รายงานวิจัยฉบับสมบูรณ์

การเตรียมตัวอย่างโดยใช้เทคนิคทางการวิเคราะห์ที่ใช้การไหลโดยเกี่ยวข้องกับอนุภาค

Sample Pretreatment Using Flow-Based Analytical Techniques Involving Particles

โดย ดร. รัตติกาล จันทิวาสน์

มิถุนายน 2548

สัญญาเลขที่ TRG4680025

รายงานวิจัยฉบับสมบูรณ์

คณะผู้วิจัย **สังกัด**

ดร. รัตติกาล จันทิวาสน์

สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยเชียงใหม่

รศ. ดร. เกตุ กรุดพันธ์ สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี และ
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มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

โครงการวิจัยนี้สำเร็จลุล่วงไปด้วยการสนับสนุนหลักจาก สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และหน่วยงานที่ให้ความอนุเคราะห์สถานที่ในการดำเนินการวิจัยคือ ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ โดยการนำเสนอผลงานวิจัยบางส่วนยังได้รับการสนับสนุนให้ไปเสนอผลงานในการประชุมนานาชาติ จากโครงการส่งเสริมการเพิ่มสมรรถนะและขีดความสามารถในการแข่งขันของประเทศ “ในโครงการการพัฒนาเครื่องมือและวิธีสำหรับวิทยาศาสตร์การสังเคราะห์ในระดับไมโครและนาโนเพื่อใช้ในงานทางสุขภาพและสิ่งแวดล้อม” ของสำนักงานคณะกรรมการอุดมศึกษา (สกอ.)

ขอขอบคุณการสนับสนุนจากห้องปฏิบัติการวิจัยเพื่อการพัฒนาเครื่องมือวิเคราะห์ สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี น้องๆ นักศึกษาบัณฑิต และนักวิจัยพี่เลี้ยง/หัวหน้าหน่วยวิจัยฯ คือ รองศาสตราจารย์ ดร. เกตุ กรุดพันธ์ ที่ได้ให้โอกาสการเรียนรู้ และข้อคิดต่างๆ ในการทำการวิจัย อีกทั้งประสบการณ์ที่สำคัญในการทำงาน และขอขอบคุณ Dr. Ronald Beckett แห่ง Water Studies Centre, Monash University, Melbourne, Australia ที่ได้ให้การสนับสนุนการเขียนบทความเพื่อตีพิมพ์ ขอขอบคุณรองศาสตราจารย์ ดร. ปรัญญา คงทวีเลิศ จาก ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ขอขอบคุณผู้อำนวยการสถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี และหน่วยการเงินและการบัญชีจากสถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยเชียงใหม่

จากการวิจัยในโครงการนี้หวังว่าจะเกิดประโยชน์แก่ผู้ที่สนใจและบางส่วนอาจสามารถนำไปพัฒนาต่อยอดและให้เกิดคุณประโยชน์ต่อไปในอนาคต

ดร. รัตติกาล จันทิวาสน์
หัวหน้าโครงการ

บทคัดย่อ

รหัสโครงการ: TRG4680025

ชื่อโครงการ: การเตรียมตัวอย่างโดยใช้เทคนิคทางการวิเคราะห์ที่ใช้การไหลโดยเกี่ยวข้องกับอนุภาค

Investigator: **ดร. รัตติกาล จันทิวาสน์** สังกัดสถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยเชียงใหม่ และ **รองศาสตราจารย์ ดร. เกตุ กรุดพันธ์** สังกัดสถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยีและภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

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ระยะเวลาโครงการ: 2 ปี (1 มิถุนายน 2546-30 มิถุนายน 2548)

งานวิจัยนี้เป็นการพัฒนาเทคนิคที่เกี่ยวข้องกับการไหลสำหรับการเตรียมตัวอย่างทางสิ่งแวดล้อมและทางชีวภาพ โดยพัฒนาเทคนิคที่เกี่ยวกับการไหล 2 เทคนิคคือเทคนิคฟิลด์โฟลแฟรกชันเนชันและเทคนิคซีเควนเชียนอินเจคชันอะนาลิซิส โดยระบบแรกเป็นการรวมเทคนิคฟิลด์โฟลแฟรกชันเนชันแบบแรงโน้มถ่วงกับการตรวจวัดของอิเลคโทรเทอร์มอลอะตอมมิกแอปซอฟชันสเปคโตรเมตรีสำหรับการหาปริมาณเหล็กในตัวอย่างดินเคลย์ที่มีขนาดอนุภาคช่วง 5-20 ไมโครมิเตอร์ โดยจากการศึกษาพบว่าได้รับข้อมูลในการกระจายของเหล็กในอนุภาคตัวอย่าง ระบบที่สองเป็นการพัฒนาระบบซีเควนเชียนอินเจคชันอะนาลิซิส-แลปแอทวาล์วบีดอิมมูโนแอสเส โดยระบบนี้เป็นการใช้บีด และใช้สารตัวอย่างและสารละลายในระดับไมโครลิตร ซึ่งได้นำระบบนี้มาทดสอบสมรรถนะกับสารสำคัญตัวหนึ่งที่ใช้เป็นตัวบ่งชี้โรคตัวคือคอนดรอยติน 6-ซัลเฟตและชาร์คเอ-1 ในตัวอย่างซีรัมคนปกติ

Keywords: gravitational field-flow fractionation, size-based speciation, sequential injection analysis, lab-at-valve, bead immunoassay

Abstract

Project code: TRG4680025

Project Title: Sample Pretreatment Using Flow-Based Analytical Techniques Involving Particles

Project: Title: Sample Pretreatment Using Flow-Based Analytical Techniques Involving Particles

Investigator: **Dr. Rattikan Chantiwas** (Institute for Science and Technology Research and Development, Chiang Mai University) and **Assoc. Prof. Dr. Kate Grudpan** (Institute for Science and Technology Research and Development and Department of Chemistry, Faculty of Science, Chiang Mai University)

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Project period: 2 Years (1 July 2003 - 30 June 2005)

This work introduces a development of flow-based analysis techniques for sample pretreatment of environmental and biological samples. Two main flow-based techniques, i.e. field-flow fractionation and sequential injection analysis, were developed. The first system, gravitational field-flow fractionation (GrFFF) coupled to UV and ETAAS detector was used for the investigation of micronsize Fe-rich clay samples in the range of 5-20 μm. The size-distribution information of particulate samples was provided by using the size-based speciation, GrFFF technique. The second system was the sequential injection-lab-at-valve (SI-LAV) bead immunoassay system. The SI-LAV system provides microfluidic handling ability to manipulate the bead, sample and reagents required to perform an immunoassay. The performance of the system was demonstrated using a potential biomarker, chondroitin 6-sulfate (C6S) and Shark A1 (Sh-A1) in a normal serum.

Keywords: gravitational field-flow fractionation, size-based speciation, sequential injection analysis, lab-at-valve, bead immunoassay

โครงการ: การเตรียมตัวอย่างโดยใช้เทคนิคทางการวิเคราะห์ที่ใช้การไหล โดยเกี่ยวข้องกับอนุภาค

1. ความสำคัญและที่มาของปัญหา

การเตรียมตัวอย่างก่อนการวิเคราะห์ในการวิเคราะห์ทางเคมีเป็นขั้นตอนที่มีความสำคัญ เช่น วิธีการเตรียมตัวอย่างให้เหมาะสมเพื่อเพิ่มความเข้มข้น (preoncentration) หรือลดความเข้มข้น (predilution) และอาจรวมถึงการแยก (preseparation) สารที่ต้องการวิเคราะห์ (analyte) ออกจากสารอื่นที่ประกอบอยู่ในสารตัวอย่าง (matrix) เพื่อให้เหมาะสมกับสภาวะในขั้นตอนการหาปริมาณ ในงานวิจัยนี้มีความสนใจที่จะพัฒนาระบบการวิเคราะห์เพื่อใช้สำหรับการเตรียมตัวอย่างก่อนการวิเคราะห์ที่ใช้เทคนิคการวิเคราะห์ที่เกี่ยวข้องกับการไหล (Flow-based analysis) ที่เกี่ยวข้องกับอนุภาค (micro particle/bead) โดยเทคนิคที่ได้มีการพัฒนาอย่างต่อเนื่องและมีประสิทธิภาพในการวิเคราะห์ที่นำมาพัฒนา 2 เทคนิคในงานวิจัยโครงการนี้ได้แก่ เทคนิค Gravitational field-flow fractionation (GrFFF) และ Sequential injection (SI)-bead injection (BI) analysis เพื่อใช้กับตัวอย่างทางสิ่งแวดล้อมและตัวอย่างทางชีวภาพ ตามลำดับ

เทคนิค GrFFF เป็นเทคนิคที่มีสามารถให้ข้อมูลในการแยกและบ่งบอกถึงลักษณะของสาร (separation and characterization) การแยกของกลุ่มอนุภาค โดยใช้ external field ที่เป็น gravitational field ร่วมกับการไหลที่เป็นลามินา (laminar flow) ใน thin channel cell โดยแยกอนุภาค (micro particle) ที่มีขนาดในช่วงไมโครมิเตอร์ โดยได้มีการพัฒนาเพื่อแยกอนุภาคก่อนการทำการวิเคราะห์ปริมาณเหล็กของกลุ่มอนุภาคเพื่อศึกษา size-based speciation โดยรวมเทคนิค GrFFF เพื่อแยกกลุ่มขนาดอนุภาค กับเทคนิค Electrothermal Atomic Absorption Spectrophotometry (ETAAS) เพื่อการหาปริมาณเหล็ก โดยการรวม GrFFF- ETAAS สามารถทำการทดลองได้ทั้งแบบ Online และ แบบ Offline โดยการทดลองแบบ Online ต้องใช้ร่วมกับ laboratory designed flow cell โดยข้อมูลที่ได้รับจากการทำ size-based speciation ของเหล็กในตัวอย่างดินเคลย์ ซึ่งสามารถนำไปใช้ประโยชน์ในเงานทางสิ่งแวดล้อมได้ (ดังรายละเอียดจาก reprint **ภาคผนวก ก1**)

Sequential injection (SI)-bead injection (BI) analysis เป็นเทคนิคของระบบการไหลที่มีการใช้กับสามารถนำมาใช้วิเคราะห์ analyte ที่สนใจร่วมกับการไหลของอนุภาคที่เป็น micro bead ได้ โดยทั่วไปงานวิจัยที่มีการใช้หลักการ BI จะต้องมีอุปกรณ์หรือเครื่องมือของ SI ที่เป็น Lab-on-valve หรือ Jet ring cell แต่ในงานวิจัยนี้ได้พยายามพัฒนาให้ได้ระบบ BI โดยใช้อุปกรณ์ของ SI ซึ่งมีในห้องปฏิบัติการและออกแบบ flow cell เพื่อนำมาพัฒนาให้ได้ระบบ sequential injection-lab-at-valve (SI-LAV) และสามารภนำไปใช้ได้ โดยนำมาทดสอบสมรรถนะของระบบ

กับสารที่ใช้เป็นตัวบ่งชี้โรคตัวคือ Chondroitin sulfate และ Shark A1 โดยได้ทดลองในตัวอย่างซีรัมคนปกติ (ดังรายละเอียดจาก manuscript drafted **ภาคผนวก ก2**)

2. วัตถุประสงค์

เพื่อพัฒนาระบบและวิธีสำหรับการเตรียมตัวอย่างในการวิเคราะห์โดยใช้เทคนิค Sequential injection-bead injection analysis และ Gravitational field-flow fractionation เพื่อการประยุกต์ทางตัวอย่างทางชีวภาพ และตัวอย่างสิ่งแวดล้อมตามลำดับ

3. วิธีทดลอง

ระบบที่ 1 Gravitational-field flow fractionation-electrothermal atomic absorption spectrometer (GrFFF-ETAAS)

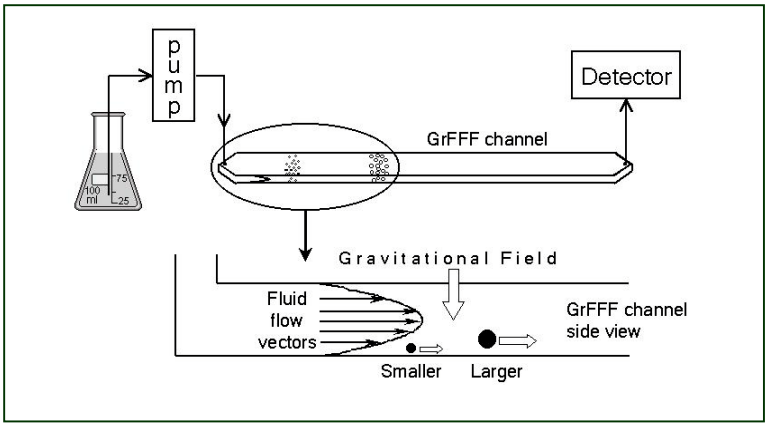

Figure 1 GrFFF instrument and steric/hyperlayer separation mechanism for micron-size of particles

