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Supramolecular Peptide/Surface Assembly for Monitoring Proteinase Activity and Cancer Diagnosis

Claire Soum,[†,∥] Sandra Rubio-Albenque,[†] Suzanne Fery-Forgues,[‡,§] Gérard Déléris,[⊥] Mohamed-Anis Alouini,[†] and Thomas Berthelot*[,∥]

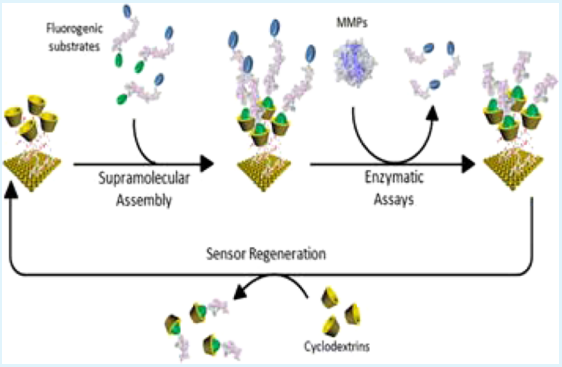
[†]Pharmacochimie, FRE3396 CNRS, University of Bordeaux, 146 rue Léo Saignat, F-33076 Bordeaux Cedex, France

[‡]Institut des Technologies Avancées en sciences du Vivant (ITAV), CNRS UMS 3039, Centre Pierre Potier, Oncopôle, 1 place Pierre Potier, BP 50624, F-31106 Toulouse Cedex 1, France

[§]LSPCMIB, UMR-CNRS 5068, Université Paul Sabatier-Toulouse III, 118, route de Narbonne, F-31062 Toulouse Cedex 9, France

[∥]Laboratory of Innovation in Surface Chemistry and Nanosciences, NIMBE UMR CNRS 3685, CEA Saclay, DSM/IRAMIS/NIMBE/LICSEN, F-91191, Gif-sur-Yvette Cedex, France

ABSTRACT: Matrix metalloproteinases (MMP) are a family of proteolytic enzymes, the expression of which in a key step of tumor progression has recently been better defined. The overexpression of one or more MMPs is thus common among malignant tumors. It may characterize tumor progression and help predict its response to chemotherapy. Consequently, the development of a device for measuring MMP activities is an important challenge for diagnosis and prognosis. In this study, we describe an innovative supramolecular peptide/surface assembly for screening MMP activities. This sensor was used to discriminate various MMP activities and to distinguish between invasive and noninvasive cancerous cell suspensions. Our results confirm the proof-of-concept of a powerful tool for the determination of the tumor aggressiveness and a technical building block for future development of MMP lab-on-chip devices.



KEYWORDS: *supramolecular assembly, regenerative surface, cancer, diagnosis, matrix metalloproteinase, cancerous cells*

1. INTRODUCTION

Matrix metalloproteinases (MMPs) are zinc-endopeptidases involved in many normal physiological and developmental events,[1] as well as in pathogenic processes such as asthma, arthritis, and cardiovascular and neurodegenerative diseases.[2] By cleaving most, if not all, of the extracellular matrix constituents,[3] they can activate signaling pathways and release quiescent factors. Above all, these enzymes play a key role in the destruction and remodeling of the stromal architecture, which is a critical cancer-related step for the growth of primary tumors and the invasion of metastases.[4,5] The overexpression of one or more MMPs is common among malignant tumors. It may characterize tumor progression and help predict its response to chemotherapy.[6,7] Consequently, the development of devices aimed at measuring MMP activities is an important challenge for diagnosis and prognosis. Currently, MMP activities have mainly been addressed by liquid assays using peptidic fluorogenic derivatives.[8] Within this context, we have previously developed a series of fluorogenic peptides to monitor their activities in vitro.[9] These peptides incorporate a Förster Resonance Energy Transfer (FRET) system based on two coumarins (3-carboxyl-7-diethylamino-coumarin (DAC) and 3-carboxyl-7-methoxy-coumarin (MC); Figure 1). However, this screening strategy presents some drawbacks, in particular, the lack of selectivity and the poor solubility of linear peptide substrates and the screening peptide quantity used for tests. To overcome some of these drawbacks, we developed a solid sensor based on covalent grafting of fluorogenic substrates on a solid support as a proof-of-concept that MMPs activities can be also monitored with solid assay.[10] However, some points such as the behavior and stability of this sensor with tumor biopsy or tumor cells, the ease of synthesis and the regeneration of the device biosensing area remained to be addressed in order to obtain an innovative and relevant device for MMP activity screening. Some of these points are also relevant for other scientific communities involved in biosensor or lab-on-chip development. For example, despite a strong academic development, the industrial success of lab-on-chip technology is currently limited by the high cost of these systems that generally arises from single-use biosensing area. In fact, biomolecules are generally covalently and randomly grafted by surface chemistry. This prevents biosensing area regeneration and can decrease the biosensor effectiveness and sensitivity.[11] Supramolecular approach, by mimicking molecular

