

End-selective functionalization of carbon nanotubes. Use of DOE for the optimization of a DNA probe attachment and hybridization using an enzymatic amplifying system†

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Self-assembling functionalized multi/single-walled carbon nanotubes (MWCNTs/SWCNTs) in a controlled manner may be an interesting route towards novel nanoscaled supramolecularly arranged MWCNT/SWCNT-based architectures as sensing constructs. Due to the presence of fullerene-like end-caps sensitive to oxidation, the oxidation of MWCNTs/SWCNTs may be readily performed to create oxidized MWCNTs/SWCNTs (MWCNT_{s_{ox}}/SWCNT_{s_{ox}}). MWCNT_{s_{ox}}/SWCNT_{s_{ox}} contain *two different areas that are well-defined topologically, i.e.* an outer hydrophobic sidewall containing oxygenated defects (COOH, CHO, and C–O–C functions), and an end-localized cluster of COOH groups. They have been readily derivatized following the simultaneous chemical activation of both end cluster and sidewall carboxylic groups by carbodiimides. This derivatization scheme and related variants were commonly used for the covalent attachment of numerous types of particles, polymers, and biomolecular probes, *but in a non-selective way*. In contrast, sidewall pegylation of MWCNT_{s_{ox}}/SWCNT_{s_{ox}} enabled the *end-selective covalent attachment* of an aminated 20-mer DNA probe and its hybridization with a fluorescein (FITC)-labeled complementary 2nd DNA sequence. End-selective DNA hybridizations of resulting pegylated MWCNT_{s_{ox}}/SWCNT_{s_{ox}} have been assayed using a blue-colored (optical readout at 620 nm) amplifying horse radish peroxidase (HRP)-based enzymatic system. The optimization of the whole functionalization sequence, *i.e.* MWCNT/SWCNT oxidation, pegylation of oxidized MWCNT_{s_{ox}}/SWCNT_{s_{ox}}, DNA attachment and subsequent hybridization, has been performed using a DOE (design of experiments) approach. The most influential factors were found to be the oxidation temperature of MWCNTs/SWCNTs, the molecular weight of PEG polymers used for sidewall passivation, and, quite unexpectedly, the commercial source of the starting MWCNTs/SWCNTs.

1. Introduction

Multi-walled and single-walled carbon nanotubes (MWCNTs/SWCNTs) are new carbon-based nanomaterials that possess a quite unique set of mechanical, electrical, and magnetic properties, making them particularly interesting for numerous (bio)nanotechnology applications.^{1–5} They are composed of one (SWCNTs) or several (MWCNTs) graphene sheets of sp²-hybridized carbon *hexagons* disposed coaxially around an internal

hollow cavity. MWCNTs/SWCNTs present end-caps that contain thermodynamically less stable *pentagon*-based structural defect sites.⁶ In addition, other types of known defects produced during industrial processing (fabrication and cleaning) mainly arise from atomic vacancies (*dangling bonds*) and from the formation of Stone–Wales defects (non-hexagonal *pentagon/heptagon*-based carbon rings).⁶ All these defect sites are quite sensitive to oxidation enabling the generation of oxygenated defect sites.^{7,8} Depending on the oxidation conditions, this results in the random introduction of hydroxyl, ether, carbonyl, nitro, and carboxyl (COOH) groups onto oxidized MWCNTs/SWCNTs (MWCNT_{s_{ox}}/SWCNT_{s_{ox}}). Accordingly, and due to the versatile derivatization chemistry of the COOH group, end and sidewall-localized carboxylated defect sites of MWCNT_{s_{ox}}/SWCNT_{s_{ox}} may then be reacted in order to covalently link biomolecular probes (DNA sequences, proteins, antibodies), natural and engineered polymers, small organic ligands, and nanoparticles.^{9–15} However, this simple basic “wet chemistry-based” oxidative approach did not allow selectivity tuning between the formation of end *versus* sidewall carboxylic groups and *vice versa*. This clear fact raises the interesting question of how *topologically selective* covalent modifications of MWCNT_{s_{ox}}/SWCNT_{s_{ox}} may be achieved. Straight

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† Electronic supplementary information (ESI) available: Table S1 contains OD and EF data for the 1st set of randomly executed DOE experiments, Fig. S1a, S2a, S3a, and Fig. S2b, and S3b show one factor plot graphs of the influence of the molecular weight of PEG polymers and the commercial source of MWCNTs/SWCNTs respectively, Fig. S4 (graphs 1–3) shows the optimal conditions from a 1st set of DOE experiments, and Scheme S1 shows the quantification of accessible COOH groups using the fluorescent probe DPEG-NH₂. See DOI: 10.1039/b818160h

answers to this question will especially address specific applications such as carbon nanotube-based nanoscaled constructs fabricated by *directionally* controlled self-assembly.^{16,17}

Here, we demonstrate that sidewall pegylation of oxidized MWCNT_{s_{ox}}/SWCNT_{s_{ox}} can chemically mask sidewall-localized carboxylic groups (chemical passivation process) *versus* end-clustered ones. Consequently, this PEG-mediated topologically selective passivation step enables the chemical derivatization of carbon nanotube ends *versus* sidewalls based on defect COOH groups. This quite unique functionalization approach of MWCNTs/SWCNTs has been successfully exploited for carbon nanotube end-selective DNA covalent attachment and hybridization (Scheme 1). For that purpose, a blue colored horse radish peroxidase (HRP)-mediated enzymatic reporting system based on double-stranded DNA hybridization^{18,19} has been used for optimization. This functionalization sequence includes the following steps, the oxidation of MWCNTs/SWCNTs (carboxylation step) affording oxidized MWCNT_{s_{ox}}/SWCNT_{s_{ox}}, the pegylation of oxidized MWCNT_{s_{ox}}/SWCNT_{s_{ox}} (chemical masking of sidewall defect COOH groups for end *versus* sidewall selectivity of derivatization), and the final end-selective covalent DNA attachment and hybridization. Due to its complexity and likely parameter interrelationship, this multi-step sequence has been optimized using statistically relevant DOE (design of experiments).^{20,21}

