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Reactive Deep Eutectic Solvents (RDESs): A New Tool for Phospholipase D-Catalyzed Preparation of Phospholipids

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Abstract: The use of Reactive Deep Eutectic Solvents (RDESs) in the preparation of polar head modified phospholipids (PLs) with phospholipase D (PLD)-catalyzed biotransformations has been investigated. Natural phosphatidylcholine (PC) has been submitted to PLD-catalyzed transphosphatidylations using a new reaction medium composed by a mixture of RDES/buffer. Instead of exploiting deep eutectic solvents conventionally, just as the reaction media, these solvents have been designed here in order to contribute actively to the synthetic processes by participating as reagents. RDESs were prepared using choline chloride or trimethyl glycine as hydrogen-bond acceptors and glycerol or ethylene glycol, as hydrogen-bond donors as well as nucleophiles for choline substitution. Specifically designed RDES/buffer reaction media allowed the obtainment of PLs with optimized yields in the perspective of a sustainable process implementation.

Keywords: phospholipids; phospholipase D; transphosphatidylation; reactive deep eutectic solvent (RDES)

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1. Introduction

In recent years, the demand for new synthetic approaches with reduced environmental impact is highly present in the political agenda. In this context, the use of new protocols involving biocatalysis as well as the investigation of greener suitable solvents in the chemical modification of organic materials fits perfectly the research focused on the achievement of the Sustainable Development Goals [1–4].

Deep Eutectic Solvents (DESs) are a relatively new class of solvents [5,6] prepared for the first time by Abbott et al. in 2003 [7] and, since then, deeply studied in many fields including, among the others, materials science [8], analytical and preparative separation [9,10], biomass processing [11,12], organic synthesis [13,14], and biocatalysis [15–19]. DESs are eutectic mixtures composed by a hydrogen–bond acceptor (HBA) and a hydrogen–bond donor (HBD) that can be easily prepared by mixing and heating the components generating clear liquid phases, which present a significantly lower melting point compared to their individual components. Clearly, these solvents are a promising alternative to ionic liquids (ILs), since they share many important features [20] such as low volatility, conductivity, high thermal stability, negligible flammability, and a broad liquid range. DESs can be prepared from a broad range of components including biomass-derivatives and renewable organic compounds. DESs have therefore a key advantage in the development of reaction media for environment-friendly and sustainable chemical processes [21]. Moreover, a distinctive feature of DESs is the extensive hydrogen bonding that, besides ensuring

very good and modulable solvating properties, can actively participate in the process as catalyst or co-catalyst [13]. Because of the very large combinatorial space of the main components [6] and the possibility to add co-solvents or anti-solvents such as water [22], DES-based process media benefit from an exceptional versatility, which allows the design of highly optimized mixtures with special characteristics such as specific catalytic activities and adjusted solvation properties. All of these qualities lead the way to the development of tailored biocatalytic processes [17,23,24], particularly with the possible addition of a water component in order to ensure the establishment of an enzyme-friendly environment [24,25]. On top of that, a recent and very interesting development is the promotion of the DESs role from solvent/catalyst to actual reactive component. Reactive Deep Eutectic Solvents (RDESs) can exploit the versatility of DESs in such a way to provide a reaction medium mixture containing one or more process reagents, resulting in several advantages that include a substantial mass effect due to the very high concentration. Moreover, the highly adaptable solvating properties allow for the designing of specifically tuned mixtures for the reactants dissolution as well as for the efficient separation of products by precipitation, enhancing the overall process yield. This can be particularly important in the optimization of enzymatic processes based on equilibrium reactions, a common case in bio-based sustainable green chemistry. RDESs are known to have been successfully employed in cellulose derivatization [26,27], free-radical polymerizations [28], heterocyclic synthesis [29,30], and in biocatalytic processes [24] such as the enzymatic synthesis of 1,3-diacylglycerols [31], glycolipids [32], and menthol fatty acid esters [33].

Phospholipids (PLs) represent the main constituents of all biological membranes and they are the topic of many areas of biomedical research due to their natural involvement in many cellular functions such as cellular regeneration processes, differentiation of neurological pathways, and promotion of molecules transportation through cell membranes [34,35]. Furthermore, they are able to promote the biological activity of various membrane linked proteins and receptors, and they have been successfully exploited as therapeutic agents, drug-delivery systems, and diagnostic markers for different diseases [36–39]. Moreover, some neurological pathologies such as stress-related disorders, schizophrenia, dementia, and Parkinson's disease have showed to be associated with a disequilibrium in PLs metabolism, signaling, and transport [40,41]. Dietary PLs are essential for preventing a broad range of human diseases such as inflammation, cancer, and coronary heart issues [42,43]. Other interesting applications involve their use as natural emulsifiers, surfactants, food stabilizers, and detergents [44,45]. From a structural point of view, as reported in Figure 1, PLs molecules present a glycerol backbone, esterified in *sn*-1 and *sn*-2 positions with saturated or unsaturated long chain fatty acids (lipophilic moieties) and in *sn*-3 with a phosphate diester (polar head group).

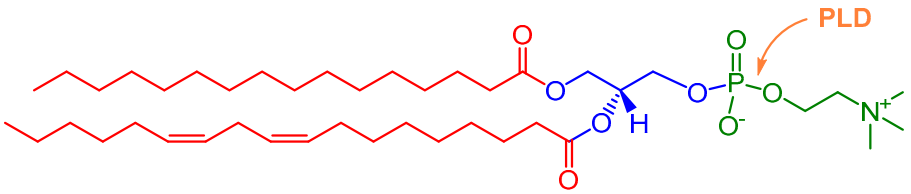


Figure 1. General structure describing the PC (here 1-palmitoyl-2-linoleyl-PC) structure composed by two acyl chains (in red), a glycerol backbone (in blue) and a polar head (in green). The attack position cleaved by PLD is also indicated with the orange arrow.

