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A Traceless Cross-linker for Photo-Cleavable Bioconjugation

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Abstract

Photo-responsive bioconjugation empowers the development of novel methods for drug discovery, disease diagnosis, and high-throughput screening, among the others. In this paper, we report on the characteristics of a traceless photo-cleavable cross-linker, di 6-(3-succinimidyl carbonyloxymethyl-4-nitro-phenoxy)-hexanoic acid disulfide diethanol ester (SCNE). The traceless feature and the biocompatibility of this photo-cleavable cross-linking reagent were corroborated. Consequently, we demonstrated its application in reversible phage particle immobilization that could provide a platform for direct single phage screening. We also applied it in protein-photoprinting, where SCNE acts as a “photo-eraser” to remove the cross-linked protein molecules at a desired region in a simple, clean and light-controllable fashion. We further demonstrated the two-tier atomic force microscopic (AFM) method that uses SCNE to carry out two subsequent AFM tasks *in situ*. The approach allows guided protein delivery and subsequent high-resolution imaging at the same local area, thus opens up the possibility of monitoring protein functions in live cells. The results imply that SCNE is a versatile cross-linker that can be used for a wide range of applications where photo-cleavage ensures clean and remote-controllable release of biological molecules from a substrate.

Introduction

Photo-cleavable cross-linkers play important roles in biological, pharmaceutical and biotechnology research¹⁻⁴ in studies involving high throughput target identification,^{5,6} kinetic analysis of bio-recognition,⁷⁻⁹ dissection of signal transduction,^{10,11} and ion transportation processes,¹² to name a few examples. The use of a photo-cleavable cross-linker as a remote control has become increasingly common in drug discovery processes for identifying the target of drugs,¹³ determining the affinity and selectivity of drug-target interaction,¹⁴ and identifying the binding site on a target.¹⁵⁻¹⁷ Among the photophores synthesized, *o*-nitrobenzyl derivative molecules show great promising. Upon photo-irradiation, the nitro group is changed to a nitroso group, and the benzyl alcohol is oxidized to aldehyde or ketone.¹⁸ Due to its rapid photoreaction, nitrobenzyl derivatives have become one of the most popular photolabile molecules in the field. Two strategies were engaged in applying nitrobenzyl derivatives. One is to deactivate a molecule by caging it with a nitrobenzyl group. Upon the photolysis of the nitrobenzyl group, the molecule is uncaged, and its function is resumed. A range of breakthrough applications have been established

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Supporting Information: Synthesis and characterization of SCNE. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

using this strategy, including a) the creation of tunable surface active layers¹⁹ and controllable protein micro/nano patterns^{20,21} by activating biotin-avidin interactions²² and nickel complex -His-tagged protein interactions;²³ b) directing cell adhesion in selected regions by activating Arginyl-Glycyl-Aspartic acid peptides;²⁴ c) manipulating the bioactivity of metals or their coordinating ligands for potential therapeutic application;²⁵ d) the intervention of DNA strand-strand interactions²⁶ and e) the establishment of near-field lithography, a new fabrication method for nanostructured patterns.^{27,28} Another strategy is to position the nitrobenzyl group in the middle of a diad molecule. The nitrobenzyl group functions as a photo-cleavable cross-linker, thus confers a photo-controllable way to dissociate the diad to two molecules. Intriguing applications established using this strategy include the generation of controllable substrates for protein adhesion and dissociation,²⁹ a precise fabrication method to generate supported bilayer structures,³⁰ and the rapid release of bioactive materials from inactive precursors.³¹

Previously, we reported the synthesis and characterization of a heterobifunctional photo-cleavable cross-linker, succinic acid succinimidyl ester 5-thioxyloxy -2- nitrobenzyl ester (SSTN).³² We demonstrated its application in manipulating protein attachment/detachment to substrates.³³ Recently, we synthesized a new multifunctional photo-cleavable cross-linker, di 6-(3-succinimidyl carbonyloxymethyl-4-nitro-phenoxy)-hexanoic acid disulfide diethanol ester (SCNE)³⁴ (see Supporting Information). The molecule was designed to include a disulfide bond, acting as the attaching group to a substrate (especially for a gold-coated substrate). SCNE is a homo-dimer containing two identical nitrobenzyl ester motifs with succinimidyl groups for photo-cleavable cross-linking of two identical or distinct biomolecules at each terminal. Importantly, we placed the succinimidyl carbonyloxymethyl group next to the nitrobenzene ring to eliminate any residual tags on the biomolecules after photo-cleavage and decarboxylation, a way to prevent any possible change of functionality of the biomolecules upon their release by SCNE photo-cleavage.

