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3D Printed Polylactic Acid (PLA) Well Plates for Enzyme Inhibition Studies: The Case of Pancreatic Lipase

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Abstract

3D printed PLA has already been demonstrated for several biotechnological applications, including enzymes immobilization. The prerequisites for an efficient screening assay include using small volumes of reagents, low cost, and rapid screening of large numbers of compounds and extracts. Hence, assays based on microtiter plates are predominant. Thus, designing and fabricating scaffolds on a similar scale, which could serve as immobilization carriers, and their recruitment in inhibitors screening studies is of great significance, adding both enzyme stability and reuse potentiality of the biocatalytic system in assay merits. In this work, pancreatic lipase was immobilized on 3D-printed PLA microwells for enzyme inhibitor screening. XPS analysis demonstrated the successful modification of the PLA scaffolds. The immobilized enzyme displayed high levels of operational, thermal, and storage stability under the tested conditions. The IC_{50} values for PPL inhibition were calculated for Orlistat, a model

lipase inhibitor, and olive leaf extract, a promising natural compound. This is the first study reporting the use of 3D-printed PLA wells with an immobilized enzyme for inhibitor screening assay.

Keywords
3D printing; lipase; well-plate assay; inhibition; olive leaf extract

1. Introduction

Obesity is a multifactorial, clinical condition associated with abnormal metabolism of triacylglycerols (TAGs) resulting from homeostatic imbalance [1]. It is characterized by excess accumulation of body fat and is linked to cardiovascular diseases, diabetes, cancer, and even mortality [2]. Hence, new treatment approaches are vital to expand the toolbox of the currently available therapeutic choices and provide improved alternatives. One of the most effective treatments for obesity involves lipase inhibitors [3]. Lipases, particularly pancreatic lipases, are prominently involved in catalyzing TAG hydrolysis and metabolism. This enzyme hydrolyzes 50% to 70% of the total dietary fats in the small intestine [1]. The porcine pancreatic lipase (PPL) is a monomeric small globular protein often used as the model enzyme for inhibition studies because of its higher amino acid sequence homology (86%) with the human pancreatic lipase [4-6].

Orlistat is the only pancreatic lipase inhibitor in use to date, approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA), while its weight loss properties along with cardioprotective, anti-diabetic and others have been discussed thoroughly [3, 7, 8]. Although orlistat is a highly selective inhibitor, several side effects have been reported, mostly gastrointestinal [9]. Therefore, nowadays natural products are in the spotlight for the identification of inhibitory agents of pancreatic lipase, which are anticipated to have fewer side effects than chemically synthesized compounds due to their natural origin [1, 10].

Biocatalysis requires enzymes in an immobilized form for long-term operational stability and cost-effectiveness [11]. Immobilization of enzymes exhibits several advantages, such as the feasibility of enzyme recovery and reuse, rapid termination of the enzymatic assay, enhanced storage, and thermal and operational stability [11, 12]. Three-dimensional (3D) printing or additive manufacturing produces immobilization carriers that are easily isolated from the reaction media and have large specific surface areas, thereby improving the mass transfer effect [13]. Polylactic acid (PLA) is a naturally-derived polymer. It is biodegradable, biocompatible, non-toxic, non-carcinogenic, and has good optical, mechanical, and rheological properties [13-15]. Moreover, its printing process is simple and consumes less energy than that required for other polymers. Hence, PLA is one of the most popular choices for biotechnological applications [13, 15]. So far, 3D printed scaffolds using PLA, carbon fiber-reinforced PLA (C-PLA), and nylon have been investigated extensively for optimizing enzyme immobilization [16-18]. Other examples of enzymes used in their free or immobilized form include the fabrication of 3D printed platforms for optical colorimetric enzymatic assays [19, 20]. Additionally, the construction of 3D-printed bioanalytical devices suitable for genetic tests has been reported in diagnostics [21-23].

In the scope of enzyme inhibition studies, microtiter plates and cuvettes are widely used [24]. Nevertheless, considering the prerequisites for an efficient screening assay, that is usage of small volumes of reagents, low cost and rapid screening of large numbers of compounds and extracts, assays based on microtiter plates are predominant. Thus, designing and fabricating scaffolds on a similar scale to function as immobilized enzyme carriers is essential. Such biocatalytic scaffolds can be applied in inhibitor screening studies because they render enzyme stability and reusability to the sustainable bioprocess.

PLA often undergoes modifications to alter specific characteristics, for instance, hydrophilicity, surface free energy, reactive functionalities, and roughness [15]. In previous studies, different methodologies, like the utilization of polyethylene glycol, polydopamine, and chitosan, were adopted [25-28]. Our research team proposed a novel protocol based on chitosan to functionalize 3D-printed PLA scaffolds. The method facilitated subsequent enzyme immobilization. Thus, biocatalytic systems with high stability, product yields, and turnover number were produced [29]. The present study proposed innovative, inexpensive, and environment-friendly 3D-printed PLA wells as sustainable matrices for enzyme inhibition studies. The well plates were designed and 3D-printed. The lipase from the porcine pancreas was immobilized in the interior of each well after appropriately modifying the scaffold surfaces. After that, spectroscopic and biocatalytic characterization of the immobilized 3D printed scaffolds was performed. Finally, the developed biocatalytic systems were successfully applied in the inhibition study of PPL. Inhibition was initially evaluated with the model inhibitor of PPL, Orlistat, and then with a promising natural compound, the extract of olive leaf . The half-maximal inhibitory concentration (IC_{50}) was determined for both reactions. The innovation of the present work stems from the fabrication of a well-plate through the utilization of the 3D printing technology, which enables the customization of the number and the volume of the wells. Moreover, the enzyme was directly immobilized on the surface of the well, adopting an eco-friendly, simple, time- and cost-efficient protocol. Interestingly, the average cost of one test (one PLA well fabrication, modification, PPL immobilization, and activity measurement) was calculated to be as low as 0.04€. Since the prepared scaffolds could be reused and stored without significant activity losses, they constitute a valuable tool in inhibition studies. To our knowledge, this is the first report involving 3D-printed PLA wells with immobilized enzymes for inhibitor screening assays.

