Biomaterials Science

Multifaceted and Route-Controlled "Click" Reactions Based on Vapor-Deposited Coatings

Journal:	*Biomaterials Science*
Manuscript ID	BM-ART-09-2015-000417.R1
Article Type:	Paper
Date Submitted by the Author:	16-Nov-2015

Complete List of Authors:	Sun, Ting-Pi; National Taiwan University, Department of Chemical Engineering Tai, Ching-Heng; National Taiwan University, Department of Chemical Engineering Wu, Jyun-Ting; National Taiwan University, Department of Chemical Engineering Wu, Chih-Yu; National Taiwan University, Department of Chemical Engineering Liang, Wei-Chieh; National Taiwan University, Department of Chemical Engineering Chen, Hsien-Yeh; National Taiwan University, Department of Chemical Engineering

Journal Name

ARTICLE

Multifaceted and Route-Controlled "Click" Reactions Based on Vapor-Deposited Coatings

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Ting-Pi Sun‡, Ching-Heng Tai‡, Jyun-Ting Wu, Chih-Yu Wu, Wei-Chieh Liang, Hsien-Yeh Chen*

"Click" reactions provide precise and reliable chemical transformations for the preparation of functional architectures for biomaterials and biointerfaces. The emergence of a multiple-click reaction strategy has paved the way to a multifunctional microenvironment with orthogonality and precise multitasking that mimics nature. We demonstrate a multifaceted and route-controlled click interface using vapor-deposited functionalized poly-*para*-xylylenes. Distinctly clickable moieties of ethynyl and maleimide were introduced to poly-*para*-xylylenes in one step via a chemical vapor deposition (CVD) copolymerization process. The advanced interface coating allows for a double-click route with concurrent copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition (CuAAC) and the thiol–maleimide click reaction. Additionally, double-click reactions can also be performed in a cascade manner by controlling the initiation route to enable the CuAAC and/or thiol-yne reaction using a mono-functional alkyne-functionalized poly-*para*-xylylene. The use of multifaceted coatings to create straightforward and orthogonal interface properties with respect to protein adsorption and cell attachment is demonstrated and characterized.

INTRODUCTION

During the past decade, new biomaterial and biointerface design has witnessed fundamental discoveries, and a more interactive and precisely controlled biological microenvironment has been created.[1, 2] The key to the success of such design is due to the development of a diversified selection of orthogonal reactions, particularly "click" reactions (e.g., the copper catalyzed azide–alkyne cycloaddition (CuAAC), Diels–Alder cycloaddition, thiol-ene, thiol-yne, and nitroxide radical coupling (NRC) reactions). These highly efficient and specific reactions have enabled the synthesis of more sophisticated and precise biointerfaces and allowed for successful conjugation in the vast array of functionalities that are present in biological systems.[3, 4] The demands for the construction of complex macromolecular structures and multifunctionality of biological responses have also advanced these click reactions to a double-, triple-, or theoretically multiple-clickable fashion.[5, 6] Future employment of click reactions for biomaterials and biointerfaces could be managed by (i) constructing a combination of different types of clickable moieties on the chemical structures or materials surfaces, (ii) relying on strategies with one single clickable moiety that is able to perform a cascade of distinct click reactions following a specific reaction route, or (iii) a combination of (i) and (ii).

In this study, we demonstrate advanced biointerfaces that undergo multifaceted and route-controlled click reactions. The surfaces are created using the functionalized poly-*para*-xylylene model system. The creation of a double-clickable surface could be synthesized in a single coating step via chemical vapor deposition (CVD) copolymerization (theoretically, multiple clickable motifs can be installed)[7] to produce two distinct clickable moieties consisting of maleimide and alkyne functionalities on the coated surfaces. The created surfaces readily underwent concurrent orthogonal click reactions consisting of copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes (CuAAC) and the thiol–maleimide click reaction. In addition, the concept of the double-click reaction was also possible through the use of a single clickable motif on the alkyne-functionalized poly-*para*-xylylene coating. Double-click reactions were performed in a cascade by controlling the initiation route with the introduction of a copper(I) catalyst or activation energy (UV irradiation/thermal energy) to the system. The accessibility to the CuAAC and/or thiol-yne reaction was controlled elegantly on the same surface.

