INTRODUCTION
Alzheimer's disease (AD), a progressive neurologic disorder, is by far the most prevalent type of dementia, accounting for 60–80% of all patients diagnosed with dementia. AD is the 6th leading cause of all deaths and the 5th leading cause of death in persons aged ≥65 years. It is characterized by a significant loss of neurons and atrophy of the hippocampus and cerebral cortex with the appearance of neurofibrillary tangles and senile plaques. [1] Patients in the disease early stages show a decline in recent memory. Afterwards they can no longer recognize children, spouses and siblings and lose the personality traits that had characterized them as individuals. Later stages show profound cognitive decline so that patients lose the abilities, interests and skills to perform simple daily life activities such as bathing, dressing, eating and toileting. These losses are coupled with the appearance of other behavioral problems that include violent behaviors, depression, delirium, various psychoses and loss of judgment and social skills. Persons with Alzheimer’s disease spend their last years institutionalized and/or in a mental exile from others. [4]

At present, there is no preventive or cure for AD. Enormous research efforts are being carried out to find a disease modifying treatment for the disorder. Only five compounds were currently approved by the FDA and EMEA for the treatment of AD. Acetylcholinesterase inhibitors (AChEI), Tacrine, Galantamine and Rivastigmine are approved for the treatment of mild-to-moderate stages of dementia. AChEI are used to counteract the functional consequences of cholinergic neurons lost in the brain. [3] Rivastigmine has been found to be beneficial in later stages of the disease. [4] Regrettably, although these pharmaceuticals can result in statistically significant improvements of cognitive and global assessment measurements of AD, Drug delivery to the brain is a challenge, because this tissue benefits from a very efficient protective barrier.

The blood-brain barrier (BBB) is a cellular and metabolic filter that regulates the exchange of materials between the blood and brain. The BBB consists of a monolayer of capillary endothelial cells surrounded by pericytes, astrocytes and microglia (perivascular macrophages). The endothelial cells are characterized by a high degree of polarization, a complete lack of fenestration and interconnection with each other by tight junctions and a diminished pinocytic activity, that together help to restrict the passage of compounds from the blood into the extracellular environment of the brain. [4, 5] P-glycoprotein (P-gp) is also present in the luminal plasma membrane of endothelial cells. P-gp is known to prevent the intracellular accumulation of an extensive variety of chemotherapeutic agents and hydrophobic compounds. [6] The BBB is largely impervious to hydrophilic substances, with the exception of certain substances such as glucose, which crosses the BBB by a mechanism of facilitated diffusion. [7] This design makes brain capillaries 50–100 times less permeable than are peripheral microvessels. [8]

In order to cross the BBB by passive diffusion, molecules should be relatively small, present a molecular mass of <400 Da, a log octanol/water partition coefficient between -0.5 and 6.0, be lipid-soluble, be either neutral or significantly uncharged at physiological pH 7.4 and be capable of forming <10 H-bonds with water. [9] Unfortunately, only few drugs fulfill these requirements and the overwhelming majority of small molecules, proteins and peptides do not cross the BBB. It has been reported that 98% of small molecules and nearly all large molecules do not cross the BBB. [10] In general, colloidal drug carriers, especially liposomes and nanoparticles aim to increase the specificity towards cells or tissues, to improve the bioavailability of drugs by increasing their diffusion through biological membranes and/or to protect them against enzyme inactivation. Moreover, the colloidal systems allow access across the BBB of non-transportable drugs by masking their physico-chemical characteristics through their encapsulation in these systems. [5] Liposomes are small vesicles composed of unilamellar or multilamellar phospholipids bilayers surrounding aqueous
Liposomes have been considered for brain targeting due to biodegradable lipids similar to biological membranes. In compartments, they are composed of biocompatible and biodegradable lipids similar to biological membranes. Liposomes have been considered for brain targeting due to their structural flexibility in size, composition and bilayer fluidity as well as their ability to incorporate a large variety of both hydrophilic and hydrophobic compounds. After intravenous administration, liposomes interact with plasma proteins, a process known as opsonization and are rapidly removed from the circulation. Consequently, the concept of “steric hindrance” has been applied to avoid the deposition of plasma proteins either by adsorbing at the surface of the colloids some surfactant molecules (such as copolymers of polyoxyethylene and polyoxypropylene) or by providing a sterical stability by the direct chemical link of polyethylene glycol (PEG) at the surface of the particles. After intravenous administration, liposomes interact with plasma proteins, a process known as opsonization and are rapidly removed from the circulation. Consequently, the concept of “steric hindrance” has been applied to avoid the deposition of plasma proteins either by adsorbing at the surface of the colloids some surfactant molecules (such as copolymers of polyoxyethylene and polyoxypropylene) or by providing a sterical stability by the direct chemical link of polyethylene glycol (PEG) at the surface of the particles.

