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3D alkyne–azide cycloaddition: spatiotemporally controlled by combination of aryl azide photochemistry and two-photon grafting†

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A novel fluoroaryl azide with an alkyne tail was synthesized and precisely immobilized within a PEG-based matrix *via* two-photon induced decomposition and nitrene insertion. Well defined 3D positioning of the terminal alkyne allows site-specific micro-patterning. The subsequent 3D alkyne–azide cycloaddition was realized using dye-functionalized molecules containing "clickable" azide moieties.

The copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) is versatile for numerous applications and well meets the criteria of a "click" reaction:[1] fast reaction rate, high yields and outstanding orthogonal reactivity. In spite of the benefits of CuAAC, the reaction is yet to fulfil the requirements for micro-patterns on flat surfaces[2] or within 3D scaffolds,[3,4] which demand very high spatial and temporal control.

Using light as a CuAAC reaction trigger is an effective strategy to realize spatiotemporal control. Bowman's group reported micropatterns of hydrogels fabricated by using standard photolithographic techniques *via in situ* generation of Cu(I) through photo-induced reduction.[5] Since such systems involved multiple species, some side effects hindering the spatially resolved character could not be avoided. Popik, Locklin, and co-workers employed surfaces functionalized with cyclopropenone-masked dibenzocyclooctynes for the light activated immobilization of azides using the catalyst-free click chemistry.[6] However, a complex synthetic procedure is required for the preparation of the cyclopropenone precursor, which restricts its broader applications. More importantly, complete spatial control, especially in 3D, is limited for the above-mentioned approaches due to the inherent limitation of traditional photolithographic techniques.

Multi-photon grafting stands out due to its potential to immobilize functional molecules within a 3D volume in a straightforward fashion.[7] Our recent work has proven the concept by employing commercial aryl azide for selective functionalization of a 3D PEG matrix under three-photon irradiation.[8] Activation under three-photon absorption usually required higher laser intensity than that required under two-photon absorption (2PA) due to the lower probability.[18] Here we report a novel two-photon active aryl azide and a strategy developed to realize spatiotemporal control of the CuAAC reaction in 3D (Fig. 1). The fluoroaryl azide with alkyne functionality was initially immobilized precisely within the PEG-matrix *via* 2PA grafting. The covalently attached alkyne moieties serve as anchors and subsequently reacted with functionalized molecules containing azide moieties *via* CuAAC "click" reaction.

A proper molecule design of aryl azide is critical for the success of a two-photon grafting process. The large 2PA cross section combined with high photografting efficiency is the most important feature for an efficient photografting reagent. Chromophores with strong 2PA normally contain a long π conjugated system with strong electron donor (D) and acceptor (A) groups, as well as good coplanarity.[9] However, the synthesis

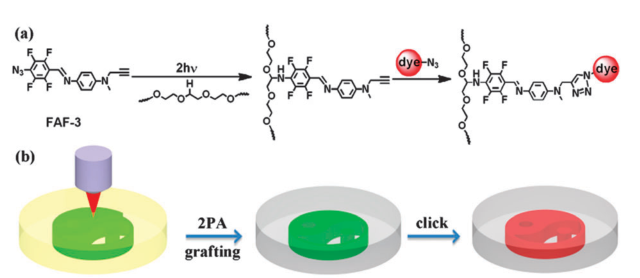

Fig. 1 (a) Two-photon absorption (2PA) induced decomposition and subsequent insertion of AFA-3 into a PEG matrix, followed by CuAAC click reaction for postmodification; (b) schematic representation of the 2PA grafting procedure and postmodification.

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of aryl azide with complex structures possessing large 2PA has remained a big challenge until now because of the high sensitivity and thermal instability of azides.[10] Therefore, we constructed a 2PA grafting agent (AFA-3) containing a C═N bond as a π bridge due to its nearly identical contribution to the 2PA cross section compared to the C═C bond analogues,[11] but based on a much simpler synthesis under mild conditions with satisfying yields (ESI†). Functionalized *N*-methylaniline and fluorinated aromatic azide were selected as D and A groups, respectively. The introduction of fluoro moieties on the aryl-azide ring not only provides strong electron drawing ability, but also greatly suppresses the ring expansion deactivation of the singlet nitrene intermediates to ensure high photografting efficiency.[12] In the open aperture *Z*-scan measurement, the novel D–π–A fluoroaryl azide AFA-3 exhibits a two-photon absorption cross section of 160 ± 8 GM at 800 nm in DMF solution, which is adequate to ensure an efficient 2PA photografting process (ESI†).

