In Silico Study of Phospholipids as An Oral Insulin Delivery System

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Abstract. Phospholipids have been applied as a material for an oral insulin delivery system that either in the form of solid lipid nanoparticles, liposomes, or microemulsions. However, the administration of this delivery system for diabetic treatment requires twenty times higher of dosage than the injection one. In the present study, we performed molecular dynamics simulation to evaluate how phospholipids can protect insulin at the molecular level and why its efficiency for the diabetic treatment is low. Phosphatidylcholine group, such as DPPC, OPPC, LPC and combine of DPPC-LPC, was used as the phospholipid material to model the delivery system in our simulations because in vitro and in vivo studies of these phospholipids as the delivery system have been well-conducted. In these simulations, the waters-phospholipid-insulin system was prepared by randomly mixing phospholipid molecules inside the solvent box containing an insulin molecule. NPT ensemble was used in all simulations with the setting temperature at 310 K and 1 atm for the pressure. The simulation results showed that DPPC and LPC delivery systems were able to maintain both secondary and tertiary structures of the encapsulated insulin within the time range of simulation. However, all phospholipid delivery system models failed to fully encapsulate insulin surface within 150 ns simulation. This study may explain the reason for the low success rate of using phospholipid as the delivery system of oral insulin in the experiment.

Keywords: Insulin, phosphatidylcholine, oral delivery system, molecular dynamics simulation

1. Background

Inability of pancreas to produce insulin in diabetes mellitus type I (DMI) patients make them rely on insulin therapy [1]. However, 60% DMI patients who used invasive method failed to achieve long-term glycemic control [2]. The pain arises due to needle injections and insulin treatment regimen complexity [3] as well as the lack of control of glucose in the blood [4] are the reason that contributes the incompliance of patients to do insulin therapy.

Oral insulin is a promising route for insulin therapy. Oral insulin offers many advantages: higher patient compliance, rapid hepatic insulination, and avoidance of peripheral hyperinsulinemia and other possible adverse effects, such as hypoglycemia and weight gain [3]. 80% of insulin that reached the intestine directly flow into the liver [5] thus it is more effective to perform their role as a regulator of glucose levels in the blood [6]. However, the main problem is on the journey before reaching the intestine. Insulin has a bioavailability less than 1% [7] because this protein is prone to the degradation by digestive enzymes and also due to its molecular size, it is difficult to pass through the epithelium intestine [8].
A phospholipid is the active ingredient that is widely used in the pharmaceutical industry. A phospholipid has amphipathic properties that can form micelles when positioned in the aqueous environment. Many studies have successfully carried out using phospholipid as a material for drug delivery system in the form of microemulsions [6], liposomes [9], solid lipid nanoparticles [10], self-emulsifying system [11], and disperse [12]. Phospholipid can increase bioavailability, absorption and release of the drug, protects it from degradation, reduce ill effects in the gastrointestinal tract, and reduce the bitter taste of the drug [12]. However, the success rate of using phospholipids as an oral insulin delivery system is low. It has been reported in the previous study that the administration of oral insulin requires twenty times higher of dosage than the injection one [6, 10, 13, 14]. In the present studies, we evaluate the effectiveness of using phospholipid of phosphatidylcholine group as the insulin delivery system at the molecular level by performing in silico analysis with molecular dynamics (MD) simulation approach. The effectiveness the delivery system was evaluated in terms of the stability of insulin structure and the condition of encapsulation. We show here that our MD simulation can provide insight into the molecular level explanation about the low success rate of phospholipid as insulin delivery system.

2. Material and Methods

2.1 Materials

MD simulations require initial coordinate of atoms of the target molecules. The initial coordinate of atoms constituted insulin structure was obtained from the crystal structure data stored in the protein data bank repository (www.rcsb.org) with the accession code of 5ENA [15]. While for the atomic coordinate of the phosphatidylcholine group, DPPC, OPPC, and LPC, were obtained from gromos database and Respiratory [2].

2.2 Procedures

All MD simulations were performed by using GROMACS software version 5.0.4 [17–21] employing GROMOS96 53a6 as a force field [22] as source of parameters for bonding and nonbonding energy calculations.

2.2.1 Preparation

The insulin-water system was constructed by randomly adding water molecules to solvate an insulin molecule that was placed in the center of 5.5 x 5.5 x 5.5 nm³ box. While the insulin-water-phospholipid system was built by randomly added phospholipid to the insulin-water system with the ratio of phospholipid:water about 1:100. Both systems were neutralized by adding counter ions.

