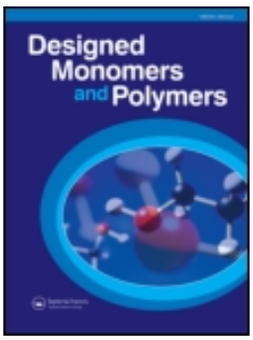

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In vitro biocompatibility and antimicrobial activity of chitin monomer obtain from hollow fiber membrane

A. V. Raut[a], R. K. Satvekar[a], S. S. Rohiwal[a], A. P. Tiwari[a], A. Gnanamani[b], S. Pushpavanam[c], S. G. Nanaware[a] and S. H. Pawar[a]

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ABSTRACT

This study for the first time shows the effective utilization and production of chitin monomers at laboratory level, with immense potential for its biomedical application. Low molecular weight (LMW) N-acetylglucosamine (GlcNAc) is prepared by depolymerization of chitin using chemical method coupled with a physical separation method. A novel filtration strategy exploiting polysulfone hollow fiber membrane is used for the preparation of GlcNAc particles with 94% yield within 8.5 ± 0.5 h. This high efficiency is analyzed using high-pressure liquid chromatography. The GlcNAc obtained was further analyzed using dynamic light scattering, first derivative Fourier transform infrared spectroscopy, and X-ray diffraction techniques. The antimicrobial properties of GlcNAc, chitin, and GlcNAc/chitin mixture were investigated using minimal inhibitory concentration against *S. aureus* and *E. coli*. Bacteriostatic property was exhibited by high molecular weight chitin, while GlcNAc and GlcNAc/chitin mixture (LMW) demonstrated bactericidal activity. Blood biocompatibility below 0.25 g/ml and cytocompatibility with NIH3T3 fibroblast cells and the proliferative efficacy suggested its utilization and suitability of these particles in biological applications.

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KEYWORDS
N-acetylglucosamine; chitin; polysulfone hollow fiber membrane; antibacterial; biocompatibility; hemocompatibility

1. Introduction

Chitin represents a class of linear polysaccharides comprising N-acetyl-D-glucosamine (GlcNAc) units linked with β-(1–4) glycosidic bonds.[1,2] A monomeric unit (GlcNAc) of the polymer chitin is naturally found in the cell wall of many fungi, mollusks, and beaks of cephalopods[3,4], which influences cell membranes, intercellular fluids and cell regeneration. GlcNAc is also a part of glycoproteins, proteoglycans, glycosaminoglycans (GAGs), and other connective tissue building blocks.[5–7] GlcNAc is extensively used to treat diseases such as osteoarthritis and inflammatory bowel disease. In addition, it has wide applications in dermatology including cosmetics as well as production of sialic acid and as a food supplement.[8]

Recent studies showed that the influence of material size are found to be good on micro- and nanostructured materials for cells' attachment and proliferation.[9,10] The degree of acetylation (DA) plays a key role in cell adhesion and proliferation of chitin and chitosan.[11,12] Lutolf et al. pointed if natural polymer such as GlcNAc is used to prepare extracellular matrix (ECM), it improvers morphology and function of stem cell growth in ECM.[13] All these points aid more value to the uniqueness of GlcNAc. Anything in high amount can be toxic, even though cytocompatible, the clearance pathway clearly showed excretion of GlcNAc in the urine which makes GlcNAc safe to be use.[14,15] Hence, the current research on the chitinous nanostructures is gaining importance.[16]

Chitin and chitosan are primarily characterized by its molecular weight (MW) and the DA for its antimicrobial activities. Commercial available chitin and chitosan are, respectively, <5% and >85% deacetylated with MW ranging between 100 and 1000 kDa.[17] Utilization of pure chitin and chitosan is good at low molecular weight (LMW) GlcNAc, especially for biomedical applications.[18,19] However, available commercial-grade LMW GlcNAc is not economically workable. Also, traditionally used chemical methods for the preparation of GlcNAc are found to be environmentally unfriendly while the enzymatic hydrolysis was time consuming. Hence in this study, we have focused on lowering the MW of chitin by physicochemical method. Use of polysulfone hollow fiber membrane (HFM) for ultrafiltration and separation of GlcNAc post chemical depolymerization is the novelty of this study to make it less expensive.

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2. Materials and methods

2.1. Materials

Chitin (DA = 90%) and chitosan (DA = 05%) were obtained from Sigma-Aldrich (USA). Standard GlcNAc was purchased from Sigma (India). Hollow fiber modules are purchased from IIT Kharagpur, India (Dr S. De Lab), with average membrane porosity of 0.22 and 0.58 µm and cutoff value 3 and 5 kDa respectively. Gram-positive bacteria *Staphylococcus aureus* ATCC 6538 and gram-negative bacteria *Escherichia coli* ATCC 25922 were obtained from Department of Microbiology, D.Y. Patil University, Kolhapur. NIH 3T3 embryonic mouse fibroblast cells were procured from NCCS, Pune, India. All other chemicals and reagents were of analytical grade and purchased from Loba Chemicals (India) unless specified.

