Journal of Applied Polymer Science

Functional hydrogels with a multicatalytic activity for bioremediation: Single-step preparation and characterization

Umile Gianfranco Spizzirri, Manuela Curcio, Giuseppe Cirillo, Nevio Picci, Fiore Pasquale Nicoletta, Francesca Iemma

Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, I-87036 Rende (CS), Italy

U. G. Spizzirri and M. Curcio contributed equally to this work

Correspondence to: G. Cirillo (E-mail: giuseppe.cirillo@unical.it)

ABSTRACT: In a single-step free radical reaction, multicatalytic hydrogels were synthesized by covalent immobilization of Pancreatin onto a film composed of Acrylamide and Polyethylene glycol dimethacrylate750. Hydrogels were characterized by determination of their dynamic swelling ratios and each catalytic activity was extensively investigated by determination of kinetic parameters K_M and V_{max}. The immobilization process was found to preserve the hydrolytic properties of Pancreatin (Protease, Lipase, and Amylase catalytic activities). Catalytic efficiencies were the highest with Protease and the lowest with Amylase. Reusability values higher than 60% after 10 repeated cycles proved the applicability of the proposed material in industrial practice. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43338.

KEYWORDS: catalysts; films; grafting; hydrophilic polymers; radical polymerization

Received 30 September 2015; accepted 14 December 2015

DOI: 10.1002/app.43338

INTRODUCTION

With the fast development of industrial practises, an intense interest has been recently devoted to the environmental pollution and its serious effects on humans, including climate changes and health risks. As a consequence of this matter, more restrictive government regulations, requiring dramatic changes in both industrial processes and wastewater treatment have been approved.[1,2]

Biotechnology offers several different approaches to answer to the increasing demand for effective bioremediation, with enzymes emerging as very promising alternatives to common catalysts. They operate under mild conditions, achieve higher product purity and avoid the use of toxic solvents,[3–5] nevertheless, the real applicability is often hindered by some limitations including a lack of long-term stability, difficulties in recovery and recycling, and protein contamination in the final product.[6,7] To overcome these drawbacks, enzyme immobilization is incoming as a valuable strategy to facilitate an efficient recovery and reuse, allowing the repeated or continuous use in a variety of reactors, such as packed bed, fluidized bed, flow-through microreactor, and membrane.[8,9]

To date, a variety of inorganic (silica, zeolites, alumina, etc.) or organic materials (hydrogels, porous acrylic resins, polymeric membranes, etc.) have been explored as supports for enzyme immobilization.[10–13] The ability to form hydrophilic aqueous microenvironments suitable for the accommodation of various size of matters,[14,15] together with their favorable properties, mainly biocompatibility, swelling ability, and resistance to dissolution,[16–18] makes hydrogels ideal supports for enzyme immobilization, since they provide improved stability of the immobilized enzyme preventing leakages or hindering the diffusion of substrate molecules and reaction products.[19,20]

Three main approaches have been employed to functionalize hydrogel networks with enzymes, namely encapsulation, binding, and cross-linking,[21,22] each of them showing key advantages and limitations. The encapsulation of enzymes in polymer networks allows an enhancement of thermal, pH, and storage stability,[23] while the low-term resistance to hard reaction conditions of some industrial processes (e.g. high ionic strengths or reactant/product concentrations) is not totally ensured, leading to a possible consistent reduction of the catalytic activity upon time.[24] As far as binding processes are concerned, both covalent and noncovalent interactions can be explored.[25] Catalytic hydrogels synthesized by weak interactions (hydrophobic and van der Waals forces) usually suffers for the same stability problems above mentioned.

The covalent linkage generally involves the use of lysine, cysteine, or aspartic and glutamic acid residues in the enzyme side chains, which undergo derivatization reaction with activated

hydrogels (by N-hydroxysuccinimide, epoxide, or diglycidyl chemistry), carbodiimide coupling, or aldehyde-mediated conjugation/reduction.[26,27] Nevertheless, reduction in the catalytic efficiency (CE) can be recorded for covalent conjugation processes as a result of the irreversible deactivation of catalytic sites during conjugation, carrying out to un-effective materials.[28]

A more valuable functionalization approach involves the modification of enzyme side chains with polymerizable groups to fabricate highly reactive macromers. The main limitation of this strategy is the need of a preliminary chemical treatment of enzyme molecules, with a possible damage of their catalytic cores.[29]

Recently, we explored the possibility to covalently functionalize hydrogel networks with pepsin by inserting the enzyme into the polymerization reaction without any preliminary chemical treatment, in a totally eco- and bio-compatible single step-reaction, with considerable advantages in terms of industrial applicability.[30] Based on previously reported results, here we have further explored this synthetic strategy with the attempt to obtain a hydrogel film with multicatalytic activity. More in details Pancreatin (PAN), a mixture of three different digestive enzymes produced by the exocrine cells of the pancreas with Amylase, Lipase, and Protease activity,[31] was immobilized by free radical reaction into a hydrogel composed of acrylamide (AAm) and polyethylene glycol dimethacrylate750 (PEGDMA). The ultimate aim of this study was the obtainment of multicatalytic hydrogels with potential industrial applications in the bioremediation processes for wastewater. In fact, such a multifunctional hydrogel could degrade α-1,4-glycosidic, triglyceride, and peptide (mainly at lysine or arginine COOH side groups) bonds exploiting the Amylase, Lipase, and Protease activities, respectively. An extensive characterization of the kinetic and reusability properties of multicatalytic hydrogels was performed to prove the applicability of the proposed macromolecular system.

