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# Diimide activated coupling of carbon nanotubes with chemically addressable peptide template for biomedical applications.

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The present work describes the preparation and application of functionalized carbon nanotubes (CNT) attached with biologically active peptide template. Multi walled carbon nanotubes (MWNT) is synthesized with stainless steel as a substrate without the use of any external catalyst by thermal chemical vapor deposition (thermal-CVD) method. The process is carried out in a cost effective way using a specially designed fabricated CVD furnace. A promising decapeptide template with free side-chain amino groups is synthesized on the PEGA support using solid phase peptide synthesis (SPPS). The sensitive biological element like cell receptors, enzymes, antibodies or nucleic acids can be attached to the side chains of this template. This deca peptide can be covalently attached to carbon nanotubes. The peptide-carbon nanotube scaffold synthesized can further be modified to detect signatures of cancer, disease-causing infectious agents and other pathological conditions.

**Keywords:** Carbon nanotubes, Multi walled carbon nanotubes biologically active peptide

Ever since its inception Carbon nanotubes (CNTs) have been the focus of scientific research due to their outstanding chemical, mechanical and electrical properties[1]. Carbon nanotubes based sensor applications involves the functional modification of surface with chemical moieties having specific recognition sites[2-4]. Major problems in this field are the viable method to synthesis less contaminated and uniform CNT tubes and finding a proper coupling strategy for immobilizing biological moieties on CNT surface. Covalent modification by the organic functionalization of end-groups and side walls of carbon nanotubes makes it ready for further modifications[5]. Multi walled carbon nanotubes (MWNT) is synthesized with stainless steel as a substrate without the use of any external catalyst by thermal chemical vapor deposition (thermal-CVD) method. The substrate is the stainless steel, a conducting substrate possessing high iron content. Techniques like plasma-enhanced chemical vapor deposition (PECVD)[6-8], thermal CVD[9-13], partial oxidation of methane[14], pyrolysis of iron phthalocyanine (FePc)[15], a flame method[16] and a liquid phase method[17] were employed to synthesize CNTs on this material. The majority of these methods require the SS substrate to be treated prior to CNT growth. It is important that in addition to the substrate treatment, these techniques require an additional catalyst to be added in order to grow CNTs on the SS surface. Here a simple procedure is followed to synthesize multi-walled nanotubes (MWNTs) directly on SS 304 by thermal CVD without any external addition of a catalyst precursor. The SS itself provides the active sites for CNT growth, enhancing in this way the CNT-substrate surface interaction efficiency. The process is carried out in a cost effective way using a specially designed fabricated CVD furnace. A promising decapeptide template synthesised on the PEGA support using solid phase peptide synthesis (SPPS)[18-20]. It consists of free side-chain amino groups which can be addressed by a variety of available chemical coupling methods[21]. Any molecule containing appropriate functionalities (peptides, oligonucleotides, carbohydrates, labels, and drugs) can be treated and attached to the template**.** The sensitive biological element like cell receptors, enzymes, antibodies or nucleic acids can be attached to the side chains of this template. This decapeptide can be covalently attached to carbon nanotubes. The peptide-carbon nanotube scaffold synthesized can further be modified to detect signatures

of cancer, disease-causing infectious agents and other pathological conditions[22]. Advantage of CNTs over other materials in biomedical sensor application is due to their small size, high strength, high electrical and thermal conductivity and high sensitivity[23-26].

An attempt to bring a confluence between peptide template and carbon nanotube (CNT) is done with a long term goal of designing an ultra-sensitive diagnostic tool from this peptide-CNT scaffold. Synthesis and purification of carbon nanotubes was also carried out to bring improvements in the reported synthetic strategy[27]. A cost effective and clean synthetic strategy is adopted. Binding of peptides to carbon nanotubes has done with various coupling strategies available and the process is optimized to get a stable conjugate. The potential of this type sensor molecule is yet to be explored by assessing the viability of the prepared peptide-CNT conjugate as a bio-sensor and/or as a tool to track cellular reactions[28-32].

