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# Clickable PEG conjugate obtained by "clip" photochemistry: Synthesis and characterization by quantitative $^{19}$F NMR

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**ABSTRACT**

In this paper, we describe a grafting methodology associated to a quantitative $^{19}$F NMR method (qNMR) for the conjugation of small molecules on a PEG building block aimed at click chemistry applications in the domain of drug delivery systems. Acetylenic PEG (PEG-yne) was first derivatized with a fluorinated benzyl amine (TagF$_6$) by means of photografting of a trifluoromethylphenyl diazirine bifunctional linker (TPD-clip). The amount of TagF$_6$ grafted on PEG-yne was calculated by NMR using an internal standard (trifluoroethanol) and adjusting of the acquisition and processing parameters. NMR is used as a valuable alternative to the complex procedures often employed for the quantification of functionalities on biomaterials. The accuracy of the qNMR methodology was attested by controlling its linearity, the determination of limits of quantification and the percentage of recovery. A good assessment of the TagF$_6$ grafting rates was obtained after taking into account the inherent unspecific adsorption that occurs on materials. This versatile methodology that combines simple chemistry and a common analytical tool was, in a second time, applied to the preparation of a PEG conjugated with a RGD (Arg-Gly-Asp) peptidomimetic in a controlled manner.

**Keywords :** PEG ; RGD peptidomimetic ; Quantitative NMR ; Diazirine ; Conjugation.

## 1. Introduction

Progress made with biomaterials nowadays requires tailored interactions of the material with its biological environment [1]. Chemically speaking, it means that the interface should display molecules that will produce the desired biological effects. For instance if one wants to target an injured tissue with a drug delivery system (DDS), an imaging agent, or to direct cell spreading for tissue regeneration, it is worth grafting the surface of the devices with ligands that can bind to cellular receptors [2-4]. Usually only a small number of unimers are functionalized with the bioactive molecules in regards of the overall unmodified ones that constitute the core of the material. This makes difficult to accurately identify the biomolecules present and to assess their functionalization rate (i.e. the number of modified monomers versus the total number of monomers). This determination is often based on rather sophisticated and expensive techniques (e.g. X-ray Photoelectron Spectroscopy (XPS), Secondary Ion Mass Spectrometry with time of flight (tof-SIMS), Isothermal Titration Calorimetry (ITC)) or sacrificial processes that preclude the use of the tested material (e.g. chemical derivatization for spectrophotometric assay, radiochemical probing for Liquid Scintillation Counting (LSC)). In addition, good characterization of functionalized materials would require the use of several techniques since they give complementary information [5-7].

Therefore there is a need to develop direct analytical tools that can lead to fast and reliable assessment of the functionality with straightforward sample preparation. For this reason we chose NMR spectroscopy which is now a routine analytical technique with moderate costs. As peak integrations are directly proportional to the number of nuclei involved in the signal, it means that quantitative measurements can be performed under appropriate conditions of recording and processing. Although poorly exemplified for the determination of functionalities on polymers [8], quantitative NMR (qNMR) is a well established technique in pharmaceutical science [9,10].

Among the polymers used for biocompatibilization, polyethylene glycol (PEG) has become a gold standard [11,12]. Typical medical applications of PEG building blocks require complex heterotelechelic PEGs synthesized through tricky multi-step procedures. To overcome these difficulties we have developed a smooth derivatization methodology, the so-called "clip photochemistry", based on the photografting of bifunctional photosensitive linkers in order to introduce activated esters along polymer backbones that can thereafter react with any compound of interest bearing primary amine functions. Photolinkers have been used intensively in photoaffinity labeling of proteins and in surface chemistry for the immobilization of small molecules and biocompatible layers on materials [13,14]. To the best of our knowledge there are very few applications of these reactive species as a convenient way to introduce functionalities along unreactive macromolecular chains (i.e. in the bulk of a material instead of its surface) [15,16]. In previous works we were able to introduce, in a controlled manner, molecular probes (labeled amino acid, fluorinated probe) or biologically active molecules (peptidomimetics, peptides and carbohydrates) on several soft, inert materials, with aryl azide photolinkers [17-19].

In the present study we illustrate how to adapt this strategy to a highly sensitive compound such as telechelic PEG with an alkyne function (MeO-PEG-yne). As above mentioned we have also developed a simple method, based on $^{19}F$ NMR, to assess the functionalization rate of an α-methoxy-ω-alkyne-PEG (MeO-PEG-yne) derivatized by means of our "clip photochemistry" process. The proof of concept was established by the conjugation of a fluorinated probe ($TagF_6$) and thereafter successfully applied to the conjugation of a peptidomimetic of the Arg-Gly-Asp (RGDp) sequence. This ligand was designed and synthesized in our laboratory to target $\alpha_v\beta_3$ integrin receptors and was exemplified in several projects for the targeting of DDS or imaging agent [20-22]. The building blocks obtained, i.e. $TagF_6$ or RGDp functionalized MeO-PEG-yne, can be engaged by click chemistry in the synthesis of a new sensitive terpolymer [23] used for the formulation of biodegradable DDS [24].

## 2. Results and discussion

### *2.1. Preparation of NHS clipped α-methoxy-ω-alkyne-PEG*

We have prepared versatile, orthogonally substituted PEG building blocks bearing one function for the PEGylation of different substrates and another one for the grafting of various biologically active compounds. Our strategy makes use of a home-expertise, namely the photografting of "molecular clips" for the introduction of molecules of interest via the substitution of an activated ester, namely the N-hydroxysuccinimidyl ester (NHS). As another anchoring point we chose to introduce an alkyne function since it opens the door to several ligation reactions via the click chemistry family (copper(I)-catalyzed azide alkyne cycloaddition (CuAAC), thiol-yne, copper free cycloadditions, etc.) [23,25].