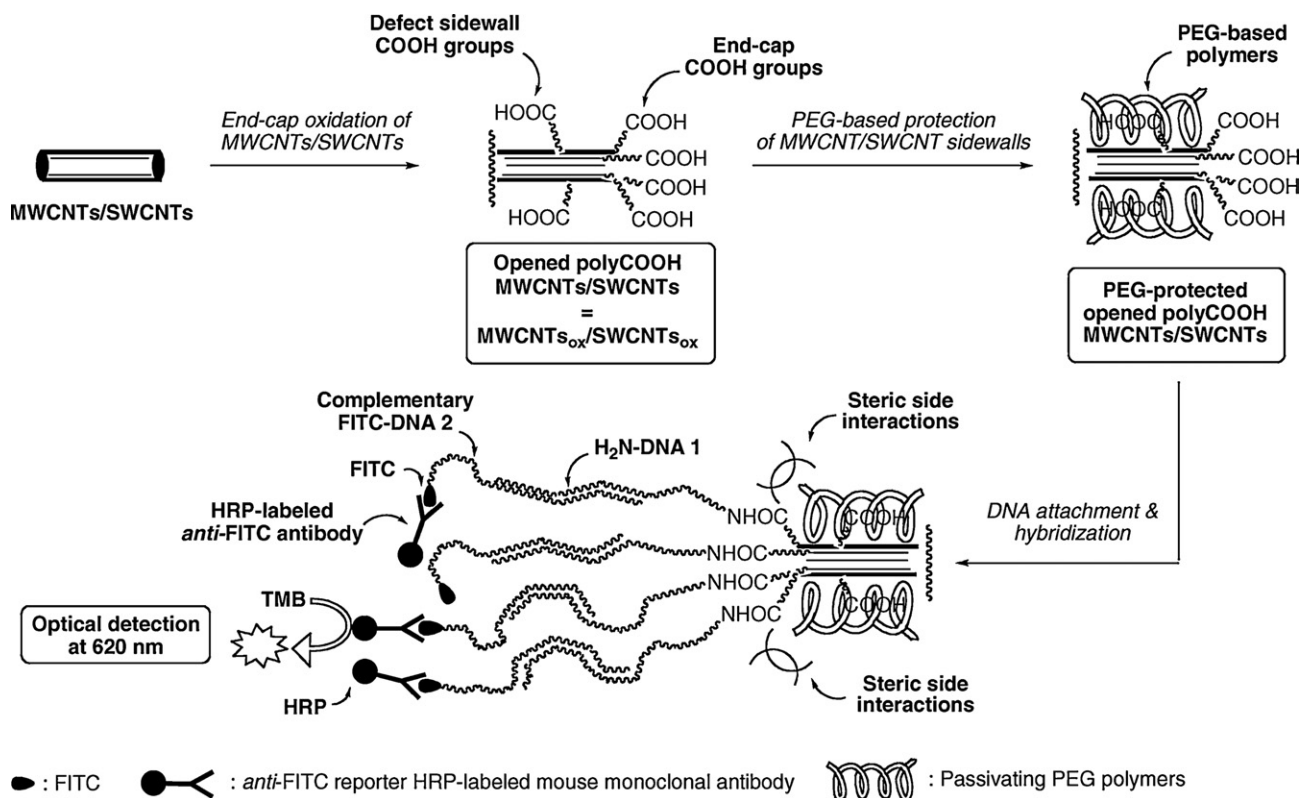
Only *three specific parameters*, the oxidation temperature of MWCNTs/SWCNTs, their commercial source, and the molecular weight of sidewall-passivating PEG polymers significantly

influenced the overall efficiency of this multi-step functionalization sequence. More specifically, the non-covalent adsorption of a 2000 Daltons PEG polymer was found optimal because of two main effects likely driven by steric hindrance. The first one concerned the successful passivation (chemical masking) of the carboxylic groups (defect groups) present on the sidewalls of oxidized MWCNT_{s_{ox}}/SWCNT_{s_{ox}}. The second one arose from the steric hindrance effects of *free pendant* PEG arms located close to the oxidized/DNA-functionalized ends of MWCNT_{s_{ox}}/SWCNT_{s_{ox}}. Both PEG-mediated effects enabled the subtle but very effective tuning of topologically selective end-localized DNA covalent attachments/complementary hybridizations. They are of major importance for the *directed* fabrication of MWCNT/SWCNT-based nanoscaled constructs to be self-assembled using DNA hybridization.^{16,17}

2. Experimental

2.1. General

Carbon nanotube specifications. Both MWCNTs and SWCNTs are commercially available from the MER Corporation Ltd and Nanocyl s. a. They have the following characteristics: MER MWCNTs, average diameter/length: 140 ± 30 nm/7 ± 2 μm, 340–530 graphitic layers, purity superior to 90% by TGA; MER SWCNTs, average diameter/length: 1.2–1.4 nm/10–50 μm, purity ~98% by TGA; Nanocyl s.a. MWCNTs, average diameter/length: 9.5 nm/1.5 μm, 5–20 graphitic layers, purity ~95% by



Scheme 1 The multi-step sequence of functionalization of MWCNT_{s_{ox}}/SWCNT_{s_{ox}} using a blue colored horse radish peroxidase (HRP)-mediated enzymatic reporting system based on double-stranded DNA hybridization

TGA & XPS, Nanocyl SWCNTs, average diameter/length: 2.0 nm/1.5 μm , purity $\sim 70\%$ by XPS).

