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Molecular interactions between Vitamin B12 and membrane models: A biophysical study for new insights into the bioavailability of Vitamin

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ABSTRACT

Vitamin B12 (VB12) deficiency is one of the most common malnutrition problems worldwide and is related to its poor bioavailability. The lipid composition of cell membranes and molecule-cell membrane lipid interactions are major factors affecting the bioavailability of nutrients. So, the study of these interactions may allow predicting the behavior of VB12 at cellular membranes and the effects on its activity. Thus, lipid vesicles with lipid composition similar to the majority of eukaryotic cell membranes were used as biomembrane models, and their interactions with VB12 molecules were evaluated. For that, different parameters were assessed such as the lipophilicity of VB12, its preferential location in the membrane and its effect on the physical properties of the bilayer. VB12 showed high affinity for the biological membranes, not inducing any biophysical changes in their properties. The interactions of VB12 with the membrane was affected by the complexity of the bilayer, since its increase in order and rigidity hinders the diffusion of molecules. Thus, the low bioavailability of VB12 is not related with its interactions with the biological membranes.

1. Introduction

Vitamin B12 (VB12) is a cobalt-containing compound involved in several cellular processes (Fig. 1). This vitamin acts as a cofactor for two enzymes, methionine synthase and methylmalonylCoA mutase, being involved in protein synthesis and cell proliferation processes. Therefore, VB12 deficiency is associated with several health complications such as megaloblastic anemia, neurological problems and cognitive impairment [1]. Humans require dietary sources of VB12, which are almost exclusively from animal sources, because we are not able to synthesize it. However, VB12 poor bioavailability limits its biological activity. Despite a balanced diet, with sufficient consumption of this vitamin, the human body is only able to use an average of 50 % of the ingested VB12. Which leads to about 15 % of the population suffering from VB12 deficiency [2].

The absorption of VB12 is a complex and multistep process that depends on its binding to different proteins. Different VB12-binding proteins mediate its transport, such as haptocorrin (HC) in the upper gastrointestinal tract, intrinsic factor (IF) in the terminal ileum and trascobalamin (TC) in the blood stream. The uptake of VB12 occurs by receptor-mediated endocytosis at two different stages. First, the IF-VB12 complex is internalized by the epithelial intestinal cells to enter the bloodstream, and then the TC-VB12 complex is uptaken in the target cells to VB12 perform its intracellular biological activity. So, in both cellular internalization processes, the protein-VB12 complex has to bind to a cell surface receptor for the endocytosis occurs [3]. Two receptors are involved in the cellular uptake of VB12, the cubilin receptor for the IF-VB12 complex [4], and the TCblR/CD320 receptor for the uptake of the TC-VB12 complex [5]. It is well established that the low bioavailability of compounds is one of the major causes of loss of biological activity [6]. Thus, as the bioavailability and biological activity of VB12 both depend on its efficient transport across cellular membranes, it is essential to study the interactions of VB12 with cell membranes.

The physical features of membranes, as order, packing, fluidity and structure, influence the transport of molecules, as other cellular processes. Until recently, membrane lipids were portrayed as passive components of the membrane mainly being responsible for the membrane structural integrity. However, now is firmly established that these lipids have also a major role in numerous functions of the membrane, as for instance transport pathways [7]. In fact, the membrane lipids play a major role with the ligand-receptor interaction regulating the endocytic

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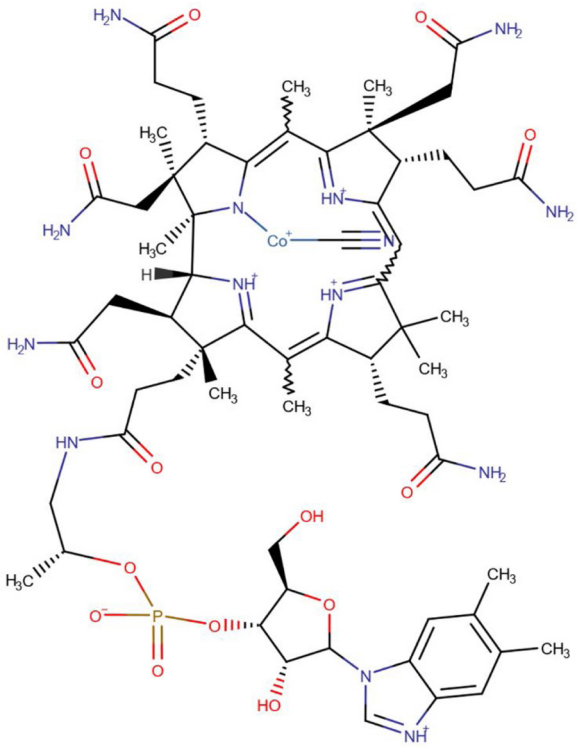

Fig. 1. Chemical structure of vitamin B12 (VB12).

function, since lipids can influence membrane receptors at a geometric/steric level and therefore affect the transport dynamics of molecules [8]. Thus, biomimetic lipid membrane models can be used to understand the role of membrane lipids in the transport of VB12.

