PCL−PEG-Based Nanoparticles Grafted with GRGDS Peptide: Preparation and Surface Analysis by XPS

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Introduction

Nanoscopic particles self-assembled from amphiphilic block copolymers have been developed over the past years for the preparation of various drug delivery systems.[1] The biodegradable carriers made of polyethylene glycol (PEG)−polyester diblock copolymers emerged as the most adequate devices due to their prolonged circulation half-life in blood, their ability to bypass natural barriers, their reduced rate of uptake by liver, and their controllable delivery potientiality.[2,3] However, the coupling of ligands to achieve targeting toward particular cell types remains a challenge.[4,5] Few methods of functionalization of polymeric micelles have been reported: they are based on the synthesis of maleimide,[6] acetal,[7] or biotin-terminated[4] PEG blocks before block copolymerization and self-assembling. The first material is thiol-reactive and can be used for the direct covalent coupling of ligands such as Arg-Gly-Asp (RGD) containing molecules.[8] The second one is used, after deprotection of the acetal, for the coupling of amine-terminated GRGDS (Gly-Arg-Gly-Asp-Ser) pentapeptide via a reductive amination standard protocol. The third one is able to fix avidin in a noncovalent, but highly strong, interaction; this enables the subsequent fixation of biotinylated ligands because several biotin binding sites remain available on the nanoparticles.

In this note, we propose an alternative strategy inspired from the photoaffinity labeling, a technique well-known in biochemistry.[9] Our method relies upon the implantation of activated ester functions in the copolymer matrix by UV irradiation of *O*-succinimidyl 4-(*p*-azido-phenyl)butanoate,[10] intimately mixed with the material. Further reaction with NH_2-terminated molecules allows the covalent coupling of ligands or molecular probes via an amide linkage resulting from NHS (*N*-hydroxysuccinimide) displacement.

This has been illustrated by the functionalization of poly-ϵ-caprolactone(PCL)-polyethylene glycol(PEG) diblock copolymers with 3,5-bis-trifluoromethyl benzyl amine (Tag F_6) and with Gly-Arg-Gly-Asp-Ser (GRGDS) pentapeptide. The Tag F_6 is a fluorinated probe easily detectable and quantifiable by X-ray photoelectron spectroscopy (XPS) and GRGDS is a biologically active ligand aiming at the binding of the cell receptors called integrins.[11,12] Those grafted PCL−PEGs were added in the formulation of PEGylated PLGA-based nanoparticles prepared by a double emulsion technique and designed for oral vaccination.[13]

Our aim was to develop a convenient method to introduce any desired compounds (ligands or molecular probes) in self-assembled, tailor-made nanoparticulate systems, constituted of an hydrophobic core of associated PLGA and PCL blocks surrounded by an hydrophilic shell of PEG chains exposing the targeting signals. By modifying the quantities and lengths of the different polymers (PLGA, PLGA−PEG, and PCL−PEG), the characteristics of the nanoparticles could be adjusted in terms of surface properties, drug encapsulation efficiency, and rate of degradation.

As we wanted to produce targetable vectors, the key point was to obtain the bioactive signals well displayed on the external shell. For that purpose, XPS analysis was performed to investigate the chemical surface composition of the freeze-dried unloaded nanocarriers in order to check the presence of the ligands we introduced via the grafted PCL−PEGs.

Here we fully describe the preparation and surface analysis by XPS of the derivatized PCL−PEGs. After formulation with PLGA and PLGA−PEG, the nanoparticles were characterized in terms of size, surface charge, ^{1}H NMR, and chemical surface composition. A more precise attention was brought to the localization of exogenous atoms that should reveal the presence of ligands or probe molecules introduced.

Experimental Section

Materials. PCL−PEG, PLGA (22000), and PLGA−PEG (11000–5000) polymers were kindly provided by professor C. Jérôme (Center for Education and Research on Macromolecules, University of Liège, Belgium).[13,14] Two batches of PCL−PEG were used, slightly differing in the block lengths: 15200–4600, polydispersity index (PDI) = 1,4, and 21200–6000, PDI = 1,15. 4-(*p*-Azidophenyl)-*N*-succinimidyl butanoate (molecular clip) was prepared according to a procedure already described.[10]

GRGDS (97.0%) was purchased from NeoMPS (Strasbourg, France). 3,5-bis-(Trifluoromethyl)benzylamine (80%, Techn) was purchased from Sigma-Aldrich (Bornem, Belgium). Water (HPLC grade) was obtained with a Milli-GQ system (Millipore, Bedford, MA). Phosphate buffer (0.1 M, pH 8) was prepared from Na_2HPO_4 (16.86 g, 94.72 mmol) and NaH_2PO_4 (0.826 g, 5.98 mmol) in Milli-GQ water (1 L). Phosphate buffer saline was prepared from NaCl (4 g, 13.7 mmol), KCl (0.1g, 0.268 mmol), KH_2PO_4 (0.1 g, 0.114 mmol), Na_2HPO_4 (0.71 g, 0.8 mmol) in Milli-GQ water (0.5 L). Sodium cholate was purchased from Sigma Chemical Co. Dichloromethane and acetonitrile (PA grade) were obtained from Acros Organics.