Considering the chemical functions we want to introduce, alkyne would be less sensitive than NHS ester or biologically active compounds. Consequently we first synthesized the α-methoxy-ω-alkyne-PEG (MeO-PEG-yne) from a commercial α-methoxy-ω-hydroxyl-PEG (MeO-PEG-OH) by esterification of the hydroxyl end with pentynoic acid via a standard carbodiimide coupling protocol. The next step was to introduce, along the MeO-PEG-yne chain, reactive ester functions that can thereafter be derivatized with amine compounds. We have already achieved in our laboratory such kind of functionalization on sensitive copolymers with the bifunctional photo-activable linker, O-succinimidyl-4-(p-azido-phenyl)butanoate ($ArN_3$ clip (2), Fig. 1), which under UV irradiation at 250 nm leads to random insertion in C-H bonds of photo-generated nitrene. This method, which we called "clip photochemistry", is easily applied on MeO-PEG-OH longer than 4 kDa since these polymers are almost insensitive to UV light and have a rigid structure due to a partial crystallinity that facilitates the insertion of triplet nitrene species [17,26]. In the present study we worked on smaller amorphous PEG with an alkyne function that absorbs light in the same UV range as the aryl azide (2) (Fig. S1, supplementary data). Moreover nitrene generated from (2) at room temperature leads to reactive di-radical triplet that might react on triple bonds. Consequently the use of triplet nitrene as reactive intermediate was not conceivable. 3-Phenyl-3-(trifluoromethyl)diazirines (TPD) are well-known linkers for photoaffinity labeling of proteins as well as for photoimmobilization on materials surface [13,14]. Under UV irradiation at 350 nm the TPD function gives a reactive carbene that leads to the desired insertion in C-H bonds [15,27] (Scheme 1) while being tolerant towards triple bond [28,29]. A recent study has established why, the withdrawing effect of the $CF_3$ group, stabilizes the singlet state of the photogenerated carbene [30]. This work had confirmed numerous experimental observations that $CF_3$ carbenes are more reactive than nitrenes [14,31 ], and that they insert into C-H bonds in a concerted mechanism. In addition, at the required excitation wavelength, no absorption of the MeO-PEG-yne is observed (Fig. S1, supplementary data). We selected O-succinimidyl-4-(1-azi-2,2,2-trifluoroethyl) benzoate (TPD clip (**1**), Fig. 1), prepared according to Nassal [32] with modifications of his initial procedure in order to obtain a fast and efficient synthesis (Scheme S1, supplementary data).

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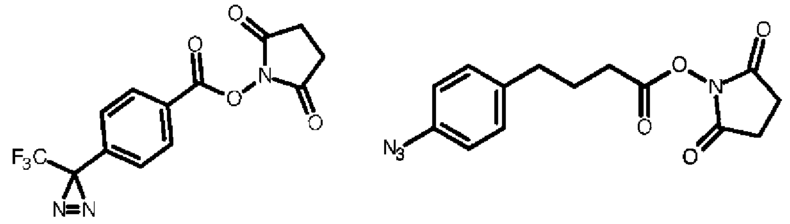
***Fig. 1.*** *Molecular clips.*



TPD clip (**1**) ArN$_3$ clip (2)

The photografting protocol was applied on MeO-PEG-yne using a clip (**1**) concentration of 65 µmol/g of polymer. We consciously chose such a low concentration for two reasons. Firstly because, based on previous work, we know that high grafting rates are not useful in regards of biological applications [22] and secondly because some literature results show that higher diazirine concentrations imply longer irradiation times with an increased occurrence of side products (most notably, persistent diazo species and homo-coupling products) [33].

The TPD clip (**1**) was mixed within the MeO-PEG-yne matrix via solvent casting and the neat mixture was irradiated with black light (350 nm) under argon atmosphere. The process was easily followed by $^{19}$F NMR spectroscopy and we observed a total disappearance of the peak at -64.77 ppm corresponding to the TPD function, after 75 min of irradiation (Fig. 2B), in comparison to the $^{19}$F NMR spectrum of both reagents before irradiation (Fig. 2A). The profile of the $^{19}$F NMR spectrum after irradiation presents several products and appears slightly different from other $^{19}$F NMR profiles found in the literature where two main fluorine products are shown [34]. This could be tentatively explained by the inherently more complex mixture of photoproducts formed in a solid matrix compared to the ones observed in solution [35].

Moreover the determination of products from carbenes insertion is not obvious and, to try to solve this question, we studied the grafting profile of the TPD clip (**1**) on a model compound of the MeO-PEG-Yne, i.e. 2-(2-methoxyethoxy)ethyl pent-4-ynoate. Two main products of grafting were identified by LC-MS which featured a preserved propargylic motif in NMR. This applied for a safe protocol towards the alkyne function (data not shown).

