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Synthesis of β-1,3-glucan esters showing nanosphere formation

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ABSTRACT

Polysaccharide nanoparticles are versatile functional materials used in drug delivery applications. Here we describe a method for the synthesis of β-1,3-glucan esters which show the formation of nanoparticles. Pyridine-soluble β-1,3-glucan formate was first synthesized as an intermediate and then reacted with various anhydrates to yield β-1,3-glucan acetate and hexaonate. The resultant esters were soluble in common organic solvents like acetone, pyridine and dimethylacetamide. By using a simple dialysis process, homogeneous hollow or solid nanospheres with diameter from 132 to 487 nm were prepared. The chemical structures of the obtained β-1,3-glucan esters were characterized and the morphologies of the β-1,3-glucan based nanoparticles were evaluated. These new types of nanoparticles could be potentially used for the encapsulation of hydrophobic drugs targeting immune cells.

1. Introduction

During the last decade, the design of polysaccharide based nanoparticles and nanocapsules has become an emerging field of research with various applications in the pharmaceutical industry, medicine, and bio and food technology (Kulterer et al., 2012). Naturally occurring polysaccharides are excellent material to fabricate such nanoparticles owe to their biocompatibility and biodegradability. Polysaccharides such as starch, alginate and β-1,3-glucan are FDA proved food additives and their degraded products are absorbable in human body (Zhang & Kim, 2012). The abundant hydroxyl groups on the anhydroglucose units could be esterified by nucleophilic substitution reaction. One simple approach to prepare polysaccharide nanoparticles is by self-assembly. Amphiphilic structure is the key for self-assembly, which can be achieved by grafting of hydrophobic groups on the hydroxyl groups of the polysaccharides (Liu, Jiao, Wang, Zhou, & Zhang, 2008b).

β-1,3-Glucans are present in a number of fungi, where they function as a structural polysaccharide like cellulose. For the chemical structure, they are essentially a linear polymer of (1 → 3)-linked β-D-glucose units (Manners, Masson, & Patterson, 1973). In the cell wall of yeast, β-1,3-glucan adopts a triple helical structure, which is unique in the polysaccharide family (Lipke & Ovalle, 1998). While cellulose is an biologically inert polymer, the biomedical properties of yeast β-1,3-glucan and its derivatives are numerous and valuable. They were reported to show anti-bacterial, anti-tumor and anti-viral effects and were also shown to accelerate wound healing and to strengthen the immune system (Chan, Chan, & Sze, 2009). Remarkably, β-1,3-glucan has a specific receptor on immune cells. It was demonstrated to be Dectin-1: a type II transmembrane protein receptor that binds β-1,3-glucan specifically, and can initiate and regulate the innate immune response (Brown & Gordon, 2001). Since β-1,3-glucan is a ligand of dectin-1 receptor, the nanoparticles derived from β-1,3-glucan may present targeting ability against immune cells (Tesz et al., 2011).

The esterification of polysaccharides is the most straightforward approach to obtain amphiphilic structures suitable for self-assembly (Liu et al., 2008b). A typical example is cellulose. It has been shown that cellulose acetate with a degree of substitution (DS) from 1.6 to 2.9 can form nanospheres or bean-shaped nanoparticles by nanoprecipitation (Hornig & Heinze, 2008). The completely acetylated cellulose failed to form nanoparticles due to the loss of amphiphilic structure. The nanoparticles with diameter down to 170 nm were obtained when the DS was about 1.9. They also found that nanoparticles can be prepared by other cellulose esters like cellulose propionate, cellulose butyrate and cellulose phthalate.

Although nanoparticles derived from polysaccharide esters were extensively studied, the esterification of yeast β-1,3-glucan and the preparation of β-1,3-glucan based nanoparticles have not been reported. A. Albrecht and U. Rau attempted to acetylate schizophyllan (a highly branched fungal β-1,3-glucan) by several chemical and enzymatic methods (Albrecht & Rau, 1994). Most of the methods only yielded schizophyllan acetate with very low DS and only strong acid catalysis with a large excess of a carboxyl

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compound led to acetylation. However, no solvents were found for the schizophyllan acetate synthesized under strong conditions, because the polymer structure might be severely damaged in the reaction. We also attempted to esterify yeast β-1,3-glucan by using the methods common for the esterification of cellulose, starch and dextran, only yielding esters with a low DS.