## Experimental Section

### Solid phase peptide synthesis

HMBA (3 equiv.) was dissolved in DMF followed by addition of TBTU (2.85 equiv.) and NEM (6 equiv.). After stirring for 3 min, the solution added to the functionally modified PEG resin (1 equiv. $NH_2$) which had been swelled in DMF (1 h) in a specially designed peptide synthesizer. The reaction mixture was kept at room temperature for 4 h with gentle shaking. The resin was filtered, washed with DMF (6 vol.) and $CH_2Cl_2$ (6 vol.) and dried on a lyophilizer for 1 day. *N*-Methyl imidazole (MeIm) (2.35 equiv.) was added to a solution of *N*a-Fmoc amino acid (2.5 equiv.) in dry DCM under argon. The resulting solution was added to a flask containing1-mesityl-sulphonyl-3-nitro-1,2,4-trizole(MSNT) (2.5 equiv.). After 1 min the activated aminoacid derivative was added to the HMBA-resin swelled in $CH_2Cl_2$. After 30 min the resin was filtered and the coupling reaction was repeated. The product resin was washed with $CH_2Cl_2$(3 vol.), DMF (3 vol.), $CH_2Cl_2$ (3 vol.) and dried on a lyophilizer. The resin loading was quantified by measuring the UV-absorption of the benzfulvene:piperidine adduct formed by treatment of 20% piperidine:DMF and relating to a standard curve. In general, peptide synthesis was carried out using fully protected *N*a-Fmoc amino acid OPfp esters (3 equiv.) in DMF with the addition of Dhbt-OH (1 equiv.) as an acylation catalyst and an indicator of the end point of acylation reaction. Side chain protection of the Lys was done with atmost care to ensure the selective deprotection and withstand the washing cycles. Cleavage from the resin was carried out by treatment with 0.1 M NaOH for 2 h and the resin washed with water (7 vol.). Finally, the filtrate was neutralized (*p*H paper) with 0.1N HCl and the crude peptide was purified by preparative HPLC. After the synthesis the resin was treated for 2 h with 95% aqueous TFA, washed with $CH_2Cl_2$ and DMF, neutralized with 10% DIPEA: DMF washed with DMF and DCM and dried (Figs 1a and b, Table 1).

### Synthesis of carbon nanotubes

Substrate (Stainless steel) was cleaned by sonicating in acetone for 30 min. followed by itching in HCl for 10 min. and was placed directly in the recrystalised alumina tube (50 OD × 40ID × 750 long) housed in a resistive furnace. Substrate was heated at 850°C for 30 min. in nitrogen atmosphere (at a flow rate of 592 SCCM) followed by a flow of acetylene (carbon source) at a flow rate of 45 SCCM at 700°C for 5 min. A growth period of 30 min. was given at 700°C in N2 (592 SCCM) followed by cool down to room temperature (Fig. 2). Carbon nano tube deposited on the substrate was characterized with SEM and TEM.

### Functional modification of Carbon nanotubes

Carboxyl group can be introduced into the purified carbon nanotube support by refluxing it in 3 M $HNO_3$ overnight; during the process, carboxyl groups are formed at the ends of tubes as well as at the defects on the sidewalls. 1-ethyl-3(3-dimethyl amino propyl) carbodiimide (EDAC)(155 mg for 35 mg of CNT, sonicated 2 h) was then added to activate the carboxylic groups present in the oxidised CNT towards the nucleophilic attack on the free amino groups on the peptide template. This reaction through o-acylisourea intermediate results in the conjugation of peptide template with CNT through stable amide linkage under mild conditions (Fig. 3).

## Results and Discussion

Carbon nanotubes synthesized are characterized with scanning electron microscopy (SEM) and transmission electron microscopy (TEM). EDXA and raman spectra gives the quality of the CNT synthesised and also the structural stability after the functionalization. The peptide synthesised is analysed by HPLC and MALDI-TOF-MS. Complexation of peptide with CNT is done with 1-ethyl-3(3-dimethyl amino propyl) carbodiimide(EDAC). A novel peptide-CNT scaffold is resulted which offers the scope for further modification into a mutifunctional diagnostic tool working on biosensor principles.