On the $^{1}$H NMR spectrum of the crude polymer mixture after irradiation, the NHS and acetylenic protons at 2.89 and 1.95 ppm, respectively are still visible (Fig. 3B) whereas the sharp peaks of the aromatic protons have become broad signals. This can be explained by a distribution of the insertion products along the PEG chain. We used the integrations of the protons as a guarantee that limited side reactions and almost no degradation occurred at the acetylenic site during the photoreaction process. Indeed, the integral ratio of the signal at 4.25 ppm (protons d in Fig. 3A) to the one at 2.89 ppm (NHS ester, protons i) remains constant (Fig. 3B), even after several washings (dissolution in DCM and precipitation in $iPr_2O$, three times) (Fig. 3C). Consequently we obtained MeO-PEG-yne bearing "clipped" NHS ester (MeO-PEG-yne-clip-NHS) which could be derivatized by compounds bearing a free amine function.

### *2.2. Grafting rates determination by quantitative $^{13}$F NMR spectroscopy ($^{19}$F qNMR)*

#### *2.2.1. Development of the qNMR methodology*

To assess the reactivity of NHS esters present along the PEG chain we chose a fluorinated probe, 3,5-bis-trifluoromethyl-benzylamine ($TagF_6$), which primary amine and $CF_3$ tag could be used as a models of RGD peptidomimetics ligands designed in our laboratory (cf. Scheme 1) [20,21]. Indeed the introduction of exogenous atoms (i.e. fluorine) is a convenient way to quantify the derivatization rates and we already used this probe in previous studies for quantification of derivatization rates by X-ray Photoelectron Spectroscopy (XPS) [36]. Although being a sensitive method, XPS only gives chemical composition of small spots on the surface of the sample. Moreover, only solids can be analyzed and the technique requires rigorous laboratory practices to avoid any contamination and the equipment is not routinely accessible (high vacuum technology).

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***Scheme 1.*** *"Clip" photochemistry to obtain NHS-ester functionalized PEG-yne (Me-PEG-yne-clip-NHS) followed by chemical derivatization with RGDp and TagF$_6$.*

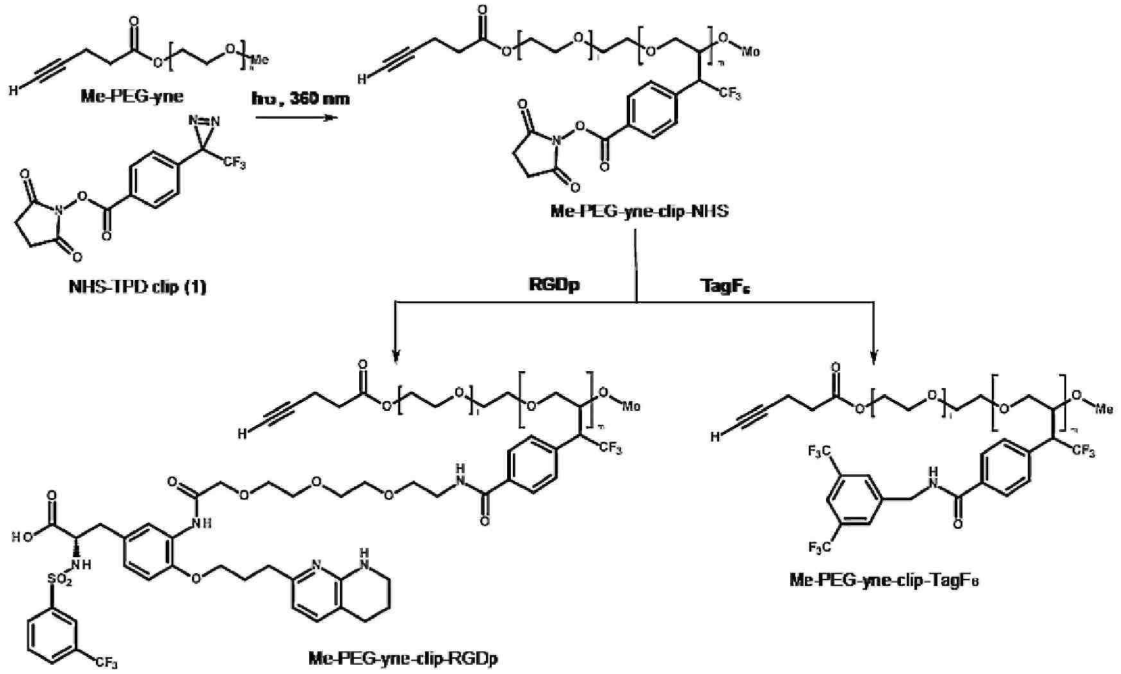


***Fig. 2.*** *ID $^{19}$F NMR (282.09 MHz, CDCl$_3$) spectra of PEG and the molecular clip at each step of the derivatization process;* (A) MeO-PEG-yne mixed with the molecular clip before irradiation; (B) crude MeO-PEG-yne-clip-NHS obtained after 75 min of irradiation at 350 nm; (C) MeO-PEG-yne-clip-TagF$_6$ (irradiated sample) with TFE (standard for quantification); (D) MeO-PEG-yne with TagF$_6$ (non irradiated sample) with TFE.

