BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
43 (2022) 102552

Original Article

nanomedjournal.com

Bioavailability by design — Vitamin D_3 liposomal delivery vehicles

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Revised 1 March 2022

Abstract

Vitamin D3 deficiency has serious health consequences, as demonstrated by its effect on severity and recovery after COVID-19 infection. Because of high hydrophobicity, its absorption and subsequent redistribution throughout the body are inherently dependent on the accompanying lipids and/or proteins. The effective oral vitamin D_3 formulation should ensure penetration of the mucus layer followed by internalization by competent cells. Isothermal titration calorimetry and computer simulations show that vitamin D_3 molecules cannot leave the hydrophobic environment, indicating that their absorption is predominantly driven by the digestion of the delivery vehicle. In the clinical experiment, liposomal vitamin D_3 was compared to the oily formulation. The results obtained show that liposomal vitamin D_3 causes a rapid increase in the plasma concentration of calcidiol. No such effect was observed when the oily formulation was used. The effect was especially pronounced for people with severe vitamin D_3 deficiency.

Key words: Vitamin D; Liposomes; Bioavailability; Digestion

In classical pharmacology, the oral bioavailability of an active compound depends predominantly on its solubility in the aqueous phase and the passive transfer across structural barriers such as the epithelium or the blood–brain barrier. Such properties impose specific structural requirements on an active compound as formulated by the rule of five, first published by Lipinski et al.[1] The approach is applicable only for active compounds that are moderately hydrophobic (logP <5). A compound hydrophobicity is typically quantitated by the log of the octanol/water partition coefficient (logP).[2,3] However, for very hydrophobic molecules, as exemplified by cholesterol and its metabolite vitamin D_3 (cholecalciferol), the classical approach cannot be applied.[4,5] Highly hydrophobic compounds cannot enter inner body compartments unassisted. This is well illustrated by the absorption and biodistribution of cholesterol, which with logP >7 requires complex supramolecular carriers in the form of lipoproteins, dedicated receptors, and complex redistribution mechanisms within the cell.[6,7] Vitamin D_3 is an essential hydrophobic compound necessary for a variety of metabolic and immunological processes. Its importance is demonstrated by the fact that it controls more than 700 genes, and its deficiency can lead to a number of pathologies.[8,9] For example, as recently demonstrated, vitamin D_3 supplementation reduces the severity of the disease caused by COVID-19 infection.[10–12] Therefore, its efficient delivery is a prerequisite for effective supplementation. Vitamin D_3 can be acquired by humans from both intrinsic and extrinsic sources. The intrinsic source requires exposure to UVB, usually from the sun, which carries the risk of melanoma; hence, it is better to consume it orally. In addition, the altered lifestyle results in an increased demand for vitamin D_3 from the diet, which is usually not satisfied by most of the available foodstuff.[13] Consequently, supplementary schemes have been

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https://doi.org/10.1016/j.nano.2022.102552
1549-9634/

Please cite this article as: Dałek P, et al, Bioavailability by design — Vitamin D_3 liposomal delivery vehicles. *Nanomedicine: NBM* 2022;43:102552, https://doi.org/10.1016/j.nano.2022.102552

proposed to counter vitamin D_3 deficiency.[14,15] The highly hydrophobic nature of vitamin D_3 causes difficulties in its effective delivery to the organism. To design an effective delivery strategy, the absorption mechanisms of hydrophobic compounds from the diet need to be considered in details.[16] For subsequent analysis, vitamin D_3 absorption and distribution processes can be functionally separated, as its absorption is mainly dependent on specialized epithelial cells in the gut, whereas the fluxes of vitamin D_3 and its metabolites inside the body are tightly controlled by dedicated transport mechanisms and metabolic processes.[17] Transfer of vitamin D_3 and its metabolites within the body cannot be altered by simple adjustments in supplement formulation and/or dose. The digestion and absorption processes, on the other hand, can be greatly altered by modifications of the vitamin formulation.[18] Water-insoluble hydrophobic compounds require complex enzymatic and/or physicochemical mechanisms to facilitate their internalization.[19] Typically, highly hydrophobic compounds remain during the digestion process within supramolecular aggregates.[20,21] The digestion and subsequent absorption of particulates with the hydrophobic compound are restricted by mucus, which contains a complex polymer matrix made of mucins.[22] Consequently, epithelial cells are only accessible to particulates smaller than 300–500 nm.[23] In addition, the absorption of a particulate carrier can be facilitated only by competent cells.[24,25] These cells can absorb intact particulates of certain sizes, therefore providing means for highly hydrophobic compound internalization. These factors together with the understanding of biologically relevant particulates can be utilized for the design of an effective formulation for highly hydrophobic compounds. In the article, the requirements for an effective vitamin D_3 formulation are identified followed by the prediction on the performance of the two different formulations of vitamin D_3. The prediction was validated in a clinical experiment.

Methods

Preparation of liposomal vitamin D3 formulation

Liposomal formulation of vitamin D_3 was prepared by mixing two solvents: propylene glycol containing lipids (20% w/w in the final preparation) with vitamin D_3 and purified water (1:1 w/w) followed by extrusion through the 100 nm polycarbonate filter. The high content of lipids in the mixture results in high viscosity and gel-like consistency of the suspension. Finally, the lipid gel was diluted 200 times with the glycerol/water (1:1 w/w) mixture and supplemented with natural flavor (0.3 w/w) and pectin (2.45 w/w). The quantity of propylene glycol (E490) in a single serving (5 g) of the final liposomal formulation is three orders of magnitude lower than the official limits considered as safe for oral formulations (Lim et al[26] and references therein). After each preparation of the liposome suspension, the size and polydispersity index were measured to confirm the formation of the monodisperse population of liposomes. Due to its high hydrophobicity (logP >7), it can be assumed that all cholecalciferol is located in the lipid bilayer forming the liposome wall (Gupta et al[27] and the citations therein); therefore, its encapsulation efficiency is expected to be high. For control studies, the commercially available oily solution of vitamin D_3 was used. In this case, the quantity of vitamin D_3 was assumed to be equal to the value declared by the producer.

