Chapter 5 Accelerating Chemical Reactions by Molecular Sledding

Abstract

Increasing the effective molarity of substrates and reagents is crucial for the development of efficient chemical transformations. The small 11-amino acid peptide pVIc is able to slide along DNA and, therefore, it bears the potential to accelerate chemical reactions in the presence of polynucleotides once two reaction partners are attached to it. In such systems, the three dimensional diffusion of substrates and reagents is reduced to one dimension, virtually increasing their concentrations. With this in mind, the speed-up of covalent chemical bond formation was achieved between a sulfhydryl group and a 2-bromopropionic acid derivative undergoing a nucleophilic substitution reaction by utilizing sliding peptide-modified substrates. The 18-fold rate acceleration was accompanied by small stereoselectivity, which indicates the potential of chiral induction within an one dimensional reaction system. Moreover, a new type of DNA cleaving reagent was developed, consisting of pVIc covalently coupled to verteporfin, a clinically relevant photosensitizer. This peptide-porphyrin conjugate allowed targeting of DNA and resulted in increased photodegradation of double-stranded nucleic acids compared to a corresponding non-sliding peptide conjugate and the pristine drug. This strategy overcomes the life-span and active radius limitation of reactive oxygen species by bringing them in close proximity to DNA targets. We believe that this successful proof-of-concept will pave the way towards more studies on one dimensional chemistry.
Chapter 5

Introduction

Proteins’ sliding, hopping, and jumping between DNA segments are believed to be essential to the remarkable efficiency of the search for specific binding sites in large amounts of nonspecific DNA sequences.[1-4] The wide-spread occurrence of one-dimensional (1D) diffusion in cellular environments facilitates many fundamental biochemical processes such as controlling transcription, unwinding, supercoiling or cleaving DNA and repairing damaged polynucleotides.[5-10] In addition to those occurring in cells, another example of 1D diffusion was uncovered in adenovirus.[11,12] During viral maturation, the viral protease is responsible for the proteolytic processing of viral proteins to render the virus infective. Recently, single-molecule experiments have shown that this protease employs a short 11-amino acid pVlc peptide (GVQSLKRRRCF) [13] to slide along DNA inside the virus particle and thus effectively processes its protein substrates by reducing the dimensionality of search from three to one.[12] Moreover, pVlc peptide has proven to be a universal ‘molecular sled’, capable of carrying heterologous cargos along DNA.[14] Thanks to this feature, we demonstrated the speed up of the non-covalent association of a pair of biomolecules by coupling each of them to a sliding peptide and adding DNA as a catalyst to induce 1D diffusion of both substrates.[15]

Besides biology, performing chemical reactions in one dimension can be equally important in chemistry because decreasing the dimensionality of 3D diffusion of reactants may drastically influence the reactivity, and thus may exhibit large potential in synthesis and catalysis. To date, studies on 1D chemistry are very rare. The few successful examples were based on topologically interlocked rotaxane-like architectures.[16, 17] However, the complex fabrication procedures of these supramolecular assemblies greatly limits their practicality and widespread application.

Here, we demonstrate that the ability of a peptide sliding along DNA can be used to perform chemistry in one dimension. First, we selected a nucleophilic substitution reaction between a thiol and 2-bromopropionamide as a model and show that this conversion is accelerated 18 times when employing pVlc conjugated substrates in the presence of DNA. Second, we present a new strategy to improve photocleavage of DNA by conjugating pVlc to a clinically approved photosensitizer, namely verteporfin.[18,19] Upon irradiation, the efficiency of DNA photocleavage by the verteporfin-pVlc (vp-pVlc) conjugate was significantly enhanced compared to the unmodified drug.
Results and discussions

Scheme 1. Schematic representation of the reaction between the sulphydryl group in the dye containing Cy3-pVIc conjugate and 2-bromopropionamide (either (S) or (R)-enantiomer) attached to the N-terminus of another pVIc molecule.

