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Choline chloride–thiourea, a deep eutectic solvent for the production of chitin nanofibers

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ABSTRACT

Deep eutectic solvents (DESs) consisting of the mixtures of choline halide (chloride/bromide)–urea and choline chloride–thiourea were used as solvents to prepare α-chitin nanofibers (CNFs). CNFs of diameter 20–30 nm could be obtained using the DESs comprising of the mixture of choline chloride and thiourea (CCT 1:2); however, NFs could not be obtained using the DESs having urea (CCU 1:2) as hydrogen bond donor. The physicochemical properties of thus obtained NFs were compared with those obtained using a couple of imidazolium based ionic liquids namely, 1-butyl-3-methylimidazolium hydrogen sulphate [(Bmim)HSO_4] and 1-methylimidazolium hydrogen sulphate [(Hmim)HSO_4] as well as choline based bio-ILs namely, choline hydrogen sulphate [(Chol)HSO_4] and choline acrylate. The CNFs obtained using the DES as a solvent were used to prepare calcium alginate bio-nanocomposite gel beads having enhanced elasticity in comparison to Ca-alginate beads. The bio-nanocomposite gel beads thus obtained were used to study slow release of 5-fluorouracil, an anticancer drug.

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1. Introduction

Nanofibers (NFs) are extensively used in nanotechnology for the preparation of functional nanocomposites (Huang, Zhang, Kotaki, & Ramakrishna, 2003). The widely used technique to produce NFs is the electrospinning process, which uses an electrical charge to draw very fine fibers from polymer solutions (Subbiah, Bhat, Tock, & Parameswaran Ramkumar, 2005). NFs are also useful in various biomedical applications and hence substantial efforts are being made to develop biodegradable polymer scaffolds suitable for tissue engineering applications (How, Guidoin, & Young, 1992; Vacanti & Vacanti, 1997). Matthews, Wnek, Simpson, and Bowlin (2002) have attempted to prepare tissue-engineering scaffolds composed of collagen nanofibers by optimizing various parameters of electrospinning process (Matthews et al., 2002). Cellulose NFs were prepared directly from wood by Abe, Iwamoto, and Yano (2007).

Chitin, the (1–4)-2-acetamido-2-deoxy-β-D-glucan, is industrially produced from marine resources (Muzzarelli, 2012; Muzzarelli et al., 2012). Chitin and chitosan activate the macrophages, and are mucoadhesive, antimicrobial, biodegradable and nontoxic and therefore they are widely used for the repair of wounded human tissues (Muzzarelli, 2009). Chitin has been processed in the form of nanofibers (CNFs) or nanocrystals (CNC) by several research groups by employing various techniques, such as acid hydrolysis (Morin & Dufresne, 2002; Lu, Weng, & Zhang, 2004; Goodrich & Winter, 2007), TEMPO mediated oxidation (Fan, Saito, & Isogai, 2008a, 2008b), ultrasonication (Zhao, Feng, & Gao, 2007) and electrospinning (Jayakumar, Prabaharan, Nair, & Tamura, 2010). More recently, Isogai et al. (2012) have reported comparative characterization of aqueous dispersions and cast films of different chitin nano whiskers (Fan, Fukuzumi, Saito, & Isogai, 2012). Nanofibrillation efficiency of α-chitin in various organic and inorganic acids by varying the pH and ionic strength was reported (Qi et al., 2013). CNFs having good dispersibility in 2,2,2-trifluoro ethanol and preparation of its nanocomposite with polycaprolactone is reported (Ji, Wolfe, Rodriguez, & Bowlin, 2012).

Ionic liquids (ILs) are low melting point salts that form liquids at temperatures below the boiling point of water. Applications of ILs in carbohydrate and polysaccharide chemistry are increasing and they are being used to prepare a number of new compounds (El. Seoud, Koschella, Fidale, Dorn, & Heinze, 2007). Biomedical exploitation of chitin is currently under way with the aid of ionic liquids (Muzzarelli, 2011). Attempts are also made to process chitin in NF form using ILs, e.g., 1-ethyl-3-methylimidazolium acetate was used to dissolve crustacean shells and highly pure high molecular weight chitin powder as well as fibers was recovered (Qin, Lu, Sun, & Rogers, 2010; Barber, Griggs, Bonner, & Rogers, 2013). Kadokawa,

