Greffage « sur »Etude du greffage de PTFALL et PBLG sur un squelette PS modifié. Observation de la structuration à l'état solide.

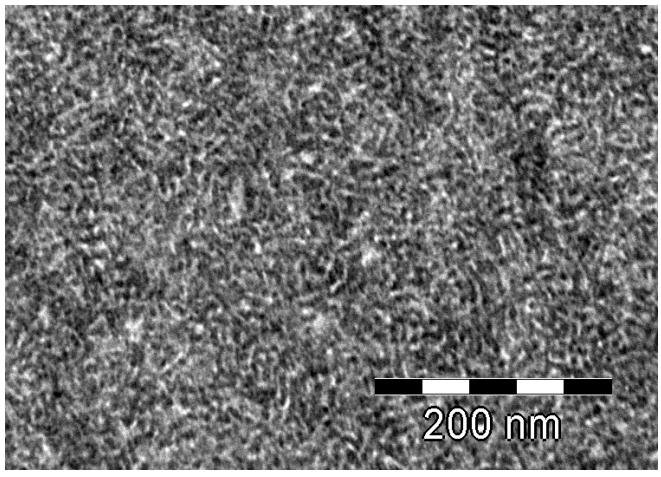

Figure 4-1. Observation TEM d'un copolymère PS-g-PTFALL. En noir apparaît le squelette polystyrène, marqué par RuO_4 ; en blanc la phase peptidique, PTFALL.

I - Introduction

Pour la synthèse de copolymères hybrides greffés au laboratoire, la voie de synthèse par greffage « sur », c'est-à-dire la préparation de copolymères hybrides par l'assemblage de segments vinyliques et polypeptidiques préformés, a été testée. A priori, cette méthode a de multiples avantages, en terme de facilité de mise en œuvre, de contrôle du taux de greffage, ou encore de richesse d'architecture possible… Elle paraît simple et modulable, par la possibilité d'ajouter plusieurs types de greffons bien caractérisés par exemple.

Par son mécanisme « normal » (cf. Chapitre 1III.1.2 -), la polymérisation par ouverture de cycle de NCA confère une terminaison amine primaire aux chaînes polypeptidiques en croissance. Les polypeptides peuvent être considérés comme monofonctionnels à leur extrémité N-terminale. Cette étude vise à utiliser cette fonctionnalité $-NH_2$ dans une réaction de greffage sur un squelette fonctionnel à travers un protocole opératoire simple. La fonction anhydride maléique a été choisit comme fonction chimique complémentaire. La réaction entre une amine primaire et une fonction anhydride est connue pour être quantitative à température ambiante.[19] La Figure 4-2 schématise le cas idéal de synthèse par greffage « sur » d'un copolymère greffé hybride.

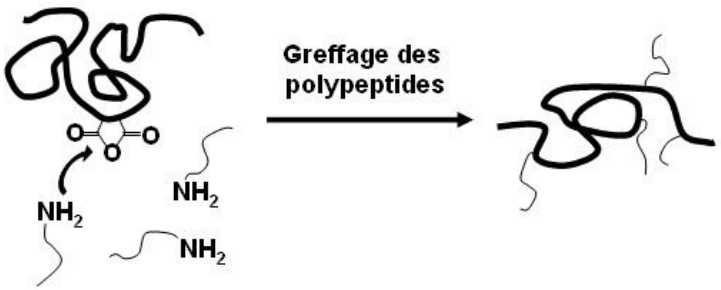

Figure 4-2. Schématisation de la réaction de greffage « sur ». La fonctionnalité NH_2 de l'extrémité N-terminale est utilisée pour greffer le polypeptide sur un squelette PS fonctionnalisé par des anhydrides maléiques.

L'objet de ce chapitre est le greffage de chaînons courts d'homopolypeptides $PTFALL_{30}$ et $PBLG_{30}$ sur un PS fonctionnalisé. Le greffage a été testé sur un squelette PS contenant 8 % (en mole) de fonctions anhydride maléique. L'étude de la réaction de greffage est réalisée par SEC.

Les $PTFALL_{30}$ et $PBLG_{30}$ présentent la particularité de se structurer en hélice-α, nous sommes particulièrement intéressés par la structuration en phase solide des copolymères greffés correspondants. En effet, la littérature montre que les segments polypeptidiques ont une grande influence sur la structuration des matériaux (cf. Chapitre 1 II.1). Les blocs structurés en hélice-α peuvent imposer la morphologie, par formation de domaines de polypeptides où les hélices sont associées parallèlement en domaines monocouches.

Dans la première partie de ce chapitre, le greffage de $PTFALL_{30}$ sur PS-co-Anh, et l'étude de la structuration du copolymère greffé obtenu, sont rassemblés dans un projet d'article. En complément, les expériences et résultats cités mais non-présentés dans l'article, sont donnés et commentés immédiatement après. Les résultats concernant les tentatives de greffage de $PBLG_{30}$ et la structuration des copolymères greffés correspondants sont donnés dans la deuxième partie de ce chapitre. Afin d'éviter les répétitions, l'essentiel des manipulations et des détails de conditions expérimentales - applicables pour le PTFALL mais aussi pour le PBLG - sont donnés dans l'article. Pour une lecture plus aisée, le lecteur est invité à lire le projet d'article avant la partie consacrée au greffage de PBLG.

Article II

Kinetics of poly(ε-trifluoroacetyl-L-Lysine) grafting onto functionalized poly(styrene).

The synthesis and solid state structuration of a hybrid graft copolymer containing polypeptide grafts are presented. We outline the synthesis of poly(ε-trifluoroacetyl-L-Lysine) (PTFALL) chains by the NCA polymerization and their subsequent grafting "onto" a functionalized PS backbone. The polypeptide N-terminus $–NH_2$ functions directly react on maleic anhydride functions on the PS backbone, without need of coupling agent or activation. The full synthesis procedure was followed by SEC. TEM observations of the solid state graft copolymers revealed a thin microphase separation. The matrix seems to be arranged into co-continuous morphology with interconnected domains of polystyrene and PTFALL.

Introduction

Hybrid copolymers attract increasing interest as they combine the properties of both synthetic and polypeptidic fragments inside of the same macromolecule. These materials mix the self-assembling of block or graft copolymers with the functionality and the ability of polypeptides to form hierarchical self-assemble nanostructures. These last self-organize at nanometer scale into regular secondary structure (such as among others, α-helices and β-sheets), or tertiary and quaternary structures (micro fibrils). For instance, driven forces for the structuration of such hybrid block copolymers were reported to be not only the microphase separation due to blocks nature, but also the aggregation into rod well-packed bidimensionnal layers domains of the polypeptidic moieties under α-helix conformation.[1][2] Polypeptides present also a wide range of functionality and stimuli responsive behaviour. Depending on the nature of side chains, polypeptidic segments can reversibly change their structure upon application of stimuli such as pH[3][4], temperature[5], solvent, chemical signal, light[6], ionic strength[7]… In accordance with their architecture, those hybrid copolymers provide a variety of applications in biomedical field, drug delivery or genetic engineering.[8][9][10][11][12][13] In particular, modified poly(ethylene glycol) - attractive for its biocompatibility, solubility and immunogenicity properties - were extensively linked to polypeptides.[8][13][14][15] As a consequence, a great development is done toward materials that can combine advantageous properties of both polypeptides and synthetic polymers (solubility, processing…), by preparation of hybrid copolymers.

The synthesis of hybrid copolymers rely strongly on the strategy adopted for the preparation of polypeptidic segments, which may be a limiting factor for the development of hybrid copolymer architecture. Various methods are available for the synthesis of polypeptide chains, the choice depending on the degree of control and on the complexity of the polypeptide sequence required.[16][17][18][19][20] For the preparation of large quantities with simple architecture, the most convenient and economical route is to polymerize α-amino acid N-carboxyanhydrides (NCA). This NCA polymerization, initiated by a nucleophile, has been widely described for 60 years[21] and permits relative chain length control. Latest developments of this technique enabled the synthesis of complex architecture such as multiblock sequences of polypeptides.[18][20][22]

Most of hybrid peptide-synthetic copolymers were synthesized by NCA polymerization initiated by functionalized synthetic macroinitiators. This grafting “from” synthesis was

mainly adopted on mono- or multi- primary amino functionalized macroinitiators. A wide range of hybrid block- and graft-copolymers was prepared according to this method.[1][2][23] Nevertheless, this grafting "from" multi-step method requires a high purification of macroinitiators to control NCA polymerization and to limit homopolypeptide contamination. [23] Another studied path for the synthesis of hybrid copolymers is the grafting "through" strategy.[1][24] In that case, peptide chains are prepared and functionalized as macromonomers, in order to be (co)polymerized with low molar mass monomer. But the chemical transformation of polypeptide chain into macromonomer with vinylic, acetylene, isocyanide or other polymerizable moieties is not easy. Moreover, the polymerization method has to be well-chosen, to avoid any side reaction with the corresponding polypeptide.

