Electronic Supplementary information (ESI)

High-Performance Cation Electrokinetic Concentrator Based on γ-CD/QCS/PVA Composite and Microchip for Evaluating the Activity of P-glycoprotein with Interference-Free from Serum Albumin

Runhui Zhang[1#], Jun Xu[1#], Jieqi Deng[1], Wei Ouyang[2], Hanren Chen[1], Qing Tang[1], Shiquan Zheng[1], Lihong Liu[1]*

[1]NMPA Key Laboratory for Research and Evaluation of Drug Metabolism, Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, 510515, China

[2]Querrey Simpson Institute for Bioelectronics, Northwestern University, Evanston, IL 60208, United States

[#] These authors contributed equally to this work.

*Corresponding Author

NMPA Key Laboratory for Research and Evaluation of Drug Metabolism, Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, 510515, China; orcid.org/0000-0003-3631-2148;

Email: lhliu@smu.edu.cn (L. Liu)

Contents

Materials and reagents

Chitosan (CS) and polyvinyl alcohol (PVA) were purchased from China National Pharmaceutical Group Corporation (Beijing, China) and Sangon Biotech Co., Ltd. (Shanghai, China), respectively. γ-cyclodextrin (γ-CD), glutaraldehyde (GA), melamine (MA), 2,3-epoxy propyl trimethyl ammonium chloride (GTAC), verapamil (VER) and 18 α - glycyrrhetinic acid (18 α - GA) were provided by Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Phosphate buffered saline (PBS, pH=7.4) was supplied by Guangzhou Alexan Biotech company (Guangzhou, China). Platinum wire electrode was purchased from Xiya Reagents (Shandong, China). Poly dimethyl diallyl ammonium chloride (PDADMAC) was supplied by Sigma-Aldrich (Shanghai, China). Sylgrd 184 silicone elastomer polydimethylsiloxane (PDMS) was provided by Dow Corning (Midland, MI, USA). Rhodamine 123 (Rho123) was purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Fluorescence was recorded using an inverted fluorescence microscope (Leica DMIL LED, Ernst & Company, Vetzlar, Germany) equipped with a CCD camera (Leica DFC 360 FX) and quantified by the free software ImageJ. A DC power supply (MP3001D, Maisheng, Dongguan, China) was used to supply DC voltages for on-line electrokinetic concentration. The physical structure of AEMs were characterized using TEM (Hitach. H, Tokyo, Japan). The chemical structures of CS, QCS and AEMs were confirmed by FTIR (Jasco Inc., Easton, MD, USA).

The Caco-2 cells were supplied by the Chinese Academy of Sciences and cultured in Minimum Essential Medium (MEM) (Procell Life Science & Technology Co. Ltd., Wuhan, China) containing 10% fetal bovine serum (FBS) (Excellbio Biological Products Co. Ltd., Shanghai, China) and 1% penicillin and streptomycin (P/S) (Gibco, Thermo Fisher Scientific, Shanghai, China) at 37 °C under a humidified 5% CO_2 conventional conditions in a cell incubator (Yiheng Scientific Instrument Co. Ltd., Shanghai, China). A 24-well Transwell chamber (Corning, USA) was used to seed Caco-2 cells. The cell viability

of Caco-2 cells was detected by CCK-8 kit (Sigma, USA) and using a SpectraMax i3x Multi-Mode microplate reader (Molecular Devices, USA) to detect the optical density.

Experimental Section

1. Modification of the channel walls with PDDAC

The microfluidic chip was loaded with 0.1% PDDAC solution and equilibrated at room temperature for 5 min, then the channels were thoroughly rinsed with water and used immediately without further treatment. PDDAC forms an electrostatic adsorption monolayer on the microchannel walls, resulting in a positive net charge on the microchannel walls and a reverse EOF in the microchannel under electric field.

