Polyphenol-lipid interactions in endothelial cells in diabetes: cellular and biophysical approaches

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2023

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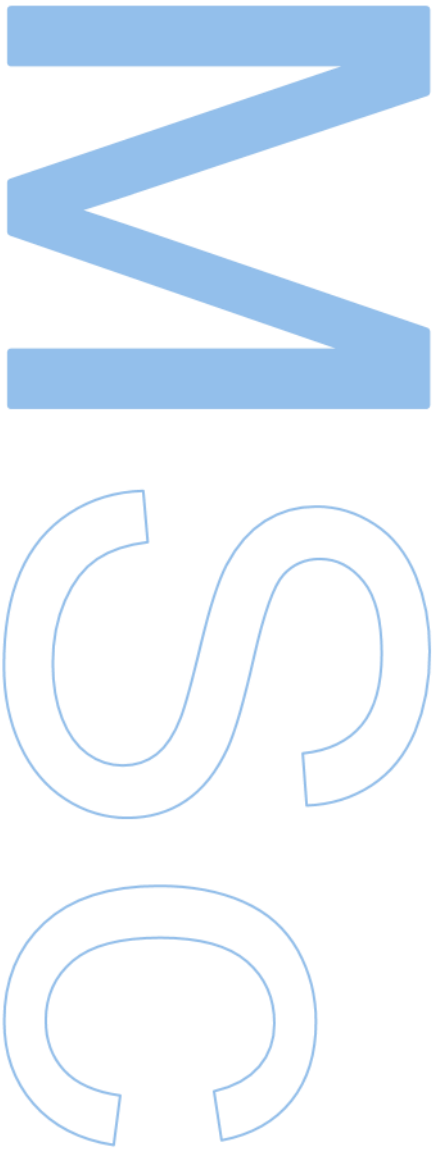


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Acknowledgments

Em primeiro lugar, gostaria de agradecer à Dra. Ana Reis por ter aceitado orientar-me neste projeto. É imperativo reconhecer a sua contribuição na conceção e supervisão do trabalho experimental, bem como na ajuda fornecida diante das dificuldades que surgiram. Da mesma forma, gostaria de agradecer ao meu co-orientador, Professor Dr. Victor Freitas, pela oportunidade que me concedeu em trabalhar neste prestigiado laboratório e pelo seu interesse em garantir o êxito geral deste projeto.

Ao Professor Dr. Orfeu Bertolami, manifesto o meu profundo reconhecimento, pelo apoio esclarecido e incondicional em todas as fases do processo.

Gostaria de agradecer aos meus colegas de laboratório pela sua presença constante e disposição para ajudar sempre que necessário. Em especial, queria destacar o papel da Inês, Joana e Margarida, cuja ajuda transcendia as paredes do laboratório.

Aos meus avós, que desempenharam um papel fundamental no meu crescimento, guiando-me nos primeiros passos e que mantiveram uma convicção inabalável no meu valor e nas minhas capacidades, desejo expressar o meu profundo agradecimento.

À Mariana e à Sofia, por, mesmo estando longe, terem estado tão presentes. Por terem tornado a minha experiência de faculdade na melhor fase da minha vida e por me fazerem acreditar que seria capaz de alcançar todos os objetivos.

Ao meu Gonçalo, desejo expressar minha profunda gratidão, por ter sido o meu maior apoio durante este último ano. A sua presença foi, verdadeiramente, inestimável e insubstituível. Obrigada por todas as palavras de conforto nos períodos mais difíceis, despontando sorrisos mesmo quando parecia não haver espaço para os mesmos.

Por último, e o mais importante, quero agradecer à minha mãe, por ser o meu maior e melhor exemplo. Por me mostrar que, por detrás de um grande profissional, deve estar sempre uma pessoa de carácter inatacável.

Este trabalho foi financeiramente apoiado pelo projeto UIDB/50006/2020, financiado pela FCT/MCTES com fundos nacionais e pelo projeto AgriFood XXI I&D&I (NORTE-01-0145-FEDER-000041).

Abstract

Over the past few decades, the increasing prevalence of diabetes, due to excessive ingestion of high-sugar and highly processed foods, has made it a global health concern. The earlier diabetes sets in, the greater the propensity to develop multiple life-threatening vascular complications. The substantial cost of drug-based therapies drives the search for alternative nutritional strategies with anti-diabetic properties, such as the Mediterranean diet, characterized by a high content of polyphenols.

To enhance our current knowledge of how polyphenols interact with epithelial lipids, this work aims to conduct membrane biophysical studies. To achieve this, complex membrane models were built to simulate both normo- and hyperglycemic conditions. Hyperglycemic conditions were mimicked by the incorporation of glycated dimyristoyl-phosphatidylethanolamine (DMPE-glyc) and adjustments in the cholesterol-to-phospholipid (Chol/PL) ratio. Mass Spectrometry (ESI-MS) and dynamic light scattering (DLS) measurements were used to characterize the DMPE-glyc adducts. The effect of this glycation process on the membrane model was also accessed by DLS measurements and fluorescence studies, namely the changes in membrane fluidity and lipid order. To evaluate the influence of polyphenol metabolites, such as hippuric acid (HYP), 4-hydroxyphenylacetic acid (HPAA), 3-(2,4-dihydroxyphenyl)propionic acid (HPPA), and protocatechuic acid (PCA), in modulating the biophysical properties of the epithelial membrane models, fluorescence quenching experiments were conducted.

The incorporation of DMPE-glyc adduct into the complex model revealed a decrease in the transition temperature, whereas fluorescence studies revealed a decrease in membrane fluidity and an increase in lipid order under hyperglycemic conditions. Furthermore, incubation of HYP, HPAA, HPPA and PCA revealed that polyphenol metabolites interacted with membrane lipids, particularly in the hydrophobic regions. However, this interaction did not significantly impact membrane fluidity.

This epithelial membrane model that mimics normo- and hyperglycemic conditions was used to investigate the impact of polyphenol-lipid interactions. Furter studies will provide insights into how Mediterranean-type diet may serve as a nutritional strategy in the prevention and management of diabetes in the pre-diabetic population.

Keywords: Mediterranean Diet, Diabetes Mellitus Type 2, Endothelial Membrane, Liposomes, Lipid Glycation, Polyphenol Metabolites, Membrane Fluidity, Anisotropy, Mass Spectrometry.

Resumo

Nas últimas décadas, a crescente prevalência da diabetes, atribuída em grande parte ao consumo excessivo de alimentos caracterizados por elevados teores de açúcar e altamente processados, tornou-se numa preocupação de saúde à escala global. A ocorrência precoce da diabetes está intimamente associada a uma maior propensão para desenvolver múltiplas complicações vasculares potencialmente fatais. O elevado custo das terapias farmacológicas impulsiona a procura por estratégias nutricionais alternativas com propriedades antidiabéticas, como a dieta mediterrânica, rica em polifenóis.

Com o propósito de aprofundar o conhecimento vigente, este trabalho tem como objetivo investigar a interação de polifenóis com lípidos analisando as suas implicações nas propriedades biofísicas. Para atingir esse fim, elaborou-se um modelo membranar complexo simulando situações de normo- e hiperglicemia. As condições de hiperglicemia foram mimetizadas através da incorporação de dimiristoil-fosfatidiletanolamina glicada (DMPE-glic) e através de ajustes no rácio colesterol-fosfolípido (Chol/PL). Técnicas de espectrometria de massa (ESI-MS) e medições de Espalhamento Dinâmico de Luz (DLS) foram utilizadas para caracterizar os aductos de DMPE-glic. O efeito do processo de glicação nos modelos de membrana foi, também, avaliado por medições de DLS e fluorescência com foco na fluidez da membrana e na ordem lipídica. A influência dos metabolitos de polifenóis na modulação das propriedades biofísicas dos modelos de membrana foi avaliada através de experiências de extinção de fluorescência.

A incorporação do aducto de DMPE-glic no modelo membranar conduziu a uma diminuição da temperatura de transição. Além disso, estudos de fluorescência revelaram uma diminuição da fluidez da membrana e um aumento da ordem lipídica em situações de hiperglicemia. Por fim, a incubação de ácido hipúrico, ácido 4-hidroxifenilacético, ácido 3- (2,4-di-hidroxifenil) propiónico e ácido protocatechuico revelou que os metabolitos do polifenol interagiam com os lípidos da membrana, particularmente nas regiões hidrofóbicas. No entanto, esta interação não induziu alterações significativas na fluidez da membrana.

Este modelo de membrana epitelial, que simula situações de normo- e hiperglicemia, foi usado para avaliar o impacto das interações entre polifenóis e lípidos. Estudos adicionais fornecerão informações sobre como a dieta Mediterrânea pode ser uma estratégia nutricional na prevenção e gestão da diabetes na população pré-diabética.

Palavras-chave: Dieta Mediterrânea, Diabetes Melitos Tipo 2, Membrana Endotelial, Lipossomas, Glicação de Lípidos, Metabolitos de Polifenol, Fluidez da Membrana, Anisotropia, Espectrometria de Massa

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List of Abbreviations

AGEs – Advanced Glycation End Products

C18-GlcCer – glucosyl-β1-10-N-stearoyl-D-erythro-sphingosine

Chol – Cholesterol

COMT – Catechol-O-methyltransferase

DAG – Diacylglycerol

DLS – Dynamic Light Scattering

DMPE-glyc – Glycated Dimyristoyl-phosphatidylethanolamine

DPH – 1,6-diphenyl-hexatriene

ECs – Endothelial cells

ESI – Electrospray Ionisation

ESI-MS/MS – Tandem Mass Spectrometry

GI – Gastrointestinal

GLP1 – Glucagon-like Peptide-1

GP – Generalized Polarization

GSL – Glycosphingolipid

HDL-C – High-density Lipoprotein Cholesterol

HPAA – 4-Hydroxyphenylacetic acid

HPPA – 3-(2,4-Dihydroxyphenyl)propionic acid

HYP – Hippuric acid

IDF – International Diabetes Federation

IR – Insulin Resistance

K_A – Affinity Constant

K_{sv} – Stern-Volmer Constant

LDL-C – Low-density Lipoprotein Cholesterol

LUVs – Large Unilamellar Vesicles

NF-κB – Nuclear Factor Kappa B

PBS – Phosphate Buffer Saline

PCA – Protocatechuic acid

PKC – Protein Kinase C

PL – Phospholipid

PLPC– 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine

RAGE – Receptor for Advanced Glycation End-Products

ROS – Reactive Oxygen Species

SFAs – Saturated Fatty Acids

SM – Sphingomyelin

SULTs – Sulfotransferases

T2D – Type 2 diabetes

TG – Triglyceride

TMA-DPH –1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate

UGTs – Uridine 5'-diphospho-glucuronosyltransferases

Introduction

1.1. Type 2 Diabetes Mellitus

According to the International Diabetes Federation (IDF), in 2021, 537 million people were living with diabetes (Figure 1). The recent projections estimate that by 2045 over 700 million people will be affected by diabetes, emphasizing the magnitude of this global problem (1). The incidence of cases among paediatric and adolescent populations is on the rise as well, due to excessive caloric intake, high-sugar, highly processed foods, low consumption of fresh fruits and vegetables and sedentary habits (2,3). This is particularly concerning because an earlier onset diabetes is associated with a longer exposure to hyperglycaemia and an increased propensity to develop long-term complications (3). These repercussions, which affect both micro and macrocirculation, are the main cause of diabetes-related morbidity and mortality (4).

The lifelong persistence of the disease and the need for ongoing treatment and management make it clear that the economic and social impact of diabetes is likely to increase (5). The primary approach to control diabetes involves the use of drug-based therapies (6). However, their costs make them less viable options for individuals and healthcare systems (5). It is imperative to develop affordable and easy-to-administer therapies to prevent and manage this disease for the entire population.

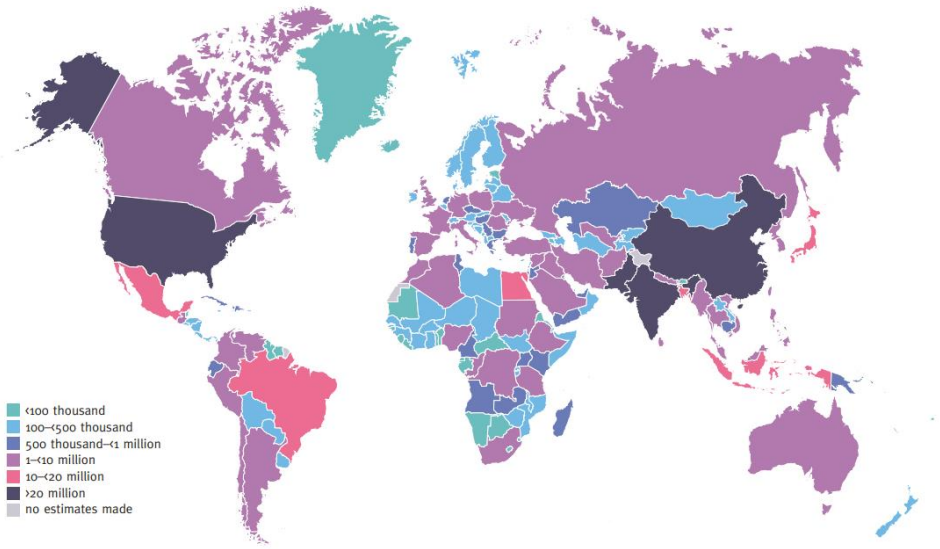


Figure 1 - Estimated total number of adults (20–79 years) with diabetes in 2021. Source: IDF Diabetes Atlas 2021 – 10th Edition.