In a last strategy to prepare hybrid copolymer, the grafting "onto" method, where preformed chains are chemically linked together, seems more adapted. This method may enable a better control of grafting rate and blocks length if combined with an extensive characterization of polypeptides before the grafting step. Thereby, a wider range of copolymer architecture may be reached with this technique, like for instance, grafting "onto" of dissimilar polypeptides onto the same backbone. Different strategies are described, by using the chemical reactivity of the polypeptide side groups as it is in classical[12][25][26], click chemistry[27][28], or with help of coupling agent.[29][30]

A particularity of polypeptide chains is that they possess a primary amino N-terminus function. This $-NH_2$ function is naturally obtained in the process of NCA polymerization at the chain N-terminus. This primary amino function can be used in reactions with a wide range of suitable chemical functions[31], and thus it can be used for the preparation of hybrid copolymers by the grafting "onto" method. In that case, polypeptides are considered as reactive and graftable chains. This grafting "onto" method is particularly well-adapted when polypeptide chains are prepared by the conventional solid-phase peptide synthesis.[16][24] In that case, the carboxylic chain end of polypeptide is immobilized onto a resin. After deprotection, the primary amino terminus end is used for the addition of a new α-amino acid or to be linked on a suitable functionalized activated chain for the grafting "onto" synthesis path of hybrid copolymer.[14][32][33] With other polypeptide synthesis methods, homopolypeptides or small sequence of peptides were reported to be conjugated in solution by their N-terminus onto activated ester moieties locate on a synthetic polymer chains.[34][35] Modification of N-terminus of polypeptide prepared by NCA polymerization were also reported for the subsequent synthesis of hybrid copolymer by the grafting "onto" method.[36]

Here, we focused on the synthesis of a poly(ε-trifluoroacetyl-L-Lysine) (PTFALL) homopolypeptide by NCA polymerization and its subsequent grafting "onto" a functionalized polystyrene. Primary amino N-terminus fonction of PTFALL chains were grafted onto a polystyrene (PS) backbone containing a low fraction of maleic anhydride units (8 mol%). The full synthesis procedure (polypeptide polymerization and grafting) was followed by Size-Exclusion Chromatography (SEC).

Rod-coil copolymer and specially hybrid copolymer containing block under α-helix structuration exhibit interesting solid-state behaviour. α-helices tend to align and self assemble into hexagonal monolayer domains that subsequently modify the copolymer structuration compare to amorphous copolymers. Lamellar structure is found over a broader range of compositions compared to amorphous copolymer.[1][2] The choice of graft, instead of block copolymer was lead by self-assembling considerations. Most studies deal with hybrid block copolymers of well defined architecture which are supposed to allow for a precise control of self-assembly.[1][2] On the other hand, graft copolymers have a less controlled molecular architecture, but in many cases their synthesis is easier. Despite their inherent polydispersity and molecular heterogeneity, graft copolymers self-assemble in nanostructures similar to those of block copolymers and in some case, they stabilize morphologies like for instance co-continuous that are difficult (or impossible) to reach with well defined block copolymers.[37]

Experimental part

Materials. ε-trifluoroacetyl-L-Lysine N-Carboxyanhydride (TFALL NCA) was purchased from ISOCHEM (Vert-le-Petit, France). Deuteriated *N*,*N*-dimethylformamide (DMF D7) (99.5 %D) and dimethylsulfoxide (DMSO D6) (99.8 %D) were purchased from Euriso-top (Gif-Sur-Yvette, France). *N*,*N*-dimethylformamide (DMF) (99.5 %), tetrahydrofuran (THF) (> 99.9 %) were purchased from SDS (Peyran, France). Deuteriated chloroform ($CDCl_3$) (99.8 %D), 3,5-bis(trifluoromethyl)benzaldehyde (BTBFA) (97 %), Lithium bromide (LiBr) (99+%), molecular sieves (4 Å) were purchased from Acros (Geel, Belgium). *n*-hexylamine (99 %), α,α,α-trifluorotoluene (TFT) (99+%) and calcium hydride (CaH_2) (99.9 %) were purchased from Sigma-Aldrich (Steinheim, Germany). DMF was distilled over CaH_2 under reduce pressure, and stored over molecular sieves under Argon atmosphere. All other chemicals and solvents were used without further purification.

Size-Exclusion Chromatography (SEC). We used a Waters 150CV apparatus equipped with differential refractometer and differential viscometer detectors. The columns set is composed of 2 linear Waters μStyragel HT6E and 1 column HT2. The eluent is DMF containing 0.1 M LiBr. The flow rate is 0.65 mL/min. All the experiments are performed at 40°C. The standards used for calibration are a set of narrow polyethylene oxides and polyethylene glycols (PSS USA) with molecular weight ranging from 1010 to 761300.[38] Unfortunately, polystyrene is not recommended as standard for calibration in DMF[39] even if it would have been more useful for comparison with the backbone partly made of styrene. Samples of polymer solutions are dilute in the eluent prior to be filtered on Millipore filters FH 0.45 μm. The polymer concentration is always the same and equal to 0.5 % w/w.

$PTFALL_{30}$ synthesis and characterisation. TFALL NCA (1.35 g, 5.04 10^{-3} mol) was dissolved in dry DMF (13.3 g) at 20°C in a two-necked round-bottomed flask. The atmosphere was kept inert by a circulation of dry Argon. A solution of *n*-hexylamine (17 mg, 1.68 10^{-4} mol) in dry DMF (1.2 g) were introduced to induce polymerization. After three hours of polymerisation, the crude solution is precipitated in water and dry under vacuum.

Study of the living character of the TFALL NCA polymerization. For the first step, solutions of TFALL NCA (2.31 g, 8.61 mmol) and *n*-hexylamine (26.94 mg, 0.267 mmol) in dry DMF (total amount of 8.08 g) were mixed in a two-necked round-bottomed flask and let at room temperature under stirring. The atmosphere was kept inert by a flow of dry Argon. The target polymerization degree was 32.3 TFALL monomers per chain. After five hours, 350

mg of the solution were sampled for SEC analysis. Then, a new solution of TFALL NCA (1.38 g, 5.16 mmol) in dry DMF (5.02 g) was added to the flask. It corresponds to an increase in the polymerization degree of 19.7 monomers per chain. After five hours, 350 mg of the solution were sampled for SEC analysis.

TFALL NCA Polymerization and grafting. TFALL NCA (1.35 g, 5.04 10^{-3} mol) was dissolved in dry DMF (13.3 g) at 20°C in a two-necked round-bottomed flask. The atmosphere was kept inert by a flow of dry Argon. A solution of *n*-hexylamine (17 mg, 1.68 10^{-4} mol) in dry DMF (1.2 g) were introduced to start polymerization. After three hours, a solution of the functionalized polystyrene (1 g, 7.73 10^{-4} mol in anhydride function) in dry DMF (10 g) was introduced. This moment corresponds to t = 0 s for the grafting reaction.

^{1}H NMR Spectroscopy. 1H NMR Spectra were recorded on a Bruker AC 300 MHz spectrometer. Polymerisation degree of $PTFALL_n$ is estimated by comparison between the *n*-hexylamine contribution and the TFALL contribution. ^{1}H NMR of $PTFALL_{30}$ in DMF D7 : δ = 0.9 (CH_3 hexylamine, 3H, I = 3), δ = 1.2-2.3 ($CH_3(CH_2)_4CH_2$ hexylamine, $CH(CH_2)_3CH_2$ TFALL, 8H + 6H**n*, I = 189), δ = 3-3.8 (CH_2NH hexylamine, $CH_2NHCOCF_3$ TFALL, 2H + 2H*n + H_2O, I = 83), δ = 3.8-4.6 (COCHNH TFALL, 1H*n, I = 30). n = 30.

^{19}F NMR Spectroscopy. ^{19}F NMR spectra were recorded on a Bruker 400 MHz spectrometer. A precise amount of $PTFALL_{30}$ purified from DMF solution by precipitation into water and dried under vacuum was weighted (20.9 mg, 3.06 10^{-6} mol) and solubilised in DMSO-D6 (0.75 g). The reference solution, containing BTBFA (49.5 mg, 2.05 10^{-4} mol) and TFT (17.6 mg, 1.2 10^{-4} mol) was prepared in DMF-D7 (5.2 g). ^{19}F NMR spectrum of the reference solution in DMF-D7 (δ (ppm), integration): TFT (56.56, 100), BTBFA (56.26, 327.1), BTBFA impurity (56.29, 20.2). BTBFA contains 5.8% of impurity (most probably 3,5-(bistrifluoromethyl)benzyl alcohol). 383 mg of the reference solution was mixed in the $PTFALL_{30}$ solution. It corresponds to 1.419 10^{-5} mol of BTBFA, after correction made for the impurity. $n_{PTFALL30}/n_{BTBFA}$ = 0.216.

FTIR. FTIR spectra were recorded on a Bruker Tensor 37. THF solutions were prepared and solvent cast on KBr windows. 32 scans were done at 3 cm^{-1} resolution.