The amphiphilic nature of these molecules, due their peculiar structure, is the key factor responsible for their spontaneous aggregation (in bilayers and micelles) in aqueous environments, a very appealing property for the applications within the drug delivery and cosmetic sectors [46]. In the last year, after the beginning of the Covid-19 pandemic, the design of new vaccines has been the major studied worldwide topic. In particular, the design considerations about the new formulations have focused on liposome preparations, which are employed in modern vaccine technologies because their design is mandatory to

ensure proper immune responses [47,48]. Liposomes serve in fact as adjuvants with the role of potentiating the immune response of the vaccines by improving their efficacy [49].

The biological activity of PLs is strictly dependent on the chemical identity of the polar head and the acyl chains. The commercial values of these compounds are highly related to the source and the purity of the products [50]. Natural PLs can be obtained from vegetable sources such as soybeans, sunflower, corn, peanut, flaxseed, rape (canola) seed wheatgerm, and animal sources like egg yolk, meat, milk and krill [51–54]. The challenging chemical preparation of the different PLs can be achieved by semi- or total synthesis starting from appropriate chiral precursors. These procedures usually require, in addition to the introduction of the desired functional groups, a complex sequence of protection and deprotection steps. For these reasons, the set-up of simpler biocatalytic approaches for the preparation of these specific PLs from natural sources is a suitable alternative. Furthermore, these greener protocols based on the use of harmless, natural, and safer reagents are especially preferred when the product's destination is for the food or pharmaceutical sectors [53,55,56].

The most abundant PL in nature is phosphatidylcholine (PC, reported in Figure 1 as 1-palmitoyl-2-linoleyl-PC). This compound can be modified in its polar head moiety by means of phospholipase D (PLD, E.C.3.1.4.4), an enzyme able to hydrolyze PC into phosphatidic acid (PA). In particular, different bacterial PLDs from *Streptomyces sp.* were studied in our research group [57–59]. Although they are extracellular hydrolytic enzymes with a broad substrate specificity, they are also able to catalyze a transphosphatidylation reaction in the presence of an alcohol (X-OH) allowing the formation of PX by the polar head modification even in the presence of water as a cosolvent [60–62]. This property is quite unique among hydrolytic enzymes, and it is essential in practical applications for the preparation of non-natural PLs or less natural abundant ones, starting from crude or purified PC (see Figure 2).

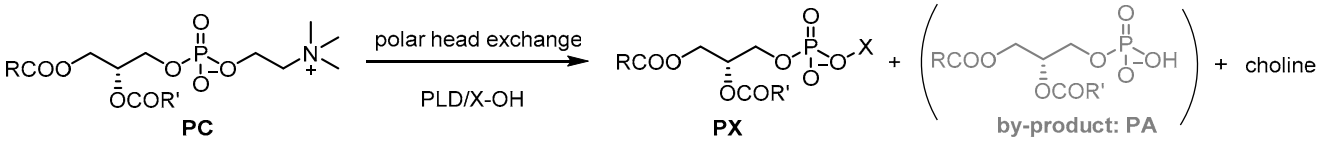


Figure 2. Phospholipase D-catalysed transformations of PC.

These reactions are usually carried out in aqueous/organic solvents biphasic systems because, due to their amphiphilic features, PLs are only soluble in organic solvents with low polarity such as chloroform, diethylether, hexane, and toluene. However, in these reaction media, the formation of PA as a by-product is quite unavoidable, but the extent of the competing hydrolytic reaction depends on the nature of the nucleophile and on the reaction conditions (see Figure 2). Hence, the competitive production of PA lowers the transphosphatidylation yield, and the need of further purification procedures becomes mandatory. The purification of the final products PX is not straightforward, and it requires various chromatographic and selective precipitation steps. For this reason, the modification and the improvement of the reaction conditions in the synthesis of polar head modified PLs have been the object of many studies exploiting new biocatalytic strategies [63]. In particular, the screening of a great number of novel eco-friendly solvents such as γ-valerolactone, limonene, and *p*-cymene were used for the synthesis of phosphatidylserine (PS), a PL with a high commercial interest because of its nutritional and medical functions [64–67]. On this topic, the authors investigated the possibility to perform the preparation of PS, exploiting a new solvent composed by the ionic liquid (IL) 1-butyl-3-methylimidazolium hexafluorophosphate [BMIm][PF_6] that allowed the preparation of the desired product with 91.4% yield with a quite complete suppression of unwanted hydrolytic side reaction [68].

The use of DESs as a solvent for the PLD-catalyzed transformations of PC was only reported by Yang et al. [69] for the preparation of PS using a commercial enzyme from

Streptomyces chromofuscus. In this work, the authors reported the screening of different DESs and identified ChCl/Glycerol (Gly) and ChCl/ethylene glycol (EG) including 5% of water as the most suitable solvents for PS synthesis.

In the present work, the use of RDES-based protocols for the biocatalytic conversion of PC in two different PLs, the natural phosphatidylglycerol (PG), and the non-natural phosphatidylethyleneglycol (P-EG) has been investigated. All conversions were catalyzed by PLD from *Streptomyces netropsis*. The glycerol and ethylene glycol needed for the transphosphatidylation reactions were supplied as the reactant part of RDESs. Tailored RDES/buffer mixtures have been developed in order to optimize the effects of the medium composition in a perspective of a more sustainable process definition. Reaction yields have been optimized both by the high reactant concentration due to the use of RDES and by tuning the solvation capability of the RDES/buffer medium in order to obtain the product separation by precipitation. Moreover, using RDES/buffer medium, unwanted PC hydrolysis appears nearly completely inhibited leading to a yield enhancement and a higher product purity.