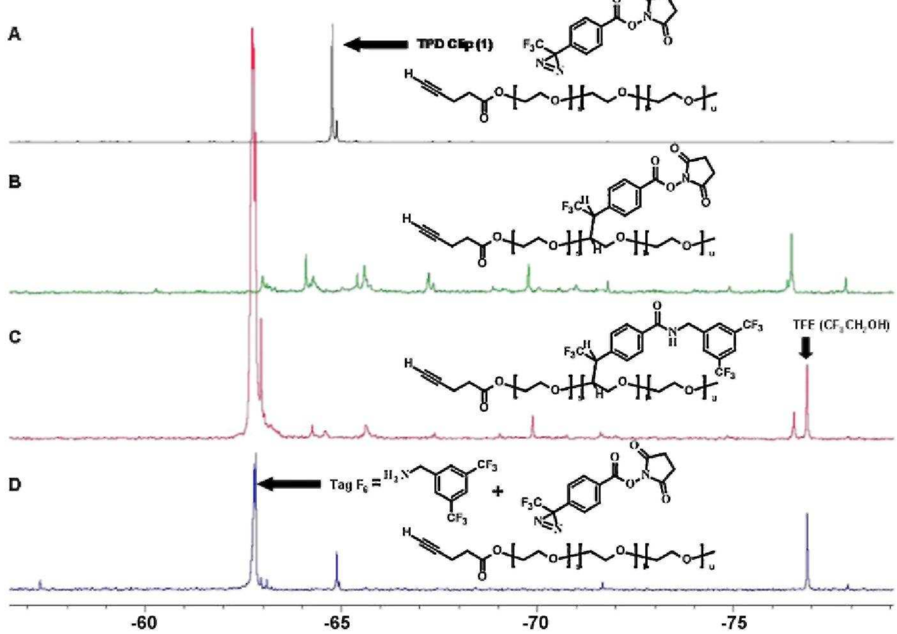

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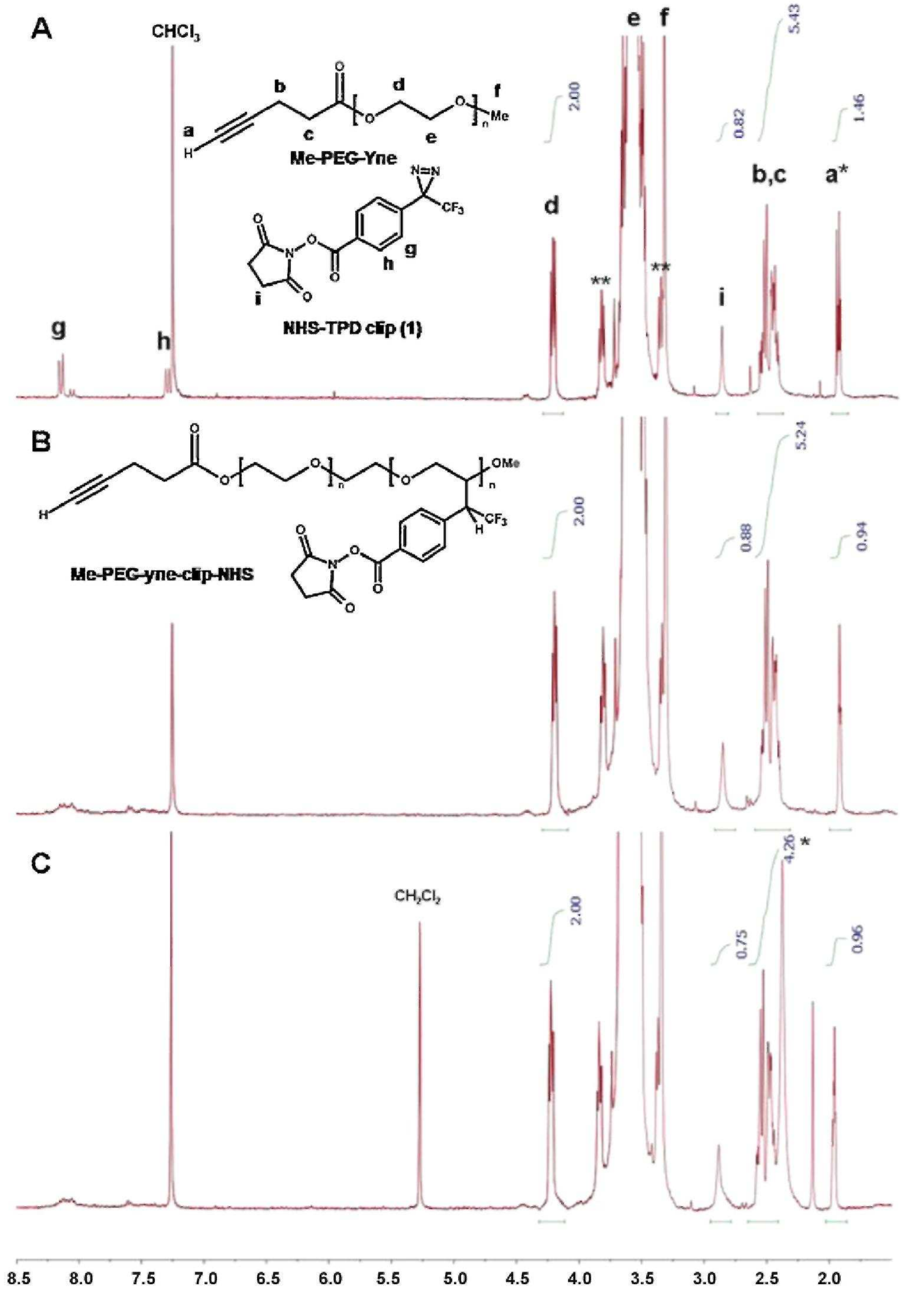
***Fig. 3.*** *ID $^{1}H$ NMR (300 MHz, $CDCl_3$) spectra of MeO-PEG-yne and the molecular clip at each step of the derivatization process;* (A) MeO-PEG-yne mixed with the NHS-TPD clip (**1**) before irradiation; (B) crude MeO-PEG-yne-clip-NHS obtained after 75 min irradiation at 350 nm; (C) MeO-PEG-yne-clip-NHS after washings; 'Integral not usable (contamination with $CH_3CN$ or $H_2O$ peaks): $^{**13}$c satellites.




