Rational Design of Carbon Nanotube Biosensors with Covalent DNA-Anchors

Justus Tom Metternich,[b] Linda Sistemich,[c] Robert Nißler,[d] Svenja Herbertz[e] and Sebastian Kruss*[a]

[a] Prof. Dr. S. Kruss
Physical Chemistry
Ruhr-University Bochum
Universitätsstr. 150, 44801 Bochum, Germany
E-mail: Sebastian.Kruss@rub.de
Biomedical Nanosensors
Fraunhofer Institute for Microelectronic Circuits and Systems,
Finkenstr. 61, 47057 Duisburg, Germany
Center for Nanointegration Duisburg-Essen (CENIDE)
Carl-Benz-Straße 199, 47057 Duisburg, Germany

[b] J. T. Metternich
Biomedical Nanosensors
Fraunhofer Institute for Microelectronic Circuits and Systems,
Finkenstr. 61, 47057 Duisburg, Germany
Physical Chemistry
Ruhr-University Bochum
Universitätsstr. 150, 44801 Bochum, Germany

[c] Dr. L. Sistemich
Physical Chemistry
Ruhr-University Bochum
Universitätsstr. 150, 44801 Bochum, Germany

[d] Dr. R. Nißler
Nanoparticle Systems Engineering Lab, ETH Zürich
Sonneggstrasse 3, 8092 Zürich, Switzerland
Laboratory for Particles- Biology Interactions, Empa
9014 St. Gallen, Switzerland

[e] Dr. Svenja Herbertz
Biomedical Nanosensors
Fraunhofer Institute for Microelectronic Circuits and Systems,
Finkenstr. 61, 47057 Duisburg, Germany

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Abstract: Semiconducting single wall carbon nanotubes (SWCNTs) are versatile near infrared (NIR) fluorophores. They are non-covalently modified to create sensors that change their fluorescence when interacting with biomolecules. However, non-covalent chemistry has several limitations and prevents a consistent way to molecular recognition and reliable signal transduction. Here, we introduce a widely applicable covalent approach to create molecular sensors without impairing the fluorescence in the NIR (>1000 nm). For this purpose, we attach single-stranded DNA (ssDNA) via guanine quantum defects as anchors to the SWCNT surface. A connected sequence without guanines acts as flexible capture probe allowing hybridization with complementary nucleic acids. Hybridization modulates the SWCNT fluorescence and the magnitude increases with the length of the capture sequence (20 > 10 >> 6 bases). Incorporation of additional recognition units via this sequence enables a generic route to NIR fluorescent biosensors with improved stability. To demonstrate the potential, we design sensors for bacterial siderophores and the SARS-CoV-2 spike protein. In summary, we introduce covalent guanine quantum defect chemistry as rational design concept for biosensors.

Introduction

Semiconducting single wall carbon nanotubes (SWCNTs) are tubular 1D nanomaterials that do not photobleach[1] and fluoresce in the near-infrared (NIR) tissue transparency window. In this spectral region scattering, autofluorescence and absorption of biological samples are minimal.[2] The optoelectronic properties of SWCNTs are highly sensitive to the chemical environment (corona phase). Therefore, SWCNTs have been used as modular building blocks in a variety of (bio-)sensing formats.[3,4]

To render them suitable for biosensing, SWCNTs are individualized and chemically functionalized using a variety of molecules. Common strategies include the non-covalent adsorption of surfactants, proteins, peptides or nucleic acids.[3,5] Due to the strong π-stacking of nucleic acids to the SWCNT surface, single stranded DNA (ssDNA) has been extensively used to prepare stable SWCNT dispersions.[6] However, the SWCNT-DNA interaction is highly dependent on the SWCNT chirality, the nucleic acid length, sequence, backbone, as well as a number of external factors such as the temperature and ion concentration.[7,8,9]

