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Novel terephthaloyl thiourea cross-linked chitosan hydrogels as antibacterial and antifungal agents

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ABSTRACT

Four novel terephthaloyl thiourea chitosan (TTU-chitosan) hydrogels were synthesized via a cross-linking reaction of chitosan with different concentrations of terephthaloyl diisothiocyanate. Their structures were investigated by elemental analyses, FTIR, SEM and X-ray diffraction. The antimicrobial activities of the hydrogels against three species of bacteria (*Bacillis subtilis*, *Staphylococcus aureus* and *Escherichia coli*) and three crop-threatening pathogenic fungi (*Aspergillus fumigatus*, *Geotrichum candidum* and *Candida albicans*) are much higher than that of the parent chitosan. The hydrogels were more potent in case of Gram-positive bacteria than Gram-negative bacteria. Increasing the degree of cross-linking in the hydrogels resulted in a stronger antimicrobial activity.

1. Introduction

Hydrogels are a cross-linked network of linear hydrophilic polymers that do not dissolve in water at a physiological temperature or pH but can absorb and retain a remarkable amount of water and swell in aqueous media drastically [1]. The swelling properties of these hydrogels have attracted the attention of researchers and technologists, and have found wide-spread applications in drug delivery systems, agriculture, separation processes and many other fields [2]. Many hydrogels are generally formed from water soluble polymers by radiation or chemical cross-linking. A variety of synthetic polymers such as polyacrylamide and polyvinyl alcohol copolymers and polymers of aspartic and acrylic acids have been employed for production of hydrogels. However, the latter is nowadays the most predominant [3–5]. Nonetheless, in response to the environmental concerns, considerable research has been recently directed toward the use of biodegradable materials derived from natural sources such as starch, cellulose and chitosan, all of which are abundant in nature. Chitosan is one of the most abundant natural polysaccharide. It is commercially produced by deacetylation of chitin, which is derived from shell fish wastes. Due to its hydrophilicity, biocompatibility, non-toxicity, biodegradability and other unique properties, chitosan found wide applications in a variety of areas such as: medicine, pharmaceutics, paper production, textiles, metal chelation, food additives, antimicrobial agents and also in cosmetic industries [6]. It is a weak base with an intrinsic pK_a of 6.5 and it can be used as biological adhesive for its hydrogel-forming ability at low pH [7]. In acidic solutions, the amino groups of a cross-linked chitosan are protonated and form a cationic hydrogel and result in the swelling of the hydrogel network. Many strategies, including cross-linking, grafting and blending are often employed for chitosan hydrogels formation. Both chemical and physical methods have been used to create chitosan hydrogels. Various reagents including gluteraldehyde [8], formaldehyde [9], glyoxal [10], dialdehyde starch [11], epoxy compound [12], diethyl squarate [13], pyromellitic dianhydride [14], genipin [15], quinine [16] and diisocyanate [17–20] have been used as chemical cross-linkers for chitosan. Thioureas have strong antifungal activities that are comparable to the activity observed for the common antifungal antibiotic ketoconazole [21,22]. Moreover, they have antibacterial and insecticidal properties [23–25]. Acetyl, chloroacetyl, and benzoyl thiourea derivatives of chitosan have shown antimicrobial activity much better than that of native chitosan [26,27].

From the aforementioned properties of both the chitosan and the thioureas, it became of interest to prepare some novel hydrogels composed of chitosan cross-linked by terephthaloyl thiourea moieties. Terephthaloyl diisothiocyanate will be used as a cross-linker for the first time in this work. The hydrogels will be characterized by elemental analyses, FTIR, SEM, XRD, solubility and swell ability in various solvents. The antimicrobial activities of these

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hydrogels against three crop-threatening pathogenic fungi namely: *Aspergillus fumigatus* (*A. fumigatus*, RCMB 002003), *Geotrichum candidum* (*G. candidum*, RCMB 052006) and *Candida albicans* (*C. albicans*, RCMB 005002) and three bacterial species namely: *Bacillis subtilis* (*B. subtilis*, *RCMB 000107*), *Staphylococcus aureus* (*S. aureus*, RCMB 000106) and *Escherichia coli* (*E. coli*, *RCMB 000103*) are also described. Further, the effect of the cross-linker content on the hydrogels characteristics will be reported.

2. Experimental

2.1. Materials

Chitosan, with a degree of deacetylation of 88% and molecular weight of 2.0×10^5, was purchased from Acros Organics, NJ, USA. All other chemicals and reagents, from Aldrich, were of analytical grade and were used as received. The crop-threatening pathogenic fungi namely: *A. fumigatus* (RCMB 002003), *G. candidum* (RCMB 052006) and *C. albicans* (RCMB 005002) and bacteria namely: *B. subtilis* (RCMB 000107), *S. aureus* (*RCMB 000106*) and *E. coli* (RCMB 000103) used for the antimicrobial assay were provided by the Reginol Center for Mycology and Biotechnology Culture Collection.

