DNA Hydrogels

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Construction of Three-Dimensional DNA Hydrogels from Linear Building Blocks**

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Abstract: *A three-dimensional DNA hydrogel was generated by self-assembly of short linear double-stranded DNA (dsDNA) building blocks equipped with sticky ends. The resulting DNA hydrogel is thermoresponsive and the length of the supramolecular dsDNA structures varies with temperature. The average diffusion coefficients of the supramolecular dsDNA structures formed by self-assembly were determined by diffusion-ordered NMR spectroscopy (DOSY NMR) for temperatures higher than 60°C. Temperature-dependent rheological measurements revealed a gel point of 42 ± 1°C. Below this temperature, the resulting material behaved as a true gel of high viscosity with values for the storage modulus G′ being significantly larger than that for the loss modulus G″. Frequency-dependent rheological measurements at 20°C revealed a mesh size (ξ) of 15 nm. AFM analysis of the diluted hydrogel in the dry state showed densely packed structures of entangled chains, which are also expected to contain multiple interlocked rings and catenanes.*

Since the first report on the formation of a pristine DNA hydrogel in 2006,[1,2] there is growing interest in the development of DNA hydrogels especially for applications as drug-release systems.[2–4] When DNA-hydrogels were generated from flexible branched double-stranded DNA (dsDNA) building blocks which were linked covalently, the enzyme-catalyzed gel formation and the release of entrapped molecules were rather slow.[2,5] As an alternative, a fast-responding pH-triggered DNA hydrogel has been formed from Y-shaped DNA building blocks, which were equipped with cytosine-rich interlocking i-motif domains.[3] At low pH values, a hydrogel was obtained, which was able to trap gold nanoparticles (AuNPs). When the pH value was changed to pH 8, the gel quickly disassembled and the AuNPs were released.[3]

More recently, DNA hydrogels have been formed by hybridization of Y-scaffolds with linear (linker) dsDNA.[4] Hydrogel formation could be reversed by heating above the melting temperature, and the temperature-dependent gel-sol transition could be monitored by rheological measurements. This type of hydrogel has been used to trap single living cells in microwells.[6]

While in previous approaches branched DNA motifs were required for the formation of DNA hydrogels,[1–6] we generated a DNA hydrogel solely from linear dsDNA equipped with sticky ends. A combined experimental study on the self-assembly of linear dsDNA building blocks using diffusion-ordered NMR spectroscopy (DOSY NMR), rheology, and atomic force microscopy (AFM) is presented.

The sequences of the investigated single-stranded DNA (ssDNA) and dsDNA monomers are shown in Scheme 1. By hybridization of two oligomers, oligo 1 (**O1**) with oligo 2 (**O2**), a monomeric dsDNA building block **O1**-**O2** with 30 base pairs (bp) is formed. This building block is further equipped with two complementary overhangs (sticky ends) of 15 bases for self-assembly. As a reference compound, a monomeric dsDNA building block **O1**-**O3** (30 bp) with two non-complementary overhangs was created, which did not allow further self-assembly. In Scheme 1 the strategy for the formation of DNA nanostructures by self-assembly is illustrated, and the sequences of the ssDNA oligomers **O1**, **O2**, and **O3** are given. The individual oligomers **O1**, **O2**, and **O3**, as well as **O1**-**O2** and **O1**-**O3**, have been analyzed by polyacrylamide gel electrophoresis (PAGE) as shown in the Supporting Information (Figure S2).

