Microarray and Surface Plasmon Resonance experiments for HPV genotyping on Au-supports

M. Banu[1, 2, *], M. Simion[1], P. Varasteanu[1, 3], L. Savu[4], and I. Farcasanu[2, 5]

[1]National Institute for Research and Development in Microtechnologies IMT Bucharest, 126A Erou
Iancu Nicolae Street, 077190, Bucharest, Romania
[2]Faculty of Biology, University of Bucharest, 91-95 Splaiul Independentei Blvd, 050095, Bucharest,
Romania
[3]Faculty of Physics, University of Bucharest, 405 Atomistilor Street, 077125, Magurele, Romania
4Genetic Lab SRL, 9 Capitan Nicolae Drossu Street, 012072, Bucharest, Romania
[5]Faculty of Chemistry, University of Bucharest, 90-92 Panduri Street, 050663, Bucharest, Romania
[*]Email: melania.banu@imt.ro

Abstract.

This paper is focused on obtaining DNA-modified gold biochips by ensuring the hybridization conditions of DNA and establishing the optimal co-immobilization ratios of DNA/ mercaptohexanol (MCH). Microarray technique and statistical analysis were employed to assess the immobilization protocol of random DNA sequences. By considering two types of probe carbon-spacer (C spacer), HPV 18 oligonucleotides were tethered on gold support with or without MCH and the probe attachment kinetics were investigated by Surface Plasmon Resonance (SPR). Further, the hybridization conditions of 4 different concentrations of real HPV-18 fragments were studied.

The probe immobilization efficiency was achieved after 2 h of incubation using buffer without MCH, or with DNA: MCH concentrations of 1:1 and 1:2, whereas the fluorescently labelled DNA and the probes with C6 spacer revealed superior hybridization capabilities. Also, SPR characterization disclosed promising hybridization results by using real DNA fragments corresponding to HPV 18.

Key-words: MCH, co-immobilization, hybridization, C spacer, HPV 18, microarray, SPR.

1. Introduction

Worldwide, virus infection and integration within human genome has been linked to carcinogenesis in 10-15% of cases, with the likelihood of this percentage to increase by improving the

detection techniques [1, 2]. In 1983, human papilloma virus 16 and 18 strains (HPV 16 and HPV 18) with high oncogenic risk were detected from cervix cells [3, 4]. Biosensors based on target DNA amplification by the Polymerase Chain Reaction (PCR) were developed for accurate detection of HPV in clinical specimens [5].

Microarray is one of the specific HPV detection methods in which PCR products are used for multiplex analysis of biological samples (genotyping of 102 HPV types) [6]. Gold films (Au) are often used in biodetection because it is an inert metal, easy to handle in various manufacturing techniques, providing covalent binding of biomolecules due to thiol-metal interactions [7]. Based on this principle (chemisorption), microarray chips were developed using thiol-modified DNA probes, where the detection was based on surface plasmon resonance imaging (SPRi) [8, 9]. Moreover, gold exhibits good plasmonic properties for wavelengths above the interband bandwidth ($\lambda \geq 580$ nm), enabling an alternative biodetection method using surface plasmon resonance (SPR) [10].

Since the first description and comprehensive analysis of Herne and Tarlov (1997) [11] where they demonstrate the use of alkanethiol-modified DNA and 6-mercaptohexanol for obtaining probe-modified gold surfaces with known and reproducible DNA coverage for obtaining high hybridization activity, there are only several literature reports on the systematic investigation regarding the optimal construction of mixed self assembled monolayers [12, 13]. Thus, it is important to systematically perform experiments of DNA hybridization to probes tethered on gold surface and proceed to optimize the parameters, since this type of support enables the possibility to verify the results by more techniques such as electrochemical approach, surface plasmon resonance (SPR), and microarray technology.

By cyclic voltammetry measurement (CV), Zhang et al. has shown hybridization performance using a ratio of 1:1 ssDNA:MCH. On the other hand, Keighley et al. [14] demonstrated by EIS that the best co-immobilization ratio for obtaining optimal probe density is ssDNA:MCH 1:4, whereas Yang et al. [15] optimized the ssDNA:MCH ratio to 1:10. To our knowledge, there are no reports regarding the immobilization and hybridization assessment by using scanning confocal microscope dedicated for microarrays and its inherent statistical analysis.

Our research is aiming for gold-based supports manufacturing and DNA attachment and hybridization efficiencies testing by microarray and SPR techniques. Herein, the immobilization and hybridization results of random sequences and real DNA-HPV samples are presented. For microarray approach, we have demonstrated best immobilization signal intensity and coefficient of variation after two hours of incubation when attaching DNA without MCH, followed by co-immobilization ratio of 1:1. The SPR study has revealed good hybridization results when employing real DNA sample, where the smallest concentration of 1 pM target DNA was precisely detected.

2. Experimental section

2.1. Materials and reagents

The immobilization buffer consists in 10 mM Tris-HCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 0.1 M NaCl and 10 mM Tris (2-carboxyethyl) phosphine (TCEP). The hybridization buffer was prepared using 10x saline sodium citrate (SSC), 2x Denhardt's solution and 200 μg/mL herring sperm DNA (Promega, USA). Nuclease-free water and microscope slides were purchased from Roth (Germany). Unless otherwise stated, the other reactives were purchased from Sigma-Aldrich (Germany).

