Sequence-Specifically Addressable Hairpin DNA—Single-Walled Carbon Nanotube Complexes for Nanoconstruction

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ABSTRACT Single-walled carbon nanotubes (SWCNTs) are attractive building blocks for molecular electronics and novel materials. Generating functional architectures with SWCNTs requires methodologies for dispersing, purifying, and binding these highly insoluble quasi one-dimensional molecules. We have previously shown that unstructured DNA strands bind to carbon nanotubes so tightly that it is difficult to address them with complementary strands. Here we show that hairpin oligonucleotides give SWCNT suspensions more concentrated than those obtainable with previously optimized DNA sequences. Further, hairpin-forming oligonucleotides and (6,5)-SWCNTs form complexes that are addressable with complementary, triplex-forming oligonucleotides. As proof of principle, we show that DNA—SWCNT complexes can be bound sequence-specifically with oligonucleotides featuring fluorophores or quantum dots. The new method brings SWCNTs of exquisite purity into the realm of DNA-based nanostructuring.

KEYWORDS: carbon nanotubes · DNA · nanostructuring · molecular recognition · triplex formation

Manipulating carbon nanotubes on a molecular level is challenging. These fascinating quasi-one-dimensional molecules form tight bundles that make it difficult to generate conventional solutions. Even for optimized organic solvents, suspensions of single-walled carbon nanotubes (SWCNTs) are metastable.[1−3] Conventional purification, for example, by crystallization, cannot be applied, and chromatography is complicated by the unusual shape of these carbon allotropes that can lead to entanglement in the pores of stationary phases.

Once sufficiently stable suspensions have been generated, for example, through the use of detergents,[4−6] a new challenge arises: addressing the nanotubes,[7−9] so that they can be incorporated site-specifically in devices. Bottom-up molecular assembly approaches require functional groups. Carbon nanotubes lack such groups, and introducing them *via* covalently attached side chains[10,11] disrupts their electronic structure. This is unfortunate, as it limits the use of carbon nanotubes, an otherwise extremely useful type of new materials, for which nanotechnological applications have been proposed ever since the discovery of their unusual electrical, thermal, mechanical, and optical properties.[12−14]

Recently, an unanticipated molecular partner has emerged for solubilizing and purifying SWCNTs, namely DNA. This biopolymer, the carrier of genetic information in the cell, gives surprisingly stable and concentrated suspensions of SWCNTs after sonication of mixtures in aqueous solution.[15−18] In fact, complexes of carbon nanotubes can be used to deliver DNA into cells.[19−25] The use of DNA-wrapped SWCNTs in nanoconstruction is less well developed. A key problem is that SWCNT—DNA complexes prepared with a probe sonicator are kinetically more stable than the corresponding Watson Crick-paired DNA:DNA duplexes, as shown in thermal denaturation studies.[26,27] Apparently, after probe sonication, DNA bases adsorb so strongly on the lipophilic surface of naked carbon nanotubes that they are no longer accessible to base pairing with bases of complementary strands.[28]

How then may DNA be used to generate a coating for SWCNTs that ensures sufficient solubility and addressability to allow for the bottom-up generation of molecular devices? We surmised that a combination of surfactants, used in the initial dispersion process, and structured DNA, introduced in a later, less denaturing step, might give addressable nanotubes. The surfactants can be expected to suppress the formation of complexes with fully adsorbed, nonaddressable DNA

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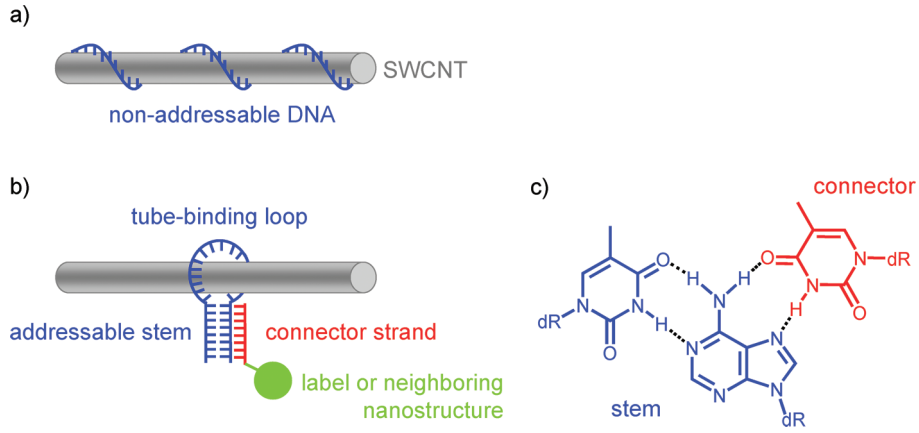

