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Notes & Tips

Effects of formamide on the thermal stability of DNA duplexes on biochips

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ABSTRACT

In molecular biology, formamide (FA) is a commonly used denaturing agent for DNA. Although its influence on DNA duplex stability in solution is well established, little is known about immobilized DNA on microarrays. We measured thermal denaturation curves for oligonucleotides immobilized by two standard protocols: thiol self-assembling and pyrrole electrospotting. A decrease of the DNA denaturation temperature with increasing FA fraction of the solvent was observed on sequences with mutations for both surface chemistries. The average dissociation temperature decrease was found to be −0.58 ± 0.05 °C/% FA (v/v) independently of grafting chemistry and probe sequence.

Numerous solvents are known to act as denaturing agents on DNA. Most denaturing agents interact with the hydrogen bonds (H-bonds)[1] in the double helix, and especially formamide (FA) is known for its ability to form H-bonds and to compete efficiently with the H-bonds between Watson–Crick base pairs [1]. Different studies have reported that FA lowers the melting temperature of free DNA in solution by 0.60% to 0.73 °C/% FA (v/v) [1–3].

Since the time of these investigations, the advent of DNA microarray technology has raised interest in the hybridization of oligonucleotide probes grafted to a solid support. Under these conditions, hybridization of DNA is influenced by the proximity of the surface, with various interactions taking place between different probes, between probes and targets, and with the surface [4–6]. This leads to the question of whether denaturing agents such as FA interact in the same way on oligonucleotide chips.

Many single nucleotide polymorphism (SNP) detection systems are based on allele-specific hybridization and rely on perfect discrimination between matched and mismatched targets [7,8]. To work at low temperatures, these assays need optimization regarding probe sequences, hybridization temperature, and buffer conditions [9]. Because we are interested in the detection of SNPs, we carried out a detailed study on the influence of FA on a model mutation system. One approach to this issue was published by Urakawa and coworkers [10] for nonequilibrium denaturation studies on polyacrylamide gel-based DNA microarrays. Our study aimed to provide predictions of the variation of DNA stability in FA for solid phase experiments with various grafting chemistries. This information concerns not only low-temperature microarray devices but also real-time microarray analysis based on nonequilibrium melting [11]. Using surface plasmon resonance imaging (SPRi), we acquired nonequilibrium thermal denaturation curves to answer the following questions. What influence does FA have on the stability of immobilized DNA? Is there a difference between perfectly matched sequences and sequences with one or two mutations? Are these findings dependent on the surface chemistry used for chip fabrication?

Gold-coated prisms (GenOptics, France) were functionalized either by polypyrrole electrospotting as described previously [12] or by spotting of thiol-modified DNA probes. For thiol self-assembling monolayers, prisms were cleaned by piranha solution (this is highly corrosive and so should be handled with care), and droplets of approximately 90 nl K_2HPO_4 of 0.5 M buffer containing 10 μM thiol-modified DNA probes (Eurogentech, France) were deposited by a robot under a controlled atmosphere of 85% humidity and left for 90 min to allow a thiol deposition to form on the surface. After overnight drying, prisms were rinsed abundantly with Milli-Q water and dried under an argon stream. If the prisms are rinsed without drying, spots show "comet tails" indicating stable probe deposition during rinsing. The surface was then covered with an aqueous 1-mM solution of 1-mercapto-6-hexanol (Sigma) in water for 90 min. Besides blocking the nonfunctionalized gold surface between the spots, this step eliminates DNA that is not attached via its thiol moiety and replaces it with the short thiol [13]. A minimum of four spots per sequence were grafted to control experimental reproducibility. Every biochip had control spots of nonfunctionalized polypyrrole and a noncomplementary DNA sequence to check the specificity of hybridization.

DNA sequences chosen for this study corresponded to the sequence of the K-ras gene (N = pyrrole-T_{10}-TGG AGC TG**G** TGG

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[1] *Abbreviations used:* H-bond, hydrogen bond; FA, formamide; SNP, single nucleotide polymorphism; SPRi, surface plasmon resonance imaging; CCND1, cyclin D1; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; MM, mismatched (duplex); T_d, dissociation temperature; PM, perfectly matched (duplex).

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CGT, M2 = pyrrole-T_{10}-TGG AGC T**CG** TGG CGT, M5 = pyrrole-T_{10}-TGG AGC TGC TGG CGT) [14] and the cyclin D1 gene (CCND1, G870A) (P1 = thiol-C6/pyrrole-T_{10}-TGT GAC CC**A** GTA AGT G, P2 = Thiol-C6/pyrrole-T_{10}-TGT GAC CC**G** GTA AGT G, P3 = Thiol-C6/pyrrole-T_{10}-TGT GAC C**TA** GTA AGT G, P4 = Thiol-C6/pyrrole-T_{10}-TGT GAC C**T**G GTA AGT G) [15] (see supplementary material for sequences). All probes had a 10-thymine spacer at the 5′ end and a hybridizing part of 15 or 16 bases. The complementary targets were 15 or 20 bases long (M5c = ACG CCA GCA GCT CCA, T1 = CTC ACT TAC **T**GG GTC ACA CT, T2 = CTC ACT TAC **C**GG GTC ACA CT). Mismatches were located in the center of the sequence to yield maximum destabilization of the duplex.