Type 2 diabetes (T2D) is a complex metabolic disorder whose hallmark is hyperglycemia (7). These high blood glucose levels can be due to insufficient insulin secretion by pancreatic β-cells, a phenomenon called insulin resistance (IR). During this IR state, there is a compensatory response to increase endogenous insulin production to maintain glucose homeostasis (8). However, at a certain point, insulin resistance continues to increase, and pancreatic β-cells fail to respond to insulin demand, thus maintaining a state of chronic hyperglycemia (9).

As the influx of glucose persists, several metabolic pathways are enhanced (Figure 2), leading to negative effects on cells (10). This includes the activation of polyol pathway and consequent accumulation of sorbitol (11), disruption of mitochondrial function and increased production of reactive oxygen species (ROS) (12). Therefore, sorbitol stimulates the *de novo* synthesis of diacylglycerol (DAG), leading to the activation of the protein kinase C (PKC) pathway (13,14), which contributes to an increase in oxidative stress and endothelial inflammation (15,16). Hyperglycemia also activates the hexosamine pathway, which is implicated in the development of insulin resistance (11). AGEs (advanced glycation end products) are formed by the covalent bonding of glucose to proteins, lipids, and nucleic acids, through a non-enzymatic process known as glycation (17). The recognition and binding of AGEs to RAGE (receptor for advanced glycation end-products) promote oxidative stress and inflammation, exacerbating microvascular and macrovascular complications (18). In concert, the activation of these pathways triggers a cascade of events that include oxidative stress, inflammation, apoptosis and insulin resistance (8,15). The cumulative damage to blood vessels and tissues in these organs can result in serious complications in highly vascularized organs such as kidneys, heart, brain, and eyes (19,20).

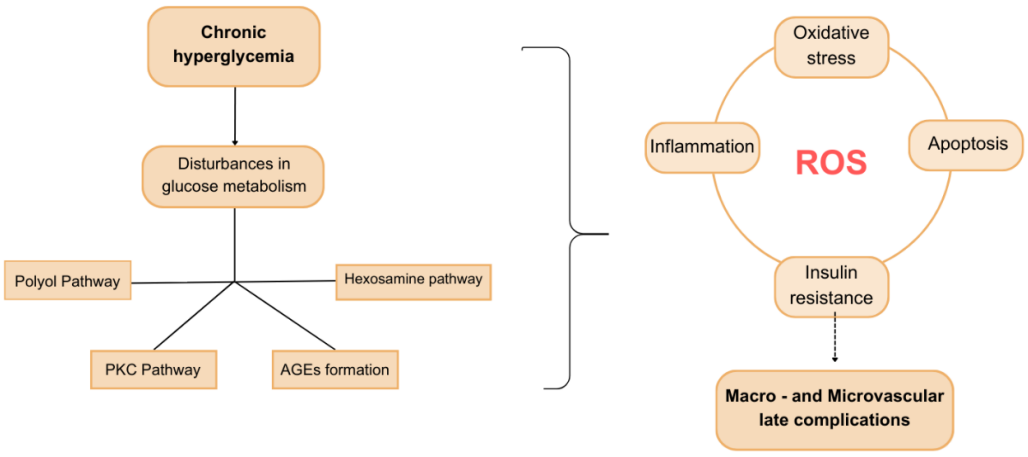


Figure 2 - Metabolic pathways involved in diabetes-related complications.

1.2. Polyphenol-rich diets as nutritional strategies in the prevention and management of diabetes

In addition to pharmaceutical interventions, nutritional strategies that include the consumption of fresh fruits and vegetables rich in polyphenols, as found in the Mediterranean and Nordic diets, offer promising alternatives for preventing and managing diabetes (21,22). Epidemiological studies have shown that diets rich in polyphenols can improve fasting glucose levels, prevent endothelial dysfunction, and have a beneficial effect on lipid metabolism (23–25). The anti-diabetic effects attributed to polyphenols are mainly due to the ability of these compounds to inhibit enzymes, such as α-amylase and α-glucosidase, responsible for cleavage and release of glucose during starch digestion, this leads to a reduction in the amount of glucose that is absorbed from food, facilitating the control of postprandial hyperglycemia (26–28). They have also the ability to influence glucose homeostasis through a variety of mechanisms that include enhancement of β-cell activity, inhibition of glucose transporters, increased insulin secretion (25,29) and the half-life of glucagon-like peptide-1 (GLP1) (30). However, the advantages of polyphenols extend to other chronic diseases (31) mostly because of their antioxidant and anti-inflammatory properties, antithrombotic and anti-atherosclerotic effects, but also because of their antimutagenic/anticarcinogenic and neuro-protective actions (24,32–34).

Polyphenols are a large group with more than 8,000 phenolic structures already identified, resulting from the secondary metabolism of plants (shikimate and acetate pathways) (35,36). These compounds are characterized by having one or more hydroxyl substituents bound with phenols and they can be grouped based on the number of phenolic groups they contain (flavonoids and non-flavonoids) (37,38).

Flavonoids share a basic 15-carbon flavone skeleton, C6-C3-C6, with two phenolic rings (ring A and ring B) usually linked by a heterocyclic ring and can be found in the form of glycosides or aglycones (Figure 3). The main distinction between the members of this group concerns the degree of oxidation of the heterocyclic oxygen and the substitution pattern of the C ring, resulting in a variety of compounds: anthocyanins, flavonols, flavones, flavanols, flavanones and isoflavones (35,37).

The majority of polyphenols present in food exist as esters, glycosides, or polymers, which cannot be absorbed in their native form (39).

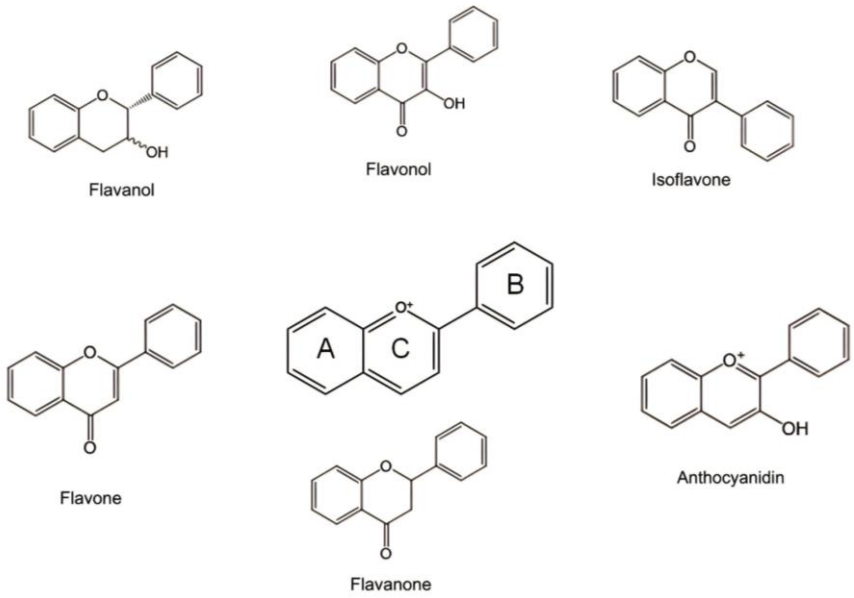


Figure 3 - The common structure of major flavonoids. Three rings are normally identified with letters A, B, and C. These chemical structures were drawn in ChemDraw.

1.2.1 Polyphenol Metabolism and the Beneficial Properties of Their Derivatives.

Polyphenols undergo biotransformation processes as they traverse the human epithelium, facing different chemical environments, including fluctuations in pH between the mouth and gastrointestinal (GI) tract, as well as variations in the intestinal microbiota, before entering the bloodstream (Figure 4) (21). Nevertheless, the polyphenol metabolome obtained from food is not similar to the metabolomic profile that is found in the bloodstream.

So, the metabolization journey of polyphenols starts in the mouth, where the mechanical mastication not only facilitates the partial release of polyphenols, potentially enhancing their bioavailability in the gastrointestinal tract but also amplifies the surface area, promoting interactions with salivary hydrolytic enzymes (40). After the oral cavity, polyphenols are transported with food into the stomach. The acidic conditions initiate the breakdown of the food matrix and polyphenols undergo hydrolysis and oxidation (phase I), (40). Depending on their chemical structures, some polyphenols, like anthocyanins, remain stable under these pH conditions, while others, such as procyanidin oligomers, undergo modifications before reaching the alkaline conditions in the intestine (21).

The second stage of biotransformation occurs when the polyphenols reach the small intestine (especially the jejunum and ileum) and the purpose of this phase is to increase

the polarity of these compounds, facilitating their excretion (41,42). This process involves three key types of conjugation: glucuronidation (catalysed by UDP-glucuronosyltransferases, UGTs), sulfation (involving sulfotransferases, SULTs), and methylation (via catechol-O-methyltransferase, COMT) (41,43). So, after undergoing phase II conjugation in the small intestine, most of the polyphenols are rapidly eliminated from the body through urine and bile as glucuronide and sulfate esters (44).

In the large intestine, gut microbiota acts enzymatically on the polyphenolic backbone of the remaining unabsorbed polyphenols, along with some excreted back into the gut lumen via bile (44,45). The metabolism of polyphenols by microbiota involves the cleavage of glycosidic linkages and the breakdown of the heterocyclic backbone leading to ring-fission polyphenol metabolites (e.g., hydroxy-benzoic acids, hippuric acid, phenylgamma-valerolactones, hydroxy-phenyl-propanoic acid and many others) with different physiological significance (44,45).

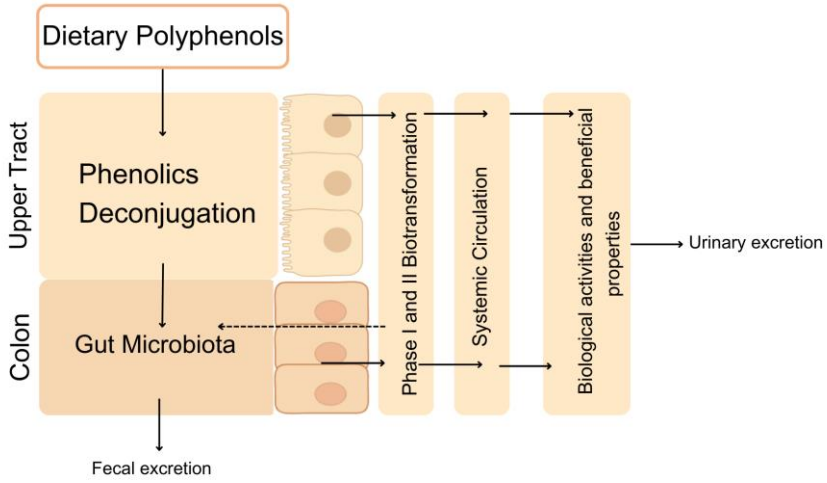


Figure 4 - Schematic representation of the processes undergone by dietary polyphenols along their journey through the gastrointestinal tract (adapted from *van Duynhoven J, Vaughan EE, Jacobs DM, Kemperman RA, van Velzen EJ, Gross G, Roger LC, Possemiers S, Smilde AK, Doré J, Westerhuis JA, Van de Wiele T. Metabolic fate of polyphenols in the human superorganism. Proc Natl Acad Sci U S A. 2011 Mar 15;108 Suppl 1(Suppl 1):4531-8.)*

It is important to highlight that, only 2% of consumed polyphenols reach the bloodstream (where they are typically found within a neutral to alkaline pH), occurring at micromolar range (44,46). This suggests that the majority of the effects observed in polyphenols are probably due to the metabolites. Despite this, the predominant focus in the literature is on dietary polyphenols and the metabolization process continues to receive little attention.

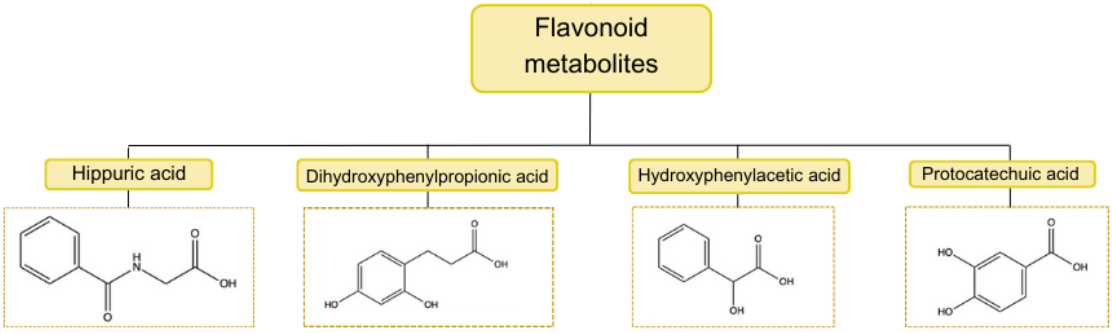


Figure 5 - Structure of polyphenol metabolites derived from gut microbiota. These chemical structures were drawn in ChemDraw.

Reis et al. (47) described that sub-micromolar amounts of certain microbial polyphenol metabolites (Figure 5) can counteract the pro-inflammatory state in glucose-challenged endothelial cells by preventing the post-translational modifications (glycosylation) of IL-6.

It has also been reported that some dietary polyphenols – quercetin, epigallocatechin gallate and curcumin – can improve the biophysical properties of the endothelial membrane, which can have beneficial effects on cells in diabetic conditions (48). At the lipid-water interface, polyphenols can expand the hydration layer and facilitate hydrogen bonding between the hydroxyl groups of polyphenols and the polar head groups of phospholipids, thereby enhancing membrane fluidity (21,49,50). Polyphenols can additionally increase membrane packing and reduce the thickness of the lipid bilayer, induce phase separation, and ultimately culminate in membrane leakage (21,49). This interaction and consequent permeation of polyphenols to the lipid bilayer are influenced by the structural characteristics and concentration of the polyphenols as well as the lipid composition of the bilayer (21,51). For example, the number of galloyl groups has a direct impact on membrane fluidity: due to the ability of galloylated polyphenols to locate in the polar head region but also to penetrate the hydrophobic core of the lipid bilayer, leading to the disruption of lipid alkyl chains and promote membrane thickness (52). All of these effects contribute to improved membrane stability, regulation of permeability, and ultimately the reversal of diabetes-induced biophysical changes in the endothelial membrane.