EXPERIMENTAL

Pancreatin Solution

About 500 mg of Pancreatin from porcine pancreas (PAN, Sigma Chemical, Milan, Italy) were dispersed in 25.0 of phosphate buffered saline (PBS, $10^{-3}M$, pH 8.0), stirred for 30 min at 4°C and then centrifuged at 9500 rpm for 30 min using an ALC multispeed centrifuge PK121 (Thermo Scientific, Milan, Italy). The supernatant was freeze dried (Micro Modulyo freeze dryer, Edwards, Albany NY, USA) and, then, reconstituted in PBS ($10^{-3}M$, pH 8.0) for the synthesis of hydrogels at a final concentration of 20 mg mL^{-1}.

Synthesis of Catalytic Hydrogels

About 534 mg of Acrylamide (AAm, Sigma Chemical, Milan, Italy) were dissolved in 3.0 mL of Pancreatin solution in PBS and, then, 466 mg of Polyethylene Glycol Dimethacrylate750 (PEGDMA, Sigma Chemical, Milan, Italy) and 2.4% Irgacure 2959 (1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, with a maximum absorption at around 275 nm, BASF, Ludwigshafen, Germany) were added as cross-linker and photoinitiator, respectively. The solution was poured in a polymerization cell formed by two 10 × 10 cm^2 glass plates, separated with Teflon spacers (thickness 1.6 mm), brought together using binder clips. The polymerization was initiated by a high pressure mercury lamp (HPK 125, Philips, Amsterdam, Netherland, 10 mW cm^{-2}, wavelength 275 nm, irradiation time 10 min). Finally, the obtained hydrogels (labelled H_{PAN}) were extensively washed with water (10 × 50 mL) to remove unreacted species and, then, dried for 12 h in an oven under vacuum at 40°C.

Blank hydrogels (labelled H_B) were also synthesized as control samples: in this case the polymerization was performed in the absence of PAN.

About 250 mg amount hydrogels were crushed, grounded into powder, sieved through a 63 μm stainless steel sieve and extensively washed with water (5 × 50 mL).

Determination of Protein Content by Lowry Assay

Standard solutions of PAN (concentrations of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, and 2.00 mg mL^{-1}) were prepared for the construction of analytical curves after appropriate dilution of a stock solution (10 mg mL^{-1}). Aliquots of 800 μL of each standard, or blank, were transferred into test tubes and 4 mL of reagent C (Na_2CO_3 2% (w/v)/NaOH $0.1M + KNaC_4H_4O_6{\cdot}4H_2O$ 2% (w/v)/$CuSO_4{\cdot}5H_2O$ 1% (w/v), all reagents from Sigma Chemical, Milan, Italy) were added. After 10 min 400 μL of Folin-Ciocalteu reagent (Sigma Chemical, Milan, Italy) were also added and solution mixed. After 30 min, absorbance was measured at 750 nm on a Jasco V-530 UV-Vis spectrometer (Jasco Europe, Milan, Italy).[32] The same procedure was performed when a fixed amount of both crushed and uncrushed hydrogels film (5.0 mg) or 800 μL of media employed in the purification step of hydrogels. The results were expressed as mg of immobilized PAN per gram of polymer.

Dynamic Swelling Behavior

The hydrogel swelling degree was measured in water media as follows: specimens of ∼ 1 cm^2 were cut from dried samples, weighted, placed in a 5-mL sintered glass filter (porosity G3), and left to swell for 12 h by immersing the filters in beakers containing the swelling medium at 37.0 ± 0.1°C. Then, at suitable time intervals (1; 15; 30; 60; 120; 240; 360 min) excess water was removed, and samples were blotted with a tissue in order to remove surface moisture and weighed.

The water content percentage, WR, was expressed by the following equation:

$$WR = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

W_s and W_d are the hydrogel weights in the swollen and dried state, respectively.

The network parameter $\overline{M}_c$ of hydrogels was calculated by the Flory–Rehner Equation in the form proposed by Kulkarni[33]:

$$\bar{M}_c = -d_p v_{m,1} \phi^{1/3} \left[\ln(1-\phi) + \phi + \chi\phi^2\right]^{-1} \quad (2)$$

$v_{m,1}$ was the molar volume of the swelling media, χ is the Flory–Huggins interaction parameter and ϕ is the polymer volume fraction in the swollen state which is a measure of the amount of fluid retained by the hydrogel.

ϕ was calculated by using the following equation:

$$\phi = \left[\left(\frac{d_p}{d_s}\right)\left(\frac{w_s - w_d}{w_d}\right) + 1\right]^{-1} \quad (3)$$

d_p and d_s were the densities of polymer and solvent (g/cm^3); w_d and w_s the weight of polymer before and after 360 min swelling, respectively.

χ was calculated experimentally from the temperature coefficient of volume fraction ($d\phi/dT$) according to the following equation:

$$\chi = \left[\phi(1-\phi)^{-1} + N\ln(1-\phi) + N\phi\right]\left[2\phi - \phi^2 N - \phi^2 T^{-1}\left(\frac{d\phi}{dT}\right)^{-1}\right]^{-1} \quad (4)$$

($d\phi/dT$) is the slope obtained by plotting the volume fraction data versus temperature (K) and N is calculated according to the following equation:

$$N = \left(\frac{\phi^{2/3}}{3} - \frac{2}{3}\right)\left(\phi^{1/3} - \frac{2}{3}\phi\right)^{-1} \quad (5)$$

The mean pore size (nm) of the polymeric network of the hydrogels (ξ) was estimated using the below equation:

$$\xi = 0.071\phi^{-1/3}(\bar{M}_c)^{1/2} \quad (6)$$

Determination of α-Amylase Activity

Fuwa method with slight modifications was employed for the determination of Amylase activity.[34] Briefly, 5 mL of PBS ($10^{-3}M$, pH 6.9) containing PAN (1.75 mg) or H_{PAN} (aliquots containing 1.75 mg of immobilized enzyme as assessed by Lowry method) were mixed with 25 mL of starch (Sigma Chemical, Milan, Italy) water solution (final concentrations of 0.50; 1.00; 1.71; 2.56; 3.41; 4.27; 5.00 mg mL^{-1}) and incubated at 37°C for 30 min. The reaction was then terminated by adding 5.0 mL of 1.0N acetic acid (Sigma Chemical, Milan, Italy). After 5 min, the mixture was transferred into a 250 mL flask and diluted to 200 mL with water, followed by the addition of 5.0 mL of iodine reagent (0.2% iodine and 2% potassium iodide, Sigma Chemical, Milan, Italy). Finally, the Amylase activity was determined by measuring the absorbance of solutions at 700 nm on a Jasco V-530 UV-Vis spectrometer.

