PREPARATION OF COMPOSITE LIPOSOMES FOR TARGETED DRUG DELIVERY

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Abstract

Nanotechnology is positively impacting the development of modern pharmaceutical industry. One of extensively studied drug delivery systems in clinical practice are liposomes, which have been used for enhancement of the ability to apply highly toxic drugs and overcoming of barriers to cellular and tissue uptake.

The objective of the present work was to obtain the stable chitosan-coated liposomes and their interaction with anti-viral drugs. Liposomes were prepared by stepwise extrusion through 0.2 and 0.1 μm pore sizes using inorganic membranes Anotop. Several series of experiments using the various liposomal compositions were conducted. The liposomal dispersions obtained were compared in terms of particle size, polydispersity index, zeta potential and stability. The optimal composition of the lipid film was selected and the effect of various additives was investigated. It was shown that the addition of the polyethylene glycol - modified natural monoacylglycerides and biocompatible choline derivatives of limiting fatty acids allows to reduce the diameter of the liposomes obtained by the extrusion to 150-155 nm with a polydispersity index of 0.11 - 0.13. Liposomes from the best lipid composition were prepared in a biggest quantity and coated by modified chitosan. It was concluded that appropriate combination of the liposomes and polysaccharide layer might be used for the increasing of colloidal stability up to 5 months and broad functional capabilities for surface modification. Obtained composite liposomes were loaded with selected anti-viral drugs and used as a model objects for the parenteral administration.

Keywords: Liposomes, chitosan, extrusion, stability, anti-viral drugs

1. INTRODUCTION

The concept of drug targeting stems from the very idea of minimizing the risk-to-benefit ratio and achieving the maximum therapeutic effect. Therefore, precise and safe drug delivery to its target site at the acceptable period of time remains a yardstick in the design and development of novel drugs. Nanocarriers, in their various forms, have the possibility of providing endless opportunities in the area of drug delivery and therefore are increasingly being investigated to harness their potential [1].

Liposomes are excellent carrier systems for a variety of applications and are particularly ideal for drug delivery to the tissues due to the similarity to natural cells. The therapeutic index of drugs can be increased through its incorporation into liposomes, which can act as a non-toxic, biodegradable system for solubilizing drugs which have low aqueous solubility; protect rapidly degrading drugs from breakdown and consequently increase the drug residence time in the body; and also decrease drug toxicity [2]. Drug delivery using liposomes also reduces the accumulation of drugs in sensitive tissues; alters the biodistribution and controls the release of an incorporated drug, therefore improving bioavailability; as well as targets the drug to specific tissue [3]. However, stability is a general problem with lipid vesicles. To minimize the disruptive influences, the formation of a polymeric membrane around the liposome has been described by several authors [4,5]. Different types of biocompatible polysaccharides such as chitosan, cellulose, starch can be employed to improve the efficiency of conventional liposomal systems [5]. Chitosan is a hydrophilic, biocompatible, and

biodegradable polymer of low toxicity. Appropriate combining of the liposomal and chitosan characteristics may produce liposomes with specific, prolonged, and controlled release [5]. Several authors showed that the chitosan-coated liposomes were formed via ionic interaction between the positively charged chitosan and negatively charged diacetylphosphate on the surface of the liposomes supporting by the hydrogen bonding and hydrophobic interaction between chitosan and neutral lipid. [4,6]. The modification of this polysaccharide enables the production derivatives soluble in water at any value of pH. These derivatives may be used without restriction to stabilize liposome suspensions. Furthermore, the side groups introduced into the polysaccharide chain provide additional steric stabilization of liposomes. Published results show that the PEG coating, which is inert in the living systems, allows for longer circulatory life for the drug delivery mechanism and contributes stabilizing of liposomes in aqueous medium [7].