Characterization of liposomal vitamin D_3 formulation

The size distribution of liposomes in the liposomal vitamin D_3 formulation was determined by the dynamic light scattering method with some modifications in the preparation of the measured samples due to the presence of pectin. Liposomes were extracted from the liposomal formulation by diluting it 100 times in purified water followed by adding 0.1 sample volume of 1 M NaOH before measurement. The effect of the procedure on the integrity of liposomes was measured in the control experiment and no alteration of the liposome parameters (average size and polydispersity (PDI)) was observed (data not shown). The quantity of vitamin D_3 was determined by RP-HPLC (reversed-phase high liquid chromatography) according to the method developed by Sazali et al[28] with some modifications in the preparation of the sample. The details of the HPLC measurements are presented in the Supplementary Materials. The vitamin D_3 concentration used in the liposome formulation was two orders of magnitude lower than that with demonstrated toxic effects on cells in culture.[29]

Determination of the vitamin D_3 encapsulation efficiency

The encapsulation efficiency (EE%) of vitamin D_3 in liposomes was evaluated using the ultrafiltration method combined with quantification of vitamin D_3 before and after extrusion. The suspension of liposomes with vitamin D_3 was diluted 50 times with purified water and placed into Amicon Ultra Centrifugal Filters (volume 0.5 ml, NMWL 50k) to separate liposomes with vitamin D_3 and free vitamin D_3. Samples were centrifuged for 15 min (14,000 RPM). Then, the solution of free vitamin D_3 (permeate) was analyzed with the RP-HPLC method described by Sazali et al.[28] Before and after ultrafiltration, the size distribution of liposomes in the sample was determined to control their stability. The encapsulation efficiency of vitamin D_3 was calculated according to the following equation:

$$EE\%_{\mathrm{VITD}} = \frac{m_{\mathrm{total}} - m_{\mathrm{free}}}{m_{\mathrm{total}}} \times 100\%$$

where m_{free} is the mass of vitamin D_3 in the permeate and m_{total} is the total mass of vitamin D_3 in the sample. Since vitamin D_3 is highly hydrophobic, when it reaches the saturation level in the lipid bilayer, it will precipitate in the aqueous phase. Consequently, to quantitate the free vitamin D_3, the monomer and precipitate forms should be accounted for. To do this, quantities of vitamin D_3 in the lipid bilayer before (multilamellar vesicles) and after the extrusion process (unilamellar vesicles) were evaluated and compared.[30] The extrusion process was carried out using membrane filters with different pores sizes (15, 100 and 200 nm), and the amount of vitamin D_3 was determined with the HPLC method described above.

Cryogenic transmission electron microscopy (TEM) imaging

Cryogenic transmission electron microscopy (cryo-TEM) images were obtained using a Tecnai F20 X TWIN microscope (FEI Company, Hillsboro, Oregon, USA) equipped with a field emission gun, operating at an acceleration voltage of 200 kV. Images were recorded on the Gatan Rio 16 CMOS 4k camera (Gatan Inc., Pleasanton, California, USA) and processed with Gatan Microscopy Suite (GMS) software (Gatan Inc., Pleasanton, California, USA). Specimen preparation was done by the vitrification of the aqueous solutions on grids with holey carbon film (Quantifoil R 2/2; Quantifoil Micro Tools GmbH, Großlöbichau, Germany). Prior to use, the grids were activated for 15 s in oxygen plasma using a Femto plasma cleaner (Diener Electronic, Ebhausen, Germany). Cryo-samples were prepared by applying a droplet (3 μL) of the suspension to the grid, blotting with filter paper and immediate freezing in liquid ethane using a fully automated blotting device (Vitrobot Mark IV, Thermo Fisher Scientific, Waltham, Massachusetts, USA). After preparation, the vitrified specimens were kept under liquid nitrogen until they were inserted into a cryo-TEM-holder (Gatan 626, Gatan Inc., Pleasanton, USA) and analyzed in the TEM at −178 °C.

Calorimetric studies

Extruded liposomes for calorimetric studies were prepared using the dry lipid film procedure followed by extrusion through the filter with 100 nm pores, as previously described.[31] In summary, 196 mg DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) lipid and 15 mg vitamin D_3 (10 mol%) were dissolved in chloroform. The organic solvent was removed by the stream of argon and the chloroform residues were removed under low-pressure storage. The resulting dry lipid film was hydrated overnight with 20 mM HEPES buffer pH 7.4 (to 50 mM lipid concentration). The liposome size distribution was determined using dynamic light scattering (Zetasizer Nano ZS, Malvern GB). The isothermal titration calorimetry measurements (Nano ITC, TA Instruments) were performed in two setups:

1. Titrating the dispersion of liposomes (5 mM DOPC concentration) to liposomes with vitamin D_3 (5 mM DOPC concentration + 10 mol% vitamin D_3).
2. Titrating dispersion of liposomes (5 mM DOPC concentration) or liposomes with vitamin D_3 (5 mM lipid concentration + 10 mol% vitamin D_3) to 50 mg/ml albumin solution.

All samples were prepared in 20 mM HEPES buffer (pH 7.4). Before each experiment, their pH was measured and adjusted if necessary. The pH difference between the solutions did not exceed the 0.02 value, so the protonation effect did not interfere with the measurement. Finally, the solutions were degassed shortly before measurements. In all experiments, the rotor speed was set at 250 rpm, the injection volume at 10 μl and the time of single injection to 300 s (control measurements) or 500 s (measurement in the presence of vitamin D_3, to detect the occurrence of any slow processes).