EXPERIMENTAL SECTION

Synthesis and CVD polymerization

The double-clickable PPX (poly[(4-ethynyl-*p*-xylylene)-*co*-(4-*N*-maleimidomethyl-*p*-xylylene)-*co*-(*p*-xylylene)]) was synthesized from 4-ethynyl-[2,2]paracyclophane and 4-*N*-maleimidomethyl-[2,2]paracyclophane via CVD copolymerization using a self-built dual-sourced CVD system. The synthesis of 4-ethynyl-[2,2]paracyclophane and 4-*N*-maleimidomethyl-[2,2]paracyclophane was conducted according to previously

Department of Chemical Engineering, National Taiwan University, Taipei 10617, Taiwan.
* To whom correspondence should be addressed: hsychen@ntu.edu.tw
‡ Ting-Pi Sun and Ching-Heng Tai contributed equally.
† Electronic Supplementary Information (ESI) available: IRRAS and XPS characterizations of double-clickable PPX and route-clickable PPX coatings; control experiments of cross immobilizations on double-clickable PPX coating; QCM analysis for the PEG and biotinylated surfaces; water condensation study; control experiments for the cell adhesion study. See DOI: 10.1039/x0xx00000x

reported protocols.[8, 9] The precursors of the two paracyclophanes with a 1:1 feeding ratio (molar) was controlled during the CVD copolymerization process. The pyrolysis temperatures were 650 ºC for 4-ethynyl-[2,2]paracyclophane and 510 ºC for 4-*N*-maleimidomethyl-[2,2]paracyclophane. The double-clickable PPX was spontaneously copolymerized on the substrates placed on top of a rotating and cooled (15 ºC) sample holder in the deposition chamber. The system pressure was maintained at 120 mTorr during the entire CVD copolymerization process. The route-clickable PPX of poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) was deposited on the studied substrates via single-sourced CVD polymerization from the 4-ethynyl-[2,2]paracyclophane precursor. The precursor was sublimated at 110 °C and transformed into reactive species by pyrolysis, which was performed at 670 °C. Then, the reactive radicals were transferred into the deposition chamber, and the route-clickable PPX was polymerized on a rotating, cooled sample holder at 15 °C. A similar system pressure of 120 mTorr was maintained during the CVD polymerization process.

Surface characterizations

Surface characterization was performed using infrared reflection absorption spectroscopy (IRRAS) to confirm the characteristic band vibrations and chemical compositions of the coatings. The IRRAS spectra were recorded using a 100 FT-IR spectrometer (PerkinElmer, USA) equipped with an advanced grazing angle specular reflectance accessory (AGA, PIKE Technologies, USA) and a liquid nitrogen-cooled MCT detector. The samples were mounted in a nitrogen-purged chamber, and the recorded spectra were corrected for any residual baseline drift. X-ray photoelectron spectroscopy (XPS) data were recorded with a Theta Probe X-ray photoelectron spectrometer (Thermal Scientific, UK) using a monochromatized AlK α X-ray source. The XPS spectra were recorded with an X-ray power of 150 kW. The pass energies were 200.0 eV and 20.0 eV for the survey spectra and the high-resolution C 1s elemental spectra, respectively. The XPS atomic analysis was reported based on the atomic concentrations (%) and was compared to the theoretical values. A QCM instrument (ANT Technologies Co., Taiwan) equipped with a flow injection analysis (FIA) device and a continuous frequency-variation recording device was used to study streptavidin adsorption. The sensing element of this instrument is a piezoelectric quartz disc with a resonant frequency of 9 MHz. First, the sensor crystals were coated with double-clickable PPX via a CVD copolymerization process followed by the immobilization of azide-terminated polyethylene glycol (average M_n = 5000, Sigma Aldrich, USA). The adsorption measurement was recorded and calculated based on a total sensing area of 0.1 cm^2. A buffer solution consisting of phosphate-buffered saline (PBS pH=7.4, Sigma-Aldrich, USA) was continuously delivered into the flow channel until a stable frequency response was obtained. Then, the streptavidin solution (312.5 μg mL^{-1} in PBS, Life Technologies, USA) was injected into the flow system, and the time-dependent change in the frequency was monitored and recorded during the detection of streptavidin adsorption. A flow rate of 36.5 μL min^{-1} was maintained using a peristaltic pump that was connected to the FIA device.