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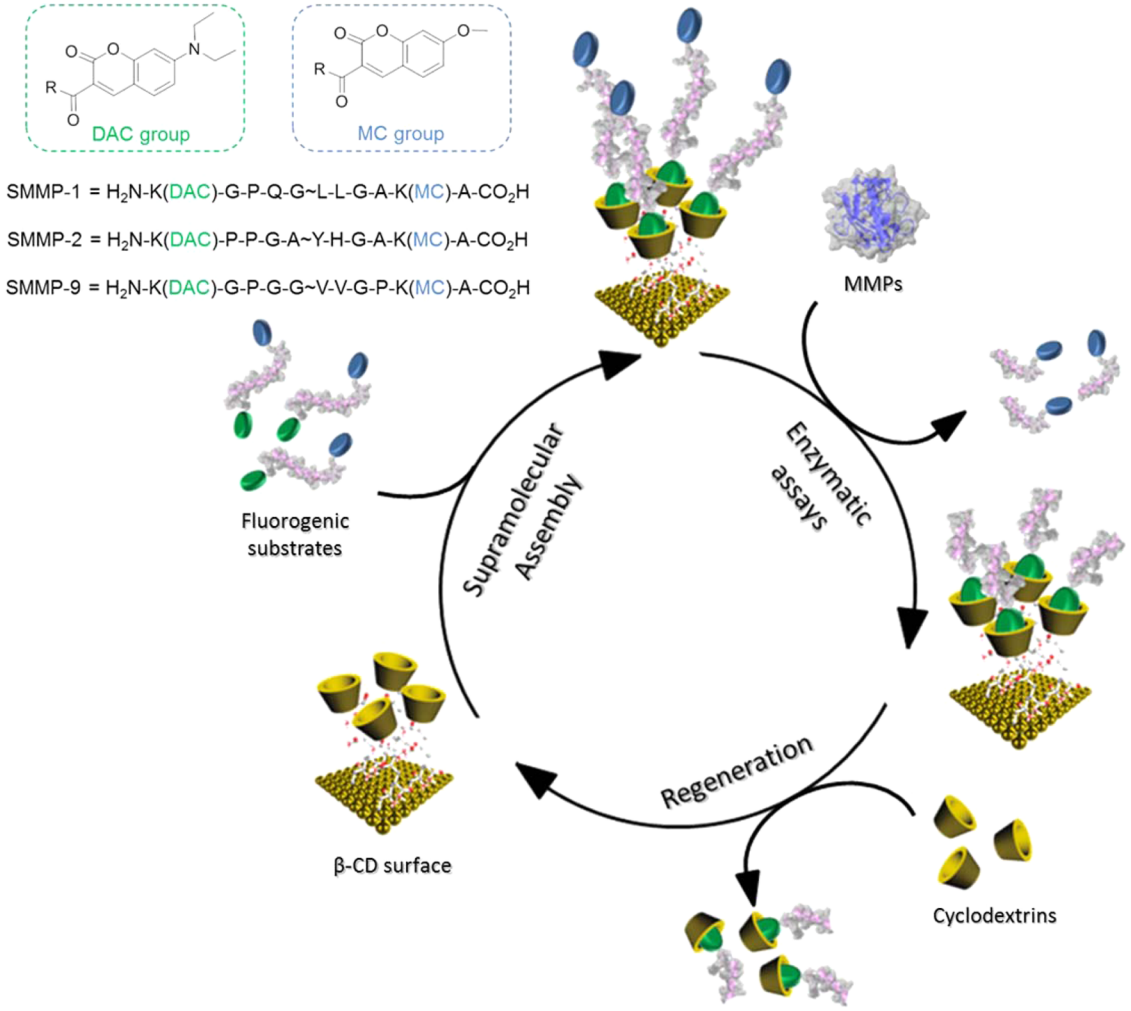

Figure 1. MMP biosensor assays strategy.

systems observed in nature,[12] can appear to resolve some of these issues. For instance, the Reinhoudt group has developed "molecular printboards" based on cyclodextrin (CD) self-assembled monolayer (SAMs) to immobilize small molecules, biomolecules, or particles.[11,13−19] Despite the ability to form complex, bionanostructures or biomolecules cannot interact by themselves and must be modified first with a synthetic PEG-bisadamantane element to form host−guest interactions with the surface and to limit steric hindrance with the surface.

Recently, we have reported that our biscoumarinic MMP substrates interact with β-CD in solution and form only a stable supramolecular complex between diethylaminocoumarin (DAC) and β-CD.[20] These results highlight intrinsic property of our peptides to potentially form supramolecular assembly with a β-CD surface. To tackle the challenges related to both MMP activity screening and biosensor development, we present an innovative supramolecular peptide sensor for screening in vitro and ex vivo MMP activities (Figure 1). This sensor was obtained by selective host−guest interactions of previously developed FRET peptides and β-cyclodextrin polymer brushes. Physical and chemical studies revealed a single and specific binding of peptides with respect to the surface support. Ex vivo data highlighted that our supramolecular peptide/surface assembly was efficient to discriminate MMP activities between invasive and noninvasive cancerous cell suspensions. These results confirm that our system is a powerful tool not only for the determination of the tumor aggressiveness but also for the resulting chemotherapy management. Surface sensor regeneration steps during in vitro and ex vivo experiments validated our approach, making our system an innovative building block for future development of lab-on-chip devices.

2. MATERIALS AND METHODS

All standard chemicals and solvents were of analytical grade and purchased from Sigma-Aldrich. All chemical reactions were carried out under nitrogen or argon with dry solvents. Dimethylformamide (DMF) was refluxed over CaH_2 overnight and distilled and dichloromethane (DCM) was distilled before use. Thin layer chromatography (TLC) was performed on aluminum plates precoated with silica gel $60F_{254}$ (Merck, Germany) with detection by UV light or by staining with $KMnO_4$ (1% aqueous solution). Column chromatography was performed on silica gel 60, 70−230 mesh (SDS, France). Human active MMP-1 and MMP-2 and Human MMP-9 (90% zymogen and 10% activated enzyme) were purchased from Calbiochem. Gold substrates were obtained by successive metallic evaporation of 5 nm of chromium underlayer and 200 nm of gold as top coat on microscopic glass slides.

2.1. Analysis. A Bruker Advance spectrometer working at 400 MHz for proton (^{1}H) was used for NMR analysis. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane as an internal standard. NMR data processing was performed using Sigmaplot 11 software. MALDI-TOF mass spectrometry was performed on a BRUKER BIFLEX III mass spectrometer (CESAMO,

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Bordeaux, France) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Infrared spectra were recorded on a Bruker Vertex 70 spectrometer controlled by OPUS software. ATR monoreflection Pike-Miracle and Hyperion 2000 microscope accessories were implemented. The detector was for both accessories MCT working at liquid nitrogen temperature. For ATR spectra, acquisitions were obtained at 2 cm^{-1} resolution after 8 scans for neat products and after 256 scans for films. All fluorescence studies and enzymatic assays were performed on a PerkinElmer Luminescence Spectrometer model LS55 with a four-position automatic cell changer including water thermostatting and stirring for each sample position. Fluorescence images were obtained using the Olympus BX 51 imaging system from Olympus (Japan). All image acquisitions were performed with an exposure time of 1/3 s.