Linear structures formed by self-assembly of **O1**-**O2** will comprise only fully base-paired dsDNA (if we neglect the two overhangs at the beginning and the end of the resulting dsDNA structure). Such structures should be more flexible and less stable than long dsDNA consisting of only two long intact single strands, since in the self-assembled dsDNA there

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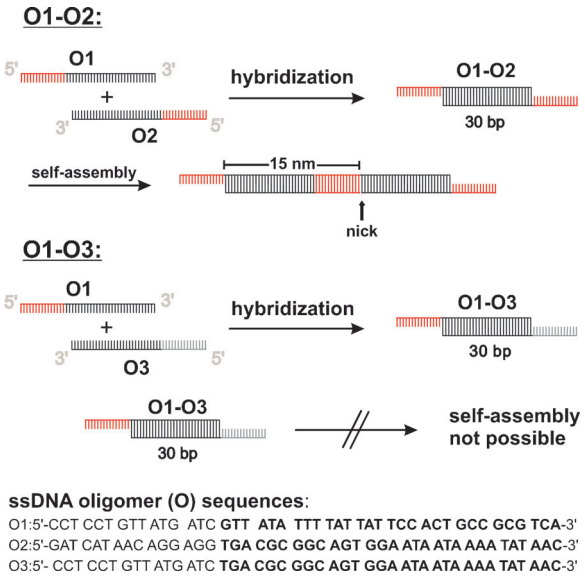

Scheme 1. Strategy for the formation of DNA hydrogels. By hybridization of **O1** with **O2**, a dsDNA monomer (**O1-O2**, 30 bp and 15 base sticky ends) is generated, which can self-assemble. The dsDNA monomer **O1-O3** (30 bp) comprises non-complementary 15-base overhangs and cannot further self-assemble.

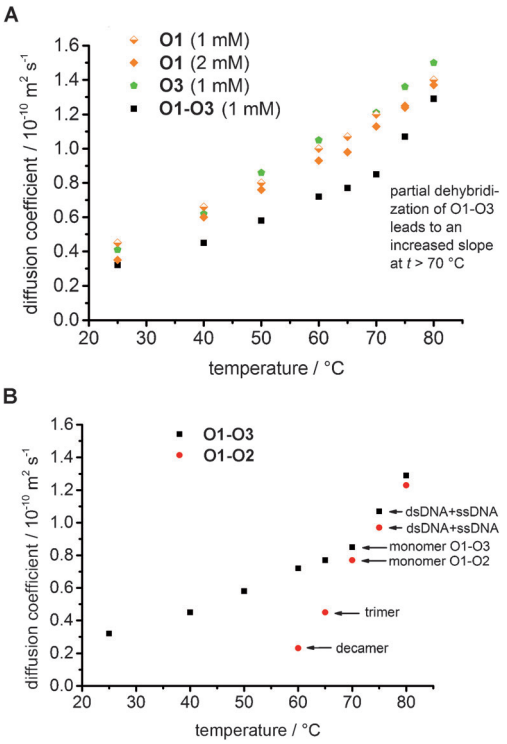

Figure 1. A) Diffusion coefficients of the ssDNA oligo **O1** (1 mM and 2 mM), oligo **O3** (1 mM), and the dsDNA oligomer **O1-O3** (1 mM) with increasing temperature (25–80 °C). B) Diffusion coefficients of **O1-O3** and the DNA hydrogel generated by self-assembly of **O1-O2** (1 mM) at increasing temperature (25–80 °C).

is a nick in the sugar–phosphate backbone after each ssDNA repeating unit of 45 bases (15 nm).

The generation of reversible polymers from linear DNA-based building blocks has been previously described.[7,8] Although an increase in viscosity upon self-assembly was observed, the resulting polymers did not behave as hydrogels. Apparently there was a strong dependence of the resulting properties of the material on the length and base sequence of the individual DNA-based building blocks.[7,8] The formation of cyclic products was also described and had been reported in ligation-closure experiments on simple flexible branched DNA molecules.[7–9] For instance, when flexible three-arm or four-arm nucleic acid junctions with two sticky-ended arms were oligomerized, trimeric cycles were formed as the smallest structure.[10,11] For dsDNA fragments of less than 100 bp, efficient loop formation has been observed.[12–14] Depending on the concentration of the building blocks from which cyclic DNA structures are generated, interlocked rings, such as catenanes,[15,16] borromean rings,[17] or olympic ring-like structures, might be obtained. The existence of interlocked rings could explain hydrogel formation in this study.