Though many studies have investigated the effect of dietary polyphenols in ameliorating T2D consequences, little is known about the changes that polyphenol metabolites—

those that successfully reach the bloodstream— may induce in the biophysical properties of endothelial membranes.

1.3. Endothelial Membrane in Diabetes

Diabetes-related macro and microvascular complications may be the result of biophysical changes in the endothelial membrane and associated endothelial dysfunction (53).

Endothelial cells (ECs) are flat epithelial cells that form a monolayer lining the inner surface of blood vessels (54,55). Due to this location between blood circulation and tissues, these cells are exposed to fluctuations in glucose concentration during pre- and post-prandial cycles (56). This exposure to varying glucose levels is a crucial factor in promoting the vulnerability of ECs to the harmful effects of diabetes. In the T2D milieu (hyperglycemia along with excess free fatty acid release and IR) a cascade of metabolic events is triggered (15). These processes, which include reduced endothelium-mediated vasodilation, release of proinflammatory cytokines and chemokines, increased oxidative stress, hemodynamic problems and abnormalities in fibrinolysis, can culminate in endothelial dysfunction (54,55,57,58). In this condition, there is a transition of the endothelium to a pro-inflammatory, vasoconstrictive and pro-thrombotic state (10,57).

In addition to the metabolic cascade and activated signalling pathways, the chemical modifications induced by T2D in various biomolecules, such as proteins, lipids, and nucleic acids in ECs, deserve considerable attention.

The glycation process, that occurs during diabetes, involves the non-enzymatic reaction between glucose and an amino group of proteins, lipids, and nucleic acids, leading to a series of chemical reactions forming a Schiff base, followed by an Amadori rearrangement and culminating in oxidative modifications to produce AGEs (59). It is known that AGEs can form cross-links with various extracellular and transmembrane proteins and alter their structure and functional properties. This can lead to the inactivation of biologically active proteins and enzymes, rendering them resistant to proteolytic degradation, providing catalytic sites for ROS generation, inciting pro-inflammatory responses, disrupting intracellular, and participating in several metabolic and biochemical perturbations (59). Consequently, the flexibility of blood vessel walls is reduced while vascular stiffness is enhanced (60,61). Moreover, by impairing the fluidity of endothelial membrane, the glycation of platelet surface proteins promotes platelet adhesion and, consequently, increases the propensity to thrombus formation (62).

Even though the poor knowledge of the endothelial lipidome under physiological and diabetic states (49), there are established lipid characteristics and chemical changes that are linked to the pathogenesis of T2D and associated complications. In diabetes, there is a dyslipidemic profile characterized by elevated serum triglyceride levels (TG)

(hypertriglyceridemia), small and dense low-density lipoprotein cholesterol (LDL-C) (hypercholesterolemia), reduced serum high-density lipoprotein cholesterol (HDL-C) and an excess of saturated fatty acids (SFAs) (63,64). It is become evident that the excess in rigidity-promoting lipids and the shift from unsaturated to saturated fatty acids in membrane phospholipids decrease the fluidity of the endothelial membrane, and consequently, impair microcirculation (64–67). It has also been reported in some studies that cholesterol has a significant ordering effect on membrane lipids (68,69). Therefore, it is plausible that in a situation of T2D and hypercholesterolemia, there will be an increase in lipid order, resulting in membrane stiffening.

1.3.1 Endothelial Biophysics in Diabetes: Insights and Implications

Although the molecular and chemical mechanisms that disrupt the normal functioning of the endothelium are well described, as previously reported, it is important to underline that few biophysical studies have been carried out on how T2D specifically alters the biophysical properties of the membrane. Some studies have stated that diabetes induces stiffness in the endothelial membrane (65,70–72), which can impair other important mechanical properties of the cell. This reduction in membrane fluidity, by promoting disturbances in blood rheological behaviour and in the microcirculation, can contribute to the underlying mechanisms of genetic hypertension (Figure 6) (73).

The thickening of the basement membrane, which is also a characteristic of hyperglycemia, can lead to an augmentation in microvascular permeability, impairing the amount and selectivity of products transported into the bloodstream (54). This happens due to alterations in the physical dimensions of the meshwork and changes in the normal electrical charge surrounding the pores between endothelial cells (74).

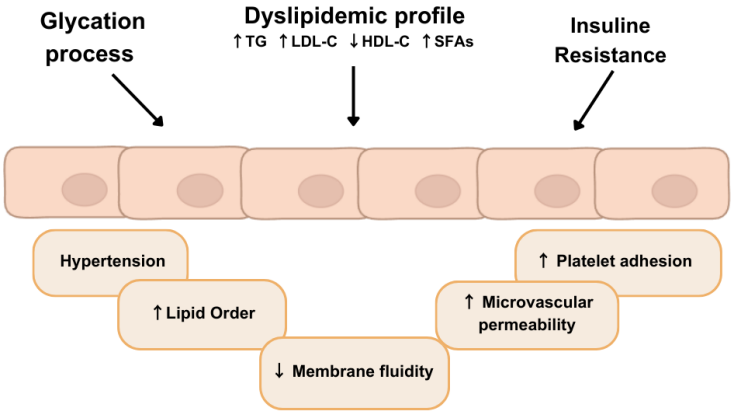


Figure 6 - T2D and Biophysical Changes in Endothelial Membrane: Consequences of glycation process, dyslipidemia and insulin resistance on biophysical properties of endothelial membranes.

Understanding how polyphenol metabolites permeate and cross the endothelial cell membrane and modulate their biophysical properties is fundamental to developing strategies to mitigate vascular complications associated with diabetes and prevent further organ damage.

1.4. Objectives

This work was conducted in order to improve our insights into the interactions between polyphenols and lipids and their effects on the biophysical properties of endothelial membranes, in hyperglycemic conditions.

Therefore, the objectives of this project included:

- Developing complex membrane models that aimed to mimic the epithelial membrane under normo- and hyperglycemic conditions;
- Characterizing the biophysical properties of these membrane models in normo- and hyperglycemic conditions;
- Evaluating the role of polyphenol metabolites in modulating the biophysical properties of the membrane models.

2. Material and Methods

2.1. Reagents and Materials

Lipid standards 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC), 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine (SLPC), Sphingomyelin (SM), 1,2-myristoyl-sn-glycero-3-phosphoethanolamine (DMPE) and glucosyl-β1-10-N-stearoyl-D-erythro-sphingosine (C18-GlcCer, GSL) were acquired from AVANTI Polar LIPIDS (Birmingham, AL, USA). Cholesterol (Chol), Hippuric acid (HYP), 4-Hydroxyphenylacetic acid (HPAA), 3-(2,4-Dihydroxyphenyl)propionic acid (HPPA) were purchased from Sigma Aldrich (Madrid, Spain) and Protocatechuic acid (PCA) from HWI group. Phosphate buffer saline (PBS) tablets were obtained from PanReac AppliChem (Darmstadt, Germany) and fluorescent probes 1,6-diphenyl-hexatriene (DPH), its trimethylamino-derivative (TMA-DPH) and Laurdan were purchased from Sigma-Aldrich. Chloroform and methanol used were HPLC grade and were sourced from Fisher.

2.2. Preparation of Large Unilamelar Vesicles (LUVs)

Firstly, PLPC, DMPE, Chol and GSL were dissolved in chloroform-methanol (1:1, v/v) mixed in a round-bottom glass flask. Depending on the experimental anisotropy procedure one fluorescent probe (DPH,TMA-DPH or Laurdan) was dissolved in chloroform and added to lipid organic mixture. The lipid: probe ratio was 300:1 (mol/mol) for DPH and TMA-DPH, and 500:1 (mol/mol) for Laurdan. Subsequently, to remove all traces of organic solvent, lipids were evaporated to dryness under vacuum at room temperature for 3 hours.

The preparation of liposomes was based on the hydrating-extrusion method described by *Hope et al*. (75). Phosphate buffer (10mM, pH 7.4) was added to a final lipid concentration of approximately 1 mM, leading to the formation of Multilamellar Vesicles (MLVs). Subsequently, MLVs were processed by interchangeable freeze/thaw cycles (more than 5) in liquid nitrogen and boiling water bath. Lipid suspensions were extruded through polycarbonate filters (100 nm pores, Whatman®, Maidstone, UK) using a LIPEX Biomembranes extruder connected to a CLIFTON thermostatic circulating water bath above the phase transition temperature (70°C) to obtain large unilamellar vesicles (LUVs). The LUVs were kept at 4°C and protected from light prior to use until spectroscopic characterization.

2.3. Synthesis of DMPE-Glucose Adducts

In order to evaluate the diabetes-induced modifications in the endothelial membrane, it was necessary to build a membrane model that could mimic a situation of hyperglycemia. For this, liposomes mimicking hyperglycemia conditions have a high PL/Chol ratio (1.6) (diverging from the normoglycemic conditions where the PL/Chol ratio stands at 2.20) and glycated dimyristoyl phosphatidylethanolamine (DMPE-glyc).

The synthesis of DMPE-glucose adducts was performed following the protocol described in *Reis et al*. (76) (Figure 7) . Briefly, 20mg of DMPE were solubilised in 90% methanol (v/v) with a few drops of ethanol. Subsequently, 282mg of glucose (corresponding to a 50 molar excess of glucose (787 µmoles) were dissolved in water and added to the DMPE solution (achieving a final concentration of 2mg/mL). The mixture was homogenized in a vortex (1 minute) and left to react at 80°C in a heating block (Stuart, SBH 130D/3, Stone, Staffordshire, UK) for 5 hours with occasional vortexing.

Afterwards, to remove the excess unreacted glucose, liquid–liquid washing steps were performed using a mixture of chloroform: water (2:1, v/v) to induce phase separation. Additional washing steps with water were done. The organic extract containing glycated and unmodified DMPE was dried under vacuum (LabConco, Kansas City, MI, USA) at room temperature until dryness. The yield of the glycation reaction is approximately 8.7% of the total PE.

The dried extracts were structurally characterised by electrospray mass spectrometry (ESI-MS) and by tandem mass spectrometry (ESI-MS/MS) and kept in an inert nitrogen atmosphere at −20°C until further incorporation in LUVs.

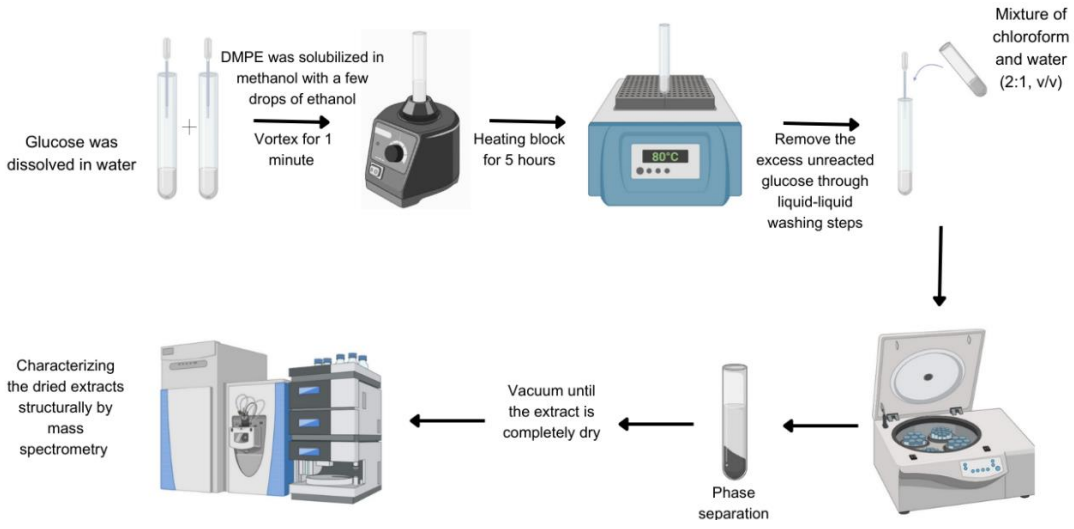


Figure 7- Schematic representation of the methods used to synthesize DMPE-glucose adducts.

2.4. Characterisation of DMPE-Glucose Adducts by Mass Spectrometry (ESI-MS) techniques

The presence of DMPE-glyc adduct in dried extracts was confirmed through electrospray mass spectrometry (ESI-MS). The characterisation of dried organic extracts was carried out by direct injection in a LCQ DECA XP ion trap mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) equipped with an atmospheric pressure ionisation (API) source using electrospray ionisation (ESI) interface (ESI-(IT)-MS).

Dried extracts were solubilized in methanol (0.1% formic acid, v/v) and analysed in the positive and negative ion modes. The capillary voltage was maintained at 5 kV and cone voltage was set at 15V; the source temperature was set at 270ºC and the sheath gas flow at 19 (arbitrary units). Samples were introduced into the electrospray source at a flow rate of 18µL/min and each spectrum produced was the result of data accumulation for 1–2 minutes. Tandem mass spectra (ESI-MS/MS) were achieved using nitrogen as the collision gas and the collision energy used for protonated molecule ($[M+H]^+$) was set at 25V (arbitrary units). Raw data was analysed Through Excalibur software (San Jose, CA, USA, version 2.2) and we obtained the respective spectra and mass-to-charge ratio values.

