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Preparation of Mammalian Expression Vectors Incorporating Site-Specifically Platinated-DNA Lesions

Wee Han Ang, **William Wesley Brown**, and **Stephen J. Lippard***
Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

Abstract

FDA-approved platinum-based anticancer drugs, cisplatin, carboplatin and oxaliplatin, are some of the most effective chemotherapies in clinical use. The cytotoxic action of these compounds against cancer requires a combination of processes including cell entry, drug activation, DNA binding and transcription inhibition resulting in apoptotic cell death. The drugs form Pt lesions with nuclear DNA, leading to arrest of key cellular functions and triggering a variety of cellular responses. DNA probes containing Pt-DNA conjugates are important tools for studying the molecular mechanisms of these processes. In order to facilitate investigation of specific Pt-DNA lesion processing within live cells, we devised a strategy for constructing plasmids containing a single site-specific Pt-DNA adduct. The method involves the use of nicking restriction enzymes to create closely spaced tandem gaps on the plasmid followed by removal of the intervening doubly nicked DNA strand to create a short single-stranded gap. Synthetic platinated oligonucleotides were incorporated into the gapped plasmid construct to generate covalently closed circular platinated plasmid in good yield. We discuss the application of this methodology to prepare plasmids containing a platinum 1,2-d(G*pG*) or 1,3-d (G*pTpG*) intrastrand crosslink, two notable adducts formed by the three clinically approved drugs.

INTRODUCTION

The simple inorganic compound *cis*-diamminedichloroplatinum(II), or cisplatin, confers one of the most remarkable treatments for cancer, particularly testicular germ-cell cancer (1,2). The cytotoxic action of cisplatin occurs by a sequence of steps involving cell entry, drug activation, DNA binding, and transcription inhibition, resulting in apoptotic cell death (3). Formation of Pt-DNA adducts is an important determinant of the anticancer activity of platinum-based drugs, leading to arrest of key cellular functions, including transcription (4–7), triggering a variety of cellular responses, including nucleotide excision repair (8–11). Cisplatin reacts with nuclear DNA to form predominantly 1,2-d(G*pG*), 1,2-d(A*pG*) intrastrand, and to a lesser extent, 1,3-d(G*pNpG*) intrastrand and interstrand cross-links, where the asterisks denote the platinated nucleosides (12,13). Understanding how the cell processes these specific lesions is important in elucidating the mechanism of action and in forming a basis for the rational design of improved drugs.

Our group along with others have synthesized and utilized a variety of platinated DNA constructs as probes to investigate the action of platinum-based drugs in vitro and the processing of Pt-DNA adducts by cellular proteins (14–17). In particular, we are interested in preparing site-specifically platinated plasmids that can be used in live cells for application as in vitro transcription probes. A widely used method involved priming a single-stranded circular DNA template with a platinated oligonucleotide followed by extension around the template

*To whom correspondence should be addressed. E-mail: lippard@mit.edu.

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with a DNA polymerase (18–20). However, we require a more efficient means of preparation that can be applied to existing mammalian expression vectors with minimal modification to the construct and which can generate the platinated plasmid in good yield. In related work, plasmids containing site-specific mismatches as substrates for mismatch repair studies were prepared by treatment with nicking endonucleases followed by incorporation of synthetic oligonucleotides into the resulting gap (21,22). This method, together with some special design elements devised during the present study, has the potential to generate plasmids containing specific Pt-DNA lesions for mechanistic work. In the present report we demonstrate the power of this methodology to generate the desired constructs using commercial mammalian expression vectors as the host. Plasmids containing the most mechanistically important Pt-DNA adducts, 1,2-d(G*pG*)-Pt and 1,3-d(G*pTpG*)-Pt, formed by the clinically relevant cisplatin, carboplatin and oxaliplatin anticancer drugs were prepared and characterized. The key design features incorporated in our study assure a high yield of the desired bioconjugates.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases (REases), Nt.BbvCI nicking enzyme, calf intestinal phosphatase (CIP), T4 polynucleotide kinase (T4 PNK) and T4 DNA ligase were obtained from New England Biolabs (NEB) and enzymatic reactions were carried out in the reaction buffers that were provided with the enzyme. Ethidium bromide (EtdBr) and nuclease S1 were purchased from Promega and supercompetent XL1BLUE *E. coli*. cells, from Stratagene. K_2PtCl_4 was obtained as a gift from Engelhard Corporation. Cisplatin and [Pt(*R*,*R*-dach)Cl_2] were prepared according to literature procedures (23,24). Desalted oligonucleotides were ordered from Integrated DNA Technologies (IDT, Coralville, Indiana, USA) unless otherwise stated.

Vector Construction and Preparation

A mammalian vector expressing gaussia luciferase, pCMV-GLuc (NEB), was used as the vector construct. pCMV-GLuc (NEB) was treated sequentially with HindIII and BamHI to digest the vector between the *CMV* promoter and *GLuc* reporter genes. A synthetic insert designed for subsequent incorporation of the 1,2-d(G*pG*)-Pt DNA lesion was prepared by annealing 5’-AGCTGCTGAGGACCGGTGCTGAGG with 5’-GATCCCTCAGCACCGGTCCTCAGC which left 5’-AGCT/GATC non-cohesive overhangs, and ligated to the HindIII/BamHI-digest of pCMV-GLuc. Similarly, the synthetic insert for the 1,3-d(G*pTpG*)-Pt DNA lesion was prepared from 5’-AGCTGAGGAGGAGCACG-TGGAGGGAGAGGCTGAG and 5’-GATCCTCAGCCTCTCCCTCCACGTGCTCCTCCTC. The ligation mixtures were transformed directly into XL1BLUE cells on LB agar plates supplemented with 100 mg/L of ampicillin. Colonies were randomly picked and plasmid extracted (Qiagen miniprep kit). Incorporation of synthetic inserts was confirmed by restriction analysis and sequencing (MWG Operon, Huntsville, Alabama, USA) prior to large scale plasmid preparation. The modified vectors for the 1,2-d(G*pG*)-Pt and 1,3-d(G*pTpG*)-Pt DNA lesions were designated as pGLuc1temGG and pGLuc2temGTG, respectively (Figure 1).

