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FABRICATION OF FUNCTIONALLY ENGINEERED CARBON NANOTUBE MATRIX SOLID SUPPORT FOR PEPTIDE SYNTHESIS

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ABSTRACT

Solid Phase synthesis is a widely exploited area for the development of biologically important molecules. Usually polymer resins are used as solid support in which suitable linkers can be covalently attached. Herein, we report a novel carbon nano tube based three-dimensional network, which can be functionally engineered and can be utilized as more efficient solid support compared to polymer resins. The proposed matrix offer unique mechanical properties combined with chemical stability. The micro and nano sized pores imbibes even thick solvents easily and facilitate the reactions with the matrix easily and efficiently. This technique is more clean and green.

Key words: Functionalization of CNT, Carbon nanotubes, Peptides, SPPS, SEM, TEM.

INTRODUCTION

Synthesis of many nanoscopic carbon entities are reported after the discovery of Carbon nanotubes by Ijima[1] followed by the developments in synthetic strategies and defining properties by the research communities. CNTs have recently gained popularity as potential drug carriers, therapeutic agents and for applications in diagnosis[2]. Relying on their electric or optical properties, functionalized CNTs have been used for ultrasensitive detection of biological species. Peptide template bearing ligands and probes for bio markers[3] can then be combined with CNTs to produce biosensors[4] for cancer-related clinical testing.

In one aspect, the invention provides a process for generating a carbon nanostructure binding a peptide template[5], which provides selectively addressable sites. The histidine

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residues can be loaded with suitable ligands for making recognition sites. Carbon nanotube matrix has been developed based on the reported synthetic strategy of CNT sponges[6] and the obtained matrix is functionally engineered and modified to synthesis biologically important peptide molecules[7] using solid phase synthetic strategy[8]. This matrix shows the preferable characteristics of solid support in SPPS like porosity, hydrophobic nature, chemical stability and mechanical strength. The novelty of work lies in the fact that the usually used polymer resins solid support can be effectively substituted in most cases, especially in the designing of peptide templates-CNT conjugate for diagnostic purpose.

Here, in this work successfully synthesized carbon nanotube based matrix, purified and functionally modified. Its applicability as a solid support was tested by successfully synthesizing a Model peptide[9]. Yield and purity was checked with HPLC and compared with that prepared in the polymer resin support followed by synthesis of the following peptide template on the matrix.

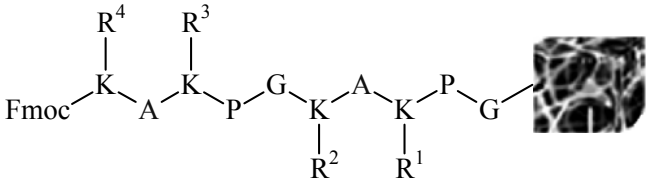


Fig. 1: Schematic representation of linear peptide template (K-A-K-P-G-K-A-K-P-G) synthesized on 3D-CNT network. Fmoc = 9-fluorenylmethoxycarbonyl R^1–R^2 are the side-chain protecting groups

EXPERIMENTAL

Instrumentation and chemicals

Furnace for synthesis of CNT matrix was well designed and fabricated. Thermal Mass Flow Controllers (FM1-V01-FAA-22-V-S) were purchased from Bronkhorst Hitec B.V., Netherlands. Ferrocene (powder), 1,2-dichloro benzene, Quartz plate (2-inch) were purchased from Alfa-easer England. Ferrocene powders were dissolved in 1, 2-dichloro benzene (0.06 g/mL).

Synthesis of CNT matrix

Solution of ferrocene with dichloro benzene was continuously injected (0.13 mL/min, using syringe pump) into quartz plate placed in a silca tube houed inside the CVD Furnace[10].

The reaction temperature was kept at 860°C. Ar and H_2 was passed at a flowing rate of 2000 mL min^{-1} and 300 mL min^{-1}, respectively. After 5 hours, sponge like CNT matrix was collected from quartz plate.