Polymer Chemistry (Table 1). *Preparation of PCL−PEGs Grafted with GRGDS (PCL−PEG−GRGDS).* PCL−PEGs were solubilized in CH_2Cl_2 or in CH_3CN (40 mL/g) containing the molecular clip (0.2 mmol/g), and the solutions were cast on clean glass plates (1 mL per plate). After solvent evaporation, the samples were dried under vacuum to constant weight. Polymer samples were removed from the plates as shavings. These were irradiated at 254 nm, under argon atmosphere, for 20 min, in a homemade reactor (rotating quartz flask of 15 mL; 3 UV lamps of 8 W placed at a distance of 4.5 cm). The samples were washed (to remove unreacted arylazide and non fixed reagent) with isopropanol:ethyl acetate (19:1, v:v) (80 mL/g; 3 times), and dried under vacuum. The activated samples were immersed into a 1 mM solution of GRGDS (80 mL/g) into phosphate buffer (PB): acetonitrile (1:1, v:v, pH = 8), and shaken for 24 h at room temperature. The peptide solution was removed by suction, and the samples were washed three times with 5 mM HCl, five times with deionized water,

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Table 1. Preparation of Polymer Samples

name	copolymer; g, mL (CH_2Cl_2)	ArN_3; g	amine; g, mL (PB/MeCN)
PCL–PEG–g–GRGDS–1 copolymer 1 grafted with peptide	(15200–4600); 4.02×10^{-1}, 16	7.4×10^{-2}	GRGDS; $\times 10^{-3}$, 6
PCL–PEG–AP1 blank A copolymer for peptide	(15200–4600); 2.5×10^{-2}, 1		GRGDS; $\times 10^{-3}$, 6
PCL–PEG–g–Tag F copolymer grafted with fluorine Tag F_6	(15200–4600); 4.5×10^{-2}, 1.8	3×10^{-3}	$C_9H_7NF_6$;3×10^{-3}, 10
PCL–PEG–AF blank A copolymer for Tag F_6	(15200–4600); 6.7×10^{-2}, 2, 7	4×10^{-3}	$C_9H_7NF_6$;3×10^{-3}, 10
PCL–PEG–1 native copolymer 1	(15200–4600); -		
PCL−PEG−g−GRGDS-2 copolymer 2 grafted with peptide	(21200–6000); 1.9×10^{-1}, 7.6	1.2×10^{-2}	GRGDS; $\times 10^{-3}$, 6
PCL−PEG−AP2 blank A copolymer for peptide	(21200–6000); 2.5×10^{-2}, 1		GRGDS; $\times 10^{-3}$, 6
PCL−PEG−B2 blank B copolymer 2	(21200–6000); 4.5×10^{-2}, 1.8	3×10^{-3}	
PCL−PEG−C2 blank C copolymer 2	(21200–6000); 5×10^{-2}, 2		
PCL−PEG–2 native copolymer 2	(21200–6000); -		

Table 2. Preparation of Nanoparticles (NP) Composed of PLGA, PLGA−PEG, and PCL−PEG (70:15:15)

name	copolymer sample used in the formulation	average size (nm)	polydispersity index	ζ potential (mV)
NP(1)	PCL−PEG-1	183 ± 1.1	0.114 ± 0.002	−9.1 ± 5.8
NP(2)	PCL−PEG-2	271 ± 4.3	0.223 ± 0.078	−7.1 ± 4.6
NP−RGD	PCL−PEG−g−GRGDS-2	192 ± 1.5	0.131 ± 0.009	−16.2 ± 5.3
NP−Tag F_6	PCL−PEG−g−Tag F_6	189 ± 2,7	0.128 ± 0.011	
NP−b(AP)	PCL−PEG−A2	182 ± 1.6	0.112 ± 0.008	−16.2 ± 5.3
NP−b(AF)	PCL−PEG−AF1	252 ± 0.8	0.243 ± 0.040	−6.1 ± 4.9
NP−b(B)	PCL−PEG−B2	181 ± 0.9	0.094 ± 0.018	−15.2 ± 6.0
NP−b(C)	PCL−PEG−C2	184 ± 1.3	0.124 ± 0.003	−14.9 ± 5.9

Table 3. XPS analysis of copolymers

	atomic composition (%)					atomic ratios			
						C(C−O)/C(C−H)		C(*C*═O)/C(C−H)	
sample (see Table 1)	C_{1s}	O_{1s}	N_{1s}	F_{1s}	Si_{2p}	theor	expt	theor	expt
PCL−PEG−g−GRGDS-1	73.66	22.65	0.98		2.71				
PCL−PEG−g−GRGDS-2	73.50	21.85	0.11		4.54				
PCL−PEG−g−Tag F_6	71.01	23.46	0.21	0.21	5.11				
PCL−PEG−AP1	71.66	23.34	0.0		5.00				
PCL−PEG−AP2	68.76	22.29	0.0		>5				
PCL−PEG−1[a]	70.37	28.91			0.73	0.64	2.21	0.25	0.23
PCL−PEG−2[a]	76.92	23.0			0.09	0.62	0.60	0.25	0.24

[a] Mean of two analyses.

shaken overnight in deionized water, rinsed with MeOH, and dried under vacuum at 40 °C to constant weight. The presence of GRGDS was established by XPS.

Preparation of PCL−PEG Grafted with 3,5-bis-(Trifluoromethyl) benzylamine (PCL−PEG−g−Tag F_6). The previous protocol was applied using a 1 mM solution of 3,5-bis-(trifluoromethyl)-benzylamine in PB:CH_3CN instead of GRGDS solution.

Preparation of PCL−PEG Blank Samples (PCL−PEG). Blank samples were prepared to control the nonspecific adsorption versus covalent grafting and the possible nitrogen contamination, at each step of the derivatization protocol.