2.2.2 Energy Minimization

The prepared initial structures above were first energy minimized to reduce a number of bad atomic contacts and to relax the bonding interactions (bond lengths, bond angles, and dihedral). Energy minimization was divided to two stages, the first is minimization of non-protein components by restraining the protein backbone with the harmonic force with the magnitude of 1000 kJ mol⁻¹ nm⁻² for 50000 steps using steepest descent algorithm. The second stages were targeting the whole system without applying the restraining force to the protein molecule using conjugate gradient algorithm. Both of the minimization stages will stop when reaching the maximum force 1000 kJ mol⁻¹ nm⁻¹.

2.2.3 Equilibration

At this stage, all of the energy components will be equilibrated at the desired conditions so it will fluctuate at a targeted value (convergent). Equilibration process is carried out in two stages, NVT and NPT. Simulated annealing was performed in this study to bring the system to desired temperature (310 K). All the conditions were analyzed using “gmx energy” to ensure the convergent of targeted value including temperature, pressure, and density.

2.2.4 Production run

The equilibrated structure prepared above was then used as the initial structure for production run. The simulation was running for 150 ns with 1 fs integration time step by maintaining temperature and pressure with a V-rescale and a Parrinello-Rahman algorithms, respectively. In the course of simulation, the coordinate of the system was stored every 100 ps resulting 1500 structures.

2.2.5 Analysis

In this study, the tertiary structure stability of insulin was analyzed in terms of radius of gyration (equation 1), while for the secondary structures analysis, we used DSSP program [23]. In order to evaluate the effectiveness of the delivery system in encapsulating insulin, we used VMD [24] to visualize the emulsion
formed by the delivery system. The interaction density between the delivery system and insulin was
evaluated by calculating the contact number between both entities.

\[ \langle R_e \rangle_E = \left( \frac{1}{N} \sum_{i=1}^{N} \left( |r_i - \bar{r}_m| \right)^2 \right)^{1/2} \]

3. Result and Discussion

In this study, we performed MD simulation to insulin free delivery system as the control to the MD simulation of
insulin encapsulated with phospholipid delivery system. Each simulation was conducted within 150 ns to see the
stability of the emulsion of insulin and the delivery system. The contacts between phospholipids as a delivery
system with insulin during the simulation to certain extent may give impact to the stability of the structure of
insulin. Therefore, it is necessary to analyze the structure of insulin during the simulation to know its stability of
tertiary and secondary structures. In addition, we also analyzed how phospholipid encapsulates insulin at the
molecular level and evaluate its stability by calculating the contact number between phospholipids and insulin.

3.1 Stability of tertiary structure of insulin

![Figure 1](image)

**Figure 1** The profile of radius of gyration in the course of MD simulation for insulin free delivery system
(control) and insulin encapsulated by phospholipid delivery system.

The tertiary structure of a protein is the 3D shape of the protein stabilized by many types of weak interactions,
such as electrostatic interactions, van der Waals, hydrophobic, and hydrogen bonds. In this study, the stability of
the tertiary structure of insulin during the simulation was evaluated through the change of 3D shape of the
protein by calculating its radius of gyration (Figure 1). The radius of gyration is the average radius from the
center mass of the protein that indicates the compactness of the protein structure.

Insulin free delivery system was unstable as indicated by the sharp increase of radius of gyration value in the
first 50 ns of the simulation. Although the radius of gyration decreased after 50 ns, the shape of the protein was
different from the initial one (Figure 2). Analysis at molecular level noted that the instability of insulin structure
was initially caused by the hydrogen bond rupture between Gly-41 and Gly-44 that stabilized the loop
connecting helix A and helix B.

Different from insulin free delivery system, insulin encapsulated by phospholipid molecules exhibited better
structural stability as seen in relatively lower radius gyrations change from the initial condition. Although, the
contact between insulin and phospholipids did not all provide optimum protection to the tertiary structures of
insulin. Among four model of phospholipid delivery system (LPC, DPPC, OPPC, and mixed DPPC-LPC) only
LPC phospholipids that seem almost optimally stabilize the insulin structure as indicated by the lowest change in
its radius of gyration for the whole simulation time range. The other three models, which were DPPC, OPPC,
and DPPC-LPC, showed considerable changes in insulin’s radius of gyration. Based on the structure of the three
phospholipids, LPC has only one hydrophobic tail, while DPPC and OPPC have two hydrophobic tails making
the former is relatively has lower sterical hindrance to encapsulate an insulin molecule compare to the later.