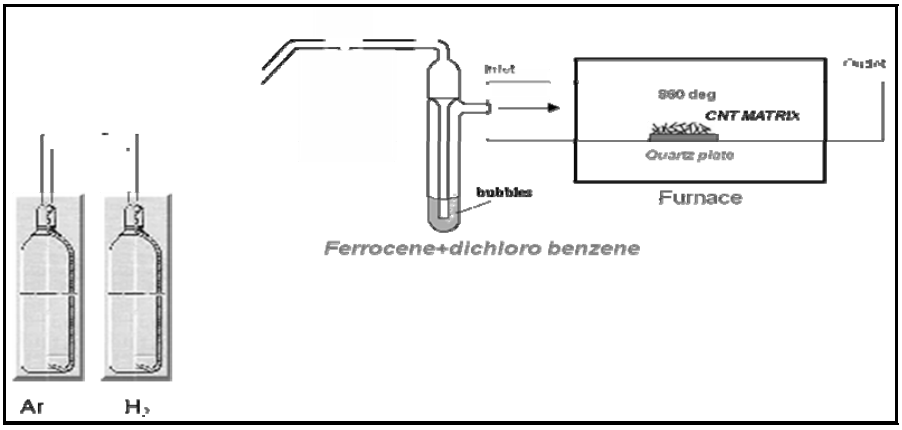


Fig. 2: Experimental set up for synthesis of carbon nanotube matrix synthesis of peptides on CNT network

Chemicals

Fmoc-Lys(Dde)-OH, Fmoc-Lys(Boc)-OH Fmoc-Gly-OH, Fmoc-Ala-OH and Fmoc-Proline-OH where purchased from Aldrich, Switzerland, all above amino acids were of the highest purity available and tested. DIC, DIPEA, HoBt, TIS, TFA, m-crosal, aceticanhydride, piperidine, pyridine, ninhydrin and dichlorodimethylsilane were purchased from Sigma-Aldrich China, Alfa-Asser England and Novabiochem Germany. NMP, DMF, DCM, diethyl ether, methanol and acetonitrile HPLC grade where purchased from E.Merk (India) and Lobachemi Mumbai. Solvents were purified by standard procedures.

Functionalisation of CNT

Carboxyl group was introduced into the purified carbon nanotube support by refluxing it in 60 mL of 3 M HNO_3 overnight[11,12], mild oxidation takes place on the side-wall of CNT. The resulting mixture was cooled to room temperature, diluted with water and filtered through polycarbonate membrane (0.2 μm). The oxidized CNT sponge thus produced was sonicated with ethylene diamine and DCC for 1 hour. Washed with methanol (3 x) and dried in vacuum. HMBA (3 equiv) linker was attached to this matrix by HOBt (6 equiv) coupling.

Synthesis of tetrapeptide L-leucyl-L-alanyl glycl-L-valine on CNT network

The model peptide was prepared by stepwise solid-phase synthesis (SPPS)[13] by using 9-fluorenylmethoxycarbonyl (Fmoc)/tBu protection strategy on both CNT network and 4-alkoxy benzyl alcohol resin that was preswollen with CH_2Cl_2 for 30 min. Standard amide couplings were performed by dissolving the Fmoc-protected amino acid (2.5 equiv) with equimolar amounts of HOBt and DIC in NMP and stirring for 5 min. The preactivated mixture was added to the resin that was swollen in CH_2Cl_2, together with a 0.1 molar (to aminoacid) amount of DIEA. Coupling times were in the range of 30–90 min and the completeness of coupling was verified by the Kaiser test and coupling was repeated if necessary. 20% solution of piperidine in DMF (2N10 min) was used for the removal of Fmoc group.

Peptide cleavage

The peptide resin was treated with a solution of trifluroracetic acid (TFA)/ triisopropylsaline (TIS)/H_2O/3,6-dioxaoctane-1,8-dithiol (DODT; 95 : 2 : 2 : 1) for 1 hr. The peptide solution was filtered, and TFA was removed using rotavapor. Cold ether was added to the solution to precipitate the peptide. The peptide was collected by filtration and washed repeatedly with cold diethyl ether. The same procedure was used for the cleavage of peptide from CNT network. After cleavage from the resin and support peptide1 (obtained from resin) and peptide 2(obtained from CNT network) were analyzed using HPLC and MALDI-TOF MS.