This interplay gives rise to complex phenomena (on different time scales) caused by binding and reorganization within the SWCNT corona, which currently restricts rational design of analyte binding and its translation into a NIR fluorescent signal.
For example, ssDNA coated SWCNTs can be used for the detection of small molecules such as dopamine with high sensitivity and spatial resolution.\textsuperscript{[10]} Among others, the contributions of analyte redox potential and the density of adsorbed nucleic acids were investigated.\textsuperscript{[8,11]} Both could not explain the changes in fluorescence, suggesting that conformational changes of the corona phase affect the exciton dynamics. To reduce unspecific effects e.g. caused by fluctuations of ion-concentrations, xeno nucleic acids (XNA) have also been used as DNA derivatives with increased rigidity.\textsuperscript{[12]}

In the case of DNA hybridization, it has been shown that the structural reorganization occurring upon DNA hybridization modulates the SWCNT emission.\textsuperscript{[13–16]} To improve the specificity of SWCNT based sensors, a non-covalent functionalization with DNA content one can pattern and tailor SWCNT surfaces.\textsuperscript{[9,16]} The perturbation caused in the corona phase upon hybridization can be amplified using surfactants or denatured proteins that compete with adsorption of ssDNA to the SWCNT surface and consequently affect the fluorescent signal.\textsuperscript{[13,14]} Due to the intricate interplay of factors determining DNA affinity to the SWCNT surface, as well as the competing binding modes of analytes of various sizes, these systems are highly sensitive e.g. to ion concentrations.\textsuperscript{[9,16]} This drawback currently restricts their use in crowded, biological environments. For diagnostic applications it is crucial to reduce unspecific signal changes caused by such processes. Covalent strategies should improve sensor robustness and other performance parameters.

However, uncontrolled incorporation of covalent bonds into the SWCNT lattice destroys the NIR fluorescence.\textsuperscript{[17,18]} In the past few years novel synthetic routes (quantum defects) have been demonstrated that do not destroy the NIR fluorescence.\textsuperscript{[18,19]} This enables creative applications for biosensing and super-resolution microscopy.\textsuperscript{[3,18,20,21]} For example, quantum (sp\textsuperscript{3})-defects decorated with maleimide linkers have been used as straightforward approach to conjugate biological recognition units on SWCNTs\textsuperscript{[22,23]} However, such quantum defects are relatively sparse and therefore co-solubilization with is still necessary.

One biocompatible surface modification that has so far not been used for the rational design of biosensors is the selective covalent reaction of certain DNA bases with the SWCNT surface.\textsuperscript{[24–26]} To generate a reactive intermediate, first singlet oxygen (\textsuperscript{1}O\textsubscript{2}) is produced by a photochemical reaction with Rose Bengal (RB). As \textsuperscript{1}O\textsubscript{2} reacts two orders of magnitude faster with guanine compared to other nucleobases, predominantly guanine endoperoxides are formed.\textsuperscript{[27]} These intermediates react in a cycloaddition between the guanosine carbon atom 8 and the nearby SWCNT surface and lead to covalent attachments of guanine bases to SWCNTs.\textsuperscript{[28]} As defect density depends on the RB concentration and the guanin content one can pattern and tailor SWCNT surfaces.\textsuperscript{[24–26,28,29]}

Here, we show that guanine quantum defects can be used to conjugate recognition units to SWCNTs, which enables several biosensing approaches. The oligonucleotides consist of a guanine containing anchor sequence that reacts with the SWCNT surface and an “inert” capture sequence. This capture sequence allows the attachment of diverse recognition units. To demonstrate its potential, we test this approach for sensing of bacterial virulence factors and the SARS CoV-2 spike protein. This functionalization strategy presents a generic covalent design concept to enable control of molecular recognition and signal transduction (photophysics).

**Results and Discussion**

We designed a new surface chemistry on SWCNTs that includes a guanine containing anchor sequence (GT)\textsubscript{15} and a guanine free capture sequence (T)\textsubscript{20} (Figure 1a). The rationale is that pinning the DNA via guanines on the SWCNT lattice would reduce unspecific movements and that molecular recognition events would be translated into meaningful and specific fluorescence changes. We first tested the influence of different defect densities on DNA hybridization to rule out that the introduction of guanine quantum defects interferes with hybridization on the capture sequence. For this, SWCNTs were non-covalently functionalized with ssDNA, incubated with 5 or 18 \textmu{}M of RB and irradiated with green light. The RB mediated generation of singlet oxygen (\textsuperscript{1}O\textsubscript{2}) resulted in concentration dependent bathochromic shifts of the absorption and fluorescence spectra associated with the incorporation of guanine quantum defects (Figure 1b).\textsuperscript{[24,28]} Additionally, the ratio between the D and G Raman modes increased (Figure S1), indicating the covalent reaction of guanine bases with the SWCNT lattice.\textsuperscript{[24,28,30]}