2. Materials and Methods

2.1 Materials

PLA filament for FDM 3D printers (PrimaValue™) in a natural form was obtained from Prima Creator (Sweden). The 3D printer used was Ender 5 from Creality 3D (China). Sodium hydroxide (NaOH) with >98% purity was obtained from PanReac Applichem (USA). The following reagents were obtained from Merck (Sigma-Aldrich, USA): chitosan from crab shells (85% deacetylated), acetic acid (>99.8%), lipase Type II from porcine pancreas (100–500 U/mg protein in lyophilized form), and p-nitrophenyl butyrate (pNPB, >98%). Orlistat (98%) was obtained from Alfa Aesar (USA). HPLC-grade acetonitrile (ACN) and dimethylsulfoxide (DMSO) were obtained from Fisher Scientific (USA). All solutions used in the present work were prepared in double-distilled (dd) water.

2.2 Methods

2.2.1 3D Design and Printing of PLA Scaffolds

PLA scaffolds were designed according to our previous reports using the Fusion 360 CAD program from Autodesk (USA) [29, 30]. Briefly, well plates were designed with a 200 µL volume capacity for each well. The number of wells on each plate depended on the number of samples for each experiment. An example of a 9-well plate is demonstrated in Figure 1. The final design was sent to the slicing software Ultimaker Cura (Netherlands). Appropriate settings were selected depending on the filament and the 3D printer model. Print settings included a 0.16 mm layer height (resolution), 100% infill density, and 80 mm/s print speed. The printing temperature was set at 200 °C, and the build plate temperature was set at 50 °C. Natural PLA filament was used as the feeding material, with a diameter of 0.75 mm, extruded from a nozzle of 0.4 mm. Printing parameters are summarized in Table 1. A copy of the .STL file is provided as supplementary material. The model printed (Figure 1) was leak free, with a rigid structure, and ready-to-use for further experimentation (no post-printing processing needed).

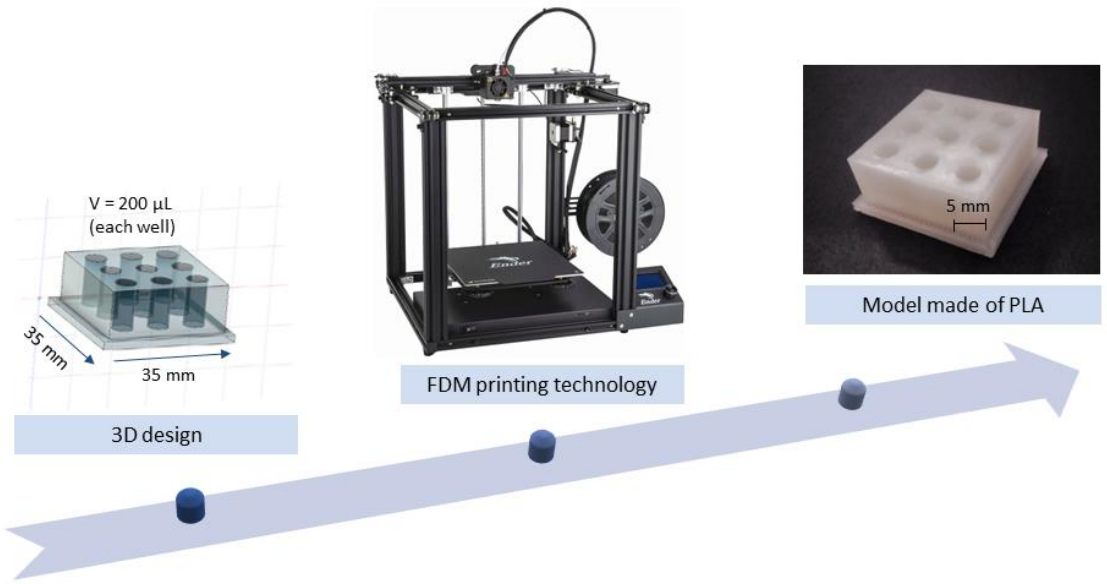

Figure 1 Demonstration of the 3D printing process. A well plate model was designed using a 3D design software, and an FDM printing technology produced the final model using natural PLA filament.

Table 1 Printing settings at the slicing software

Layer Height	0.16 mm
Infill Density	100%
Printing Temperature	200 °C
Build Plate Temperature	50 °C
Print Speed	80 mm/s
Nozzle Size	0.4 mm
Filament Diameter	1.75 mm

2.2.2 Surface Modification of the 3D Printed PLA Scaffolds

The surface of the PLA wells was modified as described in Gkantzou et al. 2022. Firstly, the 3D printed wells were incubated with a 1 M NaOH solution at room temperature for 2 h. The solution was then removed; the wells were washed twice with dd H_2O and dried under a vacuum for 30 min. After that, the wells were incubated with a 0.2% (w/v) chitosan solution for 1 min. After removing the solution, the wells were incubated with 1 M NaOH solution for 1 min. The solution was removed, and the wells were dried under a vacuum for 30 min. Porcine pancreatic lipase (PPL) was immobilized on PLA wells through non-covalent interactions. Briefly, PPL was dissolved in 50 mM phosphate buffer (pH 7) to a final concentration of 1 mg/mL and then subjected to vigorous shaking, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was used afresh as the enzyme solution. The enzymatic solution (200 µL) was added to each well, and the plate was incubated at 30 °C for 1 h. Subsequently, the enzyme solution was removed, and the wells were washed thrice with the phosphate buffer solution. The well plate was dried under a vacuum for 30 min and stored at 4 °C until further use.

2.2.3 Enzymatic Activity Measurement in the PLA Well Plates

The activity of immobilized PPL was determined with a photometric assay using p-nitrophenyl butyrate (pNPB) as the substrate. A stock solution of 50 mM pNPB was prepared in acetonitrile (ACN) so that each reaction solution contained a fixed percentage (2% v/v of ACN). Each well was filled with 200 µL of 1 mM pNPB solution in 50 mM sodium phosphate buffer (pH 7). The enzymatic activity was determined at 40 °C at regular intervals for up to 20 min, that was proved to be sufficient time for the yield of the reaction product p-nitrophenol (pNP). The reaction solution was then transferred to a microplate and the absorbance of the produced pNP was measured using an ELISA plate reader (Multiskan SkyHigh Microplate Spectrophotometer, Thermo Fisher Scientific, USA) at 405 nm.