Preparation of 13-mer Insertion Strands Containing 1,2-d(G*pG*)-Pt Lesions

Desalted 5’-TCAGCACCGGTCC oligonucleotide was ordered from IDT (Chart 1). The 13-mer non-platinated insertion strand (13-is) was obtained by purifying the desalted oligonucleotide using ion-exchange HPLC. To prepare the 13-mer insertion strand with the 1,2-d(G*pG*)-[Pt$(NH_3)_2$] lesion (13-is-Pt), desalted oligonucleotide (800 nmol) was allowed to react with *cis*-[Pt$(NH_3)_2(H_2O)_2]^{2+}$ (1200 nmol) in buffer (1 mL, 10 mM sodium phosphate pH 6.0) for 2 h at 37 °C (25). The platinated oligonucleotide was purified twice by ion-exchange HPLC. The linear gradient was 10–45% B over 13.5 min, where solvent A contained 20 mM

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Tris·HCl pH 8.0, 20% acetonitrile and solvent B contained solvent A supplemented with 1 M NaCl. The final yield was 120 nmol (15.0%). The 13-mer insertion strand with the 1,2-d (G*pG*)-[Pt(*R*,*R*-dach)] lesion (13-is-oxPt) was purified by the same method except that the conditions were 800 nmol of oligonucleotide and 1200 nmol of $[Pt(R,R\text{-dach})(H_2O)_2]^{2+}$ in buffer (1.5 mL, 10 mM sodium phosphate pH 6.0) for 4 h at 37 °C. The final yield was 10.5 nmol (1.3%).

Preparation of 27-mer Insertion Strands Containing 1,3-d(G*pTpG*)-Pt Lesions

The 27-mer insertion strand (27-is) 5'-TCAGCCTCTCCCTCCACGTGCTCCTCC was prepared by using an Applied Biosystems 392 DNA/RNA synthesizer on a 1 μmol scale by standard phosphoramidite protocols and purified with PolyPak II reverse-phase cartridges (Glen Research) in accord with the manufacturer's instructions (Chart 1). The 27-mer insertion strand with the 1,3-d(G*pTpG*)-$[Pt(NH_3)_2]$ lesion (27-is-Pt) was prepared by ligating a platinated 17-mer strand to a 10-mer in the presence of a 35-mer scaffold. The 10-mer 5'-TCAGCCTCTC and 17-mer 5'-CCTCCACGTGCTCCTCC strands were prepared on the DNA synthesizer and purified as described for 27-is. The 17-mer oligonucleotide (250 nmol) was allowed to react with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (375 nmol) in buffer (1 mL, 10 mM sodium phosphate pH 6.0) for 2 h at 37 °C and purified by ion-exchange HPLC (25). The linear gradient was 30% B for 3 min, 30–52.5% B over 13.5 min, where solvent A contained 20 mM Tris·HCl pH 8.0, 20% acetonitrile and solvent B contained solvent A supplemented with 1 M NaCl.

The platinated 17-mer (25 nmol) was phosphorylated using T4 PNK (50 U) in buffer (250 μL, 70 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP) for 6 h at 37 °C, treated with phenol/chloroform/isoamyl alcohol (25:24:1) and dialyzed against water for 4 h. The phosphorylated 17-mer, 10-mer (30 nmol) and 35-mer scaffold 5'-TTTTGGAGGAG CACGTGGAGGGAGAGGCTGATTTT (37.5 nmol) were annealed and ligated using T4 DNA Ligase (200 U) in buffer (200 μL, 50 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) at 16 °C for 36 h. The product was purified by 14% urea-PAGE, following by ionexchange HPLC. The linear gradient used was 60–77.5% B over 13 min, where solvent A contained 10 mM NaOH and solvent B contained solvent A supplemented with 1 M NaCl. The yield obtained was 0.8 nmol (3.2%). The 27-mer insertion strand containing 1,3-d(G*pTpG*)-[Pt(*R*,*R*-dach)] lesion (27-is-oxPt) was prepared using the same method and the final yield was 1.5 nmol (6.0%).

Characterization of Platinated and Non-Platinated Insertion Strands

The insertion strands were characterized by MALDI-TOF MS to determine their molecular mass, which in all cases was within 0.1% of the calculated values. Mass spectra of the synthesized oligonucleotides were recorded on a Bruker OmniFlex instrument. The analyte was mixed with a matrix containing 10 mg/mL 2,4,6-trihydroxyacetophenone with 25 mM ammonium citrate in 50% MeCN solution and applied on the target using the dried droplet method. External calibration was performed using ABI Biosystems Calibration Mixture 2. The insertion strands were analyzed for nucleotide composition by enzymatic digestion. The oligonucleotide (500 pmol) was digested with nuclease S1 (10 U) for 12 h at 37 °C in buffer (100 μL, 50 mM NaOAc, 280 mM NaCl, 4.5 mM $ZnSO_4$). Next, Tris·HCl buffer (1.5 M pH 8.8) and calf intestinal phosphatase (10 U) were added and the reaction was incubated for 4 h at 37 °C. The digests were analyzed by reverse-phase HPLC using a Supelcosil LC-18-S column. The method used was 5–15% B over 30 min, where solvent A contained 10 mM NaOAc and solvent B contained 100% methanol.