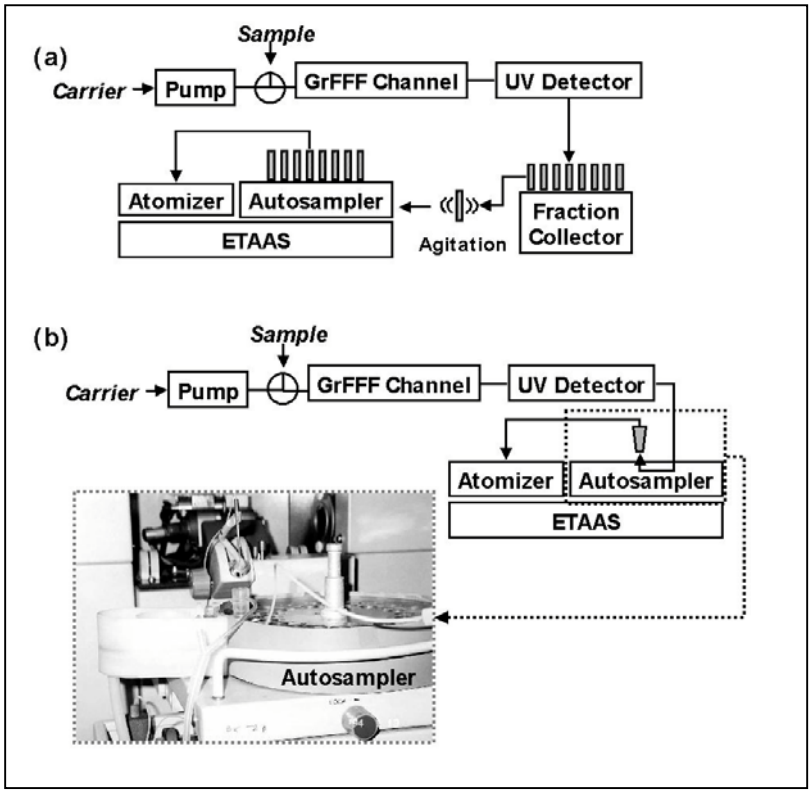

Figure 2 Instrumental set up for GrFFF coupled with ETAAS: (a) off-line GrFFF-ETAAS, (b) on-line GrFFF-ETAAS showing the flow through sampler vial

ระบบที่2 SI-LAV for bead immunoassay system

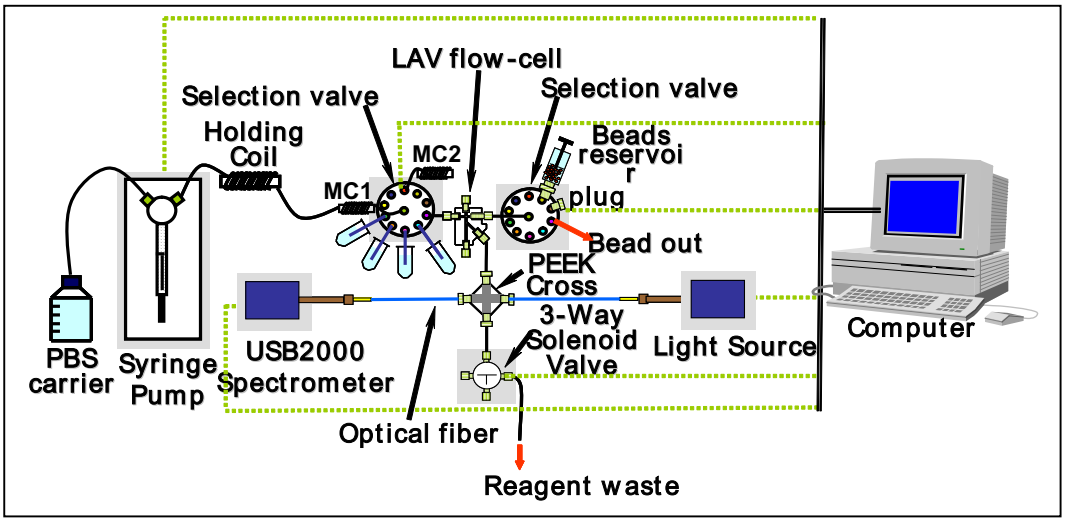

Figure 3 Schematic diagram of the SI-LAV system

4. ผลการทดลอง

ระบบที่ 1 ดังเอกสาร จาก reprint ในภาคผนวก ก1

ระบบที่ 2 ดังเอกสาร จาก manuscript drafted ในภาคผนวก ก2

5. สรุปและวิจารณ์ผลการทดลองโดยสังเขป

ระบบที่ 1

พัฒนาระบบ GrFFF-ETAAS สำหรับตัวอย่างดินเคลย์ที่มีขนาดอนุภาคช่วง 5-20 ไมโครมิเตอร์ โดยการตรวจวัดด้วย ETAAS สามารถทำได้ทั้งแบบ offline และแบบ online โดยการทำแบบ online จะใช้ร่วมกับ Flow cell ที่ออกแบบขึ้นเองในห้องปฏิบัติการและวางในตำแหน่งหนึ่งของ autosampler tray ของ ETAAS โดยได้เปรียบการผลการทดลองการหาปริมาณเหล็กของการทำ slurry injection ของ particles suspension ของวิธีการทดลองแบบ offline, online และได้ทำการทดลองหาปริมาณเหล็กรวมในตัวอย่างดินเคลย์โดยการย่อยด้วยกรดพบว่ามีค่าประมาณ 20-40 mg/kg เมื่อนำผลการหาปริมาณเหล็กมาเปรียบเทียบผลของการทดลองแบบ offline, online พบว่าโดยภาพรวมได้เปอร์เซนต์ recovery มากกว่า 90 โดยจากข้อมูลการรวมเทคนิค GrFFF-ETAAS สามารถทราบปริมาณเหล็กในแต่ละช่วงของขนาดอนุภาค (size-based speciation) โดยพบว่าความเข้มข้นของเหล็กลดลงเมื่อขนาดของอนุภาคใหญ่ขึ้น ดังเช่นที่สามารถพบได้โดยทั่วไป

ระบบที่ 2

การพัฒนาระบบ Bead immunoassay โดยใช้เทคนิค Sequential injection-lab-at-valve (SI-LAV) ซึ่งเป็นระบบที่สามารถวิเคราะห์วิเคราะห์ได้รวดเร็วและเป็นระบบอัตโนมัติและวิเคราะห์ได้แบบ real time โดยได้ประยุกต์เพื่อใช้กับการวิเคราะห์ chondroitin 6-sulfate และ shark-A1 ซึ่งเป็น biomarker ที่สำคัญสำหรับโรคข้อเสื่อม

6. ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

ในส่วนของการทดลองในระบบที่ 2 มีความน่าสนใจที่จะนำระบบไปใช้กับตัวอย่างที่หลากหลาย เช่นทำการทดลองซีรั่มในผู้ป่วยที่เป็นโรค หรืออาจประยุกต์กับตัวอย่างชนิดอื่นๆ เช่นตัวอย่างปัสสาวะ หรือน้ำลาย และอาจนำไปทดลองกับการวิเคราะห์ biomarker ตัวอื่นๆ เป็นต้น

7. หนังสืออ้างอิง

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Output จากโครงการที่ได้รับทุนจาก สกว.

ก. ผลงานตีพิมพ์ในวารสารนานาชาติ

1 R. Chantiwas, R. Beckett and K. Grudpan. *Size-based Speciation of Iron in Clay Mineral Particles by Gravitational Field-Flow Fractionation and Electrothermal Atomic Absorption Spectrometry*. Spectrochimica Acta Part B 60 **(2005)** 109-116. (impact factor 2.361) **(ภาคผนวก ก1)**

2 R. Chantiwas, P. Kongtawelert, S. Kradtap, K. Grudpan, *Development of Sequential Injection Lab-at-Valve-Bead Immunoassay System for Chondroitin Sulfate and Condroitin Sulfate Epitopes*, (manuscript in preparation) **(ภาคผนวก ก2)**

ข. ผลงานที่เสนอในที่ประชุมวิชาการระดับนานาชาติ

1. **R. Chantiwas**, P. Kongtawelert, S. Kradtap, J. Jakmunee, K. Grudpan*, The Flow-Microparticles Based Immunoassay System*, Poster Presentation. 15th International Symposium on Pharmaceutical and Biomedical Analysis (PBA 2004), 2-6 May 2004, Florence, ITALY. **(ภาคผนวก ข1)**

* *สนับสนุนโดย ทุนวิจัยโครงการส่งเสริมสมรรถนะและขีดความสามารถในการแข่งขันของประเทศ (สำนักงานคณะกรรมการอุดมศึกษา)*

2. **R. Chantiwas,** P. Kongtawelert, S. Kradtap, K. Grudpan. *Development of Sequential Injection Lab-at-Valve-Bead Immunoassay System for Chondroitin 6-Sulfate.* Poster Presentation (by KG).13th International Conference on Flow Injection Analysis (Including Related Techniques) (13th ICFIA). 24-29 April **2005**, Las Vegas, USA. **(ภาคผนวก ข2)**

3. **R. Chantiwas**, S. Suteerapataranon, H. Geckeis, R. Beckett, K. Grudpan. Gravitational Field-Flow Fractionation-Inductively Coupled Plasma Mass Spectrometry: *Exploiting Size-Based Elemental Speciation by Gravitational Field-Flow Fractionation-Coupled with Inductively Coupled Plasma Mass Spectrometry*. Poster Presentation (by RC). 2005 Asia-Pacific Winter Conference on Plasma Spectrochemistry (WC 2005). 25-30 April **2005**, Chiang Mai, THAILAND. **(ภาคผนวก ข3)**

4. **R. Chantiwas,** P. Kongtawelert , S. Kradtap, K. Grudpan, A Novel Bead Immunoassay–Sequential Injection for a Important Proteoglycan. Poster Presentation (by RC), IUPAC 2005 Innovation in Chemistry, 14-19 August 2005, Beijing, CHINA. **(ภาคผนวก ข4)**

ค. ผลงานที่เสนอในที่ประชุมวิชาการระดับชาติ

1. **R. Chantiwas**, P.Kongtawelert, S. Kradtap, J. Jakmunee and Kate Grudpan. *Development of Bead Injection System for Chondroitin 6-Sulfate Assay*. Poster Presentation. 30th Congress on Science and Technology, 19-21 October **2004**, Impact Exhibition and Convention Center, Muang Thong Thani, BKK, THAILAND. **(ภาคผนวก ค1)**

2. การนำผลงานวิจัยไปใช้ประโยชน์

งานวิจัยนี้เป็นการวิจัยที่คาดว่าจะเป็นประโยชน์ในการวิเคราะห์ทางสิ่งแวดล้อมเช่นส่วนของ ระบบ GrFFF-ETAAS สำหรับ size-based metal speciation โดยอาจเกิดประโยชน์ต่อการวิเคราะห์ข้อมูลสำหรับ fate metal transport ในงานทางสิ่งแวดล้อม และระบบ SI-LAV คาดว่าน่าจะเป็นประโยชน์หากนำไปใช้ได้จริงในการตรวจโรคหรืออย่างน้อยอาจเป็นประโยชน์ในการคัดกรองโรคสำหรับผู้ป่วย

3.อื่นๆ

จากการดำเนินโครงการวิจัยดังกล่าวได้รับการสนับสนุนเพิ่มเติมบางส่วนจากทุนเมธี-วิจัยอาวุโส โดยมี รองศาสตราจารย์ ดร. เกตุ กรุดพันธ์ เป็นหัวหน้าโครงการ นอกจากนี้ยังได้รับการสนับสนุนจากโครงการส่งเสริมการเพิ่มสมรรถนะและขีดความสามารถในการแข่งขันของประเทศ "ในโครงการการพัฒนาเครื่องมือและวิธีสำหรับวิทยาศาสตร์การสังเคราะห์ในระดับไมโครและนาโนเพื่อใช้ในงานทางสุขภาพและสิ่งแวดล้อม" ของสำนักงานคณะกรรมการอุดมศึกษา (สกอ.) เพื่อเสนอผลงานวิจัยที่ประเทศอิตาลี **(ภาคผนวก ข1)** รวมทั้งได้รับการสนับสนุนให้ไปปฏิบัติงานวิจัยระยะสั้น; Visiting Scholar ที่ University of Washington, USA และได้รับรางวัล Young Scientist Award จาก The Chinese Chemical Society สนับสนุนการเดินทางให้ไปประชุมวิชาการการประชุมนานาชาติ "IUPAC2005 Innovation in Chemistry" ระหว่างวันที่ 14-19 สิงหาคม 2005 ณ กรุงปักกิ่ง ประเทศสาธารณรัฐประชาชนจีน **(ภาคผนวก ข4)**

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Size-based speciation of iron in clay mineral particles by gravitational field-flow fractionation with electrothermal atomic absorption spectrometry

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Abstract

Gravitational field-flow fractionation (FFF) coupled to UV and ETAAS detectors has been tested for micron-size particles in the range of 5–20 μm using three Fe-rich clay samples. The iron content estimated after aqua regia extraction was about 20–40 mg kg^{-1}. The ETAAS analysis was performed both off-line from collected fractions and in an online continuous sampling mode using a specially designed flow through vial placed in the autosampler of the ETAAS. Comparison of the direct injection method with total analysis after aqua regia digestion shows that slurry injection of the dilute samples in the gravitational field-flow fractionation (GrFFF) effluent is quite efficient in these samples. In the majority of cases, more than 90% recovery was obtained for the slurry injection method. Fe mass-based particle size distributions and Fe concentration versus particle diameter plots can be generated using certain assumptions. This provides detailed information on size-based speciation of particulate samples. Generally, the Fe concentrations in the particles decreased slightly with an increase in particle size as is often found for soil and sediment samples.

