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A practical molecular clip for immobilization of receptors and biomolecules on devices' surface: Synthesis, grafting protocol and analytical assay

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Abstract—Deposition of *O*-succinimidyl 4-(*p*-azido-phenyl)butanoate (**4**) onto inorganic device (FTIR-ATR crystal) or polymer material (filtration membrane) followed by irradiation at 254 nm led to surface functionalisation with NHS esters. Further reaction with biomolecules allowed their covalent grafting. The reactivity of the photoactivated surfaces was assayed by two methods: (i) the coupling with 3,5-bis(trifluoromethyl)benzylamine (**7**) and subsequent XPS analysis; (ii) the coupling with 4,5-bis-tritiated lysine (**10**) and subsequent LSC measurement of the radioactivity.

Immobilization of molecules of biological interest on solid supports has gained a growing importance because the resulting devices are used in detection systems (biochips)[1] or as biomaterials.[2] The covalent grafting requires the presence of reactive functions on the materials' surface.[3] The techniques of surface modifications are numerous and depend on the nature of the support, namely inorganic[4] (glass, silicon, gold, germanium, etc.) or organic materials (synthetic polymers).[5]

We have developed a simple and practical method for the implanting of an activated ester function (*N*-hydroxysuccinimidyl (NHS) ester) onto both inorganic and organic surfaces; such a function is able to covalently fix (bio)molecules by nucleophilic substitution with an amine, for instance the ε-NH_2 group of outside lysines in proteins. Our strategy relies upon the use of a molecular clip, namely *O*-succinimidyl 4-(*p*-azido-phenyl)butanoate (**4**) (Scheme 1), and its photoactivation leading to insertion into surface C–H bonds via the unmasked aryl-nitrene motif,[6] while the NHS moiety remains untouched for the further coupling with biological receptors. Molecule **4**[7] was readily obtained, in 68% overall yield for three steps, from 4-(*p*-aminophenyl)butanoic acid **1** transformed into azide **3**[8] and then esterified with NHS in the presence of diisopropyl carbodiimide (DIC) and dimethylaminopyridine (DMAP) as catalyst.

In this communication, we have selected two representative examples of surface derivatisation by using the molecular clip **4**, and the related analytical methods to check the surface reactivity.

In the course of a programme devoted to the construction of a novel biosensor based on the FTIR-ATR spectroscopic detection,[9] the covalent attachment of biological receptors on the surface of ATR crystals was required. The optical elements used in this technique were made of silicium or germanium. The usual way to graft an organic layer on a silicium device consists in an oxidative pre-treatment followed by the reaction with a silane derivative such as OTS (octadecyl trichlorosilane).[10] Germanium device could be similarly treated.[10b,11] Thus, Si–OTS (or Ge–OTS) was our starting material (Scheme 2). A solution of **4** in benzene was sprayed on the ATR crystal **5**; after air-drying, the device was irradiated at 254 nm for 2 h and then rinsed with organic solvents. Immersion of the functionalised crystal **6** into an aqueous solution of streptavidin (250 μg/mL, 20 min, 20 °C), considered as a generic model of biological receptors, led to the protein grafting as previously controlled by FTIR-ATR spectroscopy