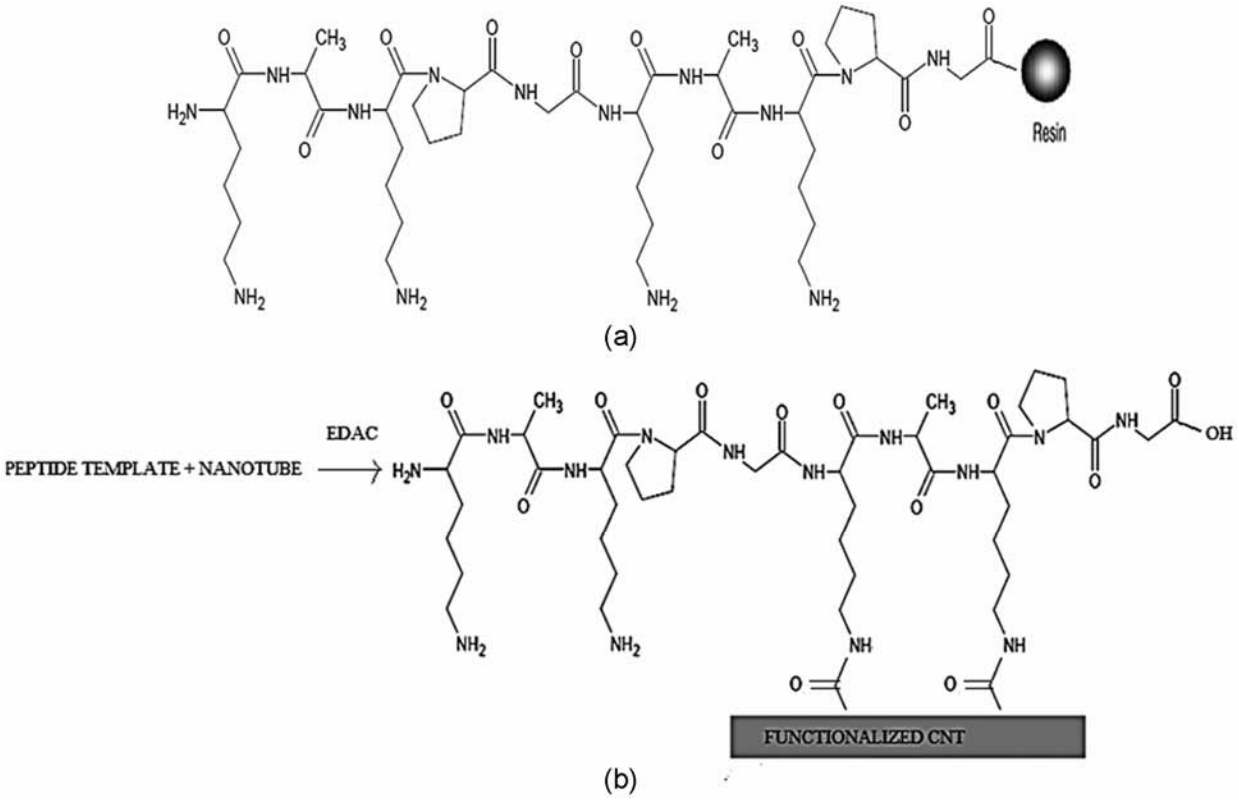



Fig. 1 — (a) Peptide template K-A-K-P-G-K-A-K-P-G (Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Pro-Gly-P); (b) attached on carbon nanotube.

