Separation characterization of a new zwitterion-bonded HILIC column

COL-31

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1. Introduction

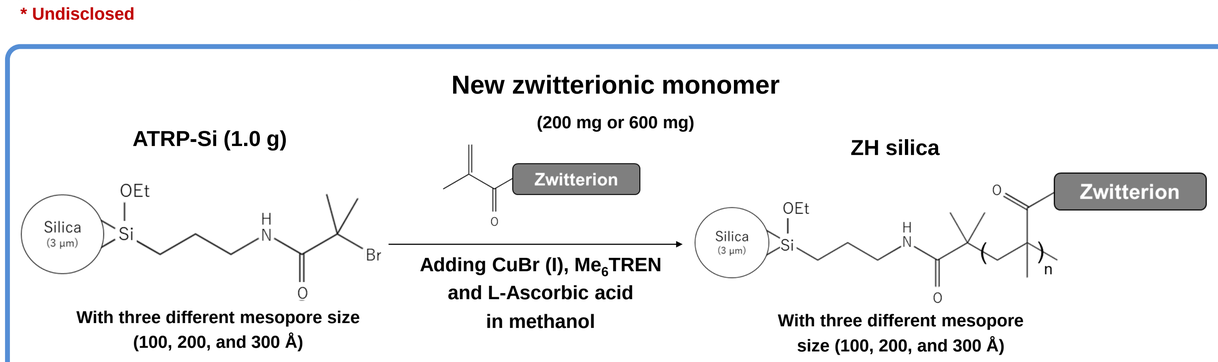
Recently, the number of the reports with hydrophilic interaction chromatography (HILIC) have been increased, especially in the metabolomics and food analysis where there are a variety of highly-polar compounds as the target compounds. Here, we address the development of a new hydrophilic stationary phase for silica particles with three different mesopore sizes, which were modified using a surface-initiated atom-transfer radical polymerization (SI-ATRP) with a home-made zwitterionic monomer. Two different monomer concentrations were examined for each silica particle to afford six different packing materials in the surface-functionalization processes, ZH series, which were packed into columns and characterized using a LC test method reported by Kawachi *et al.* [1]. Among hydrophilic analytes, the separation of nucleic bases, nucleotides and nucleosides is still challenging/meaningful [2] and [3]. These columns were applied to analysis of nucleic bases and nucleosides in this presentation.

2. Experiments

2-1. Preparation of ATRP-new zwitterion columns

We firstly synthesized a ATRP initiator silane (i.e., anchor part for polymerization) and prepared the anchor-modified 3 µm silica particles (ATRP-Si) with three different mesopore sizes (100, 200, and 300 Å). For introduction of hydrophilic stationary phase, we synthesized a newly-designed zwitterionic monomer (not shown)* and then the ATRP-Si were modified using a SI-ATRP manner with the prepared zwitterionic monomer (see Fig. 1), named ZH silica. Afterwards, the ZH silica particles were packed at 60 MPa into a 3.0 mm (I.D.) × 100 mm steel-use-stainless (SUS) column (Note that the packing procedure has been still under consideration to provide a desirable column efficiency).

*** Undisclosed**



Information of ZH silica

Name of ZH silica	ZH-100Å-200	ZH-100Å-600	ZH-200Å-200	ZH-200Å-600	ZH-300Å-200	ZH-300 Å-600
Particle diameter (µm)	3					
Mesopore size (Å)	100		200		300	
Surface area (m^2/g)	440		200		100	
Reaction amount of the new zwitterionic monomer (mg)	200	600	200	600	200	600

Fig.1_Scheme of preparation of ATRP-new zwitterion columns

2-2. Characterization: HPLC test

We examined each fabricated packed column using a LC-examination method reported by Kawachi *et al*. [1] The LC measurement conditions are shown in Table 1.

Table 1 LC measurement conditions for the separation characterization of HILIC column.

