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Single-molecule Bioelectronic Devices with Multi-Sensing Capability

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FINAL REPORT: Single-molecule Bioelectronic Devices with Multi-Sensing Capability

(FA9550-16-1-0345)

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Abstract

In this project we developed strategies to assemble bioelectronic interfaces where biomolecules of interest were interfaced to carbon nanotubes (CNTs) with control down to the single-molecule level. We employed the CNTs as nanoelectrodes and transducers in sensing platforms, investigated the coupling between proteins and aptamers to CNTs, and organized these CNT biohybrids in nanoscale biosensing devices also with multiplexing capabilities. In particular we achieved the site-specific coupling of single proteins to individual CNTs with single-molecule control, and demonstrated the importance of bioengineering optimal protein attachment sites for direct protein-nanotube communication at the single-protein level. Moreover, we demonstrated the assembly of both static and stimuli-responsive single-molecule heterostructures, where the distance and electronic coupling between an individual functional nanomoiety and a CNT are tuned via the use of DNA linkers. Finally, we fabricated reconfigurable and solution processable nanoscale real-time biosensing devices with multisensing capability based on CNTs functionalized with specific, and different, aptamer sequences employed as selective recognition elements for three different biomarkers indicative of stress and neuro-trauma conditions.

Key words: nanoscale biosensors, multiplexed sensing, single-molecule, carbon nanotubes, biomarkers, proteins.

A central challenge in nanobiotechnology is the bottom-up assembly of platforms capable of monitoring and exploiting biomolecular interactions with nanoscale or single-molecule control; this in turn can allow the investigation of ligand-receptor functions, the fabrication of next generation ultrasensitive and nanoscale biosensing devices, and the development of novel bioelectronics interfaces. Studies and platforms of these kind hold great potential for applications ranging from diagnosis of life-threatening diseases to detection of biological agents in warfare or terrorist attacks.

The primary goal of this project was to develop a new generation of bio-sensing platforms based on nanoscale and single-molecule bioelectronic interfaces, that could allow the simultaneous detection of several types of biotargets on a single biochip. We achieved this employing single-walled carbon nanotubes (SWCNTs) as nanoelectrodes and transducers when interfaced to biomolecules that could act as biorecognition elements in sensing platforms. The main results of the project have been:

1) The site-specific coupling of single proteins to individual carbon nanotubes (CNTs) in solution and with single-molecule control. Using an orthogonal Click reaction, Green Fluorescent Protein (GFP) was engineered to contain a genetically encoded azide group and then bound to CNT ends in different configurations: in close proximity or at longer distances from the GFP's functional center. Atomic force microscopy and fluorescence analysis in solution and on surfaces at the single-protein level confirmed the importance of bioengineering optimal protein attachment sites to achieve direct protein−nanotube communication and bridging. [***J. Am. Chem. Soc*. 2017, 139, 17834−17840**]
2) We developed a strategy for the assembly of both static and stimuli-responsive single-molecule heterostructures, where the distance and electronic coupling between an individual functional nanomoiety and a CNT are tuned via the use of DNA linkers. In particular, 1:1 nanohybrids were assembled where single quantum dots (QDs) were tethered to the ends of individual CNTs in solution with DNA interconnects of different lengths: stimuli-responsive CNT-QD nanohybrids were assembled, where the distance and hence the electronic coupling between an individual CNT and a single QD were dynamically modulated via the addition and removal of potassium (K+) cations; the system is further found to be sensitive to K+ concentrations from pM to mM. [***Adv. Sci.* 2018, 1800596**]
3) In collaboration with Jorge L. Chávez and Nancy Kelley-Loughnane (Air Force Research Laboratory, 711th Human Performance Wing, Wright-Patterson Air Force Base, Dayton, Ohio) we fabricated reconfigurable and solution processable nanoscale biosensors with multisensing capability, based on SWCNTs. Distinct DNA-wrapped (hence water-soluble) CNTs were immobilized from solution onto different prepatterned electrodes on the same chip, via a low-cost dielectrophoresis (DEP) methodology. The CNTs were functionalized with specific, and different, aptamer sequences that were employed as selective recognition elements for biomarkers indicative of stress and neuro-trauma conditions. Multiplexed detection of three different biomarkers was successfully performed, and real-time detection was achieved in serum down to physiologically relevant concentrations of 50 nM, 10 nM, and 500 pM for cortisol, dehydroepiandrosterone-sulfate (DHEAS), and neuropeptide Y (NPY), respectively. Additionally, the fabricated nanoscale devices were shown to be reconfigurable and reusable via a simple cleaning procedure. The general applicability of the strategy presented, and the facile device fabrication from aqueous solution, hold great