Blank A: standard protocol with omitting the arylazide reagent (molecular clip).

Blank B: standard protocol with omitting the peptide (or fluorinated Tag) reagent.

Blank C: standard protocol with omitting the arylazide and peptide (or fluorinated Tag) reagents.

Nanoparticles Preparation (Table 2). Nanoparticles were prepared by the "water-in-oil-in-water" solvent evaporation method, also called the double emulsion method, as reported previously.[13] Briefly, 50 μL of PBS were emulsified with 1 mL of methylene dichloride containing 50 mg of polymers (35 mg of PLGA, 7.5 mg of PLGA−PEG, and 7.5 mg of PCL−PEG) with an ultrasonic processor (70 W, 15 s). The second emulsion was performed with 2 mL of 1% sodium cholate aqueous solution, using an ultrasonic processor in the same conditions. The double emulsion was then poured drip into 100 mL of 0.3% sodium cholate aqueous solution and stirred at 37 °C during 45 min to evaporate the CH_2Cl_2. The solution of nanoparticles was then centrifuged at 22000*g* for 1 h and resuspended twice in PBS to eliminate the excess of sodium cholate. The formulation was composed of PLGA/PLGA−PEG/PCL−PEG (70:15:15). Nanoparticles size and ζ potential were determined using the Zetasizer Nano Series Malvern.[13] For XPS analysis, the nanoparticles were freeze–dried.

Table 4. Decomposition of C_{1s} Peak of NPs

component	binding energy (eV)	NP (1)	NP (2)	NP (2)[a] 60°	PEG
C−(C,H)	284.8 ± 0.1	42.50	44.54	46.60	
C−O (PEG)	286.1 ± 0.1	13.08	11.16	21.55	100
C−O (polyester)	287.0 ± 0.2	22.14	21.28	12.54	
O═C−O−C	288.9 ± 0.1	22.28	23.01	19.31	

[a] Lens axis was 60° between the normal to the sample surface.

Measurements. *Nuclear Magnetic Resonance (NMR).* NMR spectra were recorded on a Bruker 300 spectrometer. Lyophilized nanoparticles were suspended in D_2O (99.9 atom % D, ROCC SA) or dissolved in $CDCl_3$ (99.8 atom % D, ROCC SA). Native and grafted copolymers were dissolved in $CDCl_3$. Tetramethylsilane (TMS) was used as internal reference. A known amount of sodium benzenesulfonate was used as an internal standard to quantify the amount of PEG observed in D_2O.[15]

Size Exclusion Chromatography (SEC). SEC was carried out in THF at 40 °C with a Hewlett-Packard 1090 liquid chromatograph, equipped with a Hewlett-Packard 1037A refractometer index detector. Polystyrene and PEG were used for calibration.

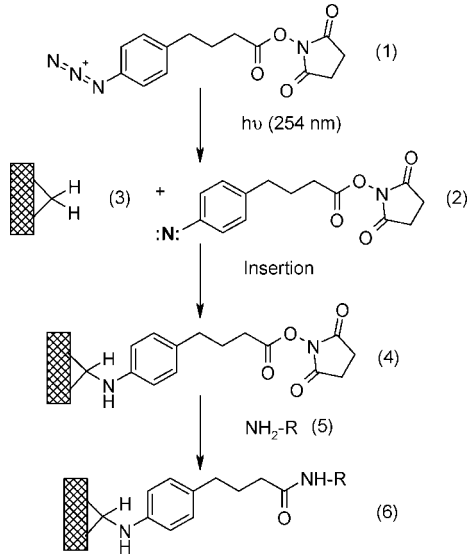
X-Ray Photoelectron Spectroscopy (XPS) (Tables 3, 4, and 5). The XPS analysis was performed in order to assess the presence of GRGDS pentapeptide and perfluorinated probe (Tag F_6) on grafted copolymers or nanoparticles surfaces. The spectra were recorded on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminum X-ray source (powered at 10 mA and

Table 5. XPS Analysis of Nanoparticles

sample (see Table 2)	atomic composition (%)						
	C_{1s}	O_{1s}	N_{1s}	F_{1s}	Na_{1s}	Cl_{2p}	Si_{2p}
NP(1)	65.77	31.39			0.95	1.07	0.82
NP(2)	56.69	30.36			4.53	8.06	0.34
NP−RGD	65.44	27.46	0.15[a]		0.65	1.52	4.78
NP−Tag F_6	66.92	29.82	0.16	0.31	0.76	0.85	1.18
NP b(AP)	60.40	31.8			2.42	4.17	1.28
NP b(AF)	55.73	28.22	0.06	0.02	4.73	7.22	0.57
NP b(B)	64.53	31.47			1.40	1.97	0.64
NP b(C)	63.89	30.86			1.71	2.32	1.23

[a] Mean of three analyses.

Scheme 1. General Principle for the Immobilization of Aminated Compounds on Copolymer[a]



[a] 4-(*p*-Azidophenyl)-*N*-succinimidyl butanoate (molecular clip) (1) decomposes under UV activation at 254 nm, giving the aryl nitrene (2) in the copolymer matrix (3). Insertion of the nitrene in C,O−H bond of (3) leads to the formation of the "activated copolymer" (4). Amino group of reagent (GRGDS or Tag F_6) (5) reacts on the *N*-hydroxy succinimidyl ester, resulting in the covalent fixation of (5) on the copolymer.