Instrument	:	Shimadzu Nexera™ XS series
Column dimension	:	3.0 mm (I.D.) × 100 mm, 3 µm (ZH-series, home-made) 2.1 mm (I.D.) × 100 mm, 3 µm (Shim-pack™ GIST Amide, Shim-pack Scepter™ Diol-HILIC and Shim-pack Velox™ HILIC, Shimadzu) 4.6 mm (I.D.) × 150 mm, 3.5 µm (XBridge BEH Amide, Waters) 4.6 mm (I.D.) × 150 mm, 5 µm (ZIC-HILIC, Merck)
Mobile phase	:	A-1) 100 mmol/L ammonium acetate buffer (pH 4.7) for evaluation of selectivity values for ion-exchange interactions A-2) 20 mmol/L ammonium acetate buffer (pH 4.7) for evaluation of other selectivity values B) Acetonitrile (ACN) A : B = 10 : 90 (v/v)
Flow rate	:	0.42 mL/min for column of 3.0 mm (I.D.) 0.21 mL/min for column of 2.1 mm (I.D.) 1 mL/min for column of 4.6 mm (I.D.)
Detection	:	UV 254 nm
Column temperature	:	30˚C
Injection volume	:	1 µL
Sample	:	1. Toluene (t_0 marker) 2. Uridine (U) 3. 2'-deoxyuridine (2dU) 4. 5-metyluridine (5mU) 5. Adenosine (A) 6. Vidarabine (V) 7. 2'-deoxyguanosine (2dG) 8. 3'-deoxyguanosine (3dG) 9. Theophylline (Tp) 10. Theobromine (Tb) 11. 4-nitrophenyl α-D-glucopyranoside (NPαGlu) 12. 4-nitrophenyl β-D-glucopyranoside (NPβGlu) 13. Sodium *p*-toluenesulfonate (SPT) *14. N,N,N*-trimethylphenylammonium chloride (TMP) (1 : 100 mg/L, 2~11 : 113 mg/L, 12 & 13 : 1000 mg/L) * All the compounds were dissolved in ACN: water = 90:10 (v/v).

2-3. Application: LC analysis of nucleic bases and nucleosides

The LC measurement conditions are shown in Table 2.

Table 2 LC measurement conditions for analysis of nucleic bases and nucleosides.

Mobile phase	:	A-1) 50 mmol/L ammonium acetate buffer (pH 4.7) A-2) 50 mmol/L ammonium formate buffer (pH 3.6) B) ACN A : B = 10 : 90 (v/v)
Column size	:	3.0 mm (I.D.) × 100 mm
Detection	:	UV 254 nm
Flow rate	:	0.42 mL/min
Column temperature	:	35˚C
Injection volume	:	1 µL
Sample	:	1. Uracil, 2. Adenine, 3. Adenosine, 4. Uridine, 5. Cytosine, 6. Cytidine, 7. Guanosine (178.6 mg/L each), dissolved in ACN : water = 50:50 (v/v).

3. Results and Discussion

3-1. Characterization of novel HILIC stationary phases

The selectivity values (α) obtained by the LC test showed no significant difference between the two prepared HILIC columns, ZH-100Å-series and ZH-200Å-series particles, functionalized applying the same monomer concentrations in the reaction solutions (see the k(U)- and α-values in Table 3)., although a specific hydrophilic retention (k(U)) for the ZH-100Å-series was obviously larger than that for ZH-200Å-series, which is consistent with the results of elemental analysis for C% and N%. Combining the obtained values for ZH-300Å-series with the aforementioned shows that a change in mesopore size of silica particles from 100 Å to 300 Å would result in an enhancement of hydrophilic retention without a drastic change in the major selectivity values for the home-made stationary phase.

Table 3 Results of the LC characterization test and elemental analysis of the fabricated HILIC silica particles.

