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Molecular Recognition Principles and Stationary-Phase Characteristics of Topoisomer-Selective Chemoaffinity Materials for Chromatographic Separation of Circular Plasmid DNA Topoisomers

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Supporting Information

ABSTRACT: We recently discovered the molecular recognition capability of a quinine carbamate ligand attached to silica as a powerful chemoaffinity material for the chromatographic separation of circular plasmid topoisomers of different linking numbers. In this paper we develop structure–selectivity relationship studies to figure out the essential structural features for topoisomer recognition. By varying different moieties of the original cinchonan-derived selector, it was shown that intercalation by the quinoline moiety of the ligand as assumed initially as the working hypothesis is not an essential feature for topoisomer recognition during chromatography. We found that the key elements for topoisomer selectivity are the presence of a rigid weak anion-exchange site and a H-donor site separated from each other in a defined distance by a 4-atom spacer. Additionally, incorporation of the weak anion-exchange site into a cyclic ring structure provides greater rigidity of the ligand molecule and turned out to be advantageous, if not mandatory, for (close to) baseline separation.

Plasmid DNA is considered as a possible breakthrough drug in a field commonly denoted as genetic medicine. The U.S. FDA has therefore published guidelines for industry on their biotechnological production, being currently the lone regulatory body to do so, and states that plasmids for human use should be predominantly present in their supercoiled isoform.[1] However, native samples of supercoiled plasmids, also called covalently closed circular (ccc) plasmids, consist of a series of individual topological isomers (topoisomers), which differ by various degrees of supercoiling.[2] While the contents of the supercoiled isoform can be controlled by the downstream process,[3] the supercoiling itself can only be regulated during upstream processing, i.e., during fermentation where the necessary enzymes are also present. We have recently described the first chromatographic separation of such topoisomers of native plasmid DNA (pDNA), employing a quinine carbamate ligand attached to a 5 μm porous silica support, which was successively used for in-process control of a plasmid fermentation.[4]

Groove-binders, intercalators, and triple-helix-forming agents are main groups of DNA binding molecules, which may recognize DNA specifically.[5] Quinine (QN) belongs to a group of intercalative substances, such as ethidium bromide, quinacrine, and chloroquine, which change the superhelical density of DNA in vitro.[6] The quinoline ring of QN satisfies the criteria for this behavior,[7] although a second binding site exists, which is thought to be an electrostatic interaction between the tertiary amino group of quinuclidine and a groove of the DNA helix.[8]

In this work, various stationary phases (SPs) were prepared and tested for pDNA topoisomer selectivity. SPs 1, 2, and 5 are based on QN, and SPs 3a–d are based on quincorine, a derivative of QN. SP 4 lacks the quinuclidine ring of SP 1. SPs 5 and 6 contain a short linkage between the ligand and the chromatographic support, while all other chromatographic ligands are connected via a long thioether linkage to the support.

Because of a successful separation of individual topoisomers with different linking numbers on SP 1 (Figure 1a), we decided

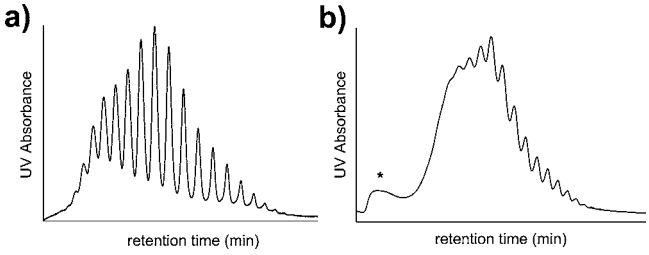

Figure 1. Separation of ccc topoisomers in a sample containing native 4.9 kbp pMCP1 pDNA on SP 1 (a) and SP 3a (b). The most abundant topoisomer has 26 negative superhelical turns, and each adjacent peak is a topoisomer differing by one superhelical turn, i.e., one linking number.[4] The open circular plasmid isoform is marked with an asterisk.

to fragment the ligand and vary individual molecule parts to find out what are the essential structural moieties to furnish topoisomer selectivity. Thus, a set of ligands was synthesized and attached to 5 μm porous (120 Å) silica particles to yield 12 different SPs (Scheme 1). pMCP1 and pAcMC1 pDNAs were analyzed on these columns employing the same mobile phase conditions, and the results are summarized in Table 1 for pMCP1 (4.9 kbp, see Supporting Information for chromatograms) and in Table 2 for pAcMC1. For the 9.9 kbp pAcMC1 plasmid, topoisomer separation was achieved only on the four columns listed in Table 2.