2.2. Synthesis of terephthaloyl thiourea cross-linked chitosan hydrogels (TTU-chitosan)

Four different predetermined amounts of terephthaloyl dichloride were dissolved in 20 ml methylene chloride and were added drop wisely to solutions of ammonium thiocyanate of appropriate concentrations in 20 ml methylene chloride. The molar ratio of terephthaloyl dichloride to ammonium thiocyanate was 1:2, respectively. Polyethylene glycol-400 (1 ml) was added drop wisely to each reaction mixture as a phase transfer catalyst. After stirring for 2 h at room temperature, the formed white precipitate of ammonium chloride was removed by filtration. Each filtrate (terephthaloyl diisothiocyanate, Scheme 1A) was added to a chitosan solution (3.22 g, 20 mmol, in 200 ml of 1% acetic acid). The reaction mixture was stirred at 60 °C for 2 h, cooled, and the homogenous cross-linked hydrogels formed (Scheme 1B) were neutralized with sodium bicarbonate solution to pH 7 to give yellowish white products which were submerged in methanol for 24 h for dewatering. The dewatered hydrogels were filtered and dried at 60 °C to constant weights. The ratios of the reactants were varied as shown in Table 1 to give four new hydrogels which were designated as TTU-chitosan-1, TTU-chitosan-2, TTU-chitosan-3, and TTU-chitosan-4 of increasing degree of cross-linking, respectively.

2.3. Measurements

The reaction between chitosan and terephthaloyl diisothiocyanate was investigated using Tescan Shimadzu FTIR spectrophotometer (Model 8000, Japan). Hydrogel samples were grounded well with KBr and then compressed under hydraulic pressure of 400 kg/cm^2 to make pellets and the spectra were recorded in the range of 500–4000 cm^{-1}. In each pellet, the amount of hydrogel sample and KBr were kept constant in order to know the changes in the intensities of the characteristic peaks with respect to the amount of terephthaloyl diisothiocyanate.

Elemental analyses of the hydrogels were performed in Perkin-Elmer (Model 2410 series II) C, H, N, S Analyzer (USA) at the Microanalytical Center, Cairo University (Egypt).

To visually examine surface morphology of the hydrogels, a Hitachi Model S-450 Scanning Electron Microscope was used to analyze the pore structure. The freeze-dried samples were loaded on the surface of an aluminum SEM specimen holder and sputter coated with gold before observation. The accelerating voltage was 20 kV.

The morphology of the pure chitosan and its terephthaloyl thiourea cross-linked hydrogels was studied at room temperature using Brucker's D-8 advanced wide-angle X-ray diffractometer. The X-ray source of 1.5406 Å wavelength was generated by a nickel-filtered CuKα radiation (40 kV, 30 mA). The dried hydrogels were mounted on a sample holder and scanned in the reflection mode at an angle 2θ over a range from 5° to 50° at a speed of 8° min^{-1}.

Determination of the soluble fraction of the hydrogels: weighed samples of each hydrogel were stirred overnight in 10 ml of each of the following solvents: acetic acid solution (1%, v/v), H_2O, DMF, DMSO, THF, NMP, chloroform, methylene chloride, acetone and methanol. The swollen samples were then dried in oven at 60 °C to constant weights. The soluble fraction was calculated according to the following equation: soluble fraction (%) = $[(W_0 - W_1)/W_0] \times 100$, where W_0, is the initial weight of the hydrogel and W_1, is the weight of the oven dried hydrogel.

Determination of the swell ability of the hydrogels in various solvents: a known weight of the dry hydrogel sample was immersed in a single solvent (acetic acid solution (1%, v/v), H_2O, DMF, DMSO, and NMP) and kept undisturbed at room temperature until equilibrium swelling was reached. The swollen sample was then removed from the immersion media, quickly wiped with filter paper to remove the droplets on its surface and reweighed. The percent swelling was calculated using the following equation: swelling (%) = $[(W_1 - W_0)/W_0] \times 100$, where W_0, is the weight of the dry hydrogel and W_1, is the weight of the swollen hydrogel. Swelling measurements were made in triplicate, and the error was estimated to be within 1%.

Antibacterial activities were investigated using agar well diffusion method. The activity of tested samples was studied against the *S. aureus* (RCMB 000106) and *B. subtilis* (RCMB 000107) (as Gram-positive bacteria) and *E. coli* (RCMB 000103) (as Gram-negative bacteria). Centrifuged pellets of bacteria from 24 h old culture containing approximately 104–106 CFU (colony forming unit) per ml were spread on the surface of nutrient agar (typetone 1%, yeast extract 0.5%, NaCl 0.5%, agar 1%, 1000 ml of distilled water, pH 7.0) which was autoclaved under 121 °C for at least 20 min. Wells were created in medium with the help of a sterile metallic bores and then cooled down to 45 °C. The activity was determined by measuring the diameter of the inhibition zone (in mm). 100 μl of the tested samples (10 mg/ml) were loaded into the wells of the plates. All compounds were prepared in DMSO, DMSO was loaded as control. The plates were kept for incubation at 37 °C for 24 h and then the plates were examined for the formation of zone of inhibition. Each inhibition zone was measured three times by caliper to get an average value. The test was performed three times for each bacterium culture. Penicilling and streptomycin were used as antibacterial standard drugs [28].

Antifungal activities were investigated by screening the tested samples separately in vitro against various fungi [*A. fumigatus* (RCMB 002003), *G. candidum* (RCMB 052006) and *C. albicans* (RCMB 005002)], on sabourad dextrose agar plates. The culture of fungi was purified by single spore isolation technique. The antifungal activity was investigated using agar well diffusion method [29] as follows: sabourad dextrose agar plates: a homogeneous mixture of glucose–peptone–agar (40:10:15) was sterilized by auto claving at 121 °C for 20 min. The sterilized solution (25 ml) was poured in each sterilized petridish in laminar flow and left for 20 min to form the solidified sabourad dextrose agar plate. These plates were inverted and kept at 30 °C in incubator to remove the moisture and to check for any contamination. Antifungal assay: fungal strain was grown in 5 ml sabourad dextrose broth (glucose:peptone; 40:10) for 3–4 days to achieve 105 CFU/ml cells. The fungal culture (0.1 ml) was spread out uniformly on the sabourad dextrose agar plates by