Microarray-Based in vitro Evaluation of DNA Oligomer Libraries Designed in silico

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We report on the microarray-based in vitro evaluation of two libraries of DNA oligonucleotide sequences, designed in silico for applications in supramolecular self-assembly, such as DNA computing and DNA-based nanosciences. In this first study which is devoted to the comparison of sequence motif properties theoretically predicted with their performance in real-life, the DNA-directed immobilization (DDI) of proteins was used as an example of DNA-based self-assembly. Since DDI technologies, DNA computing, and DNA nanoconstruction essentially depend on similar prerequisites, in particular, large and uniform hybridization efficiencies combined with low nonspecific cross-reactivity between individual sequences, we anticipate that the microarray approach demonstrated here will enable rapid evaluation of other DNA sequence libraries.

Introduction

Due to the exceptional molecular recognition properties of nucleic acids, the computational design of DNA sequence motifs is of paramount interest for a wide variety of applications ranging from DNA-based nanotechnology[1] and DNA computing[2] to the broad field of DNA microarray technologies.[3] In molecular nanosciences, for instance, DNA plays an outstanding role in the development of artificial biomolecular hybrid devices since the simple A – T and G – C base pairing specificity and its robust physicochemical nature allow the fabrication of nanostructured molecular scaffolding, surface architecture,[1] and nanomechanical devices,[4] as well as the selective positioning of proteins,[5] inorganic colloidal components,[6] carbohydrates,[7] organometallics,[8] and reactive chemical compounds[9] at the nanometer length scale. Likewise, in DNA computing the self-assembly of complementary nucleic acids is utilized to solve mathematical problems. Initiated by Adleman[10], this field is rapidly evolving to an established discipline dealing with combinatorial mixtures of DNA molecules, often attached to surfaces, which are subjected to hybridization reactions and enzymatic manipulations in order to perform logical operations.[11–17]

All the above examples rely on the specificity of Watson-Crick base-pairing and, thus, are highly sensitive to nonspecific interactions and the formation of any undesired secondary structures, which reduce the efficiency of intermolecular hybridization under given experimental parameters, such as temperature, salt concentration, and time. Since the manual creation of DNA motif libraries containing molecules that meet all these requirements is not feasible, computer programs have been developed for this task.[18–36] We here report on the use of a software tool, DNASequenceGenerator, previously developed for DNA sequence design,[37] for generating a small library of DNA oligomers for the DNA-directed immobilization (DDI)[38,39] of proteins on a DNA microarray. This library, optimized in silico with respect to uniform hybridization efficiency and avoidance of nonspecific cross-hybridization, was experimentaly tested and compared in vitro using microarray-experiments with a library taken from literature.[32]

Results and Discussion

DNA-directed immobilization (DDI) is a technique for the preparation of protein microarrays by means of molecular self-assembly. Protein microarrays are currently being explored as tools in proteome research and miniaturized multianalyte clinical diagnostics.[40] A general problem of protein biochip preparation, however, is the intrinsic instability of many proteins and the subsequent loss of functionality that interferes with the stepwise, robotic immobilization of multiple proteins at chemically activated surfaces. DDI[38, 39] of proteins provides a chemically mild procedure for the highly parallel attachment of multiple proteins to a solid support using a DNA microarray as an immobilization matrix (Figure 1). The high efficiency of protein adsorption, the reversibility and site-selectivity, and the experimental convenience of the DDI method enable a variety of applications including the fabrication and reconfiguration of biosensor surfaces and the production of microarrays containing both nucleic acids and proteins for genome and proteome research.[5, 41–43] An essential prerequisite for the application of