DNA samples used for microarray and SPR experiments are listed in Table 1.

Table 1. DNA samples

HPV 18 sequences corresponding to E1 fragment	
Probes	5'-SH-C3-TGCCGCCATGTTCGCCATTTG-3'
	5'-SH-C6-TGCCGCCATGTTCGCCATTTG-3'
Random sequence (Control probes)	5'-SH-C6-AACCAGGATATCCGCTCACAATTCC-Cy3-3'
	5'-SH-C6-AACCAGGATATCCGCTCACAATTCC-3'
Complementary sequences	5'-Cy5-CAAATGGCGAACATGGCGGCA-3'
	5'-AACAGTCCATTAGGGGAGCGGCTGGAGGTGGATACAGAG TTAAGTCCACGGTTACAAGAAATATCTTTAAATAGTGGGCAG AAAAAGGCAAAAAGGCGGCTGTTTACAATATCAGATAGGGC TATGGCTGTTCTGAAGTGGAAGCAACACAGATTCAGGTAACTA CAAATGGCGAACATGGCGGCA-3'

The oligonucleotide sequences (Biomers.net, Germany) corresponding to E1 gene fragment of HPV 18 strain were designed to have at 5'-end a -SH modification with a 3 or 6 carbon (C3/C6) spacer, in order to evaluate the influence of the probe distance in the hybridization reaction. Random probe sequences were constructed to contain at 5'-end a -SH group with C6 and at 3'-end a Cy3 fluorophore, to observe the immobilization efficiency by microarray, and unlabelled control sequences were dedicated to SPR.

Complementary synthetic oligonucleotides (with Cy5 labelling at 5' end) were used for microarray experiments, whereas real DNA amplicons prepared previously as described in Banu *et al.* [16], were employed for SPR.

2.2. Microarray

The DNA probes (50 μM) were pre-incubated for 30 to 60 minutes in 20 mM TCEP, aiming to reduce the disulfide bridges and increase the binding of Au thiolate molecules.

A condition for obtaining reproducible microarray results on the Au substrate is the optimal immobilization chemistry. The Au-S bond is relatively stable (30–40 kcal / mol) [17], but the displacement of mono-thiolate oligonucleotides constitutes a problem. Mercaptohexanol (MCH) has the role of displacing and reorganizing the nonspecifically attached DNA, keeping only the probes attached through thiol groups. In consequence, MCH determines smaller probe density which increases the hybridization efficiency [14, 18].

Controlled immobilization or co-immobilization of 10 μM DNA probes with different MCH ratios was performed according to Figure 1.

Each square represents an array type, consisting of 72 technical replicates printed for performing a comprehensive statistical analysis.

Obtaining an optimal surface density is the key in developing a wide range of DNA biosensors [14]. This aspect has been investigated by microarray after co-immobilizing DNA with different MCH concentrations for 2 h and overnight. For assessing the effectiveness of DNA tethering in the presence/absence of MCH, the biochips were scanned at a wavelength of 532 nm (excitation of Cy3 fluorophore), using a confocal scanning laser (UC4 Microarray Scanner, Genomic Solutions).
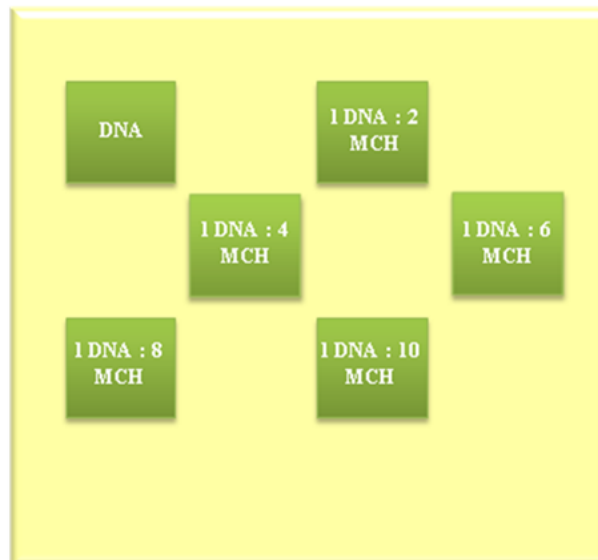
Fig. 1. Deposition of different DNA:MCH ratios on gold surface (color online).