potential for the development of the next generation of low power consumption portable diagnostic assays for the simultaneous monitoring of different health parameters. [***Nano Lett.* 2018, 18, 4130−4135**]

1. **Site-Specific One-To-One Click Coupling of Single Proteins to Individual Carbon Nanotubes: A Single-Molecule Approach:** *J. Am. Chem. Soc*. 2017, 139, 17834−17840

We directed the conjugation of single proteins selectively at the terminal ends of individual SWCNTs, for the in-solution assembly of monofunctionalized SWCNT-protein heterostructures. As a proof of concept, two different CNT-protein configurations were investigated, where green fluorescent proteins (GFPs) mutants were engineered to exhibit CNT-anchoring residues either in close proximity (short axis, GFP^{SA}), or at larger distance (long axis, GFP^{LA}), from the GFP's functional centre, the chromophore (CRO). To define the protein-SWCNT interaction, bio-orthogonal "1+1" Click chemistry was used. Notably, fluorescence investigations in solution and on surfaces at the single-protein level, showed evidence of site-specific coupling between the SWCNTs and the GFPs, i.e. only the short axis bioengineered system exhibited the expected direct protein-nanotube communication.

We employed SWCNTs mildly sonicated and dispersed in water via DNA wrapping. The DNA wrapping protects the sidewalls of the nanotubes leaving only the terminal end of the SWCNTs available for direct functionalization, via amidation reactions on the carboxylic acid groups present on the nanotubes termini. This allowed us to covalently functionalize the terminal ends of our DNA-wrapped CNTs with dibenzocyclooctyne (DBCO).

DBCO-functionalized SWCNTs were then available to readily react with azide groups, in a copper-free ring strain promoted 1,3-dipolar cycloaddition (SPAAC). We engineered two sfGFP variants to introduce an azide CNT-anchoring handle: using a reprogrammed genetic code the non-canonical amino acid (ncAA) azF (*p*-azido-L-phenylalanine) can be incorporated at defined sites in a protein of interest in response to the TAG amber stop codon. The two sfGFP mutants with modified residues allowed us to then form (in solution) SWCNT-GFP hybrids with specific protein orientations (see Figure 1a and 1b). The covalent attachment of GFP^{SA} and GFP^{LA} mutants to DBCO-functionalized SWCNTs was monitored casting our hybrids' solutions on muscovite mica, and imaging the substrate surface via Atomic Force Microscopy (AFM). Figures 1c and 1 d show representative images of sfGFP-SWCNT nanohybrids, where the proteins are tethered uniquely to the terminal ends of individual SWCNTs.

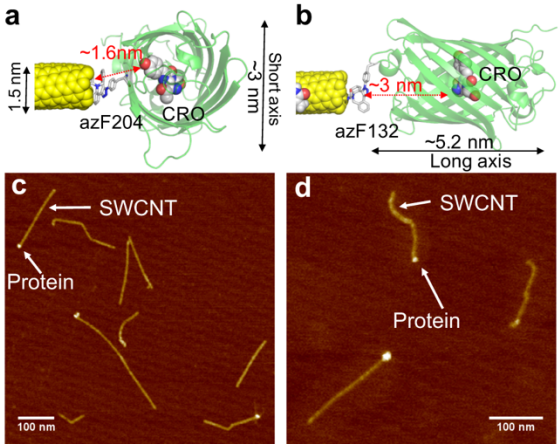

Figure 1 Schematics of the (a) short axis sfGFP^{204azF} (GFP^{SA}) and (b) long axis sfGFP^{132azF} (GFP^{LA}) CNT interface points and orientations: the chromophore (CRO) is shown as grey spheres, the DBCO-azF linkage sown as grey sticks and the CNT as gold spheres; the approximate trizole-CRO distance is highlighted in red. (c)AFM image of SWCNT-GFP^{SA} hybrids; d) AFM image of SWCNT-GFP^{LA} hybrids. Z-scales = 6nm

