Development and Evaluation of HILIC-Type Sorbents Modified with Hydrophilic Copolymers for Solid-phase Extraction

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Abstract

Hydrophilic interaction chromatography (HILIC) has attractive attention for the separation of water-soluble compounds via HPLC. There are, however, few studies on the pretreatment of HILIC-type solid-phase extraction (SPE) due to the difficulty of obtaining HILIC-type sorbent. Therefore, the development of HILIC-type sorbents for SPE is essential. In this study, four different hydrophilic copolymers, namely diallylamine–maleic acid (DAM), diallylamine–acrylamide copolymer (DAA), allylamine–maleic acid copolymer (MAM), and partly methylcarbonylated allylamine acetate copolymer (MAC), were immobilized on glycidyl methacrylate (GMA)-base resin, and their adsorptive properties were evaluated. The results of the physical and adsorptive properties indicated that a balance between the water content of the water-enriched layer on sorbent and the amount of hydrophilic copolymer immobilized on the GMA-base resin was vital for the adsorption in HILIC-type sorbent for SPE.

Keywords: Hydrophilic interaction chromatography, solid phase extraction, hydrophilic copolymer, water-soluble compounds.
Introduction

Since biological and environmental samples generally contain various contaminants, sample pretreatment procedures are an integral part of quantitative analysis. Solid-phase extraction (SPE) have generally been applied as the pretreatment method. SPE is an important pretreatment method for quantitative analyses. The SPE sorbents have commercially available separation modes, including reversed-phase (RP), normal-phase, ion exchange and adsorption. Since the RP mode is frequently used in most quantitative analyses, RP-type sorbents such as styrene-divinylbenzene (SDB) and octadecyl silica (ODS) are widely used in the analytical field. For water-soluble compounds, however, the RP-type sorbents have poor adsorptive properties because their primary mode of interaction, which is derived from the benzene ring or alkyl chain moieties in their structures, is hydrophobic. This problem can be overcome by using RP-type sorbents that possess divinylbenzene (DVB) copolymer blended with a hydrophilic monomer like N-vinylpyrrolidone or methacrylate, and these sorbents are generally called hydrophilic-lipophilic balance (HLB)-type sorbents. In our previous reports, DVB-based HLB-type sorbents containing hydrophilic methacrylate and/or N-vinylpyrrolidone were developed and possessed a higher adsorptive property for water-soluble compounds than their commercially available sorbents. Despite this step forward, there are still limitations in the adsorptive properties of these types of sorbents for water-soluble compounds.

Recently, hydrophilic interaction chromatography (HILIC) has attractive attention for its usefulness in the separation of water-soluble compounds. HILIC is a variation of normal-phase liquid chromatography (NPLC), separation mechanism is caused by the
water-enriched layer on the surface of the very polar stationary phase and the less polar mobile phase, which is composed of a water-containing polar solvent such as acetonitrile (ACN). While the separation mechanism is mainly NPLC based, secondly effect interaction like hydrogen bond, electrostatic interaction, and dipole-dipole interaction also affect the separation of water-soluble compounds.11,12 Various analytes have been applied on the HILIC separation technique, including nucleosides related compounds,13-15 antibiotics,8 and proteomics.16 The stationary phases used in HILIC, including amine-, diol-, amide-, and zwitterion-type stationary phases, are often employed for HPLC applications.17-20 Here, zwitterion-type stationary phase has a high capacity for hydration and is suitable for the separation of herbicides21 and metabolite.22

While the usage of the HILIC mode in SPE may be theoretically useful for the extraction and purification of various water-soluble compounds, there are few reports on their practical application for water-soluble compounds such as peptide23 and glycopeptide.24 This is presumably due to the scarcity of commercially available HILIC-type SPE sorbents when compared to their RP-type SPE sorbents. Amide- and NH₂-type sorbents are now commercially available as HILIC-type SPE sorbents (GL Sciences),25 whereas the sale of zwitterion-type stationary phase has been discontinued. Moreover, few studies are focused on the development of sorbents for HILIC-type SPE, whereas there are significantly more published reports on HILIC-type stationary phases for HPLC applications. Given this, effective quantitative analyses of water-soluble compounds can only be achieved with the development of more efficient HILIC-type sorbents for SPE applications.