Specific reagents, buffers and washing/assay solutions for DNA hybridization. The neutral PBS buffer (pH 7.0) was prepared from a Dubelcco's phosphate buffered saline (Sigma). The 0.4 M MES (pH 5.0) buffer was prepared using 2-morpholino-ethanesulfonic acid (hydrate form) 99%, adjusted to pH 5.0 by the addition of 10 M NaOH and stored at 4 $^{\circ}\text{C}$. The TNET buffer (pH 7.5) was prepared from a mixture of 10 mM tris-HCl, 0.5 M NaCl, 1 mM EDTA and 0.02% Tween-20. The washing solution arose from a mixture of 3 M NaCl and 2 M tris-HCl (pH 7.5). The assay solution was prepared as a mixture of 154 mM NaCl, 50 mM tris-HCl (pH 7.8), 0.5% BSA, and 0.1% Tween 20. The conjugate *anti*-FITC HRP-labeled mouse monoclonal antibody was diluted to 1/100 in an assay solution before use (Savyon Diagnostics). The aqueous solution of activating water-soluble carbodiimide EDC [*N'*-(3-dimethylamino-propyl)-*N*-ethyl-carbodiimide hydrochloride, >98% purity, Aldrich] was freshly prepared in a cold 0.4 M MES buffer (pH 5.0, 100.0 mg EDC mL^{-1} working concentration).

2.2. General protocol for the oxidative opening of MER and nanocyl MWCNTs/SWCNTs

This protocol is adapted from the known procedure reported by Dai, Smalley and co-workers.^{7,8} MWCNTs/SWCNTs (100.0 mg/experiment) were oxidatively opened at pentagon-like end caps and sidewalls (formation of acidic COOH defects) under nitrogen using a mixture of acids (12 M HNO_3 and 36 M H_2SO_4) in variable v/v ratios (see text), at variable temperatures (50, 70, and 90 $^{\circ}\text{C}$), and during variable oxidation times (1, 3, and 5 h). At reaction completion, oxidized MWCNTs_{ox}/SWCNTs_{ox} that generally formed stable suspensions were washed with bi-distilled water until neutrality using several centrifugation (9500 rps, 45 min, 25 $^{\circ}\text{C}$) and decantation steps.²²

2.3. Typical pegylation protocol of oxidized MWCNTs_{ox}/SWCNTs_{ox}

End (polyCOOH cluster) and sidewall (COOH group defects) oxidized MWCNTs_{ox}/SWCNTs_{ox} (10.0 mg) were incubated in a neutral 0.01 N PBS (phosphate) buffer containing various α,ω -bis-methoxy PEG polymers (Shearwaters Polymers, USA, molecular weights (MWs): 250, 500, 1000, 2000, 10 000, and 20 000 Daltons, 26.7, 13.3, 6.7, 3.3, 0.7, and 0.3 mg respectively/mL of a neutral 0.01M PBS buffer, 200 μL , 20 min incubation, 20 $^{\circ}\text{C}$), then washed (neutral 0.01M PBS buffer, 4 \times 200 μL) to afford PEG-protected polycarboxylated (polyCOOH) oxidized MWCNTs_{ox}/SWCNTs_{ox} (FT-IR, KBr pellet, almost total disappearance of COOH vibrations, $\nu_{\text{Csp}^2\text{-H}} = 2820\text{--}2985 \text{ cm}^{-1}$). Decantation of insoluble pegylated MWCNTs_{ox}/SWCNTs_{ox} and the elimination of PEG polymers in excess were performed using centrifugation (9500 rpm, 25 min, 20 $^{\circ}\text{C}$) and decantation. Purified pegylated and oxidized MWCNTs_{ox}/SWCNTs_{ox} were stored at 4 $^{\circ}\text{C}$ in a neutral 0.01 M PBS buffer at a 1% w/v concentration.

2.4. End-selective covalent attachment of an aminated DNA biomolecular probe to pegylated and non-pegylated MWCNTs_{ox}/SWCNTs_{ox}. DNA hybridization and enzymatic amplification using HRP (horse radish peroxidase)

