Effects of N- hydroxysuccinimide Activation on the Surface and Bulk Properties of Polyurethanes Available for Bioconjugation

LEONARD IGNAT*, ELENA GABRIELA HITRUC, MARIANA PINTEALA
Petru Poni Institute of Macromolecular Chemistry, 41 A Gr. Ghica Voda, 700487, Iasi, Romania

The polyurethane activation was made in bulk by two distinct synthetic methods, both involving the use of N-hydroxysuccinimide for obtaining water-insoluble materials susceptible to post-synthetic preparation of bioconjugates. The activated esters were obtained either during synthesis, through the direct reaction with isocyanate, or after the preliminary insertion of carboxylic groups by partial or complete substitution of the initial chain extender (1,4 butanediol) with 2,2'-bis(hydroxymethyl) propionic acid. Representative surface (hydrophilic/hydrophobic behaviour, morphology) and respective bulk (thermal and mechanical) properties were monitored in order to estimate the potential impact of succinimide activation on polyurethanes behaviour. The efficiency of resulting polyurethanes in bioconjugates formation was evaluated through the responses at papain immobilization.

Keywords: polyurethane, coupling reactions, bioconjugates

Polymers coupling with biological active compounds like drugs, peptides, grown factors, enzymes, carbohydrates, viruses or nucleic acids have known a remarkable evolution in the last decades due to the highly valuable applicative impact of resulting bioconjugate materials in almost every area of our current society, from medicine and biotechnologies to analytical chemistry and environment protection [1-4]. The progress achieved in polymer bioconjugation, initially related in particular with advances in biochemistry and molecular biology, has furthermore expanded in many fields of material science by either unveiling novel synthetic methods and macromolecules, or adapting the preexistent ones for new kinds of purposes [5-6].

On the other hand, the polyurethane-based materials are widely used in various applications, especially due to the excellent physical properties, flexible and tunable synthesis, relative biocompatibility and low toxicity [7-9]. However, until last years, they were rarely used for covalent couplings. A major drawback in the preparation of polyurethane-based bioconjugates is given by the absence of reactive groups available for post-synthetic chemical modifications. This issue was commonly circumvent either by covalent couplings with the prepolymers free isocyanate groups or by surface functionalization through physical means [10-11], but such methods have low specificity and in general do not provide enough control and reproducibility. A recent and more efficient opportunity to functionalize polyurethanes consists in the use of unconventional polyols or chain extenders that afford the final presence of reactive groups like hydroxyl and azide grafted to the main polymer chain [12-14]. Another common method involves the use of 2,2'-bis(hydroxymethyl) propionic acid (DMPA) as chain extender, but it was mainly applied for obtaining water-soluble polyurethane ionomers by reacting in situ with compounds like tertiary amines [15-17].

Taking into account these considerations, the purpose of the current work was to obtain and evaluate the N-hydroxysuccinimide (NHS) bulk-functionalized polyurethanes as water-insoluble materials susceptible to post-synthetic preparation of bioconjugates. The NHS / carbodiimide reaction system represents a classical, well known method for obtaining bioconjugates, especially by carboxyl-amine condensation [5]. NHS forms with carboxyls a labile ester, stable enough to afford stepped reactions with nucleophilic agents after the preliminary carbodiimide attack, increasing the stability of the activated group and limiting the secondary reactions. In the case of polymers it should be very important from applicative point of view to preserve the active ester for a later use. However, the applicability of pre-activated polymers is usually limited to rapid subsequent reactions due to the lability of NHS leaving group. There are only a few such products based on polystyrene or polysaccharides, like Sephadex G-10, which is applied in the preparation of bioactive surfaces and cellular affinity chromatography [18].

Experimental part

Polymer synthesis

All chemical reagents were of analytical grade and used as received from Sigma-Aldrich and Fluka. The polyurethane polymer (PU) was synthesized by the two-stage polyaddition of 4,4'-diphenylmethane diisocyanate (MDI), poly (butylene adipate) (PBA) with a molecular weight of 2000, and 1,4 butanediol (BD) in dimethyl-formamide (DMF), according to a previously reported procedure [19]. The polymers bearing carboxyl groups were obtained by partial or complete substitution of BD chain extender by DMPA (table 1).