Molecular dynamic simulations

All full-atomic simulations were performed with the NAMD[32] software and united-atom CHARMM36 force field under NPT conditions for lipid molecules. Vitamin D_3 and Soy Triglyceride (STri) force fields were created using Scigress and parameterized based on quantum calculation (optimization and single-point calculation).[33] Four different molecular systems were simulated: a single vitamin D_3 molecule with POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) bilayer, 10 vitamin D_3 molecules with POPC membrane, STri system, and STri system with 10 VTD molecules. All systems were hydrated with TIP3P water molecules. The TIP3P model describes the water molecule as a 3-atom rigid structure with point charges and Lennard–Jones parameters assigned to each of the 3 atoms. The model was selected because the CHARMM force field (on which lipids are parametrized) was developed using the TIP3P representation of water. The first system consisted of 100 POPC molecules (13,400 atoms), 1 VTD molecule (72 atoms), and 3811 TIP3P water molecules (11,184 atoms). The size of simulation box equaled 50 × 60 × 70 Å. The total simulation time was 135 ns. The second system consisted of 200 POPC molecules (26,800 atoms), 10 VTD molecules (720 atoms) and 16,503 TIP3P water molecules (47,529 atoms). The simulation box size was 80 × 80 × 120 Å. The total simulation time was 100 ns. The third system consisted of 50 Soy Triglyceride molecules (8050 atoms, 161 for each molecule). The size of the simulation box was 50× 40 × 40 Å. The total simulation time was 50 ns. The fourth system consisted of 50 soy triglyceride molecules (8050 atoms) and 10 VTD molecules (720 atoms). The size of the simulation box was 45 × 40 × 45 Å. The total simulation time was 50 ns. Membrane thickness ($d_{P\text{-}P}$) was determined from the difference between the coordinates of phosphorus atoms in the opposite monolayers along the membrane normal. The surface area per lipid molecule in the membrane (APL) was determined using Voronoi tessellation. Specifically, the membrane surface was divided into groups of n points (each point represented phosphorus and was labeled as P(x,y)) in such a way that each point was equally distant to neighboring n points. To exclude the possibility of overestimation, each membrane was multiplied in all three dimensions. Only parameters from the initial area were used for APL estimation. Every phosphorus at the distance from vitamin D_3 molecule smaller than 8 Å was considered as "perturbed".

Clinical studies and quantification of vitamin D_3 metabolites in serum

The clinical experiment has been performed on healthy volunteers (age 24-65) according to "the cross-over design". Volunteers were randomly divided into two groups. The general scheme of the clinical experiment is presented in Figure 1. Following a 12 h fasting, each volunteer consumed 10,000 IU of vitamin D_3 either in the liposomal or oily formulation. After 3 weeks, the experiment was repeated, but each group of volunteers consumed the other vitamin D_3 formulation. Vitamin D_3 absorption was evaluated by quantifying calcifediol ($25(OH)D_3$) in serum. Blood samples were drawn from each volunteer at

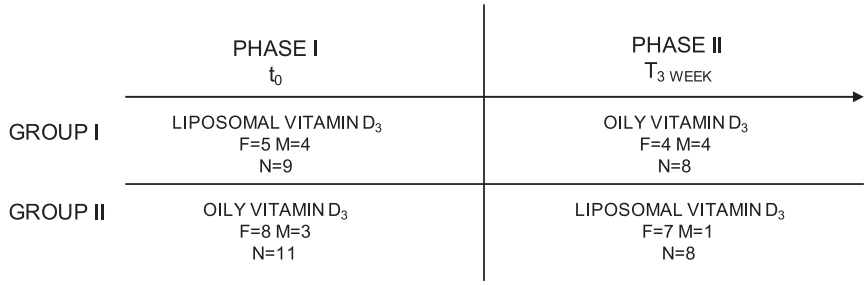

Figure 1. The design of the medical experiment. F refers to the number of women, M to number of men, and N represents the total number of participants in each group.

eight time points; the reference sample shortly before and 0.5, 1, 1.5, 2, 3, 4, and 5 h following the intake of vitamin D_3. The complex absorption of vitamin D_3 is followed by its transfer to the liver, where it is metabolically transformed. The literature data show that the AUC curve for 25(OH)D_3 reaches a maximum at three days after supplementation. However, the steep rise of the curve during the first day after supplementation justifies the proposition that 25(OH)D_3 can be evaluated even during the first few hours following supplementation. The advantage of such an approach is the elimination of possible interferences resulting from difficulty in controlling volunteer behavior when outside the medical facility (exposure to the sun and/or differences in diet). In addition, during the reduced time of the experiment, the observed rise of 25(OH)D_3 is effected exclusively by the absorption efficiency. This is because, after fasting, the supplement will pass the absorption zone in the intestine during the first hours following the supplementation.[34] Blood samples were collected by qualified personnel provided by Cambridge Diagnostics Sp. z o.o. (Poland). The company also determined the concentration of 25(OH)D_3 in blood samples using validated diagnostic methods.

Ethical approvals of the study

All procedures involving human subjects/patients were approved by the Bioethical Commission at the Research and Development Centre at the Specialized Hospital in Wrocław number: KB/07/2020. Written informed consent was obtained from all subjects. Liposomal vitamin D_3 was prepared by Lipid Systems Sp. z o.o. (Wrocław, Poland) under conditions satisfying HACCP requirements (the European Parliament Regulation No. 852/2004 from April 29 2004, Journal of Laws EU Office of 2004 as amended) and the Act on Food and Nutrition Safety from August 25 2006 (Journal of Laws from 2015, item 594).