All experiments were carried out on a homemade, temperature-regulated SPRi system as described by Fiche and coworkers [16]. The running buffer contained phosphate-buffered saline (PBS) completed with NaCl up to 450 mM, 0.05% Tween 20, and 1 mM ethylenediaminetetraacetic acid (EDTA) and volume fractions of FA from 0% to 20% in steps of 5% (all reagents purchased from Sigma–Aldrich). For hybridization, target DNA was added to the running buffer at a final concentration of 250 nM. Hybridization was carried out for 10 min at 25 °C with a flow rate of 83 µl/min. Then, after 3 min of rinsing, a controlled temperature scan was performed at 2 °C/min up to 70 °C. Thus, we obtained nonequilibrium thermal denaturation curves for all spots on the prism. Note that thiol attachment on gold became unstable at high temperatures (>75 °C) (data not published). On the other hand, control experiments showed that after more that 15 heating cycles to 70 °C, no significant decrease in hybridization signal could be found.

Because FA destabilizes the DNA duplex, the hybridization efficiency decreased with increasing percentages of FA in the buffer [10]. This effect was strongest on spots forming duplexes with two mismatches (MM) where fewer Watson–Crick base pairs are formed. At 20% FA, some probe/target duplexes with two mismatches had a hybridization signal/noise ratio below 10 and were excluded from the thermal denaturation analysis. In the case of the K-ras gene, we observed an increase in the on-rate, k_{on}, with increasing percentages of FA (see supplementary material), although the total hybridization signal decreased. We observed identical behavior for targets forming stable secondary structures hybridized at different temperatures. Formamide may be able to suppress formation of secondary structures in probes and targets or interchain interactions between identical targets. Thus, competition between secondary structures and surface hybridization was reduced, and the kinetics of hybridization to the surface was favored by the addition of FA.

To analyze the influence of FA on the DNA helix stability, we used the dissociation temperature (T_d), defined as the temperature with 50% of the initial hybridization signal on the spots [10,17]. SPRi dissociation curves were normalized to 1 and averaged over identical spots. Fig. 1A compares dissociation curves obtained for the perfectly matched (PM) duplex of T2 on P2 on polypyrrole and thiol chips. We observed higher T_d values on thiol chips compared with pyrrole grafting. This difference in T_d values must be attributed to interactions between probes, targets, and the substrate as a result of the immobilization method. In spite of the different hybridization environment, the T_d decrease due to FA on DNA duplex stability was comparable for both grafting methods. A more precise study of the stability of mismatched DNA was carried out. Fig. 1B shows the dissociation curves of duplexes with zero to two mismatches in 10% FA buffer. For the K-ras (dashed lines) and CCND1 (solid lines) probes, PM duplexes can be clearly distinguished from duplexes forming one mismatch (1 MM) or two mismatches (2 MM) with the target M5c or T1, respectively. One mismatch lowered the T_d by 6.8 to 11.5 °C in this case. Fig. 2 shows exemplarily the measured T_d values and standard errors on thiol and polypyrrole functionalized surfaces for the target T2. Thiol spots had a better spot reproducibility compared with polypyrrole spots and, thus, led to smaller experimental errors. T_d values decreased linearly with increasing volume fractions of FA, and the slope ΔT_d per percentage of FA is reported in the legend. The slope was calculated for each DNA duplex by linear regression with a determination coefficient of $R^2 \geqslant 0.98$. T_d values and slopes are present in the supplementary material.

Our results show that FA has no significant sequence-dependent destabilizing effect on the T_d, and we obtained slopes from −0.49% to −0.65 °C/% FA (v/v). No systematic dependency on the mutation type and the DNA duplex sequence could be found. Sequences with two mutations showed a slightly lower response to the increase of FA than the PM or 1-MM duplexes. This can be explained by their decreased number of H-bonds in the duplexes. We found an average slope of −0.57 ± 0.05 °C/% FA (v/v) for polypyrrole surfaces and of −0.59 ± 0.05 °C/% FA (v/v) for thiol surfaces. Thus, there was no significant difference between the two surface chemistries. From Urakawa and coworkers' data [10], one can calculate an average FA shift of −0.56 °C/% FA (v/v), extending our results to another on-chip method. These values are lower than those reported previously for DNA in solution because the results were obtained on immobilized oligonucleotides and for nonequilibrium thermal denaturation in contrast to equilibrium melting of long

