รายงานวิจัยฉบับสมบูรณ์

โครงการพัฒนาระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์สำหรับการตรวจวัดสารหลายชนิด

โดย
รองศาสตราจารย์ ดร. สุภาภรณ์ ครัดทัพ และคณะ

มีนาคม 2554

สัญญาเลขที่ RSA5180005

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หัวหน้าโครงการวิจัย
รองศาสตราจารย์ ดร. สุภาภรณ์ ครัดทัพ

ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณทุนวิจัยสนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) สำหรับเป็นค่าใช้จ่ายต่างๆ ทั้งวัสดุ สารเคมี ที่จำเป็น รวมถึงค่าใช้จ่ายในการไปนำเสนอผลงานวิจัยในต่างประเทศ ขอขอบพระคุณสมาชิกในกลุ่ม Flow Based Analysis มหาวิทยาลัยเชียงใหม่ ได้แก่ ศาสตราจารย์ ดร. เกตุ กรุดพันธ์ ผู้ให้คำแนะนำที่ดีในทุกด้าน รองศาสตราจารย์ ดร. จรูญ จักร์มุณี ที่อนุเคราะห์อุปกรณ์อิเลคโทรนิกส์บางชิ้น ผู้ช่วยศาสตราจารย์ ดร. สมชัย ลาภอนันต์นพคุณ ที่ให้ความเกื้อหนุนในด้านข้อมูลต่างๆเป็นอย่างดี นอกจากนี้ ขอขอบพระคุณ รองศาสตราจารย์ ดร. ปรัชญา คงทวีเลิศ ภาควิชาชีวเคมี คณะแพทย์ศาสตร์ และ ดร. กนกวรรณ ศรีงาม คณะเกษตรศาสตร์ มหาวิทยาลัยเชียงใหม่ ผู้ให้ความอนุเคราะห์สารชีวโมเลกุลบางชนิด ขอบพระคุณทุนสนับสนุนสำหรับ นางสาวสุภาดา คนยัง นักศึกษาปริญญาเอกในกลุ่มวิจัย จากสำนักงานคณะกรรมการการอุดมศึกษาในโครงการเครือข่ายเชิงกลยุทธ์เพื่อการผลิตและพัฒนาอาจารย์ในสถาบันอุดมศึกษา และทุนสนับสนุนนักศึกษาปริญญาโทจากทุนเรียนดีวิทยาศาสตร์แห่งประเทศไทย สำหรับนางสาวปรียาภรณ์ เรียนแพง และ รวมถึงทุน IPUS จาก สกว. สำหรับนางสาวปิยะนุช ปิ่นอยู่ นักศึกษาปริญญาตรีที่ทำปัญหาพิเศษ นอกจากนี้ ทุนสนับสนุนเพิ่มเติมจาก PERCH-CIC ได้ช่วยให้งานวิจัยต่างๆ สำเร็จลงด้วยดี ขอขอบคุณนักศึกษาในความดูแลทุกท่านที่อดทน และให้ความร่วมมือในการทำวิจัยเป็นอย่างดี และสุดท้ายนี้ขอขอบพระคุณภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่ให้โอกาสในการทำวิจัย ทั้งด้านสถานที่ สาธารณูปโภค และสนับสนุนให้กำลังใจผู้วิจัยโดยการเสนอชื่อเพื่อรับรางวัลตามความเหมาะสม

รองศาสตราจารย์ ดร. สุภาภรณ์ ครัดทัพ

บทคัดย่อ

รหัสโครงการ: RSA5180005

ชื่อโครงการ: การพัฒนาระบบซีเควนเชียลอินเจคชันแคปปิลารีอิมมูโนแอสเสย์สำหรับการตรวจวัดสารหลายชนิด

ชื่อหัวหน้าโครงการวิจัย: รองศาสตราจารย์ ดร. สุภาภรณ์ ครัดทัพ
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ระยะเวลาโครงการ: 2 ปี 11 เดือน (1 พ.ค. 2551-31 มี.ค. 2554)

ได้ทำการพัฒนาระบบซีเควนเชียลอินเจคชันอิมมูโนแอสเสย์โดยใช้แคปิลารีแก้วเป็นผิวของแข็งสำหรับการตรึงสารชีวโมเลกุล โดยทำการศึกษาระบบสำหรับการตรวจวัดสารบ่งชี้โรคทางชีวภาพบางชนิด คือ hyarolunan (HA), sialoglycoconjugates (SC) และ chondroitin sulphate (CS) พบว่า HA และ SC สามารถถูกตรึงบนผิวแก้วด้วยแรงดูดซับทางกายภาพ ส่วน CS ไม่ติดบนผิวแก้วต้องทำการเปลี่ยนผิวแก้วด้วยปฏิกิริยาทางเคมีเพื่อให้เกิดพันธะโควาเลนท์กับ CS ได้ทำการศึกษาความสามารถในการตรวจวัดสารบ่งชี้โรคทางชีวภาพโดยการสร้างกราฟความสัมพันธ์ระหว่างสัญญาณที่ตรวจวัดได้ ซึ่งแปรเปลี่ยนแบบผกผันกับความเข้มข้นของสารที่สนใจ โดยใช้วิธีการศึกษาการได้กลับคืนของสารที่เติมลงในซีรัมมนุษย์ วิธีที่พัฒนาขึ้นช่วยลดปัญหาแรงดันในระบบการไหลที่ปกติมักเกิดกับการทำอิมมูโนแอสเสย์ที่ใช้บีดเป็นของแข็งสำหรับการตรึงสารชีวโมเลกุล อีกทั้งมีความเร็วในการวิเคราะห์ต่อตัวอย่างสั้นกว่าการทำอิมมูโนแอสเสย์แบบหลุมโดยปกติ สารเคมีที่ใช้ก็น้อยลงด้วย นอกจากนี้ได้มีความพยายามศึกษาสารสกัดจากธรรมชาติ จากพืชในท้องถิ่น ทั้งสารที่ให้สี และ เอนไซม์ ซึ่งในอนาคตอาจนำมาใช้เป็นตัวติดตามสำหรับการตรวจวัด ร่วมกับการวิเคราะห์แบบไบโอแอสเสย์ ซึ่งอาจช่วยให้ระบบการวิเคราะห์มีราคาต่ำลงได้

Abstract

Project Code: RSA5180005

Project Title: Development of Sequential Injection Capillary Immunoassay System for Detection of multi-analyte

Investigators: Associate Professor Dr. Supaporn Kradtap, Department of Chemistry,
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Project Period: 2 years 11 months (May 1, 2008 - March 31, 2011)

Sequential injection-immunoassay system was developed using glass capillary as solid phase for immobilization of biomolecules. This study employed biomarkers namely hyarolunan (HA), sialoglycoconjugates (SC) and chondroitin sulfate (CS) as model analytes. It was found that HA and SC could be immobilizeded onto glass capillary with physical adsorption, while CS could not. Glass capillary needed to be chemically modified to covalently bond with CS effectively. Performance of the system for measurement of these biomarkers was valuated by construction of dose response curves and by the recovering studies of these biomarkers in spiked human sera. The proposed system helped to reduce back pressure in the flow system which normally occurred when using beads as solid surface for immobilization of biomolecules. In addition, analysis time per sample was shorter than conventional micro-plate immunoassay format. Moreover, lower amount of sample and reagents were used. Apart from this development, study on the possibility of using natural extract from local plants including pigments and enzymes were attempted. It may be possible to incorporate these natural extract as labels in bioassay in the future which should help to reduce analysis cost.

Excutive Summary

1. ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

โฟลอินเจคชัน เป็นเทคนิคการวิเคราะห์ที่อาศัยการไหลของสารแบบต่อเนื่อง โดยสารเคมีจะเข้าทำปฏิกิริยากันในท่อเล็กๆ ให้ผลิตภัณฑ์ที่ตรวจวัดได้โดยการให้ไหลผ่านเข้าไปในเครื่องตรวจวัดแล้วไหลทิ้งไป ซึ่งทำให้การวิเคราะห์มีประสิทธิภาพและรวดเร็ว สามารถใช้กับสารปริมาณน้อยและสารที่ไม่เสถียรได้ อันเป็นข้อดีที่เหนือกว่าการวิเคราะห์สารแบบดั้งเดิมที่ต้องใช้เครื่องแก้วและปริมาณสารมาก

ซีเควนเชียลอินเจคชัน เป็นเทคนิคที่พัฒนามาจากโฟลอินเจคชัน โดยผนวกเข้ากับโปรแกรมควบคุมการไหลของสารด้วยคอมพิวเตอร์ อาศัยปั๊มแบบสองทิศทางในการขับเคลื่อนสารละลายกลับไปมาได้ สามารถนำสารปริมาณน้อยๆ เข้าออกระบบได้อย่างแม่นยำ และสามารถจับเวลาการหยุดการไหลของสารละลายได้ตามต้องการ นอกจากนี้ ระบบซีเควนเชียลอินเจคชันมีวาล์วแบบหลายพอร์ต จึงมีศักยภาพที่จะพัฒนาเป็นระบบสำหรับการวิเคราะห์สารหลายชนิดได้ ซึ่งระบบดังกล่าว สามารถประกอบและเขียนโปรแกรมควบคุมเองได้ ราคาของระบบจึงไม่สูงมากนัก

เทคนิคอิมมูโนแอสเสย์ เป็นเทคนิควิเคราะห์สารที่มีความจำเพาะเจาะจงสูง แต่ประกอบไปด้วยหลายขั้นตอน ซึ่งต้องอาศัยผู้ชำนาญและมีความละเอียดรอบคอบสูง ในการทำการทดลองซ้ำจะต้องกระทำแต่ละขั้นตอนในระยะเวลาที่นานเท่ากัน และใช้สารเคมีปริมาณเท่ากันทุกครั้ง นั่นคือการจับเวลาและการใช้ปริมาณสารต้องมีความแม่นยำสูง แม้จะมีเครื่องอิมมูโนแอสเสย์แบบอัตโนมัติสำหรับเพลทหลุม แต่ราคาที่สูงมากทำให้ไม่เป็นที่นิยมใช้มากนัก

จากลักษณะการทำงานของระบบซีเควนเชียลอินเจคชันดังกล่าวข้างต้น สามารถนำมาประยุกต์เข้ากับเทคนิคอิมมูโนแอสเสย์ ให้ได้ระบบที่ทำงานอย่างอัตโนมัติ จะช่วยลดปัญหาความไม่แม่นยำ ถูกต้องของเทคนิคอิมมูโนแอสเสย์อันเนื่องมาจากความผิดพลาด และความไม่ชำนาญของผู้ทดลอง ซึ่งผู้วิจัยเคยได้รับทุนวิจัย เพื่อประยุกต์ระบบซีเควนเชียลอินเจคชันกับระบบบีดอิมมูโนแอสเสย์มาแล้ว (MRG 4580015) ซึ่งเทคนิคบีดอิมมูโนแอสเสย์มีข้อดีเหนือกว่าเพลท 96 หลุม คือเนื่องจากบีดเคลื่อนที่ได้ในสารละลาย จึงช่วยลดเวลาการบ่มเพาะให้แอนติบอดี กับแอนติเจนทำปฏิกิริยากัน เพราะระยะทางที่แอนติเจนต้องแพร่มาชนกับแอนติบอดีที่ตรึงไว้ เป็นระยะทางที่สั้นลง แต่ข้อจำกัดของการประยุกต์ระบบซีเควนเชียลอินเจคชัน กับระบบบีดอิมมูโนแอสเสย์ คือความดันย้อนกลับที่เกิดขึ้นในระบบ อันเนื่องมาจากบีดที่แออัดในระบบนำสารที่เป็นท่อขนาดเล็ก และวาล์วที่อาจเกิดการอุดตันได้ ขนาดของบีดจึงต้องจำกัด และเลือกให้เหมาะสม เซลล์กักบีดต้องออกแบบให้ลดแรงดันดังกล่าวโดยเปิดปลายข้างหนึ่งกับอากาศ

เพื่อให้การทำอิมมูโนแอสเสย์มีความง่ายขึ้น และทำงานเข้ากับระบบซีเควนเชียลอินเจคชันได้สะดวกดียิ่งขึ้น จึงจะใช้แคปปิลลารีเป็นเฟสของแข็งสำหรับการตรึงแอนติบอดีหรือแอนติเจนแทนเพลทหลุมหรือบีด ซึ่งแคปปิลารีจะสามารถต่อเข้ากับระบบได้กลมกลืนเสมือนเป็นส่วนหนึ่งของท่อนำสารซึ่งเป็นองค์ประกอบปกติของระบบวิเคราะห์ที่ใช้การไหลทั่วไป โดยไม่ก่อให้เกิดความดัน

ในระบบ อีกทั้งสามารถเพิ่มพื้นที่ผิวสำหรับการตรึงแอนติบอดีได้โดยการเพิ่มความยาวท่อแคปปิลลารี และลดระยะเวลาการแพร่ของแอนติเจนไปที่แอนติบอดีที่ถูกตรึงไว้ที่ผนังท่อได้โดยการลดขนาดเส้นผ่าศูนย์ กลางของแคปปิลลารี

ในการนี้จะทำการออกแบบสร้างระบบซีเควนเชียลอินจคชันแคปปิลลารีอิมมูโนแอสเสย์ที่สามารถตรวจวัดสารได้หลายชนิด โดยมีรูปแบบการตรวจวัดที่คล้ายหรือเหมือนกันมากที่สุดเพื่อความสะดวก ซึ่งจะมีประโยชน์มากในหลายกรณี เช่น การวิเคราะห์สารชนิดเดียวกันที่มีหลายกลุ่ม เช่น ยาฆ่าแมลงกลุ่มต่างๆ ที่ตกค้างในสิ่งแวดล้อมหรือพืชผัก และการวิเคราะห์สารต่างชนิดกันที่จะให้ข้อมูลโดยรวมที่มีประโยชน์มากกว่าผลการวิเคราะห์สารเพียงชนิดเดียว เช่น การตรวจหาฮอร์โมน ยาปฏิชีวนะ และ สารบ่งชี้โรคทางชีวภาพ เป็นต้น นอกจากนี้ระบบที่พัฒนาขึ้นจะช่วยประหยัดเวลาและสารเคมี มากกว่าการตรวจวิเคราะห์ที่มีรายละเอียดการตรวจวัดที่ต่างกันสำหรับสารแต่ละตัว นอกจากนี้ กลุ่มผู้วิจัยมีความสนใจศึกษาถึงความเป็นไปได้ที่จะใช้สารสกัดจากพืชในท้องถิ่นมาใช้ประโยชน์ในการวิเคราะห์สาร ซึ่งอาจมีประโยชน์ในการใช้เป็นเลเบล หรือช่วยในขั้นตอนการตรวจวัดไบโอแอสเสย์ได้ในอนาคต

2. วัตถุประสงค์ของโครงการ

- พัฒนาระบบซีเควนเชียลอินเจคชันควบคู่กับเทคนิคอิมมูโนแอสเสย์โดยใช้แคปปิลลารี เพื่อให้มีความเป็นอัตโนมัติสำหรับตรวจวัดสารได้หลายชนิด และไม่มีแรงดันย้อนกลับ
- ศึกษาเบื้องต้นถึงความเป็นไปได้ในการใช้สารสกัดจากพืชในท้องถิ่นสำหรับเคมีวิเคราะห์

3. ระเบียบวิธีวิจัย

- ออกแบบและพัฒนาระบบซีเควนเชียลอินเจคชัน แคปปิลลารีอิมมูโนแอสเสย์สำหรับการตรวจวิเคราะห์สารที่สนใจทีละตัว เพื่อให้ได้สภาวะการทำงานที่เหมาะสมสำหรับตรวจวัดสารแต่ละตัว ได้แก่ ความเข้มข้นสารที่ใช้ เวลาการบ่มเพาะ ปริมาณสาร อัตราเร็ว เป็นต้น
- ศึกษาและทดลองวิธีการตรึงสารที่สนใจ หรือสารที่เกี่ยวข้องบนหลอดแก้ว เปรียบเทียบแต่ละวิธี ในด้านความยากง่ายของวิธี ประสิทธิภาพ การใช้ซ้ำ เป็นต้น
- ตรวจวัดสารที่สนใจทีละตัว ในตัวอย่างจริง เปรียบเทียบกับวิธีมาตรฐาน หรือวิธีที่นิยมใช้และเป็นที่ยอมรับ เพื่อประเมินและปรับปรุงระบบ
- ใช้สภาวะตรวจวัดที่เหมาะสมสำหรับสารแต่ละชนิด ในการเขียนโปรแกรม หรือปรับเปลี่ยนระบบโดยรวมให้ดีขึ้น
- ตรวจวัดสารที่สนใจทั้งหมด จากสารมาตรฐานที่รู้ความเข้มข้น เปรียบเทียบรูปแบบความเข้มข้นของสารหลายชนิดเหล่านั้น กับรายละเอียดของสารตัวอย่างมาตรฐาน เพื่อประเมินว่าข้อมูลที่ได้จากความเข้มข้นของสารหลายชนิดในตัวอย่างเดียวกัน บ่งบอกสภาวะของตัวอย่างที่ต่างกันได้จริงหรือไม่ อย่างไร เมื่อเทียบกับข้อมูลความเข้มข้นของสารตัวใดตัวหนึ่ง

เพียงตัวเดียว

- ออกแบบและพัฒนาระบบซีเควนเชียลอินเจคชัน แคปปิลลารีอิมมูโนแอสเสย์สำหรับการตรวจวิเคราะห์สารที่สนใจหลายตัว โดยลองศึกษาแบบหลายแคปปิลารี แต่ละอันสำหรับตัวอย่างแต่ละตัว หรือใช้แคปปิลารีเดียวที่มีการตรึงสารหลายชนิดบนหลอดเดียวกัน แต่ตรวจวัดด้วยรายละเอียดวิธีที่ต่างกัน
- ตรวจวัดสารหลายชนิดในตัวอย่างจริง ประเมินการทำงานของระบบกับตัวอย่างจริง
- ศึกษาวิธีทางสถิติแบบต่างๆ และ/หรือ chemometrics ในการเปรียบเทียบและแปรผลรูปแบบสัญญาณของสารหลายชนิดที่ได้
- ศึกษาข้อมูลเกี่ยวกับการใช้สารสกัดจากพืชในเคมีวิเคราะห์ที่มีตีพิมพ์แล้ว
- พิจารณาเลือกพืชที่มีในท้องถิ่นและนำมาสกัด
- ทดสอบการใช้ประโยชน์ในด้านการวิเคราะห์ และ พิจารณาความเป็นไปได้ในการใช้ประโยชน์กับไบโอแอสเสย์ในอนาคต

4. Output ที่คาดว่าจะได้

- คาดว่าจะได้ตีพิมพ์ผลงานตีพิมพ์ในวารสารวิชาการระดับนานาชาติประมาณ 3-4 เรื่อง
- ได้นำเสนอผลงานวิจัยในงานประชุมวิชาการระดับนานาชาติอย่างน้อย 1 ครั้ง
- ผลิตบัณฑิตทั้งระดับปริญญา ตรี โท และ เอกที่มีความสามารถในการทำวิจัย

5. รายชื่อและสังกัดของผู้ร่วมวิจัย ผู้ช่วยวิจัย นักศึกษา ที่ร่วมวิจัยในโครงการ

ผู้ร่วมวิจัย	รศ. ดร. จรูญ จักร์มุณี	ภาควิชาเคมี คณะวิทยาศาสตร์
	ผศ. ดร. สมชัย ลาภอนันต์นพคุณ	ภาควิชาเคมี คณะวิทยาศาสตร์
	ศ.ดร. เกตุ กรุดพันธ์	ภาควิชาเคมี คณะวิทยาศาสตร์
	รศ.ดร. ปรัชญา คงทวีเลิศ	ภาควิชาชีวเคมี คณะแพทย์ศาสตร์
นักศึกษา	นางสาว สุภาดา คนยัง	นักศึกษาปริญญาเอก
	นาย เกรียงไกร พลหงษ์	นักศึกษาปริญญาเอก
	นางสาว นภาพร วรรณาพรม	นักศึกษาปริญญาโท
	นางสาว วรทิพย์ ศรีเปารยะ	นักศึกษาปริญญาโท
	นางสาว ปรียาภรณ์ เรียนแพง	นักศึกษาปริญญาโท
	นางสาว ชุลีกานต์ สายเนตร	นักศึกษาปริญญาโท
	นางสาว ปิยะนุช ปิ่นอยู่	นักศึกษาปริญญาตรี
	นางสาว พิมพร อินเสน	นักศึกษาปริญญาตรี
	นางสาว ธิดารัตน์ ปันอ่วม	นักศึกษาปริญญาตรี

ทั้งหมด สังกัดภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

เนื้อหางานวิจัย

1. ระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์

1.1 บทนำ

เทคนิคอิมมูโนแอสเสย์ เป็นเทคนิควิเคราะห์สารที่อาศัยการจับกันอย่างจำเพาะเจาะจงของสารชีวโมเลกุล กับสารที่ต้องการตรวจวัด โดยทั่วไปใช้แอนติบอดีที่สามารถจับกับแอนติเจนที่สนใจด้วยพันธะอื่นๆที่ไม่ใช่พันธะโควาเลนท์ (non-covalent) และใช้ประโยชน์จากรูปร่างที่เข้ากันได้พอดีกับบริเวณยึดจับของแอนติบอดี เทคนิคนี้จึงมีความจำเพาะเจาะจงสูง แต่ประกอบไปด้วยหลายขั้นตอน ซึ่งต้องอาศัยผู้ชำนาญและมีความละเอียดรอบคอบอย่างมาก ในการทำการทดลองซ้ำจะต้องกระทำแต่ละขั้นตอนในระยะเวลาที่นานเท่ากัน และใช้สารเคมีปริมาณเท่ากันทุกครั้ง นั่นคือการจับเวลาและการใช้ปริมาณสารต้องมีความแม่นยำสูง แม้จะมีเครื่องอิมมูโนแอสเสย์แบบอัตโนมัติสำหรับเพลทหลุม แต่ราคาที่ค่อนข้างแพงมากทำให้ไม่เป็นที่นิยมใช้มากนัก

เทคนิคโฟลอินเจคชัน เป็นเทคนิคการวิเคราะห์สารที่อาศัยการไหลของสารซึ่งควบคุมด้วยปั๊ม ให้สารต่างๆ เข้าทำปฏิกิริยากันในท่อเล็กๆ ระบบการวิเคราะห์สารจึงมีความเป็นอัตโนมัติมากขึ้น ใช้สารน้อยลง และมีความถูกต้องแม่นยำสูงขึ้นกว่าการทำการทดลองแบบดั้งเดิมที่ต้องใช้เครื่องแก้วจำนวนมากและสารเคมีปริมาณมาก [1] ในอดีตมักมีการทำอิมมูโนแอสเสย์จนได้ผลิตภัณฑ์แล้วจึงนำสารเข้าสู่ระบบโฟลอินเจคชันเพื่อศึกษาหาปริมาณความเข้มข้น [2] ปัจจุบันมีการพัฒนาระบบโฟลอินเจคชันที่มีความสะดวกมากขึ้น เรียกว่าระบบซีเควนเชียลอินเจคชัน โดยการควบคุมการทำงานของปั๊มสองทาง (syringe pump) วาล์วฉีดสาร และบันทึกผลตรวจวัดด้วยระบบคอมพิวเตอร์ ซึ่งทำให้สามารถควบคุมการไหลของสารด้วยอัตราที่ต้องการอย่างแม่นยำ และสามารถทำให้สารหยุดไหลในช่วงระยะเวลาที่ต้องการได้ จากคุณลักษณะดังกล่าวนี้เทคนิคซีเควนเชียลอินเจคชันสามารถนำมาปรับประยุกต์ใช้กับเทคนิคอิมูโนแอสเสย์ ตั้งแต่ขั้นแรกของการนำสารตัวอย่างมาสัมผัสกับแอนติบอดีที่ตรึงไว้ ขั้นการหยุดการไหลของสารเพื่อให้เกิดการบ่มเพาะ ขั้นการล้างสารส่วนเกิน และขั้นการตรวจวัดผลิตภัณฑ์ ได้อย่างมีประสิทธิภาพ [3-6] นอกจากนี้ ระบบซีเควนเชียลอินเจคชันมีวาล์วแบบหลายพอร์ต จึงมีศักยภาพที่จะพัฒนาเป็นระบบสำหรับการวิเคราะห์สารหลายชนิดได้ ซึ่งระบบดังกล่าว สามารถประกอบและเขียนโปรแกรมควมคุมเองได้ ราคาของระบบจึงไม่สูงมากนัก

เพื่อให้การทำอิมมูโนแอสเสย์มีความง่ายขึ้น และทำงานเข้ากับระบบซีเควนเชียลอินเจคชันได้สะดวกดียิ่งขึ้น จะต้องเปลี่ยนรูปแบบของเฟสของแข็งที่ใช้ตรึงแอนติบอดีจากแบบเพลทหลุมเป็นรูปแบบอื่นที่ใช้กับระบบการไหลได้ มีรายงานเกี่ยวกับการพัฒนาอิมมูโนแอสเสย์ที่ใช้เฟสของแข็งสำหรับตรึงแอนติบอดีในรูปแบบต่างๆ ได้แก่ บีด อิเล็กโทรด วัสดุไพอิโซอิเล็กทริก และแคปปิลลารี เป็นต้น เพื่อปรับปรุงประสิทธิภาพเทคนิคอิมมูโนแอสเสย์ให้ดีขึ้นกว่าการใช้เพลท 96 หลุมที่ใช้อยู่ทั่วไป [7-10] ซึ่งบีดอิมมูโนแอสเสย์เป็นที่นิยมมากที่สุด เพราะบีดมีพื้นผิวสัมผัสต่อปริมาตรมากกว่าพื้นผิวแบบอื่นจึงทำให้มีความสามารถตรวจวัดสารปริมาณน้อยได้ดียิ่งขึ้น อีกทั้ง

สามารถลอยตัวในสารละลายโดยการเขย่า จึงทำให้แอนติบอดีและแอนติเจนเข้าใกล้กัน ลดระยะทางในการแพร่เข้าหากัน จึงเกิดปฏิกิริยาต่อกันได้เร็วขึ้น [11-13] การประยุกต์ใช้ระบบซีเควนเชียลอินเจคชันกับบีดอิมมูโนแอสเสย์สารมารถทำได้ แต่มักมีปัญหาเกี่ยวกับความดันย้อนกลับอันเนื่องมาจากบีดในท่อเล็กๆ ทำให้เป็นอุปสรรคต่อการไหลของสารละลาย

ในที่นี้จึงจะลองใช้แคปปิลลารีเป็นเฟสของแข็งสำหรับการตรึงแอนติบอดีหรือแอนติเจนแทนเพลทหลุมหรือบีด ซึ่งแคปปิลารีจะสามารถต่อเข้ากับระบบได้กลมกลืนเสมือนเป็นส่วนหนึ่งของท่อนำสารซึ่งเป็นองค์ประกอบปกติของระบบวิเคราะห์ที่ใช้การไหลทั่วไป โดยไม่ก่อให้เกิดความดันในระบบ อีกทั้งสามารถเพิ่มพื้นที่ผิวสำหรับการตรึงแอนติบอดีได้โดยการเพิ่มความยาวท่อแคปปิลลารี และลดระยะเวลาการแพร่ของแอนติเจนไปที่แอนติบอดีที่ถูกตรึงไว้ที่ผนังท่อได้โดยการลดขนาดเส้นผ่าศูนย์กลางของแคปปิลลารี มีรายงานการทำแคปปิลลารีอิมมูโนแอสเสย์อยู่บ้าง [2,14] ซึ่งไม่ก่อให้เกิดปัญหาความดันย้อนกลับในระบบ แต่แคปปิลารีที่ใช้มีราคาค่อนข้างแพงเพราะเป็นฟิวส์ซิลิกา และไม่ใช่การทำแคปปิลลารีอิมมูโนแอสเสย์แบบอัตโนมัติตลอดทั้งขบวนการ

จุดวิจัยที่น่าสนใจอีกอย่างหนึ่งที่น่าจะเป็นไปได้ในอนาคตคือการวิเคราะห์สารหลายชนิดจากตัวอย่างเดียวกัน เพื่อให้ได้ข้อมูลที่เป็นประโยชน์เพิ่มขึ้น ในการนี้ควรใช้รูปแบบการวิเคราะห์สารหลายชนิดเหล่านี้ที่เหมือนหรือคล้ายคลึงกันมากที่สุดเพื่อความสะดวก ในการนี้จึงทำการออกแบบสร้างระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์ที่มีความเป็นอัตโนมัติ และเป็นรูปแบบที่สามารถใช้กับการตรวจวัดสารได้หลายชนิด ซึ่งจะมีประโยชน์มากในหลายกรณี เช่น การวิเคราะห์สารชนิดเดียวกัน ที่มีหลายกลุ่ม เช่น ยาฆ่าแมลงกลุ่มต่างๆ ที่ตกค้างในสิ่งแวดล้อม หรือพืชผัก และการวิเคราะห์สารต่างชนิดกันที่จะให้ข้อมูลโดยรวมที่มีประโยชน์มากกว่าผลการวิเคราะห์สารเพียงชนิดเดียว เช่น การตรวจหาฮอร์โมน ยาปฏิชีวนะ และ สารบ่งชี้โรคทางชีวภาพ เป็นต้น แม้ว่าสารแต่ละตัวอาจมีธรรมชาติการเกาะจับกับแคปิลารีแก้วได้ไม่เหมือนกัน การทำการเปลี่ยนแปลงผิวแก้วให้เกิดพันธะโควาเลนท์กับสารชีวโมเลกุลต่างๆ ก็มีความเป็นไปได้ จากรายงานมีผู้ศึกษาการตรึงลิแกนด์ต่างๆ ด้วยพันธะโควาเลนท์บนผิวแก้วผ่านหมู่คาร์โบไฮเดรต หรือหมู่เอมีนของลิแกนด์โดยใช้สารเคมีต่างๆ กัน [2,14-17] ซึ่งพันธะโควาเลนท์จะช่วยให้สารถูกตรึงแน่นไม่หลุดจากผนัง อีกทั้งจัดเรียงตัวในทิศทางที่เหมาะสมแก่การเกิดปฏิกิริยาในขั้นต่อไป นอกจากนี้ ได้มีรายงานการพัฒนาเทคนิคอิมมูโนแอสเสย์ ในด้านการตรวจวัดสารหลายชนิดโดยการใช้เลเบลชนิดต่างๆกัน การใช้แอนติบอดีหลายชนิดร่วมกัน เป็นต้น ในงานนี้ได้พยายามสกัดสารจากพืช เช่นสารเกิดสี และเอนไซม์ เพื่อศึกษาถึงความเป็นไปได้ที่จะใช้พืชท้องถิ่นเป็นแหล่งสกัดสารสำหรับทำเลเบลในไบโอแอสเสย์ ซึ่งอาจจะช่วยให้มีค่าใช้จ่ายถูกลงได้ในอนาคต

งานวิจัยนี้ ได้ทดลองใช้สารบ่งชี้โรคทางชีวภาพซึ่งเป็นที่สนใจในหมู่นักวิจัยทางการแพทย์ทั้งในและต่างประเทศ คือ hyarolunan (HA), sialoglycoconjugate (SC) และ chondroitin sulfate (CS) เป็นโมเดลในการศึกษา ซึ่งมีรายงานและข้อมูลจากผู้ศึกษาสารทั้ง 3 ชนิดนี้ ว่าสารเหล่านี้มีความเกี่ยวข้องกับโรคมะเร็ง แต่สารทั้ง 3 ชนิดไม่เฉพาะเจาะจงกับมะเร็งเท่านั้น เช่น HA และ CS มีความเกี่ยวข้องกับโรคกระดูกด้วย ส่วน SC ก็มีรายงานเกี่ยวกับโรคหัวใจเช่นกัน แต่คณะผู้วิจัย

ของคณะแพทย์ศาสตร์ มหาวิทยาลัยเชียงใหม่ เคยได้ทำวิจัยเบื้องต้น พบว่า ถ้าตรวจวัดสารทั้ง 3 ชนิดร่วมกัน โอกาสที่จะทำนายการเกิดโรคมะเร็งระยะต่างๆอาจทำได้ง่ายขึ้น แต่วิธีการตรวจวัดสารเหล่านี้มีรูปแบบที่แตกต่างกัน เช่น HA และ CS ตรวจวัดโดยวิธีอิมมูโนแอสเสย์แบบหลุม ส่วน SC ตรวจวัดโดยวิธีโครมาโทกราฟี ในงานวิจัยนี้ผู้วิจัยจึงได้พยายามพัฒนาวิธีวิเคราะห์ที่มีรูปแบบที่เหมือนกันเพื่อในอนาคตจะทำให้พัฒนาให้ใช้กับการตรวจวัดสารบ่งชี้โรคดังกล่าวร่วมกันได้ สะดวกขึ้น อีกทั้งต้องการเพิ่มความเป็นอัตโนมัติ ความรวดเร็วให้กับการตรวจวัด โดยที่ยังคงความไวและลดปริมาณการใช้สารเคมีให้น้อยที่สุด

1.2 วัตถุประสงค์

เพื่อพัฒนาระบบวิเคราะห์อิมมูโนแอสเสย์แบบอัตโนมัติ โดยอาศัยเทคนิคการไหลผนวกเข้ากับแคปปิลลารีแก้วราคาประหยัดเป็นเฟสของแข็งสำหรับการตรึงสารชีวโมเลกุล เพื่อนำระบบรูปแบบเดียวกันนี้ไปประยุกต์ใช้สำหรับการตรวจวัดสารที่สนใจหลายชนิดได้สะดวก

1.3 การทดลอง

1.3.1 ระบบซีเควนเชียลอินเจคชันแคปปิลลารีแอสเสย์สำหรับการตรวจวัด hyarolunan, sialoglycoconjugates และ chondroitin sulfate

1.3.1.1 ระบบ และอุปกรณ์

ระบบซีเควนเชียลอินเจคชันที่ใช้ในงานวิจัยนี้ แสดงอยู่ในรูปที่ 1 ประกอบด้วย

syringe pump (CAVRO) ขนาด 2.5 mL

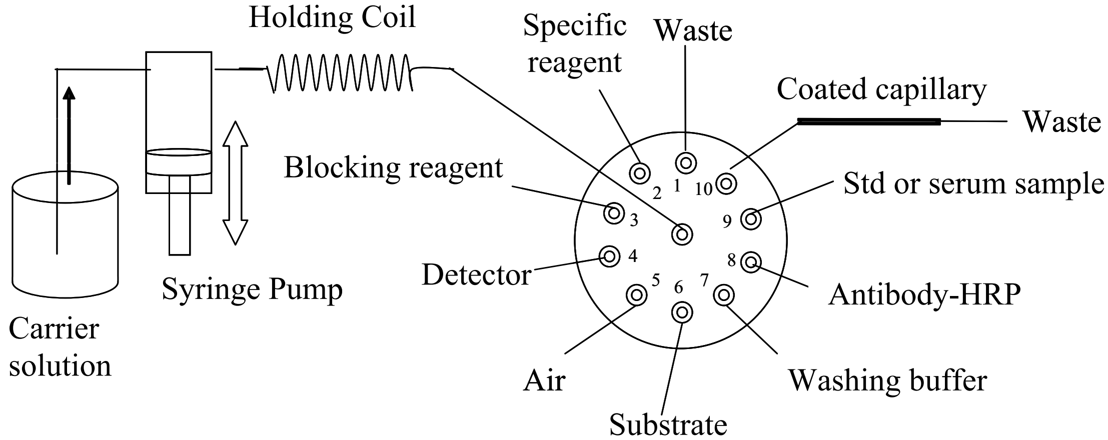
selection valve 10 ports (Valco)

spectronic 21 (Spectronic Instrument)

FIA Lab software

PTFE tubings 1/16” ID (Up Church)

Haematocrit glass capillary 7.5 cm length 1.15 mm ID (Vitrex)



รูปที่ 1 ระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์

1.3.1.2 การเตรียมแคปิลลารีแก้ว

สำหรับการตรวจวัด sialoglycoconjugates และ hyarolunan

Sialoglycoconjugate และ hyaluronan สามารถจับกับผิวแก้วด้วยแรงทางกายภาพ จึงไม่ต้องทำการเปลี่ยนแปลงผิวแก้ว เพียงนำแคปปิลลารีแก้วมาเติมสารที่จะใช้ตรึงจนเต็ม โดยใช้แรงแคปปิลลารีช่วย จากนั้นปิดทั้งสองปลายด้วยพาราฟิล์ม ทิ้งไว้ที่อุณหภูมิต่างๆ ด้วยเวลาบ่มเพาะต่างๆ กัน เพื่อหาสภาวะที่เหมาะสมสำหรับการตรึงสารแต่ละตัว ทำการปิดผิวแก้วส่วนที่ว่างด้วย blocker ที่เหมาะสม เพื่อลดการจับแบบไม่จำเพาะจากสารอื่นๆ

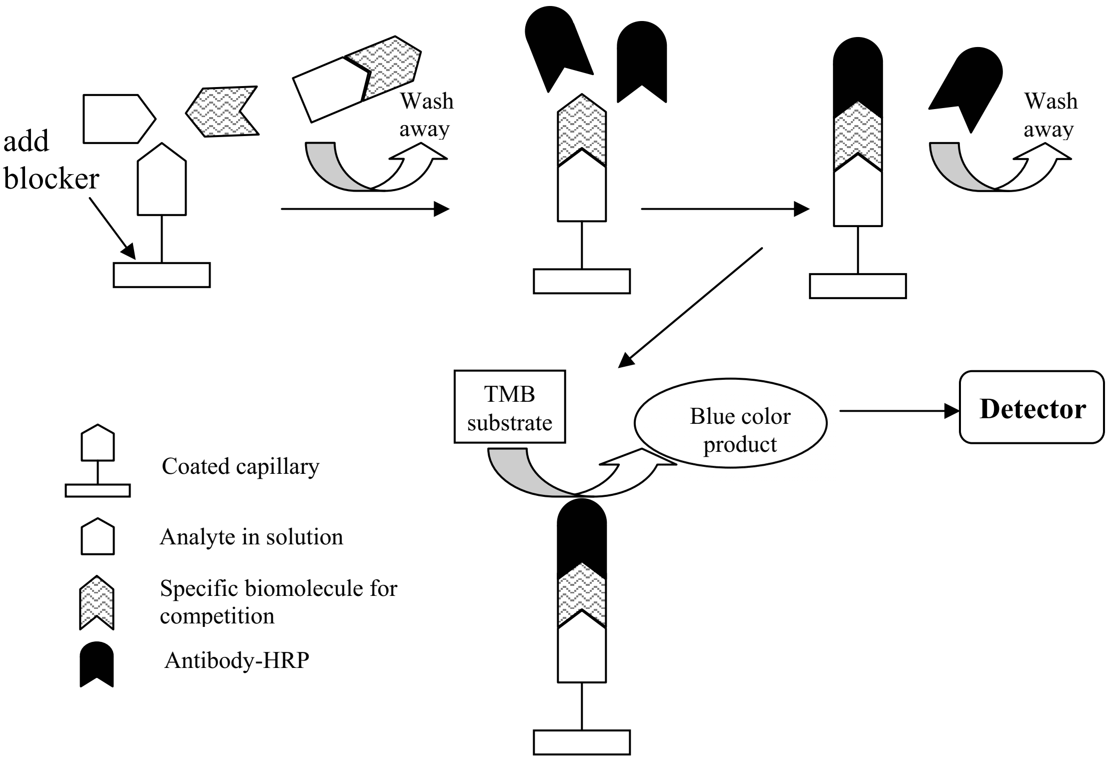
สำหรับการตรวจวัด chondroitin sulfate

Chondroitin sulfate ไม่จับกับผิวแก้วด้วยแรงทางกายภาพ จึงต้องทำการเปลี่ยนแปลงผิวแก้ว เริ่มจากการเตรียมผิวแก้วโดยการแช่แคปปิลลารีใน 6.5% v/v HNO_3 ใน sonic bath 70°C 10 นาที แล้วล้างแคปปิลลารีด้วยน้ำ DI และนำเข้าเตาอบให้แห้งที่ 100°C 2 ชั่วโมง จากนั้นทำการ silanize ผิวแก้วด้วยสารละลาย 10% 3-aminopropyltrimethoxysilane (APTES) ใน dry toluene โดยใช้อุณหภูมิ 80°C 30 นาที แล้วล้างแคปปิลลารีด้วย dry toluene ตามด้วย 95% EtOH และน้ำ DI เพื่อให้ผิวหน้าของแก้วเป็นหมู่ -OH และ เปลี่ยนหมู่ฟังก์ชันให้เป็น aldehyde ด้วยการเติม 2.5% v/v glutaraldehyde ในสารละลายฟอสเฟตบัฟเฟอร์ 0.1 M pH 7.0 ทิ้งไว้ที่อุณหภูมิห้อง 30 นาที ก่อนล้างด้วยน้ำ DI และเก็บไว้ใช้งานต่อไป ซึ่งตอนนี้แคปปิลลารีจะสามารถเกิดพันธะโควาเลนท์กับหมู่ amine บน CS ได้ เช่นเดียวกันควรทำการปิดผิวแก้วส่วนที่ว่างด้วย blocker ที่เหมาะสม เพื่อลดการจับแบบไม่จำเพาะจากสารอื่นๆ