Keywords: Clay mineral particles; Size-based iron speciation; Micron-size particles; Gravitational field-flow fractionation; Electrothermal atomic absorption spectrometry

1. Introduction

The size of particles influences many properties of solid materials. An important example is the transport and fate of particles in aquatic environments [1] through soil profiles [2] and as atmospheric aerosols [3]. Since many elements and compounds are associated with such particulate material, the study of size-based speciation is very relevant in environmental science. One approach for such work is to use conventional separation methods, such as filtration and settling (gravitational or centrifugal), to prepare subsamples with specified size ranges and to analyze these for the components of interest [4–6]. However, filtration suffers from many artefacts, and settling methods are usually quite time consuming [5,6].

For several decades, various groups have been developing methods involving field-flow fractionation (FFF) for separation purposes [7]. FFF has the advantage of generating a continuous distribution of polydisperse samples which can be either collected as discrete subsamples for analysis [8–10], or in certain cases, the FFF eluent can be fed directly into an analytical device [11]. The data generated by such experiments are often in the form of an analyte-based size distribution [8–11].

Most studies on size-based speciation of environmental samples to date have involved the use of sedimentation FFF (SdFFF) [8,12–15] or flow FFF (FlFFF) [16–22] to characterize submicron colloids. These have been coupled

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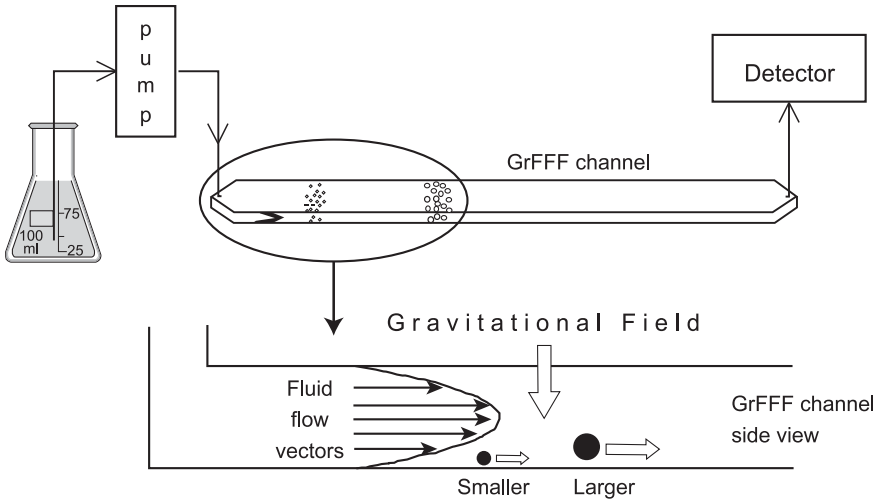

Fig. 1. GrFFF instrument and steric/hyperlayer separation mechanism for micron-size of particles.

both off-line and online with inductively coupled plasma mass spectrometry (ICP-MS) [12–22], and electrothermal atomic absorption spectrometry (ETAAS) is employed as tool [8–11] for element speciation. FFF-ICP-MS has been currently reviewed for some selected applications by Barnes and Siripinyanond [23]. One of the limitations for elemental analysis of particulate slurries is that the efficiency decreases for particles greater than about 5–10 μm. This could be due to trapping of particles in the nebulizer or incomplete atomization/ionization for these larger particles. Recently, we have demonstrated that gravitational FFF (GrFFF) can be combined with ETAAS to achieve size-based speciation data for micron-size silica particles up to 10 μm in diameter [9]. This was done off-line by collecting subfractions for Fe analysis by ETAAS. The digestion efficiency of ETAAS with slurry samples proved to be good at least up to 10-μm diameter particles.

GrFFF is carried out by applying a gravitational field perpendicular to the laminar flow direction in a thin GrFFF separation channel (see Fig. 1). One significant advantage of GrFFF is that the channel can be constructed quite easily and cheaply, and the system can be assembled using widely available components for HPLC or flow injection systems. However, the weak gravitational field restricts the size range that can be fractionated to about 2–50 μm in diameter. Nonetheless, this silt-size range is of importance for sediment transport in rivers and atmospheric aerosols.

As an extension of our work to develop analytical methods for size-based element speciation, we present for the first time data for online GrFFF–ETAAS of micron-size clay particles. This was achieved by continuously feeding the eluent from the GrFFF into a special flow through sampler set in one position of the autosampler. The stream was sampled at regular time intervals, and the Fe content was obtained by slurry introduction into the ETAAS. A major advantage of this method is that samples are withdrawn from a flowing suspension, thus avoiding the problem of settling of sample particles in a vial. This same approach may be useful in other applications where slurry injection is used.

The reason for our focus on Fe, in this and previous publications, is that hydrous Fe oxide coatings are thought to exist on aquatic and soil particles and are generally believed to be involved in the uptake of contaminants such as toxic metals [3] and phosphate [2]. In this paper, we continue with this emphasis, but instead of using pure silica with a coating of synthetic goethite [9], we study the Fe speciation of a series of natural iron-rich silt-size clay minerals.

2. Experimental section

2.1. Instrumentation

2.1.1. GrFFF

The components of the GrFFF instrument have been described in previous work [9,24]. The channel dimensions were 0.095×300×20 mm for the channel thickness, tip-to-tip length and breadth, respectively. The geometric void volume was 0.57 mL. Although the GrFFF channel was only 95 μm thick, no clogging problems were experienced with the dilute (2 mg mL^{-1}) <45-μm diameter sieved samples used in this work. A Milton Roy Constametric III pump (Pennsylvania, USA) was used to deliver the carrier, 10^{-4} M sodium hydroxide solution. Particle suspensions were injected into the carrier stream with a hypodermic syringe (40 μL) through a homemade injection port [24]. The concentration of particles in the FFF eluent was monitored by a UV detector (Model UV8, BAS, USA) at an operating wavelength of 254 nm. The Pocket Sampler (Dick Smith, Australia) with the software supplied was used as the interfacing device for digitizing the GrFFF-UV detection signal.

2.1.2. ETAAS

The ETAAS instrument was a Perkin–Elmer Model 5100 (Norwalk, CT, USA) equipped with Zeeman correction, an HGA-600 graphite furnace and AS-60 autosampler. A pyrolytically coated graphite tube with L'vov platform was used. The fast-heating program for the graphite furnace is summarized in Table 1. The peak area across the 3-s heating segment (step 2) of the program was recorded. This program enables more analysis data to be collected across the GrFFF fractogram. The total analysis time for each replicate was 86 s (including autosampler operating time and cooling step). By using this temperature program, there was no difference for the Fe atomic peak profiles from the slurry, and solution analysis was observed.

2.1.3. GrFFF–ETAAS

Off-line and online determination of iron by GrFFF with ETAAS detection can be carried out by employing the instrument set-ups, as shown in Fig. 2. Off-line GrFFF with ETAAS analysis was done by collecting fractions (ISCO, Model RETRIEVER 500 fraction collector, Lincoln, USA) of the eluent after passing through the GrFFF separation device, as shown in Fig. 2(a). Analysis of the iron content in each fraction was obtained through slurry introduction into an electrothermal atomic absorption spectrometer (ETAAS). For online GrFFF–ETAAS analysis, the eluent from FFF unit was continuously fed into the bottom of a specially designed flow through sampler vial (see Fig. 2(b)) which was placed in the autosampler of the ETAAS. Sample suspensions (10–20 μL of 2.01–2.07 mg mL^{-1} depending on the sample) were introduced into the GrFFF, and the eluted particle suspension was continuously introduced through the bottom of the sampler vial. The overflow was directed to waste (Fig. 2(b)). The autosampler arm was set always to sample from this same position so that it introduced discrete samples of the eluent into the ETAAS. A photograph of the online GrFFF–ETAAS instrumentation is shown in Fig. 2(c).

2.2. Chemicals and samples

2.2.1. Chromatographic silica particles (5 and 10 μm)

Chromatographic silica was obtained from used HPLC columns (CN packing from PARTISPHERE RTF Columns, Whatman, UK). Particle suspensions of about 2 mg mL^{-1} in water were prepared. The 5-μm particles were spherical, and the 10-μm particles had more irregular shapes. These were used as particle size standards.

Table 1
Fast heating graphite furnace temperature program

Step	Furnace temperature(°C)	Ramp time (s)	Hold time (s)	Internal Ar gas flow (mL min^{-1})
1	140	1	15	300
2[a]	2400	3	10	0
3	2600	1	5	300

[a] Peak area measurement.

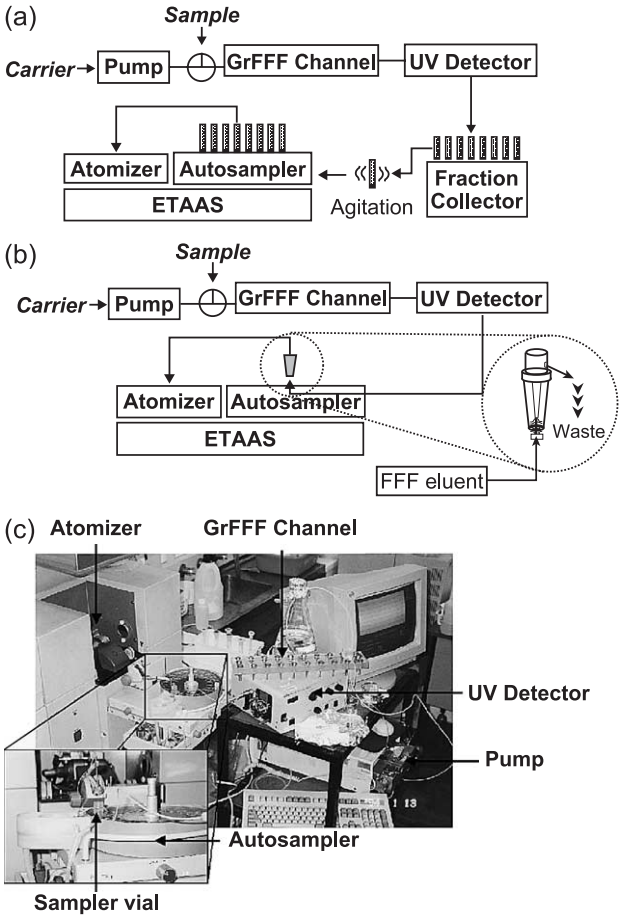

Fig. 2. Instrumental set-up for GrFFF coupled with ETAAS. (a) Off-line GrFFF–ETAAS, (b) online GrFFF–ETAAS showing the flow through sampler vial and (c) photo of the online GrFFF–ETAAS instrumentation.

2.2.2. Clay samples

All clay samples were of natural origin and were obtained from different provinces in Thailand. The clays are referred to as red clay (Kanchanaburi province), ball clay 1 and ball clay 2 (sampled from different sites in Payao province). They were dry sieved through a 45-μm sieve, then dried further at 60 °C for 12 h in an oven and stored in a desiccator until required. Each sample was suspended in water (2 mg mL^{-1}).