2.2. Preparation of GlcNAc by physicochemical method

Depolymerization of chitin by acid treatment was done with modification in Scheel and Theim (1997) derived method.[20] In brief, chitin (2 g) was ground to a fine powder, placed into a flask, dissolved in 16 ml of 10 M HCl at 30 °C, and stirred for 10 min and incubated at 40 °C under continuous stirring for 8 h. Post incubation, reaction mixture was placed in ice water bath for about 30 min and then neutralized with 50% aqueous NaOH solution under steering condition and subjected to centrifugation at 10,000 *g* for 25 min at 5 °C. The clear supernatant was collected subjected to ultrafiltration using HFM.

The feed for the separation from the centrifuge supernatant was passed through the HFM with 3 kDa cutoff. The transmembrane pressure drop value was 68 kPa and the cross flow rate selected was 15 L/h. The operation was stabilized initially at 68 kPa for 1 h with distilled water at 15 L/h, and then the oligosaccharide sample was passed through the membrane. The schematic representation of the ultrafiltration system followed in this study was shown in Figure 1.

2.3. Particle size analysis

The hydrodynamic diameters of the nanoparticles were measured using a particle size analyzer (PSS NICOMP™ 380 DLS, Santa Barbara, California, USA) with a 7.5 mW laser diode at 635 nm. The scattered intensity was detected by a photomultiplier detector. At room temperature with a scattering angle of 90 , size determination of the nanoparticles was carried out in double-distilled water.

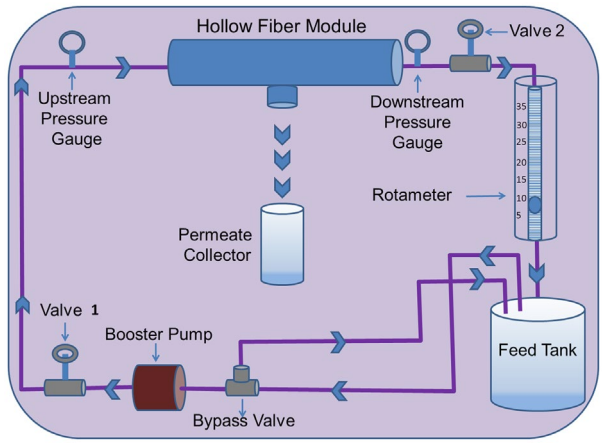

Figure 1. The schematic diagram of the experimental setup.

2.4. Fourier transform infrared spectroscopy

The DA of the samples was determined using Fourier transform infrared spectroscopy (FTIR) alpha ATR Bruker (Eco ATR). The solid samples were analyzed in the range 500–4000 cm^{-1} at room temperature. The sample was placed on the ZnSe ATR crystal and recorded at a resolution of 4 cm^{-1} with 96 scans. The standard DA values are calibrated by relating a DA-independent reference band (RB) with absorbance of a DA-sensitive probe band (MB).

2.5. X-ray diffraction

X-ray diffractograms was obtained using a Bruker AXS D8 Advance X-ray diffractometer under: 40 kV and 40 mA with Cu Kα1 radiation at 1.54184 Å and an acceptance slot at 0.1 mm. About 20 mg of the sample was spread on a sample stage, and the relative intensity was recorded in the scattering range (2 h) of 5–30° in steps of 0.1°. The crystalline index (CrI; %) was determined in two ways: $CrI_{020} = (I_{020} - I_{am}) \times 100/I_{020}$, where I_{020} is the maximum intensity below 13° and I_{am} the intensity of amorphous diffraction at 16°. For comparison, another crystallinity index was expressed as CrI_{110}, following the equation above and using I_{110}, where I_{110} is the maximum intensity at ~20°.[21]

2.6. High-pressure liquid chromatography

With reference to high-pressure liquid chromatography (HPLC), 0.1 mg of sample was taken and analyzed by HPLC using acetonitrile: water (60:40 v/v) as a mobile phase at a flow rate of 1.0 ml/min in an isocratic elution mode. Before delivering the mobile phase in to the system, it was degassed and filtered through 0.20-µm syringe filter. Injection volume was 20 µL, and the detection was

performed at 215 nm. The mobile phase is degassed by the sonication of mobile phase.[22]

Separation of analytes was carried out in Jasco HPLC-DAD system containing Kyatech C18 column (250 × 4.6 mm internal diameter, particle size 5 µm). About 20 µL sample was injected to chromatography system using Rheodyne injector. PDA detector used in this HPLC system was UV 2070 detector (Czerny turners mount monochromator) with deuterium lamp as light source.

2.7. Antimicrobial studies

Antibacterial activities of chitin and GlcNAc were examined as the inhibitory effects against the growth of gram-positive bacteria *S. aureus*, and gram-negative bacteria *E. coli*. The lowest concentration of chitin and GlcNAc required to completely inhibit bacterial growth after incubation at 37 °C for 72 h is used as minimum inhibitory concentration (MIC).[23] For determination of the MIC of chitin and GlcNAc solutions, (1% (w/w) in 1% (w/w) acid) of each substances were added to Muller Hinton agar for final chitin and GlcNAc concentrations of 0.1, 0.05, 0.01, 0.006, 0.003, and 0.001% (w/v). The sample solution of 1% (w/v) of each chitin, GlcNAc, and chitin:GlcNAc (1:1) was prepared in nutrient broth which was sterilized by autoclaving before study and incubated with shaking at 37 °C. The inhibitory effect was estimated periodically by measuring the turbidity of the cultured medium at 640 nm using a UV–visible spectrophotometer and compared using McFarland standards.

