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Physicochemical properties and blood compatibility of acylated chitosan nanoparticles

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Abstract

Chitosan was *N*-acylated with butanoic, hexanoic and benzoic anhydride under homogeneous conditions in the presence of methanol. The degree of substitution of *N*-acyl chitosans was determined by ^{1}H-NMR. The *N*-acyl chitosan nanoparticles were prepared by the addition of tripolyphosphate anions. Freeze-dried *N*-acyl chitosan nanoparticles were then analyzed for particles size, zeta potential, and blood compatibility. The *N*-acyl chitosan nanoparticles were in the range of 200–360 nm and positively charged (10–20 mV) in a saline solution after freeze-drying. It was found that long and bulky acyl groups hinder hydrogen bonding and prevent aggregation. Hemolysis and thromboelastography showed that the *N*-acyl chitosan nanoparticles were blood compatible.

Keywords: Acylation; Degree of substitution; Chitosan nanoparticles; Hemolysis; Thromboelastography

1. Introduction

Chitosan is a linear amino polysaccharide of glucosamine and *N*-acetylglucosamine and can be derived by partial *N*-deacetylation of chitin from crustacean shells (Lee, Ha, & Park, 1995; Yoksan, Aksshi, Biramontri, & Chirachachai, 2001). The term chitosan is used to describe a series of chitosan polymers with different molecular weights (50–2000 kDa), viscosities (1% chitosan in 1% acetic acid, <2000 mPaS), and degrees of deacetylation (40–98%) (Illum, 1998; Kurita, 2001). Chitosan is usually characterized by an average degree of deacetylation. It is insoluble in water, but soluble in most acids at a pH below 6.5 (Schipper, Varum, & Artursson, 1996). Aqueous acetic acid has generally been used as a standard solvent for chitosan (Oungbho & Muller, 1997). Upon dissolution, the amine groups of the polymers are protonated and the resultant soluble polysaccharide is positively charged (MacLaughlin et al., 1998). The polycationic character and ability to interact with negatively charged molecules have drawn many researchers to study chitosan particles (Hu et al., 2002; Janes, Calvo, & Alonso, 2001; Shu & Zhu, 2000).

Chitosan's unique characteristics make it potentially useful in a variety of applications (Rao & Sharma, 1997), such as a drug carrier, wound healing, implantation and gene therapy because of the following advantages (Mi, Tan, Liang, & Sung, 2002):

- Chitosan is inexpensive. It is the product of naturally abundant crustacean shells.
- Chitosan is non-toxic, biocompatible and biodegradable.
- Chitosan possesses other bioactivities. It has antacid and antiulcer activities which prevent or weaken drug irritation in the stomach (Kumar, 2000).
- Organic solvents are not required in solubilization of chitosan.

Number of authors have reported the formation of chitosan nanoparticles or microspheres for use in medical or pharmaceutical applications. Berthold, Cremer and Kreuter (1996) prepared chitosan microspheres by

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precipitation to utilize as drug carriers for anti-inflammatory drugs. Chitosan microparticles were also prepared and characterized for use in oral vaccinations (van der Lubben, Varhoef, van Aelst, Borchard, & Junginger, 2001). Remunan-Lopez studied the formation of complex coacervates between the oppositely charged polyion, chitosan and type B gelatin (Remunan-Lopez & Bodmeier, 1996). Calvo, Remunan-Lopez, Vila-Jato and Alonso (1997) reported the formation of the hydrophilic chitosan-polyethylene oxide nanoparticles and studied their potential application as protein carriers.