In the HILIC mode, the formation of the water-enriched layer is essential for the retention
of water-soluble compounds. Inoue (one of the coauthors) et al. reported HILIC-type SPE sorbents, that were modified with sulfobetaine group\textsuperscript{26} or zwitterion-type copolymer,\textsuperscript{27,28} and evaluated the adsorptive property of nucleic acids, nucleosides, sugars, and glycosides in SPE. Studies on the retention behavior of various HILIC-type stationary phases in HPLC have also been reported.\textsuperscript{29-31} Hydrophilic copolymer modified-sorbents that possess a zwitterion structure consisting of a diallylamine–maleic acid (DAM) copolymer (Fig. 1) improved the hydration capacity of the stationary phase’s surface, which facilitated the formation of the water-enriched layer. This hydrophilic copolymer-type sorbent exhibited more modified amount than its sulfobetaine-type sorbent, which led to more effective formation of the water-enriched layer. When compared with zwitterion-type stationary phases like sulfobetaine, DAM decreased electrostatic interaction between the phases due to the presence of a weak acidic substituent (maleic acid) and a weak basic substituent (diallylamine). The modification of the other hydrophilic copolymers also improved their adsorptive property.

In this study, the other hydrophilic copolymers were investigated in addition to DAM; these included diallylamine–acrylamide copolymer (DAA),\textsuperscript{32} allylamine–maleic acid copolymer (MAM), and partly methylcarbonylated allylamine acetate copolymer (MAC). There copolymers were selected as the immobilizing copolymers (Fig. 1), and the adsorptive properties of each sorbent were evaluated.

**Experimental**
Reagents

Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDM) and ACN for HPLC were purchased from Sigma-Aldrich Japan. 2,2–Azobis (isobutyronitrile) (AIBN), methyl cellulose, butyl acetate (BuOAc), sodium hydroxide, 2–propanol, and adenine (Ade) were obtained from FUJIFILM Wako Pure Chemical Corporation. Uracil (Ura), uridine (U), cytidine (C), guanosine (G) and adenosine (A) were purchased from Tokyo Chemical Industries. Methanol for HPLC was purchased from KANTO CHEMICAL CO., INC. Diallylamine–maleic acid copolymer (DAM, 40% aqueous solution) was purchased from Nitto Boseki. Diallylamine–acrylamide copolymer (DAA, 25% aqueous solution), allylamine–maleic acid copolymer (MAM, 20% aqueous solution), and partly methylcarbonylated allylamine acetate copolymer (MAC, 15% aqueous solution) were donated from Nitto Boseki.

Instrumentation

The recovery of each sorbent was evaluated by an HPLC system as follows: a GD-2080-53 degasser, two PU-2085 pumps, a MX-2080-32 dynamic mixer, an AS-4050 autosampler, a CO-2065 Plus column oven, a UV-2070 UV-Vis detector, and an LC-Net II/ADC interface box (Jasco). An InertSustain AQ-C18 (3 μm, 150 × 2.1 mm I.D., GL Sciences) was
used for the separation on HPLC. The separation conditions on HPLC were as follows: flow rate, 0.2 mL min$^{-1}$; mobile phase, a 5% methanol solution (for the evaluation of Ura, U, and Ade) or a 10% methanol solution (for the evaluation of A, C, and G); column temperature, 40 °C; and injection volume, 10 μL. Elemental analysis was performed by JM11 (J-SCIENCE LAB CO., Ltd.). The specific surface area of each base resin was measured by a Micromeritics TriStar II Surface Area and Porosity Analyzer.

**Synthesis of the GMA-base resin**

The GMA-base resins were synthesized using suspension copolymerization with GMA and EGDM at the molar ratio of either 80:20 or 60:40. BuOAc, which was used as an inert diluent, was added to each monomer mixture at the same weight ratio (in the case of GMA:EGDM = 80:20) or 1.5 fold the weight (in the case of GMA:EGDM = 60:40). AIBN (1 wt%) for the monomers (GMA and EGDM) was also added to the organic mixture. The blended organic mixture was then added to a 0.25% MC solution, which was used as a suspension stabilizer, and the suspension copolymerization was performed at 78 °C for 7 h with stirring at 250 rpm. Each base resin of 53 – 90 μm was classified by using standard sieves.