2. Establishment of Caco-2 Cell Model

A 24-well Transwell chamber was used to seed 2×10^5 Caco-2 cells and supplied with MEM (containing 12% FBS and 1% P/S) for Transwell cell culture, which added 600 μl medium in basolateral (BL) side and 300 μl medium in apical (AP) side. The medium was changed every 2 days in the first week, and changed once daily in the rest experimental days. When the cells were cultured in the Transwell chamber to the tenth day, the trans-epithelial electrical resistance (TEER) was measured every 3 days to determine the growth of Caco-2 cells. Figure S9b shows that after 21 days of growing on Transwell inserts, the Caco-2 cells had formed an intact and confluent epithelial cell layer that can be used for further studies

3. Determination of Cell viability

VER was selected as a P-gp expression inhibitor in Caco-2 cell model, and 18α-GA was used as a P-gp expression agonist in CacO-2 cell model. Next, we investigated the cytotoxicity of the two drugs as follows.

Caco-2 cells were seeded in a 96-well plate at a density of 5,000 cells per well and incubated for 24 h, then the cells were incubated with different concentrations of 18α-GA (10 μM, 50 μM, 100 μM) and VER (10 μM, 50 μM, 100 μM) in combination with Rho 123 (10 μM) for 12 h, respectively. The viability of Caco-2 cells was determined by the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instruction. Briefly, the cultured cells were rinsed with 200 μl PBS/well three times, and added 10 μl CCK-8 solution to each well, then optical density was detected at 450 nm using a SpectraMax i3x Multi-Mode microplate reader after another 2 h incubation.

4. Establishment of standard curves for Rho123

The fluorescence intensity of a series of Rho123 gradient concentration of standard solution (10-3000 pM) was measured using CEC, Image J was used to process the fluorescence intensity (y) of all the Image fluorescence bands, and the standard curve of fluorescence intensity (y) versus concentration (x) was described.

5. Statistical analysis

The fluorescence intensity of the samples obtained from our enrichment platform at a certain concentration dilution in bidirectional transport experiment was taken into the standard curve, and the concentration at each time point was calculated. The apparent permeability coefficients (Papp) were calculated in bidirectional transport experiments according to the following equation (2):

$$Papp = (dQ/dt)/\left(1/(A \times C_0)\right) \quad \textbf{(1)}$$

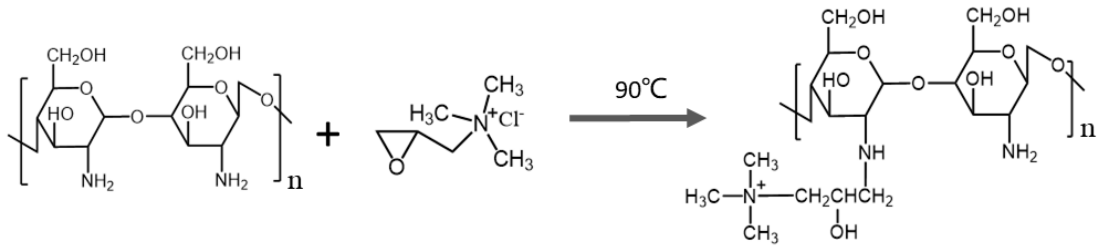
Where dQ/dt (μmol/s) is the slope of the cumulative amount transported in the receiving chamber, C_0 (μmol/L) is the initial concentration of Rho123, and A (0.33cm^2) is the surface area of the cell monolayer.

Efflux ratios (ER) were calculated according to the following equation (3):

$$ER = Papp_{BL-AP}/Papp_{AP-BL} \quad \textbf{(2)}$$

Pharmacokinetic parameters, including the area under the plasma concentration-time curve (AUC), maximal plasma concentration (C_{max}) etc. Pharmacokinetic parameters were calculated using the DAS 3.0 pharmacokinetic software (Chinese Pharmacological Association, Anhui, China).

Data were presented as mean and standard deviation, and the data analysis was performed using SPSS19.0 package (IBM SPSS Inc, Chicago, IL). The differences between the mean values were analyzed for significance using a one-way analysis of variance (ANOVA). In all statistical analyses, values of $P < 0.05$ were considered to indicate statistically significant.



Scheme S1. Schematic diagram of quaternized chitosan synthesis

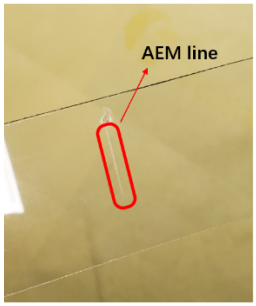


Fig. S1 AEM line on the glass substrate after the PDMS mold was removed.

