Studies on π Interactions in Liquid-phase Separations

Eisuke KANAO¹,²

¹Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan
²National Institutes of Biomedical Innovation, Health and Nutrition, 7-6-8 Saito-Asagi, Osaka 567-0085, Japan

Abstract

π interactions have recently received considerable attentions due to principal factor governing molecular recognitions and self-assemble abilities, as accumulated in the database on proximate arrangements in structures of biological systems and organic functional materials. Therefore, further deep understanding and control of π interactions will greatly facilitate the development of new functional materials. Despite the importance of π interactions, they are still challenging to study because π interactions are much weaker than most other molecular interactions, such as hydrophobic interaction, hydrogen bonding, and electrostatic bonding. On the other hand, liquid chromatography (LC) is a powerful separation technique, which is able to distinguish the partition coefficients of solutes between the mobile and stationary phases, and can sensitively reflect the strength of molecular interactions. We investigated the properties of π interactions by developing new silica-monolithic capillary columns modified with carbon materials providing strong π interactions. In this focusing review, we introduce a few specific π interactions by columns modified with fullerenes and polycyclic aromatic hydrocarbons (PAHs), which showed strong π-π interactions due to spherical recognition and multiple CH-π interactions. Furthermore, π interactions can contribute to the separation of various samples, which are difficult to achieve by the available retention mechanisms. Briefly, we applied various π interactions to specific separation analyses, and we succeeded in separating halogenated compounds, H/D isotopologue pairs, and saccharides by effective π interactions. These results indicates that π interactions contribute to practical separation science, such as removal of environmental pollutants and quantitative determination of medicinal compounds.

Keywords: π interaction; Nanocarbon materials; Polycyclic aromatic hydrocarbons; Halogenated compounds; Isotope effect

1. Introduction

Recently, weak and attractive π interactions have received considerable attention as a principal factor governing molecular recognition and self-assembly, as accumulated in the database on proximate arrangements in supramolecular structures [1-4]. π interactions involve π-orbitals of any compound and are common in aromatic rings. Depending on the target of the interaction, there are various types of π interactions, such as π-π interactions, CH-π interactions, and halogen-π (X-π) interactions. These interactions are caused by induced dipole interactions, which are very weak attractions resulting from disturbing the arrangement of electrons in the nonpolar species [5-7].

These weak intermolecular interactions are important for precise structure control because they are highly directional dependent. For example, Qing et al. successfully synthesized uniform hexamers made of phenylacetylene molecules on Au (111) surfaces and suggested that the CH-π interaction was critical for supramolecular self-assembly, dictating the number of molecules within the assembly and their relative orientation [8]. Butterfield et al. reported that π-π interactions played an important role in stabilizing monomeric α-helical structures by circular dichroism analysis of Ala-Lys host peptides [9]. As can be observed by these and many other reports, a deep understanding and the ability to control π interactions will...
greatly facilitate supramolecular chemistry.

Generally, it is challenging to study \( \pi \)-interactions, especially in the presence of other molecular interactions, because \( \pi \)-interactions are much weaker than most molecular interactions, such as hydrophobic interactions, hydrogen bonding, and electrostatic bonding. Computer simulation methods, such as Monte Carlo and molecular dynamics, have proven to be powerful tools for studying intermolecular interactions [6,10-12]. These calculations, however, only generate theoretical hypotheses that still need experimental verification. Additionally, various molecular spectroscopies, such as UV-Vis spectroscopy, fluorescence spectroscopy, and nuclear magnetic resonance spectroscopy, have been successfully applied to study strong molecular interactions, such as hydrophobic interactions and hydrogen bonding [13-15]. However, these molecular spectroscopies often lack sufficient sensitivity to detect weak \( \pi \)-interactions. Therefore, a straightforward experimental method that can directly determine \( \pi \)-interactions is strongly required.

Liquid chromatography (LC) is a powerful separation technique that is able to distinguish the partition coefficients of solutes between the mobile and stationary phases and can sensitively reflect the strength of molecular interactions. By using LC, Kimata et al. determined the differences in the strength of the \( \pi \)-interactions between allotropes by studying the retention behavior of fullerene allotropes in pyrenylethyl-modified columns [16]. In another study, Chen et al. evaluated the influence of metal species on the strength of \( \pi-\pi \)-interactions using a metalloprotoporphyrin covalently linked silica surface [17]. As these studies evaluated the retention behavior in the columns, which immobilized various aromatic compounds on the stationary phases, the strength of the \( \pi \)-interactions between these compounds and solutes can be directly elucidated.

