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Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications

Kelly Zhang [a,*], Xiaodong Liu [b]

[a] Genentech, 1 DNA Way, South San Francisco, CA 94080, United States
[b] Thermo Fisher Scientific, 1228 Titan Way, Sunnyvale, CA 94088, United States

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ABSTRACT

Mixed-mode chromatography (MMC) is a fast growing area in recent years, thanks to the new generation of mixed-mode stationary phases and better understanding of multimode interactions. MMC has superior applications in the separation of compounds that are not retained or not well resolved by typical reversed-phase LC methods, especially for polar and charged molecules. Due to the multiple retention modes that a single MMC column can offer, often MMC provides additional dimension to a separation method by adjusting the mobile phase conditions. Mixed-mode media is also an effective way to clean up complex sample matrices for purification purposes or for sensitive detection of trace amounts of analytes. In this article, we discuss mixed-mode stationary phases and separation mechanisms and review recent advances in pharmaceutical and biopharmaceutical applications including the analysis and/or purification of counterions, small molecule drugs, impurities, formulation excipients, peptides and proteins.

1. Introduction

Mixed-mode chromatography (MMC) or multimode chromatography is becoming increasingly popular in pharmaceutical and biopharmaceutical applications due to its unique selectivity and retention of a variety of compounds, especially polar and charged molecules [1–8]. MMC is a chromatographic method in which solutes interact with stationary phase through more than one interaction mode or mechanism. MMC has been used as an alternative or complementary tool to traditional reversed-phased (RP), ion exchange (IEX) and normal phase chromatography (NP). Unlike RP, NP and IEX chromatography, in which hydrophobic interaction, hydrophilic interaction and ionic interaction respectively are the dominant interaction modes, mixed-mode chromatography employs a combination of two or more of these interaction modes.

Mixed-mode phenomena in the past were considered "secondary interactions". Most stationary phases are based on rigid support matrices such as silica gel or polymers, to which specific functional groups (e.g. alkyl chain C18, diol, etc) are bonded. Often sample solutes interact differently with the matrices and the functional groups, generating "secondary interaction" characteristics [9]. Mixed-mode IEX and RP interactions can even be observed on classical silica-based RP columns without intentionally introducing an ion-exchanger. Free silanol groups on silica gel matrix are considered as sites of secondary interactions in RP chromatography. Similarly, hydrophobic interactions exist in IEX separation, and ionic interactions exist in SEC separation. While in some cases, the secondary interactions are considered beneficial for selectivity [10,11], most of the time it is considered detrimental to a separation. For example, the free silanol group on silica often contributes to peak tailing, a phenomenon that is minimized by end-capping or by optimizing mobile phase conditions.

MMC can retain and separate small, polar drugs and related substances that are not retained by typical RP HPLC. It has been used as an alternative method to traditional ion chromatography for counterion analysis [1,12,13]. MMC has been used for the purification of biological samples and allowing direct sample injection [14–17]. MMC can retain acidic and basic compounds at mild mobile phase conditions compatible with MS detection. For a given mixed-mode column, the predominant separation mechanism depends on the properties of the sample as well as the mobile phase conditions. The mixed-mode stationary phases introduced in recent years provide desirable and repeatable "secondary interaction" or "tertiary interaction" with the use of carefully designed functional groups of different retention modes and well controlled manufacturing

* Corresponding author.
E-mail address: zhang.kelly@gene.com (K. Zhang).

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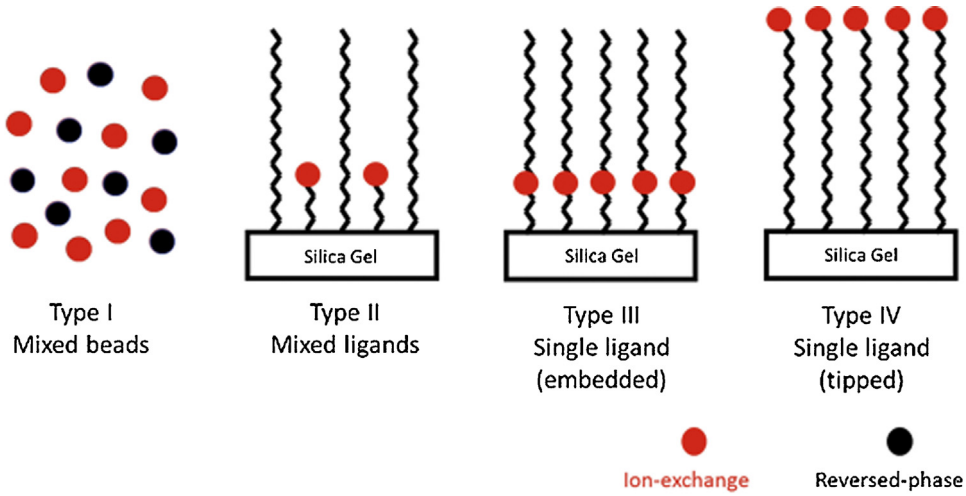

Fig. 1. Types of RP/IEX bimodal mixed-mode columns classified by the arrangement of functional groups. Adapted from Ref. [47].

process. The recent commercialization of mixed-mode columns significantly advanced the utility of MMC in pharmaceutical and biopharmaceutical applications [18–23].