2.2. Glycidyl Methacrylate-β-cyclodextrin (GMA-β-CD) Synthesis. Under inert atmosphere, to a solution of dried β-cyclodextrin (5.6 g, 4.9 mmol, 1 equiv) in anhydrous dimethylformamide (DMF; 15 mL) were added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in catalytic amount (0.05 mL, 0.33 mmol) and glycidyl methacrylate (0.82 mL, 6.16 mmol, 1.3 equiv). The reaction mixture was stirred at 98 °C for 2 h 30 min. After cooling down to room temperature (rt), toluene (200 mL) was added to the solution. A brown precipitate was formed which was filtered off, washed with toluene (1 × 200 mL) and acetone (2 × 500 mL) and dried in vacuo to give glycidyl methacrylate-β-cyclodextrin (GMA-β-CD) as a beige solid (5.71 g, 91% yield). Molecular weight: 1277.13 $g{\cdot}mol^{-1}$. TLC: Rf = 0.44; NH_4OH 6%/EtOH/BuOH = 5/5/4. ATR FTIR (cm^{-1}): 3322, 2924, 1706, 1619, 1407, 1361, 1326, 1299, 1151, 1078, 1023, 939. ^1H NMR (400 MHz, D_2O, δ ppm): 6.11 (s, 1H, H_{19}), 5.72 (s, 1H, H_{19}), 5.09−4.97 (m, 4H, H_{12}, H_{15}), 4.12−3.42 (m, 33H), 1.90 (s, 3H, H_{18}).

2.3. β-CDs Support Synthesis. In a typical experiment, 4-nitrobenzenediazonium tetrafluoroborate salt (NBD; 23.7 mg, 0.1 mmol) and GMA-β-CD (644 mg, 0.5 mmol, 5 equiv) were dissolved in 15 mL of Milli-Q water. Subsequently, iron powder (300 mg) was added and Cr/Au glass substrates were immersed in this solution for 45 min at rt. After a short sonication and repeated washing with deionized water, the resulting supports were dried with N_2 and characterized by ATR-FTIR.

For surface acoustic wave (SAW) studies, a plain gold SAM5 sensor chip including five sensor channels was used. In a typical experiment, 4-nitrobenzenediazonium tetrafluoroborate salt (NBD; 23.7 mg, 0.1 mmol) and GMA-β-CD (644 mg, 0.5 mmol, 5 equiv) were dissolved in 15 mL of Milli-Q water. The first channel (Reference) of the SAW SAM5 chip was protected with an electroplating tape (3 M 470) to prevent grafting of GMA-β-CD. Subsequently, iron powder (300 mg) was added, and the protected SAW SAM5 chip was immersed in this solution for 45 min at rt. After a short sonication and repeated washing with deionized water, the chip was dried with N_2. The channel 1 (Reference) of this chip was further functionalized by the same procedure with another monomer to make a blank channel: to a solution of NBD (17 mg, 0.07 mmol) in 10 mL of 0.5 N HCl was added 2-hydroxyethyl methacrylate (1.2 mL, 10 mmol, 143 equiv). Iron powder (300 mg) was added and the first channel of the chip was immersed in this solution for 45 min at rt. After a short sonication and repeated washing with deionized water, the functionalized chip was dried with N_2 and characterized by ATR-FTIR.

2.4. Peptides Synthesis. Linear fluorescent peptides pMC, pDAC, SMMP-1, SMMP-2, and SMMP-9 were synthesized by solid phase peptide synthesis using Fmoc strategy as described by Moustoifa et al.[9] Solid phase peptide synthesis was carried out on an Applied Biosystems 433A automated peptide synthesizer. Reverse-phase HPLC was performed on a Hitachi LaChrom Elite equipped with an Organizer, Diode Array detector L-2450, Autosampler L-2200, pump L-2130 with a Satisfaction RP18AB 5 μm 250 × 4.6 mm C18 column for analytical session. Preparative HPLC was performed on a Shimadzu instrument equipped with a SCL-10 AVP system controller, LC8A HPLC pumps and SPD-10 AVP UV−vis detector probing at 214 nm on a Satisfaction RP18AB 5 μm 250 × 20 mm C18 column (C.L.I Cluzeau) for preparation. The following solvent systems were used for the elution in a linear gradient mode at a flow rate of 1 or 15 mL/min (for analytical and preparative HPLC, respectively): (A) 0.1% aqueous trifluoroacetic acid (TFA) and (B) 0.1% TFA in 70% aqueous acetonitrile (ACN). SMMP-1: m/e $[M + H]^+$ calcd, 1484.73 Da; found, 1484.64 Da. SMMP-2: m/e $[M + H]^+$ calcd, 1541.69 Da; found, 1541.56 Da. SMMP-9: m/e $[M + H]^+$ calcd, 1411.68 Da, found, 1411.65 Da.

2.5. Supramolecular Assembly Study. Supramolecular assembly investigations were performed using a SAM5 blue SAW sensor supplied by SAW Instruments GmbH (Bonn, Germany). The functionalized chip was inserted into the tempered flow chamber (22 °C) of the SAM5 blue SAW device and HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20, pH 7.4) was used as running buffer with a flow rate of 40 μL/min. All SAW data were determined by the SensMaster software, the autosampler is controlled by SequenceMaster (both by SAW Instruments GmbH). We used the high-quality injection method and the burst-on parameter of the SAW autosampler that allows immediate exchange of the analyte solutions avoiding intermixing of running buffer and analyte during the injection. Association and dissociation events were detected as phase shifts indicating binding and unbinding events. Corresponding binding kinetics were calculated via nonlinear curve fitting using Origin (Additive, Friedrichsdorf, Germany), FitMaster (SAW Instruments GmbH), and GraphPad Prism 5.3.

A lyophilized sample of SMMP-1 was used to make dilution series and to obtain the following concentrations in HBS-EP: 50, 100, 200, and 500 μM. After equilibration, 100 μL of each sample was injected. Each SMMP-1 injection was followed by a dissociation period of 5 min. Experiments were performed in triplicate to determine the system repeatability.

Resulting data were analyzed using the steady state algorithm $P = (P_{max}C)/(K_D + C)$ where P is the phase signal at T_{stop} (136.1 s), C is the SMMP-1 concentration, and P_{max} is the maximal phase signal.

A solution of amandatine (10 mM) was used to evaluate the coumarin group involved in the immobilization of the peptide on the β-CD surface. After complete MMP proteolysis, the resulting surface was submitted to the solution of amandatine (10 mM) and the resulting solution was analyzed by fluorescence spectroscopy.