Figure 1. SWCNT complexed with DNA. (a) Single-stranded oligonucleotide helically wrapped around a nanotube, (b) hairpin DNA (blue) binding both to a SWCNT with its single-stranded loop and to a complementary connector strand (red) *via* its stem region, (c) typical base triplet of a pyrimidine−purine−pyrimidine triple helix, shown here for a T:A:T triplet.

(Figure 1a). Further, solubilization with detergents can be expected to allow for the use of density gradient ultracentrifugation protocols that yield nanotubes of essentially a single helicity. Nanotube preparations, where unstructured DNA was added after the initial solubilization step, have been shown to undergo aggregation in DNA-mediated fashion and to bind gold nanoparticles.[8] Also, nanotubes first solubilized by sonication in DMF, and then treated with fluorophore-labeled oligonucleotides have been reported to act as biosensors for DNA and proteins.[29] Still, there is a need for methods that employ structured DNA motifs, where intramolecular base pairing competes with full-length adsorption, and that utilize nanotubes of a single chirality to incorporate SWCNTs in devices *via* programmable DNA−DNA interactions.

Our study involved two stages. First, hairpin-forming oligonucleotides of varying length and sequence were screened for their solubilizing properties, in the absence of a detergent. On the second stage, a hairpin-forming sequence selected *via* the screen was allowed to form complexes with surfactant-coated nanotubes. The resulting complexes were then addressed with oligonucleotides bearing fluorescent cargo moieties.

RESULTS

Complexes of Hairpins with Naked Nanotubes. To obtain DNA−SWCNT complexes with addressable DNA, we chose hairpin DNA, consisting of complementary terminal regions forming a stem and a loop-forming sequence, initially designed to bind as shown in Figure 1b. Related oligonucleotides, labeled with a fluorophore and a quencher at each terminus, have recently been used to study fluorescence quenching.[30] In our case, the hairpin oligonucleotides consisted of 34−80 nucleotides, with stems 6−24 nucleotides in length and 10−56 nucleotide long loops (Figure 2). Since double-stranded DNA is not shape complementary to SWCNTs, and because helix formation is entropically favored in hairpins, the stems were expected not to adsorb on the tube surface. The loops were expected to either envelop the nanotube, as shown in Figure 1b or to adsorb on "side-on". For the stem, classical homopyrimidine and homopurine motifs were chosen (Figure 1c).[31] These motifs allow for triplexes made up of C:G:C$^+$ and T:A:T base triplets, such as the one shown in Figure 1c. For *all*-purine/*all*-pyrimidine triplexes *n* nucleotides in length, 2^n different sequence motifs may be designed, so that many orthogonal rule-based recognition events may be programmed into bottom-up nanostructuring protocols.

In an earlier study on the sequence and length dependence of solubilization of HiPCo SWCNTs with oligonucleotides, we had identified the mixture of the hexamers d(AC)$_3$ **1** and d(GT)$_3$ **2** as the oligomers giving the most concentrated suspensions.[26] For our current study on hairpins, we chose loops of the same sequences: d(AC)$_n$ or d(GT)$_n$ (Table 1). Further, we chose CoMoCAT instead of HiPCo nanotubes to start from material more biased toward a single chirality,[32] hoping to obtain nanotube preparations more attractive for use in molecular electronic devices, where uniform electronic properties are central. We generated aqueous suspensions of nanotubes *via* sonication of SWCNT−DNA mixtures with a probe sonicator, followed by ultracentrifugation, as described in the Methods section.

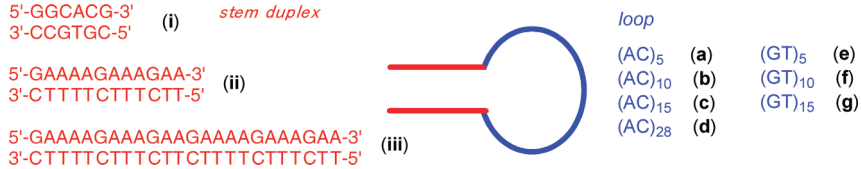

Figure 2. Sequences of stem and loop regions of hairpin oligonucleotides employed.