Because MMC is complementary to RP and other separation modes, mixed-mode columns are also used in two-dimensional liquid chromatography (2D-LC) [2,24,25]. Furthermore, the multimode retention mechanisms can add dimensionality to a single mixed-mode column by adjusting mobile phase conditions. Therefore, mixed-mode columns are frequently used as an alternative technique for 2D-LC while using a single column and conventional HPLC setup [5,26,27].

2. Stationary phase and separation mechanism

2.1. Mixed-mode LC column media

The occurrence of mixed-mode chromatography, including both RP and IEX mechanisms, has been known for decades [9]. The use of mixed-mode chromatography for HPLC separations has been widely reported [18,28–45]. Mixed-mode columns can be divided into RP/anion exchange (AEX), RP/cation-exchange (CEX), hydrophilic interaction liquid chromatography (HILIC)/AEX and HILIC/CEX bimodal phases, as well as RP/AEX/CEX and HILIC/AEX/CEX trimodal materials. According to their chemistry design, bimodal media can usually be classified into four categories (Fig. 1). Type I media are achieved by mixing different types of particulate separation media, each with a single chemistry, such as RP or IEC, and packing the mixture into a column [28,29]. Type II media consist of substrates modified at the surface with a mixture of ligands having different functionalities, RP/IEX, HILIC/IEX, or RP/HILIC [30,32,33]. More sophisticated stationary phases can be prepared using ligands that contain ion exchange functionality as a part of the hydrophobic ligand. Depending on the position of the ionizable functionality with respect to the pore surface, these phases can be "embedded" (Type III), i.e. the functionality is close to the surface and the hydrophobic chain extends in a mobile phase environment [31,34,37], or "tipped"(Type IV) with the functionality at the free end of the hydrophobic chain [34,46]. These stationary phases are advantageous in reproducibility since the chemistry is defined by the attached ligands, not by the preparation process.

In recent years, mixed-mode stationary phases have received considerable attention by both academia and industrial research organizations. Several RP/weak anion-exchange (WAX) materials consisting of a selector immobilized onto thiol-modified silica gel have been reported [38,40–43,46]. In these phases, the WAX site is located on the outer surface of the lipophilic layer and is linked to the hydrophilic silica support via a lipophilic spacer with polar embedded amide and sulfide groups. Currently, Type I and Type II bimodal columns are not commonly used due to the performance limitations. Columns using Types III and IV media have been commercialized and positioned as both general-purpose LC columns (as an alternative to C18) and application-specific products. An example of commercial Type III columns is the Primesep® column family that each column has a dual chemistry stationary phase with a hydrophobic long alkyl chain and an ionizable cationic or anionic embedded group [35–37,48,49]. When the polar group bears a charge, it effectively shields any other less polar groups of the stationary phase. As a result, the activity of silanol groups, which cause unwanted interaction in many reversed-phase columns, is completely undetectable and does not affect the peak shape or selectivity. Commercial Type IV bimodal columns are also available, such as Acclaim® Mixed-Mode WAX-1 [45], Acclaim Mixed-Mode WCX-1 [18] and Acclaim Surfactant [44]. Structures of some RP/IEX stationary phases with distinctive chemistry designs are illustrated in Fig. 2.

In addition to silica-based mixed-mode columns discussed above, polymer-based mixed-mode HPLC columns are also available [50,51,52–54]. The multimode separation mechanism of the OmniPac PAX-500 is achieved by coating a macroporous, hydrophobic polymer core for RP retention with an anion-exchange latex bead layer for anion-exchange retention. The macroporous structure provides an accessible hydrophobic core where reversed-phase retention occurs. The anion-exchange selectivity is provided by anion-exchange MicroBead™ latex that coats the outer layer of the hydrophobic core. Similarly, the multimode separation mechanism of OmniPac PCX-500 is achieved by coating a macroporous hydrophobic polymer core for RP retention with a CEX latex bead layer for cation-exchange retention.

Due to the complexity and variety of analytes in hydrophilicity and ionization, it is highly challenging but desirable to separate anionic, cationic and neutral molecules within a single HPLC analysis. This separation necessitates trimodal stationary phases that can provide CEX, AEX and RP (or hydrophilic) interactions simultaneously. Fig. 3A shows that the Scherzo stationary phase by Imtakt [23] is constructed by mixing two types of bonded silica particles: one modified with C18 and CEX functionalities, and the other one with C18 and AEX functionalities. These columns are positioned as general-purpose columns for a broad range of HPLC applications