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# Film-shaped reusable smart polymer to produce lactose-free milk by simple immersion

J. Lucas Vallejo-García [a], Ana Arnaiz [a,b], María D. Busto [c], José M. García [a], Saúl Vallejos [a,*]

[a] Grupo de Polímeros. Departamento de Química, Facultad de Ciencias, Universidad de Burgos, Plaza de Misael Bañuelos s/n, 09001 Burgos, Spain
[b] Universidad Politécnica de Madrid, Calle Ramiro de Maeztu, 7, 28040 Madrid, Spain
[c] Departamento de Biotecnología y Ciencia de los Alimentos, Facultad de Ciencias, Universidad de Burgos, Plaza de Misael Bañuelos s/n, 09001 Burgos, Spain

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ABSTRACT

In this study, we report the synthesis and characterization of a highly manageable polyacrylic film material for enzyme immobilization, using β-galactosidase (β-gal) as a model enzyme. The material is based on commercially available monomers and achieves efficient immobilization of β-gal through the formation of azo linkages between amino styrene groups in the polyacrylic material and the enzyme. The immobilized enzyme demonstrates superior performance compared to free enzyme in lactose hydrolysis of UHT milk, achieving lactose concentrations below 0.1% (<1 mg/mL), indicating its potential for lactose hydrolysis in dairy products. The film-shaped material is designed for easy submersion and removal, similar to a smart card, and offers reusability, with the ability to be reused at least 10 times without loss of enzymatic activity. Characterization of the immobilized enzyme on the polymeric material was performed using various techniques, including scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR), and Raman spectroscopy. Protein release studies confirmed the stability of the immobilized enzyme during prolonged incubation in aqueous solution without significant enzyme leakage. Overall, the polyacrylic film material demonstrates promise as a simple and efficient approach for enzyme immobilization, with potential applications in various industries, including the food industry.

## 1. Introduction

Over the past decade, lactose-free products have gained a foothold in the food market, and a wide variety of lactose-free products are currently manufactured, including milk, yogurts, cheeses, creams, margarines, butters, and ice creams, among others [1–3]. To be considered "lactose-free," a product's glucose and galactose disaccharide concentration must be below a certain threshold, as determined, for instance, by the European Food Safety Authority (EFSA) [4]. In industrial settings, lactose-free products are manufactured by adding neutral lactases (β-galactosidases) as an additive or as a processing aid [3], which irreversibly wastes the enzyme and increases production costs. As a result, immobilizing lactases on polymeric supports has become an area of growing interest, particularly in biotechnology and food science, as this technique allows reusability and often enhances enzyme thermostability and pH tolerance [5,6].

Numerous studies have been published on the immobilization of β-galactosidase (β -gal) onto a wide variety of supports, which reflects the great interest associated with this type of materials [7]. Some of the most commonly used supports include pectin-based hydrogels [6], arabic gum-based hydrogels [8], chitosan [9,10], sodium alginate gels [5,11], carbon nanotube tubular micromotors [12], ion exchange resin Duolite A568 [13], polycaprolactone and silk fibroin based nanofiber [14], natural silk fibers grafted with polyacrylonitrile [15], nanosilver reduced graphene oxide nanocomposites [16], polydopamine coated magnetic particles [17,18], epoxy resins [19,20], aminated PVC [21], polystyrene [22,23], or modified cellulose acetate [24]. However, the reusability of many of these materials has not been demonstrated, and in those that have, enzymatic activity decreases after the first use, disappearing after several cycles of washing/use [6].

Likewise, various comparative studies have been carried out between free enzymes and immobilized enzymes. Putting aside the fact that when using free enzymes, they cannot be reused, the truth is that better results are obtained in terms of lactose hydrolysis compared to immobilized enzymes [5].

Although numerous immobilized enzyme hydrolysis systems have

* Corresponding author.
*E-mail address:* svallejos@ubu.es (S. Vallejos).

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been investigated, only a few have been scaled up, and even fewer have been applied at an industrial or semi-industrial level. The most notable cases include (1) Centrale del Latte in Milan (Italy), which used SNAM Progetti technology by immobilizing the enzyme in cellulose triacetate fibers; (2) Drouin Cooperative Butter Factory in Australia, which relied on technology developed by Sumitomo Chemical (Japan) to immobilize the enzyme in phenol-formaldehyde resin; and (3) Snow Brand in Australia, which developed a rotating column reactor that could be used as a stirred tank reactor and packed bed reactor [25]. However, the main disadvantage of all these methods is the loss of efficiency in lactose hydrolysis after several cycles of use/washing, as in many cases, it does not exceed 70–80% of lactose hydrolyzed. In addition, this reuse is often made difficult by the shape/format of the supports, involving filtration processes, etc.

In this work, we describe a highly manageable polyacrylic film material that can be easily submerged and removed like a smart card, and is based on commercially available monomers. Immobilization of β -gal enzyme was achieved through the formation of azo linkages between amino styrene groups present in the polyacrylic material and the enzyme. The novelty of this material lies primarily in the possibility of reusing it at least 10 times without losing any enzymatic activity, simply by dipping the film in the milk, and surpassing the free enzyme's efficiency in the same experimental conditions. The study (summarized in Fig. 1) was concluded with a proof-of-concept experiment, in which lactose hydrolysis was performed in UHT milk to achieve lactose-free products, i.e., lactose concentrations below 0.1% (<1 mg/mL). The experiments were conducted at three different temperatures (4, 25, and 55 °C), and the results showed that the immobilized enzyme outperformed the free enzyme in terms of hydrolysis efficiency. These findings demonstrate the material's potential for lactose hydrolysis in dairy products, even by the end-user, as the procedure is as simple as "dip-wait-remove".