With prolonged self-assembly of **O1-O2** the diffusion coefficient of the resulting supramolecular structures is expected to decrease. Therefore the structures generated from **O1-O2** and a solution of **O1-O3** were analyzed by DOSY NMR spectroscopy (which allows the determination of the diffusion coefficient) at temperatures from 25 °C to 80 °C. As relatively high concentrations of the analyte are required for DOSY NMR experiments, we started our experiments by mixing **O1** and **O2**, leading to a final concentration of 1 mM using pure D_2O as solvent. After heating and cooling to room temperature, the mixture of the oligonucleotides **O1** and **O2** resulted in the formation of a stable gel-like compound (see Supporting Information, Figure S1). In contrast, the mixture of **O1** and **O3** was a liquid at room temperature.

Figure 1 A shows the temperature-dependent diffusion coefficients of the oligonucleotides **O1**, **O3**, and the monomeric dsDNA **O1-O3** generated by hybridization of **O1** and **O3**. The ssDNA strands **O1** and **O3** show similar behavior with increasing temperature (the diffusion coefficients increase).[18] With increasing temperature, partial dissociation of **O1-O3** is expected. After complete dissociation, the total concentration of ssDNA is 2 mM. For that reason we were interested in the effect of concentration on the diffusion coefficient and investigated **O1** also at a concentration of 2 mM. As can be seen from Figure 1 A, the difference in diffusion coefficients for concentrations of 1 mM and 2 mM is minor. For **O1-O3**, starting from 70 °C a faster increase in the diffusion coefficients with temperature was detected (steeper slope). This steeper slope can be attributed to the beginning of dehybridization of the oligonucleotides. During DOSY NMR spectroscopic experiments, only average values for the diffusion coefficients were detected, since the resonance signals in the ^{1}H NMR spectra of the individual compounds are completely overlapping (independent from the kinetics of DNA hybridization/dehybridization).[19]

At complete dehybridization, the diffusion coefficient measured for **O1-O3** should be the mean value between that of **O1** and **O3**. This is not the case in our experiments at 80 °C, which indicates that the dehybridization is not yet complete at that temperature. Extrapolation of the slope of the temper-

ature-dependent diffusion coefficient of **O1-O3** above 70 °C (Figure 1 A), suggests that complete dehybridization will be reached at temperatures between 85 °C and 90 °C.

In Figure 1 B the diffusion coefficients of **O1-O3** and **O1-O2** at different temperatures are shown. As observed for the monomeric dsDNA **O1-O3**, dehybridization of dsDNA **O1-O2** also begins at temperatures higher than 70 °C, and mixtures of dsDNA and ssDNA are present in solution. With decreasing temperature (less than 70 °C), the subsequent oligomerization of monomeric dsDNA **O1-O2** is indicated by a strong decrease in the diffusion coefficient. For linear molecules, the following relationship between the masses, m, and the diffusion coefficients, D, is reported [Eq. (1)]:[18,20,21]

$$\frac{D_1}{D_2} = \sqrt{\frac{m_2}{m_1}} \quad (1)$$

According to this formula, the oligomers from the monomeric dsDNA building blocks **O1-O2** consist on average of three dsDNA monomers at a temperature of 65 °C, and ten at a temperature of 60 °C, respectively. At temperatures below 60 °C, the diffusion coefficients of **O1-O2** were too low to be detectable by DOSY NMR. Figure 2 shows the ^{1}H DOSY NMR spectra of **O1-O3** and **O1-O2** between 60 °C and 80 °C in steps of 5 °C (further DOSY NMR spectra are given in the Supporting Information). At a temperature higher than 80 °C, measurement of DOSY NMR spectra was not possible with our experimental setup. To reduce convection artefacts, a DOSY pulse sequence was used according to the convection-compensation approach.[22]

To demonstrate the formation of a true hydrogel, rheological measurements were performed on **O1-O2**. The hydrogel was heated to 90 °C and applied to a rheometer. The measurements were performed during a temperature cycle cooling the sample from 60 °C to 20 °C with a cooling rate of $-4\ \mathrm{K\,min^{-1}}$. Thereafter, a second temperature ramp from 20 °C to 60 °C with a heating rate of $+4\ \mathrm{K\,min^{-1}}$ was applied.