2. Results and Discussion

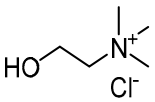
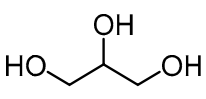
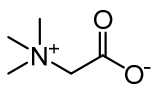
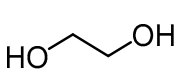
2.1. Preliminary Method Development

The first tests were carried out to screen the solubility of PC in order to select the potential DES candidates for the biocatalytic transformations aiming to produce PS, using PLD from *Streptomyces netropsis*. In particular, ChCl/Gly 1/2 (mol/mol), ChCl/acetic acid 1/2, choline *p*-toluensulphonate/acetic acid 1/2, ChCl/urea/acetic acid 1/1/1, and ChCl/levulinic acid 1/2 were tested. The starting PC was soluble in all these solvents, but only the first DES of the list was selected for the first enzymatic trials. The low pH (around 3) associated with all other DESs was in fact unsuitable for the enzymatic activity. However, using ChCl/Gly, besides the desired PS, the presence of PG (derived from the parasitic reaction with DES) was detected as well as a high quantity of unreacted PC. This leads to the decision of developing a specific RDES reaction medium, where the reacting nucleophile was provided as part of the process medium. The nature of the HBA component of RDES and the water content of the reaction medium (needed to preserve the enzymatic activity) were studied in order to increase the solubility of PC and decrease those of the products. The limitation of the product concentration in the reaction medium by precipitation allowed the enhancement of the yields. The RDES design process yielded to the formulations of four RDESs (Section 2.2) and, of the final reaction medium, composed by a RDES/water buffer mixture (1:1, *v*/*v*).

2.2. Preparation of RDES Media

In this work, four different RDESs consisting of mixture of HBA/HBD in a 1:2 molar ratio have been prepared. The quaternary ammonium salts choline chloride (ChCl) and trimethylglycine (also called betaine glycine; BetG) have been used as HBAs, whereas glycerol (Gly) and ethylene glycol (EG) constituted HBDs and reactants (see Table 1).

Table 1. Structure, name, and symbols of the HBAs and HBDs used here as components in the preparation of RDESs.

Hydrogen–Bond Acceptors (HBAs)		Hydrogen–Bond Donors (HBDs)	
	choline chloride (ChCl)		Glycerol (Gly)
	trimethyl glycine (BetG)		ethylene glycol (EG)

The RDESs have been prepared by using a defined protocol which consisted of mixing the components in a defined molar ratio and heating them at 90 °C for 2–5 h, under constant stirring in a sealed vial until a stable homogeneous colorless one phase liquid was formed (see Supplementary Materials Figure S1). They have been dried at reduced pressure for 24 h, preserved under argon, and then characterized by ^{1}H NMR spectra (see Supplementary Materials Figure S2). The selected HBAs, ChCl, and BetG possess an evident biocompatibility and an availability from natural products which constitute one of their major advantages. In addition, the HBDs Gly and EG are highly available as bulk commodity chemicals. Furthermore, Gly is produced as a by-product of different hydrolysis and transesterification processes of oils and fats most notably in the soap and biodiesel manufacturing industries.

These RDES were suitable for PL modifications because their density was in the range of 1.06 to 1.16 g/cm^3, which allowed a good mass transfer and interaction between the reaction components (see Table 2). For that reason, many other RDESs, showing higher density values, have been excluded from the screening. According to the literature, DES's densities are usually higher than water's value and are dependent on the working temperatures. As reported in Table 2, the densities of Gly-containing RDESs (RDES A1 and B1) were higher than the densities of EG-containing ones (RDES A2 and B2).

Table 2. List, composition, and density of RDESs (measured at 18 °C) and reaction mixtures (RDES/buffer 1/1) which have been prepared and used in this study.

RDES Symbol	HBA	HBD	Molar Ratio (HBA/HBD)	Density of Pure RDES (g/cm^3)	Density of RDES/Buffer Solutions (g/cm^3)
RDES A1	choline chloride	glycerol	1:2	1.16	1.09
RDES A2	choline chloride	ethylene glycol	1:2	1.06	1.03
RDES B1	trimethyl glycine	glycerol	1:2	1.16	1.08
RDES B2	trimethyl glycine	ethylene glycol	1:2	1.11	1.05

2.3. Transphosphatidylation Reactions

The substrate used for all experiments was a commercial PC from soya beans which was preliminarily purified by precipitation in cold acetone. The acyl chains pattern of PC was identified with the method by ESI/MS and GC/MS previously reported [70]. The fatty acid chains composition is reported in Figure 3 and Table 3.

Table 3. Fatty acid composition of PC (results obtained by GC analysis reported in Figure 3).

Acid	Chain	t_R	% in PC
palmitic acid	C16:0	15.7	11.2
linoleic acid	C18:2	21.3	69.3
oleic acid	C18:1	21.4	15.8
stearic acid	C18:0	22.0	3.6

Figure 3. GC/MS analysis of acyl chains in starting PC with the indication of the chemical formula of each acid (Retention time reported in min).

2.3.1. Preparation of Phosphatidylglycerol

Phosphatidylglycerol (PG) constitutes one of the most abundant PLs at the basis of natural membranes. PG possesses an excellent liposome forming ability and unique surfactant/lubricant properties, especially when mixed with appropriate proteins, and, for these reasons, it has been studied for a long time [71], especially as an emulsifier for drug delivery systems [72,73]. Moreover, in mammalian lungs, PG is highly present compared to other mammalian membranes and therefore the research has been focused on the development of new surfactant preparations based on PG derivatives for therapeutic treatment of diseases such as a neonatal respiratory distress syndrome [74]. Moreover, it has been used for the preparation of plasmid-DNA lipid and polymeric nanovaccines showing that anionic PLs could increase antigen delivery, transport, and stability [75]. Unfortunately, PG could be recovered only in small amounts from natural sources and, even if it has been prepared by different groups [76–78], the possibility to set-up new strategies of synthesis is mandatory especially for the biomedical investigations.