2.2.4 Estimation of the Immobilization Yield

To determine the amount of enzyme that was loaded during immobilization process, an activity test was conducted for the enzymatic solution before and after immobilization. A sample from the enzymatic solution before its loading on a well was measured with the pNPB protocol described above, and a sample of the enzymatic solution after incubation in the corresponding scaffold was also measured with the same process. Equation 1 was used for the calculation of the active enzyme amount loaded during immobilization in each scaffold.

$$\text{Immobilization yield } \% = 100 - \frac{\text{activity after immobilization} \times 100}{\text{activity before immobilization}} \quad (1)$$

2.2.5 Stability Studies of the Immobilized PPL

Biocatalytic characterization is critical to investigate the possible use of the developed enzyme system in further studies. Therefore, the immobilized PLA wells were studied initially in terms of operational, thermal, and storage stability. All experiments were performed in triplicates (one well-one experiment).

Operational stability The operational stability of the immobilized PPL on PLA wells was examined for ten successive cycles of use. The hydrolysis reaction of pNPB was monitored (as described in Section 2.2.3) in two different reaction media – the one containing 2% v/v DMSO and the other without added DMSO. After completion of each cycle (20 min), the reaction solution was removed, and the corresponding well was washed thrice with the reaction buffer. Fresh substrate solution was then filled in each well, and the process was repeated. The remaining activity was determined using equation 2 compared to the first cycle.

$$\text{Remaining activity } \% = \frac{\text{activity in each cycle} \times 100}{\text{activity in the first cycle}} \quad (2)$$

Thermal stability The thermal stability of the immobilized system was evaluated after 1, 2, 6, and 8 h of incubation at 40 °C. The activity of PPL in each well was measured at zero time (as described in Section 2.2.3). For every interval tested, two wells were filled with buffer solution and placed in an incubator at 40 °C. After each incubation, the remaining activity of PPL in each well was calculated with the same procedure. Finally, the percentage of thermal stability was estimated using equation 3. The same experiment was conducted for the free lipase using the same method but with some differences: the remaining activity was determined after 5 min of incubation and under stirring at 750 rpm.

$$\text{Remaining activity } \% = \frac{\text{activity before incubation } \times 100}{\text{activity after incubation}} \quad (3)$$

Storage Stability The storage stability of the immobilized system was studied after incubating for 1, 2, 3, 4, and 8 weeks at 4 °C under dry conditions. The activity of PPL in each well was measured before incubation (as described in Section 2.2.3). Subsequently, the remaining activity of the lipase in each well was calculated after each time interval, as mentioned previously. Equation 3 was used for extracting the percentage of storage stability.

2.2.6 Kinetics Study of the Immobilized PPL

The Michaelis-Menten constant (K_m) represents an essential parameter of the enzyme kinetic reaction. It indicates the affinity between the enzyme and the substrate. The value of K_m was calculated by the Michaelis-Menten equation (4):

$$V = \frac{Vmax \times [S]}{Km + [S]} \quad (4)$$

where V and V_{max} are the initial and maximal velocities of the enzymatic reaction, respectively, and [S] is the concentration of the substrate.

For determining the K_m value of the free or the immobilized PPL, 0.125, 0.25, 0.5, and 1 mM concentrations of the pNPB solution were incubated with the biocatalyst for up to 20 min at 40 °C as described before. The K_m was obtained by the nonlinear regression of the Michaelis–Menten diagrams using Prism® 9.0 (GraphPad Software, USA).

2.2.7 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) measurements were performed under an ultrahigh vacuum at a base pressure of 7×10^{-9} mbar using a FlexPS spectrometer (SPECS GmbH, Germany). The instrument was equipped with a monochromatic MgKα source (hv = 1253.6 eV) and a PHOIBOS 100 hemispherical analyzer (SPECS GmbH, Germany). The spectra were collected in normal emission, and the energy resolution was set to 1.16 eV to minimize the measuring time. The spectral analysis included a Shirley background subtraction and a peak deconvolution employing mixed Gaussian-Lorentzian functions in a least-squares curve-fitting program (WinSpec) developed at the Laboratoire Interdisciplinaire de Spectroscopie Electronique, University of Namur, Belgium.

2.2.8 Inhibition Assays

Inhibition by OrlistatOrlistat, a commercially available pancreatic lipase inhibitor, was chosen as the model compound for the inhibition studies. The stock solution of Orlistat was prepared by dissolution in DMSO, and concentrations of 0.005, 0.01, 0.02, 0.05, and 0.1 μM were used to determine the half-maximal inhibitory concentration (IC_{50}) at the fixed substrate concentration of 1 mM. In particular, the assay for evaluating the inhibition consisted of the following steps: (1) measuring the initial activity of the immobilized PPL (as described in Section 2.2.3), (2) pre-incubating the enzyme with the inhibitor solution for 10 min at 40 °C, and (3) adding the substrate and determining the remaining enzyme activity as described before. Wells with immobilized PPL were treated similarly but without any inhibitor as the controls. The percentage of DMSO in each reaction solution was fixed to 2% v/v. The inhibition rate was calculated by equation 5:

$$I(\%) = \frac{A_0 - A_i}{A_0} \times 100\% \quad (5)$$

where I (%) represents the inhibition rate, A_i and A_0 represent the enzymatic activity with or without inhibitors, respectively. The value of IC_{50} was obtained by the dose-response nonlinear regression equation using the PRISM® 9.0 software.

Evaluation of the Inhibitor Screening Assay The accuracy and reliability of the inhibition rate were evaluated by the Z' factor [31], which was calculated by equation 6:

$$Z' = 1 - \frac{3\delta_s + 3\delta_c}{|q_s - q_c|} \quad (6)$$

where q_s and q_c represent the average enzyme activity when there is no inhibitor (s, sample) and the inhibition rate is 100% (c, control), respectively. δ_s and δ_C refer to the average of the respective standard deviations of the data. q_c and δ_C are equal to 0 when the inhibition rate is 100%. Therefore, equation 6 is simplified to equation 7:

$$Z' = 1 - \frac{3\delta_s}{q_s} \quad (7)$$

A Z' value within the range of 0.5 to 1 indicates the accuracy and reliability of the proposed assay for screening inhibitors. The higher values suggest more accurate and reliable assays [31].