TABLE 1. Solubilizing Efficiency of DNA Hairpin Sequences, Based on Background-Corrected Absorption of SWCNT Suspensions at 989 nm after Sonication for 50 min, Followed by Ultracentrifugation and Five-Fold Dilution. For a Graphical Definition of Stem and Loop Regions, See *Figure 2*

compound	stem duplex	loop	nanotube content[a]
3	ii	a	1.27
4	ii	e	0.67
5	ii	b	0.94
6	ii	f	0.96
7	i	c	0.96
8	ii	g	0.47
9	ii	d	1.24
10	i	b	0.46
11	i	f	1.37
12	iii	b	0.65
13	iii	f	0.67

[a]Absorption of the SWCNT peak at 989 nm; absorbance at 1090 nm subtracted as background.

The near-infrared (NIR) absorption of the resulting suspensions was used to determine the nanotube content in the supernatant, based on the background-corrected intensity of the peak at 989 nm. Typical absorption spectra of CoMoCAT tubes suspended with hexamers **1** and **2** is shown in Figure 3. For all hairpins tested, the nanotube content was found to be very high (Table 1). All hairpins solubilize considerably more nanotubes than any of the sequences studied in our earlier study, where the most concentrated suspension gave an extinction maximum of 1.4 in the NIR in undiluted solutions,[26] which translates into $E = 0.28$ after 5-fold dilution, as used in our current study. This suggests that hairpins are favorable for solubilizing SWCNTs. For hairpin **11**, the current absorbance measurements show an approximately 5-fold increase in nanotube content over that obtained previously with **1**/**2**.

We hypothesize that these hairpins are more efficient in solubilizing SWCNTs than linear DNA because the stem can act as a large, polar "head group" that is well solvated, while the single-stranded loop region acts like a lipophilic "tail" whose nucleobases are free to adsorb on nanotube surfaces. This lipid-like model would explain the increasing solubilizing effect of the larger biomacromolecules in general. But, the sequence and length of the hairpins does not correlate in a simple way with the nanotube content in the suspensions. Instead, there appears to be a shallow structure activity landscape with subtle competition of kinetic and thermodynamic factors that govern how much SWCNT material remains in solution after sonication and ultracentrifugation. This was not unexpected, given that systematic searches in sequence space have yielded individual maxima for different tubes.[17]

It is not clear what conformation the loop region of the hairpin-forming oligonucleotides adopts when binding to the nanotubes (*vide infra*). All loops were chosen to be long enough to be able to wrap around (6,5)-SWCNTs. The length of the backbone of the shortest loops, consisting of 10 nucleotides, is at least 10 times that of the phosphate-to-phosphate internucleotide distance in B-type DNA duplexes[33] (10 × 0.7 nm). The backbone-to-backbone distance between the termini of the stem, from which the ends of the loop originate, adds another 1.5−2.0 nm, to give a total loop length of >8 nm that can be formed with the 10 nucleotides of even the very smallest loops. This is a conservative estimate, as the backbone is not fully extended in duplex DNA, and significant fraying can occur at the termini of duplexes. The estimate that 10 nucleotides suffice to span the circumference of a (6,5)-tube is also in agreement with recent models of DNA wrapped around (8,4)-SWCNTs.[17]

The thermal stability of hairpin-SWCNT complexes was found to depend on the length of the loop. Figure 3 shows the kinetics of thermal denaturation of nanotube suspensions[15] prepared with the shortest loop tested (hairpin **3**) and the longest loop tested (hairpin **9**). While the former denatures rapidly with a $t_{1/2}$ of approx 5 min, the latter is much more stable, both in terms of the half-life time (approximately 30 min) and the amount of nanotubes remaining in suspension. In both cases, a red-shift of the peak maximum was observed upon heating. This shift was 9 nm for suspension with