Synthesis of linear peptide template (K-A-K-P-G-K-A-K-P-G)

The peptide KKKPGKAKPG was built on the matrix by the same Fmoc/tBu protection strategy[14] used for the synthesis of model peptide. Since this synthesis is meant for developing a platform for CNT based biosensor development, no cleavage is done from the CNT network. Integrity of the material is tested with FT-IR and TEM.

RESULTS AND DISCUSSION

This work was mainly intended to design a peptide template on CNT but as the work progressed, we could successfully synthesis the desired peptide on the CNT matrix itself thereby it was able to skip the steps like cleavage of the peptide from the resin and again attaching the same on CNT.

The sponge-like CNT network was collected from the quartz substrate after CVD. During the growth, CNTs stack as multilayer in random manner to form 3D-CNT network of

1-2 mm thickness. SEM and TEM images of the CNT material revealed a network of nanotubes with diameters in the range 30–50 nm and lengths of tens to hundreds of micrometers.

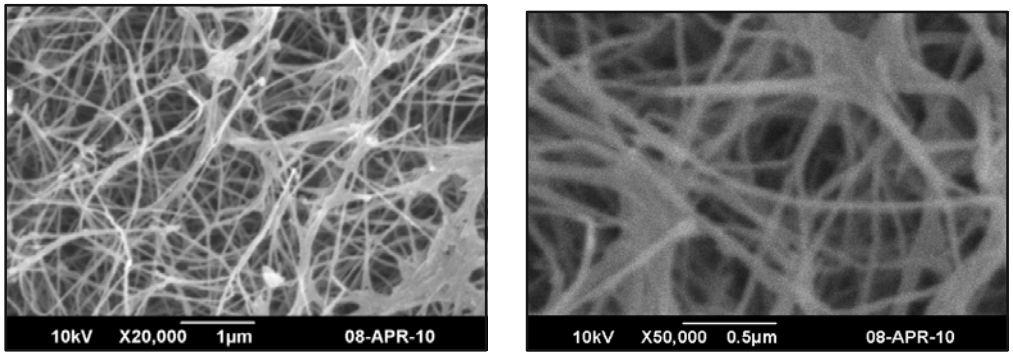


Fig. 3: Scanning electron microscope (SEM) images of CNT network

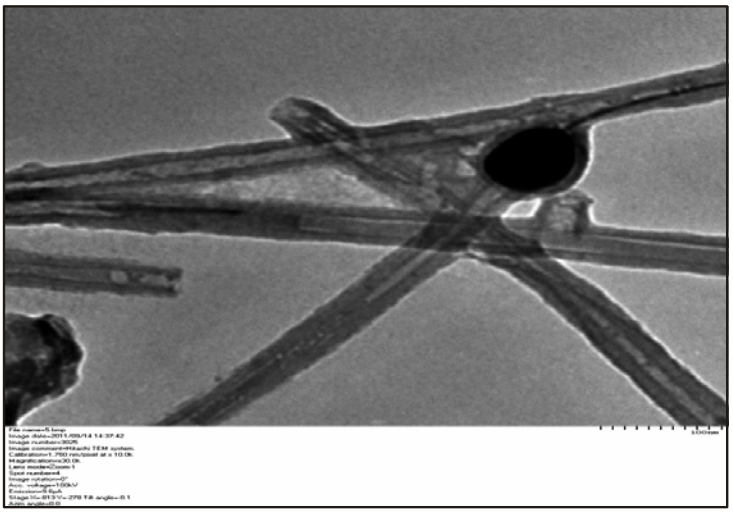


Fig.4: Transmission electron microscope (TEM) images of CNT network

Nitric acid treatment of CNT network resulted in the oxidization of the side walls[15] of the tube into carboxylic acids. Amino functionalisation was carried out as is evident from FT-IR. followed by the attachment of the linker.

The CNT network showed high absorption capacities[16] (defined by Q, the ratio between the final and initial weight after full absorption) of 80 to 180 times their own weight for a wide range of solvents and oils, with larger Q for higher-density liquids (e.g., chloroform).