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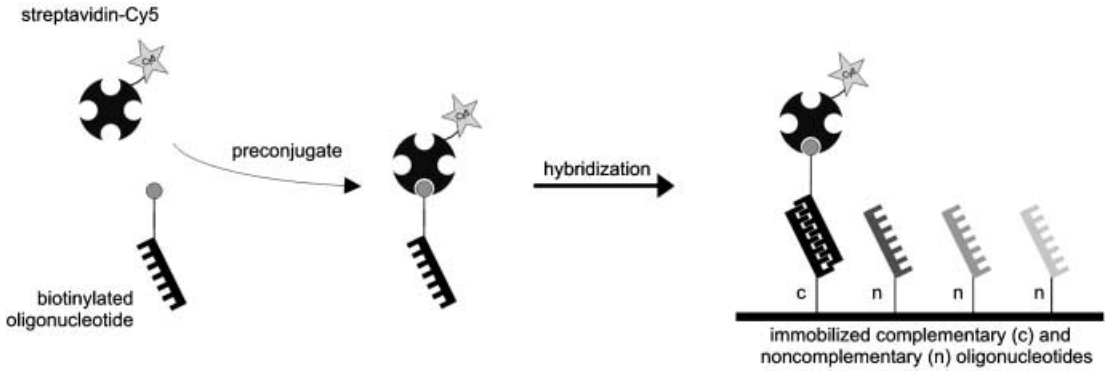

Figure 1. *Schematic drawing of the DNA-directed immobilization (DDI), as conducted in this study.*

DDI, however, is the availability of a library of designed oligonucleotide motifs that do not cross-hybridize and also have very similar hybridization efficiencies, that is the amount of material which is immobilized by nucleic acid hybridization in a certain period of time.[5, 44] Since these requirements are also necessary, amongst others, in the area of DNA computing, in particular in surface-based approaches,[15] we chose the DDI method to evaluate a software-generated library of DNA oligomer sequences experimentally.

The software DNASequenceGenerator has previously been developed for the design of DNA oligomer libraries.[37] Specificity of hybridization is achieved by limiting the length of subsequences that may appear more than once in the library to a user-defined number of nucleotides. This limit is also applied to the appearance of their complements. By forcing the oligomers to have melting temperatures within a small range, 2 °C in the case reported here, similar levels of hybridization efficiency should be obtained. A greedy graph-based search algorithm automatically assembles overlapping unique subsequences, termed "base strands", into oligomers. Several other sequence properties can be enforced automatically either on the level of the base strands or the completed sequences (Figure 2). These include the adjustment of GC-ratio, the installation of GC-base pairs at the ends of sequences to prevent fraying, the prohibition of unfavourable motifs such as three or more consecutive guanine bases, start codons, etc., as well as a maximum value for pairwise homology, which is another measure of sequence similarity. While the assembly of unique overlapping subsequences has previously been implemented in SEQUIN, a sequence design program written by Seeman[18] that supports the interactive construction of dissimilar sequences, the process is automated in DNASequenceGenerator. Modified DeBruijn sequences,[45] which also share this concept of sequence dissimilarity, do not allow for simple enforcement of additional requirements.

In this study, a library of 14 different oligomers was generated with the DNASequenceGenerator software,[37] in the following referred to as the F-library. Due to previous experimental experience with DDI,[44] the oligomers were designed to have a length of 22 bases, a GC-ratio of 50%, and a melting temperature T_M between 62 and 64 °C. The T_M values were calculated using the nearest-neighbor-method and the parameters from SantaLucia.[46] No sub-sequence of five or more nucleotides in length appears more than once in the library. Also, no more than two guanine bases appear consecutively in any of the sequences. The initial library consisted of 14 members that were manually tested for their secondary structures using the RNAFold web interface of the Vienna RNA Package, a software tool for the prediction of secondary structures based on the thermodynamic parameters of DNA.[23, 33] The ten oligomers with the least stable secondary structures predicted at 25 °C were selected for the final library. These oligomers contained essentially no secondary structure at 37 °C (Table 1).

For comparison, we chose an oligomer motif library published by Tanaka et al.,[32] in the following referred to as T-library, generated with a probabilistic optimization method termed "simulated annealing". In this approach, a solution candidate (here, a set of sequences) is improved by repeatedly applying random changes and accepting deteriorating variations only