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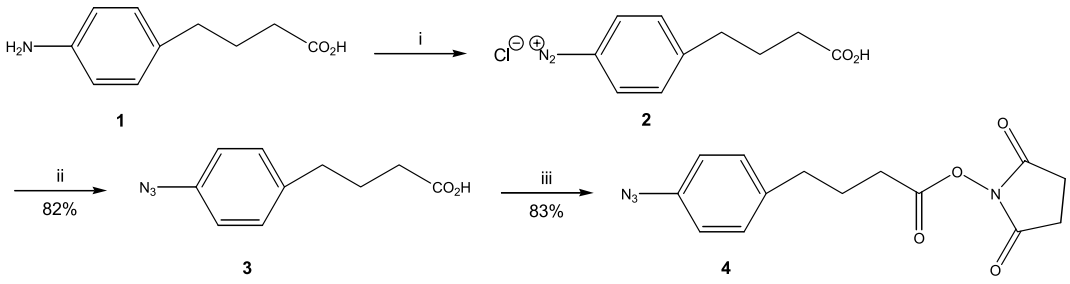
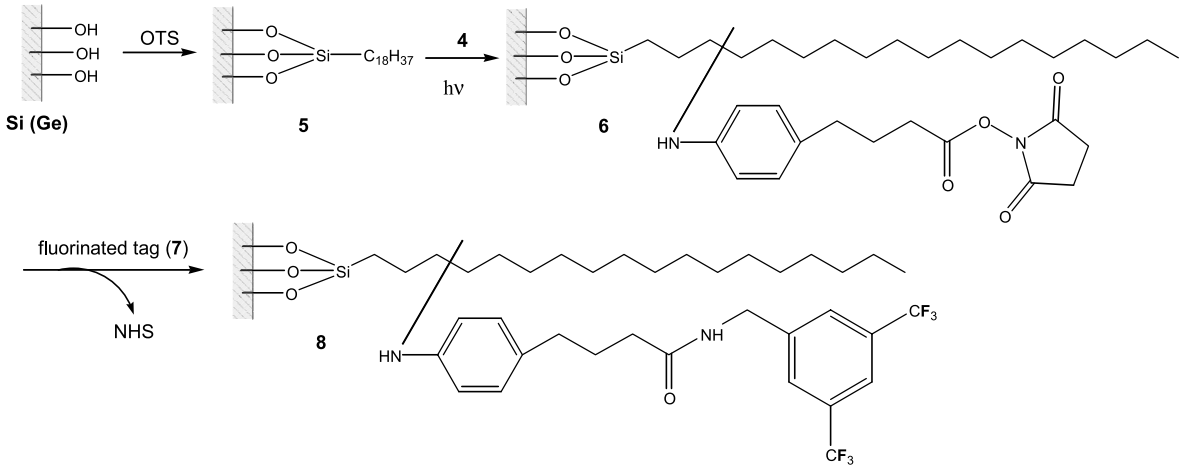


Scheme 1. Synthesis of the molecular clip. Reagents and conditions: (i) $NaNO_2$, HCl, H_2O, 15 min, 0 °C; (ii) NaN_3, H_2O, 90 min, 0 °C; (iii) DIC, NHS, DMAP cat., CH_2Cl_2, 22 h, rt.



Scheme 2. Surface functionalisation of ATR crystal.

and by the further fixation of biotin also controlled by FTIR-ATR spectroscopy.[9] The reactivity of device **6** could be assayed by the coupling of 3,5-bis(trifluoromethyl)benzylamine (**7**)[12] (Scheme 2) and subsequent XPS analysis (X-ray photoelectron spectroscopy) of the resulting ATR crystal **8**.[13] A so-called "blank" sample was also considered: this device was prepared as before, but the irradiation step was omitted. Thus, after rinsing, molecules could not be covalently grafted; only nonspecific adsorption onto Si–OTS (or Ge–OTS) **5** could occur. XPS measurements were performed with the sample **8** and the corresponding "blank" as shown in Tables 1 and 2. From the F/C atomic ratios, we concluded that about 40% of the octadecyl chains have fixed the fluorine tag **7** under our experimental conditions.[14] Interestingly, the fine structure of the C_{1s} peak showed new components regarding the Si–OTS crystal **5**; these corresponded well to the functional groups introduced by the photografting, in particular the **C**F_3 tag was visible at 292.9 eV. Si and Ge crystals, first grafted with ethylene glycol oligomers, were also successfully used in our protocol of receptor immobilization via the molecular clip **4**.[9]