Scheme 1 shows the components to enable covalent bond formation mediated by pVIc peptides sliding along DNA. As the first reactant, pVIc was conjugated with 2-bromopropionamide (either (R)- or (S)-enantiomer) at its N-terminus, while the thiol group was protected by a S-acetaminomethyl group. For simplicity, we refer to these conjugates as (S)- or (R)-Br-pVIc. The other reactant was Cy3-labelled pVIc (Cy3-pVIc) with a free thiol group acting as nucleophile. The cyanine dye Cy3 was introduced to improve the absorbance signal of the as-formed product and thus gave rise to the opportunity of tracking the reaction kinetics by High Pressure Liquid Chromatography (HPLC). Figure 1 shows a typical set of HPLC traces obtained from the reaction mixtures between 16 μM (R)-Br-pVIc and 20 μM Cy3-pVIc. Over time, the product peak at a retention time of 31.7 min increased. Formation of the product was additionally confirmed by MALDI-TOF mass spectrometry (Figure S1). Concomitantly, the intensity of the starting material peak of Cy3-pVIc at a retention time of 33.9 min decreased.
In the resulting kinetic curves, the concentrations of the formed product were plotted against time. As shown in Figure 2, the substitution on both substrates ([S]- and [R]-Br-pVIc) proceeded significantly faster in the presence of 100mer double-stranded DNA (100 bp DNA) compared to the background reactions performed in absence of DNA. This particular DNA length was chosen to ensure that the primary contributor to the rate acceleration is a 1D sliding mechanism.[15] To further elucidate the catalytic activity, the initial rates ($V_{\text{init}}$) of the substitution reactions, in the presence and absence of DNA, were calculated from the initial increase of product formation (Figure S2). With the help of the DNA track, the reaction between Cy3-pVIc and [R]-Br-pVIc was accelerated by 14.5-fold, while that applying [S]-Br-pVIc as substrate reacted with a higher rate acceleration of 17.9-fold. This slight preference for the [S]-enantiomer indicates that the chirality of DNA was transferred to the product formation to a certain extent. Although high enantioselectivities were achieved by employing DNA as a second coordination sphere in a series of chemical transformations,[20-22] we wish to emphasize that our results show the potential ability of chiral induction within an 1D synthetic reaction system, in which the distance between the reaction center and the chiral environment provided by the DNA double helix are probably further apart. More importantly, our results clearly demonstrate that the reduction of dimensionality of diffusion of the reactants from three to one makes the starting materials find each other faster and, thus, results in the overall rate acceleration of the reaction.
Encouraged by these findings, we attempted to broaden the scope of 1D chemistry based on the current system including a sliding peptide and a DNA track. As another example we decided to conjugate pVIc onto a photosensitizer, which can produce reactive oxygen species (ROS) under light irradiation.[23,24] Due to the sliding ability of pVIc along DNA, we expect that the generated ROS could be concentrated locally in close proximity to DNA and thus enhance the efficiency of DNA photocleavage. Since photodynamic therapy (PDT) has been limited by the short half-life (<0.04 μs) and limited action radius (<40 nm) of ROS in biological systems,[25,26] we also anticipated that this new strategy would offer an opportunity to possibly target DNA and improve PDT efficiency by overcoming some of these longstanding limitations.

Following our design strategy, verteporfin (vp) was chosen as photosensitizer because of two reasons: first, it exhibits an absorption wavelength at ~690 nm, which assists tissue penetration with less photosensitivity [27]; second, it exhibits a single carboxy group that allows the convenient conjugation to a peptide by amide bond formation. Verteporfin consists of two isomers which differ by the position of the ester group (either at ring C or D; Figure S3). These two isomers are often present in equal proportions and they were proven to be equally active photosensitizers either in vitro or in vivo [28]. Scheme 2 shows the synthetic route for the verteporfin-pVIc conjugate (vp-pVIc). After activation with dicyclohexylcarbodiimide and N-hydroxysucinimide, a maleimide was introduced at the carboxy site of vp. Subsequently, the resulting verteporfin-maleimide (vp-maleimide) was reacted with the thiol of pVIc to form the desired conjugate. Following the same coupling strategy, we synthesized another control verteporfin-peptide conjugate (vp-S) using a scrambled peptide (S-peptide, SFRRCGLRQVK), which contains the same residues as pVIc but in
a random order. Presumably, such alterations will affect the sliding behavior of the peptide yet preserving the net charge. The final products were purified by HPLC and characterized by UV-Vis spectroscopy. As shown in Figure S4, both conjugates show a characteristic absorption peak of verteporfin at 683 nm. The successful conjugation was further confirmed by MALDI-TOF mass spectrometry, which showed good agreement between obtained and expected molecular weights (Figure S5).