Immobilizations by click reactions

The immobilization of the fluorescein-conjugated cysteine was performed via microcontact printing (μCP) using a poly(dimethylsiloxane) (PDMS) stamp consisting of square arrays with 50 μm sides and a 100 μm center-center spacing. The stamp was inked with fluorescein-conjugated cysteine (5 mM, Yao-Hong Biotechnology Inc., Taiwan) in deionized water and used to print on top of the double-clickable PPX surfaces for 2 h. The resulting sample was washed twice with PBS (pH=7.4, contains Tween 20, Sigma Aldrich, USA) and twice with PBS (without Tween 20). Then, the Alexa Fluor® 555-labeled azide (5 mM, Life Technologies, USA) was immobilized by inking a μCP stamp (square arrays with 500 μm sides and a 1,000 μm center-center spacing) on the same double-clickable PPX coated surface for 2 h. The inking solution contained 5 mM Alexa Fluor® 555 labeled azide, sodium ascorbate (120 mM), and $CuSO_4$ (20 mM). The resulting sample was washed three times with PBS (pH=7.4, contains Tween 20, Sigma-Aldrich, USA) and one time with PBS (without Tween 20) and finally rinsed with deionized water. Similarly, azide-PEG (20 mM, average M_n = 5,000, Sigma-Aldrich, USA) and thiol-biotin (10 mM, Nanocs, USA) were immobilized on the same double-clickable PPX surfaces by first reacting homogeneous azide-PEG with double-clickable PPX in a solution containing sodium ascorbate (120 mM) and $CuSO_4$ (20 mM). Then, thiol-biotin was microcontact printed (μCPed) using a stamp consisting of square arrays with 50 μm sides and a 100 μm center-center spacing on the same surface. The same wash process was performed. All of the employed PDMS stamps were treated with a 10 W oxygen plasma for 2 min prior to the inking process to render the surface hydrophilic. The μCP process was performed at 25 °C and 55% humidity. The resulting samples were analyzed, and the fluorescent micrographs were recorded using a fluorescence microscope (Nikon TE2000-U, Japan). The immobilization of Alexa Fluor® 488 azide on the route-clickable PPX of poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) was also performed using the μCP process with a PDMS stamp consisting of square arrays with 50 μm sides and a 100 μm center-center spacing. The inking solution contained 5 mM Alexa Fluor® 488 labeled azide, sodium ascorbate (120 mM), and $CuSO_4$ (20 mM). The immobilization of thiol-terminated molecules including thiol-PEG (100 mg mL^{-1}, Nanocs, USA) or fluorescein-labeled RGDYCC peptide (5 mM, Yao-Hong Biotechnology Inc., Taiwan) was performed via a photochemical reaction in the presence of the *2,2-dimethoxy-2-phenylacetophenone* photoinitiator (50 mM, Sigma Aldrich, USA). A photomask was used during the photochemical reaction. The mask was designed using AutoCad, and the design was printed on a high-resolution emulsion transparency

(TKK, Taiwan) with a spatial resolution of 10,000 dpi. The photo-illumination process was carried out by exposing samples under UV irradiation (model S1500, OmniCure, USA) at 365 nm for 5 min. After completion of the surface reactions, the samples were incubated overnight in deionized water to rinse off the unreacted reagents. The two-step click reaction was conducted on the route-clickable PPX by first reacting RGDYCC peptide (5 mM, Yao-Hong Biotechnology Inc., Taiwan) via the previously mentioned photochemical reaction followed by reaction of azide-PEG with the same surface in a reagent solution containing sodium ascorbate (120 mM) and $CuSO_4$ (20 mM). The immobilization of fluorescein-conjugated cysteine was also performed by inking a μCP stamp (square arrays with 500 μm sides and a 1,000 μm center-center spacing) on the route-clickable PPX at elevated temperatures ranging from 55 °C to 90 °C for 30 min. The inking solution contained 5 mM fluorescein-conjugated cysteine, 2,2'-azobis(isobutyronitrile) (AIBN) (10 mM, Sigma Aldrich, USA). The samples were washed with deionized three times to rinse off the unreacted reagents, and the results were examined using a fluorescence microscope (Nikon TE2000-U, Japan).

Protein adsorption

For the protein adsorption studies, Alexa Fluor® 546 fibrinogen (Life Technologies, USA) was used to test the fouling property of the PEG-modified surfaces. The samples were incubated for 10 min with protein solutions prepared at a concentration of 75 mM. After protein incubation, a rinsing process, which involved three rinses with PBS (pH=7.4, contains Tween 20, Sigma Aldrich, USA), one rinse with PBS (without Tween 20), and one rinse with deionized water, was employed to remove the loosely adsorbed proteins. Then, the resulting samples were examined using fluorescence microscopy (Nikon TE2000-U, Japan).

Cell adhesion

Commercially available fibroblasts (3T3 clone A31, ATCC CCL-163) were purchased from Bioresource Collection and Research Center, Taiwan. Then, the cells were allowed to adhere onto the studied surfaces for 2 h at 37 °C in Dulbecco's High Glucose Modified Eagle Medium (DMEM, HyClone, USA) without serum, followed by analysis using an optical microscope (Olympus IX71, Japan). Next, the samples were rinsed with PBS after the initial 2 h of adhesion, and a new culture medium consisting of DMEM with 10% fetal bovine serum (FBS, Biological Industries, Israel) and a 1% penicillin-streptomycin amphotericin B solution (catalog # 03-033-1B; Biological Industries, Israel) was used to culture the resulting cell/surface samples for an additional 22 h. The cell culture conditions were maintained in a humidified atmosphere that contained 5% CO_2 and 95% air. The cell growth pattern was observed and recorded using an Olympus IX71 optical microscope. Finally, the cells were fixed with 10% formalin (Macron Fine Chemicals, USA) for 30 min and stained with 1 μg mL^{-1} 4',6-diamidino-2-phenylindole (Life technologies, USA) for 5 min. The resulting samples were analyzed using fluorescence microscopy (Olympus IX71, Japan).