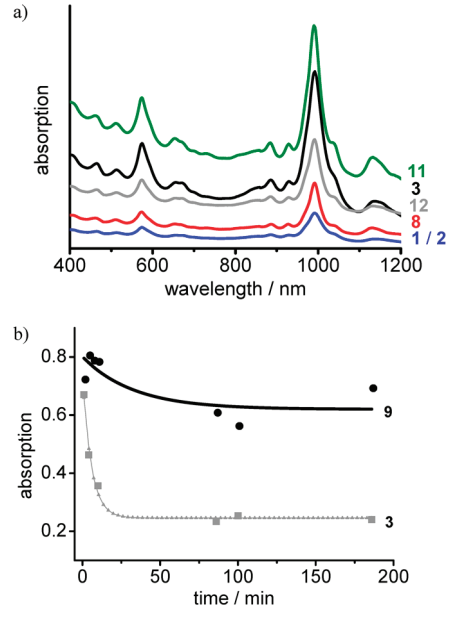

Figure 3. (a) Absorption spectra of 5-fold diluted CoMoCAT SWCNTs suspended with hexamer mixture 1 and 2 (blue line) or hairpins 8, 12, 3, or 11 (red, gray, black, or green line, respectively). Oligonucleotides (0.74 mM in nucleotides, 10 mM phosphate buffer pH = 7) were sonicated for 50 min, followed by ultracentrifugation at 90000*g* for 1 h. (b) Kinetic stability of SWCNT−DNA complexes against thermal denaturation. Samples prepared with hairpin DNA 9 or 3 and CoMoCAT nanotubes were denatured at 90 °C, and flocculation was measured *via* the change in absorbance, as described earlier.[26]

hairpin **3**, and 6 nm for suspension with hairpin **9**, followed by an additional shift of 3−4 nm at the end of the denaturation period at 90 °C (see Figure S2, Supporting Information). We note that for either of the hairpin complexes, the half-life time of denaturation is significantly shorter than those found for complexes with linear DNA, where $t_{1/2}$ values ≥2 h were found in all cases,[26] consistent with the notion that only a portion of the overall oligonucleotide length is interacting with the nanotube surface in the case of the hairpins. When the bulk of the nucleotides does not directly engage in nanotube binding, less of the nanotube surface is coated, making the suspended tubes more vulnerable toward bundling and thus flocculation. If the loop adsorbs "side-on", rather than wrapping around the tube, the fraction of the nucleotides that engage directly in tube binding gets smaller still, as some portion must bridge the distance between the curved tube surface and the two termini of the stem. We also note that without heating, the hairpin-SWCNT complexes are long-term stable, with no detectable precipitation after storage of a suspension prepared with **12** for 1 year at room temperature.

Next, we turned to the issue of addressing the SWCNT-hairpin complexes sequence-specifically with triplex-forming oligonucleotides dubbed "connector DNA". Before employing the sequences in assays with nanotubes, we first measured UV-melting curves to ensure that triple helices of the required stability do indeed form. At pH 4.5, the complex of hairpin **5** and connector strand **14** melted at 32 °C (triple helix between connector and stem), and 54 °C (stem), as shown in the Supporting Information (Figure S1). The slightly acidic pH was chosen to favor the formation of C:G:C^+ triplets.[34,35] We expect that the pH provides a possible additional parameter for fine-tuning molecular assembly protocols involving our hairpin complexes. For the complex with the partially complementary control connectors, significantly less total hyperchromicity and lack of a sigmoidal transition below the melting transition of the stem were detected.

Complexes of Hairpins with Surfactant-Coated Nanotubes. To be attractive for many practical applications, the hairpin DNA−SWCNT complexes should be both addressable and should be made up of tubes of a single chirality. Attempts to obtain DNA−SWCNT complexes that are addressable sequence-specifically in the absence of detergents have been unsuccessful in our hands. As a step toward the goal of addressable nanotube complexes, we subjected cholate-solubilized CoMoCAT tubes to density gradient ultracentrifugation.[36,37] A density gradient based on iodixanol was used, and a 2% (v/w) mixture of cholate and sodium dodecyl sulfate for the preparation of the initial nanotube suspensions.[38] Fractions containing (6,5)-tubes were isolated. A protocol was developed that allows for the adsorption of hairpin DNA onto the thus purified nanotubes through gentle sonication in a bath sonicator at 65 °C for 60 min. Figure 4 shows a representative absorption spectrum of the resulting suspension containing two peaks (λ_{max} = 571 and 983 nm) characteristic for semiconducting (6,5)-nanotubes. The main peak is sharper than those shown in Figure 2a and has shed its shoulder toward longer wavelengths. After treatment with the hairpin, the suspension retained its characteristic purple color.

