Influence of pristine SWNT in supramolecular hydrogelation: scaffold for superior peroxidase activity of cytochrome c

Tanmoy Kar, Subhra Kanti Mandal and Prasanta Kumar Das*

Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata-700032, India. E-mail: bcpkd@iacs.res.in

Electronic Supplementary Information (ESI)

Materials: Cytochrome c (oxidized), all amino acids, palmitic acid, dicyclohexylcarbodiimide (DCC), 4-*N*,*N*-(dimethyl)aminopyridine (DMAP), 1-hydroxybenzotriazole (HOBT) and all solvents were purchased from SRL India. 1,1´-carbonyl diimidazole (CDI), thionyl chloride, sodium hydroxide were purchased from Spectrochem, India. Pyrogallol was obtained from Qualigens Fine chemical Company, India. Hydrogen peroxide (30%, w/v solution) was purchased from Ranbaxy, India. Single walled carbon nanotubes (SWNT, 1-2 nm diameter) and all deuteriated solvents for NMR experiments were obtained from Aldrich Chemical Co. Milli-Q Water was used throughout the study. Thin layer chromatography was performed on Merck precoated silica gel 60-F_{254} plates. ^{1}H NMR spectra were recorded in AVANCE 300 MHz (Bruker) spectrometer. Mass spectrometric data were acquired by electron spray ionization (ESI) technique on a Q-tof-micro quadruple mass spectrometer (Micromass). Elemental analyses were performed on Perkin Elmer 2400 CHN analyzer. Probe sonication was done using Omni Sonic Ruptor 250. Bath sonication was performed with a Telsonic Ultrasonics bath sonicator. Sorvall RC 6 was used for centrifugation respectively.

Synthetic procedure: All dipeptide amphiphiles were synthesized following the reaction conditions as reported previously.[1] Briefly, methyl ester of protected L-amino acid was

coupled with acid chloride of palmitic acid in dry chloroform and dry pyridine. The ester protected long chain amide was then purified through column chromatography using 60-120 mesh silica gel and ethyl acetate/hexane as eluent. The product was hydrolyzed using 1N NaOH (1.1 equivalent) in MeOH with stirring at room temperature. Solvents were evaporated on a rotary evaporator, and the mixture was diluted with water and then washed with diethylether, followed by acidification by 1N HCl to get the corresponding carboxylic acid. This carboxylic acid was then coupled with another methyl ester protected L-amino acid using DCC (1.1 equivalent), HOBT (1.1 equivalent), DMAP (1.1 equivalent) in dry dichloromethane (DCM). The purified product was obtained by column chromatography using 60-120 mesh silica gel and ethyl acetate/toluene as eluent. The product was then subjected to hydrolysis by 1N NaOH (1.1 equivalent) in MeOH for 6h with stirring at 45-50 $^{\circ}$C to obtain amphiphilic dipeptides with free carboxylic acid end. The dipeptide carboxylic acid was then purified through column chromatography using 100-200 mesh silica gel and methanol/ chloroform as eluent. The carboxylic acids were converted to corresponding sodium salts by adding 1 equivalent 1N NaOH (standardized) to the methanolic solution of the acid. After brief stirring, the solvent was removed and dried under vacuum to get the sodium salt. The formation of sodium salt was confirmed from FTIR spectroscopy by the disappearance of the –C=O stretching peak of carboxylic acid ~ 1720-1728 cm^{-1} and also from the improved water solubility of the resultant compound.

Preparation of soft nanocomposites: 1 mg of pristine SWNT was precisely weighed in a screw cap glass vial with internal diameter of 10 mm. To it 1 mL of aqueous solution of amphiphile having required concentration was added and tip sonicated for 10 min at 30% power output. The solution was then kept undisturbed for 30 min. Now the composite material with pristine SWNT concentration 0.1% w/v formed nanohybrid gel which was stable to inversion of glass vials. The gel was then properly diluted with the required

concentration of gelator solution to get nanocomposites with different SWNT and gelator concentrations.