Preparation of Site-Specifically Gapped Plasmid

The plasmid (200 μg) was incubated with Nt.BbvCI (50 U) at 37 °C for 1.5 h. The enzyme was deactivated by heating the reaction at 80 °C for 20 min and removed by phenol/chloroform/

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isoamyl alcohol (25:24:1) treatment. The single-stranded gap was formed by trapping the nicked strand with its complement (Chart 2). Briefly, the plasmid solution was supplemented with synthetic cDNA (1:500 molar ratio) in annealing buffer (10 mM Tris.HCl pH 8.0, 2 mM $MgCl_2$, 0.4 M NaCl), and heated in a thermocycler (80 °C for 5 min then −10 °C/min to 20 °C for 5 cycles). The gapped plasmid was purified by repeated washing (6–8 times) through a diafiltration centrifugal device (Pall, MWCO 30 KDa or 100 KDa) and dissolved in buffer (10 mM Tris·HCl pH 8.0).

Incorporation of Platinated/Non-platinated Insertion Strands into Gapped Plasmid

The insertion strands were phosphorylated by T4 PNK in buffer (70 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP) at 37 °C for 1 h. The phosphorylated insertion strands were treated with phenol/chloroform/isoamyl alcohol (25:24:1), dialyzed against water and dried in vacuo. The gapped plasmid (40 μg) was added at 1:10 to 1:100 molar ratio (vs. the insertion strands) and the mixture was annealed in a thermocycler (80 °C for 10 min, then cooling to 4 °C at −1 °C/min). The reaction was supplemented with T4 DNA ligase (80 U) in buffer (50 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and incubated at 16 °C for 12 h. The mixture was supplemented with 6X loading buffer (NEB), heated at 75 °C for 15 min and separated using preparative 0.8% w/v agarose gel electrophoresis containing 0.5 μg/mL EtdBr. The DNA bands were revealed using a handheld UV lamp and the band with higher electrophoretic mobility, corresponding to covalently closed circular product, was excised. Gel extraction was carried out using a commercial kit (Promega).

The platinated plasmids were purified further by treatment with AgeI (5 U) or PmlI (40 U) at 37 °C for 30 min. The enzyme was deactivated by heating the reaction at 75 °C for 15 min and separated using preparative 0.8% w/v agarose gel electrophoresis containing 0.5 μg/mL EtdBr. The DNA bands were revealed with a handheld UV lamp and the band with higher electrophoretic mobility, corresponding to covalently closed circular product, was excised. Gel extraction was carried out as described above (Qiagen). Quantitation of the platinated and non-platinated plasmid was carried out using the picogreen assay (Molecular Probes). Overall yields range between 5–25%.

Restriction Analysis on Ligated Platinated/Non-platinated Plasmids

pGLuc1temGG plasmid (100 ng) was incubated with AgeI (0.5 U) at 37 °C for 15 min and the enzyme was inactivated by heating at 75 °C for 15 min. The plasmids were analyzed using 0.8% w/v agarose gel electrophoresis containing 0.5 μg/mL EtdBr. Pt-DNA lesions were chemically removed by treating the platinated plasmid (200 ng) with 0.2 M NaCN at 55 °C for 10 h, followed by dialysis against water for 4 h (14,26), prior to REase treatment. pGLuc2temGTG (100 ng) were treated with PmlI (2 U) in the same manner except that the mixture was incubated at 37 °C for 45 min. Control lanes containing untreated plasmids were also added for comparison. The gels were imaged using the BioRad Fluor-S MultiImager.

RESULTS AND DISCUSSION

Vector Design Considerations

The strategy used to prepare platinated plasmids is to create single-stranded gaps within the DNA and to fill them in by using synthetic platinated insertion strands containing the precise complementary sequences (Figure 2). The single-stranded gap is created by removing the DNA strand positioned between two closely spaced nicks in the plasmid. Nicking enzymes were employed to introduce single-stranded scissions site-specifically into the plasmid. These enzymes were developed from REases that recognize asymmetric DNA sequences (Type IIA), but are engineered to cleave only one strand of the sequence (27). By designing their recognition sequences in tandem into the vector, the nicking sites can be used to predefine the

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sequence and position of the gap within the plasmid. In addition, the Pt-DNA lesion is intentionally situated within a REase recognition site on the plasmid. Platination at the recognition site blocks REase cleavage and provides a facile method for discriminating between platinated and non-platinated plasmids. In order to achieve a high level of control and specificity, both the nicking enzyme and the REase for the platination site must be unique and non-cutters of the parent vector. The design of these sequences must be such that they insert with non-cohesive overhangs for directional ligation into the vector backbone (Figure 1).

An initial search for appropriate restriction sites was performed on pCMV-Gluc *in silico* to determine that the commercially available REases we selected have no restriction sites on the plasmid (28). Nt.BbvCI (CC↓TCAGC) was selected as the nicking enzyme, and AgeI (A↓CCGGT) and PmlI (CAC↓GTG) as the REases for the platinated inserts for 1,2-d(G*pG*)-Pt and 1,3-d(G*pTpG*)-Pt lesions, respectively (Chart 2). Since platinated oligonucleotides are prepared by treating unplatinated single strands with the diaqua derivatives of cisplatin or oxaliplatin, the insertion strand sequence should ideally contain G bases only at the desired 1,2-d(GpG) or 1,3-d(GpNpG) platination site. In utilizing Nt.BbvCI for tandem nicking, the gap formed necessitates an insertion strand with a 5’-TCAGC starting and a 3’-CC terminal sequence. The presence of 1,2-d(ApG) in the starting sequence, in particular, complicates the preparation of insertion strands containing 1,3-d(G*pTpG*)-Pt lesions. Therefore, additional CT-rich sequences were designed into the insertion strands that they could be constructed in modular manner from separate component strands (see below).

