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Phospholipon 90H (P90H)-based PEGylated microscopic lipospheres delivery system for gentamicin: an antibiotic evaluation

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ABSTRACT

Objective: To formulate gentamicin liposphere by solvent-melting method using lipids and polyethylene glycol 4 000 (PEG-4 000) for oral administration. **Methods:** Gentamicin lipospheres were prepared by melt-emulsification using 30% w/w Phospholipon® 90H in Beeswax as the lipid matrix containing PEG-4 000. These lipospheres were characterized by evaluating on encapsulation efficiency, loading capacity, change in pH and the release profile. Antimicrobial activities were evaluated against *Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyphii and Staphylococcus aureus* using the agar diffusion method. **Results:** Photomicrographs revealed spherical particles within a micrometer range with minimal growth after 1 month. The release of gentamicin *in vitro* varied widely with the PEG-4 000 contents. Moreover, significant (P>0.05) amount of gentamicin was released *in vivo* from the formulation. The encapsulation and loading capacity were all high, indicating the ability of the lipids to take up the drug. The antimicrobial activities were very high especially against *Pseudomonas* compare to other test organisms. This strongly suggested that the formulation retain its bioactive characteristics. **Conclusions:** This study strongly suggest that the issue of gentamicin stability and poor absorption in oral formulation could be adequately addressed by tactical engineering of lipid drug delivery systems such as lipospheres.

1. Introduction

In recent years, biocompatible lipid micro and nanoparticles have been reported as potential drug carrier systems as alternative materials to polymers[1,2]. Solid lipid particles combine several advantages and avoid the disadvantages of other colloidal carriers. The following are positive features of the potential use of solid lipid particles as drug carrier systems: they offer the possibility of controlled drug release and drug targeting; they provide protection of incorporated active compounds against degradation; their solid matrix is composed of physiological and well-tolerated lipids; they allow for hydrophilic and/or hydrophobic drugs to be incorporated[3].

The drug solubility and miscibility in melted lipid, chemical and physical structure of lipid materials, and their polymorphic state determine the loading capacity of drug in the lipid particles[4-7]. The amount of drug encapsulated can vary from 1% to 5% for hydrophilic compounds and up to 80% for lipophilic compounds[8,9]. Solid microparticles in dispersions are usually obtained using a melt dispersion method or a solvent evaporation method[10,11]. The advantage in the melt method is that no organic solvents are needed[12].

Gentamicin is an important antibacterial agent for the treatment of a wide variety of Gram-negative bacilli and Gram-positive cocci infections[13]. Clinically, it has been indicated for the eradication of bacterial pathogens involved for example in lower respiratory tract infections, skin and skin structure infections, urinary tract infections, uncomplicated gonorrheal infection, pelvic inflammatory diseases, bacterial septicemia, bone and joint infections, intra-abdominal infections, meningitis, surgical prophylaxis, as well as acute bacterial otitis media[13,14]. The antibiotic has been used successfully in both adult and pediatric patients with minimal side effects. Unfortunately,

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however, conventional formulation science has yet to provide an effective oral delivery system for the administration of gentamicin to patients[15]. Currently, effective bacteriocidal therapy using gentamicin hinges upon either intravenous infusion or intramuscular administration[16]. Parenteral administration of gentamicin has been associated with side effects that include mainly nephrotoxicity and ototoxicity[17-19].

Oral gentamicin formulations could beneficially reduce conventional burdens upon both patients and health care professionals, in addition to perhaps achieving higher blood gentamicin levels with lower dosages[20]. However, oral gentamicin administration is entirely dependent upon the ability of the dosage form to deliver permeable solubilized antibiotic directly to the intestinal mucosa, rather than premature distingration[21,22].

In this study, two different lipids were used to formulate gentamicin-lipospheres at different concentrations. The lipospheres were characterized for drug entrapment efficiency, particle size, morphology, drug release, pH change as a function of stability and drug antibacterial activity in view of its potential use in treatment of bacterial infections.