Up to now, PG, when enzymatically synthesized, was usually prepared from natural PC by a PLD-mediated process in an organic solvent/buffer biphasic system containing glycerol as the nucleophile. In this work, a new protocol was investigated aiming to avoid the presence of the organic solvent in the reaction medium by using a RDES (see Figure 4). In the first attempts, PLD had been suspended in pure RDES, but the total absence of water completely inactivated the enzyme. As already shown for ILs, a minimal concentration of at least 5% of water was mandatory for the enzyme activity [29]. The process medium composition was optimized by water addition setting the RDES/aqueous phase ratio to the value 1:1 (v/v), resulting also in a modulation of the solvating properties leading to the product precipitation. In particular, the aqueous phase was a 0.1 M NaOAc buffer solution at pH 5.6 containing 0.1 M $CaCl_2$. PC was fully solubilized in RDES and then a buffer solution containing PLD was added. After 40 h at 45 °C, the products that precipitated in the reaction mixture were recovered and analyzed. Remarkably, the extraction of the remaining reaction mixture demonstrated that most of the products were effectively in

the precipitate and just a minor amount (≈ 5%) remained dissolved in the RDES/buffer medium. This proved the effectiveness of the RDES/buffer medium design in order to minimize the product concentration in the liquid phase and thus pushing the equilibrium towards the reaction completeness.

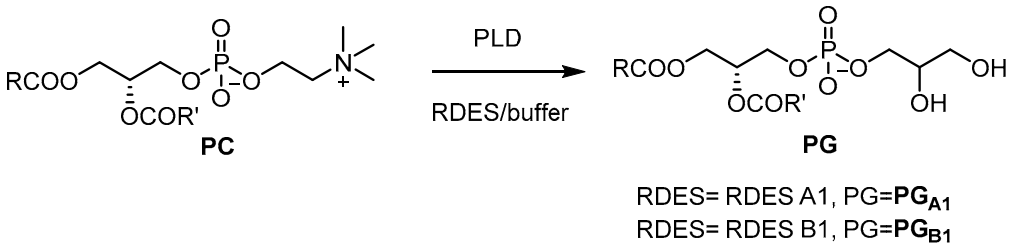


Figure 4. Transphosphatidylation of PC to PG.

When the reaction was performed with RDES A1 (ChCl/Gly 1:2) unreacted PC (~14 %, value obtained by HPLC analysis) was revealed, whereas, in RDES B1 (BetG/Gly 1:2), PC was completely converted in PG. In the first case, the presence of choline as HBA probably prevented the reaction to be completely shifted versus products because of its mass effect opposed to the HBD component (pushing the equilibrium point towards the substrate). In fact, as it resulted in the ^{1}H NMR spectrum (see Figure 5, green spectrum), PG_{A1} showed a residual peak around 3.2 ppm, which demonstrated the presence of residual unreacted PC. This peak disappeared completely in $PG_{B1,}$ because of the total conversion of PC to the final desired product.

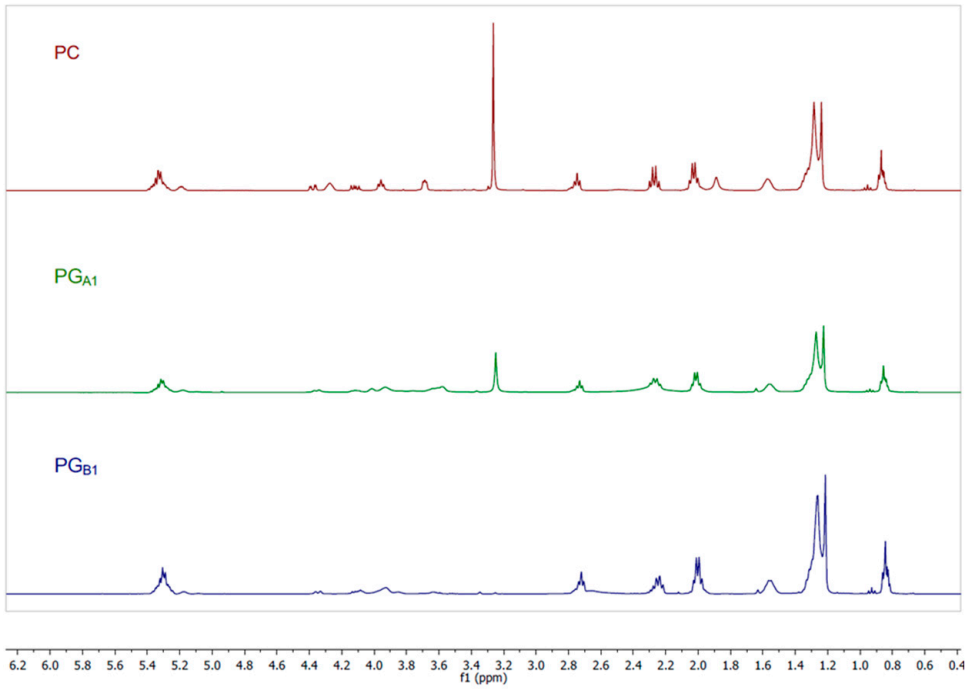


Figure 5. ^{1}H NMR spectra of starting PC (red line), PG_{A1} (green line) and PG_{B1} (blue line) in $CDCl_3/CD_3OD$.

Moreover, the presence of the RDES medium seems to inhibit nearly completely the detrimental hydrolysis side-reaction driving the conversion toward transphosphatidylation in comparison to what happened when the reaction was conducted in organic solvent/water as ESI/MS analysis confirmed (see Supplementary Materials).

2.3.2. Preparation of Phosphatidylethyleneglycol (P-EG).

Polyethyleneglycol (PEG)-grafted PLs are widely studied in the medical field, as they are one of the main components of liposomes used as carriers for mRNA, siRNA [79], and for anticancer active compounds such as paclixatel [80]. The relationship between PEG chain length and concentration in PEG-grafted PLs based vesicles has been deeply investigated [81], aiming to obtain optimized properties, in terms of water permeability and membrane strength, which resulted in a higher liposome lifetime in the blood circulation, and a better drug delivery. In this context, the authors decided to synthesize a non-natural phosphatidylethyleneglycol (P-EG), which could be an interesting building block for the production of new P-EG containing PLs. The free OH on the polar head could be then available to undergo esterification reactions, allowing the production of novel tailor-made products. In order to obtain a reference P-EG, the product has been firstly prepared with the conventional method in toluene/buffer solution. Subsequently, PC was fully solubilized in RDESs A2 (ChCl/EG 1:2) and B2 (BetG/EG 1:2) containing both ethylene glycol which constituted HBD as well as the nucleophile in the PLD catalyzed transphosphatidylation (see Figure 6). A buffer solution containing PLD was added to the solution to start the reaction. After 40 h at 45 °C, the products, precipitated in the reaction medium, were recovered and analyzed.