Preparation of Platinated Insertion Strands

Platination of the 13-mer synthetic oligonucleotide 5’-TCAGCACCGGTCC was carried out with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ or $[Pt(R,R\text{-dach})(H_2O)_2]^{2+}$, in accordance with reported procedures (7,14,16,25), to prepare oligonucleotides carrying the 1,2-d(G*pG*)-Pt lesions formed by anticancer drugs cisplatin/carboplatin and oxaliplatin (12,13). The 1,2-d(ApG) sequence competed directly with the 1,2-d(GpG) sequence for platination and resulted in an additional peak overlapping with the abundant product peak in the HPLC chromatogram. We were able to separate the major 1,2-d(G*pG*)-Pt product by repeated HPLC runs, however (Figure S1). The MALDI-TOF MS and enzymatic digestions with nuclease S1 and CIP were consistent with formation of a single 1,2-d(G*pG*)-Pt lesion on the 13-mer insertion strand (Table S1).

The 27-mer insertion strands containing 1,3-d(G*pTpG*)-$[Pt(NH_3)_2]$ or 1,3-d(G*pTpG*)-[Pt(*R*,*R*-dach)] lesions were constructed by ligating the platinated 17-mer fragment to the 10-mer fragment using a DNA scaffold (Figure 3). This strategy was adopted because direct platination of the 27-mer synthetic oligonucleotide 5’-TCAGCCTCTCCCTCCACGTGCTCCTCC might yield the 1,2-d(A*pG*)-Pt adduct as a major product rather than the desired 1,3-d(G*pTpG*)-Pt cross-link. The 17-mer 5’-CCTCCACGTGCTCCTCC synthetic oligonucleotide was therefore platinated with *cis*-$Pt(NH_3)_2(H_2O)_2]^{2+}$ or $[Pt(R,R\text{-dach})(H_2O)_2]^{2+}$. The 17-mers containing the 1,3-d(G*pTpG*)-$[Pt(NH_3)_2]$ or 1,3-d(G*pTpG*)-[Pt(*R*,*R*-dach)] lesion were readily isolated by ion-exchange HPLC (Figure S2). Next, the platinated 17-mer was phosphorylated with T4 PNK and ligated, using T4 DNA ligase, to the 10-mer 5’-TCAGCCTCTC fragment using the 35-mer 5’-TTTTGGAGGAGCACGTGGAGGGAGAGGCTGATTTT DNA as a scaffold. Although the product was obtained in modest yield (3–6%), characterization of the ligated material using MALDI-TOF MS and nucleotide composition analysis was consistent with a 27-mer sequence with platination at the desired 1,3-d(G*pTpG*) site (Table S1).

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Preparation of Site-Specifically Platinated Plasmids

A gapped plasmid was prepared by tandem nicking with Nt.BbvCI at the predefined sites and removal of the intervening nicked strand by trapping with synthetic cDNA in large molar excess (Chart 2). The plasmid was purified quantitatively by repeated washings through diafiltration devices, which separate the gapped plasmid from excess cDNA and annealed duplexes.

In order to verify that the single-stranded gap was formed, a set of control ligation experiments were carried out on an analytical scale. The gapped plasmid was mixed with water, as a control, or with the various phosphorylated insertion strands at a 1:100 ratio, annealed, and treated with T4 DNA ligase. In the absence of the insertion strands, ligation of the gapped plasmid could not be achieved; only the open circular (oc) precursor was observed by agarose gel electrophoresis. This result confirmed that the gapping procedure was successful (Figure 4, lanes 1 and 5). In contrast, ligation of the gapped plasmid with both unplatinated and platinated insertion strands yielded significant quantities of covalently closed circular (cc) DNA products (Figure 4, lanes 2–4 and 6–8). Preparation of platinated and non-platinated plasmids was carried out on a preparative scale. The phosphorylated insertion strands were annealed to the gapped plasmid by using a shallow thermal gradient of −1°C/min. Ligation with T4 DNA ligase formed the desired covalently closed circular DNA product in good yield. Ligation efficiency was estimated by comparing the fluorescence intensity of the open and covalently closed circular bands on the gel by a BioRad Fluor-S MultiImager, typically ranges lying between 40–60%. The platinated plasmids were purified further by REase treatment, which linearizes non-platinated plasmid contaminants. Purification of the plasmids was carried out by preparative agarose gel electrophoresis with commercial gel extraction kits at an efficiency of 50–70%. The overall yield from the entire procedure was typically 5–25%. Better yields were obtained on larger, preparative scales (results not shown), but purification of a greater amount of plasmid using agarose gel electrophoresis also becomes more challenging.

The incorporation of the Pt-DNA lesion site-specifically into the plasmid was verified by REase treatment of the synthesized plasmid and analytical agarose gel electrophoresis. In the absence of the Pt-DNA adduct, the plasmid was readily linearized by REase (Figure 5 and Figure 6, lanes 1 and 2). In contrast, plasmids containing the site-specific Pt-DNA lesions were not linearized by the respective REases (Figure 5 and Figure 6, lanes 3 and 4). The reagent NaCN is capable of chemically reversing Pt-DNA crosslinks from platinated DNA as $[Pt(CN)_4]^{2-}$ (14,26). Treatment of the platinated plasmids with NaCN at 55 °C for 10 h restored the susceptibility of the plasmids towards REase linearization (Figure 5 and Figure 6, lanes 5 and 6), confirming that the Pt-DNA adducts were site-specifically incorporated into the REase recognition site of the plasmid.