Pegylated and/or non-pegylated oxidized MWCNTs_{ox}/SWCNTs_{ox} (1% w/v suspension in a neutral 0.01M PBS buffer, 100 μL , 1.0 mg) were smoothly vortexed (20 s) for dispersion sake, washed by (i) the same PBS neutral buffer (3 \times 100 μL), and by (ii) a 0.4 M MES buffer (pH 5.0, 100 μL). After centrifugation (9500 rps, 5 min), and supernatant removal, at 20 $^{\circ}\text{C}$ the following were successively added: (i) EDC (0.52 M EDC in 0.4 M MES buffer, 30 μL , 3.0 mg, slow vortexing, 5 min) for COOH activation, and, (ii) the 20-mer amine-modified oligonucleotide $\text{H}_2\text{N-DNA}_1 \text{NH}_2\text{-(CH}_2\text{)}_{12}\text{-5'GCACTGGGAGCATTGAGGCT}$ (0.4 M MES buffer at pH 5.0, 70 μL , 1.68×10^{-5} M, 0.5 nmol, Operon (USA), chemical purity $\geq 98\%$). The mixture was incubated at room temperature for 2 h (smooth vortex agitation) for both carboxylate activation and an $\text{H}_2\text{N-DNA}_1$ covalent attachment. Following completion, DNA-functionalized oxidized and pegylated MWCNTs_{ox}/SWCNTs_{ox} were washed with 4 \times 100 μL of PBS buffer to remove unattached component(s)/activator(s), and inorganic salts in excess. These DNA-containing nanocomposites can be gently resuspended in 100 μL of a neutral 0.01 M PBS buffer at 4 $^{\circ}\text{C}$ for later use (1% w/v concentration). DNA-linked pegylated MWCNTs_{ox}/SWCNTs_{ox} (1.0 mg) were washed in a TNET buffer (100 μL , pH 7.5) and distributed as 5.0 μL (50.0 μg , 1% w/v per microtiter plate well) portions to the wells of a thermowell polycarbonate non-sterile 96-well microtiter plate. For total binding (TB) experiments only, each well was added with the complementary FITC-labeled *anti*-sense oligonucleotide FITC-DNA₂ FITC-triethylene glycol linker 5'-AGCCT-CAATGCTCCCAGTGC dissolved in a pH 7.5 TNET buffer (50 μL , 1×10^{-7} M, Danyel Biotech Ltd, Israel, chemical purity $\geq 98\%$), and the microtiter plate was incubated for 1 h at 60 $^{\circ}\text{C}$ for DNA hybridization. Hybridized pegylated and/or non-pegylated oxidized MWCNTs_{ox}/SWCNTs_{ox} were then washed with a TNET buffer (4 \times 100 μL , pH 7.5), and by 50 μL of a commercially available Assay Solution® (Savyon Diagnostics Ltd, Israel) before incubation with the reporter *anti*-FITC HRP-labeled mouse monoclonal antibody (commercially available from Hoffman la Roche Inc., 0.1 $\mu\text{g mL}^{-1}$, 20 min at 20 $^{\circ}\text{C}$, 50 μL of an Assay Solution®). After material decantation (Eppendorf test tube for centrifugation, 9500 rps during 5 min, 20 $^{\circ}\text{C}$), and pellet washings (4 \times 100 μL of a washing solution), the TMB substrate (0.05% w/w TMB solution in de-ionized water, 50 μL , Savyon Diagnostics Ltd) was added for color development over 1.5 min for each parallel experiment (Eppendorf test tube). Following decantation by centrifugation, supernatants were removed and 80 μL fractions for each Eppendorf test tube were transferred to a second PS 96-well microtiter plate for optical reading at 620 nm using an Elisa plate reader Anthos ht II.

3. Results and discussion

The validation and DOE-mediated optimization of the overall functionalization sequence of MWCNTs/SWCNTs exploited the effectiveness of the robust DNA hybridization system disclosed in Scheme 1.^{18,19} An amine modified 20-mer DNA capture probe

H₂N-DNA₁ [H₂N-(CH₂)₁₂-5'GCACTGGGAGCATTGAG-GCT] was covalently attached at the oxidized end caps of pegylated oxidized MWCNT_{s_{ox}}/SWCNT_{s_{ox}}. For that purpose, the water-soluble carbodiimide EDC [*N*'-(3-dimethylamino-propyl)-*N*-ethylcarbodiimide hydrochloride] chemically activated end COOH groups (0.4 M MES buffer, pH 5.0, 2 h, 20 °C) toward amine-mediated SN₂ nucleophilic substitution. DNA hybridization to the complementary fluoresceine-labeled *anti*-sense FITC-DNA₂ strand [fluoresceine (FITC)-triethyleneglycol linker-5'AGCCTCAATGCTCCAGTGC, TNET buffer at pH 7.5, 1 h, 60 °C] was readily followed by incubation with a reporter *anti*-FITC HRP-labeled mouse monoclonal antibody (0.1 µg, 20 min, 20 °C). This HRP-based enzymatic amplifying construct may be then reacted with the *colorless* TMB (3,3',5,5'-tetramethyl-benzidine) substrate of HRP. Following HRP enzymatic oxidation (1.5 min, 20 °C), its oxidized *blue* form provided blue-colored optical outputs that were read at 620 nm using an Elisa plate reader. Measured optical densities (ODs) characterized the total amount of FITC-DNA₂ recognized by pegylated and/or non-pegylated MWCNT_{s_{ox}}/SWCNT_{s_{ox}} that were functionalized by the DNA₁ capture probe (total binding: TB). In parallel, non-specific binding (NSB) experiments were also conducted by omitting the FITC-DNA₂ incubation step dealing with the same pegylated and/or non-pegylated DNA₁-decorated MWCNT_{s_{ox}}/SWCNT_{s_{ox}}. These experiments allowed the disclosure of non-covalent affinity (physical adsorption) for the reporter *anti*-FITC HRP-labeled mouse antibody. Specific and non-specific OD values were always averaged outputs of *three parallel experiments* in order to minimize data dispersion. The resulting calculated efficiency factors (EFs) that were defined by the fraction $EF = \frac{OD_{\text{total binding}} - OD_{\text{non-specific binding}}}{OD_{\text{non-specific binding}}}$ enabled quantification of the overall sequence efficiency using statistical design of experiments (DOE) as described later.