Plots were made of the optical density (OD) (i.e. absorbance at 640 nm) vs. the culture time for each of the gram-positive bacteria and the gram-negative bacteria tested by the shaking flask method. Inhibitory effects against growth due to antibacterial activities of chitin, GlcNAc, and chitin:GlcNAc (1:1) would be indicated by a levelling off of the slopes of the curves.

2.8. Biocompatibility studies

Biocompatibility assessment was made under *in vitro* conditions in a form of hemocompatibility and cytotoxicity studies.

2.8.1. Hemocompatibility

For hemocompatibility studies, 20 ml of blood was obtained from a blood bank. Erythrocytes were collected by centrifugation (2000 × *g*, 5 min, 4 °C) in the presence of a washing solution (175 g sorbitol + 8 g NaCl in 1 l water). The final pellet was resuspended in the washing solution to give a 2% (w/v) solution. About 100 µl of the obtained erythrocyte solution was then transferred to each well of a 48-well microtitration plate. To the same wells, about 200 µl of a 0.25 mg/ml solution of the test substances dissolved in the washing solution was added and incubated for 1 h. The microtitration plate was then centrifuged at 1000 *g* for 10 min, and the supernatants (100 µl) were transferred into another microtitration plate. Hemoglobin release was determined photometrically with a microtitration plate reader (Epoch; BIOTEK, United States) at 570 nm. A 0.25 mg/ml solution of ascorbic acid was used as negative control, whereas the distilled water was used as a positive control causing 100% hemoglobin release. Results were expressed as the amount of hemoglobin release induced by the test substances in percentage of the total amount.

2.8.2. Cytotoxicity

The NIH 3T3 embryonic mouse fibroblast cells cultures were maintained in DMEM supplemented with 10% fetal bovine serum, 200 mM glutamine, 2 mg/ml sodium bicarbonate, and 1 × antibiotic and antimycotic solution. The medium was replaced periodically. The cells were cultured in tissue culture flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2. About 0.05% Trypsin was used to detach the cells.

Cell viability assessment study was carried out for the sample GlcNAc, and chitin alone acts as a standard in precoated 12-well culture plates. In brief, stock solution of 1% GlcNAc and 1% chitin was prepared in PBS and added to culture plates and subsequently oxidized with periodate and subjected to air drying at 40 °C. The control wells were free from the GlcNAc and chitin particles. The dried plates were then surface sterilized with 70% alcohol for 30 min and then UV sterilized for 1 h. A cell density of 3×10^4 cells per well was seeded and incubated with the growth medium for the period of 6, 12, 24, and 48 h.

The cell viability was quantified by MTT assay. With regard to the MTT assay, the culture medium of each well was replaced with MTT (5 mg/ml diluted in serum-free medium) and incubated at 37 °C for 4 h. After the removal of MTT solution, dimethyl sulfoxide was added and the medium was then left at room temperature for two minutes. The absorbance was then measured at 570 nm using a plate reader (Epoch, BIOTEK).

2.9. Statistical analysis

Statistical data analysis was performed using 'Student *t*-test' with $p < 0.05$ as the minimal level of significance. Calculations were done using the software Xl stat Version 5.0.

3. Results and discussion

3.1. Particle size analysis

Figure 2 (a)–(d) shows dynamic light scattering (DLS) measurements of the colloidal solution of GlcNAc before and after ultrafiltration methods followed in this study. The

