# ENZYME IMMOBILIZATION ON POLYMERIC NANOPARTICLES AS A TOOL TO IMPROVE BIOCATALYTIC PERFORMANCE

L. Chronopoulou[1*], G. Kamel[2], F. Bordi[2], S. Lupi[2], C. Palocci[1]

[1]*Department of Chemistry, Università degli Studi di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy*
[2] *Department of Physics, Università degli Studi di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy*
**laura.chronopoulou@uniroma1.it*

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## Abstract
*The adsorption of Candida rugosa lipase (CRL) on different types of polymeric nanoparticles, both synthetic and derived from natural sources, was studied. Comparing to non nanostructured carriers, polymeric nanoparticles were able to induce a stabilization of the adsorbed CRL to different denaturing agents such as temperature, medium pH or organic solvents. Moreover, we evidenced that 200 nm poly-D,L-lactic acid nanoparticles could influence catalytic performance, enhancing dramatically CRL activity in aqueous medium. FTIR spectroscopy was used to characterize both free and immobilized CRL, evidencing a reproducible modification in the conformational features of CRL in different media (solid state and aqueous solutions).*

## 1 Introduction
In recent years, advances in nanofabrication have made accessible a wide variety of nanomaterials that possess peculiar electronic, photonic and catalytic properties [1, 2]. Nanomaterials are in the same size scale as biological macromolecules, such as proteins and nucleic acids. Therefore, the intersection between biotechnology and nanotechnology has lead to the development of hybrid materials, combining the catalytic and highly selective recognition properties of biological molecules to the peculiar characteristics of nanoparticles (NPs).
Enzyme immobilization is being acknowledged as a powerful strategy for improving biocatalyst stability, separation and reuse, allowing the set up of continuous operation systems [3]. Such systems, limiting biocatalyst consumption, can effectively reduce operation costs, thus removing some of the still existing limitations to the use of biocatalysts in large-scale processes.
Nonporous carrier materials, to whose surfaces enzymes are attached, are subject to minimum diffusional limitations. However, enzyme loading per unit mass of support is usually considerably low. Alternatively, high enzyme loading can be achieved with porous materials, that, however, suffer much greater diffusional limitations. Nanostructured materials should provide the upper limits in terms of balancing contradictory issues in enzyme immobilization such as high surface area, low mass transfer resistance and effective enzyme loading.

Recent research efforts have been devoted to the immobilization of lipases, among the most commonly used industrial enzymes, on nanomaterials such as magnetic nanoparticles (NPs) and nanotubes [4], with positive outcomes in terms of enantioselectivity and thermal stability. It is accepted, though not yet fully understood, that enzyme activity and stability are strongly dependent from protein structure. This is particularly important for lipases, that, in the presence of a water-lipid interface, undergo a conformational change that affords the catalytically active form of the enzyme. Therefore, it is clear that a deep understanding of the interactions of the enzyme with the immobilization surface and, more in general, with its ''nano-environment'' at the support/solvent interface is a major requirement for the advancement of technological applications. The understanding of the protein/protein and protein/surface interactions and of the whole protein adsorption process could eventually lead to the possibility of directing the self-assembly of the enzyme at the nanometer scale, necessary for the development of more efficient biosensors and/or reactors based on the controlled deposition of enzymes at the molecular level.
In this work, *Candida rugosa* lipase (CRL) non covalent immobilization was studied on different polymeric nanomaterials. The stability and catalytic activity of the bioconjugates were tested in different experimental conditions and compared to free CRL and CRL immobilized on non-nanostructured supports. Moreover, an FTIR study was conducted to elucidate the structure-performance relationships of immobilized CRL.

## 2 Materials and testing methods

### *2.1 Materials*

*Candida rugosa* lipase (CRL) type VII, tributyrin, 2-morpholino-ethane sulfonic acid (MES), methylmethacrylate, styrene, potassium persulfate, bovine serum albumin (BSA), Bradford Reagent and poly-DL-lactic acid (PDLLA) were purchased from Sigma-Aldrich and used as received. All solvents were of analytical grade and used as received.

### *2.2 Preparation of the carriers*

Polymethylmethacrylate (PMMA) and polystyrene (PS) NPs were prepared by emulsion polymerization methods described in literature [5]. PDLLA NPs were prepared by using a recently patented methodology [6]. Commercially available polymer was dissolved in DMF. The solution (5 mg/mL) was transferred into a dialysis bag and immersed into a non-solvent solution (solvent/non-solvent ratio 1:20). The system was incubated at 4°C for 5 days, and then the precipitated polymer was recovered, washed with $H_2O$, centrifuged and freeze dried.