Determination of gel-to-sol transition temperature (T_{gel}): The gel-to-sol transition temperature (T_{gel}) was determined by placing the native hydrogel or hybrid gel containing glass vial (i.d. 10 mm) in thermostatted oil bath and slowly the temperature was raised at a rate of 2 °C/min. The T_{gel} was defined as the temperature (± 0.5 °C) at which the gel melted and started to flow.

Quantification of the SWNT percent dispersions for the amphiphilic dipeptide carboxylates: 1mg of pristine SWNT was added to the aqueous solution (4 mL) of respective amphiphiles (2.5 mg/mL). This aqueous suspension was then tip sonicated for 10 min (at 40% power output) followed by bath sonication for 2h and tip sonication again for 10 min to prepare the aqueous dispersion of SWNT. After that the dispersion was centrifuged at 2300 g for 90 min. The amount of the dispersed SWNT in the supernatant was calculated from the observed absorbance value at 550 nm that was fitted in the standard calibration plot prepared using SDBS.[2] The experimental errors were in the range of 3-5 % in triplicate experiments.

UV-vis-NIR study: The nanohybrid in water obtained after the sonication was used for UV-vis-NIR experiment. In this case, a background correction was performed with the aqueous solution of the respective amphiphiles and the spectra were recorded using Varian Cary 5000 spectrophotometer. The hydrated supramolecular gel was diluted (10 times) to record the UV-vis-NIR absorption spectra.

Zeta (ζ) potential measurements: Zeta potential was measured in zetasizer Nano-ZS instrument of Malvern instrument limited using a He-Ne laser (λ = 633 nm). The hybrid gel was diluted (10 times) to measure Zeta (ζ) potential.

FTIR measurements: FTIR measurements of the xerogels were carried out in a Perkin Elmer Spectrum 100 FT-IR Spectrometer using KBr pellets.

FESEM study: A drop of native hydrogel and nanocomposites was placed on a piece of cover slip and desiccated for few hours under vacuum before imaging. Then it was layered with platinum vapour to make them conducting and analyzed on a JEOL-6700F microscope.

TEM study: Dilute solutions of hydrogel and nanohybrids were placed on a 300 mesh carbon coated Cu grid and dried for ~4h hours under vacuum before imaging. The TEM experiments were performed on a JEOL JEM 2010 high resolution microscope operating at 200 kV.

Rheology: The rheological experiments were carried out in cone and plate geometry (diameter 40 mm) on the rheometer plate using an Advanced Rheometer AR 2000 (TA Instruments, USA). The native hydrogel **3** and the CNT-gel composite of gelator **3** were scooped on the rheometer plate so that there was no air gap with the cone. Frequency sweep experiment was performed as a function of angular frequency at fixed strain of 0.01% at 25 ºC and the storage modulus ($G^{/}$) and the loss modulus ($G^{//}$) were plotted against angular frequency (ω).

Immobilization of the protein in hydrogel matrix: 50 μL of gelator solution (**3** and **4**) of 1 % w/v were taken in a 100 mL round bottom flask. Then, 25 μL of protein (cytochrome c) from stock solution (10 mg mL^{-1}) prepared using Milli-Q water was added to this solution to achieve the required amount of protein in the immobilization matrix. The protein in the gel matrix was then aged for ~30 min after which the activity was monitored. For SWNT-gel composite 50 μL of gelator solution (**3** and **4**) of 1% w/v containing 0.05% w/v SWNT were taken and the immobilization of the protein in the composite matrix was done following the same procedure as mentioned earlier.

Activity measurement of protein in water: The activity of the cyt c in water was monitored spectrophotometrically with pyrogallol and H_2O_2 as substrate. For protein activity in water, 7.5 μL of the protein solution (10 mg mL^{-1} in Milli Q water) and 4.5 μL of pyrogallol (3.3 M stock in acetone) were added to 1.5 mL of Milli Q water in a quartz cell. Lastly, required