Results

Characterization of the liposomal vitamin D3 formulation

As stated above, liposomes designed for rapid and effective delivery of vitamin D_3 should have sizes smaller than 300 nm. Figure 2 shows size distribution of liposomes containing vitamin D_3. The fitting of the experimental data shows that the average liposome size and PDI are equal to 117 nm and 0.23, respectively. In addition, the suspension of liposomes prepared according to the procedure described above is stable, both from a microbiological and physicochemical point of view, for more than six months (Table S1, Supplementary Materials). For the prepared liposomal formulation, the theoretical concentration of vitamin D_3 is declared equal to 20 μg/g. The mean concentration of vitamin D_3 in the samples was 19.83 ± 0.41 μg/g (as determined based on three measurements performed for each of the three different samples). Stability measurements after 3 months show that vitamin D_3 concentration did not change significantly and was equal to 18.56 ± 0.38 μg/g. The encapsulation efficiency was determined using the ultrafiltration method and vitamin D_3 quantification before and after extrusion. The concentration of vitamin D_3 in the permeate was below the detection limit (15 ng/g), which shows that the amount of free vitamin D_3 in the sample was below 3.4% of the total vitamin D_3. Examples of chromatograms are presented in the Supplementary Materials (Figures S1 and S2). When quantities of vitamin D_3 were measured before and after the extrusion process, the difference between the two liposome suspensions was statistically insignificant, indicating that there is no vitamin D_3 precipitation during the formation of unilamellar liposomes. The table showing the quantities of vitamin D_3 in various liposome suspensions is presented in the Supplementary Materials (Table S2). All of these show that the encapsulation efficiency of vitamin D3 in liposomes in prepared samples approaches 100%. The topological features of the liposome formulation have been investigated using CRIO-TEM microscopy. The representative image and histogram are presented in Figure 2. The results show that the liposomes are unilamellar and that their average sizes were smaller than 300 nm.

Calorimetric studies

There are three qualitatively different absorption pathways in the intestine; one for hydrophilic compounds facilitated by dedicated membrane proteins, the second for amphiphilic compounds based on passive diffusion across the epithelial barrier, and the third for highly hydrophobic compounds embedded in supramolecular aggregates. For highly hydrophobic compounds, such as vitamin D_3, there are two potential pathways leading to the systemic circulation; transcytosis and chylomicron pathway.[35–40] The two pathways are qualitatively different; transcytosis allows vitamin D_3 to enter the circulation accompanied by a lipid carrier, while the chylomicron pathway requires a series of exchange events between proteins and lipid aggregates,

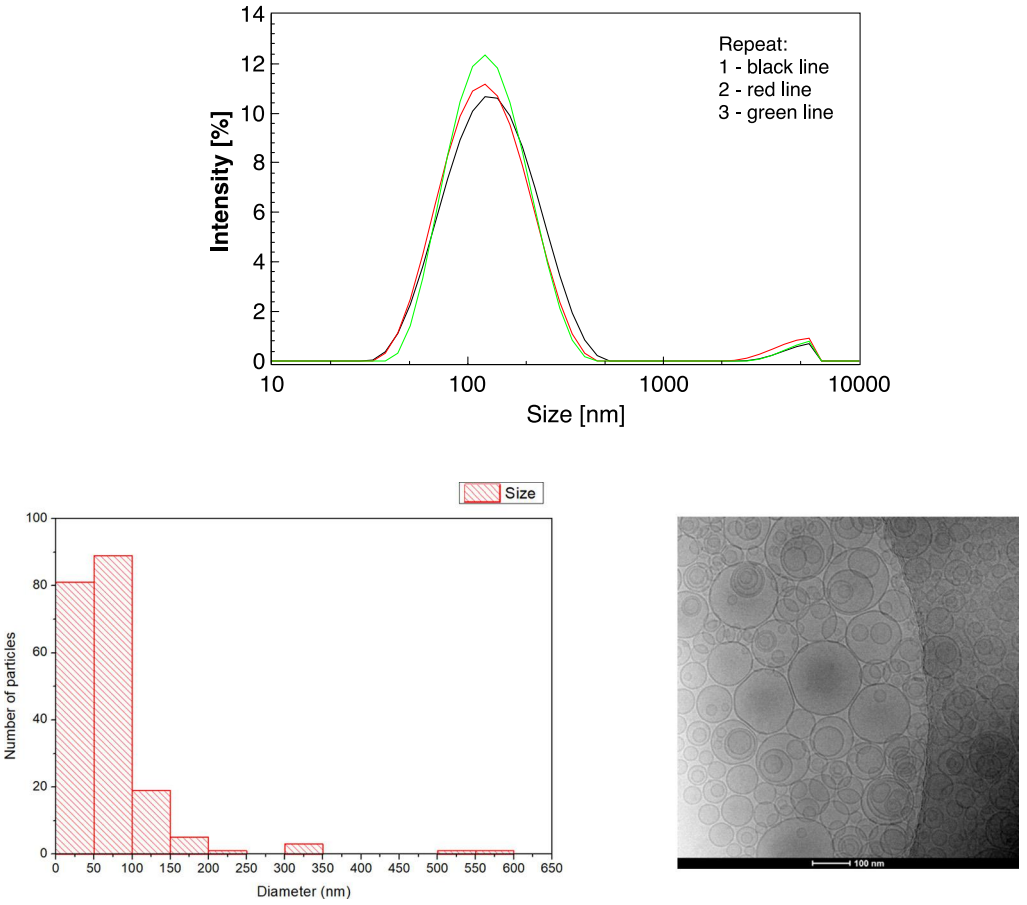

Figure 2. The size distribution of liposomes in the liposomal formulation of vitamin D_3. The top panel presents the size distribution as determined using the dynamic light scattering method, and the bottom panel presents the example of an image of liposomes with vitamin D_3 accompanied by histogram calculated from cryo-TEMP images.