3.2 Stability of secondary structure

Insulin is a protein’s hormone that may only be functional in the form of native structure. Figure 3 shows the
important residues in insulin that played an important role to bind an insulin’s receptor [26]. As shown in the
figure, several of those residues are the element of α-helical strands (helix A1, A2, and B). Therefore, besides
three-dimensional shape, the secondary structure of this protein has to be stable as well in order to maintain the
proper orientation of those key residues when interacts with the hormone’s receptor.
Figure 2 The structure of insulin free delivery system before and after the simulation. (A) At the initial structure of insulin, there is a hydrogen bond between the Gly-41 and Gly-44 residues making helix A and helix B close to each other. (B) After 150 ns of simulation, the hydrogen bond between Gly-41 and Gly-44 residues was ruptured separating apart helix A and helix B.

The secondary structural analysis was conducted in this study by using DSSP program [23]. The result of DSSP analysis to the simulation trajectory of insulin free delivery system (Figure 4a) clearly showed the condition of the three α-helical strands in the course of simulation. All the helical strands were initially partially denatured, but after 90 ns, helix B was almost completely collapse and after 120 ns all of the helical strands were also mostly collapse until the end of the simulation. This result explained the tertiary structure analysis above (Figure 1) that the increased of radius of gyration on insulin free delivery system was due to the collapse of secondary structure. Relatively less stability of insulin structure found in this study was also supported by the experimental evident that insulin tends to aggregate forming a quaternary hexamer molecules in order to stabilize its native structure. Therefore, a good delivery system is verified in our study based on its ability to preserve native structure of insulin.

Figure 3 Important residues of insulin proteins that interact with insulin receptors [26]. The red color indicates chain A, and the green color indicates chain B.

The DSSP result to the simulation trajectory of insulin in the presence of phospholipid delivery system showed the variable effect on the secondary structure of insulin (Figure 4b-e). DPPC and LPC delivery system gave better protection to the secondary structure of insulin (Figure 4(b) and (d)). However, DPPC showed the different effect to the secondary structure of insulin at the first 50 ns of simulation that in this period helix A1 initially collapse but then restored after 50 ns to the end of the simulation. Such behavior was not observed in LPC delivery system, the secondary structures of insulin were relatively stable from the beginning to the end of the simulation.

Figure 5, summarized the condition of insulin structure after 150 ns for insulin free and encapsulated with the delivery system. The insulin free delivery system was only experiencing partial denaturation after 150 ns, only helix B that apparently still exist while the rest is almost completely collapse (Figure 5a). At the encapsulated condition, it is clearly showed that only LPC delivery system that able to maintain the secondary and tertiary structure of insulin (Figure 5d). The worst impact to the structure of insulin was exhibited by OPPC delivery system, in which almost all secondary structures were almost collapse (Figure 5c). In contrast, DPPC, which has the same two hydrophobic tails as OPPC, gave better effect to the structure of insulin (Figure 5b). The prominent
different between DPPC and OPPC is the presence of oleic acid as one of the hydrophobic tail in OPPC, while DPPC has only palmitic in both tails. Oleic acid is unsaturated fatty acid containing one cis-oriented double bond making OPPC has one non-linear hydrophobic tail. In our study such difference gave different effect to the structure of insulin.

Figure 4 The result of secondary structure analysis of insulin protein (a) without delivery system and some variation of phospholipid as the delivery system: (b) DPPC, (c) OPPC, (d) LPC, (e) DPPC-LPC. The blue color in the image above shows the secondary structure of α-helix, the purple π-helical, the gray 310-helix, the red β-sheet, the yellow α-turn, the green β-turn, and the white random coil.
Figure 5 Secondary structure of insulin free delivery system (a) and encapsulated with DPPC (b), OPPC (c), LPC (d), and DPPC-LPC (e) after 150 ns MD simulation. Blue color represents for α-helix, purple for π-helical, gray for 310-helix, red for β-sheet, yellow for α-turn, green for β-turn, and white for random coil.

3.3 Analysis of encapsulation level of insulin by phospholipid delivery system

The previous analysis above focused only on the insulin structure point of view. In order to assess the effectiveness of phospholipid in encapsulating insulin, we evaluate the density of phospholipid covering the surface of insulin by visual inspection of the shape of emulsion and calculating the number of contact between phospholipid and insulin (Figure 6). The good delivery system should not only able to maintain the native structure of insulin but also must able to protect it from the digestive system. Therefore, the surface of insulin should be fully covered by the delivery system.