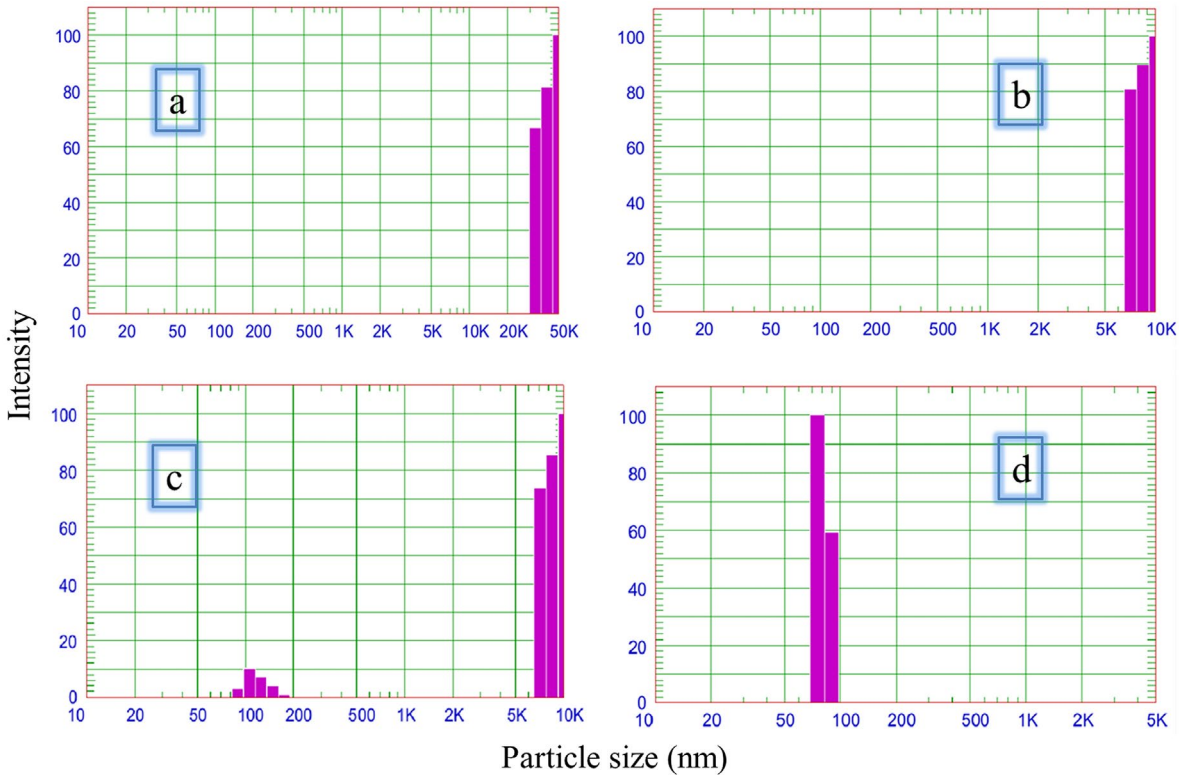

Figure 2. Size distribution of solutions containing chito-oligosaccharide/monosaccharides; (a) solution without any treatment, (b) solution after acid treatment, (c) particles separated by the membrane, and (d) Particles present in solution after filtration.

average diameter of the particles of the colloidal sample after chemical depolymerization was measured as ~42.3 µm (Figure 2(a)). The reduction in particle diameter to ~8.5 µm (Figure 2(b)) was observed after centrifugation. Additional acid treatment of the sample further reduces the particle diameter. Figure 2(c) depicts two crests corresponding to ~116.3 nm and extensive top at ~8.4 µm. The ultrafiltration method followed in this study offered particle size of ~83.0 nm (Figure 2(d)). The mean size of the most diminutive molecule obtained in this study matches with the report.[24]

The observations from DLS measurements suggested that the major particle size when taken in water was about 42 µm large. After centrifugation, the size reduced to 20%, i.e. 8 µm. Additionally, after the acid treatment, we obtained two crests, first peak stretching from 70 to 180 nm and a second one at 8 µm. The peaks at ~83.0 nm were not seen initially (Figure 2(b)). The acid treatment could give 90% small particles and rest of the major particles in one cycle. Selection of 3 kDa HFM for the separation of colloidal solution of GlcNAc was based on results obtained from HFMs of varying porosity (Table 1 and Figure 1, in supplementary data). A hollow fiber module with 3 kDa membrane was successfully used for the separation of GlcNAc monomers from larger size chitin particles. The standard deviation of the GlcNAc was found to be 2.49% which indicates a narrow size distribution.[25]

3.2. X-ray diffraction

Chitin and chitosan found in crab and shrimp shell wastes are in α-form.[21] The sample was compared for crystalline reflections with the original sample in the 2θ range of 5–35°. All samples' indexed values are found to be at (0 2 0), (1 1 0), (1 2 0), (1 0 1), and (1 3 0). The maximum peak of intensity was found to be at (1 1 0) reflection increased with the decrease in DA and moved to a higher angle, and the second maximum peak of intensity at (0 2 0) reflection also increased with the decrease in DA. These results are in accordance with Zhang et al.[21] Hence, a crystalline index (CrI;%) expressed as $CrI_{020} = (I_{020} - I_{am}) \times 100/I_{020}$ is used and another equation using I_{110} is expressed as $CrI_{110} = (I_{110} - I_{am}) \times 100/I_{110}$.[26]

Figure 3 gives the calculated peak fitting of the diffraction profiles. The calculated d-spacing and relative intensity is shown in Table 1. It was found that the d-spacing of the (1 1 0) plane was not unchanged and was 100% throughout. In Figure 4, the DA represented by CrI_{020} and CrI_{110} was found to be 84.36 and 86.23% respectively.

3.3 DA determination by first derivative ATR-FTIR

The accurate λ value was obtained using a Savitzky Golay algorithm of first derivative spectrum by smoothening FTIR spectrum of GlcNAc at 11 point.[27] The GlcNAc %DA

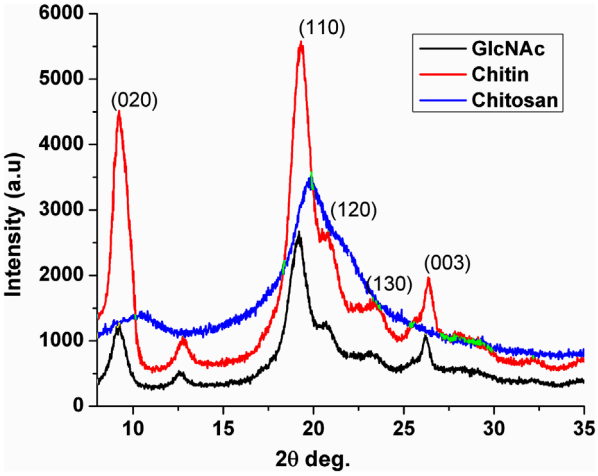

Figure 3. X-ray powder diffractograms of chitin, chitosan, and GlcNAc.

Table 1. XRD parameters of chitin, chitosan, and GlcNAc.

