Analytical Methods

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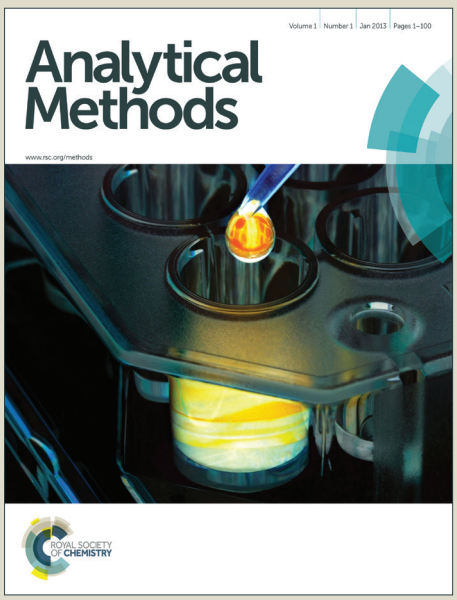

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ARTICLE

Reliable microspotting methodology for high hybridization efficiency peptide-nucleic acid layers on gold SPR imaging chips

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One-step, direct immobilization through Au-S chemistry of peptide-nucleic acid (PNA) probes onto gold surfaces is critical in terms of generating self-assembled monolayers with high hybridization efficiency. We found that this problem is more severe if the immobilization is made by contact microspotting to generate PNA arrays. Therefore, here we propose a novel microspotting-based immobilization method to generate high hybridization efficiency PNA arrays on bare gold surface plasmon resonance imaging (SPRi) chips. The essence of the approach is to spot thiol labelled PNA strands *prehybridized* with a short complementary DNA strand instead of conventionally used single stranded PNA (ssPNA) probes. After immobilization the complementary DNA strands could be easily removed to activate the surface confined PNA probes. The incubation time and the type of spotting needle had also a marked influence on the hybridization efficiency of the PNA layers. However, we show that if all other conditions the same, prehybridized PNA probes exhibit superior hybridization efficiency compared to the conventional ssPNA immobilization in all practically relevant conditions.