2.2.3. Reagents

All reagents were prepared using water obtained from a Milli-Q system (Millipore, Milford, MA, USA). Carrier of sodium hydroxide (10^{-4} M) was prepared using a reagent from Aka Chemicals (Sweden). A standard iron solution (1000 mg L^{-1}) was SpectrosoL® grade from BDH. The desired standard concentrations (10, 20, 40, 60 and 80 μg L^{-1}) were diluted appropriately from the stock standard and used to obtain the calibration line: Y=0.0049×, r^2=0.9947.

3. Results and discussion

3.1. Mass- and Fe-based fractograms obtained by GrFFF with ETAAS

Fractograms of the three clays generated with off-line and online GrFFF–ETAAS analysis for iron are given in Fig. 3. It was found that the UV and Fe profiles obtained by off-line and online operations from all samples agreed reasonably well with each other. The largest discrepancy was between the off-line and online UV fractograms for ball clay 1, which might have been caused by specific errors of the runs. In all subsequent plots, the online fractogram data were used for the calculations. The fact that the sample peak was not resolved from the void peak indicated the presence of large particles of about 20 μm or more.

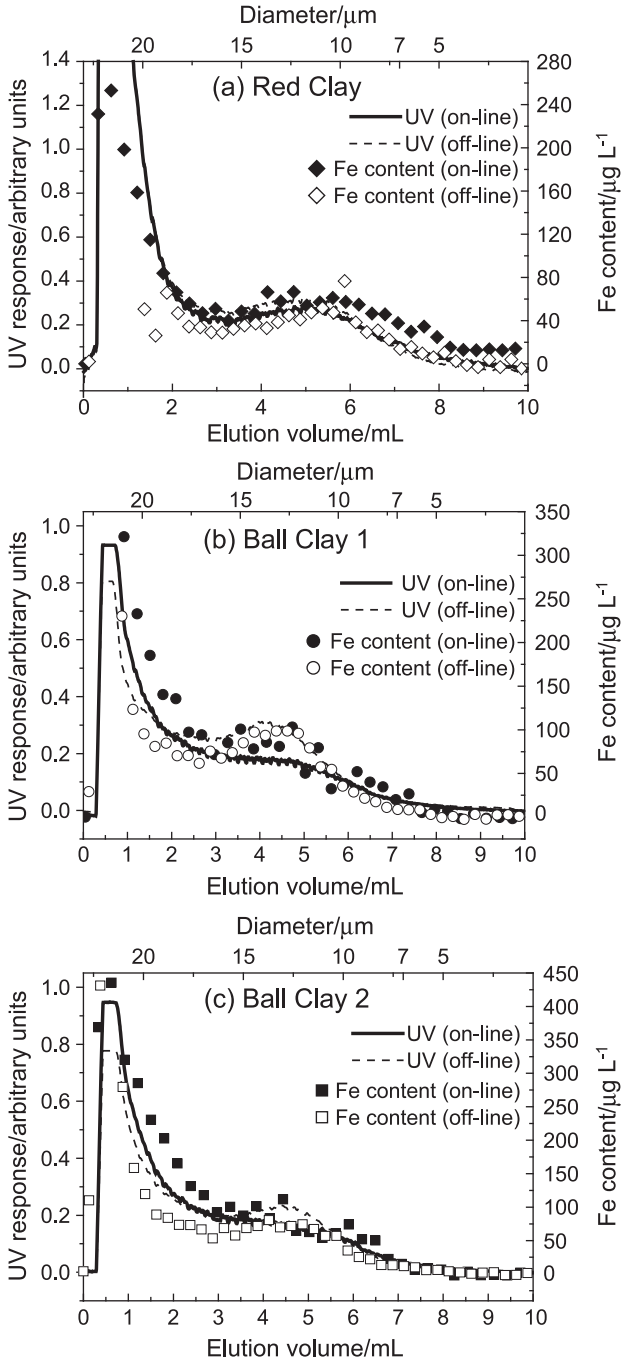

Fig. 3. UV and Fe-based fractograms of clay samples obtained by off-line/online GrFFF–ETAAS (a) red clay, (b) ball clay 1 and (c) ball clay 2.

3.2. Conversion from elution time to diameter

Due to the complications in the steric/hyperlayer model, the conversion of the elution time or volume to diameter must be done empirically via calibration with suitable standard particles. By assuming that the standard particles have the same density as the clay particles, the conversion of the diameter scale can be made employing the empirical formula [25],

$$\log t_r = -S_d \log d + \log t_{r1} \quad (1)$$

where t_r is measured retention time, d is the diameter of the particles, S_d the size selectivity, and t_{r1} is a constant equal to the extrapolated value of t_r corresponding to particles of unit diameter.

For the purposes of illustrating the general approach, a calibration line was obtained using only two chromatographic silica samples (5 and 10 μm) as particle size standards. Although it is not adequate for accurate size determination, the errors are not likely to be excessive as many previous studies have found that the calibration graph is linear [7,26]. Fig. 4 shows fractograms of the 5- and 10-μm silica particles and the plot of log t_r versus log d. This plot was used to generate the equation for converting the x-axis to a diameter scale as described above. In this case, the calculated selectivity using the data for the 5- and 10-μm silica was found to be 0.92, and t_{r1} was 98.

It should be noted that there may be a systematic error in the calibration performed because the 5-μm silica standard particles are spherical, but the 10-μm silica standard and clay particles are more platey in shape. It is known that platey particles experience higher lift forces than spheres of the same volume and hence elute at shorter times [27].

A rough estimate of the magnitude of the error introduced by shape can be deduced as follows. A survey of the literature shows that the selectivity for sedimentation FFF (including the gravitational version) usually falls between 0.7 and 0.8. The gradient for our data in Fig. 4(b) is higher (0.92) because t_r for the 10-μm nonspherical particles is smaller than would be expected for a spherical particle of the same volume. If we use a gradient of 0.75 and the single 5-μm spherical particle calibration point, we can estimate that, for a 10-μm spherical sample particle, we would obtain a diameter of

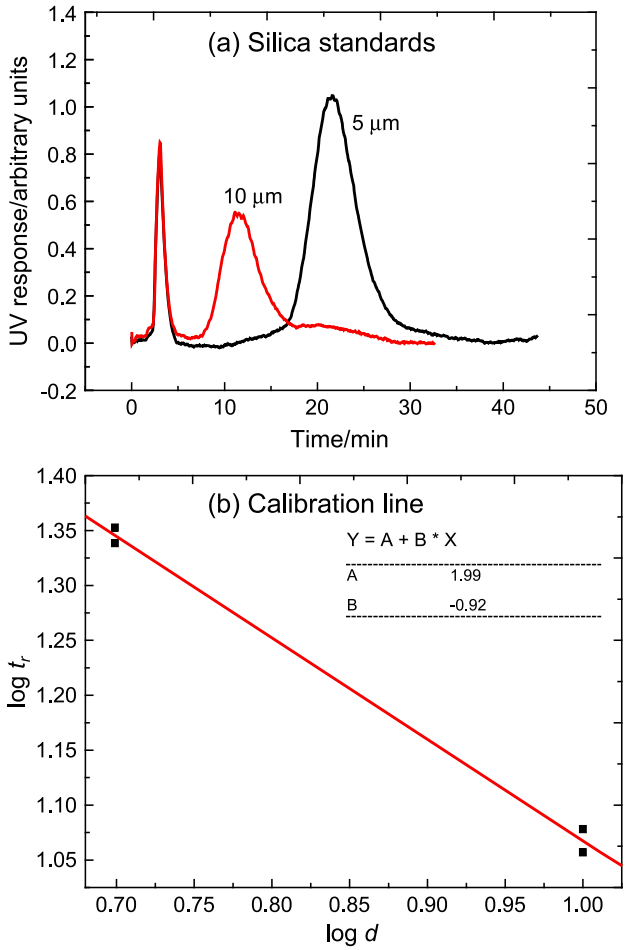

Fig. 4. (a) GrFFF Fractograms of 5- and 10-µm silica particles and (b) plot of log t_r versus log d.

8.8 µm or thus incurring an error of 12%. This simple calculation illustrates the magnitude of the shape error to be expected but should not be taken as the error in our results. Indeed, the situation here is even more complicated as the 5 µm standard was spherical, the 10 µm standard appeared to be somewhat irregular in shape, and the ball clay samples were very platey.

The density of the silica particles may also differ from the clays although this is likely to be less than 20%. Ideally, the calibration standards and samples should have the same shape and density. The calibration error encountered here are of no importance in this paper which is intended just to outline the concept of GrFFF–ETAAS approach for size-based speciation.

3.3. Conversion from UV detector signal to eluted mass

For submicron particles, the UV detector response is usually assumed to be directly proportional to the mass concentration of particles ($\mathrm{d}m^{\mathrm{c}}_{\mathrm{pi}}/\mathrm{d}V_{\mathrm{i}}$). However, according to the approach of Reschiglian et al. [28,29] for micron-size particles, the general relationship for the suspension concentration for large particles (10-fold bigger than the incident wavelength), which comes from Mie theory, can be expressed as

$$\frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{pi}}}{\mathrm{dV_i}} \propto UV_i d_i \tag{2}$$

where UV_i; is the UV detector response at point i along the FFF elution profile; $m^{\mathrm{c}}_{\mathrm{pi}}$ is the mass of sample eluted up to elution volume V_i, and d_i is the particle diameter eluting at V_i. It should be noted that the superscript c in these quantities signifies that it is the cumulative amount eluted up to point i on the fractogram.

3.4. Mass- and Fe-based particle size distributions of clay samples

The appropriate y-axis for a particle size distribution ($\mathrm{d}m^{\mathrm{c}}_{\mathrm{pi}}/\mathrm{d}d_i$) is given by [7]

$$\frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{pi}}}{\mathrm{dd_i}} = \frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{p}}}{\mathrm{dV_i}}\left|\frac{\delta V_i}{\delta d_i}\right| \propto \mathrm{UV_i d_i}\left|\frac{\delta \mathrm{V_i}}{\delta \mathrm{d_i}}\right| \tag{3}$$

where δd_i is the increment in d_i corresponding to increment δV_i in V at point i along the fractogram. The mass-based size distribution is thus a plot of $\mathrm{d}m^{\mathrm{c}}_{\mathrm{pi}}/\mathrm{d}d_i$ versus d_i.

When the GrFFF was connected to the ETAAS system, the Fe content can be evaluated. The mass concentration of the Fe present in the eluent ($\mathrm{d}m^{\mathrm{c}}_{\mathrm{Fe}_i}/\mathrm{d}V_i$) is used to plot the Fe fractogram. This is then converted to an Fe-based particle size distribution using the equation,

$$\frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{Fe_i}}}{\mathrm{dd_i}} = \frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{Fe_i}}}{\mathrm{dV_i}}\left|\frac{\delta V_i}{\delta d_i}\right| \tag{4}$$

where $m^{\mathrm{c}}_{\mathrm{Fe}_i}$ represents the cumulative mass of Fe eluted up to digitized point i on the fractogram. The Fe-based particle size distribution is obtained by plotting $d^{\mathrm{c}}_{\mathrm{Fe}_i}/\mathrm{d}d_i$ against particle diameter d_i.

Fig. 5 shows the particle mass and Fe distributions of the three clay samples. It was found that the samples contained particles in a broad size range starting from about 2 µm and extending beyond 20 µm. However, in these GrFFF runs, the larger particles (>20 µm) are eluted with the void peak where there is no resolution of particle size. Again, we caution that extrapolation beyond the 10-µm upper limit of the calibration standards is not recommended, but in this illustration of the method, it is tolerated because of the expected linear calibration graph.