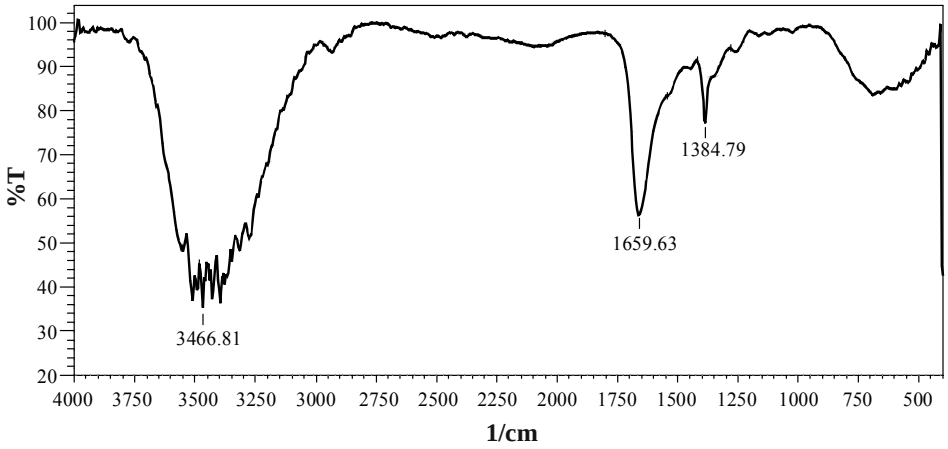


Fig. 5: FT-IR spectra of amino functionalized CNT network

Table 1: Test for Q value of pristine CNT sponge

Solvent taken = 1 mL, Residence time = 20 min

Solvent	Density g/cm^3	Initial wt. g	Final wt. g	Q value
Ethanol	0.79	0.0005	0.0678	135.6
Toluene	0.864	0.0017	0.0751	44.18
Ethylacetate	0.899	0.0129	0.1907	14.78
Water		0.0075	0.1578	21.04
DMF	0.984	0.0610	0.0964	1.58