2.5. Dynamic light scattering (DLS) measurements

The thermotropic behaviour of unmodified and glycated PE was studied by dynamic light scattering (DLS) measurements acquired in Zeta Sizer Nano ZS instrument (Malvern Instruments, Malvern, UK).

The measurements of mean count rate versus temperature were carried out in capped glass cuvettes (Starna Scientific, Hainault Essex, UK) using a helium–neon laser (λ= 633 nm) at an angle of 173◦ with fixed attenuation over a temperature range from 20 to 80°C with 2°C increments. Samples were left to equilibrate for 3 minutes at each set value before measurements.

Raw data obtained were analysed using the first-derivative plot of the experimental data (Origin software, version 2021) to determine the transition temperatures of each system.

2.6. Membrane Fluidity Studies by Fluorescence Anisotropy

Steady-state fluorescence anisotropy with TMA-DPH probe was used to evaluate the fluidity of membrane models in normo- and hyperglycemic situations itself and with the presence of different classes of polyphenol metabolites.

Anisotropy measurements were performed in a Varian Cary Eclipse Fluorescence Spectrophotometer, equipped with a temperature cell holder (Varian Cary Single Cell Peltier Accessory) coupled to a water-circulation thermostat.

For all studied systems, the liposomes (500μM) were placed in 1.5mL quartz cuvettes (Starna Scientific, Hainault Essex, UK). The steady-state fluorescence anisotropy measurements for the membrane models were recorded in a range of 16 to 80ºC (with 2ºC increments) and maintained until stabilized before each measurement. And at 37ºC for the liposomes incubated with polyphenol metabolites (25μM). The temperature of each sample was monitored using an in-house built sensor immersed inside the quartz cuvette. The excitation (λ_{exc}) and emission wavelengths (λ_{em}) used were 359 and 427nm, respectively. Anisotropy values shown are the mean of three independent measurements (n = 3) and they are defined by equation (Eq. 1) (76)

$$r_S = \frac{I_{VV} - I_{VH} G}{I_{VV} + 2\, I_{VH} G} \quad \textbf{(1)}$$

where I_{VV} and I_{VH} represent the emission intensities when the polarisers were oriented vertical–vertical (parallel) and vertical–horizontal (perpendicular) to the excitation beam. G is a correction factor and is given by the ratio of vertical to horizontal components when the excitation light is polarised in the horizontal direction, $G = I_{HV}/I_{HH}$.

For the statistical analysis of the TMA-DPH anisotropy values of the liposomes itself and with the presence of different classes of polyphenol metabolites it was used the t-test in GraphPad Prism software (version 10.0.2).

2.7. Lipid order measurements

In order to measure the lipid order of membrane models under normo- and hyperglycemic conditions, liposomes labelled with fluorescent probe Laurdan were used. Laurdan is a hydrophobic probe that has been widely used due to its high sensitivity to the polarity of the environment (77).

Fluorescence emission spectra of Laurdan-labelled liposomes were traced at an excitation wavelength of 350 nm, with emission wavelengths from 360 to 600nm. The

excitation generalized polarization (GP_{exc}) was calculated using the following equation (Eq. 2):

$$GP_{exc} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad \textbf{(2)}$$

where I_{440} and I_{490} represent fluorescence intensities at 440 (where Laurdan exhibits maximum emission in liquid-ordered (Lo) phases) and 490nm (where Laurdan shows maximum emission in liquid-disordered (Ld) phases), respectively.

2.8. Fluorescence quenching measurements

For this experiment, samples were prepared in eppendorf tubes by addition of DPH and TMA-DPH-labelled liposomes (500µM) to PBS buffer. Increasing volumes of polyphenol metabolites stock solution were added to eppendorf tubes to achieve a polyphenol concentration range of 0-100µM.

Tubes were vortexed and incubated at 37.0°C in water bath (Thermo Shaker Incubator – MS-100 / MSC-100) for 30 minutes before fluorescence readings.

Steady-state fluorescence spectra were recorded with an excitation wavelength of 359 nm, and the emission window was set from 370 to 580nm. For each experiment, 5 replicates were performed.

Fluorescence bands of the probe were integrated as the area under the curve in the absence (A_0) and the presence of the polyphenol metabolite (A) between λ_{em} = 370 and 580 nm in Origin software (version 2021). Through Stern-Volmer equation (Eq. 3), where [Q] represents the concentration of polyphenol metabolites (M), it is possible to calculate the Stern-Volmer constant (K_{sv}) for the polyphenol metabolites.

$$\frac{A_0}{A} = 1 + K_{sv} \times [Q] \quad \textbf{(3)}$$

In case linearity is not observed in the Stern-Volmer plot, a new variable, the affinity constant (K_A) corresponding to the Stern-Volmer constant of the accessible fraction, is introduced. In this case, the previous Eq. 3 is modified to the following (Eq. 4):

$$\frac{A_0}{\Delta A} = \frac{1}{fa \times KA \times [Q]} + \frac{1}{fa} \quad \textbf{(4)}$$

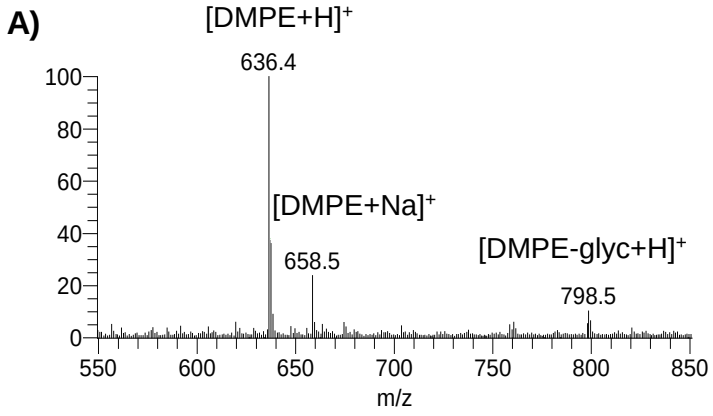
Similar to K_{SV}, this constant can also be determined through linear regression, from a graph of A0/ΔA as a function of the inverse concentration of polyphenol metabolites (1/[Q]).

3. Results

To investigate the diabetes-induced modifications in endothelial membrane, liposomes composed by PLPC, DMPE, Chol, and GSL were used. The choice of these lipids was based on the fact that they are the most abundant classes in epithelial cells, which are a type of endothelium (76). In normoglycemic conditions, the molar fractions of PLPC ranged from 0.289 to 0.322, DMPE varied between 0.135 and 0.145, Chol fluctuated between 0.234 and 0.263, SM ranged from 0.246 to 0.26, and GSL varied between 0.03 and 0.063. In hyperglycemic conditions, the molecular fractions of these lipids varied between 0.265 to 0.282 for PLPC, 0.152 to 0.165 for DMPE-glyc (which contains DMPE + DMPE-glyc), 0.271 to 0.28 for Chol, 0.234 to 0.263 for SM, and 0.028 to 0.065 for GSL.

3.1. Synthesis and Structural Characterisation of Glycated Dimyristoyl-Phosphatidylethanolamine (DMPE-Glyc) Adducts

The characterisation of glycated DMPE adduct was carried out without a purification step to avoid the extensive sample handling and thus the formation of degradation products (since glycated DMPE is highly susceptible to oxidation) (78,79). So, after the synthesis, the presence of DMPE-glyc adduct in the reaction mixture was qualitatively confirmed by mass spectrometry (ESI-MS). The ESI full mass spectrum of the reaction mixture (Figure 8A) shows the presence of the unmodified DMPE (reaction reagent) in protonated (*m/z* 636.4) and sodiated (*m/z* 658.5) form, as well as the reaction product, the protonated molecule of the glycated DMPE adduct (*m/z* 798.5).



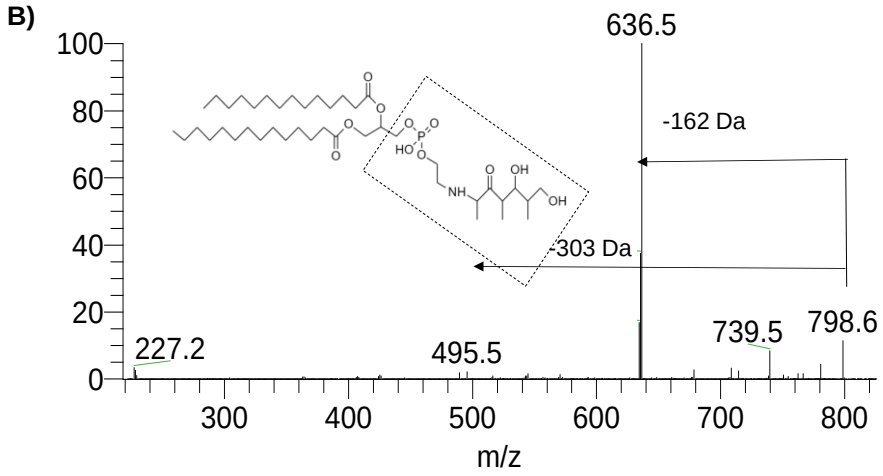


Figure 8 – Structural characterisation of DMPE-glycated adduct in the reaction mixture by electrospray mass spectrometry (ESI-MS) (A). Fragmentation spectrum of the precursor ion at m/z 798.6 (B).

To confirm that it was indeed the glycated DMPE adduct and not a contaminant, its fragmentation spectrum was analysed (Figure 8B). The fragmentation pattern revealed the presence of a product ion resulting from the loss of glucose (162 Da) from the precursor ion (m/z 798.6) and subsequently the glycated polar head (303 Da). The absence of the fragment ion formed by the loss of 120 Da, which is characteristic of Schiff bases (76), confirms that the synthesized DMPE-glyc adduct corresponds to an Amadori product.

3.2. The effect of glycation on DMPE transition temperature

The influence of glycation reaction on the transition temperature of DMPE was evaluated through DLS measurements. It is important to clarify that, although the primary goal was to evaluate the transition temperature of DMPE, the addition of PC was necessary for stability reasons. So, the DLS experiment has been made on PC:PE liposomes, and the transition temperature value for phosphatidylcholine (18:0/18:2 PC) and phosphatidylethanolamine (14:0/14:0 PE) is already established as below 6ºC and 50°C, respectively (80). SOPC's transition temperature was referenced instead of SLPC due to its structural similarity and the availability of data for meaningful comparisons. Furthermore, the liposomes employed the molar fraction of SLPC is approximately 0.3267, and the molar fraction of PE is approximately 0.6733. In the context of simulating the glycation process, these liposomes were deliberately composed with SLPC dominating the molar fraction at around 0.875, leaving the molar fraction of GlycPE at approximately 0.125.

In the graphs obtained by DLS for the unmodified and glycated PE (Figure 9), is evident that both plots show a clear gel-to-fluid transition.

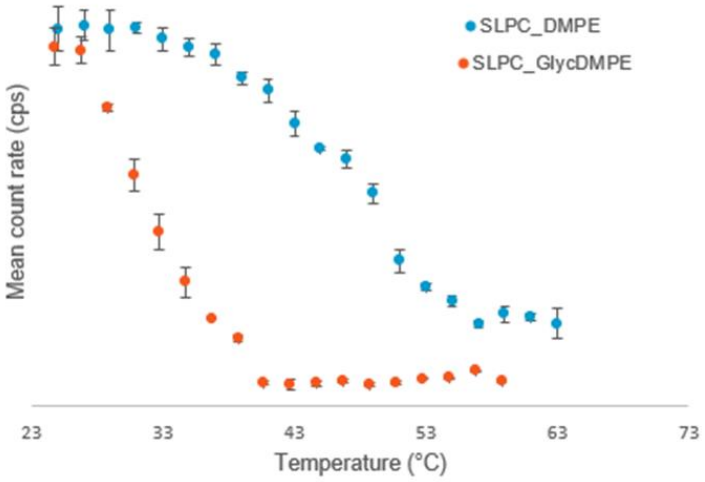


Figure 9 – Characterization of phase transition temperature of unmodified and glycated DMPE.

Therefore, it is possible to observe in Figure 10 that the transition between the two phases occurred at a lower temperature. This indicates that glycation of PE has reduced the energy required to induce phase transition.

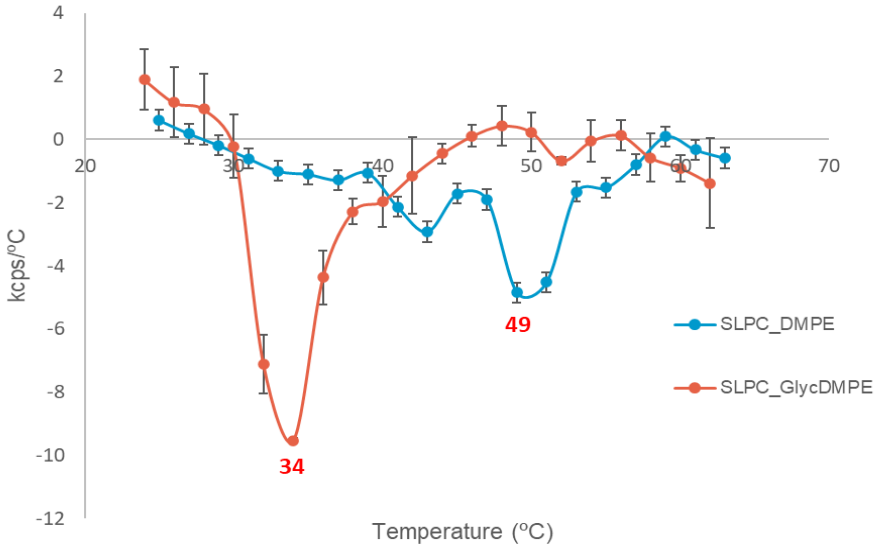


Figure 10 – Representation of the first-derivative plots of mean count rate/ ºC across the temperature range, reflecting the average of three assays.