## 2. Experimental

### 2.1. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: 1-vinyl-2-pyrrolidone (VP) (Acros Organic, 99%), methylmethacrylate (MMA) (Merck, 99%) 4-aminostyrene ($SNH_2$) (TCI, 98%), methanol (VWR-Prolabo, 99.9%), distilled water, hydrochloric acid (VWR, 37%) *d*imethylsulfoxide-$d_6$ (VWR, 99.8%), citric acid (VWR, ≥99.5%), di-sodium hydrogen phosphate anhydrous (VWR, 99%), sodium carbonate (Sigma-Aldrich, 99.9%), sodium azide (Alfa Aesar, 99%), sodium nitrite (Alfa Aesar, 98%), 2-nitrophenyl β-D-galactopyranoside (ONPG) (TCI, >98%), 2-nitrophenol (ONP) (Acros Organics, 99%), β-galactosidase from *Aspergillus oryzae* (Biolactase F Conc, BIOCON, 45 units.mg$^{-1}$) (BIO- β-gal) [26], UHT cow milk (Carrefour). Azo-bis-isobutyronitrile (AIBN, Aldrich, 98%) was recrystallized twice from methanol. The kit for lactose determination "K-LOLAC" was provided by Megazyme [27].

### 2.2. Instrumentation and methods

The polymers thermal characterization was performed by thermogravimetric analysis (Q50 TGA analyzer, TA Instruments, New Castle, DE, USA) with 10–15 mg of sample under synthetic air and nitrogen atmosphere at 10 °C·min$^{-1}$.

Infrared spectra (FTIR) were recorded with an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) with an ATR-PRO410-S single reflection accessory. RAMAN spectra were recorded with a confocal AFM-RAMAN model Alpha300R – Alpha300A AFM from WITec, using a laser radiation of 785 nm, at magnifications of 100x, 12 mW, 10 accumulations. All spectra were taken at room temperature.

Enzymatic activity assays were performed using a Synergy HT microplate reader (BioTek®, Winooski, Vermont, USA), recording

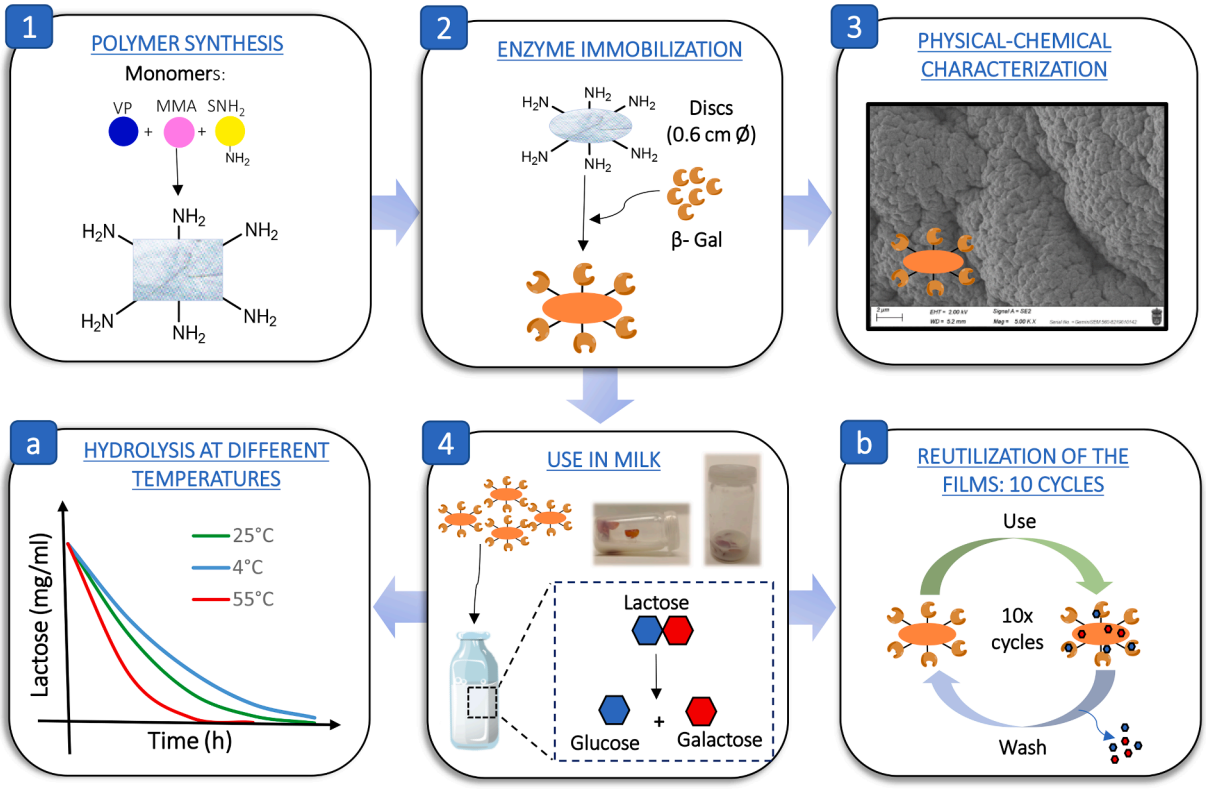


**Fig. 1.** Table of contents of the study.

absorbance data at 420 nm. Digital photographs were taken with a smartphone (Mi 9, Xiaomi, Pekín, China).

Field Emission Scanning electron microscopy (FESEM) was carried out using a model GemminiSEM560, ZEISS. Films were dried, freeze fractured, and gold coated in vacuum to ensure the electrical conductivity of the films.

The weight percentage of water taken up by the films upon soaking in pure water at 20 °C until reaching equilibrium (water-swelling percentage, WSP) was obtained from the weight of a dry sample film (ωd) and its water-swelled weight (ωs) using the following expression: WSP = 100 × [(ωs × ωd)/ωd].

The protein and fat content of milk was determined following common procedures depicted in the literature, as Bradford and Gerber methods, respectively [28,29].