Further tests consisted in analyzing the hybridization efficiency of 1 μM HPV 18-specific DNA fragments, the design of experiment being illustrated in Figure 2.
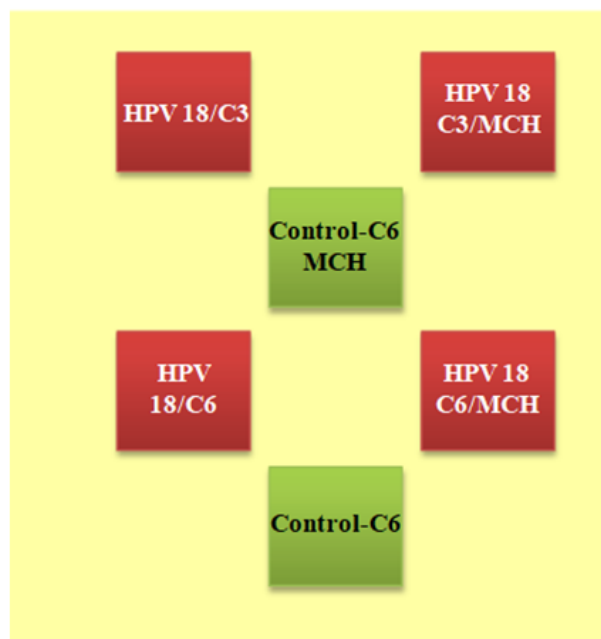

Fig. 2. DNA probe deposition on gold surface (color online).

Control probes (random sequences) were used to certify the immobilization efficiency with or without MCH. Further, we investigated the effectiveness of hybridization at 42 °C by overnight incubation of target oligonucleotides with HPV 18-specific probes containing 3 or 6 carbon spacers, previously immobilized with or without MCH. In order to examine the stability of Au-S-DNA bonding, samples washing was performed at 42 °C with 2x SSC + 0.2% SDS, 1x SSC and deionized water after hybridizing the HPV 18 specific probe types.

2.3.   Surface Plasmon Resonance

Real-time study of the immobilization and hybridization was developed at constant temperature (22 °C) using AUTOLAB – Twingle SPR (Eco Chemie., B.V., The Netherlands) in Kretschmann configuration at the fixed wavelength of 670 nm, by employing a BK7 hemicylindrical prism. The spectra were acquired by automatically varying the angle of incidence in order to achieve resonance and to record the angle with 0.1 mdeg resolution for angle sweep. The volume in the cuvette channels was 100 μL. The chip was fabricated by depositing 3 nm of Chromium (Cr) and 50 nm of Gold (Au) onto a BK7 glass disk; the gold-chips were cleaned 5 minutes in cold Piranha solution and then rinsed with deionized water. Mixing experiments using the stirring speed of 33.3 μL/s of 20 μL volume were employed, aiming to improve the immobilization time.

2.4.   Statistical analysis

GenePix® Pro 7 Software was employed for extracting the average signal intensities and the local background intensities, which underwent processing and analysis in RStudio 1.0.136 [19] environment for R 3.4.0 [20]. The graphs were obtained using background-corrected extracted values, normalized by $\log_{10}$ transformation [21]. The values placed 2 σ (standard deviation) away from the mean were treated as outliers and removed from the graphical and statistical analysis of the mismatches. The violin-plots with overlaid dot-plots were generated using ggplot2 R package [22].

3.   Results and discussion

3.1.   Microarray tests regarding the immobilization and hybridization of HPV oligonucleotides

3.1.1.   Investigation of DNA immobilization efficiency by mixed SAM formation

For a comprehensive assessment of the DNA co-immobilization effect, 5 different DNA:MCH ratios were used, considering the spotted DNA as reference. Figure 3 shows the analyzed results after 2 hours of incubation, attended by descriptive statistics.

As depicted in Figure 3, the immobilization reaction on gold surface is fast, obtaining the best results after attaching the DNA and co-immobilizing the probe sequences with doubled MCH quantity. A small decrease in immobilization signal intensity is observed after co-incubating with 1:4 C_{DNA}/C_{MCH}. By further increasing the MCH concentration, a higher variation coefficient (CV) is achieved due to data spreading, decreasing the reproducibility parameter because MCH relocates the unspecific bonded DNA outside the area defined by the printing pins.
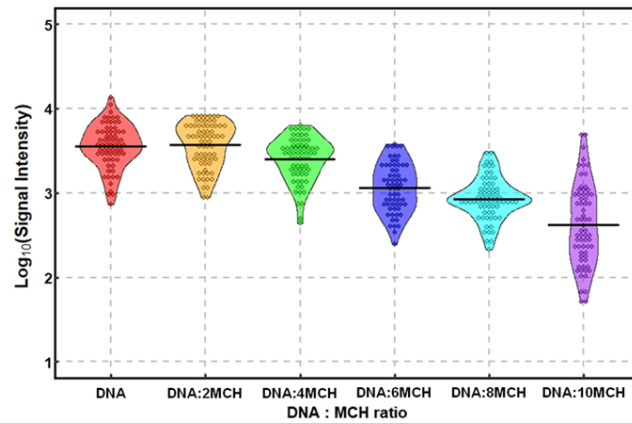
	DNA	DNA:2MCH	DNA:4MCH	DNA:6MCH	DNA:8MCH	DNA:10MCH
Average	3.552	3.566	3.398	3.063	2.93	2.622
s.d.	0.263	0.267	0.261	0.29	0.246	0.479
CV	7.4%	7.48%	7.68%	9.46%	8.39%	18.26%

Fig. 3. Signal intensities of immobilized probes and DNA co-immobilized with MCH, after 2 h of incubation (color online).