NMR spectroscopy is a practical tool for soluble compounds analysis which brings together several advantages: (i) under adequate conditions the intensity of the resonance signals are directly linked to the number of nuclei, (ii) no intensity calibration is needed since only ratios are considered, (iii) NMR samples are easy to prepare by simple dissolution with adjusted concentration for higher sensitivity, (iv) it is a non-invasive and non destructive technique, (v) it requires relatively short measuring times, (vi) analysis of crude mixtures and of multiple targets can be performed, (vii) it gives structural information, and (viii) it is routinely available in chemistry departments. One of the most cited drawback of NMR is its low sensitivity. However, recent developments of stable and high field NMR spectrometers combined with efficient treatment softwares have led to reliable NMR protocols well validated for quantification of weak samples [9]. In our case $^{1}$H NMR may not be adequate for quantitative analysis since the analyte signals would be too small compared to the main polymer chain protons. On the other hand $^{19}$F, which has a nuclear spin of 1/2, a natural abundance of 100%, a high sensitivity (83% that of proton), a large spectral window (hence minimizing signal overlap) and is an exogenous element (no interfering background signals), appears to be a candidate of choice for quantification [37,38].

To be used in a quantitative way NMR experiments should meet several criteria (i) the RF pulse excitation must be uniform on the whole spectral width (SW), (ii) all the nuclei should be able to relax between two pulses, (iii) nuclear Overhauser effect (NOE) should be suppressed, (iv) signal to noise ratio (S/N) should be higher than 250, (v) data should be properly processed, and (vi) for absolute calculation a standard is needed [9,37].

To satisfy point (ii) we had to calculate the spin-lattice relaxation times ($T1$) of fluorine in all the tested compounds. It is commonly admitted that with a delay between two scans (recycle time, D1) of at least five times the $T1$ of the slowest relaxing nuclei, 99.3% of the nuclei would have relaxed [9]. $T1$ values were determined using the inverse recovery pulse sequence method and the values obtained for the fluorinated compounds used in this study are presented in Table 1.

For absolute calculation a suitable internal standard should have most of the following characteristics: (i) a short $T1$, (ii) a single sharp and stable signal with no overlap with other chemical shifts, (iii) an optimum boiling point for easy recovery of the sample while not being too volatile, (iv) non-reactive, (vi) soluble in many solvents, (vii) same kind of fluorine function as the entities of interest, and (viii) a convenient molecular weight for using small quantities [39]. We investigated several candidates (Table 1): trifluoroethanol (TFE), trifluoroacetic acid (TFA), benzotrifluoride (BTF) and tricholorofluoromethane (CFC-11). CFC-11 is too volatile to enable reproducible experiments and is also too hydrophobic which do not fit with points (iii) and (vi). BTF might have been a good candidate but its chemical shift is too close to $TagF_6$ and thus does not fulfill criterion (ii). First we tried to use TFA but it induced precipitation of the $TagF_6$ in organic solvent and might be too aggressive for some applications and therefore does not fulfill criterion (iv). Despite having the highest $T1$, TFE was selected as the best candidate since it satisfies all the other required criteria.

The quantification of fluorine compounds required an NMR sequence that satisfies all the aforementioned criteria (see Section 4 and Table 2). The method evaluation was done using several solutions of known concentrations of $TagF_6$ and TFE in $CDCl_3$. A calibration curve (Fig. S2, supplementary data) was obtained with a correlation coefficient ($R^2$) of 0.98 which demonstrates the accuracy of our methodology in terms of linearity, precision, and limit of detection (Table 3) [9[. Based on standard calculations and using the calibration curve parameters [37], we found a lower limit of quantification ($LOQ_{min)}$ at a signal to noise ratio (S/N) of 10, at 0.25 mmol $L^{-1}$, and a recovery percentage of 105% which are acceptable standards, and comparable to other studies [10,37], a maximum limit of quantification ($LOQ_{max}$) of 0.63 mmol $L^{-1}$ can be measured, due to precipitation of the $TagF_6$ in $CDCl_3$ above that concentration. Although this does reduce the range of chloroform dissolved free $TagF_6$ that can be measured, we do not anticipate this to be limiting for the method itself because, in case of grafted $TagF_6$, the polymer solubility will be the limiting factor.

### *2.2.2. Quantification of the grafting rates*

α-Methoxy-ω-alkyne-PEG grafted with $TagF_6$ was obtained by incubation of crude mixture of MeO-PEG-yne-clip-NHS in a 10 mM $TagF_6$ solution (see Section 4). After dialysis, filtration and freeze-drying, the sample was analyzed by $^{19}$F qNMR in $CDCl_3$. In Fig. 2C, fluorine atoms of $TagF_6$ are clearly identified (-62.9 ppm) whereas those of the trifluoromethyl group from TDP clip (1) lead to a distribution of unattributed peaks. Therefore only the peak of $TagF_6$ could be used for quantification. Fig. 2D (control sample) shows that some unspecific adsorption of $TagF_6$ cannot be avoided. We checked that free $TagF_6$ and $TagF_6$ linked to TPD clip (1) (clip-$TagF_6$) have the same chemical shift in $CDCl_3$ (Fig. S3, supplementary data). Consequently, for grafting rates measurements, we have to consider that the integral of the $TagF_6$ peak also contains a contribution from unbound products. To assess this contribution we used two control samples referred to as "non-irradiated" for MeO-PEG-yne that suffers all the protocol except the UV irradiation (Fig. 2D), and "blank" for MeO-PEG-yne submitted to the protocol in the absence of clip (1). These samples allowed us to estimate the rates due to entrapment or physisorption of $TagF_6$ and of side-products arising from unbound clips coupled with $TagF_6$ (Fig. S4, supplementary data), which chemical shift could be included in the $CF_3$ peak. Hence the real amount of covalently bound $TagF_6$ can be deduced from the subtraction of the value of the non irradiated sample to the