Inhibition by Olive Leaf Extract

(i) Preparation of the Olive leaf Extract The plant material, olive (*Olea europaea* L.) leaf (*Chondrolia Chalkidikis* cultivar), were collected from an olive orchard (Serres, Greece). The leaf were rinsed multiple times with tap water and then with dd H_2O and air-dried before use. The extraction method was according to Chatzikontantinou et al. [32]. In a standard procedure, 50 g of dry olive leaf were macerated in 250 mL dd H_2O and boiled for 60 min. The extract was filtered, and the water was removed through freeze-drying to obtain 3 g of dried material of aqueous olive leaf extract.

(ii) Inhibition Study The constructed PPL wells were used to screen the inhibitory activity of the olive leaf extract. A stock solution of the extract was prepared in 50 mM phosphate buffer (pH 7). The concentrations of 1, 2, 3, 5, 8, and 10 mg/mL of the extract were tested. The inhibition was evaluated using the method mentioned in Section 2.2.8. The value of IC_{50} was calculated in accordance with section 2.2.8.

3. Results

3.1 3D Printed Scaffolds for PPL Inhibition Studies

The present study elaborated on 3D printing technology for producing ready-to-use well plates for enzymatic assays. Previous reports have demonstrated the importance of developing multi-plate bioassay methods for enzyme inhibition studies and immobilized enzymes [24, 33]. The advantages include reduced reagent volumes, rapid testing of multiple compounds, and cost-efficiency. The study immobilized PPL inside 3D printed PLA wells to screen enzyme inhibitors, providing an efficient and low-cost (approximately 0.04€ per test) analytical tool. A synopsis of this process is presented in Figure 2. After designing and printing the desired model, the surfaces of the 3D scaffolds were modified according to our previous work [30]. The lipase from the porcine pancreas was immobilized inside the modified PLA wells with non-covalent interactions. An immobilization yield of 50% ± 6% was obtained for all samples using a fixed initial enzyme concentration. The prepared well plates were used for biocatalytic studies, as described in the following sections.

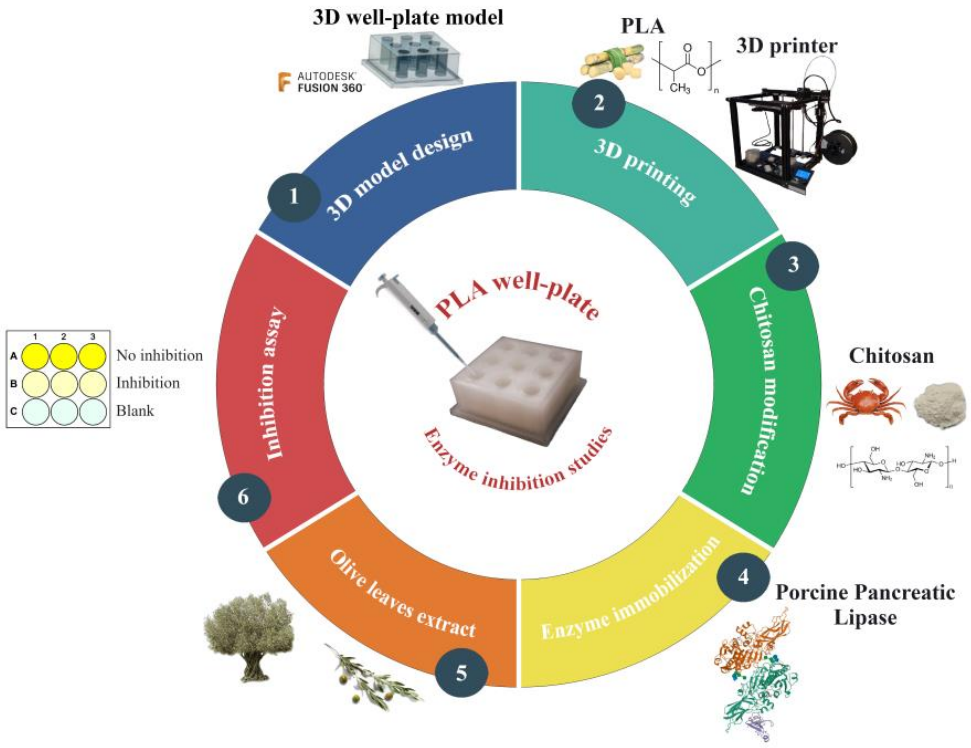

Figure 2 The process for scaffold preparation, enzyme immobilization, and enzyme inhibition studies

3.2 Spectroscopic Characterization of the Modified 3D Printed PLA Scaffolds

X-ray photoelectron spectroscopy (XPS) was performed after the NaOH-CS (0.2%) treatment for more profound information on the printed PLA scaffolds. The characteristic C-C/C-H, C-OH, C =O, and C(O)O peaks represented 65.0%, 11.6%, 13.5%, and 9.9% of the C1s photoelectron spectra, respectively (Figure 3A). These XPS analysis results are in accordance with our previous results for the same treatment [29]. The N1s peak demonstrated the existence of chitosan on the surface of the 3D-printed PLA scaffold. The atomic percentages are displayed in Table 1.