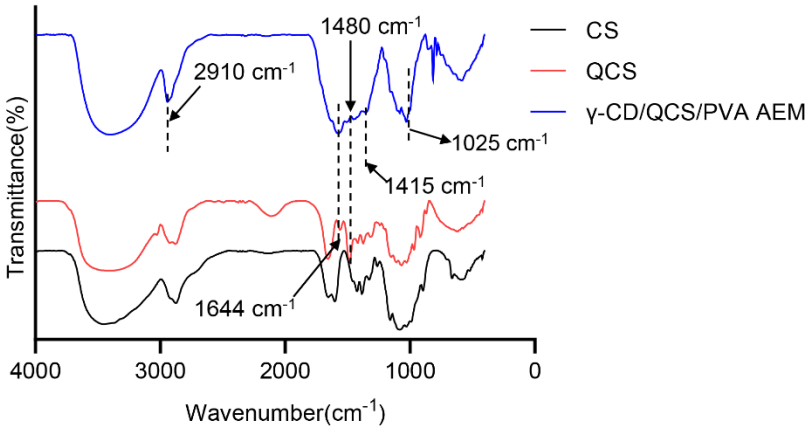
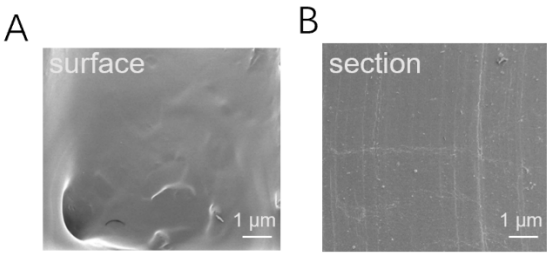


Fig. S2 FTIR spectra of CS, QCS,and γ-CD/QCS/PVA AEM. In FTIR, the absorption peak at 1480 cm^{-1} was attributed to the bending vibration of C-H in the quaternary amine group. A characteristic peak appeared at 1644 cm^{-1}, which successfully proved that the primary amine of chitosan backbone had been changed into a secondary amine structure. The appearance of the characteristic C-O-C peak at 1025 cm^{-1} can be seen after the addition of γ-CD. In addition, in FTIR of γ-CD/QCS/PVA AEM, the C-O stretching vibration peak at 1415 cm^{-1} was formed by the acetal reaction between -OH and –CHO, and the -CH stretching vibration peak of the aldehyde group appeared at 2910 cm^{-1}, which indicated that the cross-linking reaction occurred in the AEM interior.

149

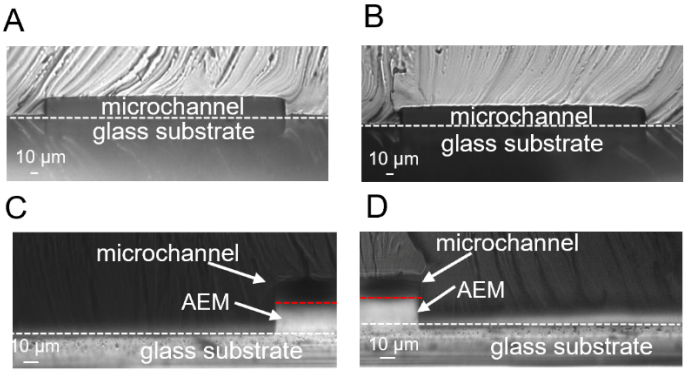


150

151 **Fig. S3** SEM of the AEM composite in hydrated state. A: SEM of surface; B: SEM of section.

152

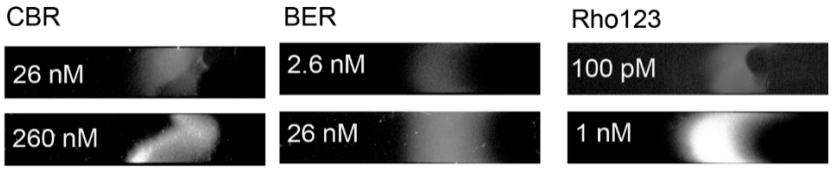
153



154

155 **Fig. S4** Images around the AFM membrane in the channel. (A) Front. (B) Back. (C) Left. (B) Right.