2.6. Supramolecular Assembly for Enzymatic Assays. Supramolecular complex between peptides and the β-CDs supports were realized by immersing the β-CDs supports in different solutions containing 2 mg of each peptide (SMMP-1, SMMP-2, and SMMP-9) in 2 mL of TRIS buffer (0.1 M TRIS, 0.1 M NaCl, 10 mM $CaCl_2$, pH = 7.46). After 1 min of immersion in peptide solutions, peptides@β-CDs supports were washed with TRIS buffer and with Milli-Q water.

2.7. Evaluation of Support Regeneration. A regeneration experiment was performed with a β-CD solution (5 mM) in HBS-EP. First, 35 μL of a 100 μM SMMP-1 solution was injected to generate supramolecular assembly on the surface. After 2 min, 35 μL of a β-CD solution (5 mM) was injected. These steps were performed in triplicate. After each enzymatic assay, peptides@β-CDs supports were restored by immersing them in a β-CD solution (5 mM) for 5 min. All regenerations were analyzed by measurement of background fluorescence (λ_{Exc}= 350 and λ_{Em}= 430 nm) signals by epifluorescence imaging.

2.8. In Vitro Enzymatic Assays. First, supramolecular complex between peptides and the β-CDs supports were realized by immersing the β-CDs supports in different solutions containing 2 mg of each peptide (SMMP-1, SMMP-2 and SMMP-9) in 2 mL of TRIS buffer (0.1 M TRIS, 0.1 M NaCl, 10 mM $CaCl_2$, pH = 7.46). After 1 min of immersion in peptide solutions, peptides@β-CDs supports were washed with TRIS buffer and with Milli-Q water.

Human active MMP-1 and MMP-2 and Human MMP-9 (90% zymogen and 10% activated enzyme) were purchased from Calbiochem. MMP assays were performed in 0.1 M TRIS, 0.1 M NaCl, 10 mM $CaCl_2$, pH= 7.46. Commercial MMP-1, -2, or -9 (12.5 μL) under 90% zymogen form was activated with 10 μL of a solution of pAPMA (4-aminophenylmercuric acetate) (1 mM) at 37 °C for 3 h. MMP assays were performed in 0.1 M TRIS, 0.1 M NaCl, 10 mM $CaCl_2$, pH = 7.46. Peptides@β-CDs supports were placed in the bottom of fluorescence cells. Slit width was 10 nm for emission and 10

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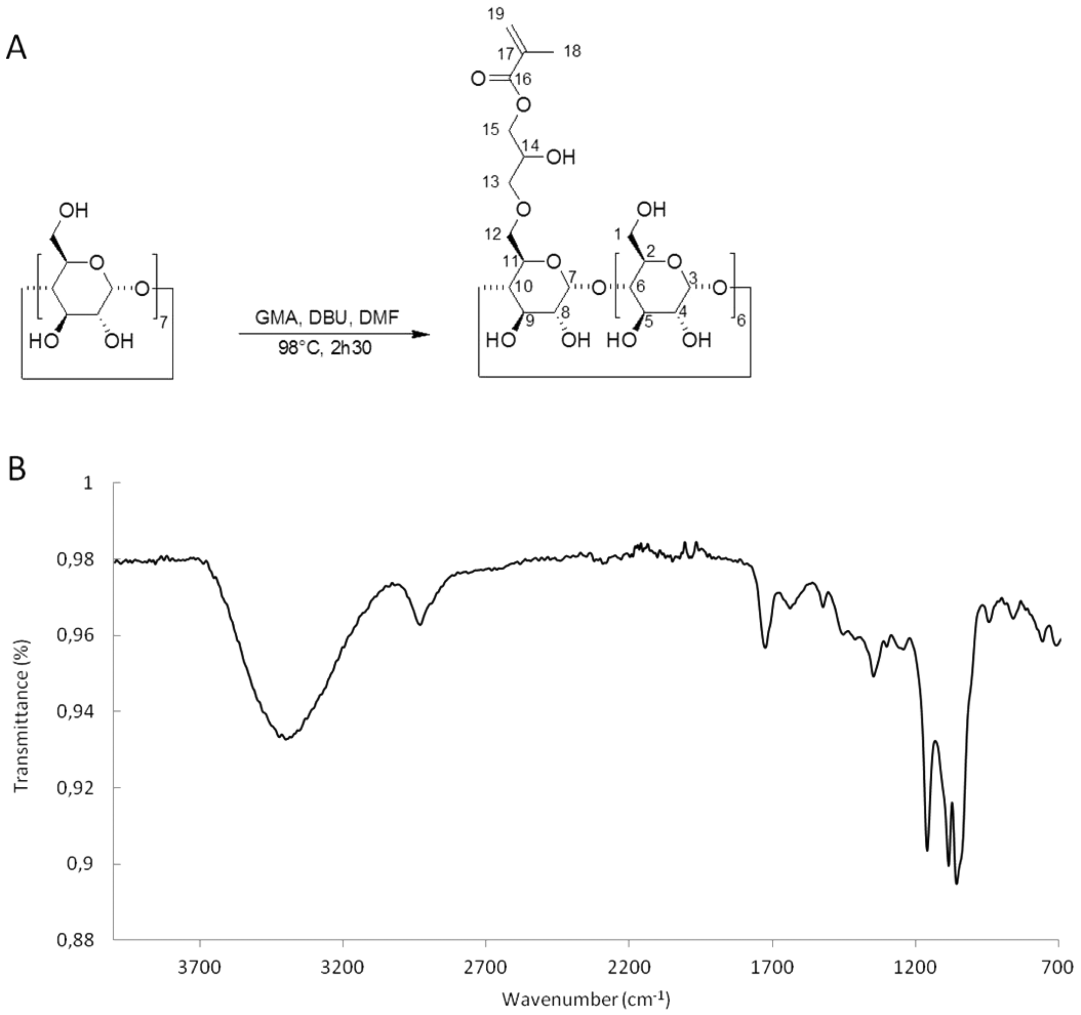

Figure 2. (A) Scheme synthesis of methacrylate β-cyclodextrin (GMA-β-CD) monomer. (B) ATR FTIR spectrum of polyGMA-β-CD layer obtained with the GraftFast process.

nm for the excitation. All assays were carried out in a total volume of 2 mL with 1 nM of pAPMA-activated MMPs. After the addition of enzyme to initiate the reaction, the initial rate of substrate hydrolysis was determined by monitoring the MC fluorescence intensity (Exc, 350 nm; Em, 401 nm) in the buffer.