Lipid biomimetic models are suitable *in vitro* tools to study the membrane properties and its interactions with external molecules. These models mimic the cell membranes in physiological conditions. Different models can be used such as lipid monolayers [9] and bilayers [10]. Lipid bilayers, such as liposomes/lipid vesicles, are the ones that best mimic the entire lipid assembly of cellular membranes [11]. Lipid vesicles as biomembrane models allow studying the lipophilicity/hydrophilicity of compounds, by determining their partition coefficient (*Kp*). Other biophysical parameters can also be evaluated using lipid vesicles as cell models such as the compound's location within the membrane, and its effect on the fluidity of the bilayer [12]. These models present an advantage over classical and computational models since they allow mimicking different levels of membrane complexity, as well as membrane curvature, ion-discrimination, osmotic swelling, different types of interactions between molecules and responses to external molecules [13].

So, in this work the interactions between the vitamin and membrane were studied to better understand the role of the biophysical properties of the membrane in the cellular uptake of VB12. These studies will also allow to assess if an eventual poor interaction of VB12 with cell membranes may be one of the reasons that leads to the deficiency of this vitamin. For that, lipid vesicles with a composition similar to biological cell membranes were used. The phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was selected for the composition of the models because phosphatidylcholine (PC) is the major phospholipid found in human cell membranes [14]. To evaluate the effect of the membrane complexity on the VB12 interactions, lipid vesicles with different degrees of complexity were proposed. Thus, lipid vesicles containing only DMPC, lipid vesicles containing DMPC and cholesterol (chol), and finally lipid vesicles composed of DMPC, chol and sphingomyelin (SM) were prepared. Cell membranes exhibit combinations of phospholipids, chol, SM and proteins organized in relatively ordered microdomains known as lipid rafts. These microdomains form functional sites for several cellular functions, such as endocytosis [15]. Hence, the addition of chol and SM to the model was used to evaluate their influence on VB12-membrane interactions

No works were reported so far for the study of VB12-membrane interactions. The aim of the present work was to evaluate the interactions between the VB12 molecules and biophysical membrane models, focusing in the lipid components of the membrane. The partition of VB12 and its preferred location within the lipid bilayer, as well as its effects on the properties of the lipid membrane such as its fluidity, were studied.

2. Materials and methods

2.1. Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC, MW 677.9), sphingomyelin (SM, MW 703.03) and cholesterol (chol, MW 386.65) were purchased from Avanti Polar Lipids (Alabama, USA). Vitamin B12 (VB12, MW 1355.38, purity ≥98 %), 1,6-diphenyl-1,3,5-1,3,5-hexatriene (DPH, MW 232.32), *N*, *N*, *N*-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenyl ammonium *p*-toluenesulfonate (TMA-DPH, MW 461.60), methanol, chloroform and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (Germany).

2.2. Preparation of lipid vesicles as membrane models

The lipid film hydration method was used for the preparation of lipid vesicles as membrane models. Vesicles with different lipid composition were synthetized at a concentration of 5 mM. For the preparation of the simpler model, only DMPC phospholipid was used. Then, two more complex models were prepared, one containing DMPC and chol (molar ratio of 85:15) and other containing DMPC, chol and SM (molar ratio 75:15:10). A lipid film was prepared by evaporating the chloroform solution of the lipid mixture, and then hydrated. PBS (0.01 M, pH 7.4) was used as the aqueous phase for lipid film hydration. After 10 min of agitation, the lipid vesicles were submitted to ultrasounds for 15 min (45 kHz, Ultrasonic cleaner, VWR, Malasya) and an extrusion step. Both steps were performed above the lipids phase transition temperature (T_m). The extrusion step, with a membrane filter with 100 nm pore size, allowed to obtain unilamellar vesicles.

2.3. Derivative UV–vis spectrophotometry

The partition coefficients (*Kp*) of VB12 between the different lipid vesicles and the aqueous phase were determined using the derivative UV–vis spectrophotometry. For this experiment, DMPC, DMPC:chol and DMPC:chol:SM vesicles were used as the lipid phase and PBS (pH 7.4) was used as the aqueous phase. VB12 dissolved in PBS at 150 μM was incubated in a 96-well plate, during 30 min with medium agitation at 37 °C, with the previously prepared lipid vesicles suspensions at concentrations ranging from 0 to 4000 μM. This incubation step at physiological temperature is essential to achieve the equilibrium of the partitioned vitamin molecules between the water and the lipid membrane phases. Lipid vesicles at the same concentrations range without incubation with VB12 were used as controls. A microplate reader (Synergy 2 Microplate Reader, BioTek, UK) was used to obtain the absorption spectra (ranging from 200 to 450 nm) of all samples and controls at 37 °C.

For the treatment of the obtained experimental data, a corrected spectrum for each sample was obtained by subtracting to it the correspondent control spectrum. Then the second derivative was applied to each corrected spectrum to eliminate the background signal of the lipids. A graph of the second-derivative data against the lipid concentration was plotted at the wavelength where the lipid noise is insignificant. The *Kp* value was then calculated by a non-linear regression

by fitting the following equation [16]:

$$D_T = D_W + \frac{(D_m - D_W)\, K_P\, [L]\, V_m}{1 + K_P\, [L]\, V_m} \quad (1)$$

D is the second-derivative of the absorbance of: the amount of total used vitamin (D_T), amount of vitamin found in the lipid (D_m), and in the aqueous (D_w) phases. *[L]* is the molar concentration of the lipid and V_m is lipid molar volume. The V_m values for DMPC, DMPC:chol and DMPC:chol:SM are 0.6630, 0.6186 and 0.6195 $L.mol^{-1}$, respectively.