CONCLUSION

The method of inserting platinated oligonucleotides into a single stranded gap provides ready access to large platinated DNA constructs, such as plasmids, in a controlled and selective fashion. The key advantage of the method is the high specificity involved in annealing the synthetic oligonucleotide into the gap since only the correct sequence length will result in successful ligation. Only two products are expected from the ligation, the unligated open circular form and the ligated covalently closed circular product, both of which are easily separated by conventional techniques. Good products yields are thus obtained, which is essential for the large-scale production of platinated plasmid for transfection studies in live cells. By comparison, the method of extending a platinated oligonucleotide primed on a single-stranded circular DNA, using DNA polymerase, can lead to formation of multiple products due to non-specific priming or incomplete extension, complicating product purification. In addition, the single-stranded circular DNA production from the plasmid template is accomplished using helper phage and the strand of plasmid produced is dictated by the

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orientation of the f1 sequence on the plasmid. Since most expression vector systems contain the f1 sequence oriented to yield the template strand to facilitate DNA sequencing, the platinated oligonucleotide can only be extended on the coding strand.

The main challenge in applying the single-stranded gapping methodology in the production of platinated plasmid is that sequence of the gap is highly dependent on the nicking enzyme used which, in turn, affects the design of the insertion strand. Ideally, the insertion strand should contain only G bases at the platination site, but due to the gap sequence it may not be possible to exclude them entirely. Nevertheless, we have shown that it is possible to overcome these limitations through more extensive purification or by applying more innovative methods to produce the platinated oligonucleotides.

In summary, the methodology of incorporating platinated oligonucleotides into synthetically gapped plasmid constructs is a versatile way of accessing site-specifically platinated plasmids with different Pt-DNA lesions. Application of the platinated plasmids as in vitro transcription and other probes in mammalian cells is currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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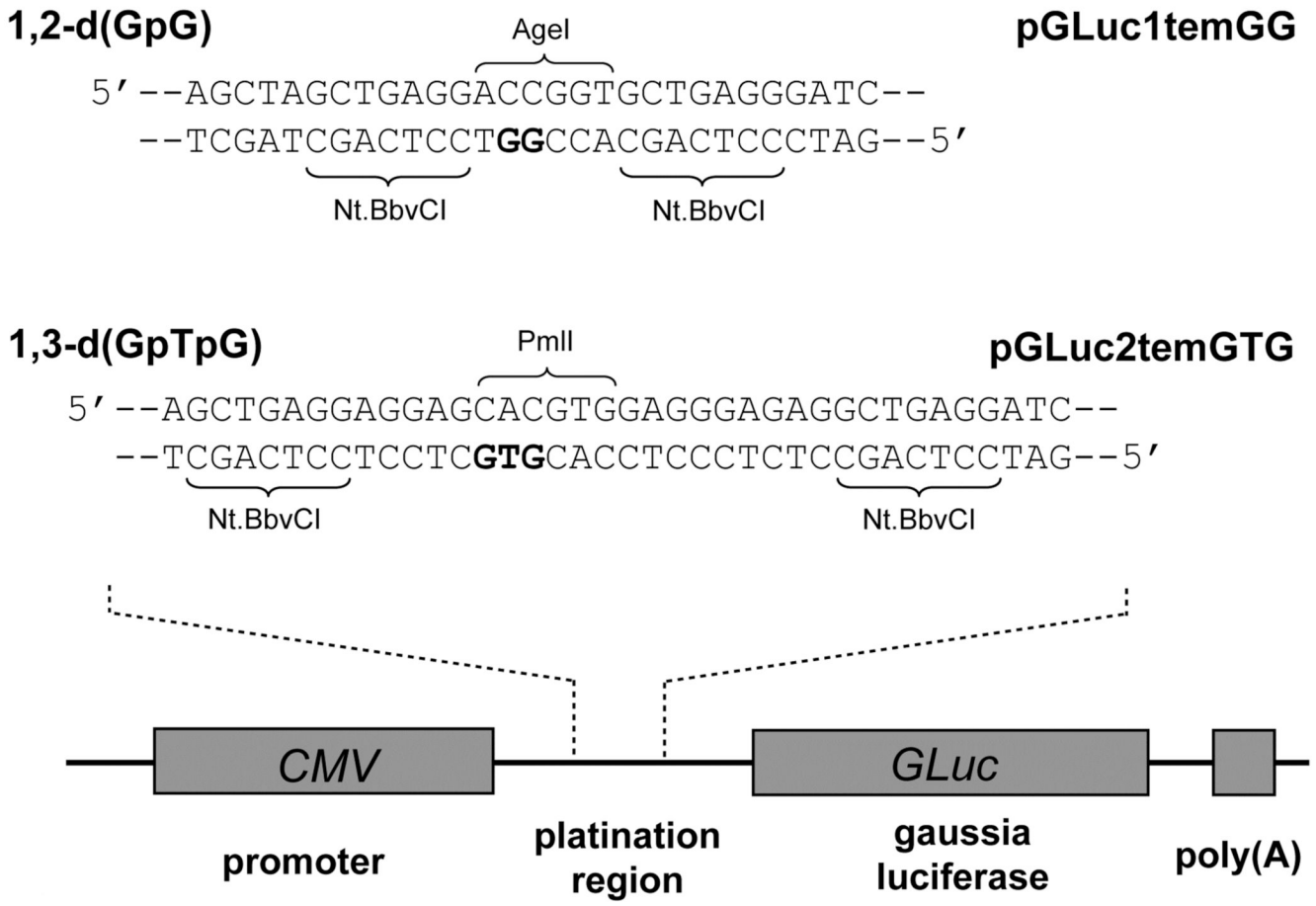

Figure 1.
DNA sequence of the platination regions in pGLuc1temGG and pGLuc2temGTG vectors; the position of the intended platination sites are highlighted in bold.