15 kV). The pressure in the analysis chamber was around 10^{-6} Pa. The angle between the normal to the sample surface and the lens axis was 0°. The hybrid lens magnification was used with the slot aperture and the iris drive position set at 0.5, resulting in an analyzed area of 700 μm × 300 μm. The constant pass energy of the hemispherical analyzer was set at 40 eV. In these conditions, the energy resolution gives a full width at half-maximum (fwhm) of the Ag 3d5/2 peak of about 1.0 eV. Charge stabilization was achieved by using the Kratos Axis device. The following sequence of spectra was recorded: survey spectrum, C_{1s}, O_{1s}, N_{1s}, F_{1s}, Si_{2p}, Cl_{2p}, Na_{1s}, and C_{1s} again to check the stability of charge compensation in function of time and the absence of degradation of the sample during the analysis. The binding energies were calculated with respect to the C−(C,H) component of the C_{1s} peak fixed at 284.8 eV. Data treatments were done with the CasaXPS program (Casa Software Ltd., UK) with a Gaussian/Lorentzian (70/30) product function and after subtraction of a linear baseline. The copolymer samples were casted, from solvent evaporation, on glass plates that were fixed on a stainless steel multispecimen holder by using double-sided conductive tape. The nanoparticles (powders) were pressed into small stainless steel troughs mounted on a multispecimen holder.

XPS calculations. *PCL−PEG C_{1s} Components Ratios.* PCL−PEG 1 (15200–4600) contains 238 monomer units divided into: 133 PCL units, $-O-CH_2-CH_2-CH_2-CH_2-CH_2-CO-(C_6H_{10}O_2)$, and 105 PEG units, $-O-CH_2-CH_2$ (C_2H_4O), hence the repeating "comonomer PCL/PEG unit" could be defined as follow:

$$1/238 \times [133 \times (C_6H_{10}O_2) + 105 \times (C_2H_4O)] = (C_{4.24}H_{7.36}O_{1.56}) \quad (1)$$

Comparison between the composition atomic ratios obtained from XPS analyses and the theoretical ones should provide evidence about a preferential or a uniform surface distribution. The components theoretical percentages were calculated from the chemical formula (eq 1). A typical example is given here for the C(C−H) component: (1/238 × [133 × 4] × 1/4.24) × 100 = 53%; similarly C(C−O) = 34%; C(C═O) = 13%.

The same calculations were done for PCL−PEG 2 (21200–6000), leading to formula (eq 2) for the "comonomer PCL/PEG unit":

$$1/322 \times [185 \times (C_6H_{10}O_2 + 136 \times (C_2H_4O)] = (C_{4.31}H_{7.43}O_{1.58}) \quad (2)$$

and the components theoretical percentages C(C−H) = 54%; C(C−O) = 33%; C(C═O) = 13%.

The peak decomposition is done in two steps: a first fitting is imposed on components placed at their corresponding energy without constraints. To improve reproducibility of the decomposition process, the peak width at half-height (fwhm) of the principal component of a peak is imposed to the others.

Tag F_6 Concentration. A Tag F_6 (3,5-bis-(trifluoromethyl)-benzylamine) coupled via the molecular clip (4-(*p*-azidophenyl)-*N*-succinimidyl butanoate) has the formula $C_{19}H_{17}F_6ON_2$. For example if **A** probes are immobilized per PCL−PEG 1 chain, the general formula of this modified chain is given by eq 3 and the *F*/*C* ratio by eq 4:

$$238 \times [C_{4.24}H_{7.36}O_{1.56}] + A \times [C_{19}H_{17}N_2OF_6] \quad (3)$$

$$\frac{F}{C} = \frac{6A}{(238 \times 4.24 + 19 \times A)} \quad (4)$$

So from the experimental *F*/*C* ratio the number of probes on a PCL−PEG chain is given by eq 5 and the derivatization percentage, which is defined as the percentage of modified "copolymer units" is obtained from eq 6.

$$A = 238 \times 4.24\left[\frac{F/C}{(6 - 19F/C)}\right] \quad (5)$$

$$\text{derivatization percentage} = \left(\frac{A}{238}\right) \times 100 \quad (6)$$

Results and Discussion

Photografting of PCL−PEG. To graft targeting ligands on copolymers used for drug-loaded nanoparticles, a novel photografting technique was used. As shown in Scheme 1, a mild and simple method was recently developed in our laboratory to functionalize nonreactive polymers.[10] It is based on the photoactivation of a so-called "molecular clip", 4-(*p*-azidophenyl)-*N*-succinimidyl butanoate, directly coated or mixed in an inert polymeric matrix. Under UV irradiation, this bifunctional photolinker generates a highly reactive nitrene that inserts into the C−H bond while the *N*-hydroxy succinimide (NHS) ester remains unchanged. In our PCL−PEG matrix, the required irradiation time at 254 nm was 20 min, after which appropriate washings and drying gave us a "NHS-activated" PCL−PEG ready to react with molecules displaying a free amino group.

