

# Facile synthesis of chitin nanocrystal decorated on 3D cellulose aerogel as a new multi-functional material for waste water treatment with enhanced anti-bacterial and anti-oxidant properties

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Gopi, Sreerag; Balakrishnan, Preetha; Divya, Chandradhara; Valić, Srećko; Govorčin Bajsić, Emi; Pius, Anitha; Thomas, Sabu

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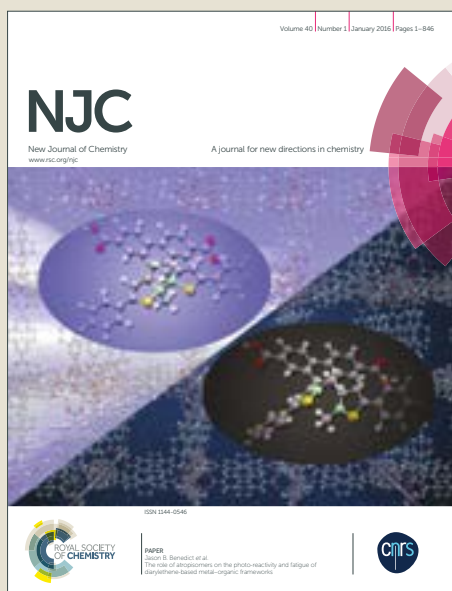


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1 **Facile synthesis of chitin nanocrystal decorated on 3D cellulose aerogel as a new multi-**  
2 **functional material for waste water treatment with enhanced anti-bacterial and anti-**  
3 **oxidant properties**

4 *Sreerag Gopi*<sup>a, b, c</sup>, *Preetha Balakrishnan*<sup>b, c</sup>, *Chandradhara Divya*<sup>d</sup>, *Srecko Valic*<sup>e</sup>, *Emi*  
5 *Govorcin Bajsic*<sup>f</sup>, *Anitha Pius*<sup>\*, a</sup>, *Sabu Thomas*<sup>\*, b, c</sup>

6 <sup>a</sup>Department of Chemistry, Gandhigram Rural Institute - Deemed University, Gandhigram,  
7 Dindigul District – 624302, Tamilnadu, India.

8 <sup>b</sup>School of Chemical Sciences, Mahatma Gandhi University, Kottayam–686560, Kerala, India.

9 <sup>c</sup>International and Inter University Centre for Nanoscience and Nanotechnology, Mahatma  
10 Gandhi University, Kottayam – 686560, Kerala, India.

11 <sup>d</sup>Bio-Agile Therapeutics Private Limited, Niran Arcade, 563/564, Ground floor, New Bel road,  
12 Bangalore 560094, Karnataka, India

13 <sup>e</sup>Center for Micro and Nano Sciences and Technologies, Radmile Matejčić 2, 51000 Rijeka,  
14 Croatia, and Rudjer Bošković Institute, Bijenička c. 54, 10000 Zagreb, Croatia

15 <sup>f</sup>Faculty of Chemical Engineering and Technology, Marulićev trg 19, 10000 Zagreb, Croatia

16  
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18 **\*Corresponding authors:**

19 Prof. (Dr.) Anitha Pius  
20 Email: dranithapius@gmail.com  
21 Ph: +91-9443108504 (Mob.),  
22 +91-4512452371 (Off.)

23  
24 Prof. (Dr.) Sabu Thomas  
25 Email: sabuthomas@mgu.ac.in  
26 Ph: +91-9447223452

27  
28 **Abstract**

29 We report fabrication and application of multi-functional hybrid bio-aerogels based on cellulose  
30 nanofibers (CNF) and chitin nano crystals (CNC) developed through environmental friendly  
31 freeze-drying process. Herein, organic non-solvent approach such as acid hydrolysis was used to  
32 extract CNF and CNC from corn husk and shrimp shell respectively followed  
33 by characterisation using IR spectroscopy and electron microscopy. Usual wired like and  
34 infrequent semi-square type of morphology were detected for CNF and CNC respectively during  
35 electron microscopic analysis. The aerogels with two different quantities of CNC on CNF were

36 made (AR1 and AR2) and compared them with reference to the neat CNF aerogel (neat AR).  
37 Interestingly, for AR2, under the electron microscopy, a maple seed like morphology was  
38 observed compared to neat AR and AR1. Their ability in water treatment were determined by  
39 conducting adsorption experiment using methylene blue and Rhodamine 6G, found that AR2 has  
40 enhanced removal percentage and adsorption capacity. To addressing their economic viability,  
41 we conducted several rounds of studies and evaluated the reusability of AR2. Anti-bacterial and  
42 anti-oxidant activities of aerogels were studied using disc diffusion method against four bacterial  
43 species such as *St. aureus*, *E. coli*, *S. typhimurium* and *B. Cereus* and carefully discussed the best  
44 condition for the determination of minimal inhibitory concentration. The prepared material  
45 having higher CNC content (AR2) shows multi-functional behaviour and in the future further  
46 studies are anticipated to produce them in industrial scale for commercial application

## 47 1. Introduction

48 Government, industries, and customers are gradually demanding products developed  
49 from sustainable and renewable resources that are biodegradable, carbon neutral, non-petroleum,  
50 and have low environmental, human or animal health and safety risks. For the past thousand  
51 years natural, cellulose based materials have been used by our society as engineering materials  
52 and their use continues in paper production and in textile field world widely. Cellulose is a linear  
53 polysaccharide which contains glucose molecules in a ringed and flat ribbon like conformation.  
54 The repeating unit of cellulose comprised of two anhydroglucose rings  $(C_6H_{10}O_5)_n$ ;  $n = 10000$  to  
55  $15000$ , where  $n$  is depended on the cellulose source material linked together through an oxygen  
56 covalently to C1 of one glucose ring and C4 of adjoining ring (1→4 linkage) and so called the  $\beta$   
57 1→4 glucosidic bond<sup>1</sup>. Cellulose mainly occurs as microfibrillated form having both amorphous  
58 and crystalline regions. The isolation of crystalline region and dissolution of amorphous region

59 makes cellulose into nanocrystals usually known as cellulose nanofibrilshaving similar properties  
60 of raw one. The low mechanical stability and charge less surface is main disadvantage of  
61 cellulose and cellulose nanofiber which can be overcome using reinforcement of other  
62 substances during the material processing time. Another polysaccharide known as chitin is a  
63 natural, renewable and biodegradable polymer, the second most abundant biopolymer after  
64 cellulose<sup>2</sup> which can be found in exoskeleton shells of arthropods such as crabs, shrimps, beetles  
65 etc and cell walls of fungi and yeasts<sup>3-5</sup>. Chitin also a linear polysaccharide containing repeated  
66 units of  $\beta$ -(1 $\rightarrow$ 4)-2-acetamido-2-deoxy- $\beta$ -D-glucose and  $\beta$ -(1 $\rightarrow$ 4)-2-amino-2-deoxy- $\beta$ -D-  
67 glucose<sup>6</sup>. Chitin has two hydroxyl groups and an acetamide group makes chitin very crystalline  
68 with surface charge and strong hydrogen bonding<sup>7</sup>. Similar to cellulose, chitin also occurs in  
69 nature as microfibrilated form having both amorphous and crystalline regions and these fibrils  
70 are typically embedded in a protein matrix depending on the origin of sources<sup>8</sup>. The crystalline  
71 regions of chitin can be extracted by excluding amorphous region and it is known as chitin  
72 nanocrystals or nanowhiskers which is widely used as reinforcing nanoparticles. Several methods  
73 can be employed for the extraction of chitin nanocrystals such as acid hydrolysis<sup>8-17</sup>, mechanical  
74 treatment<sup>7,18</sup>, electrospinning<sup>19</sup>, gelation<sup>20</sup>, and TEMPO-mediated oxidation<sup>21,22</sup>. Due to the  
75 diverse properties of chitin nanocrystals such as biodegradability, functionality, hydrophilicity,  
76 eco-friendly, and ease of processing, it is used in many field of application which includes  
77 biomedical, water purification, packing and protein immobilization<sup>23</sup>. Recently Lin et. al  
78 reported fungus – derived chitin nanocrystals and their dispersion stability evaluation in aqueous  
79 media. In their study, chitin nanocrystals which avoided possible safety risks were extracted  
80 from mushrooms using protein/mineral purification and HCl hydrolysis. Such nanocrystals of

81 chitin's were in  $\alpha$ -crystalline structure with a length and width of  $143 \pm 24$  and  $10 \pm 2$  nm  
82 respectively<sup>24</sup>.