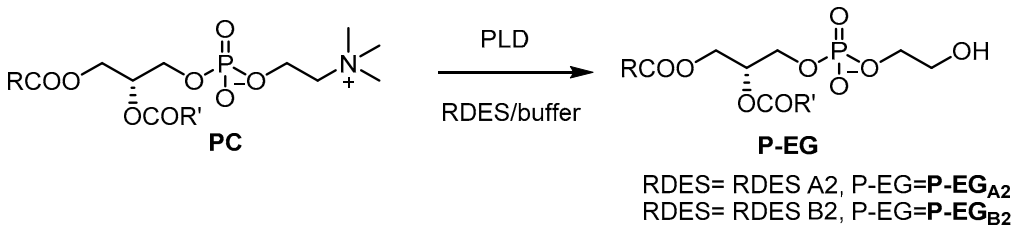


Figure 6. Scheme of transphosphatidylation reaction of PC to P-EG.

Also in this case, as described in Section 2.3.1, most of the products precipitated and just a minor part (≈10%) was recovered by solvent extraction from the RDES/buffer medium. This confirmed again the efficacy of the process medium optimization in the modulation of the reagent and products solubility. ^{1}H NMR spectra of the final products are reported in Figure 7 (P-EG$_{A2}$, green line, and P-EG$_{B2}$ blue line). As already observed in the PGs preparation in 2.2.1, when the reaction was performed in RDES containing ChCl, namely RDES A2, a small quantity of PC (~8%, value obtained by HPLC analysis) remained unreacted, whereas, in RDES B2 (BetG/EG 1:2), PC was completely converted to P-EG.

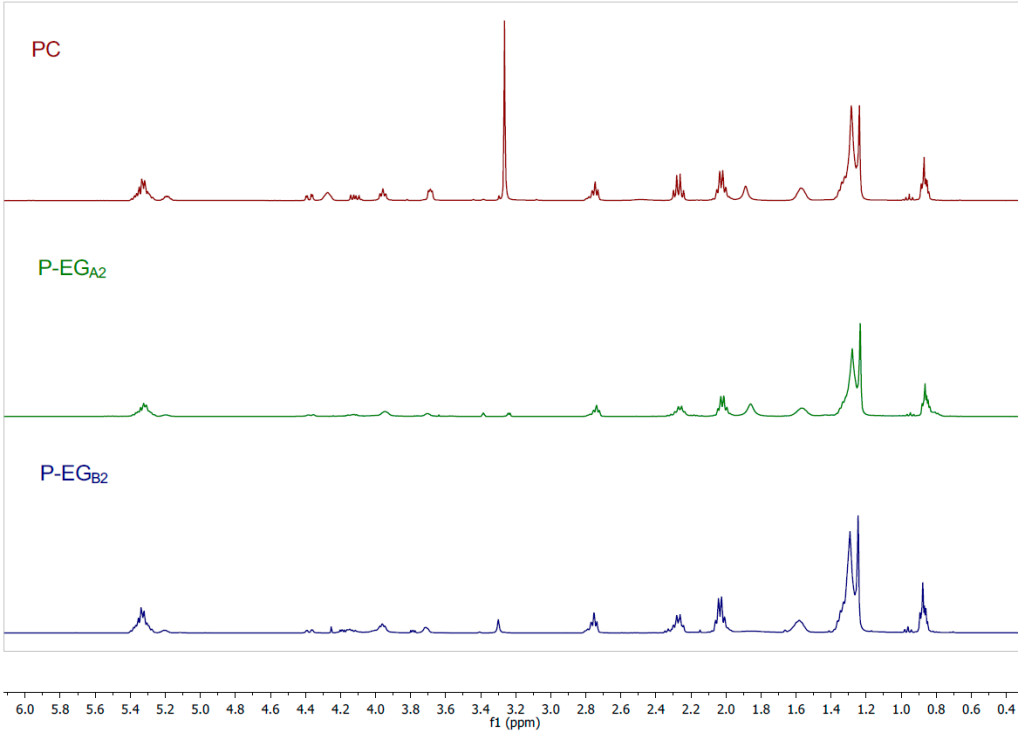


Figure 7. ^{1}H NMR spectra of starting PC (red line), P-EG$_{A2}$ (green line) and P-EG$_{B2}$ (blue line) in D_2O.

3. Materials and Methods

All chemicals were purchased from Merck (Merck Life Science S.r.l., Milan, Italy) and used without further purification. The employed solvents were of analytical or HPLC grade when necessary. Phosphatidylcholine was supplied from Lucas Meyer (Epikuron 200, soya lecithin, Hamburg, Germany) and used after precipitation in cold acetone (10 g Ep200 dissolved in 15 mL of CH_2Cl_2, added to 300 mL of cold acetone). ^{1}H NMR and ^{31}P NMR spectra were recorded on a 400 MHz Bruker Avance spectrometer (Milano, Italy). Acquisition and data treatment were performed with Bruker TopSpin 3.2 software. Chemical shifts were reported in δ units (ppm), relative to tetramethylsilane (TMS) as the internal standard and all spectra were recorded in D_2O or $CDCl_3/CD_3OD$. Deuterated solvents were purchased from Eurisotop (Saint-Aubin, France).