MATERIALS AND METHODS
Rivastigmine was kindly provided by Mepaco - Arab Co. For Pharmaceuticals and Medicinal Plant, Heliopolis, Cairo, Egypt. L-α-Lecithin was purchased from MP Biomedicals, LLC, France. Didecyldimethyl ammonium bromide (DDAB) were purchased from Sigma Chemical Co. (USA). Tween 80 (polyoxyethylene sorbitan monooleate), methanol and chloroform were purchased from Adwic, El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt. Spectra/Pore® dialysis membrane (12,000–14,000 molecular weight cut-off) was purchased from Spectrum Laboratories Inc. (USA). All other reagents were of analytical grade.

Preparation of Rivastigmine Positively Charged Liposomes
A 3² full factorial design was conducted to study the joint influence of A: DDAB molar ratio, +ve charge inducer and B: Tween 80 molar ratio. Briefly, 200 mg lecithin and 50 mg rivastigmine together with different molar ratios of DDAB and tween 80 (as shown in Table 1) were added into a round-bottom flask and dissolved in chloroform (10 ml) at 40°C. The organic solvent was evaporated at the same temperature under vacuum, using a rotary evaporator (Rotavapor, Heidolph VV 2000, Burladingen, Germany) at 90 rpm such that a thin film was formed inside the flask. The deposited thin film was then hydrated with 5 ml of phosphate buffer saline (PBS), pH = 7.4, by rotating the flask in a water bath at 40°C using rotary evaporator under normal pressure in order to ensure complete hydration of the film. The resulting vesicles were sonicated for one minute in a bath sonicator to reduce their size. The obtained dispersion was left to mature overnight at 4°C. Liposomal dispersion was then subjected to three freezing-thawing cycles, freezing at −4°C and then thawing at room temperature. The liposomal dispersion was stored at 4°C until analysis.

Characterization of Positively Charged Liposomes
The liposomal formulations were examined for their shape and morphology under optical microscope (Leica Image, Germany) and photographed at a magnification power of 40×, by means of a fitted camera (JVC, Japan). The mean particle size (PS), polydispersity index (PDI) and zeta potential (ZP), (an indirect measurement of surface charge), were determined by Zetasizer at 25°C (Malvern Instrument Ltd., Worcestershire, UK) after being diluted with PBS. Triplicates were taken for each sample. Measuring the entrapment efficiency (EE%) of Rivastigmine was conducted by ultracentrifugation at 15,000 rpm for 2 hour using a cooling centrifuge (Beckman, Fullerton, Canada) at 4°C. The precipitated liposomes were ruptured using methanol, filtered, then the concentration of the entrapped drug was measured spectrophotometrically, by first derivative (Shimadzu, model UV-1601 PC, Kyoto, Japan) at λmax = 274.8 nm.

Statistical Analysis
Design-Expert software (V. 7.0.0, Stat-Ease Inc., Minneapolis, USA) was used for the generation and evaluation of the statistical experimental design. Means were compared by ANOVA-factorial. Significance level was set at α = 0.05. Suitable regression models were driven to enable navigation of the experimental space. Response surface methodology and multiple response optimization were used to search for an optimized formula.