### *2.3 Adsorption experiments*

Adsorption experiments of CRL on polymeric NPs were performed in Pyrex tubes containing a known amount of polymer dispersed in 2 mL of phosphate buffer solution (PBS 0.1 M, pH 7.6) of lipase (50 mg/mL), under magnetic stirring and at 25°C. The amount of immobilized enzyme was obtained by standard Bradford assay [7] of the original lipase solutions, the supernatants, and the washing solutions after immobilization.

### *2.4 Evaluation of the adsorption isotherms*

Adsorption isotherms were studied at 25 and 50°C in PBS at pH 7.6. Fixed amounts of protein and carrier were mixed and incubated for 4.5 h, varying the concentration of the protein solution between 0.5 and 6 mg ml. Thereafter, the mixture was filtered, and the protein concentration in the supernatant was measured by the Bradford assay. The amount of adsorbed protein was determined from the difference between the protein concentration in solution before and after adsorption.

### *2.5 Lipolytic assays*

The activity of free and immobilized CRL was determined by a standard hydrolysis assay. In standard conditions, the reaction mixture composed of 2.5 mL of PBS (pH 7.6), 0.5 mL of tributyrin and 0.1 mL of enzyme solution (50 mg/mL) was incubated at 37°C under magnetic

stirring (600 rpm) for 30 min. Hydrolysis was stopped by adding 3.0 mL of acetone/ethanol mixture 1:1, and the reaction mixture was titrated with 0.1 M NaOH in the presence of phenolphthalein as an indicator using an automatic titrator.

*2.6 pH and thermal stability measurements*

pH stabilities of free and immobilized CRL were assayed by measuring lipolytic activity in PBS in the pH range of 5.5–7.9 at 37°C. Thermal stabilities of bound and free CRL were investigated by measuring lipolytic activities in PBS at pH 7.6 in the temperature range between 25 and 60°C.

*2.7 FTIR measurements*

FTIR absorption spectra were collected in the mid-IR range from 4000 to 400 $cm^{-1}$ using an IFS66v spectrometer (Bruker optik GmbH) equipped with a MCT (Mercury Cadmium Telluride) detector. 1000 scans were collected in transmission mode at a spectral resolution of 4 $cm^{-1}$ at a scanning speed of 8 kHz. $^2H_2O$ solutions of the pure and immobilized protein on nanostructured PDLLA were placed in a transmission cell with $CaF_2$ windows and Mylar spacers 12 mm thick. Spectra were obtained after subtraction of solvent absorption.

*2.8 FTIR data analysis*

The analysis of the protein secondary structures was based on the amide I band located in the 1700–1600 $cm^{-1}$ spectral region. Second derivative spectra were obtained following the Savitsky–Golay method after a binomial 9 points smoothing of the spectrum followed by a baseline correction. The amide I band was analyzed in terms of a linear combination of spectral components identified in the second-derivative spectrum. Those components were approximated by Gaussian functions whose peak positions, widths and intensities were adjusted iteratively in the curve-fitting procedure. The Gaussian starting values were determined following the procedure suggested by Arrondo et al. [8] and during the minimization procedure, the parameters of the Gaussian components were left free to adjust iteratively. The area of each component, expressed as a percentage of the total amide I band area, is taken as a measure of the secondary structure content assigned to it.

## 3 Results and discussion

*3.1 Preparation and characterization of nanopolymeric carriers*

Dimension control and low polydispersion of both PS and PMMA NPs were obtained by controlling the amount of emulsifier, the sorbent/water phase ratio, and reaction time. PDLLA samples were processed through the osmosis-based methodology under different temperatures, solvent/non-solvent couple, polymer concentration and process time conditions, in order to identify the optimal experimental conditions to prepare PDLLA carriers.

The morphological characterization was carried out by SEM analysis. Figure 1 shows that particles morphology is spherical. PMMA and PS NPs mean diameter is 300 nm, while PDLLA NPs mean diameter is approximately 220 nm.