Although easy to handle and efficient for the grafting on a large range of materials (organic as well as inorganic), the UV-mediated generation of aryl nitrene leads to the formation of side products, which may diminish the yields of covalent insertion and raise the rates of adsorbed residues.[16,17] As a consequence, it is useless to introduce huge quantities of the molecular clip that may result in an increase of undesirable products, hardly resorbable, with no real improvement of insertion yields. Therefore the molecular clip concentration has

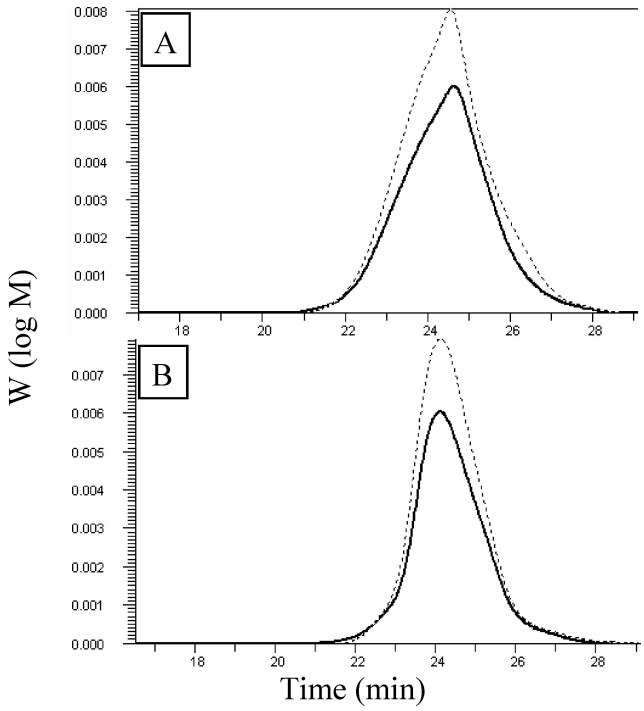


Figure 1. SEC chromatograms of PCL−PEG-1 (A) and PCL−PEG-2 (B). Plain lines are for native copolymers and dashed lines are for F_6-Tagged copolymers.

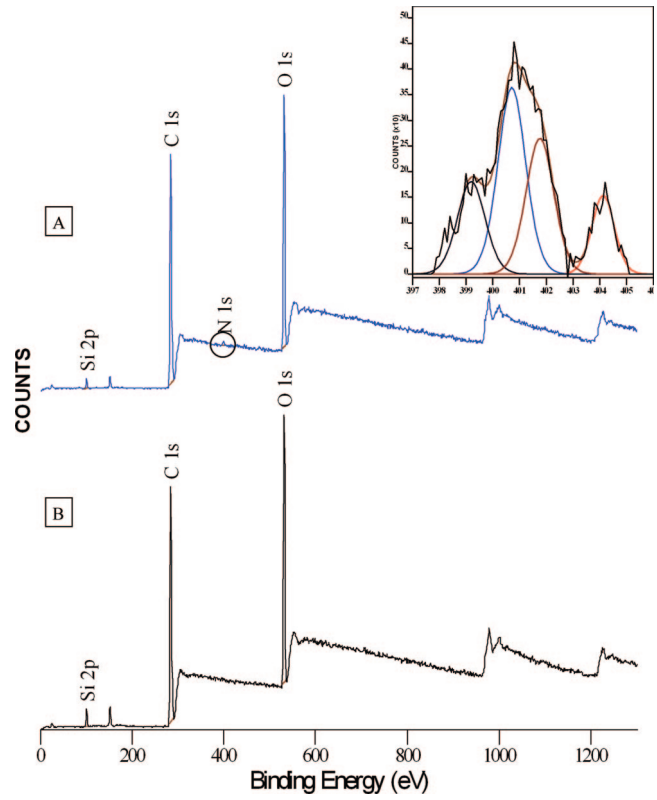


Figure 2. (A) General XPS survey scan of PCL−PEG−g−GRGDS-1: (B) general XPS survey scan of PCL−PEG−AP1; inserted spectra shows the nitrogen signal at high resolution.

been fixed at 0.2 mmol per g of the copolymer. Moreover, washings are of considerable importance to remove any unfixed generated products and their efficiency has to be controlled with several blanks prepared at each step of the process.

Table 1 presents the preparation of grafted PCL−PEG and their associated control samples. In this study, two derivatizations of the activated PCL−PEGs were carried out, first with GRGDS pentapeptide for biological applications, second with a fluorine tagged molecule, 3,5-bis-trifluoromethyl benzyl amine (Tag F_6), a probe used for the detection and quantification of grafting rates by X-ray photoelectron spectroscopy (XPS). Blanks marked as "A" were prepared to control the adsorption rates of GRGDS (or Tag F_6) independently of the molecular clip, blank B was prepared to assess the influence of side products due to the clip alone, and blank C was prepared for the estimation of treatment contamination. Native copolymers were used as control.

Because we handled hydrolytically degradable polyesters sensitive to both aqueous media and photodegradation, we had to control their post-treatment molar masses and polydispersities. SEC chromatograms of native and F_6-Tagged derivatized copolymers are presented on Figure 1. It is clear that no significant change can be detected. The post-treatment polydipersities were equal to the ones of native copolymers (1,4 for PCL−PEG-1 and 1,2 for PCL−PEG-2), meaning that no major chain cleavage occurred during our labeling procedure and potential adverse effects of irradiation or chemical treatments were not observed.