2.9. Ex Vivo Enzymatic Assays. For the ex vivo enzymatic assays, peptides@β-CDs supports were placed in the bottom of fluorescence cells. Slit width was 10 nm for emission and 10 nm for the excitation.

2.10. Enzymatic Assays with Protein Extracts. Complex biological medium assays were carried out in a total volume of 2 mL of HeLa and HUh7 protein extracts. The initial rate of substrate hydrolysis was determined by monitoring the MC fluorescence intensity (Exc, 350 nm; Em, 401 nm) in the buffer.

2.11. Enzymatic Assays with Cell Suspension. Cell assays were carried out in a total volume of 2 mL of buffer containing HeLa or HUh7 cell line suspensions with an overall number of 10^6 cells. The initial rate of substrate hydrolysis was determined by monitoring the MC fluorescence intensity (Exc, 350 nm; Em, 401 nm) in the buffer.

2.12. Cell Line Cultures. All the cell lines used were cultured in culture T75 flasks (seeding cell is 2×10^4 cells/cm^2) as monolayer culture. Cell lines were cultured in a nutritive mixture Eagle liquid modified by Dulbecco (DMEM) containing 4.5 g/L of glucose. All the culture mixture contained glutamine which was added after the use of fetal calf serum heated for 30 min at 56 °C. Cells were cultured at 37 °C in a humid atmosphere in the presence of 5% of CO_2. The culture mixture was renewed every 2 or 3 days. When cells became subconfluent, they were systematically transplanted. Thus, cells were detached from their supports by incubating them in trypsin solution for 5 min at 37 °C. Trypsin action was inhibited by the addition of DMEM (Dulbecco's minimal essential medium) with 10% of FCS (fetal calf serum) which is responsible of the trypsin inhibition. After this medium was added to the cell suspension, the resulting cell suspension was washed with PBS buffer (pH = 7.4) and collected by centrifugation. The number of obtained cells was between 5 and 6 million. A part of the cells suspension was cultured in a new support with a new culture mixture.

2.13. Protein Extracts. Cells were cultured in culture flasks in order to have enough for a protein extraction for further analysis. Cells were then washed with PBS (pH = 7.4) and treated with trypsin before being centrifuged at 1500 rpm during 5 min. After that, cell's pellet was resuspended in a freeze−thaw (FT) lysis buffer (KCl 600 mM; TRIS-HCl 20 mM pH 7.8; Glycerol 20%) in which an inhibitor of phosphatase (Sigma) was added. Cellular lysis was induced by a thermal shock involving three successive stages of freezing in liquid nitrogen and a thawing at room temperature. Lysis solution was then centrifuged for 15 min at 13 000 rpm at 4 °C, and the supernatant was collected.

Protein concentration in the cellular extracts was evaluated according to an adaptation of Lowry method's using DC protein assay (BioRad) reagents with an alkaline solution of copper tartrate (solution A) and a diluted solution of Folin reagent (solution B). The dosage was performed using a 96-well plate by successively adding 5 μL of protein extracts diluted at 1/5, 25 μL of solution A, and 200 μL of solution B.

After 20 min of incubation at room temperature, the mixture absorbance was measured at 750 nm in plate reader (PowerWaveX,

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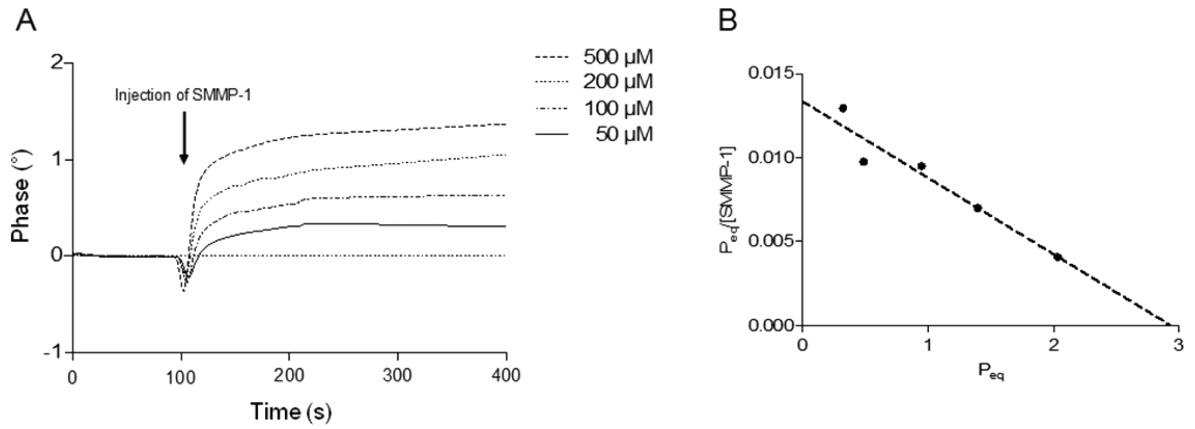

Figure 3. Supramolecular peptide/surface assembly study. (A) SAW sensorgrams of injected SMMP-1. The sensorgrams show the binding responses of SMMP-1 to the β-CD support. Concentrations of injected SMMP-1 for each sensorgram are given in the Figure. (B) Scatchard plot of SMMP-1 binding data on β-CD support. The straight line confirms the 1:1 interaction mode between the MMP substrate and the β-CD support.