amount of H_2O_2 (from stock solution of 8.82 M) was added to initiate the reaction. Overall protein concentration in the reaction mixture was 4 μM. The absorbance change was monitored instantaneously after addition of H_2O_2. The progress of the reaction was monitored by formation of purpurogallin, the oxidized product of pyrogallol, at λ_{max} = 420 nm for the initial 1-2 min ($\varepsilon_{420\ nm}$ is 2640 $M^{-1}cm^{-1}$ in water).[3] It was found that the rate of peroxidase reaction of cyt c depends linearly on the concentration of H_2O_2. The oxidation rate of pyrogallol follows the bimolecular rate law. [4]

$$v = k_{obs}\,[\text{cytochrome c}]\,[H_2O_2]$$

Activity measurement of immobilized protein in organic solvents: To the desired amount of protein entrapped hydrogel, organic solvent (50 mL toluene) was added and stirred for ~ 3 min. During this period of time the hydrogel matrix breaks down into small particles. To this stirred solution 100 μL of pyrogallol (concentration of stock 5 M in acetone) was added. Afterwards, varying amount of H_2O_2 solution (concentration of stock 8.82 M) was added in this stirred solution of organic solvent to initiate the reaction. The overall concentration of protein (cyt c) in the reaction mixture was 0.4 μM. The final concentration of the pyrogallol was 10 mM. Aliquots were taken from the upper half of the reaction solvent (the hydrogel particles prefer to localize at the bottom of the reaction vessel) to monitor the absorbance of the product. The increase of absorbance at 420 nm (production of purpurogallin) was measured at definite intervals. The concentration of purpurogallin was measured from the molar extinction coefficient at 420 nm ($\varepsilon_{420\ nm}$ is 4400 $M^{-1}cm^{-1}$ in toluene).[3] The initial rates of the formation of the product in the first one min was used to determine the activity of the immobilized protein. To check that the small hydrogel particles do not interfere through scattering, a control reaction in the exact similar way in the absence of the protein was performed. There was no change of the absorbance at 420 nm. In this context, no transfer of the product in the organic phase was observed in the control experiment in the organic

solvent at similar concentrations of substrates/protein in the absence of the gelators. Here also the rate of reaction of cyt c depends linearly on the concentration of H_2O_2. The oxidation rate of pyrogallol follows the bimolecular rate law.[4]

$$v = k_{obs}\,[\text{cytochrome c}]\,[H_2O_2]$$

Characterization of amphiphilic gelators.

^{1}H NMR of (Acid form of **1**, 500 MHz, rt, $CDCl_3$): δ = 7.04-7.03 [d, 1H], 6.64-6.51 [d, 1H], 4.61-4.51 [m, 1H], 4.42-4.36 [m, 1H], 2.35-2.19 [m, 3H], 2.05-2.00 [m, 2H], 1.63-1.59 [m, 2H], 1.35-1.15 [m, 26H], 1.00-0.86 [m, 12H]; E.A: calculated for $C_{26}H_{50}N_2O_4$: C, 68.68; H, 11.08; N, 6.16. Found: 68.62; 11.01; 6.11; ESI-MS: *m/z:* 477.1625 ($M^+ = C_{26}H_{50}N_2O_4Na^+$), *m/z* (calculated): 476.6680 ($M^+ = C_{26}H_{50}N_2O_4Na^+$).

^{1}H NMR of (Acid form of **2**, 500 MHz, rt, $CDCl_3$): δ = 7.04-7.03 [d, 1H], 6.56-6.54 [d, 1H], 4.58-4.57 [m, 1H], 4.42-4.38 [m, 1H], 2.24-2.21 [m, 2H], 2.02-2.01 [m, 1H], 1.71-1.69 [m, 1H], 1.61-1.60 [m, 1H], 1.59-1.57 [m, 2H], 1.38-1.21 [m, 26H], 0.95-0.86 [m, 12H]; E.A: calculated for $C_{26}H_{50}N_2O_4$: C, 68.68; H, 11.08; N, 6.16. Found: 68.58; 11.15; 6.23; ESI-MS: *m/z:* 477.5423 ($M^+ = C_{26}H_{50}N_2O_4Na^+$), *m/z* (calculated): 476.6680 ($M^+ = C_{26}H_{50}N_2O_4Na^+$).