AFM analysis revealed that among the nanohybrids obtained, 88% of SWCNT-GFP^{SA} heterostructures exhibited a single protein at only one end of the nanotube, while in the case of the SWCNT-GFP^{LA} nanohybrids the monofunctionalization yield obtained was 82%.

To demonstrate protein-CNT communication in our monofunctionalized nanohybrids, initially steady-state fluorescence spectroscopy was performed. In order to then monitor GFP-nanotube coupling with single-molecule resolution, we monitored, and compared, the fluorescence behavior of individual proteins and single nanohybrids via Total Internal Reflectance Fluorescence Microscopy (TIRF). GFP has a characteristic blinking behavior which can be influenced by various processes (including its local environment). A representative intensity vs time single-molecule trace for GFP^{SA}, showing "on" and off" states, can be seen in figure 2a, while the hybrid SWCNT- GFP^{SA} plot is shown in figure 3b.

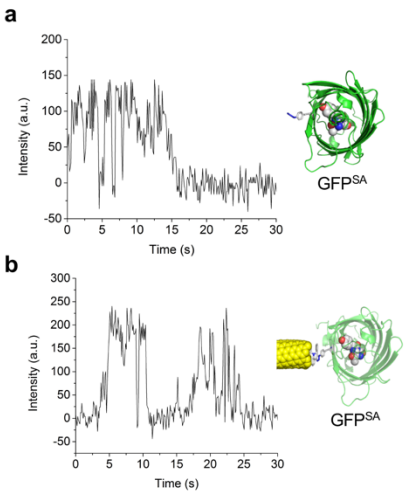

Figure 2 Representative single-molecule fluorescence traces for a) GFP^{SA}, and b) SWCNT-GFP^{SA}

Individual GFP^{SA} showed shorter off times, i.e. switching between "on" and "off" states, while the SWCNT-GFP^{SA} nanohybrids exhibited longer off time periods. This is a strong indication, at the single-molecule level, of direct coupling between the GFP^{SA} and the SWCNT in these short axis hybrids. In comparison, the SWCNT- GFP^{LA} heterostructures exhibited almost identical blinking rates when compared to the GFP^{LA} alone. This behavior was confirmed and quantified by constructing histograms of single-molecule off-times for the hybrid structures and both sfGFP variants (see *J. Am. Chem. Soc*. 2017, 139, 17834−17840)

Our protein-CNT assemblies further hold great potential for the development of solution processable single-molecule bioelectronic systems and devices (including gated GFP-based ones). Biomolecular function (e.g. GFP electronic excitation) can indeed be used to modulate conductance, and proteins have been observed to act as molecular gates. To facilitate such work, we engineered a sfGFP variant with two CNT-anchoring azide handles on opposite faces of the protein. Figure 3 shows a characteristic AFM image of the typical 1:2 protein-SWCNT junctions obtaned.

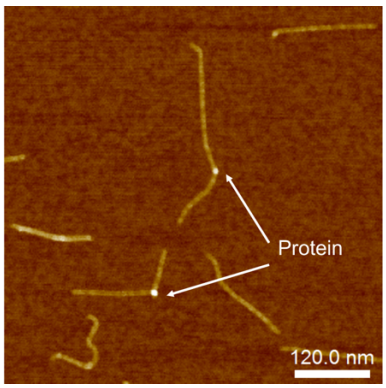

Figure 3 AFM image of a SWCNT-GFP^{SAx2}-SWCNT junction. Z-scale = 6 nm

By and large, we developed a powerful approach to generate tailored and optimal single protein-CNT hybrids that hold great potential for the development of solution-processable single-molecule bioelectronic systems and devices based on the use of carbon nanoelectrodes.