In this paper, we first verify the traceless feature and the biocompatibility of SCNE. We then demonstrate its application in reversible phage particle attachment, protein-photoprinting, and the development of a two-tier AFM method. The results imply that SCNE can effectively conjugate biomaterials to a substrate, and warrant the photo-released biomaterials to retain both chemical structure and biofunctionality.

Materials and Methods

Chemicals and reagents

All solvents as well as phosphate buffered saline (PBS) with pH 7.4, N-hydroxysuccinimidyl-11-Mercapto-undecanoate (HSC11NHS), anti-human immunoglobulin G (anti-IgG) and human IgG (IgG) proteins were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. A succinimidyl derivative dye, 6-(((4-((4-difluoro-5-(2-thienyl)-4-bora-3a, 4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino)hexanoic acid succinimidyl ester (BODIPY TR), and a permanent cross-linker, succinimidyl 3-(2-pyridyldithio) propionate (SPDP), were purchased from Molecular Probes (Grand Island, NY). Ultrapure water (18.2 M Ω) was obtained with a Barnstead Nanopure filtration system (Thermo Fisher Scientific Inc., Franklin, MA) and was used throughout the experiments.

Surface Modification

Anti-IgG or phage particles were modified on a gold-coated Si wafer substrate via the previously synthesized cross-linker³⁴ (see Supporting Information), di 6-(3-succinimidyl carbonyloxymethyl-4-nitro-phenoxy)-hexanoic acid disulfide diethanol ester (SCNE). A pre-cleaned Si substrate was coated with 7 nm of titanium, followed by 50 nm of gold using

a vacuum evaporator (Denton Vacuum, Moorestown, NJ). The substrate was immediately immersed in SCNE solution (10^{-4} M in acetonitrile) and kept for 12 h at room temperature under N₂ gas protection. After thoroughly rinsing by acetonitrile and PBS buffer, the substrate was dipped in 10^{11} pfu/mL phage particle solution or 10^{-6} M protein solution, and incubated overnight at 4° under N₂ gas protection. After thoroughly rinsing with PBS buffer, the sample was subjected to phage particle photo-release or protein photo-printing experiment.

AFM tips were modified in a similar way. Si₃N₄ tips were cleaned via UV/ozone treatment for 30 min. using a tip cleaner (BioForce Nanosciences, Inc., Ames, IA). Thinner metal coating (3 nm titanium and 15 nm gold) was applied to the tips. The freshly coated tips were modified by either SPDP or SCNE for permanent or photo-cleavable conjugation of IgG on the tip to perform lateral force measurement or to establish the two-tier AFM method.

Protein patterning was achieved by micro-contact printing. A gold-coated Si substrate was immersed in a HSC₁₁NHS solution (10^{-3} M in ethanol) overnight at 4° under N₂ gas protection. After thoroughly rinsing by ethanol and PBS buffer, the surface was printed with anti-IgG using a pre-inked PDMS stamp. It was immediately rinsed by PBS buffer and Tris-HCl buffer (pH 8.0) to passivate the hydroxysuccinimide groups at the unprinted regions of the surface.

Characterization of SCNE Photolysis

To monitor the photo-cleavage reactions, UV-Vis spectra were collected using a HP 8453A diode array spectrophotometer (Agilent Technology Inc., Santa Clara, CA). The steady-state emission-spectral measurements were conducted using 1 cm × 1 cm quartz cells in a PTI C60 spectrofluorimeter (Photon Technology International, Inc., Birmingham, NJ) equipped with a 75 W mercury lamp as an irradiation source. Potassium ferrioxalate actinometry was utilized to determine the reaction quantum yield. Photolysis of SCNE for protein or phage particle detachment was achieved using a 60 W mercury lamp equipped with an IR filter and an interference filter (330 ± 5 nm, Andover Corporation Optical Filter) at a dose rate of $1 \mu\text{W}/\text{cm}^2$.