After successful incorporation of guanine quantum defects via the anchor sequence, we tested whether the addition of complementary DNA would change the NIR fluorescence. For SWCNTs with no defects or low guanine defect densities, the addition of a complementary nucleotides (A\textsubscript{20}) to a suspension of SWCNTs caused a slight decrease of the SWCNT fluorescence (Figure 1c-d). However, the addition of non-complementary DNA or buffer led to similar effects (Figure S2-5). In contrast, addition of A\textsubscript{20} decreased the fluorescence of SWCNTs with a high guanine quantum defect density. Here, the addition of the non-complementary sequence (T\textsubscript{20}) or buffer had no pronounced effect on the fluorescence signal (Figure S2-5). This pattern suggests that conformational changes of the non-covalently bound anchors due to interactions between ssDNA, dsDNA and the SWCNT surface contribute to non-specific fluorescence changes if there are no or low defect densities. In contrast, fixing the DNA with guanin anchors reduces this effect. This reduction of anchor mobility should result in more specific translation of specific binding events into NIR-fluorescent signals. Based on this premise, we decided that the guanine defect density caused by 18 \textmu{}M RB (in the following termed: G\textsuperscript{4}-SWCNTs or (G\textsuperscript{T})\textsubscript{15}T\textsubscript{20}) presents a suitable degree of anchor fixation to reduce unspecific perturbations of the SWCNT corona while maintaining hybridization and signal transducing capabilities.
To verify the specificity of this interaction we used fluorescence anisotropy measurements. When a fluorescently labeled ssDNA strand hybridizes the rotational motion should decrease and anisotropy increase. To optimize the length of the capture sequence, we prepared (G^15T)_15SWCNTs with 6, 10 and 20 nucleotide long capture sequences (Figure 2a). Addition of the SWCNT sensors to Cyanine 3 (Cy3) labeled oligonucleotides with 10 or 20 bases increased Cy3 fluorescence anisotropy in a length-dependent fashion. In contrast, the interaction between short (6 bases) capture sequences did not affect the anisotropy of the respective Cy3 labeled oligonucleotide (Figure 2b, Table S4-S9).

The anisotropy changes revealed that oligonucleotides with a length of 20 bases displayed no time dependent changes in the anisotropy (Figure 2c). Together with the magnitude of the increase for complementary sequences, we consequently concluded stable hybridization at such lengths. Truncation of the interacting sequences to 10 nucleotides, decreased the anisotropy changes and displayed time-dependent behavior. As the on-off binding behavior of nucleotides with this length is in the timescale of minutes,[10] the observed changes might indicate a complex sequence of reorganization processes (partial binding, unbinding) until an equilibrium at the SWCNT surface is reached. Interestingly, for non-complementary nucleotide sequences the normalized anisotropy changes were strongest for small sequences (6 > 10 >> 20 bases) and increased with time. This effect was much smaller than for interactions with complementary sequences (Figure 2b). Thus, we concluded that hybridization of complementary DNA is the main interaction between nucleotides on (G^15T)_{15}T_{20} functionalized SWCNTs and increases with the length of the capture sequence (20 > 10 >> 6 bases). The reverse trend for non-complementary nucleotides might reflect the higher ability of small molecules to non-specifically adsorb on free patches at the SWCNT surface.