Table 1 — Synthesis scheme for H2N-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Pro-Gly-COOH

| Residue | Coupling | | | Ninhydrin | Washing | Deprotection (15)×2 | Washing |
|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | | | | |
| Fmoc-Gly-OH | 45 | 45 | - | -ve | Done | Done | Done |
| Fmoc-Pro-OH | 30 | 30 | - | -ve | Done | Done | Done |
| Fmoc-Lys(Boc) | 30 | 35 | - | -ve | Done | Done | Done |
| Fmoc-Ala-OH | 30 | 30 | - | -ve | Done | Done | Done |
| Fmoc-Lys(Alloc)-OH | 30 | 40 | - | -ve | Done | Done | Done |
| Fmoc-Gly-OH | 30 | 30 | - | -ve | Done | Done | Done |
| Fmoc-Pro-OH | 30 | 35 | - | -ve | Done | Done | Done |
| Fmoc-Lys(Dde)-OH | 30 | 40 | 40 | -ve | Done | Done | Done |
| Fmoc-Ala-OH | 30 | 40 | 40 | -ve | Done | Done | Done |
| Fmoc-LYS(pNZ)-OH | | | | -ve | Done | - | - |

**Texture and elemental analysis by EDXA**

The relative abundance of various elements in the deposited sample was analyzed by energy-dispersive X-ray analysis (EDX) using OXFORD make. The change in crystallographic orientation and the preferential plane for the CNT was identified by X-ray diffraction (XRD) pattern (Fig. 4).

**SEM and TEM analysis**

Microstructure characterization of CNT matrix was done by transmission electron microscope (TEM) analysis using Tecnai G220 S-TWIN instrument at an

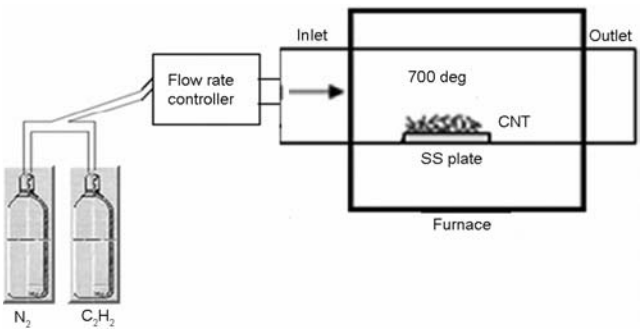



Fig. 2 — Experimental set up for synthesizing carbo nanotubes.

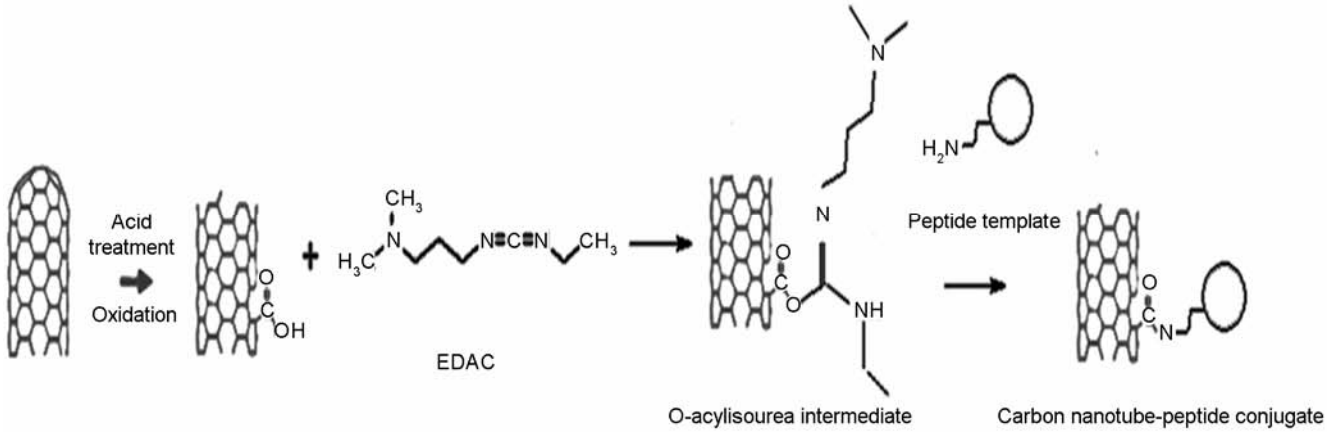
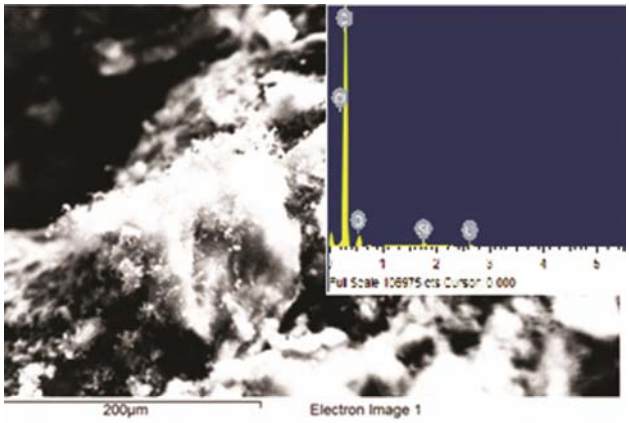