3.3. Thermotropic Characterisation of Epithelial Membrane Models under Normo- and Hyperglycemic Conditions

For mimicking hyperglycemic conditions, a membrane model was built by increasing the phospholipid-to-cholesterol (PL/Chol) ratio to 1.6, contrasting with the normoglycemic state characterized by a PL/Chol ratio of 2.20. In addition, DMPE-glyc adducts were incorporated in an amount of approximately 4% of the total PE (76).

Steady-state fluorescence anisotropy with TMA-DPH probe was used to evaluate membrane fluidity, as a function of temperature, of liposomes under normo- and hyperglycemic conditions (Figure 11). The TMA-DPH probe adjusts its rotational movement to changes in membrane fluidity (81). Based on this principle, an increase in TMA-DPH anisotropy reflects less freedom of rotation, consistent with its location in a more ordered environment (82).

Consistently, in figure 11 it is possible to notice that TMA-DPH anisotropy values were found to be higher in hyperglycemic models compared to normoglycemic models. This indicates that in a situation of hyperglycemia, there is a decrease in membrane fluidity.

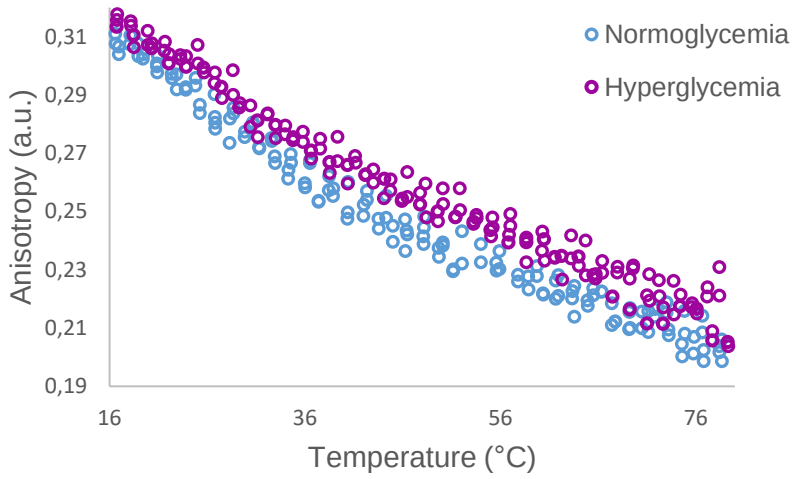


Figure 11 – Steady-state anisotropy of TMA-DPH, as a function of temperature, in liposomes under normo- and hyperglycemic conditions.

3.4. Laurdan generalized polarization measurements

Lipid order is another important biophysical property for the characterization of membrane models. The fluorescent probe Laurdan is sensitive to the polarity of the lipid bilayer and the shifts in its emission spectrum (Figure 12) can be quantified by calculating the excitation generalized polarization (GP_{exc}) (83) using Equation 2 (see section 2.7 of the Material and Methods). The GP_{exc} values are shown in Table 1.

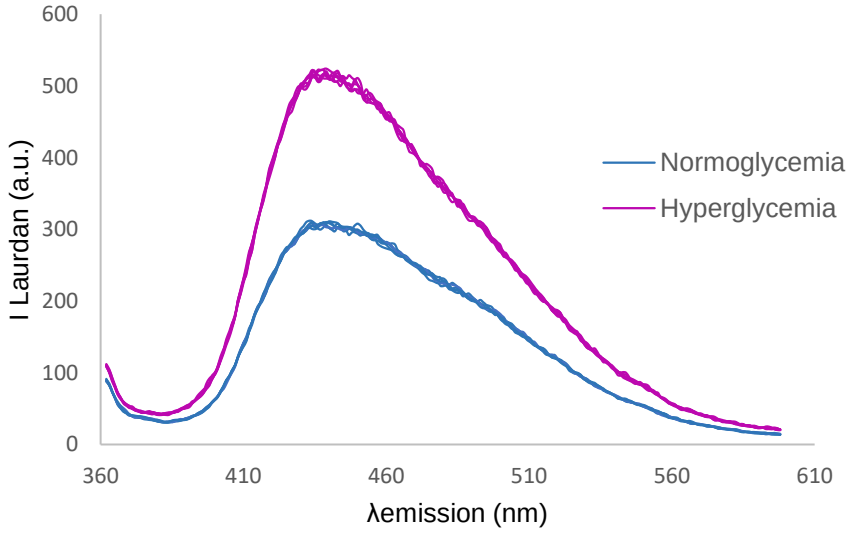


Figure 12 – Fluorescence emission spectra of Laurdan-labelled liposomes in normo- and hyperglycemic conditions.

Table 1- Excitation generalized polarization (GP_{exc} ± SD) values, at 37 ºC, determined for Laurdan- labeled liposomes under normo- and hyperglycemic conditions. These values are the result of four independent assays.

	GP_{exc}
Normoglycemia	0,220 ± 0,012
Hyperglycemia	0,245 ± 0,007

A high GP_{exc} value is indicative of an increase in lipid order and a decrease in membrane fluidity (84), visible in a situation of hyperglycemia.

3.5. Effect of Polyphenols Metabolites on the Fluidity of the Epithelial Membrane Model

Considering that polyphenol metabolites have beneficial anti-inflammatory effects in type 2 diabetes mellitus (47), we have used the metabolites HYP, HPPA, HPAA and PCA to investigate their potential in reversing the diabetes-induced biophysical membrane alterations. For this, we have conducted fluorescence anisotropy measurements using TMA-DPH labelled liposomes in normo- and hyperglycemic conditions in the presence of polyphenol metabolites at 25 µM, because it is their physiological blood concentration in normoglycemic conditions (86). Thus, we were able to understand how polyphenols interact with the plasma membrane and how they affect its fluidity.

Figure 13 shows that only HYP was able to induce alterations in TMA-DPH anisotropy values under normo- and hyperglycemic conditions. indicative of an increase in membrane fluidity (69).

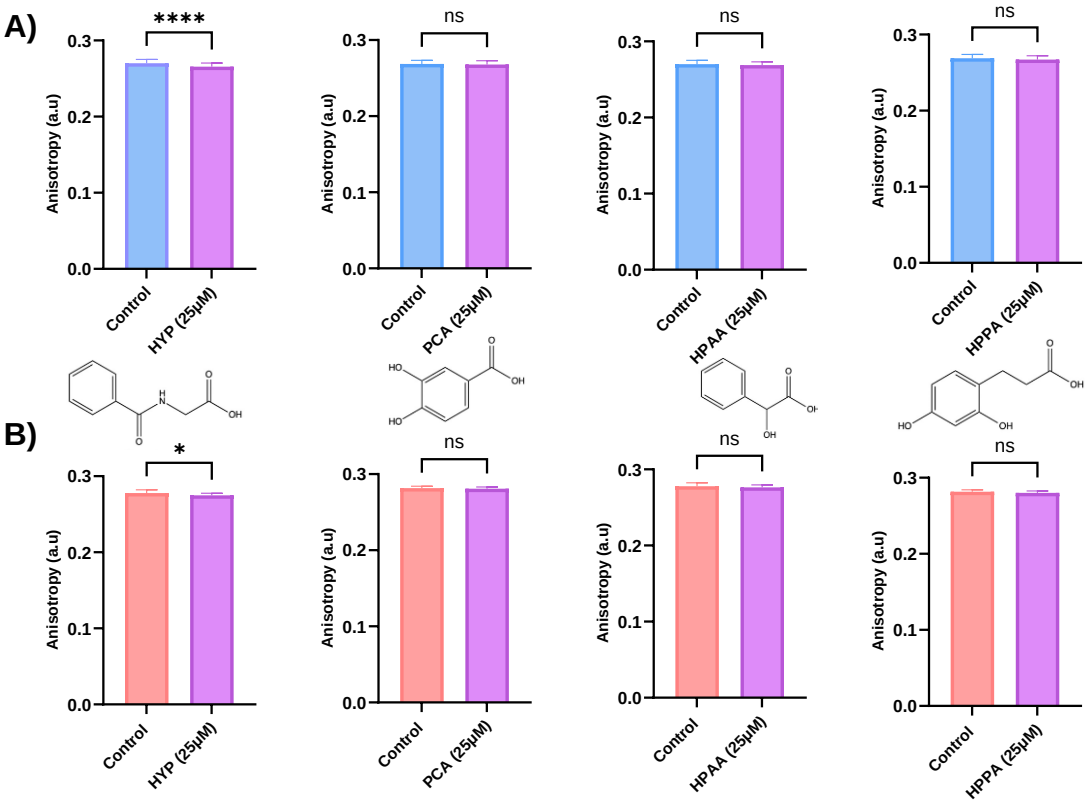


Figure 13- Anisotropy values, at 37 ºC, determined for TMA-DPH-labelled liposomes under normo- (A) and hyperglycemic (B) conditions in the absence (control) and presence of polyphenol metabolites [25µM].

ns stands for not significant

* Values are statistically different (P < 0.01)

**** Values are statistically different (P < 0.0001)

3.6. Influence of polyphenol metabolites structure on the interaction with membrane lipids

The location of polyphenol metabolites in the lipid bilayer was evaluated by fluorescence quenching of DPH and TMA-DPH probes. The location of these probes in the membrane is well known: TMA-DPH is located close to the phospholipid polar heads (87), while DPH is found deeply buried in the acyl chain region of the bilayer (88).

As an example, Figure 14 shows the steady-state fluorescence spectra obtained for DPH and TMA-DPH-labelled liposomes in normoglycemic conditions (500μM) incubated with HYP in the concentration range of 0-154μM.

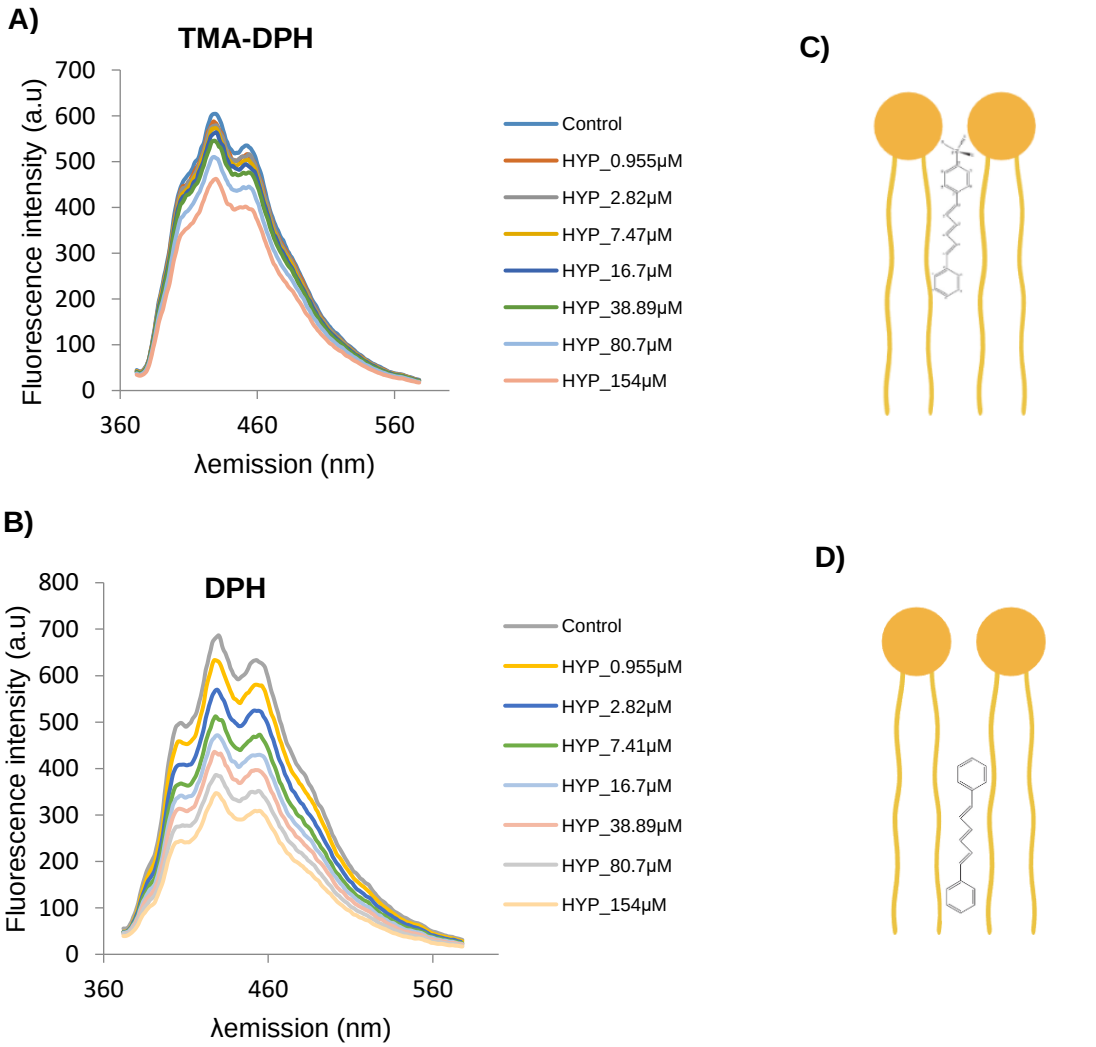
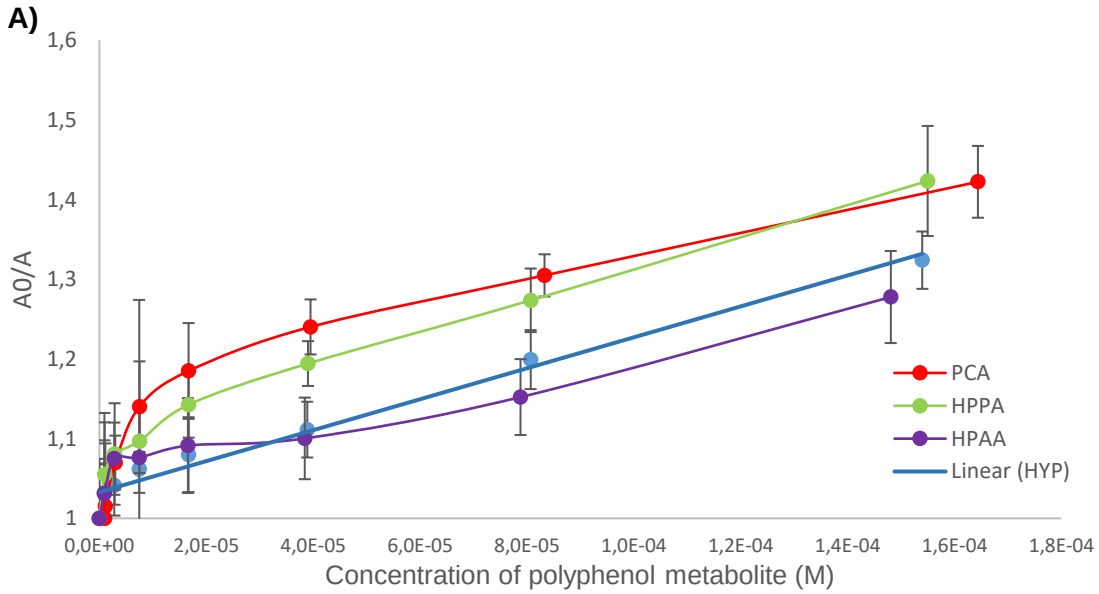