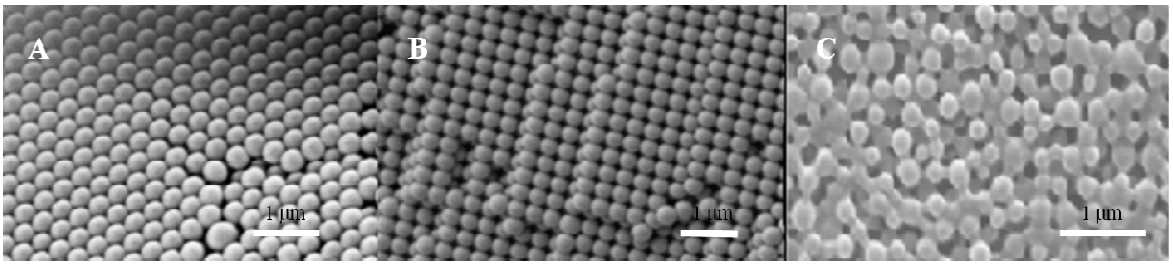


**Figure 1.** SEM micrographs of PS (A), PMMA (B) and PDLLA (C) NPs.

*3.2 Lipase immobilization on polymeric NPs*

In order to reach a better understanding of the interactions between CRL and polymeric NPs, the thermodynamics of the adsorption phenomenon were investigated. Adsorption isotherms of CRL on polymeric NPs were evaluated at two different temperatures, 25 and 50°C (Figure 2). At both temperatures, in the first part of the curves, i.e. in the low concentration range, by increasing the enzyme concentration, the enzyme loading also increases. As expected in the presence of kinetic effects, at higher temperature the enzyme loading grows faster with protein concentration. However, at both temperatures the same plateau value is reached for each system. All curves have a similar profile, corresponding to a Langmuir-type isotherm that, according to theoretical investigations, indicates a strong affinity between the adsorbent and the adsorbate that favors intermolecular interactions and the substantial irreversibility of the adsorption [9].

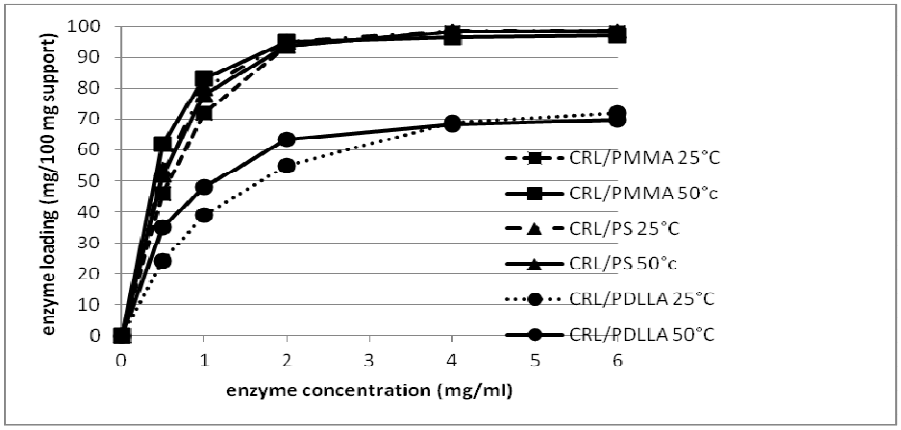


**Figure 2.** CRL adsorption isotherms on polymeric NPs at 25 and 50°C.

*3.3 Activity and stability of CRL–NPs bioconjugates in aqueous medium*

One of the most crucial issues in enzyme immobilization is activity retention after the interaction with a solid support, which, by inducing conformational changes in the adsorbed protein, may affect its activity. Nanobioconjugates lipolytic activity was measured with a standard assay and compared to those of free CRL and of CRL immobilized on non-nanostructured supports (Table 1) [10, 11]. In general, it was observed that nanocarriers were able to provide a higher activity retention. Moreover, a strong activation occurred when CRL was immobilized on PDLLA NPs.

| Sample | Specific activity (UI/mg) | Activity retention (%) |
|---|---|---|
| Free CRL | 3.90 | 100 |
| CRL-PMMA beads | 0.78 | 20 |
| CRL-PMMA NPs | 2.34 | 60 |
| CRL-PS beads | 1.13 | 29 |
| CRL-PS NPs | 2.89 | 74 |
| CRL-PDLLA beads | 3.85 | 99 |
| CRL-PDLLA NPs | 9.45 | 242 |

**Table 1.** Lipolytic activity of free and immobilized CRL in Standard Reaction Conditions.

Also, the stabilities of free and immobilized CRL to denaturing agents such as pH and temperature were studied. pH stability of the CRL/PMMA and CRL/PS nanobioconjugates

between 5.9 and 8 pH values as assessed and was comparable to free CRL stability (Figure 3). CRL/PDLLA nanobionjugate also showed a high stability.