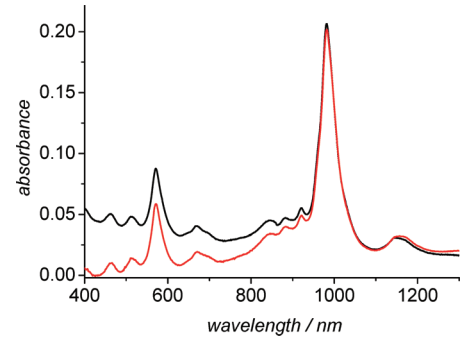

Figure 4. Absorption spectrum of surfactant-coated CoMoCAT SWCNTs after density ultracentrifugation and treatment with hairpin oligonucleotide 5, before bath sonication (black), and after bath sonication for 1 h (red). See Figure 3a for corresponding spectra without surfactant.

To test whether hairpin **5** does indeed act as an addressable binding motif, we performed binding assays shown in cartoon format in Figure 5. Suspensions of (6,5)-tubes with hairpin **5** adsorbed were either treated with connector strand **16** that is complementary to the stem region or with noncomplementary connector strand **17** under the same buffer conditions as those used for the melting curves. Both of the connector strands were fluorophore-labeled with 6-carboxyfluorescein (6-FAM). After hybridization at 20 °C, samples were applied to Microcon filters (molecular weight cutoff 30 000 Da) that retain the nanotube complexes. The complex of hairpin **5** plus fluorophore-labeled single strand **16** or **17** has a molecular weight of approximately 17 000 Da. Control experiments showed that the bulk of free DNA complexes passes through these filters. Since the complexes are not globular, this experiment was performed with Microcon filters with a molecular weight cutoff of 30 000 Da and one with a cutoff of 50 000 Da (Figure S3, Supporting Information). The filtrate of the sample with noncomplementary **17** showed strong fluorescence, whereas that of the corresponding sample with complementary **18** did not (Figure 5b). This confirmed that hairpin DNA **5** on the nanotubes can be addressed sequence-specifically. After washing, the nanotubes on the filter were resuspended and the fluorescence of the resulting suspension was measured. In the case of the complementary connector strand, significantly stronger fluorescence was observed, again confirming that the connector strand **16** had been bound sequence-specifically to the