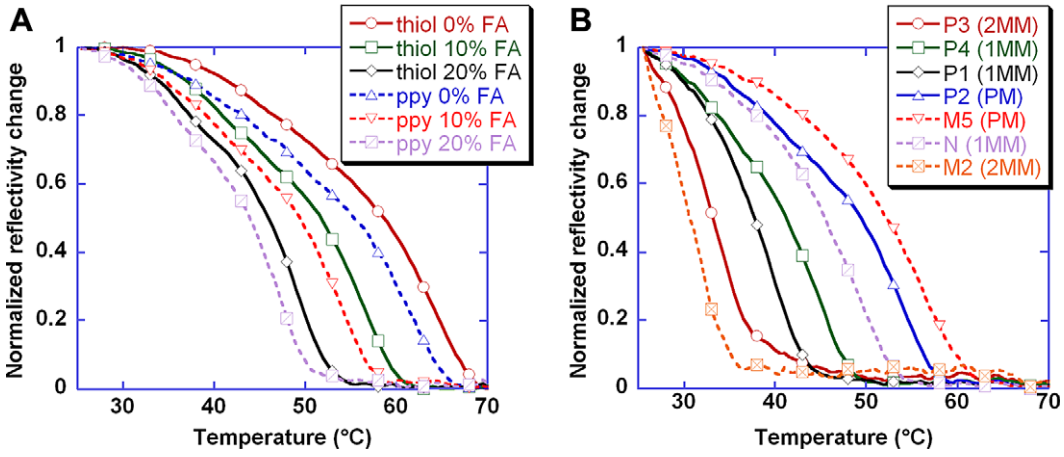

Fig. 1. (A) Comparison of thermal denaturation curves for T2 on P2 obtained in buffers of 0%, 10%, and 20% FA on polypyrrole (dashed lines) and thiol (solid lines) grafted chips. (B) Thermal denaturation curves obtained on a polypyrrole DNA chip with 10% FA buffer. Dashed lines represent the mutation system corresponding to the K-ras gene, and solid lines correspond to CCND1.

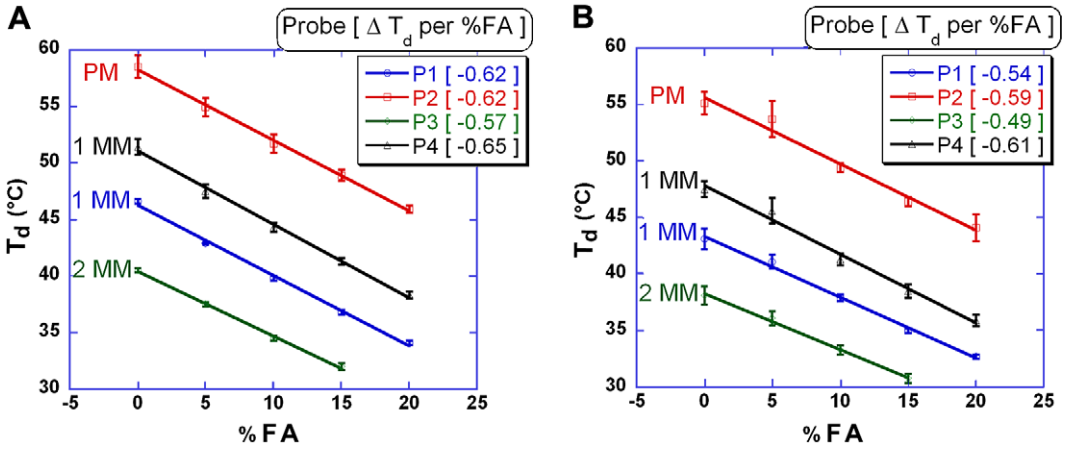

Fig. 2. Linear regression on T_d variation in dependence of the FA volume fraction for target T2: (A) on thiol-modified surfaces; (B) on polypyrrole-modified surfaces.

DNA chains (>800 bp) in solution. Thus, our results reflect the FA destabilization of DNA in solid phase experiments.

To our knowledge, this study presents for the first time the influence of FA on DNA hybridized in a surface environment. Formamide can help to suppress secondary structure in probes and targets with improved hybridization rates. The extent to which FA lowers the T_d depends little on the DNA sequence and can be fairly well approximated by an average decrease of -0.58 ± 0.05 °C/% FA (v/v) of the T_d on microarrays. We also showed that the influence of FA on oligonucleotides is independent of the grafting method. For this reason, the variation of the T_d values reported here can be applied to various biosensor and microarray platforms, saving time and cost in optimization of allele-specific DNA hybridization.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.09.044.

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