Most of the reported applications for chitosan nanoparticles have been designed for oral (van der Lubben et al., 2001) or nasal (Fernandez-Urrusuno, Romani, Calvo, Vila-Jato, & Alonso, 1999) administration of therapeutic molecules and gene delivery systems (Leong et al., 1998). Few studies to date on the intravenously injectable chitosan nanoparticles have been carried out. A novel potential application of intravenously injectable chitosan nanoparticles is drug detoxification in the emergency treatment (Morey et al., 2004). In our research, attempts have been made to develop chitosan nanoparticulate systems for intravenous administration with an assumption that engineered nanoparticle systems can adsorb overdosed drugs selectively and rapidly, and reduce its free blood concentration to a safe level (Lee & Baney, 2004; Underhill et al., 2002). It has been reported that intravenously administrated nanoparticles are taken up by the macrophages of the reticuloendothelial systems including livers, spleens, and lungs (Gaur et al., 2000). Therefore, rapid adsorption by nanoparticulate systems before organ uptake is very critical in this strategy.

In the present work, studies aimed at the chemical modification of chitosan with various functional groups were carried out to induce a hydrophobic nature to the hydrophilic chitosan backbone and prevent particle aggregation. We report the formation of *N*-acyl chitosan nanoparticles by ionic crosslinking and their physicochemical properties and blood compatibility.

2. Experimental

2.1. Materials

Chitosan was obtained from TaeHoon Bio. Co. (Korea) and used as received. The viscosity and degree of deacetylation, as determined by the supplier, were 2.8 cps (0.5% solution in 0.5% acetic acid at 20 °C) and 90%, respectively.

Butanoic anhydride, hexanoic anhydride and benzoic anhydride were purchased from Aldrich. Tripolyphosphate was obtained from Sigma, USA and used without further purification.

2.2. Modification of chitosan

Chitosan modification was performed by the method of Domszy and K.Y. Lee (Lee et al., 1995). Two grams of chitosan were dissolved in 100 ml of 1% aqueous acetic acid solution and 100 ml of methanol. An appropriate volume of 2% carboxylic anhydride solution in methanol was added to the chitosan solution. After 15 min the chitosan solution was poured into the same volume of methanol:ammonia solution (7:3 (v/v)). The precipitated chitosans were filtered, washed thoroughly with methanol and ether, then dried in a vacuum at 50 °C overnight.

2.3. Particle preparation

Chitosan solutions of 0.25% (w/v) were prepared in 1% acetic acid solution. Tripolyphosphate (TPP) solution (1.0%) was added dropwise to 20 ml of chitosan solution with magnetic stirring, followed by sonication. The resulting chitosan particle suspension was subsequently centrifuged at 15,000 rpm for 10 min. The precipitated particles were resuspended in DI water with sonication, centrifuged again and then freeze dried. The freeze-dried chitosan nanoparticles were then resuspended in a saline solution.

2.4. Characterization

^{1}H-NMR spectra were measured in D_2O containing small amount of CD_3COOD using a 300 MHz spectrometer (Gemini 300, Varian, USA). The mean size and zeta potential of the chitosan nanoparticles were determined by photon correlation spectroscopy in a Brookhaven Zeta Plus instrument.

2.5. Blood compatibility

Whole blood was obtained from three healthy male adults (22–31 years old). Red blood cells (RBCs) were collected by centrifuging whole blood. The RBCs were washed three times with a saline solution before being diluted with a buffer to prepare erythrocyte stock solutions with fixed concentrations of hemoglobin (3:11 centrifuged erythrocytes, buffer saline solution). Freeze-dried chitosan particles were redispersed and sonicated in a saline solution to give a 0.2% suspension. One hundred microliters of an erythrocyte stock solution was added to 1 ml of the chitosan nanoparticle suspension. The mixtures were left for 2 h and centrifuged. One hundred microliters of the resulting supernatant was dissolved in 2 ml of an ethanol/HCl mixture (200:5 ml (v/v)). A saline solution was used as negative control (0% lysis) and DI water used as a positive control (100% lysis). The absorption of the mixture was determined at 399 nm by an UV spectrophotometer (S2000, WPI, Sarasota, USA).

