Development and Validation of a Fluorescence Method to Follow the Build-up of Short Peptide Sequences on Solid 2D Surfaces

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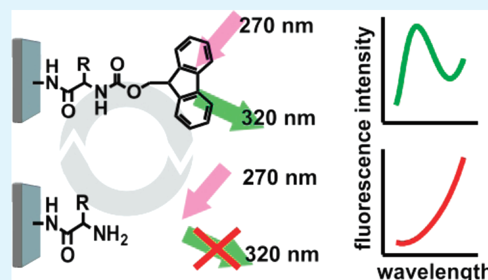
Supporting Information

ABSTRACT: The modification of material surfaces with short peptide sequences has become an essential step in many biotechnological and biomedical applications. Due to their simple architecture compared to more complex 3D substrates, 2D surfaces are of particular interest for high throughput applications and as model surfaces for dynamic or responsive surface modifications. The decoration of these surfaces with peptides is commonly accomplished by synthesizing the peptide first and subsequently transferring it onto the surface of the substrate. Recently, several procedures have been described for the synthesis of peptides directly onto a 2D surface, thereby simplifying and accelerating the modification of flat surfaces with peptides. However, the wider use of these techniques requires a routine method to monitor the amino acid build-up on the surface. Here, we describe a fast, inexpensive and nondestructive fluorescence based method which is readily accessible to follow the amino acid build-up on solid 2D samples.



KEYWORDS: *surface modification, surface analysis, peptide surfaces, solid state fluorescence, peptide synthesis*

INTRODUCTION

Surfaces that display short peptide sequences are widely used in biological and biomedical applications.[1,2] Organic and inorganic particles have been decorated with peptides to achieve targeted delivery of drugs,[3] enhance cellular uptake[4] or detect enzyme activity.[1,5] The internal surfaces of 3D scaffolds and nanostructures were modified to control drug release[6] or provide biocompatible tissue supports.[7,8] Flat 2D peptide surfaces are attractive for cell culture,[9,10] high throughput applications,[11] and — because of their simpler architecture — as models for dynamic or smart surfaces.[12,13] The advantages of short peptides for biomaterial applications have been highlighted in a recent debate[14,15] and they were deemed to hold a strong promise for future technologies because of the possibility to incorporate multifunctionality and modularity into the peptide sequence.[16]

The fabrication of 2D peptide surfaces commonly involves the separate preparation of the peptide via solid phase peptide synthesis (SPPS),[17] cleavage of the peptide from the solid support and subsequent coupling to the 2D surface. This 'grafting-to' procedure not only adds two additional steps to the sample preparation, it is also known to give lower packing densities compared to a direct build-up of the material from the substrate ("grafting from").[18] The advantage of the SPPS approach, however, is that small amounts of samples can be drawn at every coupling step to confirm the successful attachment of the amino acid.[19]

The development of the SPOT synthesis has provided the basis for a relatively simple way to prepare peptide microarrays on nitrocellulose membranes.[6] Recently, this technique has been extended to self-assembled thiol monolayers on gold by building up peptide sequences from the thiol linker, thus demonstrating the applicability of SPOT synthesis to flat surfaces.[20] The amino acid build up was monitored by MALDI-ToF mass spectrometry at each stage, relying on the ability of the matrix assisted laser desorption to detach the thiol from the metal surface to analyze the complete peptide sequence.[21] The limitation of this approach is that it can only be applied to gold substrates. Amino-acid build-up on 2D silica surfaces has been demonstrated using standard SPPS protocols[12] and has even been accomplished in a commercial microwave peptide synthesizer.[18] However, the wider use of this approach is hampered by the lack of an inexpensive, fast, and readily available technique to monitor the amino acid build-up.