TEM. Solvent cast films ~ 1 mm thick were prepared by slow evaporation from 5 wt% polymer solutions in tetrahydrofuran (evaporation during three days under a saturated atmosphere in tetrahydrofuran). Samples were annealed at 140°C, under vacuum during 48 hours, prior to be cut with a Leica Ultracut microtome in slices of 60 nm thick at room

temperature. Thin sections were collected on 400 mesh copper grids. For a selective staining of polystyrene phase, grids were exposed to the vapour of a freshly prepared aqueous RuO_4 solution for 90 seconds. Observations were carried on a Zeiss 902 microscope operating at 80 kV and equipped with a MegaviewII digital camera from Soft Imaging System.

Results and Discussion.

Synthesis of poly(ε-trifluoroacetyl-L-Lysine) (PTFALL). In the first part of the work, we synthesized and characterized the polypeptides chains. We are interested in rather small chains to be grafted on the backbone so as to minimize limitations due to steric hindrance. On the other hand we are interested in long enough polypeptide chains that can form robust secondary structure, which offer strong associativity properties. We focused our study on PTFALL of about 30 α-amino acid units per chain, which corresponds to a molecular weight slightly higher than 6500 $g.mol^{-1}$. PTFALL is prepared by ring opening polymerization of ε-trifluoroacetyl-L-Lysine *N*-carboxyanhydrides (TFALL NCA) in dry DMF. Polymerization is initiated by *n*-hexylamine which is usually employed for the synthesis of polypeptides with polymerization's degree of some decades.[40]

Polymerization is followed by SEC: a sample of the reaction medium is diluted into DMF/LiBr ([LiBr] = 0.1 $mol.L^{-1}$) and immediately injected for analysis. It is worth noting that SEC doesn't give the instantaneous composition of the reaction medium as the reaction may continue after sampling and dilution (the measurement takes one hour). It permits however to catch a tendency and to follow the evolution of the reaction, provided that the characteristic time of this reaction is greater than the measurement time. SEC allows also to estimate the end of the NCA polymerization. We observed for the polymerization degree we are interested in, that the polymerization reaction is completed in about two hours.

The "key" step for a successful PTFALL synthesis is to preserve the primary amino functionality of the polypeptide N-terminus. During the polymerization process, the $–NH_2$ function is crucial for the adding of a new monomer, as it reacts as a nucleophile on the N-carboxyanhydride function of the monomer. Therefore, our first concern was to test the living character of the polymerization, which guarantees the livingness of this primary amino function after adding of a new monomer. To perform this investigation, we used SEC, to characterize the ability of preformed PTFALL chains, to add new monomers. In a first step, we prepared a $PTFALL_{32}$ (with a polymerisation degree of 32.3) in DMF. In a second step, we added to this solution, the equivalent of 19.7 TFALL NCA monomers per chain to induce a continuation of the polymerization and to obtain $PTFALL_{52}$. Figure 4-3-(a) shows the viscosimetric signals of $PTFALL_{32}$ and $PTFALL_{52}$ obtained by SEC. The viscosity shows a clear increase when the PTFALL polymerization degree grows from 32 to 52. Figure 4-3–(b) shows the same viscosimetric signals in full scale. There is a clear shift of the signal toward

lower elution volume, corresponding to higher molar mass of the polymer. Addition of monomers results in a continuation of the polymerization reaction. There is no noticeable trace of “dead” chains.

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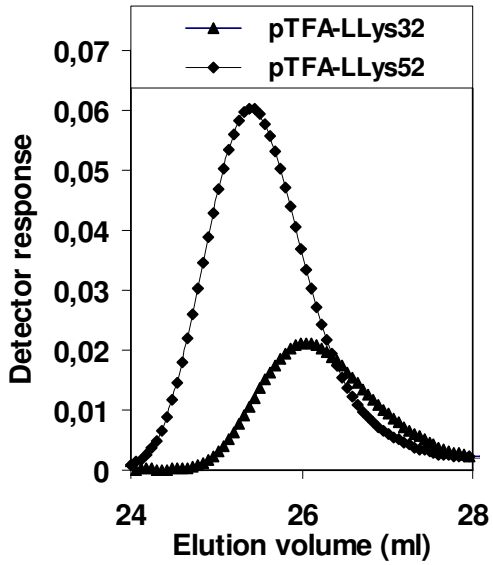

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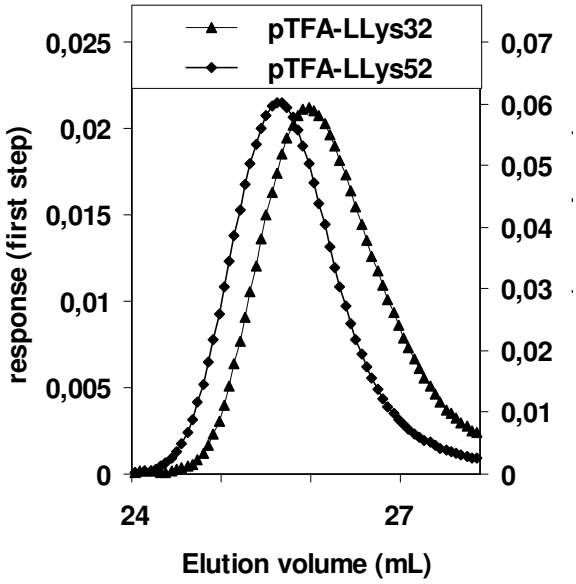

Figure 4-3. (a) SEC viscosimetric signal of PTFALL before (triangles) and after (diamonds) monomer addition. (b) Same signals in full scale. A clear shift toward higher mass and higher viscosity underlines the “living” character of these chains.

Table 1 summarizes SEC data for the polymer after the first and the second step. It is worth noting that SEC doesn't give the exact molar mass of chains but an equivalent or apparent molar mass, depending on the polymer used to establish the calibration curve (here PEO). Care must specially be taken with polypeptides as they have a strong tendency to form H-bonds inside of the main chain to adopt specific conformations (α-helix, β-strand) which are very different from the coil conformation of the polymer standards used for the calibration. These intramolecular H-bond links reduce the hydrodynamic volume of the chains leading to SEC evaluations that are noticeably lower than theoretical. Here, we use SEC as a tool to compare the relative variations of the molar mass of the chains. Theoretically, a PTFALL chain of 32.3 monomers weights around 7000 $g.mol^{-1}$ and a 52 monomer chain weights above 11 000 $g.mol^{-1}$. With 19.7 monomers added to reach 52, the expected variation in molar mass is 38%. This value is quite close to the variation of about 35%, observed experimentally for M_n and M_w using the PEO calibration (Table 1). The SEC data confirm the "living" character of PTFALL short chains and that the amino end of the chain is still active after few hours in DMF.

	$PTFALL_{32}$	$PTFALL_{52}$	Variation
M_n	4800	7300	+34 %
M_w	5600	8600	+35 %
Polydispersity	1.16	1.18	

Table 1. SEC characterization of $PTFALL_{32}$ and $PTFALL_{52}$. The molar masses were estimated by using PEO for calibration.

Determination of NH_2 functions of $PTFALL_{30}$ by ^{19}F NMR. ^1H NMR confirms the ratio between *n*-hexylamine end and polymerization degree (see Experimental Section - ^1H NMR). This is a required condition but not sufficient to conclude that chains are linear, initiated by *n*-hexylamine and ended by a primary amino function. A titration of amino end is required. To quantify the amino end, we have measured out the ratio of $–NH_2$ functions by a method developed by Macosko *et al*. and based on ^{19}F NMR.[41] This method employs a fluorinated aromatic aldehyde, the 3,5-bis(trifluoromethyl)benzaldehyde (BTBFA), which

reaction with primary amine gives an imine. The two components have distinct ^{19}F resonances. Quantification of the reaction is done by comparison with an internal standard, the trifluorotoluene (TFT). This method is precise and powerful due to the high definition of ^{19}F NMR and the quantitative reaction between molecular aldehyde and primary amined oligomers.

In the present study, the solvent utilized was a mixture of DMSO-d6 and DMF-d7. This solvent combination avoids overlapping of imine's and aldehyde's ^{19}F NMR contributions, which occurs when DMF-d7 is used as solvent. Figure 4-4 presents an expansion of the ^{19}F NMR spectrum recorded after the reaction between BTBFA and $PTFALL_{30}$. Three main contributions are observed: the TFT reference (δ = 56.56 ppm), the unreacted BTBFA (δ = 56.27 ppm and δ = 56.23 ppm) and the imine (a singulet δ = 56.13 ppm and a multiplet centred at δ = 56.21 ppm). It is worth noting that an additional ^{19}F NMR contribution, corresponding to the trifluoroacetyl group of the TFA-L-Lysine, appears as a complex multiplet at δ = 43 ppm. The BTBFA contribution presents a small amount of side product (singulet centred at δ = 56.23 ppm), which comes in a proportion of 5.8% with BTBFA. It corresponds to 3,5-bis(trifluoromethyl)benzyl alcohol derivative, as BTBFA is obtained by oxidation of this compound.