^{1}H NMR of (Acid form of **3**, 500 MHz, rt, $CDCl_3$): δ = 7.38 [br, 1H], 6.63 [br, 1H], 4.66 [br, 1H], 4.39 [br, 1H], 2.52 [br, 2H], 2.24-2.17 [m, 3H], 2.08-2.04 [m, 5H], 1.62-1.60 [m, 2H], 1.28-1.24 [m, 24H], 0.93-0.86 [m, 9H]; E.A: calculated for $C_{26}H_{50}N_2O_4S$: C, 64.16; H, 10.35; N, 5.76. Found: 64.25; 10.29; 5.79; ESI-MS: *m/z:* 509.3852 ($M^+ = C_{26}H_{50}N_2O_4SNa^+$), *m/z* (calculated): 509.3344 ($M^+ = C_{26}H_{50}N_2O_4SNa^+$).

^{1}H NMR of (Acid form of **4**, 500 MHz, rt, $CDCl_3$): δ = 7.33-7.32 [d, 1H], 6.56-6.54 [d, 1H], 4.74-4.67 [m, 2H], 2.58-2.53 [m, 4H], 2.24-2.21 [m, 3H], 2.11-2.06 [m, 8H], 2.05-1.96 [m, 1H], 1.63-1.61 [m, 2H], 1.28-1.24 [m, 24H], 0.89-0.86 [m, 3H]; E.A: calculated for $C_{26}H_{50}N_2O_4S_2$: C, 60.19; H, 9.71; N, 5.40. Found: 60.10; 9.79; 5.33; ESI-MS: *m/z:* 541.4296 ($M^+ = C_{26}H_{50}N_2O_4S_2Na^+$), *m/z* (calculated): 541.3065 ($M^+ = C_{26}H_{50}N_2O_4S_2Na^+$).

Table S1. Minimum gelation concentration (MGC, % w/v) of **1-4** in water.

Amphiphile	MGC (% w/v)
1	VL
2	0.7
3	0.8
4	1.0

VL = Viscous Liquid

Table S2. Minimum gelation concentration (MGC, % w/v) of gelator (**2-4**) in water at different SWNT (% w/v).

Pristine-SWNT (% w/v)	Amphiphile (MGC, % w/v)		
	2	**3**	**4**
0.0	0.7	0.8	1.0
0.01	WG	0.4	0.6
0.05	VL	0.4	0.6
0.1	VL	0.8	1.0

WG = Weak gel; VL = Viscous Liquid

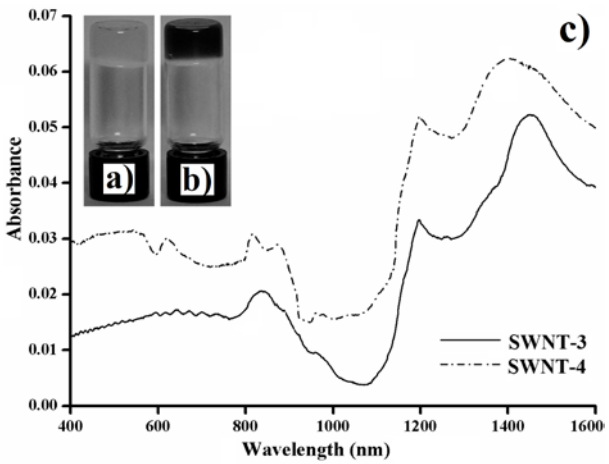

Fig. S1 Photograph of a) hydrogel **3**, b) SWNT-**3** hydrogel composite c) UV-vis-NIR spectra of SWNT-**3** and SWNT-**4** in water.

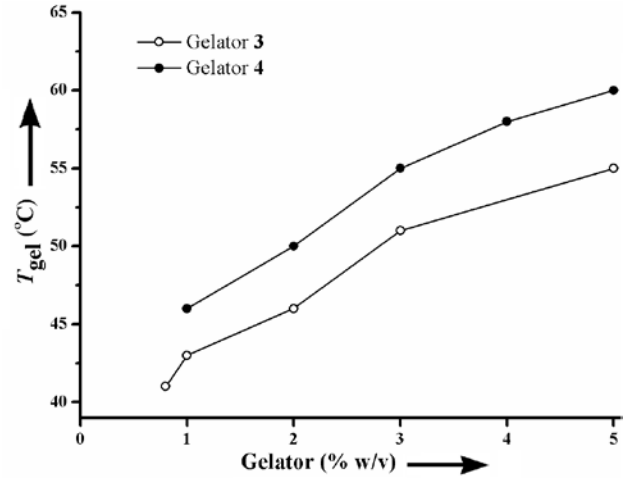

Fig. S2 Variation of gel-to-sol transition temperature (T_{gel}) with gelator concentration.