To evaluate the influence of the anchor sequence, we truncated the length of the anchor sequence from 30 nucleotides (G^15T)_{15}T_{20} to 10 nucleotides ((G^15T)_5), and evaluated anisotropy changes of a Cy3-labeled nucleotide (20 nt) upon the interaction with the respective SWCNT sensor. In case of both anchor sequences, the anisotropy of the Cy3-labeled DNA interacting with the complementary sequence increased with the SWCNT sensor concentration and saturated at SWCNT concentrations > 500 pM (Figure 2d, Table S10-S11). Based on the concentration of the Cy3-labeled DNA (10 nM), we consequently assumed, that the sensors display ≈ 20 binding sites available for binding to recognition units.

Complex biological environments contain many molecules that compete with the intended non-covalent functionalization for the SWCNT surface. We subsequently tested the colloidal stability of our nanosensors in such conditions. To this end, we chose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % Penicillin/Streptomycin, as a typical cell culture medium with high protein concentrations and phosphate buffered saline (PBS) as buffer. To evaluate the colloidal stability of the sensors, we measured the absorbance of non-covalently ((GT)_{15}T_{20}) and covalently ((G^15T)_{15}T_{20}) functionalized sensors over the period of 21 days. Incubation in DMEM decreased the absorbance, which is associated with aggregation and hence triggers the loss of colloidal stability.[11] However, for the covalent functionalized SWCNTs the absorbance solely decreased by ≈ 30 % while a decrease of ≈ 47 % was observed in the case of non-covalent functionalization (Figure S9). In the case of PBS we observed no changes in absorbance.

The loss of stability in medium indicates that displacement of ssDNA decreases the colloidal stability highlighting the importance of a stable covalent functionalization strategy.
As complex environment could also affect the sensor response, we next tested the fluorescence response to complementary DNA. Under the equivalent incubation conditions, sensors with a covalent anchor showed the similar responses to complementary DNA in PBS and cell medium, while the magnitude of the response differed for non-covalent sensors (Figure S10). Similar to the stability experiments, this demonstrates the potential of covalently functionalized sensors for long-term applications under complex biological conditions.

Subsequently, we tested if other recognition units can be immobilized via the capture sequences on the SWCNT surface. Additionally, we evaluated if guanine quantum defects allow translation of the binding events into a NIR signal. To this end, we explored the limits of our sensor design for different classes of molecules.

First, we evaluated the binding of an aptamer and addition/removal of a quenching unit to the aptamer (Figure 3a). As a model system, we selected a hemin binding aptamer (HeApt) previously used as a non-covalent functionalization for the detection of plasma porphyrins, stress associated signaling molecules in plants, as well as siderophores (virulence factors) released by bacteria. It has been known that iron complexed to the porphyrin quenches SWCNT fluorescence. The effect can be reversed in the presence of bacterial siderophores and other iron chelating molecules with the ability to remove iron from the SWCNT vicinity thereby disrupting the energy transfer process responsible for the fluorescent quenching.

Based on the general need to develop diagnostic tools for bacterial infections we chose pyoverdine as a bacterial biomarker to test our sensor design.

Figure 2: DNA hybridization on SWCNTs measured by fluorescence anisotropy. a) Simplified interaction scheme between (Cy3 labeled) DNA and SWCNT based DNA sensors. b) Influence of capture sequences on SWCNT sensors and interaction with respective Cy3 labeled oligonucleotides (lines represent visual guidance, n=3, mean ± SD). c) Anisotropy changes depend on length, time and complementarity of interacting sequences (anisotropy changes between the free and bound states (r_b – r_f) are normalized to the free state r_f. Lines represent visual guidelines). d) Anisotropy as a function of anchor length and SWCNT concentration (n=3, mean ± SD).
Addition of the hemin-aptamer (with an A₁₅ sequence to the 5’ end for hybridization) and hemin (He@HeApt-A₁₅) quenched the SWCNT fluorescence by up to 57% (Figure 3b). The subsequent addition of a chelator with a strong iron affinity dequenched the SWCNT fluorescence again. Deferoxamine is an iron chelator used for the treatment of iron-overload diseases. It increased the fluorescence again up to 75%, while the pyoverdine, as the stronger chelator, increased the fluorescence by up to 83%. This effect was concentration dependent. For DFO, we observed a saturation of this effect at concentrations > 10 µM (Figure 3c, Figure S11) and the response for pyoverdine samples saturated at concentrations > 2 µM (Figure 3c, Figure S11) as expected. The chelator-independent, fluorescence decrease over time could be attributed to a decrease in colloidal stability caused by hemin. To rule out that the observed effects in solution are related to aggregation, we coated (G⁵T₁₅T₂₀ functionalized SWCNTs on a glass surface and added He@HeApt-A₁₅ followed by DFO. Over the course of the experiment, it was noticed that some SWCNTs were desorbing from the glass surface. Nevertheless, the quenching caused by He@HeApt-A₁₅, as well as dequenching by DFO were clearly visible in larger SWCNT clusters and smaller, resolution limited spots (Figure 3d).