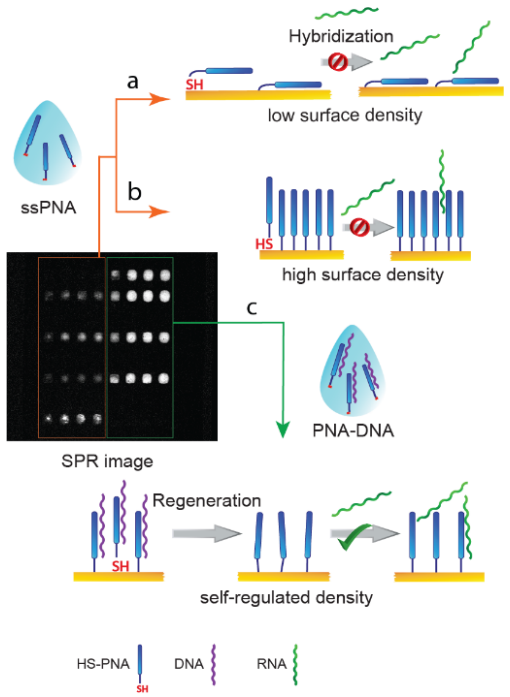
Introduction

Peptide nucleic acids (PNA)[1] are artificial nucleic acids analogs in which the nucleotide bases are attached to a peptide backbone typically formed from aminoethylglycine units. They can form Watson-Cricks base-pairing with complementary nucleic acid strands (DNA or RNA).[2] The immediate consequence of replacing the (deoxy)ribose phosphodiester backbone is that PNA lacks the negative charge of natural nucleic acids, which is a major advantage in hybridization assays as there is no charge repulsion between the hybridized strands. Accordingly, the hybridization of PNA strands in solution is not affected by the ionic strength and PNAs form stronger complexes with complementary strands as their natural counterparts. As the chemical and biochemical stability of PNA is also superior to that of DNA strands,[3] their drawbacks seems to be limited to their higher cost and need for a more careful probe design to avoid self-complementarity. PNA arrays and chips have been made using various substrates and immobilization methodologies.[4-7] However, the self-assembly of PNA strands directly attached through terminal thiol groups onto gold surfaces remains one of the preferred choices for electrochemical,[8-11] surface plasmon resonance[12-14] and quartz crystal microbalance[15] transducers. In a series of studies it was found that the direct attachment of PNA to gold via Au-S chemistry is rather critical in terms of efficiency of the subsequent hybridization step.[16-18] In fact an early study formulated fully discouraging conclusions regarding the use of Au-S chemistry for direct attachment of PNA strands to gold as opposed to biotin-streptavidin-base coupling of biotinylated PNA strands. Using quartz crystal microbalance with energy dissipation a very low energy dissipation was observed during thiol-PNA immobilization suggesting that PNA is rigidly attached with several unspecific contact points on gold. Thus the strands most likely "lie down" adsorbed on the gold surface, which hampers subsequent hybridization.[16] Similar observations were made also for DNA strands by neutron reflectivity in high salt conditions showing that the DNA strands are non-specifically adsorbed onto the gold surface.[19] In fact terminal attachment of the DNA strands through thiol groups resulting in high hybridization efficiency were only obtained if a post treatment with mercaptohexanol (MH) was performed to reduce the direct contact of the DNA strands with the gold surface. Therefore, in many studies the biotin-avidin type coupling[13, 20, 21] is still preferred over direct attachment of the thiol labeled nucleic acid probes to the gold surface.

Extensive studies by Martin-Gago and co-workers[22] on self-assembled single stranded PNA (ssPNA) layers on gold have revealed that the formation of PNA monolayers is a concentration dependent two-step process. It starts with the adsorption of ssPNAs molecules on the gold surface in a "lying down" orientation while above a certain surface coverage a phase transition occurs and the strands realign in a "stand-up" position.[22, 23] The concentration threshold was suggested to be at ca. 1 µM ssPNA in the aqueous solutions used for surface modification, resulting in ordered arrangements.[24] However, at concentrations higher than this value the surface rapidly saturates and at 5-10 µM ssPNA becomes so compact that no DNA binding was detected by X-ray photoemission spectroscopy (XPS). The repulsive interactions generated within immobilized PNA layers, upon hybridization with ssDNA probes are in fact at the core of cantilever bending in cantilever-based sensors.[25] In contrast to other studies that assign low hybridization efficiency of the PNA strands lying down on the surface Briones et al.[22] observed by XPS a close to 100 % yield for the hybridization. This discrepancy may be due to different experimental conditions as they used very high, 100 µM, concentration of complementary DNA, for their hybridization study, which is many orders of magnitude higher than used in analytical studies. The model of "lying-down" and "standing-up" PNA molecules was confirmed later also by electrochemical means using ssPNA strands labeled at C and N terminus with cysteine and ferrocene, respectively.[26, 27] Beside orientation and steric effects the ssPNA probe density on the surface can influence the hybridization efficiency in other ways as well. While the PNA-DNA hybridization *per se* is not affected by the ionic strength it was reported that in case of compact, surface confined PNA layers electrostatic repulsion can occur between the closely bound DNA strands. This effect is independent of the type of coupling chemistry and can be eliminated either by increasing the ionic strength of the hybridization buffer or by decreasing the surface concentration of the PNA probe until the hybridization of complementary strand follows the Langmuir adsorption model.[21]

While apparently the optimization of the surface concentration and orientation of thiol labeled PNA on gold for high hybridization efficiency is difficult, if successful, offers major advantages in terms of versatility and single step coupling. It eliminates the need for additional cross-linking reagents[10, 28], and consequently reduces the cost of fabrication and the structural complexity of the attached layer. Owing to the large variability in terms of length and sequence of the immobilized strands as well as co- or post immobilized spacers it is unlikely that a universally applicable optimum conditions for the immobilization can be found, which would be though preferable for the preparation of PNA microarrays.

In this study we aimed at developing a reliable preparation method of PNA receptor layers by microspotting thiol labeled PNA strands on bare gold SPR imaging chips and taking advantage of the multiplex readout for high throughput optimization. Since PNAs due to their very high affinity are ideal candidates for the determination of micro RNAs (miRNAs) the method is demonstrated through the hybridization assay of a 22-mer miRNA (hsa-miR-208a) that was identified as a biomarker of myocardial injury.[29, 30] The approach we used is based on implementing a "self-regulating"[21] mechanism for hybridization efficiency of the immobilized PNA strands (Scheme 1). Our hypothesis was that using thiol labeled PNA strands prehybridized with complementary DNA instead of ssPNA strands will automatically adjust the optimal surface conditions for subsequent hybridization. Moreover, we assumed that the non-specific surface adsorption of PNA strands on gold will be less of a problem if their duplex with DNAs are used for surface modification. This latter assumption is indirectly supported by the observation of Li and Rothberg on the differential adsorption of ss and dsDNAs on gold colloids,[31] i.e., single-stranded DNA adsorb strongly while double-stranded oligonucleotides not. The intuitive explanation for this behavior was that in case of dsDNA the nucleotide bases are involved in the formation of hydrogen bonds between the complementary strands and as such are not available for interaction with gold, which is, however, not the case for the flexible ssDNAs.