Results and discussion

Poly-*para*-xylylene surfaces with multifaceted clickable functional groups were prepared in a single step using a custom-built multifunctional CVD system,[10] which consisted of two sources containing independent sublimation and pyrolysis zones. In addition, both sources were connected to the deposition chamber. Selected precursors of [2,2]paracyclophanes were transferred from each source, and the vapor-phase copolymerization process occurred in the deposition chamber to form the multifunctional poly-*para*-xylylene copolymer with the corresponding functional groups. During the experiment, 4-ethynyl-[2,2]paracyclophane and 4-N-maleimidomethyl-[2,2]paracyclophane were used for CVD copolymerization to prepare poly[(4-ethynyl-*p*-xylylene)-*co*-(4-N-maleimidomethyl-*p*-xylylene)-*co*-(*p*-xylylene)], which is referred to as double-clickable PPX (Fig. 1(a)). The resulting double-clickable PPX coating contained both ethynyl and maleimide moieties, which provides concurrent clickable routes involving i) a copper-(I)-catalyzed Huisgen's 1,3-dipolar cycloaddition reaction (CuAAC) and ii) a Michael addition of thiol to maleimide click reaction (Fig. 1(b)). Ellipsometric analysis revealed that polymer films with thicknesses in the range of 80 – 100 nm were deposited in the study. Infrared reflection absorption spectroscopy (IRRAS) was employed to analyze the double-clickable PPX, and the spectra are shown in Fig. S1 of the ESI.† The peaks located at 1398 cm^{-1} (*C-N-C*), 1720 and 1702 cm^{-1} (*C=O*) corresponded to the characteristic band stretches for the maleimide groups, and the two peaks located at 3284 and 2100 cm^{-1} were due to the terminal alkyne groups. These adsorption characteristics were previously observed in related mono-functional coatings (poly[(4-*N*-maleimidomethyl-*p*-xylylene)-*co*-(*p*-xylylene)][9] and poly[(4-ethynyl-*p*-xylylene)-*co*-(*p*-xylylene)])[11], were also observed in the spectra of double-clickable PPX. The characterization by using X-ray photoelectron spectroscopy (XPS) for the double-clickable PPX is presented in the ESI† (Fig. S2(a)).

To confirm that both the ethynyl and maleimide groups incorporated into double-clickable PPX were available to perform specific click reactions (i.e., (i) and (ii)), a 2-step conjugation procedure was developed to immobilize multiple molecules on separate areas of the same surface. Alexa Fluor® 555 azides and fluorescein-labeled cysteines were selected as model reporter molecules for the coupling reactions. The confined reaction areas were created using a microcontact printing (μCP) technique,[12] and the selected micropatterns were used as a guide. The conjugation cascade was performed by printing Alexa Fluor® 555 azides using μCP with 500 μm x 500 μm array patterns on double-clickable PPX via the CuAAC reaction. Then, fluorescein-conjugated cysteine was printed via μCP with 50 μm x 50 μm array patterns on the same

sample surface using a thiol-maleimide click reaction. Both reactions were carried out under ambient conditions at room temperature. As shown in Fig. 1(c), the fluorescence micrographs indicated that the fluorescence molecules were addressed on designated areas of double-clickable PPX. The pronounced Alexa Fluor® 555 signals (red) were observed in the areas where the azides were printed, and the fluorescein signals (green) appeared on the cysteine (thiol)-printed areas. In addition, parallel immobilization of the two fluorescence molecules was observed in the overlaid regions, and no cross-reactions were observed. The control experiments used to verify the absence of cross-reactions are discussed in the ESI† (Fig. S3).

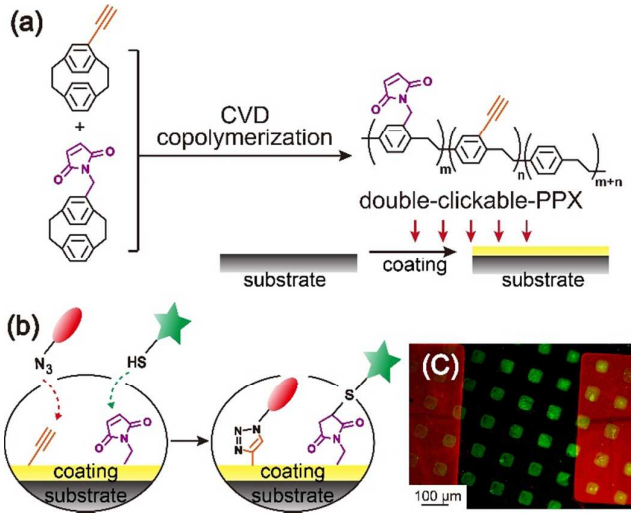


Fig. 1 (a) CVD copolymerization of a 1:1 molar ratio of 4-ethynyl-[2,2]paracyclophane and 4-N-maleimidomethyl-[2,2]paracyclophane to form double-clickable PPX (poly[(4-ethynyl-p-xylylene)-co-(4-N-maleimidomethyl-p-xylylene)-co-(p-xylylene)]) (m:n = 1:1). (b) Schematic illustration of a double click reaction via CuAAC and thiol-maleimide click reactions to immobilize Alexa Fluor® 555 azides and fluorescein-labeled cysteines, respectively, on the double-clickable PPX coating surface. The process of μCP was used to confine specific conjugates to selected locations. (c) Overlaid image of the red and green channels showing the concurrently immobilized Alexa Fluor® 555 azides and fluorescein-cysteines. The image was captured at the same location on the same sample. The polymer coatings were prepared on gold substrates for the study.