Here we describe for the first time the successful synthesis of highly substituted β-1,3-glucan esters by using β-1,3-glucan formate as an intermediate. The two esterified products β-1,3-glucan acetate and β-1,3-glucan hexaonate were synthesized and characterized by FTIR, ^{1}H NMR, GPC and TGA. The β-1,3-glucan esters are soluble in a number of common organic solvents but insoluble in water. Homogeneous nanospheres and nanocapsules were obtained by a simple dialysis process.

2. Materials and methods

2.1. Materials and reagents

β-1,3-Glucan (derived from the inner cell wall of yeast *Succhuromyces cerevisiae*; M_w: 2×10^5 Da) was obtained from Tiantian Bioengineering & Technology Co., Ltd (Zhuhai, China). Pyridine, acetic anhydride, hexanoic anhydride, formic acid, ethanol, acetone, dimethyl sulphoxide (DMSO) and other chemicals were purchased from local chemical companies. All of the chemicals are of analytical grade and used without further purification.

2.2. Synthesis of β-1,3-glucan formate (BGF)

β-1,3-Glucan was dried at 80 °C in the vacuum oven for 24 h before reaction. Two grams of β-1,3-glucan and 100 ml of formic acid were placed in a 250 mL three-neck flask. The mixture was allowed to react at 85 °C for 3 h in the oil bath. The mixture was then filtered by Durapore Membrane Filters (Millipore, Billerica, MA, USA) and the product was isolated by precipitation in 300 mL ethanol and washed two times with 50 mL ethanol and dried by lyophilization (scientz-10N freeze dryer, NingBo Scientz Biotechnology, China). Yield: 0.6 g.

2.3. Synthesis of β-1,3-glucan acetate (BGA)

Two grams of BGF (0.012 mol of anhydroglucose unit) and 50 mL of pyridine were placed in a 250 mL three-neck flask. The mixture was stirred for 1 h at 85 °C to allow BGF to dissolve and the undissolved BGF was removed by centrifuge. The BGF solution was then transferred back into the flask and 0.06 mol of cooled acetic anhydride was added dropwise to the mixture, which was then allowed to react for 6 h. By changing the reaction temperature at 20 °C (BGA20), 85 °C (BGA85) and 105 °C (BGA105) in the oil bath, it was possible to prepare β-1,3-glucan acetate with three different DS. The mixture was then filtered and the product was isolated by precipitation in 300 mL ethanol, boiled for 3 hours for de-formylation and washed two times with 50 mL ethanol and dried by lyophilization. Yield: 2.5 g.

2.4. Synthesis of β-1,3-glucan hexaonate (BGH)

Two grams of BGF (0.012 mol of anhydroglucose unit) and 50 mL of pyridine were placed in a 250 mL three-neck flask. After stirring for 1 h, 0.06 mol of cooled hexanoic anhydride was added dropwise to the mixture. The mixture was allowed to react at 85 °C in a flask in oil bath for 6 h. The mixture was then filtered and the product was isolated by precipitation in 300 mL ethanol, boiled for 3 h for de-formylation and washed two times with 50 mL ethanol and dried by lyophilization. Yield: 2.7 g.

2.5. Nanospheres formation

Preparation of the nanoparticles was carried out by a dialysis process (Nikolajski, Wotschadlo, Clement, & Heinze, 2012). Forty milligram of β-1,3-glucan ester was dissolved in 10 mL acetone and was dialyzed against distilled water (SpectraPor, MW cutoff 6000–8000 Da) for 4 days. The deionized water was exchanged 5 times in a period of three days.

2.6. Characterization

2.6.1. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded using a Nicolet 6700 spectrometer. The β-1,3-glucan and β-1,3-glucan ester samples were collected using the KBr pellet method. The resolution was 4 cm^{-1} and the total scans were 32. The samples were dried at 60 °C in the vacuum oven before measurement.