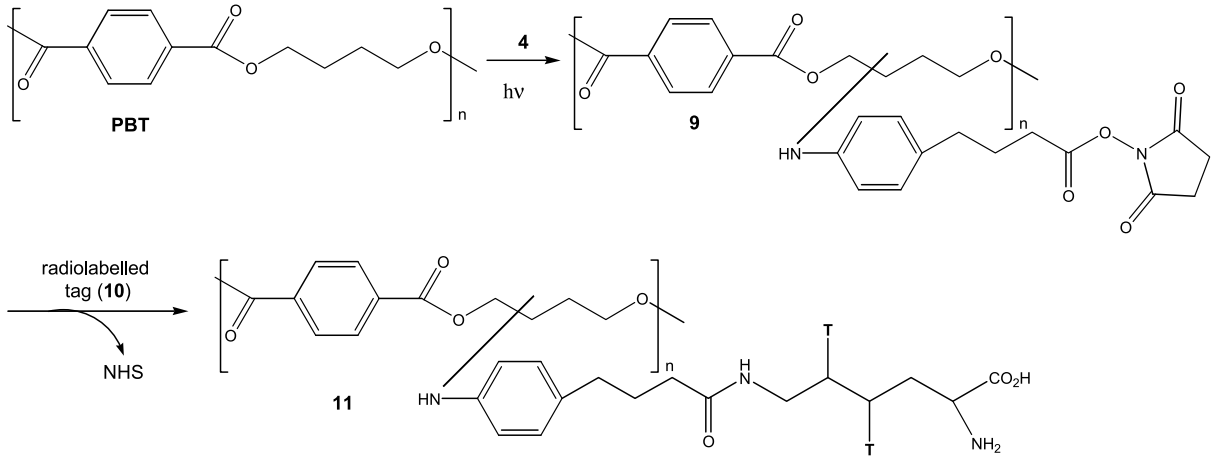
Table 1. XPS data of the grafted ATR crystal of silicium (120 min irradiation)

	Element				
	Si	C	O	N	F
Blank (%)	26.11	42.68	30.69	0.24	0.28
Crystal **8** (%)	15.09	47.72	30.81	3.02	**3.36**

In another programme aiming at the biocompatibilisation of polymer films and membranes, we used the same molecular clip **4** for the surface derivatisation with biologically active small molecules. Previously, the functionalisation of polyesters such as PET (polyethylene terephthalate) made use of the carboxyl- and hydroxyl-chain ends as anchorage points.[15] This required the pre-activation of such functions with, respectively, carbodiimide and tosyl chloride. The amounts of grafted molecules, namely integrin antagonists and enzyme inhibitors, remained quite low (20–100 pmol/cm^2 of open surface) since chain ends protruding from the polymer

Table 2. Fine structure of the C_{1s} peak

	Binding energy (eV)			
	284.7	286.2	288.5	292.9
Blank (attribution)	**C** (C, H)	—	—	—
Crystal **8** (attribution)	**C** (C, H)	**C**–X (X = O, N)	O=**C**–X (X = O, N)	**C**F_3



Scheme 3. Surface functionalisation of polymer membrane.