Figure 4-4 presents two distinct contributions for the benzylic $-CF_3$ of the imine. The first, centred around 57.02 ppm, is a well-defined singulet. The second one, between 57.06 and 57.12, is a complex multiplet. We ascribed these two contributions to a separation of the $-CF_3$ moieties between two distinct states. In the first state, the corresponding $-CF_3$ groups are free to rotate around the C-CF_3 axis, leading to an equivalence of every C-F bonds showed in a symmetric singulet. The second contribution may correspond to $-CF_3$ groups whose rotation is hindered by steric obstruction and by formation of domains of fluorinated groups. In that case, C-F bonds are not equivalent. The total amount of BTBFA consumed corresponds to the integration of the imine contribution. $I_{imine} / (I_{imine} + I_{BTBFA}) = 0.216$ which is equal to the ratio $n_{PTFALL30}/n_{BTBFA} = 0.216$ (see experimental part for details). Therefore 100% of primary amino functionality is obtained for $PTFALL_{30}$. We can conclude that our polypeptide chains are monofunctional in primary amino group.

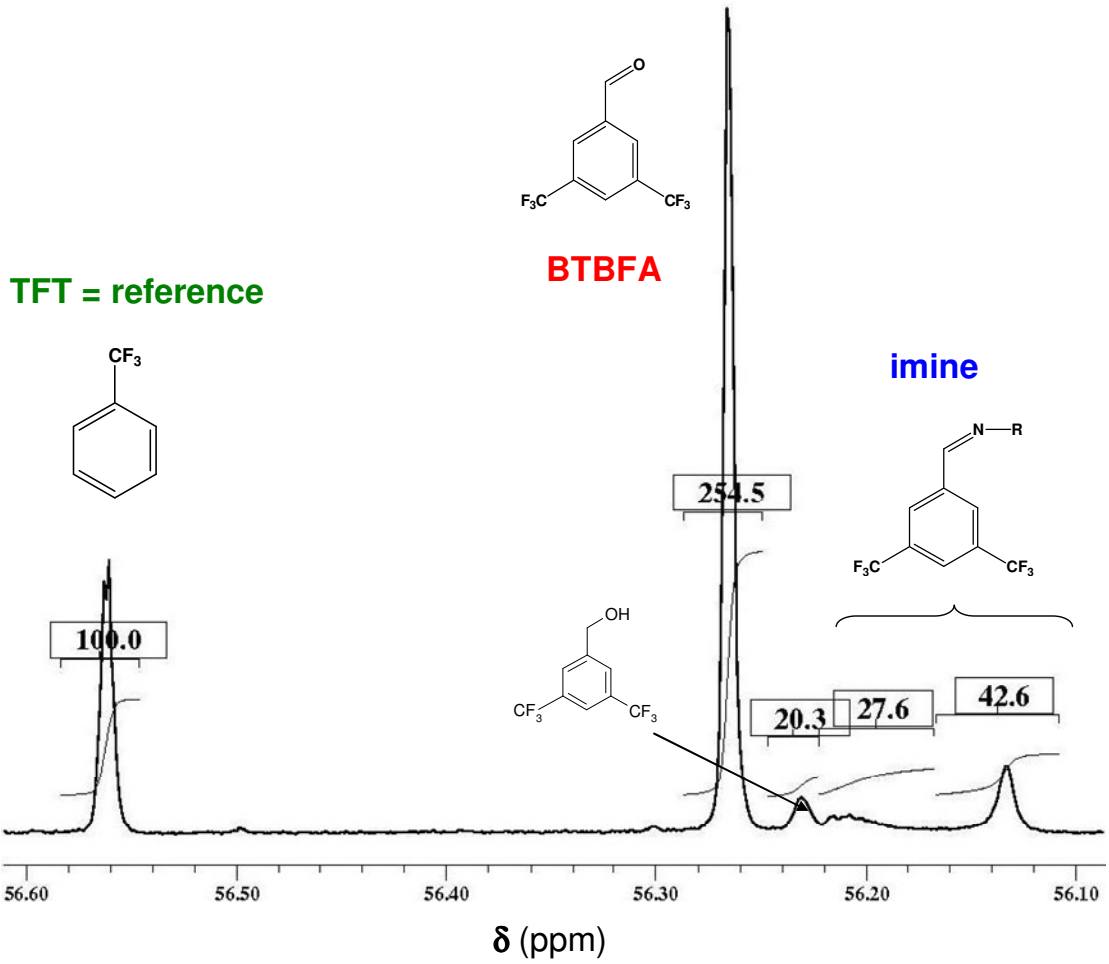

Figure 4-4. Dosage of –NH2 extremity of PTFALL$_{30}$. A fluorated aldehyde is transformed into the corresponding imine after reaction with the primary amino end-function of the polypeptide. RMN ^{19}F recorded in DMSO-D6/DMF-D7 (2/1 weight).

Grafting. The grafting of PTFALL$_{30}$ on a random copolymer containing styrene and maleic anhydride is presented in the following. Between small molecules, a primary amine reacts quantitatively on a maleic anhydride and forms an amide function connecting the two entities.[31] We wanted to perform this reaction between PTFALL$_{30}$ chain and functionalised PS. According to ^{1}H NMR, the backbone is made of 8 mol% of maleic anhydride randomly distributed onto the styrene backbone.

Before performing polypeptide grafting, the efficiency of the chemical reaction was checked between a mono-amino terminated poly(oxyethylene) (PEO) and the PS backbone. The reaction was performed at room temperature by mixing two solutions containing the two components in DMF. The reaction was followed by SEC by following the loss of PEO signal. We tested the grafting of PEO until the saturation of these 8 mol% of maleic anhydride

functions. It appeared that a fast and quantitative grafting of PEO chains is observed until the 8 mol% anhydride are grafted, which is done in less than 90 minutes.

We performed the grafting of $PTFALL_{30}$ chains by mixing DMF solutions of the backbone and the polypeptide. These solutions are adjusted to reach a rate of grafting of 22 % of anhydride functions, which correspond to 1.8 mol% grafting with respect to the backbone monomers. During the reaction, sampling of the reactive medium was performed diluted into DMF/LiBr 0.1M and directly introduced into the columns to follow the reaction progress by SEC.

Figure 4-5 and Figure 4-6 represent respectively the refractometric and the viscosimetric signals of the reaction mixture as a function of time. Both signals are composed of two well-separated contributions. At elution volumes between 24.5 ml and 27 ml, the signal corresponds to the free $PTFALL_{30}$ chains. Between 20 ml and 24.5 ml, the backbone and the grafted copolymer signals are superimposed. During the progress of the grafting, a decrease of $PTFALL_{30}$ signal was correlated with an increase of the graft copolymer signal. The shift of this last signal toward lower elution volume corresponds to an increase of the molar mass and viscosity of the graft copolymer. SEC shows distinctly the formation of the graft copolymer correlated with a recession for the signal of free polypeptide chains.

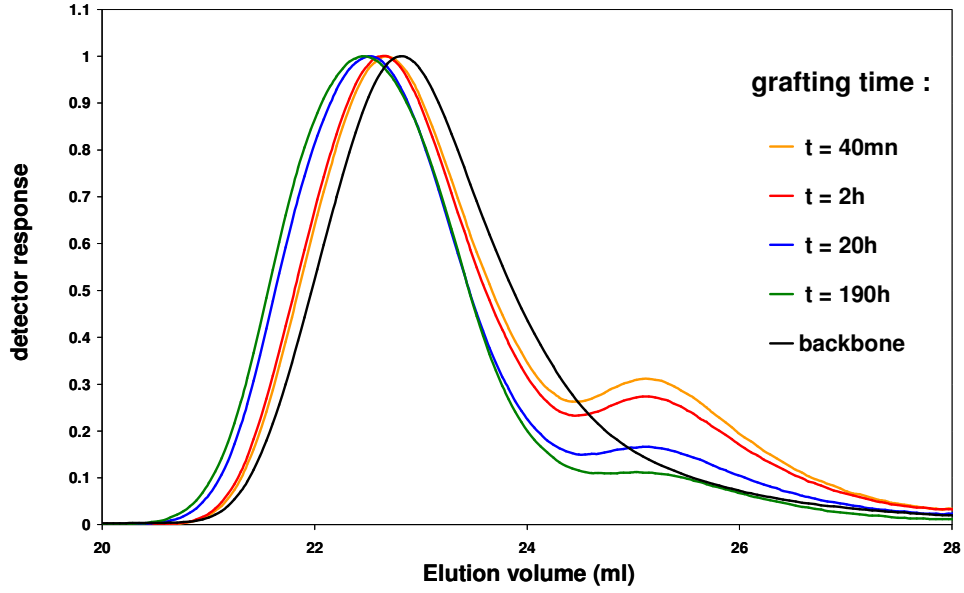

Figure 4-5. Refractometric response of $PTFALL_{30}$ grafting reaction as a function of time. In black, refractometric response of the PS backbone