Although surface analytical techniques such as water contact angle (WCA) measurements, X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) are able to provide information on the modification of the sample surface, usually by using the presence/absence of fluorenylmethyloxycarbonyl (Fmoc) as a marker,[13,22] they are not amenable for routine analysis at every

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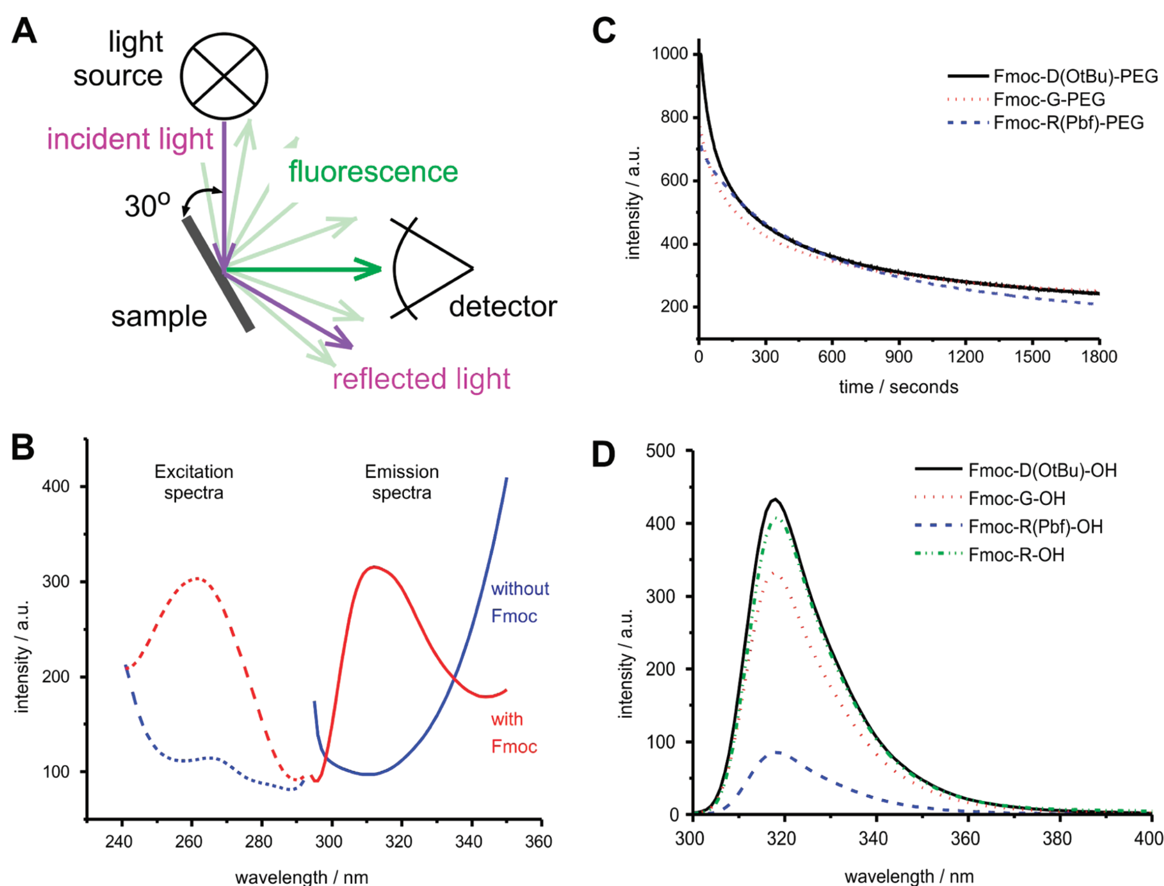


Figure 1. (A) Experimental setup of the solid state fluorescence measurements. (B) Solid-state excitation (dashed lines, λ_{Em} = 320 nm) and emission spectra (solid lines, λ_{Ex} = 270 nm) of surface attached Fmoc-GD and GD. (C) Bleaching of surface attached Fmoc-D(OtBu), Fmoc-G and Fmoc-R(Pbf) when continuously exposed to the excitation light (λ_{Ex} = 270 nm). (D) Fluorescence emission at λ_{Ex} = 290 nm of the Fmoc-amino acids in solution (10 mM in acetonitrile/water 1:1). tBu, tert-butyl; Pbf, pentamethyldihydrobenzofuran-5-sulfonyl.