83 Water pollution from industrial as well as public effluents (dyes, heavy metals, oils etc)  
84 results in acute shortage of fresh water in many parts of the world in which dyes and heavy  
85 metals can severely damage people and living things<sup>25</sup>. Consequently there is an increasing  
86 demand for advanced adsorptive materials having high selectivity, high efficiency and high  
87 adsorption capacity<sup>25,26</sup>. The dye effluents are one of major contaminants in water. Different  
88 types of adsorptive materials including activated carbon<sup>27</sup>, carbon nanotubes<sup>28</sup>, clays<sup>29</sup>, magnetic  
89 oxides<sup>30</sup> etc are being used for the adsorption of dyes from waste water. Among them, 3D porous  
90 materials seem to be the most auspicious class of high performance adsorbent due to their high  
91 porosity, light weight and large surface to volume ratio. Because of these inherent physio-  
92 chemical characteristics, they can adsorb a large quantity of dye molecules from dye polluted  
93 waste water according to their surface charges. However, complex fabrication procedures and/or  
94 high raw material cost have diminished their wide spread applicability. Therefore, there is every  
95 need to develop innovative, renewable/sustainable, eco-friendly and robust high performance  
96 adsorbent materials.

97 Xiong et.al demonstrated a facile and environmentally friendly approach to prepare  
98  $\text{Fe}_3\text{O}_4/\text{Ag}$  nanofibrillated cellulose nanocomposite aerogel having excellent catalytic properties  
99 for the reduction of 4-nitrophenol. This prepared aerogels also showed high antibacterial activity  
100 against model microbe *S. aureus*<sup>31</sup>. Zhou et. al prepared polyaniline (PANI) – decorated cellulose  
101 aerogel with strong interfacial adhesion and enhanced photocatalytic activity via  
102 dissolve/regeneration route using ionic liquid<sup>32</sup>. In this study, cellulose nanofibril-chitin  
103 nanocrystal hybrid aerogel have been developed for the first time using environmentally friendly

104 freeze drying process<sup>33</sup>. Their application in dye waste water treatment, anti-bacterial and anti-  
105 oxidant properties were evaluated very carefully. Cellulose is the richest natural, renewable,  
106 biodegradable and biocompatible polymer in the world<sup>34</sup>. Cellulose nanofibrils have high aspect  
107 ratios and high surface areas, hence they certainly form an entangled web like structure<sup>35,36</sup>.  
108 Cellulose nanofibers can be prepared using different methods including 2, 2, 6, 6-  
109 tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation<sup>36,37</sup>, mechanical shearing<sup>38</sup>, and  
110 high-pressure homogenization<sup>39,40</sup>. The aerogels made from cellulose nanofiber usually show  
111 high surface areas, reasonable mechanical, thermal properties and also low densities<sup>41-43</sup>. They  
112 have been used in a variety of applications, such as thermal insulation<sup>34</sup>, anti-bacterial agent<sup>44</sup>  
113 and oil absorbents<sup>45,46</sup>. Chitin nanocrystals is an economical polymer which contains desired  
114 properties such as water solubility, compatibility and functionality. New hybrid aerogel  
115 structures based on chitin nanocrystal and cellulose nanofiber having an innovative maple seed  
116 type morphology have been made use of for the first time for the macromolecular engineering of  
117 new class of aerogels which is an excellent advanced bio-based green material for dye adsorption  
118 from aqueous solution. Interestingly, the acetamide groups in chitin nanocrystal control anti-  
119 bacterial properties of aerogels.

## 120 2. Experimental Section

### 121 2.1. Materials

122 The cellulose extracted from local dried corn husk has been used in the present study.  
123 The chitin used for producing the chitin nanocrystal, was a commercially supplied fully bleached  
124 shrimp shell flakes. Sulfuric acid, sodium hypochlorite solution, methylene blue (MB), rhodamine  
125 6G (Rh) and other chemicals were obtained from sigma Aldrich. They were used without further

126 purification. Millipore-MilliQ distilled water was employed during the complete experiments.

127 Table 1 shows the specification of dyes used in this study.

128 **Table 1.** Specifications of MB and Rh

<b>Generic name</b>	<b>Methylene blue</b>
Color Index Number	52015
Molecular formula	$C_{16}H_{18}ClN_3S$
Molecular weight	319.85
$\lambda_{max}$ (nm)	609
<b>Generic name</b>	<b>Rhodamine 6G</b>
Color Index Number	45160
Molecular formula	$C_{28}H_{31}N_2O_3Cl$
Molecular weight	479.01
$\lambda_{max}$ (nm)	530

129

## 130 2.2.Preparation of Cellulose Nanofibers

131 Cellulose nanofiber (CNF) prepared from corn husk were collected from the local area,  
 132 Slovenia and thoroughly washed with running tap water followed by soaked in Millipore-MilliQ  
 133 water later dried under sunlight. The washed corn husk was bleached (treated) with aqueous  
 134 sodium chlorite (1.7 wt %  $NaClO_2$  in water). The bleaching treatment was performed at 90 °C for  
 135 2h followed by acetic acid was added to reach pH 4 to enhance the bleaching reaction. The  
 136 resultant fibers were repeatedly washed in distilled water until the pH became neutral and later  
 137 dried at 60 °C for 12 h in an air circulating oven. The nanofibers were prepared from treated corn  
 138 fibers by following already reported method of Khawas et.al<sup>47</sup> with suitable modifications. In  
 139 brief, the methodology is as follows: the dried corn fibers were crushed and treated with sodium  
 140 hydroxide solution (4 %) at boiling condition for 2 h under thorough stirring to solubilize lignin,  
 141 pectin and hemicelluloses. This step was done at least 3 times for complete purification of the  
 142 fibers. The resulting fibers were hydrolyzed with 65 % (w/w) sulfuric acid for about 60 min



143 using mechanical stirrer to dissolve the amorphous region and to isolate the required nanofibers.  
144 Subsequently, the suspension was diluted with ice cubes to quench the reaction followed by  
145 washing and centrifuging at 10000 rpm for 20 min. The CNF suspension was homogenized by  
146 using PRIMIX-Homomixer homogenizer for 15 min and stored in refrigerator.

### 147 **2.3.Preparation of Chitin Nanocrystals**

148 Chitin nanocrystal (CNC) were prepared by following the study reported by Gopi et  
149 al.<sup>48</sup> with suitable modifications. The powdered shrimp shells were suspended in 5 % aqueous  
150 potassium hydroxide solution for 6 h under boiling condition in order to remove the proteins and  
151 minerals from it. The obtained residue was rinsed with distilled water, filtered and kept for  
152 agitation at room temperature followed by bleaching with 17 g of sodium hypochlorite solution  
153 for 2 h at 80°C. The resulting dispersion was hydrolyzed with sulfuric acid, filtered and washed  
154 with three times and CNC solution transferred into dialysis bag. The dialysis was continued until  
155 the pH of CNC solution reached 6 followed by ultrasonicated for 5 min. The CNC solution was  
156 stored in refrigerator to prevent from bacterial growth.