156



157

158 **Fig. S5** Stacking band of different cationic analyte, including Cationic Brilliant Red (CBR),
159 Berberine (BER) and Rho 123. Conditions: 0.1% PDDAC on the inner surface of the microchannel,
160 γ-CD/PVA/QCS AEM (3% PVA, 1%QCS, 2% GA, 2% MA and 1.5% γ-CD); 1 nM Rho 123; buffer
161 0.5 × PBS (pH 3.5); AEM microchannel dimension: 200 μm width and 45 μm depth; 150 V voltage.

162

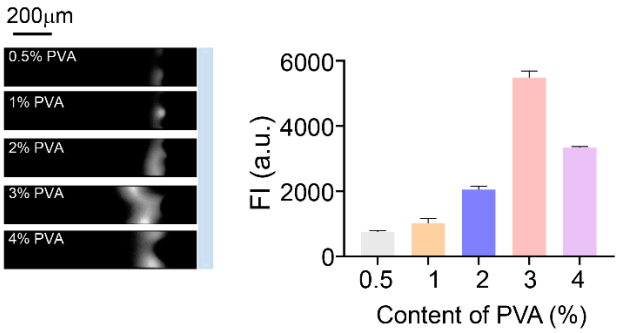
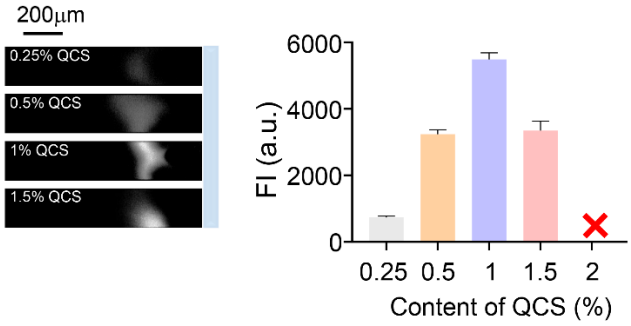


Fig. S6 Effect of the content of PVA on electrokinetic concentration performance of the system. Conditions: AEM (1%QCS, 3% PVA, 2% GA, 2% MA), other conditions as in **Fig. S5**.



168

169 **Fig. S7** Effect of the contents of QCS on electrokinetic concentration performance of the system.

170 Conditions: AEM (3% PVA, 2% GA, 2% MA), other conditions as in **Fig. S5**.

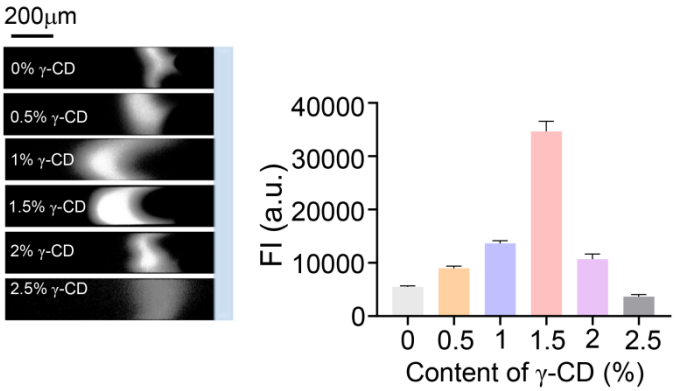
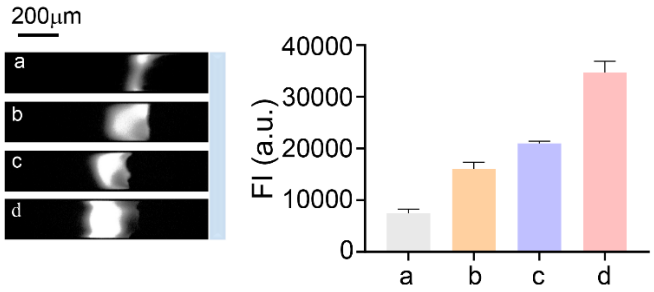


Fig. S8 Effects of different contents of γ -CD on electrokinetic concentration of CEC. Conditions: AEM (3% PVA, 1% QCS, 2% GA and 2% MA), other conditions as in **Fig. S5**.



175

176 **Fig. S9** Effects of different types of CD on electrokinetic concentration of CEC. (a: QCS/PVA
177 AEM, b: α-CD/QCS-PVA AEM, c: β-CD/QCS-PVA AEM, d: γ-CD/QCS-PVA AEM). Other
178 conditions as in **Fig. S5**.