3.1 Optimization of the functionalization sequence using design of experiments (DOE)

Sequence optimization using statistically relevant DOE^{20,21} first involved oxidized MWCNT_{s_{ox}} (Table 1). A four factor-two level partial factorial design (Res IV) $2^{(4-1)} = 8 + 1 = 9$ experiments with one center point (st. order 9) was proposed by a Design-Expert software package (Stat-Ease® software, version 6, Minneapolis, USA). The four parameters that were investigated were (i) the temperature of the oxidation medium (two values: 50 and 90 °C), the oxidation time (two values: 1 and 5 h), the relative

Table 1 1st proposed and executed statistically relevant DOE matrix of experiments for sequence optimization

St. order	T/°C	Time/h	HNO ₃ (%v)	PEG/MW	EF
1	50	1	25	2000	37.0
2	90	1	25	20 000	36.9
3	50	5	25	20 000	9.5
4	90	5	25	2000	33.0
5	50	1	75	20 000	27.5
6	90	1	75	2000	33.0
7	50	5	75	2000	48.0
8	90	5	75	20 000	32.8
9	70	3	50	10 000	34.1

volume ratio of acidic mixtures of concentrated 12 M HNO₃ and 36 M H₂SO₄ (two values: 25 : 75 and 75 : 25), and finally the molecular weight of passivating PEG polymers (two values: 2000 and 20 000 Daltons). According to the proposed DOE model, the added additional centre point corresponded to the following set of conditions: oxidation temperature: 70 °C, oxidation time: 3 h, acid v/v ratio (v/v HNO₃ : H₂SO₄): 50 : 50, and the PEG molecular weight: 10 000 Daltons. Except for the use of the PEG polymer, these oxidative conditions exactly fit a standard commonly used oxidative protocol formerly described by Dai and Smalley.^{7,8} The influence of the four tested factors has been investigated using EF values as experimental outputs. Each parallel TB and NSB experiment has been performed *in a triplicate format* and averaged. Accordingly, a set of nine corresponding experiments has been executed in a random manner as proposed in the DOE matrix of experiments described in Table 1.

EF data were obtained in a rather wide 9.5 to 48.0 (st. orders 3 and 7) range of values emphasizing a 505% increase factor between extremes. They also demonstrated that both the *oxidation temperature* and the *PEG molecular weight* are the two most influential factors with negative effects (−0.31 and −0.003 calculated factors respectively, Fig. 1 and S4).† Their combination afforded a small synergistic effect (0.410^{−4}, Fig. S4, graphs 1–2).†

Very surprisingly, both acid ratio and oxidation time parameters were found to *have no effect* in the proposed range of tested parameters (Fig. S4, graph 3).† Accordingly to the tested model, the optimal conditions for the simultaneous MWCNT oxidative opening (COOH groups introduction) and sidewall PEG-related chemical masking (passivation of sidewall defect COOH groups) have been disclosed in Fig. S4 (graph 3),† *i.e.* an oxidation temperature of 50 °C, and a PEG molecular weight of 2000 Daltons. These data clearly demonstrate that end-localized DNA attachment, hybridization, and recognition by the HRP-labeled antibody reporter at pegylated oxidized MWCNT_{s_{ox}} strongly depended on the manner in which MWCNTs were oxidized and passivated using PEG polymers of defined molecular weights. *In this regard, the literature conditions reported in st. order 9 (Table 1)^{7,8} are quite far from being optimal.²²*

Additional interesting points that should be noted: for example, OD_{non-specific binding} data for non-pegylated oxidized MWCNT_{s_{ox}} were consistently found in a 1.19–2.38 high-level range, which strongly reduced corresponding EF values (EFs in a 0.2 to 1.3 range, Table S1 in ESI).† In contrast, and due to the

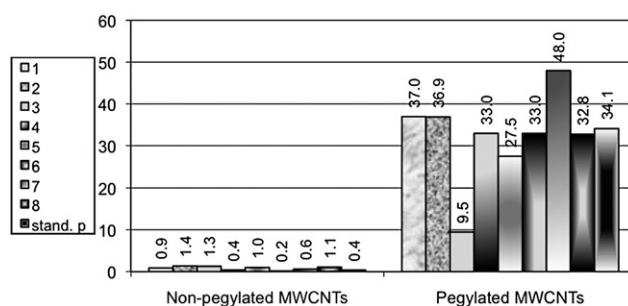


Fig. 1 The effect of the pegylation step of oxidized MWCNT_{s_{ox}}: EF data for the randomly executed set of DOE experiments described in Table 1 (histogram mode of presentation).

known protein-repulsive properties of PEG polymers, the PEG-mediated passivation of oxidized MWCNTs_{ox} was particularly effective for an optimal PEG polymer of a molecular weight of 2000 Daltons [Table 1, st. orders 1, 4 and 6–7, and Fig. 1 and S4 (graphs 2 and 3),† EF data varying in a 33.0 to 48.0 range].