Although two-photon induced reactions and one photon induced reactions might differ in the initial excited state, the subsequent grafting process is expected to be the same.[10] 2PA induced photolysis of the azide causes the dissociation of the N–N bond from the excited singlet state, followed by the generation of nitrogen and nitrene species.[13] The highly reactive nitrene intermediate could undergo various reactions and Fig. 1a depicts the major pathway which is also the desired one: direct immobilization on the matrix by insertion into a C–H bond. This single step process is very universal because it is applicable to a wide variety of matrices containing C–H or N–H bonds.[10] PEG hydrogels were chosen as model substrates here because of their excellent biocompatibility and wide applications in tissue engineering.[14] Since the PEG-network is transparent in the NIR region, the laser beams used for writing are able to deeply penetrate inside the matrix and induce the photografting reactions within 3D volume of the hydrogels. Moreover, since the two-photon interaction is only confined within the small focal point, immobilization of AFA-3 on the PEG matrix could be performed with a high spatial resolution. By moving the laser focus within the sample, arbitrary 3D patterns of immobilized molecules can be "recorded" (Fig. 1b). After grafting, chromophores which fluoresce upon excitation at 555 nm were covalently attached within the matrix. Thus, grafted patterns can be directly and tomographically observed *via* laser-scanning microscopy (LSM).

An array of squares fabricated at different laser powers and scanning speed parameters were employed to evaluate the processing window of the 2PA photografting process (Fig. 2a). The square patterns could be visualized in a laser power range from 50 mW to more than 350 mW at scanning speeds from 5 to over 555 mm s^{-1} (the highest laser power of 350 mW and a speed of 555 mm s^{-1} were the limits of the current experimental setup). The fluorescence intensity of the photografting patterns, indicating the level of immobilization, directly correlated with both laser power and scanning speed. Higher laser power combined with lower scanning speed provides more energy in the excited focal volume to produce "brighter" patterns. Unlike the 2PA photopolymerization process, which

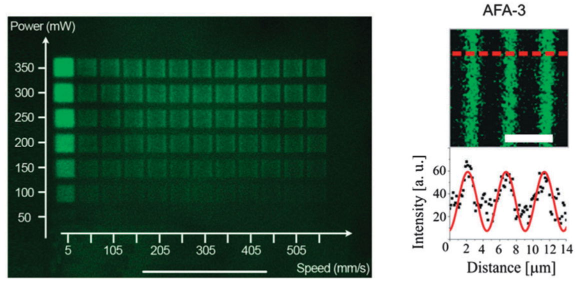

Fig. 2 (a) An array of two-photon absorption (2PA) grafted patterns produced at the different scanning speed and average laser power (scale bar: 500 µm); (b) fluorescence intensity distribution of lines (scale bar: 5 µm).

is apt to undergo overexposure at high laser irradiation dose,[9] the 2PA induced immobilization of arylazide was realized successfully with laser power as high as 350 mW at the investigated scanning rates. The good resistance to high laser power allows a fast writing speed with sufficient energy to induce 2PA photografting. Notably, the density of immobilization can be spatially controlled by simply altering the irradiation exposure time or laser intensity during the photopatterning process (ESI†). Such controlled 3D spatial gradients cannot be generated by conventional photolithographic methods. The ability to produce biochemical and biomechanical gradients in 3D is important to numerous bio-technology applications.[15]

A single-line scan (350 mW, 5 mm s^{-1}) was used to estimate the spatial resolution of the photografting process (Fig. 2b). By using a conventional 20× (NA = 0.8) microscope objective to focus the laser beam into the sample, the lateral resolution of around 3.6 µm, a highly relevant size with respect to the cellular microenvironment, was achieved. The resolvability of lines, defined as the distance below which the separate lines could not be distinguished, was confirmed by the corresponding fluorescence intensity distribution of about 4.5 µm (Fig. 2b). The developed 2PA photografting process provides comparable spatial resolution of 3D micropatterns to other 2PA lithography approaches used for micropatterning into 3D matrices, such as 2PA photopolymerization[15] and 2PA uncaging.[16] A potentially higher resolution can be realized using immersion-oil optics and shorter wavelength lasers due to the reduced light–material interaction volume.[8]