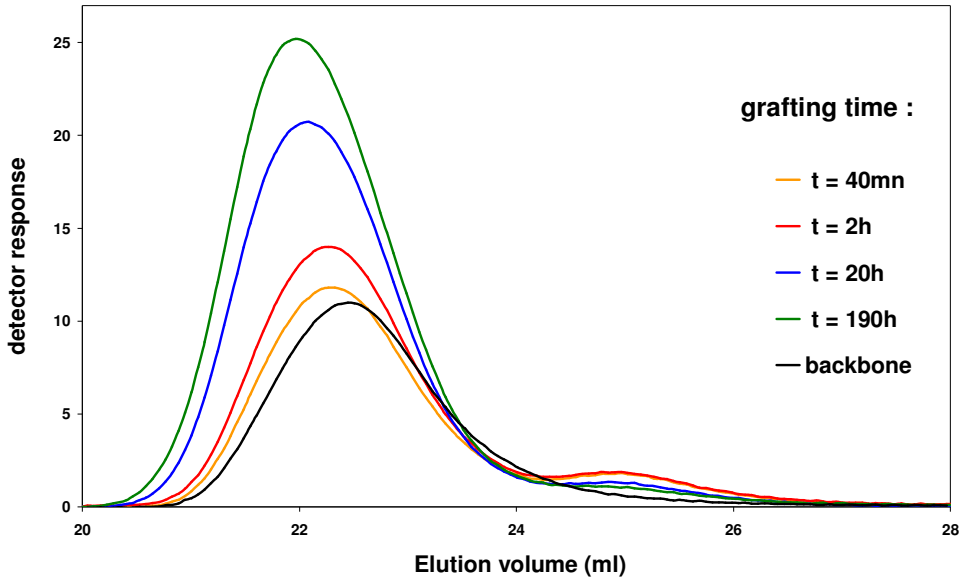

Figure 4-6. Viscosimetric response of the PTFALL$_{30}$ grafting reaction as a function of time. In black, viscosimetric response of the PS backbone

Figure 4-7 represents the surface of the viscosimetric signal of the graft copolymer as a function of time. It is noteworthy that the characteristic time for the PTFALL$_{30}$ grafting was quite slow compared to the same reaction with mono aminated PEO. Grafting rate is fast in the first 24h as can be attested by the twofold increase of the surface. After one week, the grafting rate is really slow. At long time, a weak signal for the free PTFALL$_{30}$ is still detected despite the fact that, as ^{19}F NMR showed, all the PTFALL$_{30}$ chains are –NH$_2$ functionalized and the maleic anhydride are in large excess. One explanation may be given by the difficulty for the polypeptide chains to approach maleic anhydride moieties due to steric hindrance. A second explanation is related on the reactivity of the chain end primary amino groups that depends on the nature of the polypeptide chains. The amino end group of the polypeptide may be involved in H-bonding with the amide functions of the polypeptide backbone which lowers the -NH$_2$ reactivity.

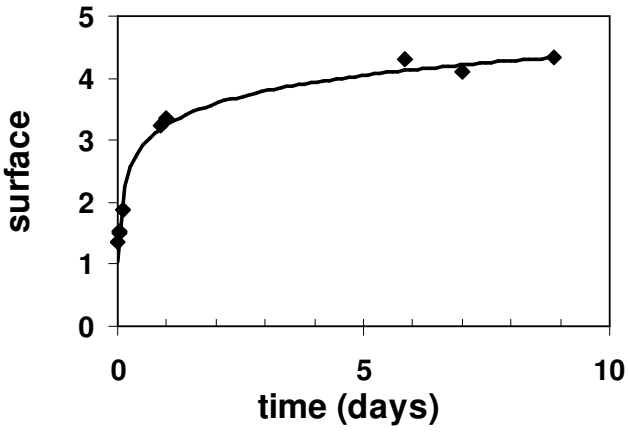

Figure 4-7. Kinetic of $PTFALL_{30}$ grafting. The surface of the viscosimetric signal corresponding to the graft (Elution volume = [20ml; 24ml]) as a function of time.

At the end of the grafting reaction, the PS-g-$PTFALL_{30}$ copolymer was purified by precipitation in water and subsequent removal of residual solvent by vacuum treatment. No solvent was found for a selective precipitation of the graft copolymer in order to remove the free $PTFALL_{30}$ chains. Thereof, the graft copolymer contained a small amount of free polypeptide.

Secondary structure of PTFALL. FTIR is powerful technique to determine the secondary structure of polypeptides. Amide I and Amide II bands provide qualitative information whether the polypeptide backbone is under α-helix, β-strand or coil conformation.[43] Figure 4-8 gives the FTIR spectra of PS-co-Anh, $PTFALL_{30}$ and PS-g-$PTFALL_{30}$ obtained by solvent casting from THF solutions.

$PTFALL_{30}$ (red curve) mainly adopts α-helix conformation with large contributions centred at 1650 cm^{-1} (amide I) and 1545 cm^{-1} (amide II). Small amounts of β-strand (1625 cm^{-1}) and coil (1670 cm^{-1}) are noticeable. α-helix is the secondary structure mainly adopted by N-substituted poly(L-Lysine) of this length.[18] β-strand contribution may come from small chains due to polydispersity, or defects in the helix structure.[44] Coil contribution is probably due to chain ends contributions, which are not negligible in our case, due to small length of the chains (DP = 30).

After grafting on PS-co-Anh, secondary structure of PTFALL is still mainly under α-helix with a rearrangement toward less β-strand and slightly more of coil (black curve). The coil contribution is probably enhanced due to the influence of the PS backbone, at the PS-PTFALL junction.

It is worth noting that FTIR spectra clearly show the decreasing of the maleic anhydride contributions (1780 cm^{-1} 1860 cm^{-1}) after the grafting (PS absorbance, blue curve, was fitted with the contributions of the typical PS vibrations at 1495 and 1455 cm^{-1}).

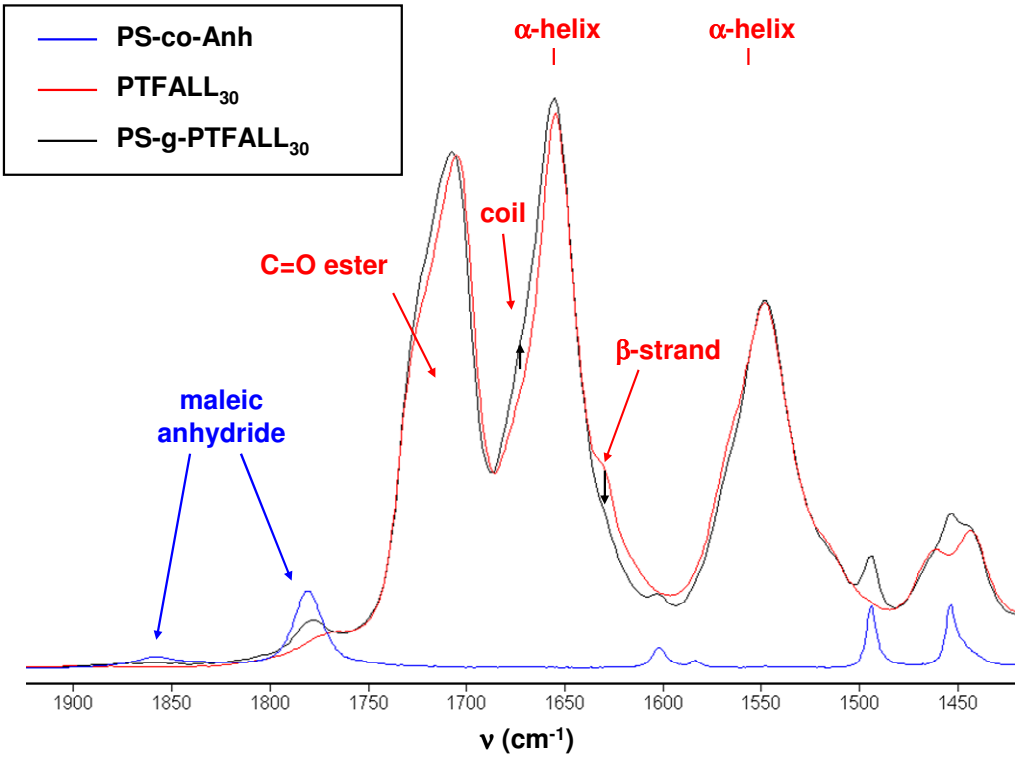

Figure 4-8. FTIR absorbance of solvent cast from THF solution of PS-co-Anh, $PTFALL_{30}$ and PS-g- $PTFALL_{30}$.

Structuration in solid phase. The solid state structuration of PS-g-PTFALL copolymer was then studied by mean of TEM. Samples were prepared by solvent casting from THF. The resulted copolymer films were annealed at 140°C under vacuum for 48h. Selective staining of the PS was successfully done using vapour of an aqueous solution of RuO_4. For the PS-g-PTFALL copolymer, only the styrene moieties were stained and lead to a good contrast for TEM observation.