### 2.3. Design of β -gal containing acrylic polymers ($F_{\beta\text{-gal-N3}}$)

From the beginning, we wanted to prepare a manageable material, without needing to handle it delicately or storing it in too careful conditions. In other words, a material to be used by non-specialized personnel in domestic environments, but also in industrial processes where there is no scope to slow down the paces of the industry. In terms of materials science, and regarding the proposed application, this means that the polyacrylic material must contain hydrophobic monomers to provide rigidity, hydrophilic monomers to allow use in aqueous media (such as milk), and aniline groups containing functional monomers for carrying out the immobilization of the enzyme β -gal.

For this study, we selected VP (hydrophilic monomer), MMA (hydrophobic monomer), and $SNH_2$ (functional monomer), using ethylene glycol dimethacrylate (E) as the crosslinking agent.

### 2.4. Synthesis of β -gal containing acrylic polymers ($F_{\beta\text{-gal-N3}}$)

Film-shaped polymers were prepared by bulk radical polymerization of the commercially available monomers VP (45 mol%), MMA (45 mol %), and $SNH_2$ (10 mol%), the polymeric was crosslinked using 0.1 mol% of E, following the experimental procedure described below.

850 mg ($7.64 \times 10^{-3}$ mol) of VP, 765 mg ($7.64 \times 10^{-3}$ mol) of MMA, 202.5 mg ($1.70 \times 10^{-3}$ mol) of $SNH_2$, 3.4 mg ($1.70 \times 10^{-5}$ mol) of E were mixed. Then, 18.2 mg ($1.1 \times 10^{-4}$ mol) of AIBN were dissolved and the solution of comonomers and initiatior were injected in a mold (90 × 120 × 0.1 mm, width, length, thickness) comprised between two silanized glasses in an oxygen-free atmosphere. The polymerization was carried out at 60 °C, overnight, and finally, the films ($F_{NH2}$) were washed with water. With the aim of removing unreacted monomers, films were washed with water:acetone mixtures ranging from 100:0 to 0:100, and finally with mixtures 0:100 to 100:0. This gradual solvent transition is performed to avoid breakages associated with sudden changes in the material's WSP.

After the preparation of $F_{NH2}$, 3 solid phase reactions were carried out: (1) generation of benzene diazonium salts on the amino groups provided by the $SNH_2$ monomer ($F_{N2+}$), (2) immobilization of the enzyme β -gal through the formation of diazo bonds (F β -gal), and (3) quenching of the leftover benzene diazonium groups with sodium azide (F β -gal-N3). The final step is carried out to prevent the highly reactive benzene diazonium groups from reacting with other components of the milk. The schemes of each material are depicted in Fig. 2.

The first step, namely, the preparation of $F_{N2+}$, began with punching $F_{NH2}$ film into 6 mm diameter discs. Then, 10 disks were dipped for 1 h in 11 mL of an aqueous solution containing 10 mL of distilled water, 1 mL of HCl (37%), and 50 mg of sodium nitrite. After that, discs were exhaustively washed with distilled water.

The second step was carried out in an Eppendorf vial, by dipping 1 disc of $F_{N2+}$ in 1 mL of the aqueous enzyme solution (25 mg of BIO- β -gal per millilitre of distilled water). The vial was stored overnight at room temperature without stirring, and finally, discs were washed five times with distilled water until no enzyme was detected in the washing water by the Bradford method [28]. It is remarkable that the enzyme solution was reused up to 6 times without losing immobilization efficiency. The enzyme quantities, temperature, and pH were optimized with the aim of achieving the highest enzymatic activity possible.

The third step was also performed in an Eppendorf, by dipping 1 disc

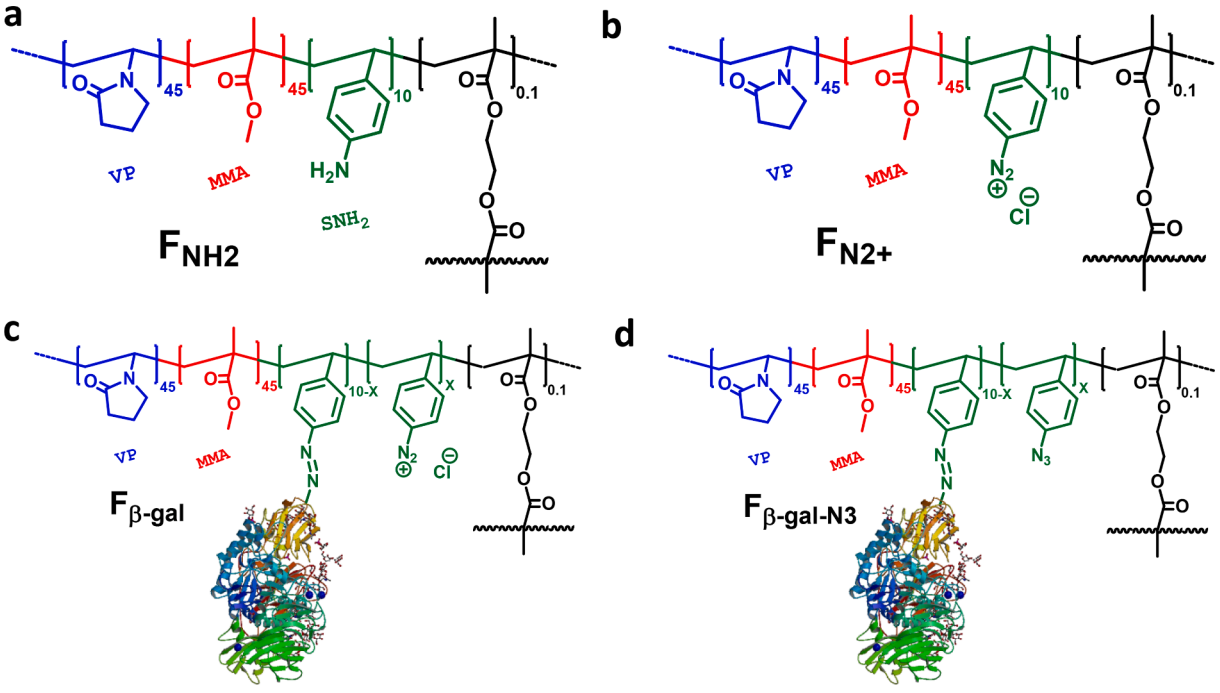


**Fig. 2.** Schematic representation of the prepared materials: a) original film with aminostyrene side groups; b) film with benzene diazonium side groups; c) film with β-gal covalently anchored through azo bonds to the polymer chain, and with remaining benzene diazonium lateral groups; and d) film with β-gal covalently anchored through azo bonds to the polymer chain, and with azidobenzene side groups (enzyme image taken from Wikimedia Commons contributors) [30].