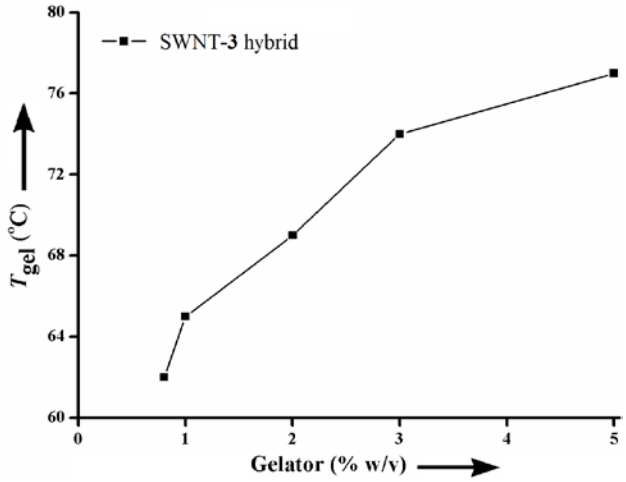

Fig. S3 Variation of gel-to-sol transition temperature (T_{gel}) with gelator concentration at fixed SWNT (0.1% w/v).

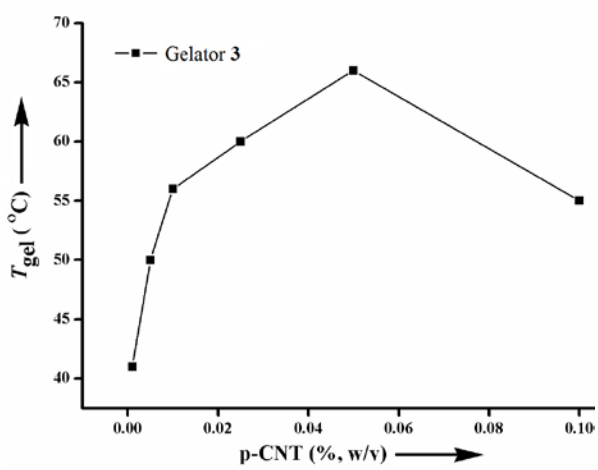

Fig. S4 Variation of gel-to-sol transition temperature (T_{gel}) with SWNT concentration at fixed gelator (**3**) concentration (0.5% w/v).

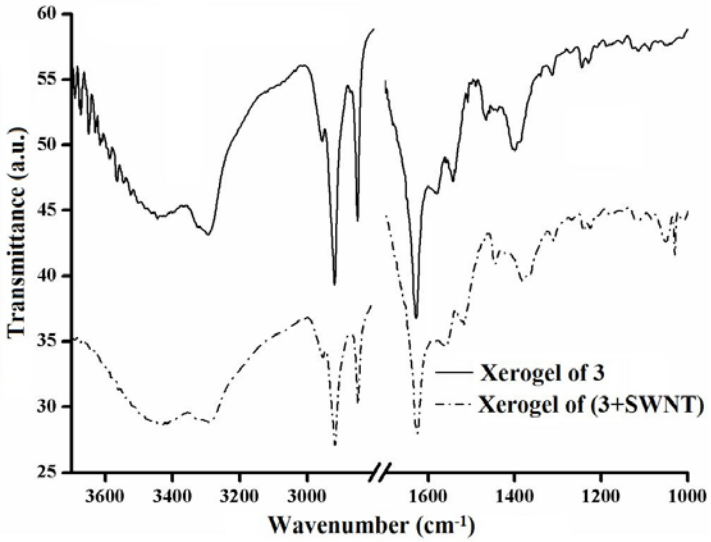

Fig. S5 FTIR spectra of xerogels **3** and SWNT-**3** gel composite.