3.1. ^{31}P NMR Sample Preparation

In addition, 50 mg of the sample was dissolved with 5 mg of triphenyl phosphate in 0.6 mL of a $CDCl_3/CH_3OH$ 2:1 solution mixed with 0.4 mL of a CsEDTA solution (prepared. by titration of 0.2 M EDTA in D_2O with $CsCO_3$ until pH 7.6 and then diluted 1:4 (v/v) with methanol). Low-soluble samples were vigorously stirring to allow the dissolution. After the complete solubilization, 0.2 mL of water was added to the mixture and, after stirring, the solution was transferred into an NMR tube and left to rest in order to achieve the correct separation of the two liquid phases. The ^{31}P uncoupled experiments were performed with the following parameters: D1: 10 s, SW: 40 ppm, O1: 10 ppm, FID size: 32768, ns: 32, T: 320 K.

3.2. Thin Layer Chromatography (TLC)

TLC plates Silica gel 60 SIL G-25 UV254 glass 250 μm (Macherey Nagel, Düren, Germany) were used for analytical TLC. The employed eluents were constituted by or $CHCl_3/CH_3OH/CH_3COCH_3/CH_3COOH/H_2O$: 50/10/20/10/5 and $CHCl_3/CH_3OH/NH_3$ (33% aq.): 65/30/2.5 (ratios reported in volume). Detection was performed by staining with a cerium molibdate reagent.

3.3. Gas Chromatography

To determine the fatty acid composition in PC from soya, fatty acid methyl esters (FAMEs) were obtained by hydrolysis of PC with methanol/ 2 M NaOH under reflux for 4 h. The reaction mixture was then cooled, evaporated to remove methanol, acidified, and extracted carefully three times with ethylacetate. The organic solutions were collected, anhydrified on sodium sulphate and evaporated at p.r. The residue has been dissolved in diethyl ether and treated with an ethereal solution of diazomethane 30 min at r.t. After evaporation, the residue was dissolved in CH_2Cl_2 and analyzed using a gas chromatograph system HP-6890 equipped with a HP-5973 mass detector and a *HP-5MS* column (30 m length × 250 μm internal diameter, 0.25 μm film thickness, Hewlett Packard, Palo Alto, CA, USA). Helium was the carrier gas with a flux of 1 mL min^{-1}; the splitting ratio was 1:30. The injector and detector temperatures were set at 250 °C. The used temperature program is reported here: 120 °C (3 min)–12 °C/min–195 °C (10 min)–12 °C/min–300 °C (10 min). The FAMEs were identified by comparison with known standards and by means of NIST 2008 mass spectral library search.

3.4. High Performance Liquid Chromatography (HPLC)

HPLC analyses were performed on a Merck Hitachi L4000 apparatus (Japan) fitted with a Sepachrom Adamas®Silica (Rho, Milano, Italy) 5μ-2 column, length/internal diameter 250/4.6, and a UV detector set to λ = 206 nm. The column temperature was maintained at r.t. PL mixtures were analyzed using an isocratic mobile phase consisting of $CH_3CN/CH_3OH/H_3PO_4$ 952/36/10.9 at a flow rate of 0.5 mL/min. The samples were dissolved in a mixture of hexane/isopropanol (1:1, *v*/*v*) with a final concentration of 1 mg/mL and filtered through a 0.45 μm filter before injection

3.5. Electrospray Ionization Mass Spectrometry

Mass spectra were recorded on a ESI/MS Bruker Esquire 3000 PLUS (ESI Ion Trap LC/MSn System, Bruker, Milano, Italy), by direct infusion of a dichloromethane solution of compounds with an infusion rate of 4 μL/min. Samples were analyzed using both positive and negative ionization modes to allow the detection of all relevant compounds.

3.6. PLD Preparation

3.6.1. Microorganism Fermentation

PLD was obtained from the fermentation broth of *Streptomyces netropsis* (DSM 40093). The medium for strain growth and maintenance was composed by glucose 4 g/L; yeast extract 5 g/L; malt extract 10 g/L.

Medium for PLD preparation: glucose 5 g/L; yeast extract 5 g/L; malt extract 10 g/L; peptone from soyabean 3 g/L; peptone from casein 1 g/L; $MgSO_4$ 0.2 g/L; NaCl 0.2 g/L.

A single colony of active *Streptomyces netropsis* culture was picked up from a Petri dish, was suspended in 1 mL of sterile water, and was then inoculated in a 500 mL conical Pyrex flask containing 150 mL of the growth medium. The flask was shaken for 5 days at 30 °C and 140 rpm. After this period, the cells were centrifuged for 15 min (4 °C, 4000× *g*) and collected, removing the medium. The cells were suspended in 10 mL of fresh medium and were added to a sterilized fermenter vessel of a 5 L bioreactor (Biostat A BB-8822000, Sartorius Stedim, Göttingen, Germany) containing 4 L of medium for PLD preparation. The temperature, the stirring speed, and the pH were set to 30 °C, 250 rpm and 7.0, respectively. The value of pH was controlled by addition of sterilized aqueous solutions (10% *w*/*w* in water) of either acetic acid or ammonia. The fermentation was performed aerobically, setting a minimum air flow of 2 L/min (0.5 *v*/*v*/min). After 48 h, the fermentation broth was filtered through a celite pad, and the clear liquid was stored at 4 °C in dark bottles.

3.6.2. Precipitation and Dialysis

To 1 L of the filtered broth, 430 g of ammonium sulfate was added over 1 h at 4 °C under stirring. Once the ammonium sulfate was completely dissolved, the broth was

left overnight at 4 °C to allow the precipitation. After 24 h, the precipitated pellet was separated from the broth by centrifugation at 8000 rpm for 15 min at 4 °C. The solid was then dissolved in a small amount of supernatant broth and dialyzed with a seamless cellulose tube (D0655 Sigma-Aldrich, Milano, Italy) against 10 mM sodium acetate buffer at pH 5. The dialyzed broth (80 mL), which had an activity of 0.525 U/mL, was finally lyophilized (240 mg, 0.175 U/mg) and used as solid powder for bioconversions.

3.7. Protein Determination

Protein concentration was determined in the dialyzed broth by the Bradford method using bovine serum albumin (BSA) as standard [82] and was 0.94 mg/mL (analysis performed in triplicate).