2.4. Fluorescence quenching studies

The preferential localization of VB12 within the lipid membrane was evaluated by fluorescence quenching studies. For this study, two fluorescent molecules, DPH and TMA-DPH, with known locations were used to label the lipid vesicles. Thus, for this experiment, 500 μM of labelled DMPC, DMPC:chol and DMPC:chol:SM vesicles were used as models. Labelled vesicles were prepared using the same experimental procedure used for the non-labelled ones. But for these experiments, DPH and TMA-DPH probes were added to the lipid mixture, at a probe:lipid molar ratio of 1:100, before the preparation of the dried lipid film.

Then, VB12 dissolved in PBS (pH 7.4) was added at increasing concentrations (0–150 μM) to the previously prepared probe-labelled vesicles. The samples were agitated for 30 min protected from light at 37 °C to mimic the physiological temperature and to achieve the equilibrium of the partitioned vitamin molecules between the water and the lipid membrane phases. Measurements were also conducted at physiological temperature (37 OC) using a microplate reader (Synergy 2 Microplate Reader, BioTek, UK). The used excitation and emission filters were 360/40 nm and 420/50 nm, respectively [17]. A graph of the obtained experimental data was plotted as the intensity of fluorescence against the quencher concentration ($[Q]_m$). For the determination of $[Q]_m$, that is the concentration of the vitamin that effectively partitioned into the lipid phase, the following equation was used [17]:

$$[Q]_m = \frac{K_P\, [Q]_T}{(K_P\, \alpha_m) + (1 - \alpha_m)} \quad (2)$$

Q_T is the total concentration of used vitamin, and α_m is the volume fraction of the lipid membrane that is obtained by the following equation:

$$\alpha_m = V_m / V_T \quad (3)$$

V_m and V_T are the volumes of the lipid membrane and aqueous phases, respectively.

For the determination of the Stern-Volmer constant (K_{SV}), an important indicator of the quenching efficiency, a linear regression was applied fitting the following equation:

$$\frac{I_0}{I} = 1 + K_{SV}\, [Q]_m \quad (4)$$

2.5. Dynamic light scattering studies

Dynamic light scattering (DLS) was chosen to evaluate VB12's effect on the fluidity of the biomembrane models. Changes in the main phase transition temperature (T_m) suggest major alterations in the lipid membrane properties, as its fluidity. So, the effect of VB12 on the fluidity of the lipid bilayer in the biomimetic models was evaluated by the determination of the T_m values. The determination of this biophysical parameter was achieved by assessing the variation of the mean count rate (average photons/second) with variation of temperature. Variations in the count rate occur because the lipid bilayer suffers major changes in its biophysical properties, due to transitions between the gel to the fluid phase, or from the fluid to more rigid phase [18]. For this experiment, DMPC, DMPC:chol and DMPC:chol:SM vesicles were used as models at a concentration of 4000 μM. To the previously prepared lipid vesicles was added VB12 dissolved in PBS (pH 7.4) at a concentration of 150 μM, and the samples were then agitated for 30 min at 37 °C. After the incubation period that allowed achieving the equilibrium of the partitioned vitamin molecules between the water and the lipid membrane phases, the samples were analyzed by DLS. Lipid vesicles without addition of VB12 were used as control. For the measurements, a Zetasizer Nano ZS (Malvern Instruments, UK) was used at a temperature range from 10.0 to 40.0 °C. Then, the experimental data was plotted as the normalized mean count rate against the temperature. A non-linear regression was used for the determination of T_m values by fitting the following equation [19]:

$$y = A_i + \frac{A_f - A_i}{1 + 10^{B(\frac{1}{T} - \frac{1}{Tm})}} \quad (5)$$

T is the temperature, B is the lipid cooperativity, and A_i are A_f are the initial and the final mean count rate of the vesicles, respectively.

2.6. Statistical analysis

All the experiments were conducted in triplicate and the obtained results are expressed as mean and standard deviation (SD). Graphpad Prism software (GraphPad Inc.) was used for the statistical analysis of the experimental data. Student's *t*-test was applied for statistical significance and a cut-off value of 0.05 was used for a 95 % confidence interval.

3. Results and discussions

3.1. Lipophilicity of Vitamin B12

Lipids vesicles were used as biophysical membrane models to study the lipophilicity of VB12. Lipid models are useful tools to predict the biological activity of molecules, since the lipophilicity influences their affinity for biological membranes, absorption, distribution, metabolism, elimination and toxicology. Lipophilicity is often expressed as the partition coefficient (*Kp*) or as its logarithm value (log *P*). For ionized molecular species, as VB12, the distribution coefficient (log *D*) is often used.

Thus, derivative spectrophotometry was used for the determination of *Kp* and log *D* values. Although, numerous techniques can be used to assess the lipophilicity of molecules, derivative spectrophotometry offers an advantage over conventional methods. Most implicate the physical separation of the two phases to determine the quantity of molecule in each phase after partition. Since methods for physical separation are time-consuming and usually poorly reproducible, methodologies such as UV–vis spectrophotometry, without need for phase separation, are useful. The partition of the vitamin molecules from one phase to the other leads to modifications in its absorbance spectrum, allowing to quantify the vitamin in each phase. Although UV–vis spectrophotometry is a suitable method to calculate the *Kp* values, derivative spectrophotometry is a superior approach allowing to eliminate the background signal created by the light scattered from the lipid vesicles. This background noise can be removed to improve the signal resolution by using the second-derivative [16].