stage of the amino acid build-up. These techniques either cannot clearly distinguish between different amino acids/protecting groups (WCA) or are too time-consuming, expensive, and not readily accessible for routine process control (ToF-SIMS, XPS).[22]

Here, we present a fluorescence technique that is able to follow the coupling of Fmoc amino acids to solid surfaces rapidly and inexpensively using a standard fluorescence spectrophotometer. Making use of the fluorescence properties of the Fmoc group, we are able to confirm the attachment of Fmoc amino acids and the subsequent Fmoc deprotection. This new method will be a useful tool which will make the preparation of peptide surfaces faster and more reliable.

■ EXPERIMENTAL SECTION

Peptide Surface Preparation. Glass substrates (round coverslips, *d* = 13 mm, VWR) were cleaned by sonication in acetone, ethanol, methanol and water for 15 min each, immersion in piranha solution (H_2SO_4: H_2O_2 = 7:3; ***Caution:*** *piranha solution is extremely corrosive and can react explosively with organic matter*) and subsequent washing with Milli-Q water three times.

The dry glass surfaces were immersed in (3-glycidyloxypropyl)trimethoxysilane (GOPTS, Sigma-Aldrich, Product Nr. 440167) and incubated at 37 °C for 1 h. Immediately after washing with acetone three times, PEG_{18}-diamine ($PEG\text{-}NH_2$, Polypure, Product Nr. 12112−1895) was melted onto the dry surfaces and left to react in the oven at 75 °C for 2 days. The samples were washed in Milli-Q water three times and blow dried under nitrogen.

For the peptide synthesis, the dry $PEG\text{-}NH_2$ surfaces are placed in a dry glass Petri dish. The Fmoc amino acid (for details, see the Supporting Information) was prepared as a 20 mM solution in dry DMF (Sigma-Aldrich, Product Nr. 227056) containing 40 mM (2 equ) ethyl(hydroxyimino)cyanoacetate (EHICA, Sigma-Aldrich, Product Nr. 233412) to suppress racemisation. Typically, 14 mL of this solution was used to immerse up to 50 samples. To this solution 2 equiv. of N,N′-diisopropylcarbodiimide (DIC, Sigma-Aldrich, Product Nr. 38370) were added. The reaction was allowed to proceed at room temperature under gentle agitation on a rotary shaker for 2 h. After this, the samples were washed with DMF, ethanol, methanol, and DMF and the same reaction was repeated and left to react overnight to maximize the yield. The samples were washed in DMF, ethanol, methanol, DMF (15 min each) and Fmoc deprotection was performed by immersing the samples in 20% piperidine (Sigma-Aldrich, Product Nr. 10409−4) in DMF for 2 h at room temperature under gentle agitation. After washing in DMF, ethanol, methanol and DMF (15 min each) the next amino acid was attached using the same procedure. After completion of the peptide sequence, the side protection groups were removed by immersion in trifluoro acetic acid (TFA, Sigma-Aldrich, Product Nr. T6508) for 4 h. For the attachment of each amino acid, samples were drawn before and after Fmoc deprotection after the methanol washing stage. The

samples were additionally washed in Milli-Q water three times and blow dried under nitrogen before analysis.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorimeter. The solid samples were mounted on a custom built sample holder with a rotating base. The samples were placed at a 30° angle to the incident light to minimize the amount of reflected excitation light on the detector (see Figure 1A). The spectra were recorded immediately after exposure of the sample to the excitation light using a slit width of 20 nm for both the light source and the detector. Emission spectra were recorded with λ_{Ex} = 270 nm and excitation spectra with λ_{Em} = 320 nm. The spectra of the Fmoc-amino acid solutions were recorded at 10 mM concentration with λ_{Ex} = 290 nm and slit widths of 3 nm.