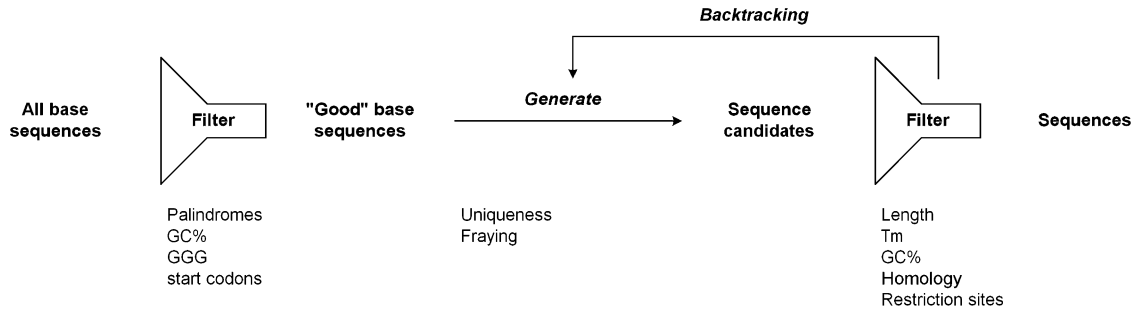

Figure 2. *Scheme of the basic sequence generation algorithm. While dissimilarity (and the avoidance of fraying) is enforced inherently by the path searching process, the other requirements are met by filtering base strands and complete sequences.*

Table 1. Oligomer sequences of the F-library generated with the DNASequenceGenerator software. Shown are the sequences in 5′ →3′ direction, the minimum free energies of secondary structure formation as predicted by RNAfold for 25 °C ($\Delta G_{ss\ 25}$) and 37 °C ($\Delta G_{ss\ 37}$), respectively. Melting temperature (T_M) and free energy at 37 °C ($\Delta G_{pm\ 37}$) of the perfect matched duplexes were calculated according to the nearest-neighbor method of SantaLucia et al.[46] Sequences printed in bold were selected for in vitro examination.

No.	Sequence	$\Delta G_{ss\ 25}$ [kcal mol^{-1}]	$\Delta G_{ss\ 37}$ [kcal mol^{-1}]	T_M [°C]	$\Delta G_{pm\ 37}$ [kcal mol^{-1}]
F1	**CCTGCGTCGTTTAAGGAAGTAC**	**−0.45**	**0.00**	**62.2**	**−26.96**
F2	**CAGCCAAGATTCTTTTACCGCC**	**0.00**	**0.00**	**63.1**	**−27.16**
F3	**CCATCATGTGTGCCGAGATATG**	**0.00**	**0.00**	**62.6**	**−26.29**
F4	**CTTCTCCTAACTGCACGGAATG**	**−0.25**	**0.00**	**63.0**	**−26.50**
F5	**GGTCCGGTCATAAAGCGATAAG**	**−0.52**	**0.00**	**62.2**	**−26.61**
F6	**GTCCTCGCCTAGTGTTTCATTG**	**0.00**	**0.00**	**62.2**	**−26.77**
F7	**GGATCTGGCGCATAGACAATTC**	**−0.23**	**0.00**	**62.7**	**−26.93**
F8	**CACGTCACTGTTAATCCGAAGC**	**−0.14**	**0.00**	**62.7**	**−27.36**
F9	**GTGGAAAGTGGCAATCGTGAAG**	**−0.24**	**0.00**	**62.4**	**−27.41**
F10	**GGACGAATACAAAGGCTACACG**	**0.00**	**0.00**	**62.1**	**−26.89**
F11	CAAGGTCTGCTTGATTTGGAGG	−2.82	−1.70	62.2	−26.55
F12	GTTTTGAACGTAGTAGAGCCGG	−0.79	−0.30	62.2	−26.96
F13	GTAGGTGTCGGTGCGAAATTAG	−1.20	−0.40	62.1	−26.89
F14	CTAGAACCGTTACGAGTTTGCG	−1.44	0.00	63.5	−27.28

with a probability that decreases over time. The objective functionalities to be optimized are several different measures of inter- and intramolecular sequence dissimilarity to prevent cross-hybridization and the proximity of GC ratio and melting temperature to preset values for homogeneous hybridization efficiency. Although this library was originally designed for DNA computing, the requirements concerning hybridization efficiency as well as inter- and intramolecular cross-hybridization are similar for word encoding in DNA computing and for oligomer design in DDI applications. Previous comparison in silico of the F-library and the T-library[32] suggested that these two bioinformatic approaches for designing sequence motifs should lead to oligomer libraries of similar performance. Thus, 10 of the 14 oligomers were selected from the T-library[32] according to their secondary structure using the procedure described above.