Starting from the promising data collected after 2 h of incubation, the reaction time influence over immobilization efficiency and reproducibility was investigated after the overnight storage of the printed biochip, as depicted in Figure 4.
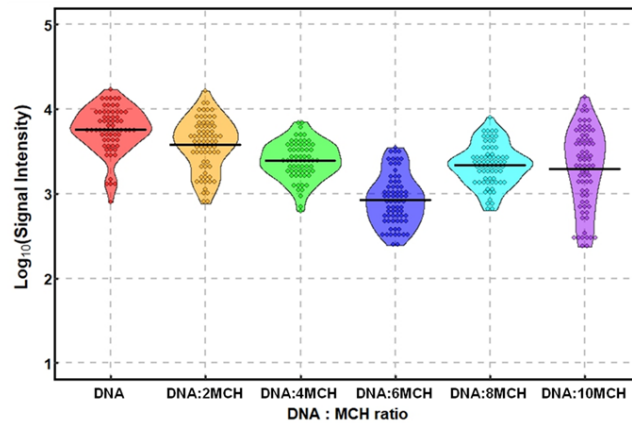
	DNA	DNA:2MCH	DNA:4MCH	DNA:6MCH	DNA:8MCH	DNA:10MCH
Average	3.754	3.581	3.391	2.93	3.335	3.29
s.d.	0.269	0.307	0.223	0.299	0.244	0.465
CV	7.16%	8.57%	6.57%	10.2%	7.31%	14.13%

Fig. 4. Log_{10} signal intensities of immobilized probes and DNA co-immobilized with MCH, after incubating overnight (color online).

The graphical representation matched with descriptive statistics shows the highest average immobilization intensity for DNA corresponding to 1:2 C_{DNA}/C_{MCH}. The overnight incubation did not improve the immobilization signal intensity nor its reproducibility marked by the variation coefficient. These findings sustain the quantitative radio-isotopic measurements of Steel et al. [18], reporting great increase of the probe attachment during the first hour of incubation, after 2 h reaching a coverage of 80% of that attained by 1 day of immobilization. Lower signal intensities are disclosed by co-incubation of DNA with 1:4 C_{DNA}/C_{MCH}, 1:6 C_{DNA}/C_{MCH}, 1:8 C_{DNA}/C_{MCH} and 1:10 C_{DNA}/C_{MCH}.

3.1.2. Investigation of hybridization parameters

Based on our previous experiments and on the investigations of Zhang et al. [12], 10 μM labeled random DNA probes were chosen to be attached or co-incubated with 10 μM MCH, to substantiate the immobilization efficiency. Figure 5 illustrates the fluorescent results after immobilizing control sequences on Au surfaces for 2 h.
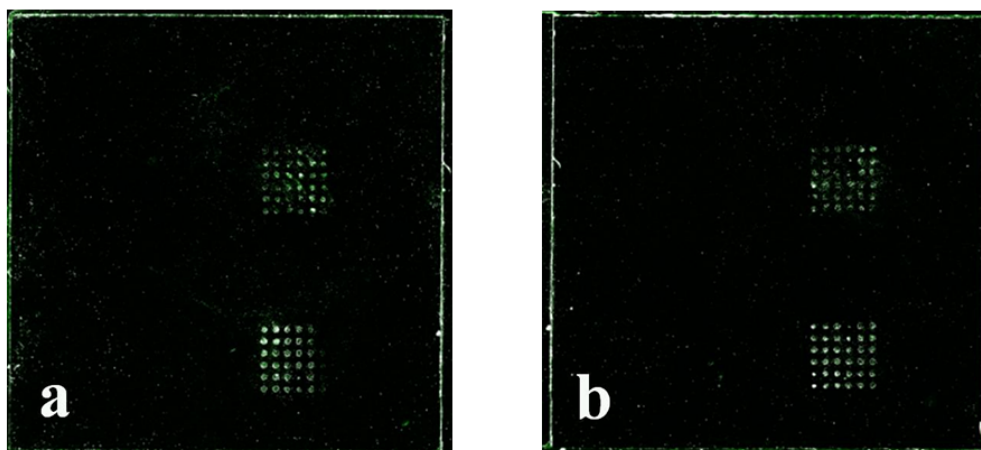

Fig. 5. Results after immobilizing fluorescent labeled probe sequences: a) first biochip and b) second biochip (color online).

From the visual analysis, the spots corresponding to sequences diluted in buffer without MCH appear to be smoother. The quantified signal intensities corresponding to the technical replicates on the two chips were translated into a graphical representation, together with statistical data (Figure 6).

The incubation of the DNA mix with mecaptohexanol leads to a smaller average signal (4.059), due to the displacement of unspecific bound oligonucleotides, noticing a minor difference between the standard deviations (s.d.$_{DNA}$ = 0.199 and s.d.$_{DNA:MCH}$ = 0.186). The coefficient of variation (CV) reveals very good reproducibility for both cases (4.60% and 4.58%).
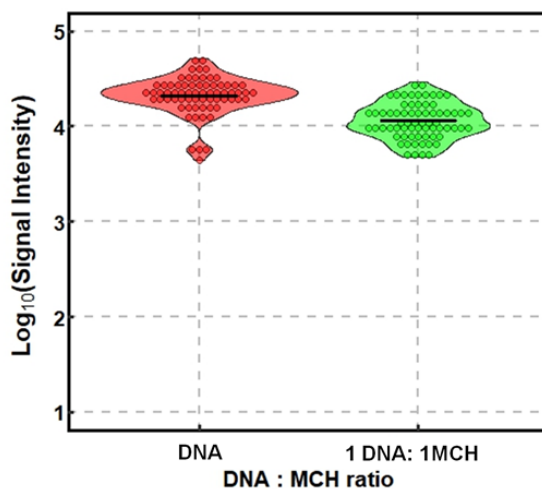
	Average	s.d.	CV
DNA	4.322	0.199	4.60%
1 DNA : 1 MCH	4.059	0.186	4.58%

Fig. 6. Immobilisation signal before processing the biochips (color online).