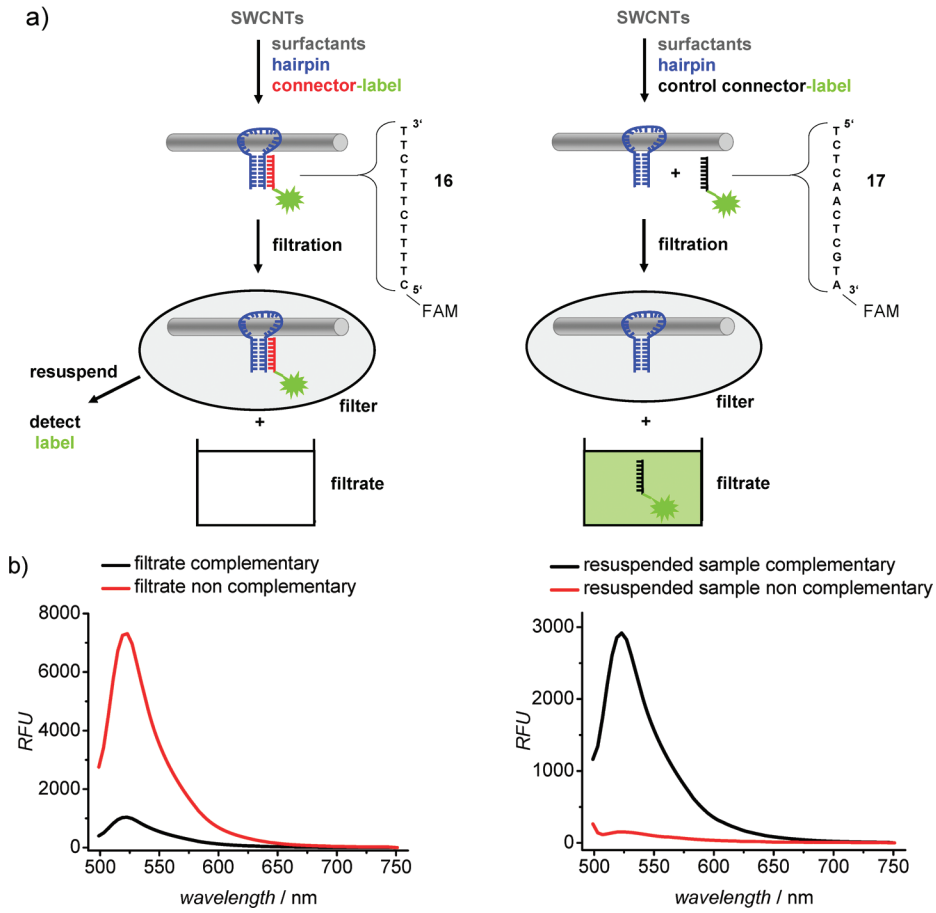

Figure 5. (a) Cartoon of binding assays demonstrating addressability of hairpin-SWCNT complexes by connector strand 16 labeled with a fluorophore (FAM). (b) Fluorescence spectra of samples from the assay with filters of a pore size cutoff of 30 kDa. Samples with complementary connector strand (red) or noncomplementary control (black) were measured at λ_{ex} = 470 nm.

hairpin-bearing tubes. Much less signal, probably induced by residual unspecific adsorption, was found for the noncomplementary control case.

We then performed exploratory experiments with CdSe quantum dot nanoparticles as cargo of the connector strand. The quantum dots can be detected

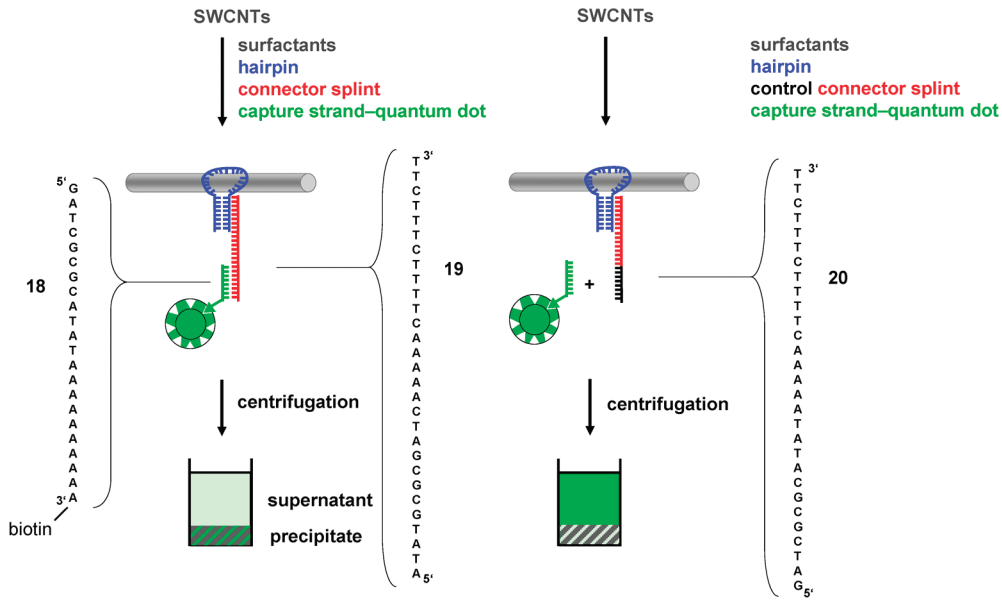

Figure 6. Cartoon of binding assays testing the addressability of hairpin-SWCNT complexes by splint connector strand 19 hybridizing to a third strand carrying CdSe quantum dots (15−20 nm in diameter, including streptavidin coating).