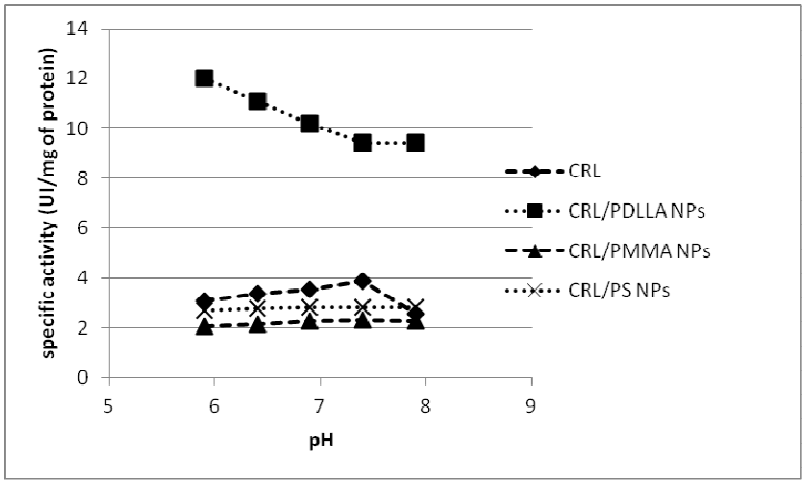


**Figure 3.** pH stabilities of free and immobilized CRL.

Temperature stability of nanobioconjugates was high between 25 and 40°C; however, CRL/PDLLA bioconjugates lost their activity above 40–45°C (Figure 4). This value is close to the PDLLA vitreous transition temperature that may be responsible for activity loss.

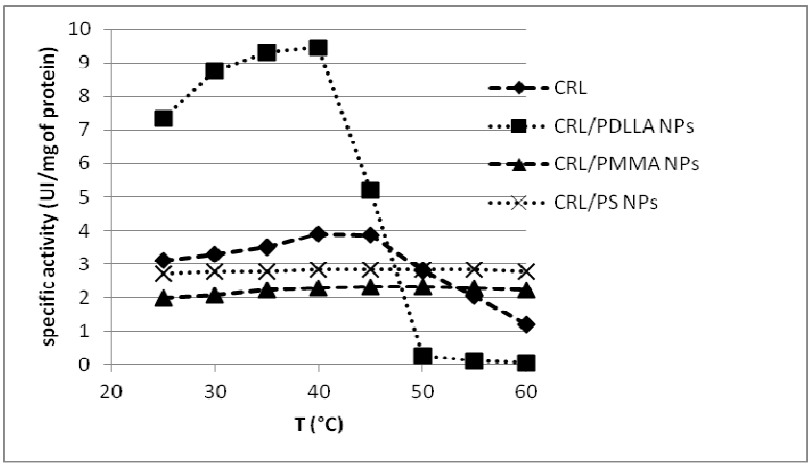


**Figure 4.** Thermal stabilities of free and immobilized CRL.

*3.4 Stability of CRL–NPs bioconjugates in organic medium*

As it is well known, lipases are useful biocatalysts in synthetic reactions because they can catalyze both esterifications and transesterifications in non-aqueous media. However, before employing biocatalysts in non-conventional media, it is crucial to evaluate their stability in such environments. Therefore, immobilized CRL was incubated in different organic solvents and residual lipolytic activities were tested after fixed time intervals. Incubation in hydrophobic solvents like n-hexane and tert-butyl-methyl ether led to high retention of lipolytic activity for all preparations (Table 2).

| | % of residual enzymatic activity | | |
|---|---|---|---|
| | **CRL/PDLLA NPs** | **CRL/PMMA NPs** | **CRL/PS NPs** |
| **n-hexane** | 59 | 80 | 100 |
| **t-but-methylether** | 64 | 59 | 70 |
| **acetonitrile** | 2 | 73 | 84 |

**Table 2.** Activity retention of immobilized CRL after incubation in organic solvents.

*3.5 FTIR spectroscopic study*

In the present study, we examined the amide I absorption band in $^2H_2O$ of free and immobilized CRL on PDLLA NPs. The assignment of amide I components of the native CRL was performed according to literature data [12]. The Decomposition of free and immobilized CRL amide I bands into their Gaussian components are shown in Figure 5 [11].