Table 1: Composition of 3² Full Factorial Design Liposomal Dispersion

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>F1</td>
<td>1 : Zero</td>
<td>1 : Zero</td>
</tr>
<tr>
<td>F2</td>
<td>1 : Zero</td>
<td>1 : 0.1</td>
</tr>
<tr>
<td>F3</td>
<td>1 : Zero</td>
<td>1 : 0.25</td>
</tr>
<tr>
<td>F4</td>
<td>1 : 0.05</td>
<td>1 : Zero</td>
</tr>
<tr>
<td>F5</td>
<td>1 : 0.05</td>
<td>1 : 0.1</td>
</tr>
<tr>
<td>F6</td>
<td>1 : 0.05</td>
<td>1 : 0.25</td>
</tr>
<tr>
<td>F7</td>
<td>1 : 0.1</td>
<td>1 : Zero</td>
</tr>
<tr>
<td>F8</td>
<td>1 : 0.1</td>
<td>1 : 0.1</td>
</tr>
<tr>
<td>F9</td>
<td>1 : 0.1</td>
<td>1 : 0.25</td>
</tr>
</tbody>
</table>

Table 2: Response (Dependent) Variables and their Respective Constraints for the 3² Factorial Design

<table>
<thead>
<tr>
<th>Responses</th>
<th>Constraints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y₁ - Particle Size</td>
<td>Minimize</td>
</tr>
<tr>
<td>Y₂ - Zeta Potential</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y₃ - Entrapment Efficiency</td>
<td>Maximize</td>
</tr>
</tbody>
</table>

[1-14]
Elucidation of Optimized Formula

Numerical optimization [21, 22] was performed using the statistical program according to the constraints listed in Table 2. The simultaneous optimization technique described by Derringer and Suich in 1980 was chosen for optimization of the responses. This method was based on the utilization of desirability functions.

Further Characterization of the Optimized Formula

1. Particle Size (PS), Polydispersity Index (PDI), Zeta Potential (ZP) and Entrapment Efficiency (EE %)

The mean PS, PDI, ZP and EE% were determined as described earlier.

2. Morphology of the Optimized Formula

For morphological examination of the liposomes, the optimum formulation was examined by optical microscope and photographed at a magnification power of 40× and by transmission electron microscope (TEM) operated at 80 kV (model JEM-1230, Jeol, Tokyo, Japan).

3. In-vitro Release Studies

The in-vitro release of rivastigmine from liposomes was performed using the dialysis bag diffusion technique. [23] Phosphate buffer (PBS, pH 7.4) was used as dissolution medium. The dialysis bags (molecular weight cutoff 12,000–14,000 Da, Sigma) were soaked in PBS for 12 h before use. 2 ml of rivastigmine liposomal dispersion were ultracentrifuged and the precipitate was reconstituted in 2 ml PBS. These 2 ml of rivastigmine loaded liposomes (equivalent to ~8-9 mg) were placed in dialysis bags with the two ends fixed by thread. Each bag was put into a flask containing 20 ml of Phosphate buffer (PBS, pH 7.4) as the dissolution medium. The flasks were placed into water bath shaker at 37±0.5°C and 100 rpm. Aliquots of the dissolution medium (500μl) were withdrawn at each time interval and the same volume of fresh PBS was added to the flask to maintain the constant volume. Drug concentrations in the dissolution medium were finally analyzed spectrophotometrically, by first derivative (Shimadzu, model UV-1601 PC, Kyoto, Japan) at $\lambda_{max} = 273.2$ nm.
release experiments were carried out in duplicates. The results are expressed as means± standard deviation.

RESULTS AND DISCUSSION
Characterization of Positively Charged Liposomes
1. Morphology of the Prepared Liposomes
The photomicrograph (40×) is shown in , respectively. It was demonstrated that all liposomes are well identified with nearly perfect lipid bilayer spherical shape. Moreover, Some pictures clearly showed multilayered structure of the formed liposomes as shown in F7.