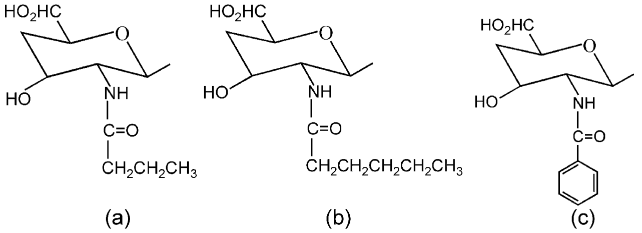

Fig. 1. Chemical structure of chitosan derivatives, (a) modified with butanoyl groups; (b) modified with hexanoyl groups; (c) modified with benzoyl groups.

Thromboelastography (TEG) was carried out using a Thromboelastograph Coagulation Analyzer (Model 3000C, Haemoscope, Co., Skokie, USA).

Before the test, the TEG cuvettes and the pin were prewarmed to 37 °C. Each chitosan particles systems in a saline solution (30 μl) was pipetted into cuvettes. The same volume of a saline solution was pippetted into cuvettes as a control. A $CaCl_2$ solution of 30 μl and whole blood of 240 μl were added into the cuvettes. The pins were immersed briefly three or four times in order to ensure uniform mixing of the blood with the samples, then lowered into the cuvettes for testing. Four drops of mineral oil were placed on the surface of the specimen to prevent air from drying out the samples.

3. Results and discussion

3.1. Characterization of acyl chitosans

The *N*-acyl chitosans were prepared under homogenous conditions using carboxylic anhydride of 0.5 mole per glucosamine residue (Fig. 1).

The structure of the substituted chitosans was identified by ^{1}H-NMR. The degree of deacetylation was calculated to be 90% from the integral ratio between proton on 2-C and the acetyl protons of *N*-acetyl-glucosamine (GlcNAc) (Kumar, 2000). The ^{1}H-NMR spectrum in Fig. 2 was obtained from hexanoyl chitosan.

The methyl protons, (CH_3), of the hexanoyl residues appear at 0.5–0.6 ppm. ^{1}H-NMR spectra analysis can also be employed for the determination of the degree of substitution. The degree of substitution was determined by comparing the integral ratio between proton of 2-C and the protons of substituted groups. During the acylation with butanoic, hexanoic or benzoic anhydride, acetylation also occurred with aqueous acetic acid which is the solvent for the homogeneous acylation reaction (Kurita, 2001). Evidence of acetylation is supported by an increased integral of the peak corresponding to acetyl groups of GlcNAc, observed at 1.8–1.9 ppm. Thus, acylated chitosan