In a separate experiment, a synergistic biointerface containing concurrent orthogonal properties (i.e., anti-protein fouling and biotinylated activity) was created by double-click reactions using the double-clickable PPX coating (Fig. 2(a)). Specifically, the modifications were performed by clicking azide-terminated polyethylene glycol (azide-PEG) to ethynyl groups on a double-clickable PPX-coated substrate via the CuAAC reaction, and then, thiol-biotin were affixed to confined areas using a μCP process via the thiol-maleimide click reaction. The double-click reaction should functionalize the surface to produce orthogonal surface properties, which included: i) functionalized PEG motifs with a fouling-resistant surface and ii) spatially immobilized thiol-biotin molecules with preferential binding affinity toward avidin or streptavidin proteins. As shown in Fig. 2(b), by introducing rhodamine (TRITC) conjugated streptavidin, the TRITC signals were only detected at the locations where previously immobilized biotin molecules were confined. These results have important implications as follows: i) the streptavidin binding was promoted only by the thiol-biotin ligand and otherwise prevented by the protein-repelling PEG-modified background and ii) the dual functional groups (4-ethynyl and 4-N-maleimidomethyl) synergistically perform orthogonal and specific conjugation reactions without cross-reacting. A possible steric effect due to the size of the immobilized molecule can be justified by tuning the composition of the functional groups during the CVD copolymerization process.[13] Quartz crystal microbalancing (QCM) analysis was also employed to quantify the streptavidin binding behavior. The double-clickable PPX and subsequently clicked PEG or biotin molecules were modified on the QCM crystal surfaces. The low adsorption of streptavidin (4.2 ng·cm^{-2} or 4.8 × 10^{10} no.·cm^{-2}) was confirmed on the modified surfaces compared to the noticeable amount of streptavidin (i.e., 139.0 ng·cm^{-2} or 1.6 × 10^{12} no.·cm^{-2}) adsorbed on the pure double-clickable PPX (without PEG) surfaces. A profoundly high amount of streptavidin (232.2 ng·cm^{-2} or 2.7 × 10^{12} no.·cm^{-2}, due to the binding affinity between the biotin and streptavidin) was detected on the biotin-clicked surface, compared to approximately 1.0 × 10^{14} no.·cm^{-2} maleimide binding sites were estimated on the surface.[13] The QCM results are shown in the ESI† in Fig. S4.

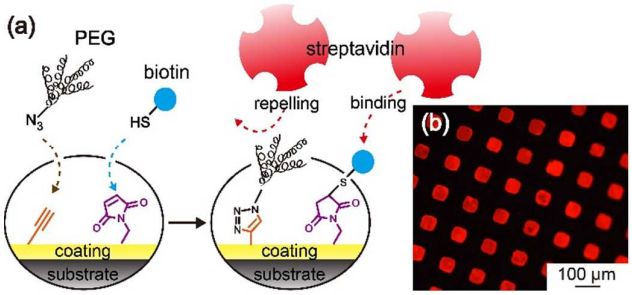


Fig. 2 (a) Concurrent display of orthogonal properties including protein-repelling (by PEG modification) and biotin-activated surfaces on double-clickable PPX. (b) Fluorescence image shows preferentially bound TRITC-conjugated streptavidin on confined locations where biotin was modified, and the streptavidin was repelled on the areas with PEG modification. The polymer coatings were prepared on gold substrates for the study.

A mono-functionalized polymer consisting of poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene, referred to as route-clickable PPX,