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Takegawa, Mine, and Prasad (2011) have demonstrated application of 1-ethyl-3-methylimidazolium bromide for the preparation of chitin nanowhiskers. Deep eutectic solvents (DES) are fluids obtained by heating two or more compounds capable of self-association through hydrogen bond interactions and have lower melting points in comparison to each of the individual components (Zhang, Vigier, de Oliveira, Royer, & Jérôme, 2012). Abbott, Boothby, Capper, Davies, and Rasheed (2004) for the first time presented DESs as suitable alternative solvents to ILs. DES has similar physicochemical properties in comparison to ILs but they are considered different from ILs mainly because (a) they do not entirely consist of ionic species and (b) they can be prepared from non-ionic species as well unlike the ILs (Zhao & Qu, 2013). Further, the DESs are more advantageous due to their cheaper cost and environmentally friendlier nature. Owing to these remarkable advantages, DESs are now of growing interest in many fields of research, e.g., catalysis, organic syntheses, dissolution media, extraction processes, electrochemistry and material chemistry (Zhang et al., 2012). We have demonstrated recently suitability of few bio-ILs and DESs for the solubilization of natural polymers such as DNA and α-chitin (Mukesh, Mondal, Sharma, & Prasad, 2013; Mondal, Sharma, Mukesh, Gupta, & Prasad, 2013; Sharma, Mukesh, Dibyendu, & Prasad, 2013).

Herewith, we report production of chitin nanofibers using a deep eutectic solvent (choline chloride–thiourea) and application of the nanofibers as reinforcement fillers for calcium alginate beads having ability to release 5-fluorourascil, an anticancer drug. The physicochemical properties of the NFs further compared with those prepared using a couple of imidazolium based ionic liquids namely 1-butyl-3-methylimidazolium hydrogen sulphate ($BmimHSO_4$) and 1-methylimidazolium hydrogen sulphate ($HmimHSO_4$) as well as choline based bio-ILs namely, choline hydrogen sulphate ($Chol.HSO_4$) and choline acrylate.

2. Materials and methods

2.1. Materials

Choline chloride, 1-methylimidazole, 5-fluorouracil and chitin powder obtained from crab shells were purchased from TCI Fine chemicals, Tokyo, Japan. The degree of polymerization of chitin from the origins was reported to be ca. 2000–4000 (Hasegawa, Isogai, & Onabe, 1994; Kurita, 2001). The degree of acetylation of the chitin sample was estimated by elemental analyses data and found to be 94.1%, which was in good agreement with that of standard chitin (Guinesi & Cavalheiro, 2006). The FT-IR spectra of the sample showed vibrational bands at 1662 cm^{-1} and 1631 cm^{-1} in the amide I region characteristic of α-chitin (Cárdenas, Cabrera, Taboada, & Miranda, 2004). The band at 1540 cm^{-1} corresponds to protein absorption, which are absent in FT-IR spectrum indicating absence of protein impurities in the sample. 1-Butyl-3-methylimidazolium chloride was purchased from Merck & Co., Germany. Sodium alginate was purchased from Sigma–Aldrich Chemical Co., USA. Thiourea, acrylic acid (AA), and $CaCl_2$ were purchased from S.D. Fine chemicals, Mumbai, India. All chemicals were of analytical grade and were used as received without further purification.