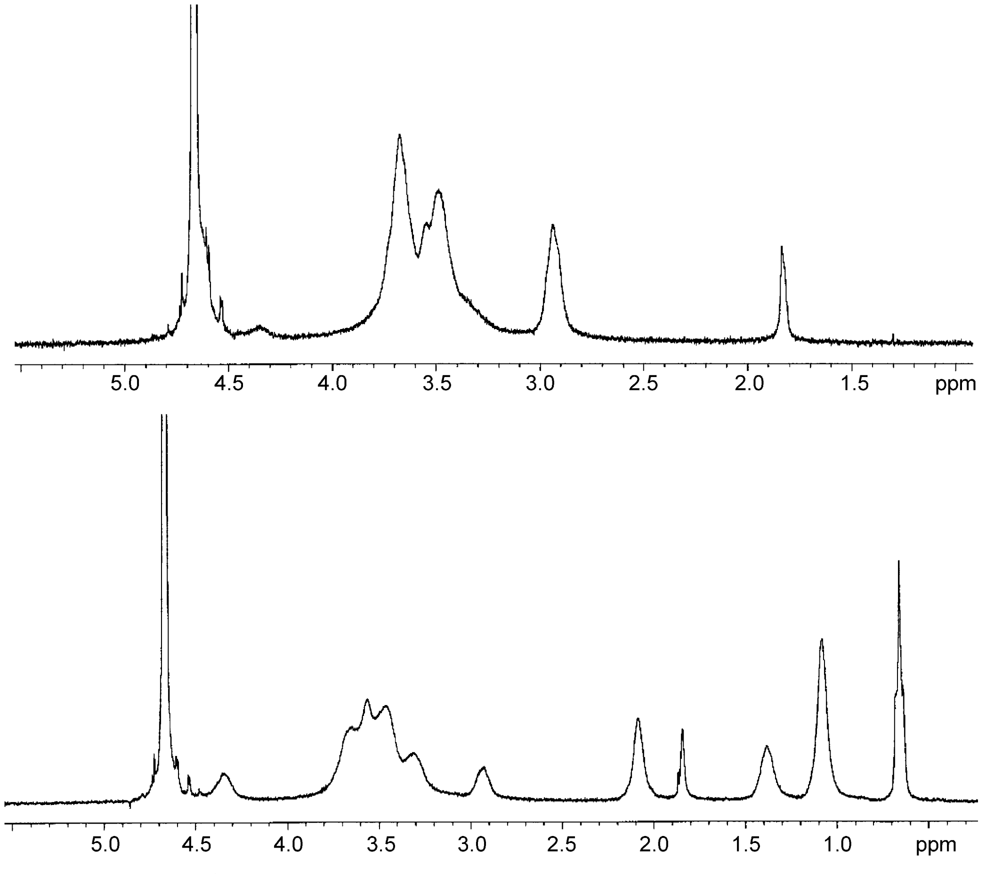

Fig. 2. ^{1}H-NMR spectra of pure chitosan (top) and hexanoyl chitosan (bottom).

Table 1
Degree of substitution and deacetylation of acylated chitosan

	Degree of substitution (%)	Degree of deacetylation (%)
Unmodified	0	90
Butanoyl	22	60
Hexanoyl	38	40
Benzoyl	27	42

has a lower degree of deacetylation after acylation has occurred (Table 1).

The degree of deacetylation is an important variable in the particle's formation. The influence of the degree of deacetylation on the formation of particles will be addressed later.

3.2. Physicochemical properties of chitosan nanoparticles

Biodistribution and fate of particles injected into the bloodstream are influenced by factors such as the particle size, surface charge and hydrophobicity/hydrophilicity (Storm, Belliot, Daemen, & Lasic, 1995) First of all, the particles should be small enough to freely circulate through the smallest capillaries (7 μm) and avoid the organ uptake (Illum & Davis, 1983; Pesko, 1996).

Preliminary experiments were performed to determine the optimal condition for nanoparticle formation. The formation of nanoparticles was only possible for specific concentrations of chitosan and TPP, i.e. 0.25 and 1%, respectively. It has been reported that the chitosan/TPP ratio controls the particle size and zeta potential (Calvo et al., 1997; Fernandez-Urrusuno et al., 1999).

In this work, the chitosan particles were prepared with the fixed chitosan/TPP ratio of 2.5:1. Table 2 shows the mean size and zeta potential of chitosan nanoparticle before and after freeze-drying. Chitosan particles are in the range of 160–200 nm before freeze-drying. A slight change in mean size is observed between unmodified and *N*-acyl chitosan nanoparticles.

Freeze-dried chitosan nanoparticles were redispersed in a saline solution by sonication. Unmodified chitosan particles aggregated due to the strong inter- and intramolecular hydrogen bonding and did not break down by sonication. However, *N*-acyl chitosan particles were readily redispersed to nano-size (∼360 nm). This result can be explained by the fact that long acyl groups lessen the inter- and intramolecular hydrogen bonding and reduce the compactness of the network. The higher zeta potential of *N*-acyl chitosan nanoparticles is also responsible for the small size and better stability at pH 6.9. We suggest that the aliphatic and aromatic acyl groups eliminate the need of stabilizers such as polysorbate 80 which are necessary to stabilize the suspension of chitosan particles (Berthold et al., 1996; van der Lubben et al., 2001).