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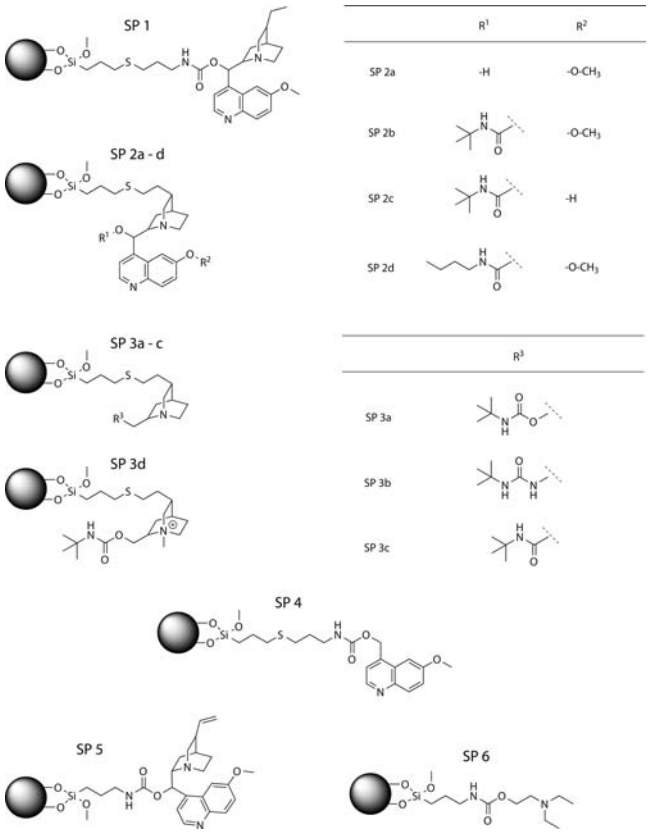
Scheme 1. Structures of Chromatographic Ligands Attached to Silica Stationary Phases Employed during This Study



The quincorine carbamate phase SP 3a, which lacks the aromatic quinoline ring, shows also reasonable selectivity (nearly the same Δt_R as compared to SP 1) (Figure 1b), and thus it can be concluded that intercalation is not involved or at least not essential for separation of pDNA topoisomers. However, the quinoline ring positively contributes, as the peak resolution is superior. pDNA elutes from columns containing quinine carbamate ligands at lower elution strength than from those with quincorine-based ligands. If intercalation were present, a shift to significantly later elution would be expected due to multivalency interaction. Also, because intercalation is a multistep and relatively slow process[9] accompanied by rearrangement of DNA,[5] significantly broader peaks would be expected as a result of such binding mode.[10]

The presence of the carbamate group is crucial for topoisomer separation. If it is removed, such as on SP 2a containing the QN ligand with an unmodified 9-hydroxyl group, no topoisomer separation is achieved, and the ccc form elutes as a single peak. On the other hand, replacement of the carbamate functionality by a urea moiety (SP 3a → SP 3b) does not much affect selectivity. However, the presence of an additional amide H-donor functionality leads to broader peaks of individual topoisomers, which in turn decreases the peak resolution dramatically. The key element of the carbamate and urea moieties is the amide H-donor/acceptor moiety adjacent to the *tert*-butyl group. When donor/acceptor functionalities are inverted (SP 3b → SP 3c), the topoisomer selectivity is lost completely. In spite of the presence of the essential amide H-donor/acceptor functionality, phase SP 3c is not capable of separating topoisomers, because the H-donor is spatially displaced and the distance between the amide H-donor functionality and the tertiary amine of the quinuclidine bicycle is only three instead of five bonds (Scheme 1). This indicates the stringent demands on spatial arrangement and orientation of functional moieties involved in simultaneous multiple interactions as expected for affinity-type molecular recognition events.

Table 2. Chromatographic Separation Parameters[a] of ccc Topoisomers of a pAcMC1 Plasmid on Different Stationary Phases[b]

SP	no. separate topoisomers	resolution[a]	peak width [min][b]
1	28	0.45	0.51
2b	27	0.78	0.38
2c	13	0.11	2.1
2d	26	0.55	0.49

[a]Between two most abundant topoisomers. [b]Of most abundant topoisomer. Chromatographic conditions are given in Table 1.