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fully treated one. From the results of $^{19}F$ qNMR presented in Fig. 4 we obtain a "corrected amount" of 62 µmol of $TagF_6$ per gram of MeO-PEG-yne, which corresponds to the functionalization of 95% of the clip initially introduced. SEC (size exclusion chromatography) analysis of MeO-PEG-yne-clip-$TagF_6$ (Fig. 5) shows the same profile and the same polydispersity compared to native MeO-PEG-yne, proving that there is neither chain cleavage nor cross-linking reaction during the photografting protocol.

***Table 1** Main characteristics of fluorine compounds.*

| | TFE | TFA | BTF | CFC-11 | $TagF_6$ | RGDp |
|---|---|---|---|---|---|---|
| Bp (°C) | 77-80 | 72 | 102 | 23.7 | Solid | Solid |
| $\delta$ (ppm), $m$[a] | -76.9, s | -75.6, s | -62.7, s | 0, s | -62.9, bs | -62.7, s |
| $T1$ (s)[a] | 3.157 | 2.186 | 2.7[b] | 2.565 | 2.029 | 1.086[c] |

[a] $^{19}F$ NMR chemical shift and multiplicity in $CDCl_3$.
[b] Ref. [8].
[c] $^{19}F$ NMR in $D_2O$ (limited solubility of free RGDp in $CDCl_3$).

***Table 2** $^{19}F$ qNMR in $CDCl_3$ main parameters.*

| | qNMR Parameters |
|---|---|
| Pulse angle | 30° |
| Acquisition time (AQ) | 0.29 s |
| Recycling time (D1) | 20 s |
| Number of scans (NS) | 200 |
| Measurement temperature (TE) | 25 °C |
| Sweep width (SW) | 402.8 ppm |
| Number of points (SI) | 65,536 |
| Line broadening (LB) | 5 Hz |

***Table 3** $^{19}F$ qNMR in $CDCl_3$ validation of the method.*

| Validation parameters from the calibration curve: Int $TagF_6 = a \times n_{TagF6} + b$ | |
|---|---|
| $a$ | 5.67 ± 0.182 µmol$^{-1}$ |
| $b$ | 0.083 ± 0.182 |
| $R^2$ | 0.98 |
| LOQmin | 0.153 µmol |
| LOQmax | 0.760 µmol |
| Recovery | 105% |

***Fig. 4.** $^{19}F$ qNMR $TagF_6$ grafting rates on MeO-PEG-yne-clip-$TagF_6$ with initial TPD clip (1) concentration of 65 µmol/g.*

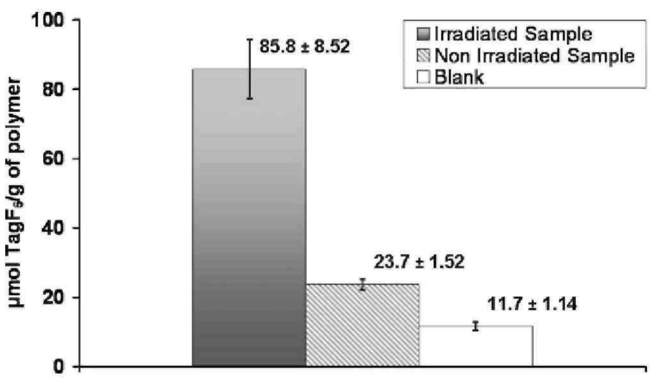
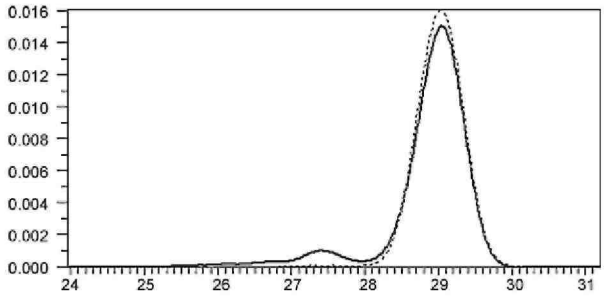


***Fig. 5.*** *SEC chromatogram of MeO-PEG-yne (dashed line) and MeO-PEG-yne-clip-TagF$_6$ (plain line).*




*2.2.3. Application to the coupling of a targeting ligand*

The methodology presented in this paper (clip photochemistry and qNMR) was developed for the conjugation and quantification of RGD peptidomimetics (RGDp) onto PEG building blocks (as depicted in Scheme 1). In that purpose TagF$_6$ was used as a model of these more valuable molecules and enabled us to develop a robust procedure that guarantee the covalent fixation of small molecules. The RGDp developed in our laboratory [20,21] have oligoethylene glycol arms ended with a primary amine that is suited to react with NHS esters. In term of $^{19}$F NMR they have a $CF_3$ tag with chemical shift comparable to the ones of the TagF$_6$, but with a lower *T*1 (Table 1) meaning that it could be quantified using the same qNMR sequence. RGDp are good ligands of $\alpha_v\beta_3$ integrins which are membrane cells receptors used by cancer cells and endothelial cells during angiogenesis. Indeed $\alpha_v\beta_3$ integrins are over expressed by angiogenic blood vessels and by cancer cells whereas poorly present in normal tissues. Thus they are an appealing target to direct DDS aimed at cancer therapies [2].