The DMPA carboxyl groups activation as NHS-ester was carried out in the classical NHS/DCC system (N-hydroxysuccinimide / dicyclohexyl carbodiimide) [5], and the residual urea eliminated by successive filtration of cold (0^0C) polyurethane solution. Direct polyurethane activation was performed through prepolymer capping with NHS-urethane ester by partial substitution of BD chain extender with a corresponding NHS amount for maintaining the NCO/OH stoichiometry. The FTIR and ^{1}HNMR surveys have confirmed the complete consumption of isocyanate groups (2270 cm^{-1}), and successful formation of NHS-activated polyurethanes (formation of 1765-1780 cm^{-1} / 1810-1820 cm^{-1} bands, respective 2.51 ppm and 2.76 ppm signals).

* *Tel.: 0232217454*

Sample code	Polymer type	Molar ratio	C_{NHCOO} (mmoli/g)	C_{COOH} (mmoli/g)
PU	PBA/MDI/BD	1.0 : 5.8 : 4.8	2.98	0
PU-NHS	PBA/MDI/BD/NHS	1.0 : 6.5 : 4.5 : 2.0	3.02	0
PUC1	PBA/MDI/BD/DMPA	1.0 : 6.0 : 3.33 : 1.67	2.98	0.359
PUC2	PBA/MDI/DMPA	1.0 : 7.1 : 5.6	3.00	0.993

Table 1
PARAMETERS OF POLYURETHANE SYNTHESIS

The resulting polymer solutions were stable after sealing for at list one month at dark, in dry and cold (<4^{0}C) conditions.

Characterization

All polymer solutions were uniform casted on glass plates, precipitated in cold (0^{0}C) and room temperature double distilled water in three successive cycles, and dried for 24 h under progressive vacuum at 30^{0}C.

The hydrophilic/hydrophobic behaviour was assessed by determination of contact angle with double distilled water on CAM 201 apparatus (KSV Instruments, Finland). Five measurements were made for each polymer sample and results averaged.

The surface morphology was evidenced by atomic force microscopy (AFM) with a SOLVER PRO-M apparatus (NT-MDT, Russia Federation).

The stress-strain measurements were performed at room temperature and 120 mm/min crosshead speed on a TIRATEST 2161 Universal Testing machine equipped with data acquisition module. The specimens for tensile testing were dumbbell cut from casted films. A 40 mm benchmark and original cross-sectional area were used to calculate the tensile properties. Three identical specimens were tested and results averaged.

Thermal analysis was carried out with a Metler 851 derivatograph (Mettler-Toledo GmbH, Switzerland). Samples of 5mg were heated under nitrogen at a rate of 10^{0}C/min, in the range of 20-900^{0}C.

Papain coupling

Papain was brought from Aldrich as a beige lyophilized powder extracted from papaya latex having a specific activity of 0.5-2 units/mg. All other chemical reagents were obtained from Roth as high purity compounds. The enzymatic coupling was done by immersion of polyurethane strips (1cm^2) obtained from thin casted films (~0.4 mm thickness) for 2 h in 2mg/mL papain solutions made in phosphate buffer (0.2M; *p*H 8.0), at 25^{0}C, with occasional shaking. Blanks were treated in similar conditions with phosphate buffer only. After incubation, all samples were washed for several times with phosphate buffer.

The amount of immobilized papain was determined from the difference between the initial and eluted amounts of enzyme, according to the Bradford method [20-21], and expressed as mg protein retained by a polymer surface of 1cm^2. The papain activity was determined from the ratio of casein degradation, by monitoring the low molecular weight compounds released from substrate and solubilized by trichloroacetic acid (10%). The concentration of these compounds was indirect measured after centrifugation (15 min, 6000 rot/min) by UV-vis quantification of resulting tyrosine at 280 nm [22-23]. Papain was pre-activated by addition of cysteine (0.05 M) and EDTA (0.02M). One unit of enzymatic activity was equivalent in these experimental conditions with the quantity of low molecular compounds expressed as tyrosine μmoles evolved in one minute from one mg of substrate.

The UV-Vis monitoring was carried out on a JENWAY 6505 spectrophotometer (Bibby Scientific Ltd., UK).