2.6.2. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy analysis

The ^{1}H NMR spectrum was recorded using a Bruker AVANCE DRX-500 spectrometer at 25 °C with a resonance frequency of 500 MHz. The solvent for all the samples is d6-DMSO. The degree of substitution could be calculated by the following Equation: DS = 7*A*/3*C*, where *A* is sum of areas of methyl protons at 1.8–2.2 ppm, and *C* is the sum of areas of seven protons in anhydroglucose unit observed at higher than 3.5 ppm.

2.6.3. Gel permeation chromatography (GPC)

Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC). The instrument was equipped with a Waters 1515 pump, a Waters 2414 refractive index detector, and three Styragel columns (HR1, HR3, and HR4; 300 mm × 7.8 mm for each) packed with 5 μm particles, and the ranges of molecular weight were 100–5000 (HR1), 500–30000 (HR3), and 5000–500,000 (HR4). N,N-Dimethylformamide (DMF) was used as a mobile phase with a flow rate of 1.0 mL/min. The measurements were carried out at 40 °C with an injection volume of 20 μL, and the sample solution was filtered through a 0.2 μm filter before the injection.

2.6.4. Thermogravimetric analysis (TGA)

TG analysis of the native β-1,3-glucan, BGF and BGA was carried out by using the thermogravimetric analysis equipment (SDT Q600, TA, USA). Samples were placed in the sealed aluminum cells and heated from 30 to 600 °C at a heating rate of 10 °C min^{-1} in nitrogen atmosphere.

2.6.5. Transmission electron microscopy (TEM)

Transmission electron micrographs of the nanoparticles formed by β-1,3-glucan esters were taken with a Hitachi 7650 transmission electron microscope with an acceleration voltage of 80 kV. BGF, BGF and BGH nanostructures were negatively stained with a 2% aqueous solution of Phosphotungstic acid, and the BGA nanospheres were deposited on a carbon-coated grid without any treatments.

2.6.6. Atomic force microscopy (AFM)

AFM images were taken using a Multimode Nanoscope-V (Veeco instruments, USA) atomic force microscope with tapping mode. The solution of samples was dried in air on a mica surface before analysis.

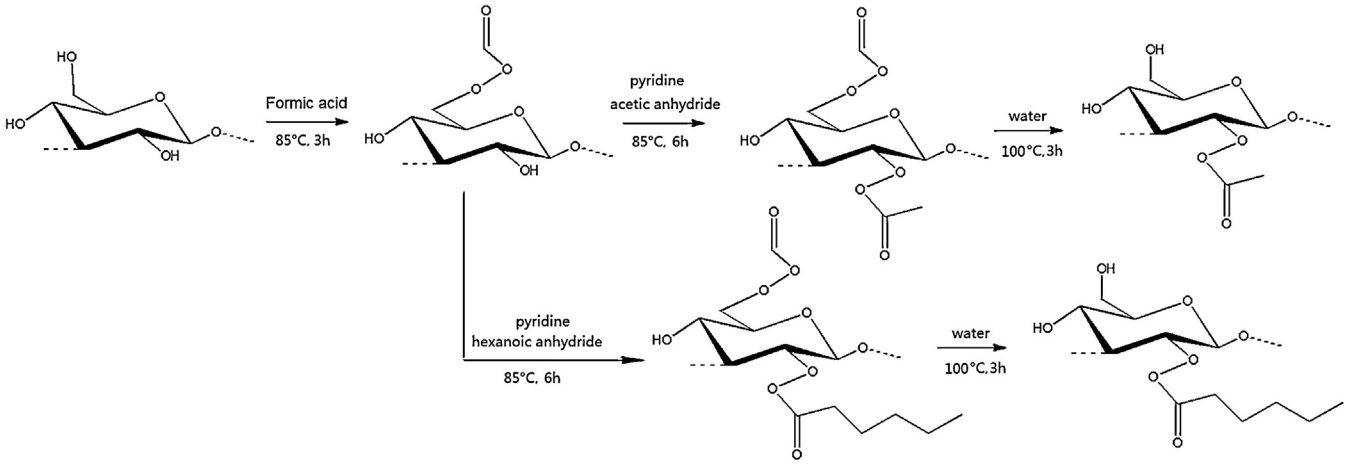

Fig. 1. The synthetic routes of β-1,3-glucan esters.