Figure 6 The shape of emulsion formed by DPPC (a), OPPC (b), LPC (c), and DPPC-LPC (d) delivery systems when encapsulated an insulin after 150 ns of MD simulation.

Our visual inspection found an interesting result, which was all phospholipids used to model the delivery system was not able to fully encapsulate the whole surface of insulin. Those phospholipids only attached to certain parts of the surface of insulin leaving uncovered part of insulin still in contact with the environment. Most parts that in contact with phospholipid molecules are the hydrophobic surfaces of insulin, the polar surfaces are mostly still free. In order to account for this behavior, we calculated the surface electrostatic potential of insulin (Figure 7). The majority of polar surfaces of insulin have a positive electrostatic potential only small fraction of the surface has the negative value. Phospholipids used in our study are negatively charged amphipathic molecule that supposed to well-attracted to the positively potential surfaces of insulin. The existence of uncovered polar surface of insulin indicating the weak interactions occurred between the polar groups of phospholipids and
insulin. This is likely that the charge density of phospholipids is too low to make stable electrostatic interactions with the polar surface of insulin. The partial encapsulation of insulin by the delivery system may cause adversely affect to the stability of insulin since when it passes through the digestive system, the uncovered parts of insulin become prone to the attack of hydrolytic enzymes and extreme environments. Our study, thus, accounts for the low success rate of using phospholipid as the delivery system of oral insulin compared to the injection method [6, 10, 13, 14].

![Surface electrostatic potential profile of insulin](image)

**Figure 7** Surface electrostatic potential profile of insulin. Blue surface indicating positive potential value, while the negative one is depicted in red.

The result of contact number calculation for all phospholipid delivery systems after 150 ns of MD simulation exhibited a relatively small variations (Figure 8). LPC delivery system has the highest contact number with insulin, while DPPC, OPPC, and mixed DPPC-LPC showed relatively similar contact number (Figure 8a). In addition, LPC also reaches the highest contact number faster than the other phospholipids (Figure 8b). It is supported by the visual progress of insulin encapsulation by LPC delivery system depicted in Figure 9. As shown in this figure, encapsulation was already established before 30 ns of the simulation progress as indicated by similar shape of the encapsulation mode captured at 30, 60, 90, 120 and 150 ns.

![The number of contacts between insulin and the phospholipid delivery system](image)

**Figure 8** The number of contacts between insulin and the phospholipid delivery system. (a) The bar chart of number of contacts after 150 ns of MD simulation. (b) The graph the number of contacts during the simulation.
The analysis above has shown that LPC is the best phospholipid compared to DPPC, OPPC and mixed DPPC-LPC to be developed as a delivery system for oral insulin. LPC offers better insulin protection as indicated by the highest contact number (Figure 8a) and stable encapsulation (Figure 8b). This analysis may account for the reason why LPC is providing better protection for the tertiary and secondary structures of insulin above (Figure 1 and Figure 4). In order to improve better protection, the use of phospholipid molecules only is not sufficient, the secondary amphipathic molecules that have better interaction with the uncovered surface by phospholipid molecules are required. The search of such secondary amphipathic molecules requires another study because some complexity may arise because those molecules may disturb interactions between phospholipids and insulin and also may induce structural change to the protein.

4. Conclusion

DPPC, OPPC, LPC and mixed DPPC-LPC are not fully suitable as the material for a delivery system of oral insulin because all of them fail to fully encapsulate the protein. However, among all phospholipid, LPC provide better protection to insulin structure both tertiary and secondary. Secondary amphipathic molecules require improving better encapsulation and protection of insulin to be used as the oral delivery system.

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References

[1] Hilsted J Madsbad S and Hvidberg A 1995 Intranasal insulin therapy: the clinical realities Diabetologia 38 680–684
[2] Lassmann Vague V and Racca, D 2006 Alternatives routes of insulin delivery Diabetes Metabolism Research and Reviews 32 513–22
[3] Gizurarson S and Bechgaard E 1991 Intranasal administration of insulin to humans Diabetes Research and Clinical Practice 12 71–84
[4] Triplitt C L Reasner C A and Isle 2008 Diabetes mellitus 1205–1241 in book: pharmacotherapy: a pathophysiologic approach 7th ed The McGraw-Hill Companies Incorporation New York 2597
[5] Matteucci E Giampietro O Covola V Giustarini D Fanti P and Rossi R 2015 Insulin administration: present strategies and future directions for a noninvasive (possibly more physiological) delivery *Dove Press: Drug Design, Development, and Therapy* 3109–3119