Scheme 1 Schematic representation of the microspotting strategies involving immobilization of ssPNA and PNA prehybridized with complementary DNA as well as their expected effects in terms of subsequent DNA hybridization. The SPR image shows side by side microspots made with the two strategies (as indicated) with the intensity of the spots being indicative of the amount of the RNA bound.

Experimental

Chemicals and materials

Twelve (N'–TGCTCGTCTTAT–C') and 18 mer (N'–GCTTTTTGCTCGTCTTAT–C') PNA strands complementary

to the microRNA hsa-miR-208a as well as a random non-complementary PNA strand (NC-PNA (18mer): N'–GCCGCTTCTTTATCTTTT–C') with a thiol group attached to the C terminus of the PNA backbones through a spacer consisting of two ethylene glycol units (C6-AEEA, ca. 2.4 nm, see Suppl. Inf., Scheme S1) were purchased from Eurogentec (Seraing, Belgium). The 22-mer hsa-miR-208a microRNA sequence (5'–AUAAGACGAGCAAAAAG CUUGU–3'), its DNA analog (C-DNA, 5'–ATAAGACGAGCAAAAAGCTT GT–3'), and a non-complementary random 22-mer RNA (NC-RNA; 5'–AGUACUAAUUCGUCUCUGUUCU–3') were from Sigma. RNAse and DNAse-free water for molecular biology (DEPC-treated and sterile filtered; Sigma) and DNA LoBind centrifuge tubes (Eppendorf) were used for preparing the RNA and DNA stock solutions in UV-cabinet for PCR operations. All other reagents such as inorganic salts and buffer components were of highest bioanalytical grade (Sigma-Aldrich). Phosphate buffer saline (PBS) solution was prepared from PBS tablets. The other buffers used for spotting, i.e., saline-sodium citrate (SSC) 3× concentrate contained 45 mM trisodium citrate, 450 mM NaCl at pH=7.0 (adjusted with 1 M HCl), while borate buffered saline (BBS) contained 10 mM sodium borate, 150 mM NaCl at pH=10.0 (adjusted with 1 M NaOH). All aqueous solutions were prepared with ultrapure deionized water (18.2 MΩ cm resistivity, Millipore).

Methods

Bare gold SPR sensor slides (HORIBA Jobin Yvon S.A.S. Palaiseau, France) were cleaned immediately before microspotting in UV generated ozone atmosphere (Novascan Technologies, Ames, IA, USA) for 15 minutes. The immobilization of PNA strands was made by microspotting using a BioOdyssey™ Calligrapher™ miniarrayer (Bio-Rad, Hercules, CA, USA) by means of either a solid pin (Stealth Solid Pin, 375 µm, Arrayit, Sunnyvale, CA, USA) or pins with an uptake channel, i.e., comprising a microcavity acting as sample reservoir (SMP15 Stealth Micro Spotting Pin, with 500 µm spot diameter and 0.25 µL uptake volume). The thiolated PNA probes were spotted onto the gold surfaces from a 384 well LD-PE plate previously blocked with protein-free TBS blocking buffer (Pierce, Thermo-Fisher, Rockford, IL USA) for 1 hour, washed with DI water, and dried. In all cases the wells were filled with 20 µL of 5 µM PNA strands. At least three parallel spots were made for each probe formulation at 65 rh% and with the spotting stage thermostated at 15°C. The spotted gold SPR sensors were incubated at 15±1°C and 65 rh% in the humidity chamber of the microspotter for periods between 4 and 19 h. In these conditions the drying of the spotted droplets was avoided. The droplets were still visible before the unspotted gold surface of the chips were blocked with 1 mM mercaptohexanol (MH) in phosphate buffer saline (PBS) for 15 min. Finally, the chips were washed with 300 mL DI water and gently dried under N_2 stream.