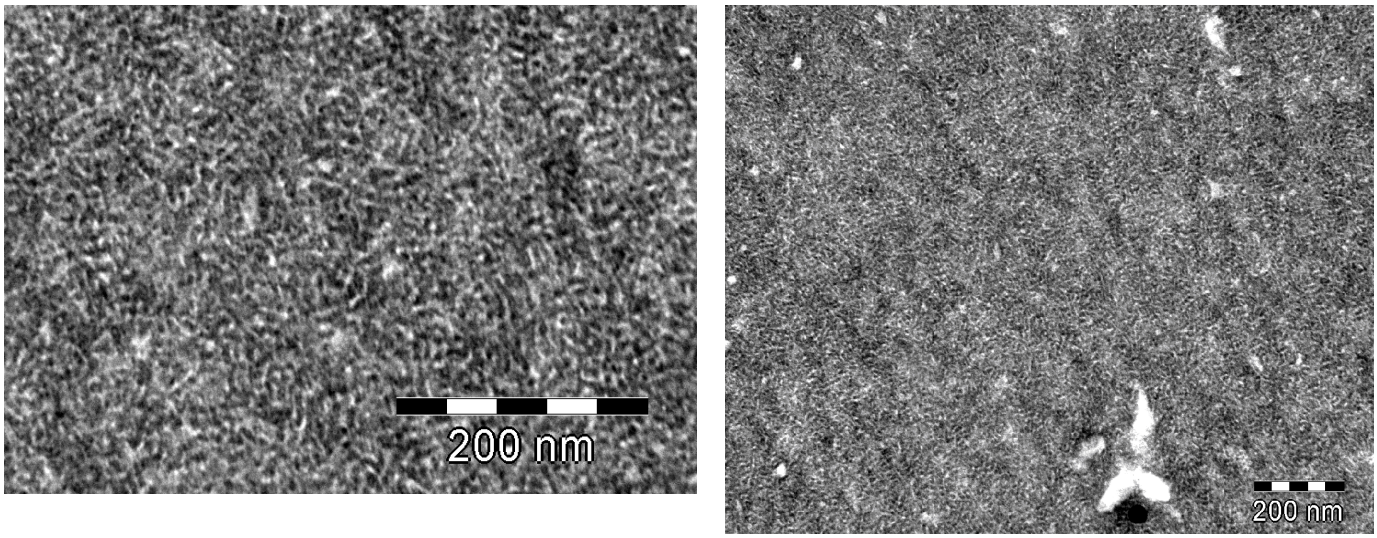

Figure 4-9 - TEM photographs of PS-g-PTFALL. Co-continuous morphology with interconnected polystyrene (in black) and PTFALL domains (in white).

Figure 4-9 shows a typical pattern of the morphology of PS-g-PTFALL. Photos show an apparent microphase separation with well defined domains of PS (black) and PTFALL (white). This nanostructured matrix coexists with a macroseparated of non-stained nodules. These white domains may correspond to aggregated free PTFALL chains. They are not stained by RuO_4 as expected if they are made of pure $PTFALL_{30}$. As expected, the fraction of these nodules is small in agreement with the conclusions from the SEC study which indicates that most of PTFALL chains are actually grafted.

The matrix's morphology is very thin and looks co-continuous with interconnected domains of polystyrene and polypeptide. In this morphology, the thickness of PTFALL domains is very small (~ 5 nm). This thickness is in agreement with the expected size (4.5 nm) of $PTFALL_{30}$ grafts under α-helix conformation. Similar morphology was shown for rod-coil block copolymer.[45]

Conclusion

We demonstrate here the synthesis of PTFALL chains by the NCA polymerization and their subsequent grafting "onto" a functionalized PS backbone. The polymerization of the polypeptide chains and the grafting are followed by SEC. The polypeptide chains are linked to the backbone by their N-terminus, without help of coupling agent or activation. Primary amino functions, naturally occurred at the N-terminus of polypeptide, are linked to maleic anhydride functions onto the backbone, and lead to the straightforward synthesis of the hybrid graft copolymer. We confirmed here the living character of the ε-trifluoroacetyl-L-Lysine NCA polymerization, by means of SEC. An additional study, led by ^{19}F NMR confirms that 100% of PTFALL chains is NH_2 terminated. Nevertheless, the grafting is not quantitative; a residual contribution of free PTFALL is still detectable after one week of reaction. In solid state, the graft copolymer revealed by TEM a thin nanostructuration which seems to be co-continuous with interconnected domains of PS and PTFALL. Thickness of polypeptide domain is in agreement with a monolayer of α-helix.

Further studies are needed to characterize properties of these copolymers after hydrolysis of PTFALL grafts into poly(L-Lysine). This particular homopolypeptide is interesting as it undergoes secondary structure transitions upon variation of pH and temperature. Such an amphiphilic graft copolymer would show interesting stimulable behaviour.

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II - Compléments de l'article II

Afin de ne pas surcharger l'article précédent, les expériences secondaires et les spectres RMN sont rassemblés ici en complément.

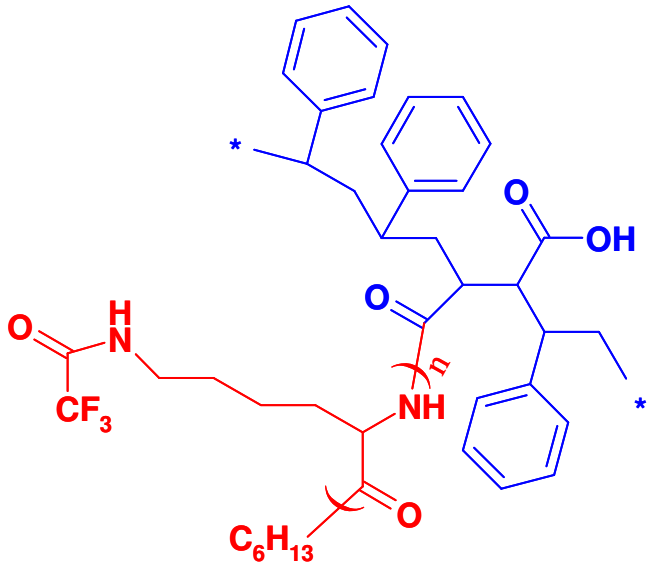

Figure 4-10. Schématisation de PS-g-PTFALL.

II.1 - RMN ^{1}H de PTFALL$_{30}$

La RMN ^{1}H permet de déterminer le degré moyen de polymérisation des chaînons PTFALL. Dans cette étude, la polymérisation des NCA TFALL a été amorcée par la *n*-hexylamine et réalisée dans le DMF. En prenant comme hypothèse que la *n*-hexylamine est le seul amorceur de la polymérisation, chaque chaîne possède à l'extrémité C-terminale un groupement hexylamine. Cette hypothèse peut être considérée comme raisonnable, étant donné que le DMF a été distillé sur CaH_2, afin d'éliminer les amorceurs potentiels tels que l'eau ou la *N*,*N*-diméthylamine.

Le spectre RMN ^{1}H de PTFALL$_{30}$ réalisé dans le DMF d7 est donné Figure 4-11. Pour déterminer ***n***, le degré de polymérisation moyen de la PTFALL, le rapport entre les contributions de la *n*-hexylamine et des monomères TFALL est déterminé.