Column name	ZH-100Å-200	ZH-100Å-600	ZH-200Å-200	ZH-200Å-600	ZH-300Å-200	ZH-300Å-600	BEH Amide	ZIC-HILIC
Mesopore size	100 Å		200 Å		300 Å		130 Å	200 Å
Hydrophilic retention k(U)	2.88	4.28	1.44	2.23	0.70	0.95	2.31	2.11
Selectivity for –OH groups α(OH) = k(U)/k(2dU)	1.57	1.79	1.57	1.80	1.54	1.70	1.70	2.03
Selectivity for $-CH_2$ groups α(CH) = k(U)/k(5mU)	1.33	1.47	1.30	1.44	1.29	1.38	1.29	1.67
Selectivity for configurational isomer α(V/A) = k(V)/k(A)	1.36	1.45	1.36	1.45	1.36	1.43	1.29	1.5
Selectivity for regio isomer α(2dG/3dG) = k(2dG)/k(3dG)	1.09	1.10	1.07	1.08	1.06	1.06	1.07	1.11
Anion-exchange property α(SPT/U) = k(SPT)/k(U)	0.25	0.31	0.24	0.33	0.20	0.31	0.19	0.33
Cation-exchange property α(TMP/U) = k(TMP)/k(U)	2.06	1.06	1.73	0.78	1.87	0.95	0.87	1.57
pH on the surface of the stationary (α > 1: acid, α = 1: neutral, α < 1: base) α(Tb/Tp) = k(Tb)/k(Tp)	1.11	1.14	1.06	1.11	1.03	1.03	1.29	1.18
Selectivity for shape (anomer) α(α/β) = k(α)/k(β)	1.16	1.14	1.15	1.13	1.14	1.13	1.17	1.14
Elemental analysis: C%	10.0	13.3	6.5	9.4	3.6	5.2	-	
Elemental analysis: N%	1.18	1.62	0.74	1.14	0.44	0.62		
Elemental analysis: Δ C/Si	0.16	0.33	0.13	0.26	0.07	0.12		