Surface plasmon resonance imaging (SPRi) measurements were made by using a SPRi-Plex II system (HORIBA Jobin Yvon S.A.S. Palaiseau, France) at fixed angle. First the working angle was selected based on recording SPR curves and then the refractive index calibration was performed to normalize the SPR response of each spot with the signal change measured in he respective location for a given refractive index change of the solution. The binding of nucleic acid strands (DNA or RNA) to the PNA microarrays (Fig. S1) were monitored at 25.00°C, at a flow rate of 50 µL min^{-1}. The activation of the immobilized phPNA strands, i.e. removal of prehybridzed complementary nucleic acid strands, was made with 100 mM NaOH solution (50 µL min^{-1}, for 4 min). The same conditions were used also to regenerate PNA surfaces between miRNA injections. The injected volume for each nucleic acid sample and regeneration solution was 180 µL. The typical durations for baseline, association and dissociation were 12, 3.6, and 8 min, respectively. The evaluation of the interaction curves was made with Scrubber 2 (GenOptics version).

Results and discussion

To determine the validity of our hypothesis that PNA strands in prehybridized form (phPNA) will provide receptor layers with higher hybridization efficiency than those formed by ssPNA the effect of various experimental parameters including the composition of the spotting buffer, the type of spotting pin (solid and stealth) as well as the length of the PNA probes (12 and 18-mer) were systematically investigated. A preliminary screening was made to identify the concentration of PNA probes and MH used for co- or post immobilization (Fig. S2). The experimental protocol featured in the Experimental section is the result of this first optimization step. Taking advantage of the multiplex capabilities of SPR imaging the effect of the experimental parameters was determined side-by-side for ssPNA and phPNA in rigorously identical conditions. The prehybridized probe solutions were prepared by mixing PNA with a 20 mol% excess of complementary DNA (the final concentration of phPNA was 5 µM assuming quantitative association with 1:1 stoichiometry). We used DNA instead of the target miRNA because is less susceptible to biodegradation. The microspotting of thiol labelled PNA probes onto the gold surface beside the self-assembly process is expected to be influenced also by the specific conditions of the spotting. Therefore, we prepared PNA chips by microspotting thiol labelled PNA probes in different spotting buffers using both solid and stealth microspotting pins. Rather surprisingly, we found that the subsequent hybridization of complementary miRNA (100 nM) as determined by SPR imaging was most severely affected by the type of microspotting pin used (see Fig. 1 A and B). The reflectance change, indicative of the amount of complementary miRNA bound to the immobilized PNA spots depending also on other experimental conditions was up to 7 times higher when using the stealth pin as compared to the solid pin. Since the essential difference between the two types of pins is the volume of the deposited droplet, apparently larger

volumes are beneficial in terms of increasing the binding capacity of the surface confined PNA probes.

The validity of our assumption that phPNA probes will provide better hybridization efficiency than ssPNAs is clearly confirmed by the results obtained for solid pin-based immobilization (Fig. 1A). For both 12 and 18-mer PNA strands as well as for all the different spotting buffers the binding to PNA spots formed from phPNA strands was in average ca. 2 times larger than to those obtained by ssPNA immobilization.