In Figure 3 A the values for the storage and loss modulus G' and G'' for **O1-O2** in the temperature region between 20 °C and 50 °C are shown. At temperatures above 50 °C, the viscosity of the samples was too low to gain accurate values. In a second cycle (not shown) the same behavior was observed, indicating that the sample behaves reversibly. The small hysteresis between the curves for cooling and heating indicates that the sample is able to adopt a new equilibrium composition relatively quickly.

As can be seen from Figure 3 A, the gel point ($G' \geq G''$) occurs at approximately 42 °C. Below this temperature, the composition behaves as a true hydrogel, evident also from the frequency-dependent scan at 20 °C (Figure 3 B). Over the entire frequency range, the value of G' was significantly higher than that of G''. For a hydrogel made from a single type of linear DNA building block, these values of G' and G'' are rather high and lead to the assumption that the resulting structures are highly interlocked. Furthermore, in contrast to

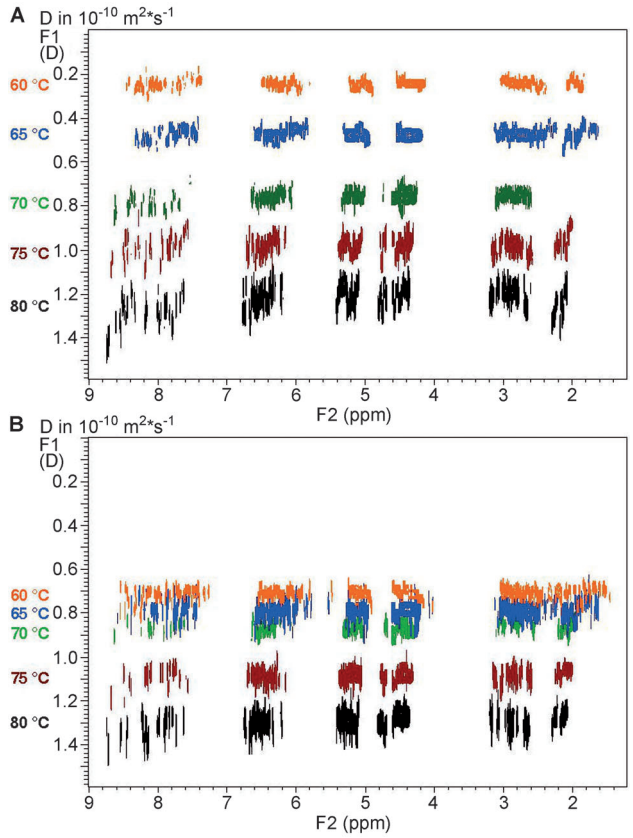

Figure 2. A) ^{1}H NMR DOSY spectra of **O1-O2** from 60–80 °C in steps of 5 °C. B) ^{1}H NMR DOSY spectra of **O1-O3** from 60–80 °C in steps of 5 °C.