Table 1. Chromatographic Separation Parameters[a] of ccc Topoisomers of a pMCP1 Plasmid on Different Stationary Phases[b]

SP	ligand density [μmol/g]	no. separate topoisomers	%B at elution[c]	resolution[d]	peak width [min][c]	selectivity[d,e]
1	318	22	58	1.23	0.36	0.76
2a	380	0	87	n.s.	2.7[f]	n.s.
2b	320	22	47	1.32	0.43	1.02
2c	336	20	48	0.64	1.40	0.79
2d	327	19	39	0.80	0.67	0.78
3a	307	18	62	0.26	2.30	0.61
3b	200	13	76	0.07	n.q.	0.75
3c	285	0	80	n.s.	3.5[f] min	n.s.
3d	236	0	68	n.s.	4.0[f] min	n.s.
4	286	0	53	n.s.	4.8[f] min	n.s.
5	260	5	49	<0.06	n.q.	n.q.
6	486	0	92	n.s.	1.7[f] min	n.s.

[a]Equal mobile phase conditions employed. [b]Based on 5 μm porous silica support. [c]Of most abundant topoisomer. [d]Between the two most abundant topoisomers. [e]Due to gradient elution, selectivity is calculated as the difference in retention times, Δt_R. [f]Of total ccc (envelope) peak; n.s. = no selectivity, n.q. = not quantifiable. Chromatographic conditions: mixed salt and isopropanol gradient from 0 to 100% B in 60 min. Buffer A was 50 mM NaH_2PO_4 titrated to pH 7.0 with 5 M NaOH. Buffer B was 50 mM NaH_2PO_4, 0.6 M NaCl, and 10% (v/v) isopropanol titrated to pH 7.0 with 5 M NaOH. Between the runs, the column was washed by a salt plug (injection of 50 μL of 3 M NaCl(aq)), followed by reequilibration at 0% B for 5 min. Flow, 0.7 mL/min; UV detection at 258 nm; column temperature, 60 °C with solvent preheating.

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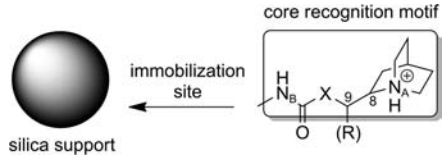
The tertiary amine of quinuclidine may act as an anion exchanger with a pK_a of 9.8,[11] but also as a H-acceptor to form H-bond-mediated ionic interactions. Such ionic interactions are directed, and in order to be formed, they require geometrically defined structural constraints, which appears to be favorable for multisite affinity-type interactions. Upon quaternization of the tertiary amine of the quinuclidine ring by methylation (SP 3d), the topoisomer separation is lost. Because quaternary ammonium groups are permanently charged but do not exhibit H-acceptor properties, it seems likely that the lack of the latter property causes also a loss of selectivity, as bifunctional directed binding is not possible anymore. However, steric effects may be in any case involved.

We have studied the effect of rigidity and conformational stability of the ligand on topoisomer selectivity by various structural variations. The *tert*-butyl group attached to the carbamate (SP 2b) is more bulky and rigid[12] and provides narrower peaks and significantly improved resolution compared to SP 2d, containing an *n*-butyl group instead. When the linker between the support and the ligand is attached at the carbamate site (SP 1), rigidity is increased and conformational adaptability is reduced, again leading to narrower peaks compared to the linker attachment to the vinyl group of quinuclidine (SP 2b). When comparing *tert*-butylcarbamoyl quinine (SP 2b) and *tert*-butylcarbamoyl quincorine (SP 3a), which differ in the presence of the quinoline ring moiety at C9, the QN-based ligand provides smaller peak widths of individual topoisomers and thus better resolution. This may also be attributed to reduced conformational flexibility of the quinine carbamate-type ligand. From NMR studies, it is known that when quinine carbamate is protonated, which applies for our mobile-phase conditions, it exists almost exclusively as a single conformer.[13] This anti-open conformer forms a defined cleft with the quinoline ring,[14] which is not so well defined for quincorine-based ligands, providing a possible explanation of higher chromatographic resolution of ccc topoisomers on quinine carbamate-based ligands. A ligand with complete rotational and torsional freedom of the tertiary amine (SP 6) but bearing all other key elements for molecular topoisomer recognition lacks ccc topoisomer selectivity completely.