was synthesized via CVD polymerization from ethynyl-[2,2]paracyclophane to create a route-controlled clickable surface. The optimized CVD conditions are discussed in the experimental section, and the surface characterizations using IRRAS and XPS are shown in the ESI† in Fig. S1(c) and Fig. S2(b), respectively. The mono-distribution of ethynyl groups can support (i) the CuAAC click reaction in the same manner using the aforementioned reaction conditions or (ii) the thermal or photochemical process of the thiol-yne click reaction through the addition of thiols across the alkyne groups on the coating surfaces. In the thiol-yne reaction, each alkyne first reacts with a thiol to produce a vinyl sulfide, followed by a subsequent reaction of the vinyl sulfide with another thiol to yield the 1,2-disubstituted thioether adduct. A summary of the click reaction routes for the route-clickable PPX is shown in Fig. 3(a). The route of the CuAAC reaction was first verified by clicking Alexa Fluor® 488 azides on the route-clickable PPX using μCP with 50 μm x 50 μm array patterns. The resulting fluorescence images exhibited the anticipated Alexa Fluor® 488 signals at the μCP-stamped regions (Fig. 3(b)), and the covalent linkage was also confirmed by μCP stamping Alexa Fluor® 488 azides on a nonfunctionalized poly-*para*-xylylene surface. With respect to the photochemically activated thiol-yne click reactions on the route-clickable PPX surfaces, the experiments were conducted using 365 nm UV irradiation for the reaction with thiol-terminated PEG (thiol-PEG), and the specific activation locations were resolved using a high-resolution emulsion photomask during the photochemical process. A water condensation study[14] was performed on the resulting surfaces to identify the hydrophilic regions containing the immobilized PEG molecules and the hydrophobic background of the unreacted route-clickable PPX (see the ESI†, Fig. S5). A subsequent protein adsorption study was performed by incubating the samples with Alexa Fluor® 546-conjugated fibrinogen. As shown in Fig. 3(c), the thiol-PEG molecules were selectively modified via the thiol-yne reaction on the route-clickable PPX surface. The adsorption of fibrinogen was demonstrated with high contrast on areas where thiol-PEGs were not present (red color regions), and low levels of protein adsorption were observed on thiol-PEG tethered areas (dark regions). The thermally activated thiol-yne reaction[15] was also confirmed by reacting the fluorescein-labeled RGDYCC peptide on the route-clickable PPX surface with temperatures ranging from 55 to 85 °C, and the results are provided in Fig. S6 of the ESI.†

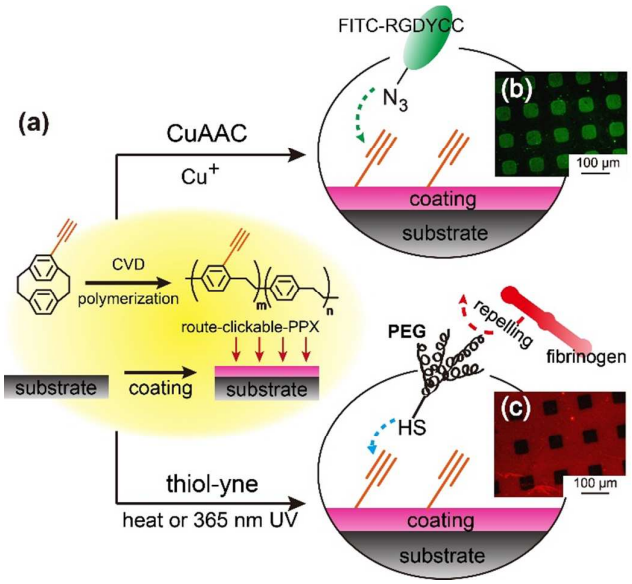


Fig. 3 (a) Schematic illustration of route-dependent click reactions on route-clickable PPX (poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene)). The initiation of the CuAAC or thio-yne click reaction was controlled by specific reaction conditions for conjugation with azide-terminated or thiol-terminated molecules of interests, respectively. (b) Fluorescence micrographs showing conjugated Alexa Fluor® 488 azides on the route-clickable PPX via CuAAC. μCP was used to confine the conjugation to selected locations. (c) Fluorescence micrographs showing selectively immobilized thiol-PEGs prepared via a photochemical thiol-yne click reaction on the route-clickable PPX, and the adsorption of Alexa Fluor® 546 fibrinogen was detected only on the areas without PEG modification. The polymer coatings were prepared on gold substrates for the study.

Finally, two click reactions (i.e., thiol-yne and CuAAC) were performed in a cascade fashion on the route-clickable PPX surface, and the initiation of the distinct reaction was controlled by supplying a copper(I) catalyst or 365 nm UV irradiation to the system, respectively. In the experiment, the thiol-rich peptide (i.e., RGDYCC) can be readily coupled with alkynes on the route-clickable PPX surface via the thiol-yne click reaction and activated at selected areas with the aid of a photomask. The remaining areas can be secondarily clicked with azide-PEGs via CuAAC to prepare an inert surface that prevents cell adhesion.[16-18] The 3T3 cells were allowed to adhere onto the modified surface for 2 h at 37 °C in serum-free DMEM. After this initial 2 h adhesion period, the samples were rinsed with DMEM and incubated with FCS-containing DMEM for 22 h. In the first 2 h of incubation, no discernible difference was observed regarding cell adhesion between the RGDYCC- and PEG-modified surfaces. A sharp boundary between cell adhesion and cell repulsion regions was observed after 24 h of incubation, as shown in Fig. 4. The absence of cell adhesion occurred in areas where azide-PEGs were clicked via CuAAC. In

contrast, cell adhesion was observed on the areas modified with RGD motifs (via the thiol-yne click reaction). Additionally, a series of control experiments with dedicated modifications of pure PEG or pure RGD indicated suppressed cell attachment or enhanced cell attachment, respectively, due to the specific modification (ESI†, Fig. S7). The cell growth pattern on this modified surface was further examined by staining the cell nuclei with DAPI. The fluorescence images unambiguously confirmed that populated cells were grown on the RGD-modified regions, and no fluorescence signal was detected on the PEG regions (Fig. 4c).