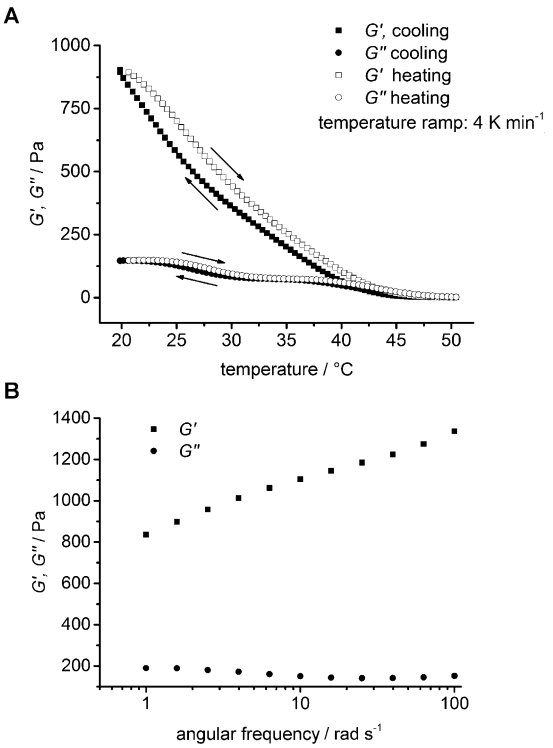

Figure 3. A) Temperature-ramp rheological measurements between 20 °C and 50 °C for the hydrogel formed from **O1-O2** in D_2O. B) Frequency-sweep rheological measurements for the same hydrogel at 20 °C.

other investigations on DNA hydrogels,[3,4] in the current study only pure water (D_2O) has been used as solvent. Since in the presence of salt the hybridization efficiency is strongly increased, the addition of salt is expected to further enhance the entanglements and elastic effects of the hydrogel.

The mesh size ξ of a polymeric, entropic, elastic network can be calculated by the following formula [Eq. (2)]:[23]

$$\xi = \sqrt[3]{\frac{(k_B T)}{G^0}} \qquad (2)$$

The corresponding value of the plateau modulus G^0 (i.e. the value of G' at the angular frequency at which the curve of G'' has a minimum) for the hydrogel formed from **O1-O2** can be taken from Figure 3B. Using a value of 1100 Pa for G^0, a mesh size of 15 nm was calculated at 20 °C. This value is in the same range as the length of the repeating unit of **O1-O2**. This indicates that the dsDNA strands of the hydrogel are strongly entangled.

The structures obtained by self-assembly of the **O1-O2** monomers were analyzed on the molecular level by AFM. The hydrogel was scanned in the dry state in ambient air after deposition on dodecylamine-modified highly ordered pyrolytic graphite (HOPG). For the undiluted hydrogel, a 3D layer with a thickness of several nm was obtained. In this case it was not possible to resolve nanometer-scale structural details. Therefore, the hydrogel was diluted after heating to 80 °C and the resulting samples underwent a heating/cooling process. In Figure 4, an AFM image of the hydrogel after 5000-fold dilution is shown. The DNA hydrogel formed a layer consisting of closely packed circular structures with a mean radius of 13.0 ± 4.7 nm (some circles measure up to 30 nm). In the center of larger rings the flat background representing the HOPG substrate is visible; the height and the width of the circular DNA strands measure 1.1 ± 0.2 nm and 15.7 ± 4.8 nm, respectively. The height is consistent with the diameter of a single molecule dsDNA measured by intermittent contact mode AFM,[24] and the lateral dimensions correspond to the diameter of approximately two molecules of dsDNA, taking into account the tip convolution effect. The differentiation of individual DNA molecules is difficult because of the close vicinity to other rings. Similar dimensions of such pore-like structures were reported for a hydrogel formed from branched X-shaped DNA molecules consisting of four oligonucleotides of comparable length.[2] This observation is consistent with a tight association, entanglements, and eventually concatenation of cyclic DNA molecules formed under the conditions used in this study. For comparison, samples of **O1-O3**, which cannot self-assemble, were prepared under identical conditions and were analyzed by AFM (Supporting Information, Figure S8). In contrast to **O1-O2** (Figure 4), the observed structures of **O1-O3** were separated and did not form an extended network.