2. Material and methods

2.1. Materials

Phospholipon® 90H (phospholipids) provided by Nattermann (Germany) is a purified, deoiled, and granulated soy lecithin with phosphatidylcholine content of 90%. Beeswax was obtained from Lavan Chemical (Enugu, Nigeria). PEG 4 000 (Sigma, UK), Gentamicin (Telyk Pharmaceutical, Nigeria), agar-agar from Qualigens Fine Chemicals Pvt. Ltd. (Mumbai,India), Polyvenyl alcohol (PVA) were purchased from Merck, Germany, and Poly(ethylene-glycol) (PEG 400) from Sigma (UK). Distilled water (Department of Biochemistry, University of Nigeria, Nsukka).

2.2. Methods

A 5.0 g quantity of P90H was carefully weighed and added to 20 mg of Beeswax (20% P90H in 80% Beeswax) in crucible and the mixture heated on water bath to melt. This was stirred thoroughly to ensure a uniformed mixing until it solidify. A 40 mg of gentamicin was added into the lipid phase and stirred thoroughly. An aqueous phase was prepared by dissolving the surfactant; 10% PEG-4 000 was carefully weighed, transferred into 100 mL beaker followed by the addition of 20 mL of double distilled water and heated to melting on a water bath at the same temperature (65 ℃) of the molten lipid phase. Hot aqueous phase was then added to the molten lipid phase and homogenized at 5 000 r/min and temperature maintained at 3 ℃ above the melting point of the lipid) with the help of a Ultra Turrax mixer (Germany) for 20 min. A dispersion of solid lipid microparticles of gentamicin was obtained by allowing the hot microemulsion to cool to room temperature. The above procedure was repeated using increasing quantities of PEG-4 000 to yield 20, 30 and 40% w/w respectively each time using 80 and 160 mg of gentamicin. The corresponding formula are shown in Table 1.

Table 1.
The composition of the investigated gentamicin lipospheres formulation (%w/w).

PEG-4 000	Drug (%w/w)	Lipid base	PVA (%w/w)	Water (%w/w)
300	80	2.7	0.10	100
300	160	2.7	0.10	100
600	160	2.4	0.10	100
600	80	2.4	0.10	100
600	00	2.4	0.10	100

2.3. Characterisation of lipospheres

2.3.1. Morphology and particles size analysis of lipospheres

The morphology and particle size of the lipospheres were determined by mounting a small quantity of the lipospheres on the microscope slide and fixed in a specialized optical microscope, which was connected to a computer. After several adjustments, the average particle sizes were calculated. The particle morphologies were also observed and photomicrographs taken. All these were done within four weeks of formulation.

2.3.2. pH analysis

With the aid of a pH metre, the pH values of the different batches of the lipospheres formulations including those of the control were measured. This was also carried out in a time dependent manner (1, 2, 3 and 4 weeks).

2.3.3. Encapsulation efficiency and drug loading

A 10 mg quantity of liposphere was dispersed in 10 mL of phosphate buffer (pH 7.4). The dispersion was allowed to stand for 2 h after which it was mixed in a vortex mixer for 5 min and then centrifuged at 5 000 r/min for 10 min. The amount of gentamicin contained in the various liposphere formulation samples was determined using spectrophotometer as discussed previously. The drug loading DL and encapsulation efficiency (EE) was then determined using the equations below:

$$EE=\frac{\text{Total quantity of the drug-quantity in supernatant}}{\text{Total quantity of the drug}}\times 100$$

$$DL=\frac{\text{Total quantity of the drug-quantity in supernatant}}{\text{Total quantity of the polymer base}}\times 100$$

2.3.4. In-vitro activity of the lipospheres on microorganisms using agar diffusion method

Biological activity of gentamicin-liposphere was measured by a bacterial growth inhibition assay using an agar diffusion method with *Escherichia coli (E. coli), Pseudomonas aeruginosa (P. auroginosa), Salmonella paratyphii (S. paratyphii) and Staphylococcus aureus (S. aureus)* as test organisms. The plate agar diffusion method was used for this study was conducted after four weeks of lipospheres preparation. This method depends on the diffusion of antibiotics from holes on the surface of the microbial seeded agar. The plate was seeded with *E. coli, P. auroginosa, S. paratyphii and S. aureus*. Using a sterile cork borer, four cups were aseptically bored at equal distances from each other. Using a sterile applicator, one drop of the formulation of a batch was applied in the holes. The plates were incubated at (37.0±0.5) ℃ for 24 h and the diameter of each inhibition zone was measured and the average determined.