To experimentally evaluate the two oligomer libraries, the sequences cF1-cF10 and cT1-cT10, were purchased as 5′-amino-modified DNA oligonucleotides (Thermo Electron) and covalently attached to chemically activated glass slides, similar as previously described.[47,48] The fully complementary oligonucleotides, F1-F10 and T1-T10, listed in Tables 1 and 2, respectively, were purchased as 5′-biotinylated DNA oligonucleotides. Each of the F1-F10 and T1-T10 oligomers was coupled with one molar equivalent of Cy5-labeled streptavidin (STV_{Cy5}) in a Tris-HCl-EDTA buffer at pH 7.3 in separate reactions. As investigated by gel-electrophoretic analysis, this coupling leads to the efficient formation of oligonucleotide-STV_{Cy5} conjugates, mainly of a 1:1 equimolar stoichiometry between the streptavidin and the biotinylated oligonucleotide attached, and the solution contains basically no free oligonucleotides which might interfere with conjugate hybridization (data not shown). Subsequently, each of the 10 F1-STV_{Cy5} - F10-STV_{Cy5} conjugates was allowed to bind to a DNA microarray containing capture oligomers cF1-cF10. Similarly, each of the 10 T1-STV_{Cy5} – T10-STV_{Cy5} conjugates was allowed to bind to an array containing capture oligomers cT1-cT10. Subsequent to hybridization for 2 h at 37 °C, the arrays were rinsed to remove unbound material, the slides were imaged with a microarray scanner (Axon), and the signals were quantified using GenPix pro 4.1 software. Typical slide images obtained are shown in Figure 3.

Table 2. Oligomer sequences of the T-library, taken from Tanaka et al.[32] Shown are the sequences in 5′ →3′ direction, the minimum free energies of secondary structure formation, as predicted by RNAfold for 25 °C ($\Delta G_{ss\ 25}$) and 37 °C ($\Delta_{Gss\ 37}$), respectively. Melting temperature (T_M) and free energy at 37 °C ($\Delta G_{pm\ 37}$) of the perfectly matched duplexes were calculated according to the nearest-neighbor method of SantaLucia et al.[46] Sequences printed in bold were selected for in vitro examination.

No.	Sequence	ΔG_{25} [kcal mol^{-1}]	ΔG_{37} [kcal mol^{-1}]	T_M/ °C	$\Delta G_{pm\ 37}$ [kcal mol^{-1}]
T1	**CGAGACATCGTGCATATCGT**	**−1.84**	**−1.00**	**61.8**	**−24.95**
T2	**TCTGTACTGCTGACTCGAGT**	**0.00**	**0.00**	**60.5**	**−24.25**
T3	**CGAGTAGTCACACGATGAGA**	**−1.42**	**−0.40**	**60.3**	**−23.93**
T4	**AGATGATCAGCAGCGACACT**	**0.00**	**0.00**	**62.1**	**−25.15**
T5	**TGTGCTCGTCTCTGCATACT**	**−1.68**	**−0.70**	**61.6**	**−24.59**
T6	**AGACGAGTCGTACAGTACAG**	**−1.41**	**−0.60**	**58.8**	**−23.92**
T7	**ATGTACGTGAGATGCAGCAG**	**0.00**	**0.00**	**60.9**	**−24.48**
T8	**ATCACTACTCGCTCGTCACT**	**0.00**	**0.00**	**61.0**	**−24.93**
T9	**GCTGACATAGAGTGCGATAC**	**−0.35**	**0.00**	**59.6**	**−23.78**
T10	**ACATCGACACTACTACGCAC**	**−0.46**	**0.00**	**59.0**	**−24.53**
T11	TATAGCACGAGTGCGCGTAT	−2.93	−2.20	62.8	−24.98
T12	GATCTACGATCATGAGAGCG	−2.76	−1.90	60.6	−23.86
T13	TCAGAGATACTCACGTCACG	−1.78	−1.10	59.5	−23.93
T14	GACAGAGCTATCAGCTACTG	−4.47	−2.60	60.0	−23.01