of F β -gal in 1 mL of aqueous sodium azide solution (5 mg/mL, distilled water). The vial was left at room temperature 1 h, and without stirring. The F β -gal-N3 discs were washed every 10 min five times, and once more overnight.

The complete physicochemical characterization of $F_{NH2}$, $F_{N2+}$, F β -gal, and F β -gal-N3 can be found in the Supporting Information (SI-Section S1, Figure S1).

### 2.5. Activity evaluation and protein release study of F β -gal-N3

Enzymatic activity of F β -gal-N3 films were confirmed using ONPG as substrate in a microplate reader with 96-well plates. 6 mm diameter discs were placed at the bottom of each well and 80 μL of a citrate–phosphate pH 5 buffer (di-Sodium hydrogen phosphate 0.2 M and citric acid 0.1 M) and 20 μL of ONPG substrate at a final concentration of 2.5 mM were added. Samples were incubated for 30 min at 37 °C and the reaction was stopped by adding 100 μL of sodium carbonate 10% (w/v). The absorbance of the supernatant was measured at 420 nm, and the enzymatic activity was represented as μmol of ONP/h·$cm^2$ (more information in SI-Section S2, Fig. S2a).p

Following the same experimental procedure, different experiments were carried out to confirm that the polymeric support was inert, that is, that the material without enzyme ($F_{SNH2}$, $F_{N2+}$ and $F_{N3}$) did not show activity. In the same way, a film with deposited enzyme (not immobilized, without covalent anchorage) was also tested and labeled as $F_{Ads}$, which for practical purposes, behaved like a film without enzyme since the successive washes after the deposition process eliminated any enzyme content, making clear the need to carry out a covalent anchorage (more information in SI-Section S2, Fig. S2a).

Protein release study was carried out with covalently anchored enzyme-containing films to verify that the enzyme is not liberated from the films in aqueous solution. For that, 10F β -gal-N3 discs (6 mm diameter) were incubated for 30 h in citrate–phosphate pH 6.8 buffer (UHT milk's usual pH). 40 μL of samples were taken at different time points (30 min, 1, 6, 20 and 30 h), and total protein content was measured according to the method of Bradford [28].

All assays were carried out in triplicate and blanks were used to account for spontaneous breakdown of the substrates (more information in SI-Section S2, Fig. S2b).

### 2.6. Design of the proof of concept

The proof of concept was designed as a comparative test when hydrolyzing commercial milk. Our main objective was to propose a realistic alternative to the current lactose-free milk production method, which is based on adding β -galactosidase directly to the milk (Method A). This means that the enzyme is not recovered, and given the boom in lactose-free products in recent years, we believe that a manageable and reusable film-shaped material with low production costs can be postulated as a viable alternative (Method B).

#### 2.6.1. Calibration. Equivalence test

First of all, it should be pointed out that an attempt was made to determine the amount of the immobilized enzyme by different methods (Bradford [28] and Bicinchoninic acid assay [31]), but none of them was satisfactory due to interference of the different methods with the polymeric material. So, since it is a comparative study ("free enzyme" vs F β -gal-N3), an experiment was carried out to establish a functional equivalence between the mg of free enzyme used in Method A, and the F β -gal-N3 discs used in Method B. To do this, different amounts of BIO- β -gal (0.5, 1, 2.5, and 5 mg) were added to 1 mL of milk. The remaining lactose in each experiment was determined after incubation at 90 rpm for 1 h at 25 °C, using the kit for lactose determination "K-LOLAC" following the manufacturer instructions. The calibration curve obtained is shown in SI-Section S3 (Figure S3).

Then, the experiment was repeated using 10 discs (6 mm diameter) of $F_{\beta\text{-gal-N3}}$ that were added to 1 mL of milk, and the system was incubated at 90 rpm for 1 h at 25 °C. The remaining lactose data was introduced in the fitted equation, and the equivalent mg of BIO-β -gal was obtained. Thus, 10 discs of $F_{\beta\text{-gal-N3}}$ are equivalent to 830 μg of BIO-β -gal (they hydrolyze the same amount of lactose, at a given time and temperature).

#### 2.6.2. Comparison. Hydrolyzing lactose from milk with both methods

10 discs (6 mm diameter) of F β -gal-N3 were added to 1 mL of milk, and vials were stirred in an orbital stirrer at 90 RPM. The remaining lactose was determined at different times (0, 1, 6, 20, and 30 h) using the kit for lactose determination "K-LOLAC". The experiment was conducted at three different temperatures (4, 25 and 55 °C). In the same way, the assays were also performed with free enzyme by adding 830 μg of BIO- β -gal to 1 mL of milk.

#### 2.6.3. Storage and reusability

Our design of the material implies a hypothetical future use in the food industry. For this reason, we understand that material reuse is a key point in our development since costs are reduced, and the material complies with the "circular economy" and "environmentally friendly" concepts. Thus, after the first use, the discs were dipped in distilled water for 10 min, and the washing process was repeated 5 times as depicted in previous works [32]. Then, the films were used again, as described in Section 2.6.2. In total, discs were used/washed up to 10 times.