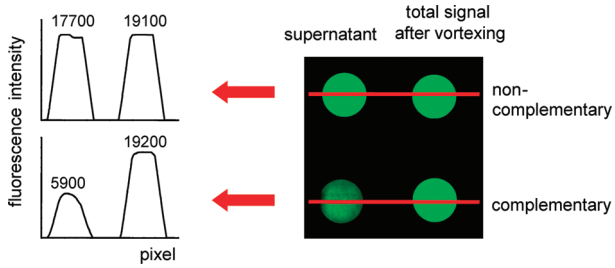

Figure 7. Results from binding assay shown in Figure 6. Supernatants were sampled after hybridization and centrifugation at 16000*g* for 30 min, and total fluorescence signal from each assay was determined after resuspending the mixture by rigorous vortexing. For quantitation, samples of 1 μL were drawn from assays with complementary splint strand 19 or noncomplementary splint strand 20, then stabilized by addition of 3 M betaine solution and 1% SDS (1 μL each), mixed, spotted in the cavities of diagnostic microscope slide, and scanned at λ_{ex} 480 ± 15 nm and λ_{em} = 530 ± 20 nm. The black square on the right shows the fluorescence images obtained by scanning. The red lines indicate where the integration traces were drawn in the scans that are shown on the left. Numbers on top of each integration trace are total fluorescence of a given spot in arbitrary units of fluorescence intensity.

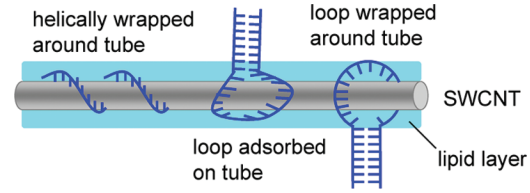

Figure 8. Possible modes of binding of hairpin-forming oligonucleotides to SWCNTs. Either of the two binding modes shown on the right-hand side allow for triplex formation.

directly by their strong and stable fluorescence.[39] To gain flexibility in the design of sequences, we decided to employ a splint connector and biotinylated capture strand **18**, as schematically shown in Figure 6. This, and the thick protein layer appear to prevent quenching of the QD fluorescence in complexes with SWCNTs. The capture strand was conjugated using a literature protocol.[40] A suspension of hairpin-bearing nanotubes was then treated with the QD-bearing DNA, followed by a complementary splint strand **19** or a noncomplementary control splint strand **20**, respectively. Splint strand **19** was designed to form a triplex with the hairpin stem and a Watson−Crick duplex with the QD-bearing **18** at its other terminus. Control splint strand **20** is not complementary to QD-bearing **18**, but the triplex-forming terminus was left unchanged. After hybridization for 1 h, either sample was centrifuged for 30 min at 16000*g*. Complexes of nanotubes and quantum dots have a high enough density to precipitate from aqueous suspension. The nanotube complexes alone remain in suspension at the centrifugal forces employed. In the presence of the complementary splint, 70% of the QDs was centrifuged down with the nanotubes (Figure 7), whereas in the presence of the noncomplementary splint strand, ≤10% of the quantum dots were spun down by centrifugation. The precipitated material formed in the former case shows bundles of nanotubes with quantum dots adsorbed in exploratory TEM images (Figure S4, Supporting Information).

DISCUSSION AND CONCLUSIONS

These results show that DNA sequence space contains motifs that bind to SWCNTs but retain their ability to form base pairs with a complementary strand. With naked nanotubes, the hairpin motif is a better solubilizing agent than oligonuleotide systems reported earlier.[26] The hairpin is also compatible with the detergent-clad nanotubes purified by density gradient ultracentrifugation, leading to stably solubilized, addressable semiconducting tubes of predominantly (6,5)-SWCNTs.[41] Structure elucidation on multicomponent soft-matter complexes of SWCNTs is difficult, but it is consistent with our data that the single-stranded loop of the hairpins interacts with the nanotube surface either with or without looping around the tube (Figure 8), so that the stem helix remains accessible for Hoogsteen base pairing with a connector strand. The remaining detergent may shield areas of the tubes that may otherwise have attracted connector strands *via* unspecific adsorption. We believe that the mild conditions developed for the adsorption of the DNA motifs on the presuspended tubes is critical for retaining their base pairing potential.