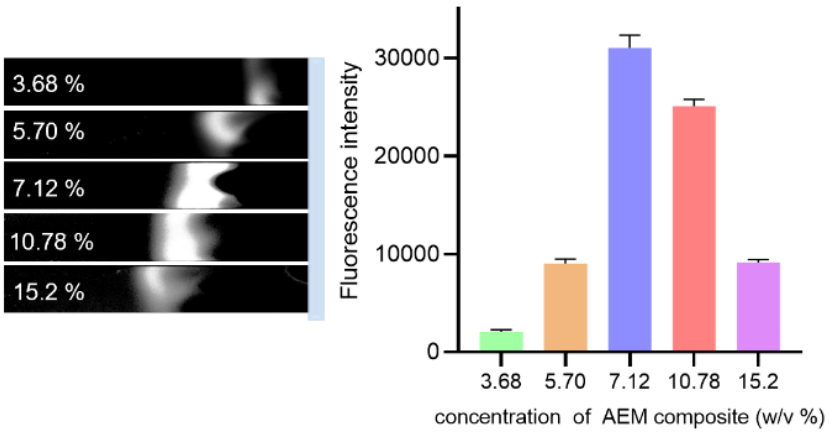


Fig. S10 Effect of the different concentration of the γ-CD/QCS/PVA composite on CEC enrichment performance**.** Conditions: 0.1% PDDAC on the inner surface of the microchannel, γ-CD/PVA/QCS AEM (PVA : QCS : GA : MA : γ-CD = 3:1:2:2:1.5); 1 nM Rho 123; buffer 0.5 × PBS (pH 3.5); AEM microchannel dimension: 200 μm width and 45 μm depth; 150 V voltage.

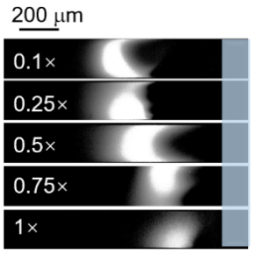
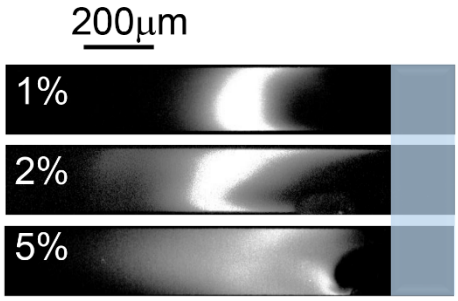


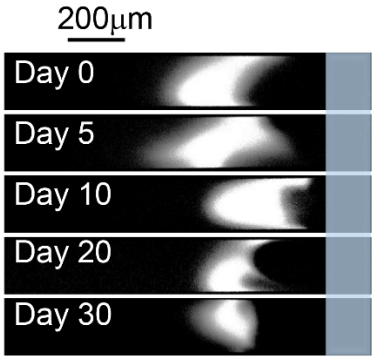
Fig. S11 Effects of PBS concentrations on fluorescent bands of 1 nM Rho 123.



190

191 **Fig. S12** Effects of serum concentrations on fluorescent bands.of 1 nM Rho 123.

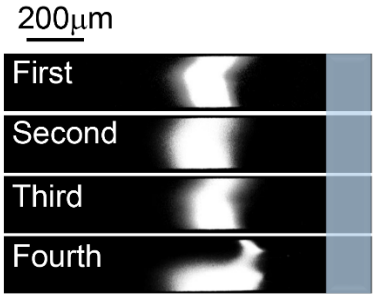
192



193

194 **Fig. S13** Effects of different storing time of γ-CD/QCS/PVA composite on fluorescent bands of 1
195 nM Rho 123.

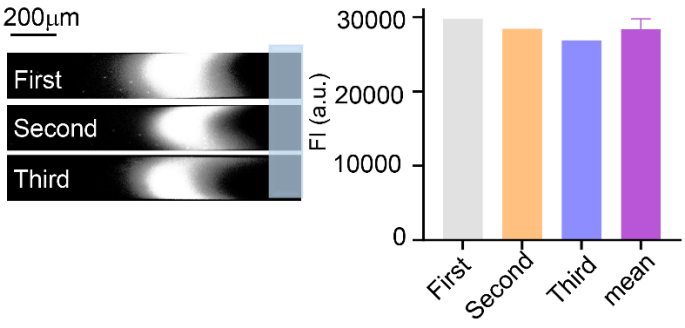
196



197

198 **Fig. S14** Reusability of one CEC-Y. The buffer was 0.5 ×PBS, the sample was 1 nM Rho 123.