One unit of enzyme (U) for the starch–iodine assay was defined as the disappearance of an average of 1 ng of iodine binding starch material per min in the assay reaction.[35]

Determination of Lipase Activity

The kinetic of Lipase activity was measured at pH 8.0 and under the standard assay conditions described in literature with some modifications, using olive oil emulsion as a substrate.[35,36] More in details, olive oil emulsions were obtained by mixing 10.0 mL of olive oil in 90 mL of 10% Arabic gum (Sigma Chemical, Milan, Italy). Different amounts of this substrate emulsion were added to the reactor containing PAN (7.0 mg) or H_{PAN} (aliquots containing 7.0 mg of immobilized enzyme as assessed by Lowry method) to raise the final concentrations of 1.81; 3.63; 5.45; 7.23; 9.15; 10.93; 12.82; 15.53; 18.24 mg mL^{-1}, and the reaction proceeded for 5 min under stirring. The released fatty acids were titrated with 0.02 mol L^{-1} of potassium hydroxide solution.

One unit of enzyme (U) for the lipase activity was defined as an average of 1 ng of oleic acid equivalents released per min in the assay reaction.

Determination of Protease Activity

The kinetic of Protease activity of PAN and H_{PAN} was determined by using casein as substrate, by the method reported in literature with some modifications.[37] Briefly, free (5.5 mg) and immobilized enzymes (5.5 mg equivalent as per Lowry method) were incubated with 20 mL of casein solutions (Sigma Chemical, Milan, Italy, final concentrations of 0.50; 0.75; 1.00; 1.25; 1.87; 2.25; 2.50 mg mL^{-1}) for 5 min in PBS ($10^{-3}M$, pH 7.5). The enzymatic reaction was stopped by adding 4 mL of trichloroacetic acid solution (10% w/w, Sigma Chemical, Milan, Italy), the mixture was allowed to stand for 30 min at 4°C and, then, centrifuged at 9500 rpm for 30 min using an ALC multispeed centrifuge PK121 (Thermo Scientific, Milan, Italy). Casein hydrolysis was estimated by the Folin-Ciocalteu assay.[38] Briefly, 1.0 mL of supernatant diluted in distilled water (6.0 mL) and Folin-Ciocalteu reagent (1.0 mL) were mixed thoroughly. After 3 min, 3.0 mL of Na_2CO_3 water solution (7.5% w/w) were added, and the mixture was allowed to stand for 2 h with an intermittent shaking. The Protease activity was determined by comparing the absorbance of solutions at 750 nm on a Jasco V-530 UV-Vis spectrometer to the calibration curve of free Tyrosine, recorded by employing five different Tyrosine standard solutions. In particular, the calibration curve was recorded by measuring the absorbance of Tyrosine solutions in the Folin-Ciocalteu system at concentrations of 10.0; 25.0; 50.0; 100.0; and 150.0 μM after 2 h from the preparation. The correlation coefficient (R^2), slope, and intercept of the obtained regression equation were calculated by using the method of least-squares.

One unit of enzyme (U) for the protease activity was defined as an average of 1 ng of tyrosine equivalents released per min in the assay reaction.

Reusability Assay

For each catalytic activity, reusability was evaluated by performing 10 consecutive reaction cycles following the above describe protocols. After each cycle, residual activities were determined and films were recovered, stored for 24 h at 5°C, and, then, reused employing fresh substrates. Furthermore, the reusability was also tested by cross-assays alternating the catalytic assays (all the possible combinations were tested).

Statistical Analysis

All experiments were performed in triplicate, and results were in agreement within ± 5% standard error. One-way analysis of variance was performed to assess the significance of differences among data. Turkey–Kramer post-test was used to compare mean values of different data treatment. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis of Catalytic Hydrogels

Several food, pharmaceutical, and textile industry effluents are rich in starches, proteins, and oil-rich compounds, thus the digestion of such organic pollutants with hydrolytic enzymes can help in wastewater bioremediation.[39–41] Here, catalytic hydrogels were developed in the form of polymeric films, bearing three activities because of the covalent immobilization of the enzymes contained in the Pancreatin complex (PAN). The immobilized Pancreatin was expected to confer Amylase, Lipase, and Protease activity to final hydrogels, which were proposed as valuable material for wastewater treatment.

The synthetic approach to fabricate multicatalytic hydrogels consisted of a single-step free radical polymerization involving PAN, AAm, and PEGDMA as catalyst, monomer, and cross-linker, respectively. Irgacure 2959 induced the photo-polymerization of acrylate monomers to form a polymer layer with PAN units randomly inserted into the network. Specifically, the disposable heteroatoms of amino acid residues in the protein structure (mainly Lysine, Histidine, Cysteine, Serine, Tyrosine, etc.) underwent radical grafting onto the growing polymer chains with the resulting covalent immobilization of enzymes. This reaction mechanism is widely explored for the synthesis of functional biopolymers showing high enzymatic or therapeutic efficiencies, and offers the key advantages of operating in mild conditions, without any preliminary chemical treatment, any toxic solvents or by-products, and preserving the key properties of the biomacromolecules.[42–44]

The reaction conditions, chosen according to the previously obtained results, consisted in a mixture of AAm and PEGDMA (molar ratio 12 : 1) in a PAN solution (60 mg mL^{-1} in PBS $10^{-3}M$, pH 8). Different molar ratios were discarded since lower amounts of PEGDMA resulted in poorly cross-linked materials, while no significant improvement in the hydrogel performance was observed increasing the PEGDMA amount. At the same time, the PAN concentration (the highest amount soluble in the system) was selected to maximize the CE of the final hydrogels, since lower protein amounts carried out to materials with lower enzymatic activity.