### 157 **2.4.Preparation of Cellulose Nanofiber/Chitin nanocrystal hybrid aerogels**

158 The Cellulose Nanofiber/Chitin nanocrystal hybrid aerogels (CNC/CNF) were fabricated  
159 by a cost effective environmental friendly freeze-drying method. 6 and 12.5 mL of 1mg mL<sup>-1</sup>  
160 CNC aqueous dispersion were mixed with 50 mL of 1.2 wt% CNF to get the final concentrations  
161 of 1 % and 2 % and named as AR1 and AR2 respectively. The resulting mixture was sonicated  
162 using a probe sonicator for 10 min to overcome the agglomerates and finally casted in  
163 petridishes. Neat CNF aerogel (neat AR) without the addition of CNC was made as a reference.  
164 The whole solutions were precooled in a 3 °C refrigerator to avoid macroscopic fracture during

165 the freezing step. Later the precooled samples were frozen at  $-73\text{ }^{\circ}\text{C}$  in a dry ice- iso-propanol  
166 solution and freeze dried in a lyophilizer at a condenser temperature of  $-88.0\text{ }^{\circ}\text{C}$  under vacuum  
167 for 4 days to fabricate aerogels. Finally, the samples of aerogel were stored in vacuum oven for  
168 further characterization.

### 169 **2.5. Fourier transform infrared spectroscopy**

170 Fourier transform infrared spectroscopy (FTIR) analysis was used to detect the chemical  
171 moieties present in CNF, CNC and CNF/CNC hybrid aerogel recorded using Shimadzu IR-470  
172 IR spectrophotometer. The FTIR spectra for every samples were attained over the wavelength  
173 ranging from  $400$  to  $4000\text{ cm}^{-1}$  using potassium bromide disk and the experiments were carried  
174 out with a resolution of  $2\text{ cm}^{-1}$ .

### 175 **2.6. X-ray diffraction analysis**

176 X-ray diffraction analysis was employed to determine the crystalline nature of CNF and  
177 CNC. The powdered materials of CNF and CNC were placed in the sample holder and leveled to  
178 get uniform and total X-ray exposure. The X-ray diffraction patterns of CNF and CNC achieved  
179 with as X-ray diffractometer (Bruker Diffractometer D8 Advanced Model) at room temperature  
180 with  $2\theta$  from  $5$  to  $60^{\circ}$  and with scan rate of  $2^{\circ}\text{ min}^{-1}$

### 181 **2.7. Transmission electron microscopy**

182 Transmission electron microscopy ((TEM) JEOL-2100 model) was used to determine the  
183 morphology and dimension of CNF and CNC. Prior to TEM analysis diluted suspension of CNF  
184 and CNC pasted on a copper grid and dried at room temperature. The analysis was carried out  
185 with accelerating voltage of  $25\text{ KV}$ .

## 186 **2.8. Field emission scanning electron microscopy**

187 The morphology of CNF/CNC hybrid aerogel was observed using field emission  
188 scanning electron microscopy ((FESEM (JEOL JSM-820 Model))with operation voltage of 20  
189 kV. All samples were dried at 40 °C and coated with vanadium to avoid following charging  
190 before SEM observation.

## 191 **2.9. BET analysis**

192 Nitrogen adsorption-desorption isotherms were measured using a Quantachrome®  
193 ASiQwin™, and Brunauer – Emmet – Teller (BET) and Barret – Joyner – Halenda (BJH)  
194 analysis were obtained using the Autosorb software (Quantachrome®). The BET analysis was  
195 performed at relative vapor pressures of 0.08 – 0.3 ( $P/P_0$ ) and the BJH analysis was done from  
196 desorption isotherm branch. The pore-size distribution and surface area were calculated using  
197 BJH and BET methods.

## 198 **2.10. Swelling studies**

199 Circularly shaped samples with diameter  $\approx$  2 cm of ASTM standard D5890 were weighed  
200 and immersed in toluene contained in test tubes with airtight stoppers kept at room temperature  
201 for 72 h. Afterwards the initial weight, swollen weight and deswollen weight were taken using a  
202 high sensitive electronic balance device. Cross link densities of the aerogel samples were  
203 calculated from swelling experiments using Flory-Rehner equation.

## 204 **2.11. Thermal gravimetric analysis**

205 Thermal gravimetric analysis (TGA) was conducted for CNF/CNC hybrid aerogels by using  
206 Perkin Elmer Pyris 1 thermogravimetric system under nitrogen purge, with a flow rate 100 mL

207  $\text{min}^{-1}$ . The scanning rate was  $10\text{ }^{\circ}\text{C min}^{-1}$  and the temperature ranging from 40 to  $700\text{ }^{\circ}\text{C}$ . All  
208 samples were left at  $40\text{ }^{\circ}\text{C}$  for 3 min before the measurements to avoid the moisture.

## 209 **2.12. Differential scanning calorimetry**

210 Thermal properties of CNF/CNC aerogels were studied by differential scanning  
211 calorimetry (DSC) with DSC instrument Mettler Toledo 822<sup>e</sup> using nitrogen atmosphere. The  
212 DSC stands as a thermal technique by which I and II order of polymer materials phase transitions  
213 can be easily determined. About 9-11 mg of samples were heated to  $200^{\circ}\text{C}$  from room  
214 temperature and stands isothermally for 15 min to exclude the effect of the thermal history  
215 during sample processing. Subsequently, the samples were cooled and reheated to  $200^{\circ}\text{C}$ .  
216 Experiments were carried out with heating/cooling rate of  $10\text{ }^{\circ}\text{C min}^{-1}$ . The phase transition  
217 temperatures were determined from the second DSC cycle.

## 218 **2.13. Performance of Cellulose nanofiber/Chitin nanocrystal hybrid aerogel on waste** 219 **water treatment**

220 The dye removal studies of aerogels were carried out using methylene blue (MB) and  
221 Rhodamine 6G (Rh 6G).  $1000\text{ mgL}^{-1}$  dye concentration in water was used as stock solution.  
222 From the stock solution, dilutions of  $10\text{ mgL}^{-1}$  concentrations of dyes were made and kept at  
223 neutral pH. All the analysis was demonstrated at room temperature with the help of UV-VIS  
224 spectrophotometer (Agilent technologies, Cary 60 UV-VIS). The samples ( $1\text{ cm}^2$ ) hanged into a  
225 glass chamber with a magnetic stirrer and 250 mL of dye solution transferred into it. The  
226 adsorption of MB and Rh 6G were measured at the absorbance maxima  $\lambda_{\text{max}} = 609\text{ nm}$  and  $\lambda_{\text{max}} =$   
227  $530\text{ nm}$  respectively. The percentage of removal of MB and Rh 6G with different adsorbents  
228 were calculated by following eq. (1)<sup>49</sup>;

$$R = \frac{C_i - C_0}{C_i} \cdot 100 \% \quad (1)$$

where  $C_i$  ( $\text{mgL}^{-1}$ ) is the initial concentration of solution and  $C_0$  ( $\text{mgL}^{-1}$ ) is the final concentration of solution.

The following eq. (2) was used for calculating the dye adsorption capacity of the adsorbents:<sup>49</sup>

$$q_e = \frac{(C_i - C_0)V}{m} \quad (2)$$

where  $q_e$  is expressed in  $\text{mg/g}$ ,  $V$  is the volume of solution and  $m$  is the mass of adsorbent.

## 2.14. Antimicrobial and antioxidant activity studies of Cellulose nanofiber/Chitin nanocrystal hybrid aerogel

Four bacterial species were employed as test organisms which include *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Bacillus cereus*. The bacteria were maintained in Mueller – Hinton (MH). Inoculate were prepared by adding an overnight culture of the organism in MH broth to obtain an OD600 0.1. The cells were allowed to grow until they obtain the McFarland standard 0.5 (approximately  $10^8$  CFU/mL). The suspension was then diluted to 1: 1000 in MH broth to obtain  $10^6$  CFU/mL. The antibacterial and antioxidant activities were carried out using neat AR, AR1 and AR2.