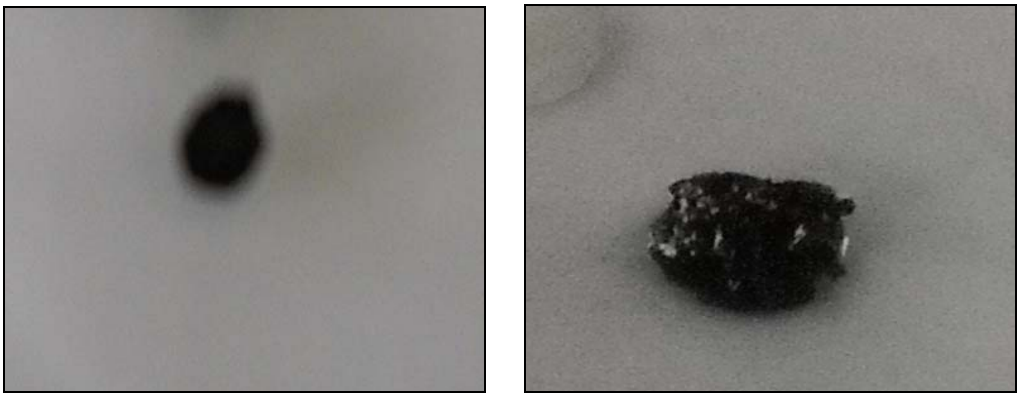


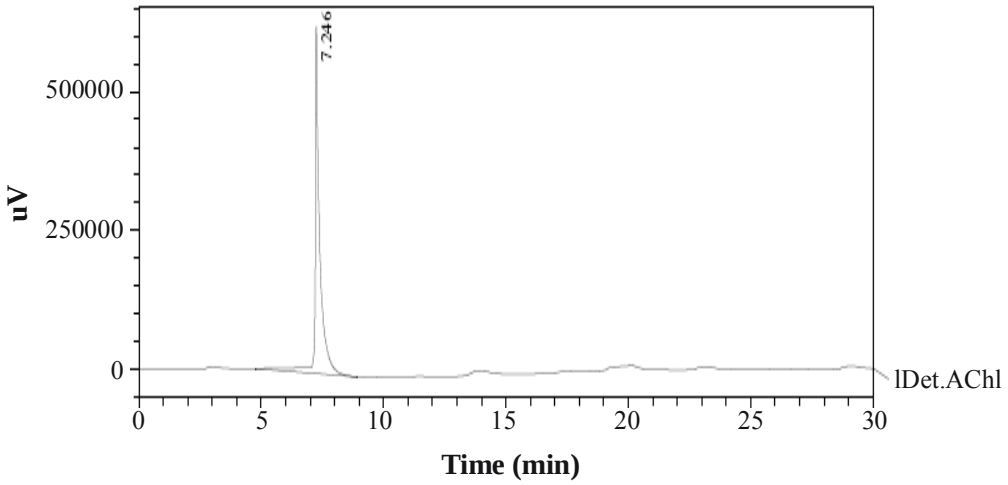
Fig. 6: Photograph of CNT matrix before and after treatment with solvents

This capacity of CNT matrix to absorb solvents facilitates the efficient peptide synthesis compared with polymer resin based solid support. Porosity measurements were carried out by N_2 absorptive analysis.

Table 2: Absorption data of CNT network with $N_{2\ as}$ analysis absorptive

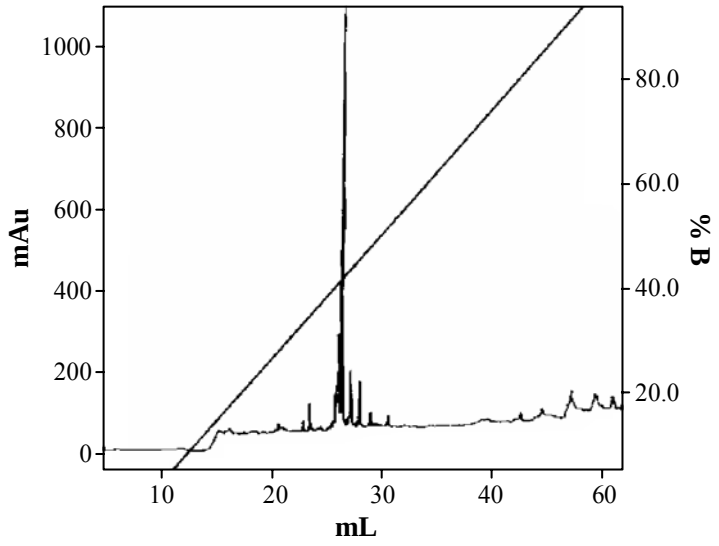
Surface area		
Single point surface area at $p/p^o = 0.316146709$	:	12.1825 m^2/g
BET Surface area	:	8.9131 m^2/g
Langmuir surface area	:	11.7687 m^2/g
t-Plot micropore area	:	91.3529 m^2/g
t-Plot external surface area	:	-82.4398 m^2/g
BJH Adsorption cumulative surface area of pores between 17.000 Å and 3000.000 Å width	:	14.0642 m^2/g
BJH Desorption cumulative surface area of pores between 17.000 Å and 3000.000 Å width	:	6.4124 m^2/g
Pore volume		
Single point adsorption total pore volume of pores less than 1522.300 Å width at $p/p^o = 0.987244411$	:	0.052433 cm^3/g
t-Plot micropore volume	:	0.048641 cm^3/g
BJH Adsorption cumulative volume of pores between 17.000 Å and 3000.000 Å width	:	0.052436 cm^3/g
BJH Desorption cumulative volume of pores between 17.000 Å and 3000.000 Å width	:	0.050249 cm^3/g
Pore size		
Adsorption average pore width (4V/A by BET)	:	235.3075 Å
BJH Adsorption average pore width (4V/A)	:	149.133 Å
BJH Desorption average pore width (4V/A)	:	313.447 Å

Synthesis of model peptide gave good yield and purity compared to that prepared in conventional solid support. MS: m/z calcd for: 268; found: 260.