แคปปิลลารีแก้วที่เตรียมได้จากสภาวะที่เลือกแล้ว จะถูกล้างเอาสารส่วนเกินออก ก่อนที่จะนำไปต่อเข้ากับท่อนำสารที่ multi-port selection valve เสมือนเป็นส่วนหนึ่งของท่อที่ใช้ในระบบการไหลทั่วไป

1.3.1.3 พารามิเตอร์ที่ศึกษา

ในงานวิจัยนี้อาศัยหลักการทำอิมมูโนแอสเสย์แบบแข่งขัน(competitive immunoassay) ซึ่งมีขั้นตอนคร่าวๆ ดังแสดงในรูปที่ 2 สารที่ใช้ตรึงบนผิวแก้ว จะแข่งขันกับสารที่สนใจตรวจวัดและมีหมู่ฟังก์ชันคล้ายกับสารที่ตรึง เพื่อจับกับสารชีวโมเลกุลจำเพาะที่เติมเข้าไปพร้อมกันด้วยปริมาณจำกัด หากมีสารที่สนใจในสารละลายตัวอย่างมาก ก็จะมีสารชีวโมเลกุลปริมาณจำกัดดังกล่าว เหลือไปจับกับส่วนที่ถูกตรึงได้น้อย ในทางตรงกันข้าม ถ้าสารตัวอย่างมีปริมาณสารที่สนใจน้อย ก็จะมีสารชีวโมเลกุลจำเพาะนั้นไปจับกับส่วนที่ถูกตรึงได้มากขึ้น สัญญาณของการทำอิมมูโนแอสเสย์แบบแข่งขันจึงแปรผกผันกับความเข้มข้นของสารที่สนใจในสารละลายตัว อย่าง



รูปที่ 2 ขั้นตอนการทำอิมมูโนแอสเสย์แบบแข่งขัน

สำหรับทุกสารบ่งชี้ทางชีวภาพที่ทำการศึกษาในที่นี้ ในแต่ละขั้นตอนมีสารชีวโมเลกุลและสภาวะต่างๆที่ไม่เหมือนกัน แต่ในขั้นตอนการตรวจวัด ใช้แอนติบอดีที่จำเพาะกับ biotin และมีเลเบลเป็นเอนไซม์ horseradish peroxidase และใช้ TMB substrate เหมือนๆกัน เพื่อให้มีสภาวะในการตรวจวัด เช่น ความยาวคลื่นแสงสำหรับวัดค่าการดูดกลืนแสง หรือ ศักย์ไฟฟ้าในกรณีถ้าใช้เทคนิคไฟฟ้าเคมี ก็จะสามารถใช้สภาวะการตรวจวัดเดียวกันได้ด้วยเครื่องตรวจวัดเครื่องเดียวกัน

สิ่งที่ต้องทำการศึกษาสำหรับสารตัวอย่างแต่ละชนิด คือ ในส่วนที่ต่าง ได้แก่ ความเข้มข้นของสารชีวโมเลกุลที่ใช้ตรึง ความเข้มข้นของสารที่ทำให้เกิดการแข่งขัน เวลาการบ่มเพาะในแต่ละขั้นตอน อุณหภูมิที่เหมาะสม สารที่ใช้ block ผิวแก้วเพื่อลดการจับที่ไม่จำเพาะ เป็นต้น ซึ่งวิธีการทดลองสำหรับแต่ละสารที่สภาวะที่เหมาะสม สรุปรวบรวมไว้ดังแสดงในหัวข้อถัดไป

1.3.1.4 ขั้นตอนการทดลองและสภาวะที่เหมาะสม

สำหรับการตรวจวัด sialoglycoconjugates

สารตัวอย่าง: sialoglycoconjugates ในสารละลายตัวอย่าง
สารที่ใช้ตรึงบนผิวแก้ว: Bovine Submaxillary Mucin (BSM) ซึ่งมีหมู่ sialic acid
สารที่มีปริมาณจำกัดสำหรับการแข่งขัน: biotinylated lectin
สารสำหรับการตรวจวัด: anti-biotin-HRP และ TMB

Bovine Submaxillary Mucin (BSM) ซึ่งมีหมู่ sialic acid ที่ตรึงบนผิวแก้วจะทำหน้าที่แข่งขันกับ sialic acid (หรือ sialoglycoconjugates) ในสารละลายตัวอย่าง เพื่อจับกับ b-lectin ที่เติมลงไปพร้อมกันที่ปริมาณจำกัด เมื่อชะส่วนที่ไม่ถูกจับบนผิวแก้วออก แล้วเติมแอนติบอดีที่จำเพาะกับ biotin และมีเลเบลเป็นเอนไซม์ horseradish peroxidase ที่ทำปฏิกิริยากับ substrate TMB ก็จะได้ผลิตภัณฑ์มีสี ที่มีความเข้มแปรผันตรงกับปริมาณแอนติบอดีที่มีเอนไซม์เลเบล หรืออีกนัยหนึ่งคือแปรผันตรงกับปริมาณ sialic acid บน BSM หรือแปรผกผันกับปริมาณ sialoglycoconjugates ในสารละลายนั่นเอง ตารางที่ 1 สรุปสภาวะต่างๆ ที่ใช้ในการทำแอสเสย์ และตารางที่ 2 แสดงขั้นตอนและสภาวะการทำงานของระบบซีเควนเชียลอินเจคชัน

ตารางที่ 1 สภาวะต่างๆ ที่ใช้ในการทำแอสเสย์สำหรับการตรวจวัด sialoglycoconjugates

พารามิเตอร์	สภาวะที่เลือก
ความเข้มข้นของ BSM ที่ใช้ตรึง	100 μg ml^{-1}
สภาวะการตรึงสาร	8°C เป็นเวลา 5-24 ชั่วโมง หรือที่อุณหภูมิห้อง เป็นเวลา 1ชั่วโมง
ความเข้มข้นของ b-lectin	1:50 dilution
ช่วงความเข้มข้นของสารตัวอย่างมาตรฐานสำหรับสร้างกราฟมาตรฐาน	1-10,000 ng ml^{-1} BSM in 6% BSA (working range 50-5,000 ng ml^{-1} BSM)
เวลาบ่มเพาะสำหรับการแข่ง	10 นาที
Washing buffer	0.01 M PBS pH 7.4
Blocking solution	0.1% Tween 20
เวลาบ่มเพาะสำหรับการ block	30 นาที
ความเข้มข้นของ anti-biotin-HRP	1:1000 dilution
เวลาบ่มเพาะสำหรับการจับเลเบลแอนติบอดี	10 นาที
Substrate	Ready to use TMB
เวลาบ่มเพาะสำหรับการตรวจวัด	2 นาที
Carrier solution	Citric phosphate buffer pH 5.0

ตารางที่ 2 ขั้นตอนและสภาวะการทำงานของระบบซีเควนเชียลอินเจคชันสำหรับการตรวจวัด sialoglycoconjugates

Step no.	Operation	Volume (µl)
1	Washing capillary with PBS	1500
2	Aspiration of air to the holding coil	20
3	Aspiration of 0.1%Tween in PBS solution to the holding coil	80
4	Aspiration of air to the holding coil	20
5	Sending 0.1%Tween in PBS solution to capillary and incubate for 3 min	80
6	Removing unbound species by washing capillary with PBS	1000
7	Aspiration of air to the holding coil	20
8	Aspiration of standard BSM solution or serum sample to the holding coil	10
9	Aspiration of biotinylated lectin to the holding coil	60
10	Aspiration of standard BSM solution or serum sample to the holding coil	10
11	Aspiration of air to the holding coil	20
12	Mixing standard BSM solution and biotinylated lectin by moving solution back and forth three times	-
13	Sending mixture to BSM coated capillary and incubate for 10 min	80
14	Removing unbound species by washing capillary with PBS	2000
15	Aspiration of air to the holding coil	20
16	Aspiration of anti-biotin HRP to the holding coil	80
17	Aspiration of air to the holding coil	20
18	Sending anti-biotin HRP to the capillary and incubate for 10 min	80
19	Removing unbound species by washing capillary with PBS	2000
20	Washing with citric phosphate buffer	1000
21	Aspiration of air to the holding coil	20
22	Aspiration of substrate TMB to the holding coil	80
23	Aspiration of air to the holding coil	20
24	Sending substrate TMB to capillary and and incubate with anti-biotin HRP for 2 min	80
25	Discarding air	-
26	Sending the solution product to the detector	50

สำหรับการตรวจวัด hyarolunan

สารตัวอย่าง: hyaluronan ในสารละลายตัวอย่าง

สารที่ใช้ตรึงบนผิวแก้ว: hyaluronan มาตรฐาน

สารที่มีปริมาณจำกัดสำหรับการแข่งขัน: biotinylated HA bining proteins

สารสำหรับการตรวจวัด: anti-biotin-HRP และ TMB

สารละลาย b-HABP จะถูกดูดเข้าสู่แคปปิลารีพร้อมๆกับ สารละลาย HA ที่ความเข้มข้นต่างๆ และ HA ที่อยู่ในสารละลายจะแข่งกันกับ HA ที่ถูกตรึงบนผิวแก้วเพื่อจับกับ HABP ที่มีจำนวนจำกัด เมื่อชะเอาสารละลายออกไป ก็จะเหลือ b-HABP ที่ถูกจับโดย HA ที่ตรึงบนผิวแก้วเท่านั้น การตรวจวัดทำได้โดยการเติมแอนติบอดีที่จำเพาะกับ biotin ซึ่งมีเลเบลเป็นเอนไซม์ hoseradish peroxidase จากนั้นเติม TMB และให้เวลาบ่มเพาะจนเกิดผลิตภัณฑ์มีสี ที่มีความเข้มแปรผันตรงกับปริมาณแอนติบอดีที่มีเอนไซม์เลเบล หรืออีกนัยหนึ่งคือแปรผันตรงกับปริมาณ HA standard บนผิวแก้ว หรือแปรผกผันกับปริมาณ HA ในสารละลายตัวอย่างนั่นเอง ตารางที่ 3 สรุปสภาวะต่างๆ ที่ใช้ในการทดลอง และตารางที่ 4 แสดงขั้นตอนการทำงานของระบบซีเควนเชียลอินเจคชัน

ตารางที่ 3 สภาวะต่างๆ ที่ใช้ในการทำแอสเสย์สำหรับการตรวจวัด hyarolunan

พารามิเตอร์	**สภาวะที่เลือก**
ความเข้มข้นของ std HA ที่ใช้ตรึง	100 μg ml^{-1}
สภาวะการตรึงสาร	8°C เป็นเวลา 24 ชั่วโมง หรือ 37°C เป็นเวลา 5 ชั่วโมง
ความเข้มข้นของ b-HABPs	1:100 dilution
ช่วงความเข้มข้นของสารตัวอย่างมาตรฐานสำหรับสร้างกราฟมาตรฐาน	1-3,000 ng ml^{-1} HA in 6% BSA (working range 25-500 ng ml^{-1} HA)
เวลาบ่มเพาะสำหรับการแข่ง	5 นาที
Washing buffer	0.1 M PBS pH 7.4
Blocking solution	1% w/v BSA
เวลาบ่มเพาะสำหรับการ block	30 นาที
ความเข้มข้นของ anti-biotin-HRP	1:1000 dilution
เวลาบ่มเพาะสำหรับการจับเลเบลแอนติบอดี	5 นาที
Substrate	Ready to use TMB
เวลาบ่มเพาะสำหรับการตรวจวัด	7 นาที
Carrier solution	Citric phosphate buffer pH 5.0

ตารางที่ 4 ขั้นตอนและสภาวะการทำงานของระบบซีเควนเชียลอินเจคชันสำหรับการตรวจวัด hyarolunan

Step No.	Operation	Volume (μl)
1	Washing capillary with PBS	1500
2	Aspiration of air to the holding coil	20
3	Aspiration of standard HA solution or serum sample to the holding coil	10
4	Aspiration of b-HABPs to the holding coil	60
5	Aspiration of standard HA solution or serum sample to the holding coil	10
6	Aspiration of air to the holding coil	20
7	Mixing standard HA solution and b-HABPs by moving solution back and forth twice	-
8	Sending mixture to HA coated capillary and incubate for 5 min	80
9	Removing unbound species by washing capillary with PBS	2000
10	Aspiration of air to the holding coil	20
11	Aspiration of anti-b-HRP to the holding coil	80
12	Aspiration of air to the holding coil	20
13	Sending anti-b-HRP to the capillary and incubate for 5 min	80
14	Removing unbound species by washing capillary with PBS followed by citric phosphate buffer	2000 (each)
15	Aspiration of air to the holding coil	20
16	Aspiration of substrate TMB to the holding coil	80
17	Aspiration of air to the holding coil	20
18	Sending substrate TMB to capillary and incubate with anti-b-HRP for 7 min	80
19	Discarding air*	40
20	Sending the solution product to the detector	50

สำหรับการตรวจวัด chondroitin sulfate

สารตัวอย่าง: chondroitin sulfate ในสารละลายตัวอย่าง

สารที่ใช้ตรึงบนผิวแก้ว: chondroitin sulfate มาตรฐาน หรือ shark A1

สารที่มีปริมาณจำกัดสำหรับการแข่งขัน: WF6 ซึ่งเป็น monoclonal antibodyสำหรับ CS-C

สารสำหรับการตรวจวัด: anti-IgM-HRP และ TMB

สารที่ใช้ตรึงบนผิวแก้วอาจเป็น CS มาตรฐาน หรือ Shark A-1 ที่มีหมู่ฟังก์ชันคล้ายกับ CS ซึ่งจะแข่งกับ CS ในสารละลายตัวอย่างเพื่อจับกับโมโนโคลนอลแอนติบอดีสำหรับ CS-C (หรือ WF6 ซึ่งเป็น IgM) ที่ถูกเติมเข้าไปในปริมาณจำกัด เมื่อชะล้างส่วนเกินออกไป แล้วเติม anti-IgM-HRP และ TMB และให้เวลาบ่มเพาะจนเกิดผลิตภัณฑ์มีสี ที่มีความเข้มแปรผันตรงกับปริมาณแอนติบอดีที่มีเอนไซม์เลเบล หรืออีกนัยหนึ่งคือแปรผันตรงกับปริมาณ CS หรือ shark A1 standard บนผิวแก้ว หรือแปรผกผันกับปริมาณ CS ในสารละลายตัวอย่างนั่นเอง ตารางที่ 5 สรุปสภาวะต่างๆ ที่ใช้ในการทดลอง และตารางที่ 6 แสดงขั้นตอนการทำงานของระบบซีเควนเชียลอินเจคชัน

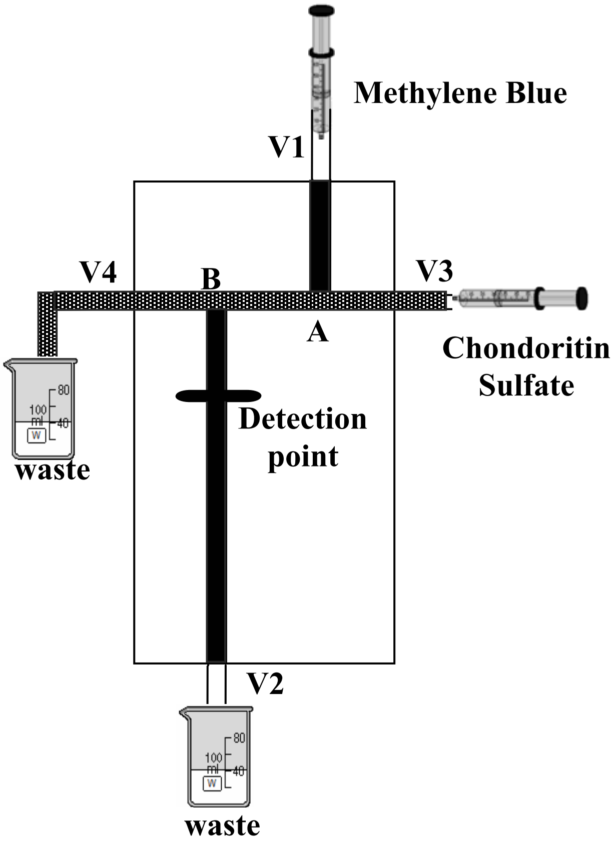
ตารางที่ 5 สภาวะต่างๆ ที่ใช้ในการทำแอสเสย์สำหรับการตรวจวัด chondroitin sulfate

พารามิเตอร์	สภาวะที่เลือก
ความเข้มข้นของ CS หรือ shark A-1 ที่ใช้ตรึง	10 μg ml^{-1}
สภาวะการตรึงสาร	8°C ข้ามคืน หรือ 25°C เป็นเวลา 5 นาที- 24 ชั่วโมง
ความเข้มข้นของ WF6	1:1000 dilution
ช่วงความเข้มข้นของสารตัวอย่างมาตรฐานสำหรับสร้างกราฟมาตรฐาน	10-100,000 ng ml^{-1} shA-1 in 6% BSA (working range 100-100,000 ng ml^{-1} shA-1)
เวลาบ่มเพาะสำหรับการแข่ง	1 นาที
Washing buffer	20 mM carbonate buffer pH 9.6
Blocking solution	1% w/v gelatin
เวลาบ่มเพาะสำหรับการ block	30 นาที
ความเข้มข้นของ anti-IgM-HRP	1:2000 dilution
เวลาบ่มเพาะสำหรับการจับเลเบลแอนติบอดี	3 นาที
Substrate	Ready to use TMB
เวลาบ่มเพาะสำหรับการตรวจวัด	7 นาที
Carrier solution	Citric phosphate buffer pH 5.0

ตารางที่ 6 ขั้นตอนและสภาวะการทำงานของระบบซีเควนเชียลอินเจคชันสำหรับการตรวจวัด chondroitin sulfate

Step no.	Operation	Volume (µL)
1	Washing coated capillary with incubation buffer	2000
2	Aspiration of air to the holding coil	20
3	Aspiration of standard Sh-A1 solution or serum sample to the holding coil	20
4	Aspiration of antibody (WF6) to the holding coil	40
5	Aspiration of standard Sh-A1 solution or serum sample to the holding coil	20
6	Aspiration of air to the holding coil	20
7	Mixing standard Sh-A1 solution and WF6 by moving solution back and forth twice	-
8	Sending mixture to Sh-A1 coated capillary and incubate for 1 min	80
9	Removing unbound species by washing capillary with incubation buffer	1500
10	Aspiration of air to the holding coil	20
11	Aspiration of anti-IgM-HRP to the holding coil	80
12	Aspiration of air to the holding coil	20
13	Sending anti-IgM-HRP to CS/Sh-A1 coated capillary and incubate for 3 min	80
14	Removing unbound species by washing capillary with incubation buffer	1500
15	Aspiration of air to the holding coil	20
16	Aspiration of substrate TMB to the holding coil	80
17	Aspiration of air to the holding coil	20
18	Sending substrate TMB to CS/Sh-A1 coated capillary and incubate for 7 min	80
19	Discarding air	30
20	Aspiration of product solution to the holding coil	50
21	Sending the product solution to the detector by using citric phosphate buffer as a carrier	1000

สำหรับงานที่เกี่ยวกับ chondroitin sulfate นี้ ได้ต่อยอดใช้กับระบบการไหล macro-chip ที่ทำขึ้นอย่างง่ายจากการเจาะท่อในแผ่นพลาสติก และได้ทดลองใช้กับการหาปริมาณ chondroitin sulfate ในตัวอย่างยา โดยใช้ methylene blue เป็นสารประกอบทำให้เห็นการเปลี่ยนแปลงความเข้มสีน้ำเงินที่ลดลงเมื่อ CS แพร่มาทำปฏิกิริยากับ methylene blue ดังแสดงในรูปที่ 3



รูปที่ 3 Macro-chip อย่างง่ายสำหรับการตรวจวัดปริมาณ CS ในยา

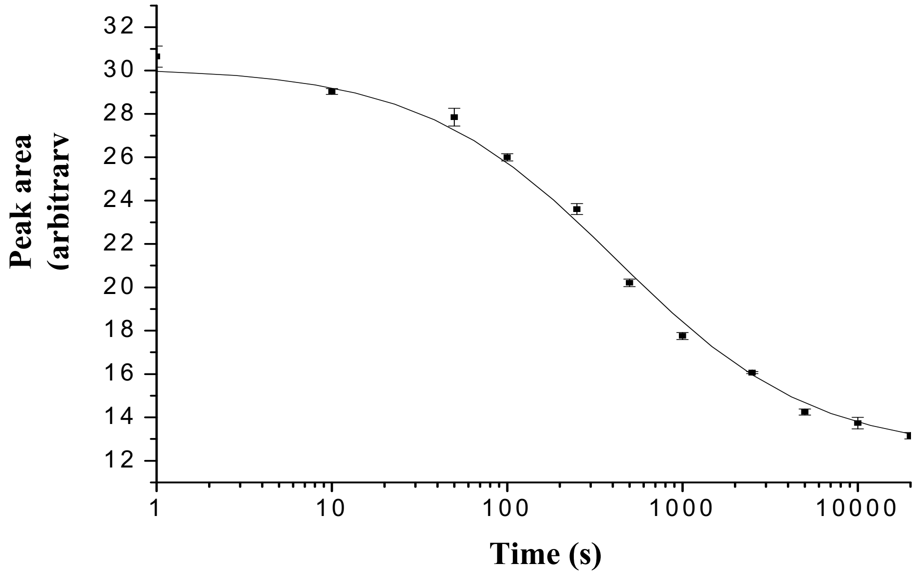
1.3.1.5 การวิเคราะห์สารหลายชนิดจากตัวอย่างเดียวกันด้วยระบบซีเควนเชียลอินเจคชันแคปปิลารีอิมมูโนแอสเสย์

ในที่นี้ได้ทดลองนำแคปปิลลารี 3 แคปปิลารีที่ตรึงด้วยสารชีวโมเลกุลแต่ละชนิด คือ BSM, HA และ Sh-A1 มาต่อเข้ากับวาล์วในรูปที่ 1 โดยใช้ท่อนำสารแยกกันโดยการต่อท่อพลาสติกแยกการไหลให้เป็น 3 ทิศทางเข้าไปในแต่ละแคปปิลารี ซึ่งมีเวลาการบ่มเพาะและกระบวนการวิเคราะห์ที่ต่างกันสำหรับแต่ละสาร จึงหวังว่าจะนำสารจากแคปปิลารีที่ใช้เวลาสั้นที่สุดเข้าสู่เครื่องตรวจวัดก่อน ตามด้วยแคปปิลารีที่ใช้เวลาบ่มเพาะและกระบวนการมากกว่า ตามลำดับ

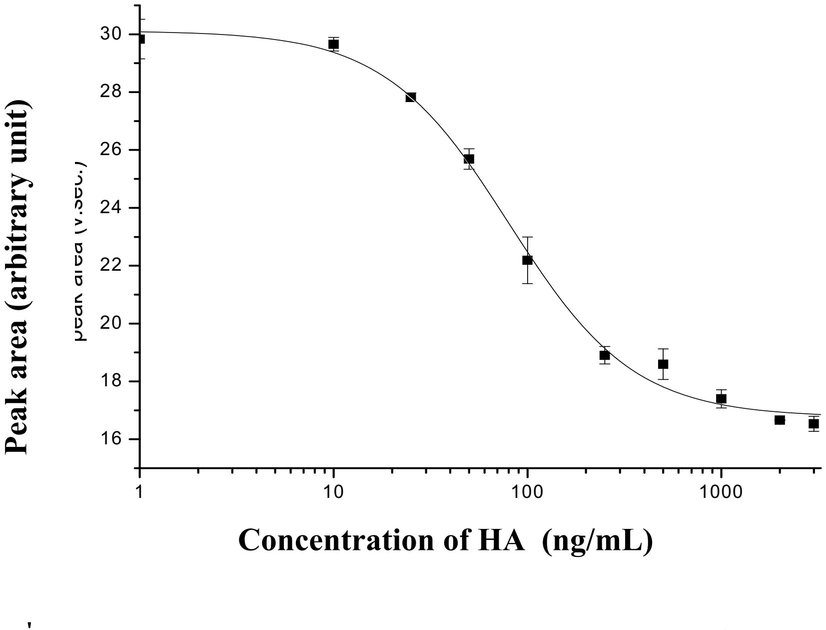
นอกจากนี้ได้ทำการทดสอบอีกแบบหนึ่งคือนำสารทั้ง 3 ชนิดมารวมกันเพื่อตรึงบนแคปปิลลารีเดียวกันไปพร้อมๆกัน โดยใช้เวลาในการตรึงตามสารที่ต้องใช้เวลานานที่สุด แล้วนำมาตรวจวัดทีละสารโดยเปลี่ยนรีเอเจนต์ที่ใช้

1.4 ผลการทดลองและวิจารณ์

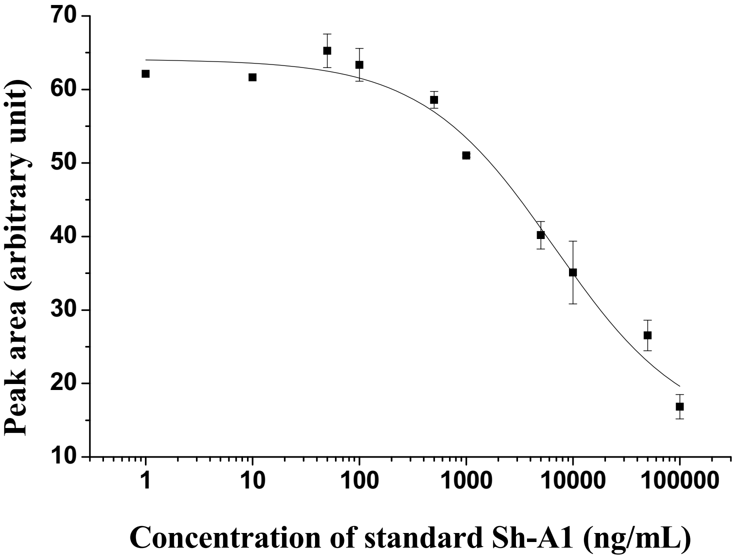
รูปที่ 4-6 แสดงกราฟมาตรฐานสำหรับการตรวจวัดสารบ่งชี้โรคทางชีวภาพ 3 ชนิด คือ sialoglycoconjugates, hyarulonan และ chondroitin sulfate ที่ได้จากระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์ (SI-CI)



รูปที่ 4 กราฟมาตรฐานสำหรับการตรวจวัดปริมาณ sialoglycoconjugates (BSM equivalent) ด้วยระบบ SI-CI



รูปที่ 5 กราฟมาตรฐานสำหรับการตรวจวัดปริมาณ hyarulonan ด้วยระบบ SI-CI

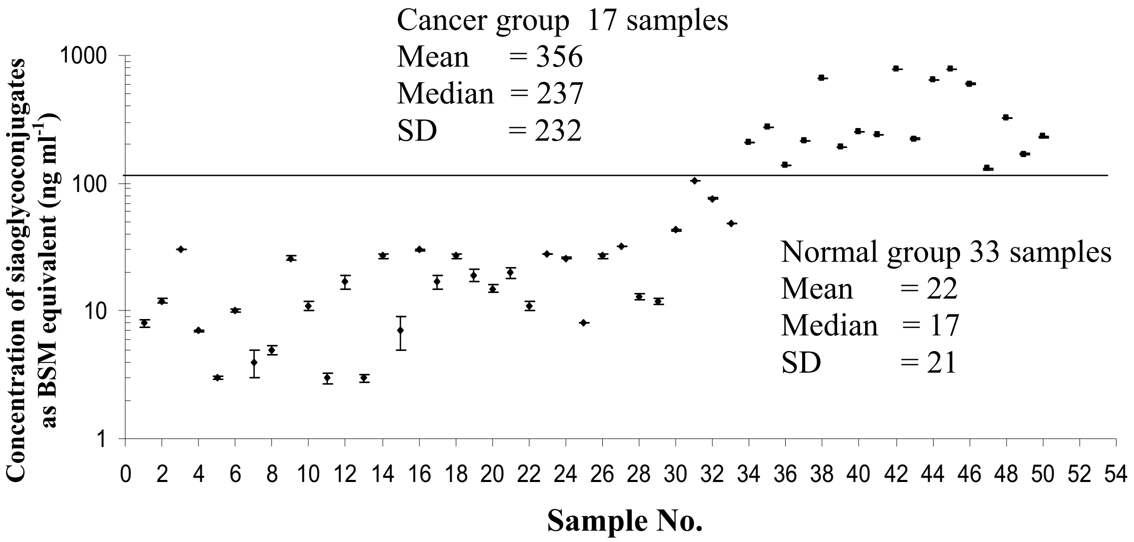


รูปที่ 6 กราฟมาตรฐานสำหรับการตรวจวัดปริมาณ chondroitin sulfate (Sh-A1 equivalent) ด้วยระบบ SI-CI

สำหรับการตรวจวัด sialoglycoconjugates

การศึกษาความแม่นยำของระบบใช้การเติม BSM ที่ทราบความเข้มข้นแน่นอนลงในซีรัมคนปกติ และทำการทดลอง 10 ครั้งในวันเดียวกัน ได้ค่าความคลาดเคลื่อนสัมพัทธ์ (%RSD) เป็น 2.3-2.8% ส่วนความแม่นยำระหว่างวัน พิจารณาจากกราฟมาตรฐานที่สร้างขึ้นในวันต่างกัน คือห่างกัน 1, 4, 10, 20 และ 33 วัน พบว่า %RSDs ที่ความเข้มข้นต่างๆ เป็น 0.7 (1 ng ml^{-1}), 0.7 (100 ng ml^{-1}), 1.1 (250 ng ml^{-1}), 2.2 (500 ng ml^{-1}) และ 2.8 (1000 ng ml^{-1})

ได้ทำการศึกษากับซีรัมตัวอย่างคนปกติ 33 ตัวอย่าง (ผู้ชาย 12 ตัวอย่าง อายุ 23-41 ปี และ ผู้หญิง 14 ตัวอย่าง อายุ 22-74 ปี และ อีก 7 ตัวอย่างไม่ทราบอายุและเพศ) และตัวอย่างซีรัมคนเป็นโรคมะเร็ง 17 ตัวอย่าง (ผู้ชาย 11 ตัวอย่าง อายุ 32-78 ปี และ ผู้หญิง 6 ตัวอย่าง อายุ 41-82 ปี) ปริมาณ sialoglycoconjugates อ่านจากกราฟมาตรฐานเทียบเป็นความเข้มข้นของ BSM (BSM equivalent) ซึ่งกลุ่มคนปกติ กับกลุ่มคนเป็นโรคมะเร็งมีปริมาณ sialoglycoconjugates ต่างกันดังแสดงในรูปที่ 7 ค่าเฉลี่ยเป็น 22 และ 356 ng ml^{-1} ในกลุ่มคนปกติ และคนเป็นมะเร็ง ตามลำดับ โดยตัวอย่างคนปกติมีระดับ sialoglycoconjugates ต่ำกว่า 110 ng ml^{-1} BSM equivalent แต่จำนวนตัวอย่างในการศึกษานี้ยังค่อนข้างน้อย ค่า cut off จึงอาจคลาดเคลื่อนไปได้ อย่างไรก็ตาม ความแตกต่างของปริมาณ sialoglycoconjugates เฉลี่ยที่พบในสองกลุ่มตัวอย่าง ก็ได้แสดงถึงความสามารถของระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์ ในการแยกแยะตัวอย่างซีรัมทั้งสองกลุ่ม

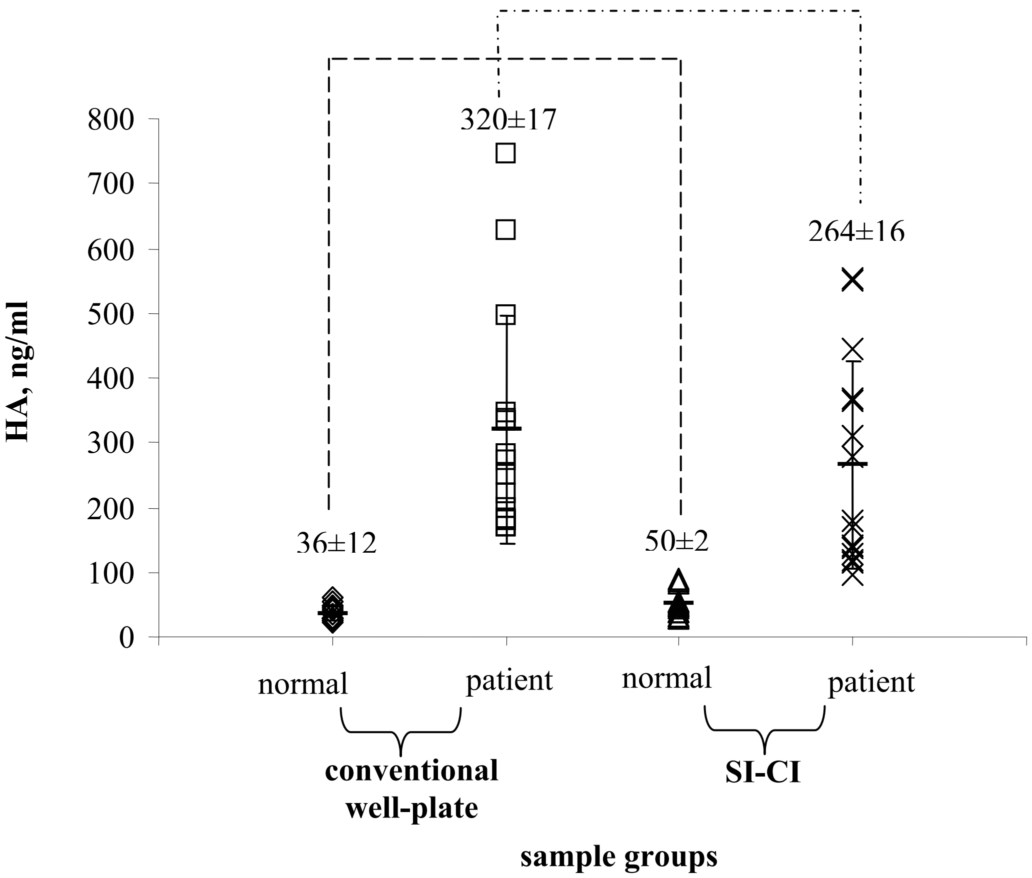


รูปที่ 7 เปรียบเทียบปริมาณ sialoglycoconjugates (รายงานเป็น BSM equivalent) ที่ตรวจพบในตัวอย่างซีรัมคนปกติ 33 ตัวอย่าง และคนเป็นโรคมะเร็ง 17 ตัวอย่าง

สำหรับการตรวจวัด hayarolunan

การศึกษาความแม่นยำของระบบใช้การเติม HA ที่ทราบความเข้มข้นแน่นอนลงในซีรัมคนปกติ และทำการทดลอง 10 ครั้งในวันเดียวกัน ได้ค่าความคลาดเคลื่อนสัมพัทธ์ (%RSD) เป็น 3.1% ส่วนความแม่นยำระหว่างวันใช้ตัวอย่างซีรัมเดียวแต่ทำการทดลองในวันต่างๆกัน 10 วัน พบว่า %RSD เป็น 5.5.

ได้ทำการศึกษากับซีรัมตัวอย่างคนปกติ 15 ตัวอย่าง และตัวอย่างซีรัมคนเป็นโรคตับ 15 ตัวอย่าง โดยทำการทดลองเทียบกับวิธีมาตรฐานแบบเพลทหลุม พบว่าทั้งระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์และระบบเพลทหลุมปกติ สามารถแยกแยะกลุ่มคนปกติจากกลุ่มคนโรคตับได้ รูปที่ 8 แสดงปริมาณ HA ที่ตรวจวัดได้จากตัวอย่างเดียวกันโดยใช้ 2 วิธี ค่าเฉลี่ยกลุ่มคนปกติคือ 36 และ 50 ng ml^{-1} และ ค่าเฉลี่ยกลุ่มคนเป็นโรค คือ 320 และ 264 ng ml^{-1} จากการตรวจวัดด้วยวิธีเพลทหลุม และระบบ SI-CI ตามลำดับ จากการคำนวณทางสถิติ (t-test) พบว่าตัวอย่างแต่ละกลุ่มที่ทำการตรวจวัดโดยสองวิธีมีปริมาณ HA ไม่ต่างกันอย่างมีนัยสำคัญ แสดงว่าระบบที่พัฒนาขึ้นให้ผลตรวจวัดที่เชื่อถือได้ไม่ต่างกับวิธีมาตรฐานที่ใช้อยู่ทั่วไป



รูปที่ 8 ปริมาณ HA ที่ตรวจวัดได้จากตัวอย่างเดียวกันโดยใช้วิธีเพลทหลุม และระบบ SI-CI (◇= normal serum samples and □ = liver patient serum samples obtained from conventional micro-plate assay, △ = normal serum samples and × = liver patient serum samples obtained from SI-CI)

สำหรับการตรวจวัด chondroitin sulfate

การศึกษาความแม่นยำของระบบ พิจารณาจากกราฟมาตรฐานที่สร้างขึ้นใน 4 วัน ต่างกัน พบว่า %RSD เป็น 3.5-5.0.