2.3.5. In vitro drug release study

It is well known fact that the release of the drug from lipospheres is influenced by the composition of the lipospheres. Hence, the *in vitro* release of gentamicin from the formulation was studied. *In vitro* release was evaluated using a dialysis bag diffusion technique as described by previous researcher[23,24]. The dialysis bags were hydrated in phosphate-buffered saline, pH 7.2 overnight before the experiment. Lipospheres equivalent to 50 mg were placed in dialysis bags. The dialysis bags were tied at both ends and were placed in the basket of USP Type I dissolution apparatus (Electrolab, Mumbai, India). The baskets were immersed in 250 mL phosphate-buffered saline, pH 7.2, maintained at (37.0±0.5) ℃. The baskets were rotated at 100 r/min. At regular intervals, 2.0 mL of dissolution medium was withdrawn and was replaced with the fresh buffer. The withdrawn sample were filtered through a 0.22 μm filter (Millipore®, USA) to avoid contamination and analyzed for gentamicin content using a spectrophotometer (Shimadzu, A160, Japan) at 242 nm.

2.4. Statistical analysis

All experiments were performed in replicates (n=3) for validity of statistical analysis. Results were expressed as mean±SD. Means of two groups were compared using non paired Student's t test. A value of P>0.05 was considered statistically significant.

3. Results

3.1. Morphology and particles size analysis of lipospheres

The microscopic images of the solid lipid microparticles (SLMs) are shown in Figure 1. The results indicate that when the ratio of drug to lipid matrix used in formulating the SLMs was low, the SLMs produced were irregular in shape but when this ratio was increased, more spherical and smooth particles were produced. Similarly, it was observed that the ratio of drug to lipid matrix used in the formulations affected the size of the SLMs, which is in the range 271.0-291.1 μm for the gentamicin-loaded SLMs and 306.1 μm for unloaded SLMs (Table 2).

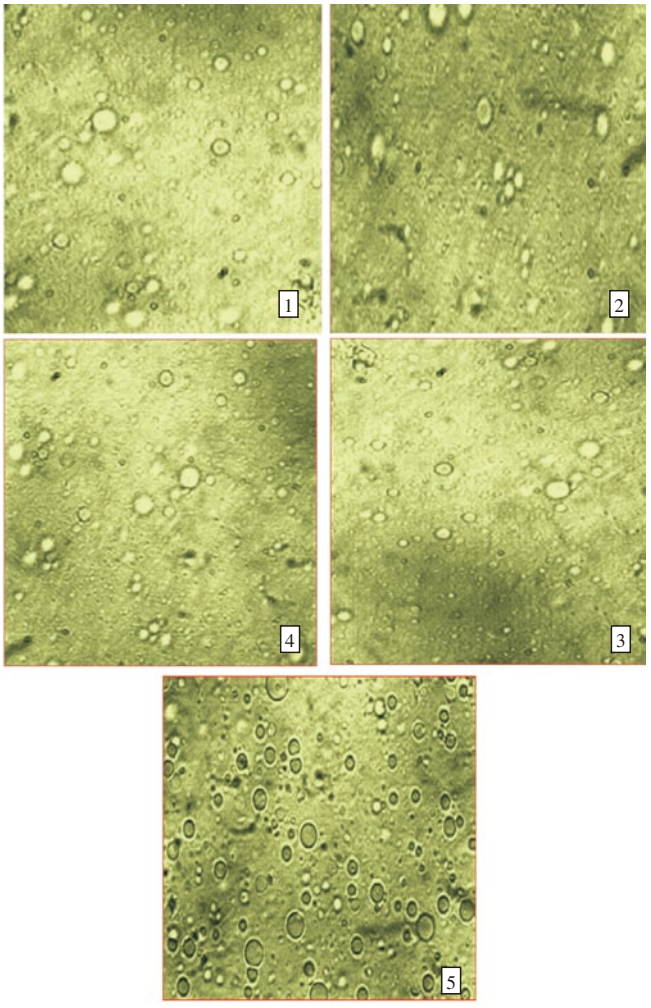

Figure 1. Microscopic images of the SLMs. 1-4: Lipospheres of gentamicin-loaded; 5: Lipospheres of unloaded lipospheres. All microscopic images are at a magnification of ×100.