**Immobilization of the hydrophilic copolymers on the GMA-base resins**

The reaction conditions in this study were slightly modified with reference to those in the
previous paper.\textsuperscript{27} Firstly, the GMA-base resin (5 g) was dispersed in 2-propanol (15 mL), to which 5 mol L\textsuperscript{-1} NaOH (16 mL), an aqueous solution of the respective hydrophilic copolymer, and water were added. To ensure that the same concentration of the hydrophilic copolymer was used during the modification reaction, the respective aqueous solutions for the hydrophilic copolymers were composed as follows: DAM (5 g) and water (44 mL); DAA (8 g) and water (41 mL); MAM (10 g) and water (39 mL); and MAC (13.3 g) and water (35.7 mL). Each slurry was then heated at 50 °C for 20 h with shaking, after which the modified sorbent was dispersed in 0.1 mol L\textsuperscript{-1} \(\text{H}_2\text{SO}_4\) and sonicated for 30 min to convert the residual epoxy group into diol group. For the evaluation of holding water and the hydration water, a diol resin (Diol-type sorbent) was also prepared from each GMA-base resin by heating at 40 °C for 4 h in 0.01 mol L\textsuperscript{-1} \(\text{H}_2\text{SO}_4\).

\textit{Measurement of the water content of the sorbents}

The water content of each prepared sorbent was determined using gravimetric method based on a previous report.\textsuperscript{27} Briefly, each sorbent (150 mg) was packed into a 3 mL cartridge for SPE (BondElut\textsuperscript{®} Reservoir, Agilent) and was dried under vacuum at 50 °C for 6 h. Once dry, each cartridge was conditioned with an ACN flush (3 mL) before being soaked in ACN for 30 min. After that, each solution was subjected to the following conditions: in the case of DAM and MAM, 3 mL of 0.1 mol L\textsuperscript{-1} NaOH and 0.1 mol L\textsuperscript{-1} HCl; in the case of DAA, and MAC, 3 mL of 0.1 mol L\textsuperscript{-1} HCl; in the case of Diol-type sorbent, 3 mL of 0.1 mol L\textsuperscript{-1} NaOH. Next, water was flushed though the cartridge to wash any excess acid or alkali, after which each cartridge
was soaked in water for 1 h. After conditioning, the excess water on each sorbent was removed by applying vacuum at -0.03 MPa for 10 min. Once dry, the weight ($W_1$) of each sorbent was measured. Then, the cartridge was washed by ACN (3 mL) and dried under vacuum at -0.03 MPa for 10 min before the weight ($W_2$) of each sorbent was measured again. After each procedure, the respective cartridge was then dried under vacuum at 50 °C for 14 h and weighted ($W_0$). The water content for each sorbent was calculated as follows:

$$holding\ water\ (%) = \frac{(W_1 - W_0)}{(W_0 - W_c)} \times 100$$

$$hydrating\ water\ (%) = \frac{(W_2 - W_0)}{(W_0 - W_c)} \times 100$$

$W_c$ was the weight of cartridge and frits without the respective sorbent.

**Evaluation of adsorptive property by SPE**

Each sorbent (50 mg) was packed into a 1 mL cartridge for SPE (BondElut® Reservoir, Agilent). ACN (10 mL), water (10 mL), and 95% ACN solution (10 mL) were passed into the cartridge for conditioning. Each 0.5 mL of 20 mg L$^{-1}$ of analyte (dividing into two groups: Ura, U and Ade, or A, C and G) dissolved in 95% ACN solution was loaded into the cartridge. Then, the cartridge was washed by 2 mL of ACN. The analyte adsorbed on each sorbent was eluted by 0.5 mL of water. The adsorptive property of sample loading, washing, and elution were calculated by a ratio of the amount in each solution to the amount in initial solution.
Results and Discussion