2.2. Measurements

Powder X-ray diffraction patterns were recorded at 298 K on a Phillips X'pert MPD system using CuK_α radiation ($\lambda = 0.15405$ nm) with 2θ range from 10° to 80° at a scan speed of 0.1° s^{-1}. The dry samples were placed on carbon coated copper grids (300 mesh sizes) and the transmission electron microscopic (TEM) images of thus prepared samples were obtained using a JEOL transmission electron microscope (Model JEM 2100, Japan) operated at accelerating voltage of 120 kV. The dry samples were dispersed in acetone and coated on aluminum stubs and evaporated to dryness followed by recording of their SEM images on an LEO 1430 VP instrument employing accelerating voltage of 18 kV. FT-IR analyses were carried out on a Perkin Elmer Spectrum GX, FTIR System, USA, by taking 2.0 mg of sample in 600 mg of KBr. All spectra were averages of two counts with 10 scans at a resolution of 5 cm^{-1}. ^1H NMR of the ILs and DES were recorded on a Bruker Avance-II, 500 MHz spectrometer. Linear viscoelastic properties (controlled deformation mode with 0.01% strain of Ca-Alg beads and Ca-Alg/CNF bio-nanocomposite beads were carried out on an Anton Paar, Physica MCR 301 rheometer, USA, using parallel plate PP50/P-PTD200 geometry (50 mm diameter; 0.1 mm gap) operating in dynamic mode. All the dynamic rheological data were checked as a function of strain amplitude to ensure that the measurements were performed in the linear viscoelastic region. The UV–vis absorption spectra of acidic ionic liquids were recorded on a Varian CARY 500 UV–Vis–NIR Spectrophotometer, USA. CNF solutions in distilled water were used for AFM sample preparation. Appropriately diluted nanofibers (0.05 mg/mL) solutions were deposited on freshly cleaved mica foil. After 2 min the solution was drained off and dried by nitrogen gas. The mica foil was then kept in dust free $CaCl_2$ desiccators for five days and then was used for AFM analysis. The AFM measurements were performed in the semi contact mode using an Ntegra Aura (Nt-Mdt, Russia) instrument at room temperature in air. The height of images was recorded from different area of each sample. The software used for image analysis was "Nova". Elemental analyses were carried out on a Perkin-Elmer CHNS analyser.

2.3. Synthesis of DESs and acidic ionic liquids

In a typical reaction for the synthesis of 1-butyl-3-methylimidazolium hydrogen sulphate [(Bmim)HSO_4], [(Bmim)]Cl (5 g, 0.0286 mol) was added into 20 mL dichloromethane in a round-bottom flask followed by drop wise addition of one equivalent of sulphuric acid (98%) (1.5 mL, 0.0286 mol) for 10 min at room temperature. The reaction mixture was refluxed for 24 h at 70 °C and the resulting IL was separated out, washed with ethyl acetate two times and dried at 70 °C for 6 h under vacuum. Similar metathesis reaction was carried out for the synthesis of the other two ILs, *viz*., choline hydrogen sulphate [(Chol)HSO_4], methyl imidazolium hydrogen sulphate [(Hmim)HSO_4] and choline acrylate. The structures were confirmed by ^1H NMR and mass spectrometry (ESI-MS).

DESs were prepared following the method described by Abbott et al. (2004). In a typical reaction, both the hydrogen bond donor (HBD) and acceptor (HBA) molecules were heated at 50 °C (reactions where urea was used as HBD) and 70 °C (reactions where thiourea was used as HBD) with constant stirring at optimized mole ratios as shown in Table 1 under argon atmosphere until homogenous and colorless liquids were formed (Supporting Fig. S1).

2.4. Preparation of α-chitin nanofibers

Pure chitin powder (at optimized concentration of 10%, w/w) was dispersed separately in DESs and acidic ILs followed by stirring at 500 rpm at 100 °C for different duration of time as shown in Table 1. The gel like materials thus obtained was diluted by adding 10 mL of distilled water. The dispersed chitin particles (Fig. 1) were collected after centrifugation at 10,000 rpm for 10 min followed by washing with distilled water to make them free from acid (Step 1). The acid free chitin was further dispersed in distilled water (30 mL) and the solution was ultra sonicated using an ultra sonication rod

Table 1
Preparation of chitin nanofibers in deep eutectic solvents and ionic liquids (chitin concentration with respect to solvent was 10% (w/w) and temperature of reaction was 100 °C).

Deep eutectic solvents		Optimized molar ratio (HBA:HBD)	Formation of nanofibers	Time (h)	Yield of nanofibers wt%	
Hydrogen bond acceptors (HBA)	Hydrogen bond donors (HBD)				DES	ILs
Choline Chloride	Urea	1:2	No	2	NA	–
Choline Bromide	Urea	1:2	No	2	NA	–
Choline Chloride	Thiourea	1:2	Yes	2	84	–
Ionic liquids						
(Chol)HSO_4			Yes	1	–	86
(Hmim)HSO_4			Yes	1	–	77
(Bmim)HSO_4			Yes	1	–	93
Choline acrylate			No	2	–	….