Table 2.
Properties of the gentamicin-loaded andunloaded liposphere with increasing concentrations of PEG-4000 and gentamicin.

Batch	Encapsulation efficiency (%) ±SD	Drug loading (%) ±SD	Percentage yield (%)	Particle size ±SD (μm)
1	86.32±0.50	43.68±1.67	94.10±1.20	291.15±0.23
2	81.25±0.13	39.25±0.05	96.12±0.12	295.14±0.12
3	75.55±0.01	44.67±0.80	89.42±0.22	267.08±0.14
4	74.70±0.25	41.67±0.80	90.52±0.23	271.13±0.21
5	-	-	89.23±0.42	306.76±1.20

1-4: Gentamicin-loaded liposphere; 5: Unloaded liposphere.

Determining the pH stability of the different batches of the SLMs when stored at room temperature and at different time intervals, is an important aspect that cannot be ignored. The pH is a strong determinant in pharmaceutical stability; it gives the formulation scientist the idea of the choice of

ingredient as well as the need for stabilizer for a particular preparation. Through the pH analysis, the shelf life and the degradation characteristics of the excipients or the drug will be properly handled. It was observed from the results that after 4 weeks, all the batches in formulation 1 had highest pH of 7.9 in the second week; those in formulation 2 had highest pH of 7.9 in third weeks, whereas the highest pH of SLMs formulations in batches 3, 4 and 5 are 7.7 in fourth week, 7.9 in first week and 6.9 in fourth weeks respectively (Table 3). It was noted that the changes in the pH values were not sequential; it was a random change. The reason for this was not known.

Table 3.
Result of pH values of gentamicin-lipospheres formulations (in weeks).

Batches	1st week	2nd week	3rd week	4th week
1	7.60±0.22	7.90±0.32	7.50±0.03	7.80±0.31
2	7.60±0.42	7.50±0.12	7.90±0.02	7.20±0.02
3	6.40±0.13	7.00±0.92	6.90±0.03	7.70±0.02
4	7.90±0.32	7.20±0.02	6.90±0.02	7.00±0.11
5	6.00±0.32	6.20±0.02	6.70±0.02	6.90±0.31

3.2. Encapsulation and drug loading

The role of the formulated lipospheres is to deliver the API to the target tissues intact. Thus, the ability of the lipospheres to accommodate active molecules is an important property. It can be expressed by the EE and DL. EE% defines the ratio between the weight of entrapped API and the total weight of API added to the dispersion, while DL expresses the ratio between the entrapped API and the total weight of the lipids. Table 1 shows the EE% and the DL obtained for the various batches of lipospheres after 1 month of storage. Both EE% and DL are dependent on several parameters, such as the lipophilic properties of the API, the screening of the most appropriate lipid composition/ratio and surfactant combination, as well as the production procedure used. DL was high (44.6%) in formulation 3 and least in formulation 2 (39.2%). The encapsulation efficiency was more in formulation 1 with (86.3%) and least in formulation 4 with (74.7%). The lipids and drug concentration played a vital role these observations (Table 1).

3.3. The release profiles of the formulations

This test was performed to establish that loaded SLMs was able to release the incorporated drug after short-term storage, and was done 4 weeks after preparation. The result of drug release studies using the dialysis membrane method is presented in Figure 2. The *in vitro* release profiles of gentamicin in phosphate buffer indicate very significant release of gentamicin from all batches of the formulation as shown in Figure 2. Batch 1 gave a release of>70%, while 2 and 3 gave a release slightly greater than 71 and 60% respectively. Similarly, batch 4 released the highest amount (> 80%) of the drug. It was observed that the release of the drug from the formulation exhibited a lag phase and that the formulations started to release the drug after 30 min in the dissolution medium.