Name	2θ (°)	d-spacing (Å)	Relative intensity (%)
Chitin	9.2233	9.58553	84.22
	12.7431	4.59539	20.16
	19.3093	6.94470	100.00
	20.8119	4.25000	19.37
	23.4509	3.75000	12.07
	26.4454	3.44000	19.65
GlcNAc	9.2416	9.56659	42.90
	12.6337	7.00459	9.97
	19.1870	4.62441	100.00
	20.7230	4.28498	40.81
	26.2753	3.39074	22.56
Chitosan	10.5054	8.418392	40.42
	19.7807	4.486926	100.00

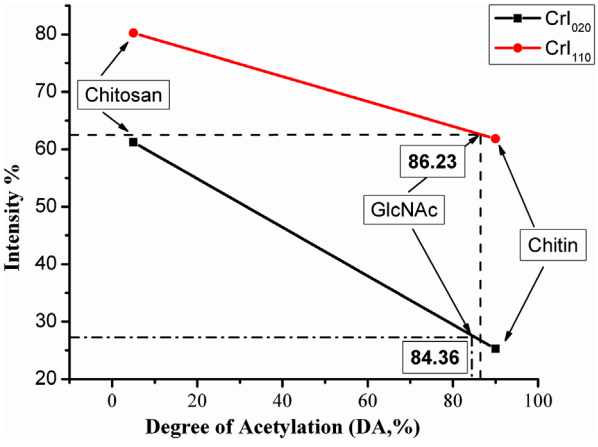

Figure 4. Crystalline index (CrI_{020}) and (CrI_{110}) as a function of the degree of acetylation (DA).

was found to be 85% after plotting with the known standard sample of chitin and chitosan with %DA, 90 and 05%, respectively (Figure 5). The values from X-ray diffraction (XRD) analysis and the values of DA give similar result. These results are consistent and validate by comparing

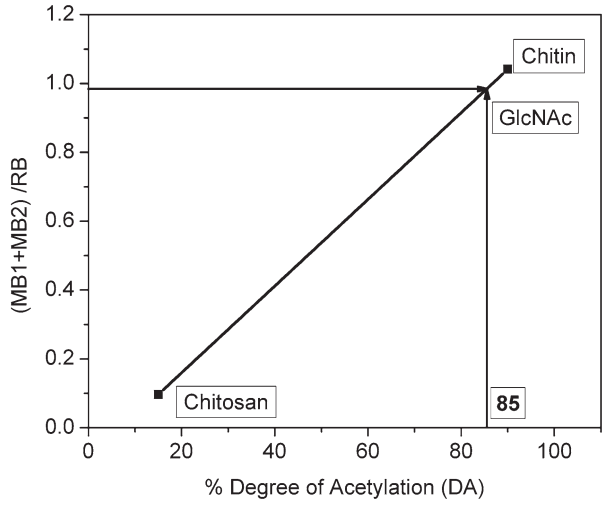

Figure 5. The calibration plot of the ratio (MB1 + MB2)/RB from first derivative ATR-FTIR spectra with the %DA of standard chitin and chitosan with the sample.

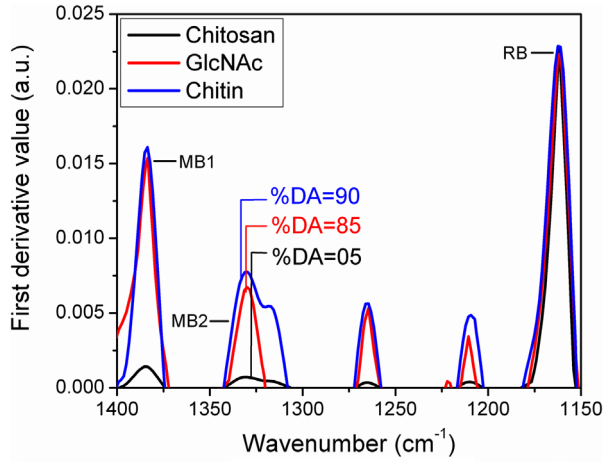

Figure 6. First derivative ATR-FTIR spectra of chitosan samples with differing DA values of chitin and chitosan.

with first derivative UV spectroscopy developed by Wu and Zivanovic.[27,28] This shows a good linear correlation between both the methods.[27,28] The RB is found at 1161 cm^{-1} which is in accordance with the Beil et al (2012); this could be because of bridge oxygen stretching in the first derivative IR spectrum. The CH-deformation band 1383 cm^{-1} (MB1) [29] and the amide III band at 1329 cm^{-1} (MB2) is considered as best probe band [27] (Figure 6).

Samples were used to determine the absorbance ratios of the amide II band at 1552 cm^{-1} to the CH-stretching band at 2878 cm^{-1} for transmission IR spectra.[30] Due to non-uniform contact with the ATR crystal, the determination of DA of chitin has poor resolution, hence the advance ATR technique was also not appropriate.[31] The main disadvantages of IR spectroscopic methods were the

amide I and the amide II band and the difficulty of drawing baselines in the IR spectra as well as the interference liability for the water content of the samples which affect the CH-stretching band of chitin.[32] Stephan et al. (2012) suggest the first derivative ATR-FTIR technique that the anhydrous mass is not needed since the bridge oxygen stretching band is used as internal standard. Also as the spectral range of the water absorption is avoided, there is no disturbance to determine by IR absorption of water.[27] The first derivative ATR-FTIR approach also avoids the drawing of baselines and allows the measurement of chitosan and chitin samples with high water content.