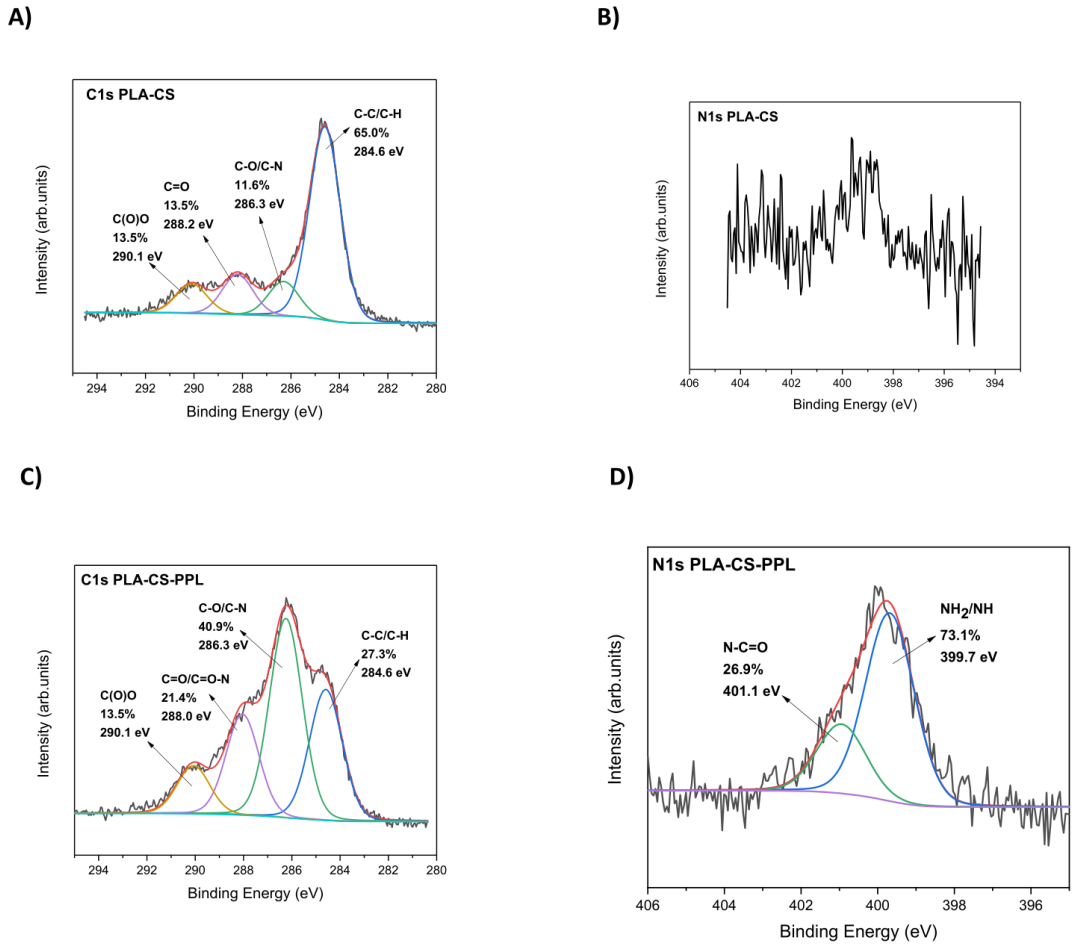

Figure 3 The C1s photoelectron spectra of (A) PLA-CS and (C) PLA-CS-PPL. The N1s photoelectron spectra of (B) PLA-CS and (D) PLA-CS-PPL.

After immobilizing PPL on the surface of PLA-NaOH-CS (0.2%), the C1s photoelectron peak changed significantly, indicating the successful incorporation of the enzyme (Figure 3C). The percentage of the major C-C peak reduced to 27.3%, while the C-OH peak at 286.3 eV dramatically increased to 40.9%. The C-N bonds from PPL also contributed to the same peak. The following peak at 288.0 eV is attributed to the C =O bonds and amide bonds of the PPL representing 21.4% of the whole carbon amount. Finally, the last peak at 290.1 eV may be due to the carboxylated groups. The N1s photoelectron peak is deconvoluted into two peaks, one main peak centered at 399.7 eV (73.1%) due to the NH_2/NH groups and one at 401.1 eV due to the amide formation of PPL (27.9%) (Figure 3B, Figure 3D). The atomic percentages are displayed in Table 2 and describe the increase of the Oxygen and the Nitrogen atoms compared to PLA-NaOH-CS 0.2%.

Table 2 Atomic percentages (%) of PLA modified with chitosan (PLA-CS), and PLA modified with chitosan and immobilized with PPL (PLA-CS-PPL).

Atom	PLA-CS	PLA-CS-PPL
C	72.9%	64.0%
O	25.9%	31.8%
N	1.2%	4.2%

3.3 Biocatalytic Characterization of the Immobilized PPL on 3D Printed PLA Scaffolds

3.3.1 Operational Stability

Enzyme immobilization plays a major partis important in catalyst stabilization, allowing for the repeated use of the active biocatalytic system. In fact, enzyme reuse is a prerequisite when establishing sustainable bioprocesses. Since organic solvents are frequently used for solubilization, their effects on enzymes have been studied for many years [34-36]. DMSO (dimethyl sulfoxide) is a well-known protein denaturant. Hence, it is essential to understand its effect on the enzyme activity in advance, especially when subjecting the enzyme to consecutive reactions. Literature reports on PPL suggest using less than 5% (v/v) of DMSO in enzymatic reactions because higher concentrations affect enzyme performance [37-40].

The reusability of the non-covalently immobilized PPL on the PLA wells was explored in terms of PPL activity in the presence of two reaction media − one without DMSO, while the other contained 2% (v/v) of DMSO. The substrate concentration was fixed at 1 mM, and ten consecutive reaction cycles were performed. After determining the activity of each cycle, the wells were washed, dried, and re-incubated with the corresponding reaction solution. The remaining (residual) activity of the immobilized PPL after each cycle is depicted in Figure 4A. Notably, at 0% DMSO concentration, the system maintained 59% of its initial activity in the 10^{th} cycle. At 2% DMSO concentration, the residual activity was reduced slightly (51%). The gradual activity loss at each cycle could be attributed to desorption, denaturation, or conformational changes in the enzyme molecules [41, 42]. Comparatively, the operational activity of the PPL immobilized non-covalently on different carriers was lower than that reported in the present work. For example, PPL adsorption on mesoporous chitosan beads retained 38% of the activity after ten cycles of reuse while hydrolyzing p-nitrophenyl palmitate (pNPP) [42]. However, its immobilization onto TiO_2/chitosan composite beads by adsorption and cross-linking exhibited nearly 50% of the activity after seven cycles of reuse while hydrolyzing p-nitrophenyl palmitate (pNPP [41]. Thus, the current study demonstrated the application of the immobilized PPL in successive reactions in both reaction media (0% or 2% DMSO for water-soluble or water-insoluble screened compounds, respectively). The lipase maintained more than half of its initial activity after ten cycles of reuse.