199



200

201 **Fig. S15** Reusability of one CEC-Y on Rho 123 (1 nM) in 1% serum sample.

202

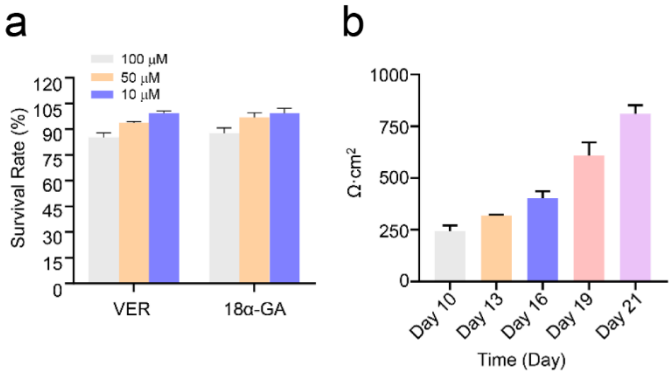


Fig. S16 The cytotoxicity of VER and 18α-GA on Caco-2 was investigated by CCK-8 (a). The relationship between the transmembrane resistance of Caco -2 monolayer cells and the growth time (b)

Table S1. Comparison of previously reported electrokinetic concentrators with our work

Nanochannel permselectivity	Nanochannel fabrication method	Permselective material	Enrichment factor	Ref.
Anion permselective	AEM solution patterning	γ-CD/PVA/QCS AEM	120000	This work
	Salt leaching technique	PPO[a)] AEM	500	[1]
	Cationic gel photopolymerization	Cationic polyacrylamide gel	6400	[2]
	Lithography and cationic polymer coating	TMSVE[d)]	500	[3]

a) Poly (2,6-dimethyl 1,4-phenylene) oxide; b) Fluorescein isothiocyanate; c) Tris(bipyridine) ruthenium (II) chloride; d) N-[3- (trimethoxysilyl)propyl]-N′-(4-vinylbenzyl) ethylenediamine hydrochloride; e) C-reactive protein; f) Bovine serum albumin

Table S2. Ranges for the examined parameters and the selected optimal mass proportion of each component of AEM based fluorescence intensity.

Parameters	Ranges (%)	Selected values (%)
PVA	0.5 − 4	3
QCS	0.25 − 2	1
γ -CD	0 − 2.5	1.5

Table S3. Comparison of the methods on the analysis of Rho 123

Methods	LOD	Ref
HPLC	105 pM (40 pg/mL)	[4]
CE-LIF	50 pM	[5]
MEKC-LIF	263 pM (100 pg/mL)	[6]
LC-ESI-MS	2.6 nM (1 ng/mL)	[7]
CEC	2.6 pM	This work

HPLC: high performance liquid chromatography; CE-LIF: capillary electrophoresis-laser-induced fluorescence; MEKC-LIF: micellar electrokinetic chromatograph-laser-induced fluorescence; LC-ESI-MS: Liquid chromatography - electrosprayionization mass spectrometry.

Table S4. Spiked results of Rho123 in 1% serum and 0.3% Cell culture medium sample

Sample	Spiked (pM)	Found (pM)	Recovery (%)	Average (%)	RSD (%)
Serum	50	56	112.0	104.7	3.7
	500	481	96.2		2.9
	2000	2174	108.7		2.7
Cell culture medium	50	55	110.7	103.5	5.80
	500	512	102.4		5.60
	2000	1946	97.3		4.10