For that, lipid membrane models with different composition were used, DMPC, DMPC:chol and DMPC:chol:SM vesicles, respectively. Fig. 2 is a schematic representation of the steps sequence to determine the *Kp* values for VB12 in the DMPC model. The absorption spectra of VB12 with increasing concentrations of lipid vesicles were obtained and are shown in Fig. 2A. In Fig. 2B is presented the second-derivative of the absorption spectra. The isosbestic points, indicated by black arrows, show that the background noise generated by the lipid vesicles was efficiently removed by the use of the second-derivative [16]. The maximum wavelength, 386 nm, between the two isosbestic points was

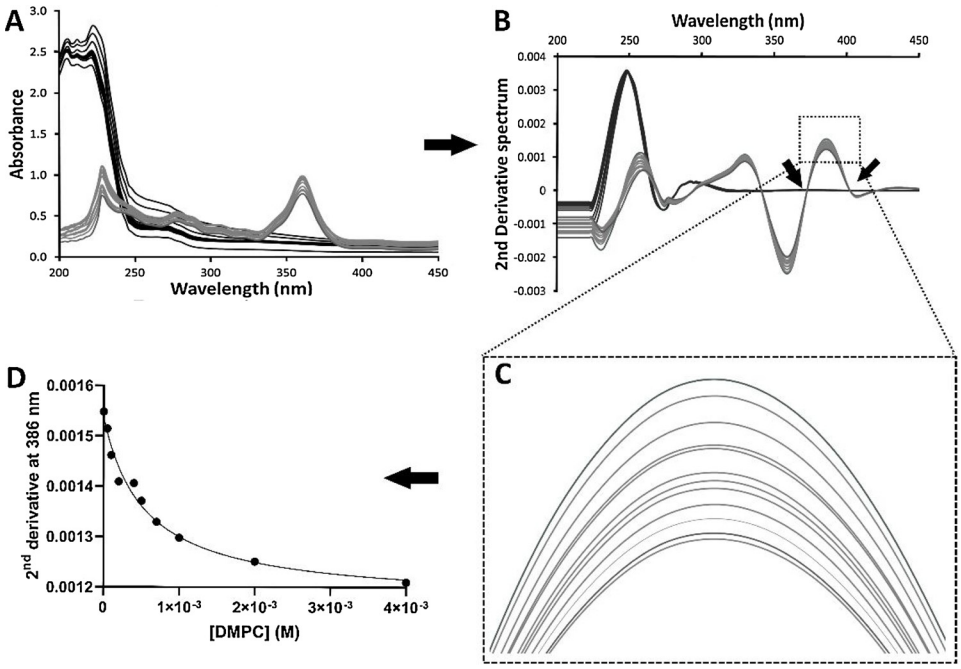

Fig. 2. Schematic representation of the process to obtain *Kp* values. (A) Absorption and (B) second-derivative spectra of VB12 at a concentration of 150 μM with DMPC vesicles with increasing lipid concentrations at 37 °C. Black lines represent DMPC vesicles without vitamin, and grey lines represent DMPC vesicles incubated with vitamin respectively. Isosbestic points are indicated by black arrows. (C) Zoom of the second-derivative spectrum at 386 nm where the scattering is eliminated. (D) The *Kp* value was obtained by a non-linear regression (Eq. 1) of the data at 386 nm.

chosen, to plot the graph in Fig. 2D. The *Kp* values were obtained by fitting the Eq. 1 to the plotted data. The *Kp* values of VB12 with the two other lipid models were determined by applying the same steps sequence.

The obtained *Kp* values of VB12, as their corresponding log *D*, are shown in Table 1. For comparison with experimental results, the MarvinSketch software (Chemaxon, Hungary) predicted the theoretical octanol/buffer log *P*. The obtained log *D* values of VB12 for all the used lipid vesicles were significantly different from the predicted log *P* value (−3.24), as shown in Table 1.

This difference can be explained since the standard two-phase system of octanol and water only acknowledges the hydrophobic interactions, while these biophysical models also consider the electrostatic and ion-dipole forces between the VB12 and the phospholipids [20]. The Marvin Sketch Calculator software also predicted that, at physiological pH (7.4), about 99 % of the VB12 molecules have positive charge. This explains the much higher experimental log *D* values when comparing with theoretical log *P* value (-3.24), because the partition of the vitamin occurs not only due to the hydrophobic forces, but also due to the ion-dipole and electrostatic interactions between the polar heads of the phospholipids and the cationic species of the vitamin. The attained results suggest that the state of ionization of the vitamin controls its distribution between the lipid membrane and the aqueous phase.

The obtained *Kp* values also suggest that the partition of the vitamin depends on the lipid composition of the biophysical models. As shown in Table 1, K*p* and log *D* values are significantly different for the three used vesicles ($p < 0.05$). *Kp* and log *D* values for VB12 significantly reduced with the increasing complexity of the model, exhibiting a higher partition for DMPC vesicles, followed by DMPC:chol and then DMPC:chol:SM vesicles. This occurs because the addition of chol and SM molecules increases the rigidity and compactness of the lipid membrane. Chol influences the phase behavior of lipid bilayers, enhancing the rigidity and order at the region where the chol molecules contact with the hydrophobic lipid tails [34]. Thus, DMPC:chol vesicles present a more packed and organized structure than DMPC vesicles, slowing the diffusion of the vitamin molecules into the bilayer. Also, the addition of SM increases the thickness, organization and packing of the lipid membrane due to the assembly of liquid-ordered SM-chol domains [21]. Thus, DMPC:chol:SM vesicles present a more packed and organized structure than DMPC:chol vesicles.