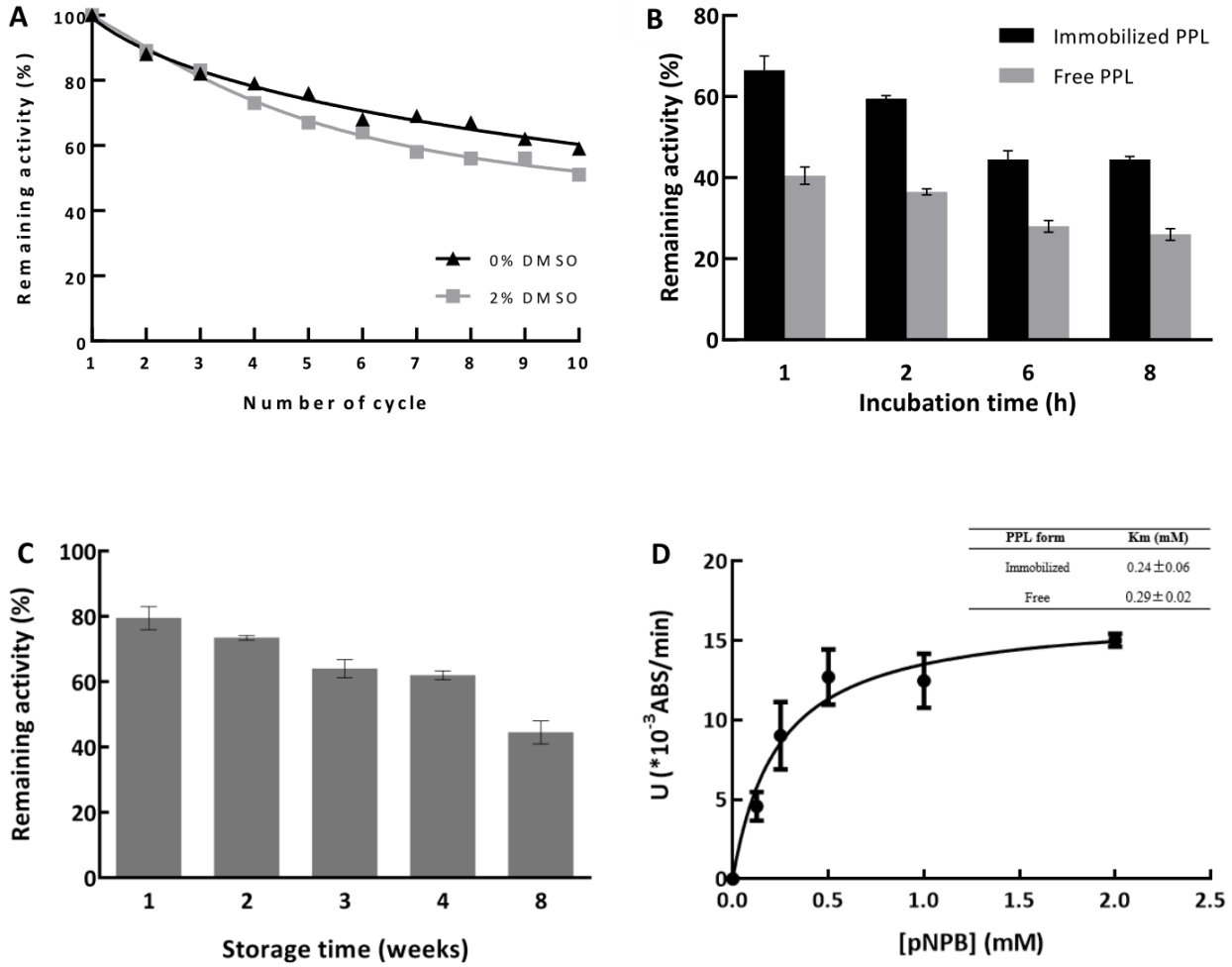

Figure 4 Biocataytic studies of the immobilized PPL on PLA wells. **(A)** Operational stability of the non-covalently immobilized PPL on PLA wells in the presence (2%) and absence (0%) of DMSO for ten cycles of reuse. The activity measured after the first use is considered 100%. (**B)** Thermal stability of the free and non-covalently immobilized PPL on PLA wells after incubation at 40 °C for 1, 2, 6, and 8 h. The activity exhibited before incubation is considered 100%. (**C)** Storage stability of the non-covalently immobilized PPL on PLA wells after incubation at 4 °C for 1, 2, 3, 4, and 8 weeks. The activity exhibited before storage is considered 100%. The reactions were performed at 40 °C for 20 min with 1 mM of the substrate (pNPB) in 0.05 M phosphate buffer (pH 7). (**D)** Michaelis–Menten plot for the PPL kinetic constant. The reactions were performed at 40 °C for 20 min with the substrate (pNPB) concentrations varying from 0.125 to 2 mM.

3.3.2 Thermal Stability

The thermal stability of a biocatalyst is its ability to withstand thermal denaturation without any substrate at high temperatures. Such a study is significant because the function of an enzyme is inseparable from its structure [43, 44]. Herein, the thermal stability of the immobilized and the free PPL was assessed for up to 8 h of incubation at 40 °C, the reaction temperature. The residual activity of the immobilized PPL indicates the activity of the free enzyme during each time interval tested. The immobilization of PPL was advantageous to lipase stability (Figure 4B). The immobilized PPL

retained 45% of its initial activity after 8 h of incubation at 40 °C, while the free PPL retained 26% under similar conditions. Indeed, other chitosan-based scaffolds support the above results [45-47]. The carefully designed immobilization carriers stabilize the biocatalytic system and offer a microenvironment that protects the enzyme from denaturation and, hence, activity loss.

3.3.3 Storage Stability

Storage stability is another aspect that should be tested when evaluating the efficiency of a biocatalytic system. Immobilization facilitates enzymatic stability, ensuring longer shelf life. The storage stability of the immobilized PPL was assessed after 1, 2, 3, 4, and 8 weeks of storage at 4 °C (Figure 4C). A sharp decline of about 20% was observed in the residual activity after the 1st week, possibly due to enzyme desorption. A comparable decrease in the activity of the immobilized form of PPL was also noted by Kilinç et al. (2002) in the first ten days of storage [48]. After eight weeks of storage, the residual activity of the PLA-immobilized PPL was 45%. Interestingly, the free enzyme lost more than half of its initial activity in the first week of storage. Thus, the biocatalytic system developed in the present study was stable and could be helpful in various applications.