Results and discussions

In the present study, we have used the NHS to obtain and evaluate two distinct types of activated polyurethanes, on the main chain and respective on the level of grafting moieties. Both functionalizations are made in bulk during and respective shortly after polymer formation. Unlike surface functionalization, the bulk ones may generates important changes on both bulk and surface properties, which could be useful or detrimental to the envisaged applications. As consequence, besides the potential reactivity of NHS-activated polyurethanes toward coupling with amine containing bioactive compounds, the effects generated by such apparent minor structural changes on the properties of interest should be also tested. Apart from these effects, the bulk modifications such as succinimide activation may be proved as very advantageous for example when the resulting polymers are processed in nanofibers through electro- or centrifugal spinning, or are used in applications and media that involve high levels of surface erosion.

In order to illustrate the potential impact of NHS activation on polyurethane properties we have chosen two representative surface (hydrophilic/hydrophobic behaviour, morphology) and two bulk (thermal and mechanical) properties.

Polyurethanes are basically segmented polymers composed from a polyol-type soft segment and a diisocyanate - chain extender hard segment that are thermodynamically incompatible and determine microphase segregation. In the current samples, the insertion of a carboxyl bearing chain extender and NHS activation both take place at the level of relative crystalline hard segments, disrupting the multiple hydrogen bonding responsible for chain packing. These effects explain for example why the use of a single chain extender conducts to more hydrophilic polyurethanes (table 2). Also, the hydrophobic activated ester gives a slightly better hydrophilicity as compared with the free carboxyl groups due to the adjacent hydrophobic aromatic moieties.

The effects of bulk functionalization in the region of hard segments are also clearly visible in the AFM images (figs.1a and 1b). In general, the hard and soft segments do not fully separate, part of the polyester being dragged into the

Table 2
POLYURETHANE CONTACT ANGLES

Sample	Contact angle
PU	85.0
PU-NHS	107.9
PUC1	117.5
PUC1-NHS	105.2
PUC2	83.4
PUC2-NHS	80.8