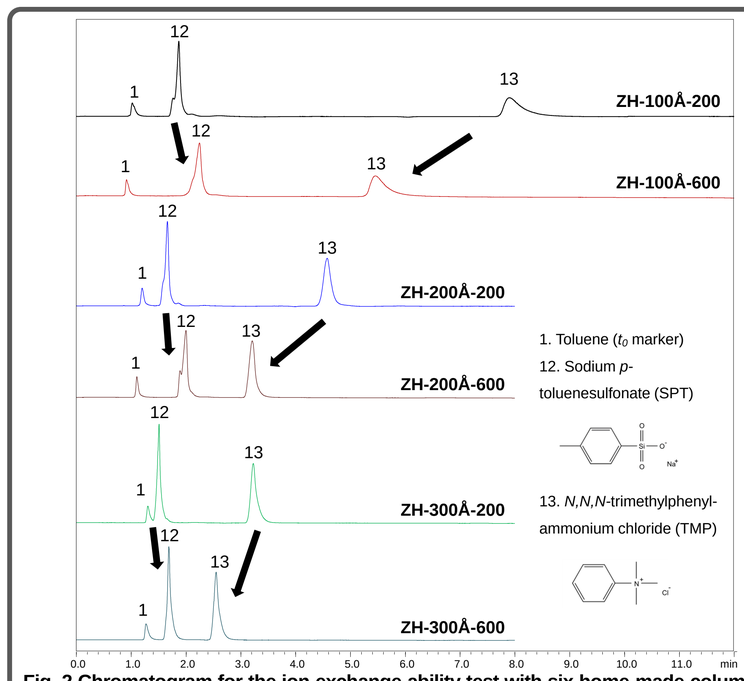
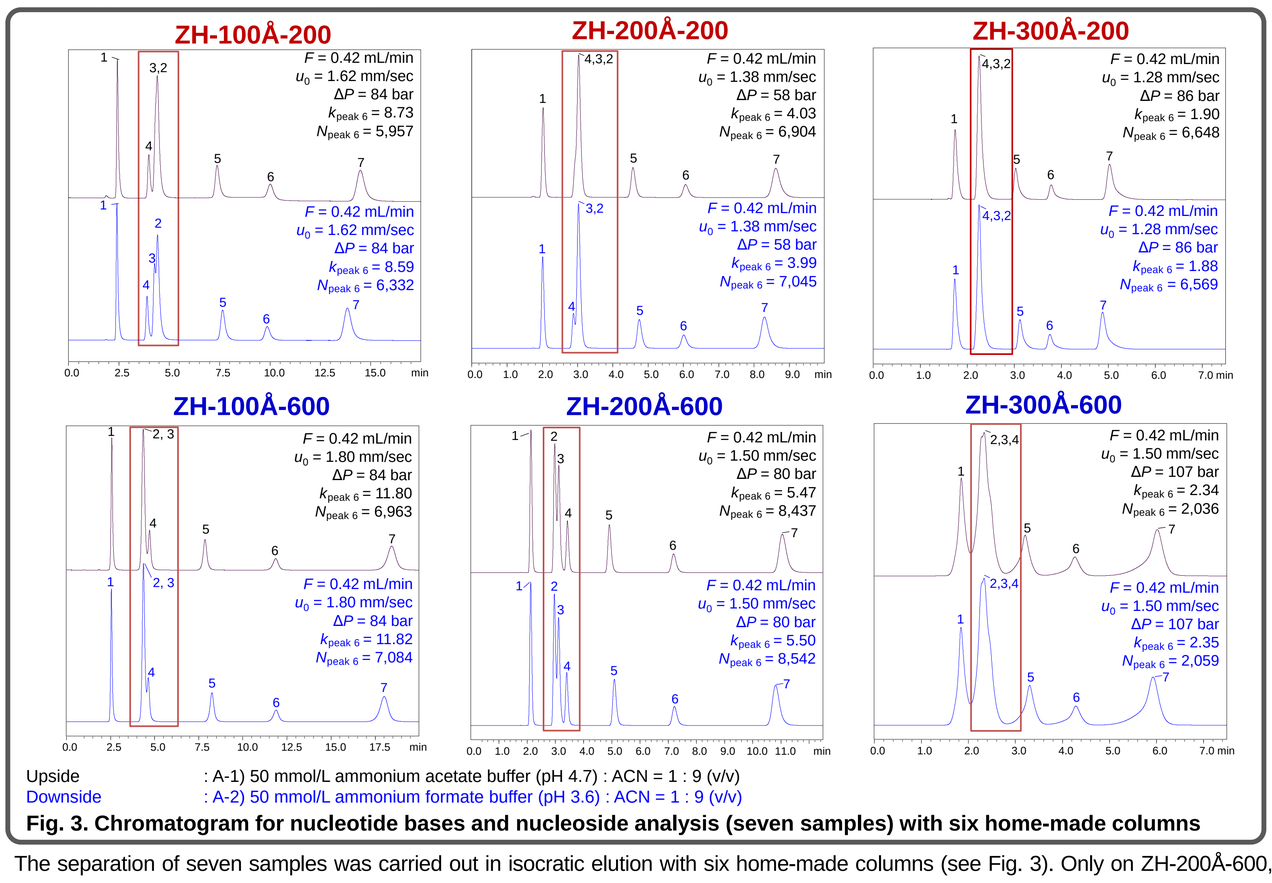


Fig. 2 Chromatogram for the ion exchange ability test with six home-made columns, mobile phase: 100 mmol/L ammonium acetate aqueous solution (pH 4.7)

Applying a larger amount of monomer led to weakening the cation-exchange property, while hydrophilic retention, selectivity for -OH groups, and the anion exchange property increased (see Table 3 and Fig.2). This indicates that elongation of the polymer chain increased the thickness of the water-enriched-layer and reduced the effect of surface silanol groups on the ion-exchange mechanism, while the electrostatic interactions derived from the zwitterionic functional groups was enhanced. Additionally, when compared to commercially-available HILIC columns, a similar ability of separation selectivity on structural difference was observed for the novel ZH columns. Through the present examination, it is suggested that as the concentration of monomer is increased via the SI-ATRP method, there is a possibility of achieving better separation selectivity than those of commercially-available HILIC columns.