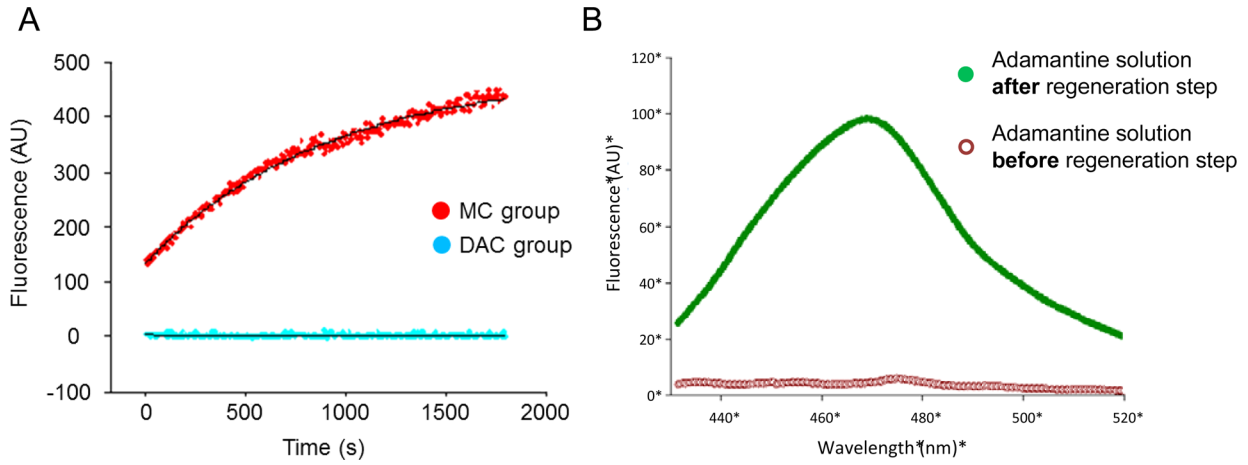

Figure 4. (A, blue) DAC and (red) MC fluorescence intensity variation during SMMP@ β-CD support proteolysis by MMP1 (λ_{ex} = 350 nm and λ_{em} = 401 nm for MC; λ_{ex} = 430 and λ_{em} = 470 nm for DAC). (B) DAC fluorescence after it release from β-CD cavities by competitive guest (adamantane; λ_{ex} = 430 and λ_{em} = 470 nm).

Bio-Tek). Protein concentration was determined by a comparison with standard range of bovine serum albumin (BSA) at 0.25, 0.5, 0.75, 1, and 1.5 μg.

3. RESULTS AND DISCUSSION

3.1. Surface Synthesis. To develop a β-cyclodextrin surface, with more flexibility and lower steric hindrance than previous β-CD SAMs, which can be easily transferred to a wide variety of support, we have used a versatile surface chemistry named GraftFast, which is based on redox grafting of aryl diazonium salts with vinylic monomers.[21,22] This process leads to the formation of thin polymer layer (a few nm) on raw conductive, semiconductive, and insulating materials. It consists of a short one-step reaction that takes place at atmospheric pressure from aqueous solutions and leads to stable, homogeneous, and covalently coated thin polymer film.

For this purpose, we have synthesized a methacrylate β-cyclodextrin (GMA-β-CD) monomer as previously described in ref 23 (Figure 2A). An aqueous solution of 4-nitrobenzediazonium tetrafluoroborate salt and GMA-β-CD with iron powder as reducer was used to produce our β-CD surface on Cr/Au glass substrates or on SAW (surface acoustic wave) SAM5 chip. The ATR FTIR spectrum confirmed the presence of a polyGMA-β-CD layer ($\nu_{C=O}$: 1 722 cm^{-1}, β-CD: 1174−1077 cm^{-1}) on the gold substrates (Figure 2B). Our attention was focused on MMP-1 for its implication in metastatic process and on MMP-2 and MMP-9 for their overexpression in numerous steps of tumor progression.[24,25] Synthetic substrates of MMP-1, MMP-2, and MMP-9 were synthesized as previously described by Moustoifa et al.[9] Peptide sequences were chosen from Nagase's work[26] and derived from sequences of natural protein cleavage sites of MMPs. These systems have demonstrated their effectiveness for measuring in vitro proteolysis.[9]

3.2. Supramolecular Assembly Study. Cyclodextrins play a key role in our system to immobilize labeled peptides on the sensor surface. These hollow structures, shaped like a truncated cone with a hydrophilic exterior and an apolar cavity, have a diameter of about 6.6 Å. They are able to form inclusion complexes in aqueous solutions with a large variety of molecules, the stability of such complexes depending on the size and hydrophobic character of the guest molecule.[27,28] In particular, β-CDs perfectly accommodate coumarin derivatives,[29−36] from 1:1 host−guest complex to higher stoichiometry.[37,38] We have previously demonstrated that our biscoumarinic MMP substrates interact with β-CD in solution and form only a stable supramolecular complex between diethylaminocoumarin (DAC)/ β-CD with a K_a constant of 3236 M^{-1}.[20] To

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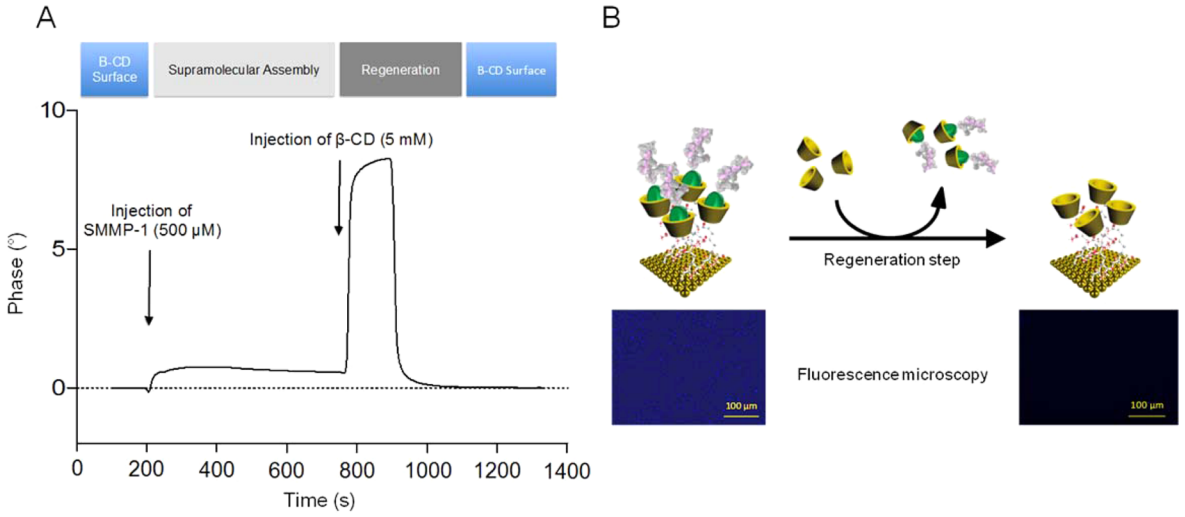