The protocol developed with TagF$_6$ was easily adapted to the RGDp changing only the washing steps. Starting with the same amount of 65 µmol of TPD clip (1) per gram of MeO-PEG-yne, we first irradiated the mixture to graft NHS esters that were secondly incubated in a solution of RGDp (Fig. S5, supplementary data). After washings to eliminate the unfixed RGDp, we obtained, by $^{19}$F qNMR, an amount of 59 µmol of RGDp per gram of MeO-PEG-yne which corresponds to the functionalization of 89% of the TPD clip (**1**) initially introduced (Fig. S6, supplementary data). This result is comparable to the one obtained with TagF$_6$ (i.e. 95% of functionalization).

## 3. Conclusion

In conclusion, we have developed a versatile methodology, usable by non-specialists, for the elaboration of orthogonally functionalized PEG building blocks and their characterization by qNMR, a non-destructive technique. We are able to attach dedicated molecules on an ω-alkynyl-PEG without damaging the triple bond which can further be involved in click chemistry for the preparation of smart materials [24]. The use of a non expensive commercial source of PEG and straightforward chemistry (clip irradiation, click chemistry), combined with a routinely available spectroscopic instrument, provide a practical way to produce PEGs for biomedical applications.

## 4. Experimental

### *4.1. Materials*

Solvents were of analytical grade and obtained from Aldrich-Fluka, Acros or Rocc. Reagents were distilled or recrystallized before used. Trifluoroethanol (TFE) 99%+ (GC), 3,5-bis-(trifluoromethyl)benzylamine (80%, Techn) and α-methoxy-ω-hydroxypoly(ethylene glycol) (1000 g/mol) were purchased from Aldrich. Phosphate buffer (PB, 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). Dialysis membrane (CE, MWCO 500 Da, 31 mm) was obtained from Spectra/Por. $^1$H (299.80 MHz) and $^{19}$F (282.09 MHz) NMR spectra were recorded on a Bruker Avance II spectrometer. Spectra were obtained in $CDCl_3$, $D_2O$ or $CD_3OD$ at room temperature. Chemical shifts $\delta$ are reported in ppm and are calibrated on the solvent signal at 7.26, 4.79, 3.31 ppm for $CDCl_3$, $D_2O$ or $CD_3OD$, respectively. Coupling constants *J* are given in Hz. Relaxation times *T*1 were measured using a BBFO probe on a Bruker Avance II operating at 282.09 MHz for $^{19}$F. UV spectra were recorded on a UV-vis-NIR Varian-Cary spectrophotometer. Size exclusion chromatography (SEC) was performed at 45 °C with a refractive index detector and UV-visible detector and two polystyrene gel columns (columns HP PL gel 5 µm, porosity: 102, 103, 104, and 105 Å, Polymer Laboratories) that were eluted by THF at a flow rate of 1 mL/min. The columns were calibrated with polystyrene standards (Polymer Laboratories). The RGD peptidomimetic was synthesized in our laboratory following a protocol published elsewhere [20,21]. The preparation of O-succinimidy1-4-(1-azi-2,2,2-trifluoroethyl)benzoate (NHS-TPD-clip) is given in the supplementary data.

### *4.2. Preparation of α-methoxy-ω-alkyne-poly(ethylene glycol) (MeO-PEG-yne)*

3 g of α-methoxy-ω-hydroxyl-poly(ethylene glycol) (1000 g/ mol) were dried by repeated azeotropic distillation of toluene (three times). 147 mg of 4-pentynoic acid (1.498 mmol), 18 mg of N,N-dimethyl aminopyridine (DMAP) (0.147 mmol), 309 mg of dicyclohexyl carbodiimide (DCC) (1.497 mmol) and 35 mL of dry $CH_2Cl_2$ were added. The solution was stirred at room temperature for 36 h. The solvent was evaporated in vacuo, the solid residue was dissolved in THF, the solution filtered to remove the dicyclohexylurea byproduct and poured in $Et_2O$. After filtration, the solid was dried in vacuo at room temperature to reach 74% (yield 80%) of alkyne functionalization from $^1$H NMR.

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.95 (*m*, 2H, C≡CH), 2.50 (*m*, 4H, CH2-C≡C), 2.60 (*m*, 2H, CH2-C(=O)), 3.35 (*s*, 3H, O-(CH3)), 3.85 (*m*, 4H, 2CH2-O), 4.25 (*t*, 2H, CH2-O-C(=O))

### *4.3. Standard protocol for the preparation of conjugated PEG (MeO-PEG-yne-clip-TagF$_6$ or RGDp)*

PEG was solubilized in $CH_3CN$ (20 mL/g) with the desired amount of the molecular TPD clip (1) (65 µmol/g of polymer) and the solution was cast on clean glass plates. After solvent evaporation, the samples were dried under vacuum, removed from the plates as shavings and irradiated (3 W x 8 W BLB lamps, 360 nm, placed at a distance of 4.5 cm) in a home-made reactor (rotating quartz flask of 15 mL) under argon atmosphere for 75 min. The activated samples were incubated in a solution of the desired compound (3,5-bis-trifluoromethyl-benzylamine 10 mM or RGDp 5 mM) in 0.1 M phosphate buffer (PB):$CH_3CN$ (1:1, v/v) at pH 8.0 and shook for 24 h at 20 °C.