To assess the complete removal of any trace of unreacted enzyme, H_{PAN} was extensively washed till no protein can be detected by Lowry assay in the washing media. The same results were obtained when the Lowry assay was performed on the washing media of the crushed H_{PAN}, proving the covalent immobilization of PAN into the hydrogel network, and that no encapsulation phenomena occurred.

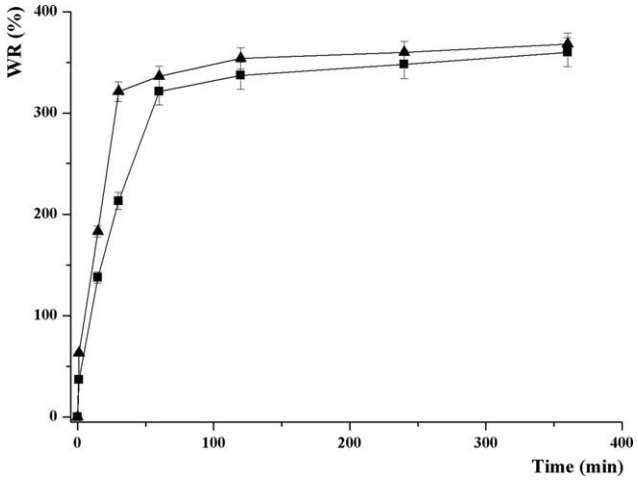

Figure 1. Dynamic swelling profiles of H_{PAN} (■) and H_B (▲).

Swelling behavior and kinetic studies of the catalytic activities were performed to characterize the H_{PAN}, while noncatalytic materials (H_B, synthesized in the same reaction conditions of H_{PAN}, but in the absence of PAN) were used as control.

The dynamic swelling behavior of H_B and H_{PAN} is reported in Figure 1 H_B showed a high water affinity and a fast swelling upon time, while the presence of PAN was found to reduce the swelling rate, while not effecting the WR values at the equilibrium (368% and 360% for H_B and H_{PAN}, respectively, see Table I).

Flory-Rehner Equation was applied for the extrapolation of network parameters, namely the molecular weight of the polymer chain between two neighboring cross-links ($\overline{M}_c$) and the corresponding mesh size (ξ, see Table I).[33] The average pore diameter of H_{PAN} was found to be 27 nm, consistent with the hydrodynamic diameter of the substrate employed in the catalytic determinations.[45–47] Interestingly, the incorporation of the enzyme in the hydrogel network did not significantly modify the calculated pore size.

Evaluation of Catalytic Activity

The catalytic performance of H_{PAN} was investigated as Amylase, Lipase, and Protease activities by employing starch, olive oil, and casein as substrates for each determination and comparing the found activities with those obtained with free PAN (Figure 2).

Before the evaluation of the catalytic activities, the H_{PAN} functionalization degree (expressed as milligrams of PAN per gram of hydrogel) was assessed by the Lowry method (Table I), and found to be 45.1 ± 1.1. After that, the kinetic determinations

Table I. Chemical Composition and Characterization of Hydrogels

	Composition			Immobilization Efficiency	Swelling Behavior	Networks Parameters	
Code	PAN (mg)	AAm (mg/mmol)	PEGDMA (mg/mmol)	Immobilized PAN (mg/g)	WR (%)	Mc (g/mol)	ζ (nm)
H_B	-	534/7.51	466/0.62	-	368 ± 5	35,957	28
H_{PAN}	60	502/7.06	438/0.58	20.1 ± 1.3	360 ± 3	34,643	27

PBS Volume = 3 mL; Hydrogel thickness = 1.6 mm; PAN = Pancreatin; AAm = Acrylamide; PEGDMA = poly-(ethylene glycol) dimethacrylate 750. WR = Water content percentage at equilibrium (after 360 min).