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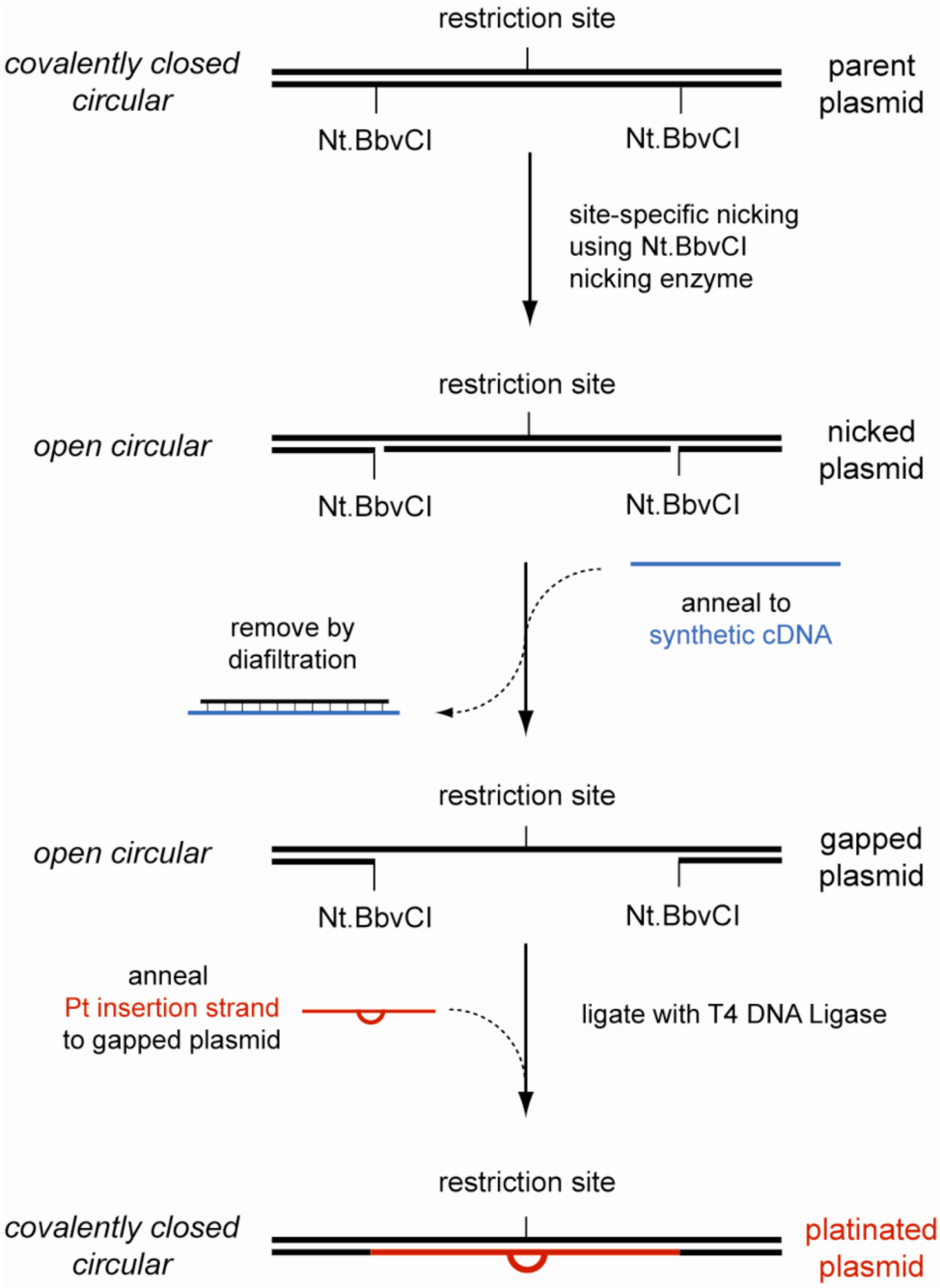

Figure 2.
Overall scheme for preparing platinated plasmids.

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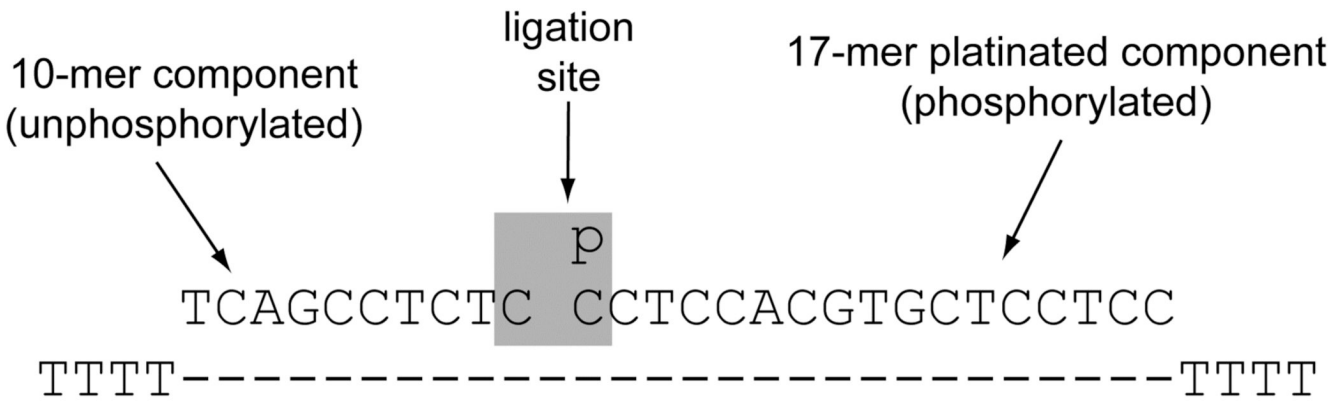

Figure 3.
Preparation of platinated 27-mer insertion strands.