Water Contact Angle. Static WCA measurements were performed with a DSA30 (Krüss) using Milli-Q water. The drops were placed on the surface and high contrast images captured after 10 s. A circular fit was applied to the drop outline from which the WCA was determined. The data is an average from 3 samples with 5 measurements from each surface.

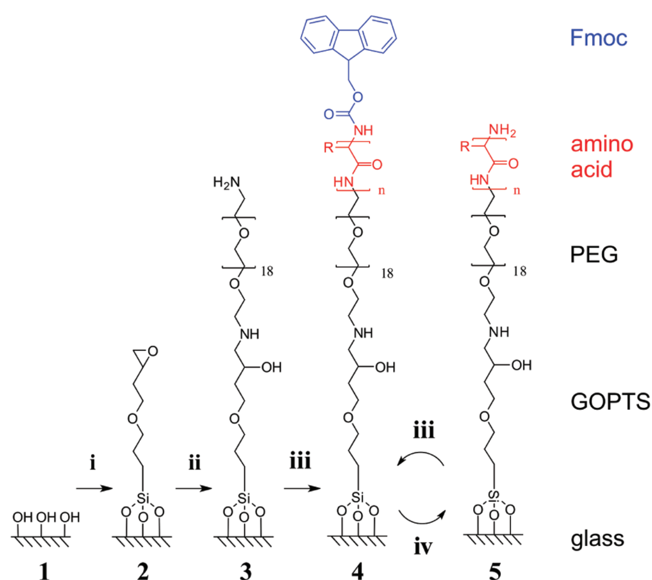
Time of Flight Secondary Ion Mass Spectrometry. ToF-SIMS analysis was performed using a ToF-SIMS IV time-of-flight instrument (ION-TOF GmbH). A 25 kV Bi_3^+ liquid metal ion gun (25 kV, 1.0 pA pulsed target current, 10 kV post acceleration) was used as the primary ion source and the mass fragments were analyzed with a single stage reflectron analyzer. A flux of low energy electrons (20 eV) was used for charge compensation. Images were obtained by rastering the surface (256 × 256 pixels); the displayed spectra were normalized to the total ion intensity.

RESULTS AND DISCUSSION

The synthesis of short peptides on flat, solid surfaces is shown in Scheme 1. It followed a previously described procedure that has been developed using surface analysis to optimize each step.[12] Before the peptide build-up, a short amine-terminated PEG chain is attached to the surface via an epoxy-terminated silane (GOPTS) to provide a more flexible linker to the substrate which facilitates the attachment of amino acids to the surface and provides a suitable platform for bioassay applications. The tethering of the PEG amine results in the functionalization of the surface with amine groups that cannot exceed a monolayer coverage. Because of this low amount of material on the surface, routine monitoring of subsequent surface modifications is difficult and requires the development of a suitably sensitive method.

The amino acid build-up is based on standard solid phase peptide synthesis on solid particles.[17] However, in the case of flat surfaces the choice of the coupling procedure has to satisfy different requirements. In addition to maximizing the yield and driving the reaction at a solid−liquid interface, the byproducts have to be soluble in the solvent to prevent precipitation on the substrate. It is known that the byproducts of a DIC mediated amide bond formation are highly soluble.[23] In initial screening experiments we have observed that the reaction with DIC proceeds with apparently higher yields (higher fluorescence intensities) compared to HBTU (O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate) and NHS (N-hydroxysuccinimide) activation, making DIC the reagent of choice. This method was employed to prepare short amino acid sequences on the amine terminated surface and follow the amino acid build-up on the solid substrate. Arg-Gly-Asp (RGD) was chosen as an example peptide because of its

Scheme 1. Direct Preparation of Peptide Surfaces on Glass Substrates[a]



[a](i) (3-glycidyloxypropyl)trimethoxysilane (GOPTS), 37°C; (ii) PEG_{18}-diamine, 75°C; (iii) Fmoc-amino acid, ethyl(hydroxyimino)-cyanoacetate (EHICA), N,N′-diisopropylcarbodiimide (DIC), DMF, 2h; coupling reaction repeated over night; (iv) 20% piperidine in DMF.

biological importance and widespread use in biomaterial surface modifications.