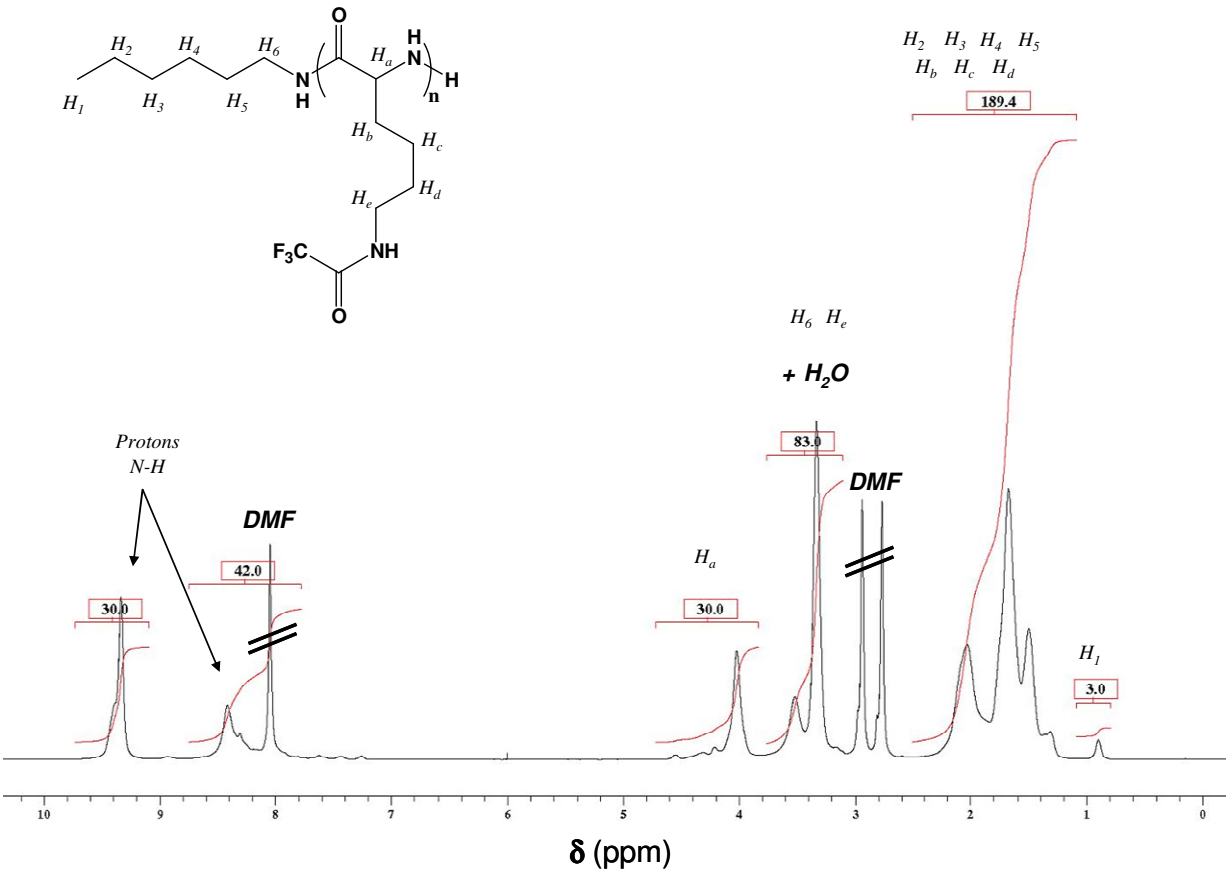

Figure 4-11. RMN ^{1}H de PTFALL$_{30}$ dans DMF D7.

H_1, correspondant au groupement CH_3 de la *n*-hexylamine. Il intègre à δ = 0.9 ppm pour une valeur arbitraire de 3. Chaque proton de l'extrémité hexylamine intègre ici pour une valeur de 1. H_a correspond au proton du squelette peptidique. Il intègre pour une valeur de 30. Soit ***n*** = 30 pour une première estimation. Le massif intégrant entre δ = 1.2 et 2.4 ppm, correspond aux protons H_2, H_3, H_4, H_5 et H_b, H_c, H_d, soient 6 protons par monomère de TFALL et 8 protons appartenant à la *n*-hexylamine. Ce massif a une intégration de 189.4. D'où une deuxième estimation de ***n*** :

$$\boldsymbol{n} = \frac{189.4 - 8}{6} \sim 30.2$$

Aux approximations d'intégration et de ligne de base près, ***n*** est donc de l'ordre de 30.

Les chaînes PTFALL ont été purifiées par précipitation dans H_2O suivit d'un séchage sous vide de 24 h. Néanmoins, la RMN met en évidence des traces d'eau, en plus de celles de DMF, provenant elles du milieu de polymérisation mais aussi contenues dans le DMF deutérié.

II.2 - Dosage des fonctions amines terminales

Une expérience de dosage des fonctions amines primaires de l'extrémité N-terminale des chaînes de $PTFALL_{30}$ a été présentée dans l'article II. Le spectre RMN ^{19}F de la solution de BTBFA et de la référence TFT dans DMF D7 est donné ci-dessous. La position relative du pic correspondant à l'alcool 3,5-(bistrifluoromethyl)benzylique a changé. Cela s'explique par le fait que la réaction de dosage de PTFALL se fait dans un mélange DMF D7 - DMSO D6. La même expérience, juste dans le DMF D7 montre un épaulement des contributions de l'imine et de la BTBFA. A cause de l'introduction du DMSO D6, le déplacement chimique de l'alcool 3,5-(bistrifluoromethyl)benzylique est modifié.

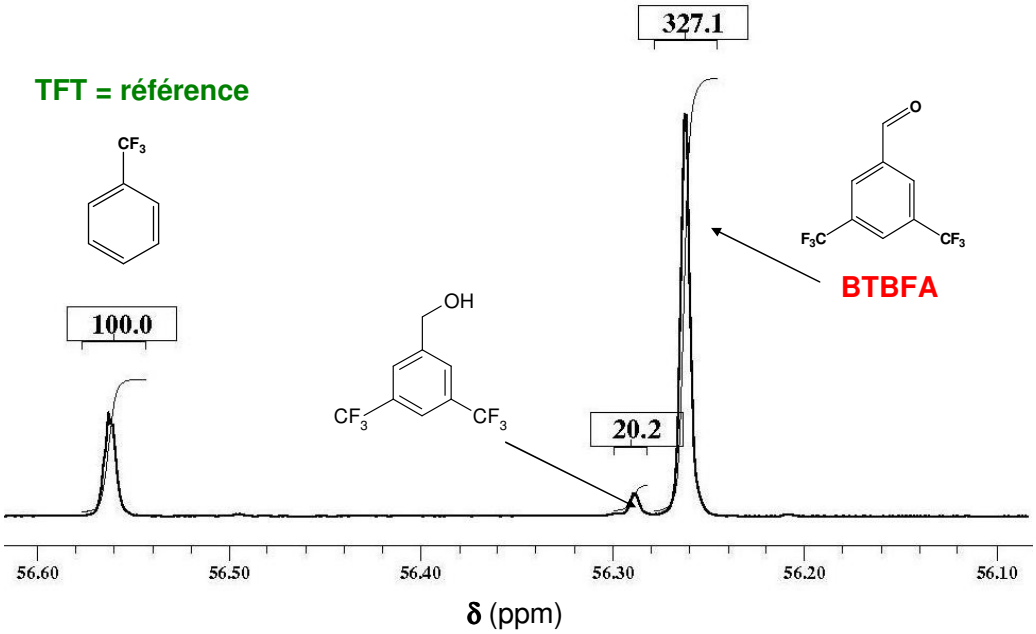

Figure 4-12 – Spectre RMN ^{19}F dans DMF D7, de la solution de référence (BTBFA et BTF) utilisée pour le dosage des fonctions $-NH_2$ de l'extrémité N-terminale de $PTFALL_{30}$.

II.3 - Détermination du pourcentage d'anhydride maléique du squelette PS

La RMN ^{1}H permet de quantifier la proportion de fonction anhydride maléique au sein du squelette. Un spectre RMN ^{1}H a été réalisé dans $CDCl_3$, solvant dans lequel le copolymère est très bien solubilisé. La Figure 4-13 présente le spectre ainsi que l'attribution des intégrations pour chaque proton.

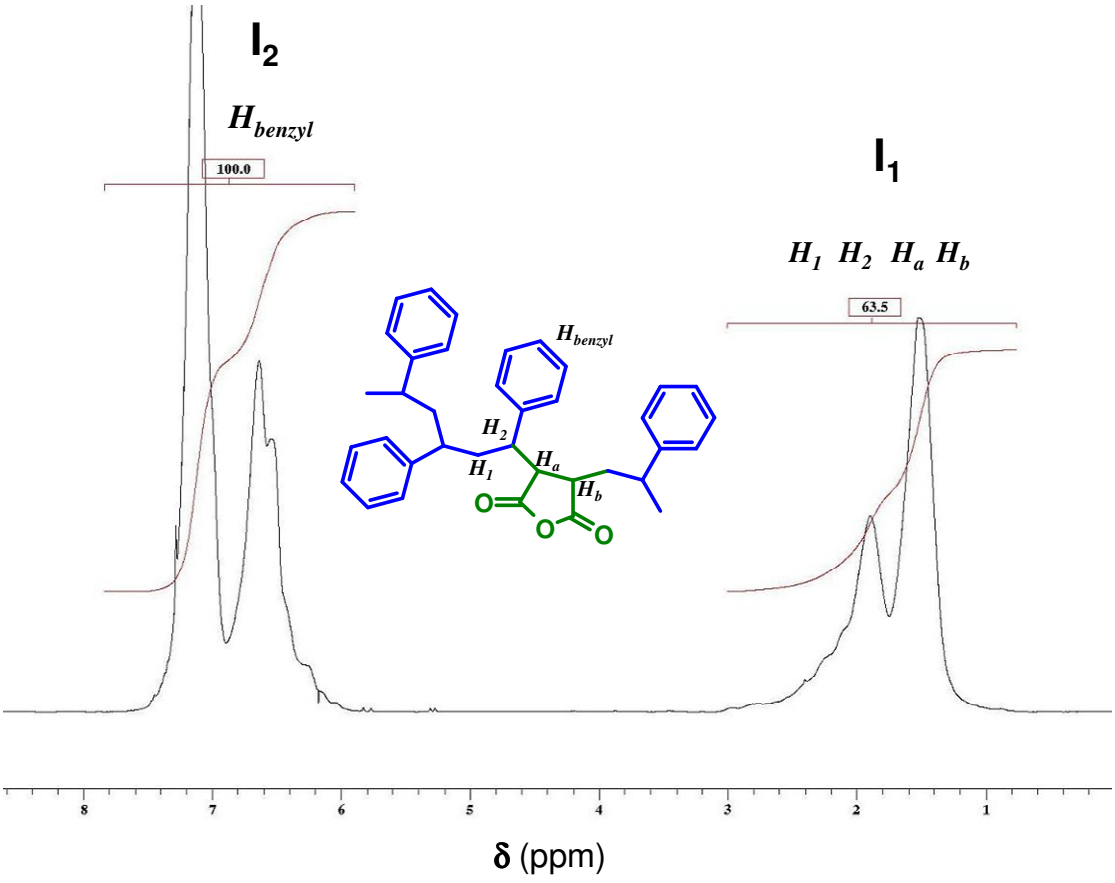

Figure 4-13 – Spectre RMN ^{1}H du squelette poly(styrène-co-anhydride maléique) dans $CDCl_3$.