In all samples, the size distributions decrease rapidly in the range from 4 to 2 µm. This truncation of the distributions is expected in these runs since no stop flow relaxation step was used. Stop flow relaxation is sometimes required in order that the particles travel across the channel to the accumulation wall under the influence gravity. Thus, some of the particles <2 µm would be eluted in the void peak as their relaxation time is >35 s compared to the mean residence time of the carrier in the channel of 171 s [7]. The

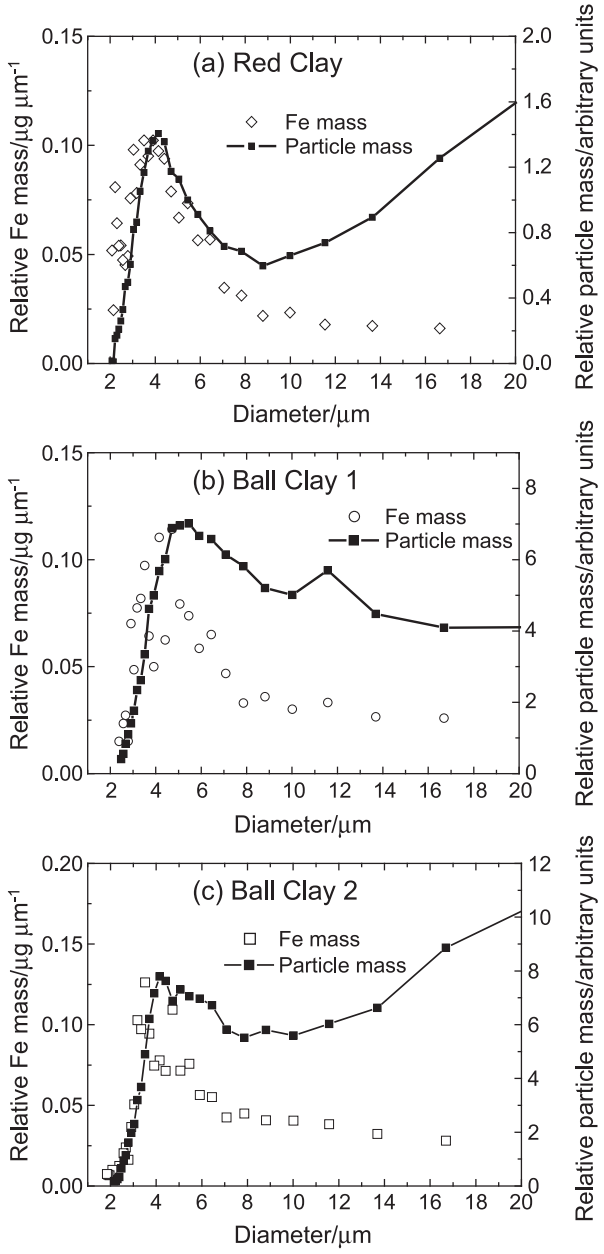

Fig. 5. Mass- and Fe-based particle size distributions (a) red clay, (b) ball clay 1 and (c) ball clay 2.

relaxation time is estimated as the time for the smallest particle of interest to settle across the channel width, w. The relaxation time (t_{relax}) is calculated by transposing the Stokes settling velocity equation to give $t_{relax}=18\eta w/g\Delta\rho d^2$, where η is the carrier liquid viscosity, w is the channel thickness, d is the particle diameter, g is the gravitational acceleration and $\Delta\rho$ is the density difference between the particle and the carrier liquid. However, optical microscope observation of these samples revealed that only small amounts of material were present with diameter <2 μm. The lack of sample relaxation will cause some spreading of the smaller particles but should be insignificant for particles greater than about 4 μm which have relaxation times <9 s.

3.5. Fe content distributions

The Fe concentration in the particles is given by

$$\frac{dm^c_{Fe_i}}{dm^c_{p_i}} = \frac{dm^c_{Fe_i}/dV_i}{dm^c_{p_i}/dV_i} \propto \frac{dm^c_{Fe_i}}{dV_i}\frac{1}{UV_id_i} \quad (5)$$

The Fe concentration distributions were obtained by plotting $\frac{dm^c_{Fe_i}/dV_i}{UV_id_i}$ i.e $\frac{[Fe_i]}{UVd}$ against particle diameter.

The distribution of the Fe concentration in the particles is plotted in Fig. 6. The data are only plotted between 5 and

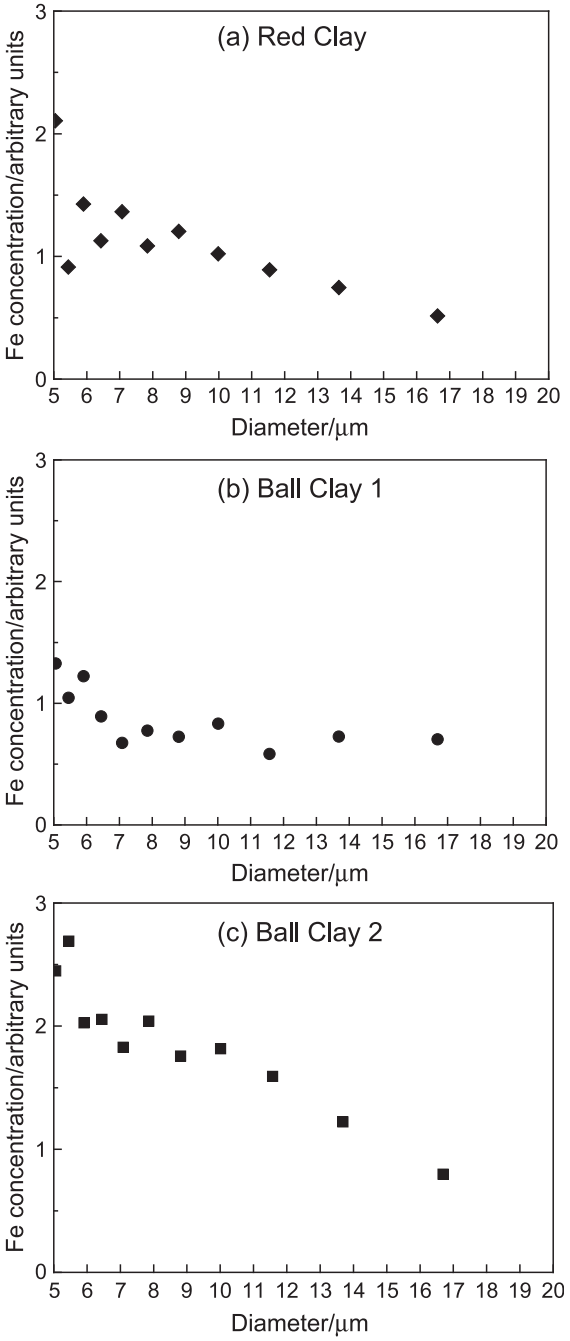

Fig. 6. Distribution of Fe concentrations in the particles as a function of diameter (a) red clay, (b) ball clay 1 and (c) ball clay 2.

Table 2
Total Fe concentrations in the three clay samples

Samples	Fe content (mg g^{-1})		
	Digested	Online	Off-line
Red clay	41.7	32.6	38.6
Ball clay 1	19.9	18.2	18.3
Ball clay 2	21.7	23.9	21.7

20 μm. For >20-μm particles, no resolution from the void peak is obtained at the run conditions employed. The data below 5 μm are not shown as both the UV and Fe concentration values are small, which can result in a large swing in the graph due to errors in setting the correct baselines in the fractograms.

The Fe content increases in the smaller particles, particularly for ball clay 2. This is commonly found for soil and sediment samples [1,11,14]. This may indicate that a significant proportion of the Fe is present as a surface coating on the particles, or that there is an increase in the proportion of Fe containing particles in the smaller size range.

3.6. Efficiency of Fe analysis by GrFFF–ETAAS

An evaluation was carried out on the effectiveness of the slurry ETAAS method for Fe analysis after GrFFF separation. Summation of the iron amounts in each fraction across the entire Fe-based fractogram (including the void peak) provides an estimate of the total mass of Fe injected into the GrFFF with the samples. This was done by integration of the area under the Fe-based fractograms obtained by both off-line and online operations (Fig. 3).

The results were compared with the total iron contents of the original samples analyzed by flame atomic absorption spectrometry after being digested with aqua regia. This comparison is presented in Table 2. It can be seen that the total Fe contents obtained by the GrFFF–ETAAS methods (both off-line and online slurry injections) agreed reasonably well with the digestion analysis. Thus, it would appear that the slurry ETAAS analysis for Fe does not suffer from the same loss of efficiency. This agrees with some previous studies [30].

Using the mean value of all three methods for a given sample, the percentage deviations of each method from this mean can be calculated, as shown in Table 3. It was found that the percent deviations from the mean for the digestion, online and off-line methods were 3–11%, 3–13% and 3%, respectively.

Assuming that the digestion method gave the most accurate estimate of the total Fe content of the sample, the percentage deviations from this value for the slurry methods were found to be 8–22% for the online method and 0–8% for the off-line method. The mean deviation (discounting the sign) was 9%, and only one value was greater than 10%. By far, the most significant outlier was the online red clay result (22% deviation). One contributing factor may be the fact that only 10 μL of sample was injected compared to 20 μL for the ball clays.

It was found that for ball clay 2, the slurry results were higher than those obtained from the digestion analysis. This suggested that there could be other errors in the analysis in addition to inefficient slurry atomization. Thus, it is reasonable to conclude from the results in Tables 2 and 3 that the slurry efficiency is quite good. However, optimization of the slurry method is required to improve the overall accuracy of the Fe analysis.

4. Conclusion

GrFFF–ETAAS was demonstrated to be an effective method for investigating the Fe size-based speciation of micron-size particles. Direct injection of the dilute suspensions of GrFFF eluent into the graphite furnace was shown to be quite efficient. The average deviation of the Fe contents from the values determined after aqua regia digestion was 9%.

The method was illustrated using some Fe-rich clay samples. The Fe concentration in the particles increased slightly with decreased particle size, perhaps indicating that significant amounts of the Fe exists as surface hydroxy oxide coatings. An alternative explanation is an increase in the proportion of Fe-rich minerals in the smaller particles.

GrFFF is the most cost effective of the FFF suite of separation techniques and could be constructed in the simplest workshops. However, it is only applicable over a size range of about 2–50 μm. The ETAAS can be operated

Table 3
Percentage deviations of the total mass of Fe in the three clay samples obtained by various ETAAS methods from either the mean Fe content value or the Fe content of the total digested sample

Sample	%Deviation of Fe content from the mean value[a]			%Deviation of Fe content from the digested value[b]	
	Digested	Online	Off-line	Online	Off-line
Red clay	11	13	3	22	7
Ball clay 1	6	3	3	8	8
Ball clay 2	3	6	3	10	0

The mean value was for the three methods.
"methodi" refers to either the digest, online or off-line method for ETAAS analysis.
[a] %Deviation of Fe content the from mean value=(Total $Fe_{(methodi)}$−Total $Fe_{(mean)}$×100/Total $Fe_{(mean)}$).
[b] %Deviation of Fe content from digest value=(Total $Fe_{(methodi)}$−Total $Fe_{(digest)}$×100/Total $Fe_{(digest)}$).

online using a specially designed sampler vial which was used in the autosampler. GrFFF–ETAAS has been demonstrated for Fe speciation here but should also be useful for a range of other elements.

Acknowledgments

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Development of Sequential Injection Lab-at-Valve-Bead Immunoassay System for Chondroitin 6-Sulfate and Chondroitin Sulfate Epitopes†

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ABSTRACT

This work introduces a development of a sequential injection-lab-at-valve (SI-LAV) immunoassay system. The SI-LAV system provides microfluidic handling ability to manipulate the bead, sample and reagents required to perform an immunoassay. Chondroitin 6-sulfate (C6S) and Shark A1(Sh-A1) fraction assay are important for diagnosis of cartilage disease. The C6S/Sh-A1 analyte is mixed with a fixed amount of WF6 specific antibody. The analyte in a sample competes with the C6S coating on beads for the binding with the WF6 antibody. The C6S coating beads and the reagents are aspirated and trapped in a specially designed device attached to a selection valve. Flow manipulation is made using a syringe pump. The investigation on assay conditions and it advancetages compared with the conventional method are discussed.

1. INTRODUCTION

Immunoassays are widely used in the molecular biology field, providing the molecular basis for many clinical applications (refs). Currently bead immunoassay is one of the most topics widely used. Bead immunoassay, in which antigen or antibody are supported on a solid phase are of interested. A flow-based with bead immunoassay

Up to date, there are some reports on bead injection with sequential injection method (refs). They included sequential injection (SI) with a jet ring cell in the early works (ref) and a lab-on-valve technique in the recently works (ref). We report a new method for automated bead-based immunoassay using lab-at valve (LAV) (refs). It is based on the sequential injection (SI) providing the required microfluidically manipulated ability to handle the microparticles/beads, sample and reagents to perform an immunoassay. The flow-based immunoassay offers an automatic assay carrying out rapidly in an integrated unit. The beads/microparticles were discarded after use, thus avoiding regeneration step. The fresh particles were introduced.

Chondroitin 6-sulfate assay (C6S) which is important for diagnosis of cartilage disease was chosen to be a model compound for developing the system based on competitive immunoassay.