2.6.7. Scanning electron microscope (SEM)

SEM images of the resultant nanospheres were recorded by a ZEISS Ultra55 field emission scanning microscope operating between 5 and 15 kV (ZEISS Germany).

2.6.8. Dynamic light scattering (DLS)

The particle size and polydispersity of the nanospheres in deionized water were measured by dynamic light by a 90 Plus particle size analyzer (PSA; Brookhaven) at room temperature. The suspensions were diluted with deionized water to a concentration of about 0.01%.

3. Results and discussion

3.1. Synthesis

So far, DMSO is known as the only solvent for yeast β-1,3-glucan. However, direct esterification of β-1,3-glucan in DMSO only resulted in β-1,3-glucan esters with very low DS. Also, the esterification in water catalyzed by strong acid failed to achieve meaningful esterification (Albrecht & Rau, 1994). Since the solvent plays an important role in esterification reaction, we therefore developed a pyridine-soluble β-1,3-glucan derivative as an intermediate because pyridine is a common catalyst for electrophilic substitution reaction. The synthetic route is shown in Fig. 1. Although β-1,3-glucan is difficult to be esterified by acetic anhydride or other higher anhydride, it is very easy to be formylated in formic acid to yield β-1,3-glucan formate (Adachi et al., 1988). Adachi et al. studied the formolysis of β-1,3-glucan derived from fungus *Grifola frondosa*. They found that β-1,3-glucan was formylated and degraded in formic acid in an endo-mode fashion and the degraded product was soluble in water. The formolysis did not change the biological properties and the triple-helical conformation of native β-1,3-glucan. The formyl groups could be easily removed by water-boiling for 3 h. Schnabelrauch, et al. also synthesized cellulose formate as intermediates for the regioselective derivatization of cellulose (Schnabelrauch, Vogt, Klemm, Nehls, & Philipp, 1992). They found that cellulose can be formylated in formic acid to reach a DS up to 1.2 and the cellulose formate was soluble in many organic solvents like DMF and N,N-dimethylacetamide. Remarkably, the NMR spectroscopy revealed a strongly preferred introduction of formate ester groups into the C-6 position of the anhydroglucose units. In our experiments, the reaction conditions used were 85 °C/3 h in aqueous 90% formic acid. The yeast β-1,3-glucan gradually dissolved into formic acid as the reaction proceeded. If the reaction lasted longer than 4 h, the yield would drop greatly because of the extensive degradation of β-1,3-glucan. BGF was unstable and should be dried in a lyophilizer instead of a vacuum oven. If dried at 40 °C or above, the color of the polymer would change to dark brown. The resultant BGF was a white powder and was soluble in a number of organic solvents like pyridine, DMF, DMSO or Li/DMAc. The esterification can be achieved in a homogeneous environment when acetic anhydride or hexanoic anhydride was added into the β-1,3-glucan formate/pyridine solution.

3.2. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the unmodified β-1,3-glucan, BGF, BGA, BGH are shown in Fig. 2. For the spectra of the unmodified β-1,3-glucan (Fig. 2a), several discernible absorbances at 1157 cm^{-1}, 1081 cm^{-1} and 1015 cm^{-1} are present and can be attributed to C—O