The strength of Au-S-DNA bonding was verified by applying thorough biochip washing steps, highlighting the best results in Figure 7.
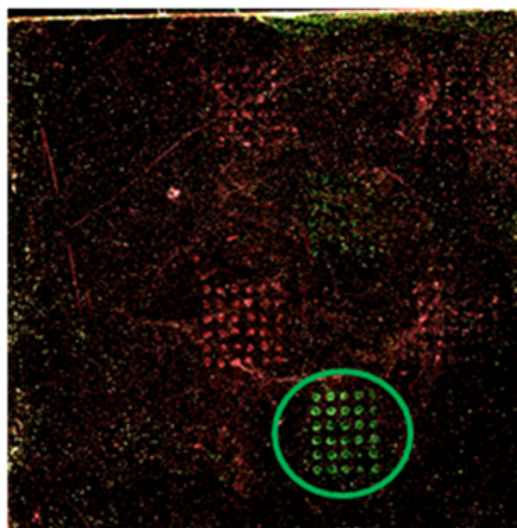

Fig. 7. Immobilization signal after 15 hours of incubation with hybridization solution at 42 °C and rigorous washings (color online).

A loss in signal intensity is observed due to the removal of the unstable probes, farther assessed by the graphical analysis coupled with descriptive statistics in Figure 8.
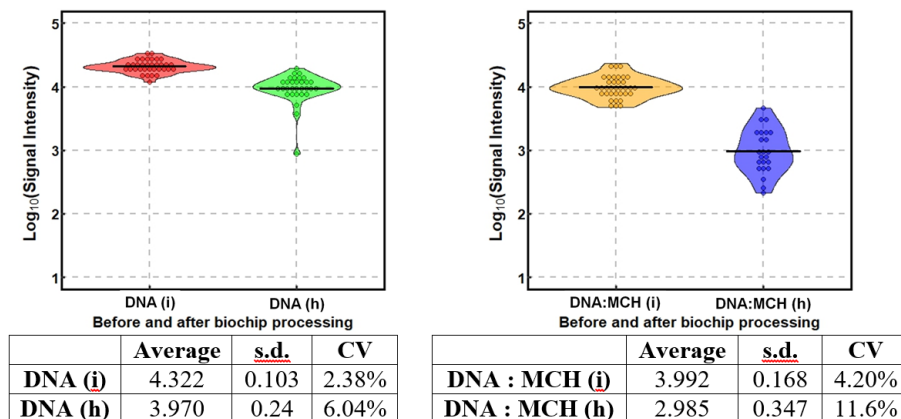

	Average	s.d.	CV
DNA (i)	4.322	0.103	2.38%
DNA (h)	3.970	0.24	6.04%

	Average	s.d.	CV
DNA : MCH (i)	3.992	0.168	4.20%
DNA : MCH (h)	2.985	0.347	11.6%

Fig. 8. Modification of immobilization intensity after additional stages of biochip processing (color online).

The intensities of the Cy3-labeled probe immobilization signals decrease after the additional biochip processing, in both cases: incubation with or without MCH. The CV correlated with the graphical analysis also shows the spreading of values and the loss in reproducibility, thus, stringent washing conditions are not recommended for gold-coated biochips.

The vertical coordinate of probe attachment on solid support is an important parameter, having a decisive role in facilitating the access of complementary oligonucleotides to probes during hybridization. The spacer is positioned between the probes and the support, its role being to "lift" the probe, exposing better the nucleotide sequences that will participate in the hybridization event. Using small oligonucleotides ($\sim$25 bases), it is essential to optimize the hybridization signal by adjusting the length of the spacer or probe concentration [23]. In Figure 9 are highlighted the best hybridization results between Cy5-fluorescently labeled target DNA and the oligonucleotides corresponding to E1 gene fragment from HPV 18.
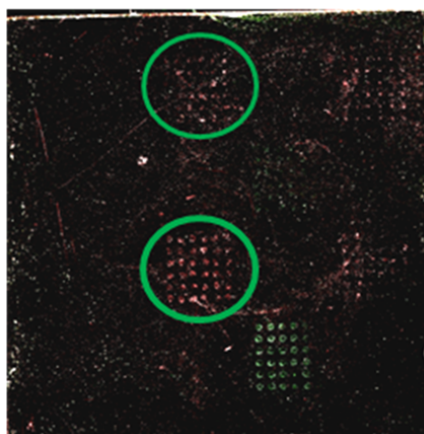

Fig. 9. Hybridization signal intensity after overnight incubation with target DNA (color online).