เนื่องจากข้อจำกัดในการหาตัวอย่างซีรัม ในที่นี้จึงทำการศึกษาจากการนำซีรัม 3 ตัวอย่าง มาผสมรวมกัน (pooled serum) เพื่อให้มีปริมาณมากพอสำหรับการทดลอง และเติม CS มาตรฐานลงในปริมาณต่างๆ เปอร์เซ็นต์การได้กลับคืน (%recovery) คำนวณได้ดังแสดงในตารางที่ 7 พบว่าสามารถใช้กราฟมาตรฐานได้อย่างมีประสิทธิภาพในช่วงความเข้มข้น Sh-A1 100-10,000 ng ml^{-1} ซึ่งให้ค่าการได้กลับคืนในช่วง 89-120% ที่ความเข้มข้นสูงกว่า 10,000 ng ml^{-1} ค่าการได้กลับคืนมีความผิดพลาดสูงมาก

ตารางที่ 7 การศึกษาการได้กลับคืนของ CS ที่เติมลงในซีรัม 3 ตัวอย่าง โดย S1 เป็น single serum และ S2 กับ S3 เป็น pooled samples

Sample	**[CS] as Sh-A1 equivalence**		**%Recovery**
	Expected (ng ml^{-1})	**Found (ng ml^{-1})**	
S1	-	82	-
S1 + 100 ng ml^{-1}	182	185	103
S1 + 1,000 ng ml^{-1}	1082	968	89
S2	-	5652	-
S2 + 500 ng ml^{-1}	6152	6255	120
S3	-	4244	-
S3 + 5,000 ng ml^{-1}	9244	10043	116

หมายเหตุ ตัวอย่างซีรัมทั้งหมดที่ใช้ในงานวิจัยนี้ มีทั้งที่ได้รับบริจาคจากเพื่อนร่วมงาน และได้รับจากกลุ่มวิจัยของ รองศาสตราจารย์ ดร. ปรัชญา คงทวีเลิศ ภาควิชาชีววิทยา คณะแพทย์ศาสตร์ มหาวิทยาลัยเชียงใหม่ เจ้าของซีรัมทั้งหมดได้รับการบอกกล่าวถึงจุดประสงค์การนำซีรัมไปศึกษา และส่วนที่ได้รับจากคณะแพทย์ศาสตร์ ได้ผ่านการตรวจสอบและอนุมัติตามขั้นตอนกระบวนการของการใช้ตัวอย่างจากมนุษย์ของคณะแพทย์ศาสตร์ มหาวิทยาลัยเชียงใหม่แล้ว

การตรวจวัดสาร 3 ชนิดพร้อมกัน

จากผลการทดลองพบว่า การต่อแคปปิลารี 3 อัน เข้ากับเครื่องตรวจวัดเดียวกันโดยใช้ท่อพลาสติกรวมการไหลเข้าสู่ inlet เดียวของเครื่องตรวจวัด แม้สารแต่ละแคปปิลารีจะไหลเข้าสู่เครื่องตรวจวัดที่เวลาต่างกัน แต่มีปัญหาการปนกันของสารที่จุดรอยต่อเชื่อม ทำให้สัญญาณที่ได้ผิดรูปไปจากที่ควรเป็น

การทดสอบอีกแบบหนึ่ง คือนำสารทั้ง 3 ชนิดมารวมกัน แล้วตรึงบนแคปปิลารีเดียวกัน แล้วนำมาตรวจวัดทีละสารโดยเปลี่ยนรีเอเจนต์ที่ใช้ วิธีนี้ไม่ค่อยสะดวกเพราะพอร์ตที่วาล์วเลือกสารมีจำกัด ไม่สามารถต่อเข้ากับสารทุกชนิดที่ต้องการใช้สำหรับ 3 สารตัวอย่างได้ ต้องเปลี่ยนสารเข้าออก ซึ่งก่อให้เกิดการกวนกันของสาร และอาจเนื่องมาจากการใช้พื้นที่แคปปิลารีร่วมกัน ทำให้ปริมาณสารที่ตรึงอาจน้อยเกินไป ไม่สามารถตรวจวัดได้ถูกต้อง

การตรวจวัด chondroitin sulfate ในยาด้วย macro-chip อย่างง่าย

สำหรับงานที่เกี่ยวกับ chondroitin sulfate นี้ ได้ต่อยอดใช้กับระบบการไหล macro-chip ที่ทำขึ้นอย่างง่าย สังเกตสีที่เปลี่ยนแปลงของ mehtylene blue จากการแพร่ของสารเข้าทำปฏิกิริยา

ซึ่งอัตราการแพร่ขึ้นกับความเข้มข้นของสาร แม้ methylene blue ไม่จำเพาะกับ CS แต่สำหรับตัวอย่างที่มีสิ่งเจือปนน้อย ก็สามารถใช้ประโยชน์ได้ดี ในที่นี้ได้ทดลองใช้กับการหาปริมาณ CS ในตัวอย่างยา ซึ่งให้ผลเป็นที่น่าพอใจ ดังแสดงในตารางที่ 8

ตารางที่ 8 ผลการตรวจวัดปริมาณ CS ในตัวอย่างยาด้วย methylene blue ใน macro-chip อย่างง่าย

Sample	**Labeled amount CS mg/tablet**	**Found amount CS mg/tablet**	**% Difference**
I (Japan)	260	255	98 %
II (Germany)	300	318	106 %

1.5 สรุป

ระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์สามารถใช้ตรวจวัดสารบ่งชี้โรคทางชีวภาพ คือ sialoglycoconjugates, hyarulonan และ chondroitin sulfate ในตัวอย่างซีรัมมนุษย์ได้ ระบบที่พัฒนาขึ้นใช้เวลาบ่มเพาะในขั้นต่างๆ สั้นกว่าอิมมูโนแอสเสย์เพลทหลุมปกติมาก ทำให้เวลาวิเคราะห์สำหรับแต่ละตัวอย่างสั้นลงมาก เป็น 25-45 นาทีต่อตัวอย่าง จากเดิม 3-5 ชั่วโมง แม้ว่าวิธีเพลทหลุมมีข้อได้เปรียบคือ สามารถทำการตรวจวัดหลายตัวอย่างในคราวเดียว อย่างไรก็ดีระบบที่พัฒนาขึ้นจะเหมาะกับตัวอย่างจำนวนน้อยที่ต้องการความเร็วนอกจากนี้ ระบบที่พัฒนาขึ้นมีความเป็นอัตโนมัติมาก เพราะควบคุมการทำงานด้วยระบบคอมพิวเตอร์ ทำให้การทำอิมมูโนแอสเสย์ที่ยุ่งยาก สามารถทำได้ง่ายขึ้น ซึ่งจะเหมาะกับที่ที่ขาดแคลนบุคลากรที่ชำนาญทางการแพทย์

การประยุกต์ใช้ระบบซีเควนเชียลอินเจคชันแคปปิลลารีอิมมูโนแอสเสย์ สำหรับการตรวจวัดสารหลายชนิดพร้อมๆ กัน ยังคงต้องมีการพัฒนาต่อไป วิธีการแยกการไหลแล้วรวมเมื่อเข้าสู่เครื่องตรวจวัดยังควรต้องมีการพัฒนาระบบต่อเชื่อม เพื่อลดปัญหาการปนกันของสารจากแคปปิลลารีที่ต่างกัน ส่วนการใช้สารหลายชนิดตรึงบนแคปปิลารีเดียวกัน สารแต่ละตัวมีพื้นที่บนแคปปิลลารีน้อยลง สารที่เข้าสู่เครื่องตรวจวัดก็น้อยลงด้วย จึงยังคงต้องพัฒนาระบบตรวจวัดเช่นเลเบลและเครื่องตรวจวัดที่มีความไวสูงมากขึ้น

การใช้ macro-chip อย่างง่าย สำหรับการตรวจวัดปริมาณสารที่สนใจจากอัตราการแพร่สามารถทำได้ โดยอุปกรณ์มีราคาถูก ไม่ต้องอาศัยเครื่องมือตรวจวัดราคาแพง เพียงสังเกตด้วยตาเปล่าและใช้นาฬิกาจับเวลาเท่านั้น เป็นระบบที่มีขนาดเล็ก พกพาได้ เหมาะกับการตรวจคัดกรองในสถานที่ที่ต้องการได้

1.6 แผนงานวิจัยในอนาคต

- พัฒนาระบบเชื่อมต่อให้มีประสิทธิภาพมากขึ้น เพื่อลดปัญหาการไหลปนกันของสารจากหลายแคปปิลลารี
- ใช้ระบบการตรวจวัดที่มีความไวสูงขึ้น เช่น เทคนิคทางไฟฟ้าเคมี ฟลูออเรสเซนต์เป็นต้น

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2. การศึกษาเบื้องต้นถึงการใช้ประโยชน์สารสกัดจากธรรมชาติสำหรับเคมีวิเคราะห์

2.1 บทนำ

ในปัจจุบัน นักวิจัยด้านเคมีได้ให้ความสนใจกับการทำการวิจัยที่มีความปลอดภัยกับผู้ทดลอง และสิ่งแวดล้อมมากขึ้น โดยจุดประสงค์เพื่อลดอันตราย และผลกระทบต่อสุขภาพอันเนื่องมาจากการใช้สารเคมีอันตรายหรือใช้สารเคมีที่มากเกินไป แนวคิดนี้เรียกว่า เคมีสีเขียว ซึ่งมีวิธีการทำในหลายแนว เช่น ใช้สารเคมีที่มีอันตรายน้อยหรือไม่มีเลยทดแทนสารเคมีอันตราย ลดปริมาณสารเคมีโดยการออกแบบกระบวนการทำงาน ทั้งด้านเครื่องมือ และปฏิกิริยาเคมีแบบใหม่ เป็นต้น [1-4]

ในที่นี้ ผู้วิจัยร่วมกับกลุ่มวิจัย Flow Based Analysis มหาวิทยาลัยเชียงใหม่ ได้ร่วมกันทำการศึกษาเบื้องต้นถึงการใช้ประโยชน์จากสารสกัดจากพืชในท้องถิ่นและผลิตภัณฑ์ธรรมชาติที่ราคาประหยัดและหาได้ง่ายในท้องตลาด เพื่อนำมาใช้ในการทำเคมีวิเคราะห์ ผู้วิจัยมีแนวคิดที่จะรวบรวมข้อมูลเหล่านี้ เพื่อศึกษาถึงความเป็นไปได้ในการใช้สารสกัดทางธรรมชาติหล่านี้ในขั้นตอนการตรวจวัดของไบโอแอสเสย์ เช่น ใช้เป็นเลเบลของแอนติบอดี เป็นต้น หากเป็นไปได้ก็จะช่วยลดค่าใช้จ่ายการทำไบโอแอสเสย์ได้

ในที่นี้ กลุ่มผู้ร่วมวิจัยได้ทดลองศึกษาสารสกัดที่มีสีเปลี่ยนตามค่า pH เช่น ดอกกล้วยไม้ ดอกอัญชัน บีทรูท [5] และสารที่เกิดสารประกอบที่มีสีกับโลหะ เช่น ใบฝรั่ง มาแล้ว [6] ผู้วิจัยได้มาต่อยอดศึกษาสารที่สามารถเกิดสารประกอบกับโลหะบางชนิด เช่น ใบชาเขียว สารให้สีจากดอกบัว และเอนไซม์ เช่น persoxidase จากหัวไชเท้า มาใช้ร่วมกับเทคนิคการไหลและการตรวจวัดในรูปแบบต่างๆ

2.2 วัตถุประสงค์

เพื่อทำการศึกษาเบื้องต้นของการใช้ประโยชน์จากสารสกัดธรรมชาติจากพืชในท้องถิ่นในการวิเคราะห์ทางเคมี ซึ่งอาจใช้ประโยชน์ในขั้นการตรวจวัดไบโอแอสเสย์ได้ในอนาคต

2.3 การทดลอง

2.3.1 ระบบการไหลรูปแบบต่าง ๆ

ระบบการไหลที่ใช้ได้แก่ ระบบโฟลอินเจคชันอย่างง่าย ระบบโฟลอินเจคชันร่วมกับ web cam สำหรับตรวจวัดความเข้มสีจากค่า RBG ของสาร ระบบการไหลที่สังเกตการแพร่ของสารด้วยตาบน macro-chip และระบบซีเควนเชียลอินเจคชันแลบออนวาล์ว ระบบเหล่านี้ได้รับการพัฒนาอย่างต่อเนื่องโดยผู้ร่วมวิจัย คือ ศาสตราจารย์ ดร. เกตุ กรุดพันธ์ ผู้ช่วยศาสตราจารย์ ดร. สมชัย ลาภอนันต์นพคุณ และนักศึกษาปริญญาเอก นายวศิน วงศ์วิไล ผู้วิจัยได้ร่วมศึกษาสารสกัดธรรมชาติโดยใช้ระบบโฟลอินิเจคชันอย่างง่าย และระบบซีเควนเชียลอินเจคชัน ในงานวิจัยนี้

2.3.2 วัตถุดิบสำหรับสกัด

ได้ทดลองใช้ ใบชาเขียวแห้งเป็นแหล่งสารที่สามารถเกิดสารประกอบมีสีกับไอออนของโลหะ กลีบดอกกุหลาบแดงและดอกบัวสัตตบงกชเป็นแหล่งสารที่เปลี่ยนสีตามสภาพกรดเบส และหัวไช้เท้าเป็นแหล่งเอนไซม์เปอร์ออกซิเดส โดยการสกัดสารเหล่านี้ใช้น้ำเป็นตัวสกัดเพื่อความง่าย ประหยัดและปลอดภัย

2.4 ผลการทดลองและวิจารณ์

ได้ลองสกัดชาเขียวในตัวกลางต่างๆ คือ น้ำ สารละลายอะซิเตทบัฟเฟอร์ และกรดไนทริก พบว่าสารละลายบัฟเฟอร์ pH 4.8 เหมาะสมที่สุด เกิดสารประกอบมีสีกับไอออนของเหล็ก วัดค่าการดูดกลืนแสงได้ที่ 570 nm ซึ่งเมื่อนำไปประยุกต์ใช้กับระบบโฟลอินเจคชันซึ่งทำให้สามารถทำการตรวจวัดได้ถึง 180 ครั้งใน 1 ชั่วโมง และสามารถใช้หาปริมาณเหล็กในตัวอย่างยาวิตามินได้ โดยสามารถทนการรบกวนของไอออนบางชนิดที่อาจพบในยา เช่น Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cr^{3+}, Cu^{2+} และ Mn^{2+} ได้อย่างน้อยในอัตราส่วนความเข้มข้น 1:1 เมื่อคำนวณทางสถิติ พบว่าผลการวิเคราะห์โดยใช้ระบบการไหลร่วมกับสารสกัดชาเขียว กับวิธีวิเคราะห์เหล็กในยาโดยวิธีมาตรฐาน AOAC ไม่มีความต่างกันอย่างมีนัยสำคัญ ดูจากตารางที่ 9

ตารางที่ 9 ปริมาณเหล็กในยาตัวอย่าง ที่ได้จากการวิเคราะห์โดยระบบการไหลร่วมกับสารสกัดชาเขียว และวิธีมาตรฐาน AOAC เทียบกับค่าที่ระบุบนฉลาก

Sample No.	Form of iron	Amount of Fe(II) / mg $tablet^{-1}$			% Deviation from AOAC
		Label amount	AOAC method	The proposed FI –green tea extract	
1	Fumarate	200	183	184 ± 4	0.5
2	Fumarate	200	209	208 ± 7	-0.5
3	Fumarate	200	204	201 ± 16	-1.5
4	Fumarate	90	94	100 ± 3	6.4
5	Sulfate	135	116	107 ± 4	-7.8
6	Gluconate	250	244	246 ± 5	-0.8

สารมีสีจากดอกบัวและกุหลาบซึ่งคาดว่า คือแอนโทไซนานิน ซึ่งเปลี่ยนสีตามสภาพกรดเบสที่เปลี่ยนไป สามารถนำมาใช้เป็นอินดิเคเตอร์หาปริมาณกรดในผลิตภัณฑ์น้ำยาทำความสะอาด ได้อย่างมีประสิทธิภาพดีพอๆ กับอินดิเคเตอร์ทางการค้า เช่น ฟีนอล์ฟทาลีน สีของสารสกัดจะเปลี่ยนชัดเจนจากชมพูแดงในกรดเป็นสีเหลืองในเบส เหมาะแก่การใช้กับการไทเทรตอย่างง่าย

สำหรับการศึกษาการใช้ประโยชน์เอนไซม์ peroxidase จากพืชนั้น ผู้วิจัยเห็นว่า ไบโอแอสเสย์ที่อาศัยเอนไซม์เป็นเลเบล มักใช้ horseradish peroxidase จึงต้องการลองใช้เอนไซม์ที่สกัด

อย่างหยาบจากพืชที่มีในท้องถิ่นและราคาไม่สูงมากเพื่อเป็นอีกทางเลือกหนึ่ง โดยในการศึกษาเบื้องต้นนี้ ต้องการให้แน่ใจว่าเอนไซม์ peroxidase สกัดหยาบ สามารถนำมาใช้ได้จริงในปฏิกิริยาเอนไซม์-ซับสเตรท ซึ่งพบว่าสามารถใช้ตรวจวัดปริมาณไฮโดรเจนเปอร์ออกไซด์ในตัวอย่างจริงได้ดังแสดงในตารางที่ 10

ตารางที่ 10 ปริมาณไฮโดรเจนเปอร์ออกไซด์ในตัวอย่างน้ำยากัดสีผม ที่ตรวจวัดได้จากระบบระบบซีเควนเชียลอินเจคชัน และเอนไซม์สกัดหยาบจากหัวไชเท้า

Samples	%w/v H_2O_2 found with SI-LAV radish extract	% label
1	2.8±0.0	3
2	2.6±0.1	3
3	5.4±0.1	6
4	5.8±0.1	6
5	5.3±0.1	6

2.5 สรุป

สารสกัดจากชาเขียวสามารถนำมาหาปริมาณเหล็กในยาวิตามินได้ ในขณะที่เอนไซม์จากหัวไชเท้าใช้หาประมาณไฮโดรเจนเปอร์ออกไซด์ในผลิตภัณฑ์กัดสีผมได้ โดยสารสกัดทั้งหมดเป็นการสกัดแบบง่ายโดยใช้น้ำและบัฟเฟอร์ที่ไม่มีอันตราย ไม่ได้ผ่านการทำบริสุทธิ์ แต่สามารถนำมาใช้กับระบบการไหลในรูปแบบต่างๆ ได้ดีเป็นที่น่าพอใจ สารสกัดจากดอกบัวและกุหลาบนำมาใช้ในการไทเทรตอย่างง่ายหาปริมาณกรดได้อย่างมีประสิทธิภาพ ในแง่การใช้ประโยชน์กับไบโอแอสย์ เอนไซม์จากพืชท้องถิ่นเป็นสิ่งที่น่าสนใจที่อาจนำไปต่อยอดได้มากที่สุด สารสกัดที่สามารถเกิดสารประกอบมีสีได้กับไอออนของโลหะบางชนิดก็อาจนำไปใช้ประโยชน์ในด้านนี้ได้ ถ้าอยู่ในสภาวะที่เหมาะสมและมีความจำเพาะต่อโลหะใดโลหะหนึ่ง โดยเฉพาะกับกรณีที่ใช้โลหะเป็นเลเบล เช่น quantum dot immunoassay จึงยังเป็นสิ่งที่น่าศึกษาค้นคว้าต่อไป และสารจากดอกบัวที่เปลี่ยนสีตามสภาพกรดเบส อาจใช้ประโยชน์โดยตรงด้านการตรวจวัดไบโอแอสเสย์ได้ไม่ดีนัก แต่งานวิจัยนี้ก็ได้ผลพลอยได้ที่น่าสนใจ คือความเป็นไปได้ที่จะใช้ดอกไม้อันเป็นสัญลักษณ์ในทางพุทธศาสนาซึ่งจะสามารถเก็บดอกบัวที่ใช้บูชาพระแล้วมาทำประโยชน์เพิ่มมูลค่าได้

2.6 แผนงานวิจัยในอนาคต

ควรมีการศึกษาต่อในด้านการเก็บรักษาสารสกัดจากธรรมชาติเพื่อใช้งานได้นานขึ้น และศึกษาความสัมพันธ์ของปริมาณเอนไซม์ที่สกัดได้กับส่วนต่างๆ ของพืชต่อไป

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Output ที่ได้จากโครงการ

งานวิจัยตีพิมพ์ในวารสารวิชาการระดับนานาชาติซึ่งมีทุนวิจัยนี้เป็นผู้สนับสนุนหลัก จำนวน 6 เรื่อง

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ผลงานตีพิมพ์อื่น ๆ จำนวน 1 เรื่อง

1. ชาเขียว เคมีสีเขียว โดย ปิยะนุช ปิ่นอยู่ และ สุภาภรณ์ ครัดทัพ บทความสำหรับตีพิมพ์ในวารสารคณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ เพื่อเผยแพร่ความรู้ให้เยาวชนระดับมัธยมศึกษา

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ผลงานนำเสนอแบบโปสเตอร์ในงานประชุมวิชาการระดับนานาชาติ จำนวน 8 เรื่อง

1. Sequential Injection-Capillary Immunoassay for Chondroitin Sulphate Proteoglycans, S. Khonyoung*, J. Jakmunee, P. Kongtawelert, K. Grudpan, S. Kradtap Hartwell, P-34, 15th International Conference on Flow Injection analysis (ICFIA 2008), Nagoya, Japan, September 28- October 3, **2008**

2. Simple Labs on Chip Approach with Time-based Detection, K. Grudpan*, J. Jakmunee, W. Jangbai, K. Jitmanee, J. Junsomboon, T. Kanyanee, S. Kradtap Hartwell, W. Kumutanant, A. Laokuldilok, S. Lapanantnoppakhun, P. Nuntaboon, S. Pencharee, K. Ponhong, W. Siriangkhawut, W. Sripaoraya, S. Suphareok, S. Tontrong, K. Watla-iad, W. Wongwilai, P-105, 15th International Conference on Flow Injection Analysis (ICFIA 2008), Nagoya, Japan, September 28- October 3, **2008**
3. Green Tea Extract as an Alternative Reagent for Flow Injection Determination of Iron, P. Pinyou*, J. Jakmunee, S. Lapanantnoppakhun, K. Grudpan, S. Kradtap Hartwell, Pure and Applied Chemistry International Conference (PACCON), Phitsanulok, Thailand, January 14-16, **2009**
4. Assay of Chondroitin Sulfate with Time Based Detection in a Simple Macro-Channel Reaction Flow Cell, W. Sripaoraya*, S. Lapanantnoppakhun, K. Grudpan, S. Kradtap Hartwell, Pure and Applied Chemistry International Conference (PACCON), Phitsanulok, Thailand, January 14-16, **2009**
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7. Online Capillary Based Competitive Enzyme-Linked mmunosorbent Assay for Detection of Zeatin Riboside, S. Konyoung*, K. Sringam, J. Jamunee, K. Grudpan, S. Kradtap Hartwell, 16th International Conference on Flow Injection Analysis (ICFIA 2010), Pattaya, Thailand, April 25-30, **2010**
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ผลิตและพัฒนานักศึกษาให้เรียนรู้งานวิจัย โดยทุนวิจัยนี้ได้ให้การสนับสนุนบางส่วนแก่นักศึกษาเพื่อต่อยอดงานวิจัย จำนวน 9 คน

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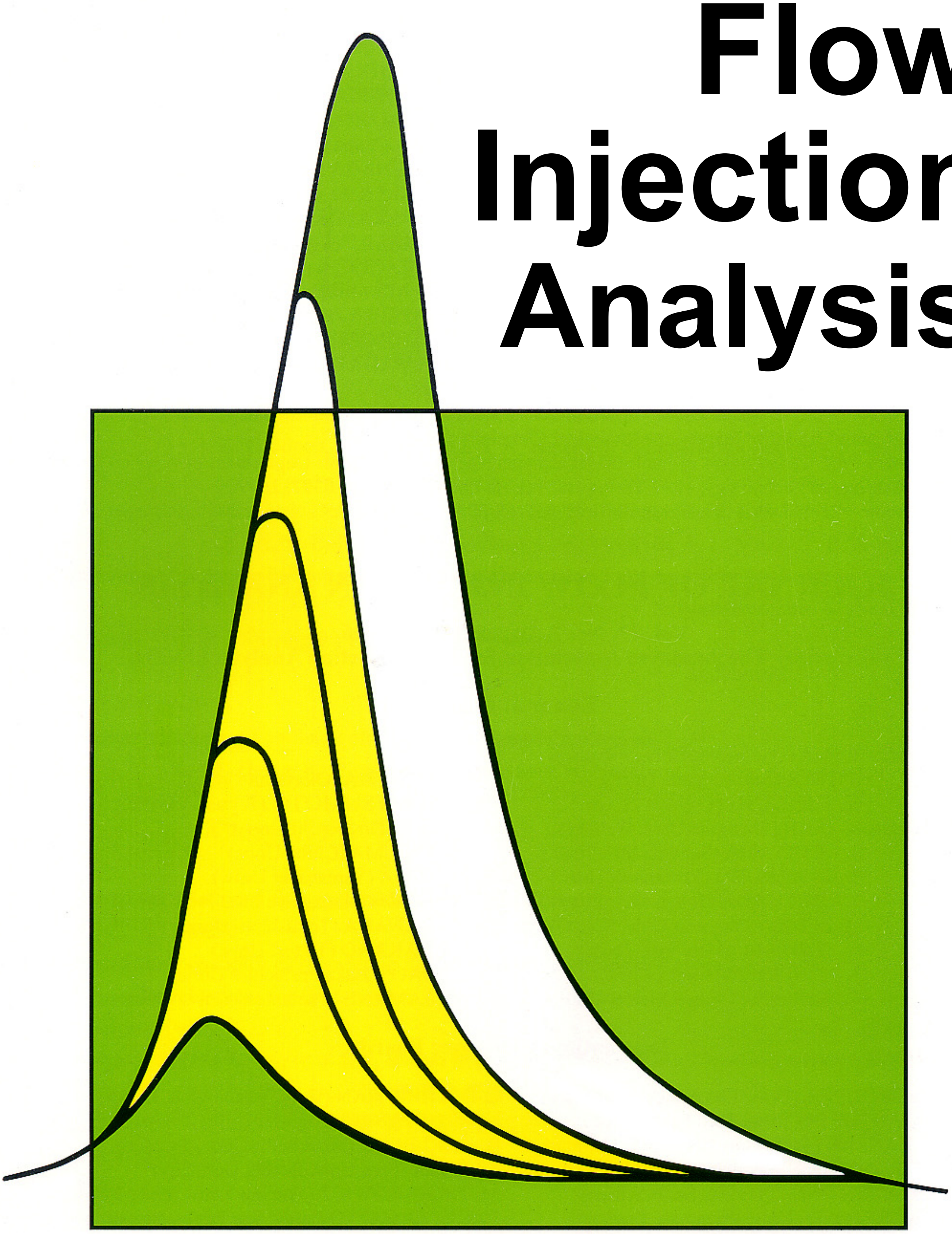

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Flow Based Clinical Analysis Systems: Potential Applications for Telehealth

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Abstract

Telehealth and telemedicine are the approach to provide health care coverage to people in rural areas or patients who cannot conveniently travel to meet with doctors at a hospital. The telehealth system requires a multidisciplinary collaboration involving various sectors such as telecommunications, information technology, medical experts, hospitals, equipment suppliers and social workers. The advantages of automatic flow based systems in reducing the volume of sample, the analysis cost, the analysis time and the need of having highly trained personnel, make them suitable for use where the healthcare facility budget is inadequate. Therefore, flow based techniques have potential for applications in clinical analysis as part of the telehealth system in the developing countries.

Keywords telehealth, flow based techniques, clinical analysis, flow injection analysis, sequential injection analysis

1. Introduction

Telehealth

Definition of telehealth and telemedicine is a delivery of health-related services and medical information via telecommunication technologies for the purpose of consulting, examinations and remote medical processes. [1] This idea was introduced several years ago as a new approach to provide health care coverage for people who cannot visit doctors in person. It began to take place in the USA in the mid-90's. [2] Some activities that can be adapted for use in telehealth/telemedicine include health advisory by phone in emergency cases, live video conferencing, and transmission of medical images.

The possibility of having health service without actually seeing doctors will especially benefit people who are facing a natural disaster, soldiers that are patrolling in the remote areas and people who live far away from any hospital. Even people who live in urban areas but whose busy life style results in an unmatched schedule with their doctors will benefit from a telehealth system.

Although, the idea of telehealth has been around for a long time, the development of an effective telehealth system has not yet been reported. This might be due to the need of collaboration between various sectors such as doctors, social services, telecommunication technologies experts, health insurance companies and government.

Why do we need a telehealth system?

In highly advanced countries such as the USA and some European countries, although there is no limitation on technologies, people may still want to have a telehealth system for better choices of doctors that may not live in close by areas. It has also been proven that in many psychology treatment cases, telephone consulting is better than confrontation between patient and doctor. [3-4] People who live far from a hospital may also prefer not to travel such a great distance to the nearest city for a health check up.

The need of telehealth systems in Thailand and other developing countries may arise from different concerns. The lack of technologies, inadequate number of hospital facilities and medical personnel, and the lack of safe and effective transportation from rural area to the urban hospital are the main limitations in the health service system. Some statistics on the number of facilities and doctors in Thailand are shown in Table 1. Comparison of the number of doctors per 1000 people in different countries around the globe, reported by WHO, is also shown in Table 2. It is alarming that in most developing countries including Thailand, this number is less than 0.5 doctor per 1000 people. [5,6]

Thailand actually has become known for world class medical services. A recent report showed that each year there are as many as 1.2 million tourists from Japan, Europe and the Middle East have come to Thailand and scheduled to have their health checked while on vacation. [7] These services are conducted by highly skilled doctors and with advanced machineries located in hospitals in big cities. These services are available for people who have adequate financial support and accessibility. However, for people in the rural areas and low income group, the problem of ineffective healthcare coverage exists. An effective telehealth system should help to shrink down this gap and make healthcare available to more people.

Telehealth in Thailand

In Thailand some attempts have been made to support the development of telehealth. Some software companies have promoted specialized software for long distance data transfer.[8] Some scientific research works that gear toward development of instrumentation, robots and other devices for the purpose of medical care have been proposed. [9,10] In the southern area where there are situations that make

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traveling to the hospital unsafe, telephone consulting services have been set up for medical and health treatment.[11] The main project involves the set up of a satellite communication link for the remote villages near the Myanmar border to serve the telehealth program.[12] However, there has not been any concrete national telehealth project going on. They are in the trial period and it is still not clear how much it costs or who should pay for it. In the developing countries like Thailand, apart from collaboration of different sectors and people as previously mentioned, the effective telehealth system should be composed of simple and cost effective screening/diagnosis systems. If these systems are set up and operated at health centers near where people live, they will help cut down the analysis and transportation costs and make traveling to crowded urban hospitals unnecessary. Results can be transferred via telecommunication technologies and the decisions on further treatment can be made by doctors in town.

Table 1 Statistics on medical facilities in Thailand (Reported by Thailand Ministry of Public Health in 2004) [5]

Region	No. of hospital with overnight beds	No. of bed	Bed:Patients	No. of doctors	Doctor:Population
Bangkok	111	25,596	1:4	6,526	1:879
Central	360	38,211	1:8	4,752	1:3,134
Northeast	345	28,736	1:10	2,875	1:7,466
North	259	23,807	1:9	2,639	1:4,534
South	203	16,895	1:8	2,126	1:3,982
TOTAL	**1,278**	**133,245**	**1:7**	**18,918**	**1:3,305**

Table 2 Number of doctors per 1000 people in different selected countries (Reported by WHO) [6]

Countries	No. of doctors	No. of doctors : 1000 people
Europe		
Belgium	46,268	4.49
Denmark	15,653	2.93
France	203,487	3.37
Germany	277,885	3.37
Spain	135,300	3.30
Sweden	29,122	3.28
UK	133,641	2.30
North America		
Canada	66,583	2.14
USA	730,801	2.56
Asia/ Oceania-Asia Pacific		
Australia	47,875	2.47
Cambodia	2,047	0.16
China	1,364,000	1.06
Indonesia	29,499	0.13
Japan	251,889	1.98
Malaysia	16,146	0.7
Singapore	5,747	1.4
South Korea	75,045	1.57
Thailand	22,435	0.37
Vietnam	42,327	0.53
Africa		
Uganda	2,209	0.08
Zambia	1,264	0.12

2. Possibilities of flow based techniques for telehealth in Thailand

Flow based techniques have many features that are suitable for bioassay in clinical samples. Being closed systems helps to reduce the chance of sample contamination as well as decrease the risk of the operator having direct contact with body fluid samples. Reactions are at a small scale which require only a minute amount of sample and produce little waste. Capability of detection for chemical product at non-equilibrium state helps to shorten the analysis time. The whole analysis process can be made automatic which can reduce personal error and has less requirement for well trained medical personnel to operate the test. Parts of flow based systems can be built and adapted separately as needed, resulting in lower cost systems as compared to most instrumental based chemical analysis techniques. Data are normally collected by a computer which enables them to be easily transferred online. All these benefits make flow based systems have high potential to be put to use in a telehealth system.

Many groups around the world have reported flow based analysis systems for various biochemical substances in clinical samples. In Thailand, the Flow Based Analysis (FBA) Group at Chiang Mai University has pioneered the flow based screening/diagnosis system for some diseases based on different biomarkers. These systems are developed based on the interest of medical doctors in Thailand which can also be adapted for the international use. The following are examples of flow based disease screening systems that have been developed by the FBA Group with the aim of improving the performance of the existing batch technologies in terms of automation, analysis time, sample volume and cost. The comparison of features and performances of the flow based systems and batch systems are summarized in Table 3. All these systems were evaluated with real blood samples which indicates their potential for the future use in a telehealth system.

Table 3 Comparison of features and performances of some flow based systems and batch systems for disease screening/diagnosis

Flow based systems	**Conventional techniques**	**Disease (Analyte/Sample)**	**Comparison of performances Flow based system vs Batch**	**Ref. No.**
FI-reduced volume anion exchange chromatography	Batch micro-column	β-thalassemia (HbE/packed red cells)	35 min /run vs 4 h 80 μL of 50 fold dilution packed red cells (1.6 μL of undiluted sample) vs 2 mL undiluted sample	13
FI-immunoaffinity micro-chromatographic column	Well plate ELISA	Ovarian cancer (chondroitin-6-sulphate/serum)	30 min/run vs 5-8 h 200 μL of 2 fold dilution serum vs 300-400 μL Reusability of column for 90 runs	14
FI-bead injection	Well plate ELISA	Osteoporosis (Bone ALP/serum)	30 min/run vs 4 h 200 μL vs 300-400 μL Better differentiation of bone diseases from healthy cases as compared to commercial ELISA kit	18
SI-bead based immunoassay	Well plate ELISA	Cancer, Liver and Bone diseases (hyaluronan/serum)	30 min/run vs 5-8 h 30 μL vs 300-400 μL	19

2.1 Flow injection (FI) based system

Some simple first generation flow injection systems have been coupled with various standard analytical techniques to scale down the amount of time and reagents while improving precision, the ease of operation and interpretation of results.

FI- reduced volume anion exchange chromatographic system for hemoglobin typing

One of the screening techniques for HbE thalassemia commonly used in Thai hospitals is micro-column anion exchange chromatography. A plastic syringe with the plunger removed is used as a column for packing DEAE sephadex resin. Packed red cells are passed through the column and each type of hemoglobin, with different degree of net negative charge, can be eluted by pH gradient buffers. Fractions of eluate are collected to measure for absorbances with a spectrophotometer. When plotting absorbances with elution time or volume, a chromatogram of peak separation can be obtained. Order of hemoglobin eluted are in the order of HbA2, followed by HbA and HbF. HbE will co-elute with HbA2, causing an abnormally large HbA2 peak as compared to a normal blood sample. By coupling a smaller plastic column of 3 mm i.d. x 2 cm length with the flow injection-spectrophotometric detection system, this hemoglobin typing technique can be done more automatically with much smaller blood sample volume and much less analysis time. [13]

FI-immunoaffinity micro-chromatographic column system for chondroitin sulphate proteoglycans (CSPGs)

The flow injection system was also successfully coupled to a mini-immunoaffinity column made of a 3 mm i.d x 5 cm length persplex glass packed with WF6 coupled beads. WF6 is specific antibody against chondroitin 6 sulphate. This separation technique can be used as an alternative method for determination of CSPGs by ELISA. The system is based on selective retention of CSPGs before elution with different buffers. Quantitative analysis was done indirectly from the assay of protein contents of proteoglycans by Bradford reagent. Relative amount of CSPGs in total amount of proteoglycans was compared for serum samples of patients with various cancers. The results show the same trends as the previous preliminary study using ELISA which is that CSPGs seem to more specifically indicate ovarian cancers. This flow based immunoaffinity chromatographic system involves easier operational steps as compared to standard (well) ELISA and it is more economical because of the reusability of the column. [14]

FI-bead injection (FI-BI) system for bone alkaline phosphatase (BALP)

Bead injection (BI) technique utilizes beads as solid surfaces to selectively retain analyte of interest and accommodate chemical reaction. The first generation flow injection system is successfully coupled to the bead injection technique for various uses.[15] The early reported FI-BI systems used Fe as a model analyte.[16,17] The flow injection-bead injection system was developed for the assay of bone specific alkaline phosphatase as an alternative to other more complicated techniques such as HPLC and ELISA. The system utilized wheat germ coated beads to specifically capture BALP. The subsequent introduction of substrate PNPP into the bead retention cell yielded yellow product PNP that flowed into and was detected by a spectrophotometer. The used beads were discarded to eliminate the memory effect. Interpretation of the results is based on peak height or peak area which is more precise and easier as compared to observation of the degree of precipitation which is normally observed with bare eyes in the conventional wheat germ agglutination technique. The system is also superior to a commercial ELISA kit in that it can better differentiate bone osteoporosis patients from healthy people which is probably due to the higher surface area of beads as compared to a micro-well.[18]

2.2 Sequential injection (SI) based system

Sequential injection system with advanced small volume manipulation capability and precise timing is very suitable for automation of an analytical technique with multi-steps operation such as immunoassay. An example is described here.

SI-bead based immunoassay for hyaluronic acid (HA)

An automatic immunoassay system was developed to determine the amount of HA in serum of patients with cancer and liver diseases. The system was designed to 1) reduce back pressure from beads that act as solid surfaces for immobilization of the target substance by using opened end cell, 2) reduce dispersion and dilution of the reagent during incubation by separation the reagents zone with air segment, and 3) maximize signal while minimizing incubation time by employing a sensitive detector. Competitive ELISA was performed with HA coupled bead, biotinylated HABPs and anti-biotin-HRP. Product from substrate TMB was electrochemically detected with an amperometer. Sample volume and analysis time per sample were dramatically reduced as compared to the standard well ELISA. The automatic immunoassay system clearly demonstrates the benefit of SI system on reducing the need of having highly experienced personnel to operate a complicated multi-steps analytical technique. [19]

3. Conclusion remarks

Having presented some examples, there are many steps yet to be done on further development of the flow based clinical analysis systems to enable them to be put to use for telehealth system. These steps include miniaturization and standardization of equipment parts, stabilization of reagents for long storage duration, and collaboration of doctors for study and evaluation of the system in real medical situation. However, we hope that our on going attempts on the development of simple and cost effective flow based clinical analysis systems will help in completing the picture of an effective telehealth system in Thailand and elsewhere in the future.