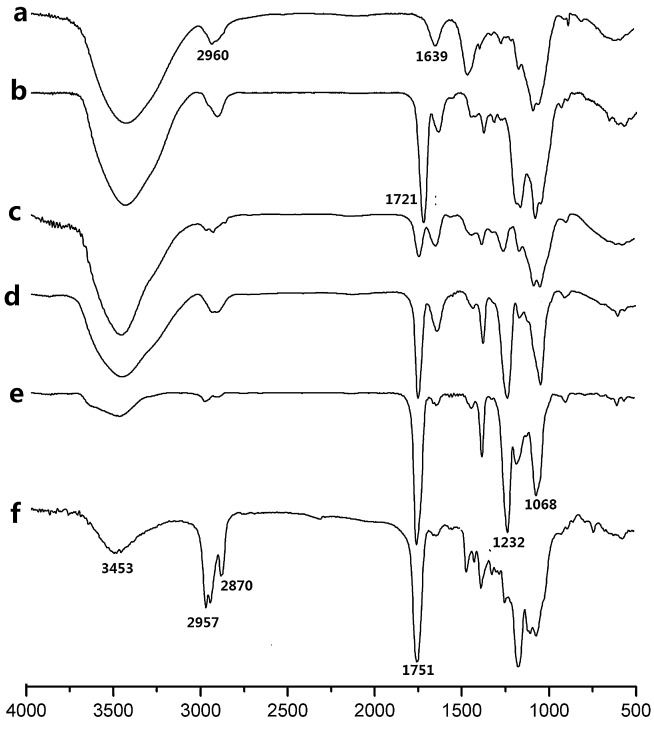

Fig. 2. FITR spectra for β-1,3-glucan (a), BGF (b), BGA20 (c), BGA85(d), BGA105(e) and BGH (f).

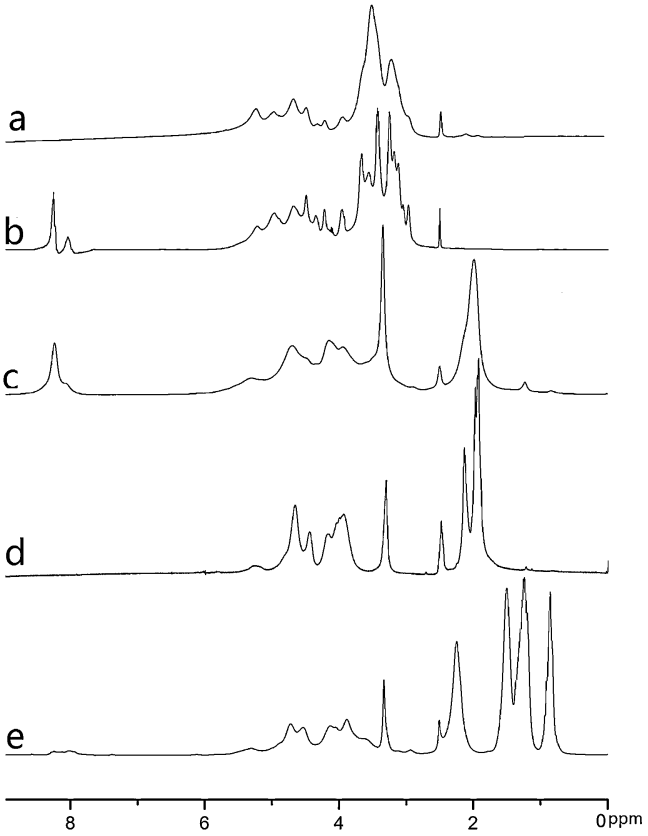

Fig. 3. ^{1}H NMR spectra for β-1,3-glucan (a), BGF (b), BGA85 without deformylation (c), deformylized BGA85 (d) and BGH (e).

bond stretching (Goheen & Wool, 1991). Additional characteristic absorption bands appeared at 991 cm^{-1}, 928 cm^{-1}, 867 cm^{-1} and 763 cm^{-1} can be attributed to the anhydroglucose ring stretching vibrations. An extremely broad band at 3453 cm^{-1} can be attributed to the hydrogen bonded hydroxyl groups (Fang, Fowler, Sayers, & Williams, 2004). For the spectra of BGF (Fig. 2b), the band at 1721 cm^{-1} can be assigned to the carbonyl C=O moiety on the formyl group. For the spectra of BGA and BGH, the band at 1751 cm^{-1}, 1431 cm^{-1}, 1372 cm^{-1} and 1232 cm^{-1} can be assigned to carbonyl C=O, CH_3 antisymmetry deformation vibration and carbonyl C—O stretch vibration, respectively. The spectra of BGH also shows strong absorption band at 2870 cm^{-1}, which can be assigned to be the —CH_2— methylene bridge on the hexanoyl group. These strong absorption bands indicated that highly substituted β-1,3-glucan esters were successfully synthesized during the esterification process.