3.8. PLD Activity Determination

In addition, 360 mg of phosphatidyl-*p*-nitrophenol (PpNP), prepared as described in our previous work [83], was dissolved in diethylether (1 mL) and added with 15 mL of 0.1 M Tris buffer and 15 mL of a solution of 10% Triton X-100 in the same buffer, giving a final concentration of 16 mM. The mixture was heated gently to 60 °C until it became clear, the organic solvent was removed under reduced pressure, and the solution was stored in dark vials at −18 °C. PLD activity was determined spectrophotometrically using a UV-Vis Double Beam spectrophotometer V-730 Jasco (Jasco Europe, Cremella, Italy) in 0.05 M Tris Buffer, pH 8, by monitoring the hydrolysis of PpNP to phosphatidic acid and *p*-nitrophenol at 405 nm using a molar extinction coefficient of 18.450 $mmol^{-1}$ L cm^{-1}. One unit (U) is defined as the amount of enzyme able to hydrolyze one µmol of PpNP in 1 min.

3.9. RDES Preparation

HBA (choline chloride or glycine betaine) and HBD (glycerol or ethylene glycol) were previously dried for 10 h under vacuum. They were then mixed in a 1:2 molar ratio (see Table 2 for detailed composition) and heated under magnetic stirring for 2–4 h at 70–90 °C until a homogeneous and clear liquid phase was obtained. The synthesized RDESs were left to cool slowly to r.t., and they were stored in a sealed vial under argon until used. They were analyzed by ^{1}H NMR (spectra are reported in Supplementary Materials Figure S2).

3.10. RDES Density Measurement

The density of the RDESs prepared in 3.9 has been measured at atmospheric pressure and at 18 °C with the following procedure. In addition, 2 mL volumetric flasks have been weighed on an analytical balance with a resolution of 0.1 mg. Then, they have been filled with the opportune RDES and thermostated at 18 °C for 1 h. Then, the volume was adjusted to the right value and the flasks were weighted. Each measure had been performed in duplicate. The density has been calculated for each RDES as the ratio between the mass of RDES (mass of filled flask–mass of empty flask) and the volume of the flask. The final value reported in Table 2 is the average of the two measurements.

3.11. RDES Preparation of Phosphatidylglycerols PG_{A1} and PG_{B1} in RDES A1 and B1

Furthermore, 150 mg of PC was dissolved at 45 °C in 10 mL of RDES (A1 or B1). Once full solubilization of the substrate in RDES was achieved, 10 mL of buffer solution containing 0.1 M of sodium acetate, 0.1 M of calcium chloride, and 15 mg (2.6 U) of solid PLD adjusted to pH 5.6 were added to the solution. This mixture was left stirring at 45 °C for 40 h. The product precipitated in the reaction mixture and was isolated by filtration on a Buchner under vacuum. The liquid solution was extracted with toluene, in order to check the presence of residual PLs. The precipitated products for RDES B1 accounted for 60% (*w*/*w*, 90 mg) of the reaction yield, while the products recovered from the medium extraction were instead 3% (*w*/*w*, 5 mg) of the reaction yield. The product was then purified from residual RDES: it was dissolved in 1 mL of toluene, and the solution was left stirring with 5 mL of water for 30 min at r.t. The organic phase was then separated and evaporated

under reduced pressure. The PLs mixture recoveries were 130 mg (86% *w*/*w*) for PG_{A1} preparation and 95 mg (63% *w*/*w*) for PG_{B1}. The final mixtures were analyzed by ESI/MS and NMR.

PG_{A1} ESI/MS negative ion spectrum m/z values: 16:0/18:2-PG $[745.6]^-$, 18:2/18:2-PG $[769.5]^-$. Residual PC was seen in the positive ion spectrum with m/z: 16:0/18.2-PC $[780.7 + Na]^+$, 18:2/18:2-PC $[804.6 + Na]^+$. ^{1}H NMR: δ_H 0.84−0.85 (m, 6H, CH_3), 1.23−1.27 (m, 34 H, CH_2 acyl chains), 1.50−1.60 (m, 4H, $\underline{CH_2}CH_2CO$), 1.99−2.04 (m, 7 H, $\underline{CH_2}$−CHCH), 2.23−2.30 (m, 4H, $\underline{CH_2}CO$), 2.72−2.77 (m, 3H, CHCH−$\underline{CH}$−CHCH), 3.58−3.64 (m, 3H, $\underline{CH}OH\underline{CH_2}OH$), 3.93−4.12 (m, 5H, O−$\underline{CH_2}$−CHOH−$\underline{CH_2}O$ and $\underline{CH_2}$−OPO_3−), 4.34−4.37 (m, 1H, $\underline{CH_2}$−OPO_3−), 5.18 (m, 1H, $\underline{CH}$−OH), 5.29−5.35 (m, 6H, $\underline{CHCH}$). ^{31}P NMR δ: 0.59 ppm.

PG_{B1} ESI/MS negative ion spectrum—the desired product was identified with the following m/z values: 16:0/18:2-PG $[745.5]^-$, 18:2/18:2-PG $[769.5]^-$. No residual PC was observed in the positive ion spectra. ^{1}H NMR: δ_H 0.82−0.86 (m, 6H, CH_3), 1.21−1.27 (m, 34 H, CH_2 acyl chains), 1.50−1.60 (m, 4H, $\underline{CH_2}CH_2CO$), 1.98−2.03 (m, 7H, $\underline{CH_2}$−CHCH), 2.22-2.29 (m, 4H, $\underline{CH_2}CO$), 2.71-2.76 (m, 3H, CHCH−$\underline{CH}$−CHCH), 3.59−3.64 (m, 3H, $\underline{CH}OH\underline{CH_2}OH$), 3.85−4.13 (m, 5H, O−$\underline{CH_2}$−CHOH−$\underline{CH_2}O$ and $\underline{CH_2}$−OPO_3−), 4.33−4.36 (m, 1H, $\underline{CH_2}$−OPO_3−), 5.17 (m, 1H, $\underline{CH}$−OH), 5.27−5.35 (m, 6H, $\underline{CHCH}$). ^{31}P NMR δ: 0.59 ppm.

