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Beta-sitosterol/polyethylene glycol complexes as drug delivery vehicles

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β-sitosterol/polyethylene glycol complexes were prepared by solution blending in 1,2-dichloroethane. 1,2-Dichloroethane is a good solvent for the two components. The complexes were studied by Nuclear Magnetic Resonance (NMR) spectroscopy and Differential Scanning Calorimetry (DSC). These complexes have the possibility of reducing the swelling of benign prostatic hyperplasia and diminishing inflammation. β-sitosterol is hydrophobic and thus it is difficult to deliver the sterol into aqueous systems. Aqueous system delivery is required for effective blood circulation. Polyethylene glycol was used because of its amphiphilic properties. Proton NMR (¹H NMR) of the complexes shows that the methylene (CH₂) protons of the PEG are slightly shifted because of its non-covalent interaction with β-sitosterol. The complex formation was supported by 2-D NMR (NOESY) spectroscopy. NOESY spectra show cross peaks, indicating interaction between the two components. DSC of the complexes show thermal characteristics that
are different from the individual components. In particular, the PEG in the complexes shows a lower melting point and decreased crystallinity compared to the pure PEG. The melting point is lowered from 62 °C to 55 °C for the PEG 35,000/β-sitosterol (10%) complex. The NMR and DSC studies suggest the formation of a relatively stable β-sitosterol/polyethylene glycol complex.
BETA-SITOSTEROL/POLYETHYLENE GLYCOL COMPLEXES AS DRUG DELIVERY VEHICLES

A THESIS
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY
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DEPARTMENT OF CHEMISTRY

ATLANTA, GEORGIA

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LIST OF ABBREVIATIONS

CES: Carboxyethyl-β-sitosterol

Da: Dalton

DSC: Differential Scanning Calorimetry

FT-IR: Fourier Transform Infrared

IP: Intraperitoneal

IPCS: International Programme on Chemical Safety

LD50: Lethal Dose, 50% or median lethal dose

Log P: The logarithm of the ratio of the concentrations of unionized solute in the solvent

MP: Melting Point

NMR: Nuclear Magnetic Resonance

PEG: Polyethylene Glycol

PCL: Poly(ε-carolactone)

PLA: Polylactic acid

PLL: Poly(L-lysine)

PP2A: Protein Phosphatase 2A

RES: Reticuloendothelial system

SIT: β-sitosterol
CHAPTER 1
INTRODUCTION

1.1 Prostate Cancer

Prostate cancer is the second most diagnosed cancer in the world.\(^1\) However, the number of prostate cancer survivors has been increasing in the past 10 years by an estimated 200,000 men every year.\(^2\) The prostate cancer survivor population in the United States approached 2.8 million survivors in January 2012.\(^2\) Most of them (62%) are aged 70 and older with multiple comorbid conditions.\(^3\) This increase in prostate cancer survival rates is due to the availability of different treatment options including radiotherapy, chemotherapy, surgery, hormone therapy, immunotherapy, and in many cases, a combination is needed to produce the intended curative results.

A patient’s prognosis depends on early detection, complete surgical removal and effective radiotherapy, chemotherapy, or other treatments. Unfortunately, each of the treatments has advantages and disadvantages. For example, chemotherapy induces many adverse effects such as anemia, hair loss, fatigue, appetite change, nausea, vomiting, diarrhea, and constipation. An effective approach to decrease or minimize side effects is via multiple or combined treatment options.

The most important factor in prostate cancer survival is early and precise detection by regular screening examinations. It is difficult to determine who is at risk of developing any type of cancer. However, eliminating tobacco and alcohol usage, sun
exposure and obesity; having a healthy diet, and engaging in physical activity can decrease the incidence of cancer.\textsuperscript{4}

Once cancer is detected, it is important to reduce the swelling related to the existing inflammation before starting any type of treatment. Phytosterols are medications that diminish inflammation.