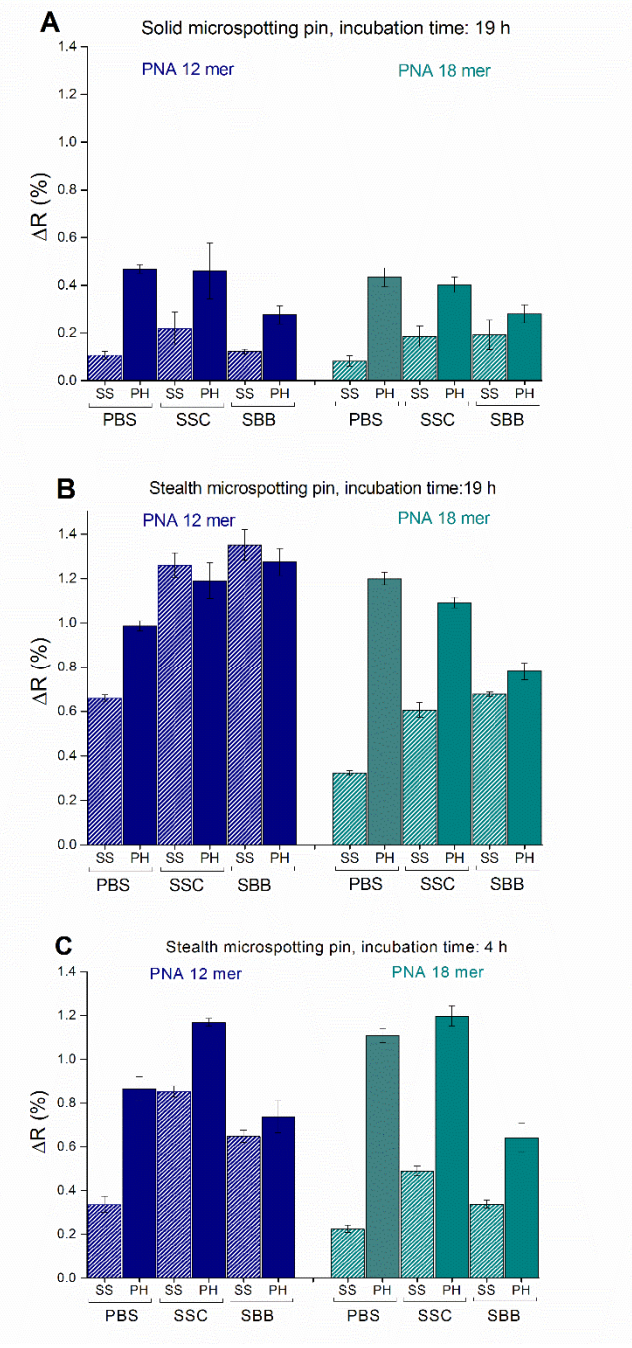


Fig. 1 Reflectance changes indicative of the amount of complementary RNA bound upon injecting 100 nM RNA for differently immobilized PNA spots: (A) solid pin and incubation time of 19 h, (B) stealth pin and incubation time of 19 h, and (C) stealth pin and incubation time of 4h. PBS, SSC and SBB abbreviate the spotting buffers (phosphate buffer saline, sodium saline citrate 3x, and sodium borate saline, respectively) in which the ssPNA (SS) and the phPNA strands (PH) were formulated.

In case of stealth pins the same trend is obvious for the 18-mer PNA strands, however, in case of the 12-mer PNAs the effect is only noticeable when using PBS as spotting buffer (Fig 1B).Such a length dependent behaviour could be explained if shorter PNA strands would assemble easier on the gold surface to enable subsequent hybridization and/or their non-specific interactions with gold are weaker than of longer PNA strands. If so it is reasonable to expect that the hybridization efficiency will depend on the time allowed for self-assembly. Therefore, to test the feasibility of these assumptions we have reduced significantly the incubation time of the spots, i.e., the time after spotting allowed for the formation of PNA SAMs in controlled relative humidity atmosphere. Using an incubation time of only 4 hour the difference between phPNA and ssPNA spots was visible also for the shorter 12-mer PNA strand. This suggests the immobilization of shorter PNA strands is less critical especially if enough time is allowed for the SAM to arrange. Since the length of the PNAs and immobilization time can vary in a wide range this may explain, at least in part, the controversy in the literature regarding the performance of PNAs immobilised through terminal HS groups to gold.

In all cases the difference between the hybridization efficiency of the two type of immobilized PNAs is the largest for strands spotted from PBS buffer. Otherwise, is difficult to choose a single optimal spotting buffer between SSC and PBS for the different length PNAs, but overall the SSC buffer seems to offer the most consistent results. Thus the hybridization efficiency of the PNA spots depends on the volume of the spotted solution, the time allowed for immobilization and spotting buffer used, but very importantly in all instances using the prehybridized form for spotting provides the best results in the given conditions. The superiority of PNA spots immobilized from phPNA using stealth pins was confirmed for a wide concentration range of miRNA as shown in Fig. 2.

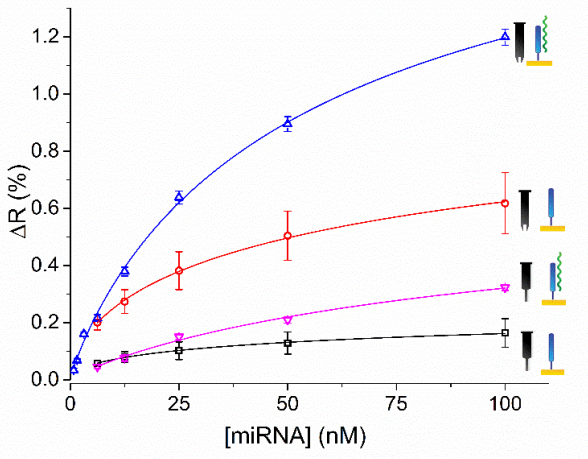


Fig. 2 SPR response of various PNA spots to miRNA. The PNA probes were immobilized from 5μM 18-mer ssPNA or phPNA in PBS using either solid or stealth pin as indicated on the graph.