[6] Rachmawati H Haryadi B M Anggadireja K and Suendo V 2014 Intraoal Film Containing Insulin-Phospholipid Microemulsion Formulation and In Vivo Hypoglycemic Activity Study *American Association of Pharmaceutical Scientist*

[7] Lee VH 1991 Oral route of peptide and protein drug delivery In Peptide and Protein Drug Delivery Chapter 16 *Marcel Dekker Incorporation* New York 691–738

[8] Lassmann Vague V and Racca D 2006 Alternatives routes of insulin delivery *Diabetes Metabolism Research and Reviews* 32 513–22

[9] Kisel M A Kulik L N Tsybovsky I S Vlasov A P Vorobyov M S Kholodova E A and Zabarovskaya Z V 2001 Liposomes with phosphatidylethanol as a carrier for oral delivery of insulin: studies in the rat *International Journal of Pharmacy and Pharmaceuticals Sciences* 216 105–14

[10] Zhang Z Huixia L and Zhou J 2009 Novel solid lipid nanoparticles as carriers for oral administration of insulin *Pharmazie* 64 574–578

[11] Cui F Shi K Zhang L Tao A and Kawashima Y 2006 Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: preparation, in vitro characterization and in vivo evaluation *Journal of Controlled Released* 28 242–50

[12] Fricker G Kromp T Wendel A Blume A Zirkel J Rebmann H Setzer C Quinkert O Martin F and Müller C 2010 Phospholipids and Lipid-Based Formulations in Oral Drug Delivery *Pharmaceutical Research* 27 1446–1486

[13] Cilek A Celebi N Tirmaksiz F Tay A 2005 A lecithin-based microemulsion of rh-insulin with aprotinin for oral administration: Investigation of hypoglycemic effects in non-diabetic and STZ-induced diabetic rats *International Journal of Pharmaceutics* 298 176–185

[14] Zhang N Ping Q Guihua H Xu W Cheng Y and Han X 2006 Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin *Pharmaceutical Nanotechnology* 327 153–159

[15] Mandal K Dhayalan B Avital-Shmilovici M Tokmakoff A and Kent SB 2016 Crystallization of Enantiomerically Pure Proteins from Quasi-Racemic Mixtures: Structure Determination by X-Ray Diffraction of Isotope-Labeled Ester Insulin and Human Insulin *Chembiochem* 17 421–5

[16] Koziarz K B Stroet M Malde AK and Mark A E 2014 Testing and validation of the Automated Topology Builder (ATB) version 2.0: prediction of hydration free enthalpies *Journal of Computer-Aided Molecular Design* 9713–9717

[17] Pronk S Páll S Schulz R Larsson P Bjelkmar P Apostolov R Michael R Shirts Jeremy C Spoel D Hess B and Lindahl E 2013 GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit *Bioinformatics* 29 845–854

[18] Spoel D Lindahl E Hess B Groenhof H Mark E A Herman J C and Berendsen 2005 GROMACS: Fast, flexible, and free *Journal of Computational Chemistry* 6 1701–1718

[19] Berendsen D Spoel R and Drune V 1995 GROMACS: A message-passing parallel molecular dynamics implementation *Computer Physics Communication* 91 43–56

[20] Hess B Kutzner C Spoel D and Lindahl E 2008 GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation *Journal Chemical Theory and Computation* 4 435–447

[21] Lindahl E Hess and B Spoel D 2001 GROMACS 3.0: a package for molecular simulation and trajectory analysis *Journal of Molecular Modeling* 7 306–317

[22] Oostenbrink C Villa A Mark A E and Gunsteren W F 2004 A biomolecular force field based on the free enthalpy of hydration and solvation: the GROMOS force-field parameter sets 53A5 and 53A6, *Journal of Computational Chemistry* 13 1656–76

[23] Kabsch W and Sander C 1983 Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features *Biopolymers* 22 2577–2637

[24] Humphrey W Dalke A and Schulten K 1996 VMD - Visual Molecular Dynamics *Journal of Molecular Graphics* 14 33–38

[25] Hassiepen U Federwisch M Iders T and Wollmer A 1999 The Lifetime of Insulin Hexamer *Biophysical Journal* 77 1638–1654

[26] Wirahadikusumah 2008 Biokimia: Protein, Enzim dan Asam Nukleat *ITB Bandung*

[27] Ward Colin Lawrence and Mike 2014 Insulin Binding and Activation of the Insulin Receptor [internet] *Diapedia* Available from https://doi.org/10.14496/dia.51040851468.30