When the acylation was performed using carboxylic anhydride of 1.0 mole per glucosamine residue, chitosan nanoparticles did not form with the addition of anions. This can be attributed to the low extent of protonation of highly substituted acylated chitosan. As mentioned above, chitosan particles are formed by the ionic crosslinking (ionotropic gelation) between oppositely charged ions (Calvo et al., 1997; Remunan-Lopez & Bodmeier, 1996). The production process of nanoparticles is dependent on the number of positively charged groups left on the dissolved chitosan molecules. In the case of highly acylated chitosan, there are not enough protonated amine groups remaining for ionic crosslinking to occur.

The surface charge is the critical parameter on the stability of suspensions and adhesion of particle systems onto biological surfaces. Consequently, investigation of the zeta potential is an important part of nanoparticle characterization. Chitosan nanoparticles are all positively charged which is typical characteristic of chitosan/TPP particles. This can be explained by the particle formation mechanism, the positively charged amine groups are neutralized by their interaction with the negative charge in tripolyphosphate molecules (Calvo et al., 1997; Fernandez-Urrusuno et al., 1999). The residual amino groups would be responsible for the positive potential. It was observed that *N*-acyl chitosan particles have a lower zeta potential than unmodified chitosan nanoparticles. This result is possibly due to the lower degree of deacetylation of acylated chitosan. The acylated chitosans have a lower number of free amine groups which can be protonated in an acidic condition and therefore participate in particle formation through association with the TPP.

In a saline solution, pH 6.9, the zeta potential of the unmodified chitosan particles decreased significantly by deprotonation and/or adsorption of anions. The *N*-acyl

Table 2
Mean size and zeta potential of chitosan particles before and after freeze-drying

	Mean size (nm)		Zeta potential (mV)	
	Before freeze-drying[a]	After freeze-drying[b]	Before freeze-drying[a]	After freeze-drying[b]
Pure CS/TPP	160.1 ± 2.3	Aggregated	29.9 ± 3.6	1.4 ± 4.0
Butanoyl CS/TPP	157.2 ± 10.3	352.4 ± 16.6	22.6 ± 3.9	17.9 ± 6.0
Hexanoyl CS/TPP	189.0 ± 9.3	347.5 ± 74.6	18.1 ± 3.5	11.2 ± 3.8
Benzoyl CS/TPP	213 ± 17.3	368.8 ± 16.6	19.5 ± 3.1	9.3 ± 3.2

[a] pH of suspension before freeze drying was 4.5.

[b] Freeze-dried chitosan particles were redispersed in a saline solution (pH 6.9).

chitosan nanoparticles undergo less reduction in zeta potential as compared to unmodified chitosan nanoparticles. The higher zeta potential implies that chitosan nanoparticles are more stable. It seems likely that the long acyl groups hinder the anion adsorption and keep the high value of the electrical double layer thickness, suggesting the prevention of aggregation.

3.3. Blood compatibility

Previously, the blood compatibility of unmodified chitosans in microspheres and emulsions were evaluated in terms of hemolysis (Jumaa, Furkert, & Muller, 2002; Remunan-Lopez & Bodmeier, 1996; Richardson, Kolbe, & Duncan, 1999). Lee et al. (1995) investigated the blood compatibility of acylated chitosans in the formation of films with the measurement of dynamic viscoelasticity by a rheological method. No research has been reported on the TEG of chitosan particles. In this work, hemolysis and thromboelastography were utilized to evaluate the blood compatibility of *N*-acyl chitosan nanoparticles.