Physical properties of HILIC-type sorbents modified with four hydrophilic copolymers

First, HILIC-type sorbents for SPE were prepared by immobilizing each hydrophilic copolymer on GMA-base resin consisted of GMA and EGDM at the molar ratio of GMA:EGDM = 80:20. Immobilization of the copolymers was carried out via epoxy-ring-opening reaction between GMA and each copolymer that possess at least one amino group. The physical characteristics of each synthesized sorbent were established by the nitrogen content, the holding water content, and the hydrating water content parameters. The nitrogen content of each hydrophilic copolymer modified-type sorbent was determined via elemental analysis to reveal the amount to which modification occurred on each sorbent. Table 1 shows that all modified sorbents had nitrogen atoms, which was indicative of modification of each sorbent with the respective hydrophilic copolymer. DAA and MAC have two nitrogen atoms in the repeating unit of the copolymer structures, whereas DAM and MAM have one nitrogen atom. Therefore, the amounts of modified DAA and MAC were estimated as half. Table 1 shows that the DAA- and MAM-modified sorbents had a higher nitrogen content than the DAM- and MAC-modified sorbents, whereas the DAM- and MAC-modified sorbents had almost the same nitrogen content. Based on the nitrogen content, the order of the modified amount for each hydrophilic copolymer with considering DAA and MAC having two nitrogen atoms in the repeating unit of the copolymer structures was as follows: MAM > DAA > DAM, MAC.
Next, the water-enriched layer on the surface of a modified sorbent was investigated by measuring the water content. Inoue et al. previously reported that the water contents were classified into two types: one type contributed the partition in HILIC (referred to the “holding water”), and the other layer did not contribute to the partition in HILIC (refer to “hydrating water”). Both the holding and the hydrating water contents of each modified sorbent and the Diol-type sorbent are shown in Table 1. The hydrating water content of each sorbent was higher than the Diol-type sorbent. This change was attributed to the effective adsorption of water molecules onto the sorbent by the respective immobilizing hydrophilic copolymer. The holding water contents of the DAM-, DAA-, and MAC-modified sorbents were lower than that of the Diol-type sorbent while the MAM-modified sorbent is comparable. The reason for the decrease in the holding water content of the DAM-, DAA-, and MAC-modified sorbents may arise from smaller surface area available to capture water molecules. Additionally, the DAM-, DAA-, and MAC-modified sorbents had a lower holding water content than MAM-modified sorbent. This may be due to differences in the amount of modification of the hydrophilic copolymer. The holding water content of the DAA-modified sorbent was intermediate between that of the DAM- and MAM-modified sorbents and its immobilizing amount was relatively high. This was because DAA possessed both a cation moiety (diallylamine) and neutral moiety (acrylamide), unlike the zwitterion-type copolymers, which had a decreased capacity for holding water. On the other hand, the hydrating water content of the DAA-modified sorbent was the highest of all the sorbents, which is derived from highly immobilizing amount of DAA. The MAM-modified sorbent had the highest immobilizing amount and holding water content. The MAC-modified sorbent had the lowest hydrating water content in the hydrophilic copolymer modified sorbents.
The reason for this may be neutral moiety (methylcarbonylated allylamine) on the copolymer structure of MAC, which made it difficult to the capture of water molecules due to the existence of the methyl group. The differences in the immobilizing amount, the holding water, and the hydrating water content were observed using these measurements, which, in turn, influenced the adsorptive properties of each sorbent.

Comparison of the adsorptive property of each HILIC sorbent.

Fig. 2 shows the recoveries of the SPE in each process for the four hydrophilic copolymer modified sorbents. Each bar graph from left to right (e.g., from Ura to G) is the elution order of the separation on commercially available HILIC column (TSKgel Amide-80, Tosoh Corporation). Excluding the conditioning process, the evaluation of the SPE processes were categorized in three steps; the “loading” step represented the ratio of the amount that was not adsorbed on the sorbent in the sample loading process; the “washing” step represented the ratio of the amount that was washed by ACN (2 mL) in washing process; and the “elution” step represented the ratio of amount adsorbed on the sorbent.