1.2 Phytosterols

Phytosterols, as the name indicates, are a group of steroids that are produced by plants. They have a cholesterol-like molecular structure and are present in all plant based foods, especially in vegetable oils. Phytosterols are structurally similar to the cholesterol produced by animal cells. Due to their structural similarity to cholesterol, the function of phytosterols in plant cells is similar to that of cholesterol in animal cells.\textsuperscript{5} Phytosterols are triterpenes that act as structural components and stabilize phospholipid bilayers in plant cell membranes.\textsuperscript{6}

Phytosterols were initially studied as a cholesterol-lowering agent because they reduce the absorption of dietary cholesterol and thus offer protection from cardiovascular diseases. Later, they were found to have many other beneficial uses for human health. Most phytosterols have 28 or 29 carbon atoms with one or two carbon–carbon double bonds, one is in the sterol nucleus and another one may be present in the alkyl side chain.\textsuperscript{7} The most commonly occurring phytosterols are \(\beta\)-sitosterol, campesterol, and stigmasterol (Fig. 1).
One of the most abundant dietary phytosterols is β-sitosterol (24-ethylcholesterol), which is found in many nuts, seeds, and beans. It has been used in many natural remedies such as stinging nettle, devil’s claw, and saw palmetto. As it is one of the phytosterols it has a structure similar to cholesterol. Therefore, it has been studied as a blood cholesterol-lowering agent, which may be due to its ability to prevent the intestinal absorption of cholesterol. β-sitosterol has also shown anti-inflammatory and analgesic properties in various animal models. It was determined that it has an anti-inflammatory activity, similar to hydrocortisone and oxyphenbutazone, and an antipyretic effect similar to acetyl salicylic acid. In another study, it was found to have an anthelmintic property.
More importantly, β-sitosterol has been found to reduce the symptoms of benign prostatic hyperplasia.\textsuperscript{20,21} Moreover, it decreases the incidence of colon, prostate and breast cancers among Asian men and women who consume significant amounts of β-sitosterol.\textsuperscript{8,9,10} β-sitosterol has been found to inhibit growth and also shown to have cytotoxic effects against a range of cancer cell lines.\textsuperscript{22,23}

1.4 Polyethylene Glycol

Unfortunately, the free form of β-sitosterol is not soluble in water.\textsuperscript{24} It has a molecular weight of 414.7067 Da and a log P value of 9.3, which indicates high hydrophobicity.\textsuperscript{25} Due to its insolubility in water, β-sitosterol has limited applications. Many studies have been carried out to increase its solubility either in oil or in water. One study has reported that the chemically modification of the 3-hydroxy group with fatty acids increases its solubility in fats.\textsuperscript{26} The ester compounds resulting from the esterification of β-sitosterol were studied and found to have the same cholesterol-lowering effects as the parent molecule.

Because of the beneficial effect of β-sitosterol in the treatment of prostate cancer and due to its limited hydrophilicity, β-sitosterol has been covalently conjugated with PEG (Fig. 2). The conjugated structure has shown increased solubility in aqueous medium. The conjugation required a two-step chemical synthesis and is shown in Figure 3. The first step was to synthesize an intermediate (carboxyethyl-β-sitosterol, CES), which imparts a carboxylic functionality to β-sitosterol, and the second step was the coupling of CES to PEG with a molecular weight of 1,500 Da.\textsuperscript{24}
An easier approach to increase water solubility would perhaps be to prepare a β-sitosterol and PEG complex by solution blending of the two components. The resulting complex would have a hydrophobic moiety of β-sitosterol and a hydrophilic part of PEG. This approach was utilized to improve water solubility of a co-poly(ester amide) by complexation of the hydrophobic poly(ester amide) with PEG. 

Figure 2. Chemical structure of polyethylene Glycol.

Figure 3. Synthesis of β-sitosterol and PEG conjugate. 

β-Sitosterol + Succinic anhydride → CES

CES + HO(\text{CH}_2\text{CH}_2\text{O})_n\text{OH} → HPS
Several other approaches have been proposed to increase the water solubility of β-sitosterol and they were mainly by preparing formulations with emulsifiers. Lecithin emulsified micelles of β-sitosterol were found to have a cholesterol-lowering effect similar to pure β-sitosterol. An other attempt to increase β-sitosterol’s water solubility was the formulation of β-sitosterol containing liposomes and this increased its chemopreventive effect. In addition an inclusion complex of β-sitosterol in β-cyclodextrin has been studied and found to have improved the water solubility of β-sitosterol.