(35 amplitude, 0.5 cycle, 40 min). The NFs were isolated from this solution by lyophilization (Step 2). The water present in the ionic liquid/DES and water mixture obtained in step 1 was evaporated using a rotor vapor to obtain the recycled ILs/DESs. The purity of thus obtained solvents was checked by ^{1}H NMR.

2.5. Preparation of Ca-alginate/chitin nanofibers bio-nanocomposite (Ca-Alg/CNF)

Homogeneous mixture of 1% (w/v) of Na-alginate and 0.4% CNF in deionized water was taken in a surgical syringe attached to a needle. The mixture was then poured drop wise into 5% $CaCl_2$ aqueous solution under stirring. The Ca-alginate beads thus formed were isolated from the solution by filtration and washed several times with distilled water to remove adhered salts on the bead surfaces.

2.6. Release of 5-fluorouracil (5-UF) from Na-Alg/CNF gel beads

Homogeneous mixture of 1% (w/v) of Na-alginate, 0.4% CNF and 0.5 mg/mL of 5-UF in deionized water was taken in a surgical syringe attached to a needle. The mixture was then poured drop wise into 5% $CaCl_2$ aqueous solution containing 0.5 mg/mL of 5-FU under stirring (Supporting Fig. S2). The beads were lyophilized and known amounts of the thus obtained beads (500 mg) were accurately weighed and added to 25 mL of pH 7.4 phosphate buffer (PBS). The sample was placed in an ultrasonic bath (Elma, Germany) for 10 min to ensure maximum extraction of encapsulated 5-FU in the buffer. The sample was then made up to 10 mL using pH 7.4 phosphate buffer. An aliquot sample was assayed at 266 nm using an UV-spectrophotometer.

3. Results and discussion

We have recently observed that DES consisting of the mixture of choline chloride and urea or thiourea can solubilize chitin between 5 and 9% (w/w) (Sharma et al., 2013). Hundred to 600 mg of chitin (2–12%, w/w) was added into vials containing 5 g of solvent (separately for DES and ILs) placed in an oil bath with vigorous stirring at 100 °C for different duration of time (Table 1). It was observed that, chitin with concentrations up to 8% (w/w) produced dilute solutions making the isolation of the nanofibers very difficult. Upon dissolution, 9% (w/w) chitin gave formation of a viscous solution, while 10% (w/w) chitin produced soft gel which could be processed to the form of nanofibers following method shown in Fig. 1. However, the concentration of chitin higher that 10% (w/w) yielded relatively stronger gel and nanofibers having enhanced length and

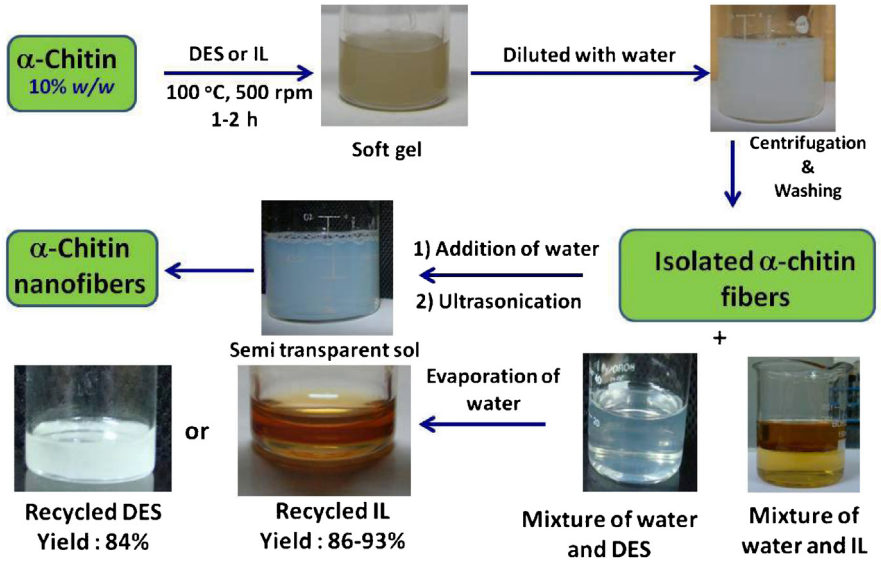

Fig. 1. Photographic demonstration for the preparation of α-chitin nanofibers and recycling of ionic liquid or deep eutectic solvents.