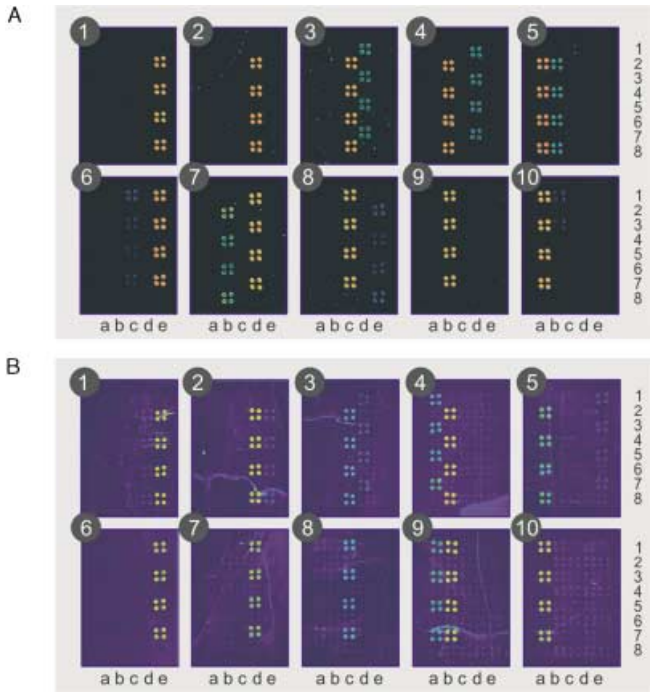

Figure 3. *Microarray fluorescence images obtained from DDI experiments using oligomer-STV$_{Cy5}$ conjugates which hybridize to DNA arrays containing capture oligonucleotides from two different libraries, generated either with DNASequenceGenerator (A) or taken from Tanaka et al.[38] (B). Each capture oligomer was spotted on the array in quadruplets, and each oligomer-STV$_{Cy5}$ conjugate was allowed to hybridize to an array containing all capture oligomers, listed in Tables 1 and 2, respectively. The fully-matched signal runs from e/8/6/4/2 to a/8/6/4/2 (1 – 5) and from e/7/5/3/1 to a/7/5/3/1 (6 – 10). Each position encodes for a quadruplet of identical capture oligomers.*

The quality of the sequence design is immediately evident from a naked-eye comparison of the signal intensities obtained from the two libraries F1-F10 and T1-T10 (Figure 4). The numerical signal intensities of the two libraries are summarized in Tables 3 and 4, respectively. As can be seen from the signals of the fully-matched hybridization, library F1-F10 yielded much higher signals than library T1-T10 with average intensities of 29849 ± 8395 a.u. and 4727 ± 3550 a.u., respectively. This corresponds to deviations in hybridization efficiency for perfectly matched oligomer pairs of $\pm 28\%$ (F1-F10) and $\pm 75\%$ (T1 – 10). In particular, library T1-T10 shows maximum deviations of about 156%, while this value is only 52% in library F1-F10. It is not yet clear why the T-library shows much lower total fluorescence signals compared to the F-library even though the calculated T_M values are not much different. Further studies will investigate this issue.

The second point of major importance concerns the extent of cross-hybridization of the oligonucleotide-STV$_{Cy5}$ conjugates to noncomplementary capture oligomers. On the basis of data shown in Tables 3 and 4, we set a threshold value for cross-reactivity of 15% of the lowest signal intensity obtained from the fully-matched probes, that is, 3019 a.u. for library F1-F10 and 79 a.u. for library T1-T10. This threshold value is of practical importance for DDI protocols since in biomedical diagnostics one needs to discriminate signals resulting from non-specific hybridization and background immunosorption from the perfectly matched oligomer pairs. Taking into account these threshold values, the T-library showed five false-positives whereas the F-library had no peak due to nonspecific hybridization. Although this result clearly demonstrates how careful design of oligomer sequences can improve the in vitro performance of such a library, we note that additional rounds of design and evaluation will be necessary to further reduce cross-reactivity in order to meet the dynamic range performance criteria of routine immuno-assays.[43]

The less homogenous distribution of hybridization efficiencies of the T-library might originate, in part, from the estimation of duplex melting temperature by mere use the of GC ratio.[32]