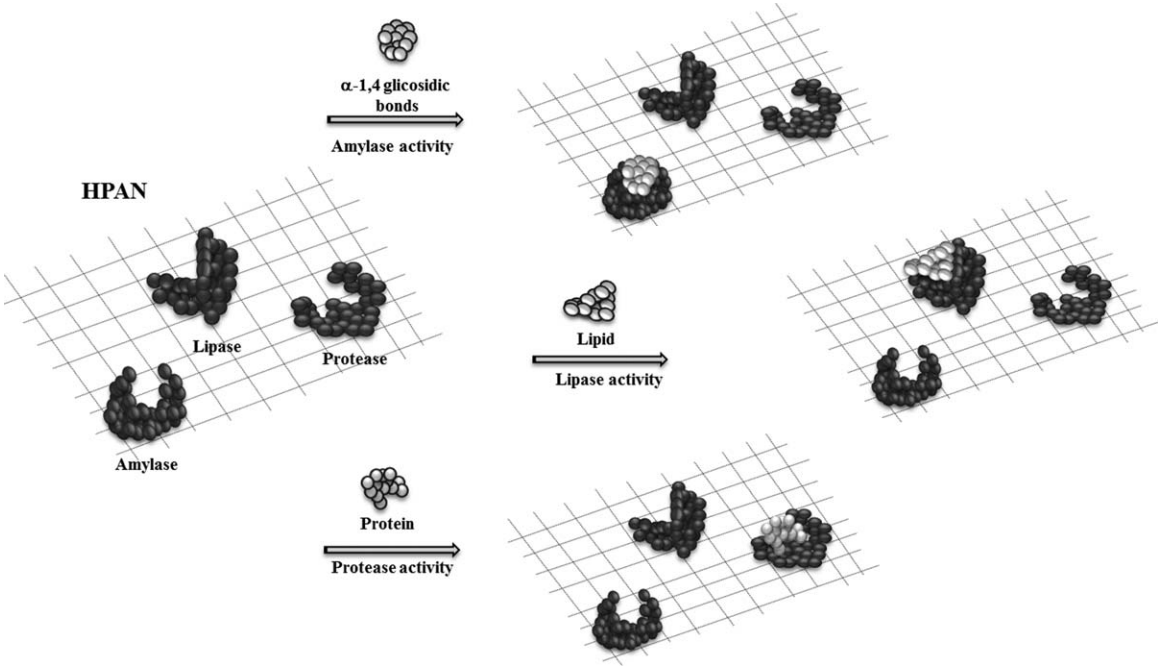

Figure 2. Schematic representation of a multicatalytic hydrogel.

were performed by measuring initial reaction rates at different amounts of substrates.

Michaelis-Menten kinetic behaviors were evaluated according to the following equation:

$$V = \frac{V_{\max}[S]}{K_M + [S]} \tag{7}$$

V is the reaction rate, $[S]$ the substrate concentration, $V_{\max}$ defines the highest possible velocity when the enzyme is saturated with substrate. K_M is the Michaelis-Menten constant and is defined as the substrate concentration that gives a reaction velocity of $^1/_2 V_{\max}$.[48,49]

The kinetic parameters K_M and $V_{\max}$, calculated via the Lineweaver–Burk linear fitting according to the following equation, are collected in Table II.

$$\frac{1}{V} = \frac{K_M}{V_{\max}}\frac{1}{[S]} + \frac{1}{V_{\max}} \tag{8}$$

The units of free and immobilized enzyme (U) were expressed as ng min^{-1} of hydrolyzed substrate (Amylase) or released product equivalents (Lipase and Protease) per mg of enzyme.

The CE was evaluated by considering the $V_{\max}/K_M$ ratio, while the effect of the immobilization process on the CE was better highlighted by introducing the ratio, R_{CE}, between CE of free enzyme to CE of immobilized enzyme according to the following equations:

$$CE = \frac{V_{\max}}{K_M} \tag{9}$$

$$R_{CE} = \frac{CE_{H_{PAN}}}{CE_{PAN}} \tag{10}$$

It was found that blank hydrogels, H_B, were not able to interfere with all the catalytic determinations.

Amylase Activity

Pancreatic α-Amylase catalyses the initial step in the hydrolysis of starch by cleavage of α-1,4-glycosidic linkages, to form a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and a number of α-(l-6) and α-(1-4) oligoglucans.[50]

According to the literature, the determination of Amylase activity is based on the decreased staining value of blue starch–iodine complexes, since Amylase reduces the concentration of starch polymers that are able to bind iodine and the obtained oligosaccharides are too short to efficiently interact with iodine.[34]

Table II. Kinetic Characterization of Free and Immobilized Pancreatin

Activity	$V_{\max}$ (U mg^{-1})		K_M (mg mL^{-1})		CE		R_{CE}
	PAN	H_{PAN}	PAN	H_{PAN}	PAN	H_{PAN}	
Amylase	23.14×10^6	0.58×10^6	4.73	1.30	4.89×10^6	0.45×10^6	0.09
Lipase	0.91	0.44	4.20	6.61	0.22	0.07	0.32
Protease	0.73	0.30	1.15	1.24	0.63	0.24	0.38

Catalytic efficiency $CE = \frac{V_{\max}}{K_M}$; Catalytic efficiency ratio $R_{CE} = \frac{CE_{H_{PAN}}}{CE_{PAN}}$.