After the fourth use and washing, discs were stored in the conditions that have turned out to be the most optimal (in the swelled state, packed in zip bags, and at 4 °C), and the reusability study was resumed one month later. This proof of concept has been designed to assess the hydrolytic functionality of the material after 10 cycles of use and a storage period.

## 3. Results and discussion

### 3.1. Physicochemical characterization of $F_{\beta\text{-gal-N3}}$

The immobilization of β -gal on the polymeric material was characterized by several techniques, being the SEM images shown in Fig. 3 the most visual one. The images from $F_{NH2}$, and $F_{N2+}$ materials revealed dense structures. Fig. 3b shows folds due to the high WSP of the $F_{N2+}$ sample. Unlike $F_{NH2}$, $F_{N2+}$ is a polymer that contains 10 mol% of a salt-type co-monomer, which could be understood as a polyelectrolyte with a high water affinity. In fact, $F_{NH2}$'s WSP is 30 ± 1 %, while $F_{N2+}$'s is 1,526 ± 71 %. Our interpretation is that the conditions of the SEM analysis (ultra high vacuum) abruptly eliminate the water within the polymeric structure, and wrinkle the material.

Regarding $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N3}}$, Fig. 3c and 3d show a rough surface due to enzyme immobilization. Similarly, the cross-section of the materials was also analyzed, but no enzyme was found. That is, immobilization occurs only on the surface, which makes sense due to the size of β-gal (464 KDa), and the dense structures observed before immobilization. The WSP of $F_{\beta\text{-gal}}$ and $F_{\beta\text{-gal-N3}}$ is 516 ± 26 % and 139 ± 4 %, respectively, since after the immobilization process, there are still active sites (benzene diazonium salt groups) that provide hydrophilicity, which disappear after the reaction of active sites with sodium azide. Therefore, folds can be seen in Fig. 3c (still high WSP) but not in Fig. 3d.

The presence of enzyme on the surface of the polymeric material was also corroborated by FT-IR and RAMAN, as shown in Fig. 4 and SI-Section S1 (Figure S1), respectively.

Materials $F_{\beta\text{-gal}}$ and $F_{\beta\text{-gal-N3}}$ present a wide band at 3300 $cm^{-1}$ due to the N–H vibration band corresponding to the amide bonds of the enzyme. This band is also present in $F_{NH2}$, and $F_{N2+}$ materials, but much less intense. In the first case, the two bands are attributed to the asymmetric and symmetric stretching of amine groups (only 10 mol%), while in the second case, the band is unrelated to the chemical structure of the

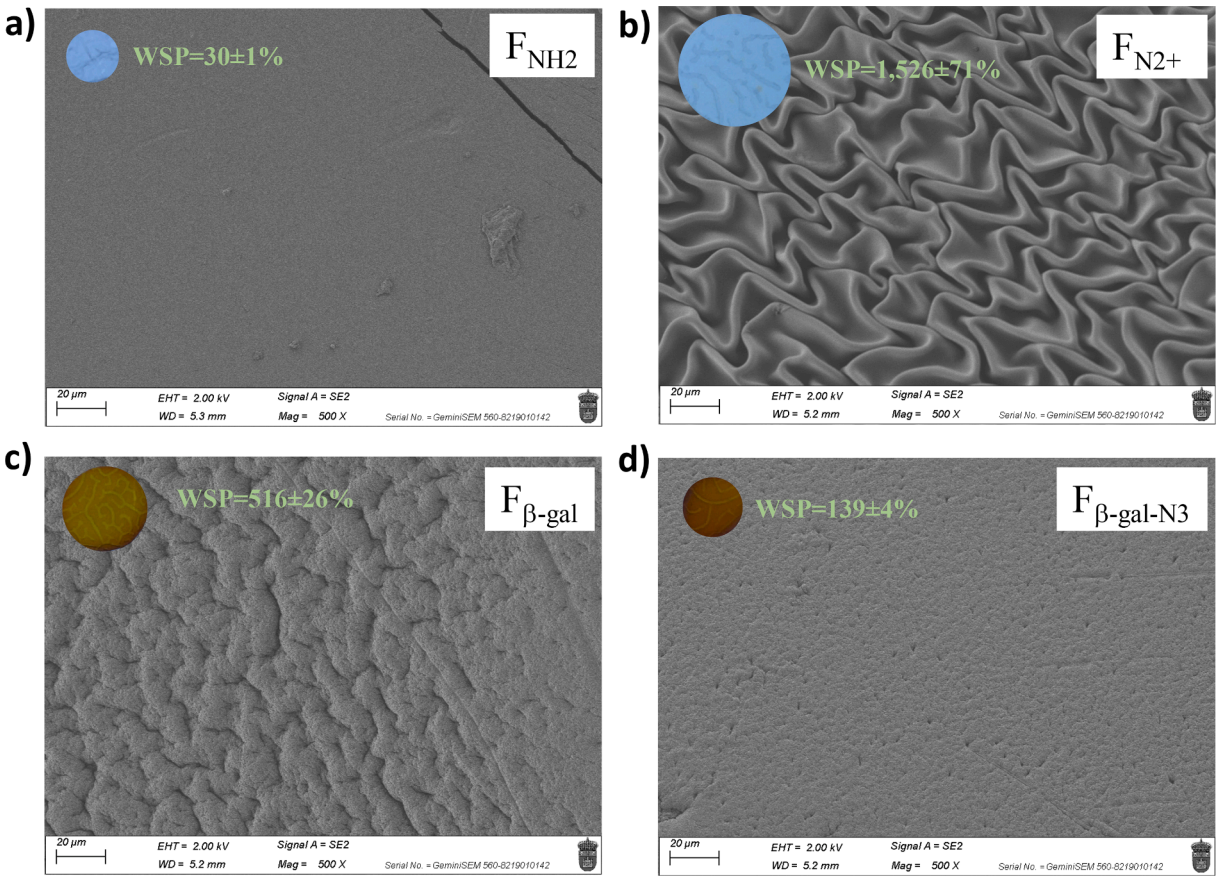


**Fig. 3.** SEM images of the developed material in the different production stages: $F_{NH2}$, $F_{N2+}$, $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N3}}$. In the upper left corner, the materials' real images (photographs) are shown, visually denoting the WSP and the size differences.