Figure 5. (A) SAW sensorgram of the regeneration of the β-CD support. First, SMMP-1 (500 μM) was injected to form the supramolecular assembly. After reaching equilibrium, a solution of β-CDs (5 mM) was injected to disrupt the supramolecular assembly and regenerate the β-CD support. (B) Epifluorescence pictures of peptides@β-CD supports. Left picture depicts fluorescence emission (DAC emission) of the supramolecular assembly after proteolysis by MMPs. Right picture shows no fluorescence emission after immersion in a β-CDs solution which confirms the regeneration of the β-CD support.

validate the ability of our biscoumarinic MMP substrates to form a stable supramolecular assembly with our β-CD surfaces, were performed surface acoustic wave (SAW) studies to investigate their interactions. A SAW SAM5 chip (5 channels, one for reference and the others for binding studies) was modified with the GraftFast process with two different monomers: 2-hydroxyethyl methacrylate (HEMA) for the reference channel and GMA-β-CD for the measuring channels. The modified chip was mounted in the microfluidic cell of the SAM5 blue SAW sensor. HBS-EP buffer with Surfactant P20 (0.005%, v/v) was used as running buffer. As a peptide model, addition of SMMP-1 (50, 100, 200, and 500 μM) to the microfluidic cell resulted in significant binding being observed in the SAW sensorgram (Figure 3A). The affinity of our MMP substrate with our β-CD surface was calculated by fitting the resulting data at different concentrations of SMMP-1 using a 1:1 Langmuir Model, which gave a K_a constant of 5228 M^{-1}. To validate the 1:1 host−guest complex between our biscoumarinic MMP substrate and the β-CD surface, a Scatchard plot (P_{eq}/[SMMP-1] = $f(P_{eq})$) was performed (Figure 3B). The plot gives a straight line that confirms the 1:1 interaction mode between the MMP substrate and the β-CD support. In order to confirm that DAC group is only involved in the host−guest complex, a β-CDs support was immersed in a solution of SMMP-1 at 1 mM during 15 min, and then rinsed with Milli-Q water to remove nonfixed peptides. Afterward, the SMMP-1@β-CDs support was submitted to proteolysis by MMP-1 (1 nM) for 30 min.

Fluorescence emission in solution of the two coumarin groups was monitored by fluorescence spectroscopy during enzymatic reaction (Figure 4). It was clearly observed that the fluorescence signal due to the MC fluorophore was increased, while that of DAC could not be monitored (Figure 4A). During proteolysis, the peptide fragment that bears the MC group was released in solution, whereas the peptide fragment that bears the DAC group seems to remain complexed with β-CD. After proteolysis, the resulting peptide@β-CDs support was submitted to a solution of amantadine, a well-known high-affinity guest for β-CD, to destruct the complex formed between coumarinic residue and β-CD support. The fluorescence spectroscopy study of the resulting solution clearly indicated the presence of the DAC group (Figure 4B). This result confirms that only one complex (DAC/β-CD) takes place in our supramolecular assembly. All of these results highlight that our doubly labeled peptides formed a 1:1 supramolecular assembly through only their DAC moiety with the β-CD support, as expected from the previous studies in solution.[20] Due to similar peptide structure and our previously published data in solution[20] of these peptides (SMMP-1, 2, and 9), we expect that K_a values for SMMP-2 and SMMP-9 are in the same range as those for SMMP-1.

To validate the sensor regeneration, a supramolecular assembly between β-CD modified SAW SAM5 chip and SMMP-1 (500 μM) was performed. After reaching equilibrium, a solution of β-CD (5 mM) was injected, and the phase variation was measured. Resulting data show a return to a zero phase signal as before the SMMP-1 injection (Figure 5A). This result was confirmed by fluorescence microscopy after MMP proteolysis of SMMP-1. As demonstrated previously, only the peptide fragment that bears the DAC group acts as a guest on β-CDs support. After excitation, only DAC fluorescence emission was observed after proteolysis and disappeared after supramolecular system dissociation by β-CD solution (Figure 5B). These results again validate the facile regeneration of our biosensing area by simple incubation. Thereby, the same β-CD support has been used for all further in vitro and ex vivo enzymatic assays using this rapid and easy regeneration step.

3.3. In Vitro and Ex Vivo Enzymatic Assays. Peptide@β-CDs supports were submitted to proteolysis with purified commercial human MMPs. β-CDs supports were immersed in peptides substrate (SMMP-1, -2, and -9) solutions and finally were rinsed to remove unanchored peptides. Peptide@β-CDs supports were then submitted to enzymatic proteolysis. Each MMP substrate supramolecular assemblies were cleaved by its own enzyme at an optimum concentration of 1 nM. Monitoring MC fluorescence vs time shows that MMPs recognize and cleave substrates engaged in β-CDs supports. Specificity constant k_{cat}/K_m was calculated from the increase of MC

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fluorescence in solution. The apparent first-order rate constant was calculated by fitting the progress curves according to eq 1:

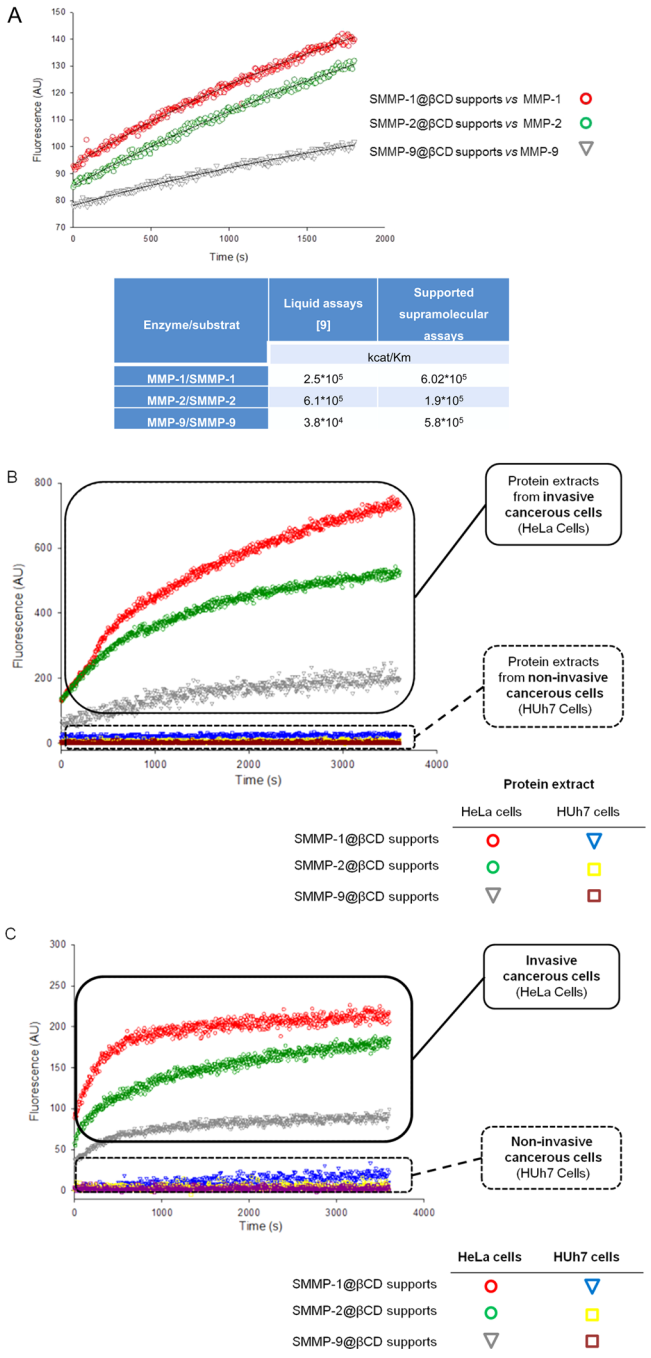
$$F(t) = F_0 + \Delta F(1 - \exp(-k_{obs}t)) \quad (1)$$

where $\Delta F = F_{max} - F_0$ and k_{obs} is the slope of the curve. The second order rate constant k_{cat}/K_m was then calculated using 2:

$$k_{cat}/K_m = k_{obs}/[E_0] \quad (2)$$

where k_{cat} is the catalytic constant, K_m is the Michaelis constant, k_{obs} is the observed constant (slope of the hydrolysis curve), and E_0 is the total enzyme concentration in the solution. The table in Figure 6A presents the second-order rate constant (k_{cat}/K_m) obtained with peptides@β-CDs supports compared to solution assays with the same substrates.[9] Substrate specificity is maintained by supramolecular surface assembly and can be eventually increased compared to solution assay as show the results for SMMP-9@β-CDs support.

To validate the effectiveness of our MMP sensor toward tumoral biopsy, peptide@β-CDs supports were first incubated with protein extracts (20 μL of a protein solution with a concentration of 4 μg/μL) from HeLa and from HUh7 cells as a control. In fact, HeLa cells are well-known to present invasive and metastatic characters[39,40] and to overexpress several MMPs, among them MMP-1, -2, and -9.[41] Unlike these cells, liver cancer cell lines HUh7 show noninvasive and non-metastatic properties which result in basal or very low expression of MMP-2 and MMP-9.[42,43] Proteolysis monitoring of peptide@β-CDs supports with protein extracts of HUh7 or HeLa cells show an obvious difference (Figure 6B) with a major proteolysis for HeLa experiments and a very low peptide degradation for HUh7 experiments. These results are in agreement with the relation between the quantity of secreted MMPs and the invasive properties of cancer cells. Nevertheless, this result also highlights a quite surprising and important fact: the supramolecular assembly between linear peptide and the β-CDs supports resist unspecific proteolysis. In fact, it is well-known that linear peptides are not stable in biological medium resulting in unspecific proteolysis. Currently, to improve their stability, linear peptide cyclization is used, as described in our previous work on cyclic synthetic MMP substrates.[9] In concordance with cell line models used in this study, Figure 6B shows that peptide@β-CDs supports can discriminate the high level of MMPs activities in the HeLa protein extracts compared to those of HUh7. Next to these results, the peptide@β-CDs supports were submitted directly to proteolysis by HeLa and HUh7 cell line suspensions with an overall number of 10^6 cells in order to mimic biopsy conditions. Similar results to those obtained with protein extracts are displayed in Figure 6C and highlight the great potential of our concept for screening MMP activities from in vitro conditions to tumoral biopsy, even with a limited number of cells (10^6 cells).



Enzyme/substrat	Liquid assays [9]	Supported supramolecular assays
	kcat/Km	
MMP-1/SMMP-1	$2.5*10^5$	$6.02*10^5$
MMP-2/SMMP-2	$6.1*10^5$	$1.9*10^5$
MMP-9/SMMP-9	$3.8*10^4$	$5.8*10^5$

Figure 6. (A) Fluorescence variations of MC in solution after proteolysis of peptide@ βCD supports (fluorescence excitation at 350 nm and Fluorescence emission at 401 nm) and specificity constant k_{cat}/K_m for supported supramolecular assemblies compared to liquid assays. Fluorescence variation of MC during incubation of peptide@β-CDs supports with (C) HeLa cells, HUh7 cells, and (B) their respective protein extracts.

4. CONCLUSION

Our results demonstrate that a supramolecular peptide/surface assembly based on specific association between FRET linear peptides and a β-CDs support can determine specific MMP activities from in vitro conditions to tumoral biopsy, even with a limited number of cells (10^6 cells). In vitro activity measurements are in agreement with those obtained in liquid assays from our previous works. Moreover, for some cases, we also observe that resulting supramolecular assemblies can improve substrate specificity. Ex vivo data highlight that our system is efficient for screening MMP activities and distinguishing between invasive and noninvasive cancerous cell suspensions. These results confirm that our system is a powerful tool for the determination of not only the tumor aggressiveness but also for the resulting chemotherapy management. Due to the versatility of the surface chemistry used for the β-CDs support synthesis, our system could be easily transferred to a large variety of transducer systems, for

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example, surface plasmon resonance and quartz crystal balance devices. Surface sensor regeneration during in vitro and ex vivo experiments validates our approach as an innovative technical building block for future development of lab-on-chip devices.

AUTHOR INFORMATION

Corresponding Author

*E-mail: thomas.berthelot@cea.fr.

Notes

The authors declare no competing financial interest.

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DEDICATION

[⊥]In memory of Professor Gérard Déléris (1950−2012).

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