surface are rare events. A commercial membrane (for blood product filtration) made of PBT (polybutylene terephthalate) was chosen to illustrate our photochemical method because this material typically represents a problematical case due to its sensitivity towards air-oxidation under UV irradiation. However, by using short irradiation times (less than 30 min), the PBT filter was easily functionalised by the direct photografting of molecule **4** (Scheme 3) under air atmosphere. Two "blank" samples were considered: (i) PBT incubated with **4**, but nonirradiated, then rinsed (blank 1); (ii) PBT without pre-treatment by **4** and irradiated (blank 2). The grafted filter **9** (Scheme 3) was incubated into a solution of homemade synthetic inhibitors of thrombin (10^{-3} M in phosphate buffer/CH_3CN 1:1, pH 7.2, 20 °C, 2 h); the covalent grafting of the inhibitors was controlled by XPS (1–3% of surface derivatisation; data not shown), and by the inhibition of blood coagulation on the modified polymer devices.[16] The open surface reactivity of membrane **9**[17] could be assayed by coupling with 4,5-bis-tritiated lysine (**10**)[18] and measurement of the radioactivity associated to the sample **11**[19] by liquid scintillation counting (LSC). Results given in Table 3 (procedure A) showed a good level of lysine grafting, about two times higher than the value recorded with the previous method (PBT activation by tosylation; data not shown).[16] The lysine fixed on the blank 1 sample could result from nonspecific adsorption and/or from covalent reaction with PBT ester functions (transesterification–transamidation). The increased level of lysine fixed on the blank 2 could result from the photo-oxidation of the material surface that enhanced the polymer hydrophilicity and therefore its capacity for adsorbing lysine. The reproducibility of this procedure was well established and lysine immobilization via the molecular clip **4** was always found to be greater than the unspecific adsorption (between 50 and 100 pmol/cm^2). Modifying the protocol (diluted lysine solution, prolonged incubation time and increased number of washings) it was possible to almost completely eliminate the adsorption phenomenon and highlight the covalent fixation due to our photochemical activation strategy (Table 3, procedure B). Other polymers (PET, PP, PVDF, PS, PMMA, etc.) are actually involved in our protocol of surface photoactivation for the coupling of various molecules of interest. Such an approach was scarcely considered in the previous literature.[20]

Table 3. LSC data (pmol of **10** per cm^2 of apparent surface) of the grafted PBT membrane

	Procedure A[a]	Procedure B[b]
Blank 1	58 (±28)	9 (±5)
Blank 2	101 (±20)	17 (±1)
Membrane **11**	153 (±23)	59 (±9)

[a] Each value is the average of three independent measurements performed with three samples similarly treated.
[b] Each value is the average of three samples similarly treated. Standard deviation is given in parentheses.

In conclusion, we have disclosed a general method for organic and inorganic surface derivatisation based on the photografting of *O*-succinimidyl 4-(*p*-azido-phenyl)butanoate (**4**) as a versatile molecular clip. Two complementary methods to check the surface reactivity of the activated devices (**6**, **9**)[21] are also presented. They consisted in the coupling with a fluorinated or tritiated amine tag (**7**, **10**), followed by analysis by XPS or LSC, respectively. The first method that samples the apparent outermost surface layer (about 50–100 Å in depth) is recommended in the case of rigid, dense and smooth samples. The second method that analyzes the total open surface (with variable in-depth analysis, depending on the material swelling property) is particularly well adapted to soft, porous and tortuous materials. The levels of surface derivatisation (from 40% to a few percent) that could be reached mainly depended on the irradiation time in the presence of azide **4**, and this time had to be adapted considering the device photostability under air atmosphere.[22]