Cross-linking of Homodimeric pilM protein (3HG9)

Homodimeric pilM protein (Protein Data Bank code 3HG9) of 3.65×10^{-5} M was prepared in PBS (pH 7.4) and cross-linked by SCNE. To 300 μL of freshly-prepared 3HG9 solution, 50 μL SCNE (5 mM in acetonitrile) was added and the mixture was kept at room temperature for 8 h followed by the addition of 250 mM Tris-HCl buffer (pH 8.0) to passivate any unreacted SCNE. Prior to MALDI-TOF analysis, the samples were desalted/concentrated with a C4 Zip Tip (Millipore Corporation, Billerica, MA) according to the manufacturer's instruction. To carry out the photolysis and recover the proteins, 20 μL of cross-linked protein solution was transferred to a quartz cuvette and irradiated under UV light at 330 nm for 2 h. Samples were desalted/concentrated with a C4 ZipTip after 30 min., 1 h, and 2 h, respectively, followed by MALDI-TOF analysis.

MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry analysis of the cross-linked peptides was performed on a Voyager Biospectrometry DE-STR Workstation (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser (330 nm). The instrument was operated in positive ionization mode and the measurements were performed in linear mode (mass range m/z 10000 to 40000) using sinapinic acid as the matrix. A saturated matrix solution was prepared in 80% acetonitrile, 19.9% water and 0.1% TFA (v/v/v). Samples were prepared using the dried droplet method by spotting 1 μL of matrix solution and 1 μL of zip-tipped sample solution

onto the target. Spectra from 100 to 300 laser shots were accumulated to produce one spectrum. The instrument was calibrated using the monoisotopic masses of standard proteins (New England Biolabs Inc., Ipswich, MA): RNase A (Bovine), average MW 13690.29 Da, monoisotopic MW 13681.32; triose phosphate isomerase (E. coli), average MW 26971.81 Da, monoisotopic MW 36954.82; bovine serum albumin, average MW 66462.98 Da, monoisotopic MW 66419.87.

Plaque assay

A plaque assay was applied in determining the phage activity. Serial dilution of the phage solution was conducted by gradually diluting the phage solution by 100-fold. The mixture of phage dilution, bacteria, and agarose was loaded on top of the LB/Agar. After incubation overnight at room temperature, individual plaques appeared as clear dots in the dish. The concentration of the original phage solution (pfu/mL) can be calculated by:

$$\text{number of plaques} / (d \bullet v),$$

where d is the dilution factor, and V is the volume of the diluted phage added to the plate.

AFM analysis

The AFM study was carried out using a multimode Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA), equipped with a J-type scanner. Topographic imaging was performed using oxide sharpened Si₃N₄ tips in PBS buffer in a fluid cell. The images were acquired in the fluid-tapping mode at 512 pixels per line at a thermal resonance frequency of 8-10 kHz. The scan rate was less than 1.00 Hz. To detect the photo-printed anti-IgG, imaging in lateral force (LF) mode was carried out using an IgG modified tip at a scan angle of 90°. Line direction was set for both “trace” and “retrace” to compare and corroborate the high frictional force attributed to the specific interaction between IgG and anti-IgG. Alternatively, an IgG modified tip was utilized to scan a surface at a resolution of 32 pixels per line; a force-distance curve was collected at each pixel to detect the presence of anti-IgG via the specific interaction, indicated by an adhesive force in the retraction force curve.³⁵⁻³⁸ The spring constant of the AFM cantilever was 0.07 ± 0.01 N/m, calibrated by using reference cantilevers with known spring constants.³⁹

Protein delivery was achieved by applying direct irradiation to SCNE that conjugates IgG molecules to an AFM tip. The tip was positioned at an anti-IgG patterned region, engaged and scanning at a pre-set area of 0.0001×0.0001 nm² and a scan rate of 0.02 Hz (in practice, the tip was fixed in position and in contact with the substrate). 20 min. irradiation was applied to photo-release IgG molecules from the AFM tip. To minimize the light penetration through the sample, we applied irradiation parallel to the sample surface. The protein-free tip was then used to scan this local area to examine the delivered proteins.