Peak #	Ret. time	Area	Height	Area %	Height %
1	7.246	8271888	626757	100.000	100.000
Total		8271888	626757	100.000	100.000

(a)



(b)

Fig. 7: HPLC of peptide synthesized on (a) CNT network (b) PS-DVB resin

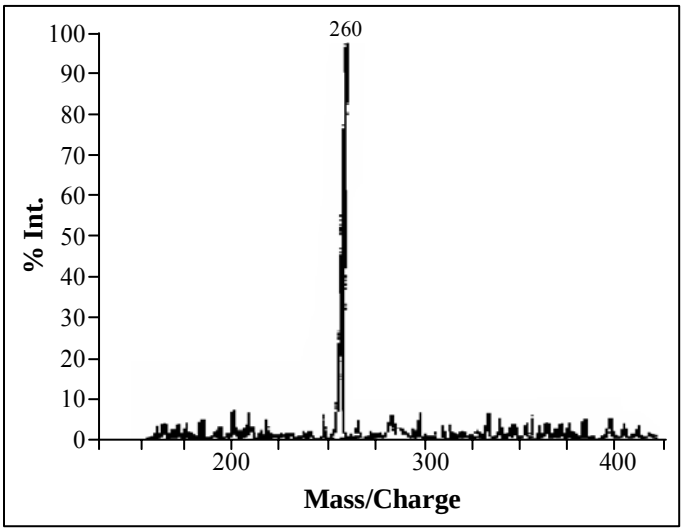


Fig. 8: MALDI TOF-MS of L-leucyl-L-alanyl glycl-L-valine

Peptide sequence K-A-K-P-G-K-A-K-P-G was synthesized successfully on CNT network and no cleavage was done. The free side-chain amino groups on the template molecules can be addressed by a variety of available chemical coupling methods. 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.

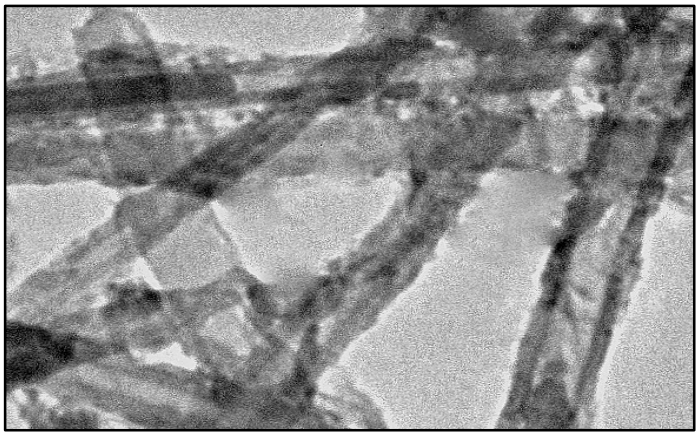


Fig. 9: Transmission electron microscope (TEM) images of CNT network attached with Peptide template

CONCLUSION

The pristine CNT network is hydrophobic, have optimum surface area and porosity, which help more solvation and this help to mimic liquid phase reaction[17]. Its nano-sized pores can soak up heavy, sticky oils or fluids without difficulty. Like the polymer resin beads, this newly proposed matrix could also be functionalized with suitable linkers. CNT matrix satisfies all the properties that are required for a solid support for peptide synthesis. Most importantly, the light-weight, high porosity, and large surface area, high absorption capacities and optimum chemical resistance[18] of CNT-based materials make them a promising candidate as a solid support for peptide synthesis. Synthesis of model peptide gave good yield and purity.

The peptide-carbon nanotube scaffold synthesized can further be modified to detect signatures of cancer, disease-causing infectious agents and other pathological conditions. 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. The attached template provides a multiple site which is a promising step towards the development of ultra sensitive diagnostic tool for biomedical application[12].

Abbreviations

CNT - Carbon nano tube

CVD- Chemical vapor deposition

HMBA- Hydroxy methyl benzoic acid

HOBt -1-Hydroxybenzotriazole.

HPLC- High performance liquid chromatography

MALDI TOF MS- Matrix Assisted Laser Desorption/ionization- time of flight-Mass spectroscopy.

SEM-Scanning electron microscope

TEM-Transmission electron microscope.

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