## 3. Results and discussions

### 3.1. Fourier transform infrared spectroscopy

The fourier transform infrared (FTIR) spectroscopy was used to analysis the chemical moieties present in CNF, CNC, neat AR and AR2 shown in the figure 1. The FTIR spectra of CNF and neat AR show a broad peak at a frequency of  $3300 \text{ cm}^{-1}$  which corresponds to polysaccharide

249 hydroxyl group. The peaks at 2900-2800  $\text{cm}^{-1}$  represent the C-H symmetrical stretching  
250 vibrations. The FTIR spectrum of CNC in which, peaks at 1663  $\text{cm}^{-1}$ , 1630  $\text{cm}^{-1}$  correspond to  
251 amide I and peak at 1556  $\text{cm}^{-1}$  corresponds to amide II bands. These are typical well  
252 characterized peaks for chitin already reported in the scientific literature<sup>15</sup>. Peak at 3451  $\text{cm}^{-1}$   
253 represents -OH group and peak at 3259  $\text{cm}^{-1}$  attributed to -NH group. The FTIR spectrum of  
254 AR2 having 2 wt.% of CNC shows the same peaks as of CNF or neat AR and also along with  
255 the peaks of CNC such as 1663  $\text{cm}^{-1}$ , 1630  $\text{cm}^{-1}$  and 1556  $\text{cm}^{-1}$  which correspond to amide I and  
256 amide II bands respectively. AR2 also shows peak at 3259  $\text{cm}^{-1}$  attributed to -NH group. To the  
257 better understanding about the interaction between CNC over neat AR, FTIR spectra of the same  
258 were compared. One thing can be observed that neat AR have a well-defined single peak around  
259 3300  $\text{cm}^{-1}$  corresponds to -OH functionality. But in AR2, the peak multiplies and a combined  
260 multiple peak of both -OH (3300  $\text{cm}^{-1}$ ) and amide group (3259  $\text{cm}^{-1}$ ) observed which possibly  
261 due to the hydrogen bonding interaction between the CNF and CNC. Compared to neat AR, AR2  
262 have new peaks at 1663  $\text{cm}^{-1}$ , 1630  $\text{cm}^{-1}$  correspond to amide I and peak at 1556  $\text{cm}^{-1}$   
263 corresponds to amide II bands of CNC which were absent in AR. This confirms better interaction  
264 and nanoscale reinforcement of CNC over AR2. The similar observation has been reported by  
265 Sun et.al in chitosan (chitin)/cellulose composite biosorbents prepared using ionic liquid for  
266 heavy metal ions adsorption. They reported that hydroxyl stretching vibrations for chitosan  
267 (chitin) and cellulose located at 3436  $\text{cm}^{-1}$  and 3343  $\text{cm}^{-1}$  were broadened and shifted to 3413  
268  $\text{cm}^{-1}$  indicated that stronger intermolecular hydrogen bonds interaction<sup>50</sup>. From the demonstrated  
269 FTIR spectra of neat and nanocomposites hybrid aerogel it can be confirmed the presence of  
270 CNC in AR2 and the reinforcement doesn't make any changes in chemical moieties present in  
271 CNF based aerogel.

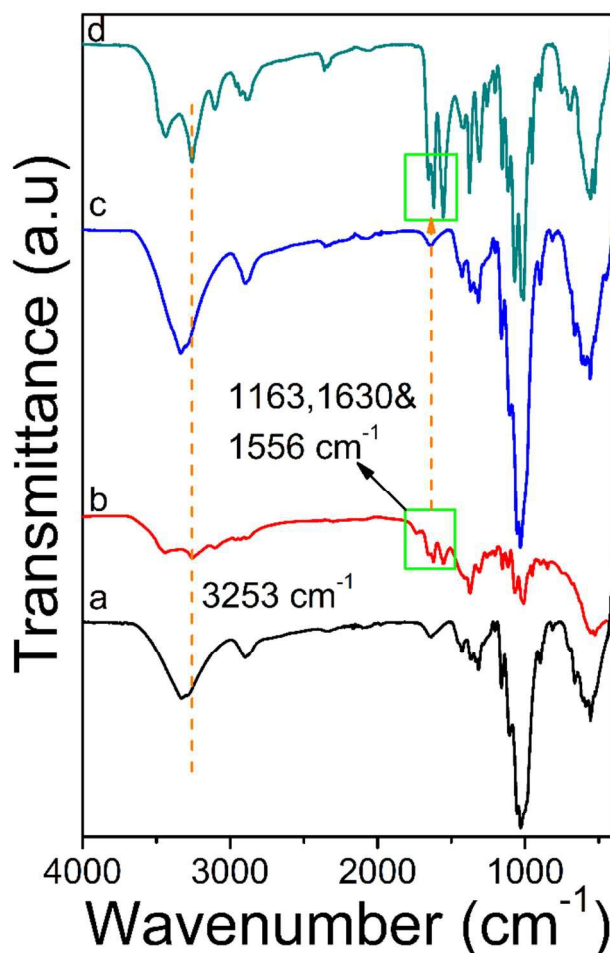
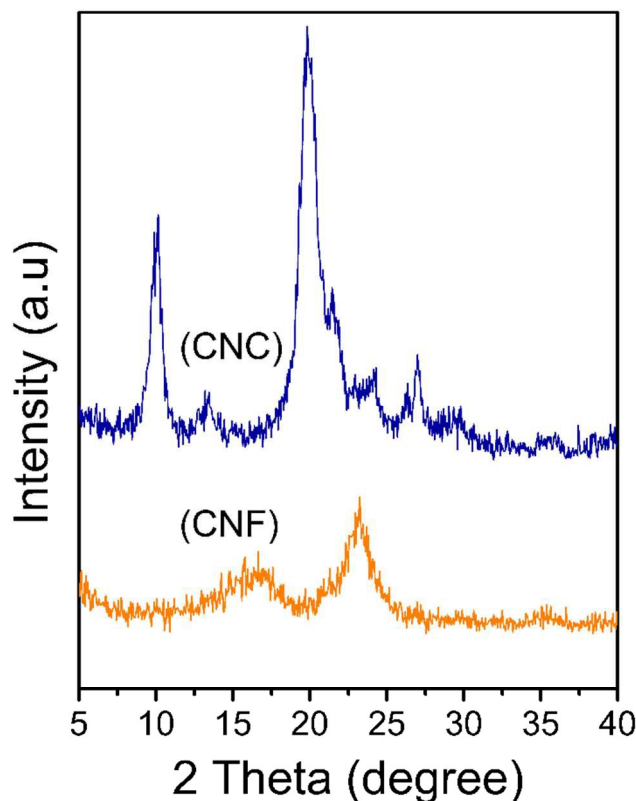


Figure 1. FTIR spectra of a) CNF, b) CNC, c) neat AR and d) AR2.

### 3.2. X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) analysis were employed to reveal crystallinity of CNF and CNC shown in figure 2. The XRD profile of CNF have two major peaks corresponds to  $2\theta = 16.1^\circ$  and  $22.8^\circ$  which is characteristically structure of cellulose I and sharp peak at  $22.8^\circ$  is due to higher crystallinity of cellulose. In the case of CNC, two major diffraction peaks at  $2\theta = 9.8^\circ$  and  $19.5^\circ$  along with three minor peaks at  $12.6^\circ$ ,  $23.9^\circ$ , and  $26.8^\circ$  were observed. In CNC, the high crystalline nature and structure is due to the van der Waals and hydrogen bonding interaction in it.