Characterization of the Photografted Copolymers. XPS was used for the detection of nitrogen on the two batches of PCL−PEG−g−GRGDS. Both grafted PCL−PEGs present a nitrogen peak due to GRGDS fixation (Table 3), but no accurate quantification can be calculated because of fluctuating rates and the possibility of contamination with reagent residues. However the decomposition of N_{1s} peak (Figure 2) on PCL−PEG−g−GRGDS-1, confirmed the presence of an amine bond at 399 eV, an amide bond around 400 eV, and a protonated nitrogen around 401 eV. The attribution of the nitrogen peak at 404,1 eV was uncertain because it could be due to a guanidinium motif in the peptide, azide residue as well as a contaminant. For PCL−PEG−g−GRGDS-2, the N_{1s} peak decomposition was less clear but exhibited a main region at 399.02 eV (data not shown). As evidenced by the absence of nitrogen on the native copolymers and blank samples (Table 3), the levels of adsorbed products is negligible. Consequently, the presence of nitrogen on the PCL−PEG−g−GRGDS samples was likely due to covalently bound compounds. It has to be noticed that our protocols of washings allowed a complete removal of the side products but unfortunately was unable to get rid of the silicon pollution.

The determination of grafting yields is a tricky problem to overcome that usually needs the development of specific methodologies. We used an XPS method based on the coupling of a perfluorinated probe (Tag F_6),[10,17] which introduced fluorine as an exogenous element that could not be a contaminant. The XPS analysis of PCL−PEG−g−Tag F_6 showed a significant presence of fluorine with a $F/C \times 100$ ratio of 0.3 (Table 3). This result corresponds to the fixation of almost 0.5 Tag F_6 per chain of PCL−PEG and a surface derivatization percentage of 2.1% (from eqs 3 and 4, see Experimental Section). It has to be noticed that the F/C ratio is underestimated due to an organic silicon contamination that may have increased the C_{1s} rates.

Our result is in good accordance with examples found in the literature for the coupling of biological probes on PCL−PEGs displaying conventional end-chain strategy derivatizations. Xiong and co-workers[7] used PCL−PEG terminated with an aldehyde function, made it react with GRGDS for 72 h, and after reductive amination, they calculated that 50% of their copolymers were functionalized. Gref et al.[4] synthesized biotin-

terminated PCL−PEG and measured, with a spectrophotometrical technique, that almost 60% of their polymer had biotin groups available for further coupling.

Although some qualitative information were inferred from the XPS chemical composition of our derivatized PCL−PEGs, we had to face several limitations during this study. The first one came from the preparation of XPS samples from solvent casting. In fact, during solvent evaporation, the tensioactive parts of the copolymers (PEG) will migrate to the surface in order to minimize the air/CH_2Cl_2 interfacial energy. Following this idea and as evidenced by Viville and co-workers, if some organic silicon grease (such as PDMS) is present within the sample, they will also migrate to the surface.[18] As a consequence, sometimes a non-negligible surface pollution of organic silicon had appeared and the detection of hydrophilic compounds (such as small peptides) might have been hindered.

The second limitation is due to the fact that PEG and PCL blocks are known to form separate crystalline microdomains, leading to the creation of surface chemical heterogeneity with sometimes a predominance of one of the blocks over the other.[19] Those phenomena could be estimated by the decomposition of the C_{1s} peak of the native PCL−PEGs, leading to the relative intensities of the C(C−H), C(C−O), and C(C═O) regions. The first one belongs to the methylene groups of PCL, the second one is mostly due to PEG, and the third one comes from the carbonyls of the polyester. The comparison between XPS atomic ratios and theoretical ones, obtained from the bulk composition (see Experimental Section), should provide evidence about a preferential or a uniform surface distribution. The C(C−O)/C(C−H) ratio should be a good indicator of the PEG/PCL ratio, whereas the C(C═O)/C(C−H) ratio should remain constant (as influenced exclusively by the PCL block). In the case of PCL−PEG 1, a clear PEG predominance could be identified because the C(C−O)/C(C−H) ratio is more than 3-fold higher than the theoretical one for the same C(C═O)/C(C−H) ratio. On the contrary, PCL−PEG 2 seems to exhibit a uniform distribution thanks to atomic ratios that correspond to the theoretical ones (Table 3).

The question of determining if the insertion of the molecular clip is preferentially oriented toward PEG blocks rather than PCL blocks would be hardly addressed. Indeed, the small amount of grafted materials renders the localization of the bonding site on the copolymer chain very difficult. Nevertheless several qualitative considerations claim that grafting should be preferentially "PEG-directed". The first factor is the well-known higher reactivity of nitrenes toward the C−H bond next to an ether function than the C−H bond of a hydrocarbon chain.[20] For example, Shingaki et al. found that αC−H bonds of cyclic ethers have a relative reactivity up to 12-fold higher compared to equivalent cyclic hydrocarbons in the insertion of ethoxycarbonyl-nitrene.[21] This phenomenon was explained by a different mechanism than the classic one admitted for hydocarbons. These observations were confirmed by experiments run in our laboratory on PEG and PCL homopolymers. We found that, for equal amounts of molecular clip, the grafting rates on PEG are six times higher than that on PCL (unpublished results). The second factor that may influence PEG selectivity should be a better solubility of the molecular clip in the soft part (PEG) than in the hard crystalline parts (PCL) of the copolymer.[22] Moreover, as exemplified by the XPS results of the previous paragraph, the PEG segments should be predominantly located on the outer part of the copolymer and therefore more exposed to UV irradiation. These combined effects would more likely lead to higher yields of insertion in PEG.

Nanoparticles Preparation and Characterization. The grafted PCL−PEGs were then introduced in the preparation of targeted nanoparticulate systems.[13] The particles were obtained by a double emulsion technique and constituted PLGA, PLGA−PEG, and PCL−PEG in the proportions 70/15/15, respectively. The different particles prepared are presented in Table 2. As we performed an investigation of the surface properties, the nanoparticles were unloaded to avoid any external contamination with the enclosed material.