While it is interesting that the addressable DNA-bearing nanotubes produce a new material when treated with quantum dot nanoparticles decorated with complementary DNA, we feel that the more important aspect of our technique is that it provides access to the nanostructuring potential of DNA for nanotubes.[42−45] DNA is perhaps the most versatile biomacromolecule for soft matter nanostructuring known, producing both nano-objects of a wide range of shapes and new nanostructured materials and three-dimensional lattices.[46−48] The nanotubes employed here are unperturbed in their electronic structure because they are without covalent modification. They are attractive for applications because they are essentially of a single chirality.[49] The chromophore labeling demonstrated in our first binding assay (Figure 5a) produces tube complexes interesting for spectroscopic studies and electron transfer work. Materials, such as the one formed with quantum dots may also be useful for photovoltaic applications.[50−53] It will be interesting to see whether the addressable nanotubes can also be combined with DNA-bearing electrodes,[54] multi-walled carbon nanotubes, polymers,[55] or redox-active groups, and what the properties of the resulting complexes will be.

METHODS

Preparation of DNA−SWCNT Suspensions. DNA−SWCNT suspensions were prepared by sonicating a mixture of the oligonucleotide (0.74 mM nucleotide concentration) and 1 mg mL^{-1} CoMoCAT material in 10 mM phosphate buffer (pH = 7, 1 mL) for 50 min at 0 °C and at a duty cycle of 30% with a probe sonicator HD 2070 (Bandeline, 200 W, 20 kHz). Ultracentrifugation for 1 h at 20 °C and 90000*g* yielded a black residue and

a clear gray supernatant. The supernatant was harvested by aspiration.

Cholate-coated SWCNTs enriched in (6,5)-tubes were prepared using a literature protocol.[36]

Binding Assay with Fluorophore-Labeled DNA Strand. To a solution of hairpin **5** (500 μL, 20.8 μM) in 10 mM phosphate buffer (pH = 4.5, 1 w/v % sodium cholate) was added to a suspension of SWCNTs enriched in (6,5)-nanotubes (100 μL, 1.05 mg mL^{-1}), followed by bath sonication for 1 h at 65 °C. The resulting suspension was split in two halves, which were either treated with a solution of noncomplementary fluorophore-labeled strand **17** (0.9 μL, 80 μM, in water) or a solution of its complementary counterpart 16 (0.9 μL, 80 μM, in water), respectively. After **16** h at 20 °C, either sample was applied to a Microcon filter (50 000 NMWL: Nominal molecular weight limit in Dalton). Prior to this, the filters had been washed with phosphate buffer (200 μL). After centrifugation at 16000*g* for 5 min, the filter residue was washed with phosphate buffer (200 μL) and centrifugated at 16000*g* for 5 min. The nanotubes were recovered by inversion of the filter and elution with phosphate buffer (200 μL) by centrifugation at 82*g* for 1 min.

Samples (1 μL) from the binding assay (filtrate, washing solution, resuspended tubes) were mixed with 3 M betaine solution (1 μL) and 1 w/v % SDS solution (1 μL) to avoid evaporation and to decrease surface tension. The resulting mixtures were spotted into the wells of diagnostic microscope slides, followed by fluorescence read-out on an ArrayWoR$_x^e$ Biochip Reader with an exposure time of 10 ms and the following filters: $\lambda_{ex} = 480 \pm 15$ nm, $\lambda_{em} = 530 \pm 20$ nm. Additionally, fluorescence spectra were recorded on a NanoDrop ND-3300 fluorospectrometer with $\lambda_{ex} = 470 \pm 10$ nm.

Binding Assay with Quantum Dots. To a solution of hairpin **5** (150 μL, 35 μM) in phosphate buffer (10 mM, pH = 4.5, 1 w/v % cholate) a suspension of SWCNTs enriched in (6,5)-tubes (25 μL, 1.05 mg mL^{-1}) was added, followed by sonication at 65 °C for 1 h in a sonication bath. An aliquot of the resulting suspension (8.6 μL) was added to 50 μL of the QD−DNA suspension, prepared as described in Supporting Information. After mixing, the suspension was split into two samples. Each was treated with a solution of complementary **19** or noncomplementary splint strand **20** (each 2 μL, 145 μM, in water). After 1 h at 25 °C, both sample was centrifuged for 30 min at 16000*g*, and the fluorescence of a sample of the supernatant was measured, as described above ($\lambda_{ex} = 480 \pm 15$ nm, $\lambda_{em} = 530 \pm 20$ nm). Then, the remaining mixture was vortexed for approximately 5 s at 2500 rpm (Heidolph Reax control vortexer) and analyzed for fluorescence.

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Supporting Information Available: Additional protocols, figures and data. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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