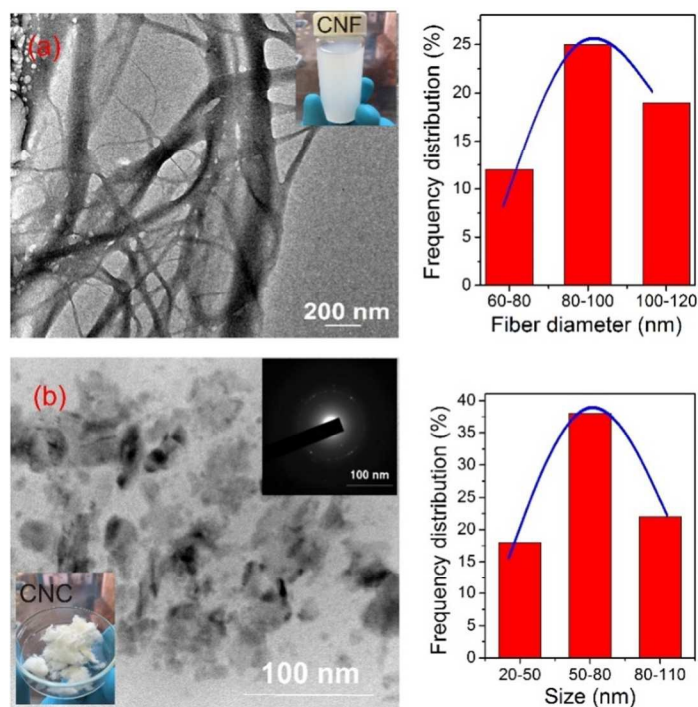
282  
283 Figure 2. XRD spectra of CNF and CNC

### 284 3.2. Transmission electron microscopy

285 The transmission electron microscopy (TEM) images and size distribution graphs of both  
286 CNF and CNC in different resolutions given in figure 3. Let us examine the TEM images of CNF  
287 which is shown in figure 3 a. Uniform suspension of CNF was achieved after mild acid  
288 hydrolysis of purified corn husk which is shown in the insight of figure 3 a. A wire like  
289 morphology can be observed for CNF with slight agglomeration which may due to the  
290 insufficient ultra-sonic treatment. Agglomeration also could be possible due to the water  
291 evaporation from the CNF suspension during the sample preparation. The average diameter from  
292 the size distribution graph was found to be in the range of 60-120 nm. Similar CNF morphology  
293 were reported by Asha et. al<sup>51</sup> in their studies.



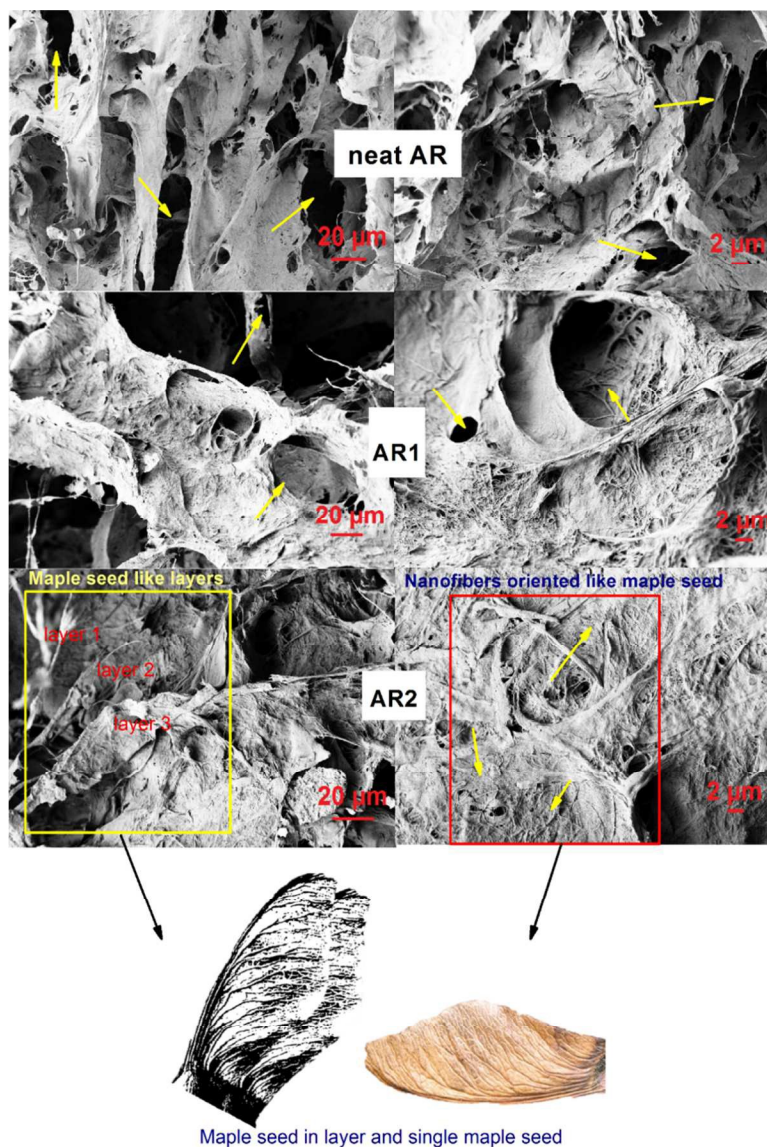
294 Let us now examine the TEM images of CNC. The CNC isolated from shrimp shell using  
295 sulfuric acid hydrolysis shows a different morphology compared to other reported studies. The  
296 TEM image of CNC show a needle type or rod like structure in most of the reported studies<sup>15,52</sup>.  
297 In our present study, CNC shows semi-square crystal morphology which is shown in the figure  
298 3. The crystalline nature and morphology of the product depends on the raw materials and  
299 chemical treatment process. An inconsiderable agglomeration also can be observed from the  
300 TEM images of CNC which can influence dispersion behavior on CNF suspension. We also  
301 observed bright crystalline nature of powdered CNC (achieved by lyophilizing) by visually,  
302 which is shown the inset of figure 3 b. The average size from the size distribution graph was  
303 found to be in the range of 20-100 nm.



304  
305 **Figure 3.** TEM images of a) CNF, b) CNC and their size distribution diagrams.

306 **3.3. Field emission scanning electron microscopy**

307 The morphologies under different resolutions of neat aerogel, AR1 and AR2 were  
308 observed and analysed using field emission scanning electron microscopy (FESEM) as shown  
309 figure 4. The images of neat AR show random, non-oriented porous structure having distribution  
310 in size and these pores were large in size. The fibers were not well interconnected and  
311 aggregation of fibers also can be observed. Nanofibers have a tendency to attract each other and  
312 this could be a reason for the above-mentioned aggregations. While the images of AR1 show,  
313 smaller pores compared to neat AR due to the small addition of CNC which may influence the  
314 morphological behaviour of CNF aerogel. In the other case, the images of AR2 containing higher  
315 amount of CNC show a notable morphological behaviour compared to neat AR and AR1. The  
316 morphological character of AR2 can be compared with layer-by-layer of maple seed. From a  
317 clear observation, it can be understood that a single maple seed contains a well oriented vein,  
318 these veins interlocked between each other and also a rough texture on the surface. The FESEM  
319 images of AR2 is very similar to multi-layer maple seed type morphology demonstrated here.  
320 The fiber orientation in AR2 is also mimicking the maple seed which clearly observed from the  
321 FESEM image of the same. Upon higher amount of CNC reinforcement, it has a tendency to  
322 locate in between CNF that reduces the intermolecular interaction between the fibers.  
323 Consequently, it hinders the chances of aggregation resulting to obtain fiber networking leading  
324 to branch like maple seed morphology. This type of morphology is expected to have potential  
325 applications in various fields such as water treatment (adsorption, filtration), drug delivery, tissue  
326 engineering etc.



327

328

**Figure 4.** FESEM images of aerogels

329

### 3.4. BET analysis

330 BET surface area, pore diameter and pore volume of neat AR, AR1 and AR2 given in  
 331 table 2. It can be observed that the pore volume is decreasing gradually from neat AR (0.24 cc/g)  
 332 with an increasing in levels of CNC content. AR2 has lesser pore volume (0.029 cc/g) compared  
 333 to AR1 (0.039 cc/g) since higher level of CNC content in AR2 significantly changes the pore  
 334 structure. A decrease in pore diameter can also be observed from neat AR to AR1 and then AR2,

335 this arises by filling the pores of AR2 with the higher CNC compared to AR1. BET surface area  
336 value of AR2 was 17.508 m<sup>2</sup>/g which is higher than AR1 and neat AR, this increase in surface  
337 area can improve the efficiency of adsorption performance.