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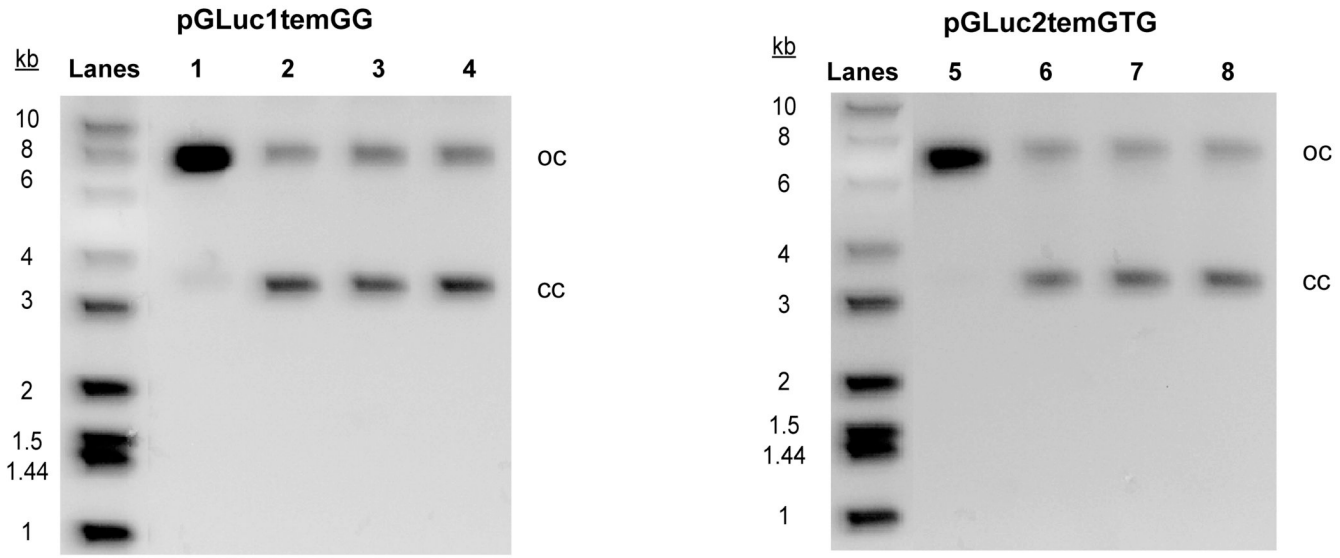

Figure 4.

Ligation experiment of gapped pGLuc1temGG (left) and pGLuc2temGTG (right) in presence of different insertion strands with T4 DNA Ligase at 16°C for 12 h; lane 1: gapped pGLuc1temGG alone, lane 2: plasmid + 13-is, lane 3: plasmid + 13-is-Pt, lane 4: plasmid + 13-is-oxPt; lane 5: gapped pGLuc2temGTG alone, lane 6: plasmid + 27-is, lane 7: plasmid + 27-is-Pt, lane 8: plasmid + 27-is-oxPt.

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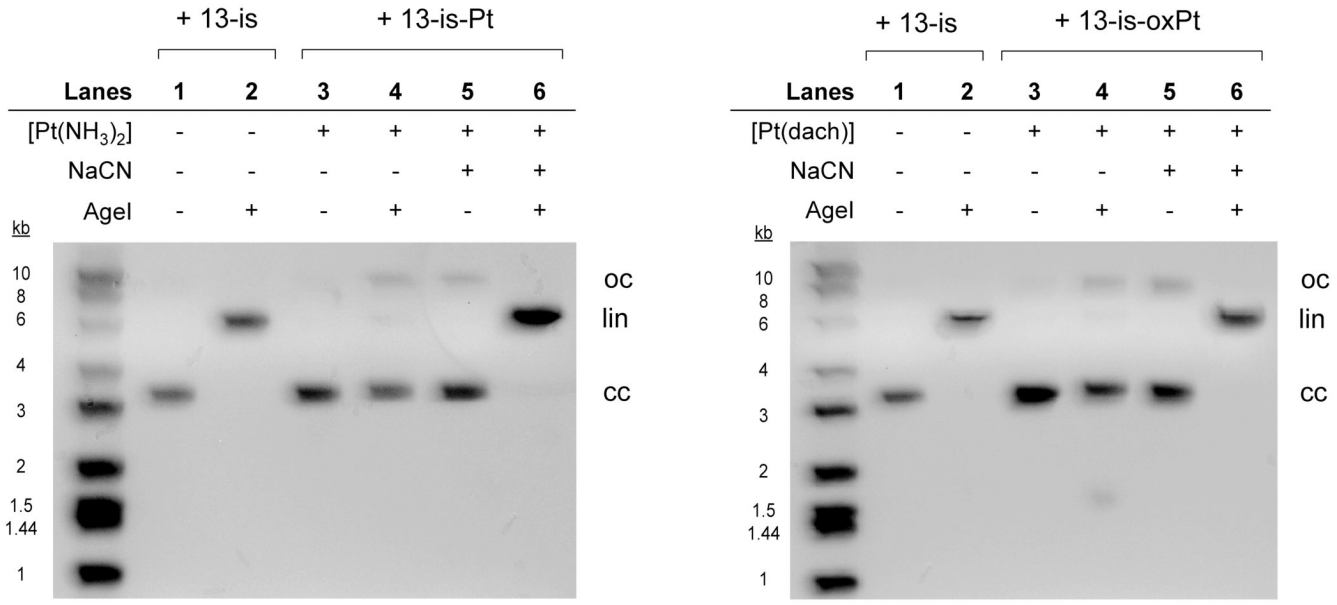

Figure 5.