The samples treated with TagF$_6$ were dialyzed for 100-130 h against water, lyophilized, solubilized in $CH_2Cl_2$, filtered on PTFE Acrodisc filters (0.2 µm) and dried by azeotropic distillation of dry $CH_3CN$. Several reference samples were prepared to control the non specific adsorption versus covalent grafting at each step of the derivatization protocol. They are designed as non irradiated (standard protocol omitting the UV irradiation) and blank (standard protocol omitting the molecular clip).

The samples treated with RGDp were lyophilized, extracted with DCM and dried under vacuum. Then they were solubilized at room temperature in a minimum amount of MeOH and precipitated in isopropyl ether at -20 °C twice, followed by another solubilization in a minimum amount of MeOH and precipitation at -20 °C twice. The samples were dried by azeotropic distillation of dry $CH_3CN$ before being submitted to $^{19}$F qNMR.

### *4.4. Quantification by $^{19}$F NMR ($^{19}$F qNMR)*

#### *4.4.1. NMR sample preparation*

Polymer samples were carefully weighted, solubilized in 600 µL of a 1 mM solution of trifluoroethanol (TFE) in $CDCl_3$, transferred in a 5 mm NMR tube and submitted to $^{19}$F qNMR experiment. Each preparation and measurement was repeated at least three times.

#### *4.4.2. $^{19}$F qNMR experiment*

The $^{19}$F NMR spectra were acquired in $CDCl_3$ at 25 °C using a Bruker Avance II spectrometer operating at 282.09 MHz for $^{19}$F with inverse-gated waltz-16 1H-decoupling. The experimental settings were as follows: 30° pulse width, 3.6 µs; sweep width 402.8 ppm; acquisition time of 0.29 s; 200 scans, delay between scans of 20 s in $CDC1_3$ zero filling to 65,536 final points; line broadening (LB) 5 Hz.

#### *4.4.3. Measurement of grafted TagF$_6$ amounts by $^{19}$F qNMR*

Processing and spectra handling were performed using the TOPSPIN 1.3 program suite (Bruker Biospin GmbH, Rheinsteten, Germany). Spectra were referenced to the signal of the internal standard (i.e. TFE) at -76.85 ppm.

Phasing was done manually, baselines were fitted to zero with a fifth order polynomial correction algorithm and integrations around the signal regions were as follow: from -62 to -64 ppm for $TagF_6$ and from -76.6 to -77.2 ppm for TFE.

Grafting rates of $TagF_6$ were calculated with Eq. (1):

$$n_{TagF_6} = \left[\frac{f_{TagF_6}}{f_{std}}\right] \times \left[\frac{V \times C_{std}}{m}\right] \times \left[\frac{\text{Int TagF}_6 \times nF_{std}}{\text{Int std} \times nF_{TagF_6}}\right] \quad (1)$$

where $n_{TagF6}$ is the amount of molecular probe $TagF_6$ in µmol/g of sample; $f_{TagF6}$ and $f_{std}$ are the purity factors of TFE internal standard and $TagF_6$, respectively; $V$ is the volume of solvent in the NMR tube in mL, $C_{std}$ is the concentration of the TFE internal standard in µmol/mL and m the mass of the analyzed sample in g; Int std and Int $TagF_6$ are the integral values of TFE internal standard and $TagF_6$, respectively, $nF_{std}$ and $nF_{TagF6}$ are the number of fluorine atoms of the TFE internal standard and $TagF_6$, respectively.

### *4.4.4. Measurement of spin lattice relaxation times (T1)*

After calibration of the 90° pulse, a series of experiments with varying relaxation times of 0.05, 0.1, 0.2,0.3, 0.5,0.7,0.9,1.5,2 and 3 s were set up using the Bruker "t1ir" pulse sequence for each compound whose *T*1 needed to be determined. These different relaxation times were measured scrambled. Each spectrum was run over a sweep width of 402 ppm, with 8 scans (plus 4 dummy scans) and a delay of 30 s between scans. Processing (LB = 2 Hz) and analysis of the data were performed in the TOPSPIN 1.3 program suite (Bruker).

Determination of *T*1 was done on free, in solution, probes and ligands since it was not possible when conjugated to PEG due to broadening of the fluorine peaks corresponding to the $CF_3$ tags. Nevertheless it can be expected that *T*1 of conjugated molecules would be lower than the free ones.

### 4.4.5. *Validation parameters*

A calibration curve was obtained by plotting the integrations calculated with the $^{19}F$ qNMR method for various amounts of $TagF_6$ mixed with a constant quantity of internal standard in a fixed volume of $CDCl_3$. Each point was the mean of at least three independent preparations. The slope a (Eq. (2)) and other statistical parameters such as the correlation coefficient ($R^2$) and the standard error were calculated with referenced statistical methodologies based on the least-squares linear regression analysis using Microsoft Excel DROITEREG function.

$$\text{Int TagF}_6 = a \times n_{TagF_6} + b \quad (2)$$

The accuracy of the measured values to the weighed ones is expressed in term of recovery percentage. qNMR Results are expressed as the mean of three independent measures ± the standard deviation (SD). The limit of quantification (LOQ) at a signal to noise ratio (S/N) of 10 was calculated with Eq. (3).

$$\text{LOQ} = b + 10 \times (\text{standard error}) \quad (3)$$

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfluchem. 2012.05.006.

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