diameter (outside nano meter range) could be isolated. Considering these, chitin with 10% (w/w) was considered as optimized concentration for the preparation of the CNFs. After the isolation of the nanofibers the corresponding DES or IL could be recycled with reasonably good yield (90–92%) and was reused in the NF preparation process. It was observed that, the recovery of DES was bit lesser in comparison to the recovery of the ILs. It should be noted that, the DESs comprising of choline chloride/bromide and urea did not result formation of nano fibers. The isolated chitin from these solutions showed presence of agglomerated structures (Supporting Fig. S3) rather than fibers. On the other hand, chitin isolated from choline chloride–thiourea 1:2 (CCT 1:2) showed formation of nanofibers under SEM (supporting Fig. S4). The morphology of chitin before treatment showed presence of thicker fibers (Supporting Fig. S5). CNFs also could be isolated from all the other ILs except choline acrylate used in the investigation. Choline acrylate gave formation of fibers with agglomerated morphology, perhaps due to the thermal polymerization of the IL. Since the acid hydrolysis was the prime reason behind formation of CNFs, the acidity of the ILs as assessed using Hammett functions was responsible for the formation of the CNFs (Table S1). Further, the acid character of DESs perhaps helped the formation of the CNFs. The yield of CFNs prepared in CCT 1:2 was 84% (w/w), while the highest yield of 93% (w/w) was observed for the NFs prepared in (Bmim) HSO_4 followed by (chol)HSO_4 (86%) and (Hmim)HSO_4 (77%) (Table 1).

The FT-IR spectra of the CNF prepared using CCT 1:2, (Bmim)HSO_4, (Hmim)HSO_4 and (chol)HSO_4 showed vibration bands at 1663 and 1631 cm^{-1} characteristic of α-chitin (amide-I region), similar to the pure α-chitin, which indicates preservation of backbone structure of chitin in the NFs (Supporting Fig. S6). Powder XRD pattern of the CNFs prepared in the DES and the ILs (Supporting Fig. S7) mainly showed four diffraction peaks at around 9.5°, 19.5°, 20.9°, and 23.4°, which were assigned to 020, 110, 120, and 130 planes of chitin and corresponds to the crystalline structure of α-chitin and is in good agreement that with chitin powder (Supporting Fig. S7a). These data indicated that the crystalline structure of α-chitin remained intact during the preparation of the NFs and their reconstruction from the solutions (Kadokawa et al., 2011). ^{13}C solid NMR (SSNMR) showed presence of characteristic carbonyl band of the amide (δ176 ppm) for chitin in all the CNFs prepared in the above solvent systems. The representative spectra are shown in Supporting Fig. S8. All the above results confirmed the preservation of structural integrity of the biopolymer in the NFs.

The TEM images of the regenerated NFs from CCT 1:2 showed formation of NFs of diameter 20 ± 6 nm having length 1 μm ± 0.5 μm (Fig. 2a) (average of fifteen NFs). On the other hand as evident from the images (Fig. 2b–d) the NFs prepared in the ILs had a bit higher diameter and lesser length in comparison to the NFs prepared in the DES [e.g., diameter and length of 28 ± 6 nm and 1.5 ± 0.5 μm for NFs isolated from (Bmim)HSO_4.

The AFM images of the CNFs prepared from CCT 1:2 showed formation of fibers of width 25–45 nm and length 162–450 nm. The height of the fibers was ca. 40 nm. On the other hand the CNF prepared in (Hmim)HSO_4 had marginally higher width (30–40 nm) and substantially lower length (110–250 nm) in comparison to the NFs prepared in the above DES (Fig. 3). The NFs prepared in the rest of the ILs also showed the similar trend.

After the isolation of the NFs, the corresponding DESs or ILs were recycled and the purity of the recycled solvents was found to be equivalent to that with respective unprocessed solvents. The recycled solvents thus obtained were reused for the preparation of the CNFs following the same scheme shown in Fig. 1. The SEM and TEM images of the fibers showed formation of NFs of similar dimensions as obtained for the pure solvents proving the efficiency of the recycled solvents for reuse in the process (Supporting Figs. S9 and S10).