Results

SCNE photolysis, traceless nature and biocompatibility

The photolytic reaction of SCNE was carried out by irradiating a SCNE solution in CH₃CN with a high-pressure mercury lamp equipped with a band-pass filter centered at 330 nm. Based on NMR, elementary analysis and mass spectroscopic experimental results³⁴ (also see Supporting Information), we concluded the photolysis scheme as outlined in Fig. 1A. The photolysis process was also monitored by UV-vis spectroscopy. Figure 1B illustrates the absorption spectral changes of the SCNE solution during photolysis with 10-minute

irradiation time intervals. The presence of an isosbestic point at 274 nm indicates a clean conversion from *o*-nitrobenzyl ester to nitrosobenzaldehyde.⁴⁰ The reaction is characterized quantitatively by monitoring the reduction of the peak at 313 nm. Using a potassium ferrioxalate actinometry,^{32, 41} the quantum yield of the photoreaction was calculated at 0.020 ± 0.005 under the irradiation at 330 nm.

SCNE conjugates to amine groups in a target molecule via the N-hydroxysuccinimidyl terminal. It was anticipated that the photo-cleavage of SCNE results in the recovery of the amine groups without any residues. We thus designed experiments to illustrate the reinstatement of the amine groups after SCNE photolysis. As shown in Fig. 2A, a primary amine modified substrate was prepared by forming a monolayer of 2-aminoethanethiol on a gold coated substrate. SCNE was then anchored to the substrate by amine-succinimidyl reaction to form a covalently bound monolayer. Such SCNE-anchored substrates were subjected to two separate treatments: (1) after exposure to irradiation of 330 nm light for 2 h, the substrate was rinsed (sample i) and then incubated with a succinimidyl derivative dye solution (BODIPY TR, 1×10^{-6} M in acetonitrile) for 2 h at room temperature, followed by thoroughly rinsing to remove any unbound dye molecules (sample ii); (2) without irradiation, the substrate was treated with the same dye solution (sample iii). In sample i, no fluorescence signal was detectable. Strong fluorescence signal peaked at 625 nm was observed in sample ii (Fig. 2B, green line); whereas negligible fluorescence signal was detected in sample iii in the absence of irradiation (Fig. 2B, blue line). Apparently, the clean photo-cleavage of SCNE led to restoration of the amine groups on the substrate, and permitted the same amine-succinimidyl chemistry to couple the dye. The fluorescence intensity in sample ii is close to that obtained in sample iv (red line in Fig. 2B), in which the amine modified substrate incubated directly with the dye solution under the same conditions. It implies that ~100% amine group recovery was achieved after photolysis of SCNE. We also carried out a negative control experiment, in which SCNE was replaced by a permanent cross-linker, SPDP, to react with the amine modified substrate (data not shown). After the surface was irradiated and treated with dye solution under the same experimental conditions, dye molecules were undetectable. It provides further evidence that SCNE is a traceless, photo-cleavable cross-linker.

The traceless nature of SCNE was also examined when SCNE was applied in protein cross-linkage. The homo-dimeric pilM protein from *Pseudomonas aeruginosa* 2192 (3HG9) was chosen as a model system because of its simplicity, possessing only two lysines per monomer for convenient Mass spectroscopic data analysis. Although the X-ray crystallographic analysis indicated that 3HG9 is a homodimer,⁴² the protein was in the forms of both monomer (*m/z* 13673) and dimer complex (*m/z* 27348) when dissolved in solution as indicated by the MALDI data analysis (Fig. 3A). Cross-linking by SCNE caused the increase of the monomer mass from 13673 to 14411 (Fig. 3B), indicating that two lysines in the monomer were intra-cross-linked by SCNE. A shoulder peak at *m/z* 15122 is likely a result of the reaction of SCNE with the amino group at the N-terminal, and the additional mass of 712 Da is attributed to the hydrolyzation of the carbonated ester at the other end of SCNE while a CO₂ is lost. In the dimer complex, there are six primary amines: four in lysines, and two in N- terminals. The cross-linking gave rise to several peaks. Among them, the peak at *m/z* 28823 is associated with dimers with two intra-complex cross-links. Other peaks ranging from *m/z* 29483 to *m/z* 29615 are likely associated with dimers having various numbers of intra-cross-links and N-terminal labels. After 30 min. irradiation (Fig. 3C), we observed the partial recovery of the cross-linked proteins in both monomers and dimers. After 2 h irradiation (Fig. 3D), the proteins were fully recovered, as evidenced by the clean peaks at *m/z* 13673 and *m/z* 27384 for the original protein monomer and dimer, respectively. Note that the laser of MALDI-TOF is significantly weaker than the UV light