Fig. 3 — Schematic representation of peptide template conjugation with CNT.

accelerating voltage 200 kV. The solution was drop-casted on to the grid and was dried under vacuum. SEM analysis was performed using Hitachi model S3000-H and TESCAN model VEGA3TEM analysis of the CNT was performed to know the side wall functionalization and the modification in the tube morphology, (Fig. 5). TEM analysis shows that the outer walls of CNT were functionalized and there was no destruction to the tube morphology. Although there occur tube breakage due to agitation or sonication employed during bath preparation. The layers of CNT were not thinned, tube structure was not damaged and the agglomeration of CNT was controlled through functionalization.

**FT-IR and FT-Raman analysis**

FT-IR spectra of the samples were recorded using a Tensor FT-IR spectrophotometer from Bruker Optics. All the samples were recorded from 400 to 4000 $cm^{-1}$. The various functional groups were identified from the characteristic absorption frequency of the specific groups present in the functionalized MWCNT sample. Raman spectra of the sample was analyzed using RenishawInvia Raman microscope having He-Ne laser 633 nm as the source and the wave number in the range 100-3300 $cm^{-1}$. Noticeable peak in the range from 2800-2950 $cm^{-1}$ and 2850 $cm^{-1}$ corresponds to C-H stretching vibration. It implies to the stability of CNT suspension in the aqueous medium. The peak at around 3400 $cm^{-1}$ corresponds to -OH stretching. This peak can be assigned to the hydroxylic group of moisture, alcohol or carboxylic groups. From FT-IR analysis it is clear that funtionalization of CNT was effective. In Raman spectra, the peak at ~1480 $cm^{-1}$ show the disorder induced D band and that for the tangential G band the peak is at ~1670 $cm^{-1}$. Absence




| | Weight % | Atomic % | Compd % | Formula |
|---|---|---|---|---|
| C K | 27.26 | 33.31 | 99.88 | $CO_2$ |
| Si K | 0.05 | 0.03 | 0.10 | $SiO_2$ |
| Cl K | 0.02 | 0.01 | 0.00 | |
| O | 72.67 | 66.66 | | |
| Totals | 100.00 | | | |

Fig. 4 — EDX Analysis of CNT

of prominent radial breathing modes in the Raman spectra was noted for all scans. The ratio of G and D band is a good indicator of quality of CNT. In the present case the peak intensity of D and G band are comparable revealing that the structural defects within the CNT were less after functionalization.

**HPLC and mass spectra of peptide synthesized**

The HPLC system for peptide and biological samples (Make: M/s Shimadzu Corporation, Japan) RP-$C_{18}$ column diameter 150 mm × 2.6 mm, 25 cm