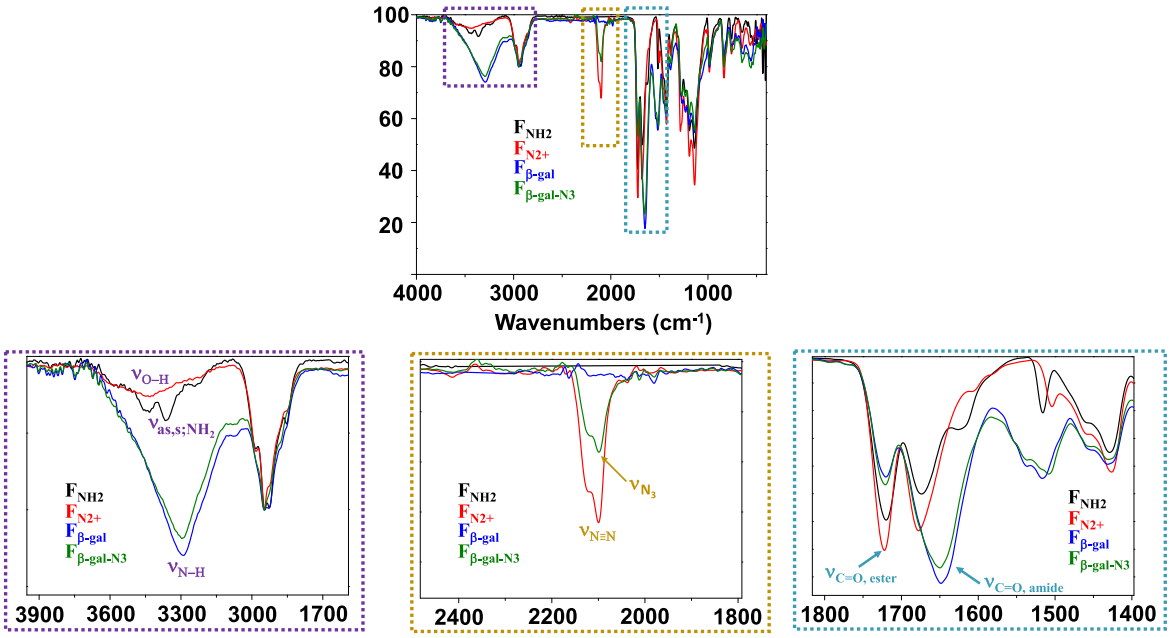


**Fig. 4.** Characterization of polymeric films $F_{NH2}$, $F_{N2+}$, $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N3}}$ by FTIR.

material but rather to the moisture it absorbs. In fact, the band is a result of water presence in the polymeric material, stemming from its high hydrophilicity. The spectra of the two materials without enzyme ($F_{NH2}$, and $F_{N2+}$) present two vibration bands of C═O at 1721 $cm^{-1}$ and 1674 $cm^{-1}$, corresponding to MMA and VP, respectively. On the other hand, the materials with enzyme ($F_{\beta\text{-gal}}$ and $F_{\beta\text{-gal-N3}}$) present one extra $\nu_{C=O}$ band, at 1647 $cm^{-1}$, due to the enzymés peptidic bonds. It is also noteworthy the presence of the $\nu_{N=N}$ band at 2100 $cm^{-1}$ only in $F_{N2+}$ and

$F_{β\text{-gal-N3}}$, due to benzene diazonium salt and azide groups, respectively.

### 3.2. Evaluation of β-galactosidase activity using the acrylic polymer $F_{β\text{-gal-N3}}$

The immobilization of the enzyme in the polymeric material does not imply that it has activity. Therefore, in the first stage of validation of the immobilization process, several experiments were conducted. First, we confirmed the β-galactosidase activity of $F_{β\text{-gal-N3}}$ disc using ONPG as substrate (SI-Section S2, Fig. S2a), verifying that only the film with the enzyme covalently bound ($F_{β\text{-gal-N3}}$) presents enzyme activity.

As these materials are intended for use in the hydrolysis of lactose in milk samples over long periods of time, a protein release study was carried out to validate that the enzyme is not released from the support after long periods in aqueous solution. No protein released to the medium was detected during the 30 h of incubation of the $F_{β\text{-gal-N3}}$ discs in citrate-phosphate pH 6.8 buffer (SI-Section S2, Fig. S2b), confirming strong binding of the enzyme to the polymeric matrix.

Furthermore, the Km values for the free and immobilized enzyme were determined (SI-Section S2), and showed no significant differences suggesting that enzyme immobilization did not affect the affinity of the enzyme for the substrate.

### 3.3. Proof of concept

The recommended storage temperature for milk is 4 °C, so it was one of the temperatures chosen for the study and the most realistic one when considering everyday use. However, two additional temperatures were included to provide a comprehensive characterization of the material. On the one hand, 25 °C was selected as the temperature under normal conditions. On the other hand, the highest temperature was chosen as the optimal working temperature for the enzyme as indicated by the manufacturer (optimal conditions: 55 °C and pH = 5) [26].