Restriction analysis of platinated/non-platinated plasmids with AgeI; pGLuc1temGG + 13-is (lanes 1–2); pGLuc1temGG + 13-s-Pt (left, lanes 3–6); pGLuc1temGG + 13-is-oxPt (right, lanes 3–6).

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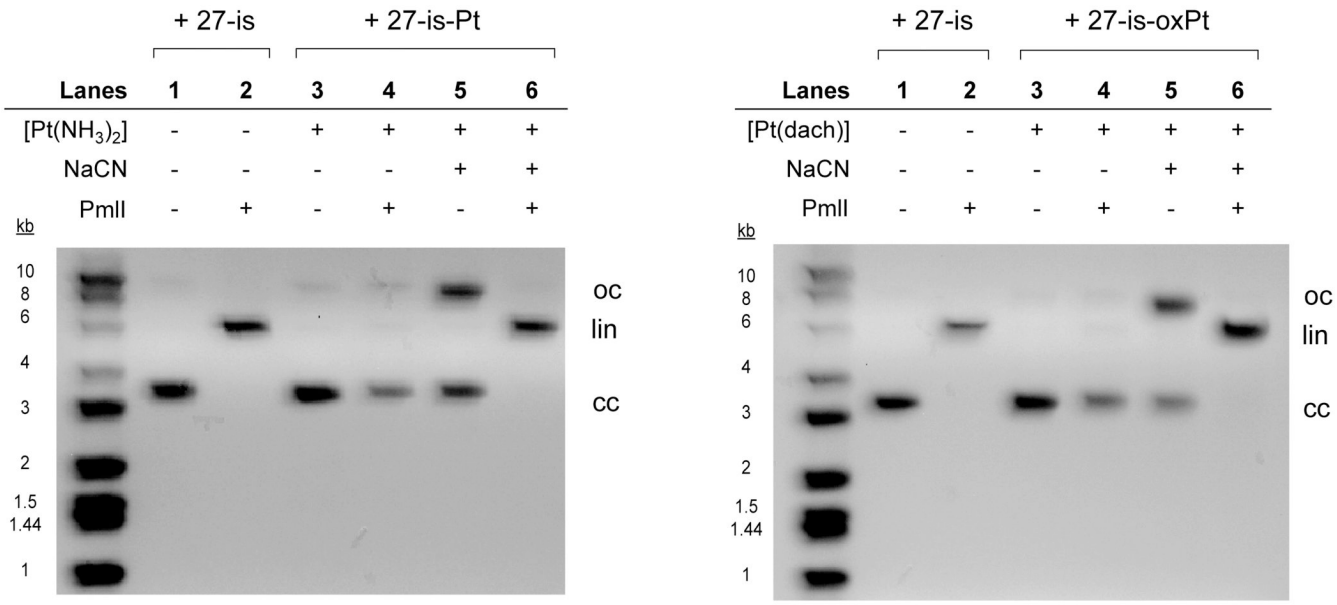

Figure 6.

Restriction analysis of platinated/non-platinated plasmids with PmlI; pGLuc2temGTG + 27-is (lanes 1–2); pGLuc2temGTG + 27-is-Pt (left, lanes 3–6); pGLuc2temGTG + 27-is-oxPt (right, lanes 3–6).

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	1,2-d(G*pG*)-Pt lesion	1,3-d(G*pTpG*)-Pt lesion
unplatinated	5′-TCAGCACC**GG**TCC (13-is)	5′-TCAGCCTCTCCCTCCAC**GTG**CTCCTCC (27-is)
[Pt(NH$_3$)$_2$]$^{2+}$	5′-TCAGCACC**GG**TCC (13-is-Pt)	5′-TCAGCCTCTCCCTCCAC**GTG**CTCCTCC (27-is-Pt)
[Pt(*R*,*R*-dach)]$^{2+}$	5′-TCAGCACC**GG**TCC (13-is-oxPt)	5′-TCAGCCTCTCCCTCCAC**GTG**CTCCTCC (27-is-oxPt)

Chart 1.
Sequences of platinated and unplatinated insertion strands.

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pGLuc1temGG

```
                AgeI
        Nt.BbvCI------Nt.BbvCI
         =======      =======
5'--AGCTGCTGAGGACCGGTGCTGAGGGATC--
  --TCGACGACT             CCCTAG--5'

             CCTGGCCACGACT
             |||||||||||||
      5'-TTTTGGACCGGTGCTGATTTT (cDNA)
```

pGLuc2temGTG

```
                PmlI
Nt.BbvCI     ------         Nt.BbvCI
 =======                    =======
5'--AGCTGAGGAGGAGCACGTGGAGGGAGAGGCTGAGGATC--
  --TCGACT                           CCTAG--5'

        CCTCCTCGTGCACCTCCCTCTCCGACT
        ||||||||||||||||||||||||||||
5'-TTTTGGAGGAGCACGTGGAGGGAGAGGCTGATTTT (cDNA)
```

Chart 2.

DNA sequence at gapping site of pGLuc1temGG (left) and pGLuc2temGTG (right) with nicked strand annealed to synthetic cDNA.