Figure 14- Fluorescence emission spectra of TMA-DPH (A) and DPH (B) labelled liposomes in normoglycemic conditions (500μM, 37°C), in the presence of increasing amounts of hippuric acid (of 0-154μM). Schematic representation of TMA-DPH (C) and DPH (D) probes in the lipid bilayer.

The fluorescence spectra obtained with increasing additions of polyphenol metabolites for the liposomes labelled with TMA-DPH probe under normo- and hyperglycemic conditions exhibited reduced quenching of fluorescence, as shown in Figures 1 and 2 of Attachments.

Under the same experimental conditions tested, and contrary to what was observed for its cationic form, a significant decrease in the fluorescence intensity of the DPH probe (fluorescence quenching) was observed with increasing additions of polyphenol metabolites (Figures 3 and 4 of Attachments). These results suggest an interaction between polyphenol metabolites and membrane lipids in the hydrophobic region of the plasma membrane.

The quenching of fluorescence can be quantified through the Stern−Volmer plots. Figure 15 shows the Stern–Volmer plot, the result of the steady-state fluorescence measurements (A0/A) of TMA-DPH labelled liposomes in normoglycemic (A) and hyperglycemic (B) conditions in the presence of HYP, PCA, HPPA and HPAA at different concentrations.



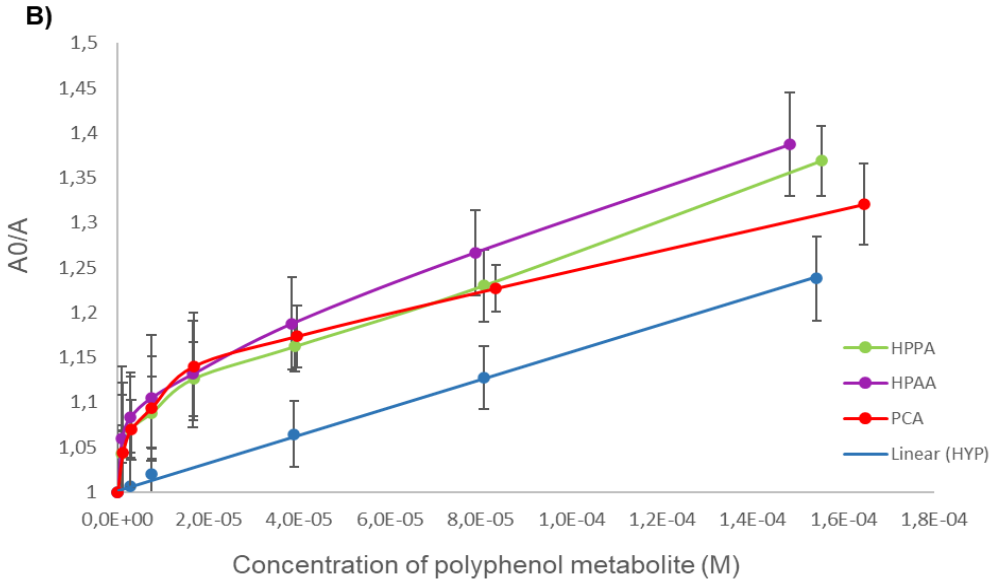
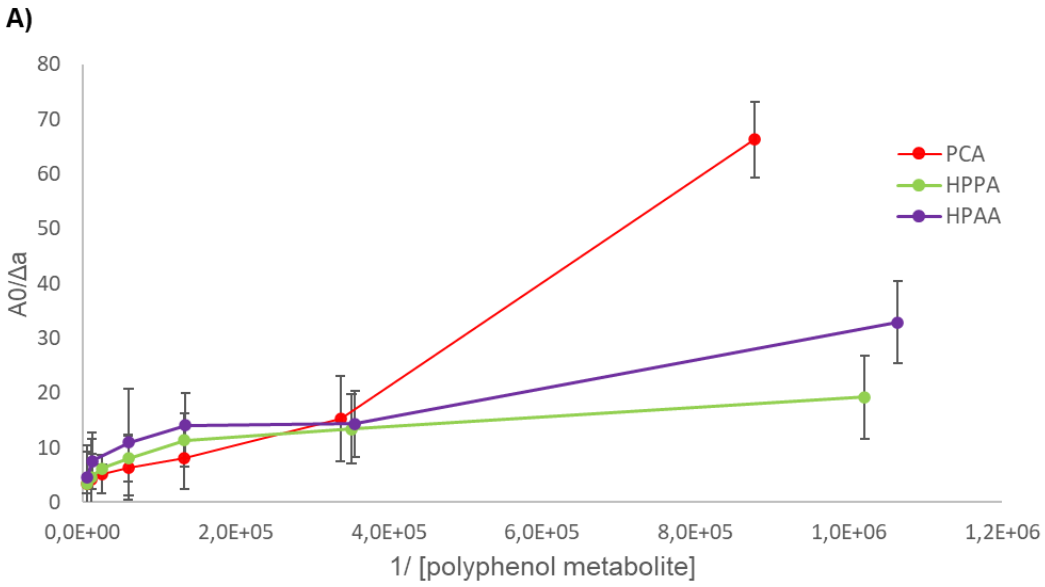


Figure 15- Stern-Volmer plot of the TMA-DPH labelled liposomes in normo- (A) and hyperglycemic (B) conditions with increasing concentrations of HYP, PCA, HPPA and HPAA (from 0 to 164 µM).

Linear behaviour was exclusively observed in the case of hippuric acid under both conditions. However, in the remaining cases (PCA, HPPA, and HPAA), the plots exhibited a deviation from linearity. Despite rigorous attempts to apply the modified Stern-Volmer equation (Equation 4, Materials and Methods) to linearize each of these curves, a linear plot could not be achieved (Figure 16).



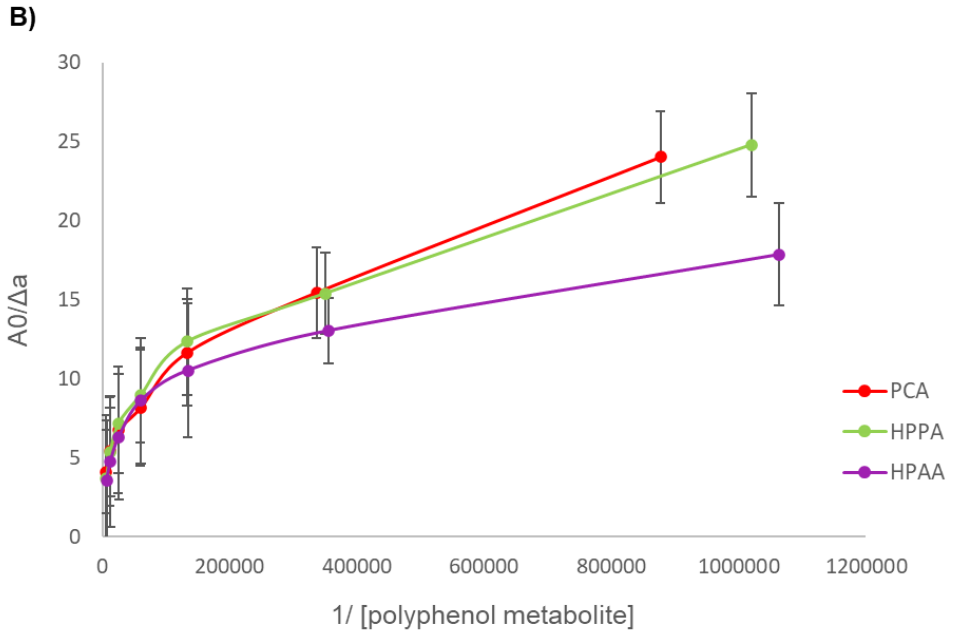
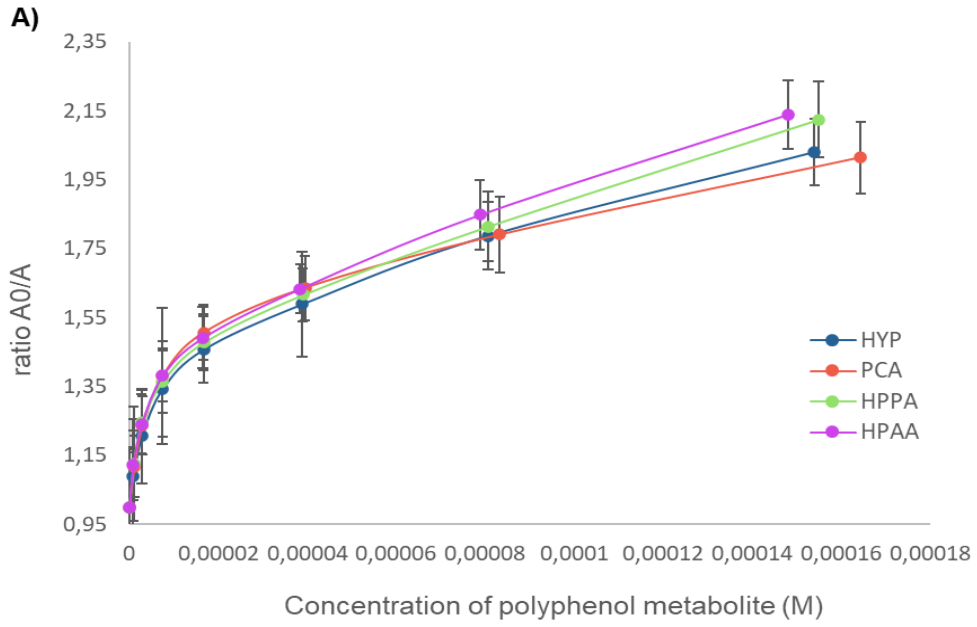


Figure 16- Modified Stern-Volmer plot of TMA- DPH labelled liposomes in normo- (A) and hyperglycemic (B) conditions with increasing concentrations of PCA, HPPA and HPAA (from 0 to 164 μM).

Figure 17 shows the Stern–Volmer plot, the result of the steady-state fluorescence measurements (A0/A) of DPH labelled liposomes in normoglycemic (A) and hyperglycemic (B) conditions in the presence of HYP, PCA, HPPA and HPAA at different concentrations.