The ratio of the sensitivities (slope of the linear range of ΔR vs. log [miRNA]) of the spots showing the highest and lowest hybridization efficiency exceeds an order of magnitude, i.e. spots of phPNA with stealth spotting pin and ssPNA with solid pin, respectively. However, even when using stealth pins for spotting there is a factor of two between the sensitivity of PNA spots immobilized from phPNA and ssPNA, which is remarkable. Using the optimized spotting procedure for PNA immobilization a miRNA amount as low as 140 fmol could be detected label-free, without amplification (Fig. S3). There is another less obvious advantage of using phPNA for immobilization. Namely, the signal change (ΔR) during removal of the DNA strand to activate the immobilized PNA probes is a good estimate of the ΔR_{max} value (Fig. 3A inset) corresponding to saturation of the PNA receptor layer with complementary RNA strands. This value is difficult (or at least unpractical and costly) in many cases to be determined by hybridization assays due to the very high concentration of complementary RNA required. Knowing the signal at saturation enables a more exact fitting of the binding curves (Fig. 3).

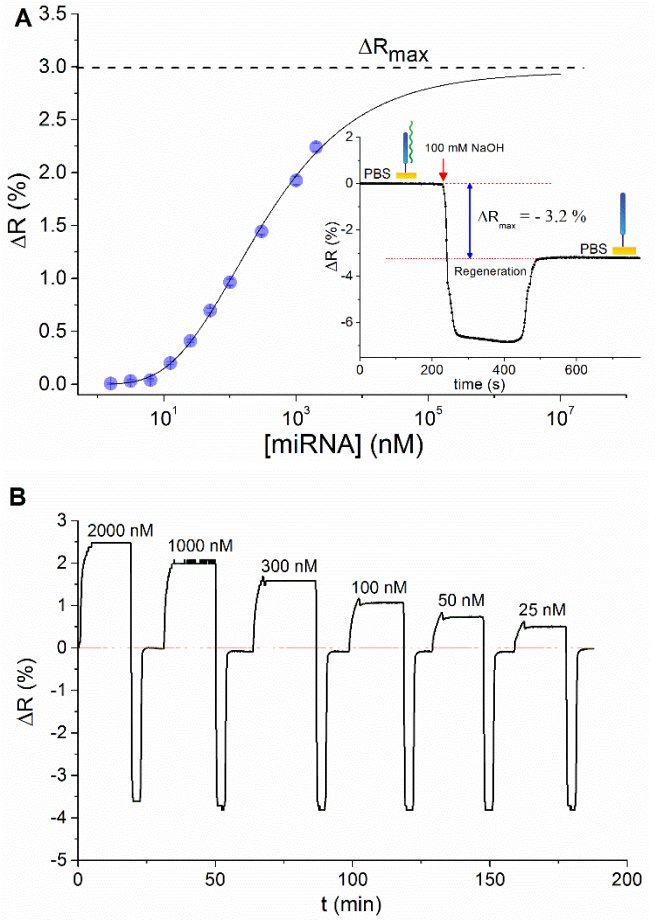


Fig. 3 (A) SPR calibration curve for miRNA using 18-mer PNA probes immobilized using the optimized microspotting procedure (using phPNA, stealth pin). The inset shows the SPR signal change during the activation of the PNA probes with 0.1 M NaOH that is used to calculate the ΔR_{max} values. (B) Real-time SPR signal transient upon injection of various concentration miRNA samples. Between successive samples the PNA layer is regenerated with 0.1 M NaOH.

