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Fabrication of magnetic and fluorescent chitin and dibutyrylchitin sub-micron particles by oil-in-water emulsification

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ABSTRACT

Chitin is a carbohydrate polymer with unique pharmacological and immunological properties, however, because of its unwieldy chemistry, the synthesis of discreet sized sub-micron particles has not been well reported. This work describes a facile and flexible method to fabricate biocompatible chitin and dibutyrylchitin sub-micron particles. This technique is based on an oil-in-water emulsification/evaporation method and involves the hydrophobization of chitin by the addition of labile butyryl groups onto chitin, disrupting intermolecular hydrogen bonds and enabling solubility in the organic solvent used as the oil phase during fabrication. The subsequent removal of butyryl groups post-fabrication through alkaline saponification regenerates native chitin while keeping particles morphology intact. Examples of encapsulation of hydrophobic dyes and nanocrystals are demonstrated, specifically using iron oxide nanocrystals and coumarin 6. The prepared particles had diameters between 300–400 nm for dibutyrylchitin and 500–600 nm for chitin and were highly cytocompatible. Moreover, they were able to encapsulate high amounts of iron oxide nanocrystals and were able to label mammalian cells.

Statement of Significance

We describe a technique to prepare sub-micron particles of highly acetylated chitin (>90%) and dibutyrylchitin and demonstrate their utility as carriers for imaging.
Chitin is a polysaccharide capable of stimulating the immune system, a property that depends on the acetamide groups, but its insolubility limits its use. No method for sub-micron particle preparation with highly acetylated chitins have been published. The only approach for the preparation of sub-micron particles uses low acetylation chitins.
Dibutyrylchitin, a soluble chitin derivative, was used to prepare particles by oil in water emulsification. Butyryl groups were then removed, forming chitin particles.
These particles could be suitable for encapsulation of hydrophobic payloads for drug delivery and cell imaging, as well as, adjuvants for vaccines.

1. Introduction

Chitin is a biopolymer of N-acetyl-D-glucosamine and is found in the cell walls of fungi, the exoskeletons of crustaceans and the digestive tract lining of many insects. This ubiquitous distribution makes chitin the second most abundant polysaccharide on earth; the first being cellulose. Though structurally similar to cellulose, chitin owes its unique properties to its acetamide groups. The degree of acetylation of chitins varies between 85% and 95%, with its solubility properties being dependent on this percent. Chitin has found widespread use in biomedical pursuits and has been used in drug delivery systems and tissue engineering [1–3].

Moreover, chitin is a potent trigger of innate immune responses. Consequently, several mammalian immune cells such as macrophages produce chitinases that degrade chitin. Recent studies have shown that chitin is a pathogen-associated molecular pattern (PAMP) that activates TLR-2 and regulates macrophage function and acute innate inflammation [4]. Chitin fragments have been shown to exert size-dependent effect on macrophage activity [5], demonstrating untapped potential for use as immunoadjuvants [6–8]. Smaller-sized chitin fragments (<40 μm) have been shown

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to activate both TNF and IL-10 in macrophages, eliciting an immune response in the absence of excessive inflammation-induced tissue damage [9].

Further, chitin nanocrystals particles are efficient in stabilizing oil-in-water emulsions. The particles can form a layer around the interface of the emulsion globules, hindering the coalescence of the globules (Pickering emulsions) [10,11].

Whereas it is recognized that chitin is an important and versatile biomaterial, chitin is not broadly used due to its unwieldy chemical nature. Chitin is insoluble in water and most organic solvents, due to its high crystallinity, mostly due its network of intra- and intermolecular hydrogen bonds through acetamide and hydroxyl groups. There are only a few known solvent systems for chitin, such as dimethylacetamide/LiCl, NaOH/(thio)urea, N-methylpyrrolidone/LiCl, ionic liquids (1-butyl-3methyl-imidazolium chloride, 1-allyl-3-methylimidazolium chloride, etc), and saturated $CaCl_2$/methanol [3,12–17]. Because of the difficulty in performing discrete chemistry with chitin and the inability to synthesize well-formed particles, a chitin derivative called chitosan has been more often used in the development of new drug and gene carriers [18–20]. Chitosan is obtained from the partial deacetylation of chitin under alkaline conditions or enzymatic hydrolysis (using chitin deacetylase). This reduction in the degree of acetylation allows its solubility in acidic aqueous solutions. Other carbohydrate polymers similar in chemical composition (glucose base chains) have also been used for preparing particles, such as starch and cellulose [21,22]. However, neither starch nor cellulose particles exhibit vaccine-like immunological properties, making chitin a unique carbohydrate material.