A simple method for increasing the hydrophilicity of hydrophobic agents is to form a complex with polyethylene glycol (PEG). Many of the past and current studies in pharmaceutical research focused on the ability of PEG to modify the pharmacokinetic properties of drugs and drug carriers. Shielding or bonding the administered drugs to PEG changes their pharmacokinetics resulting in prolonged blood circulation times. This, in turn, prevents the recognition of the drug as foreign and allows it to reach its site of action before being cleared from the body. Therefore, most liposomal and micellar formulations as well as conjugated drugs, on the market or in advanced clinical trials, are PEG-containing products (PEGylated).
CHAPTER 2

BACKGROUND

2.1 Theories of Cancer Protection

The exact mechanism of cancer protection by β-sitosterol is not known. Several theories have been developed to explain this effect and the findings in these areas are as follows:

- **Effect of β-sitosterol on Membrane Structure**

  Due to its similarity to cholesterol, an integral lipid component of biological membranes, β-sitosterol incorporation into membranes has been studied. β-sitosterol incorporation resulted in a 50% decrease in sphingomyelin and an 8% increase in phosphatidyl choline. This may cause an alteration in some signal transduction pathways.

- **Effect of β-sitosterol on Membrane Fluidity**

  Membrane fluidity is influenced by the lipid composition of membranes. Fluidity should be kept within a very narrow range for the proper function of membranes. Incorporation of β-sitosterol into membranes decreased fluidity.
• Effect of β-sitosterol on Membrane-Bound Enzymes

β-sitosterol incorporation into membranes increases the activities of some fatty acid desaturases in the liver. However, it decreases the activities of hepatic and prostatic 5-α-reductase and prostatic aromatase, membrane-bound enzymes involved in the metabolism of testosterone in rats. These two enzymes metabolize testosterone into androgens and estrogens, which play a role in the development of prostate hyperplasia and prostate cancer.

• Effect of β-sitosterol on Signal Transduction Pathways

As previously mentioned, incorporation of β-sitosterol into cell membrane causes changes in membrane phospholipid, mainly in sphingomyelin, which may indicate that β-sitosterol has an effect on the sphingomyelin cycle (Fig. 4). By investigating this pathway in HT-29 cells and LNCaP cells, it was found that there is an activation of the cycle and an increase in the production of ceramide. Ceramide has been suggested to activate protein phosphatase 2A (PP2A) as an intermediate step for the action of ceramide on cell growth and apoptosis. β-sitosterol supplementation caused an increase in the activity but not the amount of PP2A in LNCaP cells.
Effect of β-sitosterol on Apoptosis

The rate of tumor growth depends on the balance between the rates of cell proliferation and apoptosis. The effect of β-sitosterol on apoptosis, or programmed cell death, has been studied in two tumor cell lines, MDA-MB-231 and LNCaP cells. In both cell lines, it was found that β-sitosterol stimulated apoptosis by four to six folds above control levels after 3–5 days of treatment.

Effect of β-sitosterol on immune function

It was found that a mixture of β-sitosterol and its glucoside at a mass ratio of 100:1 potentiate human peripheral blood lymphocyte proliferation in vitro and enhanced
T-cell proliferation upon stimulation in vitro. The exact mechanism by which β-sitosterol may stimulate immune system function is not fully understood.

- Effect of β-sitosterol on Neutral and Acidic Sterols in the Colon

Primary bile acids and cholesterol are converted to secondary bile acids and coprostanol, respectively, in the large intestine by bacterial action. A high level of these modified sterols in the colon plays a role in the development of colon cancer. Dietary β-sitosterol alters the level of fecal sterols by acting on colonic bacteria and causing alteration of cholesterol absorption.

2.2 PEGylation Concept

The concept of PEGylation was not known until the late 1970s, and by the 1990s, it was being used in many drug carrier systems. Abuchowski et al first reported PEGylation of proteins in 1977. They showed that PEGylated albumin was non-immunogenic and PEGylated liver catalase had a long blood circulation time (48h) while maintaining the activity of the enzyme.

Many PEGylated DNA, RNA, proteins, polypeptides, and small molecules have been found to be more stable and efficient compared to native drugs and have reached the market as commercial products. The pharmacokinetics of PEG surface covered poly(lactic-co-glycolic acid) microspheres was reported by Gref et al in 1994. It was found that the liver removed 66% of then on-coated particles in only 5 minutes after injection while it captured less than 30% of the 20 kDa PEG-coated nanospheres 2 hours after injection.
Since the early 1960s, liposomes have been used as versatile drug-delivery systems. In 1990, it was reported that the PEGylation of liposome had enhanced blood circulation times of liposomes. Conventional liposomes were reported to be completely cleared from blood after 5 hours, whereas 49% of PEGylated liposomes were still circulating in the blood after the same amount of time.