The validity of the approach is based on the assumptions that (i) all PNA probes bound to the surface are in hybridized form, i.e., the equilibrium dissociation constant (K_D) is sufficiently small, and (ii) the first regeneration step removes all the DNA hybridized to the PNA strands. Kinetic analysis of the PNA-DNA interaction revealed a K_D value of 0.9 nM for the PNA-DNA complex that makes the first assumption reasonable. The kinetic curves for miRNA interaction (Fig. 3B) confirm the efficiency of the regeneration step using 0.1 M NaOH in terms of excellent baseline recovery. This suggest that the first regeneration step that activates the PNA probes after spotting effectively removes the hybridized DNA strands. The real time monitoring of the binding cycles further support the strong interaction between the PNA and complementary miRNA as there is no detectable loss of miRNA whatsoever in the timeframe allowed for dissociation (Fig. 3B).

The selectivity of the optimized PNA layers for hsa-miR-208a was assessed by using a 22-mer random sequence RNA at 100 nM concentration. In case of PNA arrays it must be also ensured that there is no cross-talk between the different surface confined probes, therefore, a preliminary test was performed by spotting also a random 18-mer PNA in the optimized conditions. In all cases when the spotting was made according to the optimized protocol the non-specific interactions were low (Fig. 4).

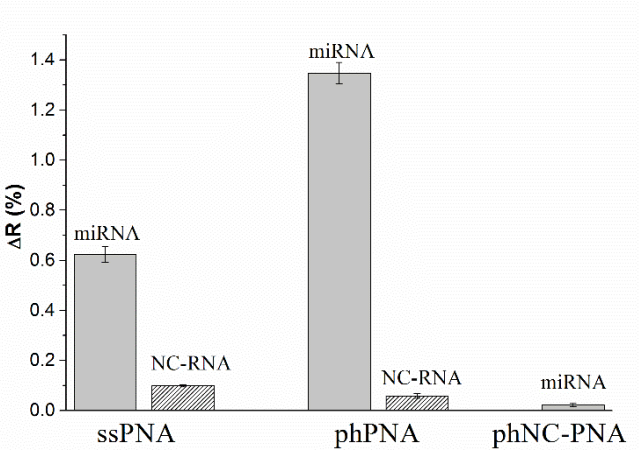


Fig. 4 Selectivity of various PNA probes tested by injection of 100 nM hsa-miR-208a miRNA and a 22-mer random sequence RNA. The 18-mer PNA probes were immobilized by microspotting using stealth pins from 5 μM ssPNA or phPNA solutions in PBS buffer. The ssPNA and phPNA denote the complementary probes for hsa-miR-208a miRNA, while the phNC-PNA is a random sequence PNA probe immobilized in prehybridized form.

Interestingly, and most importantly, we found that the selectivity towards complementary miRNA stands is somewhat enhanced by immobilizing the probes in prehybridized form as compared to the single stranded ones.

Conclusions

While this aspect has received little awareness our study showed that thiol labelled PNA probes immobilized in one step via Au-

S bond to gold are extremely sensitive in terms of subsequent hybridization to complementary nucleic acids to the immobilization conditions. The sensitivity was clearly influenced by the length of the PNA probe. The optimization of the immobilization was more critical for longer probes. The main finding of the paper was that in all practically relevant conditions the microspotting of PNA strands in prehybridized form with a complementary DNA strands result in layers that are superior (or equal) in terms of binding capacity to those obtained by ssPNA microspotting. The use of prehybridized PNA strands is also beneficial in terms of determining the signal corresponding to the maximum binding capacity of the respective layers upon their activation, i.e., by measuring the signal change due to the removal of the hybridized complementary strands. The immobilization strategy is potentially applicable to a wide range of gold made transducers as those use in electrochemical, SPR, quartz crystal microbalance sensors. We believe that the fact that if all other conditions are the same the use of phPNA gives superior result as compared with ssPNA relieves the need for lengthy optimization of PNA immobilization to gold surfaces.

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Notes and references

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Electronic Supplementary Information (ESI) available: typical SPR images and layout of the PNA arrays, results of prescreening the spotting condition and unprocessed SPR curves. See DOI: 10.1039/b000000x/