before vitamin D_3 enters chylomicrons formed in the enterocyte cytoplasm.[41] The topological integrity of liposomes during digestion and absorption processes will likely affect vitamin D_3 bioavailability. Hence, the exchange of liposome-forming compounds (lipids and/or vitamin D_3) with surrounding structures, in addition to enzymatic activity and solubilization by bile acids, can be used as predictors of liposome fate during absorption. To test the tendency of liposomes to exchange materials between various structures, the ITC technique was used. Specifically, the heat flow after mixing vitamin D_3-containing liposomes with liposomes without vitamin D_3 or with albumin was measured. Quantitatively, the structures used in the experiments correspond to the biological context, i.e. 5 mM lipids and 50 mg/mL albumin. Examples of thermograms and the cumulative heat flow for the respective experiments are shown in the supplementary materials (Figures S3-S8). There is no significant difference between heat flow in control experiments and when liposomes containing vitamin D_3 were added to vitamin D_3-free liposome suspension or albumin solution. When the experimental values were fitted to the theoretical model of independent binding sites (enthalpy vs mole ratio between titrant/titrate), there was no interaction between liposomes and liposomes with vitamin D_3 ($\Delta H = 0.20 \pm 0.02$ kJ/mol). Similar results were obtained when albumin was used as a receptor. Values determined using fitting of the theoretical binding model to the experimental data were $\Delta H = 0.45 \pm 0.03$ kJ/mol when liposomes without vitamin D_3 were titrated to albumin suspension, while the respective values when albumin was titrated with liposomes with vitamin D_3 equal to $\Delta H = 0.25 \pm 0.04$ kJ/mol. Based on calorimetric data, it can be concluded that vitamin D_3 remains within the lipid matrix and is unable to cross the unassisted water barrier between the host and acceptor membranes or the host lipid bilayer and albumin. The result is quantitatively similar to that presented elsewhere, where the energy associated with the compound exchange between host and acceptor structures depends on its hydrophobicity and decreases with increasing logP.[42–45] Therefore, these results justify the assumption that the fate of vitamin D_3 in the gastrointestinal tract depends on the fate of the host particulate, as its spontaneous exchange between various biological structures is unlikely.

Molecular dynamic simulations

To better understand molecular-level interactions between vitamin D_3 and the lipid bilayer or triglycerides, molecular dynamics simulations have been performed. First, the incorporation of vitamin D_3 into the lipid bilayer was analyzed. As expected, when in the lipid bilayer, the cholecalciferol molecule positions

itself in such a way that its hydroxyl group is located in the lipid bilayer interface. The high energy cost required to remove the cholecalciferol molecule from the lipid bilayer is in good agreement with other simulations and shows that the exchange of cholecalciferol molecule between lipid bilayers is very unlikely.[46,47] Figure 3, *A* illustrates the hypothetical pathway of cholecalciferol internalization into the lipid bilayer. Figure 3, *B* shows the time course of the internalization process, where cholecalciferol is represented by its center of mass. Even in the presence of an external force, hydrophobic cholecalciferol does not rapidly enter the lipid bilayer. This is likely due to the large conformational changes within the lipid bilayer required to accommodate the bulky cholecalciferol molecule. Furthermore, during the subsequent simulation for another 100 ns, the cholecalciferol molecule does not flip spontaneously to the other side of the lipid bilayer. This indicates that vitamin D_3 spontaneous transfer across the lipid bilayer is unlikely due to the massive conformation changes required in both membrane monolayers.[48,49] Interestingly, when the cholecalciferol incorporation into the DOPC lipid bilayer is completed, the presence of the cholecalciferol molecule does not alter the lipid organization, as evaluated by the bilayer thickness and area per lipid molecule (two parameters frequently used to evaluate the perturbation caused by exogenous molecules[50]). All these lead to the conclusion that cholecalciferol when in the lipid bilayer is not likely to change its location due to the prohibitive energetic barriers required for movement along the membrane normal. The behavior of vitamin D_3 is similar to that of cholesterol.[51]

In another simulation, several cholecalciferol molecules were positioned in the aqueous phase adjacent to the lipid bilayer, as shown in the first image in Figure 4. After 50 ns simulation, no single vitamin D_3 molecules entered the lipid bilayer; they rather formed a stable aggregate in the aqueous phase. To test the behavior of cholecalciferol molecules in the triglyceride matrix, a