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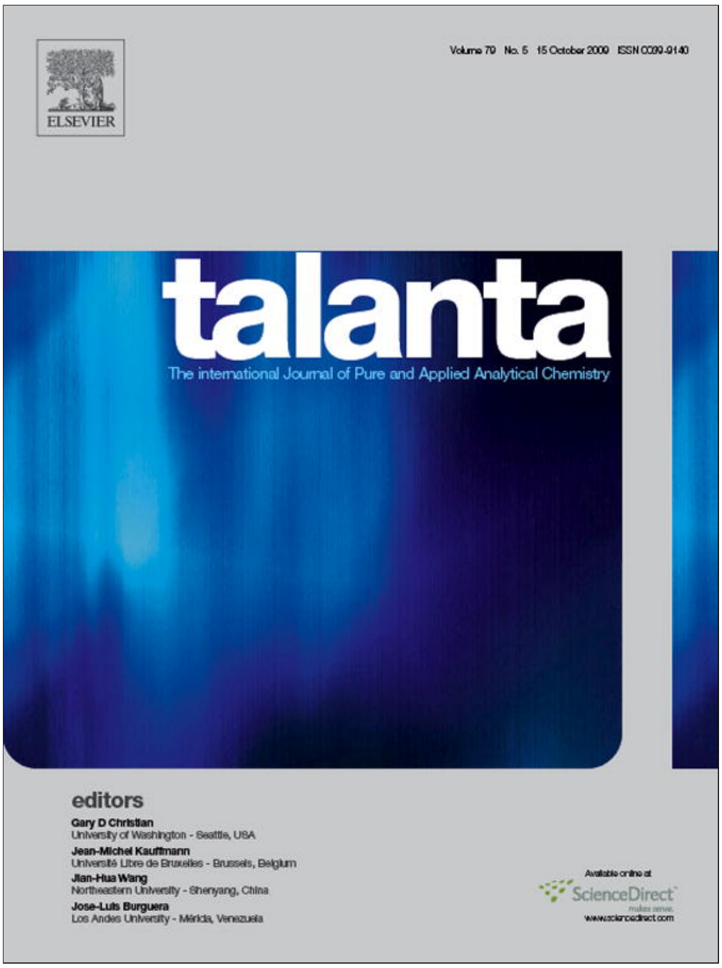

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Sequential injection-capillary immunoassay system for determination of sialoglycoconjugates

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ABSTRACT

An automatic immunoassay system for an assay of sialoglycoconjugates was developed based on the sequential injection technique. A cost effective plain glass capillary tube was used as a solid surface for immobilization of biomolecules via a simple physical adsorption which is adequate to tolerate the force of solution flowing through the capillary during the multi-steps immunoassay process. Immunoassay could be performed with many improvements—rapidity per sample as compared to the conventional micro-plate format (40 min vs. 5–8 h); lower cost and simpler as compared to fused silica capillary with covalent immobilization; and without problem of back pressure as compared to flow injection-bead based immunoassay. Performance of the sequential injection-capillary immunoassay was demonstrated by assay of sialoglycoconjugates level in human serum to differentiate cancer patients from healthy people.

1. Introduction

Sialic acid in human body is presented in the form of N-acetylneuramic acid, a nine carbon sugar, as a component of glycoproteins, glycolipids and polysaccharides found in the outer cell membrane [1,2]. Here the term "sialoglycoconjugates" is used rather than "sialic acid" because most of the sialic acid binds to glycoprotein and glycolipid and free sialic acid is normally only found in very small concentration. It has been reported that cancer cells are composed of sialic acid rich glycoproteins and it might contribute to the ability of cancer cells to adhere to various organs and decrease the ability of host defense mechanisms to destroy cancer cells. It was found that cancer patients, especially ones with tumor growth status, have an elevated level of sialoglycoconjugates in their serum. Thus, sialoglycoconjugates are thought to be a potential biomarker for various cancers, though other conditions such as heart disease, inflammation and pulmonary disease have also been reported to have some relationship with this biomarker [3,4].

A variety of techniques including colorimetry [5,6], fluorimetry [7], enzymetry [8], HPLC [9], CE-MS [10] and immunochemical methods [11,12] have been used for quantitative analysis of sialic acid and sialoglycoconjugates. Among all these techniques, immunoassay is superior to other techniques in terms of high specificity and requirement of small amount of sample and reagents. In conventional 96-well micro-plate immunoassay, operation still involves many steps including incubation and washing which are time consuming and subjected to human errors.

Sequential injection analysis (SIA) is the second generation of flow injection analysis (FIA) technique where chemical reaction takes place in small tubing using a bi-directional pump to control the order of reagent aspiration. A computer program is used to manage the various operational steps with high precision in timing and small volume manipulation. SIA has been applied for various bioassays, especially with a special unit integrated to a multi-selection valve called a "lab-on-valve" (LOV) [13,14]. Solid phase for immobilization of antibody or ligands has evolved from micro-well into forms that are more suitable to operate with the flow system. Beads are the most popular new immobilization surfaces due to their mobility in solution and their retainability to facilitate the separation process of bound and unbound components. Beads may be packed into the channel of the LOV which acts as a bioassay reactor. However, using beads in a flow system may cause a back pressure problem which would alter flow rate and precision of the analysis. An open tubular surface such as fused silica capillary has been reported as successfully used in immunoassay [15,16]. While the use of high cost fused silica material is essential for immobilization processes that require high heat activation, a plain glass capillary may be adequate for some biomolecules with high physical affinity to glass surface.

In this study, a low cost plain glass capillary tube was introduced as an alternative solid surface for the immobilization of the specific receptor used in the competitive immunoassay of

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sialoglycoconjugates. A capillary tube is well suited for use in the flow system because it acts as part of the tubing where the solution flows through. This helps to eliminate the back pressure problem and reduce cost of analysis. Bovine submaxillary mucin (BSM) which has sialic acid as the main acidic carbohydrate constituent was chosen for immobilization onto the inner wall of a glass capillary tube and also for the competition standard to construct a calibration curve. Sialic acid in BSM and sialoglycoconjugates in serum sample competed to bind with a limited amount of lectin conjugated to biotin (b-lectin). Anti-biotin conjugated with enzyme horseradish peroxidase (anti-b-HRP) and substrate TMB were used for detection of bound b-lectin which in turn inversely relates to the amount of sialoglycoconjugates in the sample. This SIA system which was developed for better flow using a capillary tube as reactor, instead of a bead reactor, also provides automatic operation of the immunoassay steps. The system was applied to determine the amount of sialoglycoconjugates in healthy and cancer subjects.

2. Experimental

2.1. Materials and apparatus

Normal glass capillaries used as a solid phase for immobilization of mucin were commercial plain haematocrit tubes with the dimension of 75 (±0.5) mm in length and 1.15 (±0.05) mm i.d. (Vitrex). Stock solution of mucin from bovine submaxillary glands, Type I-S (BSM) was prepared at the concentration of 0.0100 g ml^{-1} in 0.02 M carbonate buffer pH 9.6. Working solution used for coating the capillary was diluted to 100 μg ml^{-1}, except for the study on suitable blocking reagent to reduce non-specific binding, 10 μg ml^{-1} BSM solution was used. BSM was immobilized onto the inner wall of glass capillary by physical adsorption, followed by incubation of a suitable blocking reagent. These two steps were done off-line in order to prepare many capillaries in one batch. Various blocking solutions (0.1% Tween20, 1% gelatin, 5% non-fat milk, 5% skim milk, and 1% BSA) prepared in 0.01 M phosphate buffer, pH 7.4 (PB) were tested. Biotinylated *Maackia amurensis* Lectin II (MAL II, Vector Laboratories) was diluted to 1:50 ratio with PBS (0.01 M PB with 0.15 M NaCl, pH 7.5). Mouse anti-biotin conjugated with horseradish peroxidase (Zymed) was diluted to 1:1000 ratio with PB. Ready to use substrate, 3,3′,5,5′-tetramethylbenzidine (TMB, KLP) was employed. Standard calibration curve was constructed from a series of BSM standard solutions prepared in 6% BSA (Sigma) to compensate for the protein matrices of real serum samples. Carrier solution in the flow system was citric phosphate buffer pH 5.0, prepared by mixing 0.05 M citric acid with 0.10 M di-sodium hydrogen phosphate dehydrate.

Serum samples were prepared by centrifugation of fasting bloods, collected in the Vacutainer tube without EDTA (BD), to separate out the blood cells. They were kept at −20 °C and were used without further dilution. Serum samples of patients with cancer were obtained from the Thailand Excellence Center for Tissue Engineering, Department of Biochemistry, Faculty of Science, Chiang Mai University Hospital. Serum samples of healthy people were from volunteers.

The SI system was set up using a syringe pump (XL 3000, CAVRO Scientific Instrument) with 1 ml barrel. A 10 ports selection valve (C25-3180 EMH, Valco Instrument) was used to accommodate all the reagents, air, detector and waste connections. All tubings were PTFE tubings. Spectronic 21 (Spectronic Instrument) with a flow through cell of 8 μl (Pye Unicam) was set at 650 nm. SIA control software (FIA Lab3000, FIA Instrument) is used for controlling the flow rate, volume of reagents and incubation time.

3. Results and discussion

3.1. Manifold and operation steps

The manifold design is shown in Fig. 1. To perform the competitive immunoassay illustrated step by step as in Fig. 2, the operational steps controlled by a computer software program are summarized in Table 1. Volume, flow rate and time for each step were obtained from optimization as described in more detail in the section “optimization”.

3.2. Blocking solution

High non-specific binding of either b-lectin and/or anti-b-HRP onto the uncoated-capillary (capillary without immobilized BSM) was observed. This could cause high background signal and error in the use of a coated capillary if there was empty space where BSM was not bound. To reduce this problem, various blocking reagents were tried. The detergent based blockers such as Tween20 are believed to prevent hydrophobic adsorption of the other proteins that may cause non-specific binding to the solid surface. The small protein based blockers such as BSA, milk protein, and gelatin are believed to fill in the empty solid surface not occupied by the immobilized receptor [17].

The results compared between uncoated capillary (blank) and BSM-coated capillary (analytical signal) were used to identify the best blocking reagent. Fig. 3(a) shows the differences between the assay systems that gave blank and analytical signals. Fig. 3(b) clearly indicates that 0.1% Tween20 was the most suitable blocking solution because the analytical signal is much higher than blank which means that Tween20 did not interfere with the assay. This also implied that the non-specific binding in this assay system may be due mainly to hydrophobic binding of non-specific components to the glass surface which could be reduced by detergent based blocker.

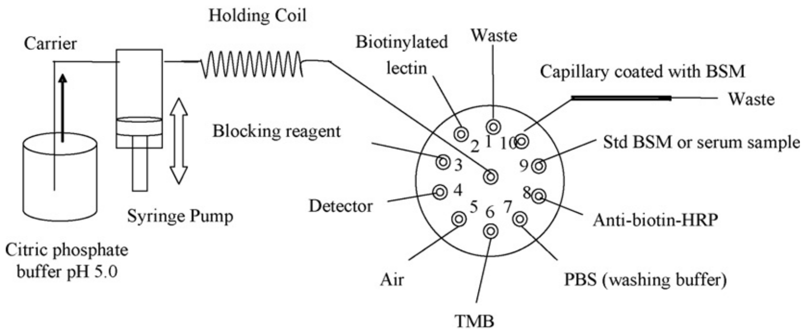

Fig. 1. Sequential injection-capillary immunoassay (SI-CI) system.

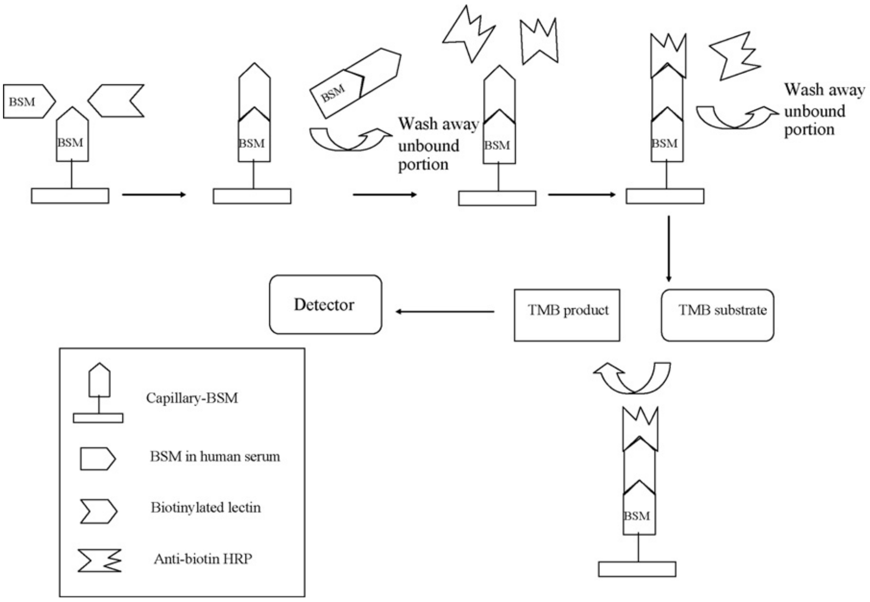

Fig. 2. Diagram illustrating the competitive immunoassay process of BSM.

Table 1
Summarization of operational steps.

Step no.	Operation	Volume (μl)	Flowrate ($\mu l\, s^{-1}$)
1	Washing capillary with PBS	1500	50
2	Aspiration of air to the holding coil	20	10
3	Aspiration of 0.1%Tween in PBS solution to the holding coil	80	10
4	Aspiration of air to the holding coil	20	10
5	Sending 0.1%Tween in PBS solution to capillary and incubate for 3 min	80	10
6	Removing unbound species by washing capillary with PBS	1000	50
7	Aspiration of air to the holding coil	20	10
8	Aspiration of standard BSM solution or serum sample to the holding coil	10	10
9	Aspiration of biotinylated lectin to the holding coil	60	10
10	Aspiration of standard BSM solution or serum sample to the holding coil	10	10
11	Aspiration of air to the holding coil	20	10
12	Mixing standard BSM solution and biotinylated lectin by moving solution back and forth three times	–	–
13	Sending mixture to BSM coated capillary and incubate for 10 min	80	10
14	Removing unbound species by washing capillary with PBS	2000	50
15	Aspiration of air to the holding coil	20	10
16	Aspiration of anti-biotin HRP to the holding coil	80	10
17	Aspiration of air to the holding coil	20	10
18	Sending anti-biotin HRP to the capillary and incubate for 10 min	80	10
19	Removing unbound species by washing capillary with PBS	2000	50
20	Washing with citric phosphate buffer	1000	50
21	Aspiration of air to the holding coil	20	10
22	Aspiration of substrate TMB to the holding coil	80	10
23	Aspiration of air to the holding coil	20	10
24	Sending substrate TMB to capillary and incubate with anti-biotin HRP for 2 min	80	10
25[a]	Discarding air		30
26[a]	Sending the solution product to the detector	50	10

[a] To completely prevent an air bubble from entering the detection flow cell, the air bubble was discarded before sending the product zone into the flow cell. The discarded volume (30 μl) needs to be higher than its aspiration volume (20 μl). Therefore, the volume (50 μl) of the product zone left to be sent to the detector was lower than the aspirated solution volume (80 μl).

Note that the signal profiles were peak down due to the electronic data acquisition system used in this work that gave the signal in voltage which relates to transmittance.

3.3. Optimization

3.3.1. Concentrations of BSM, b-lectin, and enzyme conjugates

The suitable concentration of BSM for coating the capillary was obtained from the previous independent studies of coating BSM on the surface of the conventional micro-well plate. The surface area of a micro-well is approximately the same as that of the glass capillary used in this study (2.7 cm^2) [18]. Therefore, the same optimum concentration of BSM at 100 $\mu g\, ml^{-1}$ was used in the immobilization step to ensure an excess amount of BSM. Amounts of other reagents and the incubation time for each step were optimized as follows.

The amount of biotinylated lectin (b-lectin) is an important parameter. An inadequate amount would be used up when binding with a small amount of sialoglycoconjugates in serum and therefore, would not be able to differentiate various higher sialoglycoconjugates concentrations. In contrast, having too much excess b-lectin would lead to unnecessary expense and increase in analysis cost. It was found that the signals were increased with the increased amount of b-lectin. However, in this study, the highest concentration used was limited at 1:50 dilution ratio to save reagent.

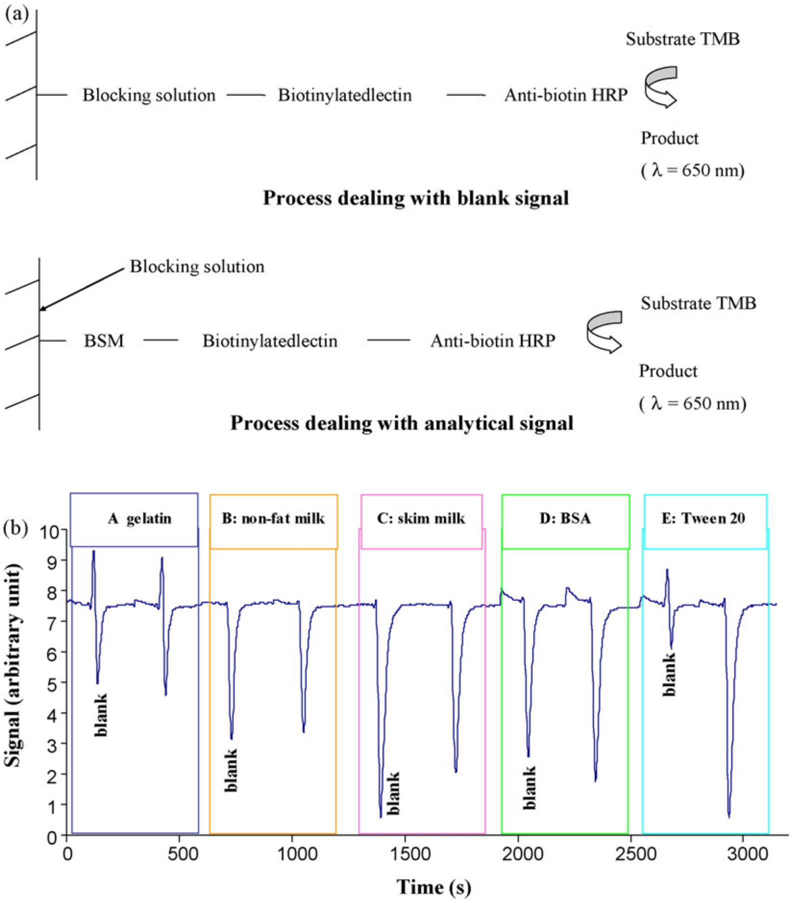

Fig. 3. Study on suitable blocking reagent to reduce nonspecific binding in the BSM coated glass capillaries using the SI-CI system. (a) Diagram illustrating the process of obtaining blank signal and analytical signal. (b) Comparing blank and analytical signal profiles when using (A) 1% gelatin in PBS, (B) 5% non-fat milk in PBS, (C) 5% skim milk in PBS, (D) 1% BSA in PBS and (E) 0.1% Tween20 in PBS, as blocking reagents. The first peak and the second peak of each signal set are blank and analytical signals, respectively. Each capillary was coated off-line with 10 μg ml^{-1} BSM at 8 °C for 24 h, followed by addition of 1:200 dilution b-lectin and 1:2000 anti-b-HRP and TMB. Product was detected with the SI system.

The optimum amount of anti-biotin conjugated with HRP (anti-b-HRP) was determined by varying the dilution ratio in the range of 1:5000, 1:2000 and 1:1000. Anti-b-HRP should be present in sufficient amount to bind to all bound b-lectin. However, too much excess anti-b-HRP would require extensive washing and unnecessary waste of time and reagents. The signals were found to increase with the increase of the amount of anti-b-HRP and leveled off at the concentration between 1:2000 and 1:1000 dilution ratios. Although dilution of 1:2000 seemed to be adequate, dilution ratio of 1:1000 was chosen for further experiments to compensate for the loss of enzyme activity which might occur due to unfavorable storage conditions. (i.e. storage temperature not maintained).

3.3.2. *Incubation time*

There were four different steps in which incubation time should be optimized; immobilization of BSM onto the glass capillary, incubation of the mixture of b-lectin and BSM in serum/standard to the immobilized BSM, incubation of anti-b-HRP to the bound b-lectin, and incubation of the bound anti-b-HRP with substrate TMB. The longer incubation time yielded better sensitivity but reduced sample throughput. Incubation times of each step were chosen to compromise sensitivity and analysis time. Immobilization of BSM onto the glass capillary was done off-line for the convenience in preparation of many capillaries at once and to reduce overall analysis time in the flow system.

Efficiency of immobilization of BSM onto the glass capillary depended on both time and temperature. Three sets of conditions were studied; in the refrigerator (8 °C) for 1, 5 and 24 h; at room temperature (25 °C) for 1, 5 and 8 h; and in the incubator (37 °C) for 1, 3 and 5 h. The results are shown in Fig. 4. Immobilization was best at 8 °C for 5–24 h or, alternatively, at room temperature for 1 h. The higher temperature of 37 °C was not suitable, possibly owing to

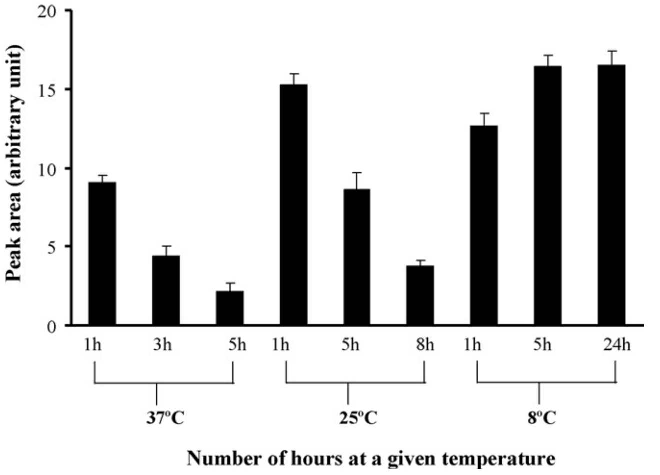

Fig. 4. Effect of temperature on efficiency of immobilization of BSM onto glass capillary. Each capillary was coated with 100 μg ml^{-1} BSM at various times and temperatures, followed by addition of 1:100 dilution b-lectin in 6% BSA and 1:1000 dilution anti-b-HRP and TMB.

degradation or conformational change of BSM. Here immobilization of BSM at 8 °C between 5 and 24 h was chosen for the convenience of preparation of many capillaries. However, if online immobilization is to be done one at a time, the performance of immobilization of BSM at 25 °C for less than 1 h should be explored.

The b-lectin was introduced into the sequential injection-capillary immunoassay (SI-CI) system and mixed with standard BSM prior to being sent to stop in the capillary. Immobilized BSM and BSM in serum/standard competed for a limited amount of b-lectin. The degree of binding depends on incubation time. Longer incubation time allows for higher interaction and better analytical sensitivity. Incubation times of 1, 5, 10, 20 and 40 min were studied. Signals increased from 1 to 10 min, after that the signals remained unchanged. This indicated that the interaction between b-lectin and BSM was completed in 10 min and therefore this incubation time of 10 min was chosen for further experiments.

Similarly, the longer incubation time between bound b-lectin and anti-b-HRP led to higher sensitivity. The increasing of sensitivity was observed from 1 to 10 min. After that (10–40 min), the signal was stable. This implied that the binding between b-lectin and anti-b-HRP reached equilibrium at 10 min, so this was selected for further experiments.

The longer incubation time between anti-b-HRP and excess amount of TMB yielded more enzyme-substrate product which resulted in higher analytical signal. However, to avoid auto-oxidation of TMB and to shorten up the analysis time, incubation time of 2 min was chosen for this step as it gave sufficient sensitivity.

It should be noted that the fused silica capillary with small diameter (e.g. 0.53 mm [16]) has advantages in high ratio of the surface area to the volume and flexible length of choice. The plain glass capillary used in this work is relatively larger in diameter (~1.15 mm) with length limited in commercial availability. Therefore, this glass capillary may provide lower sensitivity and require longer reaction time as compared to fused silica capillary. However, the much cheaper plain glass haematocrit capillary makes its usability worth the investigation. In addition, the bigger diameter of the glass capillary would actually facilitate the flow of solution and reduce back pressure in the system.

3.4. Performances

3.4.1. Precision

Within-run precision or repeatability was determined by analyzing a normal serum sample spiked with a 50 μl of 100 ng ml^{-1} BSM standard. Analysis was done repeatedly 10 times within a day. This study was carried out using 2 different samples. The relative standard deviations (RSDs) were practically the same in both samples (2.8% and 2.3%).

Between-run precision or reproducibility was determined by considering the calibration curve of standard BSM equivalent constructed in 5 different days (1, 4, 10, 20 and 33 days apart). The investigated concentrations were in the range of 1–1000 ng ml^{-1}. The percentages of relative standard deviation (%RSDs) were 0.7 for 1 ng ml^{-1}, 0.7 for 100 ng ml^{-1}, 1.1 for 250 ng ml^{-1}, 2.2 for 500 ng ml^{-1} and 2.8 for 1000 ng ml^{-1}.

The precision of system decreased as the concentrations of BSM increased. This may be due to the fact that in this competitive immunoassay, when the concentration of analyte increased, less b-lectin was bound to BSM on the capillary wall, leading to lower signal (peak area). The precision of measurement was therefore decreased. However, all were in an acceptable range.

3.4.2. Stability of the coated capillary

The aim of this study was to estimate the stability or shelf life of BSM coated glass capillary tubes kept in different conditions. This was done by incubating BSM solution with glass capillaries 24 h and dividing them into four groups; the first group was kept in phosphate buffer (PB) pH 7.4, the second group was kept in PB containing 0.05% Tween20 pH 7.4, the third group was kept in carbonate buffer pH 9.6, and the last group was kept dry. All capillaries were sealed with parafilm on both ends. The capillaries were refrigerated at 8 °C for future use. The stability of capillary was investigated in 1, 3, 5, 7 and 15 days as compared to freshly prepared coated capillary. The peak areas obtained from capillaries kept at various conditions were calculated against the peak areas obtained from freshly prepared capillaries which were set at 100%.

Table 2 shows that the signals obtained from the capillaries kept in PB pH 7.4 for 1 and 3 days were not significantly changed as compared to the freshly prepared capillaries. Capillaries kept in PB longer than 3 days tended to be degraded. This might be because the immobilization of BSM with glass capillary was not through covalent bonding. Passive adsorption may be too weak of a force to hold the BSM on the capillary surface for a long time. It also

Table 2
Efficiency of BSM coated capillaries kept in various conditions.

Day	Efficiency relative to freshly prepared capillaries			
	PB pH 7.4	PB with 0.05% Tween20	Carbonate buffer pH 9.6	Dry
1	100	99	84	99
3	97	94	76	82
5	92	89	65	70
7	82	79	62	59
15	72	70	41	33

Percentages of signal were compared to those obtained from freshly prepared capillaries. Results from freshly prepared capillaries are set as 100%.

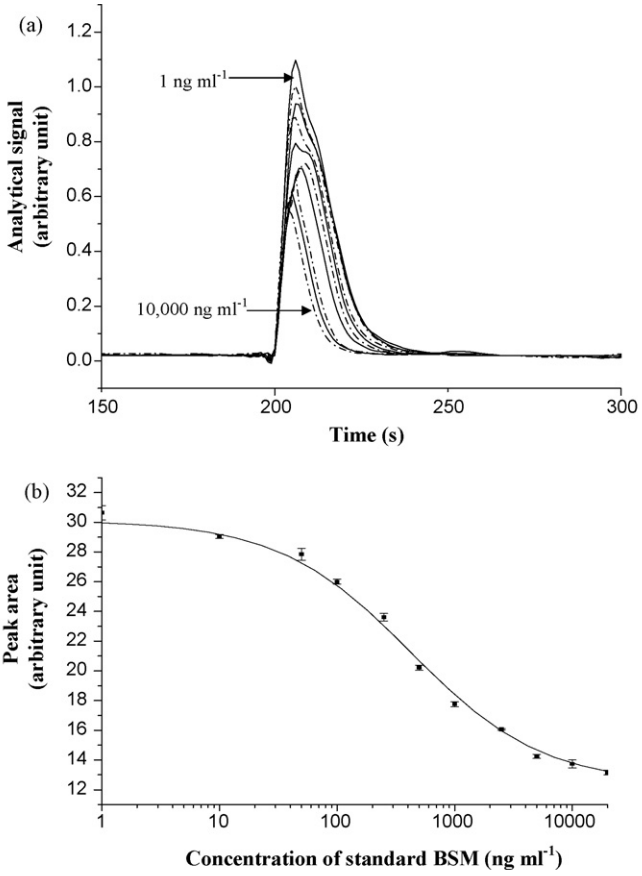

Fig. 5. (a) Analytical signal profiles of standard BSM of various concentrations: 1, 10, 50, 100, 250, 500, 1000, 2500, 5000 and 10,000 ng ml^{-1}, obtained from the SI-CI system (peaks were rotated for easier viewing) and (b) the corresponding calibration curve for determination of sialoglycoconjugates (BSM equivalent) using SI-CI system.

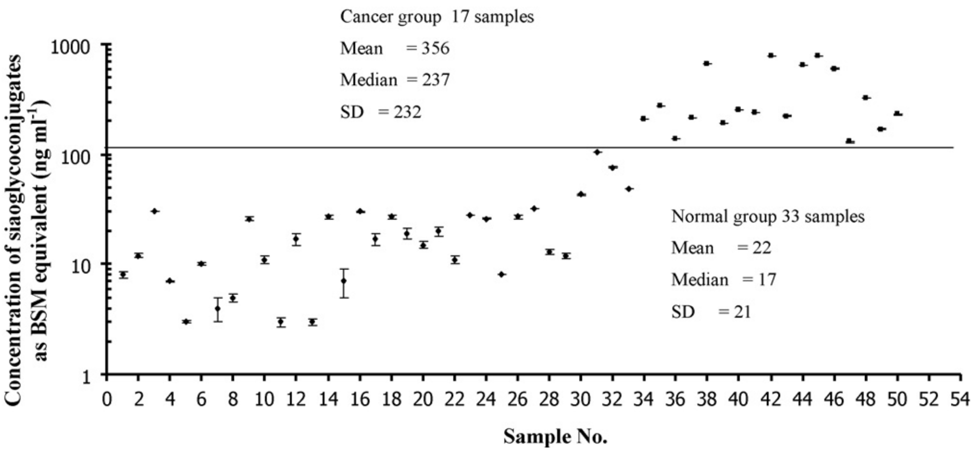

Fig. 6. Comparison of the concentrations of sialoglycoconjugates as BSM equivalent obtained from 33 healthy and 17 cancer diseases samples. All normal subjects have sialoglycoconjugates lower than 110 ng ml^{-1} BSM equivalent determined using the proposed SI-CI system. Note that *Y*-axis is in log scale.

showed that Tween20 was not necessary for keeping capillaries. The capillaries that were kept dry showed the decrease in signal after 1 day. This might be because the BSM was degraded and lost the specific binding abilities when the capillaries were kept dry. The BSM coated onto glass capillary could not be kept in carbonate buffer pH 9.6 because the conformation of BSM might be altered and could no longer bind to the b-lectin.

Since the binding of BSM on the glass capillary is through physical adsorption, the ability to recycle of BSM coated capillary is not expected to be effective. However, reusing of the plain capillary after removing all reagents may be possible and this will be investigated further.

3.4.3. Calibration curve

Calibration curve of BSM standard (sialoglycoconjugates equivalent) was investigated in the concentration range of 1–10,000 ng ml^{-1} to cover the amount of sialoglycoconjugates normally found in a normal person and various types of cancer patients. The profiles of analytical signals and the corresponding calibration curve are as shown in Fig. 5(a) and (b), respectively. Here, the signals were turned up side down as compared to those shown in Fig. 3 for easier viewing and relating the concentration of product to the absorbance.

Calibration curve is the sigmoidal (logistic) fit obtained from the computer software (Origin version 7.0). The calibration curve is expressed in sigmoid equation as follows:

$$Y = [(A_1 - A_2)/(1 + (X/X_0^p))] + A_2 \quad (R^2 = 0.9967),$$

where Y is analytical signal as peak area, X is concentration of BSM equivalent in ng ml^{-1}, A_1 is initial Y value (30.12), A_2 is final Y value (12.37), X_0 is X value at Y equal to half of the limit A_1 and A_2 (424.48), and P is power (0.77).

From this calibration curve, the working range was found to be 50–5000 ng ml^{-1}. The lowest detectable concentration of BSM equivalent was 10 ng ml^{-1}, determined as the lowest distinguishable analytical signal ± 2S.D. with 95% confidence from blank signal while the highest detectable concentration of BSM equivalent was 5000 ng ml^{-1}, determined similarly on another end of the calibration curve. This range very well covers the sialoglycoconjugates level found in normal human and at various elevated levels in patients with cancer diseases.

3.4.4. Real samples

The SI-CI system was developed for determination of sialoglycoconjugates in serum samples. Normal human serum samples (no. 1–33) were from 12 male (age 23–41 years old), 14 female (age 22–74 years old) and 7 subjects with unknown gender and age. Cancer serum samples (no. 34-50) were from 11 male (age 32–78 years old) and 6 female (age 41–82 years old) subjects. The concentrations read from the calibration graph are not true concentrations of sialoglycoconjugates because the calibration graph was constructed from BSM standard. Therefore, the sialoglycoconjugates concentration is reported as BSM equivalent. The concentrations of BSM equivalent obtained from healthy people and cancer patients using the proposed SI-CI system are shown in Fig. 6. The over all trends showed that the concentrations of sialoglycoconjugates as BSM equivalent in normal samples are much lower than that found in cancer subjects (average 22 vs. 356 ng ml^{-1}). All normal subjects have sialoglycoconjugates lower than 110 ng ml^{-1} BSM equivalent. However, with the limited number of samples, it is not possible to pinpoint the cut-off value. The use of a higher number of samples and chemometrics may help in better categorization of the two groups with the suitable cut-off value. Nevertheless, the distinctive difference between the average levels of sialoglycoconjugates found in the two groups demonstrated the performance of the SI-CI system for automatic quantification of sialoglycoconjugates in real clinical matrices.

4. Conclusion

The SI-CI system for determination of sialoglycoconjugates in human serum samples was developed. The ordinary commercially available glass capillary which is cost-effective can be used as a solid surface to immobilize BSM. The opened tubular glass capillary can eliminate the backpressure which is a drawback of packed bead column. In addition, the glass capillary is much cheaper than other solid surfaces such as fused silica capillary and bead. BSM could be adsorbed directly onto the glass surface without the need of modification, but careful selection of blocking reagents is necessary. The small size of the capillary can reduce the diffusion time for lectin to reach the immobilized BSM on the surface, resulting in a shorter assay time per sample as compared to the conventional microplate assay. Therefore, the glass capillary is suitable to be coupled to the sequential injection system to make the immunoassay process more automated. The results obtained from the SI-CI system can differentiate cancer disease serums from normal healthy serums based on the amount of sialoglycoconjugates, though the cut-off value should be further investigated with a larger number of samples and the aid of chemometrics. The system may be used as an alternative analysis system for sialic acid and the authors hope that the results from this work would benefit the researchers that are evaluating the use of this biomarker.

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REVIEW ARTICLE

Flow based immuno/bioassay and trends in micro-immuno/biosensors

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Abstract The term immuno/bioassay refers to analytical techniques that utilize the specific molecular recognition between antibodies and antigens or between biomolecules and specific receptors. Features such as high selectivity and low sample consumption make them very useful for analysis of samples with complex matrices. However, immuno/bioassays involve time-consuming (multi-step) operations which usually consist of steps of multiple incubation and washing. These are tedious and may result in large errors. Automatted immuno/bioassay systems can ease and shorten these processes and thus are highly beneficial. The hyphenation of flow-based techniques (i.e. flow injection related techniques and micro-fluidic systems) with immuno/bioassay protocols paves a new way for performing such assays. Compared to conventional microplate formats, flow-based immuno/bioassays can reduce the time needed for analysis, the volumes of samples and reagents consumed, and the need for trained personnel. In order to transform immuno/bioassays from conventional to flow-based formats, the solid surfaces used for the immobilization step has to be changed in order to meet the specific requirements of flow systems. To further develop the on-site analytical systems in micro-fluidic platforms, improvements in detection methods are necessary for high-sensitivity and rapid measurement. This review overviews the advantages and disadvantages of flow-based immuno/bioassay formats, the various types of solid surfaces for immobilization, and the methods of detection. Trends to improve sensitivity, speed and robustness are emphasized.

Keywords Flow injection · Immunoassay · Bioassay · Sensors

Introduction and practical integration of flow based techniques to immunoassay

Immunoassay is known as a highly specific analytical technique. The basic principle of the technique involves the molecular recognition between an antibody and a specific antigen, similar to the immune system, and this makes it naturally very useful for clinical analysis. The capability of modern laboratories in raising antibodies for various types of antigen including enantiomers [1], compounds containing metal ions [2], or other haptens, has extended its application to many areas of studies such as food technology [3–5], agricultural products [6, 7], and environmental studies [8, 9]. The typical immunoassay process, especially heterogeneous or label immunoassay, involves many steps. These normally include incubation (among antibody, antigen and relevant reagents) followed by washing/separation to remove excess reagents. Precise timing and precise volume or amount of reagents used for each assay replication are critical for good precision and accuracy. Therefore, it requires a certain level of training to operate these tedious steps. Attempts in automating the immunoassay process have resulted in the development of commercial immunoassay machinery based on robotics or mechanical movable parts that can introduce and remove reagents into or from the standard micro-well plate [10, 11]. These machines are useful for massive sample assays.

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However, they are at high price and do not give any benefit in shortening up the analysis time or simplifying the operational steps. Most laboratories own only a plate reader for the automatic spectrometric detection step rather than the automatic immunoassay machinery. In many situations, analysis involves low to medium numbers of samples of which results are preferred within a short time to better serve the clients and analysis purposes. Simplifying and shortening of immunoassay procedures would become important.

Strip tests such as those for pregnancy and glucose testing have gained popularity for home healthcare. Its rapidity and self testing serves the privacy needed by clients. These strips are based on immunoassay with pre-immobilization of antibody and relevant reagents onto the specially designed membrane. Results can be visualized with the easy to understand format as one/two lines or positive/negative signs. Similarly, immunoassay in the form of a disposable dipstick for environmental and food testing has also been introduced. They are very convenient, easy to use, rapid and reasonably priced. However, most test strips normally give just a yes/no answer or semi-quantification which is only adequate for the detection of substances that have significant effect or information when they are either present in high amount or absent [12–14]. This is not suitable for the analysis of substances where detailed quantities are necessary. Examples are biomarkers of many diseases. These biomarkers are present in the body fluids but the levels are normally different in healthy people as compared to patients with diseases. Therefore, it is important to know how much they are present [15–17].