2. Tuning the Coupling in Single-Molecule Heterostructures: DNA-Programmed and Reconfigurable Carbon Nanotube-Based Nanohybrids: *Adv. Sci.* 2018, 1800596

We developed a strategy for the controlled formation of reconfigurable single-molecule heterostructures, where the spacing between a functional nanomoiety and an individual carbon nanostructure is controlled and dynamically tuned by a DNA spacer, employed as a molecular ruler. As a proof of concept, we assembled individual single-walled CNTs (SWCNTs) coupled to single colloidal semiconductor nanocrystals (Quantum Dots, QDs), chosen as model systems due to their tunable emissions and broad absorbances that further make them ideal candidates for novel light harvesting systems in photovoltaics and light emitting diodes. A bio-inspired approach was pursued via the use of DNA as the linking moiety, due to its demonstrated ability to chemically program the assembly of nanoparticle-based materials. In particular, we altered the number of bases in a double stranded (ds)DNA, in order to regulate the nanoscale distance between a SWCNT and a QD, in 1:1 nanohybrids. This in turn allowed us to modulate the coupling between the two nanostructures with single-molecule control, as demonstrated via static and time-resolved

photoluminescence investigations, as well as single-molecule measurements: the ability to control the electronic coupling in such heterostructures is an essential attribute for future device implementation. Figure 4 and 5 show this (see also additional figures in *Adv. Sci.* 2018, 1800596).

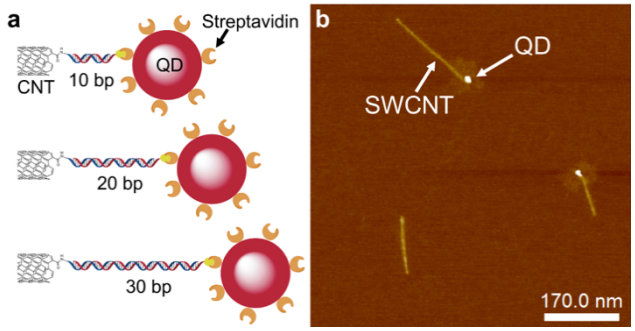

Figure 4. (a) Schematics for the SWCNT-QD nanohybrids with DNA linkers of different length; (b) representative AFM image of the SWCNT-10bp-QD heterostructures.

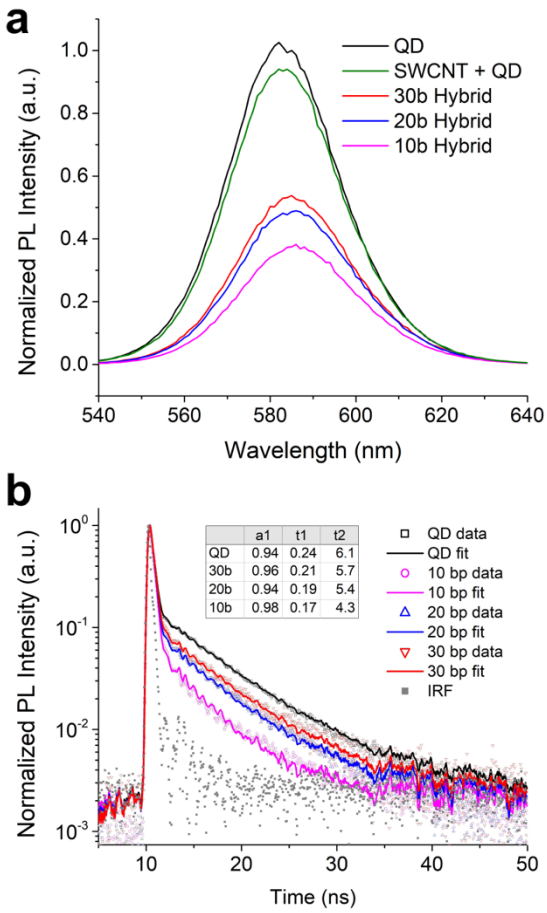

Figure 5. Photoluminescence spectra of pristine QDs and each SWCNT-QD nanohybrid: (a) SSPL spectra (as a control experiment, shown in green, SWCNTs underwent the same treatment as the hybrids but with no linker present); and (b) time-resolved PL spectra (the data were fitted with an