3.12. RDES Preparation of Phosphatidylethyleneglycols P-EG_{A2} and P-EG_{B2} in RDES A2 and B2

In addition, 150 mg of PC was dissolved at 45 °C in 10 mL of RDES (A2 or B2). Once full solubilization of the substrate in RDES was achieved, 10 mL of buffer solution containing 0.1 M of sodium acetate, 0.1 M of calcium chloride, and 15 mg (2.6 U) of PLD powder adjusted to pH 5.6 were added to the solution. This mixture was left stirring at 45 °C for 40 h. The liquid solution was then separated by filtration from the solid product. The solution was extracted with toluene, whereas the solid was dissolved in toluene. The precipitated products for RDES B2 accounted for 63.3% (*w*/*w*, 95 mg) of the reaction yield, while the products recovered from the medium extraction were instead 6.6% (*w*/*w*, 10 mg) of the reaction yield. All the organic phases were collected and evaporated under reduced pressure. The product containing RDES residues was dissolved in 1 mL of toluene, and the solution was left stirring with 5 mL of water for 30 min at r.t. The organic phase was separated and evaporated under reduced pressure. The PL mixture recoveries were 140 mg (93% *w*/*w*) for P-EG_{A2} and 105 mg (70% *w*/*w*) for P-EG_{B2}. The final mixtures were analyzed by ESI/MS and NMR.

P-EG_{A2}: ESI/MS negative ion spectrum—the desired product was identified with the following m/z values: 16:0/18:2-P-EG $[715.6]^-$, 18:2/18:2-P-EG $[739.6]^-$. Some PC was recorded in the positive ion spectrum with m/z: 16:0/18.2-PC $[780+Na]^+$, 18:2/18:2-PC $[804.7+Na]^+$. ^{1}H NMR: δ_H 0.84−0.88 (m, 6H, CH_3), 1.23−1.27 (m, 34 H, CH_2 acyl chains), 1.56 (m, 4H, $\underline{CH_2}CH_2CO$), 2.00−2.05 (m, 7 H, $\underline{CH_2}$−CHCH), 2.24−2.29 (m, 4H, CH_2CO), 2.72−2.76 (m, 3H, CHCH−$\underline{CH}$−CHCH), 3.38 (m, 1H, $CH_2\underline{CH_2}OH$), 3.64−4.24 (m, 6H, O−$\underline{CH_2}$−CHO−$\underline{CH_2}O$ and $\underline{CH_2}$−OPO_3−), 4.35−4.38 (m, 1H, CH_2−OPO_3), 5.19 (m, 1H, $\underline{CH}$−OH), 5.31−5.37 (m, 6H, $\underline{CHCH}$). ^{31}P NMR δ: 0.35 ppm.

P-EG_{B2}: ESI/MS negative ion spectrum—the desired product P-EG was identified with the following m/z values: 16:0/18:2-P-EG $[715.8]^-$, 18:2/18:2-P-EG $[739.6]^-$. No PC was recorded in the positive ion spectrum. ^{1}H NMR: δ_H 0.86−0.89 (m, 6H, CH_3), 1.25−1.29 (m, 34 H, CH_2 acyl chains), 1.58 (m, 4H, $\underline{CH_2}CH_2CO$), 2.01−2.06 (m, 7 H, $\underline{CH_2}$−CHCH), 2.24−2.30 (m, 4H, $\underline{CH_2}CO$), 2.74−2.77 (m, 3H, CHCH−$\underline{CH}$−CHCH), 3.30 (m, 1H, $CH_2\underline{CH_2}OH$), 3.71−4.25 (m, 6H, O−$\underline{CH_2}$−CHO−$\underline{CH_2}O$ and $\underline{CH_2}$−OPO_3−), 4.36−4.40 (m, 1H, CH_2−OPO_3), 5.20 (m, 1H, $\underline{CH}$−OH), 5.30−5.38 (m, 6H, $\underline{CHCH}$). ^{31}P NMR δ: 0.35 ppm.

4. Conclusions

The present work constitutes an innovative approach for the biocatalytic preparation of polar head modified PLs based on the use of RDESs/buffer mixtures, which allows the conversion of natural PC in high yields. RDESs act both as solvents and reactants because they contain the alcoholic nucleophiles that will substitute the choline moiety in the transphosphatidylation reaction. The best results have been obtained using two RDESs constituted by glycine betaine as HBA and glycerol or ethyleneglycol as HBD with a total conversion of starting PC due to the absence of choline in the composition of RDES. The implementation of specifically designed reaction media based on RDESs and buffer resulted in several distinctive advantages, including the yield enhancement by the synergic combination of a substantial mass effect (due to the high concentration of nucleophile reactant in the RDES) and the product precipitation (which maintains at a low value its concentration in the medium). Moreover, the implementation of RDES/buffer reaction medium demonstrates the inhibition of the parasitic competitive hydrolysis of PC to PA, improving the reaction yields and easing the product recovery and purification. Detailed studies about the interaction of RDESs with PLD catalysis are under way. In fact, the effect of RDESs (and DESs) on the phospholipase's active site as well as on PLs aggregates is hardly predictable, and further studies are needed to fully exploit this promising approach to biocatalysis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/catal11060655/s1, Figure S1: RDES preparation example (left image: initial mixture of ChCl (HBD) and ethylene glycol (HBA), right image: RDES A2). Figure S2: ^{1}H NMR spectra of the four RDES A1, A2, B1 and B2 in D_2O. Figure S3: ^{1}H NMR Spectrum of PC. Figure S4: Mass spectra of PG_{A1} obtained in RDES A1. Figure S5: Mass spectra of PG_{B1} obtained in RDES B1. Figure S6: Mass spectra of P-EG_{A2} obtained in RDES A2. Figure S7: Mass spectra of P-EG_{B2} obtained in RDES B2.

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