used for photolysis. Thus the laser-induced SCNE cleavage during MALDI measurement is negligible.

One concern in SCNE application is that, the irradiation at a wavelength of 330 nm for SCNE photolysis may cause degradation of bio-functions of biomolecules (such as DNA, proteins) or living organisms. The results from MALDI analysis suggest that cross-linked proteins can fully resume their primary chemical structures upon 2 h irradiation by the light of 330 nm. To demonstrate the safety of SCNE photocleavage towards biofunctions, we further investigated the bioactivity of T7 phage particles under the same irradiation condition. Using a plaque assay, we found the number of infectious T7 phage particles remained constant after 2 h irradiation with light of 330 nm. However, the number decreased dramatically within 30 min. when the phage particles were irradiated with light of 297 nm (Fig. 4A). The result implies that T7 phage particles well survived the irradiation condition for SCNE photolysis. Taken together, SCNE is a traceless cross-linker for protein conjugation; the irradiation condition of its photolysis has negligible impact on the function of biomaterials. These are further evidenced when we demonstrate the application of SCNE in the following three areas.

Photo-release of covalently immobilized phage particles

In this study, we applied SCNE in achieving the photo-release of conjugated phage particles from a micro-channel array (Fig. 4B) with the potential application in direct phage screening (see Discussion section). The microchannel array has a total of 2682 channels. Each channel is 5 μm in diameter, 25 μm long, and the adjacent channels are separated 25 μm apart (center-to-center distance). A gold ring with a width of 1 μm is embedded in the micro-channel for anchoring phage particles via SCNE. The effective volume of the compartment within each channel is ~ 20 femtoliters. T7 phage particles were loaded at a solution concentration of 1.9×10^{11} pfu/mL, and conjugated to the gold ring via SCNE. Statistically only one or two phage particles can be accommodated in each channel at this loading concentration. After thoroughly rinsing, the final wash solution was collected as the control sample. As shown in Fig. 4C, less than 15 phage particles presented in the final washing elute before the photolysis, providing a baseline for counting the number of particles photo-cleaved from the surface. After irradiation with the light of 330 nm, the elutes from the experiment were collected for plaque assay analysis. 1340 ± 30 and 2970 ± 20 T7 phage particles (out of at least five measurements) were present in the post-irradiation elute upon 30 min. and 2 h irradiation, respectively. It suggests efficient detachment and recovery of phage particles after 2 h irradiation. Control experiments were also conducted using SPDP, a permanent cross-linker, to anchor the T7 phage particles. Due to the non-cleavable nature of SPDP, most phage particles remained even after two hours of irradiation (Fig. 4C). Note that, when SPDP was used, the number of phage particles was higher in the final wash elute prior to irradiation. We speculate that the shorter chain in SPDP impedes its ordered monolayer assembly on gold, leaving more defects for physical adsorption of phage particles and consequently a larger number of particles in the wash elute.