Table 1
Partition coefficient (Kp) and respective distribution coefficient (log D) values of VB12 between the lipid vesicles and the aqueous phase (PBS, pH 7.4). The results are expressed as mean ± SD (n = 3).

	DMPC	DMPC:chol	DMPC:chol:SM
K_P	2796 ± 69	2084 ± 113	1488 ± 54
log *D*	3.45 ± 0.01	3.32 ± 0.02	3.17 ± 0.02

3.2. Preferential location of Vitamin B12 in the membrane

Steady-state fluorescence quenching assay was used to determine the preferential location of VB12 molecules within the lipid models. Quenching refers to the reduction in the florescence intensity of a fluorophore, induced by molecular interactions with a molecule that acts as a quencher. Therefore the quenching processes requires molecular contact between the quencher and the probe molecules [22]. For the experiments, DMPC, DMPC:Chol and DMPC:Chol:SM lipid vesicles were used as membrane models, and DPH and TMA-DPH were used as fluorescent probes. Since the position of these probes within the bilayers is well described, it is possible to assess the position of the vitamin molecules. While TMA-DPH molecules are situated next to the polar heads of the phospholipids, DPH is found deeply in the bilayer intercalated with the acyl chains of the phospholipids. Stern-Volmer graphics with increasing concentrations of vitamin were drawn as shown in Fig. 3 (Eq. 4). The Stern-Volmer constant (K_{SV}) values are given by the slope of the linear fitting and are present in Table 2. These are important indicators of the quenching efficiency, and high K_{SV} values indicate a great quenching effect. This is because, as the distance between the vitamin molecule and the probes decreases, the fluorescence intensity also decreases, yielding higher K_{SV} values [23].

K_{SV} values for DPH are significantly higher than for TMA-DPH in all the studied vesicles ($p < 0.05$). As proved by these Stern-Volmer constants, the quenching effect was stronger for DPH probe in all the lipid vesicles, suggesting that the vitamin is preferentially located deeply within the bilayer.However, the obtained K_{SV} values for TMA-DPH indicate that VB12 can also be found near to the hydrophilic region of the phospholipid.

As also shown in Table 2, the K_{SV} values for both probes decreased

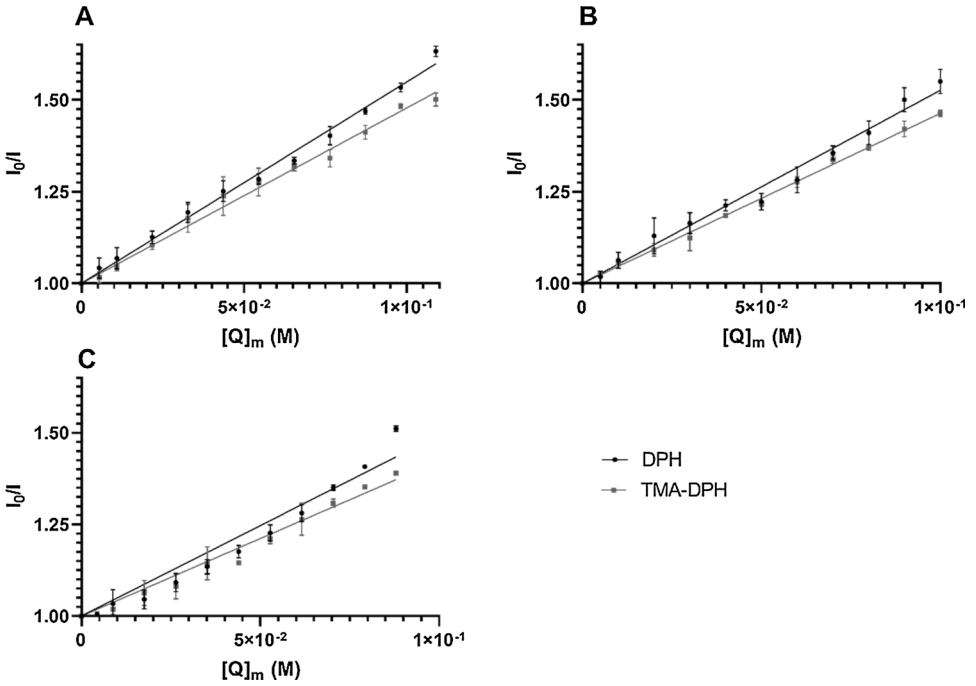

Fig. 3. Stern-Volmer graphs at pH 7.4, 37 °C for the probes DPH and TMA-DPH in (A) DMPC, (B) DMPC:chol and (C) DMPC:chol:SM vesicles by increasing concentrations of VB12. Data expressed as mean ± SD (n = 3).

Table 2
Stern-Volmer constants (K_{SV}) values of VB12 for the used lipid vesicles. The results are expressed as mean ± SD (n = 3).