Nowadays design of antiviral drugs becomes increasingly important. There are several reasons: emergence of new viral strains, spreading of dangerous diseases of viral etiology (AIDS, viral hepatitis, viral fever etc.) and reduction of the general immune status of the population [8]. Therefore, the aim of the present work was to obtain the stable chitosan-coated liposomes and their interaction with anti-viral drugs. Liposomes were loaded by derivatives of triazolo-pyrimidine and triazolo-triazine. These are nucleoside analogs of nucleic acid, the principle of their action is inhibiting synthesis of viral RNA and DNA and replication of genomic fragments. In this study we investigated the possibility of obtaining stable liposomal preparations of these derivatives and the effect of various additives on main parameters of suspension.

2. MATERIALS AND METHODS

2.1 Materials

Epikuron 200 (composed of phosphatidylcholine (min. 95%) was obtained from Cargill, Incorporated (USA). Cetylpyridinium chloride was from Merck & Co., Inc., (Germany). Oxyethylated monoalkilfenol (Igepal) was from Aldrich chemical company, Inc. (USA). Tocopherol acetate, glycol-modified additives PEG-7 glyceryl cocoate, PEG-6 glycerides caprilinic and capric acids were purchased from Galaxy Surfactants Ltd.(India). Standart phosphate buffer (pH=6,86), chloroform were of analytical grade from Ekros (Russia). Palmitic acid and stearic acid were from BASF (Germany), palmitoylamide trimethylethanammonium iodide was synthesized according to the well-known method [9]. Chitosan derivatives were synthesized by method which based on the Ugi four-component condensation.

2.2 Synthesis of charge-forming components

Palmitic acid (1.8 g) (or stearic acid (2.0 g)) was dissolved in methylene chloride with heating, then N-hydroxysuccinimide (0.80 g) and dicyclohexylcarbodiimide (1.44 g) were added to flask. The mixture was placed under magnetic stirrer at 50 °C for 2 hours. The precipitate was filtered and discarded. 5 ml of solvent was added to the filtered product, the flask was placed under magnetic stirring and 0.77 g of dimethylethylenediamine was added, the reaction mixture was allowed to stir for 1 hour. The solvent was distilled off by rotary evaporation to obtain a dry powder. The powder was washed with distilled water at the pump 3 times. The precipitate was dried at room temperature. The powder was dissolved in 5 ml of chloroform, methyl iodide (0.520 ml) was added to solution with stirring. After 3.5 hours chloroform was distilled off by rotary evaporation. The product was dried at room temperature.

2.3 Preparation of liposomes

Samples of soy phosphatidylcholine, charge-forming component, active substance and glycol-modified additive were dissolved in 50 ml of chloroform. Then, a thin lipid film was formed on the sides of a 100-ml round-bottom flask by subsequent removal of the solvent by rotary evaporation at 40 °C to complete dryness. The resulting film was dried at room temperature by placing the flask on a vacuum pump for nearly 3 hours. Thereafter, the lipid film from the previous stage was filled by standard phosphate buffer solution pH

6.86 and placed in a shaker-type stirring device. Hydration was carried out for 2 hour at 40 °C of temperature. The resulting multilamellar vesicles were extruded two times through inorganic membranes Anotop for ultrafiltration with 0.2 and 0.1 μm pore sizes sequentially. Thus obtained solution of liposomes was stored in a dark place at 4 °C of temperature. The final lipid concentration was 10 mg/ml.

2.4 Synthesis of modified chitosan

For stabilizing the liposomal suspensions we selected a sample of chitosan with a molecular weight of 1.9×10^5 Daltons and 5% degree of acetylation. Thus, this polysaccharide contains free amino groups which can be used to modify the polymer chain. We chosed the Ugi-reaction, which had previously been used for the synthesis of cross-linked microgels based on pectinic acid [10].

Chitosan(1) (1.1 g) and carboxylic acid (250 mg) was dissolved in 0.4 l of 0.01 M HCl by using a magnetic stirrer at room temperature. The resulting solution was titrated with sodium hydroxide solution (1 g / l) until the pH 6.8. Isocyanide (20 mol % in relation to the free amino group of chitosan) and formaldehyde (1 ml of a 30% solution in water) were added to the chitosan suspension. Microgel suspension was incubated for 30-40 minutes under magnetic stirring and an excess of triethylamine was added to pH 8.0 and was incubated for another 1 hour. Then the reaction mixture containing the product (2) was filtered through ultrafiltration membrane Millipore (0.45 μm). General reaction scheme is shown in Fig. 1.