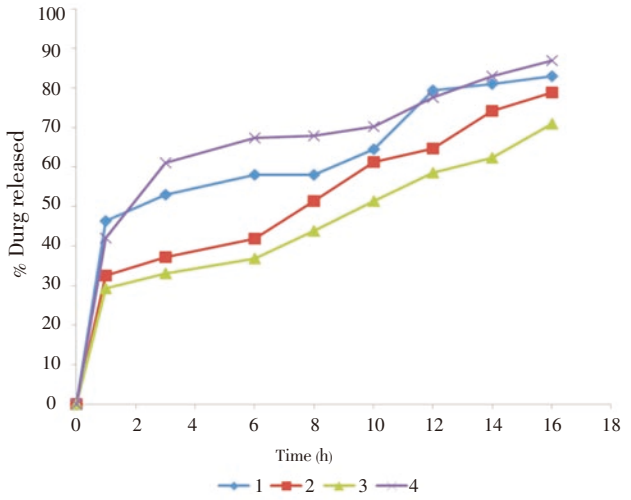

Figure 2. Release behaviour of gentamicin-loaded lipospheres from the formulations. 1, 2, 3 and 4 are liposphere batches.

3.4. In vitro activity against test agents

The determination of inhibition zone diameter (IZD) using agar plate method is based on the diffusion of an antibiotic agent or formulation thereof through a solidified nutrient agar inhibiting the growth of test organism. From the result presented in Figure 3, the liposphere formulations (1-4) show activities in highest order against the various test agents: Pa>S>Ec>Sa. The result here indicate that the activity of the antimicrobial agent from the formulation was very high. In order words the agent did not lose its activities when formulating as a lipospheres.

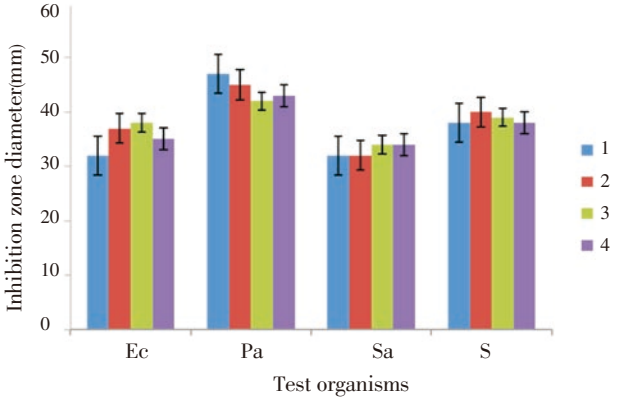

Figure 3. Antibacterial effect of the formulation as a function of IZD against *E. coli, P. auroginosa, S. aureus* and *Salmonella* as test organisms obtained after 1 month of storage (n=3). 1, 2, 3 and 4 are liposphere batches. Ec: *E. coli*; Pa: *P. auroginosa*; Sa: *S. aureus*; *S: Salmonella.*

4. Discussions

4.1.Surface morphology

The surface morphology of the gentamicin-loaded liposphere was investigated by computerized specialized microscope (Lieca, Germany). Micrographs revealed homogeneous and spherically shaped particles with fairly round smooth surfaces for all the formulations. Occasionally, small discrete shapes were observed on the unloaded liposphere. Conversely, the surface of the loaded lipospheres appeared clustered together, which depended on nominal drug loading. The liposphere size was affected by the quantity of the polymer (PEG), lipids and drug content. The result of the particle size analysis of gentamicin-loaded lipospheres formulations vary according to the variation in the lipid base and polymer concentration, and the sizes were in the range of 267-306 μm. As concentration of PEG 4 000 was increased (Batch 3 and 4), the particle size was decreased which may be due to the formation of small globules during emulsification by PVA. However, a different pattern was observed for batch 5 (unloaded lipospheres). The reason for this is uncertain. The variation in the size is not dependent on the emulsification time and speed, since they are kept constant in all the formulations.

The pH of the different gentamicin-lipospheres was evaluated after 1, 2, 3 and 4 week of preparation to ascertain if there is any change in pH with time, which could be a function of degradation of the drug or excipients. Scientifically, it is very important to know the likely changes in the pH of any pharmaceutical formulation in order to avert possible drug toxicity and loss of therapeutic value. This will also inform the formulation scientists or the pharmaceutical formulators or designers on the necessary step to take in the course of the formulations. The pH varied from between 6.40 ±0.13 to 7.90±0.02 within 4 weeks of preparation, the highest pH was found in batch 1 and 2 after 1 month (pH=7.94± 0.32). The results here showed that there were no significant changes in the pH of the formulations. All the formulations were considered stable based on the fact that the pH of the gentamicin-lipospheres formulations were within the value given for a conventional marketed gentamicin (pH 6-8) which is in accordance to the literature[23]. The slight change in the pH is neither from the lipids nor the drug, because a similar change was also observed in the unloaded lipospheres (no drug).