Regarding the formulation of chitin particles, in general, chitin fragments are formed by mechanical means without the capacity for encapsulating payloads or for forming sub-micron particles [4,7]. Currently, the only approach for the preparation of chitin nanogels consists of the dissolution of the polysaccharide in saturated $CaCl_2$/methanol [23] and intense probe sonication. This method prepares nanogels ~70–150 nm diameter, depending on the payloads [12,24–28]. However, this approach has only been achieved with chitins with low acetylation degree (DA, close to 70%) [3]. For example, amorphous chitin with a low DA (~60%) was used to prepare particles with a diameter <300 nm. Its low DA allows the solution of the polysaccharide in a dilute acid solution, allowing the preparation of the particles by ionic cross-linking with pentasodium tripolyphosphate [29]. Also, polyelectrolyte complexation was also used for the preparation of particles of <500 nm with hyaluronic acid and chitin nanofibrils [30]. Importantly, chitin particles have been shown to be suitable for encapsulating active agents such as anti-cancer, anti-aging, or imaging agents [12,25,28–30].

To date, there is no method for the preparation of sub-micron particles with chitins of high acetylation degrees. As the unique immune properties of chitin are attributed to the acetamide groups, it is important to fabricate chitin particles with high acetylation. Additionally, chitin particles encapsulating high amounts of magnetic and fluorescent payloads would enable the use of imaging to track labeled cells in many biological applications, from vaccination to cell therapies.

The main objective of this work was to devise a robust strategy of preparing discreet sized DBC and chitin sub-micron particles. To do this, we borrowed a technology originally used to fabricate chitin fibers. Chitin starting material is first functionalized to dibutyrylchitin (DBC) (Fig. 1). DBC presents two butyryl groups, one in position C3 and another in C6, and is soluble in many organic solvents. Taking advantage of the organosolubility of DBC, an oil-in-water emulsification/evaporation method was used to prepare particles (Fig. 2). The butyryl groups could then be removed by alkaline saponification, allowing the regeneration of chitin [31–34].

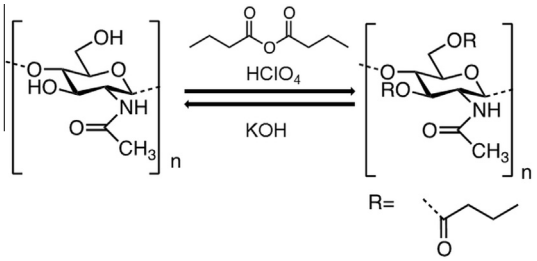

Fig. 1. Reversible acylation of chitin.

While chitin is a promising biomaterial, DBC is also highly biocompatible and it can be degraded by enzymes [35]. Moreover, it has wound-healing and antibacterial properties [36]. Thus, DBC is an interesting new material as well. The only DBC particles prepared to date were synthesized by tip-sonication of DBC solution in ethanol [35].

To prove the suitability of this method for encapsulating hydrophobic compounds, a fluorescent dye (coumarin 6) and iron oxide nanocrystals were loaded into the particles. Finally, its suitability for labeling cells and contrast agents was demonstrated.

2. Materials and methods

2.1. Materials

Agarose, ammonium hydroxide solution (NH_4OH; 28–30%), butyric anhydride (BA; 98%), chitin from shrimp shells, coumarin 6 (C6; 98%), dibenzyl ether, diethyl ether anhydrous (99%, containing BHT as inhibitor), N,N-dimethylacetamide (DMAc; 99.8%), Dulbecco's Phosphate Buffered Saline (DPBS), 1,2-hexadecanediol (90%), hydrochloric acid (HCl; 37%), iron (III) acetylacetonate (97%), lithium chloride (LiCl, 99%), manganese (II) chloride tetrahydrate (MnCl), oleic acid (90%), oleylamine (70%), paraformaldehyde (95%), penicillin/streptomycin solution (P/S; penicillin 10,000 units and streptomycin 10 mg/mL), perchloric acid ($HClO_4$; 70%), poly (vinyl alcohol) (PVA, Mw 13,000–23,000, 87–89% hydrolyzed), potassium hydroxide (KOH; 85%) and sodium hydroxide (NaOH; 98%) were purchased in Sigma Aldrich (USA). Phalloidin (Alexa Fluor® 647 phalloidin), Prolong® diamond antifade mountant with DAPI, Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine serum (FBS) were obtained in Life Technologies (USA). Acetone, dichloromethane (DCM), MTT and Triton X100 were bought at Fisher Scientifics (USA), Macron Fine Chemicals (USA), Roche (USA) and Research Product International (USA), respectively. Macrophages Raw 264.7 (ATCC® TIB-71™) was obtained from ATCC (USA).

2.2. DBC synthesis and characterization

Chitin was purified to remove residual proteins and calcium carbonate. Firstly, chitin was deproteinated for 30 min in 1 M NaOH (1% w/v) at 80 °C, then demineralized and hydrolyzed with 3 M HCl (1% w/w) at ambient temperature for 24 h. Pure chitin was isolated by centrifugation, and then washed several times with water until the pH was neutral. Finally, it was freeze-dried (overall yield 81.5%).

Afterwards, the chitin was functionalized as described before with slight modifications [32]. Briefly, a fresh acylation mixture of $HClO_4$ and BA at −17 °C was slowly added to chitin powder on ice at a molar ratio of 1:10:1 (chitin:BA:$HClO_4$). The reaction was