3.3. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

The chemical structure of the β-1,3-glucan esters were further characterized by ^{1}H NMR. All samples of the β-1,3-glucan esters were dissolved in d-DMSO. The peaks of the polymer ^{1}H NMR spectrum are broad, due to the overlap of signals from different chemical environments and are also due to the viscosity of the samples. Generally, the signals at 3.8–5.5 ppm can be assigned to be the β-1,3-glucan ester backbones. For BGF, The chemical shift at 8.3 ppm is assigned to the proton on the formyl group (Fig. 3b). For BGA, the chemical shift at 1.8–2.2 ppm is assigned to methyl protons on the acetyl group. For BGH, the chemical shifts at 0.8 ppm (CH_3), 1.2 ppm 1.5 ppm 2.3 ppm (—CH_2—) are assigned to the protons on the hexanoyl group. Compared Fig. 3d with c, it can be seen that the formyl group have been removed completely after boiling for 3 h in water.

Table 1
Characterization of β-1,3-glucan esters and their assemblies in water.

Samples	Degree of substitution (DS)	Diameter (nm)	Polydispersity
BGF	0.71	270	0.337
BGA20	0.92	–	–
BGA85	1.73	160	0.115
BGA105	2.46	132	0.089
BGH	1.87	487	0.154

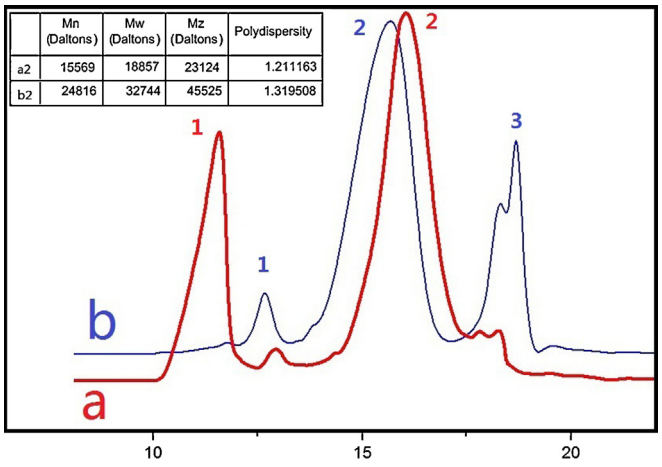

Fig. 4. GPC spectra for β-1,3-glucan (a) and BGA85 (b).

The DS of β-1,3-glucan esters (determined by ^{1}H NMR) are summarized in Table 1. The esterification reaction was very efficient, because a highly substituted BGA (DS: 2.46) could be synthesized within 6 h.

3.4. Gel permeation chromatography (GPC)

Fig. 4a shows GPC result of BGF and two polymer peaks are resolved by ma.a.l.l.s. photometry. Peaks 1 and 2 represent 25.6% and 68.1% of the total polymer mass respectively. Peak 2 has a weight-average M_w of 1.89×10^4 Da and a polydispersity of 1.2. Fig. 4b shows the result for BGA, three polymer peaks are resolved. Peaks 1, 2 and 3 represent 5.2%, 73.2% and 21.7% of the total polymer mass respectively. Peak 2 has a M_w of 3.27×10^4 Da and a polydispersity of 1.3. After formic acid treatment, the molecular weight of the formylated β-1,3-glucan is decreased to about 1/10 that of native β-1,3-glucan. A moderate molecular weight may favor nanoparticle formation, because the shorter polymer chain is more flexible, avoiding the excessive chain entanglement.

3.5. Thermogravimetric analysis (TGA)

Fig. 5 shows the TGA curves of the native β-1,3-glucan, BGF and BGA. The thermal degradation of polysaccharides esters such as cellulose acetate are known to take place in various steps such as removal of water and solvents (50 °C ~150 °C), deacetylation at 320 °C and the thermal pyrolysis of the cellulose skeleton at 370 °C (Shaikh, Pandare, Nair, & Varma, 2009). For the native β-1,3-glucan, the weight loss region at 50.4 °C can be attributed to the loss of bond water, because the native polymer is very hydrophilic. The weight loss region at 268.7 °C can be attributed to the pyrolysis of β-1,3-glucan skeleton. Compared Fig. 5b with c, it can be seen that the thermal stability of BGA is greater than β-1,3-glucan formate, due to the higher stability of acetyl group compared with formyl group.