3.3.4 Kinetic Study of the Immobilized PPL on PLA Wells

The effect of immobilization on the catalytic behavior of PLL was determined from the Michaelis-Menten plot. Several substrate concentrations (0.125–2 mM) were used to derive the Michaelis-Menten constants (K_m) of the free and immobilized enzymes (Figure 4D). The K_m value of the immobilized PPL was 0.24 ± 0.06 mM, which was similar to the K_m value of the free PPL (0.29 ± 0.02 mM). Thus, lipase adsorption on chitosan-modified PLA wells did not initiate steric hindrance of the active site. Hence, substrate accessibility and binding occurred without significant limitations.

3.4 Inhibition Studies

3.4.1 Inhibition by Orlistat

Evaluating PPL inhibition is clinically significant in obesity and related diseases. Also, establishing more efficient inhibition assays is critical. Hence, the prepared immobilized PPL wells were further applied in inhibitor screening assays. Orlistat, a commercially available pancreatic lipase inhibitor, was employed as the positive control to verify the proposed method. Varying concentrations (0.005–0.1 μM) of Orlistat were tested at a fixed substrate concentration (1 mM) to determine the half-maximal inhibitory concentration (IC_{50}) according to the dose-response nonlinear regression equation (Figure 5A, Figure 5B). The IC_{50} value for the immobilized PPL was 31.73 ± 6.52 nM, whereas that of the free lipase was 8.53 ± 1.68 nM. Therefore, the immobilized system detected the inhibitory effect exerted by the model compound, but 50% inhibition of PPL required a higher concentration. The observed difference between the two values could be attributed to the immobilization of the lipase on chitosan-modified PLA wells. Similarly, different inhibition rates between the free and immobilized form of cathepsin D were realized in some cases when the same extract or compound was incubated with both forms of the enzyme [49]. Notably, the IC_{50} values of Orlistat reported for PPL in the literature often are not comparable. They deviate largely among a variety of studies, ranging from (0.38 ± 0.05) × 10^{-3} μM to 60.48 ± 0.02 μM [39, 50]. These fluctuations may be due to the variations between the distinct assays. Hence, a more reliable

criterion was required to assess the inhibitory capacity of the immobilized enzyme. The Z' factor is a parameter that extensively reinforces the accurate detection and reliability of enzyme inhibitors in an assay procedure (Section 2.2.8.2). The present study identified 0.86 (n = 8) as the value of the Z' factor, indicating that the proposed method was ideal for recognizing PPL inhibitors [31]. The results mentioned above summarize that the immobilized PLA wells could be successfully incorporated in inhibition studies of various possible inhibitors of natural origin.

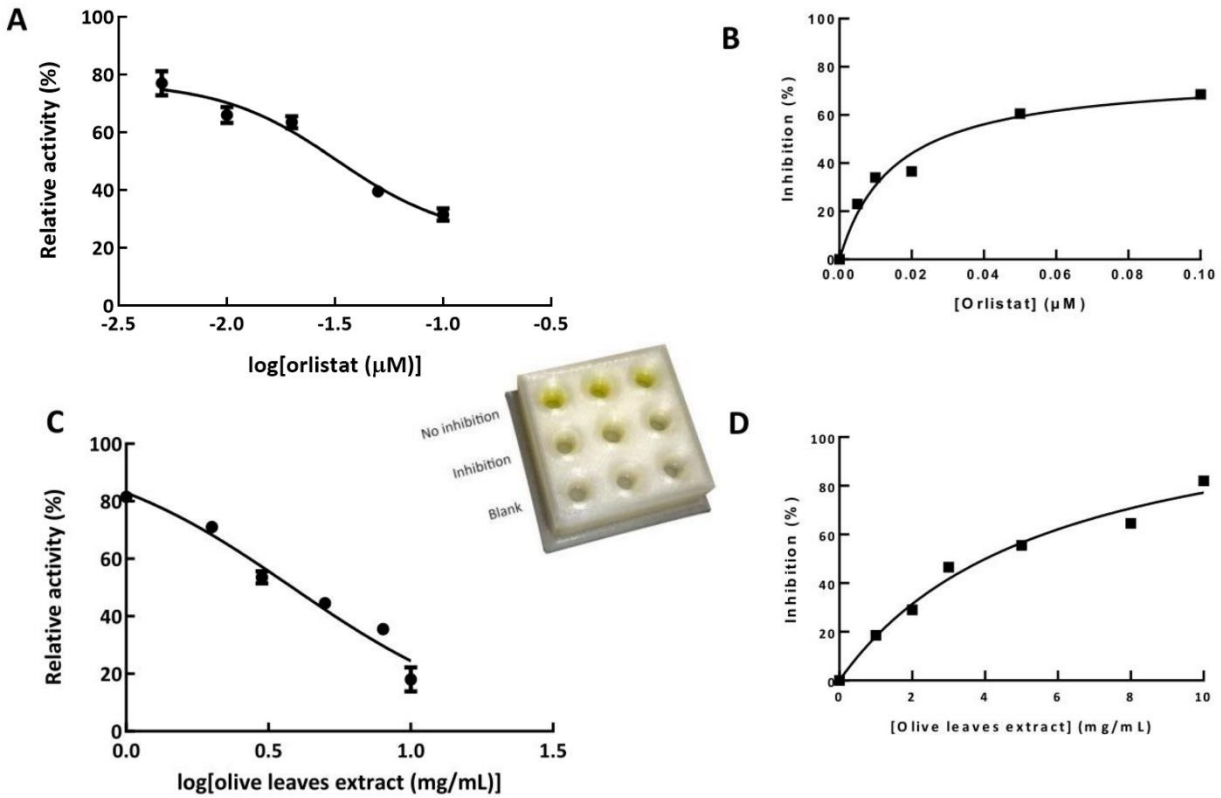

Figure 5 Inhibition studies of the immobilized PPL using Orlistat and olive leaf extract. **(A-B)** Determining the values of the inhibitory concentration (IC_{50}) of Orlistat for inhibiting immobilized PPL. The reactions were performed at 40 °C for 20 min using 1 mM of the substrate (pNPB). The orlistat concentrations ranged from 0.005–0.1 μM (2% DMSO) in 0.05 M phosphate buffer (pH 7). (**C-D)** Determining the values of the inhibitory concentraton (IC_{50}) of the olive leaf extract for inhibiting immobilized PPL. The reactions were performed at 40 °C for 20 min using 1 mM of the substrate (pNPB). The concentration of the olive leaf extract ranged from 1–10 mg/mL in 0.05 M phosphate buffer (pH 7). There was a 10 min pre-incubation period between the lipase and the inhibitor at 40 °C, after which the substrate was added.