An increased background noise is observed due to the unspecific binding of Cy5-labelled target molecules. From the fluorescent image analysis, the spots corresponding to the hybridized probes not incubated with MCH and having C6 spacer appear to be best defined. This observation is confirmed by the graphical and statistical analysis of the technical replicates, depicted in Figure 10.
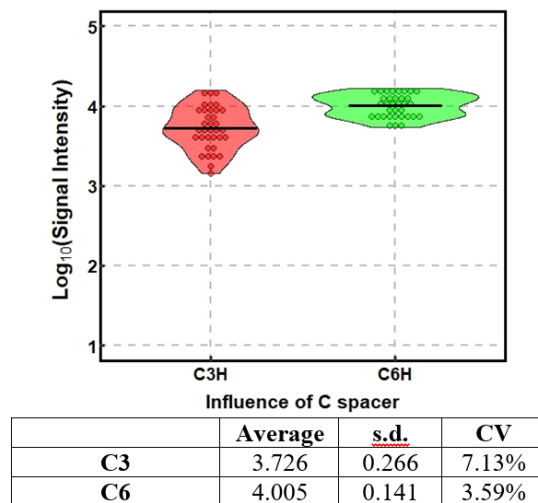

	Average	s.d.	CV
C3	3.726	0.266	7.13%
C6	4.005	0.141	3.59%

Fig. 10. Influence of the carbon-spacer length in the efficiency of the hybridization reaction (color online).

From the graphical analysis it is observed that the 6 carbon spacer had a positive impact on the hybridization, where the fluorescent signal intensity was higher (more target molecules were attached to the DNA probes) and more uniform, revealed by the CV=3.59%.

HPV 18 specific probes with C6 spacer tethered with/without MCH were considered further for comparative investigation of hybridization efficiency (Figure 11).
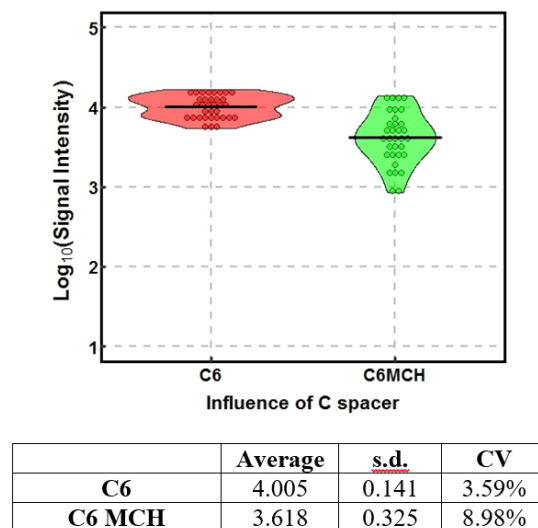

	Average	s.d.	CV
C6	4.005	0.141	3.59%
C6 MCH	3.618	0.325	8.98%

Fig. 11. Influence of MCH in the hybridization efficiency (color online).

The best results were exhibited by the hybridized probes tethered without MCH, having the highest average of signal intensity (4.005). The average signal intensity is lower (3.618) and the values are more dispersed (CV=8.98%) when hybridizing the sequences co-immobilized with MCH, due to the displacement of unspecific bound probes.

3.2. SPR tests regarding the immobilization and hybridization of HPV oligonucleotides

To determine the fastest approach for creating DNA self assembled monolayers (SAM), we have studied the influence of stirring the solution on the concentration of adsorbed molecules (Figure 12).
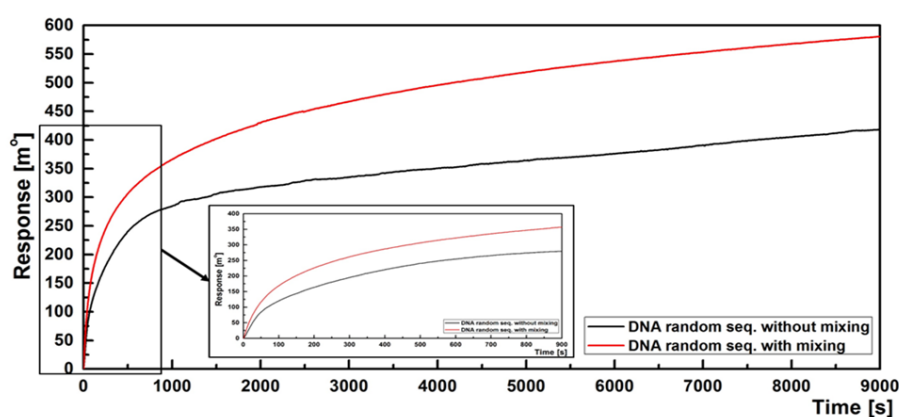

Fig. 12. Influence of solution mixing in oligonucleotide tethering reaction (color online).

The sensogram reveals an elevated resonance angle shift related to a higher number of attached DNA probes for the mixing procedure (4.71 ng/mm^2) and, for the experiment without mixing is 3.27 ng/mm^2. The nonspecific bonding to the surface is easily removable by mixing, thus creating more sites for the specific attachments of DNA. Thus, mixing guarantees the homogeneity of DNA probes in the cuvette channel and reduces the number of nonspecific interactions.