Various flow based techniques (i.e. flow injection (FI), sequential injection (SI) and micro-fluidic systems) can be incorporated with immunoassay to automate the process [18, 19]. Since the year 2000, miniaturized bio-analysis systems such as micro-total analysis system (μ-TAS) [20], and micro-fluidic sensors have emerged as new state of the art developments in chemical/biochemical assays [21, 22]. Numerous developments in micro-fluidic immunoassay chip including unique formats such as credit card, digital style flow lateral unit and compact disc have been reported [23–25]. However, the meso- and micro- systems such as FI and SI are still used routinely in many laboratories while the cutting edge technologies of micro-fluidics systems are on-going. Therefore, this review on flow based immunoassay will include flow injection and sequential injection based immunoassays as well as micro-fluidics immunoassays, but with different categorization and emphasis as compared to the previously published reviews [18, 19, 23, 24].

Flow injection technique, where reagents are mixed in small tubings while traveling into the detector, has brought about a new way of performing rapid chemical analysis. Numerous FI applications have been presented through a large number of research articles and commercially available instrumentations that have been accepted in many modern laboratories. Later generations of flow injection technique including stopped-flow injection [26], bead injection [27], sequential injection (SI) with lab-on-valve (LOV) [28, 29] and lab-at-valve (LAV) [30] have expanded the testing possibilities with many unique analysis systems. Most of these systems share a common feature of detection of products or signal changes at non-equilibrium state which is different from the way in which batch-wise process is conducted. Even though at non-equilibrium state, where the degree of reaction is not yet maximized, it is possible to detect the changes using commonly available detectors which now are capable of adequate sensitivity. Without the waiting period for equilibrium to occur, analysis time can be dramatically shortened. SI offers better repeatability with a computer program for system control [1]. Automatic operation of the pump and valve at precise volume and time eliminates the imprecision that may occur when detection is done at non-equilibrium state. When coupling flow based techniques with immunoassay, more detailed quantification of substance can be performed more rapidly with better precision. Automation also helps to minimize the need of having well trained personnel to operate the immunoassay test. FI/SI based immunoassay is therefore a very useful integration.

In this review, various flow based immunoassay formats utilizing different immobilization surfaces and unique ways of sample introduction/detection will be discussed. This will also include bioassays that utilize specific interaction between bio-molecules other than antibody-antigen, but with the same principal operations. Future trends toward miniaturization/micro-fluidics and unique developments including those reported for off-line operation (batchwise, non-flow system), but possible to be adapted for using with on-line (flow based) systems, are emphasized.

Various flow based immuno/bioassay formats

Immunoassays may be categorized in many different ways. Those that are based on the requirement for separation of the free and bound antigen/antibody are classified as either homogeneous or heterogeneous immunoassays. Also, they can be considered according to the order in which the reagents are added. This affects the order of interaction between the reagents and therefore the method can be considered as either a competitive or non-competitive immunoassay. Immunoassays can also be categorized based on the different types of labeling and detection system used. For example, they may be classified as radio isotope immunoassay (RIA), enzyme linked sorbent immunoassay

(ELISA), or as chemiluminescence immunoassay. Another way to classify immunoassay techniques is based on the format of the stationary phase, such as micro-plate, bead, membrane and capillary immunoassays. Differences in these solid substrate formats will be discussed and emphasized in this review. It is the transformation of the solid substrate from a conventional micro-plate to various other formats that has made possible automatic flow based immunoassays, while types of labeling and detection unit may remain the same as in the conventional immunoassays. Comparison of various flow based immunoassay formats is summarized in Table 1.

Bead based immuno/bioassay

Micro-particles are probably the most popular solid surface alternative to the micro-well plate for immobilization of antibody, antigen or relevant reagents. One of the benefits is the high surface area per volume of the micro-particles and the possibility to increase the immobilized surface area as compared to the restricted area of the fixed size micro-well. The ability to accommodate higher numbers of immobilized molecules helps to improve sensitivity and detection limit of the assay. A common way of using micro-particles is in the form of packed column as in chromatography [31–36]. This format is also known as immunoaffinity extraction, used mostly to extract the analyte from impurities by immuno-capturing which does not involve as many operational steps as in a normal immunoassay process. A more advanced system was reported using a multi-immunoaffinity column array [37], in which each column was packed with different specific antibodies. With a single flow of sample solution through this array of columns, detection of multi-analytes in the sample can be done simultaneously.

Other formats of bead-based immunoassay take advantages of short diffusion distance between molecules in bulk solution (i.e. antigen) and the immobilized ones on the bead surface (i.e. antibody) suspended in the solution, see Fig. 1a and b for comparison with the conventional micro-well plate. At the early stage of development, the bead-based immunoassay format was operated off-line (non-flow based system). Magnetic beads were used to facilitate the separation step by accumulating the beads at one spot with a magnet while the liquid phase was removed for detection [38–41]. The flow injection system could be employed to automate bead separation and improve precision [42].

The need for rapid analysis has led to a replaceable bead-based immunoassay with on-line operation. This can be done successfully with bead-injection technique. It is useful

Table 1 Comparison of advantages of various immunoassay formats. The relative amount of advantage of each format per feature is indicated by positive (+) symbol, where relatively more advantageous format is represented by a higher number of (+) symbols. Sensitivity depends mainly on types of label. However, enlargement of surface area and reduction of dilution by performing on-surface detection also affect the amount of detectable product

Advantages/ Various immunoassay formats	Adjustable surface area	Ease of replacement	Commercial availability/ Ease of modification	Rapidity of process/detection
Bead	+ + + + +	+ + + + +	+ + + + +	+ + + + +
	By increasing number of beads	By flushing out with flow	Various modified beads commercially available	Detection can be done directly on transparent beads
Capillary	+	+	+ + +	+ + + +
	By increasing length or diameter of capillary but may be limited in availability	Need mechanical movement	Various modification methods but not commercially available	Detection can be done directly on transparent capillaries
Membrane	+ + +	+	+ + +	+ +
	By choosing higher porosity membrane	Need mechanical movement	Several types commercially available	Requires extensive washing
Electrode	+	+	+ +	+ + + + +
	By increasing size or surface area	Need mechanical movement	Restriction is on the need of facilitating electron transfer	Detection is done directly on surface
Conventional micro-plate	+	N/A	+ + + + +	+ + +
	By selecting bigger size well		Widely available	Excellent for parallel massive sampling but highly time consuming per sample

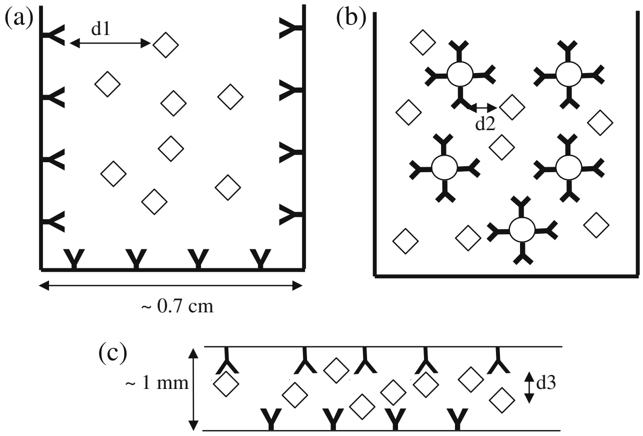


Fig. 1 Comparison of diffusion distances (d1, d2 and d3) between antigen (◇) in solution and the immobilized antibody (Y) on **a** the wall of the conventional micro-well plate, **b** the bead suspended in solution, and **c** the wall of the narrow capillary

for cases where regeneration of bead surfaces is impossible or difficult to be performed on-line. The first generation flow injection system can be coupled with bead injection technique to carry out bioassay with simple operational steps [16]. However, sequential injection is more suitable for immunoassay where many reagents and operational steps are required. Reaction cells, where beads are trapped while solution flows in and out during incubation and washing steps, can be designed in many different ways.

The jet ring cell concept [43], also known as "leaky tolerance cell" [44], was introduced to trap rather big size particles in the flow line while solution is passed through the small gap, as shown in Fig. 2a. For small size beads i.e. <10 μm that may escape through the small gap, the additional use of larger sized particles to be mixed with the small working beads to create void volume for trapping of the small beads, as illustrated in Fig. 2b, has been reported [44]. A unique micro-fluidic piece, called Lab-on-valve (LOV) [45], that can be integrated onto a sequential injection (SI) valve to accommodate both chemical reaction and detection process is now commercially available. The LOV can also be adapted to trap beads. Direct on-bead measurement could be done with a fiber optic detection unit. This helps to increase sensitivity because of less dilution effect, as compared to downstream solution detection. However, some limitations of bead based immunoassay, especially in the form of packed column and direct on-bead detection, may be encountered. The main problems of beads are irreproducibility of bead packing and light scattering from beads which interferes with the spectrometric based detection [46]. Bead size and uniformity are important factors for reproducible packing. Beads that are too small may clog the valve while beads that are too large would increase void volume, and reduce uniformity of packing.

If the bead size is not suitable for operation in a micro-channel or if the LOV piece is not available, a micro-reservoir can be used. The open end of the reservoir eliminates the back pressure which can easily occur in the flow injection-bead based immunoassay system [47]. The micro-reservoir can be attached directly onto one of the ports of the normal multi-port selection valve. The name Lab-at-Valve (LAV) [30] was later introduced for this kind of direct arrangement as an alternative to a LOV.

Beads made of various materials are commercially available. Magnetic beads have gained much popularity because of their ease in being retained in the flow system

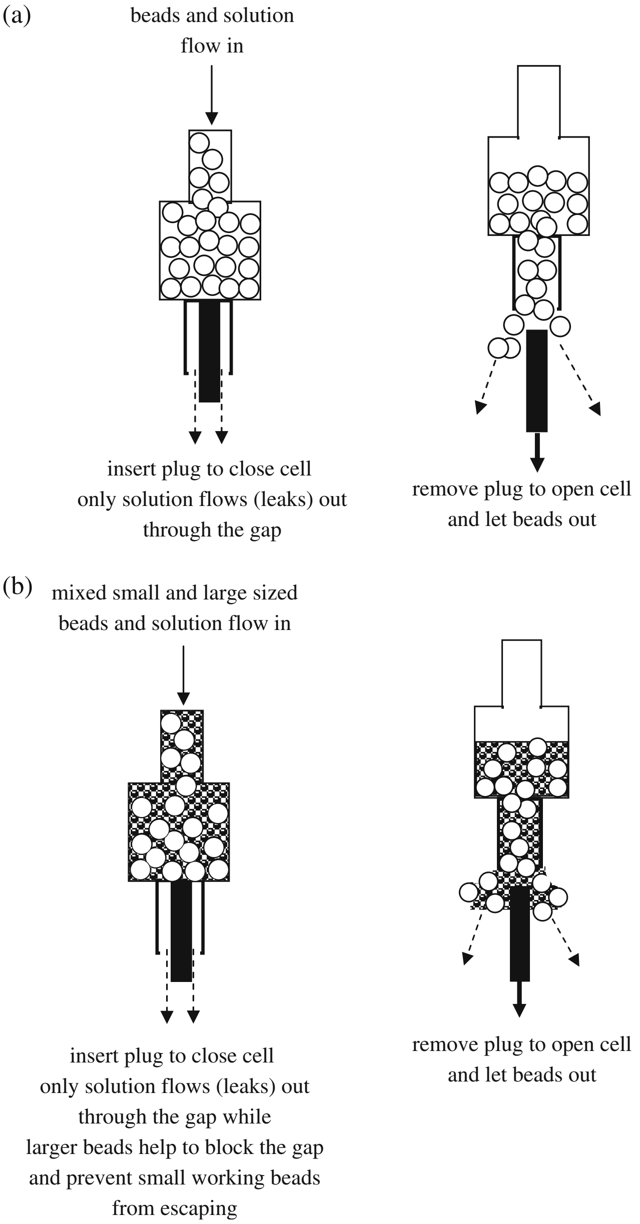


Fig. 2 Diagrams showing the concept of **a** jet ring cell or leaky tolerance cell and **b** the use of large size beads to help trap small working beads

during washing and incubation steps as compared to non-magnetic beads [48]. The works on magnetic bead based immunoassays, including the pioneering works of the use of magnetic beads with rotating disk electrode (RDE) [38–41, 49–52] have been reviewed [53]. The effectiveness of online magnetic trapping systems in some applications of flow injection bead based immunoassay using chemiluminescence and electrochemical detections has been demonstrated [54, 55]. A multi-channel flow injection system combined with magnetic collection enables multiple immunoassays [56]. The combination of electrochemical detection with bead-based immunoassay has been increased. The interdigitated electrode array (comb electrodes) enabled redox cycling of the product from enzyme labeled on magnetic beads that were trapped on the array electrodes by a magnet [57]. Although this latter work was performed batch-wise with a drop of the solution, it appears that this method could also be adapted for use within the flow system.

The main point that should be considered for further improvement of flow bead based immunoassays may be how to keep bead suspension from settling down over time. Homogeneity of the bead suspension affects the precision of the number of bead being introduced into the system for each assay.

Capillary based immuno/bioassay

Another solid substrate format that is very useful for flow based technique is capillary. Antigen or antibody can be immobilized onto the inner wall of the capillary. The use of the antibody pre-coated capillary for capturing analyte, by employing capillary force, prior to redissolving and collecting antibody-antigen complex for detection was reported for clinical application [58]. Flow injection capillary immunoassay was started with off-line incubation of reagents in the capillary reactor and followed by injection of product, collected from the capillary, into the flow system for detection [59–62]. Another approach is based on incubation of reagents in a test tube or micro-plate and passing the mixture into the flow system (FI or SI) equipped with the protein A coated capillary. Antibody-antigen complex can bind to Protein A on the capillary surface, while antigen alone cannot. After separation of bound and unbound species, the amount of antigen can be estimated [60]. Connecting capillary to the FI system, as part of the tubing flow line, offers the benefit of eliminating back pressure that may occur with bead-based packed column immunoassay [63]. The fused silica capillary, as that used for gas chromatography, and specially treated glass capillary were reported as suitable substrates for covalent binding with biomolecules through chemical reactions that need heat activation [59, 60, 64]. When dealing with chemical reaction without heating process or when physical adsorption is adequate, a lower cost uniformly sized glass capillary such as that used for hematocrit [65, 66] or a plastic/polymer capillary [67, 68] can be employed. Glass capillary is more popular because on-capillary optical detection and extended application such as waveguide immunosensor [69], can be accomplished more easily at lower cost as compared to the more opaque plastic capillary [64, 70].

As compared to beads, capillary has not been used as much. This is probably because capillary does not provide as high of a surface area. In addition, there is no modified surface capillary commercially available to facilitate the immobilization of biomolecules as there is in bead format. Modification and immobilization of biomolecules on the capillary surface normally involve multi-steps and many reagents [64]. Furthermore, removing/replacing the used capillary is not as easy and automatic as removing beads. However, shorter distance between immobilized molecules on the capillary inner wall and the molecules in solution in the narrow capillary, as shown in Fig. 1c, yields better mass transportation efficiency as compared to that of a conventional micro-plate. Capillary also allows for lower volume consumption of solution (i.e. 20 μL vs 300–400 μL) [65, 66]. Antibody immobilized monolithic capillary has also been demonstrated to improve rapidity and reduce flow resistance [71]. In addition, glass capillary is also much lower cost than beads.

Capillary immuno/bioassay is the most convenient format to be transformed into micro-fluidic format. The electrophoresis technique, normally carried out on capillary, can easily be coupled with immunoassay to perform immuno-electrophoresis [72, 73]. Capillary force itself can be utilized for sample/reagents introduction without other sample introduction unit [74, 75].

The main limitation of flow based-capillary immunoassay probably lies on the inconvenience in changing the capillary. A suitable mechanical approach is still needed. Some unique developments for multi-analytes detection were reported and would help to decrease the frequency of capillary changing. A common approach is to fabricate a micro-fluidic platform with embedded multi-capillaries, each of which accommodates different immobilized antibodies [70, 76]. Specific site multi-antibody immobilization is also possible that enables detection of multi-analytes in one capillary [64, 77]. Other convenient alternative ways for rapid changing of capillaries would be helpful.

Membrane based immuno/bioassay

The use of membrane has become popular in immuno/bioassay applications since it mimics the cell membrane, which offers some advantages over other formats of solid phases. The porous nature of a membrane makes available a surface area greater than that of a nonporous plastic surface. Several manufacturers offer micro-well plates with nitrocellulose bottoms and some have assemblies for clamping membranes into micro-plate [78, 79]. The porous characteristics also enable a membrane to adapt well within flow based techniques as it will not cause high back pressure.

Various types of membrane are available. Most unmodified membranes are hydrophobic. Choices should be selected based on suitability with solvent and flow rate required. Nylon and polysulfone work well with aqueous based biological samples while polyvinylidene fluoride (PVDF) is resistant to most solvents [78]. Other membrane materials such as lipid membrane [80] and membranes produced from easy to find raw materials such as shrimp, crab shells (i.e. chitosan) [81] and egg shell [82] are interesting low cost products. Nano-structured membranes have been reported that help to lower detection limit. Some nano-fibrous polymer membranes such as PVDF and PDMS with dendrimer PMMA could be prepared using a low cost electrospinning technique [83, 84]. When nanomaterials such as gold are incorporated onto the nano-pored membrane, antibody could be easily immobilized through disulfide bond [85]. Development of membranes that are stable and more tolerant to the environmental and operational conditions, e.g. air and shear flow, aiming to extend the shelf life and ease the storage, is also an interesting research trend [86]. Silicon membrane with arrays of micropores is one example recently reported [87].

Utilization of membrane in flow injection immunoassay is possible in different ways. Membrane can be used to support immobilization of antibody or bio-receptor to retain analyte of interest, a method known as immuno-filtration [88]. Immobilization can be done in small spots to create a microarray for multiplex assays [89]. Membrane may be placed on the electrode and the change in electrical signal due to antibody-antigen interaction can be measured directly at the electrode surface [90, 91]. Membrane can also be used as part of an extraction unit, similar to liquid–liquid extraction technique. The technique is also known as immuno-supported liquid membrane extraction (ISLM) [92, 93]. The principle is as shown in Fig. 3. Analyte (antigen) in the flowing aqueous stream (donor) is extracted into an organic phase immobilized on the membrane by adjusting of pH. Then, owing to concentration gradient, the analyte is further extracted into another aqueous phase on the other side of the membrane (acceptor) containing a specific bioligand such as antibody. The antibody-antigen complex formed will be trapped and accumulated in the acceptor due to its low solubility in organic phase. In a flow injection system, an acceptor can also be conveniently used as an injection loop. The simultaneous enrichment and clean up of analyte from this pre-concentration/extraction process helps to improve sensitivity of the analysis as compared to the FIA system alone.

Unique applications would depend on the selection of suitable membrane properties. A successful work on the immunoassay of protein isoforms, using a membrane with ion exchange and affinity that is specific for a certain isoform, was reported as membrane assisted isoform immunoassay [94]. Searching for other new membrane with specific properties is still an interesting trend of research.

The porous nature of the membrane offers continuous flow which enables continuous extraction. However, this characteristic may also cause some drawbacks. Large surface area has a higher possibility of nonspecific binding toward proteins. Therefore, when using a membrane in immunoassays, extensive washing steps and blocking agents are essential. The research on minimizing these drawbacks would be very useful.

Label free immuno/bioassay

Label free immuno/bioassay is useful in shortening analysis time by eliminating some incubation steps such as incubations for binding of secondary antibody, label, and substrate. Some problems on alteration of affinity of the biomolecules by the label, may also be reduced. When labels are not used, quantitative analysis must be done through the changes that relate to physical properties such as mass, refractive index, electrical parameters, etc. using various methods of detection (plasmonic, photonic, electronic and mechanic) [95–97]. Some new developments with surface plasmon resonance (SPR) technique, the optical detection method commonly used in label free

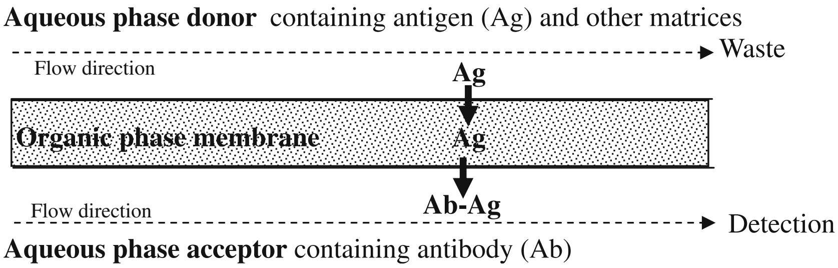


Fig. 3 Diagram showing extraction of antigen (Ag) from donor to membrane and to acceptor containing antibody (Ab)

immunoassay, are still on going to improve its optical sensitivity [98], reduce interference signals [99], and to make it suitable for multiplex immunoassay [100, 101]. Other novel developments in optical detections [97, 102] have been reported but not as many as in electrochemical detection. Here, the authors would like to emphasize the aspects of materials and solid surfaces used for immobilization that enhance the performance of the label free immunoassays.

Quantitative binding of analyte onto piezoelectric surface can be measured based on the degree of the resonant frequency shifting due to mass changing. Selectivity of the quantitative analysis is possible by immobilization of specific molecules such as antibody on the surface of the piezoelectric material while sensitivity depends upon viscosity of the solution medium and the immersion angle in the solution [103, 104]. When incorporating immunoassay with the FI technique, a stronger link between the receptor molecules (e.g. antibody) and any solid surface is preferred to prevent the loss of the receptor within the flowing stream of solution. Reported works on piezoelectric detection based immunoassays have placed emphasis on this issue. Various covalent binding procedures such as glass silanization, thiol-gold bonding and bifunctional cross-linking have been reported [105, 106]. However, the processes involve complicated multi-steps and many chemicals. Therefore, trends of research in this area consist of attempts on simplifying the immobilization process while maximizing reusability, by searching for new processes or materials. Examples of reusable piezoelectric immunosensor are amine terminated plasma-polymerized film (PPF) with alginate [107] and conductive polymer entrapment (CPE) [108].

Porous aluminum oxide films can be used as immobilization surface in the interferometric immunosensor [109]. White light reflection from the film gives a spectral pattern that is changed when there are changes due to refractive index or thickness of the film i.e. from binding of the biomolecules on the immobilized receptor on the Al film. It was also found that some aluminium nano-structure when placed close to tryptophan containing proteins can help to enhance fluorescence from proteins without using additional labels [110].

Electrodes may be modified in various ways to have specific binding with the analyte of interest. Extended applications on study of interaction between protein and small molecules [111] and identification of specific protein in a so called "biobarcode" technique [112] were reported with DNA modified electrode. Direct measurements of the change in conductivity, capacity, and impedance upon binding have been reported successfully in both FI and micro-fluidics formats [113–118]. Although the background signal from ions in real sample matrices can present major interferences for the conductivity measurement, this problem could be solved by designing the system in such a way so that the difference between signal from the sample and from the background matrices can be compared in one run [113]. The capability of the newly introduced immuno-transister to detect low molecular weight antigen has been demonstrated [119]. The new label-free immunoassay systems that involve simple preparation are still most wanted.

Electrochemical based immuno/bioassay

Generally, this type of immunoassay refers to the assay that can be detected using electrochemical techniques. An overview on electrochemical immunoassay was presented in 2000. [120] Electrochemical detection can be applied to any of the formats of immunoassay previously mentioned. However, here we will concentrate on the unique modified electrode and the immunoassay where the electrode itself is a support material for immobilization of reagents as part of an immuno-reactor. The label free aspect of the electrochemical based immunoassay was already mentioned in the previous section and will be excluded from this section.

Miniaturization or down scaling of the system is the trend in developing an analysis system. Electrochemical techniques play an important role since the parts needed for the system, such as the conductor, electrode and wire, can all be made very small and the techniques are conveniently adaptable for multiplex immunoassay [121–124]. By applying an electrical field during the incubation process, the immuno-binding could also be done in a shorter time [125].

Various types of electrodes have been proposed that can be employed directly as a support element for bioassay. The most popular type is screen printed electrode due to simplicity of preparation, low cost and suitability for mass production [126]. Most works have suggested disposable sensors [123, 127–129] while some preffered the renewal of the surface for reusable purposes [130]. The latter comes with the drawback of having a long renewal time which adds to the overall analysis time. If materials used are at low cost, disposable immunosensors such as those made of chitosan membrane [131] can be developed and used without the need of regeneration/reuse which would indirectly help to cut down analysis time.

Nano-materials (such as nano-gold [132, 133] and hydroxyapatite nanopowder [134]), polymer film [135], and membrane with ion-exchange sites [136] can be used to modify the electrode and make it ready for reagentless immobilization. The combination of nano-metal particles such as nano-gold with mediated charge transport reagent helps to catalyze the electrochemical process [137]. The unique 3D nanogold hollow microspheres on chitosan membrane glassy carbon electrode helped to increase immobilization surface area to improve sensitivity [138].

There has been an increased interest of nanotechnology to produce nano-sized electrodes in the forms such as nanowire [139], nano-tube [140, 141], and nano-pipette [142]. This enables multiplex immunoassay in a reduced size device. Immobilization of bio-molecules onto magnetic material prior to attaching them onto the electrode surface via an external magnet [143] eases the electrode regeneration process, which can be accomplished just by removing the magnet. Overview on the use of metal and semiconductor nanoparticles in electrochemical sensors can be found in the recent review [144].

Chemiluminescence is a detection technique that is very popular for immunoassay because it offers high sensitivity. Its combination with the electrochemical technique to yield electrochemiluminescence was reported to extend its applications. For example, an uncommon enzyme label, i.e. glucose oxidase with low turnover number as compared to peroxidase and alkaline phosphatase, could be utilized as an enzyme label in immunoassay when being used with electrochemiluminescence [145]. Addition of long chain molecules such as dextran [146, 147] or nano-particle such as gold [148] between the electrode surface and the molecules to be immobilized further enhances the performance of immuno/bioassay by increasing flexibility and reducing sterric hindrance in the later steps of immunoassay binding.

Elimination of some steps would help to simplify and shorten analysis time. Examples are the attempts to modify electrode surfaces with materials that undergo redox-cycling i.e. electroactive polymer [127] or substances that act as electron transfer mediator [149] to eliminate addition/incubation of substrate or other electron transfer mediators in the detection process. The ease of the process in which biomolecules are immobilized onto the electrode surface is also in demand. A one-step cross linking reaction was reported for the immobilization of antibody onto electrodes at room temperature, using the mixture of antibody with BSA and glutaraldehyde [126, 138].

Research trends in this area deal with investigations into the seemingly endless alternative possibilitites for immobilization of biomolecules onto the electrode surface with the aim of offering robustness, reusability, and a fast response. The monolayer immobilization is highly desired [150] for improving analysis time and efficiency of analyte transportation to accommodate micro-/nano- technologies. If these electrode modification/immobilization process were used in conjunction with automatic operation of flow based technique, the whole immunoassay process could be much more rapid.

Trends in micro-immuno/biosensors

Similar to chemical analysis, immunoassay is developing toward 1) miniaturization for portable and personal/on-site analysis, 2) multi-analytes detection capability for better information, 3) rapid analysis, 4) regeneratable/reusable device, or 5) disposable device. However, in immunoassay as compared to normal chemical analysis, there are some additional points to be considered due to non-specific binding commonly found with protein based analysis. In many cases, the blocking reagents may not be sufficient due to the pronounced effect of nonspecific binding in a micro-system and the non-permanent nature of a blocking reagent which may be washed off with the flow of reagents. To overcome the nonspecific binding, surface modification and improved detection sensitivity are very important. Therefore, new trends of immunoassay involve not only development of small hardware, but also new chemicals, materials, and techniques that will enhance sensitivity of detection.

Novel sample introduction and flow control systems

A simple flow manipulation system with controllable flow rate but without external power supply is of high demand for use with miniaturized chemical/biochemical analysis systems. Since it is important that sample introduction and flow control systems can be integrated into the same unit with the reaction chamber/reactor and detection unit, having small sized parts is therefore beneficial [151]. Various types of micro-pump and valves for flow injection analysis in micro-fluidic flow injection system, including gravity driven, acoustic wave, ultrasonic and electroactivated pumps, have been reported [152–156]. To construct an analysis system that can be used as a stand alone device, pumps that do not require external power supply are of particular interest.

Utilization of the natural vibration of piezoelectric material is an example way to create a miniaturized peristaltic/diaphragm pump [157–160]. In the micro-channel, the unique laminar flow characteristics of the liquid and the capillary force enable the transportation of reagents without an external pump or injection system [161, 162]. In the early development of a micro-fluidic system for chemical analysis, H and I shaped systems, example shown in Fig. 4, were introduced for chemical extraction of biomolecules from one liquid stream into another liquid stream that was flowing into the same micro-channel, based on different diffusion rates of small and large sized molecules [163]. Later developments, involving the unique characteristics of fluids interfaces and a modified micro-channel solid surface with immobilized ligands, enabled additional steps such as capturing, washing and detecting in heterogeneous immunoassay [164].

High solubility and rapid diffusion of air in polydimethylsiloxane (PDMS), normally considered as a drawback in preparation of PDMS film, was reported to be useful for

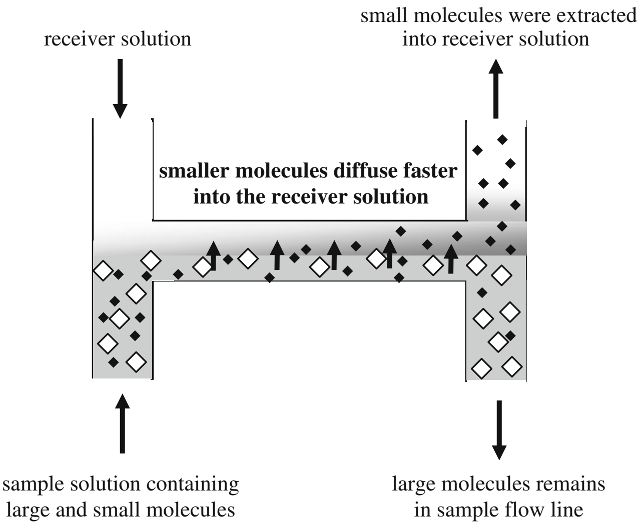


Fig. 4 Example of an H shaped microfluidic system for extraction of small molecules from the mixture of large and small molecules based on the different diffusion rates

pumping of solution in a PDMS-glass based micro-channel [165, 166] when moving it in and out of a vacuum desicator. The related work also demonstrated that the diffusion at the boundary of the two streams of laminar flow is sufficient to provide the mixing and capturing process involved in immunoassay [167].

One limitation of pumping systems which are based solely on laminar flow and capillary action is their inability to change flow rate due to the fixed size of the channels. Electro-osmotic flow is a more well known approach for reagent introduction with variable flow rate within the same size channel and this may be a required feature in some cases [168]. However, the limitation of the electro-osmotic flow system lies in the requirement of having an electrical power source and that the liquids being transported as well as the surface of the channel must be charged to some extent to allow the attraction with the oppositely charged electrode to pull the solution in the desired direction. Recently, a time controllable flow in a microchannel using an electrowetting technique was introduced [169]. By embedding working and reference electrodes at 2 sites across the micro-channel and enlarging the glass channel at the specific site designated as a valve, the liquid reagents can travel forward with capillary action and can be stopped at the electrode because of the wider dimension of the channel and hydrophobic nature of the electrode. When the suitable potential was applied to the electrodes, making them to be more hydrophilic, liquid continued to flow past over the electrode surface, and after that the capillary force was resumed, leading the solution to flow into the reactor. As an alternative to electrical power pumps, the magnet can activate the suspension plug of ferromagnetic particles in the channel to create the ferrofluid pump [170].

The search for micro-pumps without the need of electrical supply that offers pulse free, controllable flow rate and flow time with no restriction of types of solution is still on-going.

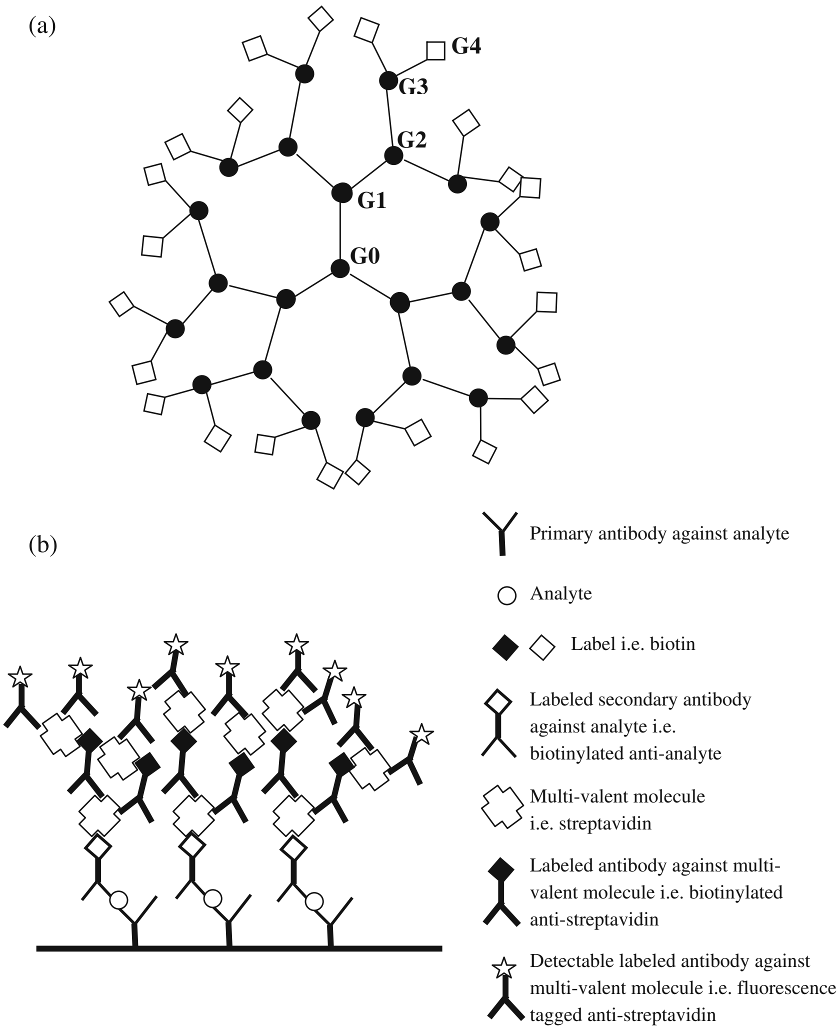
Rapid/specific immunoassay with immobilization/conjugation through flexible long chain and highly branched molecules

The addition of a chemical chain to extend the immobilized antigen or antibody from the solid surface has increasingly been applied. Extension of the antibody from the surface is thought to provide more freedom of movement and less chance of losing its activity. It is expected that the long flexible structure (spacer arm) can extend the antigen or antibody out into the solution, decreasing the diffusion time for the component in the solution to reach and be captured by the immobilized ones. Another main function of the linker is to orient the antigen or antibody and maximize the accessibility of the binding sites or epitopes. For example, if immobilization of antibody was done through the fragment crystallizable (Fc) region, then the antibody should be oriented in a way that allows binding sites to be available for the next binding steps, as compared to random immobilization which may orient the antibody in such a way that the binding sites cannot be accessed.

There are many linkers, especially with biotin conjugate, commercially available that can be used as spacer arms to link molecules of different functional groups [171]. Other linkers reported for their use in the immobilization step are dextran [146, 147] and polyethylene glycol (PEG) [60, 70, 71]. It has also been reported that PEG added in the label conjugation step, between the thiol-specific quantum dot and the secondary antibody, can also help to increase sensitivity and specificity [172, 173].

Dendrimer or dendron, a highly branched synthetic macromolecule as shown in Fig. 5a, is another interesting tool that can be used to create a high capacity immobilization surface [174, 175] or to accommodate high capacity labels [176, 177]. In combination with some conductive materials such as nano-gold, a dendrimer electrode can be constructed [90, 178]. Dendrimer is different from polymers because it does not have molar mass distribution owing to its symmetrical structure and monodispersion characteristics. Dendrimer is synthesized to have tree-like branches. Each branching point is called a generation (G). More numbers of branches are created when more generations are added. The ability of dendrimer to bind to high numbers of ligand or antibody was reported to be useful as an effective blocking reagent [179]. This should also be useful in increasing sensitivity and rapidity of the assay. The important property of the dendrimer depends on the functional groups at the end of the branches which can

Fig. 5 **a** A dendrimer. G0–G4 are the generations of branching points from low to high generations **b** formation of dendrite structure using multi-valence molecules



be engineered as required. This should make it possible to couple the dendrimer with various ligands or antibodies to enhance immunoassay performance. However, reports on immunoassay using the dendrimer molecule in this way are still rare. Even though the actual dendrimer molecule is not used, its branched structure is usually mimicked to improve sensitivity of the assay as will be described next.

Highly sensitive immunoassay and possibility of multi-analytes analysis with unique detection systems

Building dendrite structure to accommodate a higher number of labels

Another approach for better detection performance is signal amplification using modified assay procedure. Dendrite structure, not a dendrimer molecule, is used for amplification of signal by accommodating a higher number of labeled molecules. By employing two multi-valent molecules or particles, antibody labeled with dendrite structure could be constructed on the bound antibody-antigen complex. The difference between the dendritic structure Fig. 5b and a dendrimer molecule Fig. 5a is compared. Examples of multi-valent combinations are streptavidin conjugated fluorescence label with biotinylated anti-streptavidin [167], biotinylated liposome with avidin [180], DNA or antibody-modified gold particles [181, 182], and biotinylated protein with streptavidin [183, 184]. The limitation of additional tedious multi-incubation steps during dendrite structure formation has been addressed. Utilization of the unique laminar flow characteristic of liquids in a micro-channel to reduce incubation steps during dendrite construction has been demonstrated [167] by using two continuous laminar streams to mix the reagents and allow the bonding to take place while flowing.

Multi-enzymes or substrates

For the assay with enzyme label, multi-enzymes or multi-substrates can be used to amplify the detection signal.

These appear to be used in static formats such as in microplate immunoassay format offline or in a specially made reactor, however, online use should also be possible. Some unique examples are described next.

The employments of two substrates or enzymes, named as bienzyme-substrate-recycle, were reported [185–187]. This helped to increase the detection signal by producing an intermediate product that reacts with the 2nd enzyme and can re-start the production of the final product again and again. The detection could be done when more enzyme-substrate product had accumulated in order to obtain a better sensitivity. By adding enzyme as part of the electrode, a unique way to integrate enzyme amplification with electrochemical detection was achieved [188]. This approach is different from the bienzyme-substrate-recycle in that amplification is due to the direct electron transfer process occurring at the electrode surface with the embedded enzyme rather than by enzyme-substrate-recycling.

Encapsulated labels

Another way to amplify the detection signal is by encapsulating the vast amount of detectable molecules (such as fluorophores) and release them at the detection step. Liposome and Nano-encapsulated organic micro/nanocrystalline particle labels are described here.

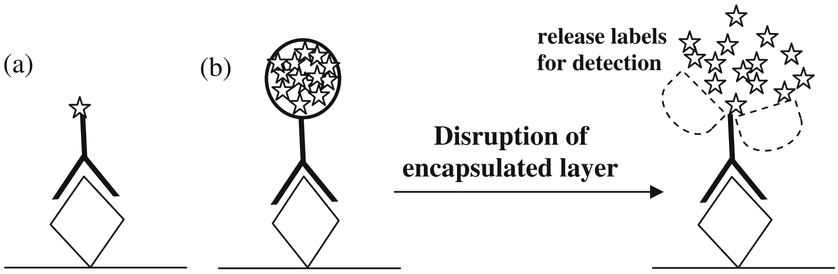
Liposome is a highly complex, microscopic lipid sphere with a membrane, composed of phospholipids and a cholesterol bilayer, and core that usually contains aqueous solution. It has been applied as a technology for drug delivery. The use of liposomes in immunoassay was reviewed in 1997 [189]. Since that time, work on liposomal immunoassay has progressed.