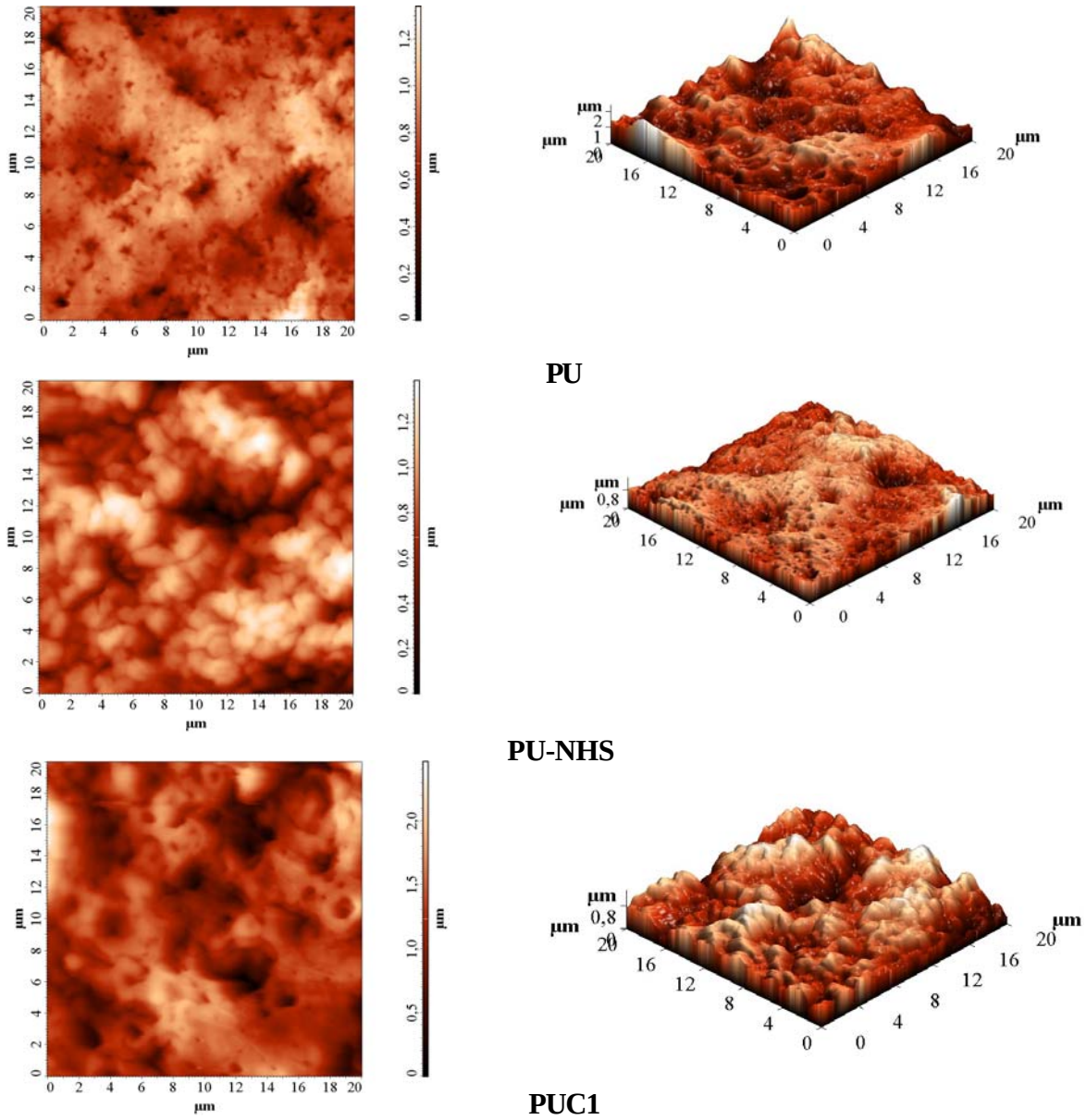

Fig. 1a. 2D and 3D topography of polyurethane samples. Data obtained in tapping mode

crystalline domains with a consequent reduction of their crystallinity. The most clear spherulitic structures appear at PU-NHS, while the PUC1-NHS and PUC2-NHS samples show the lowest crystallinity. So, the NHS increase the phase separation and roughness when is used for capping, but acts as a compatibilizer when is used for active ester formation with DMPA, phenomenon that exhibit the importance of the specific positioning of the supplementary small molecular structures on the overall polymer morphology.

The bulk properties, and in particular the mechanical ones, were also influenced by each structural modification of polyurethane samples (figs. 2 and 3). The poorer strength and elongation at break were shown by PUC1 and PU-NHS samples, as an effect of the severe disruption exerted at hard segment level accompanied by the reduction of inter-chain cohesion realized by hydrogen bonding. The complete substitution of BD by DMPA has increased the resistance at stress and the viscoelastic behaviour, but compensates by a lower elasticity as compared with PU. The compatibilizer role of NHS active esters observed at the examination of surface properties could be also clearly noted in the stress/strain diagrams, especially in the case of PUC1-NHS. The thermal behaviour of analyzed polyurethane species seems to be less influenced than the other monitored properties. However, the onset of thermal decomposition is sensible lower (130-140^{0}C versus 220^{0}C) for polyurethanes containing NHS esters than for unmodified ones. In addition, the earliest mass loss start (70-80^{0}C) presented by PUC2-NHS may be rather due to an increased retention of water.

PUC2-NHS has performed significantly better than the other two succinimide activated polyurethanes at papain coupling tests, and especially in retaining the enzymatic activity at very high levels (about seven times higher than free papain) (fig .4). This efficiency may be the common result of a higher hydrophilicity and an optimum exposure of the active ester groups at surface. Also, the hydrophilicity and viscoelasticity contribute to the soaking and swelling of PUC2-NHS, which in turn impart some limited sponge-like features to the respective polymeric materials. Such features are usually very convenient for the unfolding of enzymes activity.