Indeed, both OD_{total binding} and OD_{non-specific binding} parameters for pegylated oxidized MWCNTs_{ox} varied synergistically at very high 0.51–1.33, and very low, 0.03–0.05, ranges respectively. The last data emphasized the overall efficiency of the PEG-mediated topologically selective masking process of MWCNT_{ox}/SWCNT_{ox} sidewalls. This critical feature of topological end selectivity for DNA attachment and hybridization induced by PEG passivation was checked using *two different approaches*. Indeed, the non-covalent adsorption of PEG polymers onto carbon nanotube sidewalls has been demonstrated for *non-oxidized* carbon nanotubes, while remaining questionable for *oxidized* MWCNTs_{ox}/SWCNTs_{ox}.^{23–25}

Therefore, exploiting the known strong affinity of thiols for Au surfaces, both non-pegylated and pegylated oxidized MWCNTs_{ox}/SWCNTs_{ox} were separately functionalized by L-cysteine using the EDC activation of COOH groups (EDC, 0.04 M MES buffer, 20 °C, 30 min). The resulting non-pegylated and pegylated thiolated MWCNTs_{ox} were then reacted with 20–30 nm-sized Au nanoparticles (deoxygenated H₂O, 24 h, 20 °C) produced by the citrate reduction of AuCl₄[−] anions (HAuCl₄, 17 %w HCl, H₂O, 60 °C).^{26–29} These Au nanoparticles were stabilized against aggregation by tannic acid. On the completion of the reaction, the resulting Au particle-carbon nanotube composites were visualized by high resolution SEM (JEOL JSM-7000P apparatus, Oxford Instruments, Gatan CCD Camera), and analyzed by EDAX elemental analysis.

Corresponding microphotographs (Fig. 2a–c) show a very characteristic *end-selective* aggregation/attachment of Au nanoparticles at thiolated ends of pegylated surface-smooth MWCNTs_{ox}/SWCNTs_{ox} (Fig. 2b). Very few isolated Au nanoparticles were detected on the pegylated sidewalls, this is likely to be due to non-specific binding. The corresponding EDAX compositional analysis of these samples (INCA Software, Oxford Instruments) indicated globular end areas strongly enriched with Au (Fig. 2c). In contrast, non-pegylated MWCNTs_{ox} possessed a quite rough aspect due to linked Au nanoparticles (Fig. 2a). These particles appeared uniformly and densely distributed on MWCNTs_{ox} sidewalls as well as at oxidized ends (absence of topological selectivity for particle attachment).³⁰

Additionally, oxidized PEG_{20 000}-passivated MWCNTs_{ox} (Table 1, st. order 2) have been incubated in the presence of a deoxygenated 1 : 2 molar ratio Fe²⁺ (FeCl₂) : Fe³⁺ (FeCl₃) basic (KOH) aqueous solution for the *in situ* hydrolytic generation and COOH-mediated attachment of 20–40 nm-sized magnetite (Fe₃O₄) nanoparticles. Resulting magnetically responsive biphasic magnetite-PEG_{20 000}-passivated MWCNTs_{ox} have been examined by high-resolution TEM (JEOL-JEM2010 instruments, Oxford Instruments, accelerating voltage 200 kV, Gatan CCD camera, Fig. 3) including compositional energy-dispersive X-ray EDAX analysis for the presence or absence of the Fe element (15 nm-sized focused electron beam). MWCNTs_{ox} sidewalls did not reveal bound Fe₃O₄ nanoparticles (irradiated area showing the absence of the Fe element, Fig. 3a), while such particles have been readily detected at polycarboxylated extremities (Fe-rich area, 7.72% atomic concentration, Fig. 3b).

Emphasizing the PEG-mediated passivating effect of MWCNTs_{ox}/SWCNTs_{ox} sidewalls, end-accessible COOH

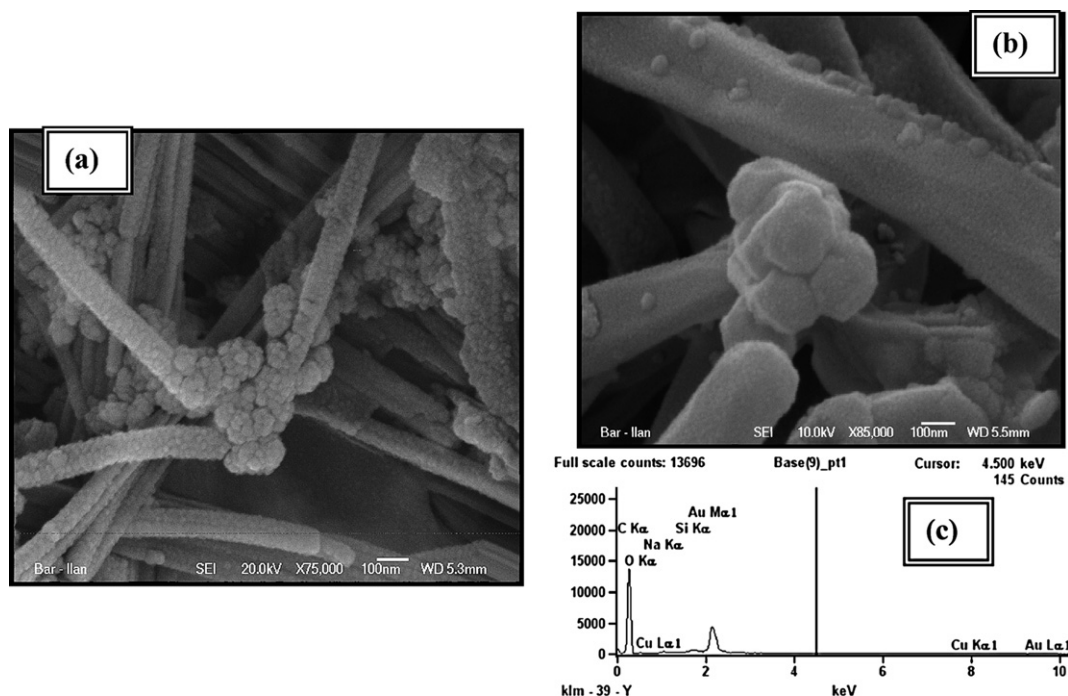


Fig. 2 SEM microphotographs of non-pegylated (a) and pegylated (b) oxidized MWCNTs_{ox} after reaction with 20–30 nm-sized Au nanoparticles. (c) EDAX analysis for Au presence (samples did not include Au evaporation bore imaging).