2. Particle Size (PS), Polydispersity Index (PDI) and Zeta Potential (ZP)
The values of PS, PDI and ZP of all formulation are shown in Error! Reference source not found.. It is clear that all formulae showed a PS within the nano-range. Regarding the PDI, it ranged between 0.357 to 0.662 which implies that the liposomes were relatively heterogeneous. [24] As for the ZP, upon addition of DDAB (positive charge inducer) a slight decrease in the negative charge was observed. Surprisingly, the presence of tween 80, non ionic

<table>
<thead>
<tr>
<th>Formulae No.</th>
<th>EE%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>55.16±2.56</td>
</tr>
<tr>
<td>F2</td>
<td>60.00±1.96</td>
</tr>
<tr>
<td>F3</td>
<td>53.71±1.65</td>
</tr>
<tr>
<td>F4</td>
<td>47.67±0.25</td>
</tr>
<tr>
<td>F5</td>
<td>54.1±2.52</td>
</tr>
<tr>
<td>F6</td>
<td>45.24±1.35</td>
</tr>
<tr>
<td>F7</td>
<td>33.67±3.57</td>
</tr>
<tr>
<td>F8</td>
<td>47.31±0.60</td>
</tr>
<tr>
<td>F9</td>
<td>44.39±2.11</td>
</tr>
</tbody>
</table>

*Each value represents mean ± standard deviation (SD) of two determinations (n = 2)
surfactant, leads to a significant decrease in the negative charge that was discussed later in the statistical analysis.

Post Hoc test, Tukey’s test showed that this decrease is insignificant at low level of DDAB (0.05 M), while at higher level (0.1 M) the decrease in EE% is significantly different at 5% significance level ($\alpha = 0.05$). This decrease in EE% might be attributed to the chemical structure of both Rivastigmine and positive charge inducer, DDAB, where

3. **Entrapment Efficiency (EE %)**

The EE% of all prepared formulations are shown in Table 4 and Figure 2. It was observed that there is a decrease in the EE% upon increasing DDAB molar ratio. Applying the

**Figure 4:** Line plots for the main effects and interaction of different factors on zeta potential, a) Effect of DDAB molar ratio, b) Effect of Tween 80 molar ratio, c) Interaction between DDAB and Tween 80 molar ratios

**Figure 5:** Line plots for the main effects and interaction of different factors on Entrapment efficiency, a) Effect of DDAB molar ratio, b) Effect of Tween 80 molar ratio, c) Interaction between DDAB and Tween 80 molar ratios

**Figure 6:** Response surface plot for the effects of DDAB and Tween 80 molar ratios on, a) Particle size, b) Zeta potential, c) Entrapment efficiency
Rivastigmine belongs to the phenethylamines. These are compounds containing a phenethylamine moiety, which consists of a phenyl group substituted at the second position by an ethan-1-amine. Moreover, it has a carbamide moiety which additionally contributes to its repulsion from the positive charge inducer-DDAB (being a quaternary ammonium compound). Subsequently upon addition of increasing molar ratio of DDAB, a charge repulsion occurs between rivastigmine and DDAB provoking the escape of rivastigmine from the surrounding environment that is, the liposomes. This theory is supported by the results of EE% of formulae containing tween 80 only (F1-3). Being a non ionic surfactant, tween 80 showed no significant change in EE% as there was no repulsion between tween 80 and rivastigmine.

Statistical Analysis
Each response, that was aforementioned in Table 2, was analyzed individually and fitted quadratic model using linear regression. Sequential model sum of squares, lack of fit test and model summary statistics were used to elucidate the best model to describe the relation between the response under question and the variables studied. In the present study the highest order unaliased model with highest prediction $R^2$ and lowest PRESS was the one of choice. Further improvement of the chosen model was done by model reduction, by removing any non significant model terms that are not needed to support hierarchy. The significant factors and the significant interaction appearing in the equation are represented as line plots in Figure 3 to Figure 5. Response surface plot of PS, ZP and EE% against the pair of significantly influential factors appearing as an interaction in the final equation is shown in Table 5: Theoretically Optimized Levels of the Factors Used and the Calculated Desirability

<table>
<thead>
<tr>
<th>Factors</th>
<th>Optimized levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: DDAB molar ratio</td>
<td>0.02</td>
</tr>
<tr>
<td>B: Tween 80 molar ratio</td>
<td>0.25</td>
</tr>
<tr>
<td>Desirability</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Table 6: Predicted and Actual Values of Particle Size, Polydispersity Index, Zeta Potential and Entrapment Efficiency Responses for the Optimized Formula

<table>
<thead>
<tr>
<th>Response Variables*</th>
<th>Predicted</th>
<th>Optimized Formula</th>
<th>Actual</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>445.13</td>
<td></td>
<td>375.3±4.95</td>
</tr>
<tr>
<td>ZP</td>
<td>-10.25</td>
<td></td>
<td>-11.6±0.28</td>
</tr>
<tr>
<td>PDI</td>
<td>------</td>
<td></td>
<td>0.589±0.03</td>
</tr>
<tr>
<td>EE%</td>
<td>50</td>
<td></td>
<td>42.355±0.67</td>
</tr>
</tbody>
</table>

* Each value represents mean ± standard deviation (SD) of two determinations (n = 2)
Figure 6.