The calcium alginate beads embedded with the CNFs prepared in the DES were prepared as described above. The phase contrast

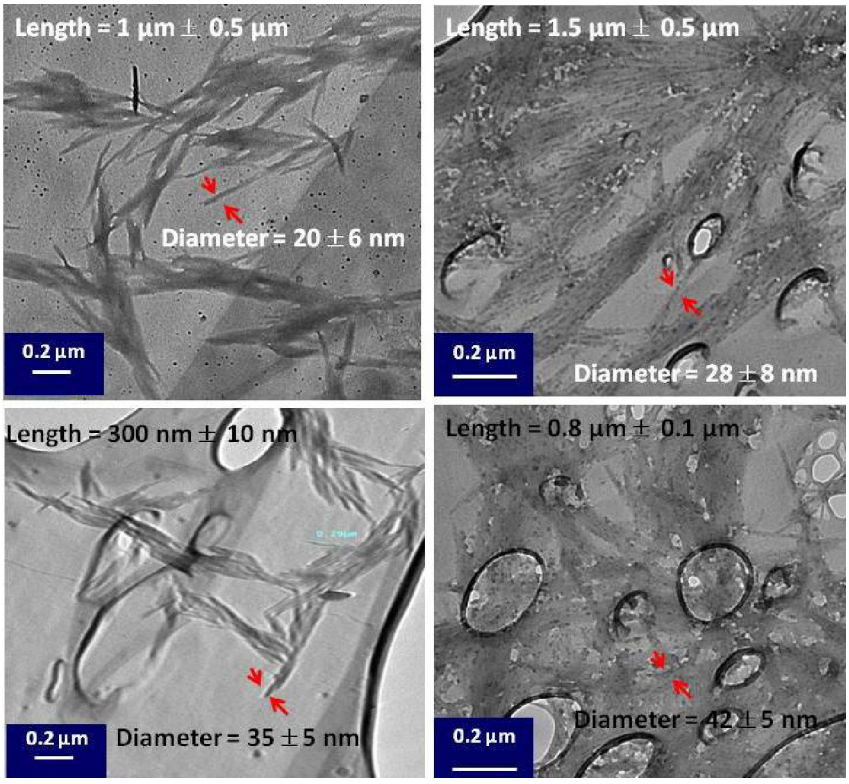

Fig. 2. Transmission micrographs of α-chitin nanofibers prepared using (a) choline chloride–thiourea 1:2 (b) (Bmim)HSO_4 (c) (Hmim)HSO_4 (d) (Chol) HSO_4.

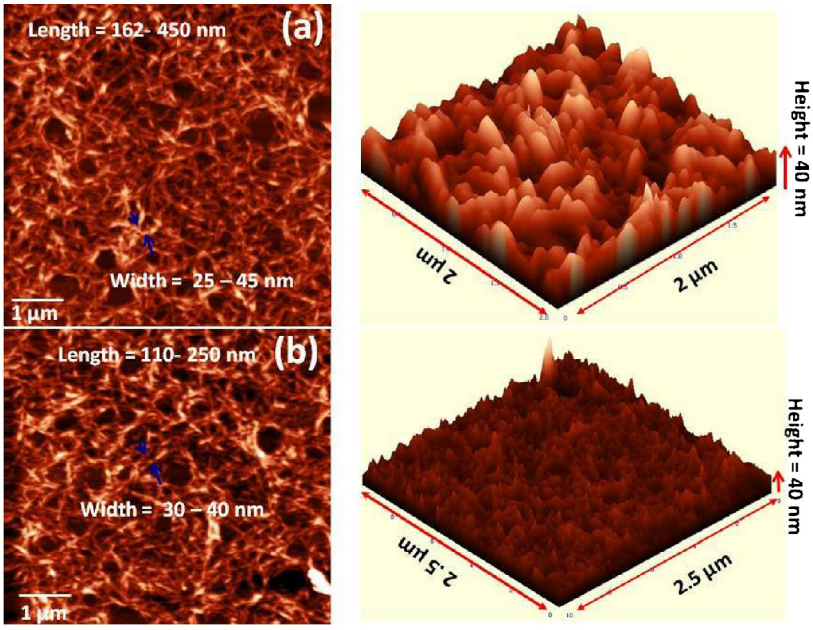

Fig. 3. Atomic force micrographs of α-chitin nanofibers prepared using (a) choline chloride–thiourea 1:2 and (b) (Hmim)HSO_4.

optical micrographs (100×) of the cross section of the beads showed presence of clusters of the NFs (Supporting Fig. S11). The FT-IR spectra of the bio-nanocomposite gel beads showed presence of CNFs in the beads (Supporting Fig. S12) evident from the appearance of the characteristic 1640 cm^{-1} band of the CNFs in bio-nanocomposite gel beads confirmed formation of the bio-nanocomposite. The elemental analyses of gel beads showed presence 65.5% (w/w) of CNFs in the gel bio-nanocomposite beads.