Protein photo-printing

We combined the use of SCNE with the photolithographic technique to achieve simple and clean protein photo-printing. Anti-IgG molecules were anchored on a gold-coated substrate via SCNE. As shown in the AFM images in Fig. 5A, the surface is flat and featureless, except some bright dots which are likely protein aggregates or gold particle aggregates. A 1 h irradiation of 330 nm light was carried out through a Cr mask that possesses arrays of dots with a diameter of 3 μm . SCNE cleavage occurred at areas exposed to the irradiation, causing detachment of the antibody molecules; whereas antibodies in the shadow of the mask (within the circle in Fig. 5B) remained intact. After thoroughly rinsing, protein

patterns were visualized as shown in Fig. 5B. The height of the anti-IgG patterns is 7.1 ± 0.9 nm above the surrounding areas, indicating a monolayer of anti-IgG covalently anchored on the substrate.^{37, 38, 43-45}

To investigate the binding activity of the photo-printed protein layer, an IgG-modified AFM tip was used to perform measurements in LF mode. In this mode, when the line direction is set to “trace”, high lateral (frictional) forces are indicated by bright contrast; the relative signal strength inverts as the scan direction is reversed to “retrace”, thus high lateral forces are indicated by dark contrast.^{46, 47} Reversed contrast of the patterns is shown in the “trace” (Fig. 6B) and “retrace” (Fig. 6C) LF images. In contrast, when a BSA modified tip was applied in repeating the experiment, no clear pattern was observed in LF images even though the patterns were clearly visible in topographic images. The results imply that the origin of the high friction force in Fig. 6 is the anti-IgG - IgG specific interaction. It verifies the binding activity of the photo-printed proteins.

Two-tier AFM

A two-tier AFM assay was established based on the use of SCNE in the AFM probe modification. It permits two sequential AFM tasks at the same site to locate specific protein species on a surface (tier 1) and to deliver its binding partner at a targeted position (tier 2). In addition, the post-delivery event can be monitored *in situ*.

We modified an AFM tip with human IgG via SCNE. Anti-IgG was patterned on a surface by micro-contact printing, as shown in the height image in Fig. 7A. When the tip scanned the surface in force mode, a force-distance curve was collected at each pixel. Typical adhesion force curves at the bright (M) and dark (N) pixels in Fig. 7B are shown in Fig. 7C. The adhesion force measured on the anti-IgG patterns was 1.2 ± 0.2 nN, in contrast to the low adhesion force of 0.2 ± 0.1 nN at the surrounding regions where anti-IgG was absent. Hence the strong adhesion force is attributed to the specific interaction between IgG and anti-IgG, which identifies anti-IgG at the bright-contrasted regions.³⁵⁻³⁸ This was verified by locating the same area in the height image in Fig. 7A (highlighted region), in which the anti-IgG patterns (in bright contrast) elevate above the surrounding areas. Note that the tip apex of a gold-coated AFM probe was 35 nm in radius, much greater than the dimension of IgG.⁴³⁻⁴⁵ Hence multiple anti-IgG molecules were immobilized on the AFM probe. The measured adhesion force is ascribed to the specific interaction between multiple pairs of anti-IgG and IgG. Adhesion force based protein identification is particularly important when a surface contains a mixture of unknown proteins, as in the case of live cell membranes.³⁵⁻³⁸ In the current experiment, we demonstrated that the IgG-modified tip provided guidance to identify the anti-IgG pattern for the follow-up IgG photo-delivery. By selectively modifying IgG at the tip apex, we were able to restrict IgG delivery to a desired region of the anti-IgG pattern upon SCNE photo-cleavage (Figs. 8A & B). After photo-cleavage, the protein-free tip was then employed to collect images to monitor the IgG and anti-IgG binding event. As illustrated in the height profile analysis (Figs. 8C & D), the IgG is characterized by a 7.7 nm elevation above the round-shaped platform of anti-IgG pattern, and the anti-IgG in the pattern is 7.2 nm above the surrounding area. The measured height of IgG is consistent with the literature reported value.^{43-45, 48} IgG binding was not detected on other patterns of the same surface, indicating the successful local protein delivery and the effective monitoring of the post-delivery event. The photo-delivered IgG molecules are stable even after continuous scans of the local area, and remained for days when the sample was kept in PBS buffer. In the absence of anti-IgG on the substrate, the delivered proteins were visualized instantly, however, disappeared from the surface upon continuous scans. It provides further evidence that the delivered proteins retained the biofunctionality to recognize the binding partners on the substrate.