Chondroitin sulfate is one of cartilage proteoglycan which is a major constituent in various connective tissues (ref). There were several works reported that chondroitin sulfate and epitopes on chondroitin sulfate chains are important biomarker of cartilage destruction (ref).

The purpose of the present work is to develop the novel SI-LAV bead based immunoassay system for chondroitin 6-sulfate (C6C) and epitopes on chondroitin sulfate chains from Shark A1 (refs). The system was simply composed of the auxiallry apparatus to facilitate the development of higher throughput for more automated immunoassay. The detection system used was the spectrometric measurement which commonly used in ELISA. The model compounds of chondroitin 6-sulfate (C6C) and C6S epitopes based on competitive immunoassay are therefore chosen for developing the system. The assays are important for diagnosis of cartilage disease. It is a promising performance to use the system for disease screening.

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2. EXPERIMENTAL

2.1 Chemical and Regents

Chondroitin 6-Sulfate sodium salt from shark cartilage (Product No. C4384) was a chemical from Sigma-Aldrich (Saint Louis, MO).

Shark cartilage proteoglycan (A1 fraction); (Shark A1): The shark A1 (Sh-A1) is a highly associated form shark proteoglycan molecules from which separated other small one by CsCl gradient centrifugation. Al fraction was the largest major component in cartilage containing the bottom two fifths of the extractant after centrifugation. The detail for the method of preparation was described previously (ref. 2 MS thesis). The A1 fraction was purchased from Assoc. Prof. P. Kongtaweelert, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, according to the general procedure reported previously (ref.thesis MS).

Monoclonal Antibody WF6; (mAb WF6): The specific monoclonal antibody WF6 (IgM) was synthesized by immunization of female Balb/c mice with the embryonic shark PG-

A1 fraction by using a standard hybridoma technique. The monoclonal antibody WF6 was gifted from Assoc. Prof. P. Kongtaweelert, according to the method description reported previously (ref.thesis MS). Dilution of the WF6 antibody used was 1 mg/mL.

Goat Anti-Mouse IgM Horseradish Peroxidase Conjugate (A8786), IgM-HRP, was purchased from Sigma-Aldrich (Saint Louis, MO). The working dilution used was 1:2,000.

The TMB Peroxidase substrate, TMB-H_2O_2 (3,3',5,5'-tetra-methylbenzidine in an acidic buffer) was purchased from SureBlue™ TMB Microwell Peroxidase Substrate (1-Component) (Cat. No. 52-00-02), KPL (Gaithersburg, MD). The TMB-H_2O_2 solution is ready to use.

Carrier used is phosphate buffer saline (PBS, 10 mM, pH 7.4), 1 L of PBS contain 0.26 g KH_2PO_4, 2.17 g $Na_2HPO_4{\cdot}2H_20$ 1.44 g, 8.71 g NaCl, 0.5 mL of TWEEN 20. Dilution of WF6 antibody and Anti IgM-HRP were made with PBS.

Working dilution of standard C6S and Sh-A1 was spiked in 6 g/L bovine serum *albumin* (in 10 mM PBS) to imitate a real biological fluid which had a total protein at around 6 g/L.

2.2 Pre-coupling C6S /Sh-A1 on beads

Sepharose 4B™ was purchased from Amersham Biosiecnes (Uppsala, Sweden). Beads were washed following the manufacurer suggested procedure. The Carbodiimide method was used as coupling procedure. The target antigen (C6S or Sh-A1) was dissolved in coupling solution, deionized water pH 4.5 adjusted, to obtaind 2.5 mg/mL

and 5 mg/mL for C6S and Sh-A1, respectively. The antigen solution (10 mL) was added to the beads, 5.0 g (wet weight), and followed by adding carbodiimide power 0.1951 g. The mixture was end-over-end rotated for 18 hrs at 4 ^{o}C. The pH of the mixture was measured by pH paper during the first hour and adjusted to pH 4.5. Acetic acid (1.0 M) was used as a blocking solution by rotation with beads for 4 hrs at 4 ^{o}C. The coupled bead was washed throughly with three cycles of 0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl. The C6S/Sh-A1 coupling beads was kept in 10 mM PBS at 4 ^{o}C until use.

2.3 Serum sample

Serum samples were obtained from the volunteer students, age 22-26 years (Flow-based analysis group, Chiang Mai University). A sample of fastingmorning serum was collected from a voluteer in the laboratory. Serums were kepted at -20 ^{o}C freezer before analysis required.

2.4 Instrumentation

2.4.1 SI-LAV apparatus

The syringe pump (Cavro XL3000) was purchased from FIAlab Instruments Inc. (Bellevue, WA). The ten-port selection valve (C25-3180EMH) was a product from VICI Valco Instruments Co. Inc. (Houston, TX). The spectrometric detection system used a model LS-1 light source, a USB2000 spectrometer and two 400-μm (FIA-P400-SR ($^{1}/_{16}$ inch-o.d. or 1.587 mm diameter)) from Ocean Optics, Inc. (Dunedin, FL). The laboratory designed flow cell was connected with a PEEK cross 0.020 inch thru-hole (P-729) (Upchurch Sciencetific, Oak Harbor, WA). The three-way isolation solenoid valve (A-0136772) was purchased from Cole Parmer International, USA. All connecting $^{1}/_{16}$

inch-o.d. tubing were FEP Teflon and PEEK (Upchurch Sciencetific, Oak Harbor, WA). The instrument was controlled by the FIAlab for windows 5.0 (version 5.9.158) software. The signal out put was recorded by the LabView software (v.7). The data evaluated and graph generated was made by OriginLab v.7 (Northampton, MA).

The automated instrument for bead immunoassay was developed using the LAV apparatus for spectrophotometric measurement. The instrument (Figure 1) consists of a syringe pump (syringe volume 1000 μL), a holding coil, ten-port selection valves, a three-way solenoid valve, a lab-at-valve flow cell, a bead reservoir (plastic syringe 1 mL), a PEEK cross flow cell with a fiber optics light source and a detector. Sample and bead introduction are carried out by combinations of reversible flow pump motion, valves port-position selected and solenoid valve switching direction. The LAV flow cell schematic is shown in Figure 2. All connecting tubing was shortened as much as possible to minimize dilution and reagent consumption.

2.4.2 Operation sequences

The SI-LAV operating sequences was designed and modified from a typical ELISA batch-well competitive immunoassay for chondroitin 6-sulfate and Sh-A1 (ref.). The beads and the reagents were aspirated via the selection valves by a controlled-flow motion of the syringe pump. The certain beads amount was achieved by the LAV flow-cell cavity. Solenoid valve was needed in order to retain the microparticles within the LAV flow-cell and to bypass the flowing reagents to carry out an immunoassay. The operation sequences were drawn in Table 1.

3. RESULTS AND DISCUSSIONS

3.1 Developed LAV flow-cell

A LAV flow-cell for bead immunoassay with spectrometric measurement was designed. It was made of Perspex and comprised a cylindrical cavity (1.5 mm i.d., 4.5 mm long) with three flow connections. One connection, the frit 5 μm was placed to retain the bead in the cavity. Bead inlet, bead outlet and beads blocking connections were made by using the same connection via MV2. Beads were introduced into the cavity of the LAV cell from MV2 (beads reservoir). Beads were retained in the cell by changing valve position MV2 (plug), to block the beads in the cell and by switching 3-way solenoid valve to by pass the flowing reagents. Bead discarding was done by selection of MV2 (bead outlet) and stopper of the solenoid valve. The whole cavity of the flow cell was filled with beads, approximately 8 μL.

3.2 Assay condition

Assay condition was investigation to perform bead-based SI-LAV immunoassay as following.

Incubation: A slow flow rate (1 μL/min) incorporating a pulsed flow was performed in the incubation steps (Table 1, steps 10, 15). These flow patterns prolonged the contact time of the sample zone, thus would lead the more effective binding between beads and reagents. Air segments were aspirated to prevent dilution of the sample and reagent zones (Table 1, steps 3, 7, 13, 18).

Reagent concentration: The amounts of mAb WF6 (0.25, 0.5, 1, 2 mg/mL) were varied while amounts of the others were fixed (beads 8 μL, 100 μg/mL C6S). The suitable concentration was found to be 1 mg/mL.

Fresh bead introduction: Used beads were discarded and the fresh beads were introduced into the LAV flow-cell after each run. Thus, there is no need to regenerate the beads. However, the used beads can be collected for further refreshing.

3.3 Monitoring Signal

Signal due to the bead immunoassay was monitored in real-time by an optical fiber spectrometer. The response was monitored for the entire spectra at peak maximum (Figure 4 (a)). The sensitivity of the measurement in the UV at 375 nm, and the Visible at 675 is the highest, however the signal at 375 nm could suffer from low light intensity, the response in the visible 675 nm is therefore monitored as a detected wavelength. The SI profiles of the various standards C6S competed with the packed bead-C6S (Figure 4 (b)). During the runs, a well reproducible level of zeroed baseline was obtained.

3.4 Competitive binding curve

The competitive binding curve was typical generated using the expression B / B_0, which is the unitless ratio *vs.* log [Analyte]. B is the signal from the amount of the label bound to the beads when the sample analyte is present. B_0 is the signal from the amount of the label bound to the beads when the sample analyte is absent.

The C6S/Sh-A1 analyte was mixed with a fixed amount of WF6 specific antibody, and the analyte in the sample competed with the coating C6S/Sh-A1 on beads for the binding with specific antibody.

The analyte C6S/Sh-A1 binds to the WF6 specific antibody thus preventing the antibody from binding with the C6S/Sh-A1 coating on the bead surface. Therefore, an increase in amount of C6S/Sh-A1 in sample solution resulted in a decrease of the signal.

The two model of competitive binding immunoassay of C6S and Sh-A1 were investigated. The same conditions were used for the two models. The competitive binding curves are shown in Figure 4. Concentrations of C6S standards over range from 100-3200 μg/mL (curve detail…), the Sh-A1 range from 500-200 000 ng/mL were obtained. Sh-A1 gave the better sensitivity of the assay. This could be due to the larger size for Sh-A1 than C6S, which could make it possible to have a high binding property with mAb WF6. Therefore Sh-A1 could be used as a potential standard antigen as a relative equivalent of chondroitin sulfate epitopes in a serum (ref).

3.5 Comparison of the operation steps of the convenentioal ELISA and the developing system

3.6 Preliminary analysis of Cs epitopes in human serums

Sh-A1 was used as a potential standard antigen for the assay of a relative equivalent of chondroitin sulfate epitopes in human serum. The data for chondroitin sulfate epitopes in human serum by the developed SI-LAV method is given in Table 2.

4. CONCLUSION

A microfluidic flow-microparticles based immunoassay has been developed. It is rapid, automatic and can be used for real time analysis. Apart from C6S epitope assay, applications to other analytes should be possible using this instrument.

These results demonstrate the potential of the developed system for assay where the C6S epitope levels are lower for normal case. The high levels from patient serums are currently under investigation.

REFERENCES

ACKNOWLEGEMENTS

This project was supported by grants from the Thailand Research Fund. Department of Chemistry, Faculty of Science, Chiang Mai University was thanked for the laboratory facility needed. Thanks are due to Dr. Siripat Suteerapataranon for helping in the early designed of the flow cell, Mr. Lucksagoon Ganranoo for assisting with solenoid valve assembly.

List of Tables

Table 1 Operation sequences

Step	Port (MV1)	Port (MV2)	Volume*/ μL	Flow rate/ μL/sec	3-way Solenoid valve	Description
1	Beads	Beads resevior	~ 8	NA	On	Manual fill beads inlet to LAV cell
2	Beads (PBS)	Plug	-100	1	On	Flush beads with PBS
3	MC2	Plug	+20	20	On	Load air segment-A1
4	WF6 antibody	Plug	+25	20	On	Get WF6 antibody
5	Standard solution	Plug	+50	20	On	Get a compettive standard
6	WF6 antibody	Plug	+25	20	On	Get WF6 antibody
7	MC2	Plug	+20	20	On	Load air segment-A2
8	MC2	Plug	-/+100	20	On	Third times reversible flow for mixing
9	Waste	Plug	-25	20	On	Discard air-A2
10	Beads	Plug	-80	1	On	Inject the mixture to packed beads
11	Waste	Plug	-50	20	On	Discard air-A1
12	Bead (PBS)	Plug	-300	1	On	Flush PBS carrier to beads
13	MC2	Plug	+20	20	On	Load air segment-B1
14	Anti-IgM HRP	Plug	+60	1	On	Get Anti-IgM HRP
15	Beads	Plug	-50	1	On	Inject Anti-IgM HRP to beads
16	Waste	Plug	-50	20	On	Discard air-B1
17	Bead (PBS)	Plug	-300	1	On	Flush PBS carrier to beads
18	MC2	Plug	+20	20	On	Load air segment-C1
19	TMB-H_2O_2	Plug	+60	1	On	Get TMB-H_2O_2
20	Beads	Plug	-50	1	On	Inject TMB-H_2O_2 to beads
21	Waste	Plug	-50	20	On	Discard air-C1
22	Bead (PBS)	Plug	-650	1	On	Flush PBS carrier to beads
23	Bead (PBS)	Waste	-1000	100	Off	Clear packed bead in LAV cell

* Postitive (+) means syringe aspiration, negative (-) means syringe dipensing

3-way solenoid valve ‘On’; in order to bypass the flowing reagents, ‘Off’; in order to retain the beads with in the cell.