Kabanov et al. were the first to use PEG as a hydrophilic part of linear diblock copolymers for the formation of micelles. Kwon and Kataoka used PEG-containing block copolymer micelles as drug-delivery carriers. Polymers used as vectors for gene transfection in gene therapy must have special properties due to the charged nature of DNA. However, the cationic non-viral vectors are toxic and have a short half-life in the body. The PEGylation of these carriers decreased their depositions in the lung and lowered initial toxicities compared to unmodified complexes. These beneficial effects may be due to a lower aggregation of the complexes, a decreased interaction with blood constituents, and a lower rate of filtration by pulmonary capillaries. Moreover, PEG covered carriers have a slower uptake by the organs (liver and spleen) of the reticulendothelial system (RES). PEGylated poly(l-lysine) (PLL) showed an increased amount of polyplex (15% to 69%) circulating in the blood.

PEGylation of drugs, liposomes, and nanocarriers reduces their renal filtration, decreases enzymatic degradation, and diminishes uptake by the RES. Therefore, PEGylated drugs have a prolonged half-life in the body and an enhanced bioavailability. This decreases the frequency of drug administration and lowers the amount of the drug
administered, which leads to an improvement in the life quality of the patient and reduction in clinical costs.\textsuperscript{31,51}

\textbf{2.3 Polyethylene Glycol Applications}

The molecular weights of PEG used in different pharmaceutical and medical applications ranges from 400 Da to about 50 kDa. To conjugate with low-molar-mass drugs such as small molecules, oligonucleotides, and siRNA, PEG with a molar mass of 20 kDa to 50 kDa is often used. This avoids fast renal clearance because the size of the conjugates is above the renal clearance threshold. For larger drugs, such as antibodies or nano particulate systems, PEGs with lower molar weights of 1 kDa to 5 kDa are mostly used. This decreases the opsonization and subsequent elimination by the RES.\textsuperscript{52}

Pharmacokinetic studies of PEG on a wide range of animal species such as rats, mice, guinea pigs, monkeys, and dogs have been carried out. These studies determined increasing the molar mass of PEG decreased its gastrointestinal absorption. Therefore, PEGs with a molar mass of 4 kDa to 6 kDa are not absorbed over 5 hours in rat intestines, and lower molar-mass PEGs of about 1 kDa have a slight absorptive effect of about 2%. PEG is mainly excreted by the kidneys. After intravenous injection of 1 g of 1 kDa and 6 kDa PEG, 85\% and 96\% of PEGs were excreted in human urine in 12 hours, respectively.\textsuperscript{53} As reported by IPCS, LD\textsubscript{50} values for 6 kDa PEG (50\% solution in water) in mice, rats, rabbits, and guinea pigs after oral intake were higher than 50 g/kg of body weight. After intraperitoneal (i.p.) administration, 6 kDa PEG showed LD\textsubscript{50} value of 5.9 and 6.8 g/kg in mice and rats, respectively.\textsuperscript{54}
Due to its success in drug-delivery applications, PEG has been used in other medical fields. PEG reduces the aggregation of red blood cells and improves the blood compatibility of poly(vinylchloride) bags. Therefore, it is used in blood and organ storage.\textsuperscript{55,56} Cardiovascular devices, such as stents made from PEG copolymers showed decreased thrombosis.\textsuperscript{57} Furthermore, PEG is used in pharmaceutical preparations as an excipient for parenteral, topical, nasal, and ocular applications, and it is used as the active ingredient in laxatives.

PEG has been extensively used in the formation of micelles by covalent bonding to a hydrophobic moiety to from a hydrophobic core and a hydrophilic shell. The hydrophilic shell allows the micelles to escape uptake by the reticuloendothelial system allowing the micelles more time in circulation as in the case of PEG/PLA and PEG/PCL micelles.\textsuperscript{58} PEG has been also used to increase the stability of liposomes and increase their residence time in circulation by allowing them to remain undetected by the RES system.\textsuperscript{59} Solid lipid nanoparticles having a PEG surface coverage has been studied and found to increase the half-life of hydrophobic drugs such as Noscapine.\textsuperscript{60} Moreover, PEG covered gelatin nanoparticles have been used to increase the residence time of gelatin nanoparticles.\textsuperscript{61}
CHAPTER 3

EXPERIMENTAL

3.1 Materials

Polyethylene glycol (MW 2,000, 8,000, and 35,000) was obtained from Sigma-Aldrich Chemical Company. β-sitosterol (Practical Grade M.W. 414.7) was purchased from MP Biomedicals Inc. 1,2 Dichloroethane (99%) was obtained from Sigma-Aldrich Chemical Company. Benzene was purchased from Fisher Chemical Company. Chloroform-D, (99.8%), for NMR was acquired from Acros Organics Company. All materials were used as received.