On the other hand, the PUC1-NHS presents a higher immobilization capability than expected given de reduced number of activated esters, but was quite inactive in enzymatic reactions. The most plausible explanation for such behavior lies on the higher hydrophobicity and

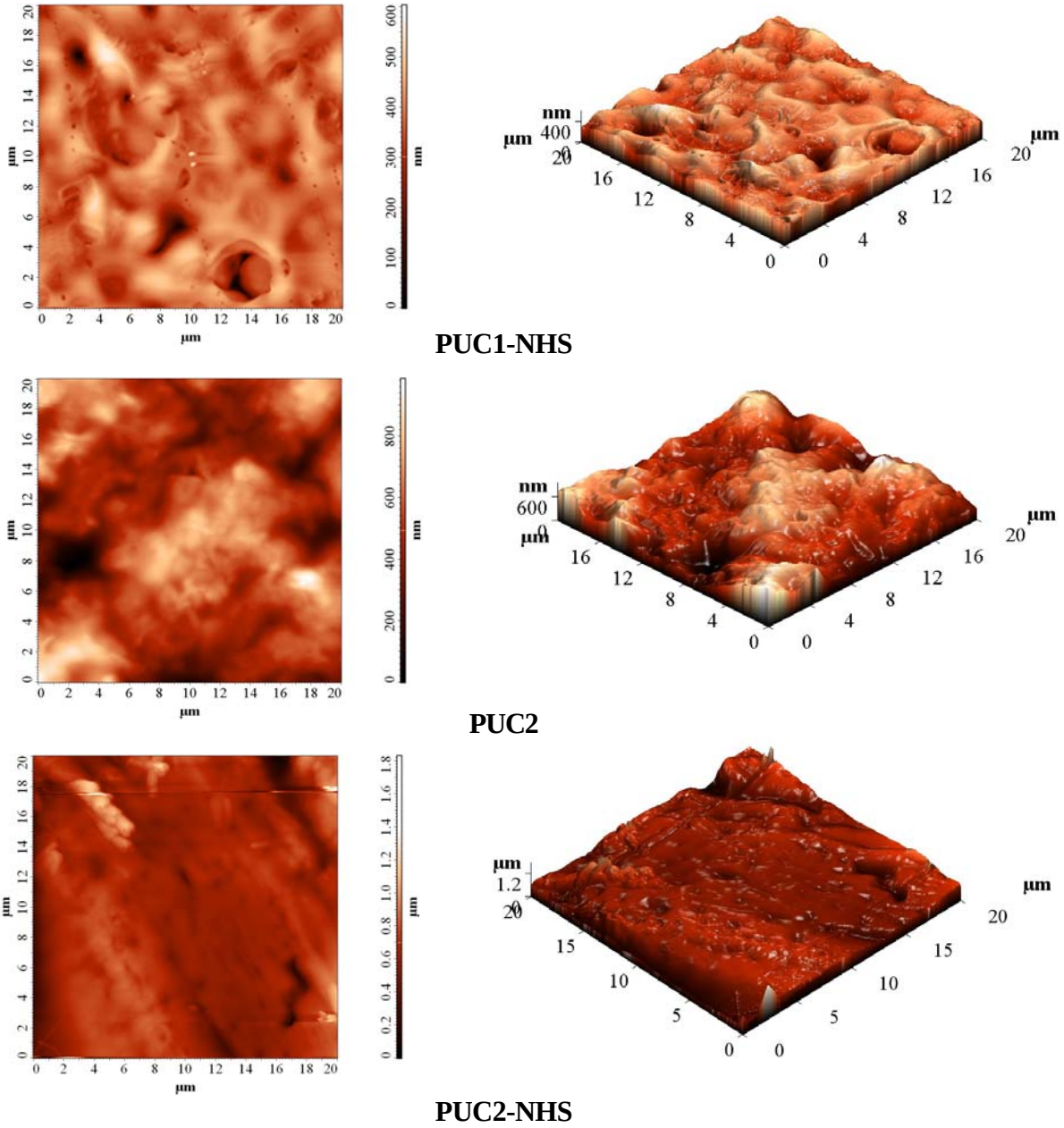

Fig. 1b. 2D and 3D topography of polyurethane samples. Data obtained in tapping mode