338 **Table 2.** Specifications of MB and Rh

Aerogels	Pore volume (cc/g)	Pore diameter (nm)	BET surface area (m <sup>2</sup> /g)
Neat AR	0.24	1.595	11.473
AR1	0.036	1.564	13.608
AR2	0.029	1.555	17.508

339

### 340 3.5. Cross-link density studies

341 According to the theory of swelling behavior of cross-linked polymers, strong bonding such  
342 as chemical cross link between the cellulose nanofiber and chitin nanocrystal chains prevent the  
343 molecules from completely surrounded by the fluids, but they can cause swelling. The  
344 percentage of increase in swollen mass was calculated by the following equation.

$$345 \text{ Swollen mass increase (\%)} = [(m_1 - m_0)/m_0] \times 100 \quad (3)$$

346 where  $m_0$  initial weight of the sample and  $m_1$  is weight of sample after immersing into the  
347 solvent in which the solvent specimens cleared by using filter paper.

348 Cross-link density is usually calculated from equilibrium swelling data by means of the Flory-  
349 Rehner equation<sup>53-55</sup>.

$$350 \text{ Cross-link density } V = 1/2 M_c \quad (4)$$

$$351 M_c = [-\rho_r V_s V_{rf}^{1/3}] / [\ln(1 - V_{rf}) + V_{rf} + \chi V_{rf}^2] \quad (5)$$

$$352 V_{rf} = [(d - fw)\rho_r] / [((d - fw)\rho_r) + (A_s/\rho_s)] \quad (6)$$

353 where  $M_c$  is the molecular weight of polymer between cross-links,  $\rho_r$  is the density of polymer,  
354  $V_s$  and  $V_{rf}$  are molar volume of the solvent and volume fraction of the polymer in the swollen  
355 sample respectively. In which  $d$  represent deswollen weight  $f$  is the volume fraction of filler,  $w$   
356 is the initial weight of the material,  $A_s$  is the adsorbed amount of solvent by the material. The  
357 interaction parameter  $\chi$ , shown in eqn (4) and is given by Hildebrand equation

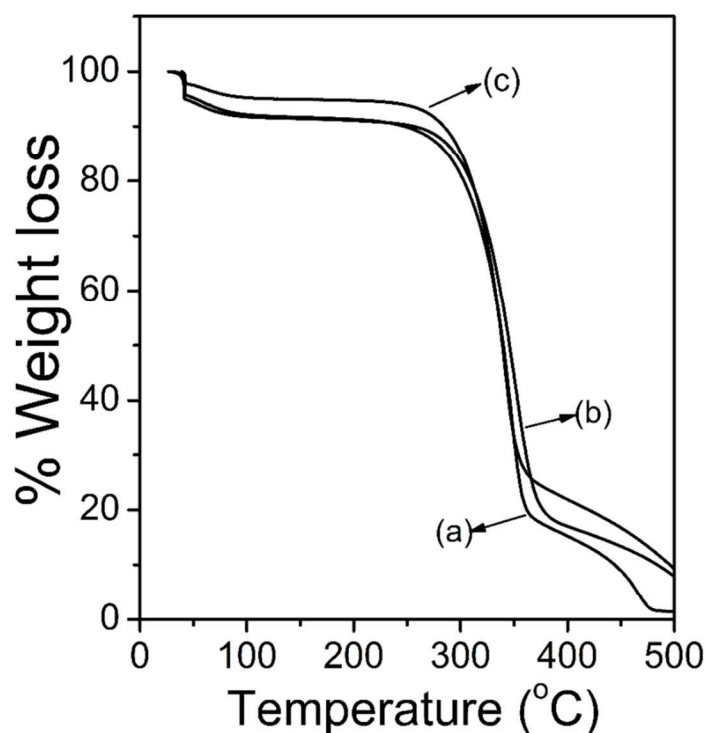
$$358 \quad \chi = \beta + \left[ V_s (\rho_s - \rho_p)^2 / RT \right] \quad (7)$$

359 here  $\beta$  is the lattice constant,  $R$  represent the universal gas constant,  $T$  is absolute temperature,  $\rho_s$   
360 and  $\rho_p$  are the solubility parameter of solvent and solubility parameter of the polymer  
361 respectively. Table 1 shows the cross-link density values obtained by swelling experiments.  
362 Aerogel containing higher amount of CNC (AR2) exhibit higher cross-link density value except  
363 AR1 such as  $V = 0.420 \times 10^4$  (mol cm<sup>-3</sup>) and  $V = 3.4124 \times 10^4$  (mol cm<sup>-3</sup>) for AR1 and  
364 AR2 respectively. The functional groups (hydroxyl and acetamide groups) of CNC on cellulose  
365 nanofiber can act as a network forming agents able to react with the CNF chains leading to the  
366 formation of strong networking structure in the aerogel.

### 367 **3.6. Thermal gravimetric analysis**

368 The thermal gravimetric analysis (TGA) curves for AR1 and AR2 in comparison with  
369 reference neat AR are shown in figure 5. All materials show decrease in mass at 100°C which  
370 obviously due to the evaporation of adhering moisture from the materials. By careful evaluation  
371 of obtained data, we can observe that AR2 losses only 5.2 % of mass at this temperature which is  
372 low compared to other reported studies<sup>56,57</sup>. This may due to the freeze-drying process that  
373 sublimate the whole water moieties from the sample. Also, the mass loss of AR2 at 100 °C lower  
374 than neat AR and AR1 (8.4 % and 7.8 % respectively) probably due to fine dispersion of CNC

375 on CNF thus leaving no vacant space for entrapment of water molecules. The AR2 with  
376 relatively higher CNC content unveiled an onset weight loss at around 290 °C, which was higher  
377 than neat AR and AR1 having lower CNC content. The TGA results showed that thermal  
378 stability of AR2 aerogel was the best among all the samples (neat AR and AR1). This suggested  
379 that there exist strong interaction between CNF and CNC in AR2.



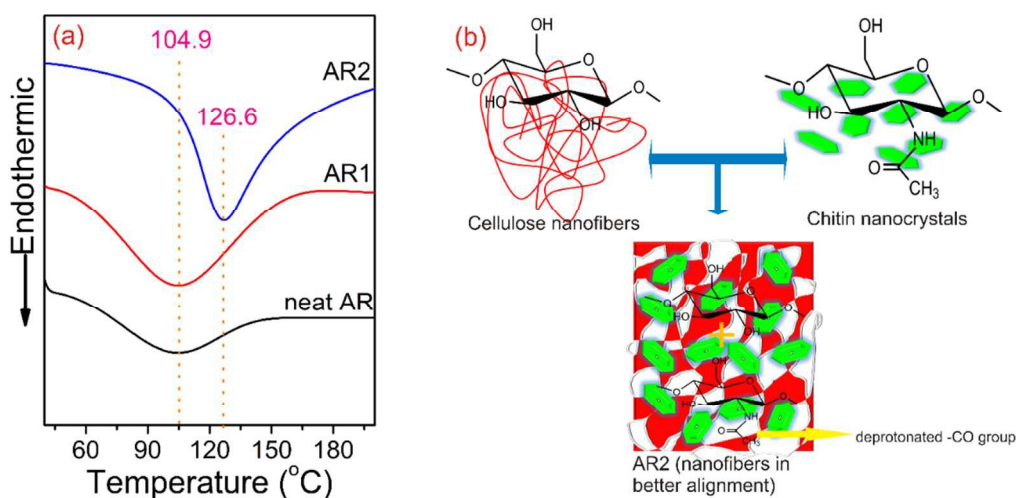
380  
381 **Figure 5.** TGA curves of a) neat AR, b) AR1 and c) AR2.