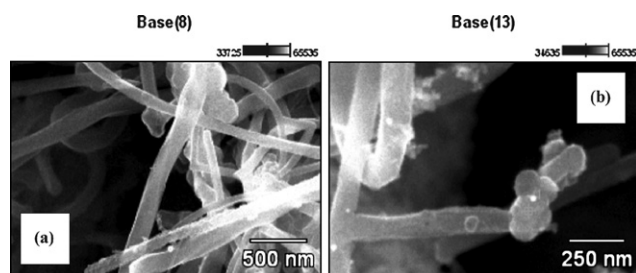


Fig. 3 High-resolution TEM microphotographs of oxidized PEG₂₀₀₀-passivated MWCNT_{s_{ox}} end-functionalized by magnetite (Fe₃O₄) nanoparticles. (a) Sidewall-focused compositional EDAX analysis (Fe element was not detected); (b) end-localized compositional EDAX analysis showing the presence of a Fe-rich area (7.72% atomic concentration).

groups of optimally oxidized PEG₂₀₀₀-passivated MWCNT_{s_{ox}} (Table 1, st. order 7) have been quantified in the following manner. EDC-mediated COOH activation of both oxidized MWCNT_{s_{ox}} and PEG₂₀₀₀-passivated MWCNT_{s_{ox}} (Table 1, st. order 7, 10.0 mg, see section 2.4 for experimental details) afforded EDC-activated *amine-sensitive* MWCNT_{s_{ox}} and PEG₂₀₀₀-MWCNT_{s_{ox}} (Scheme S1, ESI p. 6).[†] They have been separately reacted with the ω -aminated PEG-like fluorescent dansylated probe DPEG-NH₂ in excess (1×10^{-2} mmol, 7 : 3 v/v H₂O : CH₃CN, 20 °C, 18 h). Amounts of unreacted DPEG-NH₂ present in H₂O : CH₃CN washings (7 : 3 v/v, 7 \times 20 mL) for both series of experiments have been measured by analytical HPLC in a *triplicate format* (reverse phase silica Merck Purospher® RP-18e, 125 \times 3 mm, injection volume = 50 μ L, time/solvents/gradient: 0–10 min, 90 : 10 v/v H₂O : CH₃CN to 30 : 70 v/v H₂O/CH₃CN (linear gradient), 1.0 mL min⁻¹, UV detection/quantification at $\lambda = 276$ nm using a MERCK diode array detector, probe retention time: 8.0 min) and averaged after system calibration (Scheme S4, calibration graph).[†] In addition, the same sequence performed while omitting the EDC activation step enabled to check that the DPEG-NH₂ probe had no affinity (non-covalent adsorption) for starting non-activated MWCNT_{s_{ox}} and PEG₂₀₀₀-passivated MWCNT_{s_{ox}} (probe recovery better than 99.1%, HPLC measurement). Respective concentrations of accessible probe-reactive COOH groups for MWCNT_{s_{ox}} and PEG₂₀₀₀-passivated MWCNT_{s_{ox}} were found to be 13.0 and 8.3 nmol mg⁻¹ of nanomaterial respectively corresponding to sidewall and end-localized *versus* end-localized concentrations of COOH groups.

3.2 Optimization of the functionalization sequence using design of experiments (DOE)

Final refinement step. A 2nd last step of sequence optimization aimed at investigating the influence of PEG polymers of molecular weights *lower* than the optimal 2000 Daltons value discovered previously. The influence of two different commercial sources of starting MWCNT/SWCNT materials has also been included in this study. They were chosen in order to present similar average size, diameter, and purity level of MWCNT/SWCNT samples to be tested. Three parameters were specifically examined during this final refinement series of DOE-mediated experiments. They were (i) the molecular weight of passivating

non-functional PEG polymers (five values: absence of PEG, 250, 500, 1000 and 2000 Daltons), (ii) the commercial source of MWCNTs/SWCNTs (two sources/companies: MER Corporation Ltd and Nanocyl s.a.), and, lastly, the carbon nanotube type (two values: MWCNTs and SWCNTs).

The influence of these three factors was investigated using similar EF outputs obtained from DNA-based experiments. Each parallel experiment has been performed *in a triplicate format* and the resulting TB and NSB data averaged.

A five-level partial factorial design (Res IV) $5 \times 2 \times 2 = 20$ experiments has been proposed by the same Design-Expert software package. The corresponding set of *twenty* experiments (2nd DOE matrix of experiments, Table 2) was again executed in a random manner. For that purpose, SWCNTs and MWCNTs were oxidatively opened using the best temperature value 50 °C disclosed during the 1st optimization step (Fig. S4, graph 3).[†] Since both oxidation time and v/v HNO₃ : H₂SO₄ acid ratio were not influential parameters, the 2 h and 1 : 1 values were chosen close to the ones defined for the commonly used literature protocol.^{7,8}

Similar to the 1st set of DOE experiments, EF values varied considerably in a wide 0.4 to 23.0 range (Table 2, st. orders 1, 17 and 11, and Fig. 4). These data emphasized that both the PEG molecular weight and the CNT commercial source are the most influential factors with a positive effect (Fig. 4, S1a, and S2a/b, ESI).[†] Effectively, MWCNTs or SWCNTs from the MER Corporation Ltd were found to be the optimal starting materials. The influence of this factor was quite unexpected, but critical for this sequential functionalization system. Certainly, different industrial processes and/or end cleaning steps were likely to be involved in the fabrication of MWCNT/SWCNT substrates. This would have resulted in different reactivities of end caps and sidewalls of MWCNTs/SWCNTs toward oxidation *via* the introduction of corresponding structural defects (COOH groups in our case).