Confirming the attachment of each amino acid in a routine, cheap and easily accessible manner is essential. The fluorescence method we have developed takes advantage of the fluorescence properties of the Fmoc group which is a commonly used protection group for the amine terminus of amino acids in peptide synthesis. In solution, Fmoc amino acids are known to have adsorption maxima near 290 nm and fluorescence emission maxima around 320 nm (Figure 1). To measure the Fmoc fluorescence in the solid state on a flat substrate, the sample was mounted in the light path of the spectrometer such that the path of the incident light was at a 30° angle to the surface (Figure 1A). This ensured that only a small amount of reflected light reaches the detector while collecting a maximal amount of the fluorescence emission from the sample. As the amount of material on the sample is expected to be close to a monolayer, the amount of Fmoc on the surface is very small. Therefore, to obtain a reasonable fluorescence signal, the slit width for both the light source and the detector was set at its maximum value (20 nm) to ensure a detectable fluorescence emission.

In many instances, solid state fluorescence has different characteristics than the same material in solution because of the absence of solvent effects on the energy states of the molecule. Figure 1B shows the excitation and emission spectrum of Fmoc-GD on the surface. Compared to the solution, both the excitation and emission maximum shifted to lower wavelengths, with λ_{Ex} = 262 nm and λ_{Em} = 312 nm. However, to clearly distinguish the Fmoc fluorescence from the background and obtain a good signal contrast between these two, the samples were excited at a slightly higher wavelength (270 nm) than the excitation maximum. Removal of the Fmoc protection group from the surface resulted in a loss of the Fmoc fluorescence.