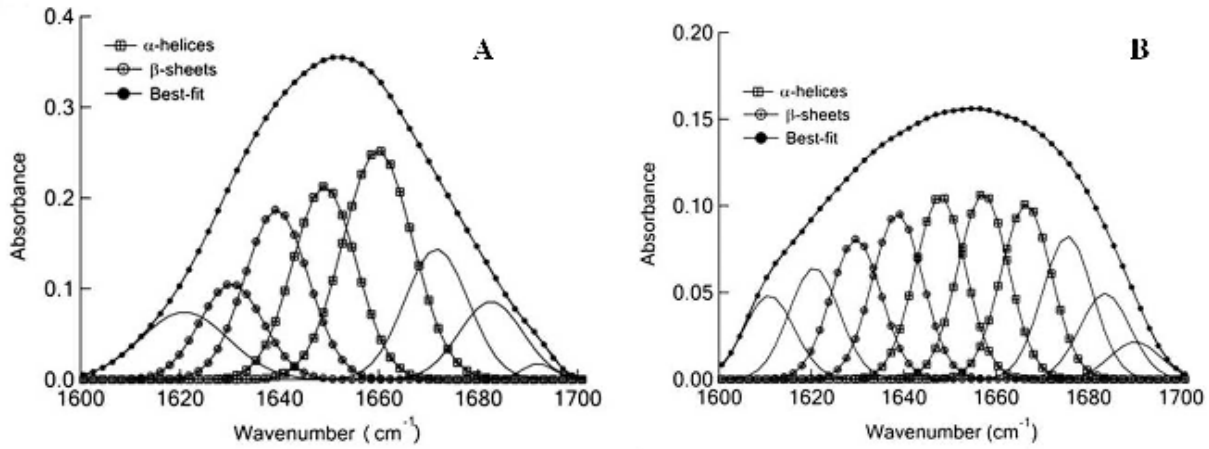


**Figure 5.** Decomposition of free (A) and immobilized (B) CRL amide I bands in $^2H_2O$ into their Gaussian components.

CRL is a single domain protein whose secondary structural elements have been already published [13]. Two conformations have been identified for the lipase molecule in its crystal form, depending on the active site accessibility to the substrate; when the active site is shielded from the solvent by a part of the polypeptide chain known as the lid, the lipase is said to occur in the inactive ‘‘closed’’ conformation, CRLc. In the presence of a water–lipid interface, the active site is exposed to the solvent and the lipase occurs in the active ‘‘open’’ conformation, CRLo.

| | FTIR | | XRD | |
|---|---|---|---|---|
| | **Free CRL** | **CRL/PDLLA NPs** | **CRLc** | **CRLo** |
| **α-helices** | 44% | 42% | 45% | 30% |
| **β-sheets** | 26% | 23.4% | 14% | 12% |

**Table 3.** Secondary-structure relative contents derived from FTIR and XRD studies.

A comparative study between published X-ray diffraction data of the two conformational states of CRL in its crystal form and experimental FTIR results was performed to investigate the conformational changes occurring in the free CRL upon its adsorption on PDLLA NPs. Based on the listed IR data in Table 3, free CRL has about 44% of α-helices and 26% of β-sheets. XRD data revealed in the CRLc a similar content of 45% α-helices and 14% of β-sheets. On the other hand, free CRLo, as obtained from XRD, contains 30% of α-helices and

12% β-sheets. For the immobilized CRL on PDLLA NPs, the α-helices content was found to be 42% against 23.4% of β-sheets. The discrepancy in β-sheets content is due to the different sensitivity of the techniques.
On this basis, we suggest that PDLLA NPs could simulate the effect of a hydrophobic interface, converting a fraction of the whole population of the lipase molecules from their closed to the open form.

## 4 Conclusions

Nanometric polymeric supports proved to be useful carriers for lipase immobilization, as they were able to maintain high activity and stability of the biocatalyst. Moreover, the bioconjugation of CRL to PDLLA NPs proved very interesting for its ability to enhance dramatically enzymatic activity. FTIR spectroscopy, successfully employed in the proteins characterization to elucidate the secondary structures of free and immobilized CRL, evidenced the occurrence of a conformational change upon CRL interaction with PDLLA NPs.
This work gives further evidence of the role of immobilization at an interface to enhance the catalytic activity by favoring the active conformation of the enzymatic protein.

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