Discussion

SCNE is a tri-functional cross-linker. The N-hydroxysuccinimidyl and the disulfide groups permit conjugation of proteins to a gold-coated substrate. The photolysis of the nitrobenzyl ester group in SCNE offers clean cleavage and reinstatement of the amine group of the conjugated protein. Thus SCNE is capable of attaching proteins or organisms to a substrate, and subsequently releasing them from the substrate without a trace of alteration in structure and function. Note that the ether group in the 5' position places the quantum yield of SCNE photolysis at the lower end among the *o*-nitrobenzyl compounds.¹⁸ However, the presence of an ether group in the 5' position renders SCNE a longer-wavelength absorption (absorption maximum at ~310 nm) when compared to an absorption maximum at ~260 nm for a pure *o*-nitrobenzyl molecule. This effectively prevents the photodamage of proteins and other biomolecules when irradiation is employed for photocleavage, as was substantiated in our experiments (Figures 3-5).

We have demonstrated the application of SCNE in attaching and detaching phage particles from a micro-channel array (Fig. 4). The experiment was inspired by the need of new methods for phage particle based screening. Phage display has been the workhorse for mapping the protein-protein interactions of proteins of interest. In phage display, proteins or peptides are expressed as fusions to a capsid protein. The target-specific phage particles can be isolated by a biopanning process. The key advantage of phage display or a phage-particle based screening method is the capability of linking the binding proteins with the genetic information that encodes them within the phage. It enables the identification of a protein sequence at the single molecule level, a capability that no other method can achieve so far. To fully attain this potential, it is critical to develop a method that is capable to collect individual phage particles from a phage mixture. One strategy for such a challenge is 1) to compartmentalize the phage mixture at the single phage particle per compartment level; 2) to immobilize the phage particle to the compartment for binding reactions and subsequent washing; and 3) to release and recover the selected phage particle for corresponding protein identification. To implement this strategy, we designed the micro-channel array with a total of 2682 channels that served as well-separated compartments. 2970 ± 20 phage particles were photo-cleaved from the channels, indicating an average of one to two phage particles were accommodated in each channel. Individual phage particles are expected to be revealed against the target in each compartment for effective screening. This can be accomplished by introducing fluorescent-tagged target to the substrate and visualize the target-specific phage complexes by a fluorescence microscope. The efficient release of phage particles from the microarray, as demonstrated in Figure 4, permitted the recollection of selected phage particles from the compartments for further amplification and sequencing. Such a system has the potential to screen a mixture of phage particles in a one-by-one fashion to eliminate the tedious multi-step enrichment procedure in conventional biopanning processes. The same principle can be applied to the screening of sub-population cells within a cell mixture to understand the collective behavior of communicative cells. This research has been undertaken in our group.

Protein chips play increasingly important roles in modern biology and biotechniques.⁴⁹⁻⁵¹ One essential feature of chips is to array the proteins in a desired pattern. Current approaches rely on the fabrication of patterned substrate via lithography or micro-contact printing, followed by selectively anchoring protein molecules at the patterned regions through specific chemistry. Herein we introduced a simple and direct way to generate protein patterns applicable on a large scale. Starting with a uniform protein layer via SCNE conjugation to a substrate, we selectively removed unwanted proteins with light to transfer a geometric pattern from a photo-mask to the protein molecules on the substrate. Desired proteins within a pattern remain intact throughout the process, and the binding activity

remains unchanged (Fig. 6). While UV irradiation at a shorter wavelength can also remove the proteins by photodecomposition, products of photodecomposition will be left behind as small debris and intrude upon the protein patterns via random physical adsorption. Due to the traceless nature of SCNE, the proteins cleaved from the substrate are in their native form, thus can be cleaned and eliminated from the surface by thoroughly rinsing. This has been demonstrated in the clear and reproducible protein patterns as shown in Fig. 5.

One of the greatest challenges in cell biology is the ability to visualize and make measurements of individual proteins of an identified species in a living organism. We expect to tackle this challenge by the two-tier AFM assay established in the current study. Living cell membranes consist of a number of cell-surface proteins, generating a sophisticated surface. An antibody-modified AFM tip can distinguish a target protein from other species via force measurement, thus provide guidance to register a target protein. As demonstrated in the model system of the biomimic surface (Figs. 7 & 8), the IgG-modified tip identifies anti-IgG patterns. Due to the use of SCNE as the cross linker, natural IgG can be released from the tip and delivered to a local region by remotely switching on the light. Importantly, the post-delivery event can be *in situ* investigated immediately. This approach offers great potential in live cell studies, as it permits the monitoring of the initial ligand-receptor binding events, such as protein redistribution and rearrangement, associated with the protein function. We expect that the application of two-tier AFM will open up new avenues to elucidate cellular functions at the molecular level, and provide novel strategies for correcting cell dysfunction and for drug design.