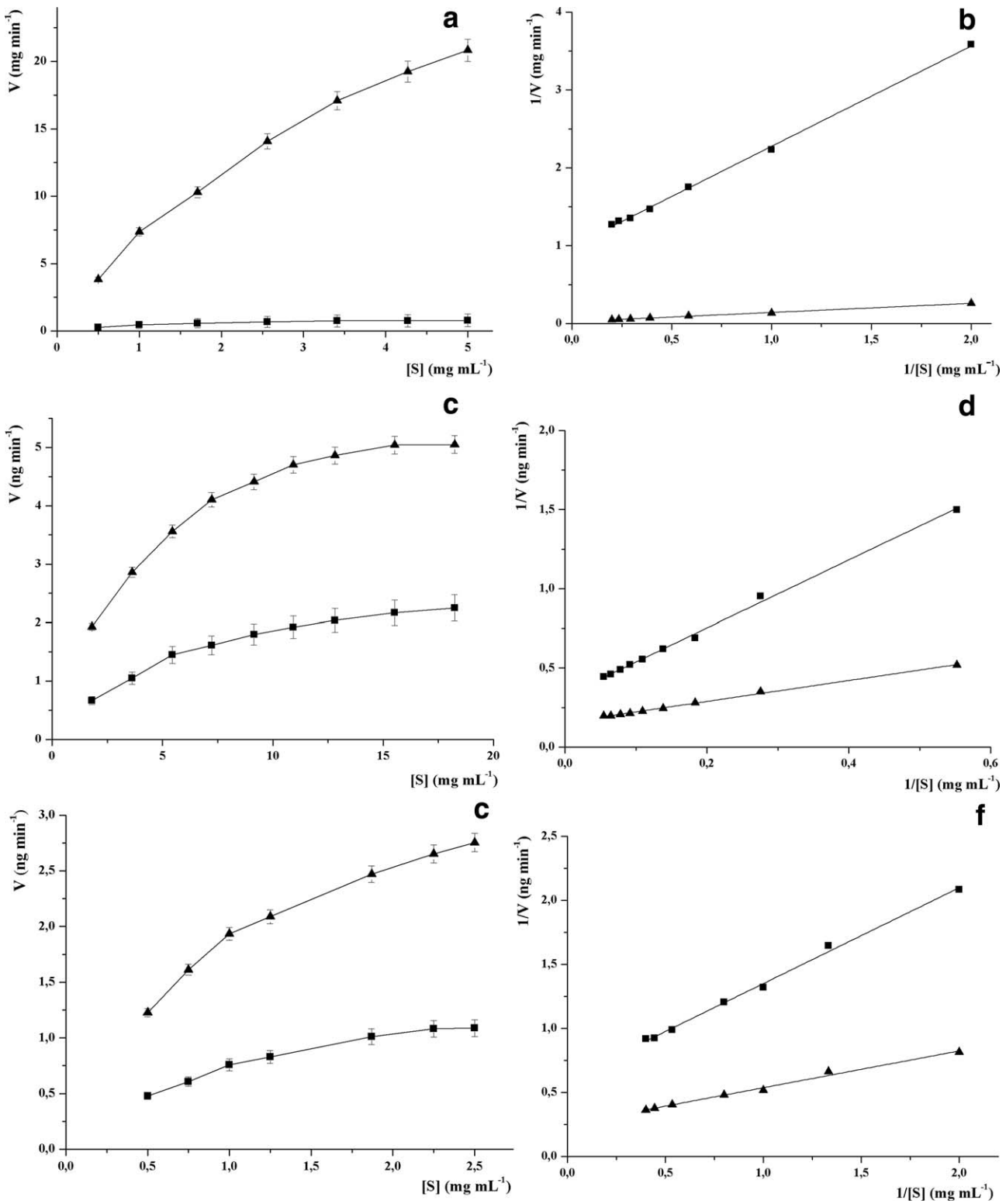

Figure 3. Kinetic characterization of catalytic activity of PAN (▲) and H_{PAN} (■). (A–B) Amylase; (C–D) Lipase; (E–F) Protease; (A–C–E) Initial reaction rate; (B–D–F) Lineweaver–Burk plots.

As shown in Table II and Figure 3(A,B), the immobilization of PAN carried out to a catalytic hydrogel able to hydrolyze starch. As expected, the covalent immobilization reduced the CE (by almost 10 folds), with H_{PAN} showing a lower reaction rate (V_{max} changed from 23.14×10^6 to 0.58×10^6 U mg^{-1}) and a faster saturation as a consequence of changes in the affinity for the substrate (K_M varied from 4.73 to 1.30 mg mL^{-1}).

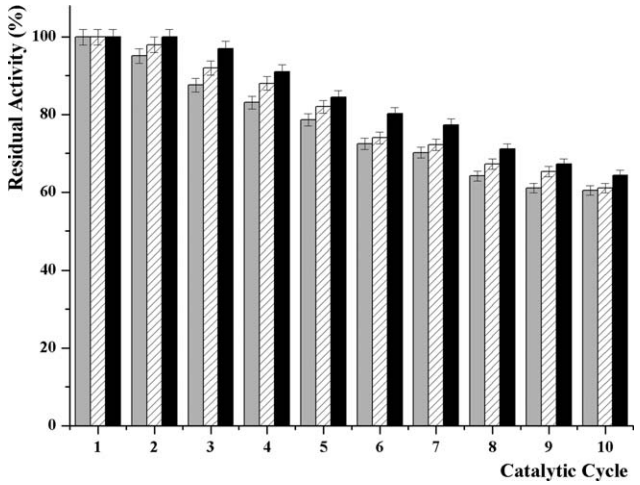

Figure 4. Reusability of H_{PAN} by repeated batch process (10 cycles). Amylase (grey bar), Lipase (dashed bar); Protease (black bar).

Lipase Activity

Lipases are defined as triacylglycerol acylhydrolases catalysing the hydrolysis of fats and oils at the interface between water and lipidic substrates,[36] thus for the determination of the Lipase activity of both free and immobilized PAN, olive oil was used as a substrate in the presence of Arabic Gum as emulsifying agent.[35]

The results of the kinetic determinations [Figure 3(C,D)] clearly proved that the lipolytic activity was well preserved upon immobilization, since the R_{CE} value was 0.32. A decrease of V_{max} and increase of K_M values from 0.91 to 0.44 U mg^{-1} and from 4.20 to 6.61 mg mL^{-1} (for free and immobilized enzyme, respectively) is recorded, as a consequence of a slight reduction of the affinity between enzyme and substrate.

Protease Activity

Proteases are a class of hydrolytic enzyme widely used in industrial and biomedical applications. Trypsin is the Protease found in PAN and hydrolyzes peptide, ester, and amide bonds at the carboxylic groups of Arginine, Lysine, and Ornithine.[37] Casein was employed as the substrate for the determination of Protease activity [Figure 3E,F)], which was estimated by the color development involved in the redox reaction between the Folin-Ciocalteu reagent and Tyrosine released by the proteolytic cleavage.[30] The Protease activity was found to be close to that obtained for the free enzyme, with a R_{CE} of 0.39.

Enzyme Reusability

The possibility to reuse the proposed hydrogels without a significant loss in the CE loss is a key item for their possible application in wastewater bioremediation processes. Thus, 10 repeated hydrolytic cycles were performed to determine how the reuse can affect each activity, and data were collected in Figure 4.

The results proved that the hydrogel performance was well retained: the residual activity after 10 cycles was higher than 60% in all cases, with protease showing the best score (65%).