The comparison of two zwitterion-type sorbents (e.g., the DAM- and MAM-modified sorbents) revealed that the adsorptive property of all evaluated compounds on the DAM-modified sorbent was higher than that of the MAM-modified sorbent. From the viewpoint of the normal-phase partition, the MAM-modified sorbent was expected to have more adsorptive property than the DAM-modified sorbent due to its higher holding water content. However, the higher holding water content of the MAM-modified sorbent may have caused the low adsorptive property, this, in turn, reduced the expression of secondary effect interaction in
the water-enriched layer that were caused by MAM’s polar functional group after extraction via normal-phase partitioning during the sample loading process. The balance between the holding water content and the amount of polar functional group of hydrophilic copolymers was shown to be crucial for the adsorption of water-soluble compounds in the HILIC mode.

Other comparisons between the sorbents also supported this consideration. A comparison of the DAM- and DAA-modified sorbents indicated that the DAA-modified sorbent, which had a higher holding water content than the DAM-modified sorbent, displayed more adsorptive property than the DAM-modified sorbent. The high adsorptive property of the DAA-modified sorbent was derived from the imbalance between holding water content and the modified amount of DAA, which further facilitated secondary effect interactions. Thus, the high adsorptive property of the DAA-modified sorbent caused the specific adsorption of Ura, U, Ade, and G, that could not be eluted by a small amount of water (for instance, the recovery of Ade given by the DAA-modified sorbent is only ca. 10% as shown in Fig. 2). A comparison of the MAM- and MAC-modified sorbents also supported this observation as the MAC-modified sorbent, which was shown to have a more balanced holding water content vs. the amount of modified hydrophilic copolymer than the MAM-modified sorbent, displayed higher adsorptive property. Additionally, even though the DAM-modified sorbent had more adsorptive property than the MAC-modified sorbent, both sorbents had the same modified amounts and holding water content. The hydrating water content in the MAC-modified sorbent was lower than that seen in the DAM-modified sorbent. This was due to the difficulty in forming stable water-enriched layer since MAC had a property for easily releasing water molecules. These results indicated that the balance between the holding water content and the amount of the
modified hydrophilic copolymer was important for adsorption in the HILIC-type sorbent for SPE. Moreover, the structure of the functional group may also influence the adsorptive properties of water-soluble compounds. From these evaluations, it was concluded that the modification of DAM and DAA was an effective approach for improving the adsorptive property for water-soluble compounds in HILIC-type sorbents for SPE.

*Improvement of adsorptive properties by changing the GMA-base resin*

Specific surface area is one of the important factors for improving adsorptive property in SPE. In the previous section, GMA-base resin at the molar ratio of GMA:EGDM = 80:20 (80GMA) was used because it contained many epoxy groups for modification. On the other hand, the degree of cross-linkages from the EGDM monomer was relatively low, which, in turn, decreased the specific surface area of the GMA-base resin. At the molar ratio of GMA:EGDM = 60:40 (60GMA), GMA-base resin was also synthesized as a means of increasing the specific surface area available; DAM and DAA were modified. Analysis of the specific surface area for each GMA-base resin revealed that 60GMA had a higher specific surface area than 80GMA (60GMA: 90.3 m²g⁻¹ vs. 80GMA: 54.4 m²g⁻¹). This result indicated that adsorption was generally improved by immobilizing the hydrophilic polymer onto 60GMA.

The nitrogen content, the holding water content, and the hydrating water content for each sorbent are shown in Table 2. Here, both the holding and hydrating water contents of the diol resin (i.e., Diol-type sorbent) were approximately doubled due to the increase in the number of crosslinks (i.e., an increase in the EGDM content). When compared to the DAM- and DAA-modified sorbent that was prepared using 80GMA, the DAM- and DAA-modified sorbent
that had been prepared by 60GMA showed higher holding and hydrating water contents. From the holding water content of the Diol-type sorbent, the larger specific surface area facilitated an increase in the water content in the resin’s pores when compared with that of the Diol-type sorbent of 80GMA. This change also increased the holding and hydrating water contents of the DAM- and DAA-modified sorbents. From this result, an increase in adsorptive property was expected.