Additionally, and whatever the commercial source, the carbon nanotube type, multi- *versus* single-walled, has practically no

Table 2 2nd proposed (executed) statistical DOE matrix of experiments for sequence optimization involving oxidized MWCNT_{s_{ox}}/SWCNT_{s_{ox}}

St. order	PEG (MW/Daltons)	CNT source (MER or Nanocyl)	MWCNTs or SWCNTs	EF
1	250	Nanocyl	SWCNTs	0.4
2	1000	MER	MWCNTs	20.0
3	0	Nanocyl	SWCNTs	1.2
4	500	Nanocyl	SWCNTs	0.5
5	0	MER	MWCNTs	3.6
6	2000	MER	SWCNTs	22.9
7	500	Nanocyl	MWCNTs	0.5
8	0	MER	SWCNTs	2.4
9	500	MER	SWCNTs	1.1
10	500	MER	MWCNTs	13.8
11	2000	MER	MWCNTs	23.0
12	1000	Nanocyl	MWCNTs	0.6
13	1000	Nanocyl	SWCNTs	0.6
14	250	MER	MWCNTs	9.2
15	2000	Nanocyl	MWCNTs	3.1
16	1000	MER	SWCNTs	17.6
17	250	Nanocyl	MWCNTs	0.4
18	0	Nanocyl	MWCNTs	1.5
19	250	MER	SWCNTs	6.6
20	2000	Nanocyl	SWCNTs	3.1

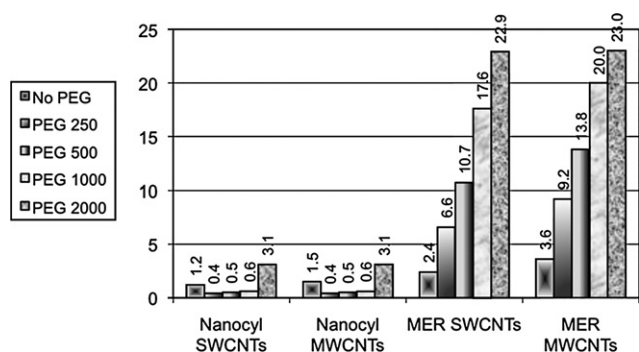


Fig. 4 EF data for the randomly executed 2nd set of DOE experiments: influence of the pegylation step involving oxidized MWCNTs_{ox} and SWCNTs_{ox} (effect of PEG MWs).

effect within the given range of tested parameters (Table 2, Fig. S3a/b, ESI).[†] This surprising result may nevertheless be rationalized in the following manner. End-localized pending chains of adsorbed PEG polymer chains may sterically interact with one or several steps of the functionalization sequence (steric non-bonding side interactions), *i.e.* with the covalent attachment of the capture probe H₂N-DNA₁, with its hybridization to the complementary FITC-DNA₂ sequence, and/or with its recognition by the reporter HRP-labeled monoclonal antibody. For MWCNTs_{ox}, this hindering effect should be less effective for the *most internal* polyCOOH oxidized layer/rolled oxidized graphene sheet. This situation much resembled the one occurring for similarly processed pegylated SWCNTs_{ox}. Finally, sidewall passivation of oxidized MWCNTs_{ox}/SWCNTs_{ox} sidewalls by the same PEG polymer of a 2000 Daltons molecular weight was again found to be the most effective (Table 2, st. order 11, MER MWCNTs_{ox}, EF = 23.0, Fig. S1a, ESI).[†] It is worth noting that, to the best of our knowledge, pegylations of MWCNTs/SWCNTs reported in the literature commonly used PEG polymers of 10 000 or 20 000 Daltons molecular weights. The question of how variable PEG molecular weights may optimally influence the fabrication, use, and response tuning of corresponding MWCNT/SWCNT-based sensing constructs was never examined. In contrast, this study, which involved a biologically relevant DNA-based capture system as a validating model, clearly demonstrates the critical importance of this variable amongst others for model optimization.

4. Conclusion

A multi-step functionalization sequence involving the *end-selective* covalent attachment of oxidized pegylated MWCNTs_{ox}/SWCNTs_{ox} by a 20-mer DNA capture probe, followed by its hybridization using an HRP-based enzymatic amplifying system has been optimized using a design of experiments (DOE) approach. Accordingly, the *three* most influential factors for the optimization of the overall sequence were identified, namely, the oxidation temperature of starting MWCNTs/SWCNTs, the molecular weight of PEG polymers enabling the sidewall passivation of oxidized MWCNTs_{ox}/SWCNTs_{ox} (chemical masking of sidewall defect COOH groups), and, quite unexpectedly, the commercial source of starting MWCNTs/SWCNTs. Such optimized parameters must be taken into

consideration for the successful *directed* fabrication of MWCNT/SWCNT-based nanoscaled constructs to be self-assembled using DNA hybridization.^{16,17}

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