Liposome can be formed naturally or artificially. It can bind specifically to antibodies, specific antigen or vitamin by insertion of biomolecules with specific interaction to those targets into the phospholipids bilayer during liposome synthesis. Alternatively, conjugation of liposome label with antibody could also be done via non-covalent binding between streptavidin and biotin. Matrix for detection is encapsulated inside the cavity core. This requires the liposome disruption, normally by adding detergent, in the detection step. In the past, liposome-released peroxidase was reported with potentiometric detection [190]. Currently, liposomes filled with dye or chemiluminescent/bioluminescent solution are mainly used for better sensitivity. Because a liposome vesicle contains a large number of molecules, it can be detected with very high sensitivity as compared to a conventional fluorophor label [191], see Fig. 6. The exceptional performances of liposome immunoassays with some that offer detection limit as low as zeptomole (10^{-21}) level have been demonstrated [192–194].

Nano-encapsulated organic microcrystalline particle label is based on the similar idea as the liposome label where a large amount of fluorescent molecules are released from the label in the detection step, except that the materials used for encapsulation are not the same. For example, instead of a phospholipids layer, ultra-thin poly-electrolyte layers such as poly(allylamine hydrochloride) and poly(sodium 4-styrenesulfonate) are used to trap the fluorescein molecules, using a layer-by-layer technique [195]. These layers can be covalently bound to antibodies that are needed to be labeled. By adding DMSO and NaOH, trapped particles can be released for detection. The attempts to simplify the encapsulating process have been reported using amphiphilic polymers such as distearoylphosphatidyl- ethanolamine modified with amino(polyethylene glycol), and alkylated poly(ethylene imine) to coat the nanocrystalline fluorescence particles [196, 197]. The technique can replace the layer-by-layer technique but with the lower sensitivity due to smaller overall size and therefore, lower numbers of trapped fluorescein molecules. The interaction of the nano-encapsulated nanocrystalline label and the antibodies was also through adsorption which is less specific as compared to the covalent bonding when using the layer-by-layer coating. However, the incorporation of non-covalent yet strong and specific bonding between Avidin/Streptavidin/Neutravidin and biotinylated biomolecules can overcome this problem [198, 199].

One limitation of using a large amount of fluorescence particles or aggregates is known as the self quenching effect. A new class of photoluminescence particulate label called nanocrystalline siole such as hexaphenylsiole has been introduced to be used as an alternative to fluorescein molecules [200]. Siole has an increased luminescence intensity when presented in aggregate form while has no emission when dissolved in organic solvent at room temperature. Therefore, it can be used as nanocrystalline labels without quenching effect. Sensitivity of up to 40–140 folds as compared to the direct FITC-label was reported. DNA prime probe encapsulating liposome has recently been reported with some unique detection methods such as rolling circle amplification (RCA) where repeated sequence of DNA helps to improve sensitivity and “biobarcode” where unique sequences of DNA can be used to label each particular analyte [201]. The research trends in liposome FI-immunoassays still focus on searching for compounds to be entrapped inside the liposome for the best detection power. Enzyme label can be a good candidate due to its amplification power but it is important to stabilize its native structure for the best efficiency. A cross-linked 3D hydrogel network was claimed to be suitable for entrapping enzyme label because it helps to reserve the micro-environment for immobilization of enzyme [202].

Fig. 6 Comparison of **a** conventional labeled antibody and **b** encapsulated labeled antibody that can accommodate a large number of labels. (◊ is antigen, Y is antibody, ☆ is label)



Quantum dot labels

Semiconductor nano-particles known as quantum dots (QDs) have gained popularity for sensitive labels. They are composed of atoms from groups II–VI or groups III–V of which CdSe, CdTe and ZnS are commonly used [203, 204]. QDs have unique properties of size dependent fluorescence which means a single excitation wavelength can excite the same type of QD of various sizes and yield different emission wavelengths. QDs have been used as alternatives to organic fluorophores for molecular and cellular labeling in biological imaging, in studying of virus localization in living cells and immunoassay [205]. Strong fluorescent emission enhances sensitivity of the assay while multiple emission wavelengths enable multiplex immunoassay in various types of sample [206–212]. QDs are also more tolerant to harsh conditions such as heat [213]. However, the limitation and poor performance of QDs as compared to the organic fluorophores were also reported when using QDs with a normal flow cytometry on an environmental sample. This might be due to interference from other fluorescence species in the matrices, irreversible aggregation and reduction in stability of QDs in aqueous environments [214, 215]. The trends of research in immunoassay with QD labeling include attempts to overcome the difficulties involving the simultaneous detection of fluorescent signals, to improve solubility of QD in aqueous solution by using proper coated (capped) material, and to explore alternative detection enhancement methods.

Normally, QDs are capped with carboxylic functional groups such as mercapto acetic acid, mercaptosuccinic acid or dihydrolipoic acid to provide negative charges on the QDs surfaces for bio-conjugation with the positive charges of the protein. To make QDs safer to handle and to make them water soluble for easier conjugation with protein, amphiphilic polymers such as poly(maleic anhydride) were used for coating instead [203, 216]. Bio-conjugation, which can be achieved via the carboxylic group of the polymer coated onto QDs and the amino group of protein, is more stable than electrostatic bonding. Apart from polymers, silica has also been used as an interesting capping material to prevent the oxygen accelerated photobleaching process and to prevent the loss of fluorescent properties in common buffer solutions, which are normally encountered with semiconductor based QDs [204, 217]. The transparent nature of SiO_2 enhances and stabilizes fluorescence. Other materials such as gold particles [218] and InP/ZnS [219] have also been proposed to be used as QDs as alternatives to potentially toxic semiconductive materials.

The most popular detection method for QDs labeling is fluorescence spectrometry. Improving optical resolution of the fluorescent detection is desired, especially for the research trends in simultaneous detection of multiplexes [220]. Coupling of QDs onto micro-particles such as latex beads, referred to as “nano-on-micro” (NOM) was suggested [221]. This work utilized the improved light scattering intensity of the NOM for additional immunoreaction detection along with identification of multi-analytes from fluorescent emission signals.

Alternative ways of detection other than fluorescence spectrometry is also of interest. Electrochemical immunoassay such as ones that detected electrical signal changes due to the releasing of Cd from the Cd semiconductor based QDs, [222–224], or one with the combined electrochemistry with chemiluminescent spectroscopy (electrochemiluminescent) [225] have been reported. Instead of using them as conjugated labels, QDs can also be used to modify the electrode surface leading to the development of label-free immunoassay for the measurement of the change in signal due to sterric hindrance of binding [226]. Other less common ways of detection for QDs labels such as acoustic optic-tunable filter [215] and atomic force microscopy [227] are also possible.

Robust and longer shelf life solid substrate using molecular imprinting

The objective of molecular imprinting is to create a mold of a specific antigen onto a substrate that will act as an artificial antibody or enzyme. With this manipulation as an alternative way to produce specific binding for an analyte, the ethical and time consuming issues involved in raising antibodies in living animals can be avoided. The longer shelf life, the high tolerance to matrix effects, the easy storage condition and the robustness of the artificial antibodies are also important benefits.

As illustrated in Fig. 7, by adding the molecules of a target analyte (template) into the polymer during its production process and then extracting those molecules out, the polymer that is formed will have cavities to bind with the target molecules through electrostatic attractions and/or complimentary physical shape. Successful imprinting of variously sized molecules has been reported and some are commercially available [228]. Most of the developed molecular imprinting polymers are in the form of microbeads which are used as packing materials in a micro-column for the separation of analyte.

Now that the development of biosensors are increasingly of interest, the recent trends of research in molecular imprinting have been focused on improving the production of protein imprints, especially in the thin film and nanotube formats that can be easily adapted with the sensor device. This will also enable the imprinted molecules to be used more conveniently with the flow based system. So far molecular imprinting based immunoassays incorporated with the flow based system are still rare. One report [229] indicated the benefits of reducing non-specific binding by utilizing epitope imprinting rather than protein- engaged imprinting. The work was applied with quartz crystal microbalance (QCM) for detection of Dengue virus. Another work [230] reported the polymerization of acrylic based imprinting onto the inner wall of glass capillary. The advantage of reuseability of the capillary was demonstrated. This resulted in a low cost and automatic system with long term stability that is suitable for unattended monitoring [228, 230].

New materials other than polymer that are compatible with the easier imprinting process are also in demand. Sol-gel material has been reported as successfully used to create imprints [231–233]. Generally, sol-gel is a wet chemical process used for fabrication of metal oxide materials. Mesoporous sol-gel glass could be used for immobilization of biomolecules [231]. Sol-gel bioglass can be produced in various forms such as monoliths, thin film, powder and fiber. The advantages of using sol-gel are owing to the ability to create the imprint at ambient conditions and in aqueous solution. This greatly helps to eliminate the problems of losing biological activity of the protein being imprinted which results when using high temperature and harsh organic media.

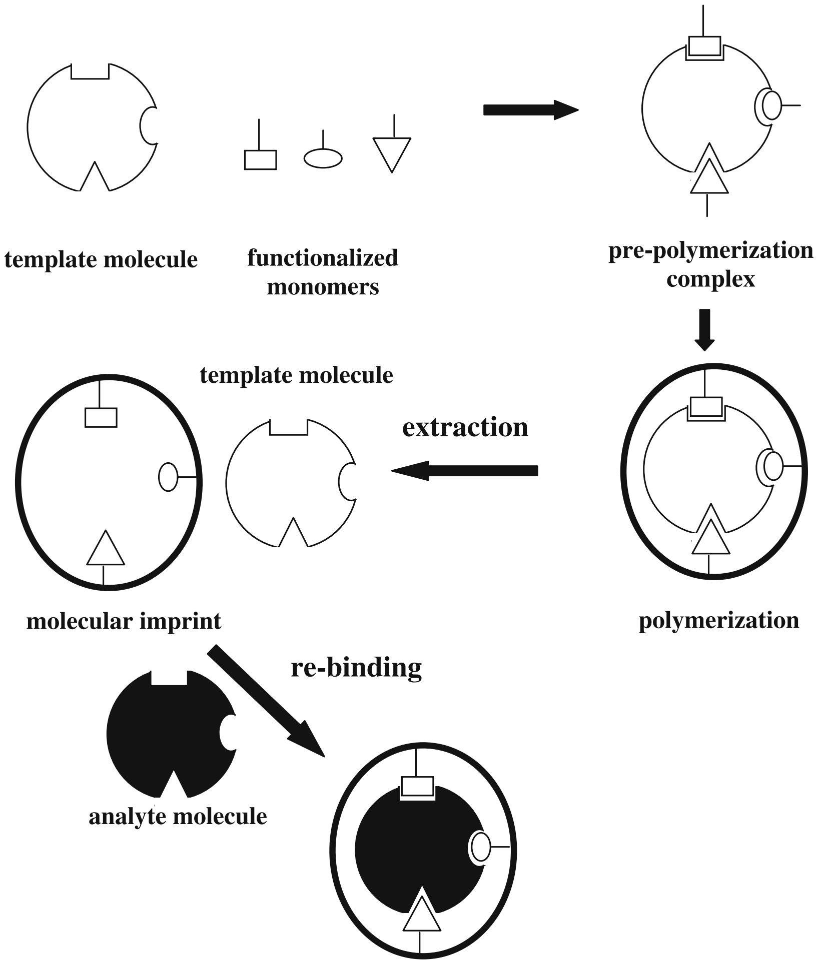


Fig. 7 Diagram showing molecular imprinting production process and its use for binding the target analyte

Conclusion

The combination of various flow based systems with immunoassay has led to improved performances as compared to the conventional batch-wise immunoassays. This integration requires major changes on the format of the solid surface for immobilization of bio-reagents. Rapidity and precision are the major benefits gained, making the highly selective immunoassay technique even more superior. However, the ability of the conventional micro-plate immunoassay to perform massive samples in parallel still remains the great benefit of the conventional batch-wise system [234]. The FI/SI based immunoassays are suitable for small to medium number of samples where the results are required in a short time. Research trends in flow based chemical analysis have led to Lab-on-Chip and down scaling micro-fluidics devices, many of which can be applied to perform immuno/bioassays. Attempts in developing small sized liquid introduction, flow control and detection systems as well as high sensitivity detection systems have been continuously progressing to the same goal of making a cost effective and easy to operate single unit immuno/bioassay based micro-total analysis device.

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Flow Injection and Related Techniques in Blood Studies for Clinical Screening and Analysis: A Review

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Flow and Sequential Injection—General Approaches

FLOW INJECTION AND RELATED TECHNIQUES IN BLOOD STUDIES FOR CLINICAL SCREENING AND ANALYSIS: A REVIEW

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Blood studies for clinical screening/analysis are geared toward point of care testing. Flow based techniques have expanded their applications with unique approaches that may be adaptable for use as alternative disease screening/diagnosis. Many newly developed systems for solution based chemical analysis can be easily adapted for use with plasma and serum. However, cell and intracellular analyses are different. Blood cell analyses require a particular way of sample introduction and detection. This review emphasizes the applications of flow based techniques, especially those that were coupled with FI/SI, in clinical studies through analysis of red blood cells and their intracellular substances.

Keywords: Blood studies; Flow injection; Intracellular substances; Microfluidics; Red blood cell; Review; Sequential injection

FLOW INJECTION AND RELATED TECHNIQUES

Flow based techniques may refer to a group of techniques that carry out analytical chemistry processes in the flowing stream of solutions, normally accommodated in small tubing or channels. Analytical processes (i.e., sample introduction, sample pretreatment, chemical reaction/analysis process, and detection) done in this dynamic manner while flowing into the detector, can decrease the analysis time. Flow based techniques include a wide variety of techniques such as flow cytometry (FC), field flow fractionation (FFF), electrophoresis, chromatography, flow injection (FI) analysis, and micro-fluidics sensors. Some techniques (e.g., FC, FFF, electrophoresis, and chromatography) normally do not involve chemical reaction between

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sample and reagents. They are useful for separation and/or biophysical analysis of samples. In clinical analysis, FC and FFF may particularly be used for particles in suspension, whereas electrophoresis and chromatography are used mainly with sample in liquid form. These techniques are different from flow injection analysis based techniques that normally involve on-line chemical reactions between sample and reagents.

Flow injection based techniques, referring to various formats of flow injection (FI) and sequential injection (SI) analysis techniques (including stopped flow injection (Grudpan et al. 2002; Hodder, Blankenstein, and Ruzicka 1997), segmented flow (Buch-Rasmussen 1990), bead injection (Hartwell et al. 2007), and hydrodynamic injection (Somnam et al. 2008)), have been established as tools for automatic operation of chemical/biochemical analysis. The wet chemistry procedures can be carried out without glassware. A cost effective FI system may be composed of a peristaltic pump for introduction of carrier solution, tubing for mixing and transporting sample and reagent, an injection valve for introduction of precise volume of sample or reagent, and a detector with a recorder, as shown in Figure 1(a). Later generations of flow injection systems aim for multi-commutate and multi-sample approaches (Llorent-Martinez et al. 2010), which are composed of multi-pumps (Santos et al. 2007; Fajardo et al. 2008), multi-valves (Lavorante, Pires, and Reis 2006), and multi-tubing channels (Oliveira et al. 2007) to accommodate more reagents/samples and complicated reactions. A newer generation and more sophisticated SI system is

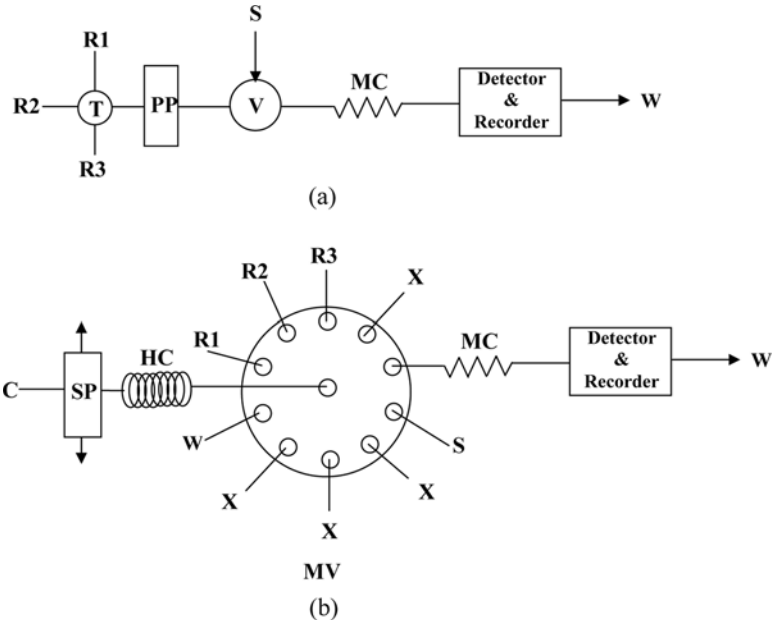


Figure 1. Schematic diagram of (a) a simple flow injection (FI) system and (b) a sequential injection (SI) system. R1, R2, R3 = reagents; HC = holding coil; C = carrier; T = 3-way valve; PP = peristaltic pump; SP = syringe pump; V = 6-port injection valve; MV = multi-ports selection valve; X = extra ports for more samples or columns; S = sample; MC = mixing coil; W = waste.

composed of a bi-directional syringe pump, a holding coil, a multi-selection valve, and a detector with a recorder, as shown in Figure 1(b). Various manifolds could be created for different applications (Economou, Tzanavaras, and Themelis 2007). The operation of the SI system is controlled by computer and the system requires much lower volume of solutions as compared to a FI system. In both FI and SI systems, reagents and sample are transported by pump and are mixed in small tubing while flowing into the detector. In the flow injection based techniques, the measurement of the changes related to chemical/biochemical reaction may be done simultaneously and normally at non-equilibrium state. Even though the analytical signal measured at the nonequilibrium state may not be at its maximum, the constant flow rate ensures the precise timing for each measurement. Therefore, signals obtained still relate to concentrations of the analyte. This helps to greatly reduce the total analysis time as compared to the conventional non-flow based method where reactions often need to be observed at equilibrium for the best precision and sensitivity. In the case of slow kinetic reaction, the flow can be stopped (known as "stopped flow" technique) or a mixing coil/chamber can be added to promote mixing and extend the reaction time. With these extra components/operations, the flow based processes are still much more rapid and more precise than the conventional batch wise processes. The theories on the dilution and flow characteristics have been well studied and documented (McKelvie 2008). The FI/SI system is not limited to liquid phase study, it can also be applied for the study of suspensions of particles and live cells (Ruzicka 1994; Hartwell et al. 2007).

Now that materials science and fabrication technology have grown, various flow based techniques can be put into a micro-fluidics format. Work on the development of sample introduction and detection units that can be applied with microfluidic systems has been on-going. The aim is to develop a portable device for on-site analysis at low cost.

BLOOD STUDIES FOR CLINICAL SCREENING/ANALYSIS

One of the main objectives of clinical screening is for prediction of diseases or to reveal certain body conditions. Clinical screening is normally performed on unclear or asymptomatic subjects. Screening is done to cut down on the number of samples that are unnecessarily tested using the high cost or highly time consuming extensive diagnostic methods. Screening processes are very important for early detection of some diseases, for indication of the stage of diseases, decision on further treatment, and for monitoring the effectiveness of the treatment. Clinical screenings of various substances, microbes, and characteristics of certain cells are carried out routinely. Drug abuse (Weinmann and Svoboda 1998) is commonly tested for in forensic science and regulated sports. Contamination of individuals with heavy metals (Yantasee et al. 2007; Gotoh et al. 2003; Jimenez de Blas et al. 1994; Hernandez, Hernandez, and Losada 1986) may be related to environmental and food safety problems. Chemicals secreted into body fluids such as creatinine (Carducci et al. 2006), prostate specific antigen (PSA) (Lankford, Pollack, and Zagars 1997), carcinoembryonic antigen (CEA), or α-fetoprotein (AFP) (Bekci, Senol, and Maden 2009) may be used as early indicators for some diseases such as cancer. Some illness can be indicated from an antibody being produced (Kadival, Kameswaran, and

Ray 2000) or abnormal blood conditions such as bile acidity (Oda et al. 1989). Effectiveness or progress of the treatment may need to be evaluated through blood testing during the treatment process or when a new treatment and new drug are being studied (Boscaro et al. 2002).

Testing of body secreted substances can be carried out using either body fluids (e.g., blood serum, saliva) or cells (e.g., hair, oral epithelial tissue). The most commonly used sample for clinical testing is blood because it contains so much of the body's chemical information, and it is easier to obtain as compared to other types of samples such as body tissues and cartilage liquid. Various substances in blood, e.g., glucose, uric acid, and cholesterol, are widely acceptable as biomarkers for some diseases such as diabetes, gout, and heart problems, respectively. Numerous other substances in blood and conditions of blood components (i.e., white blood cells, red blood cells, and platelets) are being studied and evaluated to be used as new diagnostic tools for some diseases that are difficult to be diagnosed at an early stage such as cancer (Hartwell, Wannaprom, et al. 2009), liver (Hartwell, Boonmalai, et al. 2010), heart (Chugh et al. 2009; Schwartzenberg et al. 2007), and Alzheimer's diseases (Holland et al. 2009; Verwey et al. 2009; de Barry, Liegeois, and Janoshazi 2010).

Most blood tests deal with plasma and serum (blood fluids after removing cells and fibrin clot). However, much information can be gained from the study of blood cells and their intracellular substances. Many newly developed technologies and methods of solution-based chemical analysis can be adapted for use with plasma and serum analysis. On the other hand, cell and intracellular analyses are different. In some studies, cells need to be kept intact; whereas, in some studies, cells need to be broken but with certain time/controlled conditions. Systems used for cell analysis should be adaptable for these different study purposes.

FLOW INJECTION AND RELATED TECHNIQUES FOR BLOOD STUDIES

Regardless of the objectives, clinical screening/analyses usually all have some desired features in common, including the need for rapidity, the use of only a small amount of sample per test, ease of operation, and low cost. Rapidity is important because the appropriate further extensive test or treatment may need to be done urgently to stop the progress of disease. Many screening tests may need to be carried out at the same time; therefore, the requirement of using a low amount of sample is essential in order to have adequate sample left for other tests. This is especially critical for the cases where patients have no sample to spare, such as under-weight and premature babies. The cost may not be the most important issue in certain places, but many areas of the world where economic and limited budget problems are the healthcare barrier, it would be most beneficial to have low cost screening tests for the purpose of disease control and prevention.

The disadvantages of most existing screening methods are due to the over-simplified processes that provide only crude yes/no answer. Most are not automatic operations, and they have room for human errors and low precision. Most screening processes, even though they are easy to perform, require well trained personnel to translate the results. For example, the observation of abnormal cells under a micro-scope or distinguishing results based on a particular shade of colors requires a well trained eye.

Owing to the automatic chemical process and closed system features of the flow based techniques, these drawbacks can be minimized with the improvement of the analysis time, precision, volume of sample/reagents used, and safety in sample handling. The FI/SI systems have another useful feature in that they have the ability to be coupled with various detection techniques. In particular, SI, which is capable of handling small volumes of various reagents has been addressed as a very useful tool for performing clinical and biochemical analysis (Economou et al. 2007). The use of FI/SI enables the screening/analysis to be enhanced with the various newly developed detection methods. Most of the existing detectors now offer very satisfactory sensitivity, and they help to reduce the consumption of reagents and sample, which, in turn, also reduces the production of bio-hazardous wastes. In addition, the results obtained from the flow based system are normally in the form of peak signal, which can be translated into graph or number values for easy understanding and comparison.

The FI/SI applications for analysis of serum and plasma are numerous because they can be adapted from newly developed solution based chemical analyses. These will not be included in this review. The applications of FI/SI for analysis of whole blood are described briefly in the next section. This is to indicate the feasibility of flow injection with various sample pretreatment and detection systems, especially those that were applied for blood analysis. However, as mentioned earlier, blood cell analyses involve various aspects that are not always the same as solution based analyses of plasma and serum. Blood cell analyses require a particular way of sample introduction and detection. The rest of this review emphasizes the application of flow based techniques, especially those that were coupled with FI/SI, in clinical studies through analysis of red blood cell (RBC) and substances inside RBC.

APPLICATIONS OF FLOW INJECTION AND RELATED TECHNIQUES FOR ANALYSIS OF WHOLE BLOOD

Whole blood contains both liquid and cell/particulate components. Analyses of whole blood normally aim for quantitation of total amount/concentration of analyte in blood, regardless of whether the chemical sources are plasma or cell. Whole blood is different from solution based samples like plasma and serum in that it contains particulates. Therefore, the analysis system must be capable of suppressing or removing the interference effect from particulate suspended in the samples. Examples of FI/SI analysis systems for various analytes in whole blood are presented in Table 1. The role of flow based system for whole blood analysis is to integrate online sample pretreatment, such as digestion, dialysis, and extraction, with the detection process. This helps to automate the whole analysis process with much more convenient operation as compared to carrying out each individual step batch-wise.

APPLICATIONS OF FLOW INJECTION AND RELATED TECHNIQUES FOR ANALYSIS OF HEMOLYSATE

To study the substances inside the RBC, the destruction of cells is required to release the substances of interest prior to detection. The process, called hemolysis, usually employs chemical reagents, or mechanical force from sonication and centrifugation. Some basic applications of FI in hemolysate analysis for automation of

Table 1. Examples of FI and related techniques reported for analysis of whole blood

Analyte	Flow system	Special role of flow system	Detection method	References
Metals				
Hg	FI	– online digestion	– Cold vapor AAS	Barbosa et al. 2004; Burguera, Burguera and Gallignani 1992; Guo and Baasner 1993
			– AAS (commercial system)	Albalak et al. 2005; Chen et al. 1998
Ca, Mg, Cu, Zn	FI	– online chemical treatment and ultrasonic agitation	– FAAS	Rondon et al. 1995
Zn, Cu, Fe	FI	– online digestion	– AAS	Burguera, Burguera and Brunetto 1993; Burguera and Burguera 1991; Burguera, Burguera and Alarcon 1986
Mo	FI	– online digestion/precipitation	– ETAAS	Burguera, Burguera and Rondon 2002
Sb	FI	– online extraction	– Hydride generation AAS	Petit de Pena et al. 2001
Pb	FI	–	– Stripping potentiometry	Almestrand et al. 1988
Co	FI	– online digestion	– ETAAS	Burguera et al. 1995
Se	FI	– online digestion	– Hydride generation AAS	Alegria et al. 1998
Glucose	Stopped FI	– online enzymatic reaction	– Chemiluminescence	Lee and Choi 2001
	FI	– online dialysis	– Chemiluminescence	Petersson 1989
	FI	–	– Amperometry	Johnson and Kriz 1998; Buch-Rasmussen 1990; Gunasingham, Tan and Aw 1990; Gunasingham and Tan 1990
	FI	– online enzymatic reaction	– Thermal biosensor	Harborn et al. 1997
	FI	–	– Absorbance	Ayupe de Oliveira et al. 2005

NADH, glucose	FI	– online enzymatic reaction	– Amperometry	Yao et al. 1995
Urea	FI	–	– Selective electrode	Petersson 1988
	FI	– online gas diffusion	– Optosensing	Petersson, Anderson and Hansen 1987
Ammonia	FI	– online diffusion	– Photometry	Svensson and Anfaelt 1982
EtOH	Stopped FI	– online enzymatic reaction	– Fluorometry	Ruz, Luque de Castro and Valcarcel 1987
	FI		– Voltammetry	Fernandez, Luque de Castro and Valcarcel 1987
Cyanide	FI	– online extraction	– Chemiluminescence	Lu et al. 2005
Electrolytes (H, K, Cu)	FI	–	– ion selective membrane on field effect transistor	Harrow et al. 1980
Malondialdehyde and ofloxacin	FI	– micro-dialysis	– HPLC	Chen, Wu and Huang 2009
Protein and Hb	micro-fluidics	– online capturing of protein	–	Wen et al. 2007
	SI	– online solid phase extraction with multi-walled carbon nanotubes	–	Du et al. 2007
Guanidinoacetate, Creatine (in dried blood spot)	FI	–	– electrospray tandem mass spectrometry	Carducci et al. 2006

sample/reagents introduction and manipulation are presented in Table 2. A few novel developments and applications of flow based systems are described in more detail in the following sub-sections.

Cost Effective Hemoglobin (Hb) Typing System

The Hb typing system is the separation of Hbs to investigate for the presence of any abnormal Hb type or abnormal amount of certain Hb in the sample. It is commonly done by HPLC analyzer. The main disadvantage of HPLC is its relatively higher instrumentation cost. Some HPLC analyzers are made specifically for thalassemia (Tosoh Biosciences 2010), but the buffer ingredients are normally unrevealed and need to be ordered from the manufacturer at a high price. Examples of FI/SI system combined with small sized ion-exchange columns (2 cm length, 3 mm i.d.) were demonstrated for Hb typing. A simple FI system could operate Hbs separation with buffer gradient created by mixing of buffers of different pHs using low cost 3-way valves (Srisawang et al. 2003). More automatic control for buffer gradient could be achieved using a more sophisticated SI system (Hartwell, Sripaoraya, et al. 2010). The possibility of parallel sampling can also be done with an SI system, employing a multi-selection valve. Either anion or cation exchanger could be used successfully for separation of human adults' hemoglobin (i.e., HbF, HbA, and HbA_2). Abnormally high amounts of HbA_2 indicate the presence of HbE (which is co-eluted with HbA_2). Even though the separation time obtained from the FI/SI systems is not as good as HPLC due to the slower operational characteristics of the pump, the main benefits of the FI/SI systems are the lower instrumentation cost, and

Table 2. Examples of FI systems reported for analysis of hemolysate

Analyte	Flow system	Detection method	Special features	Ref. No.
Glutathione	FI	Chemiluminescence	Use glutathione to enhance chemiluminescence of luminal and sodiumperiodate reaction	Ensafi, Khayamian, and Hasanpour 2008
Hb	FI	Chemiluminescence	facilitate the study on enhancement of chemiluminescence intensity of the reaction between Hb and H_2O_2 by addition of β-cyclodextrin	Zhang and Ju 2003
Hb	FI	Electrochemistry	with modified electrode using organic substance and dyes (i.e., lipoic acid, thionin, toluidine blue, methylene blue) to promote redox reaction of Hb)	Zhang et al. 1998; Zhou and Wang 1992; Zhou and Wang 1991; Ye and Baldwin 1988
Hb	FI	Electrochemistry	with modified electrode using nanomaterials (i.e., CdS quantum dots and cobalt hexacyanoferrate (CoHCF) nanoparticle)	Liu et al. 2006; Liu et al. 2005

having a simpler known buffer ingredient. The search for suitable ingredients of the mobile phase buffer solution to better separate Hbs of various types should be very useful.

Rapid Low Volume Multi-Parameters/Multi-Samples Analysis Systems

In flow based systems, performing a sequence of multi-reaction/detection is possible. An example of this research, dealing with red blood cell (RBC), is the simultaneous quantitation of total Hb and glycol-Hb (Nanjo, Hayashi, and Yao 2007). By placing two detectors, one at the entrance to the sample loop and another at the exit port of a normal six port injection valve, sample was detected by one detector during the normal sample loading mode and then was detected by another detector when switching to the injection mode, see Figure 2. Total Hb was detected spectrophotometrically followed by the detection of glycol-Hb electrochemically. Sample throughput of 40 samples/h was achieved.

An example of a down scaling device for analysis of multi-RBC samples is a simple polydimethyl siloxane (PDMS)/glass microfluidic chip with parallel channels platforms, fabricated for the assay of glutathione in normal and type 2 diabetes RBCs (Oblak, Meyer, and Spence 2009). A number of samples could be analyzed at the same time. The microfluidic multi-analyses systems decrease the analysis time

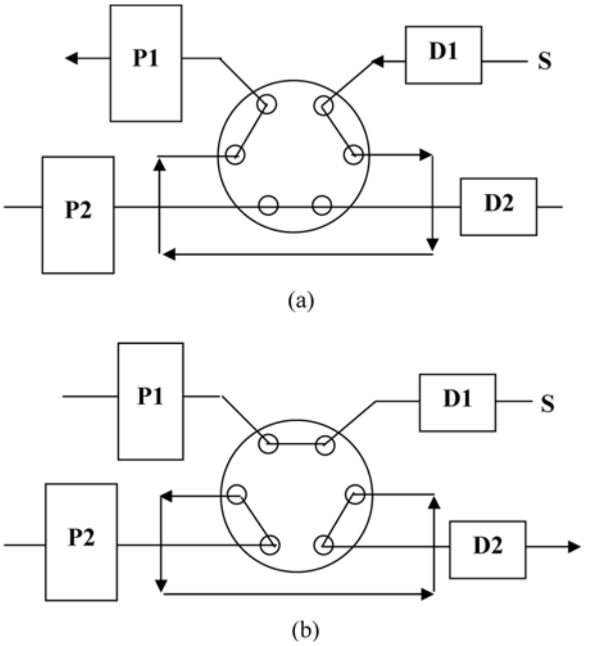


Figure 2. Arrangement of dual detectors for multi-parametric detection using a normal FI system (a) detection using the first detector while loading sample and (b) detection using the second detector in the injection mode. P1, P2 = pumps; D1, D2 = detectors.

and sample/reagent consumption. However, the technology involved is rather new and at high expense. The low cost disposable chip and the ease of fabrication process are in demand.

APPLICATIONS OF FLOW INJECTION AND RELATED TECHNIQUES FOR ANALYSIS OF RED BLOOD CELL (RBC) THAT REQUIRES RUPTURING IN CONTROLLED TIME/CONDITIONS

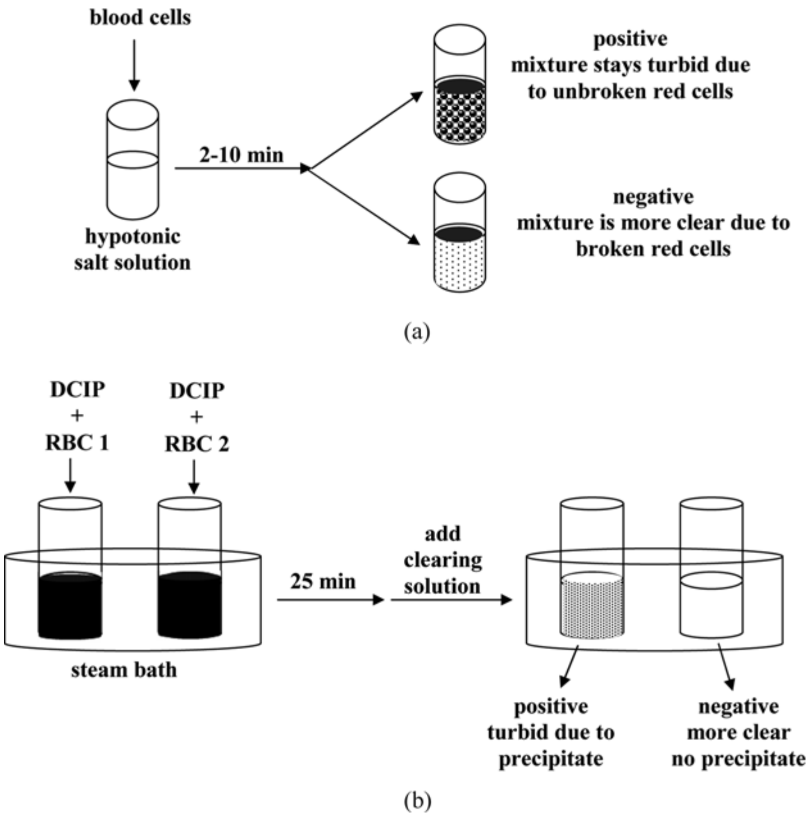
In some studies, RBC is required to be hemolyzed but at controlled times and conditions. The analysis system must be able to delay or prevent the hemolysis, until needed. Some unique flow based systems developed for this purpose are described in this report.

Stopped Flow Hydrodynamic Injection for Automatic Screening of Abnormal Red Blood Cell (RBC)

Abnormality of RBC shape and cell wall can be detected indirectly from their osmotic pressure resistance as compared to the normal RBC with the screening method called osmotic fragility test (OFT). Some hemoglobinopathies involving unstable Hb with exposed chemical groups can be detected from the precipitation reaction at high temperature with dichlorophenol indophenol (DCIP). Figure 3 shows the diagram of the processes of these screening tests. The results of both OFT and DCIP screening tests can be observed from turbidity by naked eyes but with the limitation that the detection must be done in the restricted time. If the test tubes were left too long, both normal and abnormal subjects would show no differences. In OFT, normal red cells will eventually rupture and turn clear if they stay in a hypotonic salt medium for a long time. In DCIP, normal Hb might also expose chemical groups that can be precipitated and cause turbidity when they were heated too long. In addition, since the results are observed by the naked eye, there is no evidence or record for second opinion. There is a greater chance of human errors when carrying out many tests.

By incorporating a stopped flow injection system with OFT and DCIP (Khonyoung et al. 2009; Hartwell, Kochasit, et al. 2009), more precise detection time for each test can be controlled. The most important point of OFT is that RBC must only be broken because of osmotic pressure caused by the medium. Likewise, in DCIP, precipitation of Hbs should only occur due to naturally exposed precipitable chemical groups. To ensure this condition, the sample introduction unit has to be developed to avoid cell rupturing and Hb deformation due to the switching of a normal sample injection valve as used in the FI system. Here, a hydrodynamic injection system was employed in place of a normal six-port switching valve. Dispersion and dilution effect must be brought into account when studying for the suitable hypotonic salt concentration and its pH for OFT, and the suitable recipe for DCIP reagents.

A spectrometer, which is more sensitive than human eyes, showed the differences in turbidity between positive and negative cases in a shorter time as compared to naked eyes observation (i.e., 15 s vs. 2 min for OFT and 3 min vs. 25 min for DCIP at higher temperature). The analytical signals obtained from the stopped flow hydrodynamic injection based OFT and DCIP were shown as slopes and peaks,



(a)

(b)

Figure 3. Diagram illustrating the screening processes for (a) osmotic fragility test (OFT) and (b) dichlorophenol indophenol precipitation test (DCIP).

respectively. These results could be recorded for further reference. The need for further developments in this area should aim for multisamples analysis to accommodate massive screening in parallel.

Flow Based System for Analysis of Single Cell Lysate

Cell studies are often carried out with a group of cells or cell cultures. Novel designs of cell trapping devices to be coupled with FI/SI systems are useful. Some could be used for the study in the genetic transcription levels of cells (Hillard and Stewart 1998). Currently, the need for analysis of single cell has become a challenge for many study purposes. An example of a successful integration of a flow based system for injection of intact RBCs into a capillary electrophoresis system was reported (S. Chen and Lillard 2001). An electroosmotic flow pump introduced cells into an interface capillary that provided mechanical force and caused an individual cell to be lysed on-column without the need of chemical reagents. Major proteins in hemolysate were migrated and separated by capillary electrophoresis simultaneously after online hemolysis.