226

227 **Table S5.** The approximate material cost of the CEC

	Description	Supplier	Order unit		CEC	
			Quantity	Price	Quantity	Material Cost
γ-CD/PVA/QCS composite	PVA	Shanghai Aladdin Biochemical Technology Co., Ltd., China	1 Kg	￥203	0.039mg	￥0.0000079
	CS	China National Pharmaceutical Group Co., Ltd., China	500 g	￥252	0.007 mg	￥0.0000035
	GTA	Shanghai Macklin Biochemical Co., Ltd., China	250 g	￥116	0.007 mg	￥0.0000016
	GA	Shanghai Macklin Biochemical Co., Ltd., China	500 mL	￥47	0.052 ul	￥0.0000030
	MA	Shanghai Macklin Biochemical Co., Ltd., China	500 g	￥73	0.026 mg	￥0.0000038
	γ-CD	Shanghai Macklin Biochemical Co., Ltd., China	5g	￥ 184	0.02 mg	￥0.000736
Chip	glass slide	Yancheng Feizhou Glass Co., LTD, China	50 pieces	￥8	1 piece	￥0.16
	Sylgard 184	DOW SILICONES CORPORATION, USA	19.9 Kg	￥13500	2 g	￥1.36
CEC						**￥1.52**

229 **Table S6.** Pharmacokinetic parameters of Rho123 in rats after intragastrical administration of
230 Rho123 (5 mg/kg; n= 3, mean ± SD) with or without treatment of VER.

Parameters	Negative control group	Verapamil 5 mg/kg	P value
$AUC_{(0-t)}$ (μg/L h)	38.41±4.16	104.29±5.14	0.00002
$AUC_{(0-\infty)}$ (μg/L h)	57.15±9.80	129.45±25.18	0.027
CLz(L/h/kg)	87.48±11.07	39.69±6.90	0.012
Vz (L/kg)	2008.32±1040.21	583.96±273.20	0.047
C_{max} (μg/L)	8.46±1.80	20.85±1.62	0.002

231
232

233 **Table S7 List of Abbreviations**

234

Abbreviations	Difinition
AEM	anion-exchange membranes
CEC	cation electrokinetic concentrator
AP-BL	Apical-to-basolateral
BER	Berberine
BL-AP	Basolateral-to-apical
CBR	Cationic Brilliant Red
CE	Cationic electrophoresis
CEM	Cation exchange membranes
CS	Chitosan
EF	Enrichment factor
EOF	Electroosmotic flow
ER	Efflux ratio
GA	Glutaraldehyde
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
ICP	Ion concentration polarization
IDZ	Ion depletion zone
MA	Melamine
MDR1	Multidrug resistance protein 1
MS	Mass spectrometry
PDDAC	Poly dimethyl diallyl ammonium chloride
P_{app}	Apparent permeability coefficient
P-gp	P-glycoprotein
PVA	Polyvinyl alcohol
QCS	Quaternized chitosan
Rho 123	Rhodamine 123
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
VER	Verapamil
18α-GA	18α-glycyrrhetinic acid
γ-CD	γ-cyclodextrin

References

[1] M. Lee, H.J. Kwon, G. Lim, A Multiscale-Porous Anion Exchange Membrane for Convenient and Scalable Electrokinetic Concentration of Cationic Species, Adv Funct Mater 31 (2021).

[2] S. Yamamoto, F. Okada, M. Kinoshita, et al., On-line microchip electrophoresis-mediated preconcentration of cationic compounds utilizing cationic polyacrylamide gels fabricated by in situ photopolymerization, Analyst 143 (2018) 4429-4435.

[3] I.H. Shin, K.J. Kim, J. Kim, et al., Cation-selective electropreconcentration, Lab Chip 14 (2014) 1811-1815.

[4] N. Hashimoto, N. Nakamichi, S. Uwafuji, et al., ATP binding cassette transporters in two distinct compartments of the skin contribute to transdermal absorption of a typical substrate, Journal of Controlled Release 165 (2013) 54-61.

[5] E. Yu, E. Ban, M.K. In, et al., Development for the analysis of reactive oxygen species using capillary electrophoresis with laser-induced fluorescence detection, Journal of Microcolumn Separations 13 (2001) 327-331.

[6] R. Li, R.a. Wu, M. Wu, et al., MEKC-LIF analysis of rhodamine123 delivered by carbon nanotubes in K562 cells, Electrophoresis 30 (2009) 1906-1912.

[7] L. Xiu, M. Yuan, Y. Sun, et al., Jiangxi Journal of Chinese Traditional Medicine 45 (2014) 11-13.