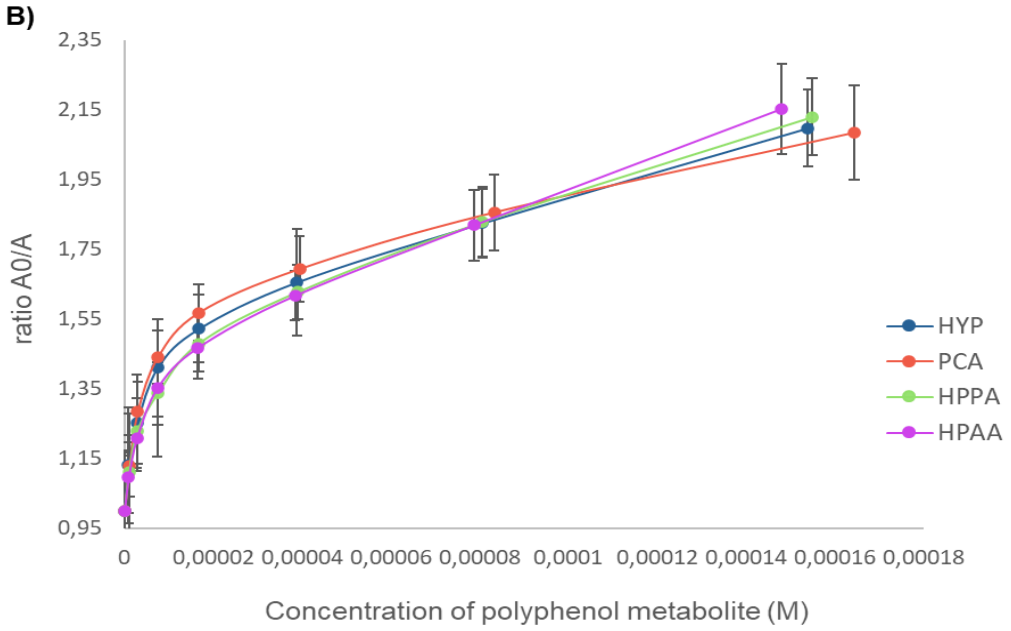
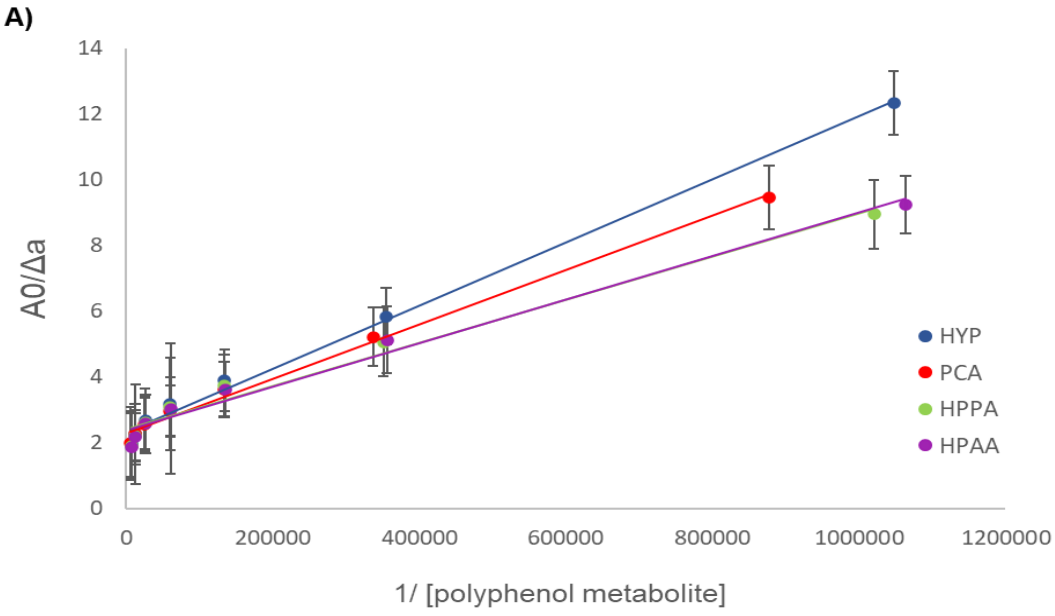


Figure 17- Stern-Volmer plot of the DPH labelled liposomes in normo- (A) and hyperglycemic (B) conditions with increasing concentrations of HYP, PCA, HPPA and HPAA (from 0 to 164 μM).

In contrast to the findings with TMA-DPH-labelled liposomes, the application of the modified Stern-Volmer equation demonstrated its efficacy in producing linear plots for liposomes labelled with the DPH probe. Therefore, figure 18 shows the resulting plot of A0/ΔA as a function of the inverse concentration of polyphenol metabolites for DPH-labelled liposomes incubated with diverse concentrations of HYP, PCA, HPPA, and HPAA under normoglycemic (A) and hyperglycemic (B) conditions.



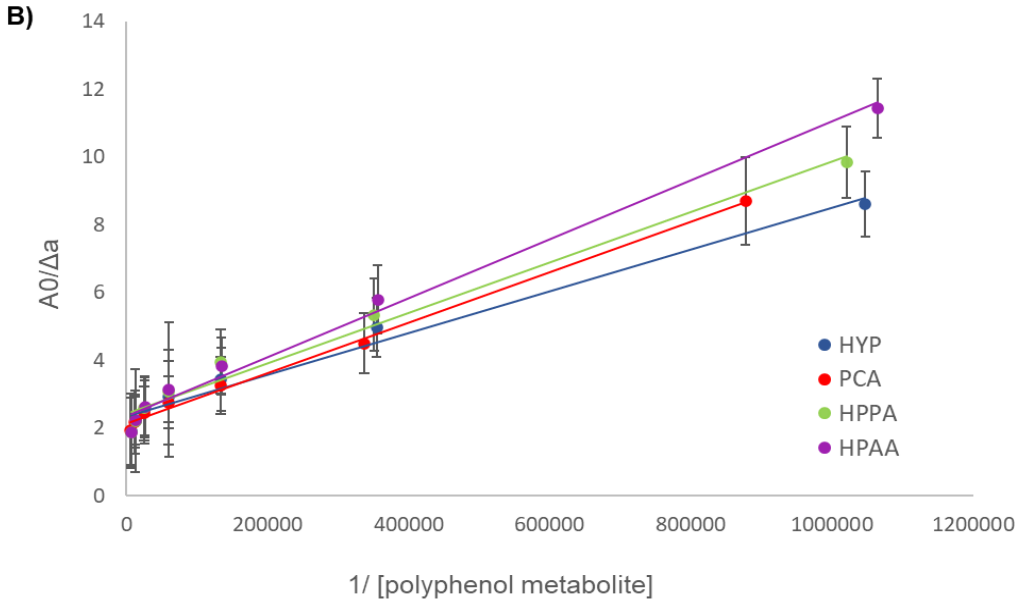


Figure 18- Modified Stern-Volmer plot of the DPH labelled liposomes in normo- (A) and hyperglycemic (B) conditions with increasing concentrations of HYP, PCA, HPPA and HPAA (from 0 to 164 μM).

The deviation from linearity is observed in Stern-Volmer plots indicates that both dynamic and static quenching may be contributing to the reduction of the fluorescence intensity of the probes. In dynamic quenching, the fluorescence of a fluorophore is diminished due to collisions between the fluorophore and the quencher. Static quenching is a mechanism where the fluorophore interacts with a quencher, forming a stable complex before the excitation of the fluorophore. Contrary to dynamic quenching, where the fluorescence intensity decreases as the quencher concentration increases, in static quenching the formation of stable complexes does not follow a direct linear relationship with the quencher concentration (89).

The K_A observed for the polyphenol metabolites in DPH labelled liposomes in normo- and hyperglycemic conditions are obtained from the modified Stern-Volmer equation. Just the opposite is verified in the case of HYP in TMA-DPH labelled liposomes in normo- and hyperglycemic conditions, where the K_{SV} are obtained through the linear Stern–Volmer plot.

Table 2- Affinity constant (K_A) values of HYP, PCA, HPPA and HPAA in DPH labelled liposomes in normo- and hyperglycemic conditions, obtained through the equation $\frac{A0}{\Delta A} = \frac{1}{fa\ x\ KA\ x\ [Q]} + \frac{1}{fa}$

DPH labelled liposomes				
PP Metabolites	**Normoglycemia**		**Hyperglycemia**	
	Ka	fa	Ka	fa
HYP	$2,4\times 10^5$	0,43	$3,9\times 10^5$	0,42
PCA	$2,9\times 10^5$	0,44	$3,1\times 10^5$	0,47
HPPA	$3,5\times 10^5$	0,41	$3,5\times 10^5$	0,41
HPAA	$3,4\times 10^5$	0,42	$2,6\times 10^5$	0,43

Table 3- Stern-Volmer constant (K_{SV}) values of HYP in TMA-DPH labelled liposomes in normo- and hyperglycemic conditions, obtained through the equation $\frac{A0}{A}$ = 1+ Ksv × [Q]

TMA-DPH labelled liposomes		
PP Metabolites	**Normoglycemia**	**Hyperglycemia**
	K_{SV}/M^{-1}	K_{SV}/M^{-1}
HYP	$1,94 \times 10^3$	$1,53 \times 10^3$

All polyphenol metabolites in the tested panel showed different K_A values, suggesting that each metabolite interacts differently with the probe. These interactions are also contingent on the glycemic state. Under hyperglycemic conditions, HYP proved to be the most effective metabolite in inducing quenching of the DPH probe, while under normoglycemic conditions, HPPA outperformed the rest of the panel.

For TMA-DPH labelled liposomes, it was only possible to determine the K_{SV} value for HYP, as it exhibited a linear plot. A reduction in TMA-DPH probe quenching was observed in hyperglycemic situations (increased PL/Chol ratio and presence of DMPE-glyc), indicated by a lower K_{SV} value.

Discussion

Mediterranean and Nordic diets, both rich in polyphenols, have been shown to mitigate diabetes-associated complications by improving vascular health and glucose metabolism, and reducing inflammation and oxidative stress (21,22,24,32).

However, it is important to highlight that throughout the digestive tract, polyphenols undergo an intense metabolization process, with only 2% of dietary polyphenols reaching the circulation (44,46). Therefore, it is believed that most of the beneficial effects associated with chronic diseases are due to polyphenol metabolites and not dietary ones. Although the metabolic journey of dietary polyphenols must be considered when studying their beneficial properties, most studies in membrane models carry out investigations with polyphenols found in food (51,52,90,91).

In order to understand the polyphenol-lipid interactions and their impact on the biophysical properties of the endothelial membrane in T2D, a membrane model was built with a complex lipid composition characteristic of the apical domain of epithelial membranes. The decision to opt for this approach was due to the significant limitation that this study faces from the beginning: restricted knowledge of the endothelial lipidome, which plays a crucial role in understanding diabetes-induced vascular damage. To overcome this limitation, the adopted model includes modifications in the PL/Chol and PC/PE ratios along with a more physiologically relevant amount of glycated DMPE (92,93), in an attempt to replicate the endothelial membrane in a T2D condition (76).

After the synthesis of the glycated DMPE adduct, its fragmentation spectrum (Figure 8B) was analysed to confirm its suitability for incorporation into the membrane model. The fragmentation pattern revealed the presence of a product ion resulting from the neutral loss of 303 Da, corresponding to the glycated polar head group, consistent with findings in prior studies (78). Therefore, having confirmed the presence of glycated DMPE adduct as an Amadori product rather than a contaminant, it was possible to evaluate the impact of glycation on the transition temperature through DLS measurements.

In fact, the glycation of DMPE reduced the energy required to induce the gel-fluid phase transition (Figure 10) in agreement with what *Annibal et al*. reported through differential scanning calorimetry curves (79). This can have substantial repercussions for membrane fluidity and cellular function in diabetes. During long-standing hyperglycemia, there is an increase in the amount of AGEs formed. AGEs can interact directly by cross-linking of membrane proteins, causing significant changes in their structure and stability (94,95). AGE-RAGE signalling promotes an increase in oxidative stress and the production of

pro-inflammatory cytokines by triggering NF-κB activation (59). Additionally, AGE-RAGE interaction also impairs insulin signalling and glucose transport in cells, contributing to the development of insulin resistance (96). In summary, the glycation process can play a significant role in the pathophysiology of T2D, either by promoting insulin resistance or by inducing vascular stiffness.

Another crucial factor that significantly influences membrane fluidity is lipid composition. Prolonged hyperglycemia can lead to changes in the lipid profile, leading to an increase in stiffness-promoting lipids, with cholesterol being a notable example (64,67). To understand the influence that T2D may have on membrane fluidity, steady-state fluorescence anisotropy with TMA-DPH labelled liposomes in normo- and hyperglycemic conditions was carried out. The outcomes (Figure 11) reveal that the TMA-DPH anisotropy values exhibited a greater increase with the elevation of temperature in liposomes under hyperglycemic conditions compared to those in normoglycemic situations. The fluorescence anisotropy values of TMA-DPH are inversely proportional to cell membrane fluidity (97). Therefore, under hyperglycemic conditions, there is a decrease in membrane fluidity, which is in line with what has been published in the literature (65,71,72,98). This can be attributed to several factors, including changes in the Chol/PL ratio and the presence of glycated DMPE molecules in a situation of T2D. Cholesterol, due to its rigid tetracyclic structure, can intercalate between phospholipids in lipid bilayers thereby modifying the order and decreasing the fluidity of the membrane (99–102). It is important to emphasize that changes in membrane fluidity are not just a consequence of diabetes but may accelerate the progression of associated vascular complications. Understanding how hyperglycemia, glycation, lipid profile changes, and other factors impact membrane fluidity can show valuable insights into the disease's mechanisms and its long-term consequences.

In hyperglycemic conditions, liposomes revealed an increase in lipid order (Table 1) in clear contrast to membrane models in normoglycemic states. These findings, obtained due to the sensitivity of Laurdan probe to changes in lipid order, are in agreement with previously published studies (68,69). The ordering effect mentioned can be associated with an increase in membrane stiffness that has already been reported. These factors can lead to a less permeable membrane (103) and thicker, thereby affecting the conformation and function of membrane proteins and, ultimately, the proper functioning of the cell (64).

The hypothesis under consideration was whether polyphenol metabolites derived from gut microbiota, known for their inherent anti-inflammatory properties (85), could reverse

these diabetes-induced biophysical alterations in the cell membrane. For this purpose, commercially purchased polyphenol metabolites (HYP, HPPA, HPAA, and PCA) were used to simulate the metabolites produced by gut microbiota, at a concentration of 25 µM. The decision to employ a 25 µM concentration in this study was driven by the objective of closely mimicking the concentrations typically found in the bloodstream of individuals, who regularly consume a polyphenol-rich diet (86). While there is research demonstrating that dietary polyphenols can influence membrane fluidity (48,50,90,91), little is known about the specific effects of polyphenol metabolites on membrane fluidity.

The data, obtained by steady-state anisotropy measurements using TMA-DPH labelled liposomes under normo- and hyperglycemic conditions, following incubation with polyphenol metabolites, indicate that among the tested compounds, only HYP exhibited a significant ability to increase membrane fluidity in both conditions (Figure 13). These results can be explained by considering that the impact of polyphenol metabolites on membrane fluidity is influenced by multiple factors. These factors encompass the chemical properties of polyphenols as well as their concentration. Furthermore, the composition of the lipid bilayer itself, especially the cholesterol content, can also influence how polyphenols interact with the membrane and exert their beneficial properties (51). However, it should be noted that this approach is not a fully reliable representation, as the biotransformation reactions occurring in the organism are always much more complex.

