Gamma Irradiation of Liposomal Phospholipids

Suna ERDOĞAN*, A. Yekta ÖZER**, Melike EKIZOĞLU***, Meral ÖZALP**, Şeyda ÇOLAK***, Mustafa KORKMAZ***

**Lipozomal Fosfolipidlerin Gama İşınlanması Özet**

**Anahtar Sözcükleri:** Gama işınlanması, fosfolipidler, surfaktanlar, stearylamin, disetil fosfát, kolesterol, kimyasal stabilite

**INTRODUCTION**

A liposome is a spherical vesicle composed of naturally-derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine), or of pure surfactant components like DOPE (dioleoylphosphatidylethanolamine) and cholesterol bilayer. Liposomes are used for drug delivery due to their unique properties. Because of the several advantages of these systems, liposomes have been recognized as promising...
carriers for drugs and diagnostic agents [1]. These delivery systems are becoming increasingly utilized by the pharmaceutical industry to deliver certain vaccines, enzymes, or drugs (e.g., hormones, proteins, antibiotics and antifungals and some cancer drugs) for treatment or prevention of a variety of diseases with a different route of application, such as parenteral, oral, or topical. Niosomes have similar properties.

As with the conventional pharmaceutical product, when the particulate carrier systems such as liposomes and niosomes are applied parenterally, one of the most important parameters is to produce sterile systems. Historically, the pharmaceutical industry has relied on steam, dry heat, ethylene oxide gas, filtration and chemical processes to accomplish microbial reduction requirements for parenteral products.

Liposomes are complex products usually containing lipids and/or polymers with glass transition temperatures below the temperature required for heat sterilization. Consequently, these products would break down if subjected to heat sterilization. Gamma irradiation is becoming more and more accepted as a sterilization method of pharmaceuticals, particularly for those showing heat sensitivity during autoclaving [2-4]. According to guidelines [5], ionizing radiation can be used in the manufacture of medicinal products, but effects of irradiation on the stability of the product and formation of degradation products should be claimed, and furthermore, after irradiation, toxicological risk should be evaluated.

Historically, the dose of 25 kGy (=2.5 Mrad) was generally accepted as a suitable sterilization dose [6,7]. The choice of this dose was based on the radiation response of radiation-resistant bacterial spores of the Bacillus pumilus [8].

Nowadays, several pharmaceuticals, raw materials and finished products are being sanitized and/or sterilized successfully with gamma radiation [9], and it is becoming an interesting and promising technique for the sterilization of liposomes as well.

The effects of gamma irradiation on liposomes have been reviewed, and it has been reported that peroxidation of unsaturated phospholipids and formation of radiolysis products like lysophospholipids, free fatty acids, phosphatidic acid and different hydrocarbons takes place during gamma irradiation. The physical stability is affected to a lesser extent [11-13]. In spite of the minor physical changes and the promising toxicological results, the chemical degradation and the subsequent presence of high amounts of degradation products might restrict the use of gamma irradiation as a sterilization method for aqueous liposome preparations. To overcome this problem, solid phospholipids or the lyophilized product can be sterilized prior to liposome production or prior to hydration, respectively [11,14,15]. Another means is using lower sterilization dose for achieving $10^{-6}$ Sterility Assurance Level (SAL) according to the AAMI (Association for Advancement of Medical Instrumentation) recommendations. Little work has been done thus far in this field, and these options therefore need to be further evaluated.

In this study, components of liposomes and niosomes were exposed to gamma irradiation as a solid powder in an air environment. Extent and nature of physicochemical and microbiological changes resulting from the action of ionizing radiation at the selected radiation dose determined with microbiological studies were investigated.

**MATERIALS AND METHODS**

**Materials**

Dimyristoyl phosphatidylcholine (DMPC) and hexadecyl-poly-(3)-glycerol (SUR I) were gifts from Phospholipid GmbH (Germany) and L’Oreal (France), respectively. Stearylamine (SA), dicetyl phosphate (DCP) and cholesterol (CHOL) were obtained from Sigma (USA). These and all other chemicals were of analytical grade.

**Irradiation process**

Irradiation was performed at ambient temperature using a $^{60}$Co Gamma Cell 220 available at Turkish Atomic Energy Agency at a dose rate of 2.84 kGy.
Solid lipid samples in Type I glass vials were irradiated at the doses of 5, 7.5, 10, 15 and 25 kGy.

**Determination of SAL (Sterility Assurance Level)**

To determine the maximum radiation dose required for the sterilization of these dispersions, accelerated conditions were applied. For this purpose, all dispersions were contaminated by B. pumilus spores, the most resistant microorganism against gamma radiation. All contaminated dispersions were irradiated at the dose rates of 5, 7.5, 10, 15 and 25 kGy. The actual doses received by samples were determined by Red Perspex, which gave readings within 3%.