In vitro erythorocyte-induced hemolysis is considered to be a simple and reliable measure for estimating blood compatibility of materials. The behavior of chitosan nanoparticles in vivo can be predicted by investigating the degree of hemolysis in vitro (Jumaa et al., 2002). The concentration of particles in suspension was fixed at 0.2% (w/v). DI water and saline solution were used as a positive and negative control, respectively. Hemolysis results demonstrated in Fig. 3 show that a slight hemolysis was produced in all chitosan nanoparticle systems after 2 h incubation.

Unmodified chitosan nanoparticles show a negligible hemolysis (less than 2%) (Jumaa et al., 2002). The *N*-acyl chitosan nanoparticles revealed a relatively higher extent of breakdown of the RBCs. The long hydrophobic acyl groups on the surface may damage RBCs, leading to enhanced release of hemoglobin. Unmodified chitosan nanoparticles and *N*-acyl chitosan nanoparticles were compared using One Way ANOVA with a significance level of $P<0.05$. There was a statistically significant difference between unmodified chitosan nanoparticles and *N*-acyl chitosan nanoparticles in hemolysis. However, the largest observed hemolytic activity was lower than 10% which indicates a wide safety margin in blood-contacting applications and suitability for intravenous administration (Jumaa et al., 2002; Richardson et al., 1999).

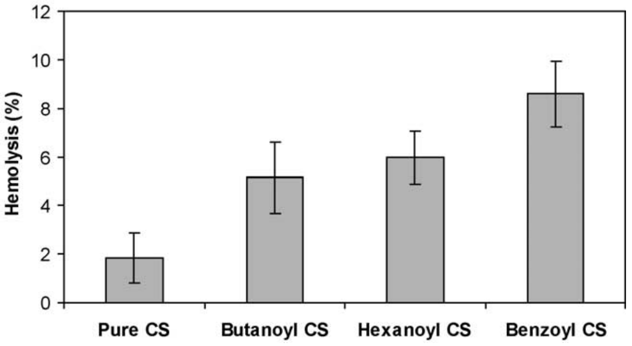

Fig. 3. Hemolysis of chitosan particle systems, $n=6$.

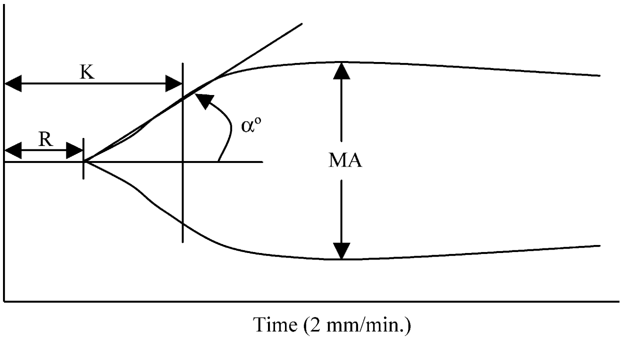

Fig. 4. Interpretation of TEG tracing.

TEG measures the viscoelastic properties of blood as it is induced to clot under a low shear environment which is similar to a sluggish blood flow. TEG also characterizes the formation and strength of the blood clot over time. Strength of a clot is graphically represented over time as a characteristic cigar shape profile which is obtained on a chart running at 2 mm/min (Fig. 4).

There are some principle parameters of TEG tracing (Tobias, Henry, & Augostides, 1999; Wenker, Wojciechowski, Sheinbaum, & Zisman, 2000). Reaction time, *R*, is the period of time from initiation of the test to the initial fibrin formation. *K* is a measure of time from beginning of clot formation until the amplitude of thromboelastogram reaches 20 mm and represents the dynamics of clot formation. An α angle is an angle between the line in the middle of the TEG tracing and the line tangential to the TEG curve. Maximum amplitude (MA) is the maximum amplitude of the tracing and represents the strength of a clot.

Fig. 5 illustrates TEG profiles of blood with benzoyl chitosan nanoparticles and saline solution.