### 382 3.7. Differential scanning calorimetry

383 The differential scanning calorimetry (DSC) curves of neat AR, AR1 and AR2 are shown  
384 in figure 6 a. It can be seen that the melting temperatures ( $T_m$ ) of neat AR and AR1 (104.9 °C) are  
385 much lower than AR2 (126.6 °C). The high melting temperature of AR2 is due to the uniform  
386 dispersion of CNC in CNF as mentioned in morphological part, which prevents the motion of  
387 CNF chains. As a result, more energy is needed for the molecular motion of CNF chains. The



388 more is the crystalline region, the higher will be the melting temperature. A graphical  
389 representation given in figure 6 b showing better organization and alignment of nanofibers in  
390 AR2 having higher amount of CNC that can be leads to higher crystallinity and melting point.

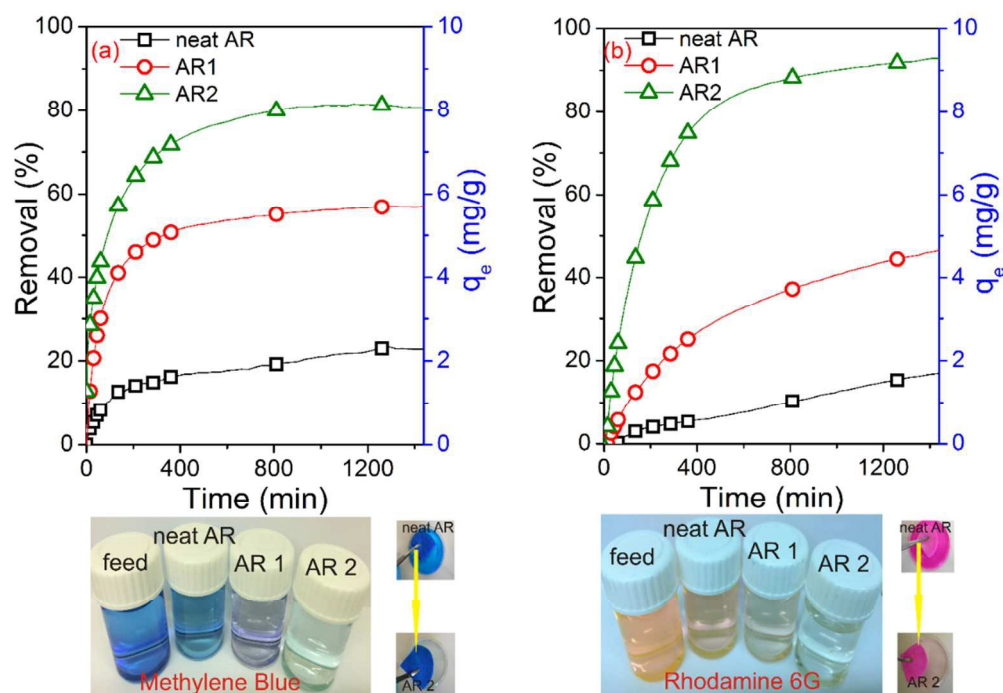


391  
392 **Figure 6a)** DSC curve of aerogels **b)** Graphical representation of aligned fibers in AR2

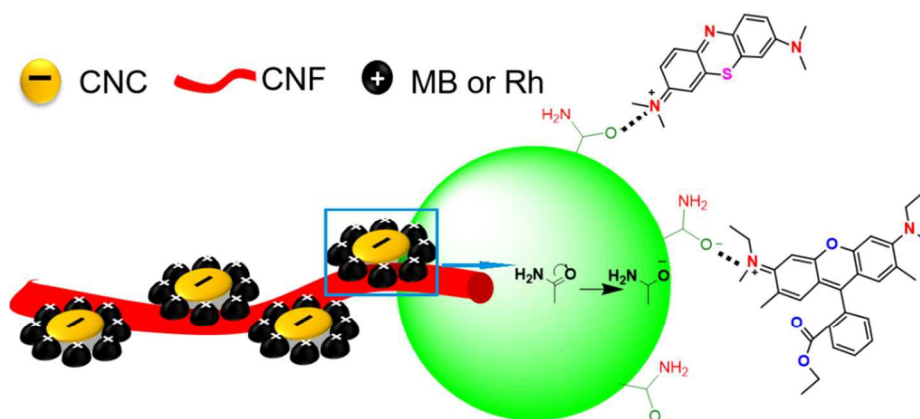
393 **3.8. Performance of Cellulose nanofiber/Chitin nanocrystal hybrid aerogel on waste**  
394 **water treatment**

395 The effect of contact time on the dye water treatment using MB and Rh are shown in  
396 figure 7. It is clearly observed that the percentage of removal (removal (%)) and adsorption  
397 capacity ( $q_e$ ) of neat AR and AR1 are low compared to AR2. The percentage of removal and  
398 adsorption capacity of AR2 were found to be 80.07 % and 8 mg/g for MB, 92.89 % and 9.2 mg/g  
399 for Rh respectively. A schematic representation of mechanism of MB and Rh adsorption onto  
400 AR2 is shown figure 8. The AR2 material contains higher amount well dispersed CNC on CNF  
401 and the CNCs are enriched with acetamide groups. During the course of adsorption, the  
402 deprotonated carbonyl group present in acetamide (reactive group in CNC) creates a negative  
403 charge species as shown in the scheme (inside the green one). These negatively charged CNCs

404 an electrostatic interaction between positively charged dye molecule which is shown as dotted  
 405 lines. These electrostatic interactions favour efficient dye adsorption over the CNC decorated  
 406 AR2. It is also important to consider the well-designed morphological texture in AR2 as already  
 407 discussed under morphological part which could be another possibility of the enhanced dye  
 408 removal from water.



409  
 410 **Figure 7.** Removal( $R$ ) and adsorption capacity ( $q_e$ ) of neat AR, AR1 and AR2 on a) MB and b)  
 411 Rh.



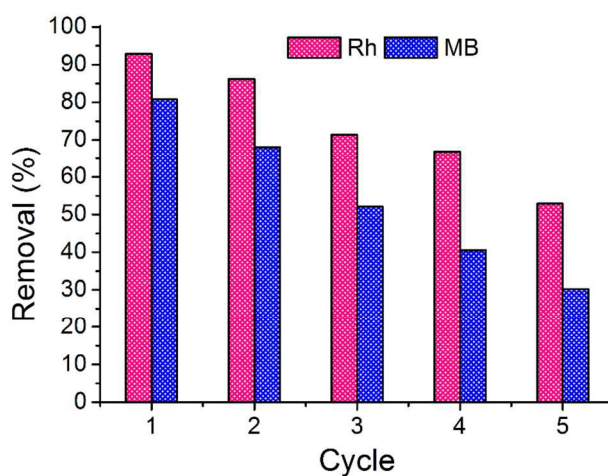
412



413 **Figure 8.** Schematic representation of mechanism of MB and Rh adsorption onto AR2.

### 414 **3.9. Round studies of material**

415 Round studies were carried out using AR2 on MB and Rh to understand the reusability of  
416 material. After the first-round of adsorption experiment the sample AR2 was dried at room  
417 temperature and washed several times using distilled water. Totally 5 round experiments were  
418 conducted with the same drying and washing protocols and the results are shown in figure 9.  
419 During the first round, 80.07 % MB and 92.87 % Rh could be removed and the removal efficiency  
420 was gradually found decreased and in the final round i.e. in 5<sup>th</sup> round 30.2 % MB and 52.9 % Rh  
421 could be removed using AR2. The decreasing efficiency up reuse could be explained as follows:  
422 During the several adsorptions process the active sites of material were occupied with dye  
423 molecule and few of them only could be removed during thorough washing procedure<sup>58</sup>. As a  
424 result, subsequent adsorption process, active sites required to adsorb dye molecules reduces.  
425 Thereby a reduction in adsorption takes place in the round studies. Even though AR2 can be  
426 reused by simple washing and drying process in several times, one has to sacrifice the efficiency.