Next, photocleavage activity of the conjugates was evaluated by using supercoiled pUC19 plasmid DNA as a model substrate (Figure S6). It is worth to note that in the following experiments 35% (v/v) DMF was present as co-solvent in the buffer under physiological pH because of the poor solubility of verteporfin in aqueous medium. We first tested the DNA cleavage efficiency of the photosensitizers in dark, because an ideal photosensitizer should not be harmful to the target until the treatment beam is applied. For that purpose, pUC19 DNA was incubated with verteporfin conjugates overnight in the dark. The resulting samples were analyzed by agarose gel electrophoresis. As shown in Figure S7, without irradiation, no detectable cleavage of DNA was observed with all photosensitizers.

Subsequently, the efficiency of DNA cleavage by verteporfin derivatives was investigated in the presence of light. The samples containing DNA and different photosensitizers at various concentrations ranging from 1 to 5 μM were irradiated by light through a 680 nm band-pass filter (excitation power: 0.5 mW/cm²). Each sample was irradiated for 30 minutes and then subjected to gel electrophoretic analysis. For all concentrations, only negligible DNA cleavage was observed using pristine verteporfin as photosensitizer (Figure 3). Larger photodamage was achieved in the presence of vp-S conjugate, which can be attributed to the electrostatic interactions between the

**Scheme 2.** Synthesis route of verteporfin-pVlc conjugate (i: DCC, NHS, CH₂Cl₂, 1h; ii: THF, MeOH, overnight; iii: Et₃N, THF, MeOH, overnight). Verteporfin functionalized with a scrambled peptide vp-S was synthesized analogously.
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positively charged S-peptide and the negatively charged DNA backbone. The most efficient photosensitizer was verteporfin conjugated to the pVIc sliding peptide. Notably, in the presence of 5 μM of each verteporfin compound, almost no pUC19 DNA was detected for vp-pVIc, while there was still 50% and 90% DNA substrate left for vp-S and pure verteporfin, respectively. It is noteworthy that in our experiments, vp-pVIc conjugates cut supercoiled DNA into small pieces and linear or nicked DNA strands were not obtained,[29-31] as evidenced by the disappearance of these DNA bands in the agarose gel. These results indicate that the sliding ability of pVIc might play an important role in the way of cleaving DNA, namely, pVIc shuttles verteporfin along DNA which results in multiple damage sites as well as high efficiency of cleavage.

We further tested the effect of irradiation time on DNA cleavage using 2 μM of each verteporfin derivative. As shown in Figure 4, with only 5 min irradiation time, the majority damage of DNA was achieved. For real clinical treatment, short time exposure to light is an appealing feature because it will decrease the side effects of PDT.

Figure 3. a) Photodamage of supercoiled pUC19 DNA with different verteporfin derivatives in a 35% DMF (v/v) solution (pH=7.4) after 30 min irradiation employing a 680 nm band-pass filter (excitation power: 0.5 mW/cm²). b) Gel electrophoretic analysis of DNA cleavage: lane 1-6, DNA control, verteporfin 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, respectively; lane 7-12 DNA control, vp-S 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, respectively; lane-13-18, DNA control, vp-pVIc 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, respectively.
A question that still remains is whether vp-pVIc also cuts conventional double-stranded DNA equally well as supercoiled one. To give an answer, the disappearance of nicked DNA, which is the first product of the photodamage of the corresponding supercoiled DNA substrate, was followed (see SI for further information, Figure S8). Again, this analysis revealed that verteporfin attached to the functional peptide sled degraded this DNA substrate more efficiently than the two controls, i.e. pristine verteporfin and vp-s.

In previous work, the speed up of the kinetics of supramolecular interactions with the help of pVIc sliding on DNA was demonstrated exemplified for the acceleration of the binding between streptavidin and biotin as well as for the search of primers during the annealing phase of PCR.[15] In this contribution, the sledding of pVIc peptide on DNA was for the first time exploited in the context of speeding up covalent bond formation and photocleavage. The degree of acceleration of the nucleophilic substitution in the current work is comparable to the one observed for streptavidin-
biotin binding reported previously.[15] On the other hand, an artificial catalyst for DNA cleavage in a processive fashion has been described by Nolte et. al. before.[32] This elegant hybrid catalyst system contains a large clamp protein (ca. 680 amino acids) acting as a processivity mediator and a synthetic ligand and metal center. However, the biological part needs to be produced by laborious recombinant expression followed by difficult conjugation and purification steps. In this respect, we have significantly reduced the size of the sliding scaffold to only 11 amino acids, which renders our catalytic porphyrin-peptide hybrid appealing from a synthetic point of view.

