide Gly_6 soluble in DMSO was immobilised onto amino modified glass slides. Cy3 labelled streptavidin binds strongly with biotins via complexation. It should be noticed that Cy3 fluorescence behavior is rather independent of molecular neighborhood [21,22], either in air or aqueous PBS buffer. Surface density of grafted peptides is then considered directly proportional to the fluorescence signal. In the typical results shown in Fig. 5, activated peptide spots yield a fluorescence signal four-fold higher than non activated peptides spots. Spots without peptides yield a fluorescence signal eight-fold lower than activated peptides, indicating a low non-specific adsorption of streptavidin onto the surface. Glycine residues do not exhibit lateral chains, and therefore bind to aminated surface only via their NHS ester activated C-terminal end.

These results are in good agreement with the IR analyses concerning the elaboration of a robust and oriented covalent coupling of peptides onto silanised glass slides. Moreover, the Cy3-streptavidin indirect labeling is validated for our glass slide/silane/biotinylated peptide system. This labeling method was used to assess different immobilisation conditions (time of coupling, oligo-peptide concentration, solvent).

3.4. Influence of coupling time on immobilisation yield

A 2×10^{-3} M biotinylated Gly_8 peptide solution was activated overnight in DMSO, and spotted at regular time in-

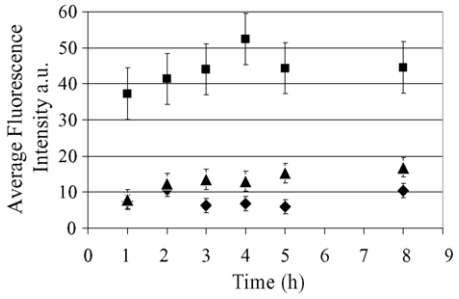

Fig. 6. Effect of time of coupling reaction on peptide immobilisation yield. Square: Activated peptide solution, (triangle) non activated peptide solution, lozenge: solvent only. Lateral error bars indicate experimental standard deviation.

tervals on amino modified glass slide. After washing and labelling, fluorescence signals were analysed to follow the influence of coupling time on peptide grafting density. Similarly, a solution of the peptide Gly_8 without activation and a solution of solvent without peptide were deposited onto the same slide and compared to the activated peptide spots.

Fluorescence data in Fig. 6 show that immobilisation of activated peptides is effective after 1 h of reaction time and no significant increase in peptide density occurs for longer reaction times. The signal to noise ratio (activated/non activated) measured for the different times of reaction is approximately of 5, which is consistent with results of Fig. 5 for DMSO. This result indicates a low non-specific adsorption of non activated peptides on amino-modified slides. Similar behaviours were obtained for reaction times of up to 50 h.

3.5. Influence of oligo-peptide concentration on immobilisation yield

Different concentrations (10^{-4} M to 10^{-2} M) of biotinylated Gly_6 peptide solutions were activated overnight and spotted onto amino-modified glass slide (coupling time: 12 h). After washings and Cy3-streptavidin labelling, the fluorescence spots were analysed. Fig. 7 represents the mean fluorescence signal detected in function of the concentration of oligo-probes in solution. The grafting density increases with

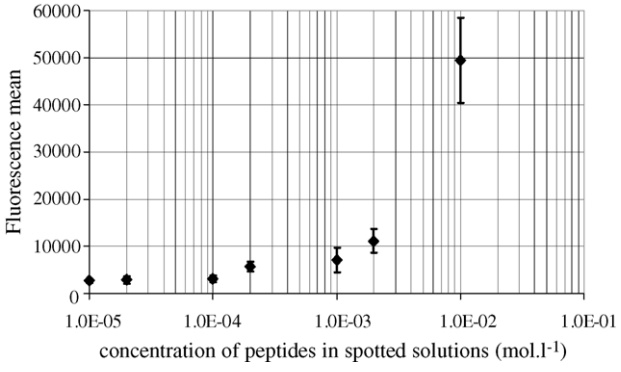

Fig. 7. Influence of peptide concentration in spotted solutions on immobilisation yield.

Table 1
Influence of solvent on immobilisation yield of biotinylated peptides onto silanised glass slides

Solvent	Activated peptide	Non activated peptide	Signal to noise ratio
Water	214 (13.2)	40 (9.0)	6.8
NMP	377 (67.4)	14 (3.8)	91.75
DMF	27 (8.9)	11 (2.2)	17
DMSO	52 (9.5)	20 (7.7)	4.2

For each solvent, results correspond to average fluorescence intensities (± experimental standard deviation). They are obtained from 3 to 6 experimental data points.

the concentration of oligo-probes in solution. Since a concentration of 10^{-2} M is difficult to attain due to the peptide synthesis scale, a work concentration of 2×10^{-3} M provides a good compromise for optimising peptide surface density.

3.6. Influence of solvent on immobilisation yield

Several solvents (water, NMP, DMF, DMSO) were investigated to optimise the peptide grafting efficiency on aminated surfaces. 5 μl drops of peptide solutions (Gly_8 at 2×10^{-3} M in the different solvents) were left to react during 3 h at 22 °C. They were then washed and revealed by Cy3-streptavidin labelling. The fluorescence intensities measured for each solvent were reported in Table 1. The Signal to noise ratio (SNR) compares the fluorescent signals from activated peptide and non activated peptide after subtraction of the local background signal (background level is approximately 10 ± 2).

The grafting of oligo-peptides in DMF, DMSO and water gives SNR higher than 3 (17, 6.8 and 4.2, respectively), indicating that the chemical grafting is preponderant in relation to adsorption at interfaces. Spots with water and NMP exhibit the highest densities of grafted peptides. It may be surprising that water allows for a fair immobilisation yield. Poor results are expected to occur because two competitive reactions take place in water: (i) condensation of NHS activated esters onto surface primary amines, leading to covalent binding of peptides onto silanised glass and (ii) hydrolysis of NHS activated esters, leading to their deactivation and regeneration of the carboxylic acid moieties. This competition should decrease peptide immobilisation yield in an aqueous medium. NHS ester is known to be a hydrolysis-resistant active intermediate [21,22] in relation to the water-soluble carbodiimide reagents. The NHS active esters hydrolyse slowly in aqueous media compared with their rates of reaction with amino groups [23]. Moreover, the in situ formation of NHS active esters is assumed to retain the NHS-ester groups preponderant during the coupling step. The highest grafting density is obtained with the NMP (SNR = 91.7), it can be assume a catalytic activity of the solvent upon the coupling reaction (i.e. dimethylaminopyridine is known to catalyze the conjugating of carbodiimide intermediate).

4. Conclusion

A three-step method for immobilising oligo-peptides onto glass and Si/SiO_2 was presented here. A systematic investigation of the bio-functionalisation was realised on silicon dioxide and porous silica support in order to define and control each step of the oligo-peptides grafting. Topological AFM measurements have showed the formation of a dense and homogeneous silane monolayer at the silica surface. FTIR analysis proved attachments to be covalent at each step of the process. The oligo-peptides grafting step allow to attach peptide in a covalent and oriented manner. Indirect labelling of biotinylated peptides with Cy3-streptavidin conjugate proved to be a simple and efficient method to measure relative densities of peptides on glass slides, and to compare different conditions of immobilisation.

Further papers will present biological recognition of antibodies using model peptide microarrays, and implementation of peptide microarrays by direct chemical synthesis.

Acknowledgments

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