Next, we wanted to test our chemical design for another biomolecule class (proteins). Proteins serve as biomarkers in numerous diseases. We selected two aptamers (Aptamer1: SP34 from [20], Aptamer2: MSMA2 from [20]) with known binding affinity to the spike protein of the severe acute respiratory syndrome coronavirus 2 (SARS CoV-2). Both aptamers were modified by the addition of an A₁₅ sequence to the 5’ end and incubated with complementary (G⁵T₁₅T₂₀ functionalized SWCNTs (Figure 4a). The addition of SARS CoV-2 spike protein (CoV-2 S) decreased the SWCNT fluorescence in all cases (Figure 4b-c). This effect was again concentration dependent and saturated at concentrations ≥ 10 nM (Figure 4d-e), which corresponded to the estimated concentration of the aptamers attached to the SWCNT surface. Bovine serum albumin (BSA) served as a negative control for the unspecific interaction of the SWCNT surface with a protein. No clear concentration dependent increase or decrease was observed for BSA. In the case of Aptamer1, the fluorescence signal of sensors decreased slightly over the course of two hours (Figure 4f, Figure S12a-c). In contrast, the sensor with Aptamer2 slightly increased the fluorescence and remained almost stable for the observed period (Figure 4g, Figure S12d-f). This could indicate that the binding mode between the aptamer and the protein and the aptamer and the SWCNT plays an important role. Additionally, it is possible that slow reorganization on this time scale influences the signal.

To illustrate the robustness of the guanine functionalized sensors, we compared the fluorescent responses of both sensors with the anchor sequence being covalently and non-covalently linked to the SWCNT. In all cases, the addition of CoV-2 S resulted in a pronounced decrease of the SWCNT fluorescence (Figure S13). Although, the non-covalently functionalized sensors (Figure S13a) showed a stronger response to the analytes compared to the guanine functionalized sensors (Figure S13b), their non-specific responses to the tested negative controls (PBS and BSA) were also elevated. We explain the reduction of non-specific responses in G⁵-functionalized SWCNTs by the fixation of the DNA anchor causing a higher order in the corona phase. As for the earlier introduced sp²-defects, [20,22,23] this approach allows a controlled patterning and a generic route towards the rational design of SWCNT sensors. In contrast to the sp²-defects, G⁵-functionalized SWCNTs are soluble without an additional surfactant. Pinning guanine bases on the SWCNT allows to control non-specific interactions interfering with the signal-translation of SWCNT sensors in complex environments.

We conclude that the covalent attachment of DNA anchors via guanine quantum defects offers promising opportunities in terms...
of stability and the reduction of unspecific ‘noise’ resulting from other molecules interacting with the SWCNT.

**Conclusion**

In summary, we introduce a generic covalent functionalization approach for SWCNT-based biosensors. The concept is based on the covalent attachment of guanine (quantum defect) containing anchor sequences connected with non-guanine containing capture sequences to the SWCNT surface. The fluorescence signal of the SWCNTs changes in response to complementary DNA. We identify how long a capture sequence should be to guarantee a fluorescence change also when additional recognition units such as aptamers are linked to it. Due to the stability provided by the anchor sequences, this approach allows a new level of control over the SWCNT corona. It furthermore increases colloidal stability and a reduction of non-specific sensing effects. Therefore, the concept provides a generic chemical strategy beyond trial-and-error approaches and rationalizes the design of SWCNT-based sensors. We envision broad usage in fundamental research as well as next-generation biomedical applications.

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