**Conclusions**

In summary, we successfully demonstrated performing chemistry enabled a small peptide, which slides over DNA to accelerate covalent bond formation and to efficiently induce photocleavage of DNA. The speed-up of the chemical conversions was achieved by reducing the dimensionality of diffusion of substrates from three to one. As a proof-of-concept, the reactivity of a bimolecular nucleophilic substitution reaction was significantly enhanced by utilizing sliding peptide-modified substrates. The rate acceleration was accompanied by a small preference for one enantiomeric substrate, which indicates the potential of chiral induction within an 1D reaction system. Moreover, we have developed a new type of DNA cleaving reagent consisting of pVIc covalently coupled to the clinically approved photosensitizer verteporfin. This gives the opportunity to overcome the life-span and active radius limitation of reactive oxygen species by bringing them in close proximity to DNA targets. Upon irradiation, vp-pVIc exhibits the highest DNA cleavage activity, and more interestingly, it cleaves DNA into short fragments which might be attributed to the diffusional behaviour of pVIc sliding along the DNA substrate. Considering the fact that a series of artificial molecular sleds were successfully developed recently,[33] we envision that this work will pave the way towards more types of 1D chemistry.

**Experimental section**

**Materials**

Peptides (>95% purity) were purchased from CASLO ApS (Denmark). Cy3 labelled pVIc was acquired from Bio-Synthesis Inc (United States). All peptides were used without further purification. 100 bp DNA was purchased from Integrated DNA Technologies. Verteporfin was purchased from MedChem Express Company (United States). All other chemicals were obtained from Sigma-Aldrich and used without further purification. During all experiments, ultrapure water (18.2 MΩ) purified by a MilliQ-Millipore system (Millipore, Germany) was used.
Chapter 5

**General methods**

Reaction kinetics were analyzed by reverse-phase High-Performance Liquid Chromatography (HPLC) performed on a Shimadzu VP instrument using a C18 X Bridge BEF column. Verteporfin-peptide conjugates were purified on the same instrument. Mass spectrometric analysis of the synthesized conjugates was performed using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) with 3-hydroxypicolinic acid (HPA) as matrix. All UV-vis spectra were measured on a JASCO V-630 spectrophotometer. Analysis of DNA cleavage was carried out by agarose gel electrophoresis (1.5 weight%) with subsequent ethidium bromide staining, and photographs of the gels were taken with a GenoSmart Image Reader (VMR, The Netherlands).

**Synthesis**

**Standard procedure for substitution reactions:**

(S) or (R)-bromide-pVIc, pVIc-cy3 and 100 bp double-stranded (ds) DNA (if needed) were mixed in 50 mM Tris-HCl buffer (pH 8.5) containing 2 mM sodium chloride and 5 mM ethylenediaminetetraacetic acid (EDTA). The final concentration of pVIc-Cy3, bromide-pVIc and DNA are 20 μM, 16 μM and 4.4 μM, respectively. The mixtures were degased by purging with argon and reacted at room temperature. After reaction times ranging from 30 min to 24 hours, the reaction mixtures were treated with DNase I to digest DNA catalyst, and then they were analyzed by HPLC.

**Syntheses of verteporfin-peptide conjugates:**

Verteporfin (6 mg, 8.35 μmol) was dissolved in 1 ml anhydrous dichloromethane, to which 1 mg (8.67 μmol) N-hydroxysuccimide and 1.8 mg (8.72 μmol) N,N'-dicyclohexylcarbodiimide were added. The reaction was stirred at room temperature and was completed after one hour as monitored by TLC (5% methanol in dichloromethane). The dichloromethane was evaporated under reduced pressure and the crude mixture was re-dissolved in tetrahydrofuran and methanol (v/v = 4:1, 1.6 ml). 2 equiv. of N-(2-aminoethyl)maleimide trifluoroacetate salt (4.5 mg) was added to the mixture, which was kept overnight at room temperature. The verteporfin-maleimide (verteporfin-M) compound was purified by silica gel column chromatography, using 2.5% methanol in chloroform as eluent. To obtain verteporfin-peptide conjugates, veteporfin-M (2 mg, 2.46 μmol) and pVIc or scrambled peptide (S) (2 mg, 1.48 μmol) were mixed in tetrahydrofuran and methanol (v/v = 1:3, 600 μL). Then 6 μL trimethylamine was introduced, and the reaction mixture was shaken overnight.
The crude products were purified by gradient reverse phase HPLC (buffer A: 0.1% trifluoroacetic acid (TFA) in 5% acetonitrile and 95% MilliQ water, buffer B: 0.1% TFA in 95% acetonitrile and 5% MilliQ water). The final conjugates were lyophilized and kept in freezer for further use (yield:~60%).