3.4. HPLC detection

The sample and the standard chitin solutions were analyzed by HPLC method. The results showed that GlcNAc was the only product obtained at 2.0 min retention time, which is similar to standard (Figure 7(a), (b)). This result confirms that the HFM with 3 kDa cutoff is able to separate GlcNAc monomers after high acid treatment. The said peak also matches with the result of Waghmare and Ghosh.[22]

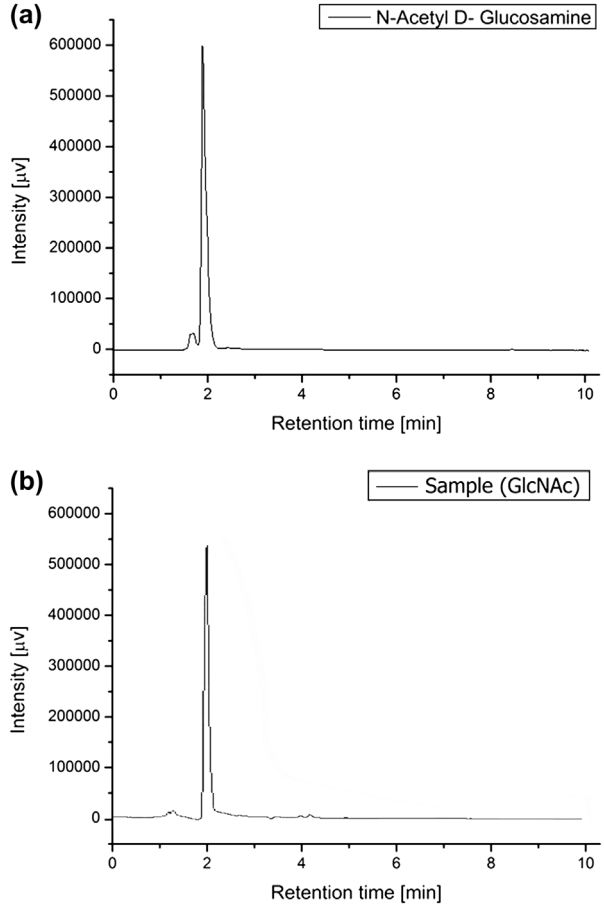

Figure 7. HPLC analysis of filtrate and GlcNAc detected by monitoring absorbance at 215 nm.

Current physiochemical method is compared with different types of production methods and their characteristics are shown in Table 2.

Production of GlcNAc from chitin has been carried out by several chemical,[33] physical,[34] enzymatical [35], and biotransformation [36,37] methods with some advantages and disadvantages. Irrespective of the hydrolysis methods followed, separation of monomers is challenging, the ultrafiltration technique is the most promising method to get monomers. The ultrafiltration technique involves various membrane modules in the form of plate and frame, tubular, spiral wound, etc. Ultrafiltration using HFM modules has not been approached yet for the monomers. The unique advantages in using HFM module include (i) large membrane area per unit volume leading to saving of space, (ii) ease of back flushing after the operation, (iii) ease of handling and operation, and (iv) less time consumption.[38,39]

3.5. Antibacterial activity

Figure 8 shows the plots of the OD (i.e. absorbance at 640 nm) versus the culture time for 0.1% (w/v) chitin, 0.1% (w/v) GlcNAc, and 0.1% (w/v) chitin: GlcNAc (1:1) against one gram-positive *S. aureus* ATCC 6538 bacteria and one gram-negative pathogenic *E. coli* ATCC 25922 bacteria as measured by the shaking flask test method. Fewer the microbial cell, smaller the OD of the medium at 640 nm absorbance, which suggest the higher antimicrobial activity of tested material.

The bacterial numbers increased rapidly with time in the control group. The stationary phase was reached after only 3 h of incubation in *S. aureus* and 4 h in *E. coli*. Chitin shows similar growth activity with inhibitory effect against both organisms, and stationary phase was reached at 3 and 4 h of *S. aureus* and *E. coli*'s incubation period. At 640 nm absorbance, the OD in the control *S. aureus* group was 0.39 (i.e. 1.17×10^8 cell/ml) and control *E. coli* group was 0.33 (i.e. 0.99×10^8 cell/ml), but was only 0.27 (i.e. 0.81×10^8 cell/ml) in both *S. aureus* and *E. coli* groups, for the experiments with chitin. So it is clear that chitin has inhibitory activity against *S. aureus* and *E. coli*. The antibacterial activity of chitin is evident from 30.76 to 18.18% killing of the *S. aureus* and *E. coli* bacteria, respectively, compared with the untreated control. The studies by Gerasimenko et al. give reproducible results reported on antimicrobial activity of chitosan on *S. aureus*.[46] Obtained results clearly indicate that MW of chitosan is inversely proportional to the cell growth. Similar results have been shown by various groups.[46,47,50] In the current study, treatment with the GlcNAc, alone and in combination of chitin:GlcNAc (1:1), completely inhibited the growth of *S. aureus* after 3 and 5 h, respectively, and *E. coli* is inhibited after 3.5 and 6 h,

Table 2. Comparison of different types of production methods and their characteristics.