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7. Synthesis and characterisation of **4**: in the dark, and under argon atmosphere, to acid **3**[8] (0.103 g, 0.5 mmol) in CH_2Cl_2 (3 mL) were added successively *N*-hydroxysuccinimide (0.111 g, 0.96 mmol), dimethylaminopyridine (catalyst, 0.005 g), and then, dropwise, diisopropylcarbodiimide (0.153 mL, 0.98 mmol) in CH_2Cl_2 (2 mL), at 0 °C, under stirring. The mixture was stirred for 1 h at 0 °C and left overnight at 20 °C. After filtration, washing with 0.05 N HCl (three times) and brine (three times), the organic layer was dried over $MgSO_4$ and concentrated. Addition of ethyl acetate to the solid residue and storage at −20 °C allowed the remaining diisopropylurea to crystallize. After filtration, the crude ester **4** was chromatographed (silica gel; EtOAc). Yield: 0.123 g (81%) as white crystals. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.05 (quint, J = 7.4 Hz, 2H), 2.60 (t, J = 7.4 Hz, 2H), 2.72 (t, J = 7.4 Hz, 2H), 2.85 (s, 4H), 6.97 (d, J = 8.5 Hz, 2H), 7.20 (d, J = 8.5 Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) ppm 169.6, 168.9, 138.9, 138.1, 130.6, 119.9, 34.6, 30.8, 26.9, 26.3; IR (KBr) ν 2944, 2111, 1814, 1785, 1739, 1507, 1368, 1289, 1206, 1068, 909, 732, 647 cm^{-1}.
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13. Procedure for the preparation of ATR crystal **8**: a solution of **4** (1.1 mg) in benzene (0.5 mL) was sprayed onto the crystal **5** (5.2 cm × 2.0 cm). After solvent evaporation, half of the crystal surface was longitudinally masked with a black paper and the other half surface was irradiated during 2 h with three UV lamps of 8 W (254 nm), placed at a distance of 7 cm. The crystal was rinsed, under shaking, with chloroform (5 min) and tetrahydrofuran (10 min). The resulting crystal **6** was sprayed with a solution of amine **7** (6.2 mg) into benzene (0.5 mL). After solvent evaporation, the crystal was left in the dark for surface reaction (nucleophilic displacement of the NHS ester) during 100 min at 20 °C. Rinsing with benzene (2 × 3 min), under shaking, gave crystal **8** (one half part) and the blank (non-irradiated half part) for XPS analysis.
14. Calculation of the amount of fixed tag from the XPS data, assuming that each octadecyl chain has fixed one molecule **4** to give crystal **6**: theoretical atomic composition of the organic layer of crystal **8**: $C_{18} + C_{19}N_2F_6 = C_{37}N_2F_6$; thus, F/C for 100% grafting of **7** = 0.1622. Experimental F/C = 0.0704, corresponding to 43% of grafting.
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17. Procedure for the preparation of PBT membrane **9**: we used polybutylene terephthalate Meltblown, Goodfellow, of 0.133 mm thickness and with a mean flow pore of 5 μm. Polymer discs (13 mm of diameter) were individually immersed into a solution of **4** (0.025 g) in acetonitrile (10 mL) during 5 s. The so-wetted samples were air-dried in the dark for 30 min. Both sides were then irradiated during 10 min with three UV lamps of 8 W (254 nm) placed at a distance of 7 cm. The samples were individually washed with acetonitrile (2 mL, 10 min, three times) and air-dried. Blank 1 samples were similarly prepared but without UV exposition. Blank 2 samples were exposed to UV without any other treatment.
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19. Preparation of PBT membrane **11**. Procedure A: activated and blank samples were individually incubated for 2 h at 20 °C with 1 mL of 10^{-3} M [^{3}H]lysine in a phosphate buffer (PBS)–CH_3CN mixture (1:1); the stock-solution of lysine was prepared as follows: 250 μL of 0.1 M unlabelled lysine and 187.5 μL of labelled lysine at 37 MBq/mL in 25 mL PBS buffer (pH 7.2); the samples were washed successively with PBS–CH_3CN (1:1) (1 mL, 2 × 10 min), water (1 mL, 2 × 5 min), 5×10^{-3} M HCl (1 mL, 2 × 5 min) and water (1 mL, 2 × 10 min). Procedure B: activated and blank samples were individually incubated for 12 h at 20 °C with 1 mL of 10^{-4} M [^{3}H]lysine in a phosphate buffer (PBS)–CH_3CN mixture (1:1); the stock solution of lysine was prepared as follows: 25 μL of 0.1 M unlabelled lysine and 18.75 μL of labelled lysine at 37 MBq/mL in 25 mL PBS buffer (pH 7.2). The samples were washed successively with PBS–CH_3CN (1:1) (1 mL, 3 × 10 min), water (1 mL, 2 × 5 min), 5×10^{-3} M HCl

(1 mL, 2 × 5 min) and water (1 mL, 2 × 10 min, then 1 mL overnight). Finally, the samples were drained over filter paper and directly analysed by LSC.

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21. The air-dried activated devices (**6**, **9**) can be stored in a dry box for several weeks, without loss of reactivity; reproducible values are recorded in the XPS and LSC assay. Very long time durability is still under investigation.
22. Irradiation under argon atmosphere is possible (by placing the samples in a home-made Pyrex reactor, flushed with inert gas[16]); however, when possible, we recommend the simplest techniques.