The analysis of fluorescence spectra, Figures 14 and Figures 1, 2, 3 and 4 of Attachments, provided insights into the localization of the tested compounds within the lipid bilayer's hydrophobic region, mediated by the DPH probe. Notably, these findings challenge existing literature, which typically describes dietary polyphenols as being positioned at the lipid-water interface (49,51). The data obtained sheds light on the location of polyphenol metabolites in the lipid bilayer.

The affinity constant (K_A) values varied for all polyphenol metabolites in DPH-labelled liposomes. The K_A values of PCA and HPAA decreased under hyperglycemic conditions, suggesting that these metabolites are less effective in promoting the quenching of the DPH probe in these circumstances. The HPPA values remained relatively constant. In the case of HYP, the K_A values increased in a hyperglycemic situation (Table 2). Conversely, the Stern-Volmer constant (K_{sv}), determined through fluorescence quenching studies for TMA-DPH-labelled liposome incubated with HYP was higher in normoglycemic situations than in hyperglycemic ones (Table 3).

Although the calculation of the partition constant (K_p) for the panel of metabolites was not performed in this study, according to existing literature, K_{sv} and K_p are typically proportional to each other (81).

Considering these outcomes, it can be inferred that the tested polyphenol metabolites interact with the membrane models, under both normo- and hyperglycemic conditions. However, this interaction does not translate into a significant change in membrane fluidity.

Conclusion

In overview, this work presents, for the first time, insights into the interaction between polyphenol metabolites derived from gut microbiota and membrane lipids, in a hyperglycemic condition.

The elevated Chol/PL along with the glycation process impacted the biophysical properties of these membrane models, namely membrane fluidity and lipid order.

Moreover, our data revealed that polyphenol metabolites derived from gut microbiota interacted with membrane lipids, especially within the hydrophobic regions. Even though this interaction did not translate into a significant change in membrane fluidity.

This epithelial membrane model can be further employed in investigating polyphenol-lipid interactions within endothelial cells. This will improve our knowledge of the molecular mechanisms underlying the beneficial effects of polyphenol-rich diets on the biophysical properties of the endothelium.

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Attachments

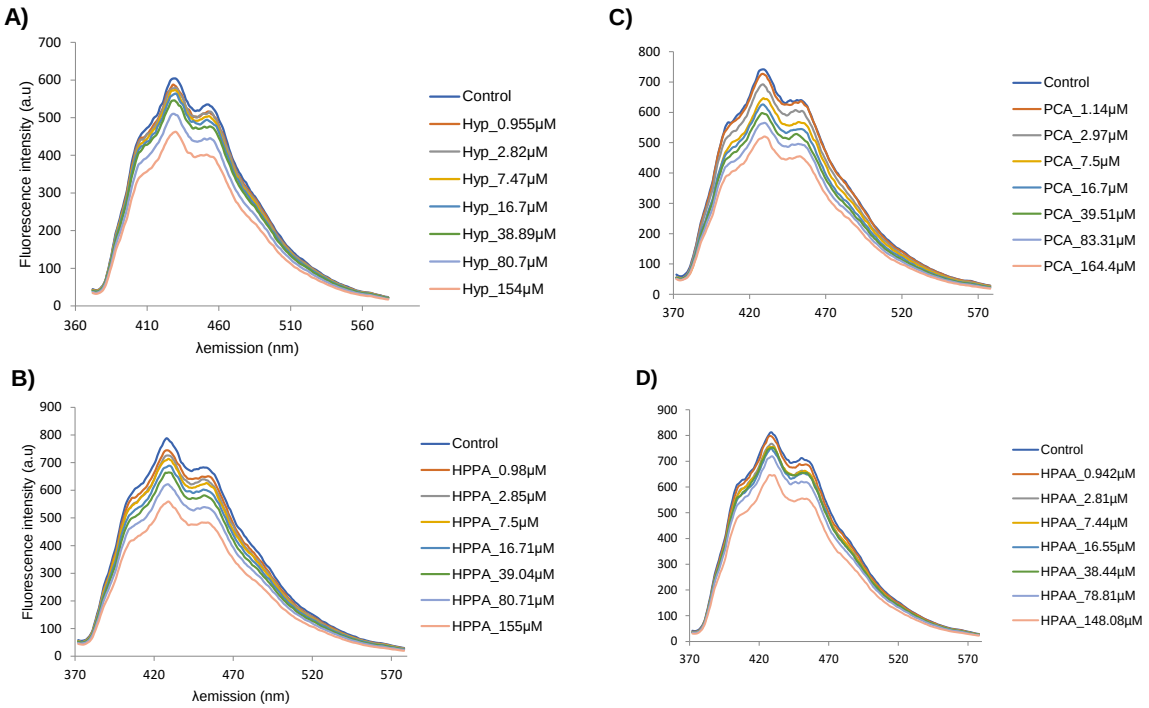

Figure 1- Fluorescence emission spectra of TMA-DPH labelled liposomes in normoglycemic conditions (500μM, 37°C), in the presence of increasing amounts of HYP (A), HPPA (B), PCA (C) and HPAA (D).

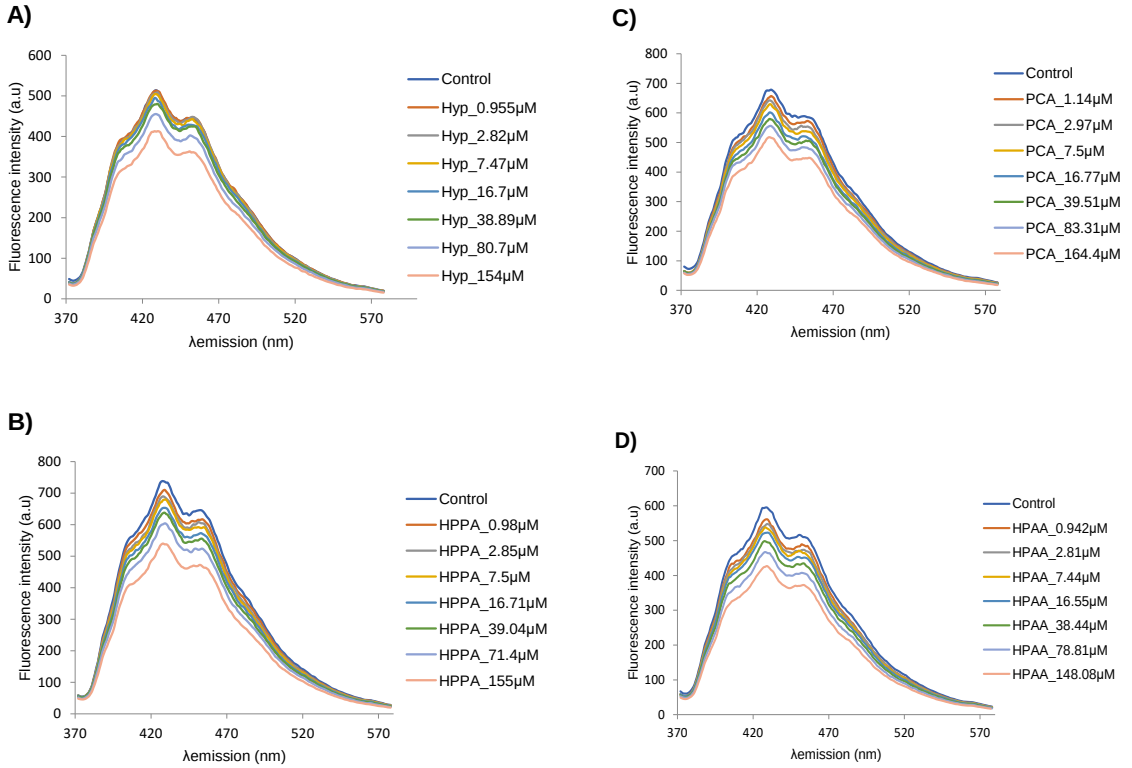

Figure 2- Fluorescence emission spectra of TMA-DPH labelled liposomes in hyperglycemic conditions (500μM, 37°C), in the presence of increasing amounts of HYP (A), HPPA (B), PCA (C) and HPAA (D).

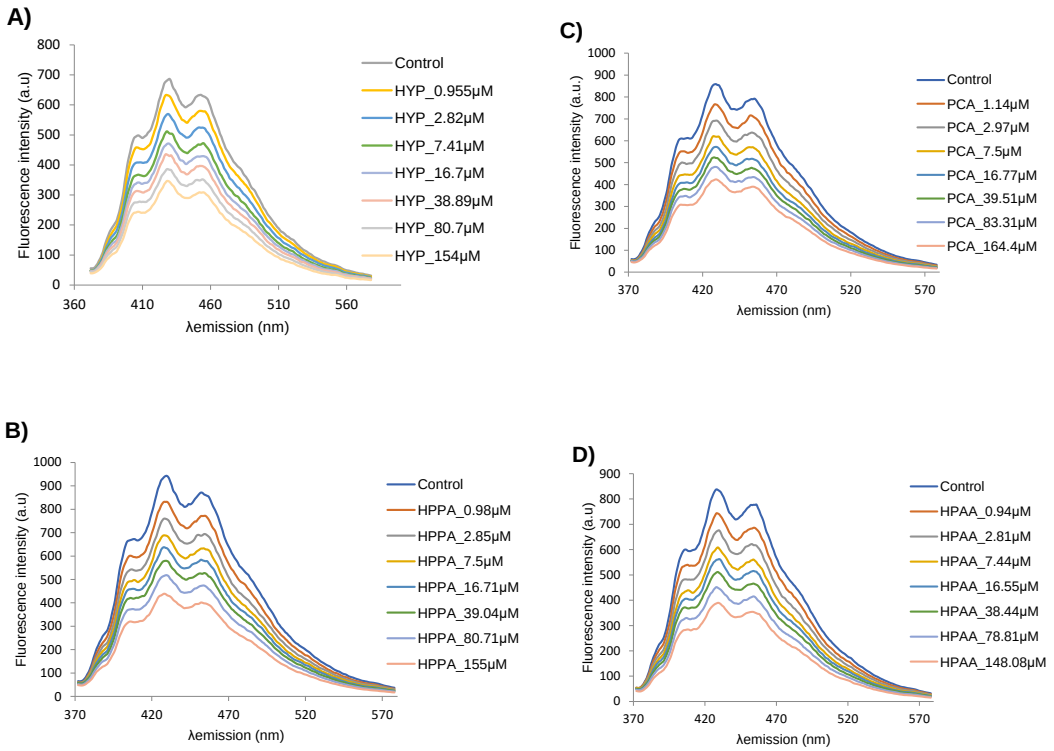

Figure 3- Fluorescence emission spectra of DPH labelled liposomes in normoglycemic conditions (500μM, 37°C), in the presence of increasing amounts of HYP (A), HPPA (B), PCA (C) and HPAA (D).

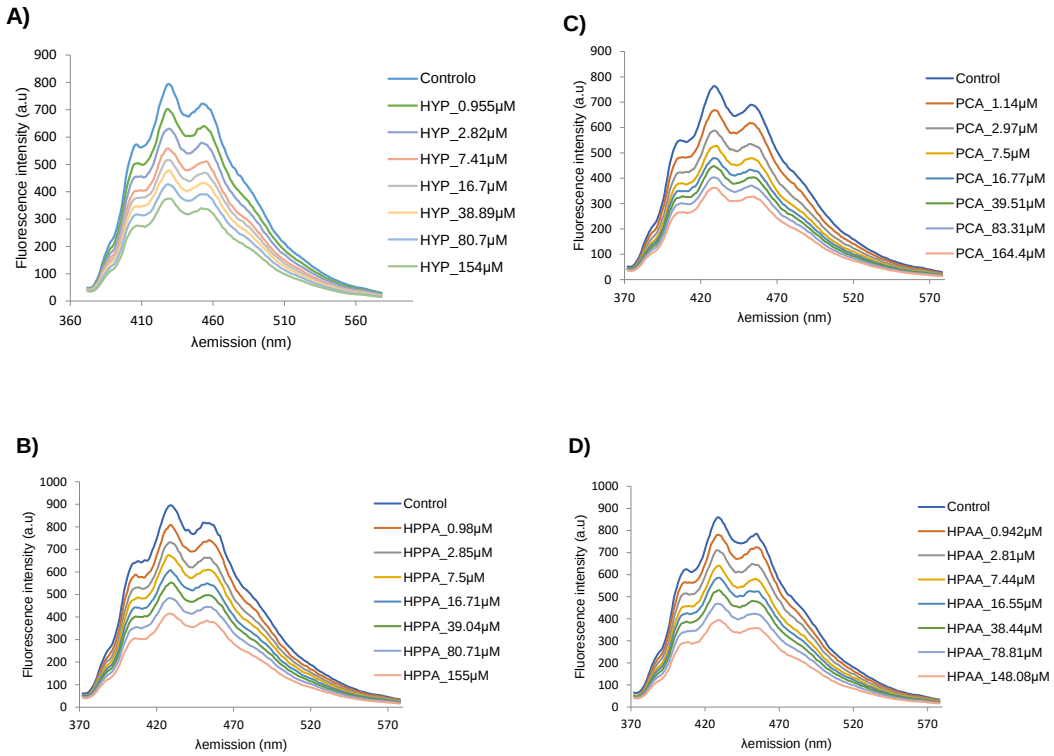

Figure 4- Fluorescence emission spectra of DPH labelled liposomes in hyperglycemic conditions (500μM, 37°C), in the presence of increasing amounts of HYP (A), HPPA (B), PCA (C) and HPAA (D).