More interestingly, it was found that residual activities did not influence with each other, with exciting practical appeals. The same residual activities were indeed recorded alternating the hydrolytic assays in all possible combinations. This is of tremendous importance, since the wastewater bioremediation often involves multifactorial contamination where, for an effective treatment, the hydrolysis of proteins, polysaccharides, and oils is needed. An apparent drawback arising from above reported results is the reduction in the catalytic activity which is higher than those reported in the available literature, reporting data on the immobilization of either PAN (38% vs 85% of protease activity) or individual enzymes on different supports.[51–54] The decrease in the catalytic activity could arise from the particular components (AA and PEGDMA used in the preparation of hydrogels), and the proposed synthetic approach, which could reduce enzyme efficiency. In particular, literature data[51] refers to the PAN immobilization on a preformed polymer (Carboxymethyl Cellulose), and the covalent linkage did not affect the internal sites (and thus the catalytic pockets) of enzyme molecules. In our condition, it can be hypothesized that, during the immobilization process, PAN is randomly linked to the growing polymer chain, with a possible co-cross-linking process reducing the accessibility of the catalytic sites.[55] Nevertheless, the proved reusability, together with the easy and scalable fabrication procedure, are the key advantages making the proposed idea a valuable tool to design a new generation of multifunctional catalysts with versatile applications.

CONCLUSIONS

To the best of authors' knowledge, this work is the first example of covalent immobilization of an enzyme mixture (i.e. Pancreatin) into an acrylate cross-linked polymer by a single step green process involving a UV-induced radical reaction.

The key finding of this study is the possibility to obtain a biocatalyst with multifunctional activity, able to work on the cleavage of α-1,4-glycosidic, triglyceride, and peptide bonds by virtue of the intrinsic activities of the immobilized enzymes, namely Amylase, Lipase, and Protease.

The catalytic characterization of multifunctional hydrogels allowed the determination of the kinetic parameters, proving the suitability of the synthetic approach for the insertion of enzyme moieties into the macromolecular systems without a significant loose of the catalytic performance. More in detail, Protease activity was found to be highly preserved, while the reduction in the CE was slightly higher for Lipase and Amylase.

The good reusability properties and the ease of handling confer considerable appeal to the whole system for industrial applications like wastewater bioremediation.

ACKNOWLEDGMENTS

This work was financially supported by University of Calabria funds.

REFERENCES

1. Spizzirri, U. G.; Cirillo, G.; Picci, N.; Iemma, F. *Curr. Org. Chem.* **2014**, *18*, 2912.
2. Moiseev, I. I. *Russ. Chem. Rev.* **2013**, *82*, 616.
3. Long, J.; Li, X.; Wu, Z.; Xu, E.; Xu, X.; Jin, Z.; Jiao, A. *Colloids Surf. A: Physicochem. Eng. Aspects* **2015**, *472*, 69.