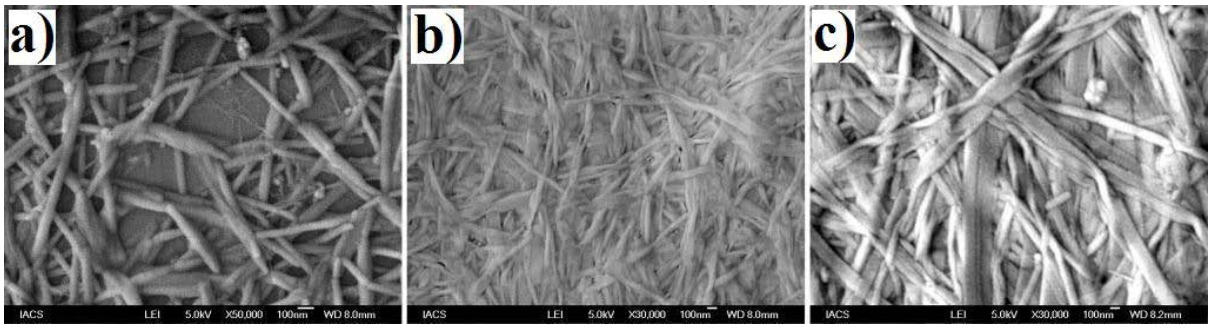

Fig. S6 FESEM images of a) magnified image of SWNT-**3** composite b) hydrogel **4** and c) SWNT-**4** hydrogel composite.

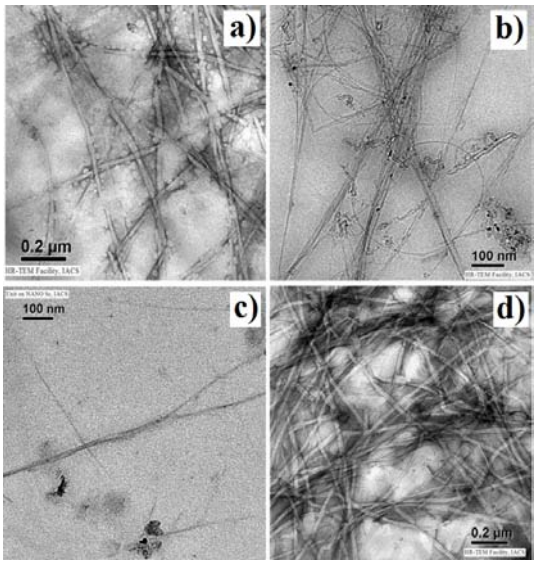

Fig. S7 TEM images of a) hydrogel **3**, b) pristine-SWNT, c) SWNT dispersion with **3** d) SWNT-**3** hydrogel composite.

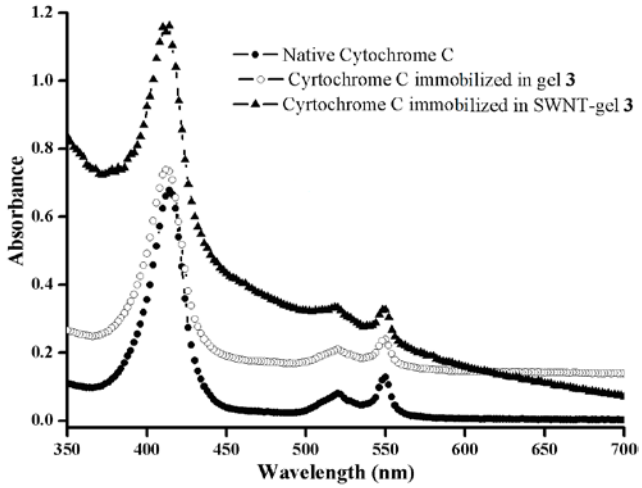

Fig. S8 Absorbance spectra of cyt c in water, in immobilized state within hydrogel of **3** and SWNT-**3** hybrid gel.