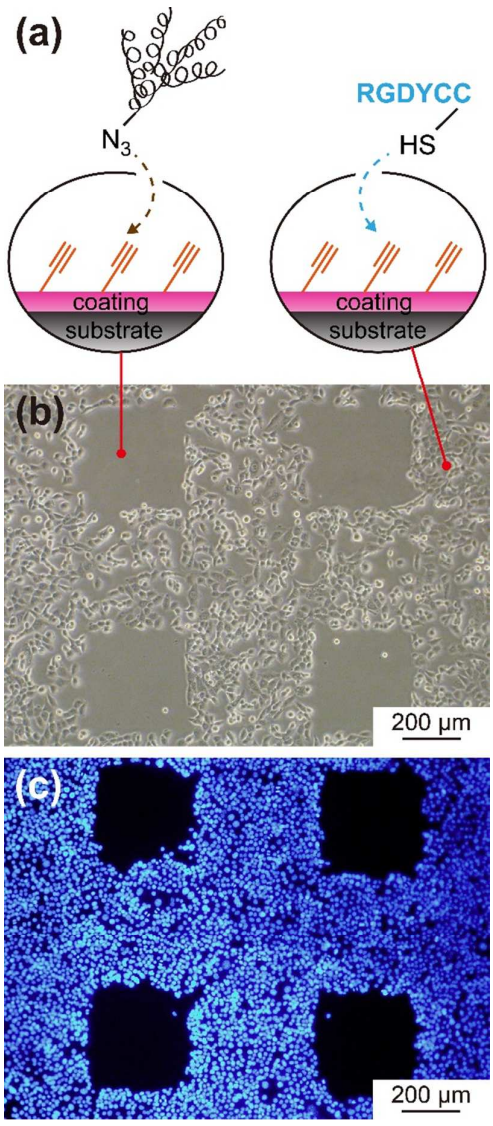


Fig. 4 (a) Concurrent immobilizations of azide-PEG and RGDYCC peptide were performed in a cascade fashion via CuAAC and thiol-yne click reactions, respectively, on the same route-clickable PPX surface. (b) Microscopic images 3T3 cells growth after 24 h of incubation on the previously mentioned (a)-modified surface. The cells are excluded from the azide-PEG passivated background and adhere to the cell-adhering peptide (RGDYCC) immobilized regions. (c) DAPI-stained image of 3T3 cell growth after 24 h of incubation on the previously mentioned (a)-modified surface. The polymer coatings were prepared on tissue culture polystyrene (TCPS) microplates for the study.

Conclusions

Multifaceted and route-controlled click reactions were demonstrated with advanced interface coatings consisting of multifaceted clickable poly-*para*-xylylenes. In a concurrent manner, straightforward and orthogonal biological activities (i.e., protein adhesion to protein resistant properties or a cell-adhered state to a cell-repelled state) were precisely controlled and/or in spatially defined regions. Unlike the conventional synthesis approach of preparing clickable functional groups in multiple complex steps with low product yields, vapor deposition was employed in a facile and versatile fabrication process to prepare multifaceted clickable surfaces in a single step with a nearly complete synthesis yield. In addition, the fundamental aspects of the CVD coating technology translate well from one substrate/application to another.[19, 20] Extensions of applications that employ clickable poly-*p*-xylylenes are envisioned to attract more clickable moieties, such as strained alkyne groups[21] or Diels–Alder groups, to enable metal-free and more biocompatible reaction conditions. Additionally, the creation of more sophisticated orthogonal properties, such as distinct differentiation pathways for stem cells, diversified combinations of drug potencies, as well as active/positive and passive/negative signaling pathways for tissue engineering and diagnostic studies, are also foreseeable and will take advantage of these multifaceted coatings.