Fig. 3 shows the recovery values of the DAM- and DAA-modified sorbents using 60GMA as the GMA-base resin. Quite unexpectedly, we noted a decrease in adsorptive property of the DAM-modified sorbent. When compared with the DAM-modified sorbent of 80GMA, the modified amount of DAM was low, whereas an increase in the holding water content was observed in the DAM-modified sorbent of 60GMA; this meant that the decrease in adsorptive property of the DAM-modified sorbent results from the difficulty of the expression of secondary effect interaction caused by the increased water content. A decline in the adsorptive property was also seen in the DAA-modified sorbent of the 60GMA, in other words the DAA-modified sorbent of the 60GMA can more easily desorb Ura, U, and Ade in the elution process than the DAA-modified sorbent of the 80GMA. Here, the observed decrease resulted from the higher holding water content. In the case of the DAA-modified sorbent, the increase in the holding water content promoted desorption, thereby leading to higher recovery values for the evaluated compounds. These results provide further evidence for the importance of the balance between the amount of modified hydrophilic copolymer and the water content in ensuring better adsorption of HILIC-type sorbents for SPE.
Conclusions

In this study, novel HILIC-type sorbents for SPE were synthesized. The adsorptive tendencies of each synthesized sorbent were evaluated. Comparisons of each sorbent revealed that a balance between the modified amount of the respective hydrophilic copolymer and the water content is important in order to facilitate effective adsorption of water-soluble compounds. The DAM-modified sorbent had the high adsorptive property in the developed sorbent. In contrast, the DAA-modified sorbent, which is the imbalance between the holding water content and the modified amount of DAA, has specific adsorption. The degree of cross-linkage in the GMA-base resin was also important as the sorbents with a higher ratio of the crosslinking monomer (EGDM). GMA-base resin having a higher specific surface area was also studied. Quite unexpectedly, an increase in the adsorptive property was not observed due to the higher water content of each sorbent. From these results, it was clear that a balance between the water content of water-enriched layer and the amount of modified hydrophilic copolymer must be maintained to ensure adequate secondary effect interaction for the adsorption in HILIC-type sorbent for SPE. We will study on the effect of mixing of DAM and DAA to improve the adsorption property of HILIC-type sorbent for water-soluble compounds.

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Table 1 Nitrogen content and water content of the Diol-type and four hydrophilic copolymer modified sorbents prepared using 80GMA* as the GMA-base resin.

|                      | units | Diol | DAM | DAA | MAM | MAC |
|----------------------|-------|------|-----|-----|-----|-----|
| nitrogen contents    | [N%]  | -    | 0.40| 1.52| 1.13| 0.80|
| holding water contents | [%]  | 85.0 | 65.5| 73.1| 85.8| 60.8|
| hydrating water contents | [%]  | 3.3  | 6.6 | 8.4 | 6.4 | 4.4 |

* 80GMA: GMA:EGDM = 80:20
Table 2 Nitrogen content and water content of the Diol-type, DMA-, and DAA-modified sorbents prepared using 60GMA* as the GMA-base resin.

|                      | units | Diol | DAM  | DAA  |
|----------------------|-------|------|------|------|
| nitrogen contents    | [N%]  | -    | 0.32 | 1.93 |
| holding water contents | [%]  | 166.1| 142.4| 118.9|
| hydrating water contents | [%]  | 7.1  | 10.7 | 12.3 |

* 60GMA: GMA:EGDM = 60:40
Figure Captions

Fig. 1 Structure of each hydrophilic copolymer

Fig. 2 Comparison of adsorptive property (loading, washing, and elution) among four hydrophilic copolymer modified sorbents prepared using 80GMA as the GMA-base resin.

Fig. 3 Comparison of adsorptive property (loading, washing, and elution) between DAM- and DAA-modified sorbents prepared using 60GMA as the GMA-base resin.
Fig. 1 Structure of each hydrophilic copolymer
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Graphical Index

hydrophilic copolymer

DAM

DAA

MAM

MAC

water-enriched layer

HILIC-type SPE sorbent