The topoisomer peak resolution decreases with increasing plasmid size when using the same gradient. It turned out that none of the quincorine-type phases was able to resolve the topoisomer peak pattern, and only the more rigid QN-type ligands provided topoisomer separation for the pAcMC1 plasmid, which is about 2 times the size of the pMCP1 plasmid (see Table 2). Just as with the smaller plasmid, SP 2c showed the highest peak width, resulting in the lowest peak resolution. SPs 1, 2b, and 2d all were able to resolve a similar number of topoisomers; however, the pAcMC1 topoisomers were best separated on SP 2b. Topoisomers of a 14.5 kbp plasmid (pGNA3) have not been separated on SP 2c at all, nor on phases bearing quincorine-type ligands. A slight separation has been observed on SPs 1 and 2d, however being insufficient for calculating chromatographic parameters. Only SP 2b provided a separation good enough for calculating the peak resolution (0.13) and the peak width at half-height (1.48 min), supporting the fact that the separation power decreases with increasing plasmid size. In general, topoisomers of larger plasmids might also be resolved on SPs 1, 2b, and 2d by increasing the gradient time or by isocratic elution with step gradients, if necessary.

From the combination of these results, a general ligand lead structure for topoisomer recognition was concluded (Scheme 2). It is based on a sterically defined arrangement of a H-donor (amide group) and a H-acceptor-supported weak anion-exchange site (quinuclidine nitrogen). It is interesting that a similar arrangement of structural elements can be found in minor groove binders such as netropsine[15] or hairpin polyamides designed for specific sequence recognition.[16] Minor groove binders bind preferentially to A·T base pairs,[17] groove binders in general bind to double-stranded DNA sequence-specifically, while hydrogen-bonding and other non-covalent interactions are thought to be key modulators for molecular recognition.[18] A binding model between an A·T base pair and a hairpin polyamide, which satisfies our proposed structural requirements, suggests the formation of two hydrogen bonds to the N3 of the purine and the O2 of the pyrimidine, respectively.[19] The exact binding mode of quincorine- and quinine-type ligands to double-stranded DNA, however, remains to be evaluated by specialized (e.g., NMR) methods.

Scheme 2. General Molecular Recognition Motif of a Chromatographic Ligand with Topoisomer Selectivity[a]



[a]The arrow represents a suitable linker between the particulate silica support and nitrogen atom N_B. X = −NH− or −O−. (R) = H in quincorine-based ligands or quinoline in QN-based ligands.

SPs 2b and 5 with different stereochemistry were synthesized, starting from quinidine (QD) instead of QN. These stereochemical variants are "pseudo-enantiomers", actually diastereomers, with a configuration of 8*S*,9*R* for QN and 8*R*,9*S* for QD[20] (see Scheme 2 for numbering). These phases are known to possess enantiomer selectivity for derivatized amino acids, while the relative enantiomer affinity and thus elution order observed on the QN phase are inverted on the QD phase.[21] Both phases were, however, equivalent in terms of topoisomer selectivity, and one may attribute the observed molecular recognition phenomena hence as "topological selectivity for large (bio)molecules".

All SPs satisfying the structure in Scheme 2 as well as free QN (SP 2a) possess selectivity between the supercoiled topoisomers and the open-circular (oc) isoform (see Supporting Information). Thus, the topological analysis can be carried out reliably regardless of the content of the oc isoform, which is obtained by nicking one DNA strand, and the linear isoform, which often coelutes with the oc isoform (data not shown). Additionally, the good separation of oc and ccc isoforms on SPs 1, 2b, and 5 shows a possible alternative to existing chromatographic[22] and electrophoretic[23] methods for ccc homogeneity determination.

In this paper we have derived a general ligand structure that is able to specifically interact with differently supercoiled topoisomers of circular pDNA. SPs 1 and 2b were able to resolve the largest number of topoisomers and thus are most suitable for topological analysis, and should be based on QN rather than the more expensive QD. We have identified four

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structural key elements: (1) the presence of an H-acceptor-supported weak anion-exchange site, (2) an H-donor, (3) the specific distance between these moieties, and (4) a conformational stability of the H-acceptor moiety. When either of those elements was not present in the ligand structure, no topoisomer separation was obtained. The presence of all mentioned elements defines a nearly constant chromatographic selectivity. However, using different types of the key moieties and, of course, the insertion of further functional groups, may heavily influence the chromatographic resolution. This is the key toward a baseline separation and subsequently for a successful validation of this topology-selective chromatography in order to be used for regulatory purposes and in-process control of upstream processing.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of chromatographic ligands, substance characterization data, procedures for synthesis of SPs, and chromatograms obtained on each SP. This material is available free of charge via the Internet at http://pubs.acs.org.

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