	K_{SV} (M^{-1})		
Model	**DMPC**	**DMPC:chol**	**DMPC:chol:SM**
DPH	5.4 ± 0.3	5.3 ± 0.1	4.9 ± 0.1
TMA-DPH	4.71 ± 0.08	4.6 ± 0.1	4.2 ± 0.2

with the increasing of complexity of the membrane model, suggesting that the quenching of the probes is influenced by the bilayers' fluidity and packing/organization. Indeed, the K_{SV} values for DMPC:chol:SM significantly decreased when comparing to the K_{SV} values for DMPC vesicles with both probes ($p < 0.05$). Since chol-SM domains increase the packing and decrease the fluidity of membrane, the diffusion of the vitamin through the bilayer is more difficult, as previously mentioned. These values result from a decreased probe-vitamin interaction leading to less fluorescence quenching of both probes.

The quenching of fluorescence can be attributed to different mechanisms, such as collisional/dynamic and static quenching. In static quenching, the probe and quencher molecules form a complex in the ground state (before excitation), and in dynamic quenching the fluorescence deactivation occurs due to diffusive encounters when the quencher molecules collide with the probe. The observed linear Stern–Volmer plot indicates that the fluorescence deactivation by VB12 occurs due to a collisional process, *i.e.* dynamic quenching. Non-linear Stern Volmer plots are usually associated to a combination of both dynamic and static quenching [24].

3.3. Effect of Vitamin B12 on the membrane fluidity

The effect of VB12 on the fluidity of the membrane models was evaluated by DLS analysis. Interactions between a molecule and the phospholipid vesicles can disturb the characteristics and the physical state of the membrane. The conformation and organization of the lipid bilayer depend on its physical state. At different temperatures, the lipid bilayer can be either in a liquid–crystalline or a solid-gel state. In the liquid phase, the phospholipids diffuse more freely due to a more disordered assembly. T_m is the temperature at which the transition between the solid-gel and the liquid disordered states occur, and it is influenced by the arrangement and fluidity of the membranes [25]. Since changes in the fluidity of the bilayers usually lead to variations in T_m, the effect of VB12 molecules on the membrane fluidity was evaluated in terms of modifications in the T_m values.

For the determination of T_m of lipid vesicles with or without VB12, the normalized mean count rate was plotted against the temperature (Fig. 3). The mean size of the lipid vesicles remained constant with the increase of temperature, securing the validity of this method (data not shown).

T_m values were obtained by a non-linear regression (Eq. 5) and the obtained values are shown in Table 3. T_m for DMPC model is 23.5 ± 0.1, within the reported range [26]. As indicated by the T_m values for the three models (without vitamin), the fluidity of the membrane is significantly altered with the membrane composition ($p < 0.05$). In fact, the addition of chol molecules to the model, significantly decreased the T_m values from 23.5 ± 0.1 to 20.0 ± 0.2 °C ($p < 0.05$), since it enhanced the packing and ordering of the bilayer, leading to a more rigid membrane. Inserting SM molecules in the lipid model, also significantly change the T_m value to 21.2 ± 0.1 °C ($p < 0.05$). This increase may be explained by the main transition phase temperature of SM, near 37 °C, being higher compared to DMPC and chol model [27]. This can introduce lateral heterogeneity by creating domains in the bilayer, leading to changes in the T_m of the membrane model.

The insertion of chol and SM molecules also affected the thermal

Table 3
Phase transition temperature (T_m) values of the lipid vesicles with and without VB12. The aqueous phase is PBS (0.01 M, pH 7.4). The results are expressed as mean ± SD (n = 3).

	T_m (°C)		
	DMPC	**DMPC:chol**	**DMPC:chol:SM**
–	23.5 ± 0.1	20.0 ± 0.2	21.2 ± 0.1
VB12	23.7 ± 0.4	20.3 ± 0.9	21.1 ± 0.2