3.2 Methods

3.2.1 Preparation of β-sitosteroland Polyethylene Glycol (MW 2,000,8,000, and 35,000) complexes

One gram (1g) of polyethylene glycol (MW 2,000, 8,000, 35,000) of different molecular weights was added to three different round bottom flasks equipped with magnetic stirrers and dissolved in 1,2-dichloroethane (10 mL). The solutions were stirred until the polymers were completely dissolved and this process took 24 hours at room temperature. Also, 0.1 g of β-sitosterol powder was dissolved in 1,2-dichloroethane (10 mL) in three separate flasks by stirring for 24 hours at room temperature. Each of the flasks containing polyethylene glycol was mixed with a flask containing β-sitosterol and stirred overnight. Most of the 1,2-dichloroethane was removed by nitrogen flushing for 6
hours. Lastly a few drops of benzene were added to the flasks and the samples freeze dried to obtain white powders. The powders were dried on the vacuum line for 48 hours.

3.3 Instrumentations

3.3.1 Nuclear Magnetic Resonance Spectroscopy

Proton NMR ($^1$H-NMR) spectra in solution state were obtained using a Bruker AVANCE 500 MHz spectrometer at room temperature with chloroform (CDCl$_3$) as the solvent. 2D NMR experiments (NOESY) were also carried out using the AVANCE 500 NMR spectrometer.

3.3.2 Fourier Transform-Infrared Spectroscopy

The β-sitosterol/polyethylene glycol complexes were crushed in a mortar and pestle with 98% potassium bromide (KBr) until a fine powder was obtained. The samples were molded into pellets and IR spectra recorded.

3.3.3 Differential Scanning Calorimetry

Differential Scanning Calorimetry was carried out on a TA instruments DSC Q2000 at a heating rate of 10°C per minute between the temperature ranges of 25°C to 200°C. The DSC was calibrated using Indium under a nitrogen gas atmosphere. All samples were quenched cooled before acquiring the thermograms. The samples were heated to 200°C and cooled to 25°C before obtaining the heating cycle at 10 °C. The
glass transition temperatures were reported from the second heating cycle. The heating rate in the first and second heating cycles remained the same.

3.3.4 Carbon T$_{1p}$Relaxation

Rotating frame carbon spin lattice relaxation (T$_{1p}$) of solid samples was determined at room temperature on a Bruker 500 MHz AVANCE NMR instrument at a spinning rate of 5 KHz with 5 mm zirconium spinners. A 4 µs 90° pulse sequence (p1) was employed followed by variable durations. Typically, the spin locking pulse sequence was applied ranging from 0.001 ms to 5 ms (16 different pulses) to the samples within the expected range of relaxation time. The value of pulse power p111 used was the same as p1 (= 4 µs) for relaxation measurement purposes. The number of pulse sequences corresponded to the number of data points collected for relaxation calculation. Relaxation data was directly fitted to a binomial equation to determine the relaxation time (topspin analysis).
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Solid State Characterization of the Complexes

The solid state characterization studies suggest the formation of water soluble complexes of β-sitosterol/polyethylene glycol. The solid state complexes were studied by FT-IR and DSC.

4.1.1 Fourier Transform-Infrared Spectroscopy

The FT-IR spectra of the pure β-sitosterol, pure polyethylene glycol, and the complex are shown in (Fig. 5). The FT-IR of the complex shows that the intensity of the aliphatic tail of β-sitosterol (i.e. the C-H stretching 2876 -2900 cm⁻¹) was slightly decreased compared to the free β-sitosterol. This observation is consistent with observation for the β-sitosterol/β-cyclodextrin complexes.