The results of the experiments using $F_{β\text{-gal-N3}}$ were better than using free enzyme in terms of hydrolyzed lactose (or remaining lactose), regardless of the temperature (4, 25 and 55 °C). As shown in Fig. 5a, the dashed curves corresponding to the free enzyme are, in all cases, above solid curves corresponding to $F_{β\text{-gal-N3}}$. European Food Safety Authority (EFSA) established that the lactose concentration must be below 0.1 g per 100 mL for milk to be considered "lactose-free" [4]. Using $F_{β\text{-gal-N3}}$, that objective is met after 20 h at 25 °C, and after 6 h at 55 °C. When the experiment was carried out at 4 °C, a final lactose concentration of 2.5 mg/mL was achieved after 30 h, insufficient to be considered "lactose-free milk", but low enough to be considered "low lactose milk" [4], which gives rise to future improvements of the material to achieve the objective of lactose concentration <0.1 g per 100 mL, working under refrigerated conditions.

This experiment was also used to verify the nutritional content of milk as described by other authors [14]. The milk was analyzed before and after the hydrolysis process with $F_{β\text{-gal-N3}}$ carried out at 4 °C for 30 h, and it was found that there was no variation in either protein content or fat content (more information in SI-Section S4).

In addition, as shown in Fig. 5b, the discs were washed/reused 4 times at 25 °C, then stored for 1 month in a zip bag, and finally reused/washed another 6 times at 25 °C, maintaining the same enzymatic activity throughout the process. This point is key to calculating the material's effective costs since although the initial material costs are low, they are reduced even more considering materiaĺs reusability (reusable at least 10 times), and the enzyme containing solutioń s reusability (reusable at least 6 times). In this way, the manufacturing costs for 1 g of $F_{β\text{-gal-N3}}$ are estimated in 0.7 euros (1 g of material is equivalent to 400 discs).

### 3.4. Comparison with other studies

Table 1 summarizes the most relevant papers in the field in recent years, in terms of type of immobilization (covalent/noncovalent), source of the enzyme, whether it has been tested under real conditions (namely, in milk), and whether it is reusable.

Some of the works expose materials with many reuse cycles, but they have not been tested in milk, therefore, it cannot be ensured that other components of the food matrix interfere in the lactose hydrolysis efficiency. Other analyzed materials have been tested in milk, but the efficiency of lactose hydrolyzation in the best case is 82%, and only with 2 use/wash cycles. On the other hand, our material hydrolyzes > 99% of lactose after 10 cycles of use/washing in milk, so we consider it a great contribution to the field.

## 4. Conclusions

In conclusion, we have developed a polyacrylic film material for enzyme immobilization, exemplified by the successful immobilization of

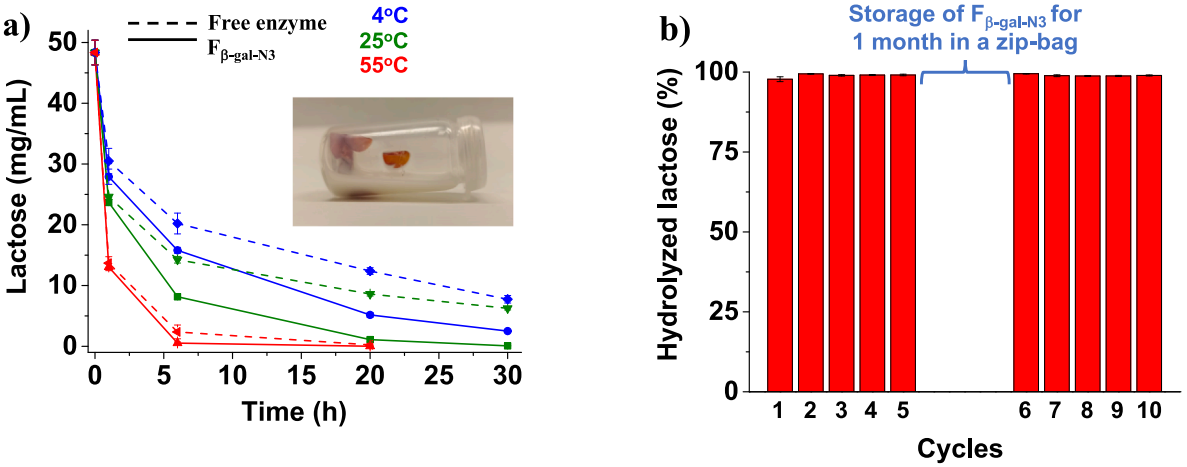


**Fig. 5.** Lactose hydrolysis tests carried out with 1 mL of UHT milk, by adding 830 μg of BIO-β-gal (dashed lines), or with 10 $F_{β\text{-gal-N3}}$ discs (solid lines). a) The graph represents the remaining lactose using both BIO-β-gal and $F_{β\text{-gal-N3}}$ discs (inset), and was quantified with the "K-LOLAC" kit (Megazyme) at different times (0, 1, 6, 20 and 30 h) and different temperatures (4, 25 and 55 °C). b) The experiments were carried out at 25 °C, and the hydrolyzed lactose (using $F_{β\text{-gal-N3}}$ discs) was quantified after 30 h with the "K-LOLAC" kit (Megazyme). The films were washed with water and reused ten times following the same experimental procedure, including a one-month storage period after cycle 5. Films were stored in the swelled state, packed in zip bags, and at 4 °C.