After photografting, the immobilized chromophores still contain C═N bonds, which are potentially hydrolysable under acidic conditions. In order to evaluate the stability of the clickable patterns, the 2PA functionalized pellets were transferred from DMF to aqueous solutions with different pH values and incubated at room temperature for 24 h. Then the pellets were re-immersed in DMF for LSM observation. Considering that the PEG matrix can be destroyed under strong acidic or alkaline conditions, the stability tests were performed with pH values ranging from 3 to 10, at which the matrix has been proved to be stable for that period of time. If hydrolyzation of the photografting patterns occurred, the intensity of the fluorescence would shift due to the change in the electronic structure of the immobilized molecules derived from the

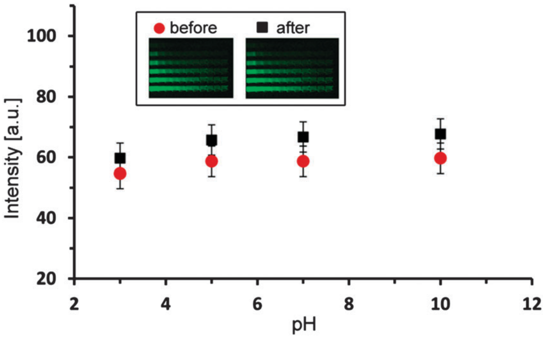

Fig. 3 Fluorescence intensity before and after the stability tests at different pH values; the inset shows laser scanning microscopy (LSM) images of the fluorescent patterns before (left) and after (right) the tests at pH = 7.

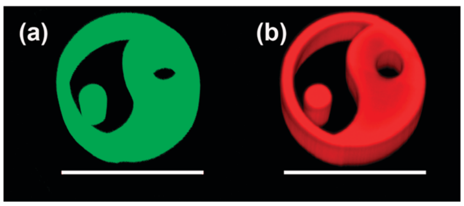

Fig. 4 Laser scanning microscopy (LSM) images of the 3D photografted patterns: (a) before click reaction (λ_{exc} = 488 nm); (b) after click reaction (λ_{exc} = 555 nm). Scale bar: 100 μm.

hydrolysis of the C=N bond. Actually, under excitation at 555 nm, no fluorescence was observed for the model patterns obtained by UV photografting of 4-azido-2,3,5,6-tetrafluorobenzaldehyde (data not shown). Therefore, a decrease in fluorescence was expected if immobilized molecules underwent the hydrolyzation process. As shown in Fig. 3, the unchanged intensities of fluorescence indicated that 2PA grafting patterns exhibit remarkable stability at investigated pH values. Considering that most biological applications are performed under mild conditions in buffer solutions, the 2PA photografting patterns demonstrate sufficient stability to fulfil the requirement.

Due to the distinct character of the CuAAC click reactions, various functionalized molecules with azide moieties could be conjugated onto the formed ''clickable'' patterns containing alkyne functionalities produced by 2PA grafting. Azide MegaStokes dye 673 was selected as a model precursor for the click reaction because the strong fluorescence emission of the dye lies at a long wavelength of 673 nm, at which the fluorescence of the ''clickable'' patterns produced by 2PA grafting could not be observed. Therefore, an excellent differentiation of the fluorescent patterns before and after click reaction could be achieved. Good hydrophilicity of the dye[17] allows the click reaction to occur in PBS solution (pH 7.4) in the presence of $CuSO_4$–sodium–ascorbate and triethylamine (ESI†). Before click reaction, the complex 3D patterns (yinyang) obtained *via* 2PA grafting exhibited green fluorescence (Fig. 4a). The color of the fluorescent pattern changed from green to red indicating the successful 3D site-specific immobilization of the azide-fluorophores *via* CuAAC click reaction (Fig. 4b).

In conclusion, we have reported a versatile and straightforward strategy to realize the 3D CuAAC click reaction *via* combination of arylazide photochemistry and two-photon grafting. A novel fluoroaryl azide with a terminal alkyne moiety, serving as an efficient 2PA photografting agent, was immobilized within a PEG-matrix to generate 3D ''clickable'' micropatterns with a lateral resolution down to 3.6 μm. The successful 3D CuAAC click reaction was confirmed by the localized conjugation of the azide-fluorophores onto the ''clickable'' covalently attached alkyne moieties. The presented strategy to realize 3D alkyne–azide cycloaddition with highly spatiotemporal control is simple and versatile, which reveals great potential applications in microarray-based proteome analysis, biosensors, cell patterning, and drug screening.

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