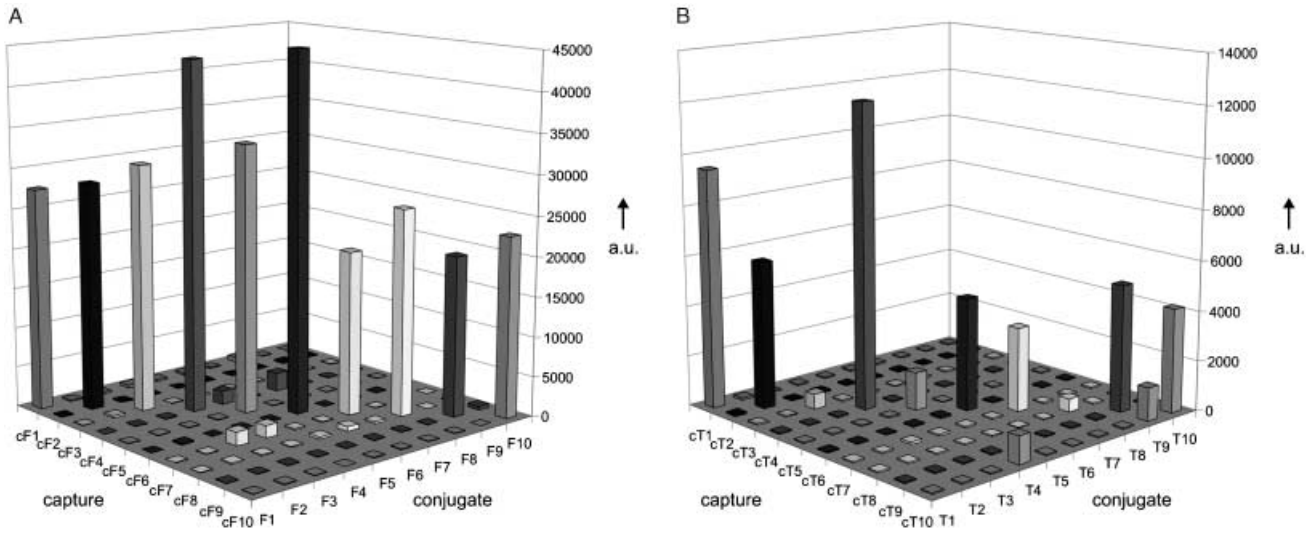

Figure 4. *Hybridization signals obtained in DDI experiments with the two different oligonucleotide libraries, generated either with DNASequenceGenerator[37] (A) or taken from Tanaka et al.[32] (B). Shown are the fluorescence signal intensities obtained from hybridization of oligomer-STV$_{Cy5}$ conjugates, similar as described in the legend of Figure 3. Note that the library in (A) has higher and more homogeneous signal intensities than the library in (B) with average signals of $29849 \pm 28\%$ and $4727 \pm 75\%$, respectively.*

Table 3. Fluorescence signal intensities of DDI experiments using the oligonucleotide library F1-F10, generated with DNASequenceGenerator.[37] Numbers printed in bold represent the hybridization signals obtained for the perfect-match complementary oligonucleotides (average signal: 29849 ± 28 %). Note the lack of cross-reactivity (values greater than 15 % of the lowest perfect-match signal, that is, 3019).

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
cF10	56	110	68	70	110	111	73	91	60	**22655**
cF9	58	61	84	72	64	82	142	73	**20124**	469
cF8	57	180	115	132	293	591	249	**25847**	59	57
cF7	56	77	1722	1545	176	117	**20459**	66	59	59
cF6	56	171	75	59	101	**45428**	65	195	66	57
cF5	58	115	89	113	**33477**	80	198	65	59	62
cF4	58	89	110	**43646**	1703	72	2328	62	54	58
cF3	58	326	**30814**	76	62	82	128	396	58	56
cF2	71	**28430**	144	57	59	89	62	60	219	79
cF1	**27605**	203	203	356	243	277	161	690	63	59

Table 4. Fluorescence signal intensities of DDI experiments using the oligonucleotide library T1-T10 taken from Tanaka et al.[38] Numbers printed in bold represent the hybridization signals obtained for the perfect-match complementary oligonucleotides (average signal: 4727 ± 75 %). Please note the larger deviation in average signal intensity and the larger number of cross-reactive oligomers (bold and underlined entries) as compared to that observed in the F-library (Table 3).