Table 2 Comparison of ELISA method and SI-LAV method

Operation steps*	**ELISA method (ref)**	**SI-LAV method**
1. Coupling antigen	Onto well-plate ELISA (>18 hr)	Onto Sepharose bead[a] (~4 hr)
2. Introducing C6S/Sh-A1 and WF6 antibody mixture	100 μL mixture/well (1 hr, 4 ^{o}C and 37 ^{o}C, manual)	50 μL+50 μL of C6S/Sh-A1 and WF6 (100 s, R_T, air automatic flow with air segmented)
3. Conjugation of Anti-IgM HRP	100 μL (1:4000)/well (7 hr, 37 ^{o}C, manual)	50 μL (1:4000) (50 s, R_T, automatic flow with air segmented)
4. Loading substrate TMB-H_2O_2	100 μL (1:4000)/well (7 hr, 37 ^{o}C, manual)	50 μL (1:4000) (50 s, R_T, automatic flow with air segmented)
5. Stop reaction	100 μL (2 M H_2SO_4)/well (20 min, 37 ^{o}C, manual)	Not required

Table 3 C6S epitopes in human serum samples

Sample no.	Pre added Sh-A1 (ng/mL)	Sh-A1 added (ng/mL)	Sh-A1 found (ng/mL)	% Recovery
S1	571	2500	2994	97
S2	1129	2500	3038	84
S3	1095	2500	3110	87

List of Figures

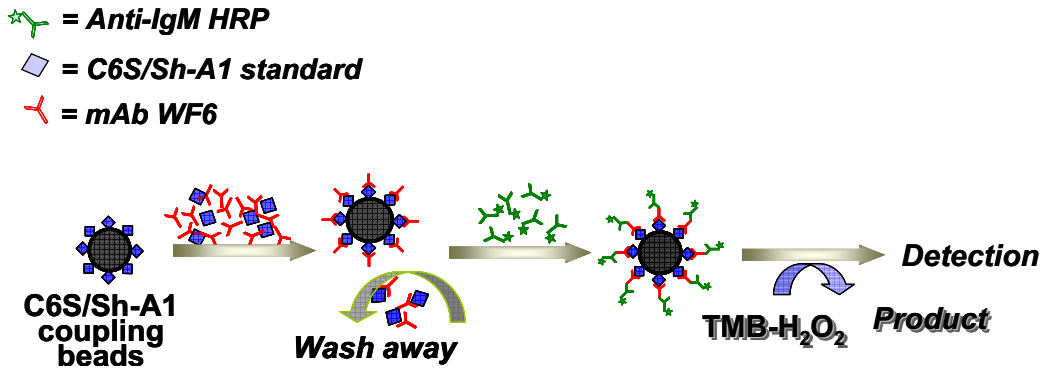

Figure 1 Competitive formate for C6S/Sh-A1 using identical C6S/Sh-A1 coupling beads

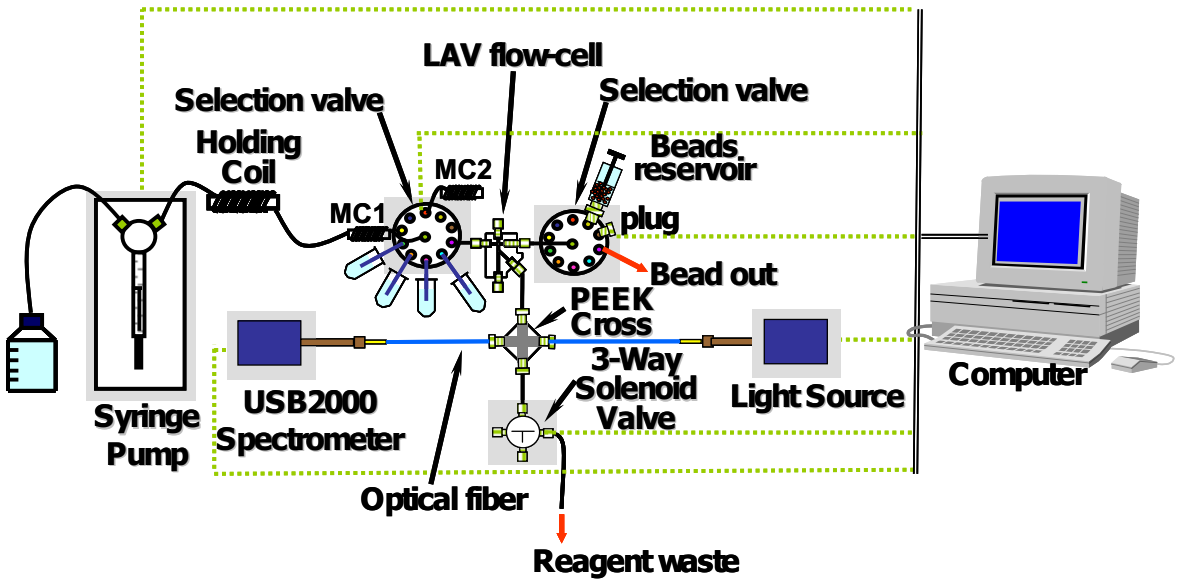

Figure 2 Schematic diagram of the SI-LAV system

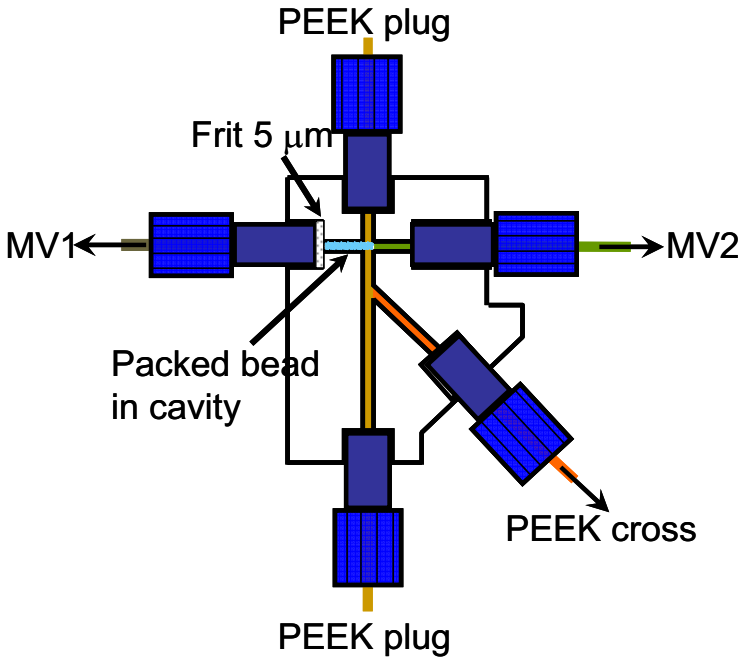

Figure 3 LAV-flow cell

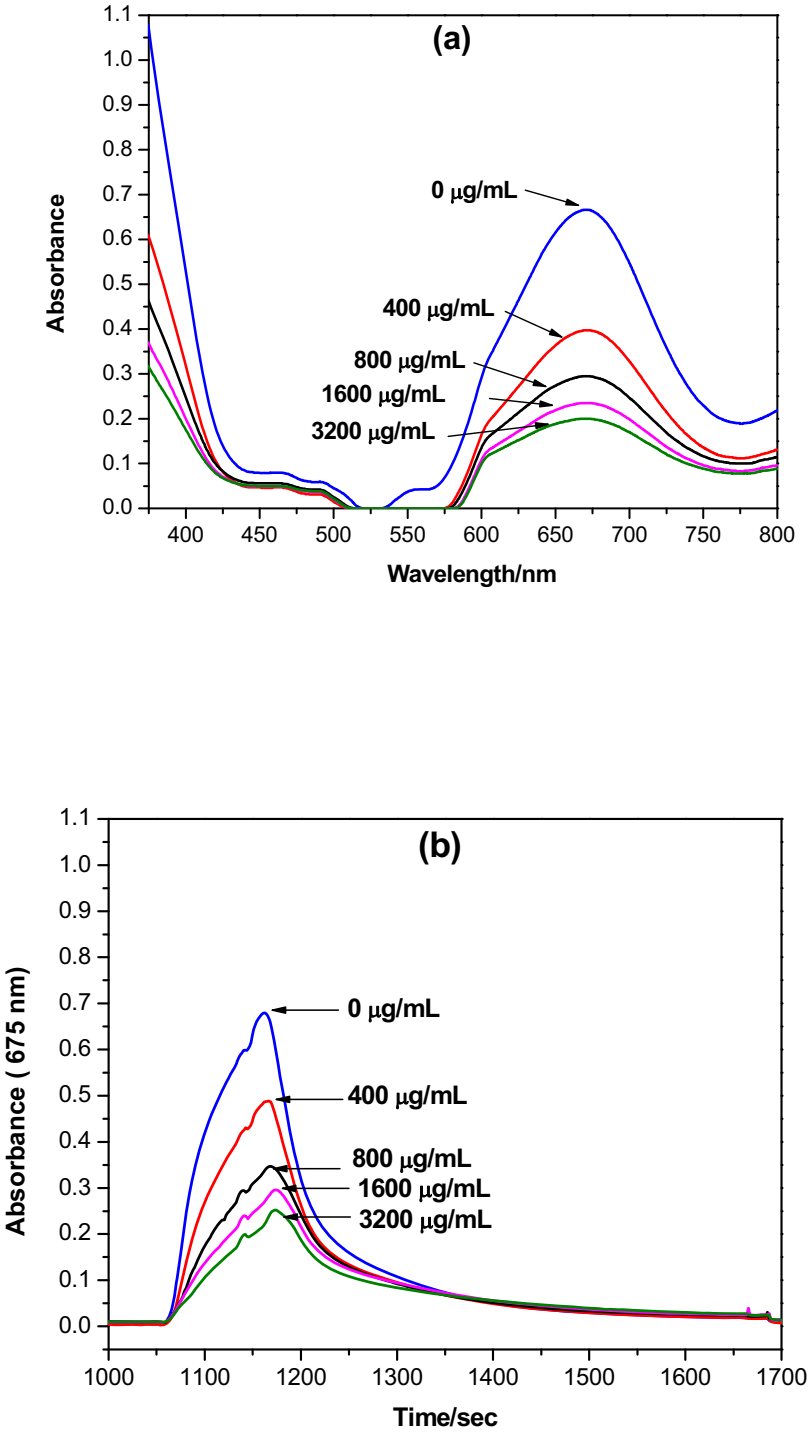

Figure 4 (a) Spectra at peak maximum from different C6S standards competed, (b) SI profiles