Inference of the Statistical Analysis of the Different Responses
DDAB molar ratio has a significant quadratic effect on PS, ZP and EE%. A decrease in particle size as the molar ratio increase might be attributed to the positive effect of the DDAB that leads to repulsion between formed liposomes, preventing aggregation and consequently yielding smaller particle size (from 632.4 nm to 373.35 nm). An increase in ZP was observed due to positive charge effect. While the decrease in EE% was explained earlier.

Concerning Tween 80 molar ratio, it showed a significant quadratic effect on PS, ZP and EE%. An increase in particle size is shown at 0.1M Tween 80. This might be that Tween 80 molecules acting as amphiphilic molecules deposited at the particle surface resulting in increment of particle size. [25] After wards any further increase in tween 80 molar ratio resulted in the increase of the liposome stability in the dispersion. Currently, the mechanism of tween 80 on the liposome stability is unknown. This might be attributed to penetration of the hydrocarbon tail in the lipid bilayer thus leaving the polyethylene oxide groups on the surface of the liposomes thereby introducing a steric barrier on the surface of the liposomes, which might decrease liposome fusion and consequently decrease particle size. The same reason contributed in the significant decrease in ZP, as this steric barrier on the surface masks the negative charge of the phospholipid. [26]

A positive significant interaction existed between DDAB and tween 80 molar ratios for the responses PS, ZP and EE%. It was proven that upon increasing tween 80 molar ratio a greater decrease in PS and ZP was observed than in its absence, while the decrease in EE% caused by DDAB was ameliorated by the presence of tween 80. This may be explained by the solubilizing effect imparted by tween 80 which help in increasing the entrapment of rivastigmine that overlaid the repulsive effect of DDAB.

Elucidation of Optimized Formula
After generating the final polynomial equations relating the dependent and independent variables, optimization was done for the three responses studied. Numerical optimization searched the design space using the final models created for each studied response. It aimed at finding factors' levels that meet the constraints set for different responses. The levels of the combination chosen for the optimized formula are shown in Table 5. The overall desirability of the chosen formula was 0.75, which means that it satisfies to a great extent all the listed constraints previously stated in Table 2.

Further Characterization of the Optimized Formula
1. Particle Size (PS), Polydispersity Index (PDI), Zeta Potential (ZP) and Entrapment Efficiency (EE %)
The values of PS, PDI, ZP and EE% of the optimized formulation (predicted and actual), are shown in Table 5. The actual results showed close relevance to the predicted one with a 15 % deviation.

2. Morphology of the Optimized Formula
The photomicrograph (40×) and the TEM micrographs of the optimized formulation are shown Figure 7 and Figure 8, respectively. It was demonstrated that the liposomes are well identified with nearly perfect spherical shape.

3. In-vitro Release Studies
The results of the in-vitro release of rivastigmine from the selected formula compared with that of rivastigmine solution are graphically represented in Figure 9. It was clear that the release of the drug from the solution was faster than that from the selected formulation. This confirms that a sink condition was accomplished and that the dialysis membrane did not limit the drug release. For the selected formula, it exhibited a burst release of about 30%, resulting from the un-entrapped drug. The percentage of drug released after 2 hrs were 87.86% in case of optimized formula, respectively. At the end of the
eight hours, 96.83% were released from the optimized formula.

CONCLUSION
This research aimed at formulating a brain targeted liposomal dispersion containing DDAB, to avoid repulsion between the negatively charged liposomes and BBB, Tween 80, to provide enhanced solubility and stability for the liposomal dispersion. Therefore, a promising new application of positively charged rivastigmine liposomes was being introduced in this work.

REFERENCES AND NOTES