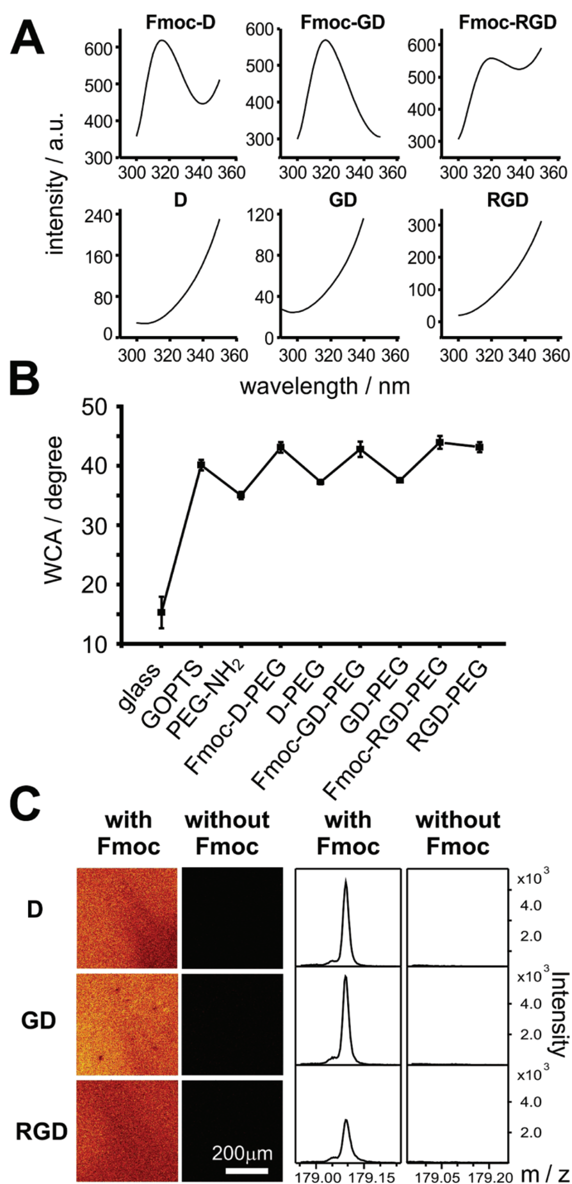


Figure 2. Comparison of different methods to follow the amino acid build-up using the RGD sequence as an example. (A) Solid state fluorescence at λ_{Ex} = 270 nm. (B) Water contact angle measurements. (C) ToF-SIMS analysis showing the intensities of an Fmoc marker fragment, $C_{14}H_{10}^{+}$, at m/z = 179; left, surface scans; right, mass spectra.

Figure 1B shows that only non specific background intensities are observed in the emission spectra.

The intense illumination necessary to generate a fluorescence signal of reasonable strength and the small amount of material present on the surface render the material susceptible to bleaching. To investigate the stability of the surface immobilized Fmoc, each of the three amino acids (Fmoc-Arg, Fmoc-Gly and Fmoc-Asp) were coupled individually onto the amine surface and exposed to the excitation light continuously for 30 min. When following the fluorescence intensity of these samples at λ_{Ex} = 270 nm over time (Figure 1C) a sharp decrease in intensity is observed within the first 5 min which continues over the whole period monitored. The strong susceptibility of the samples to bleaching is a combined effect of the low amount of fluorophore on the surface, high excitation light intensity and the inability to recover bleached areas by diffusion that would be possible in solution. Therefore, fluorescent detection of the Fmoc in the solid state requires fast data acquisition which should be kept within 5 min after exposure to the light source and repeated measurements of the same sample should be avoided. Under these premises, the solid state fluorescence method to detect the presence/absence of amino acids on solid surfaces is suitable for qualitative analysis to confirm the successful Fmoc-amino acid coupling and Fmoc deprotection, but it cannot be used quantitatively to assess the amount of Fmoc-amino acid immobilized on the surface.

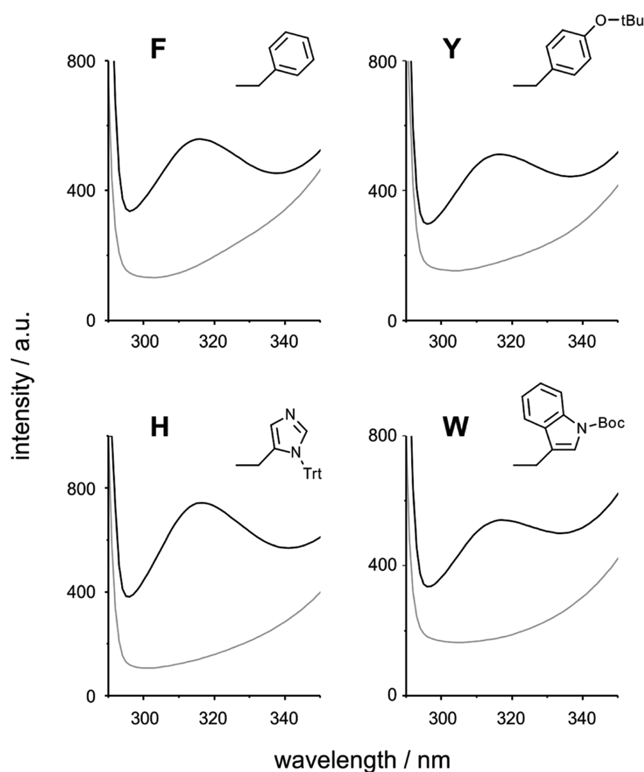


Figure 3. Solid-state fluorescence spectra of side group protected aromatic amino acids with (black line) and without (gray line) the Fmoc protection group. The amino acids are directly attached to the PEG amine surface. tBu, tert-butyl; Trt, triphenylmethyl; Boc, tert-butyloxycarbonyl.