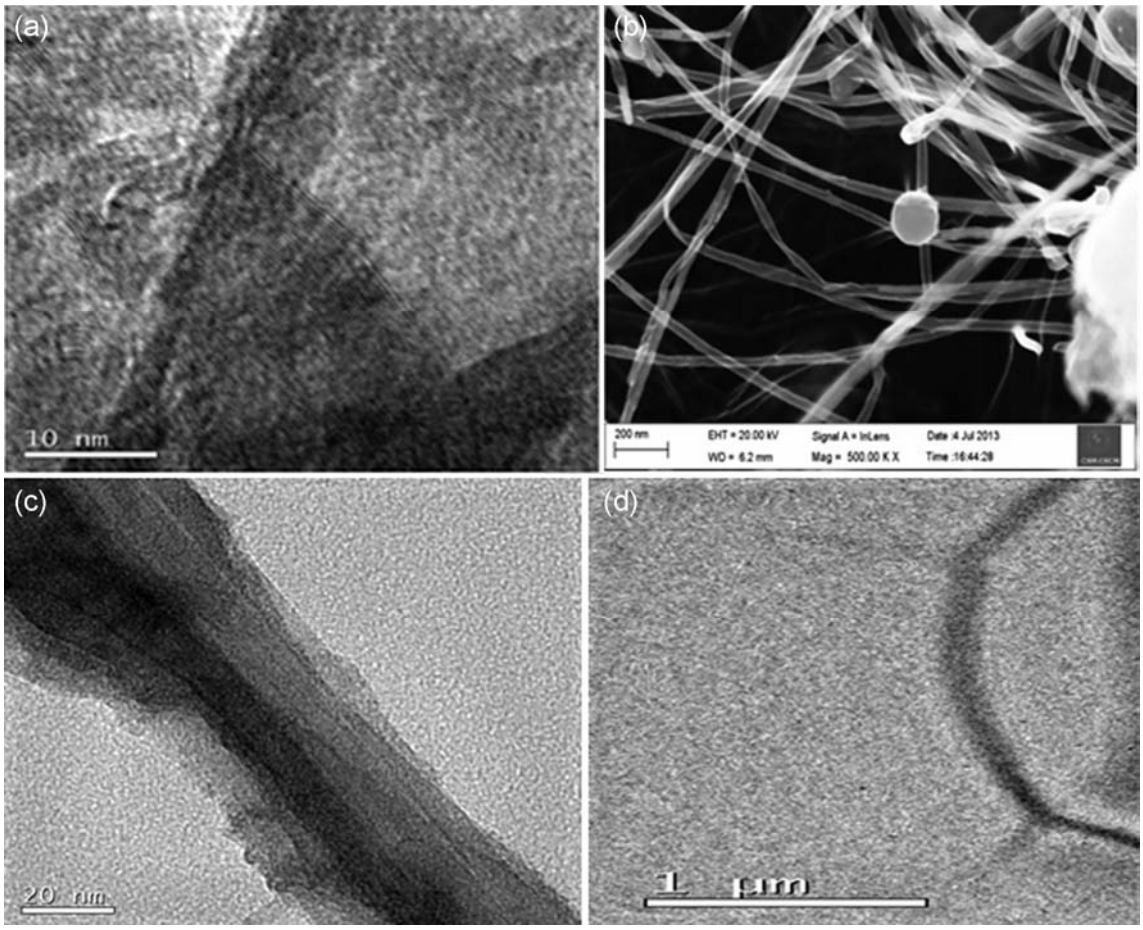



Fig. 5 — SEM (a) and TEM analysis of pristine CNT (b), (c) and CNT-Peptide conjugate (d).

length, 5 µm particle size, run time 30 min, injection volume : 20 µL, linear gradient 5% acetonitrile : 95% water at 0 min, 100% acetonitrile : 0% water at 30 min, flow rate 1 mL per min was used. The HPLC analysis report shows a sharp single peak (ret. time 7.34 min) which indicates the target peptide with >99% purity.

## Conclusion

A novel peptide CNT scaffold has been synthesized and the synthetic strategies of both peptide template and CNT have been done with improved general strategy. Selective deprotection is done during the synthesis of chemically addressable peptide template. Nucleophilic addition of carboxy functional group of oxidized CNT and amino group on the peptide is carried out in the presence of diimide. This work was done with a long term goal to synthesize a peptide - CNT conjugate with biomarker identifiers for fabricating a platform for developing ultra-sensitive diagnostic tool.

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