Sr. no.	Type of production	Time	Different processes	% yield	Antibacterial and biocompatibility studies of material
1	Chemical method	–	15–36% HCl for 40–80 °C [33]	Bellow 65% [33]	N acetyl chito-oligosaccharides (12 kDa) affects *S. aureus* at MIC 0.003 in 2.5 h and *E.coli* at MIC 0.003 in 3.5 h [40]
		–	10% methanol Used as solvent and filtration and crystallization [34]	43% [34]	
		–	Using pyridine as a solvent and further purification [41]	Higher than 70% [41]	
			Ozone treatment and recrystallization process [42]	Higher than 70% [42]	
2	Enzymatic method	10 days	Crude enzymes derived from Aeromonas hydrophila H2330 processed with α-chitin [43]	77% [43]	Chitosan with molecular weight <5–300 kDa shows complete inhibition of *E.coli* as concentration of chitosan increases from 0.25 to 1% for 2 h [44]
		–	Crude chitinases from Bacillus licheniformis SK-1 were used to digest α-chitin powder [45]	41% [45]	
		–	Enzyme was used to digest hydrolysis of β-chitin [6]	~ 100% [6]	
3	Improved enzymatic method	8 days	1% β-chitin hydrolysis with various commercial enzymes, such as cellulose, hemicellulase, papain, lipase, and pectinase [33]	76% [33]	*D*-glucosamine residues and a few *N*-acetyl-glucosamine units (5–27 kDa) exhibit 38.0% *E.coli* cell death for 5 kDa and 80.0% *E.coli* cell death for 27 kDa in 2 h. With increasing %DD, there is an increase in cell number (less toxic with high %DD) [46]
		10 days + 28 h (1 cycle)	Continuous production system with purity greater than 98% [35]	Higher than 76% [35]	
4	Biotransformation method	–	Novel chitin-degrading microbe named Chitinibacter tainanensis act on α-chitin [37]	75% [37]	Chito-oligomers (4 kDa) (78% Yield) executes all *E.coli* cell in 35 h and *S. aureus* have little effect on death [47]
		–	Novel chitin-degrading microbe named Chitinibacter tainanensis act on β-chitin [48]	98% [48]	
		8 h	GlcNAc was generated from S. coelicolor A3(2) (ScHEX) as final product with a yield over 90% after 8 h incubation.[49]	90% [49]	
5	Physiochemical method	8 h + (20–60) min for filtration	HCl used to depolymerize the chitin and HFM separated GlcNAc with purity greater than 90% (Current paper Raut et al.)	Higher than 94% (refer supplement Figure 2) (Current paper Raut et al.)	GlcNAc (N acetyl glucosamine) (yield 94%) particles are completely bactericidal to *E.coli* and *S. aureus* with 3.5 and 3 h time rate, respectively. Hemostasis was observed in all samples, and there was no hemolysis with an increase in fibroblasts' cell growth.

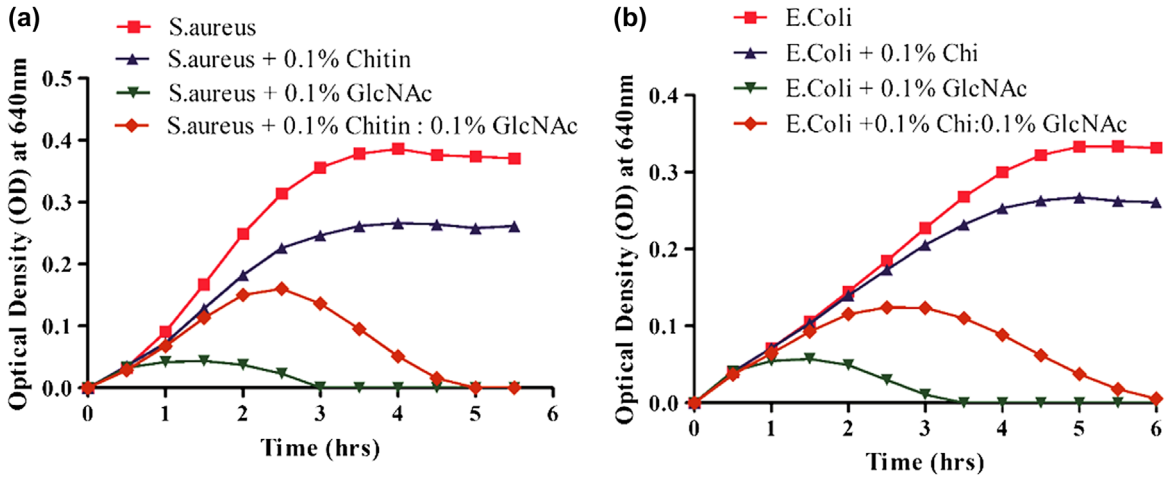

Figure 8. Effect of chitin, GlcNAc, and chitin:GlcNAc (1:1) on the growth of (a) *Staphylococcus aureus* ATCC 6538 and (b) *Escherichia coli* ATCC 25922.

respectively, of incubation time (Figure 8). Benhabiles (2013) also reported similar result, where *S. aureus* and *E. coli* when treated with N-acetyl chitooligosaccharides (NAc-COS) and chitooligosaccharides (COS) has completely inhibited the growth within 2 h.[40,51] Chitosan with a higher degree of deacetylation tends to have a higher antimicrobial activity.[52] Other have reported that COS with LMW has higher antimicrobial effect against *E. coli* than

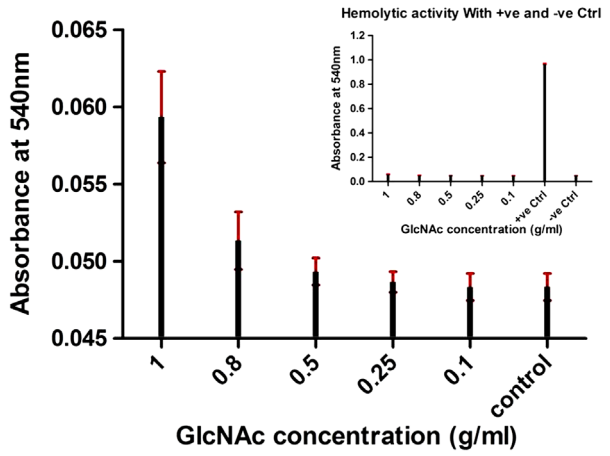

Figure 9. Hemolysis measured at the concentration range of 0.1–1 g/ml incubated with RBCs at 25 °C for 2 h at 540 nm absorbance. In insert figure, hemolytic activity is compared with positive and negative controls with different concentrations.