In conclusion, SCNE is a versatile cross-linker that can be used for a wide range of applications where photo-cleavage ensures clean and remote-controllable release of biological molecules from a substrate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Photolysis reaction of SCNE. (A) Scheme of the photolysis of SCNE. (B) Absorption spectra of SCNE solution ($5.6 \times 10^{-5} \text{M}$) under different period of irradiation: (a) 0 min.; (b) 10 min.; (c) 20 min.; (d) 30 min.; (e) 40 min.; (f) 50 min.; (g) 60 min.; (h) 70 min.; (i) 80 min.; (j) 90 min.; (k) 110 min.; (l) 120 min.

Figure 2.

Surface reactions to demonstrate the traceless feature of SCNE. (A) Illustration of the paths of surface reactions. (B) Fluorescence spectra of substrates modified by SCNE and BODIPY TR. Red line: direct BODIPY TR modification without SCNE involvement (sample iv); blue line: BODIPY TR introduced to SCNE modified substrate without irradiation (sample iii); green line: BODIPY TR introduced to SCNE modified substrate after irradiation (sample ii).

Figure 3.
MALDI TOF spectra of protein cross-linking and recovery via irradiation of 330 nm light.
(A) Un-treated Protein 3HG9; (B) Cross-linked 3HG9 by SCNE; (C) Cross-linked 3HG9 after 30 minutes irradiation; (D) Cross-linked 3HG9 after 2 hours irradiation.

Figure 4.

Response of phage particles to irradiations. (A) Irradiation time-dependence of the bioactivity of phage particles. Circles: under 330 nm irradiation; triangles: under 297 nm irradiation. (B) Illustration of the micro-channel array, the surface modification and reactions. (C) Phage recovery from a SCNE or SPDP modified micro-channel array under various conditions of irradiation. Blue: no UV irradiation with SCNE cross-linker; green: 30 min. irradiation with SCNE cross-linker; pink: 120 min. irradiation with SCNE cross-linker; yellow: no UV irradiation with SPDP cross-linker; 120 min. irradiation with SPDP cross-linker.

Figure 5. Tapping mode AFM images of a gold coated Si wafer substrate functionalized by anti-IgG via SCNE cross-linker before (A) and after (B) 330 nm light irradiation through a Cr mask. Image size: 30×30 μm^2 ; irradiation time: one hour.

Figure 6. AFM images of Anti-IgG patterned surface collected by an IgG functionalized AFM probe. (A) height image; (B) LF mode image in “trace” direction; (C) LF mode image in “retrace” direction. Image size: $16 \times 16 \mu\text{m}^2$.

Figure 7.

AFM study of an anti-IgG patterned surface. (A) 16×16 μm² height image of anti-IgG patterns in round-shape with a diameter of 2.5 μm, captured at 512 pixels per line; (B) 12×12 μm² force map collected at the highlighted region in (A) at 32 pixels per line using an AFM tip conjugated with IgG via SCNE; (C) Typical force curves collected at bright (M) and dark (N) contrasted pixels in (B).

Figure 8. Protein delivery to an identified anti-IgG pattern followed by surface characterization. (A) $4 \times 4 \mu\text{m}^2$ height image of the identified anti-IgG pattern (highlighted in Fig. 7(B)), where IgG was locally delivered by SCNE photo-cleavage; (B) Higher resolution height images, captured at the highlighted region in (A), showing the structural difference before and after IgG local delivery (i.e., in the circled area); (C) Height profile at regions marked in (D), with the height differences of 7.7 nm and 7.2 nm between the pairs of red and green marks, respectively; (D) $5 \times 5 \mu\text{m}^2$ height image of the same anti-IgG pattern after IgG delivery.