After the irradiation, about 100 µL samples were inoculated to nutrient agar plates and incubated at 35°C for 24-48 h. Those providing 10^-6 of SAL were determined with the logarithmic reduction graphics.

**Sterility test**

For sterility test, irradiated samples were inoculated to two different media, FTM (Fluid Thioglycolate Medium) and SCDM (Soybean Casein Digest Medium) and incubated for 2 weeks at 35°C and 25°C, respectively.

**Organoleptic properties**

After the irradiation, organoleptic properties (i.e. color, odor and appearance) of DMPC, SUR I, SA, DCP and CHOL were investigated.

**Analytical methods**

Changes in spectral properties of control and irradiated solid samples were studied using IR (Infrared), NMR (Nuclear Magnetic Resonance) and ESR (Electron Spin Resonance) techniques. Changes of the thermotropic behavior of the lipids were also investigated.

For DSC (Differential Scanning Calorimetry) studies, lipids were put into an aluminium pan, an empty aluminium pan was used as a reference. Calorimetric scans from -30 to 150°C were performed on a Dupont DSC (Dupont DSC 910 Instrument, USA). The scanning rate was 10°C.min^-1 and flow rate was 10 mL.min^-1.

IR spectra were recorded using Perkin Elmer FT-IR Spectrometer (Model 1720X, UK) for control and irradiated powders in KBr matrix. NMR analysis was performed using proton NMR spectrometer (Bruker FT 80MHz NMR Spectrometer, Germany) on unirradiated and irradiated samples dissolved in chloroform-d. Tetramethylsilane was used as an internal standard.

ESR measurements were carried out using a Varian 9 E-L X-band ESR spectrometer (USA) equipped with a TE104 rectangular double cavity. The following spectrometer settings were adopted throughout the experiments: Central field, 326.0 mT; sweep width, 10 mT; microwave frequency, 9.1 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; receiver gain, 2.5x10^3 - 10x10^3; scan time, 240 s; time constant, 1s and temperature, room. All measurements were performed using a DPPH reference sample placed in the front cavity. The position of the standard sample in the cavity was not changed throughout the experiments to avoid any possible changes in the cavity filling factor. The spectra were double integrated over the magnetic field range of 320.0-332.0 mT, which gives a figure proportional to the number of radicals in the sample. Each spectrum was corrected for variation in the amount of material in the “active length” of the ESR tube. A simulation study based on possible radical species was also carried out.

**RESULTS AND DISCUSSION**

Even though 25 kGy is suggested by pharmacopoeias as the radiation sterilization dose, previous studies reported excessive degradation of liposomal phospholipids after gamma irradiation of aqueous liposome dispersions with this dose [3]. Therefore, the required radiation dose achieving 10^-6 of SAL was first determined with microbiological studies according to recommendations of the AAMI and pharmacopoeias [6,7].

To determine the maximum radiation dose required for the sterilization of liposome and niosome dispersions, these dispersions infected by B. pumilus spores (10^6) were irradiated by different radiation doses (5,
7.5, 10, 15 and 25 kGy, and after irradiation, microorganism concentration in the samples was recalculated. Dose-Microorganism Survival Ratios were plotted and SAL $10^{-6}$ doses were calculated with the help of these graphics (data not shown). As a result of these microbiological studies, 15 kGy was determined as achieving $10^{-6}$ of SAL, and this dose level was used in the further studies.

Irradiation at the dose rate of 15 kGy did not produce any significant changes in organoleptic properties of DMPC, SUR I, DCP, SA and CHOL. However, the odor of DMPC samples changed after the irradiation. After gamma-irradiation, the thermotropic behavior of the lipids changed as measured by DSC. Obtained DSC scans are shown in Figure 1 (a-e). In some cases,
the pre-transition peak was decreased or disappeared. The pre-transition has been reported to be highly sensitive to perturbations of the lipid bilayer [16]. The main phase transitions of lipids broadened after gamma irradiation. The broadening of the peaks of the main phase transition might be partly explained by the presence of degradation products. The melting characteristics of the lipids are in good agreement with data reported in the literature [15]. It was found that the melting peaks appearing in all scans were very sensitive towards gamma irradiation.

Proton NMR spectra of the samples dissolved in chloroform-d containing tetramethylsilane as an internal reference showed different chemical shifts that varied from 1.63 to 11.0, depending on the chemical environment of the related protons. In general, irradiation of solid samples at the dose of 15 kGy did not produce any significant changes. The fatty acid/main chains remained intact after irradiation. Only proton-NMR spectra of SUR I revealed irradiation-induced degradation, but the structures of these molecules are not known (Figure 2 a-e).

Figure 2 a-e: NMR spectra of (a) DMPC (b) SUR I (c) DCP (d) SA and (e) CHOL.
After the gamma irradiation, IR spectra of samples were taken and compared to those of unirradiated samples (Figure 3 a-e). The FTIR spectra for the non-irradiated and irradiated samples showed no significant differences.