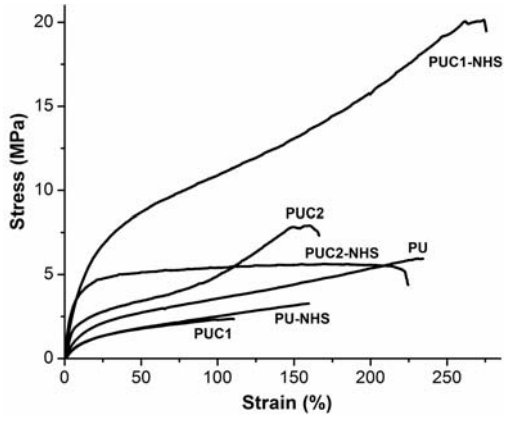

Fig. 2. The mechanical behaviour of polyurethane samples

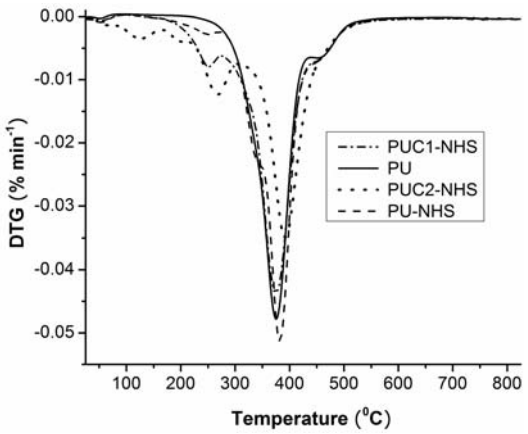

Fig. 3. The thermal behaviour of polyurethane samples

smoothness of PUC1-NHS surfaces that may facilitate the excessive binding of both papain and casein macromolecules by strong adsorptive forces, blocking the enzymatic activity. This assumption seems to be confirmed by the characteristics of papain coupling on PU-NHS. PU-NHS has a comparable hydrophobicity while exhibits a large phase separation and a rough, spherulitic surface that is quite inappropriate for strong and nonspecific adsorption, but favors the exposure of urethane activated esters. Therefore the degree of immobilization, albeit lower, still affords a specific activity more than twice higher than free papain.

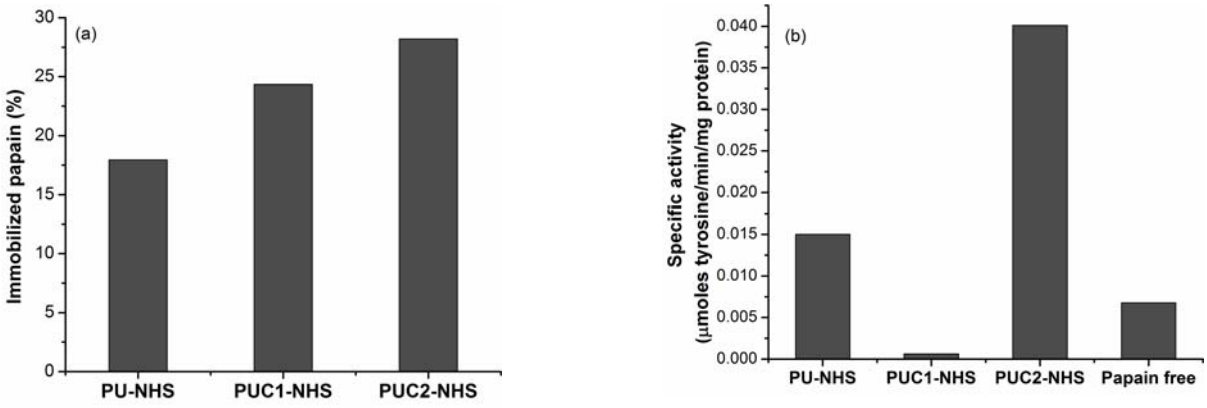

Fig. 4. The comparative immobilization efficiency (a) and remanent activity (b) of succinimide activated polyurethane samples

Conclusions

Bulk functionalization of polyurethanes was successfully attained by changing the chain extenders traditionally used for synthesis. The succinimide activation furthermore affords the synthesis of preactivated polyurethanes that contain NHS esters stable enough to be used in bioconjugation after a preliminary polymer processing by filtration and even film formation by precipitation in water. However, these relative minor structural modifications have a strong effect on the both surface and bulk properties of the resulting material. The significant changes brought to these properties made from the resulting polymer a virtually new type of material. Moreover, function of their type and specific positioning regarding the hard segment and in particular the diisocyanate moieties, the added functionalities may also enhance or compromise the initial aim of making efficient polymers for coupling reactions.

Overall, the results obtained strongly suggest that bulk functionalisation of polyurethanes not only open the way of preparing new bioconjugate derivatives, but also could afford better and more versatile biomaterials, even for complete different fields of material science.

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