4. Chakraborty, D.; McClellan, E.; Hasselbeck, R.; Calabrese Barton, S. *J. Electrochem. Soc.* **2014**, *161*, H3076.
5. Liu, Q.; Hua, Y.; Kong, X.; Zhang, C.; Chen, Y. *J. Mol. Catal. B Enzym.* **2013**, *95*, 89.
6. San, B. H.; Ha, E. J.; Paik, H. J.; Kim, K. K. *Nanoscale* **2014**, *6*, 6009.
7. Brady, D.; Jordaan, J. *Biotechnol. Lett.* **2009**, *31*, 1639.
8. Oriero, D. A.; Jabal, J. M. F.; Deobald, L.; Weakley, A. T.; Aston, D. E. *React. Funct. Polym.* **2011**, *71*, 870.
9. Starke, S.; Went, M.; Prager, A.; Schulze, A. *React. Funct. Polym.* **2013**, *73*, 698.
10. Zhu, X.; Ma, Y.; Zhao, C.; Lin, Z.; Zhang, L.; Chen, R.; Yang, W. *Langmuir* **2014**, *30*, 15229.
11. Rodrigues, R. C.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Fernández-Lafuente, R. *Chem. Soc. Rev.* **2013**, *42*, 6290.
12. Cipolatti, E. P.; Silva, M. J. A.; Klein, M.; Feddern, V.; Feltes, M. M. C.; Oliveira, J. V.; Ninow, J. L.; De Oliveira, D. *J. Mol. Catal. B Enzym.* **2014**, *99*, 56.
13. Zhou, Z.; Hartmann, M. *Chem. Soc. Rev.* **2013**, *42*, 3894.
14. Tibbitt, M. W.; Anseth, K. S. *Biotechnol. Bioeng.* **2009**, *103*, 655.
15. Parascandola, P.; Branduardi, P.; de Alteriis, E. *Enzyme Microb. Technol.* **2006**, *38*, 184.
16. Curcio, M.; Spizzirri, U. G.; Cirillo, G.; Spataro, T.; Picci, N.; Iemma, F. *Int. J. Polym. Mater.* **2015**, *64*, 587.
17. Fejerskov, B.; Smith, A. A. A.; Jensen, B. E. B.; Hussmann, T.; Zelikin, A. N. *Langmuir* **2012**, *29*, 344.
18. Curcio, M.; Spizzirri, U. G.; Cirillo, G.; Vittorio, O.; Picci, N.; Nicoletta, F. P.; Iemma, F.; Hampel, S. *RSC Adv.* **2015**, *5*, 44902.
19. Sato, R.; Kawakami, T.; Tokuyama, H. *React. Funct. Polym.* **2014**, *76*, 8.
20. Pérez, J. P. H.; López-Cabarcos, E.; López-Ruiz, B. *Biomol. Eng.* **2006**, *23*, 233.
21. Homaei, A. A.; Sariri, R.; Vianello, F.; Stevanato, R. *J. Chem. Biol.* **2013**, *6*, 185.
22. Sheldon, R. A.; Van Pelt, S. *Chem. Soc. Rev.* **2013**, *42*, 6223.
23. Tang, D. W.; Yu, S. H.; Wu, W. S.; Hsieh, H. Y.; Tsai, Y. C.; Mi, F. L. *Colloids Surf. B Biointerfaces* **2014**, *113*, 59.
24. Schoffelen, S.; Van Hest, J. C. M. *Soft Matter* **2012**, *88*, 1736.
25. Dicosimo, R.; McAuliffe, J.; Poulose, A. J.; Bohlmann, G. *Chem. Soc. Rev.* **2013**, *42*, 6437.
26. Jia, F.; Narasimhan, B.; Mallapragada, S. *Biotechnol. Bioeng.* **2014**, *111*, 209.
27. Barbosa, O.; Torres, R.; Ortiz, C.; Berenguer-Murcia, A.; Rodrigues, R. C.; Fernandez-Lafuente, R. *Biomacromolecules* **2013**, *1414*, 2433.
28. Brena, B.; González-Pombo, P.; Batista-Viera, F. *Methods Mol. Biol.* **2013**, *1051*, 15.
29. Contractor, A. Q.; Sureshkumar, T. N.; Narayanan, R.; Sukeerthi, S.; Rakesh, L.; Srinivasa, R. S. *Electrochim. Acta* **1994**, *39*, 1321.
30. Cirillo, G.; Nicoletta, F. P.; Curcio, M.; Spizzirri, U. G.; Picci, N.; Iemma, F. *React. Funct. Polym.* **2014**, *83*, 62.
31. Phan, S.; Salentinig, S.; Hawley, A.; Boyd, B. J. *J. Pharm. Sci.* **2015**, *104*, 1311.
32. De Lourdes Mendes Finete, V.; Gouvêa, M. M.; De Carvalho Marques, F. F.; Netto, A. D. P. *Food Chem.* **2013**, *141*, 3649.
33. Kulkarni, A. R.; Soppimath, K. S.; Aminabhavi, T. M.; Dave, A. M.; Mehta, M. H. *J. Control. Release* **2000**, *63*, 97.
34. Xiao, Z.; Storms, R.; Tsang, A. *Anal. Biochem.* **2006**, *351*, 146.
35. Batista, K. A.; Purcena, L. L. A.; Alves, G. L.; Fernandes, K. F. *J. Mol. Catal. B Enzym.* **2014**, *102*, 25.
36. Smichi, N.; Gargouri, Y.; Miled, N.; Fendri, A. *Int. J. Biol. Macromol.* **2013**, *58*, 87.
37. Anirudhan, T. S.; Rejeena, S. R. *J. Colloid Interface Sci.* **2012**, *381*, 125.
38. Cirillo, G.; Vittorio, O.; Hampel, S.; Spizzirri, U. G.; Picci, N.; Iemma, F. *Int. J. Pharm.* **2013**, *446*, 176.
39. Ali, I.; Akbar, A.; Yanwisetpakdee, B.; Prasongsuk, S.; Lotrakul, P.; Punnapayak, H. *Biomed. Res. Int.* **2014**, *2014*, Art. n. 106937.
40. Lee, L. P.; Karbul, H. M.; Citartan, M.; Gopinath, S. C. B.; Lakshmipriya, T.; Tang, T.-H. *Biomed. Res. Int.* **2015**, *2015*, Art. n. 820575.
41. Inacio, F. D.; Ferreira, R. O.; Araujo, C. A. V. D.; Brugnari, T.; Castoldi, R.; Peralta, R. M.; Souza, C. G. M. D. *Biomed. Res. Int.* **2015**, *2015*, 290161.
42. Spizzirri, U. G.; Hampel, S.; Cirillo, G.; Mauro, M. V.; Vittorio, O.; Cavalcanti, P.; Giraldi, C.; Curcio, M.; Picci, N.; Iemma, F. *Int. J. Polym. Mater.* **2015**, *64*, 439.
43. Vittorio, O.; Brandl, M.; Cirillo, G.; Spizzirri, U. G.; Picci, N.; Kavallaris, M.; Iemma, F.; Hampel, S. *RSC Adv.* **2014**, *4*, 31378.
44. Cirilo, G.; Iemma, F. Antioxidant Polymers: Synthesis, Properties, and Applications; Scrivener Publishing LLC: Beverly, **2015**.
45. Zhu, K.; Jutila, A.; Tuominen, E. K. J.; Kinnunen, P. K. *J. Protein Sci.* **2001**, *10*, 339.
46. Parker, R.; Ring, S. G. *J. Cereal Sci.* **2001**, *34*, 1.
47. O'Connell, J. E.; Grinberg, V. Ya.; de Kruif, C. G. *J. Colloid Interface Sci.* **2003**, *258*, 33.
48. Huang, X. J.; Ge, D.; Xu, Z. K. *Eur. Polym. J.* **2007**, *43*, 3710.
49. Betigeri, S. S.; Neau, S. H. *Biomaterials* **2002**, *23*, 3627.
50. Sudha, P.; Zinjarde, S. S.; Bhargava, S. Y.; Kumar, A. R. *Altern. Med.* **2011**, *11*, art. n. 5.
51. Neklyudov, A. D.; Ivankin, A. N.; Baburina, M. I. *Appl. Biochem. Microbiol.* **1998**, *34*, 57.
52. Sun, Y. M.; Chen, J. P.; Chu, D. H. *J. Biomed. Mater. Res.* **1999**, *45*, 125.
53. Park, S.; Kim, S. H.; Won, K.; Choi, J. W.; Kim, Y. H.; Kim, H. J.; Yang, Y. H.; Lee, S. H. *Carbohydr. Polym.* **2015**, *115*, 223.
54. Li, J.; Cai, J.; Zhong, L.; Dud, Y. *Carbohydr. Polym.* **2012**, *87*, 2697.
55. Curcio, M.; Altimari, I.; Spizzirri, U. G.; Cirillo, G.; Vittorio, O.; Puoci, F.; Picci, N.; Iemma, F. *J. Nanopart. Res.* **2013**, *15*, art. n. 1581.