Figure 13 presents the SPR analysis of 1 μM thiol-modified ssDNA corresponding to HPV 18 fragment, immobilized on Au support.

The overall immobilization time was 170 minutes, where a maximum resonance angle shift of 700 m° was obtained (5.73 ng/mm^2 probes attached on Au). During the first hour of incubation, the immobilization reaction occurs very fast, with a slower increase of the curve, due to the partial occupation of Au surface with DNA probes.

The detection sensitivity of the hybridization illustrated in Figure 14 involved 4 concentrations of real DNA fragments corresponding to HPV 18 strain: 1 pM, 10 pM, 0.1 μM and 1 μM).
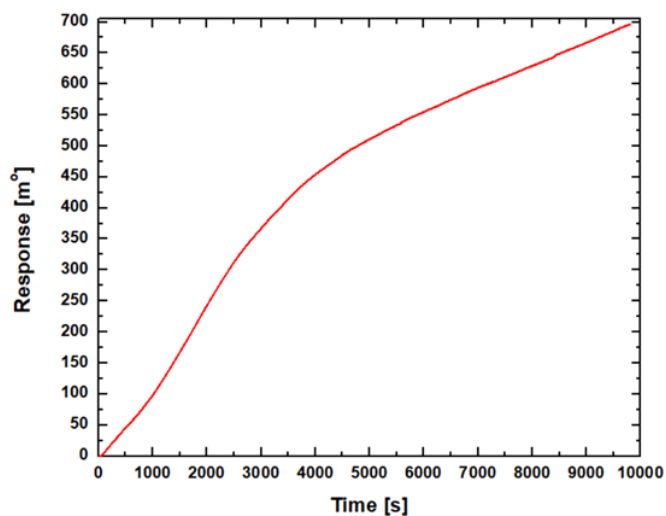
Fig. 13. DNA immobilization of HPV 18 strain monitored in real time using SPR (color online).
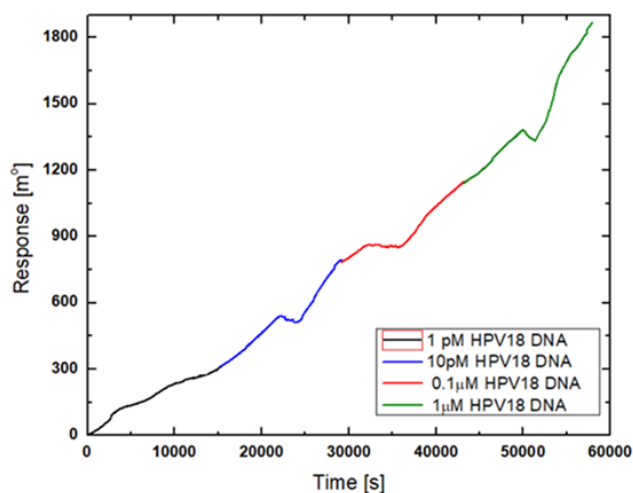

Fig. 14. Hybridization curves for different concentrations of real DNA fragments corresponding to HPV 18 strain (color online).

For the most diluted DNA sample (1 pM), a maximum deviation of 331 m° corresponding to 2.71 ng/mm^2 of hybridized sequence was obtained over 15000 seconds. For the second concentration (10 pM), we obtained the maximum angle shift of 484 m° equivalent to 3.97 ng/mm^2 target DNA in 15000 s. For the third DNA concentration (0.1 μM), the maximum was reached at 513 m° matching to 4.2 ng/mm^2 amplicons in 13000 s, and for the last sample (1 μM), a deviation of 732 m° (6 ng/mm^2 hybridized target DNA) was obtained in 15000 s. The use of real DNA samples disclosed good hybridization results, with impressive detection of the lowest

experimental concentrations.

Future research is aiming to decrease the HPV-specific probes' incubation time by applying continuous mixing and to determine the detection limit of the hybridized amplicons.

4. Conclusions

The microarray data analysis revealed fast immobilization reaction (2 h) and best signal intensities when attaching DNA without Mercaptohexanol, or after co-immobilizing 1:1 C_{DNA}/C_{MCH} and 1:2 C_{DNA}/C_{MCH}.

The highest average of hybridization signal intensity - 4.005 - was achieved by employing probes with C6 spacer immobilized without MCH. Lower hybridization intensity - 3.618 - was revealed by the oligonucleotides co-immobilized with MCH, due to the displacement of unspecific bound probes.

More specific and faster attachment were achieved after solution stirring, disclosing 4.71 ng/mm^2 tethered probe sequences, according to the SPR study.

Good hybridization results were obtained by employing real DNA sample, where the smallest concentration of 1 pM target DNA was precisely detected.

References

[1] C. J. CHEN, W. L. HSU, M. H. LEE, H. C. CHEN, Y. C. CHIEN and S. L. YOU, *Epidemiology of virus infection and human cancer*, in: Viruses and Human Cancer, M.H. Chang and K.T. Jeang (Eds.), Springer, **193**, pp. 59–74, 2014.

[2] F. HOPPE-SEYLER and K. BUTZ, *Molecular mechanisms of virus-induced carcinogenesis: the interaction of viral factors with cellular tumor suppressor proteins*, Journal of Molecular Medicine, **73**, pp. 529–538, 1995.