In this review, we introduce our recent advancement in elucidating the nature of \( \pi \)-interactions in LC and applying \( \pi \)-interactions to specific separation analyses.

2. Development of fullerene-coated silica-monolithic capillary columns to evaluate \( \pi \)-interactions by liquid chromatography

Nanocarbon materials, e.g., graphene, fullerene, corannulene (Crn) and carbon nanotubes, have specific properties, such as a high specific surface area, physical strength, and conductivity [18-21]. Therefore, a variety of applications have been carried out for electronic materials [22,23], reinforced materials [24], medicinal materials [25], and others [26,27]. Furthermore, carbon-based nanomaterials have many \( \pi \)-electrons and show strong \( \pi \)-interactions [28,29]. In analytical chemistry, they are expected to fabricate a separation medium that exhibits an unprecedented strong \( \pi \)-interaction by immobilizing the nanocarbon material onto the surface of separation substrates. For example, Kubo et al. succeeded in immobilizing \( \text{C}_{60} \)-fullerene (C60) onto the surface of a silica monolith in a capillary, which effectively interacted with aromatic hydrocarbons [30,31]. They evaluated the C60-coated column by LC, and then several polycyclic aromatic hydrocarbons (PAHs) were successfully separated by the effective interaction based on the \( \pi \)-electrons arising from C60 in the capillary. Therefore, the weak \( \pi \)-interaction can be accurately evaluated by using a new separating media with the immobilized nanocarbon material.

Furthermore, we focused on \( \text{C}_{70} \)-fullerene (C70), which is an allotrope of C60 and has more \( \pi \)-electrons. We expected that the column prepared by immobilization of C70 on a silica-monolith capillary would retain PAHs more effectively. Additionally, C70 is not spherical like C60 but has asymmetric elliptic spheres [32,33], so C70-coated columns may show different molecular recognition abilities compared to C60-coated columns.

A C60/C70-coated column was prepared via a thermal reactive compound, perfluorophenyl azide (PFPA), which effectively reacts with nanocarbon materials, generating covalent bonding [34,35]. Briefly, an \( N \)-hydroxysuccinimide (NHS)-PFPA composite was synthesized, and then the mixture of the composite and fullerene was heated at 108 \(^\circ\)C for 5 days to obtain a PFPA-fullerene composite. Additionally, we could improve the reaction efficiency by utilizing a microwave reaction [36]. The silica-monolithic capillary column was modified with 3-aminopropyltrimethoxy-silane in methanol solution to introduce amino groups, and then a toluene solution of PFPA-fullerene was passed through the column for modification through a reaction between amino groups and NHS.

The prepared C70-coated column allowed strong \( \pi-\pi \)-interactions toward a variety of PAHs even in normal-phase LC mode with \( n \)-hexane as the mobile phase. Additionally, the C70-coated column showed a higher retention ability for Crn, which is a structure of the edge of the fullerene structure similar to that of C60 (Fig. 1). The computer

![Fig. 1. Chromatogram of PAHs with C70-coated column and schematic diagram of the spherical recognition on C70. Conditions: column, C70-coated column (12.1 cm × 100 \( \mu \)m i.d.); flow rate, 2.0 \( \mu \)L min\(^{-1}\); detection, UV 254 nm.](image-url)
simulation and the evaluation of absorption spectra for the interaction between fullerenes and Crn suggested that C70 effectively interacted with Crn by the induced-dipole interaction [37]. These results proved the difference in characteristics of \( \pi \) interactions depending on the structures of fullerenes and suggested that LC separation media with nanocarbon materials were effective for evaluating weak \( \pi \)-interactions.