The OH group intensity of pure polyethylene glycol was weak because there are only two terminal OH groups compared with many C-O and C-H bonds. Additionally, the OH peaks in the PEG/β-sitosterol is diminished in intensity compared to the free β-sitosterol. This suggests the formation of a complex. The formation of the complex lowers the possibility of intermolecular hydrogen bonding among β-sitosterol molecules because; in the complex the β-sitosterol is interacting with the PEG via van der Waals interactions. This observation also suggests the formation of a complex between the two components.
4.1.2 Differential Scanning Calorimetry

The melting point for PEG 2,000, 8,000, and 35,000 are 51.1°C, 61.5°C, and 62.1°C respectively (see figures 6, 7, and 8). The melting point decreased when 10% (by weight) of β-sitosterol was mixed with different molecular weights of PEG (Table 1). The decrease in the melting point is most likely because the β-sitosterol interferes with the crystallization of PEG. The insertion of the β-sitosterol in between polymer chains inhibits the alignment required for crystallization. The largest difference in melting point was between 35,000 PEG alone and the mixture of 35,000 PEG with β-sitosterol. When the 35,000 PEG interacted with 10% of β-sitosterol through hydrophobic interaction, the formation of well-defined complexes inhibit crystallization. The interaction between the β-sitosterol and lower molecular weight PEGs (2,000 and 8,000) is weaker and had a smaller decrease in the glass transition temperature. The β-sitosterol was better distributed within the 35,000 PEG because the longer chains permitted better van der Waals interactions between the β-sitosterol and the PEG methylene groups.
Figure 6. DSC thermograms of 2,000 PEG and 2,000 PEG/β-sitosterol complex.

Figure 7. DSC thermograms of 8,000 PEG and 8,000 PEG/β-sitosterol complex.
Figure 8. DSC thermograms of 35,000 PEG alone and 35,000 PEG/β-sitosterol complex.

Table 1. Comparison of the melting point of the PEG alone and PEG with the 10% of β-sitosterol.

<table>
<thead>
<tr>
<th>PEG MW</th>
<th>Polymer MP (°C)</th>
<th>Complex MP (°C)</th>
<th>Decrease in MP (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,000</td>
<td>51.13</td>
<td>48.86</td>
<td>-2.27</td>
</tr>
<tr>
<td>8,000</td>
<td>61.49</td>
<td>57.05</td>
<td>-4.44</td>
</tr>
<tr>
<td>35,000</td>
<td>62.06</td>
<td>55.75</td>
<td>-6.31</td>
</tr>
</tbody>
</table>

To find the maximum possible amount of β-sitosterol that can be loaded into complexes with the 35,000 PEG, the percentage of β-sitosterol was increased to 20% and 30%. Increasing the β-sitosterol content past 10% increased the PEG melting point after a lowering of the melting point was observed at 10%. The melting point of the complex with the higher β-sitosterol content was closer to the pure PEG, suggesting that
increasing β-sitosterol past 10% results in a microphase separated system with separate phases of PEG and β-sitosterol present (Fig. 9). Therefore, the results of our study indicate that 10% β-sitosterol was distributed very well distributed in matrix and interacted well with the PEG through hydrophobic interactions. However, the PEG with 20% and 30% of β-sitosterol formed clusters (almost phase separated) in a PEG matrix and thus had poor distribution within complex (Table 2).

Figure 9. DSC thermograms of 35,000 PEG and 35,000 PEG/β-sitosterol (10%, 20%, 30%) complexes.
Table 2. Difference in MP between PEG 35,000 (62.06 °C) and the complex of the drug with PEG 35,000 as a function of β-sitosterol content.

<table>
<thead>
<tr>
<th>β-sitosterol by weight</th>
<th>Complex MP (°C)</th>
<th>Difference in MP (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>55.75</td>
<td>-6.31</td>
</tr>
<tr>
<td>20%</td>
<td>62.84</td>
<td>0.78</td>
</tr>
<tr>
<td>30%</td>
<td>63.91</td>
<td>1.85</td>
</tr>
</tbody>
</table>

4.1.3 Carbon $T_1p$ Relaxation

The $T_1p$ of β-sitosterol had the longest relaxation time (11.42 ms). The $T_1p$ of polyethylene glycol was 4.99 ms and the complex with 10% β-sitosterol had the shortest $T_1p$ of 3.08 ms (Table 3). The short $T_1p$ of the complex suggests that the system is more amorphous and this observation supports the DSC results, which indicated that the crystallinity is lower in the complex compared to the pure PEG. Additionally, it further suggests that the β-sitosterol is embedded in the amorphous region of the PEG in the matrix of the complex.

Table 3. $T_1p$ for β-sitosterol, PEG, and the complex.