**Table 1**
Figures of merit: a comparative table of some of the most relevant papers in the field.

| Support material | Immobilization (Covalent/Noncovalent) | Enzyme source | Tested in milk(Y/N) | Cycles of use/ wash | Relative activity after last cycle (%) | Hydrolyzed lactose in milk | Incubation Time /Temp (°C) | Ref. |
|---|---|---|---|---|---|---|---|---|
| Hydrogels | Noncovalent | *Kluyveromyces lactis* | Y | 6 | 16 | – | 20 min/37 | [6] |
| Sodium alginate | Noncovalent | *Bacillus circulans* | N | 8 | 60 | – | 60 min/30 | [5] |
| Arabic-gum hydrogels | Noncovalent | *Lactomax 200 s* | N | 3 | 94 | – | -/37 | [8] |
| Carbon nanotubes micromotors | Covalent | *Kluyveromyces lactis* | Y | 2 | – | 82 | 20 min/37 | [12] |
| Ion exchange duolite A568 + GA | Covalent | *Aspergillus Oryzae* | N | 30 | 100 | – | 1 day/RT | [13] |
| Polycaprolactone and silk-fibroin based nanofiber fibroin | Noncovalent | – | Y | 1 | – | 41.8 | 24 h/4 | [14] |
| Silk fibers | Covalent | *Escherichia coli* | N | 6 | 92 | – | 6 h/50 | [15] |
| Nanosilver reduced graphene oxide | Noncovalent | *Aspergillus oryzae* | N | 10 | 85 | – | 15 min/37 | [16] |
| Polymer coated magnetic particles | Covalent | *Aspergillus oryzae* | N | 20 | 58 | – | 5 min/30 | [17] |
| Epoxi based polymeric film | Covalent | *Escherichia coli* | N | 12 | 51 | – | 15 min/37 | [19] |
| PVC + GA | Covalent | *Aspergillus oryzae* | N | 10 | 98 | – | 30 min/40 | [21] |
| Polystyrene nanofiber | Noncovalent | *Kluyveromyces lactis* | N | 9 | 30 | – | 10 min/37 | [22] |
| Polystyrene microspheres attached L-alanine | Covalent | *Escherichia coli* | N | 10 | 73 | – | 10 min /30 | [23] |
| Polyacrylic film $F_{\beta\text{-gal-N3}}$ | Covalent | *Aspergillus oryzae* | Y | 10 | >99% | >99% | 30 h/25 | This Work |

β-galactosidase (β-gal) enzyme through the formation of azo linkages. The immobilized enzyme exhibits superior efficiency in lactose hydrolysis in UHT milk compared to the free enzyme, highlighting the promising potential of our polyacrylic film material for application in dairy products. The material was prepared in a film shape for easy submersion and removal, and showed excellent reusability, with the ability to reuse it several times without loss of enzymatic activity. Notably, this polyacrylic film material holds promise for broader applications beyond lactose hydrolysis in dairy products. The general immobilization of enzymes using this material presents potential perspectives in various areas of biocatalysis and biotechnology. Moreover, the potential for tailoring the material properties by modifying the monomer composition or incorporating functional groups could offer new opportunities for fine-tuning the performance of the immobilized enzymes. Additionally, the film shape and reusability of the material make it attractive for continuous and batch processes, providing practical advantages in industrial settings.

## 5. Open data

Open Data is available at https://riubu.ubu.es/handle/10259/5684 (https://doi.org/10.36443/10259/7647).

## CRediT authorship contribution statement

**J. Lucas Vallejo-García:** Methodology, Conceptualization, Validation, Investigation. **Ana Arnaiz:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **María D. Busto:** Validation, Investigation, Writing – original draft, Resources, Supervision. **José M. García:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Saúl Vallejos:** Conceptualization, Funding acquisition, Project administration, Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Characterization of polymeric films $F_{NH2}$, $F_{N2+}$, $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N3}}$ by RAMAN and TGA; calibration curve of the equivalence test; biochemical characterization of $F_{\beta\text{-gal-N3}}$; determination of the nutritional content of milk. Authors partially generated the graphical abstract with DALL-E, OpenAI's large-scale image-generation model from natural language descriptions. Upon generating part of the image (the white surface with small balls), the author reviewed, edited, and revised the final image to their own liking and takes ultimate responsibility for its content. Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2023.112495.

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**J. Lucas Vallejo-García** received his Bachelor degree in Biotechnology from the Polytechnic University of Madrid (Spain, 2020) and his Master of Science in Food Security and Biotechnology from the University of Burgos (Spain, 2021). In 2019 he was at the Health Institute Carlos III of Madrid doing his curricular internship. Currently, he is doing his PhD in Food Science and Biotechnology. His research interests center on immobilizing biomolecules in polymeric synthetic supports for their use as biosensors or for biomedical and industrial purposes.

**Dr. Ana Arnaiz** (F) completed her PhD in Biotechnology and Genetic Resources in Plants and Associated Microorganisms at the Universidad Politécnica de Madrid in 2019. In addition, she has a postdoctoral Margarita Salas Grant at the University of Burgos to expand her research in the development of sensory polymeric materials oriented to detect biological targets.

**Prof M.D. Busto (F)** is Full Professor of Biochemistry and Molecular Biology at University of Burgos (Spain). She received the PhD degree in Chemistry at the University of Valladolid (Spain) in 1992. She has developed part of her activity, in the Biological Laboratory of the University of Kent (UK), and in the Research Laboratory of Biochemical Procter Food Science Department of Leeds (UK). Prof. Busto has published 72 scientific articles, 58 indexed in SCI (42 in Q1), along with 6 book chapters and one book co-edition. Her research is focused on the development of biotechnological systems and processes for applications in the agri-food, environmental and health fields, with emphasis on immobilization techniques for biocatalysts (enzymes and cells).

**Prof. José Miguel García** (M) is Full Professor at the Department of Chemistry at the University of Burgos, Spain. He carried out his doctoral studies at the Institute of Polymer Science & Technology, Spanish National Research Council (CSIC), receiving his PhD in Chemistry at the Complutense University of Madrid, Spain in 1995. Prof. Garcia is a co-author of 120+ peer-reviewed scientific publications and has a number of patents, along with co-author books and book chapters. His principal research areas are high-performance materials, functional polymers, and sensory polymers as sensing materials for food, biomedical, environmental, and civil security applications.

**Dr. Saúl Vallejos** (M) received his PhD in Chemistry in 2014 from the University of Burgos, Spain. Now he is a Senior Postdoc Researcher at the Department of Chemistry, University of Burgos. Dr. Vallejos is the author/co-author of 60+ peer-reviewed scientific publications and has 23 patents, along with co-author books and book chapters. His research interests are functional polymers with receptor motifs as sensory materials for anions, cations, and neutral molecules.