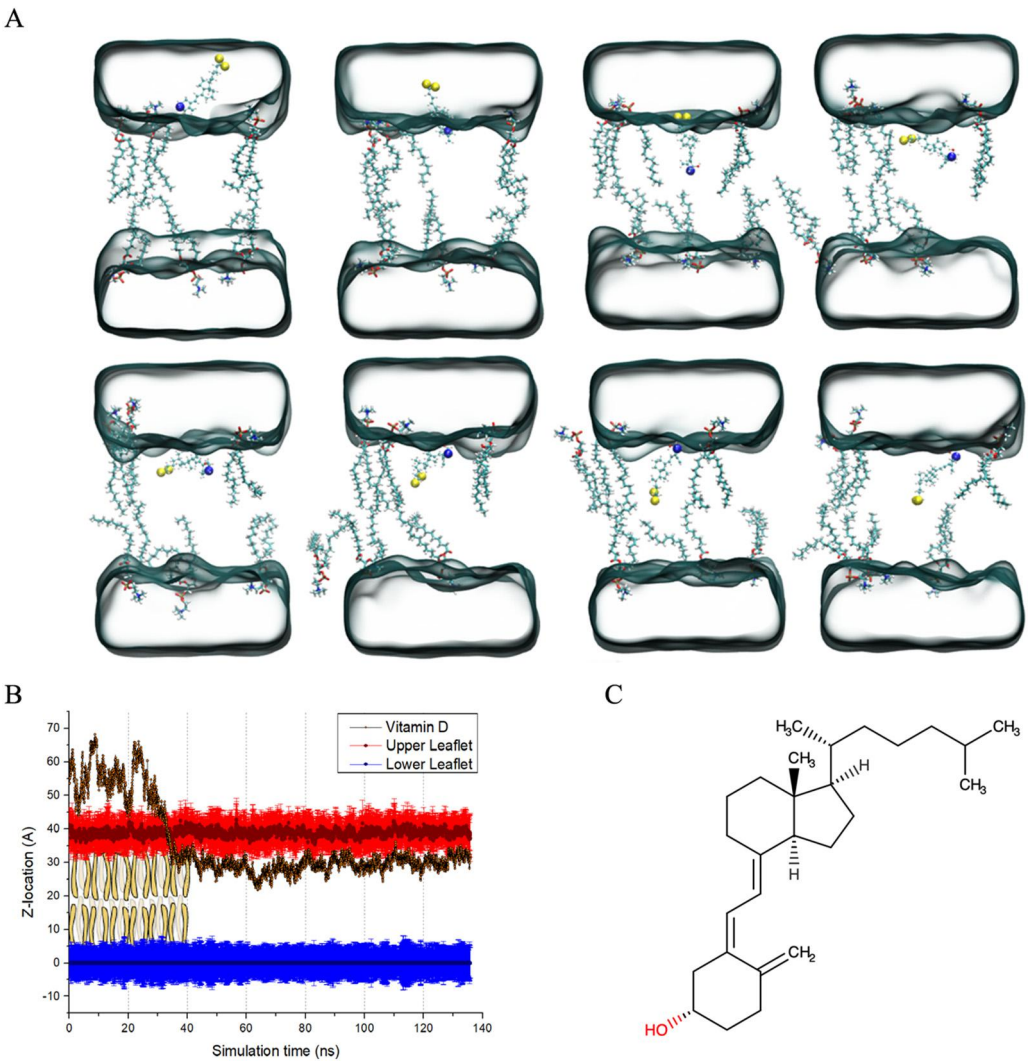

Figure 3. Molecular dynamic simulation of the incorporation pathway of the cholecalciferol molecule into the DOPC bilayer. **(A)** Selected images of a single cholecalciferol molecule forced into the lipid bilayer. The hydroxyl group (blue beads) and methyl groups on the hydrocarbon side chain (yellow beads) are highlighted. The lipid bilayer context is indicated by a few lipid molecules and adjacent aqueous phases. **(B)** The cholecalciferol internalization into the lipid bilayer is shown as the position of its center of mass (brown points) with the respect to the locations of lipid monolayers represented as the mean values of phosphorous atoms positions (red and blue points). **(C)** The cholecalciferol chemical structure with the highlighted hydroxyl group.

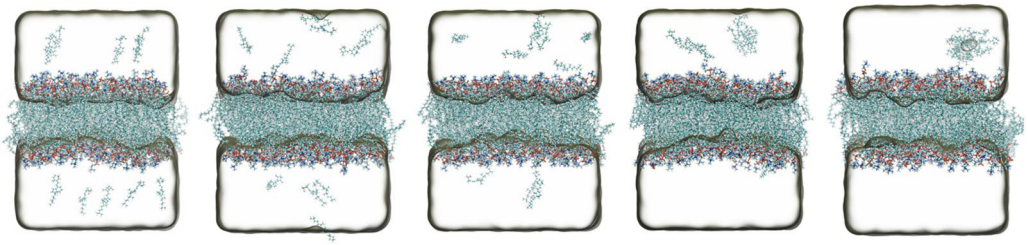

Figure 4. Images from the MD simulation where cholecalciferol molecules were positioned in the aqueous phase in at the vicinity of the lipid bilayer. After 50 ns of simulations, all cholecalciferol molecules formed stable aggregates. Despite several contacts between cholecalciferol molecules and membrane surface throughout the simulation time, not a single event of cholecalciferol molecule incorporation was observed.

stable triglyceride molecular system was constructed followed by the incorporation of 10 cholecalciferol molecules. Throughout the 50 ns simulation time, the system was stable, i.e., no aggregation of cholecalciferol in oil or other redistribution within the oily phase was detected. The results of computer simulations confirm the conclusion derived from the calorimetric data that cholecalciferol would remain within the particulate, in which it is initially incorporated. The digestion of oil involves hydrolysis by lipases and/or emulsification by bile acids. The emulsification process depends on the ratio of bile acids and triglycerides. When the ratio is low, large particles would form. The activity of lipases will produce monoglycerides and free fatty acids, which can reach enterocytes where chylomicrons are formed.[24] In the case of an oily vitamin D_3 formulation, the evolution of the emulsion towards reduced-size particulates will be greatly affected by the dietary fats from a foodstuff, which would reduce the ratio of bile acids and triglycerides, therefore altering (slowing) the emulsification process. In addition, increased volume of substrate will also slow the digestion by lipases. When cholecalciferol is delivered in liposomes, with a size enabling the rapid mucus penetration and absorption assisted by proteins or endocytosis in enterocytes, its absorption should be accelerated and enhanced. Consequently, vitamin D_3 will rapidly enter the lymphatic system, followed by the metabolic transformation in the liver.[52,53] The presented molecular arguments lead to the conclusion that vitamin D_3 delivered in liposomes will result in the appearance of calcidiol in the blood circulation much earlier than when delivered in the oily formulation.

Clinical studies

The prediction that the liposomal formulation of vitamin D_3 will be absorbed in the gastrointestinal tract much faster than the oily formulation was tested in the medical experiment. In the experiment, vitamin D_3 was applied orally using two formulations: oil-containing capsules and liposomes with sizes satisfying physiological requirements, i.e., their diameters are smaller than 300 nm. Size restriction reflects the structure of mucins in mucus and anticipated endocytic processes in enterocytes.[22,54–57] The hypothetical sequence of events for each formulation can be proposed, and the outcome of the experiment predicted. Specifically, triglycerides with cholecalciferol after swallowing will form an ill-defined emulsion. Before entering the intestine, where the fat absorption is taking place, it will interact with foodstuff in the stomach. The inherently unstable oily emulsion can phase separate in the stomach which would increase the time of release to the intestine.[58] In addition, the phase separation will result in the reduced time of exposure of the oily emulsion to enterocytes due to its unequal distribution in the foodstuff. In the intestine, the oily emulsion will be digested and emulsified by the joint action of bile acids and lipases. Due to digestion processes, the emulsion will slowly evolve into a suspension consisting of oil droplets and micelles, both containing vitamin D3. The digestion process will be greatly affected by the foodstuff, which will delay absorption. The ill-defined size of the oily solution and a tendency to coalescence will likely produce a wide range of heterogeneous particles that reduce their effective transport across mucus.[57] Both transendocytosis and receptor-mediated intake of vitamin D_3 require close particulates proximity to the intestine wall.[59,60] Eventually, due to bile acids and the activity of enzymes, a fraction of the oily solution will be reduced to particulates smaller than 300 nm, including micelles capable to pass the mucus barrier and making vitamin D_3 available for internalization.[53,57] The inherent uncertainty of the process and the limited time available for digestion will result in the outcome where only a fraction of vitamin D_3 will be absorbed. Suspension of uniform liposomes containing vitamin D_3 with sizes smaller than 300 nm, on the other hand, is predicted to behave differently. Firstly, the inherent stability of liposome suspension will mix well with the aqueous phase and will be little affected by foodstuff in the stomach. Consequently, liposomes will spread uniformly throughout the whole volume of the stomach content, enhancing the effective exposure time of liposomes for absorption in the intestine. Liposomes are not easily destabilized by low pH or mechanical stress and their topology is preserved even during enzymatic digestion.[31,61] Only a sufficiently high quantity of surface-active substances such as bile salts, which will appear in the intestines, will destabilize the lipid bilayer. The action of detergents will result in much smaller mixed micelle formation, therefore speeding up the vitamin D_3 transfer across the mucus layer even more.[62,63] Consequently, it is expected that cholecalciferol absorption in liposomes will be superior to the oily solution with respect to the onset time of absorption.