Table 2 presents the characterization of the nanoparticles in terms of size and ζ potential. The negative values are attributed to ionized carboxyl groups. With the increase thickness of the PEG shell, a shift in the surface charge should appear due to a shielding effect of the neutral PEG segments.[23] Accordingly, the presence of PEG was attested by potentials between −6.12 and −16.2 mV compared to simple PLGA nanoparticles, which exhibited lower ζ potentials.

The PEG crown was quantified with ^{1}H NMR.[15] When the nanoparticles were dissolved in $CDCl_3$, which is a nonselective solvent, all the signals of PEG, PLGA, and PCL blocks were visible. Contrarily, when dispersed in D_2O, the signals of PEG segments that were in a solvated state were observed, whereas the signals of the hydrophobic PLGA and PCL blocks were no more present in the spectra. Those results confirmed the fact that these self-assembled systems are constituted of a hydrophobic core where water can not penetrate, surrounded by a PEG shell displayed in the aqueous media and stabilizing the system. It was possible to calculate the molar concentration of PEG in D_2O by adding a known quantity of an internal reference (benzene sulfonate). On the basis of the initial weight of PEG introduced in the whole formulation, it was calculated that only 21% was seen in water.

XPS Investigation of Nanoparticles Surface. XPS was carried out to further investigate the chemical composition of the particles surface. In the literature, previous works have detailed the practice of XPS analysis on lyophilized unloaded biodegradable nanocarriers. This technique was exemplified with the qualitative detection of a PEG shell on nanoparticles made of amphiphilic block copolymers[2,24,25] or composite nanoparticles of PIBCA and PCL−PEG.[26] It was also used for the analysis of surface engineered particles; Mu et al. studied different coatings,[27] and Constantino et al. used it to evidence the presence of peptidic ligands on the surface of PLGA−PEG nanoparticles.[28] But to our knowledge, the detection and quantification of covalently grafted probes on the surface of nanoparticles has been scarcely reported.

Parts A and B of Figure 3 and Table 4 show a typical decomposition of nanoparticles C_{1s} peak based on registered references[29] and on our own results on pure homopolymers. Four main envelops were found: a peak at 284.8 eV corresponding to the C−(C,H) region and mainly due to the methylene groups of PCL, a peak around 286.1 eV corresponding to the electrons of the C−O region of the PEG moieties, a peak around 287.0 eV coming from electrons of the C−O region next to a carbonyl belonging to PLGA and PCL segments (these are differenced from the former because of the so-called "second nearest neighbour effect" of carbonyl group which induces a decrease of the net charge on the carbon), and the contribution at 288.9 eV corresponding to the carbon of the carbonyl function O═C−O.

These calculations exhibited a PEG contribution around 10–13% of the whole surface atomic composition. This could be explained by the fact that during lyophilization, the PEG corona is hanging down and, with an analysis depth of ±5 nm,

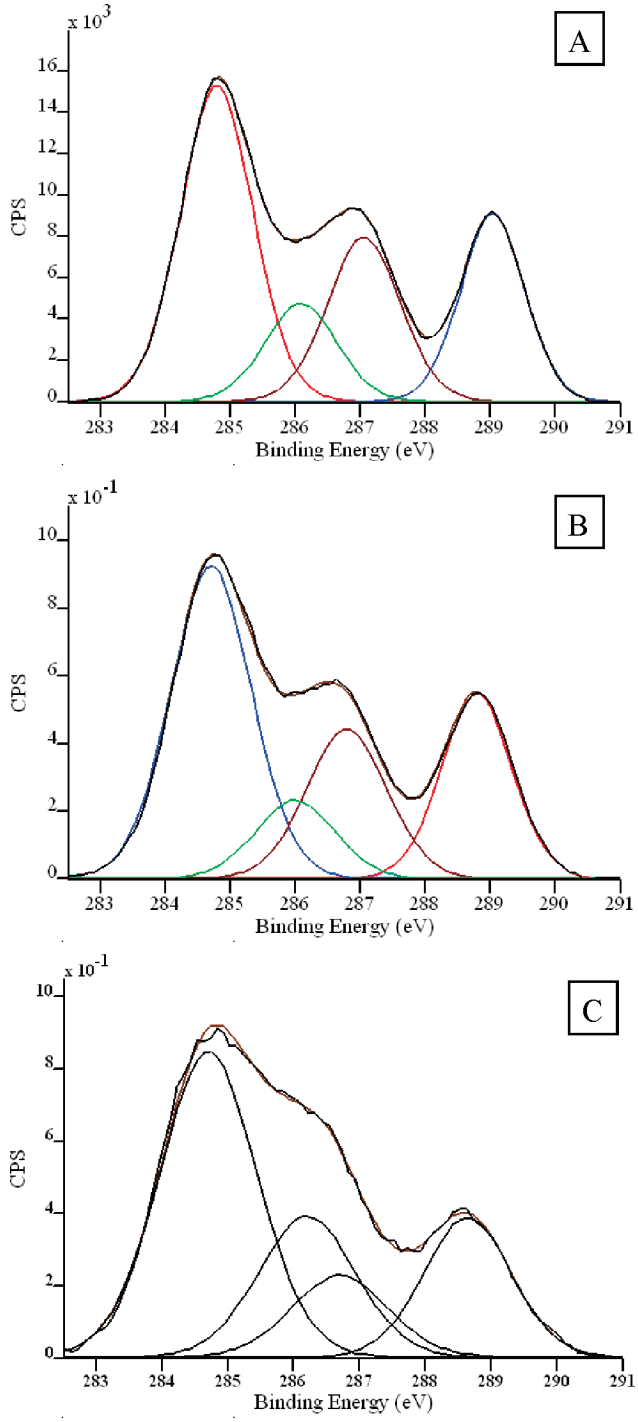


Figure 3. XPS C_{1s} peak decomposition: (A) on NP(1), (B) on NP(2), (C) on NP(2) with a R−X incidence of 60° (Table 4).