iterative convolution of the instrument response function, IRF in gray, and a bi-exponential trace; the emission wavelength is 585 nm).

In addition, reversibly reconfigurable heterostructures were assembled where a Guanidine(G)-rich sequence was used as a linker. The QD's position relative to the end of the SWCNT could then be controlled by the addition and removal of K^+, which induces the folding of the sequence into a G-quadruplex (G4) and shortens the distance between the two components (cryptand 222 allowed us to revert the linker back to its extended conformation restoring the original distance between the two nanostructures). We demonstrate how this stimuli-responsive strategy allows real-time control over the coupling between the SWCNT and QD, and can be further exploited for the sensing of K^+ from mM to pM concentrations: see figure 6.

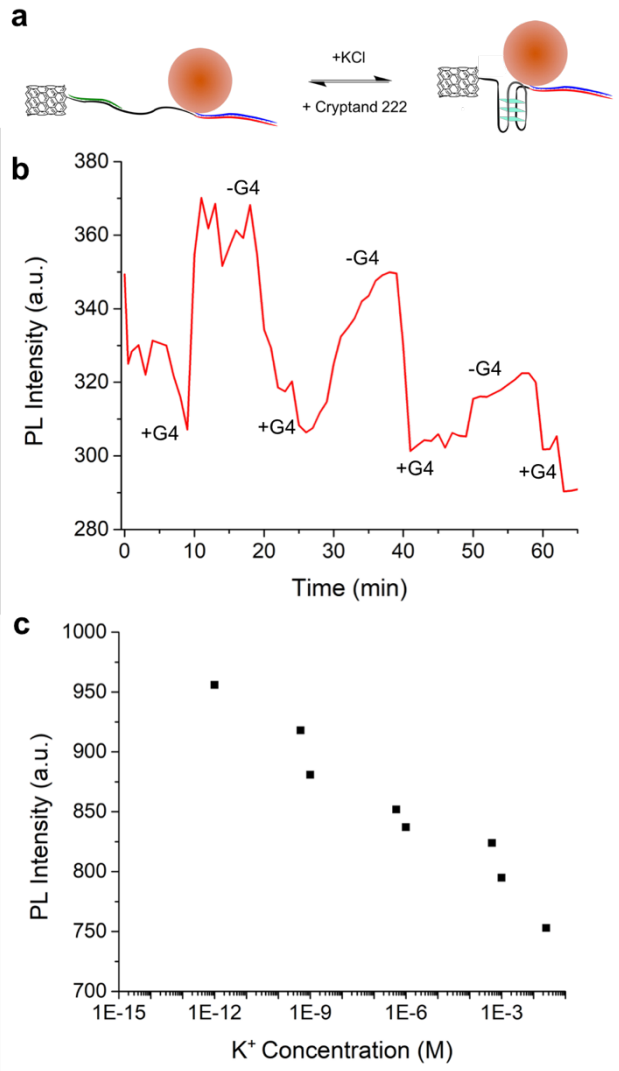

Figure 6. (a) Scheme for the conformational changes of the SWCNT-QD nanohybrid with G4 aptamer upon addition of K^+ or cryptand 222; (b) PL intensity plotted against time with alternating additions of K^+ and cryptand 222. (+G4) indicates the formation of the G-quadruplex, while (-G4) indicates the reversion to the linker's extended conformation; (c) PL intensity plotted against the concentration of K^+ indicating the range of sensitivity.