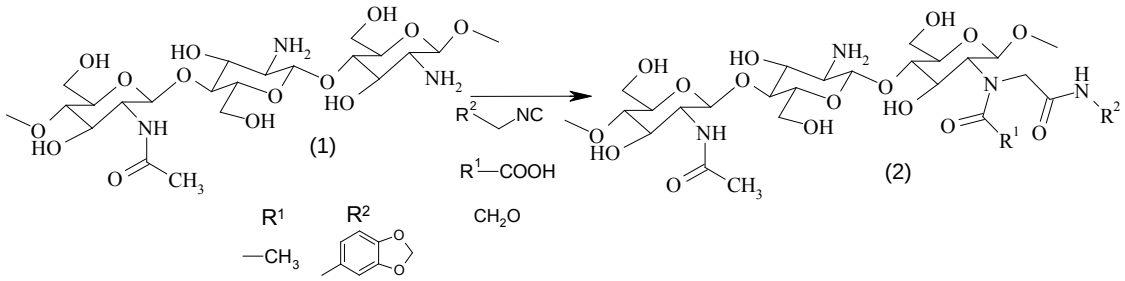

Fig. 1. Synthesis of modified chitosan (2)

2.5 Preparation of chitosan-coated liposomes

For the preparation of chitosan-coated liposomes, Liposomes from the best lipid composition were prepared in a biggest quantity and coated by modified chitosan. Chitosan derivative solution of various weight ratios (chitosan: lipids) 1:15, 1:5, 1:1 was added drop-wise to 10 mg/ml of liposomal suspension under magnetic stirring at room temperature. After addition of the chitosan, the mixture was left under stirring for approximately 1 hour, then incubated at 20 °C for stability test.

2.6 Characterization of liposomes

Particle size and zeta potential of liposomes and chitosan-coated liposomes were measured by dynamic light scattering spectrometer Zetasizer Nano SL (Malvern Instrument, UK) at scattering angle of 173°, 670 nm, and 25.0 °C. Polydispersity index was calculated from a Cumulants analysis of the DLS measured intensity autocorrelation function.

2.7 Characterization of synthesized compounds

The structures of the synthesized compounds were confirmed by Bruker DRX-400 (400 MHz) NMR Spectrometer and Bruker Alpha IR spectrometer.

3. RESULTS AND ITS DISCUSSION

3.1 Selection of optimal lipid composition

The content of the charge-forming component has a great influence on the distribution of liposome size. All samples with high content of cetylpyridinium chloride are characterized by two maxima of the size distribution in the range of 50 and 300 nm (diameter). Thus reducing of the amount of cetylpyridinium chloride from 5 to 1% was resulted in a decrease in the average particle diameter from 260 to 200 nm by changing the nature of the bimodal distribution to monomodal. Surface charge was decreased from 85 to 35 mV.

In subsequent experiments, the effect of various polyethylene-glycol additives to lipid compositions was studied. As such additives were used oxyethylated monoalkylfenol (Igepal), PEG-7 glyceryl cocoate, PEG-6 glycerides caprilinic and capric acids. Effect of 5% PEG additives on diameter of liposomes after extrusion through a membrane Anotop (200nm) is shown in fig. 2. These data suggest that all the selected components reduce the average diameter of the obtained liposomes, but the most effective compound is the PEG-7 glyceryl cocoate, which has been selected for further studies.