part of the hydrophobic core is also attained. Accordingly, to further prove the major presence of PEG at the surface, a variable X-ray angle experiment, with an incidence of 60°, was performed on NP(2) (Figure 3C). In this condition, the X-ray beam is skimming over the surface and thus is less penetrating, and consequently, only the outermost surface atoms are reached. This experiment showed a clear increase of PEG ratio (up to 20%) and a diminution of PCL and PLGA contributions (Table 4). This was in good agreement with the NMR and ζ potential results that had also evidenced the presence of a PEG shell. On the other hand, as lactide contributions were still present, this confirmed that the PEG shell is thin and that hydrophobic parts are close to the surface level. No significative difference in terms

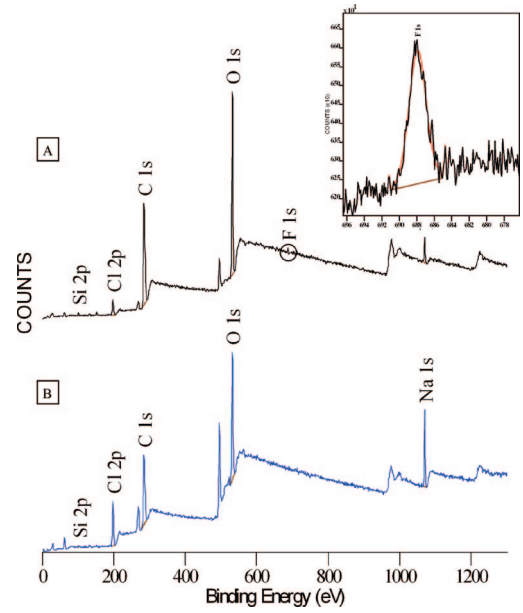


Figure 4. (A) General XPS survey scan of NP−Tag F_6 and (B) NP−b(AF) inserted spectra show the fluorine signal at high resolution.

of quantity of PEG could be pointed out between NP(2) and NP(1) made of two different PCL−PEG batches.

In our strategy for the preparation of targeted vehicles, the key point was to be sure that the ligands are well exposed on the outermost parts. Therefore XPS was performed on nanoparticles, prepared with PCL−PEG−g−GRGDS-2 (NP−RGD), and an amount of 0.15% of nitrogen (Table 5) was detected. The control experiments, on blank samples, have shown no nitrogen peak even after long accumulation times. On the basis of these results, we concluded that there were no evidence of peptide adsorption (NP−b(AP)) or molecular clip presence (NP−b(B)) or treatment contamination (NP−b(C)) (Table 5). Thus the nitrogen detected, in a depth of ±5 nm of the nanoparticles surface, is likely due to covalently grafted ligands. The ratio obtained could appear quite low, but we have to keep in mind that initial grafting yields on the PCL−PEGs are low too and that the X-ray beam penetrates deeper than only the outermost surface. To confirm the presence of nitrogen without any doubt, experiments were run on three different zones of the NP−RGD sample, all confirming an amount of 0.15%. Because there is no need of displaying large amount of ligands to have an effective targeting, this result was considered as satisfactory to fulfill our biological objectives.

Furthermore as fluorine has a higher atomic sensitivity factor (ASF) than nitrogen and cannot be a contaminant, we found it interesting to explore the surface composition of NPs prepared with PCL−PEG−g−Tag F_6(noted NP−Tag F6). Figure 4 illustrates a survey scan of NP−Tag F_6 and its corresponding blank. On this sample, ratios of 0.31% of fluorine and 0.16% of nitrogen (Table 5) were detected with negligible contamination due to the adsorption of the Tag F_6 (NP−b(AF), Table 5). This result confirmed the external presence of ligands or tags introduced in the formulation via the modified PCL−PEGs.

Conclusion

Functionalized PCL−PEGs were prepared by the photografting of a bifunctional photolinker thereafter modified with a biologically active ligand (GRGDS) or perfluorated XPS probe (Tag F_6). The grafted PCL−PEGs obtained were characterized

by XPS for the detection of nitrogen on PCL−PEG−g−GRGDS and fluorine on PCL−PEG−g−Tag F_6, respectively. We were able to prove the GRGDS fixation and to assess that almost 0.5 molecules were immobilized per PCL−PEG chain. This original method was aimed at the production of targeted nanoparticles for the oral delivery of vaccines. Using the required formulation for the encapsulation of antigenic materials,[13] we prepared unloaded nanoparticles and studied their surface properties. The presence of a PEG shell was attested by a shielding in ζ potential and by ^{1}H NMR spectroscopy in D_2O. Moreover, as evidenced by the XPS detection of nitrogen (or fluorine) on the surface of targeted particles and negligible rates of adsorbed products on blank samples, the ligands appeared to be favorably disposed for an effective targeting. Our XPS methodology has provided biologists with a reliable analytical tool for confirming that the targeting effects observed toward M cells are truly "RGD dependent" and not simply due to modifications of the surface polarity of nanoparticles.[13]

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