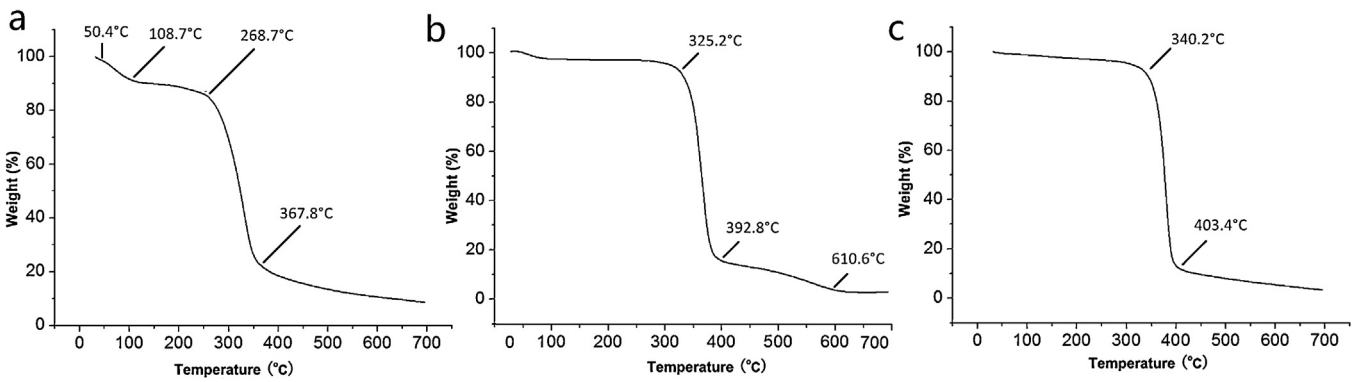

Fig. 5. TGA results for β-1,3-glucan (a), BGF (b) and BGA (c).

3.6. *Morphology analysis by atomic force microscopy (AFM), scanning electron microscope (SEM) and transmission electron microscopy (TEM)*

The nano or micro structures formed by the native β-1,3-glucan and its esters were characterized by AFM, SEM and TEM. Fig. 6a shows a SEM images of the powder of the native β-1,3-glucan. The native β-1,3-glucan appears to be granule-shaped microparticles in diameter about 10–100 μm, which are in good accordance to other report in the literature (Hunter, Gault & Berner, 2002). Fig. 6b shows a TEM image of nanoparticles found in the water solution of BGF, which are rod-like stiff particles in length about 50–500 nm. We assume that the rod-like particles are β-1,3-glucan triple helices (Kitamura et al., 1996). Each rod is consisted of three strands of β-1,3-glucan single chains and is stabilized by hydrogen bonding among the O-2 hydroxyl groups (Numata & Shinkai, 2008). This structure was also found in the formylized β-1,3-glucan derived from fungus *G. frondosa* (Adachi et al., 1988). We speculated that the formyl group was mainly grafted on the C-6 position on the anhydroglucose unit, in a same regioselective fashion as the case of cellulose formate (Fox, Li, Xu, & Edgar, 2011). The helical characteristics might not change during the formylation because O-6 hydroxyl groups are located on the exterior of the triple helices (Numata, Sugikawa, Kaneko, & Shinkai, 2008). To precisely measure the diameter of the resultant triple helices, we performed AFM examination, the image of which was shown in Fig. 6c. The diameter of the helices were determined to be 1.7 nm, which was much greater than that of β-1,3-glucan in native form (1.21 nm) (Wang, Xu, & Zhang, 2008). The increase of diameter might be attributed to the attachment of formyl groups. Fig. 6d and e shows images of the nanoparticles formed by BGA85 by a dialysis process. These nanoparticles appear to be spherical and have a diameter about 160 nm. This size was very suitable for drug delivery applications. Fig. 6e shows an image of the nanoparticles formed by BGH. Interestingly, these nanoparticles were hollow spheres with a diameter about 500 nm. The shell of the nanospheres might consist of two layers of BGH in the same way as the shell of liposomes. The structure of the anhydroglucose unit of the BGH is very similar to lipid,