1. P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, *Science*, 1991, **254**, 1497-1500.
2. M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden and P. E. Nielsen, *Nature*, 1993, **365**, 566-568.
3. V. V. Demidov, V. N. Potaman, M. D. Frank-Kamenetskil, M. Egholm, O. Buchard, S. H. Sönnichsen and P. E. Nlelsen, *Biochem. Pharmacol.*, 1994, **48**, 1310-1313.
4. M. Beier and J. D. Hoheisel, *Nucleic Acids Res.*, 1999, **27**, 1970-1977.
5. J. Weiler, H. Gausepohl, N. Hauser, O. N. Jensen and J. D. Hoheisel, *Nucleic Acids Res.*, 1997, **25**, 2792-2799.
6. N. Winssinger and J. L. Harris, *Expert Rev. Proteomics*, 2005, **2**, 937-947.
7. S. Y. Lim, W. Y. Chung, H. K. Lee, M. S. Park and H. G. Park, *Biochem. Biophys. Res. Commun.*, 2008, **376**, 633-636.
8. T. H. Degefa and J. Kwak, *J. Electroanal. Chem.*, 2008, **612**, 37-41.
9. H. Aoki and Y. Umezawa, *Nucleic Acids Symposium Series*, 2002, **2**, 131-132.
10. D. Ozkan, A. Erdem, P. Kara, K. Kerman, J. Justin Gooding, P. E. Nielsen and M. Ozsoz, *Electrochem. Commun.*, 2002, **4**, 796-802.
11. N. Husken, M. Gebala, W. Schuhmann and N. Metzler-Nolte, *ChemBioChem*, 2010, **11**, 1754-1761.
12. J. Liu, L. Tiefenauer, S. Tian, P. E. Nielsen and W. Knoll, *Anal. Chem.*, 2006, **78**, 470-476.
13. K. K. Jensen, H. Ørum, P. E. Nielsen and B. Nordén, *Biochemistry*, 1997, **36**, 5072-5077.
14. N. Prabhakar, K. Arora, S. K. Arya, P. R. Solanki, M. Iwamoto, H. Singh and B. D. Malhotra, *Analyst*, 2008, **133**, 1587-1592.
15. J. Wang, *Anal. Chem.*, 1997, **69**, 5200-5202.
16. F. Hook, A. Ray, B. Norden and B. Kasemo, *Langmuir*, 2001, **17**, 8305-8312.
17. G. Jágerszki, R. E. Gyurcsányi, L. Höfler and E. Pretsch, *Nano Lett*, 2007, **7**, 1609-1612.
18. C. Ananthanawat, T. Vilaivan and V. P. Hoven, *Sens. Actuator B-Chem.*, 2009, **137**, 215-221.
19. R. Levicky, T. M. Herne, M. J. Tarlov and S. K. Satija, *J. Am. Chem. Soc.*, 1998, **120**, 9787-9792.
20. Y. Sato, K. Fujimoto and H. Kawaguchi, *Colloid Surf. B-Biointerfaces*, 2003, **27**, 23-31.
21. D. F. Yao, J. Kim, F. Yu, P. E. Nielsen, E. K. Sinner and W. Knoll, *Biophys. J.*, 2005, **88**, 2745-2751.
22. C. Briones, E. Mateo-Marti, C. Gomez-Navarro, V. Parro, E. Roman and J. A. Martin-Gago, *Phys. Rev. Lett.*, 2004, **93**.
23. E. Mateo-Marti, C. Briones, E. Roman, E. Briand, C. M. Pradier and J. A. Martin-Gago, *Langmuir*, 2005, **21**, 9510-9517.
24. S. Ghosh and R. Mukhopadhyay, *J. Colloid Interface Sci.*, 2011, **360**, 52-60.
25. S. Ghosh, S. Mishra and R. Mukhopadhyay, *J. Mater. Chem. B* 2014, **2**, 960-970.
26. A. Paul, R. M. Watson, P. Lund, Y. J. Xing, K. Burke, Y. F. He, E. Borguet, C. Achim and D. H. Waldeck, *J. Phys. Chem. C*, 2008, **112**, 7233-7240.
27. N. Husken, M. Gebala, F. La Mantia, W. Schuhmann and N. Metzler-Nolte, *Chem.-Eur. J.*, 2011, **17**, 9678-9690.
28. D. Kambhampati, P. E. Nielsen and W. Knoll, *Biosens. Bioelectron.*, 2001, **16**, 1109-1118.
29. E. Wang, Y. Nie, Q. Zhao, W. Wang, J. Huang, Z. Liao, H. Zhang, S. Hu and Z. Zheng, *Eur. J. Cardiothorac. Surg.*, 2013, **8**, 165.
30. X. Ji, R. Takahashi, Y. Hiura, G. Hirokawa, Y. Fukushima and N. Iwai, *Clin. Chem.*, 2009, **55**, 1944-1949.
31. H. Li and L. Rothberg, *Proc. Natl. Acad. Sci. U.S.A*, 2004, **101**, 14036-14039.