3.4.2 Inhibition by Olive Leaf Extract

In a further study, a natural product, olive leaf extract, was also assessed for its inhibitory effect on PPL. When the assay was first performed with free PPL, the enzyme activity was reduced. Subsequently, the extract was used to determine the IC_{50} values of both forms. Varying concentrations (1–10 mg/mL) of the extract were investigated at a fixed substrate concentration (1 mM). The IC_{50} value of the olive leaf extract was 3.84 ± 0.48 mg/mL for the immobilized PPL (Figure

5C, Figure 5D, Table 3). This value was similar to that obtained for the free form (3.46 ± 0.34 mg/mL). Thus, the potentiality of the present assay for screening inhibitors was validated.

The inhibitory effect of extracts derived from *Olea europaea* L. on PPL has been also previously demonstrated. Buchholz and Melzig (2016) studied the PPL inhibitory activity of two olive leaf extracts. They obtained 0.186 ± 0.012 mg/mL and 6.772 ± 0.479 mg/mL as the respective IC_{50} values for the methanol and the aqueous extracts [50]. Consequently, the extraction solvent causes differences in the active components, thereby causing differences in the inhibitory potency of each extract. The differences in the content of the olive leaf extract used in the present study due to environmental and climatic conditions and the extraction method applied could be responsible for the different IC_{50} values of the aqueous extract.

Table 3 Inhibitory potency (IC_{50}) of Orlistat and olive leaf extract on PPL for both its immobilized and free form.

	IC_{50} for the immobilized PPL	**IC_{50} for the free PPL**
Orlistat	31.73 ± 6.52 nM	8.53 ± 1.68 nM
Olive leaf extract	3.84 ± 0.48 mg/mL	3.46 ± 0.34 mg/mL

Summing up, this study established a method for the development of a screening assay elaborating immobilized enzymes. The requirements for the development of a drug screening assay have been discussed thoroughly elsewhere [51]. Briefly, an inhibition assay must be able to detect inhibitors accurately and reliably; thus, which contributes to the assurance of assay quality as is denoted by Z' factor. In addition, the developed assay must be reproducible and of low cost. Another aspect that should be investigated is the influence of the presence of organic solvents, such as DMSO, to the assay performance, because this class of solvents are commonly used for chemical compounds preservation. Herein, the effect of the DMSO solvent on the enzymatic activity was evaluated and the value of Z' factor was determined as 0.86 (n = 8), which clarifies that the proposed method is accurate and reliable; therefore, appropriate for the identification of PPL inhibitors. Regarding the reproducibility and cost of the proposed method, a key feature of this study lies on the fabrication of the PLA well-plates through 3D printing, which allows for the adjustment of the number and volume of the wells, with a naturally-derived and low-cost feeding material. The surface modification of PLA with an eco-friendly, simple, time- and cost-efficient protocol based on chitosan, and the immobilization of PPL, which was found to be highly stable, allow for a highly reproducible method to develop biocatalytic systems.

4. Conclusions

The present work describes a method for enzyme immobilization and elaboration for inhibitors screening. 3D printed well-plates, served as immobilization scaffolds allowing for fast production of identical models of high quality and functionality to facilitate parameter testing. PLA was utilized as the immobilization matrix, being a material of agricultural origin, biocompatible and biodegradable, while pancreatic lipase was studied for being an important enzyme related to metabolic diseases. The study showed that the immobilized enzyme retains its catalytic characteristics presenting high

operational, thermal and storage stability; thus, demonstrating the potential of the enzyme-immobilized well-plates for a series of biocatalytic studies.

The prepared biocatalytic system was evaluated as an analytical tool for inhibitors screening, both for a model inhibitor-orlistat, and for a novel potential inhibitor-olive leaf extract. For the inhibition study, the value of Z' factor was determined as 0.86 (n = 8), which clarifies that the proposed method is appropriate for the identification of PPL inhibitors. The IC_{50} factor calculated for the olive leaf extract was 3.84 mg/mL, presenting a promising effect as an inhibitory agent of natural origin.

Thus, designing and fabricating scaffolds in a similar scale, which could serve as immobilization carriers, and their recruitment in inhibitors screening studies could be a promising bioanalytical tool, adding enzyme stability and reuse potentiality of the biocatalytic system, as essential prerequisites for sustainable bioprocess design.

Author Contributions

Anastasia Skonta: Conceptualization, Methodology, Investigation, Writing-Original draft, Elena Gkantzou: Conceptualization, Methodology, Investigation, Writing-Original draft-Review and Editing, Konstantinos Spyrou: Investigation, Data curation, Stamatia Spyrou: Investigation, Data curation, Angeliki Polydera: Writing-Review and Editing, Dimitrios Gournis: Writing-Review and Editing, Haralambos Stamatis: Conceptualization, Fund raising, Writing-Review and Editing, Supervision.

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Competing Interests

The authors have declared that no competing interests exist.

Additional Materials

The following additional material is uploaded at the page of this paper.

.STL file of well-plate model

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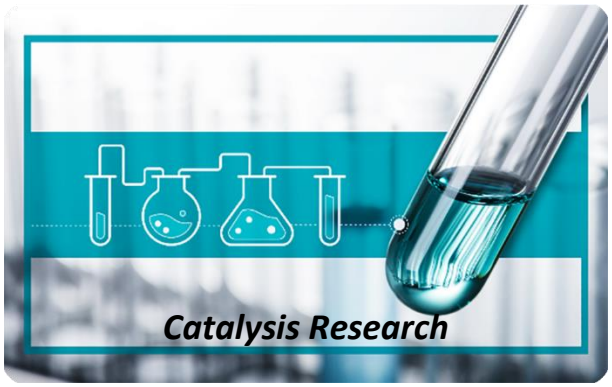

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