Monitoring of the $G'G''$ vs time of the 1% (w/w) Ca-Alg gel bead and Ca-Alg/CNF bio-nanocomposite gel beads showed a three fold enhanced values of both the moduli for the bio-nanocomposite beads in comparison to the Ca-Alg beads indicating higher elasticity for the composite gel beads resulted from the enforcement by the CNFs (Supporting Fig. S13).

Alginates have been widely used in pharmaceutical industry for controlled drug release. Arica, Calis, Kas, Sargon, and Hincal (2002) have used Ca-Alg beads as a drug slow release matrices for 5-FU and studied effect of polymer concentration and the drug loading on the release profile of the drug and observed about 80% release of the drug (Arica et al., 2002). To study the slow release of 5-FU from the Ca-Alg/CFN bio-nanocomposite and Ca-Alg gel beads, weighed amount of 5-FU loaded samples were put into a glass vessel containing 25 mL of phosphate buffer solution (pH 7.4) at 37 ± 0.5 °C. At scheduled time intervals, 1 mL of sample was removed from the vessel and the amount of 5-FU released from the matrices was determined by UV–vis spectrophotometer at 266 nm upto 24 h. It was observed that, bio-nanocomposite gel beads were able to release about 70% of the drug after 24 h at this pH, whereas the control Ca-Alg beads rapidly released 39% of drug after 3 h as shown in Fig. 4. The higher release rate and extended duration of release characteristics of the composite gel beads indicated the crucial role of the CNFs in the sustained release of the drug at pH 7.4 (pH of the colon).

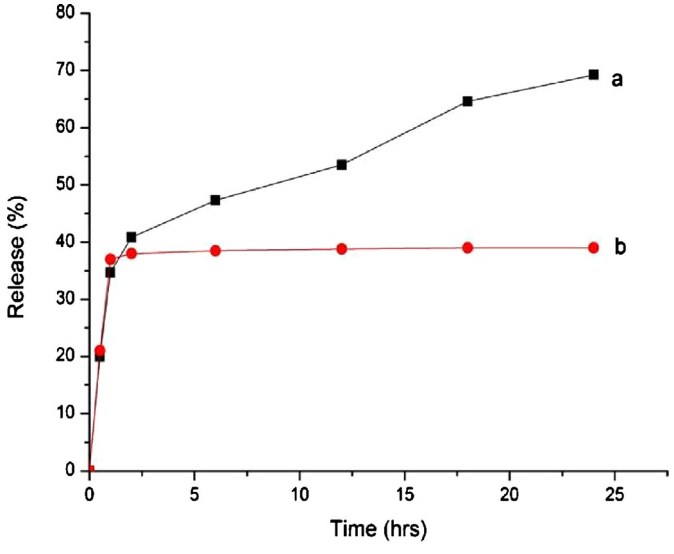

Fig. 4. In vitro release profile of (a) 5-FU loaded Ca-alginate/chitin nanofibers bio-nanocomposite gel beads and (b) calcium alginate beads.

4. Conclusions

α-Chitin nanofibers were prepared by solubilizing chitin in deep eutectic solvents comprising of the mixture of choline chloride and urea or thiourea as well as in a couple of imidazolium based and choline based bio-ionic liquids. DES comprising the mixture of choline chloride and thiourea yielded nanofibers, while the mixture with urea did not produce nanofibers. Formation of chitin nanofibers in all the acidic ionic liquids except choline acrylate was observed. The results demonstrated the suitability of the DESs for the preparation of chitin nanofibers. The efficiency of the nanofibers as enforcement filler for calcium alginate gel beads was also demonstrated by preparing chitin nanofiber embedded calcium alginate bio-nanocomposite gel beads with enhanced elasticity and ability to release 5-fluorouracil, an anti cancer drug at pH 7.4 (pH of the colon).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.12.082.

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