APPLICATIONS OF FLOW INJECTION AND RELATED TECHNIQUES FOR ANALYSIS OF WHOLE INTACT/NON-RUPTURED RED BLOOD CELL (RBC)

Studies on the characteristics and chemistry of intact cells are also very important. The FI/SI manifolds can be designed in various ways to manipulate the order and direction of flow. Various aspects of flow based techniques for the applications on cell studies are presented.

FI-Flow Cytometry for Single Cell Study

One main use of flow cytometry (FC) is for detection of cancerous cells which are labeled by a specific dye (Goodale et al. 2009). Thousands of cells can be analyzed in a short time. The unique combination of FC with field flow fractionation (FFF) technique has been reported for additional information on cell study (Cardot et al. 2002).

The multiparametric FC, by itself, may already be capable of qualitative and quantitative single cell analysis. However, if it is connected to the FI system, the analysis performance can be improved. The flow based nature of FI and FC techniques enable them to be easily combined with the capability of online sample pretreatment (e.g., online dilution, online reagent addition, mixing, cell fixation, staining, washing, and temperature controlling for cell suspensions) and improved kinetic measurement (Zhao, Natarajan, and Srienc 1999; Ruzicka and Lindberg 1992; Lindberg et al. 1994; Blankenstein et al. 1996; Lindberg, Ruzicka, and Christian 1993). It should also be worthwhile to mention that, on another aspect, the cytometry sheath flow system helped to enhance the performance of the stopped flow injection technique (Hodder et al. 1997).

So far, most works reported the FI system as a sample pretreatment unit for flow cytometry (FC) and it would be of interest to couple SI with FC. Using FI or SI for post-separation chemical analysis/biochemical assay should also be possible. Confirmation or supporting information may be gained with these integrations.

FI-Field Flow Fractionation (FFF) Blood Cells Separation Systems

The chromatography-like field flow fractionation (FFF) technique is a size based particle separation technique, which may be operated using either gravimetrical, electrical, or magnetic forces that are applied perpendicularly to the direction of the flow of the particle suspension stream. Its theory and development of various formats of instrumentation set ups has been on going. The basic set up for sample introduction for FFF is similar to that of the FI system which consists of a pump and an injection valve. The parabolic flow profile of the particulate suspension sample stream in a small channel is also the same as what occurs in the flow of the liquid sample zone in the small tubing of the FI system (Chianéa, Assidjo, and Cardot 2000). Now the applications of FFF have been extended for the possibility of differentiation of normal cells from abnormal ones, such as hemolytic anemia cells (Andreux et al. 1993), cancerous cells (Yang et al. 1999), and cells of different ages (Cardot, Launay, and Martin 1997), which usually have deformed shape, density and rigidity (Cardot et al. 1994).

Only a few works reported the coupling of FI technique in conjunction with FFF, as an interface for chemical analysis after cell separation. Examples found are for elemental analysis with ICP-MS (Cizdziel et al. 2008) and for chemiluminescence reaction of heme (Melucci et al. 2004). Actually, the combination of FI with FFF seems to have more room to grow, both for sample pretreatment and for post-separation analysis.

Flow Based Magnetophoresis for Cell Sorting

Magnetophoresis is the particle separation technique based on differences in their movement or migration in the magnetic field due to the magnetic properties of the particles. In many aspects, this technique is very similar to FFF performed under magnetic force, but it has been reported under its own name, possibly owing to differences in the detailed instrumentation set up.

Sorting of cells with different magnetic properties such as deoxy/methemoglobin (found in blood poisoning) with paramagnetic property and oxyhemoglobin with diamagnetic property could be done. Iron and hemoglobin contents could also be quantitated in this process (Winoto-Morbach, Tchikov, and Müller-Ruchholtz 1995). The ability of cells to bind with a certain antibody can also be studied through magnetically labeled antibodies (McCloskey et al. 2003; Hartig et al. 1992). The FI technique should be able to facilitate sample handling and detection for the magnetophoresis system. Trends in analysis of this field aim to enhance the magnetic field gradient, reduce the size into capillary format (Watarai and Namba 2002), and to develop reproducible instrumentation for a more detailed separation of a wider range of magnetic moments for extended applications (Espy et al. 2006).

FI Immunosensor for Red Blood Cell (RBC) of Specific Antigen

Noncompetitive immunoassay of specific antigen (Ag) on RBC was demonstrated with the immunosensor composed of dual glassy carbon electrodes (B. Lu et al. 1997). One was employed as a solid surface for immobilization of antibody against the Ag of interest. Another one was for study of non-specific binding. The captured blood cells were sandwiched by the enzyme labeled secondary antibody for amperometric detection. Although the work only demonstrated the assay of RBC with Ag A, it shows the possibility of further applications for automatic immunoassay of other blood groups and other substances on a cell surface.

APPLICATIONS OF FLOW INJECTION AND RELATED TECHNIQUES FOR BLOOD SAMPLE PREPARATION AND AS MODEL SYSTEMS FOR STUDIES RELATED TO BLOOD FLOW

Microfluidics Systems for Separation of Plasma from Blood Cells

Microfluidic devices were reported for separation of plasma from blood cells. In the cross-flow system, blood cells, and plasma were separated by size exclusion (VanDelinder and Groisman 2006). Plasma could be delivered to the device outlet at the rate of 0.65 µL/min after 30 s of blood introduction. It was also claimed that the flow in the device caused very little hemolysis. Another microfluidic system utilized hydrodynamic forces for the separation of plasma from blood cells with the emphasis on flow rate and temperature studies (Rodriguez-Villarreal et al. 2009). A wide range of operational conditions (i.e., 23–50°C, 50–200 µL/min) was possible for various capabilities of cells removal. These microfluidic devices have potential for possible integration with an on-chip assay for point of care diagnosis.

FI System as a Model of Blood Flow for Evaluation of Potential New Drug

In the process of developing an intravenous drug, it is important to evaluate its hemolytic influence (Dal Negro and Cristofori 1996; Obeng and Cadwallader 1989). A flow based system can be used as a simulation model for blood stream flowing in the vein at the injection site. The suitable size tubing can be selected as an artificial capillary vessel. Controllable flow rates of the blood stream in the tubing and of the injected solution can mimic the flow rate of blood flowing in the vessel and of drug injection flow rate. The flow based system could give primary information on the degree of hemolysis, which would be very useful for development of suitable formulations of the new injected drugs.

CONCLUSION AND REMARKS ON ANALYSIS TRENDS

Flow injection based systems with more automatic operation in a closed system help to facilitate sample handling, reduce the consumption of sample/reagents, decrease analysis time, and increase safety for the operator by minimizing the direct contact with hazardous samples/reagents. This is especially useful for clinical analysis where rapid results, good accuracy and precision are required. The low cost instrumentation set up of an FI system may be a suitable alternative for some screening tests in a small hospital with low budget. However, these flow injection based system may not be able to replace some existing analysis systems that are capable of parallel massive analyses. Down scaling and microfluidic devices are the trends of analytical device development. These trends are very attractive for blood analysis in order to reduce blood sample consumption and enable point of care analysis. Similar to the development of microfluidic systems for other chemical analyses, the aims of development of blood analysis devices should not only place emphasis on down scaling of the reaction unit, but also on the sample introduction and detection units, in order to create a true micro-total analysis device. In addition, for blood cell and intracellular analyses, the influence of sample flow in the micro-channel should be thoroughly evaluated since this can cause false results. Studies using blood samples for the purpose of clinical screening/diagnosis must pay special attention to the possible source of any false results due to the newly developed methods because they may adversely affect the decision for further treatment. A few studies of blood cells deformation in the micro-channel under gravity (Yamada et al. 2008; Hardy et al. 2009) and under pressure driven flow (Hardy et al. 2009) have already been reported, but studies of more aspects concerning blood samples used in these methods should be carried out.

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ARTICLES

Assay of Chondroitin Sulfate Using Time-Based Detection in a Simple Lab-on-Chip[1]

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Abstract—Amount of chondroitin sulfate in supplementary tablets is determined using the reaction with methylene blue in a proposed simple lab-on-chip platform. An easy approach of detection by timing the migration of the reaction zone, which depends upon concentration of chondroitin sulfate, is described. This simple assay system does not require any complicated or expensive instrumentation and has a potential of further development for on-site analysis.

Keywords: lab-on-chip, chondroitin sulfate, time-based detection

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In a previous work [1], we introduced a simple lab-on-chip with channels easily fabricated within a piece of acrylic plastic. The system is manually operated without the requirement of any complicated devices. Detection was done by observing migration of the reaction zone by the unaided eye and the migration time was recorded with a stop-watch. This work shows an extended application of this simple lab-on-chip approach for nutritional supplements.

Chondroitin sulfate (**CS**) is a member of the glycosaminoglycan polysaccharides which is composed of repeating disaccharide (β-glucuronic acid and N-acetyl-β-D-galactosamine sulfate) units [2]. It has been reported as a potential biomarker for some diseases such as bone and ovarian cancer [3, 4] due to secretion of CS at elevated level into body fluids of patients. In addition, CS has been used for treatment of bone disorders such as rheumatism, arthritis and lumbago. Consumption of CS helps to improve and restore bone condition of the patients [5].

A simple way to quantitate CS is by monitoring its reaction with some dyes such as Azure B, methylene blue (**MB**), dimethylmethylene blue (**DMMB**), and brilliant cresol blue (**BCS**) [2, 6–8]. Among these dyes, MB is the reagent most commonly found in laboratories. Therefore, it was chosen to demonstrate the proposed system. CS and MB form a complex that causes the reduction of absorbance of MB at wavelengths 640 and 614 nm while producing blue shifted band with increasing absorbance at 570 nm [6]. Detection of CS can be normally done with a spectrophotometer at any of these three wavelengths. However, for this simple lab-on-chip with bare eye detection of the reaction zone, it is possible to observe the reduction of the intensity of dark blue MB solution as the reaction progresses. When the concentration of MB and the parameters such as angle of the chip are fixed, the rate of migration of the faded color reaction zone depends directly on the concentration of the CS that reacts with MB. Although these dye binding reactions are not specific for CS and cannot discriminate different glycosaminoglycan sulfates, they are widely used due to rapidity and simplicity. When used in combination with other techniques such as ELISA, quantitative determination of various sulfated glycosaminoglycans such as chondroitin sulfate, hyaluronan and keratin sulfate could be done [9, 10]. In this case, the simple system was demonstrated with two supplements which contain CS as the main ingredient with different matrices.

EXPERIMENTAL

Reagents. Stock solutions of chondroitin sulfate (Sigma) and methylene blue (Fluka) were prepared at 1000 mg/L and 5×10^{-3} M (1780 mg/L), respectively. Working solutions were prepared fresh daily by diluting the stock solutions to appropriate concentrations with deionized (DI) water. Calibration graphs were constructed from both chondroitin sulfate 6 and chondroitin sulfate 4.

The time based calibration graph was constructed from chondroitin sulfate standard solutions of 200, 250, 300, 350, and 400 mg/L. Supplementary pill sample solutions were prepared by dissolving sample powder with a representative weight of 1 tablet (averaged from 20 tablets) in DI water to the concentration of approximately 300 mg/L (determined from product

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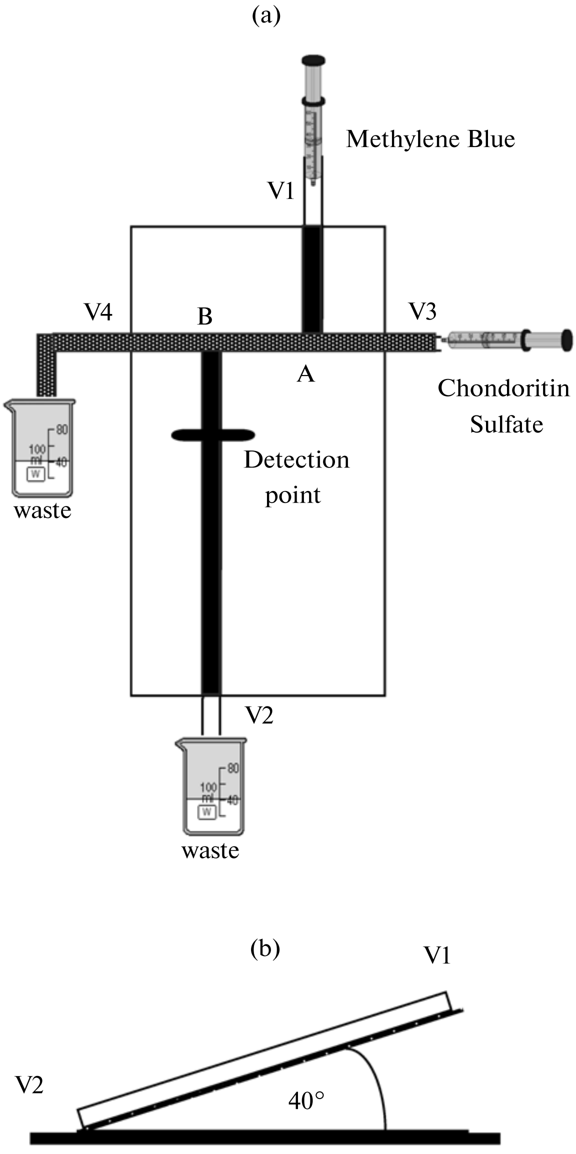

Fig. 1. Diagram of a simple chip (a) showing ports and direction of each solution (V: 3-way valve; A and B: crossing points of the horizontal and vertical channels) and (b) showing how the chip is tilted with respect to the horizontal plane.

label) to ensure that the concentration falls in the calibration working range. Two samples were used here for demonstration of the concept.

Apparatus. A macro-channel chip was made by drilling channels through the side of an acrylic piece 2.5 cm × 5.0 cm × 1.2 cm (width × length × thickness) with a 1 mm drillbit, as described in the previous work [1]. The format of the channels indicating the crossing point of the vertical and horizontal channels is shown in Fig. 1a. Total internal volume of the channels is approximately 55 μL.

The injection and exit ports at the end of the channels were made to fit normal nuts used in flow injection assemblies. Three way valves (MEDITOP) were connected to each nut (Upchurch) with a piece of 0.03" i.d. PTFE tubing (Upchurch). Syringes were connected to 2 out of the 4 ports, one to a horizontal channel and another to a vertical channel, for injecting sample and reagent solutions into the channels. The tubings connected to the other 2 ports led to waste containers.

Operational step. The chip was tilted as shown in Fig. 1b. When MB was injected into the vertical channel and CS into the horizontal channel, migration of the reaction zone could be observed from the crossing point B to the detection mark point. The reaction between CS and MB causes the change in color of MB from dark to light blue. The operational steps are as follows;

Step 1: MB solution was injected into the vertical channel through valves V1 and V2 while valves V3 and V4 were closed.

Step 2: Then valves V1 and V2 were closed and valves V3 and V4 were opened for introduction of CS solution.

Step 3: Valves V3 and V4 were closed (now all the valves were closed) Stop watch was started immediately.

Step 4: Once the reaction zone reached the detection mark point, the stop watch was stopped. Migration time (i.e. the period since CS and MB first contact (point B) till the decolorized zone arrival at the detection point) was recorded for each CS concentration.

RESULTS AND DISCUSSION

Optimum angle of the chip. Migration of reaction zone was in vertical downward direction. However, many forces including convection, gravity, capillary force, and diffusion are expected to have roles in migration. The explanation of this relationship may be complicated and need further investigation. In this work, the effect of the angle of tilting the chip (as shown in Fig. 1b) was investigated and selected based on analysis time and sensitivity. Figure 2 shows the time-based calibration graphs obtained from various angles of the chip position. It was found that tilting the chip at too high an angle (i.e. 80°) slowed down the migration. A tilt of 40° offered the shortest migration time with acceptable sensitivity. Therefore, it was chosen for further studies.

Optimum concentration of methylene blue. Concentration of the MB solution is expected to affect the detection process. If it is too concentrated, it would be difficult to observe the reaction zone that should have lighter color. This actually causes the analysis time to

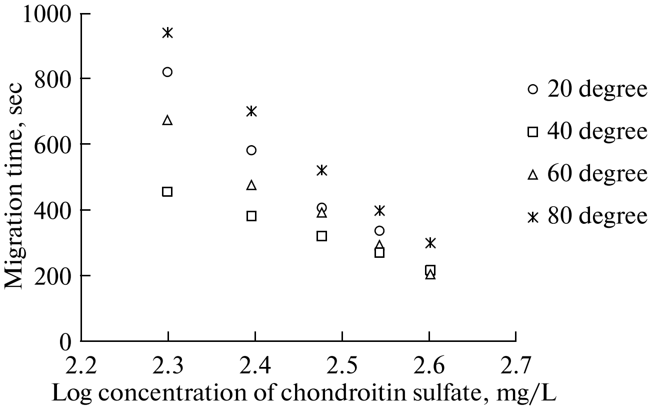

Fig. 2. Time based calibration graphs obtained from various degrees of chip tilting with respect to the horizontal plane. Concentration of methylene blue was 17.8 mg/L.

be longer. As shown in Fig. 3, higher concentrations of MB at 21.5 and 32.0 mg/L (or 60 and 90 μM) prolonged the detection time. At a lower concentration of MB, reaction zone could be observed easier and faster. However, if MB is too dilute, working range may be too narrow and sensitivity would decline. Therefore, concentration of MB at 10.7 mg/L (or 30 μM) was chosen for further studies.

Comparison of CS6 and CS4 calibration graphs. The two major types of chondroitin sulfate are CS4 (or CS-A) and CS6 (or CS-C), depending on the position of sulfonate group on N-acetyl-galactosamine [2]. However, in nutritional supplement products, there is no label of specific type of chondroitin sulfate. Therefore, calibration graphs of both CS6 and CS4 were constructed and compared. Linear calibration graphs were obtained in both cases as $Y = -871X + 2524$, $R^2 = 0.9953$ and $Y = -873X + 2533$, $R^2 = 0.9936$, for CS6 and CS4, respectively (Y: migration time; X: log concentration of CS). Their slope, intercept and R^2 values indicate that calibration graphs obtained from CS6 and CS4 are not significantly different. We selected CS6 as a standard CS in further studies due to its availability in our laboratory.

Precision. Within a day precision was found to be 3.4% RSD from 10 replicate runs of 300 mg/L CS standard solution. Precision of the system for the determination of CS at 200, 250, 300, 350, and 400 mg/L prepared from the same stock solution on 3 different days (10 and 30 days apart) was also investigated. Calibration graphs obtained on those days are compared in Table 1. Relative standard deviation (RSD) of the slope was 2%.

Determination of chondroitin sulfate in nutritional supplement samples. Although there was no significant deviation of calibration graphs obtained on different days, the time based calibration graph was always constructed on the same day as the sample analysis to ensure the best accuracy.

Two brands of nutritional supplement tablets were used to evaluate the proposed simple assay system. The ingredients in each supplement are as follows; Sample I: chondroitin sulfate 260 mg/tablet without other ingredients reported; and Sample II: chondroitin sulfate 300 mg/tablet with 400 mg glucosamin sulfate, 400 mg gelatine hydrolysate, 10 mg vitamin E, 5 mg zinc, and 0.02 mg selenium.

This simple time-based detection system could estimate the amount of chondroitin sulfate at 98 and 106% label, respectively (Table 2).

In conclusion, the proposed simple Lab-on-Chip with easily available reagent could correctly estimate the important ingredient of bone supplementary tablets (chondroitin sulfate) without the need of any complicated or expensive instrumentation. Assay of chondroitin sulfate in biological samples such as serum and urine is possible by appropriate pre-treat-

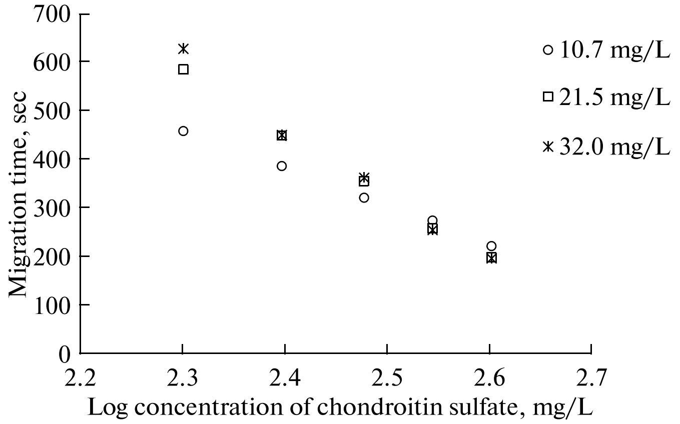

Fig. 3. Time-based calibration graphs of CS obtained from various concentrations of MB at 10.7, 21.5, and 32.0 mg/L when the chip was tilted at 40°.

Table 1. Comparison of time-based calibration graphs obtained on different days using the same conditions (Y is migration time and X is log concentration of CS)

Days	Calibration graph	R^2	Avg slope	%RSD of slope
1	$Y = -871\,X + 2524$	0.9953	−882	2
10	$Y = -874\,X + 2523$	0.9872		
30	$Y = -901\,X + 2610$	0.9900		

Table 2. Assay of CS in supplement tablets using the simple lab-on-chip with time-based detection

Sample	Labeled amount of CS, mg/tablet	Found amount of CS, mg/tablet	% of labeled amount
I (Japan)	260	255	98%
II (Germany)	300	318	106%

ment of samples [9, 11]. This application may be extended further for screening of some diseases or following up the treatment. The system has a potential to be developed for on-site analysis. Migration time-based detection in a simple Lab-on-Chip should be applicable for the assay of various substances, although the detail of parameters that affect the migration may be different.

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Trends in Analytical Chemistry, Vol. 27, No. 9, 2008 Trends

Recent developments in automatic solid-phase extraction with renewable surfaces exploiting flow-based approaches

Manuel Miró, Supaporn Kradtap Hartwell, Jaroon Jakmunee, Kate Grudpan, Elo Harald Hansen

Solid-phase extraction (SPE) is the most versatile sample-processing method for removal of interfering species and/or analyte enrichment. Although significant advances have been made over the past two decades in automating the entire analytical protocol involving SPE via flow-injection approaches, on-line SPE assays performed in permanent mode lack sufficient reliability as a consequence of progressively tighter packing of the bead reactor, contamination of the solid surfaces and potential leakage of functional moieties.

This article overviews the current state-of-the-art of an appealing tool for overcoming the above shortcomings, so-called bead-injection (BI) analysis, based on automated renewal of the sorbent material per assay exploiting the various generations of flow-injection analysis. It addresses novel instrumental developments for implementing BI and a number of alternatives for on-line chemical-derivatization reactions, and it pinpoints the most common instrumental detection techniques utilized. We present and discuss in detail relevant environmental and bioanalytical applications reported in the past few years.

Keywords: Bead injection; Bioanalysis; Bioassay; Chemical-derivatization reaction; Detection; Environmental analysis; Flow-injection analysis; Preconcentration; Sample processing; Solid-phase extraction

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1. Introduction

The complexity of the matrices of samples in environmental, biological, industrial and biotechnological fields along with the low concentration levels of target species that might be encountered pose severe dilemmas for their direct analysis, even when exploiting modern analytical instrumentation. This is a result of the dependence of the analytical read-outs upon concomitant matrix components, and the fact that the concentration of analytes is often below the dynamic linear range of the detection device. Hence, there is a need, prior to the quantification step, for the development of simple, robust and reliable sample-pretreatment procedures, which are aimed at removing interfering matrix constituents and at the same time improving analyte detectability by preconcentration. When performed in a manual fashion, these preliminary operations are labor intensive and time consuming, difficult to control systematically, and a source of major bias and accidental errors (e.g., sample contamination) that might have a decisive impact on the accuracy and the precision of the analytical results.

The advent of the various generations of flow injection (FI) (i.e. FI, sequential injection (SI), lab-on-valve (LOV)) and

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hybrid techniques for on-line or in-line sample processing has opened new perspectives as regards automation and miniaturization of sample handling with the extra advantage of saving sample and reagent consumption and reducing waste generation [1,2].

This review illustrates the current state-of-the-art of the so-called bead-injection (BI) technique, which performs the entire solid-phase extraction (SPE) procedure in a renewable fashion in flowing systems. In contrast with earlier reviews dealing with BI [3–6], this article critically discusses the overall platforms described in the literature for BI-based SPE analysis and illustrates alternative approaches for uptake and detection of target species. We also describe in detail pros and cons of BI assays, compared with conventional particle-packed microcolumns, for flow-based SPE, via selection of representative examples in the environmental and bioanalytical fields, as reported within the past decade.

2. On-line solid-phase extraction

Sorptive extraction is the predominant sample-processing method to be automated by resorting to flow systems as a consequence of its straightforward operation, high separation and preconcentration capabilities and minimum consumption of organic solvents. Most often, it is employed by using packed-bed or disk-phase-based microcolumns, which are filled with appropriate sorptive materials and placed within the flow network prior to the detection device.

The ultimate goal is to improve the sensitivity of the analytical procedure and/or overcome the inherently low tolerance of the detection system to sample constituents by preconcentrating the analyte or removing interfering components from harsh milieus (e.g., high-salt or protein-content matrices) with no need for sample dilution. Several solid-phase reactors with different designs, mainly uniformly-bored or conical microcolumns, have been successfully assembled in flow-based set-ups. The temporary retention of low-level concentrations of individual metal ions, nutrients and charged (bio)molecules by electrostatic interactions onto ion-exchange microcolumns or chelating reactors has been a common practice in flow-through SPE methods [7]. Derivatized non-polar metal chelates (e.g., iminodiacetates, dithiocarbamates, dithiophosphates or quinolinates) or hydrophobic species (e.g., organic pollutants, dyes and drugs) have been preconcentrated on reversed-phase materials (e.g., octadecyl-chemically modified silica gel, polytetrafluoroethylene (PTFE) beads or turnings, or multimodal polymeric sorbents) by partitioning, hydrophobic, or π-π interactions [1,7]. The target compounds are either directly retained on the reactive surfaces after appropriate sorbent conditioning or in-line derivatized into a suitable chemical form. This extraction mode circumvents interfering effects from alkaline and alkaline-earth elements when determining hazardous trace metals via intelligent selection of the chelating reagent [1].

As detailed above, flow-through SPE procedures have been implemented using permanent packed-column reactors. However, these sorptive columns are prone to problems in long-term operation due to the progressively tighter packing or clogging of the column material resulting in increase of back pressure, which might be alleviated to some extent by back-flushing elution protocols. Further drawbacks of on-line SPE with reusable surfaces are analyte carry-over effects, shrinking or swelling of the sorbent bed, malfunctions of the active entities including loss of functional moieties (the last of these being a common problem for reagent-impregnated bead materials) and, finally, surface deactivation due to irreversible sorption of interfering species. All these problems can be eliminated by exploiting the concept of renewable surfaces, or, as it has been termed, BI, i.e. where the solid-phase material, if called for, can be renewed for each analytical cycle.

3. The concept of bead injection

The BI approach originally adapted to optical sensing in SI analyzers was introduced as a powerful tool for the automation of immunoassays using diffuse reflectometric detection [4]. The miniaturized BI chemosensors use minute amounts of beads carrying the derivatization reagent or functional moieties, whereby the analyte becomes preconcentrated and can be monitored via optical fibers in custom-built flow-through cells that capture the active sensing surfaces while allowing the perfused liquid to flow freely [4]. After each analytical run, the sorbent particles are disposed by flow reversal and the sensor surface is renewed by injecting a new plug of a fresh bead suspension. This concept of optical sensing at solid surfaces, also termed solid-phase optosensing or BI spectroscopy, constitutes an attractive alternative to on-line SPE procedures with eluate detection for preventing the partial loss of the preconcentration capabilities gained during the sorption step as a result of dilution of the eluate plug when delivered to the detector [8]. Moreover, BI fosters real-time monitoring of the sorption process and thus detection relying on initial rate measurements can be readily accomplished. A further advantage of BI derived from its characteristic renewability is its inherent flexibility in accommodating reagent-based SPE assays with no stringent demands as to the full reversibility of the sorption or elution procedure. This is the case in uptake of Fe(II)-1,10-phenanthroline onto chelating beads because eluents, such as nitric acid or EDTA, are inefficient for quantitatively stripping out the chelate from the beads [9].

Notwithstanding the fact that both optical paths and reaction times in BI-based schemes are shorter, compared with steady-state liquid-phase spectrophotometry, no deterioration in sensitivity is observed as a result of immobilizing the analyte on a small surface area of the sensor wherein the reaction is driven by an excess of solid reagent [4].

In order to handle the bead material reproducibly within the automatic system, it is imperative to ensure bead-size homogeneity and the spherical shape of the reagent-supporting entities so as to prevent compact settlement into the conduits of the flow set-up. Hence, reversed-phase, chemically-modified, silica-gel lumps are not really suited for this purpose as a result of their irregular shape and size distribution [5]. However, bead material with a backbone of poly(styrene-divinylbenzene), poly(vinylpirrolidone) or agarose (e.g., Sephadex or Sepharose-type beads) fulfils the foregoing demands, inasmuch as they are perfectly spherical and have a uniform size. The use of either micellar media or ancillary continuous recirculation schemes for the bead suspension [10] might be needed for reliable manipulation of hydrophobic sensing entities with higher density than water within the flow manifold.

4. Development of instrumentation

4.1. *Jet-ring cell and rotating-rod flow cell*

The instrumentation development for BI, initiated by Ruzicka and co-workers [4], is based on an SI network furnished with the so-called jet-ring cell. The name ‘‘jet-ring’’ cell comes from the configuration and operation of the bead-retention cell made as part of the flow conduit by inserting a solid rod to block the beads in the assigned space. As shown in Fig. 1, the channel of the cell has an inner diameter slightly larger than the outer diameter of the blocking rod, forming the *O*-ring gap between the rod and the wall of the channel all around the rod. Only solutions are able to flow out while beads whose sizes are larger than the gap are packed due to the continuous incoming flow pushing the beads against the blocking rod. Used beads are discarded by the jet of solution reversing and rapidly flowing upstream.

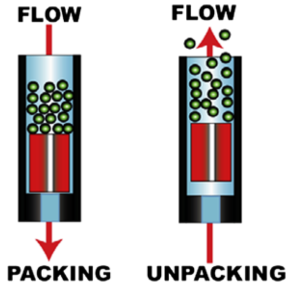

Figure 1. Jet-ring cell and operation of bead injection (packing and renewal steps) (Reproduced with the author’s permission from J. Ruzicka, Flow Injection Analysis CD-ROM Tutorial, 3rd Edition, 2004; Published by FIAlab Instruments, Inc.).

For capture and release of beads as small as 5–7 μm in SI manifolds, a novel bead-trapping strategy, the so-called rotating-rod flow cell [11], was designed. The renewable sorbent microcolumn is created by first trapping larger beads (>15 μm) in the flow cell, and then smaller beads bearing functional moieties are captured either within or on top of larger beads, thus acting as a filter layer [12].

4.2. *Lab-on-valve*

The LOV approach hyphenated to SI set-ups should be viewed as a judicious advance towards automation and miniaturization of BI schemes but within integrated microbore units [13]. The microconduit unit, made initially of Perspex, but more recently of hard polyvinylchloride, polyetheretherketone (PEEK) or polyetherimide (ULTEM), is a single monolithic structure mounted atop the multiposition valve of an SI assembly, as illustrated in Fig. 2. Designed to incorporate all necessary laboratory facilities for a variety of analytical chemical assays, including in-valve manipulation of sorbent materials, hence the name *lab-on-valve*, it is made to contain microchannels working as the bead-retention cell along with mixing points for chemical derivatization of the analyte, if needed, and a multipurpose flow-through cell for real-time interrogation of analyte-containing beads [13,14].

In short, packed-column reactors are generated in situ by aspirating beads from a peripheral port of the valve, advantage being taken of the fact that the sorbent in the LOV unit can be manipulated in exactly the same way as when handling liquids. The solid entities can even be automatically transported between different column positions within the micromachined unit, their retention within the columns being facilitated by fitting the column positions with appropriate stoppers (see Fig. 2), namely, up-down movable rods or optical fibers themselves, which will keep hold of the beads, yet allow solutions to flow freely. In-valve detection could be realized at the eluate in the same way as conventional SPE or by solid-phase optosensing [15].

As opposed to lab-on-chip (LOC) microdevices, the open architecture of the SI-LOV approach facilitates implementation of BI protocols at will with no need for redesign of the unit. Readers are referred to a recent comprehensive review by Miró and Hansen [16] for a critical discussion of the pros and cons of LOV compared to LOC microsystems for automated SPE.

4.3. *FI-BI with home-made and commercial flow cells*

BI has also been adapted to FI systems as a cost-effective alternative to more sophisticated SI set-ups. The

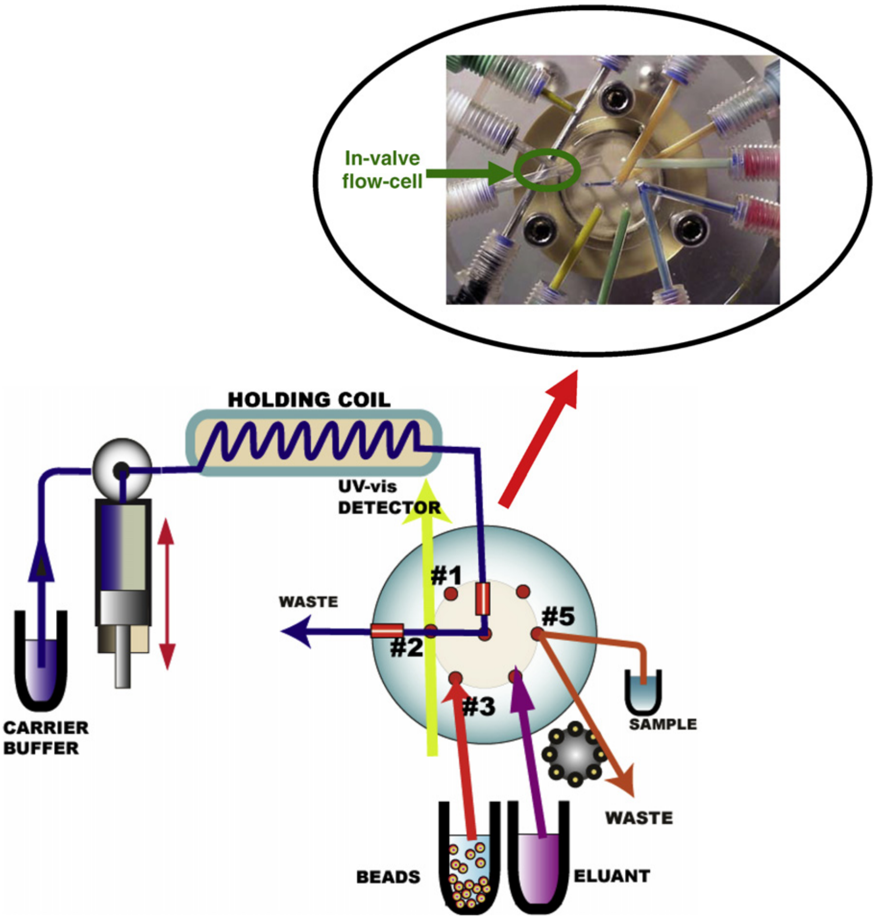

Figure 2. An SI-LOV set-up for BI optosensing. The inset shows a magnified close-up of the micromachined LOV unit (Reproduced with the author's permission from J. Ruzicka, Flow Injection Analysis CD-ROM Tutorial, 3rd Edition, 2004; Published by FIAlab Instruments, Inc.).

home-made bead-retention cell introduced by Grudpan's group [9,17] was modified from the jet-ring cell to suit the uni-directional flow normally employed in FI systems. The dedicated cell was furnished with an LED-based light source and a phototransistor detector at the opposite ends of the cell windows. Similarly to the jet-ring cell and LOV unit, the blocking rod leaves a minute space between the rod and the exit channel to let solution flow through while beads are retained. A mechanically movable blocking rod is used to control bead trapping and discarding instead of flow reversal. A solenoid actuator connected to the rod controls its movement. After each analysis, the rod is pulled back far enough to open the outlet and let the used beads flow out.

Commercially-available flow-through cells commonly used in FI liquid-phase spectrophotometric detection have been adapted to BI spectroscopy by Molina-Díaz and co-workers [18–20]. Glass wool is used to plug the outlet of the flow cell to keep beads inside. Bead discarding is done by backward flushing the beads out with a high flow-rate solution using an ancillary uni-directional peristaltic pump. Two Hellma-type flow cells, namely, 138 OS (1 mm light path, 50 μL inner volume) and 176 QS (1.5 mm light path, 25 μL inner volume), were used for spectrophotometric [18,20] and fluorometric [19] optosensing, respectively. The configurations of both flow cells can be found in Hellma's cell catalog and in works published by Molina Díaz's group [18–20]. However, according to the current Hellma catalog, the flow-cell model 138-OS is no longer available. A drawback of the commercial flow cells is that the detection window is smaller than the bead volume, so not all product sorbed is monitored.

4.4. Magnetic flow-through cell

Ruzicka and co-workers [21] proposed in the 1990s a tubular BI cell design for magnetic bead-based SI immunoassays with detection of unbound labeled antibodies in the liquid phase, which was later extended to solid-phase optosensing by Imato's group [22,23] using a dedicated configuration for BI with luminescence detection. The magnetic beads involving functionalized agarose-coated iron-oxyhydroxide particles are suitable for assays (e.g., immunoassays) involving several operational steps. A neodymium magnet or magnetic wire located under the (immuno)reactor is moved up and down to trap the magnetic beads in place or let them flow with carrier solution. Fig. 3 shows the configurations of the magnetic flow cell and SI network for BI-based chemiluminescence bioassays. In this open tubular cell design, the continuously flowing stream of solution is less likely to cause bead packing or clumping during the multi-steps of washing and incubation required in immunoassays. The most important limitation of this flow configuration for BI stems from the metallic nature of the bead core, which impedes monitoring of the development of chemical reactions at low pH values.

5. Analytical procedures for bead injection

5.1. Bead modification

Although commercially-available ion exchangers or (co)polymeric beads can be directly utilized for BI, raw sorbents might be modified in the batch mode to incorporate appropriate moieties by physical or chemical binding with the bead surface or matrix for selective sorption or reaction with the analyte. As a consequence of the renewable nature of beads, BI is well suited to overcoming the most severe drawback of SPE procedures involving physical immobilization of reactants, i.e. the short lifetime of the loaded sorbent because of the gradual flushing out of the reagent during sample percolation.

For bioassays, antibodies or antigens [15,21–25] have been immobilized on magnetic [21,22] or polysaccharide-based [24,25] beads. The latter have also proved suitable as substrate for cell cultivation [26,27].