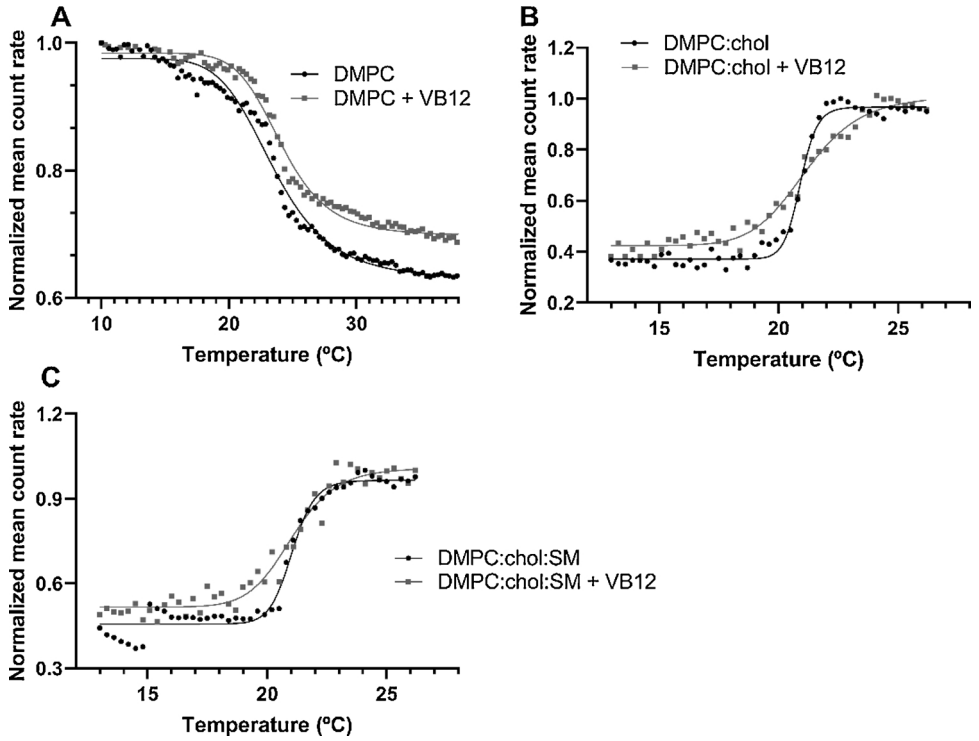

Fig. 4. Normalized mean count rate of lipid vesicles (A) DMPC, (B) DMPC:chol and (C) DMPC:chol:SM, with and without VB12 (150 μM), as a function of temperature. The aqueous phase is PBS (0.01 M, pH 7.4).

behavior of the bilayer as shown in Fig. 4. While for DMPC vesicles, the increase in temperature caused a variation from an ordered to a disordered phase [28], for DMPC:chol and DMPC:chol:SM vesicles an opposite effect was verified where a transition from a disordered to an ordered phase occurred. In these two models, at temperatures below T_m the acyl chains of the phospholipids are randomly oriented, and at temperatures above T_m the lipids are closely packed. This effect occurs due to chol and SM molecules insertion in the bilayer intercalating between the DMPC phospholipid molecules, filling in the free spaces. This increased the thickness and rigidity of the bilayer [29].

As also shown in Table 3, T_m values did not change by adding the VB12 to the models ($p > 0.05$), proving that this vitamin does not produce modifications in the fluidity and packing of the lipid membrane. This was expected due to the preferential location of VB12 in the hydrophobic region of the bilayer, near to the acyl chains of the phospholipids, as indicated by the previously determined K_{SV} values. This region exhibits a more fluid and disordered structure, while the region near to the hydrophilic headgroups of the phospholipids exhibit a more rigid and ordered packing. Therefore, as observed, the insertion of the VB12 molecules in the more disordered region of the membrane do not induce significant variations in the fluidity of the bilayer.

4. Discussion

Lipid vesicles were used as models to study the interactions of VB12 with biological membranes. These present several advantages over conventional models, allowing to better understand the biodistribution and biological activity of VB12. Since the biophysical features of the membranes affect its permeability and endocytic activity, the effect of VB12-membrane interactions was evaluated to assess if these interactions could be a factor for VB12 insufficiency. Although the VB12 molecules have bind to the membrane receptors to be transported, the membrane lipids also exhibit an important role. This because the biophysical properties of the lipid membrane as its shape, packing and fluidity, influence the membrane processes [15].

First, the developed models were used to study the lipophilicity of VB12. These lipid models present an advantage over the classic octanol/water systems that only acknowledges the hydrophobic interactions. In fact, several other interactions such as electrostatic and ion-dipole forces must be considered, since they are involved in more than a few membrane binding phenomena such as the transport of molecules [30]. Although the theoretical log *P* values (-3.24) suggest a low lipophilicity for VB12, the attained experimental log *D* values (Table 1) show that VB12 has high affinity for the three membrane models. VB12 is a ionic compound, and it is well established that ionic/ionizable molecules are able to partitioning through the lipid bilayer due to establishment of hydrogen bonds and electrostatic interactions [31]. In fact, some studies have shown log *D* values very different from the predicted log *P* values that are determined for the neutral species of molecules [32]. Though, it is not possible to determine the preferential localization of the VB12 molecules within the bilayer from the obtained log *D* values, because the variations in the absorbance of VB12 can occur due to its partition deeply into the membrane or to its adsorption at the vesicle surface [33]. So, fluorescence quenching experiments were necessary to determine the VB12's preferred location within the biomembrane. The higher K_{SV} values for DPH revealed that VB12 molecules are preferentially located deeply in the bilayer, due to hydrophobic interactions with the hydrocarbon chains of the phospholipid. These results are important to understand the role of the lipid phase in the endocytosis of VB12, because TCblR/CD320 receptor, its receptor at the target cells, is a transmembrane protein. TCblR/CD320 receptor exhibits a hydrophobic segment aligned with the acyl chains of the membrane's phospholipids. Hydrophobic forces between the hydrophobic segments of the receptor and the hydrophobic regions of the lipid bilayer are responsible for the stabilization of receptor within the bilayer [34].Thus, VB12 must have affinity for the lipid phase of the membrane and interact with the hydrophobic region to effectively bind to its receptor.

The obtained results for the location studied of the vitamin were in agreement with the membrane fluidity experiments. In fact, the absence of modifications in T_m values of the membranes indicates that VB12 should be preferentially found in the more fluid and disordered deeper

area of the bilayer not significantly affecting the packing of the membrane. The more fluid regions of the bilayer are the least disturbed by the insertion of molecules This area is able to better accommodate the vitamin molecules due to its more disordered-state [35]. Thus, these results prove that this vitamin does not produce perturbations on the biological membranes.

The biophysical features of the biological membranes are critical aspects for the membrane processes. Hence, changes in these properties may compromise the membrane processes such as endocytosis. Modifications in the biophysical characteristics of the membrane, such as its fluidity, organization and packing lipid can reduce the diffusion of a molecule through the membrane, hindering the binding to its receptor. Also, the curvature of the bilayer, a critical aspect for endocytosis, is influenced by the fluidity of the membrane [8]. So, changes in the fluidity if the membrane could affect that endocytic transport of VB12. Since VB12 does not alter the properties of the bilayer, its endocytic transport should be not affected by VB12 interaction with the membrane lipids. On the contrary, this interaction should facilitate the binding with the membrane receptor.