[3] J.S. BUTEL, *Viral carcinogenesis: revelation of molecular mechanisms and etiology of human disease* Carcinogenesis, **21** (3), pp. 405–426, 2000.

[4] P. S. MOORE and Y. CHANG, *Why do viruses cause cancer? Highlights of the first century of human tumour virology*. Naure Reviews of Cancer, **10**, pp. 878–889, 2010.

[5] S. TASOGLU, C. H. TEKIN, F. INCI, S. KNOWLTON, S. Q. WANG, F. WANG-JOHANNING, G. JOHANNING, D. COLEVAS and U. DEMIRCI, *Advances in nanotechnology and microfluidics for human papillomavirus diagnostics*, Proceedings of IEEE, **103**(2), pp. 161–178, 2015.

[6] S.T. ARRON, P. SKEWES-COX, P. H. DO, E. DYBBRO, M. Da COSTA, J. M. PALEFSKY, and J. L. De RISI, *Validation of a diagnostic microarray for human papillomavirus: coverage of 102 genotypes*, Journal of nucleic acids, pp. 1–6, 2011.

[7] H. HAKKINEN, *The goldsulfur interface at the nanoscale*, Nature Chemistry, **4**, pp. 443–455, 2012.

[8] S. NIMSE, K. SONG, M. SONAWANE, D. SAYYED and T. KIM, *Immobilization Techniques for microarray: challenges and applications*, Sensors, **14**, pp. 22208–22229, 2014.

[9] B. P. NELSON, T. E. GRIMSRUD, M. R. LILES, R. M. GOODMAN and R. M. CORN, *Surface plasmon resonance imaging measurements of DNA and RNA hybridization adsorption onto DNA microarrays*, Analytical Chemistry, **73**, pp. 1–7, 2001.

[10] M. OLIVERIO, S. PEROTTO, G. C. MESSINA, L. LOVATO and F. De ANGELIS, *Chemical functionalization of plasmonic surface biosensors: a tutorial review on issues, strategies, and costs*, ACS Applied Materials & Interfaces, **9**(35), pp. 29394–29411, 2017.

[11] T. M. HERNE and M. J. TARLOV, *Characterization of DNA probes immobilized on gold surfaces*, Journal of the American Chemical Society, **7863**, pp. 8916–8920, 1997.

[12] L. ZHANG, Z. LI, X. ZHOU, G. YANG, J. YANG, H. WANG, M. WANG, C. LIANG, Y. WEN and Y. LU, *Hybridization performance of DNA/mercaptohexanol mixed monolayers on electrodeposited nanoAu and rough Au surfaces*, Journal of Electroanalytical Chemistry, **757**, pp. 203–209, 2015.

[13] Z. LI, L. ZHANG, H. MO, Y. PENG, H. ZHANG, Z. XU, C. ZHENG and Z. LU, *Size-fitting effect for hybridization of DNA/mercaptohexanol mixed monolayers on gold*, Analyst, **139**, pp. 3137–3145, 2014.

[14] S. D. KEIGHLEY, P. LI, P. ESTRELA and P. MIGLIORATO, *Optimization of DNA immobilization on gold electrodes for label-free detection by electrochemical impedance spectroscopy*, Biosensors & Bioelectronics, **23**, pp. 1291–1297, 2008.

[15] Z. YANG, E. CASTRIGNANO, P. ESTRELA, C. G. FROST and B. KASPRZYK-HORDERN, *Community sewage sensors towards evaluation of drug use trends: detection of cocaine in wastewater with DNA-directed immobilization aptamer sensors*, Scientific Reports, **6**, pp. 1–10, 2016.

[16] M. BANU, M. SIMION, L. SAVU and I. C. FARCASANU, *Optimization of detection parameters on microarray Au-support for genotyping HPV strains*, International Semiconductor Conference (CAS), Sinaia, 2017, pp. 59–62, 2017.

[17] J. C. LOVE, L. A. ESTROFF, J. K. KRIEBEL, R. G. NUZZO and G. M. WHITESIDES, *Self-assembled monolayers of thiolates on metals as a form of nanotechnology*, Chemical reviews, **105**(4), pp. 1103–1169, 2005.

[18] A.B. STEEL, R.L. LEVICKY, T.M. HERNE and M.J. TARLOV, *Immobilization of nucleic acids at solid surfaces: Effect of oligonucleotide length on layer assembly*, Biophysical Journal, **79**, pp. 975–981, 2000.

[19] RStudio: Integrated development environment for R (Version 1.0.136) [Computer software]. Boston, MA. Retrieved February 15, 2017.

[20] R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

[21] S. DRAGHICI, Multiple comparisons, in: *Data analysis tools for DNA microarrays*, Chapman and Hall/CRC Mathematical Biology and Medicine Series, Boca Raton, Florida, 2003.

[22] WICKHAM, H., ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2009.

[23] C. C. CHOU, C.H. CHEN, T. T. LEE and K. PECK, *Optimization of probe length and the number of probes per gene for optimal microarray analysis of gene expression*, Nucleic Acids Research, **32**(12), pp. 1–8, 2004.