**Preparation of supercoiled pUC 19 DNA:**

Escherichia coli strain DH5α (Life Technologies) was transformed with the circular 2686 base pair vector pUC19 (New England Biolabs) as described by Sambrook et al. [34] The vector was isolated from a 2 L bacterial culture in LB Lennox Broth (Sigma-Aldrich) using the GenElute HP Plasmid DNA Maxiprep Kit (Sigma-Aldrich). pUC 19 DNA was characterized by 1.5% agarose gel electrophoresis (Figure S6).

**Photocleavage of supercoiled plasmid DNA:**

DNA photocleavage by all verteporfin derivatives was conducted in Tris buffer containing 21 μM pUC 19 plasmid DNA. The samples were irradiated by a white light through a band-pass filter (680±10 nm, 0.5 mW/cm²). After different irradiation times, the efficiency of photocleavage was analyzed by gel electrophoresis on 1.5% agarose gel.
Supplementary figures

Figure S1. MALDI-TOF mass spectra of pure (R)-bromide pVlc (calculated M.W. 1554), (S)-bromide pVlc, pVlc-cy3 (calculated M.W. 1784) and their corresponding products (calculated M.W. 3257).
Figure S2. Determination of initial rates for a) (R)- and b) (S)-product formation in the presence of DNA, c) (R)- and d) (S)-product formation in the absence of DNA.
Figure S3. Structures of two regioisomers (A1 and A2) of verteporfin (differing by the position of the propionic ester group either at ring C or D).

Figure S4. UV-vis spectra of verteporfin and verteporfin-peptide conjugates. All compounds show a typical absorption wavelength at 683 nm.
Figure S5. ESI-MS spectra of vp-maleimide, vp-pVlc and vp-S conjugates.
**Figure S6.** Gel electrophoretic analysis of prepared pUC 19 DNA. The top faint band on the right is attributed to nicked plasmid DNA.

**Figure S7.** Incubation of verteporfin-conjugates with DNA overnight in dark. a) Agarose gel electrophoresis analysis; lane 1: pUC 19 DNA, lane 2: DNA+vp, lane 3: DNA+vp-s, lane 4: DNA+vp-pVIc. b) Recovery of pUC19 DNA as determined from gel electrophoretic analysis.
Figure S8. Monitoring of nicked pUC19 DNA during photodamage induced by verteporfin, verteporfin-scrambled peptide and verteporfin-pVIc conjugates at different photosensitizer concentrations. a) During the photodamage of supercoiled DNA (lower band in b), all experiments contained a smaller amount of nicked pUC19 plasmid DNA (upper band in b). The amount of nicked pUC19 DNA without photosensitizer was set to 100% and photocleavage was followed as a function of concentrations of verteporfin derivatives after 30 min light irradiation (680 nm, 0.5 mW/cm²). The increasing amounts of nicked DNA in the presence of pristine vp can be explained by the fact that the photosensitizer induces nicks in the supercoiled plasmid leading to its accumulation. This behaviour is to a lesser extent observed for reactions involving vp-s. In the case of vp-pVIc, a small increase in nicked pUC19 is detected up to concentrations of 2 μM. For higher concentrations of the photosensitizer conjugate the DNA cleavage is so efficient that smaller fragments are obtained. These observations clearly indicate that this vp conjugate also efficiently degrades non-supercoiled double stranded DNA. b) Gel electrophoretic analysis of DNA cleavage: lane 1-6, DNA control, verteporfin 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, respectively; lane 7-12 DNA control, vp-S 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, respectively; lane-13-18, DNA control, vp-pVIc 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, respectively.
References