3. Reconfigurable Carbon Nanotube Multiplexed Sensing Devices: *Nano Lett*. 2018, 18, 4130−4135

In collaboration with Jorge L. Chávez and Nancy Kelley-Loughnane (Air Force Research Laboratory, 711th Human Performance Wing, Wright-Patterson Air Force Base, Dayton, Ohio) we developed a strategy for the facile fabrication of reconfigurable and solution processable nanoscale multiplexed biosensors, based on SWCNTs. DNA-wrapped (hence water-soluble) SWCNTs functionalized with specific nucleotide sequences were employed as selective recognition elements. Figure 7 shows an AFM image of the biohybrids assembled.

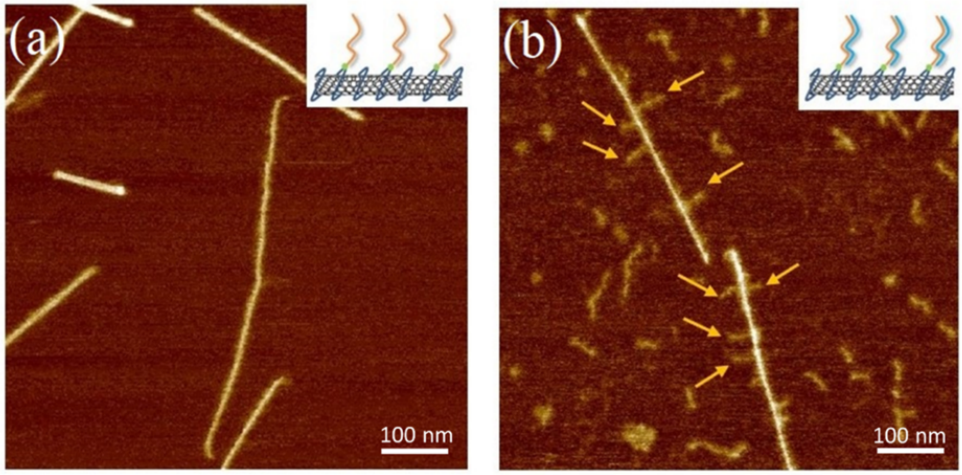

Figure 7. AFM images, and cartoon insets, of aptamer-functionalized SWCNTs (a) without and (b) with hybridized ss-DNA sequences. The yellow arrows show the hybridized aptamers along the nanotubes. Z-scales = 2.5 nm.

Distinct SWCNT-aptamer hybrids were then immobilized on the same chip from solution onto pre-patterned electrodes via dielectrophoresis (DEP); this allowed us to fabricate a multisensing platform for the simultaneous electrical detection of different biomarkers: see figure 8. As a proof-of-concept, we employed our devices for both the selective detection of ss-DNA (i.e. hybridization events) and, most notably, the label-free multiplexed sensing of cortisol, neuropeptide Y (NPY), and dehydroepiandrosterone-sulfate (DHEAS), due to the roles of these biomarkers in various physiological processes such as energy metabolism, blood pressure regulation, cognitive function, post-traumatic stress disorder and traumatic brain injury.