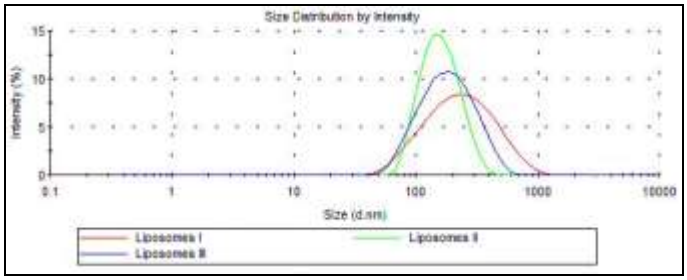

Fig. 2. Effect of PEG-modified complexes on liposomes size: ethoxylated monoalkylfenol (liposomes I), PEG-7 glyceryl cocoate (liposomes II), PEG-6 glycerides caprilinic and capric acids (liposomes III)

Then we have studied the dependence of the average size of the liposomes from the percentage input of PEG-7 glyceryl cocoate into liposomes produced by extrusion through Anotop membranes with a pore diameter 200 nm and 100 nm respectively. The resulting graphs (Fig.3) show that the optimal concentration of PEG-7 glyceryl cocoate in lipid composition is 5%. Addition of this component provides a liposome size 150-155 nm and a polydispersity index of 0.11-0.13 that satisfies the requirements of parenteral pharmaceuticals.

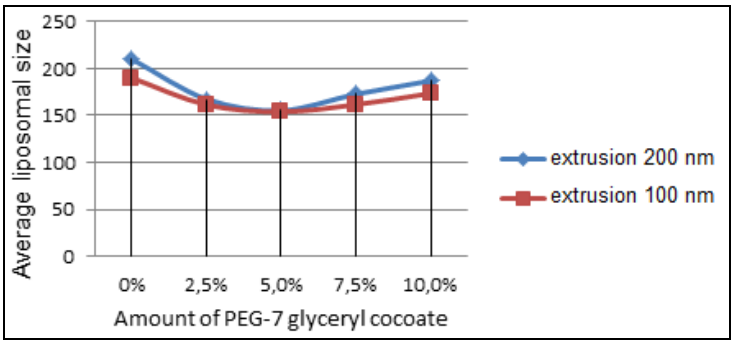

Fig. 3. Effect of PEG-7 glyceryl cocoate in different concentrations on liposomes size

3.2 Synthesis of non-toxic charge-forming compound

In continuation of this work we have decided to synthesize biocompatible amides as a charge forming additive to the lipid composition [9]. Stearoylamide trimethylethanammonium iodide (3) and palmitoylamide trimethylethanammonium iodide (4) were obtained according to the reactions shown in Fig. 4.

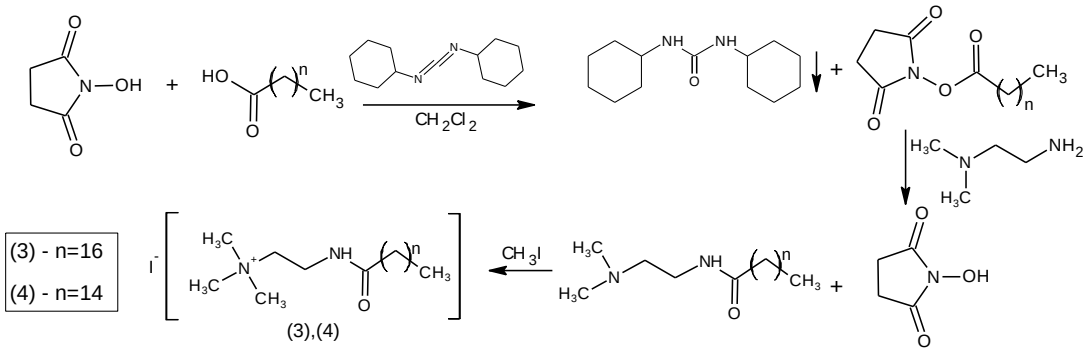

Fig. 4. Synthesis of stearoylamide trimethylethanammonium iodide (3) and palmitoylamide trimethylethanammonium iodide (4)

Liposomes (IV,V) with the synthesized substances (3,4) were obtained by standard techniques. As a result, after double extrusion through membranes Anotop 200 and 100 nm monodisperse liposomes were turned, its average diameter and the polydispersity index were 163.4 nm, PdI = 0,15 (IV) and 154.5 nm, PdI = 0,13 (V).