Acknowledgements

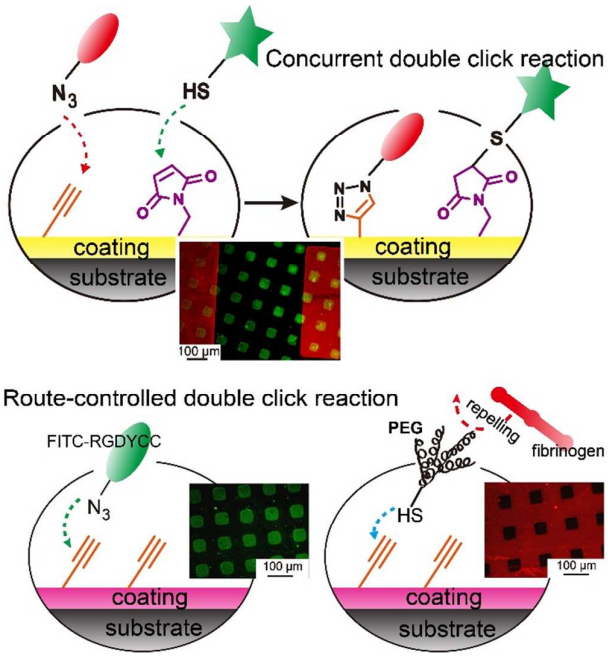
H.-Y. Chen gratefully acknowledges support from the Ministry of Science and Technology of Taiwan (MOST 104-2628-E-002-010-MY3 and MOST 101-2628-E-002-034-MY3) and the National Taiwan University (102R7745 and 103R7745).

Notes and references

1. W. L. Murphy, T. C. McDevitt and A. J. Engler, *Nat Mater*, 2014, 13, 547-557.
2. T. Sun, G. Qing, B. Su and L. Jiang, *Chemical Society Reviews*, 2011, 40, 2909-2921.
3. H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angewandte Chemie International Edition*, 2001, 40, 2004-2021.
4. E. M. Sletten and C. R. Bertozzi, *Angewandte Chemie International Edition*, 2009, 48, 6974-6998.
5. H. Durmaz, A. Sanyal, G. Hizal and U. Tunca, *Polymer Chemistry*, 2012, 3, 825-835.
6. U. Tunca, *Macromolecular Rapid Communications*, 2013, 34, 38-46.

7. H.-Y. Chen, T.-J. Lin, M.-Y. Tsai, C.-T. Su, R.-H. Yuan, C.-C. Hsieh, Y.-J. Yang, C.-C. Hsu, H.-M. Hsiao and Y.-C. Hsu, *Chemical Communications*, 2013, 49, 4531-4533.
8. H. Nandivada, H.-Y. Chen, L. Bondarenko and J. Lahann, *Angewandte Chemie International Edition*, 2006, 45, 3360-3363.
9. M.-Y. Tsai, C.-Y. Lin, C.-H. Huang, J.-A. Gu, S.-T. Huang, J. Yu and H.-Y. Chen, *Chemical Communications*, 2012, 48, 10969-10971.
10. M.-Y. Tsai, Y.-C. Chen, T.-J. Lin, Y.-C. Hsu, C.-Y. Lin, R.-H. Yuan, J. Yu, M.-S. Teng, M. Hirtz, M. H.-C. Chen, C.-H. Chang and H.-Y. Chen, *Advanced Functional Materials*, 2014, 24, 2281-2287.
11. X. Deng, C. Friedmann and J. Lahann, *Angewandte Chemie International Edition*, 2011, 50, 6522-6526.
12. S. Alom Ruiz and C. S. Chen, *Soft Matter*, 2007, 3, 168-177.
13. J.-T. Wu, T.-P. Sun, C.-W. Huang, C.-T. Su, C.-Y. Wu, S.-Y. Yeh, D.-K. Yang, L.-C. Chen, S.-T. Ding and H.-Y. Chen, *Biomaterials Science*, 2015, 3, 1266-1269.
14. C. Wendeln, S. Rinnen, C. Schulz, H. F. Arlinghaus and B. J. Ravoo, *Langmuir*, 2010, 26, 15966-15971.
15. M. Uygun, M. A. Tasdelen and Y. Yagci, *Macromolecular Chemistry and Physics*, 2010, 211, 103-110.
16. E. Wischerhoff, K. Uhlig, A. Lankenau, H. G. Börner, A. Laschewsky, C. Duschl and J.-F. Lutz, *Angewandte Chemie International Edition*, 2008, 47, 5666-5668.
17. M. Q. Zhang, T. Desai and M. Ferrari, *Biomaterials*, 1998, 19, 953-960.
18. H. Du, P. Chandaroy and S. W. Hui, *Biochim. Biophys. Acta-Biomembr.*, 1997, 1326, 236-248.
19. H.-Y. Chen, M. Hirtz, X. Deng, T. Laue, H. Fuchs and J. Lahann, *Journal of the American Chemical Society*, 2010, 132, 18023-18025.
20. J.-T. Wu, C.-H. Huang, W.-C. Liang, Y.-L. Wu, J. Yu and H.-Y. Chen, *Macromolecular Rapid Communications*, 2012, 33, 922-927.
21. A. J. Inglis and C. Barner-Kowollik, *Macromolecular Rapid Communications*, 2010, 31, 1247-1266.

TABLE OF CONTENTS ENTRY



Multifaceted and route-controlled click reactions are realized using functionalized poly-para-xylylenes coatings, and the concurrent display of orthogonal interface properties is demonstrated.