### 3. Evaluation of \( \pi \) interactions on convex-concave \( \pi \)-conjugated surfaces by liquid chromatography

Convex-concave \( \pi \)-conjugated surfaces in hemispherical bucky bowls, such as Crn, show increasing utility in constructing self-assembled new functional materials owing to their unique \( \pi \) electrons and strong dipole. Therefore, a deep understanding and the ability to control \( \pi \) interactions on convex-concave \( \pi \)-conjugated surfaces will greatly facilitate the development of new functional materials.

Therefore, we developed new Crn-coated silica monoliths for the precise understanding of \( \pi \) interactions on curved \( \pi \)-conjugated surfaces using LC. Crn is known to have convex and concave surfaces, which is expected to lead to different molecular recognition at each surface. To this end, we developed two kinds of Crn-functionalized silica monoliths, namely, Crn-ester columns and Crn-PFPA columns (Fig. 2). The Crn-ester column was prepared from a Crn derivative that was edge functionalized with a \(-\text{CH}_2\text{OH}\) group [38], which was then conjugated to a carboxy-functionalized silica monolith. It was anticipated that both surfaces of the Crn structure could interact with solutes in the Crn-ester column. The Crn-PFPA column was prepared from a Crn derivative that was functionalized with PFPA to form an aziridine on a spoke of Crn. In this case, aziridine formation converts two sp\(^2\) hybridized carbon atoms of Crn into pyramidal sp\(^3\) centers, and it breaks the possibility of inversion of the convex-concave surface. Using these two new columns, we evaluated the strength of \( \pi \) interactions between Crn and several PAHs by normal-phase LC analysis employing carbon material-coated columns, in which the hydrophobic interaction was completely suppressed, and thus the \( \pi \) interaction could be simply examined [45]. As a result, higher retentions were observed as the number of Cl-, Br- or I-substituted benzenes increased on all columns; in particular, the C70-coated column showed higher efficiency. The tendency of the retention might be due to the X-\( \pi \) interaction between the halogen atoms and \( \pi \)-electrons of the stationary phase. Furthermore, to clarify the retention mechanism of halogenated benzenes by carbon-material coated columns, we measured UV absorption spectra of a mixed solution of halogenated benzenes and C70. The UV absorption spectra of mono- to tribrominated benzenes were critically changed so that these compounds showed \( \pi \)-\( \pi \) interactions with C70 (Fig. 3). On the other hand, the UV absorption spectra of the further brominated benzenes were changed; therefore, we anticipated that multiple

### 4. Separation of halogenated aromatic compounds by X-\( \pi \) interaction in liquid chromatography

The X-\( \pi \) interaction, which is expressed between an electron-poor region of the halogen atom and an aromatic ring, plays an important role in molecular recognition and enzymatic processes in biological systems [40-42]. Many computational approaches to study the X-\( \pi \) interaction using quantum mechanical models have been reported in recent years [43,44], whereas there are few reports by the experimental approaches because of the weakness of the X-\( \pi \) interaction compared to any other intermolecular interaction.

We experimentally evaluated the strength of the X-\( \pi \) interaction between the carbon materials and the variety of halogenated benzenes by normal phase LC analysis employing carbon material-coated columns, in which the hydrophobic interaction was completely suppressed, and thus the \( \pi \) interaction could be simply examined [45]. As a result, higher retentions were observed as the number of Cl-, Br- or I-substituted benzenes increased on all columns; in particular, the C70-coated column showed higher efficiency. The tendency of the retention might be due to the X-\( \pi \) interaction between the halogen atoms and \( \pi \)-electrons of the stationary phase. Furthermore, to clarify the retention mechanism of halogenated benzenes by carbon-material coated columns, we measured UV absorption spectra of a mixed solution of halogenated benzenes and C70. The UV absorption spectra of mono- to tribrominated benzenes were critically changed so that these compounds showed \( \pi \)-\( \pi \) interactions with C70 (Fig. 3). On the other hand, the UV absorption spectra of the further brominated benzenes were changed; therefore, we anticipated that multiple

![Fig. 2. Schematic diagram of Crn-ester column and Crn-PFPA column.](image)