Also, evidences indicate that the function of membrane proteins is dependent on the lipid composition of the cellular membranes. Thus, lipid vesicles with different composition were used to evaluate how the bilayer complexity would affect the VB12-membrane interactions. Chol and SM molecules were added to the lipid model to better mimic the biological membranes composition, due to being major components of the membranes of eukaryotic cells. Studies report that cell membranes contain in their lipid composition approximately 15 % of chol and 10 % of SM, respectively [36]. An increase in the membrane levels of chol and SM leads to changes in its biophysical properties, leading to a transition to a more organized and rigid state. These molecules assemble in specific microdomains known as lipid rafts that are in an ordered phase and are surrounded by other lipid molecules in a more disordered phase. Studies reported that chol molecules have higher affinity to SM than PC phospholipids, and consequently leading to the assembly of these chol- and SM-enriched domains [37]. These lipid rafts are specialized structures involved in several membrane processes, such as receptor-mediated endocytosis. In fact, evidences show that receptors for endocytosis-mediated transport are located in lipid rafts [38]. The use of three different membrane models with different lipid composition proved in this work that the membrane composition, fluidity and organization influence the VB12 interactions with the bilayer. Hence, the distribution of the vitamin molecules within the membrane depends on both the VB12 and the membrane physicochemical characteristics. As observed, the affinity of VB12 for the lipid models, decreased with the increasing model complexity. This occurs because SM and chol molecules increase the thickness and rigidity of the bilayer, hampering the diffusion of VB12 through the membrane [21]. The fluidity of bilayers affect the ability of the ligands to bind to the membrane receptor because, as some studies reported, membrane proteins usually exhibit higher affinity for ligands in more rigid bilayers [8]. Thus, despite VB12 exhibiting lower affinity for more rigid membranes, its endocytosis should be facilitated. In fact during endocytosis, both membrane lipids and proteins are uptaken by the endocytic vesicles, and studies reported that the more rigid domains of the membranes are selectively included in endocytic vesicles [39].

The results obtained in this work suggest that the low bioavailability of vitamin B12 it is not due to its interaction with cell membranes. Since vitamin B12 exhibits high affinity to the biological membranes, not inducing changes in their biophysical properties, other causes must be related with the insufficiency of this vitamin, such as deficiency of the intrinsic factor, and other factors that hamper its gastrointestinal absorption such as age, gastritis and usage of oral antidiabetics and gastric protectors [4]. Strategies aiming to enhance its gastrointestinal absorption could be a suitable approach to increase VB12's bioavailability. The use of nanoparticles for VB12 delivery could be a potential solution [40], since these drug delivery systems allow overcoming the low bioavailability while maximizing the activity potential of several molecules [41,42].

5. Conclusion

Deficiency of VB12 is a common nutrition problem with significant impact on nutrition and health of world population. Malabsorption and malnutrition have been pointed as main causes for this vitamin deficiency. Being the VB12's low bioavailability an underlying cause for its deficiency, in this work it was evaluated if its affinity for biological membranes regulates its low bioavailability and consequently low cellular uptake.

The biophysical features of the cell membranes regulate several membrane phenomena such as transport and endocytic functions. In fact, although the cellular uptake of VB12 occurs by a receptor-mediated endocytosis mechanism, lipid membrane microdomains known as lipid rafts are essential for several membrane phenomena. These lipid rafts are involved in the binding of the ligand to its receptor, therefore playing an important role in the endocytosis. Thus, understanding the role of membrane lipids in cellular processes, may allow predicting the interaction of the vitamin with biological membranes and its effects on the biodistribution and cellular uptake of the vitamin.

Hence, lipid vesicles were used as biomembrane models to assess the role of the molecular interactions of VB12 with biological membranes on its low bioavailability. These models were used to calculate the partition coefficient of the vitamin, as well as its location in the bilayer and its effects on the fluidity of lipid membrane. VB12 have shown high affinity for the biomimetic models suggesting that the low bioavailability of the vitamin is not due to a poor interaction with the biological membranes and that reduced endocytic function should not be a major cause for VB12 low bioavailability. Also, the vitamin proved not to induce any modifications in the biophysical characteristics of the membranes.

Finally, the complexity of the membrane is a major factor regulating the vitamin-membrane interactions. Thus, the choice of the models to be used is a critical aspect because the biodistribution of the vitamin depends not only on its molecular properties but also depends on membrane complexity and structure such as the packing of the lipid molecules. DMPC:chol:SM vesicles are a more complex model and therefore these vesicles are the most similar to the biological membranes, providing more representative predictions.

Author contributions

M.J. Ramalho conducted the experiments, data analyses, and the writing of the manuscript. All authors contributed to the conceptualization of the experiments, review and editing of the manuscript. All authors have given approval to the final version of the manuscript.

CRediT authorship contribution statement

Maria João Ramalho: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Stéphanie Andrade:** Conceptualization, Writing - review & editing, Visualization. **Manuel A.N. Coelho:** Resources, Supervision. **Joana A. Loureiro:** Resources, Writing - review & editing, Supervision, Funding acquisition. **Maria Carmo Pereira:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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