If the formation of a highly viscous hydrogel originates from the flexibility of the supramolecular dsDNA structures formed by self-assembly, then an increase in flexibility should result in a further increase in viscosity. To confirm this assumption, we carried out rheological measurements on **O4-O5**, which are shown in Figure 5. Building block **O4-O5** has the same sequence as **O1-O2** except for one base, which has been added to each oligomer. These additional bases remain unpaired upon self-assembly, thereby introducing additional flexibility. As seen from the rheological data, the viscosity is further increased, and in comparison to **O1-O2**,

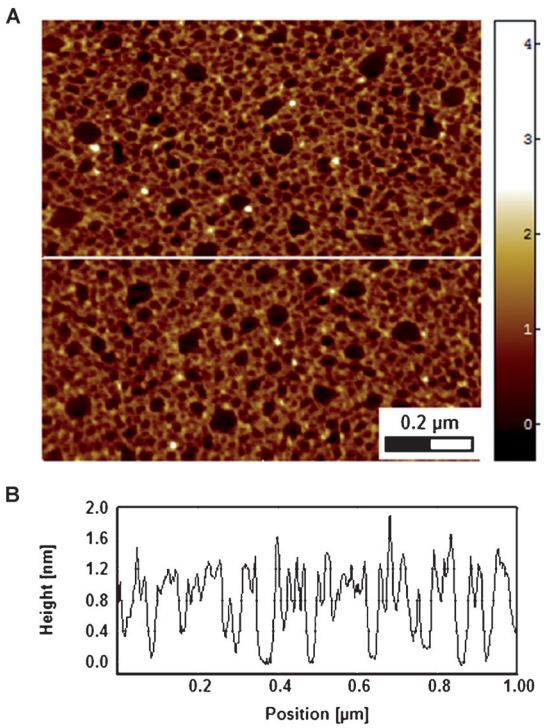

Figure 4. A) Intermittent contact mode AFM (constant amplitude) height image displaying the topography of **O1-O2** deposited onto dodecylamine-modified HOPG after 5000x dilution and heating/cooling (the color bar indicates the height in nm). B) Section along the horizontal line indicated in (A).

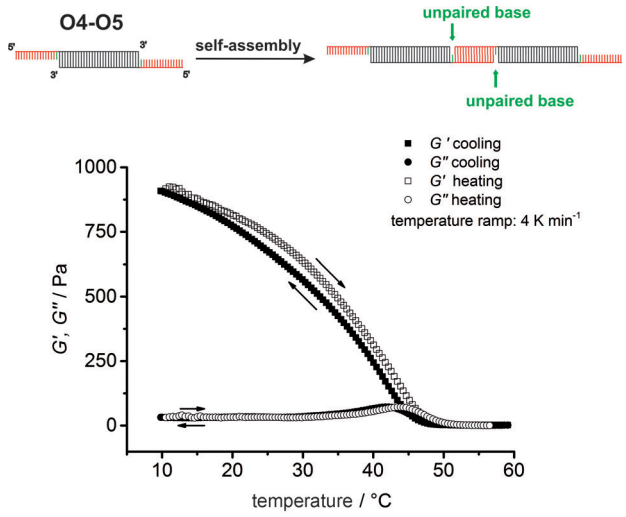

Figure 5. Self-assembly of **O4-O5** and temperature ramp rheological measurements between 10 °C and 60 °C for the hydrogel formed from **O4-O5** in D_2O. Oligomer **O4-O5** has the same sequence as **O1-O2** except for one additional T and one G (green), which have been added in **O4** and **O5**, respectively.

the gel point of **O4**-**O5** is shifted to higher temperatures by about 5 °C.

In this work we presented a highly entangled DNA hydrogel generated by self-assembly of linear dsDNA equipped with sticky ends. The rheological properties of our hydrogels were comparable to those of DNA hydrogels built from more complicated branched components,[3,4] even though our experiments were carried out in pure D_2O. In the presence of salt an even higher viscosity of the gels would be expected. The unexpectedly high viscosity of the investigated hydrogels can be explained by the high flexibility of the DNA building blocks. AFM analysis showed densely packed structures of entangled chains, which are also expected to contain structures such as multiple interlocked rings and catenanes. Since the DNA hydrogel was not covalently linked (which could be achieved by the addition of ligase), the material is thermoresponsive. From the rheological experiments, it can be seen that the hydrogel is able to adopt a new equilibrium composition rather quickly after a change in temperature. In this study, DOSY NMR analysis was used for the first time to characterize the supramolecular structures present in DNA hydrogels at different temperatures.

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