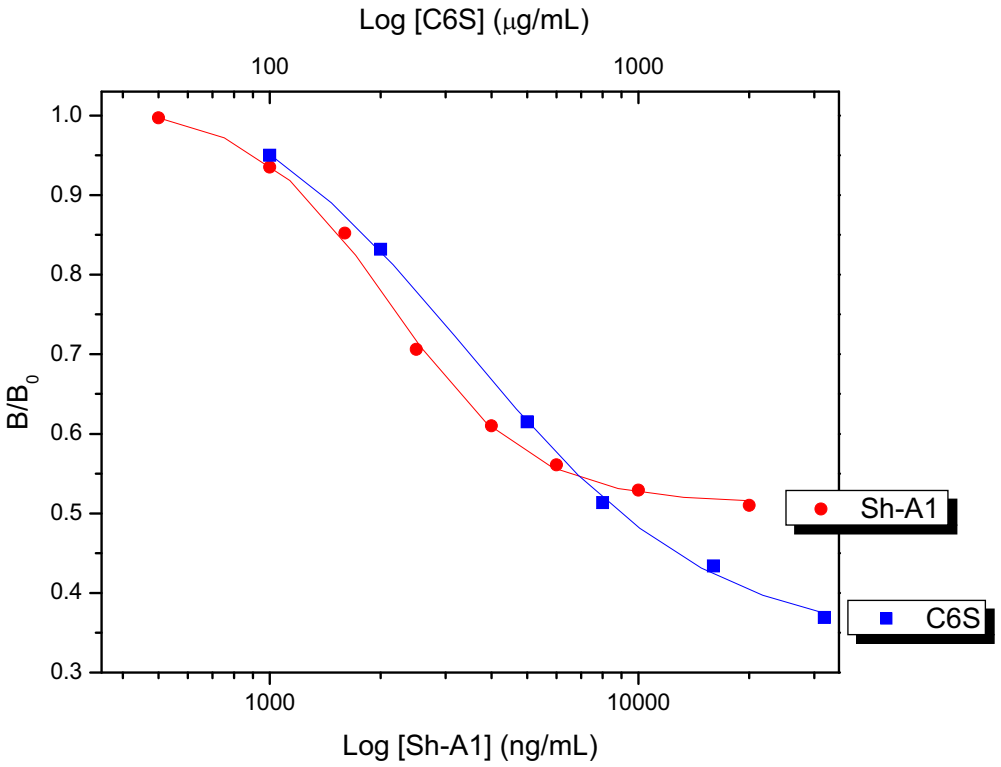

Figure 5 Competitive binding curve for Shark A1 (Sh-A1) and chondroitin 6-sulfate (C6S) standards

THE FLOW-MICROPARTICLES BASED IMMUNOASSAY SYSTEM

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Keywords: Microparticles; Immunoassay; Flow system

This presentation will be discussed on utilizing microparticles as mobile solid-phases for immunoassay. The larger surface area/volume of such microparticles as compared to a microtiter well-plate makes immunoassay ease of miniaturization and mobility with high sensitivity and rapidity. Investigation on some flow systems will be discussed. Antibody against specific proteoglycans is chosen to be a model for flow-enzyme liked microparticles based immunoassay system. Some selected microparticles, specific antigen, specific antibody, peroxidase-conjugated antibody and peroxidase substrate were employed for flow systems with spectrophotometric detection. Performance of flow-microparticles based immunoassay systems and advantages will be discussed.

(1) E. Diamandis, T. Christopoulos (Eds.), Immunoassay, Academic Press, San Diego, 1996.

Development of Sequential Injection Lab-at-Valve-Bead Immunoassay System for Chondroitin 6-Sulfate

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This work introduces a development of a sequential injection-lab-at-valve (SI-LAV) immunoassay system. The SI-LAV system provides microfluidic handling ability to manipulate the bead, sample and reagents required to perform an immunoassay. Chondroitin 6-sulfate (C6S) assay is important for diagnosis of cartilage disease. The C6S analyte is mixed with a fixed amount of C6S specific antibody. The analyte in a sample competes with the C6S coating on beads for the binding with the specific antibody. The C6S coating beads and the reagents are aspirated and traped in a specially designed device attached to a selection valve. Flow manipulation is made using a syringe pump. The investigation on assay conditions will be discussed.

EXPLOITING SIZE-BASED ELEMENT SPECIATION BY GRAVITATIONAL FIELD-FLOW FRACTIONATION COUPLED WITH ICP-MS. **Rattikan Chantiwas**, Institute for Science and Technology Research and Development, Chiang Mai University, Muang, Chiang Mai 50200 Thailand, Siripat Suteerapataranon, School of Science, Mae Fah Luang University, Muang, Chiang Rai 57100 Thailand, Horst Geckeis, Institut für Nukleare Entsorgung, Postfach 3640, D-76021 Karlsruhe, Germany, Ronald Beckett, Water Studies Centre, Department of Chemistry, Monash University, Victoria 3800 Australia, Kate Grudpan, Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200 Thailand; rattikan@chiangmai.ac.th

Gravitational field-flow fractionation coupling with ICP-MS for size-based element speciation of clay mineral particles has been investigated. The mass concentration of particles was monitored by using UV detector. The eluent from the UV detector was merged with Rh standard and then introduced directly into the ICP-MS nebulizer. Mass and elemental based particle size distribution can be estimated under some certain assumptions. The results showed that decrease in efficiency of ICP-MS signal was observed for particles larger than 10 μm. This could be due to either loss of larger particles in the nebulizer or incomplete atomization and ionization of elements in the micronsize- particles larger than 10 μm. In additions, it was observed that band broadening of the element GrFFF-ICP-MS fractograms was apparently greater than that of flow FFF-ICP-MS of nanosize humic substances.

ภาคผนวก ข4

A NOVEL BEAD IMMUNOASSAY-SEQUENTIAL INJECTION SYSTEM FOR AN IMPORTANT PROTEOGLYCAN

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This work introduces a development of a novel sequential flow based-immunoassay system. The sequential injection (SI) system provides microfluidic handling ability to manipulate the beads, sample and reagents to perform an immunoassay. Bead-based immunoassay can be made by a flow manipulated of a required reagent through the beads using the SI system with a simple lab-designed flow-cell. A specific proteoglycan assay is important for diagnosis of cartilage disease. The competitive immunoassay for an important proteoglycan was demonstrated. A proteoglycan analyte was mixed with a fixed amount of a specific antibody, and the analyte in a sample competed with the proteoglycan coating on beads for the binding with the specific antibody. The investigation on assay conditions for developing the system will be discussed.

การพัฒนาระบบบีดอินเจคชันสำหรับการหาปริมาณคอนดรอยติน 6-ซัลเฟต

DEVELOPMENT OF BEAD INJECTION SYSTEM FOR CHONDROITIN 6-SULFATE ASSAY

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บทคัดย่อ: งานวิจัยนี้ได้พัฒนาระบบบีดอินเจคชันสำหรับการหาปริมาณคอนดรอยติน 6-ซัลเฟต (ซีเอสซี) โดยใช้บีดเป็นเฟสของแข็งเคลื่อนที่ในระบบที่ใช้การไหลแบบซีเควนเชียล โดยการหาปริมาณปริมาณคอนดรอยตินซัลเฟต มีความสำคัญในการวินิจฉัยผู้ที่เป็นโรคกระดูก งานวิจัยนี้ได้นำบีดที่เคลือบด้วยซีเอสซีเข้าสู่โฟลเซลล์ จากนั้นฉีดสารผสมของสารซีเอสซีมาตรฐานกับแอนติบอดีที่จำเพาะต่อซีเอสซี แอนติบอดีคู่คอนจูเกตของเปอร์ออกซิเดส และซับสเตรทเปอร์ออกซิเดส ในระดับไมโครลิตรเข้าสู่โฟลเซลล์ และผลิตภัณฑ์ที่เกิดขึ้นบนบีดจะถูกตรวจวัดที่ความยาวคลื่น 630 นาโนเมตร ระบบซีเควนเชียลโฟลนั้น ประกอบด้วย ปั๊มแบบซิรินจ์ ซีเล็กชันวาล์ว สวิทชิงวาล์ว และออพติคอลดีเทคเตอร์ ระบบบีดอินเจคชันที่พัฒนาขึ้นนี้เป็นระบบกึ่งอัตโนมัติ จากผลการทดลองเบื้องต้น พบว่าได้ช่วงความเข้มข้นของสารซีเอสซีมาตรฐาน 500-6000 ไมโครกรัมต่อมิลลิลิตร (ค่าสมการถดถอยเชิงเส้น 0.99) และความสามารถในการทำซ้ำ (ที่ความเข้มข้นของซีเอสซี 1000 ไมโครกรัมต่อมิลลิลิตร, จำนวน 4 ครั้ง) ซึ่งแสดงโดยค่าเปอร์เซ็นต์ของความเบี่ยงเบนมาตรฐานสัมพัทธ์เป็น 5 เปอร์เซ็นต์

Abstract: Bead injection system for determination of chondroitin 6-sulfate (CsC) was developed by utilizing bead as a mobile solid-phase in a sequential flow method. Chondroitin sulfate assay in body fluids is important for diagnosis of cartilage diseases. CsC coated bead was injected to the laboratory-designed flow cell. Then microfluid amounts of a mixture of

CsC standard, CsC specific antibody, peroxidase-conjugated antibody and peroxidase substrate were sequentially introduced. The product of colored beads was monitored in real time at 630 nm. The sequential flow system composed of a syringe pump, a selection valve, a switching valve and an optical detector. The developed bead injection system was operated semi-automatically. Calibration range of 500-6000 µg/mL CsC (R, correlation coefficeint = 0.99) and reproducibility (1000 µg/mL CsC, n=4) of 5 %RSD were obtained.

Methodology:

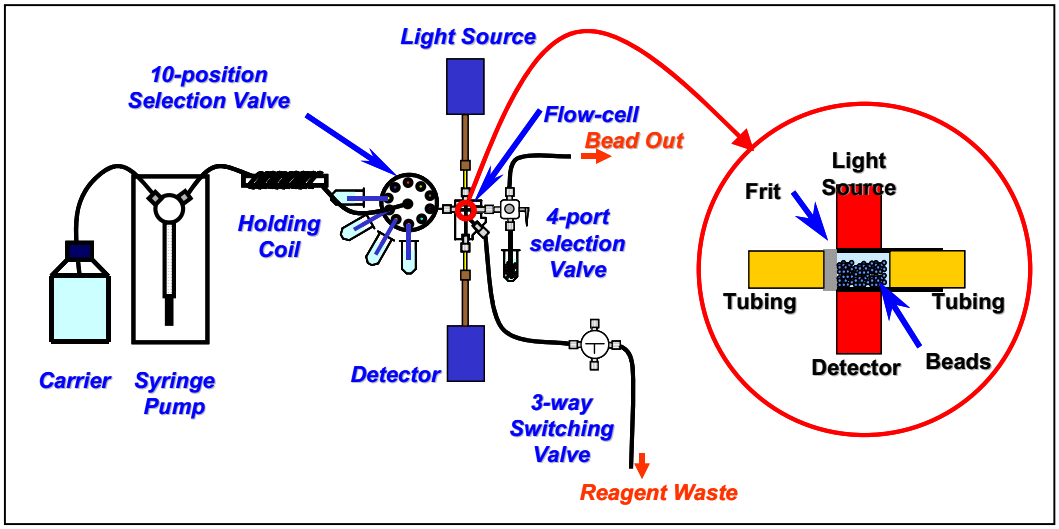

Figure1. Scheme diagram of the developed bead injection system.

Results, Discussion and Conclusion: Bead injection system based on competitive immunoassay was developed for the determination of CsC. Calibration for CsC is demonstrated in Figure 2. Reproducibility (1000 µg/mL CsC, n=4) of 5 %RSD were obtained. The system will be further developed for fully-automatic operation and will be optimized to improve sensitivity.

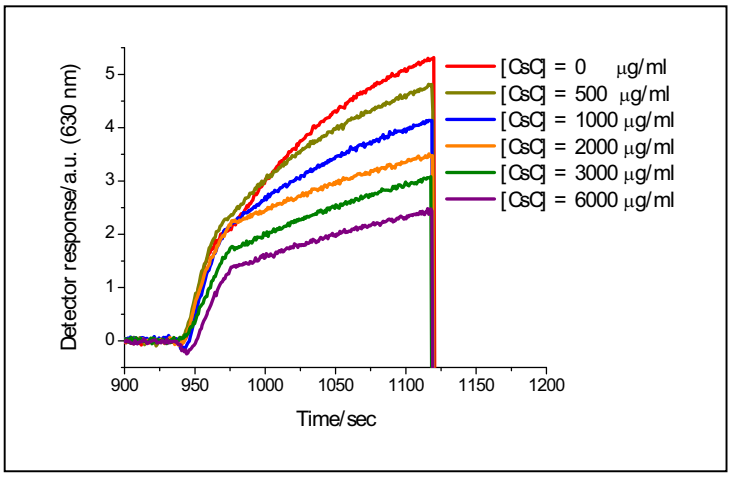

Figure 2 Profiles of bead injection for different chondroitin 6-sulfate concentrations

Acknowledgement: The Post-doc Research Grant to RC and the Senior Research Scholar to KG from the Thailand Research Fund (TRF) are acknowledged for financial support, the Department of Chemistry, Faculty of Science, Chiang Mai University is thanked for laboratory facilities.

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[2] A.D. Carroll , L. Scampavia , D. Luo , Åke Lernmark and J. Ruzicka, *Analyst*, 2003, 128 (9), 1157 - 1162

Keywords: Bead Injection, Sequential Flow System, Chondroitin 6-Sulfate