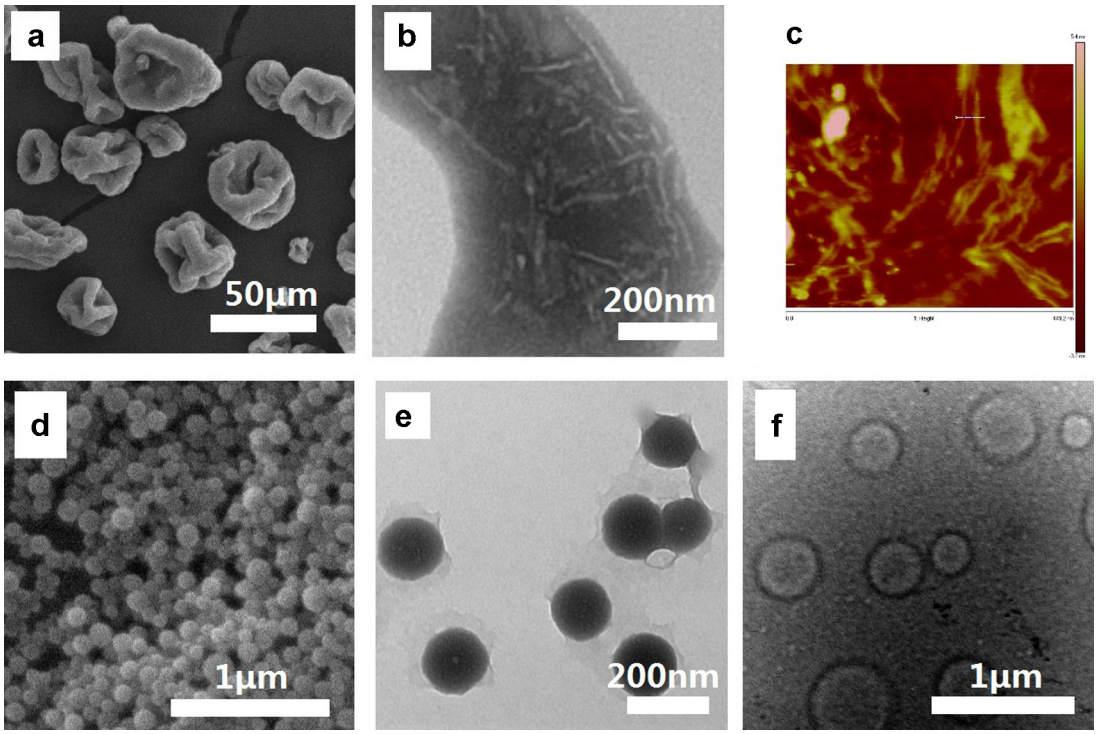

Fig. 6. Morphological characterization for the assemblies by β-1,3-glucan and its esters: native β-1,3-glucan (a), BGF (b, c), BGA85 (d,e), BGH (f). SEM image: a and d; TEM image: b,e and f; AFM image: c.

because there is a long hydrophobic tail (hexanoyl group) and a small hydrophilic head (O-6 hydroxyl group) on each anhydroglucose unit. Actually, similar structure was also proposed in the case of carboxylmethyl hexanoyl chitosan (Liu, Chen, Liu, & Liu, 2008a), which can self-assemble into hollow nanospheres in a similar fashion.

4. Conclusions

In this work, β-1,3-glucan derived from yeast cell wall was studied for esterification. β-1,3-glucan was formylated by formic acid. β-1,3-glucan acetate and hexanote were successfully synthesized by reacting β-1,3-glucan formate with acetic anhydride and hexanoic anhydride respectively. The esterified β-1,3-glucans exhibited enhanced hydrophobicity and were soluble in several common organic solvents. Spherical nanoparticles with size ranging from 132 to 487 nm were prepared by a simple dialysis procedure. These new types of nanospheres will be further studied as drug delivery carriers targeting macrophages.

Acknowledgements

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