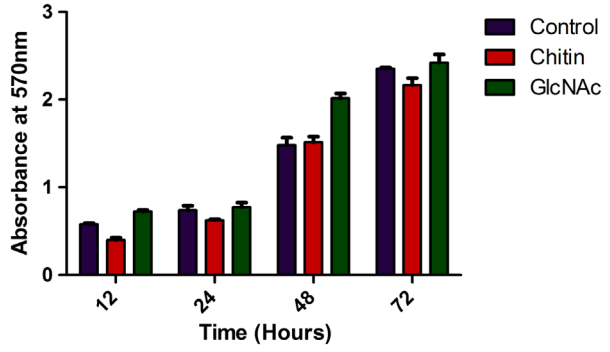

Figure 10. Biocompatibility of GlcNAc and chitin. NIH 3T3 fibroblasts incubate with 1 mg/ml concentrations of GlcNAc and chitin. 12–72 h incubation time noted by MTT assay for determining cell viability.

water insoluble high molecular weight (HMW) chitosan.[44] Moreover, chitosan with MW of 224 and 1106 kDa possessed weak or no antibacterial activity against microbe, compared to chitosan with a MW = 28 kDa is observed by Jeon et al.[53]

3.6. Hemocompatibility

When polymer comes in physical contact with blood, they interact with the RBCs which can lead to RBC lysis or RBC disfunctioning, because of which the hemocompatibility is one of the essential tests to be performed. Interactions of negatively charged red blood cell membrane with the monomer of the different concentration were studied using blood biocompatibility. The quantification of hemoglobin release finds the membrane-damaging property of the tested compound. Results of this study are shown in Figure 9. After 1 h of incubation of RBC's with GlcNAc, a direct correlation between membrane-damaging effect of RBC's and concentration of GlcNAc is observed. Visually, there was hemostasis involvement in each concentrations tested. Red blood cell lysis depends on the concentration of the compound in the solution.

3.7. In vitro *cytotoxicity evaluation*

Biocompatibility is thought to be influenced by diverse properties of the polymers such as (i) structure and sequence (linear, branched, block, random), (ii) charge density and type of the cationic functionalities, (iii) MW, and (iv) conformational flexibility.[54–56] Figure 10 shows biocompatibility of the GlcNAc with respect to relative cell viability of NIH 3T3 fibroblast cells. Both the test compounds support the adherence and proliferation of fibroblast cells.

A quick effective method (MTT assay) was used for testing the cell viability, and this correlates well with cell proliferation. Thus in recent years, it has been often used as a preliminary screen to evaluate *in vitro* cytotoxicity of polymeric components.[54] No significant difference in the cell count was observed between the samples. With respect to concentration, the polymer displays toxicity compared to the monomer. An increase in cytotoxicity as a function of the MW, noticing in this study for chitin, was also reported for other polymers, for example, poly-L-lysine [56,57] dendrimers [58] and poly(ethylenimine).[59]

The ability of mammalian cells to respond to exogenous GlcNAc has not been well studied. Increase in cell viability in the presence of GlcNAc observed in this study shows connection with the cellular mechanisms to regulate their expression. This conclusion has been arrived based on the presence of respective genes in the chosen cells to catabolize GlcNAc, according to Naseem et al.[60] Changes in nutrition elevates GlcNAc synthesis leading to the increase in formation of UDP-GlcNAc which is reported as a substrate for the enzyme O-GlcNAc transferase (OGT).[61] These OGT modifies proteins by catalyzing the transfer of the GlcNAc moiety of UDP-GlcNAc to Ser or Thr residues on proteins. Many of the substrates that have been identified for OGT play key roles in cellular regulation, such as the transcription factors c-myc, p53, and NFk-B.[62]

4. Conclusion

Modified chemical method followed by physical separation techniques using 3 kDa HFM successfully yields chitin monomer GlcNAc, in the form of monodispersed colloidal solution in nano range in short duration (480 min + 20–60 min). This study is an attempt to simplify

the industrial process of LMW chitin preparation, yet to achieve high purity without compromising on yield. With 90% purity and 94% yield obtained, GlcNAc was found to be biocompatible and hemocompatible. The activity of the chitin revealed bacteriostatic condition, whereas in the presence of GlcNAc, shows bactericidal activity against gram-positive and gram-negative microorganism. The utilization of GlcNAc as compared to chitin was significantly good. GlcNAc prepared by employing this method has vast potential to support various biomedical applications. A versatile bioactive biomaterial (GlcNAc) prepared by physiochemical method and separated by HFM module is a novel and efficient method to obtain LMW chitin to our knowledge.

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Disclosure statement

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