The stability of the liposome samples with stearoylamide (IV) and palmitoylamide (V) trimethylethanammonium and PEG additives described above was measured by dynamic light scattering weekly (Table 1). All measurements shown smaller liposome size and polydispersity index for samples with palmitoylamide (V). From these results it can be concluded that the liposomes with palmitoylamide trimethylethanammonium are the best for use in medical practice.

Table 1 Comparison of liposome stability

Days	Sample name	Averege size, Z-Ave d.nm	Polydispersity index, PdI
0	IV	163,4	0,150
0	V	155,1	0,143
7	IV	212,2	0,236
7	V	188,7	0,178
14	IV	217,6	0,298
14	V	193,0	0,221
21	IV	225,5	0,352
21	V	220,7	0,317

3.2 Preparation of chitosan-coated liposomes

Modified chitosan samples prepared as described above were used for stabilization of liposomal suspensions. It was found that input of the water-soluble chitosan containing carboxylic acid groups allows to extend shelf life of the liposomes till 3 months or longer. For this study we used liposomal suspensions prepared by standart technique including double extrusion through inorganic membranes Anotop 200 nm. Obtained liposomes were not stabilized by addition of charged compounds or by other methods, that explains fast coagulation of the liposomes at the room temperature during 2 weeks. After the preparation of the 10 g/l liposomal suspension it was divided into 3 parts. One of these parts was used as a standart sample, to the remaining parts water solution of modified chitosan (2) was added in weight ratio chitosan: lipid composition 1:15 and 1:5. To research the accelerated aging of the samples they were put into dark place and held at room temperature during 3 months. Stability of suspensions was estimated by increase in average size which was measured with the help of dinamic light scattering. The results of the observation for one of the modified chitosan derivatives are summed in table 2. Analisis of these data allows to make a conclusion that the addition of derivative (2) in ratio 1:5 results the change of liposome size less than 2 % per month. Polydispersity of this sample remain almost unchanged during the observation period. These results

significantly differ from the nonmodified liposomes, which is rapidly destroyed in solution. After two weeks exposure of the standart sample a precipitate formed, which was not possible to dissolve by shaking or sonication of the sample. Stability of chitosan-coated liposomes increases sharply in the range of 1:10 - 1: 5 and further remains constant (we conducted an experiment with a 1:1 ratio for one of the suspensions). We attribute this result to the fact that this ratio allowes to achieve full coverage of the liposomes surface by the chitosan microgel. It was shown that small fraction of stabilized liposomes precipitate can be formed during storage. However, this precipitate easily becomes colloidal solution by manual shaking.

Table 2 Change of liposome characteristics after stabilization by chitosan derivative (2)

Time, days	Average size, nm			Polydispersity of sample 1:5
	without chitosan	1:15	1:5	
1	221	242	266	0.215
5	269	265	267	0.217
10	336	320	267	0.221
15	Precipitate	436	270	0.225
30	Precipitate	Precipitate	272	0.227
60	Precipitate	Precipitate	278	0.230
90	Precipitate	Precipitate	283	0.236

4. CONCLUSION

This study was dedicated to the research of influence of different factors on the liposomes stability, such as input of charge-forming components, PEG-modified acylglycerides and modified chitosan coating. The increase in the content of charge forming component in liposome composition above 1% leads to the change in the character of liposomes distribution by size from monomodal to bimodal. We managed to replace the cytotoxic cetylpyridinium chloride, which is used to create a surface charge on the liposomes, with biocompatible amides of trimethylethanammonium and limiting fatty acids. It was also shown that the addition of the 5 % polyethylene glycol - modified natural monoacylglycerides allows to reduce the diameter of the liposomes obtained by the extrusion through the 100 nm membranes to 150-155 nm with a polydispersity index of 0.11 - 0.13. That enables us to use such samples for the parenteral administration. The obtained liposomal form with modified chitosan coating was shown to be stable during 3 months and longer. Thus, we managed to find the optimal parameters of the liposomal suspension for drug delivery. The obtained results are the basis for the development of laboratory regulation of liposomal suspensions preparation.

5. ACKNOWLEDGEMENTS

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