4.2. Encapsulation efficiency (EE%) and loading capacity (LC)

The drug-encapsulation efficiency increased as the concentration of the drug increase, and the highest drug loading was found in the liposphere containing 160 mg of gentamicin, while formulation containing 80 mg of the drug was found to show least encapsulation efficiency.

The entrapment efficiency of gentamicin also depended on the polymer (PEG) and increased generally with increasing the polymer. The enhanced microencapsulation may be due to the PEG. PEG being a hydrophilic polymers may be ascribed to enhanced molecular interactions between the drug and the polymer[24]. In addition, the low crystalline nature in one of the lipid matrix used especially the phospholipon 90H, may also be a contributing factor[25]. The yields varied from 89.42%±0.22% to 96.12%±0.12%, the result here suggesting that the processing parameters did not affect the yield from the solvent-emulsion technique. However, if all the necessary precautions are put in place, there is every likely hood of getting close to 100%.

4.3. In vitro drug release studies

A biphasic release pattern of drug release was observed in all the formulation. The formulations released substantial of the drug at the initial time, and further maintained a steady release up to 16 h the experiment lasted. The *in vitro* release of the lipospheres (batch 1-4) showed an initial release of 39 to 45% for all the batches within 1 h and 33 to 47% of the drug was released in a slow manner over 16 h. The highest released (83%) was found to be batch 1 at the end of 16 h. The release of the formulations followed the same pattern and were predictive of sustained released formulation. The influence of the lipid matrix on the release was evident from the batch that show a very slow release (batch 3), and it could be attributed to the complex formed between lipid and the drug. It was predicted that these release patterns could be related to gentamicin that was loosely or poorly bound (burst release), trapped within the lipid matrix, or encapsulated by the lipid. However, the sustained release profile observed up to 16 h suggests the diffusion of gentamicin from the core of the lipid matrix to the release medium.

4.4. Antibacterial activity of gentamicin-loaded lipospheres studies as a function of IZD

Effects of gentamicin-liposphere encapsulation and storage on the biological activity of the formulated drug were studied by an antibacterial assay using agar diffusion method. The assay measured growth inhibition of the test organisms. The determination of IZD using agar plate method is based on the diffusion of an antibiotic agent or formulation thereof through a solidified nutrient agar. The effect of the lipospheres formulations on the tests organism gave the zones of inhibition in decreasing order of magnitude: Pa>S>Ec>Sa. There was no zone of inhibition in unloaded lipospheres (result not shown). This indicates that all the test formulations containing the gentamicin, retained its antibacterial potential and demonstrated significantly greater zone of inhibition ($P<0.05$). Batch 1 was very much active against *P. auroginosa* as compared to other test organisms, when compared to the rest of the formulations, it is worthy of noting that, the lipid base has effect on the release of the loaded drug. In batch 2, the preparation has more effect on *P. auroginosa* and *S. paratyphii*. This may

be as a result of increase in the drug content from 80 mg to 160 mg. However, it shows that more of the drug was accommodated in the lipospheres, hence the increase in the release and subsequently, more effect on salmonella as compared to other formulations. Batch 3 and 4 showed a similar effect, though batch 3 proved very effective in *E. coli and S. aureus* than other test organisms, which may be associated to the increase in the polymer and the decrease in lipid base used. In general, the gentamicin-lipospheres preparations were very effective and retained their bioactivity.

In conclusion, the present work suggests that the highly hydrophilic and cationic gentamicin can be entrapped with acceptable efficiency into liposphere and drug bioactivity maintained as observed in this work. However, variation in lipid concentration and polymer all influenced the encapsulation efficiency, liposphere morphology and particle size. This formulation may constitute an appropriate delivery system for this drug.

Conflict of interest statement

We declare that we have no conflict of interest.

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