![Fig. 3. Difference of absorbance in maximum wavelength (205 nm) of brominated benzenes with/without C70.](image)
substitutions of halogen atoms (>3) contributed to the effective X-π interaction toward fullerenes. To confirm the X-π interaction between multiple substitutions of halogen atoms and C70, the 1H NMR spectra of o-dibromobenzene and pentabromobenzene in the presence of C70 were measured. No significant peak shifts were observed in o-dibromobenzene, while the aromatic peak in pentabromobenzene was shifted upfield. These results clearly suggested that pentabromobenzene behaved as an electron acceptor for C70. Finally, we challenged the separation of halogenated benzenes in hydrophobic mobile phases. We optimized the mobile phase conditions in consideration of the dielectric effect and succeeded in one-pot separation of all isomers of bromo benzenes. LC separation of halogenated benzenes is very important in the field of environmental science; therefore, this result showed the possibility of π interactions to separate harmful halogenated aromatic compounds, such as polychlorinated biphenyls and polybrominated biphenyls.

5. Liquid chromatographic strategies for the separation of deuterated isotopologues by π interactions

Deuteration of drugs, as a simple approach for enhancing pharmacological effects, has attracted attention for more than 50 years. In particular, since the Food and Drug Administration (FDA) approved deuterated tetrabenazine, which is a symptomatic treatment of hyperkinetic movement disorders and was developed by Teva®, competition for the development of deuterated drugs among pharmaceutical companies is expected to become more intense [46,47]. However, the chemical properties of isotopologues are exactly the same, and it is difficult to distinguish them. Therefore, the demands for purification techniques of deuterated compounds are expected to explosively expand.

π interactions can contribute to the separation of various samples, which are difficult to achieve by the existing retention mechanisms. For example, π-π interactions, which are the most famous and common π interactions, are effective for the separation of aromatic compounds. Kayillo et al. reported that phenyl group-bonded silica provided more effective separation of PAHs than a typical hydrophobic adsorbent, octadecylsilyl bonded silica [48]. Croes et al. compared the retention selectively in phenyl group-bonded silica versus alkyl silica columns and proved the importance of π-π interactions in the phenyl stationary phase [49]. In particular, there were some reports of using the CH-π interaction for the LC mechanism. For example, Turowski et al. succeeded in the separation of protiated/deuterated (H/D) isotopologue pairs with aryl group-bonded silica-based adsorbents in reversed-phase LC [50].

Therefore, we shed light on the isotope effect in LC to clarify the detailed separation mechanism and to provide a rational strategy for the effective separation of deuterated isotopologues. We evaluated the deuterated and protiated isotopologues regarding aromatic compounds by LC along with various combinations of mobile phases and stationary phases. We calculated free energies in chromatographic partition equilibrium on LC, and then it was found that the stationary phase immobilized with aromatic compounds showed larger differences in free energy between deuterated and protiated isotopologues due to CH/CD-π interactions (Fig. 4) [51]. Furthermore, the separation media that had polar functional groups, such as silanol groups, allowed for higher separation efficiencies for the pairs of aromatic H/D isotopologues. In the case of silanol functional groups, aromatic rings of the analyte acted as donors through OH-π interactions with hydrogen atoms in the silanol groups. Thus, the deuterated analytes were able to be greatly retained by the stronger OH-π interactions. Finally, applying these opposing H/D isotope effects, we were able to demonstrate effective H/D isotopologue separations by utilizing the complementary action of OH-π and CH-π interactions [52].

6. Separation of saccharides via OH/CH-π interactions on fullerenes

Antibodies are a group of proteins that play a central role in immune responses and maintaining homeostasis in life. In recent years, precise molecular recognition and biocompatibility for the development of drugs to satisfy unmet medical needs cannot be solved with only small molecule drugs [53]. Generally, antibody drugs are produced by animal cells, such as those derived from Chinese hamster ovaries, and they are subject to glycosylation. The precise control of these glycans is nearly impossible because the type and amount of glycans vary greatly depending on the cell substrate and culture conditions, although the glycans greatly affect the bioactivity of antibodies. Therefore, the analysis of the
glycan species contained in the antibodies could be the key to the next generation of antibody research [54-56].

Glycan analysis of glycoproteins is mainly performed by enzymatic extraction and analyzed by LC with highly sensitive tandem mass spectrometry [57-60]. Thus, the development of separation techniques for glycans, which have more branched structures than nucleic acids and proteins, as well as diverse conformations and linkage styles, is still a difficult issue. We reported the separation of proteins, as well as diverse conformations and linkage species contained in the antibodies could be the key to the next generation of antibody research [54-56].