To investigate if the Fmoc fluorescence depends on the amino acid attached to it, we measured the emission of Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, and Fmoc-Arg(Pbf)-OH (Figure 1D). This experiment was performed in solution since the fluorescence intensities on the surface bleach too quickly to allow a reliable comparison between the samples. While the emission intensities of Fmoc-Asp(tBu)-OH and Fmoc-Gly-OH are in the same order of magnitude, the fluorescence of Fmoc-Arg(Pbf)-OH is 3 to 4 times lower. This appears to be related to the aromatic nature of the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protection group on Arg since the fluorescence of the unprotected Fmoc-Arg-OH is comparable to that of the other two amino acids. To investigate if this effect is also present when the Fmoc is not directly attached to Arg but to an intermediate amino acid, we measured the fluorescence emission of Fmoc-Gly-Arg-OMe and Fmoc-Gly-Arg(Pbf)-OMe (see the Supporting Information, Figure 2). We found that Pbf inducing quenching of the Fmoc fluorescence occurs to a similar extent, indicating that the effect is

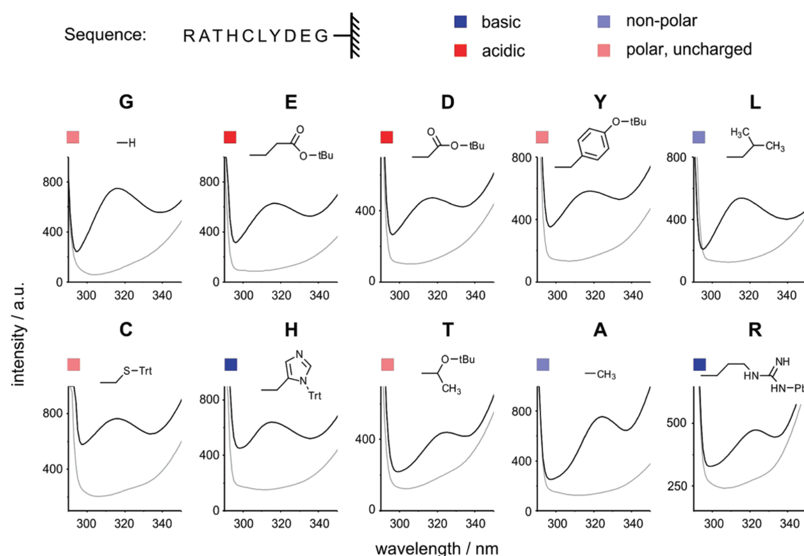


Figure 4. Fluorescence monitoring of the build-up of a sequence of ten different side group protected amino acids from the PEG-amine surface. Black line, Fmoc protected; gray line, after Fmoc deprotection; tBu, tert-butyl; Trt, triphenylmethyl; Pbf, 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl.

independent of the structure of the amino acid. Hence, it can be expected that Fmoc-Arg(Pbf)-OH and Fmoc amino acids subsequently attached to it will also display a less intense fluorescence emission in the solid state.

To validate the solid-state fluorescence method against other techniques, we compared the fluorescence data for the build-up of RGD to water contact angle (WCA) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis (Figure 2). Figure 2A shows how the RGD build up can be followed via solid-state fluorescence. The presence or absence of the Fmoc group during the peptide synthesis can be monitored as an on/off behavior of the Fmoc fluorescence on the surface. Based on the effects of the Pbf group observed in solution, the less distinct peak shape for Fmoc-RGD may be attributed to the reduction in Fmoc fluorescence induced by the Pbf group on Arg, as well. However, the significantly different acquisition parameters (slit width, excitation wavelength) compared to the solution phase fluorescence spectra in Figure 1D prohibit further quantitative comparison between the two data sets.