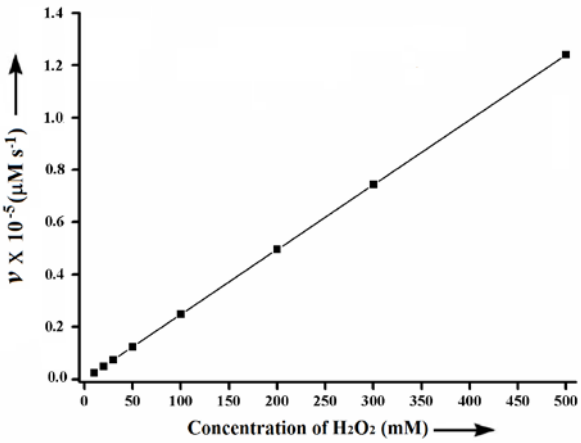

Fig. S9 Plot of product formation rate catalyzed by cytochrome c with varying H_2O_2 concentration in water at 25 °C. Concentration of the cyt c = 4 μM and pyrogallol = 10 mM.

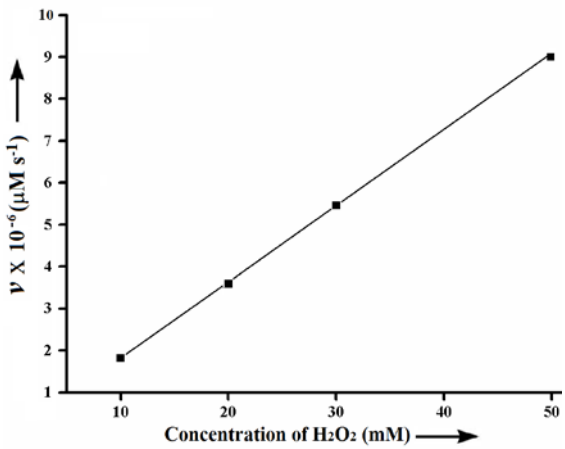

Fig. S10 Plot of product formation rate catalyzed by cytochrome c immobilized in toluene at 1% w/v of gelator **3** with varying H_2O_2 concentration at 25 °C. Concentration of the cyt c = 0.4 μM and pyrogallol = 10 mM.

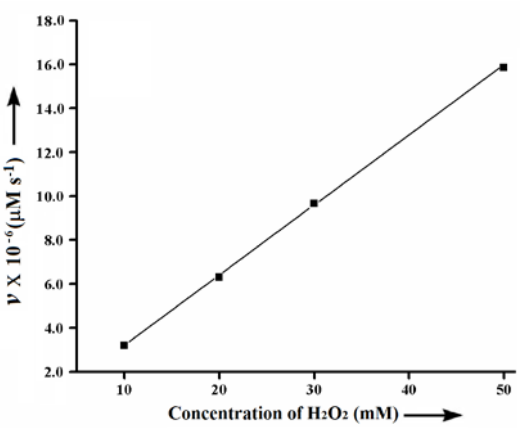

Fig. S11 Plot of product formation rate catalyzed by cytochrome c immobilized in toluene at SWNT-**3** hybrid gel (0.05% w/v SWNT, 1.0% w/v **3**) with varying H_2O_2 concentration at 25 °C. Concentration of the cyt c = 0.4 μM and pyrogallol = 10 mM.

References:

1. D. Das, A. Dasgupta, S. Roy, R. N. Mitra, S. Debnath, P. K. Das, *Chem. Eur. J.* **2006**, *12*, 5068; T. Kar, S. Debnath, D. Das, A. Shome, P. K. Das, *Langmuir* **2009**, *25*, 8639.

2. S. Brahmachari, D. Das, P. K. Das, *Chem. Commun*. **2010**, *46*, 8386.

3. D. Das, S. Roy, S. Debnath, P. K. Das, *Chem. Eur. J.* **2010**, *16*, 4911; Q. Wang, Z. Yang, L. Wang, M. Ma, B. Xu, *Chem.Commun*. **2007**, 1032.

4. R. E. M. Diederix, M. Ubbink, G. W. Canters, *Eur. J. Biochem.* **2001**, *268*, 4207.