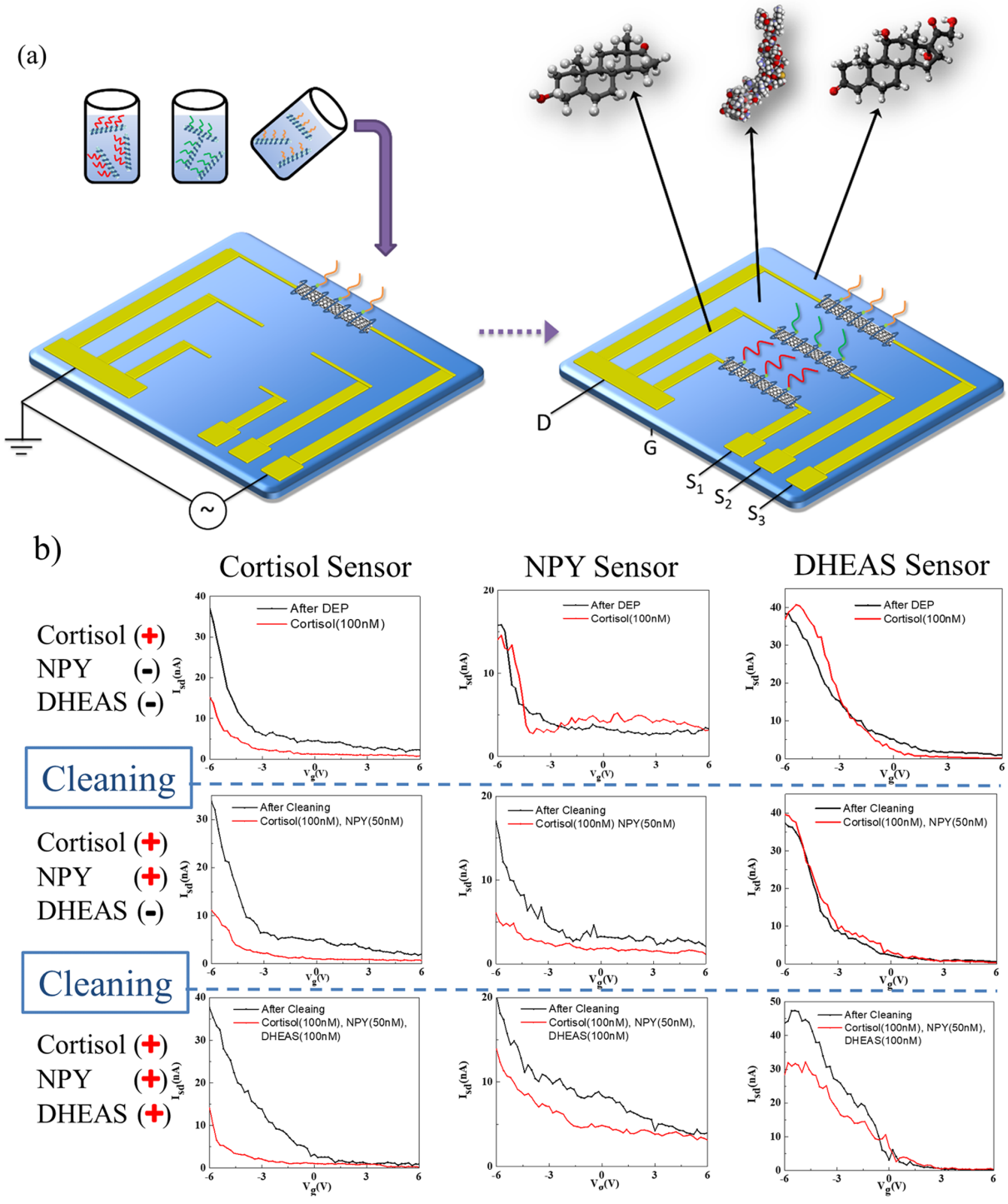

Figure 8. (a) Schematic of the DEP strategy employed for the fabrication of multi-sensing devices: a drop of the chosen SWNCT-aptamer solution is cast on the chip mainly over one electrode pair , a voltage is then applied only across this electrode pair in order to direct the assembly of SWCNT-aptamer hybrids only across this pair of electrodes, and not the others (see also the SI); (b)

multiplexed sensing: electrical responses of the different biosensors on the same chip (V_{sd} =100 mV): the + sign indicates the addition/presence of the analyte of interest; the "cleaning step" indicates the addition of 8M of urea in order to regenerate the sensor after each detection; "after cleaning" indicates the measurements performed after this step.

We further demonstrated the real-time detection of these hormones at their physiological relevant concentrations, from pM to μM: see figure 9. Additionally, we show how the platform developed is reconfigurable and reusable via a simple cleaning procedure.