To test this assumption, a cross-over clinical experiment has been performed. Healthy volunteers consumed an equal amount of vitamin D_3 preceded by 12 h fasting. This will reduce somewhat the effect of gastrointestinal track content. The clinical experiment consisted of two stages, separated by three weeks, where two groups of volunteers received an oily or liposomal

formulation of vitamin D_3, as schematically presented in Figure 1. The blood of each volunteer was drawn to determine cholecalciferol metabolites. The sampling time points were selected in such a way that the early events of absorption, "onset time", will be measured (up to 5 h after supplementation).[64] All experimental data were treated equally regardless of the $25(OH)D_3$ patient status (an indicator commonly used for the diagnosis of vitamin D3 deficiency) before supplementation. To measure the $25(OH)D_3$ rise after supplementation, the following quantifier was devised. The initial quantity of $25(OH)D_3$ was subtracted from its values for samples collected at later time points and normalized to the initial value $[(A(t) - A_0)/A_0]$ (Figure 5). Next, the tendency of $25(OH)D_3$ change was approximated with the linear function using the least square fitting. The average values of slopes were equal to 7.03×10^{-5} 1/min ± 3.02×10^{-4} 1/min and 4.01×10^{-4} 1/min ± 7.12×10^{-4} 1/min for oily and liposome formulations, respectively. The difference is statistically significant, as evaluated with the null hypothesis, and shows that on average the liposome formulation delivers vitamin D_3 within hours of supplementation, whereas within the same time interval the oily formulation does not produce any effect. This indicates that "the onset time" required to elevate the $25(OH)D_3$ concentration in serum is shorter for the liposomal formulation and may reflect the existence of different and slower absorption pathways for the oily formulation. Figure 5 shows the dependence of the rate of blood concentration of $25(OH)D_3$ increase versus the initial $25(OH)D_3$ concentration in the serum as determined for each person. The rate of $25(OH)D_3$ rise following the supplementation increases with decreasing initial $25(OH)D_3$ concentration in the patient's serum. When the trend for each formulation is quantitated with linear regression, the following quantitatively data evaluation can be offered. The slope (expressed as $tg\alpha$, where α is the angle between the derived linear approximation and the ordinate axis) calculated for the oily solution equals to 2×10^{-7} 1/(min*ng/L), whereas the value for the liposomal formulation equals to -5×10^{-5} 1/(min*ng/L). This result demonstrates that the liposomal formulation might be superior (two orders of magnitude) to the oily solution, considering the ability to rapidly deliver vitamin D_3 to patients with

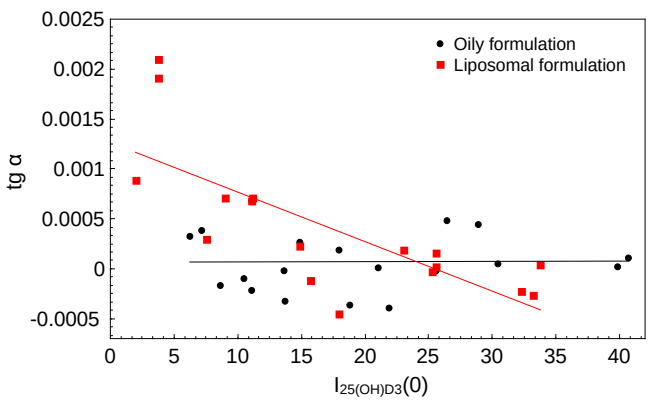

Figure 5. The dependence of the rate of $25(OH)D_3$ increase in plasma on its initial value, prior to the supplementation with vitamin D_3 in oily (black) and liposomal (red) formulations. The trend lines for each formulation are indicated.

significant deficiencies. The observation requires further clinical evaluation before conclusion.

Continuous efforts are made to evaluate the bioavailability and pharmacokinetics of active substances as early as possible during the drug development process.[65] The introduction of targeted drug delivery systems opens new possibilities to modify the absorption and/or biodistribution of active substances without the need for its chemical modifications.[19,66,67] The effectiveness of the approach is elegantly demonstrated by the example of DOXIL™. In this case, the structural features of the formulation are designed to achieve passive targeting, based on the structural properties of solid tumors and interaction with proteins in the circulation (long circulation time).[68] Since then, a variety of delivery systems have been developed to improve an active substance solubility, enhance its affinity to target cells, reduce metabolic elimination, or achieve accumulation in specific locations within the body.[69–71] As shown in the paper, a similar approach can be employed to enhance the bioavailability of the orally delivered compounds which are not considered as drugs.[4,36] The spatial and topological limitations imposed by the digestive processes can be implemented in the design of a delivery vehicle for highly hydrophobic compounds to improve their performance in vivo.