Radical formation was observed due to rupture of the chemical bands of materials. To detect the radicals formed by gamma radiation, ESR studies were carried out in unirradiated (control) and irradiated solid samples. ESR spectra are given in Figure 4 (a-e). Control samples exhibited no ESR signal. All irradiated samples were found to present a singlet resonance line centered at about g = 2. Simulation calculations have shown that different radicals in samples are responsible for the induced ESR spectra. Characteristic

![Figure 3 a-e: IR spectra of (a) DMPC (b) SUR I (c) DCP (d) SA and (e) CHOL.](image-url)
The most important radicals were oxygen radicals (\( .O \)) formed by irradiation of DCP and hydroxyl radicals (\( .OH \)) formed by irradiation of CHOL. (\( .O \)) radicals are important due to the high affinity of the oxygen radicals to the biological compounds. The superoxide radical has been suggested to represent a major factor of oxygen toxicity in biological systems. (\( .OH \)) radicals are also important as they can easily get into chemical reactions. They may cause electron transfer reactions with organic and inorganic compounds or break of the hydrogen bonds that are connected to the carbon atom (as a result of which, lipid peroxidation starts), and they incorporate into...
Table 1. Characterization of radicals detected by ESR techniques

<table>
<thead>
<tr>
<th>Sample</th>
<th>Radical</th>
<th>( I_a ) (a.u)</th>
<th>( G_b ) (G)</th>
<th>( g_c )</th>
<th>( A_d ) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>• N</td>
<td>59.15</td>
<td>10.01</td>
<td>2.0189</td>
<td>51.78</td>
</tr>
<tr>
<td></td>
<td>• CH2</td>
<td>16.55</td>
<td>7.55</td>
<td>2.0119</td>
<td>13.73</td>
</tr>
<tr>
<td>SUR I</td>
<td>• CH2</td>
<td>35.47</td>
<td>7.61</td>
<td>2.0006</td>
<td>31.76</td>
</tr>
<tr>
<td></td>
<td>• CH2OH</td>
<td>46.60</td>
<td>16.13</td>
<td>2.0025</td>
<td>2.44</td>
</tr>
<tr>
<td>SA</td>
<td>• N</td>
<td>1.04</td>
<td>0.27</td>
<td>2.0037</td>
<td>AN = 4.22</td>
</tr>
<tr>
<td></td>
<td>• CH3</td>
<td>181.20</td>
<td>16.93</td>
<td>1.9881</td>
<td>AH = 2.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>DCP</td>
<td>• O</td>
<td>998</td>
<td>9.46</td>
<td>2.0131</td>
<td></td>
</tr>
<tr>
<td>CHOL</td>
<td>• OH</td>
<td>18.53</td>
<td>5.07</td>
<td></td>
<td>g // = 2.0304</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>g^ = 2.0074</td>
</tr>
</tbody>
</table>

\(a\) Contributing weight \(b\) Half-height at half-width \(c\) Lande g factor \(d\) Hyperfine splitting constant

the structure of double bonds. In addition, they are able to form secondary radicals of high reactivity. These radicals, especially unsaturated lipids, cause lipid peroxidation by the interaction of fatty acids that results in destabilization of the membrane. One of the suggested ways to prevent the decomposition is addition of radical scavengers such as superoxide dismutase, thioredoxin or \(\alpha\)-tocopherol. It was reported that addition of these substances reduces the lipid peroxidation [17].

CONCLUSION

According to IR, NMR spectra, DSC thermograms and ESR studies, gamma radiation of raw materials as solids used in liposome and niosome dispersions resulted in induced degradation of phospholipids/lipids. Still, it seems that these changes represent only minor chemical degradation when compared to chemical degradation caused by irradiation of aqueous dispersions. The peaks belonging to the fatty acid chains remained the same (or showed small changes) before and after irradiation. From this point of view, gamma irradiation seems to be a suitable sterilization technique for solid phospholipids/lipids. However, changes in the physical behavior of the subsequently produced liposomes and niosomes should be investigated. In addition, studies concerning toxicological risks caused by the irradiated products should also be evaluated.

ACKNOWLEDGEMENTS

This project has been supported by Hacettepe University Research Foundation (Project No: 99.02.301.001) and TUBITAK (Project No: SBAG-2303).

The gift of the phospholipids and surfactants by Phospholipid GmbH (Germany) and L’Oreal (France), respectively, were greatly appreciated.

The authors would like to thank Assoc. Prof. Dr. Murat Tien for his help in the DSC studies. We also thank the Turkish Atomic Energy Agency, especially Chem. Ömer Kantölü, for performing the irradiation treatment.

REFERENCES

6- European Pharmacopoeia, Second Edition, Part II,