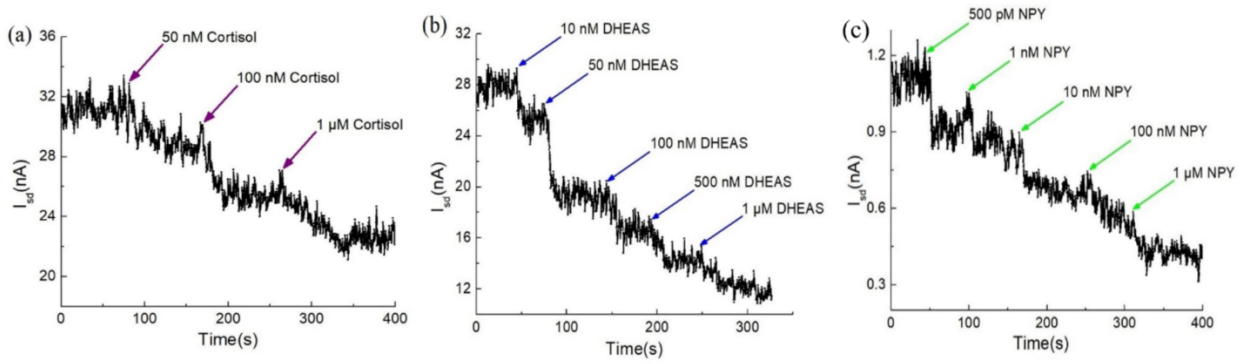

Figure 9. Real time detection of (a) cortisol (from 50 nM to 1 μM), (b) DHEAS (from 10 nM to 1 μM) and (c) NPY (from 500 pM to 1 μM) at various concentrations, in serum (V_{sd} = 100 mV, V_g = –2V).

To the best of our knowledge, these results represent the first example of solution-processable and reconfigurable nanoscale multiplexing sensing devices based on the use of carbon nanostructures. By and large, the general applicability of the strategy developed, and the solution processability of the nanoscale multiplexing biosensing devices we fabricated, hold great potential for the development of the next generation of portable, point of care and home diagnostic assays for the continuous and simultaneous monitoring of different health parameters.

General Conclusions

The relevance and impact of the outputs obtained in this project include: i) improved analysis of biological and chemical agents down to single-molecule resolution; ii) the use of carbon nanoelectrodes for the construction of biosensors employing either aptamers or proteins: this opens the possibility of exploring different kind of biosensing, from biomarkers associated to stress and neurotrauma, to antimicrobial resistance as demonstrated in this report and the associated publications; iii) novel platforms for the low-cost fabrication of miniaturized multipurpose biosensing devices, that thanks to their intrinsic biocompatibility and flexibility hold great potential as wearable and implantable body nano-sensors. By gaining fundamental insight on the effect of biomolecular recognition on the properties of carbon nanostructure-based nanosensors we have contributed at enhancing the development and use of nanoscale devices as biosensors of Airman biosignatures. This will allow: (i) the optimization of the sensor performance in biofluids, (ii) the integration of multiple analyte signals to characterize Airman Biosignatures, as well as (iii) the tuning of sensitivity ranges of sensor elements thereby extending the ranges of arrays.

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- M. Freeley, H. L. Worthy, R. Ahmed, B. Bowen, D. Watkins, J. E. Macdonald, M. Zheng, D.D. Jones*, M. Palma*, "Site-Specific One-To-One Click Coupling of Single Proteins to Individual Carbon Nanotubes: a Single-Molecule Approach"
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  Advanced Science, 2018, 5, 1800596
- X. Xu, P. Clement, J.Eklˆf-Ostenberg, N. Kelley-Loughnane, K. Moth-Poulsen, J. L. Chavez, M.Palma* "Reconfigurable Carbon Nanotube Multiplexed Sensing Devices"
  Nano Letters, 2018, 18, 4130-4135
- X. Xu, B. J. Bowen, R. E.A. Gwyther, M. Freeley, B. Grigorenko, A. V. Nemukhin, J. Eklöf-Österberg, K. Moth-Poulsen, D. D. Jones,* and M. Palma* "Tuning Electrostatic Gating of Semi-Conducting Carbon Nanotubes by Controlling Protein Orientation in Biosensing Devices"
  In preparation, 2020