Discussion

When designing an effective oral vitamin D_3 formulation, two qualitatively different processes need to be considered, absorption and redistribution. While absorption is greatly affected by vitamin D_3 formulation, redistribution is not. After internalization, transport and metabolic processes determine the quantity of vitamin D_3 and its metabolites redistributed throughout the body. In short, its active form is generated after its metabolic transformation in the liver to calcidiol ($25(OH)D_3$) by CYP2R1 followed by transformation to calcitriol ($1a,25(OH)2D_3$) by CYP27B1 in the kidney or other extra-renal sites.[66,72] The spatial distancing of locations critical for vitamin D_3 metabolism requires effective distribution mechanisms. The transport of highly hydrophobic compounds is complex by nature. It requires the participation of proteins or supramolecular carriers for the transfer of hydrophobic compounds between tissues[72] and membrane receptors for its internalization by cells.[8] Similar mechanisms are expected when vitamin D_3 absorption from foodstuff in the gastrointestinal tract is considered.[8,73]

The development of an effective formulation for a highly hydrophobic compound (logP >5) requires consideration of both the compound properties and the physiological barriers it encounters. The high hydrophobicity makes its absorption via passive transport or membrane proteins assisted transport difficult. The cholecalciferol can be suspended in the aqueous phase only in the form of crystals or in emulsions, where it is dissolved in nonpolar solvent (oil, micelles or liposomes[25]). Consequently, cholecalciferol will remain in the nonpolar solvent during the entire digestion process or will precipitate. Therefore, its absorption will depend mainly on the properties of particulates and their fate during the digestion process.[19,23,52]

The hydrophobic compound such as vitamin D_3 can be delivered in three forms: as crystals, oily solution or preformed

particulates in the aqueous suspension. Its low solubility in an aqueous phase makes crystal form impractical since its absorption is expected to be negligible.[25] When hydrophobic vitamin D_3 is dissolved in oil (triglycerides), during oral administration, the suspension of particulates with broad size distribution would likely form.[36,73,74] Such suspension, with its natural tendency to coalescence, may phase separate leading to the delayed release from the stomach and possible interference with the foodstuff.[75–77] The subsequent exposure to bile leads to the formation of a heterogeneous oily emulsion. The fate of the emulsion depends predominantly on three factors; the relative quantity of fats and bile acids, the composition of bile (amphiphiles and lipases are relevant in this case), properties of triglycerides, such as length and saturation of fatty acids or surface charge, and time.[25,53,73] The time available for emulsification and/or absorption of particulates containing vitamin D_3 in the small intestine depends on the peristaltic activity and the particulate propensity to reach the intestine wall.[20] Due to the presence of mucus, only particulates of certain sizes will reach the intestine wall where enterocytes or M cells are located. These cells would incorporate micelles composed of phospholipids, bile acids, vitamin D_3, and perhaps other particulates as well.[38,53,67,78] Based on the available literature data, only particulates smaller than 300 nm can pass the mucus barrier.[18,19,23,79] The other factor that needs to be considered is the enzymatic hydrolysis of the oily solution, which will alter the vitamin D_3/oil ratio. Since vitamin D_3 solubility in oil is limited, the progression of hydrolysis may cause vitamin precipitation, making its absorption unlikely.[75] Based on the abovementioned facts, one can propose the fate of the oily formulation of vitamin D_3 in the gastrointestinal tract. Specifically, the oil with vitamin D_3 will form a heterogeneous emulsion with particulate sizes ranging from submicron up to hundreds of microns in diameter.[80] The emulsion being inherently unstable would tend to form a separate phase in the stomach, delaying its transfer to the intestine, hence slowing and/or limiting the absorption. A similar analysis may provide functional and topological requirements for the effective vitamin D_3 delivery vehicle. Specifically, the vehicle in the form of preformed suspension of stable particulates with well-defined sizes should be beneficial. Liposomes satisfy all these requirements. They can be prepared in such a way that the stable suspension of small vesicles can be formed.[81] The technologically available sizes of liposomes match the physiological requirements imposed by mucus and endocytic processes. They also do not coalesce, so their size in the gastrointestinal tract will not increase. More importantly, the action of bile will rather reduce their sizes, therefore further accelerating their absorption. Consequently, it can be predicted that liposomes with vitamin D_3 will be internalized without delay after entering the intestine. In summary, the active hydrophobic compound will stay in the gastrointestinal tract in two forms: as a precipitate or solubilized in nonpolar solvent, i.e., fats.[27] It can be absorbed only when it is dissolved in the nonpolar phase, which prevents its precipitation. In such a case, the digestion process can be considered as the evolution of the two-phase system, oil/water emulsion or liposome suspension. The resulting particulates are altered by mechanical agitation, enzymatic transformation, and modification by detergents (bile acids). The detailed analysis presented in the paper leads to the conclusion that to achieve efficient absorption of hydrophobic compounds such as vitamin D_3, the preformed, stable in time, particulate suspension such as liposomes with a well-defined size distribution should be used. The preliminary clinical experiment shows that while the liposomal vitamin D_3 formulation delivered orally elevates rapidly calcidiol concentration in the serum, no such effect was observed when the oily solution was used. In addition, the clinical experiment shows for the first time that vitamin D_3 intake may depend on the patient status with respect to the vitamin D_3 level.

Author Contributions

Paulina Dałek: methodology, formal analysis, investigation, resources, writing – review & editing, visualization. Dominik Drabik: software, formal analysis, data curation. Halina Wołczańska: methodology, validation, formal analysis, investigation. Aleksander Foryś: formal analysis, investigation. Małgorzata Jagas: conceptualization, resources, investigation. Natalia Jędruchniewicz: conceptualization, resources, investigation. Wojciech Witkiewicz: conceptualization, supervision, funding acquisition. Magdalena Przybyło: conceptualization, resources, investigation, funding acquisition. Marek Langner: conceptualization, writing – original draft, supervision, project administration.

Funding

This work was supported by the Faculty of Biomedical Engineering at Wrocław University of Sciences and Technology and by the National Centre for Research and Development [grant number POIR.04.01.04-00-0159/17].

Conflict of interest

ML and MP are partners in Lipid Systems Sp. z o.o. PD and HW are employed by Lipid Systems Sp. z o.o.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2022.102552.

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