427

428 **Figure 9.** Reusability of AR2 on removal of Rh and MB

429 **3.10. Antimicrobial and antioxidant activity studies of Cellulose nanofiber/Chitin**  
430 **nanocrystal hybrid aerogel**

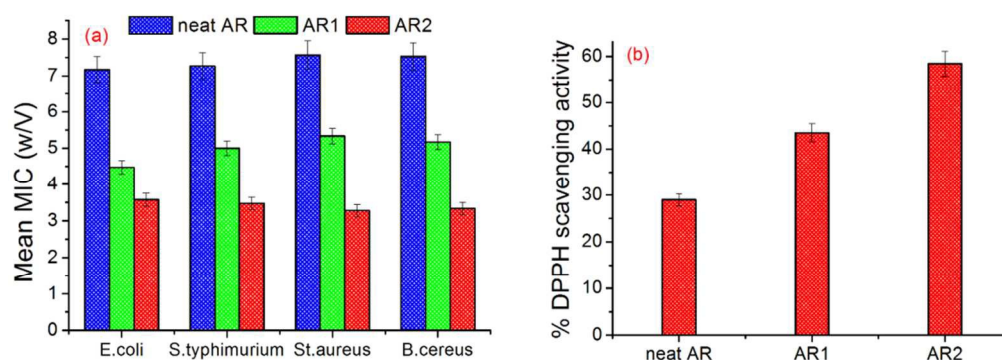
431 **Determination of antibacterial activity.** The discs such as neat AR, AR1 and AR2 were  
432 placed on Mueller-Hinton agar plates, previously swabbed with the target bacterial isolate at a  
433 concentration of  $10^6$  CFU/mL. In one disc, the respective organic solvent was added as negative  
434 control to determine possible inhibitory activity of the solvent. This preparation was incubated  
435 for a period of 24 h at 30 °C. Antibacterial activity was defined as the diameter of the clear  
436 inhibitory zone formed around the discs. The MIC of the extract was determined by tube dilution  
437 technique in Mueller-Hinton broth (supplied by Merck) according to NCCLS. The range of  
438 concentration used was 156.25 to 5000  $\mu$ g/mL. The four last vials of each bacterium with no  
439 growth from the MIC procedure were streaked onto nutrient agar (NA) plates. The antibacterial  
440 activities of aerogels neat AR, AR1 and AR2 against both gram-positive and gram-negative  
441 bacteria strains were examined by detecting of the minimum inhibitory concentrations (MICs).  
442 Each sample were assayed three times and the results are shown in the figure 10 (a) and table 3.  
443 From these results, it can be observed that the antibacterial activity of AR2 is the best among all  
444 other samples (neat AR and AR2) that produced zones of inhibition against *E. coli*, *S.*  
445 *typhimurium*, *St. aureus* and *B. cereus*.

446 **Determination of antioxidant activity.** The antioxidant activity of the aerogels neat AR,  
447 AR1 and AR2 was evaluated using DPPH (2, 2-diphenyl-1-picrylhydrazyl from Merck,  
448 Damstadt, Germany) radical scavenging assay. Briefly, 3 mL of aerogel dispersions were mixed  
449 with 1 mL of 1mM methanolic solution of DPPH. The mixture was vortexed and incubated in  
450 the dark at ambient temperature for 30 min. When the DPPH solution was mixed with the sample  
451 mixture which is acting as a hydrogen atom donor, a stable non-radical form of DPPH is

452 obtained with simultaneous change of the violet color to pale yellow. The absorbance was then  
453 measured at 517 nm. The percentage of DPPH free radical quenching activity was determined  
454 using the following equation

$$DPPH \text{ scavenging activity}\% = \frac{A_{DPPH} - A_{Extract}}{A_{DPPH}} \times 100 \text{ --- (8)}$$

455 where  $A_{DPPH}$  is the absorbance value at 517 nm of the methanolic solution of DPPH and  
456  $A_{Extract}$  is the absorbance value at 517 nm for the sample extract. Each sample was assayed two  
457 times and results shown in the figure 10 (b) and table 4. From this results, it can be observed that  
458 the antioxidant activity of AR2 was high compared to neat AR and AR1 as evaluated by free  
459 radical scavenging assay.



460  
461 **Figure 10.** neat AR, AR1 and AR2 for a) antimicrobial b) antioxidant studies

462 **Table 3.** Antibacterial properties of aerogels

Sample	Bacterial strains	MIC % (w/V)			Mean MIC % (w/V)
		Assay 1	Assay 2	Assay 3	
neat AR	<i>E. Coli</i>	6.2	7.5	7.8	7.17
	<i>S. typhimurium</i>	6.8	7.2	7.8	7.27
	<i>St. aureus</i>	7.1	7.6	8	7.57
	<i>B. cereus</i>	6.9	7.9	7.8	7.53
AR1	<i>E. Coli</i>	5	3.8	4.6	4.47
	<i>S. typhimurium</i>	4.8	5	5.2	5.00

	<i>St. aureus</i>	4.6	6.1	5.3	5.33
	<i>B. cereus</i>	5.2	4.8	5.5	5.17
AR2	<i>E. Coli</i>	3.2	4.1	3.4	3.57
	<i>S. typhimurium</i>	3.5	3.8	3.1	3.47
	<i>St. aureus</i>	3.1	3.6	3.1	3.27
	<i>B. cereus</i>	3.6	3.2	3.2	3.33

463

464

**Table 4.** Antioxidant properties of aerogels

Sample	DPPH scavenging activity %		Mean DPPH scavenging activity %
	Assay 1	Assay 2	
neat AR	25	33	29
AR1	41	46	43.5
AR2	55	62	58.5

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The CNC embedded bio-aerogels of cellulose (mainly AR2 which contains more CNC) in which the cells stick to each other and often these cells adhere to a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS). Furthermore, from all the analysis it is revealed that AR2 is showing a promising result of antibacterial and antioxidant properties compared to neat AR and AR1 which may be due to the presence of acetamide group in chitin. Hence it would be interesting to investigate the potentiality of this aerogel for possible applications in different fields.

472

#### 4. Conclusion

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A facile green method was used to fabricate multi-functional bio-hybrid aerogel based on CNF decorated with CNC. The morphology and properties of the new nanostructured hybrids were characterised using TEM, FESEM, DSC and FTIR. The chemical moieties of CNF, CNC and AR2 were analysed using FTIR spectroscopy and confirmed the presence of CNC on CNF aerogel. The CNF fibers showed wire like morphology having 60-120 nm in diameter while the CNC showed semi-square morphology having 20-100 nm in size. This semi-square morphology

479 for CNC has been reported for the first time as compared to other studies in the literature.  
480 Subsequently bio-hybrid aerogels are made with different percentage of CNC (1 % and 2 %)  
481 decorated on to CNF using environmentally friendly freeze drying method. Their morphologies  
482 were investigated with respective the reference (neat AR) and found maple like morphology for  
483 AR2. The multi functional efficiency of AR2 was evaluated using various studies such as dye  
484 water treatment, anti-bacterial and anti oxidant studies and realized that AR2 is a promising  
485 aerogel material for the above mentioned applications. Further studies to be anticipated in the  
486 future for industrial scale applications.

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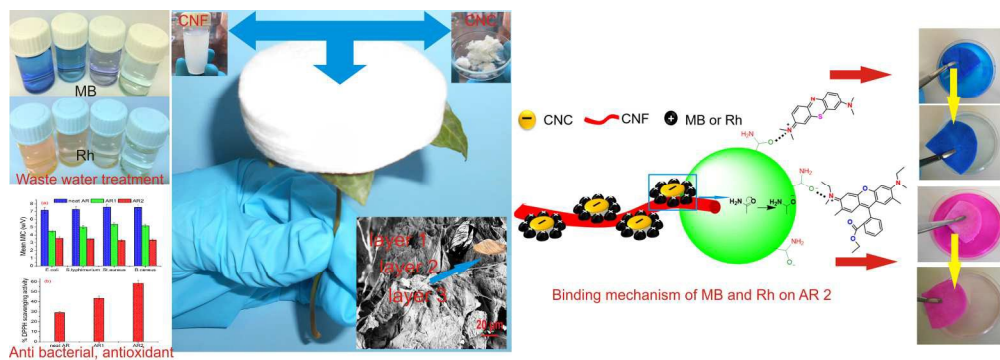
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