<table>
<thead>
<tr>
<th>Substance</th>
<th>$T_1p$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sitosterol</td>
<td>11.42 ($T_1p$ of the carbon with longest relaxation time)</td>
</tr>
<tr>
<td>PEG</td>
<td>4.99 (methylene carbon)</td>
</tr>
<tr>
<td>(10%) β-sitosterol/PEG complex</td>
<td>3.08 (methylene carbon of PEG)</td>
</tr>
</tbody>
</table>
4.2 Solution State Characterization of the Complexes

The administration of the complex as a therapeutic has to be carried out in the solution state and therefore, it is necessary to characterize the formation of the complex in the solution state. The complex in the solution state was studied by one-dimensional (proton) and two-dimensional (2D) NMR spectroscopy (NOESY).

4.2.1 Proton Nuclear Magnetic Resonance (1H NMR)

1H NMR is beneficial for studying complexes in solution. β-sitosterol is insoluble in H2O. The aqueous solubility of β-sitosterol was increased by the presence polyethylene glycol. This observation is consistent with the polyethylene glycol’s capability of solubilizing hydrophobic compounds in aqueous media as a consequence of the formation of a complex or a non-covalent adduct.64 1H NMR spectra of polyethylene glycol as a function of β-sitosterol are displayed in Figure 10. As the β-sitosterol was added in the same ratio to different molecular weights of polyethylene glycol, a downfield shift the methylene protons of the PEG was observed (Table 4). The downfield shift of the methylene proton suggests that the PEG is an electron donor to the β-sitosterol.
Figure 10. $^1$H NMR chemical spectra (a) PEG 8,000 and (b) PEG with 10% $\beta$-sitosterol content.
Table 4. The $^1$H NMR chemical shift of the PEG methylene groups as a function of molecular weight at 10% β-sitosterol content.

<table>
<thead>
<tr>
<th>PEG (MW)</th>
<th>Methylene protons (ppm)</th>
<th>Methylene protons (ppm)</th>
<th>Δ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pure PEG</td>
<td>Of PEG with 10% β-sitosterol</td>
<td></td>
</tr>
<tr>
<td>2,000</td>
<td>3.54</td>
<td>3.58</td>
<td>0.04</td>
</tr>
<tr>
<td>8,000</td>
<td>3.55</td>
<td>3.58</td>
<td>0.03</td>
</tr>
<tr>
<td>35,000</td>
<td>3.55</td>
<td>3.58</td>
<td>0.03</td>
</tr>
</tbody>
</table>

4.2.2 2D (NOESY) Proton Nuclear Magnetic Spectroscopy

Further evidence for the formation of a complex or a non-covalent adduct was found in the 2D Nuclear Overhauser Effect Spectroscopy (NOESY) as shown in Figure 11. The NOESY spectrum displays cross peaks between the hydrophobic part of polyethylene glycol (the methylene groups) with the aliphatic tail and with the cyclic head of the β-Sitosterol. The NOESY results suggest that the β-sitosterol may have different possible orientations as it interacts with the polyethylene glycol chain.62-63 Possible orientations include one of the two ends interacting with the methylene groups of the PEG and also an orientation in which both ends simultaneously interact with the PEG. The two ends simultaneously interacting with the PEG will make for a more stable non-covalent adduct or complex because of two site of interactions is most likely stronger than just one interacting site.
Figure 11. 2D NOESY (500 MHz) of β-sitosterol/polyethylene glycol complex in CDCl₃.
CHAPTER 5
CONCLUSION

The findings of this study indicate that hydrophobic interactions between the β-sitosterol and polyethylene glycol results in the formation of a complex or a non-covalent adduct. The complex or non-covalent adduct is water soluble whereas the pure β-sitosterol is water insoluble. The differential scanning calorimetry studies suggest that the complex with 10% (by weight) β-sitosterol is well distributed throughout the matrix. Increasing the β-sitosterol content to greater than 10% by weight in the complex results in the formation of β-sitosterol clusters, which diminishes the distribution of β-sitosterol throughout the matrix. The NOESY NMR results demonstrate different possibilities for the orientation of how the β-sitosterol interacts with the polyethylene glycol. β-sitosterol can interact through either the cyclic head or the aliphatic tail or both head and tail can simultaneously interact with the methylene groups of the PEG. The level or strength of interaction increases with increasing PEG molecular weight.
REFERENCES


41. Abuchowski, A.; McCoy, J.R.; Palczuk, N.C.; van Es, T.; Davis, F.F., Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating