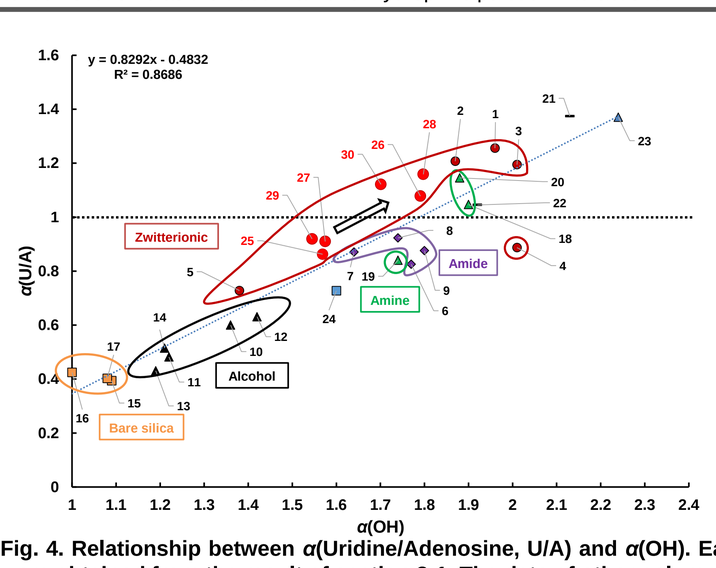
3-2. Application: LC analysis of nucleic bases and nucleosides



Upside : A-1) 50 mmol/L ammonium acetate buffer (pH 4.7) : ACN = 1 : 9 (v/v)
Downside : A-2) 50 mmol/L ammonium formate buffer (pH 3.6) : ACN = 1 : 9 (v/v)

Fig. 3. Chromatogram for nucleotide bases and nucleoside analysis (seven samples) with six home-made columns

The separation of seven samples was carried out in isocratic elution with six home-made columns (see Fig. 3). Only on ZH-200Å-600, seven peaks can be observed. Although there was no significant difference between ZH-100Å-600 and ZH-200Å-600 for the α-values except for the cation-exchange property (see Table 3), the co-elution (peak 2~4) was found on ZH-100Å-600. It is suggested that the difference in the peak resolution may be given by that in column efficiency due to mesopore size and/or by ion-exchange ability. Fig.3 also shows, when comparing the elution order of adenosine (**3**) and uridine (**4**), the modified columns with a higher monomer amount result in an elution order of **4** < **3**, opposite to the case using a lower amount of monomer. These results and the preceding report [4] imply that for polymer-modified columns the water-enriched-layer becomes thicker as polymerization progresses, thereby increasing retention of uridine as the trace-marker of hydrophilic partition interactions.



No.	Type	Column name
1	Zwitterion	ZIC-HILIC (Merck)
2	Zwitterion	ZIC-cHILIC (Merck)
3	Zwitterion	ZIC-pHILIC (Merck)
4	Zwitterion	Click XIon (Acchrom Technologies)
5	Zwitterion	PC HILIC (Osaka Soda)
6	Amide	SunShell HILIC-Amide (ChromaNik)
7	Amide	TSKgel Amide-80 (Tosoh)
8	Amide	XBridge BEH Amide (Waters)
9	Amide	Shim-pack GIST Amide (Shimadzu)
10	Alcohol	Lichrospher Diol (Merck)
11	Alcohol	Shim-pack Scepter Diol-HILIC (Shimadzu)
12	Alcohol	Ascentis Express OH5 (Supelco)
13	Alcohol	ULTRON AF-HILIC-CD (Shinwa Chemical Industries)
14	Alcohol	CYCLOBOND I (Shinwa Chemical Industries)
15	Bare silica	Shim-pack Velox HILIC (Shimadzu)
16	Bare silica	Chromolith Si (Merck)
17	Bare silica	HaloHILIC (Advanced Materials Technology)
18	Amine	ULTRON AF-HILIC-DA (Shinwa Chemical Industries)
19	Amine	COSMOSIL Sugar-D (Nacalai)
20	Amine	COSMOSIL $5NH_2$-MS (Nacalai)
21	Others	PolySULFOETHYL A (PolyLC)
22	Others	PolyHYDROXYETHYL A (PolyLC)
23	Tetrazole	DCpak HILIC (a prototype of DCpak PTZ) (Daicel)
24	Triazole	COSMOSIL HILIC (Nacalai)
25	Zwitterion	ZH-100Å-200 (Home-made)
26	Zwitterion	ZH-100Å-600 (Home-made)
27	Zwitterion	ZH-200Å-200 (Home-made)
28	Zwitterion	ZH-200Å-600 (Home-made)
29	Zwitterion	ZH-200Å-200 (Home-made)
30	Zwitterion	ZH-300Å-600 (Home-made)

Fig. 4. Relationship between α(Uridine/Adenosine, U/A) and α(OH). Each value of No.1, 8, 9, 11, 15 and 25-30 columns were obtained from the result of section 3-1. The data of other columns follow those from the previous reports [1] and [4].

Additionally, we recommend that α(U/A) may be more informative as a new factor indicative of water-enriched-layer instead of k(U). Fig.4 displays the relationship between α(U/A) and α(OH) for both commercially-available and home-made columns. The α(OH) has been reported to corelate to the thickness of water-enriched-layer [4], and it appears that as the α(OH) of commercial HILIC columns increases, the α(U/A) value also tends to increase. By considering that adenosine possesses the positively-charged structure in the applied LC measurement conditions [1], it would be predictable that the α(U/A) could be low when the effects of silanol are pronounced in silica-based columns because of the cation-exchange effects. When a simultaneous analysis of nucleic bases, nucleotides and nucleosides is required, one can see that columns with α(U/A) around 0.95–1.05 would show incomplete separations of adenine, adenosine, and uridine. On the other hand, columns with lower α(U/A) are likely to exhibit weaker hydrophilic interactions (retention), suggesting only a narrower range of organic solvent/water ratios is applicable during gradient analysis, i.e., their impracticability for simultaneous analysis. Therefore, it would be essential to select a HILIC column with an adequately high α(U/A)-values (e.g., the home-made columns prepared via the high monomer concentration) when conducting simultaneous nucleic acid analysis, meanwhile one needs to keep it in mind that columns with high α(U/A) may cause slower molecular diffusion in water-enriched-layer leading to slower mass transfer of solutes, which can show a loss of column efficiency for fast separation. Future investigations will focus on compromising the trade-off relationship.

4. Conclusions and future perspectives

We developed new zwitterionic HILIC columns and evaluated them. The columns showed the similar ability of separation in term of selectivity for structural difference, compared to commercially-available HILIC columns. It was suggested that higher monomer concentration at the stage of SI-ATRP functionalization method, there is a possibility of achieving better structural selectivity than those of commercially-available HILIC columns.

For the comprehensive exploration for a desirable column performance in HILIC, we will examine a correlation between column efficiency (plate height) and the immobilization amount of polymer (carbon content), and optimization for the packing conditions. For the analysis of nucleotides, columns packed in an inert-column hardware will also be investigated.

5. References

[1] Y. Kawachi, *et al., J. Chromatogr. A*, 2011, 1218, 5903–5919. [2] D. Garcia-Gomez, *et al., Trends Anal. Chem*., 2013, 47, 111–128.
[3] S. Arase, *et al., J. Pharm. Biomed. Anal*., 2018, 158, 307–316. [4] T. Ikegami, *et al., J. Chromatogr. A*, 2021, 1638, 461850.

6. Acknowledgments

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