	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
cT10	42	47	44	**1092**	51	44	49	70	**1326**	**4174**
cT9	43	48	47	59	54	45	51	59	**5087**	73
cT8	45	47	45	57	52	51	52	**525**	56	58
cT7	50	51	**100**	64	52	52	**3360**	46	57	68
cT6	45	46	50	54	**88**	**4506**	58	40	48	56
cT5	41	44	45	57	**1522**	44	48	45	60	52
cT4	45	49	51	**12128**	54	48	59	50	63	79
cT3	52	49	**604**	62	55	49	53	60	59	67
cT2	75	**5881**	52	65	56	53	58	47	56	75
cT1	**9483**	**105**	56	64	57	53	62	45	53	66

However, the calculation of T_M values using nearest-neighbor methods, in our hands, did not reveal a significantly larger standard deviation for the T-library (± 1.13 °C) than for the F-library (± 0.36 °C). In agreement with experimental studies carried out earlier,[44] this again underlines the fact that a narrow range of duplex stability, and thus T_M values, is not the only prerequisite for homogeneous hybridization efficiency. According to in silico evaluation, the pairwise homology (a Hamming-distance-based measure) of both libraries were quite similar, that is 0.38 ± 0.05 in T-library and 0.36 ± 0.05 in F-library. The more striking difference between the two libraries concerns the increased length of unique subsequences – nine nucleotides in T-library as compared to five nucleotides in F-library. This difference should be responsible for the higher rate of cross-hybridizations observed in vitro for the T-library. Thus, the concept of sequence dissimilarity, implemented in the DNASequenceGenerator software,[37] appears to be a realistic approach to minimize cross-hybridization.

In summary, we here reported on the in vitro evaluation of oligonucleotide sequences, designed in silico. To the best of our knowledge, this is the first study which is devoted to the comparison of sequence motif properties theoretically predicted with their performance in real-life. Thus, the study is one crucial step towards the systematic development of software tools for DNA sequence design for applications in supramolecular self-assembly. Since DDI-based microarray technologies,[5, 41] DNA computing,[2] and DNA nanoconstruction[1] essentially depend on similar prerequisites, in particular, large and uniform hybridization efficiencies combined with low nonspecific cross-reactivity between individual sequences, we anticipate that the microarray approach demonstrated here will enable rapid evaluation of other DNA sequence libraries. Repeated cycles of design and evaluation will expand the number of oligomer members within a library and simultaneously improve their in vitro performance. Our future work will include further comparative studies of published sequence data as well as the improvement of our software's performance, for instance, to predict values for mismatch hybridization and for absolute signal intensities. Moreover, development of longer capture oligomers, capable of specifically assembling two and more different DNA-protein conjugates into well-defined supramolecular aggregates,[5, 49] is under way.

Experimental Section

Covalent immobilization of oligonucleotides: For the attachment of oligonucleotides, typically 0.3 nL of a solution of the 5′-amino-modified capture oligonucleotide in water (10 μm, Thermo Electron) were deposited onto 3DProtein Slides (Chimera Biotec, Dortmund) using a piezo-driven spotting device (GESIM), and the slides were incubated overnight. The slides were stored at − 20 °C until use.

Conjugate preparation: 10 pmol of streptavidin-Cy5 (Amersham Bioscience) and 10 pmol of the biotinylated oligonucleotide (Thermo

Electron) were coupled for 10 min at room temperature in a reaction volume of totally 10 μL TE buffer (10 mM Tris, 1 mM EDTA). Subsequently, the preconjugates were diluted in TETBS buffer (120 mM NaCl2, 20 mM Tris, 5 mM EDTA, 0.05 % Tween20) supplemented with 800 μm dBiotin (Sigma) to a final conjugate concentration of 10 nm.

Hybridization experiments: To reduce nonspecific binding of the Cy5-labeled STV-DNA conjugate, the microarray was pretreated for 30 min with blocking solution (Chimera Biotec). Subsequently, the microarray was dried with nitrogen, a hybridization chamber (ABGene) was fixed on top of the slide and the resulting chamber was filled with the conjugate solution. Hybridization was carried out at 37 °C for two hours. The chip was then washed twice for 5 min with TETBS followed once by rinsing with deionized water. The microarray was dried with nitrogen. The fluorescent signal intensities were measured with a microarray laser scanning system (Axon), and the signals were quantified using GenePix pro 4.1 software (Axon).

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Keywords: bioinformatics · microarrays · nanostructures · nucleic acids · self-assembly

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