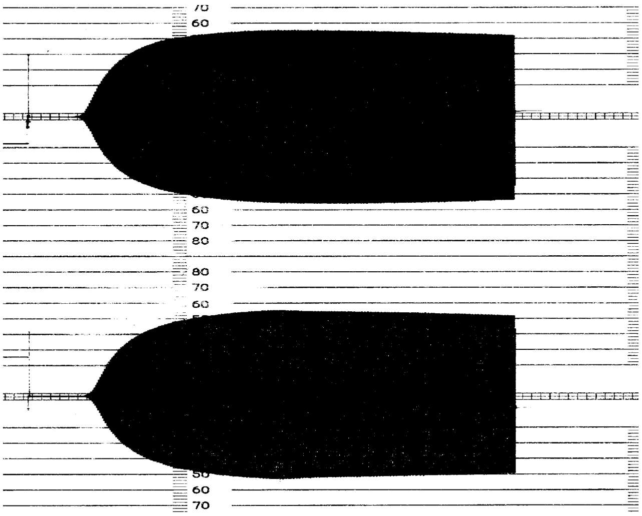

Fig. 5. TEG profiles of benzoyl chitosan nanoparticles treated blood (top) and saline-treated control (bottom).

Table 3
TEG variables for chitosan nanoparticle-treated whole blood ($n = 3$)

Groups	*R*-time (min)	*K*-time (min)	α Angle	MA (mm)
Pure chitosan	7.9 ± 1.6	2.5 ± 0.5	57.6 ± 5.8	52.6 ± 1.5
Butanoyl	8.4 ± 0.6	2.5 ± 0	55.5 ± 4.9	51.6 ± 2.5
Hexanoyl	5.3 ± 2.8	1.7 ± 0.6	65.5 ± 10.1	56.0 ± 3.6
Benzoyl	5.9 ± 0.7	1.8 ± 0.2	61.0 ± 1.0	54.0 ± 2.0

No significant change was observed, illustrating that benzoyl chitosan nanoparticles are compatible with blood. TEG data from different acyl groups were compared by the ‘Two Way Repeated Measures ANOVA’ (Table 3).

A *P*-value less than 0.05 was considered significant. There was no significant difference between chitosan nanoparticle-treated groups and saline-treated control. In addition, no significant difference was observed between unmodified chitosan nanoparticles and *N*-acyl chitosan nanoparticles at the concentration of 0.2% in a saline solution.

Lee et al. (1995) reported the in vitro blood compatibility of *N*-acyl chitosans based on rheological measurement and blood clotting test. One of the possible explanations for the blood compatibility of *N*-acyl chitosans is the balance in the hydrophobic and hydrophilic properties (Hirano, Noishiki, & Kinugawa, 1985). Hirano et al. explained that the hydrophilic nature of chitosans was altered by hydrophobic groups such as long chain acyl groups and, thus the balance in the hydrophobic and hydrophilic properties improve the blood compatible properties.

4. Conclusion

Chitosan was *N*-acylated with butanoic, hexanoic and benzoic anhydride under the homogeneous condition in the presence of methanol. The degree of substitution was determined to be 22–38% by ^{1}H-NMR. The *N*-acyl chitosan nanoparticles were prepared by ionic crosslinking with tripolyphosphates. The particle formation of *N*-acyl chitosans was found to be dependent on the degree of substitution. After freeze-drying, unmodified chitosan nanoparticles (~160 nm) aggregated because of strong inter- and intramolecular hydrogen bonding. As a result, there was a significant reduction of zeta potential. *N*-acyl chitosan nanoparticles were in the range of 200–360 nm and positively charged (10–20 mV) in a saline solution after freeze-drying. Long and bulky acyl groups seem to hinder hydrogen bonding and prevent aggregation. Preliminary blood compatibility tests showed that the long aliphatic and aromatic acyl groups did not significantly influence the hemolytic activity and blood clotting behavior, suggesting that *N*-acyl chitosan nanoparticles are compatible with blood.

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