L'intensité relative d'un proton styrènique se déduit de la valeur de l'intégration du pic entre 6 et 7.6 ppm. $I_{H\ styrène} = I_2 / 5 = 20$. L'intensité relative d'un proton des fonctions anhydrides maléiques se déduit de l'intégration du pic entre 0.7 et 3.1 ppm. $I_{H\ anhydride\ maléique} = (I_1 - 3*I_{H\text{-}styrène}) / 2 = 1.75$.

La proportion de fonction anhydride maléique est donc égale à :

$$I_{H\ maleic\ anhydride} / (I_{H\ maleic\ anhydride} + I_{H\ styrene}) = 8\ \%$$

II.4 - Greffage de PEO-NH_2 sur PS-co-Anh

II.4.1 - Commentaires préliminaires sur la SEC

Dans ce chapitre, le suivi des réactions de greffage est réalisé par SEC. Un échantillon de la solution de greffage est prélevé et dilué dans l'éluant SEC (DMF/LiBr 0.1M), afin de quantifier l'avancement de la réaction. Ceci permet de travailler à des concentrations de l'ordre de 0.5 à 0.6 % en masse.

Les réactions de greffage sont réalisées dans le DMF pur : cette dilution entraîne une baisse de la concentration en LiBr dans l'échantillon en analyse, par rapport à l'éluant. Cela se traduit par une contribution négative, visible sur les signaux réfractométriques et viscosimétriques. La Figure 4-14 présente le signal obtenu par le réfractomètre, pour une solution de DMF à 6.2 % (diluée environ 16 fois) dans l'éluant. Le défaut de LiBr est mis en évidence par le pic négatif centré à 30.5 mL.

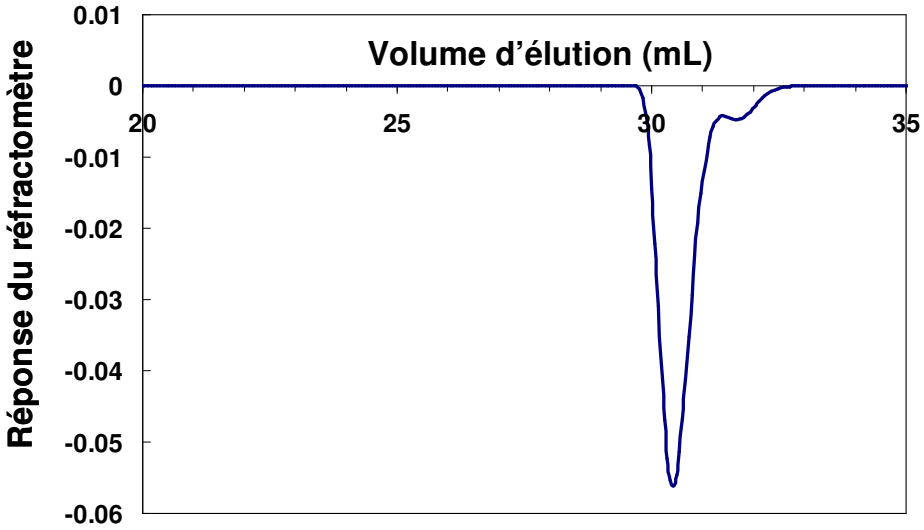

Figure 4-14. Signal du réfractomètre du DMF séché sur CaH_2 à 6 % dans l'éluent de la SEC DMF/LiBr (0.1 $mol.L^{-1}$).

Une deuxième contribution négative, d'intensité plus faible et centrée vers 31.7 mL s'explique par la présence de gaz dans l'échantillon a étudié. Le DMF utilisé comme éluant est dégazé, contrairement à celui utilisé lors des réactions de polymérisation ou de greffage.

II.4.2 - Réaction de greffage de PEO-NH_2

Afin de tester la réactivité des fonctions anhydrides maléiques du polystyrène, des tests de greffage de chaînes courtes monofonctionnelles PEO-NH_2 (M = 1000 g.mol^{-1}) ont été réalisés. Deux solutions de DMF, l'une contenant le squelette, l'autre le PEO-NH_2, sont préparées séparément et mélangées. La concentration en polymère est de l'ordre de 10 % dans le milieu réactif. Après 1h30 à température ambiante, un échantillon est prélevé et dilué dans DMF/LiBr (concentration finale ~ 0.6 % en masse) et analysé par SEC. La Figure 4-15 donne les réponses réfractomètriques du squelette seul (en vert), des chaînons PEO-NH_2 seuls (en rouge) et des expériences de greffages de PEO-NH_2 pour un équivalent de PEO-NH_2 par fonction anhydride maléique du squelette (mélange stœchiométrique en gris) et pour trois équivalents (en noir).

Les deux contributions négatives discutées ci-dessus sont confondues avec le signal des greffons PEO-NH_2 seuls (courbe rouge), empêchant une analyse quantitative des greffages.

Pour le mélange stœchiométrique (1 équivalent de PEO-NH_2 par fonction anhydride maléique du squelette), la contribution du copolymère greffé (courbe grise, 20 à 26 mL) a été translatée vers les grandes masses molaires par rapport au squelette seul (courbe verte). La réponse réfractométrique entre 28 et 33 mL de volume d'élution, correspondante aux chaînons libres de PEO-NH_2, est presque superposée à celle du squelette seul, confirmant que la quasi-totalité des chaînons PEO-NH_2 sont greffés. Dans l'expérience où 3 équivalents de PEO-NH_2 par fonction anhydride maléique du squelette ont été introduits (courbe noire), la contribution du greffé est sensiblement à la même place que pour le mélange stœchiométrique, et le PEO-NH2 en excès est retrouvé.

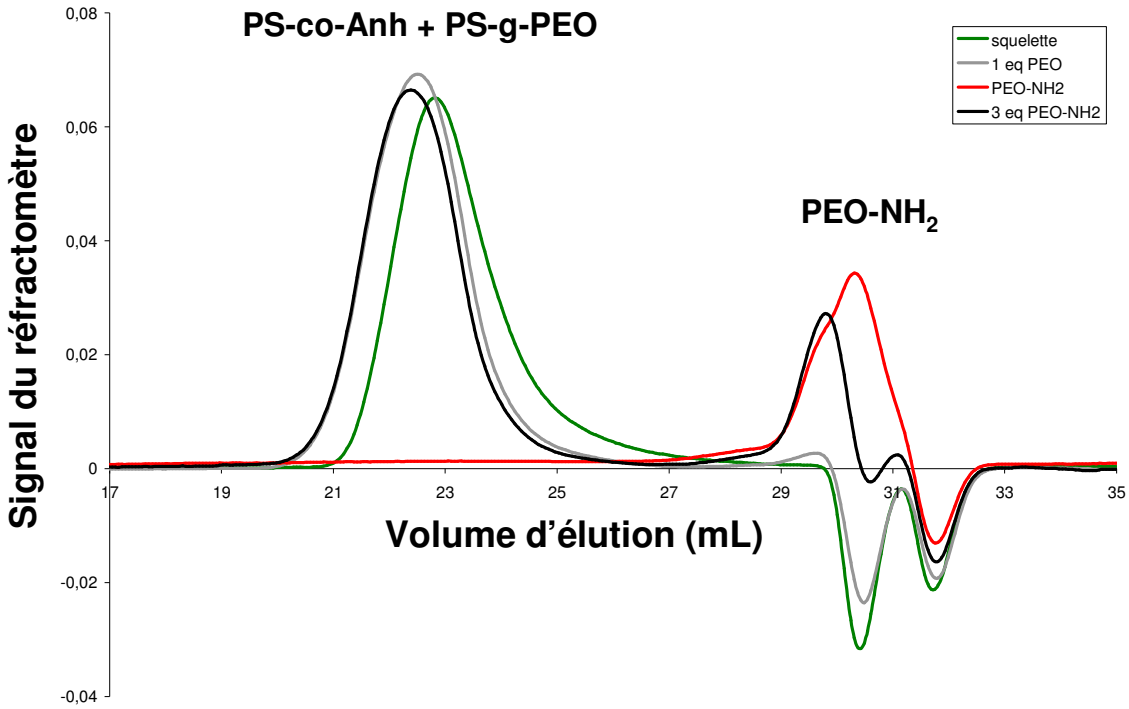

Figure 4-15. Chromatogrammes SEC. Signaux réfractomètriques de PEO-NH_2, du squelette PS-co-Anh, et des réactions de greffage de PEO-NH_2 sur le squelette.

Ces expériences confirment que le greffage de chaînons courts de PEO-NH_2 est quantitatif et rapide sur le squelette PS-co-Anh, confirmant par la même occasion la fonctionnalité de ce dernier.