The WCA measurements (Figure 2B) show an increase in hydrophobicity after the attachment of the linker (GOPTS) which is reduced again when amide functionalities are introduced to the surface via the PEG-diamine. The subsequent attachment of the Fmoc amino acids is evident as a WCA increase of approximately 5°. Removal of the hydrophobic Fmoc generates a free amine surface again, causing the WCA to decrease to a similar value as on the PEG-NH_2 surface. An exception for this is the deprotection of Fmoc-RGD, where the WCA remains almost unchanged even though the RGD surface is also expected to present free amines. Hence, even though WCA measurements are a cheap and easily accessible tool to monitor Fmoc deprotection, the wettability is not only affected by the presence/absence of the Fmoc group but also depends on the amino acid side chain.

ToF-SIMS is able to identify the presence of Fmoc via a specific marker fragment ($C_{14}H_{10}^+$, $m/z = 179$).[24] The spectra in Figure 2C show the presence/absence of Fmoc at each stage of the peptide build-up. The associated chemical maps for these samples display the same trend and demonstrate that the surface has been modified uniformly with the peptide.

Both WCA and ToF-SIMS support the data from the solid state fluorescence. However, they also demonstrate the advantages of the cheap and fast fluorescence method. Compared to the WCA measurements, solid-state fluorescence specifically detects the presence/absence of Fmoc with little influence of the amino acid side chains, whereas the wettability of the surface is heavily dependent on the nature of the amino acid. In contrast to WCA measurements, ToF-SIMS is more specific to the Fmoc group, but it is expensive and not rapidly accessible for those outside specialist surface analysis facilities and therefore not suitable as a routine method to stepwise follow peptide synthesis on surfaces. Although we have shown that solid-state fluorescence is a convenient tool for routine monitoring of the Fmoc amino acid build-up, it has to be noted that it does not replace the need for more in-depth chemical surface analysis of the final material to confirm its chemical composition and the homogeneity of the surface.

Although the RGD peptide sequence investigated is of biological relevance, it does not contain any aromatic amino acids which might interfere with the fluorescence properties of the Fmoc. Tryptophan in particular is known to display minor fluorescence and may interfere with the measurement. To investigate any effect of aromatic Fmoc amino acids on the fluorescence signal, we attached phenylalanine (F), tyrosine (Y), histidine (H), and tryptophan (W) to the PEG-amine terminated substrates. Figure 3 shows the spectra of these four surfaces before and after Fmoc deprotection. The on/off behavior due to presence/absence of Fmoc is clearly visible and the Fmoc deprotected amino acids do not display any fluorescence signals. This confirms that the build-up of aromatic amino acid surfaces may also be monitored without interference from the aromatic side groups of the molecules. The broader application of the technique to monitor the build-up of a variety of amino acids into a longer peptide sequence is shown in Figure 4. A random sequence containing charged, polar uncharged, and nonpolar amino acids was prepared and the build-up monitored via solid state fluorescence. The data shows that even though the fluorescence intensities may change depending on the amino acid (and likely affected by different

degrees of bleaching), the on/off Fmoc fluorescence can be conveniently monitored for the build-up of longer sequences.

In summary, we have demonstrated that the direct build up of short amino acid sequences directly on a solid surface can be monitored via solid-state fluorescence. The method is convenient because it is inexpensive and readily available compared to other surface analytical techniques such as ToF-SIMS. This method will facilitate the wider use of peptide synthesis from surfaces by allowing routine monitoring of the amino acid coupling at every stage of the sample preparation, circumventing the more time-consuming and expensive approach to prepare peptides on resins and transferring and attaching them onto the surface after cleavage from their support.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of the sample holder and its setup for the measurement. Experimental details for the synthesis of the dipeptides Fmoc-GR(Pbf)-OMe and Fmoc-GR-OMe and their fluorescence spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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