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The findings obtained throughout these studies will greatly advance the understanding of π interactions, which should aid the development of novel functional materials and the apprehension of biosystems in our body. Furthermore, the newly developed separation media based on nanocarbon materials allowed for selective and effective separations based on various strong π interactions. These studies will contribute to practical separation science, such as the removal of environmental pollutants and quantitative determination of biomedical compounds in the future.

Acknowledgments
The author would like to express his great appreciation to Professor Koji Otsuka (Graduate School of Engineering, Kyoto University), Dr. Takuya Kubo (Graduate School of Engineering, Kyoto University) and Dr. Toyohiro Naito (Graduate School of Engineering, Kyushu University) for their support and encouragement throughout the research.

The author is grateful to Professor Yasushi Ishihama (Graduate School of Pharmaceutical Sciences, Kyoto University) and Professor Jun Adachi (National Institutes of Biomedical Innovation, Health and Nutrition) for their splendid comments and discussions. The author wishes to acknowledge Professor Nobuo Tanaka (Graduate School of Engineering, Osaka University), Dr. Tomoharu Sano (National Institute for Environmental Studies), and Professor Mingdi Yan (University of Massachusetts Lowell) for their valuable suggestions and experimental instructions. The author is also grateful to all the coworkers for their constant encouragement during the course of his research.

Finally, the author would like to thank the Society for Chromatographic Science for selecting him as a recipient of the Encouragement Award in 2021 and giving him an opportunity to publish this focusing review.

References
[1] Zang, L.; Che, Y.; Moore, J. S. Acc. Chem. Res. 2008, 41, 1596-1608.
[2] Schenning, A. P. H. J.; Meijer, E. W. Chem. Commun. 2005, 3245-3258.
[3] Metrangolo, P.; Resnati, G. Chem. Eur. J. 2001, 7, 2511-2519.
[4] Gibson, H. W.; Yamaguchi, N.; Hamilton, L.; Jones, J. W. J. Am. Chem. Soc. 2002, 124, 4653-4665.
[5] Wheeler, S. E. Acc. Chem. Res. 2013, 46, 1029-1038.
[6] Tsuzuki, S.; Honda, K.; Uchimaru, T.; Mikami, M.; Tanabe, K. J. Am. Chem. Soc. 2000, 122, 3746-3753.
[7] Riley, K. E.; Hobza, P. Acc. Chem. Res. 2013, 46, 927-936.
[8] Li, Q.; Han, C.; Horton, S. R.; Fuentes-Cabrera, M.; Sumpter, B. G.; Lu, W.; Bernholc, J.; Maksymovych, P.; Pan, M. ACS Nano 2012, 6, 566-572.
[9] Butterfield, S. M.; Patel, P. R.; Waters, M. L. J. Am. Chem. Soc. 2002, 124, 9751-9755.
[10] Wells, R. A.; Kellie, J. L.; Wetmore, S. D. J. Phys. Chem. B 2013, 117, 10462-10474.
[11] Huber, R. G.; Margreiter, M. A.; Fuchs, J. E.; von Grafenstein, S.; Tautermann, C. S.; Liedl, K. R.; Fox, T. J. Chem. Inf. Model. 2014, 54, 1371-1379.
[12] Senthilkumar, K.; Grozema, F. C.; Bickelhaupt, F. M.; Siebbeles, L. D. A. J. Chem. Phys. 2003, 119, 9809-9817.
[13] He, A.; Kang, X.; Xu, Y.; Noda, I.; Ozaki, Y.; Wu, J. Spectrochim. Acta. A 2017, 185, 343-348.
[14] Kohn, S. C.; Dupree, R.; Smith, M. E. Nature 1989, 337, 539-541.
[15] Del Bene, J. E.; Perera, S. A.; Bartlett, R. J. J. Phys. Chem. A 1999, 103, 8121-8124.
[16] Kimata, K.; Hosoya, K.; Araki, T.; Tanaka, N. J. Org. Chem. 1993, 58, 282-283.
[17] Chen, S.; Meyerhoff, M. Anal. Chem. 1998, 70,