For environmental assays of trace metals, polymeric beads have been loaded with ligands (e.g., carbazides or azo compounds) off-line [28–30] because the time frame for quantitative impregnation of the bead surface can then be selected at will regardless of ligand-sorption kinetics. Compared with on-line protocols, higher reagent concentration on the sorbent material is then ensured, which, in turn, should lead to improved enrichment factors and analyte-breakthrough volumes. However, optimization of immobilization protocols (pH and reaction time) by measuring the rate and the yield of reactions could be performed at-line in SI-LOV by attaching a stirred bead microreactor to a peripheral port

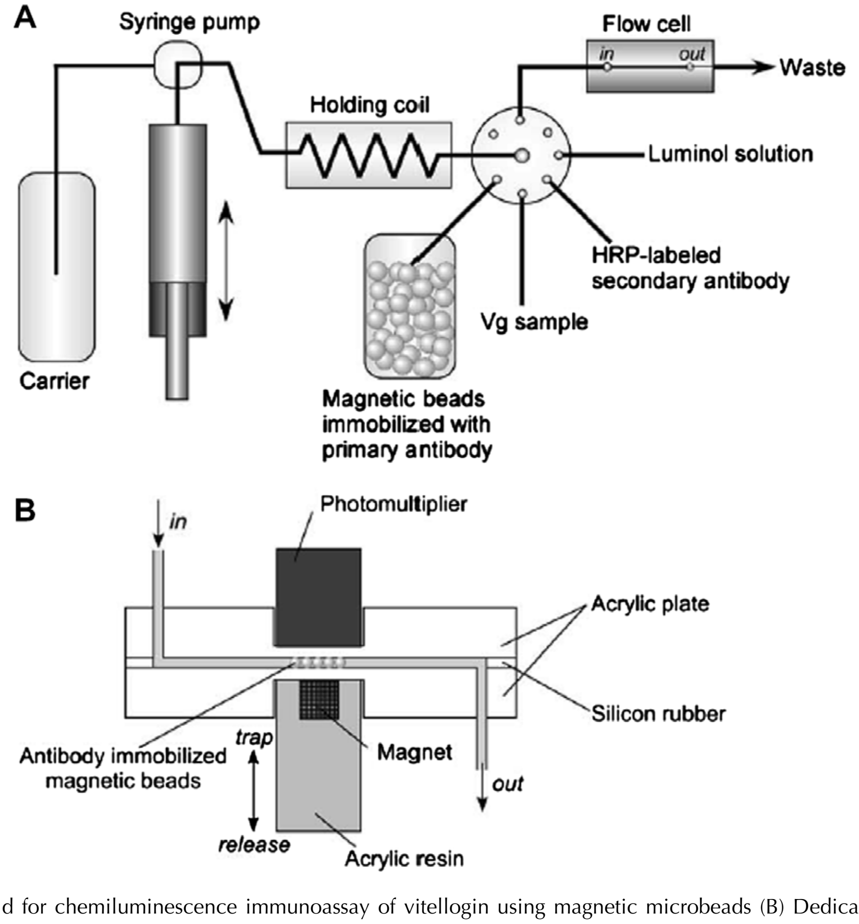

Figure 3. (A) SI manifold for chemiluminescence immunoassay of vitellogin using magnetic microbeads (B) Dedicated flow-through cell for retention of antibody bearing magnetic beads. Vg, Vitellogin; HRP, Horseradish peroxidase (Reproduced from [22] by permission of Elsevier Ltd.).

of the selection valve and real-time monitoring of ligand uptake on bead surfaces [31].

5.2. *Analyte uptake*

Sorption of the analyte itself onto renewable ion-exchange resins has been common practice for SI-LOV-BI determination of trace-metal ions [5], although it could equally be exploited for uptake of charged drugs or biomolecules followed by UV-Vis or fluorometric measurements [32,33].

Analytes could in principle also be retained on the sorbent prior to addition of a chromogenic agent for heterogeneous on-bead derivatization in solid-phase spectrophotometric measurements. For example, copper [34], iron [9] and mercury [35] were separated from sample matrices via sorption onto chelating resins prior to derivatization with ammonium pyrrolidine dithiocarbamate, 1,10-phenanthroline and dithizone, respectively. However, the approach is limited to those applications where the color-development reaction proceeds rapidly with no elution of the chelate formed.

To tackle the above drawback, researchers have exploited homogeneous phase derivatization, i.e. the analyte reacts in-line with the ligand before sorption of the resulted products on beads. This reaction might lead to improved retention selectivity and increased affinity of the analyte to the sorptive material. For example, trace metals after derivatization into a non-charged chelate or (co)precipitate are selectively sorbed on reversed-phase (e.g., PTFE or octadecyl-chemically-modified copolymeric) packing materials [18,36,37]. Indirect determination of drugs (e.g., promethazine and trifluoperazine) could be effected by on-line oxidation with Fe(III) with subsequent complexation of the resulting Fe(II) with ferrozine followed by solid-phase spectrophotometric detection of the chelate onto anion exchangers [18].

5.3. *Detection techniques*

A notable asset of BI is the inherent capability for hyphenation to many instrumental devices. Though most frequently used in combination with spectrophotometry [9,15,17,20,25,35], BI spectroscopy also has applications involving fluorometric [12,19] and chemiluminescence [22,23] detection. The most important requirement for BI spectrophotometry is the optical transparency of beads to prevent high signal background and light-scattering effects. It should be noted that not merely single-parameter but biparametric BI-based spectrophotometric or spectrofluorometric sensors have been designed for determination of trace elements (e.g., Zn and Cu or Al and Be) using a non-selective chelating reagent and selective elution or sorption protocols [19,20]. Unfortunately, sequential assays were required because of the limited chromatographic resolution of the BI columns.

Coupling BI to electrothermal atomic absorption spectrometry (ET-AAS) with direct introduction of metal-loaded sorbent into the graphite furnace is feasible for polymeric organic beads (e.g., Sephadex or Sepharose) because of pyrolysis of the beads prior to metal atomization [5]. Besides the aforementioned detectors, BI has been coupled to voltammetric [26], inductively coupled plasma-mass spectrometry (ICP-MS) [5], atomic fluorescence spectrometry (AFS) [37,38], MS [39] and radiometric [40] instruments, although eluate detection was called for in all instances.

6. Applications of flow-through bead injection

In this section, we review relevant applications of BI in both environmental and bioanalytical fields. Table 1 shows the analytical performance of such methods, including the sorbent material, the flow approach, the detection technique, the dynamic linear range, the detection limit, the precision and the sample matrix.

6.1. *Environmental assays*

Most of the work conducted in this field has focused on determination of trace-level concentrations of inorganic elements in harsh environmental matrices containing high levels of dissolved salts, such as seawater, brines or soils [29,36,41]. To this end, matrix separation with concomitant metal/metalloid enrichment has been effected using SI-LOV-BI preconcentration and eluate detection by ET-AAS or AFS. SI-BI is also a suitable platform for accommodation of trace-metal-speciation assays (e.g., Cr(III) and Cr(VI)) via selective retention of oxidation states in appropriate sorbent materials (e.g., cation and anion exchangers) or via sequential determinations of a single species and total inorganic metal following in-line homogeneous/heterogeneous redox reactions and preconcentration in a given sorptive microcolumn [42]. Readers are referred to a comprehensive review article [43] for further details as to the designs and analytical performance of SI-BI-LOV methods for trace-metal determinations in environmental matrices.

Recent trends are devoted to the exploitation of hybrid flow systems (e.g., multi-syringe flow injection (MSFI) analysis) in combination with BI-LOV to expand the scope of the miniaturized systems [38,44]. It should be borne in mind that SI-BI-LOV manifolds are not well suited to implementation of post-LOV derivatization reactions because of the need in SI-LOV to aspirate the overall solutions in a holding coil. However, interfacing BI-LOV with MSFI enabled the sorptive preconcentration and determination of hydride-generating species by on-line derivatization of eluate with sodium tetrahydroborate prior to AFS detection [38]. Despite the divergent kinetics of analyte sorption and hydride generation,

Table 1. Analytical performance of representative flow-based sorbent extraction methods involving bead injection analysis

Analyte	Flow system	Bead type	Detection technique	Detection limit	Linear range	Precision	Sample/ Application	Ref.
Metal and metalloid species								
Fe	FI	Iminodiacetate chelating resin (Chelex-100)	Spectrophotometry	0.2 μg/L	0.5–6 μg	NR	Beer	[9]
Cd	SI-LOV	C_{18}-PS/DVB and PTFE	ET-AAS	5 ng/L (PTFE) and 135 ng/L (C_{18})	0.05–1μg/L (PTFE) and 0.2–1.5 μg/L (C_{18})	4.3% (PTFE) and 3.4% (C_{18})	CRM 279 (Sea Lettuce), CRM 320 (River sediment), NIST 1640 (Natural water) and human urine	[10]
Fe	FI	Chelex-100	Spectrophotometry	25 μg/L	Up to 217μg/L	4%	Spiked tap and drinking water	[17]
V(V)	FI	Sephadex QAEA-25 loaded with ARS	Fluorometry	0.45 μg/L	2–60 μg/L	4.22%	Water, serum, urine and mussel tissues	[19]
Cu and Zn	FI	Sephadex QAEA-25 loaded with Zincon	Spectrophotometry	29 μg/L Cu and 40 μg/L Zn	50–1000 μg/L Cu and 100–1800 μg/L Zn	< 5%	Water, pharmaceutical preparation, soil and hair	[20]
Co	FI	Dowex 50 W loaded with PAN	Spectrophotometry	19 μg/L	50–2000 μg/L	4.16%	Water, pharmaceutical formulation, steel	[28]
Cr(VI)	SI-LOV	C_{18}-PS/DVB impregnated with DPC	ET-AAS	0.03 μg/L	0.12–1.5μg/L	3.8%	NIST 1640 (Natural Water), tap water and seawater	[29]
Pb	SI-LOV	Sephadex G-25 impregnated with dithizone	ET-AAS	0.3 ng	1–4 ng	1.9%	Standard solutions	[30]
Cu	FI	Chelex-100	Spectrophotometry	10 μg/L	0.01–1.0 mg/L	NR	Swimming pool water and supplement tablets	[34]
Cu and Hg	SI	Chelex-100	Spectrophotometry	0.63 μg/L Cu and 0.25 μg/L Hg	5–500 μg/L Cu and 2.5–30 μg/L Hg	5.2% Cu and 9.4% Hg	River water	[35]
Ni	SI-LOV	poly-DVB-co-N-vinylpyrrolidone	ET-AAS	0.05 μg/L	0.2–2 μg/L	< 5.6%	Hard tap water, seawater and NIST 1640	[36]
Cd	SI-LOV	C_{18}-PS/DVB	Hg-AFS	3.5 ng/L	NR	1.6%	CRM 320 (River sediment), CRM 279 (Sea Lettuce) and GBW 09139 (Cattle blood)	[37]

(*continued on next page*)

Table 1 (*continued*)

Analyte	Flow system	Bead type	Detection technique	Detection limit	Linear range	Precision	Sample/ Application	Ref.
Metal and metalloid species								
As	SI-LOV- MSFI	Q-sepharose (anion exchange)	HG-AFS	20 ng/L	0.05–2.0 μg/L	5.7%	Tap and underground water and SRMs (TMDA-54.3 Lake water and ERM- CA010-Hard drinking water)	[38]
Cr (VI)	SI-LOV	Q-sepharose (anion exchange)	ET-AAS	NR	0.02–0.6 ng	< 6.8%	SRM 2709 San Joaquin Soil/On-line leaching investigations	[41]
Cr(III) and Cr(VI)	SI-LOV	Chelating Sepharose	ET-AAS	0.010 μg/L Cr(III) and 0.020 μg/L Cr(VI)	0.02–0.28 μg/L Cr(III) and 0.035–0.4 μg/L Cr(VI)	4.7% Cr(III) and 4.5% Cr(VI)	Environmental waters, NIST 1640 and CRM 320	[42]
Radionuclides								
^{90}Sr, ^{241}Am, and ^{99}Tc	SI	Sr-resin, TRU-resin, TEVA resin (Eichrom industries)	Liquid scintillation	NR	NR	3% ^{90}Sr	Nuclear waste	[40]
Bioanalytical applications								
Biotin, DNA, rabbit IgG	SI	Lumavidin-coated beads, peptide nucleic acid-modified beads, Protein A-coated beads	Flow-cytometer fluorometry	NR	1–100 μg/L (biotin) and g/L level for DNA and IgG	< 25%	Binding assays/ sandwich immunoassays/ multiplexed analysis	[12]
IgG	SI-LOV	Sepharose with immobilized protein G	Spectrophotometry	5 mg/L (AC) and 50 mg/L (BIS)	0.100–1.00 g/L (AC) and 0.100–0.400 g/L (BIS)	NR	Human IgG, rabbit IgG, horse IgG	[15]
Mouse IgG	SI	Magnetic beads with immobilized polyclonal sheep anti mouse IgG	Fluorometry	155 μg/L	1–10 mg/L	2.1%	Competitive binding assays of IgG	[21]
Vitellogenin	SI	Magnetic beads with immobilized anti-vitellogenin antibody	CL	2 μg/L	2–100 μg/L	NR	Standard solutions	[22]

Goat, anti-mouse IgG	SI-LOV	Protein G-coated Sepharose 4B and Protein A-coated Sepharose 4B	Spectrophotometry	470 ng	0–0.4 g/L	NR	Interaction of antibodies with protein A and protein G via label dilution	[24]
GAD 65 autoantibody (type I diabetis)	SI-LOV	Streptavidin-coated Sepharose 4B	Spectrophotometry	20 μg/L	100–400 μg/L	< 5%	Human serum/ELISA	[25]
H_2O_2	SI-LOV	Live cells on Cytodex-2 beads	Voltammetry	9 μM	10–1000 μM	NR	Degradation rate of H_2O_2 by wild-type cells or catalase-overexpressing mouse embryonic fibroblasts	[26]
pH	SI-LOV	Sephadex G-50 with impregnated pH indicator/ Cytopore with immobilized live cells	Spectrophotometry	NR	7.2–7.4	< 8.38%	Investigation of extracellular acidification rates of the mouse hepatocyte cell line TABX2S	[27]
Albumin, ovalbumin, lysozyme, human IgG, ribonuclease A and cytochrome C	SI-LOV	Aminolink agarose beads	Spectrophotometry	NR	g/L level	NR	Examination and optimization of protein immobilization protocols	[31]
Biotin-containing conjugates and β-galactosidase	SI-LOV	Streptavidin-coated agarose beads	UV/Vis and ESI-MS	NR	mg/L level	NR	Determination of β- galactosidase activity in human cell homogenates/ Kinetic measurements of dissociation rates of model biotin- containing conjugates	[39]
Lactate and glucose	SI-LOV	Cytopore with immobilized live cells	Spectrophotometry	NR	0.05–1.00 mM (lactate) and 0.1–5.6 mM (glucose)	< 7.3% (lactate) and 7.1% (glucose)	Lactate extrusion and glucose consumption by mouse hepatocyte cell line TABX2S and TABX1A	[47]

(*continued on next page*)

Table 1 (*continued*)

Analyte	Flow system	Bead type	Detection technique	Detection limit	Linear range	Precision	Sample/ Application	Ref.
Bioanalytical applications								
DNA purification	SI-LOV	Silica beads	Fluorometry	NR	mg/L level	3.8%	Human whole blood	[48]
DNA	SI-LOV	Streptavidin-coated Sepharose 4B	Fluorometry	1 pmol	1–1000 pmol synthetic DNA sequence	< 9.9%	Surrogate organism	[49]
DNA	SI-LOV	Streptavidin-coated Sepharose 4B	Fluorometry	111 pg	0–993 μg/L	NR	Standards of biotinylated single stranded DNA	[50]
Bone Alkaline phosphatase	FI	Wheat germ lectin coated beads	Spectrophotometry	10 U/L	10–1000 U/L	6%	Human serum	[51]
Organic pollutants and drugs								
Promethazine and trifluoperazine	FI	Sephadex QAEA-25 (anion exchange)	Spectrophotometry	0.09 mg/L (promethazine) and 0.14 mg/L (trifluoperazine)	0.5–8 mg/L (promethazine) and 0.5–10 mg/L (trifluoperazine)	< 2%	Commercial pharmaceutical preparations	[18]
Alkylphenol polyethoxylates	SI	Magnetic beads with immobilized anti-alkylphenol polyethoxylates	CL	10 μg/L	1–1000 μg/L	3%	River water	[23]
Ketoprofen, naproxen, bezafibrate, diclofenac, ibuprofen (NSAID)	SI-LOV-MSFI	poly-DVB-co-N-vinylpyrrolidone	HPLC-UV	0.02–0.67 μg/L	0.4–40 μg/L	< 11%	Urban wastewater, urine and surface water	[44]
Muscarinic receptor agonists (carbachol, acethylcholine and pilocarpine)	FI	Cytodex-2, CultiSpher, Biosilon and Cytodex-3 with immobilized Chinese hamster ovary cells	Fluorometry	1 μM Ca	NR	NR	Evaluation of drug candidates	[46]

FI, Flow injection; SI, Sequential injection; LOV, Lab-on-valve; MSFI, Multi-syringe flow injection; ET-AAS, Electrothermal atomic absorption spectrometry; CL, Chemiluminescence; ESI-MS, Electrospray ionization-mass spectrometry; HG-AFS, Hydride generation-atomic fluorescence spectrometry; C_{18}-PS/DVB, Poly(styrene-divinylbenzene) copolymer alkylated with octadecyl groups; PTFE, Polytetrafluoroethylene; ARS, Alzarin Red S; PAN, 1-(2-pyridylazo)-2-naphthol; DPC, 1,5-diphenylcarbazide; AC, Affinity chromatography; BIS, Bead-injection spectroscopy; ELISA, Enzyme-linked immunosorbent assay; NSAID, Non-steroidal anti-inflammatory drug; NR, Not reported.

optimal experimental conditions for both reactions were affixed in a sole flow network due to the discontinuous flow nature of the MSFI technique and the flexibility of the approach for providing variable flow rates at will [38].

BI in an LOV approach has not been merely applied to sorptive preconcentration of metal species but organic pollutants as well prior to reversed-phase liquid chromatographic (LC) separations, as demonstrated by determination of anti-inflammatory drugs in raw wastewaters using hydrophobic/hydrophilic co-polymeric beads with no need for ancillary sample treatment [44]. The marriage of BI-LOV with MSFI proved suitable for quantitative elution of sorbed drugs whilst efficient LC band focusing of eluate was also ensured.

6.2. Cellular assays/activities

Cellular assays are crucial for studies of new pharmaceutical candidates and their reaction protocols. These studies normally involve loading of various doses of a given drug on cells, monitoring the reaction and detecting cell responses in batch mode. These procedures require techniques that can handle living cells reliably by preventing deterioration of cells, while assuring reproducible timing and precise injection of minute volumes of reagents. BI has played an outstanding role in the improvement of cellular assays [45].

In 1999, Hodder and Ruzicka [46] introduced a novel application of SI-BI by using beads as micro-carriers of living cells to measure elevation of intracellular calcium. A high density of cells could be trapped in the small inner volume of a jet-ring cell, which was placed above a fluorescence-microscope-objective lens. The beauty of this approach is that a fresh portion of cells is exposed to a given concentration of chemical and replaced for each assay. The investigation of cell response to a given drug or chemical is thus done more accurately than classical approaches because of the absence of carry-over effects and loss of cell activity over time. The continuous monitoring of signal changes at the beads provided peak profiles that yielded valuable information on the kinetics of cellular assays and the concentration threshold of reagent, agonist in this case, that causes cell response.

Similar uses of beads as micro-carriers for living cells were extended to LOV [26]. For example, consumption of glucose and extrusion of lactate could be detected sensitively and rapidly due to the high concentration of immobilized cells [47]. The exploration of cellular activities based on the release of protons from cells in LOV was also reported [27]. Though BIs were done manually here, two micro-packed bead columns were uniquely created within one section of the LOV micro-channel, as shown in Fig. 4A. Beads carrying cells were retained upstream to the beads with immobilized pH indicator. Protons extruded from cells were accumulated during the stopped flow and were pushed to react with the immobilized indicator, causing the color change on the indicator beads which could be in-valve monitored by solid-phase spectrophotometry (see Fig. 4D). The flow set-up illustrated in Fig. 4B was proposed as an alternative to more complicated molecular devices and to replace the light-addressable potentiometric sensor for measurement of

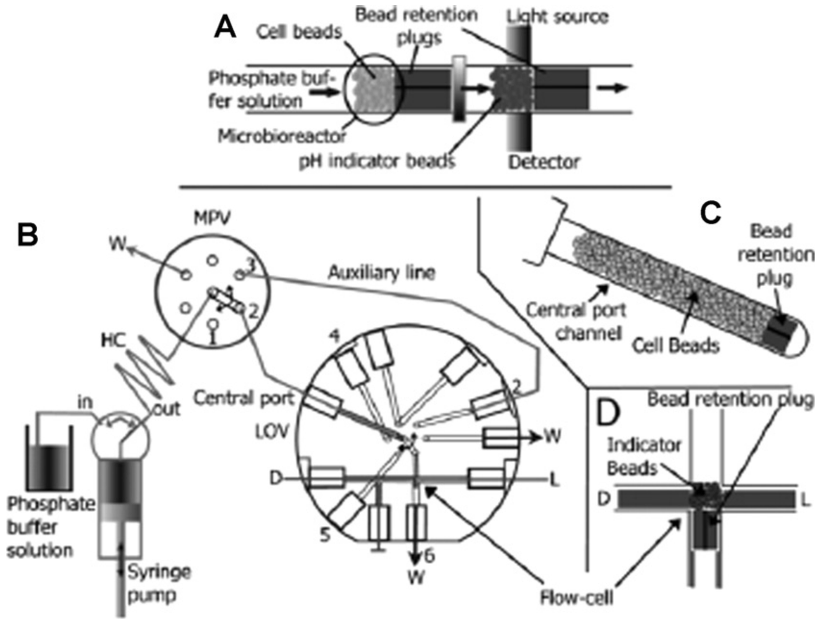

Figure 4. An SI-LOV manifold for measurement of proton extrusion from living cells using two different packed-bed columns. A), C), and D) Magnified views of bead columns (cell beads and pH indicator beads) implemented within LOV microconduits. B) Detailed diagram of SI components for BI spectroscopy of pH indicator beads (Reproduced from [27] by permission of Royal Society of Chemistry).

extracellular acidification rates, which has been utilized frequently for evaluation of metabolic functions, but which is no longer commercially available.

6.3. Kinetic affinity studies and affinity chromatography

In 2002, Ogata et al. [39] demonstrated that BI-LOV could be used effectively as an automatic system for investigation of kinetics of affinity capture and release of biomolecules. Model studies were conducted with a well-known non-covalent binding between streptavidin-coated beads and biotinylated conjugates. This group devoted further work to develop the beta-galactosidase assay in human-skin fibroblasts using on-line ion-exchange and electrospray ionization-mass spectrometry (ESI-MS) [39]. This work revealed the flexibility of LOV as a front end to many analytical instruments besides optical-fiber spectrophotometers.

A similar miniaturized set-up was exploited by Ruzicka and co-workers for critical comparison of BI spectroscopy with affinity chromatography (or more precisely ''affinity extraction''), which involved eluate detection, in the course of bioassays [15]. Preliminary studies showed that results obtained from the two different modes of detection complemented each other and gave a complete picture of biomolecular association and dissociation between target biomolecules and bioligands immobilized onto bead surfaces. However, while affinity chromatography offers improved sensitivity, compared to BI spectroscopy (detection limits are >10-fold better), BI spectroscopy offers unique possibilities for monitoring biomolecules irreversibly bound to the chromatographic column.

6.4. Immunoassays

The precise micro-fluidic manipulation and timing inherent in SI-LOV, combined with the concept of renewable solid surfaces, make it possible to perform multi-step protocols (e.g., immunoassays) automatically [24,25]. In-valve bead retention was accomplished using jet/nozzle plug and fiber optics, as previously designed for other LOV applications. Both label dilution immunoassay for determination of goat, anti-mouse IgG [24] and ELISA for detection of GAD65 antibodies [25] were demonstrated. Although low sample throughput is a limitation, compared to standard 96-well plate immunoassay, the much shorter time per assay cycle (less than 30 min vs. 5–8 h) is suitable for immediate clinical feedback and low-to-medium numbers of samples. In addition, the micro-fluidic, computer-controlled analyzer requires much lower sample volume (50 μL or less vs. 200–400 μL) and precludes the need for experienced medical technicians.

6.5. Purification and quantitation of DNA and specific biomarkers

BI-LOV has recently been proposed as a platform for determination of nucleic acids. The use of silica gel as a renewable SPE material for purification of DNA from whole human blood was demonstrated [48]. Beads were packed within an LOV cavity to retain DNA, which was later eluted for PCR amplification and electrophoretic separation. DNA purification was monitored on-line by post-column fluorimetry using ethidium bromide as a derivatization reagent. Other researchers used specially functionalized beads for elucidation and quantitative determination of specific DNA sequences [49,50]. The improved sensitivity gained by BI-spectroscopic detection allowed determination of DNA at the pmol level [49]. This system might ultimately be applied to any assay involving identification of life-threatening micro-organisms.

Hartwell et al. [51] incorporated wheat-germ-bearing beads in an FI-BI assembly for determination of bone-specific alkaline phosphatase. Detection can be done easier than with the conventional wheat-germ-agglutination technique. In addition, the increase in surface area of micro-beads for immobilization of reagent with respect to the inner surface of the micro-well used in ELISA permits better differentiation between the osteoporosis-patient group and the normal group.

Readers are encouraged to peruse critical reviews [32,33] for a comprehensive overview of LOV applications within the bioanalytical field.

7. Future trends

It is to be expected that BI in the different formats will be further developed in the years to come for high-throughput analysis or screening in various analytical fields. Coupling of LOV with affinity chromatography with various detection systems, including BI-spectroscopy, MS, amperometry or fluorometry, is expected to be well suited to current demands in bioanalytical sciences, including natural-product research, drug discovery, and protein and DNA analyses, as well as in clinical and medical analyses for rapid screening and diagnostics.

SI-LOV-BI offers significant potential in the environmental field for removal of matrix ingredients in leachates and concomitant analyte enrichment following on-line leaching tests of environmental solids (e.g., soils or sediments) packed in microcolumns attached to the peripheral ports of a multi-position valve [41]. It should be noted that there has not yet been any report on any attempt to investigate the sorptive capabilities of materials (e.g., soil layers for trace elements) by utilizing flow systems furnished with on-line solid-column reactors. Leaching or sorption schemes might be extended to radioisotopes as tracers or essential ingredients of isotope dilution in geochemical studies or as analytes in nuclear technological studies involving nuclear waste storage and management. Applications of BI systems with radiochemical detection for automation in handling radioactive materials in a closed system would also be very useful practice for nuclear medicine and nuclear reactor management.

It would be also interesting to explore the combination of BI with field-flow fractionation as a tool for gaining knowledge on size and distribution of low-level concentrations of macromolecules. This should be useful for environmental and clinical investigations.

As regards to new sorptive materials, we expect molecularly-imprinted polymeric spheres, prepared by suspension polymerization, precipitation polymerization or multi-step swelling polymerization [52], which feature improved selectivity for a given target analyte or group analogous, to be exploited for BI spectroscopy or BI sorbents prior to LC separations.

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Sequential Injection System with Modified Glass Capillary for Automation in Immunoassay of Chondroitin Sulfate

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Flow and Sequential Injection—General Approaches

SEQUENTIAL INJECTION SYSTEM WITH MODIFIED GLASS CAPILLARY FOR AUTOMATION IN IMMUNOASSAY OF CHONDROITIN SULFATE

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Sequential injection was introduced to perform a multi-step immunoassay. Modified low cost hematocrit glass capillary was employed as the immobilization surface for a competitive immunoassay of chondroitin sulfate (CS), a potential biomarker for cancer. Glass capillary is low cost and adapts well to the flow system without causing back pressure. The analysis time per sample run with automation of the multi-step immunoassay is improved as compared to the conventional batch-wise micro-plate format. The performance of the sequential injection capillary immunoassay (SI-CI) system for CS was evaluated with spiked human serum samples.

Keywords: Capillary immunoassay; Chondroitin sulfate; Sequential injection

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INTRODUCTION

Despite being a highly specific technique, especially suitable for analysis of bio-molecules in complex matrices, batch wise immunoassay has the disadvantage of being a tedious process due to multiple incubation and washing steps. Attempts to reduce error and tediousness have led to the construction of automatic immunoassay machinery (Tosoh Bioscience, Inc. 2009; PerkinElmer 2009). However, even with automatic operation, the analysis process is still highly time consuming, similar to that of the conventional batch-wise micro-plate immunoassay format, due to the waiting period to perform detection at equilibrium.

Sequential injection, which is a computer controlled flow based system equipped with bi-directional syringe pump and multi-selection valve, has been employed successfully for various rapid chemical analyses (Llorent-Martínez et al. 2008; Anthemidis 2008; Segundo, Lima, and Rangel 2004; Ruzicka and Marshall 1990). Apart from being able to perform analysis at nonequilibrium state, which helps to reduce total analysis time, its capacity for many reagents at low volume and capability of precise time control make it suitable to be adapted for rapid immunoassay. Various formats of solid substrate for immobilization of biomolecular receptors have been reported for use in flow based systems (Li, Lan, and Zhang 2008; Yang et al. 2008; Dietrich and Kräme 1995; Zhang et al. 2007; Lin, Qu, and Zhang 2007). Capillary is a particularly convenient format to use as it can be coupled with the tubing generally used in a flow injection/sequential injection system. Even though the surface area of the capillary cannot easily be expanded as compared to that of beads and membrane, capillary has the benefit of being able to eliminate back pressure in the flow line owing to its open tubular shape. Sufficient sensitivity can be obtained from capillary-based immunoassay, as it has been reported for some analytes (Nistor and Emnéus 2003; Surugiu et al. 2001; Jiang, Halsall, and Heineman 1995).

Biomarkers are of interest for early prediction of some difficult to diagnose diseases (Stephan et al. 2007; Anderson et al. 2008; Petri et al. 2009; Terenghi et al. 2009; Heiss et al. 2008). However, none of these biomarkers are completely specific for a certain disease. For example, the amount of sialic acid was reported to correlate with various cancers and also with heart disease (Nigam, Narain, and Kumar 2006; Surapaneni and Vishnu 2009), while elevated levels of hyaluronan and chondroitin sulfate were found in patients with liver disease, bone disease, inflammation, and some cancers (Tangkijvanich et al. 2003; Sun et al. 2006; Tammi et al. 2008). Preliminary studies by our co-workers revealed that considering various biomarkers (i.e., hyaluronan, sialic acid and chondroitin sulfate) at the same time seemed to be more effective in predicting the stage of cancer. To reduce total analysis time, especially for multi-analytes in complex matrices, an immunoassay system that is more rapid and easier to perform should be useful for this task.

Our group has reported the use of plain glass capillary in place of high cost fused silica capillary with a sequential injection system. Sialic acid and hyaluronan, potential biomarkers for cancers and bone diseases, could be immobilized onto the plain glass surface via a simple physical adsorption and could be used in the system with flowing reagent (Hartwell et al. 2009, 2010). Another biomarker of interest, chondroitin sulfate (CS), requires different immuno-biochemistry. Determination of CS using a flow injection system with a micro-affinity column has been done (Hartwell et al. 2007). However, the valve switching and pump were manually controlled. To reach the final goal of developing a rapid multi-biomarkers analysis system, use of the same analysis method (sequential injection-capillary immunoassay (SI-CI), in this case) would be most convenient. Preliminary study showed that, unlike HA and sialic acid, CS cannot be physically adsorbed onto the plain glass capillary. Mastichiadis et al. (2008) have summarized strategies for glass capillary activation for both covalent and non-covalent bonding of various bio-molecules. Covalent bonding through APTES and glutaraldehyde was selected for use in our CS study.

EXPERIMENTAL

Reagents and Buffer Solutions

Phosphate buffer of 0.1 M, used for the capillary modification process, was prepared by dissolving 3.39 g $Na_2HPO_4 \cdot 2H_2O$ (Scharlau, Spain) and 4.82 g $NaH_2PO_4 \cdot 2H_2O$ (Merck, Germany) in 400 mL deionized (DI) water and the pH was adjusted to 7.0 with NaOH (Merck, Germany). Then, the volume was brought up to 500 mL with DI water.

Shark A1 (Sh-A1) prepared from shark cartilage as described elsewhere (Pothacharoen et al. 2006) and chondroitin sulfate (Sigma) for immobilization onto the modified glass capillaries were prepared as solutions in a coating buffer [20 mM sodium carbonate buffer composed of 0.33 g Na_2CO_3 (Carlo Erba) and 1.42 g $NaHCO_3$ (Merck)]. Their pHs were adjusted to 9.6 by adding a few drops of NaOH (Sigma) prior to making up volume to 1 L with DI water.

Monoclonal antibody (IgM) against chondroitin 6-sulfate (named as WF6), which was laboratory prepared by The Thailand Excellence Center for Tissue Engineering, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, as previously described elsewhere (Pothacharoen et al. 2006) was diluted to 1:1000 ratio with incubation buffer. It was found experimentally that WF6 has high specific recognition toward CS and Sh-A1 through two epitopes octasaccharide sequences which, evidenced by computer modeling, govern similar shape and distribution of electrostatic potential that are different from other nonbinding octasaccharide sequences (Pothacharoen et al. 2007).

The μ-chain specific goat-antimouse IgM conjugated with horseradish peroxidase (anti-Ig-HRP, Sigma-Aldrich) was diluted to 1:2000 ratio with an incubation buffer.

The incubation buffer (THST) was 0.01 M Tris-HCl saline buffer pH 7.4, which was composed of Tris-HCl (USB) and NaCl (Carlo Erba), and containing 0.1% Tween 20 (USB). The Sh-A1 and CS standard solutions for competitive binding with immobilized CS and Sh-A1 were also prepared in this incubation buffer in the presence of 6% BSA (Sigma) to compensate for protein rich serum matrices. Substrate for peroxidase was 3,3′,5,5′tetramethylbenzidine (TMB) ready to use solution (KLP). Carrier solution was citric phosphate buffer pH 5.0, composed of 0.05 M $C_6H_8O_7 \cdot H_2O$ (Carlo Erba) and 0.1 M $Na_2HPO_4 \cdot 2H_2O$ (Scharlau) and HCl (Sigma).

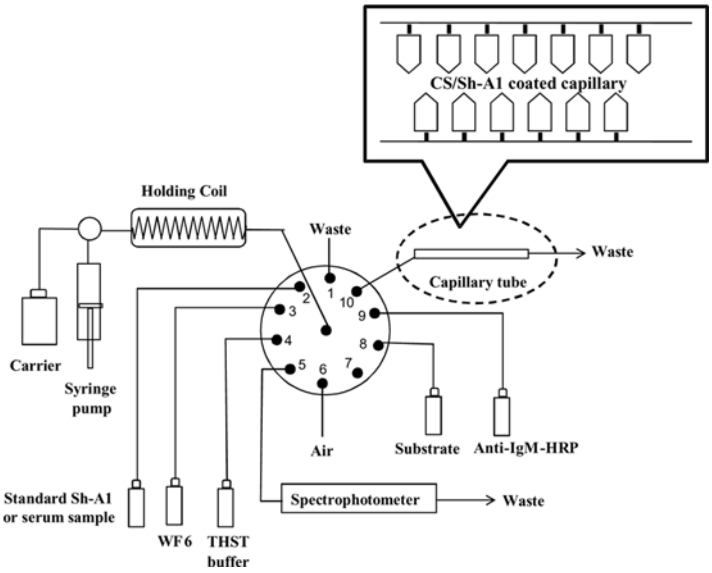

Figure 1. Diagram of the SI-CI system for chondroitin sulfate.

Preparation of Capillary Reactor

Preparation of glass capillaries for immobilization of CS and Sh-A1 through covalent bonding was conducted using the process modified from Torabi et al. (2007). The glass capillaries of 7.5 cm in length and 1.15 mm i.d (micro- hematocrit tube, BRIS) were cleaned by submerging them in a small closed glass bottle containing 6.5% (v/v) HNO_3 (Sigma). The bottle was placed in an ultrasonic water bath (Bandelin electronic) set at 70°C for 10 min. Then, the nitric acid solution in the bottle was discarded and changed to DI water. Capillaries were washed with DI water four times, by shaking on a Lab-shaker for 5 min each time. Finally, capillaries were dried in an oven (Memmert) at 100°C for 2 h.

Modification of glass capillaries started with silanization. Capillaries were submerged in the solution of 10% 3-aminopropyltrimethoxysilane (APTES, Sigma-Aldrich), prepared in dried toluene (Sigma). The bottle containing APTES solution and capillaries was placed in a water bath set at 80°C and was left for 30 min. After that, capillaries were washed once with dried toluene, followed by ethanol 95% (Merck), and then three times with DI water. The capillary washing step was done on a shaker and dried in a heated oven as described previously.

Capillaries were filled with a solution of 2.5% v/v glutaraldehyde (Ajax), prepared in 0.1 M phosphate buffer pH 7.0. Glutaraldehyde was allowed to react with the silanized surface at 25°C for 30 min, and then it was removed by washing with DI water three times. Capillaries were stored in 0.1 M phosphate buffer pH 7.0 at 8°C for further use. These treated capillaries were reported to be able to be kept for many months (Torabi et al. 2007).

The capillaries were filled with 10 μg/mL (approximately 80 μL) of either Sh-A1 or CS solution, prepared in 20 mM carbonate buffer pH 9.6. One end of each

Table 1. Summarization of the operational steps of the SI-CI system for chondroitin sulfate

Step no.	Operation	Volume (μL)	Flow rate (μL/s)
1	Washing coated capillary with incubation buffer	2000	100
2	Aspiration of air to the holding coil	20	10
3	Aspiration of standard Sh-A1 solution or serum sample to the holding coil	20	10
4	Aspiration of antibody (WF6) to the holding coil	40	10
5	Aspiration of standard Sh-A1 solution or serum sample to the holding coil	20	10
6	Aspiration of air to the holding coil	20	10
7	Mixing standard Sh-A1 solution and WF6 by moving solution back and forth twice	–	10
8	Sending mixture to Sh-A1 coated capillary and incubate for 1 min	80	10
9	Removing unbound species by washing capillary with incubation buffer	1500	50
10	Aspiration of air to the holding coil	20	10
11	Aspiration of anti-IgM-HRP to the holding coil	80	10
12	Aspiration of air to the holding coil	20	10
13	Sending anti-IgM-HRP to CS/Sh-A1 coated capillary and incubate for 3 min	80	10
14	Removing unbound species by washing capillary with incubation buffer	1500	50
15	Aspiration of air to the holding coil	20	10
16	Aspiration of substrate TMB to the holding coil	80	10
17	Aspiration of air to the holding coil	20	10
18	Sending substrate TMB to CS/Sh-A1 coated capillary and incubate for 7 min	80	10
19	Discarding air	30	10
20	Aspiration of product solution to the holding coil	50	10
21	Sending the product solution to the detector by using citric phosphate buffer as a carrier	1000	10

Note: Steps 17–20, the discarded air volume (30 μL) needs to be higher than the aspirated air volume (20 μL) to ensure the prevention of air entering the detection flow cell. Therefore, the volume of the reaction zone left to be sent to the detector (50 μL) was lower than the aspirated TMB solution volume (80 μL).

capillary was submerged in the Sh-A1 or CS solution at a 45° angle with respect to the solution surface. The capillaries were filled by using capillary force. Then, both ends of the capillaries were sealed with Parafilm® and left for incubation overnight at 8°C. The capillaries were washed with 2 mL incubation buffer prior to use in order to remove any unbound species.

Blood Samples

With the limited access to blood samples, some studies used pooled blood samples from many individual subjects to obtain sufficient amount of samples for study. All blood donors gave their informed consent before participation in this study.

Apparatus and Operational Step

The sequential injection capillary immunoassay system (SI-CI) set-up is similar to that previously reported for sialoglycoconjugates and hyaluronan (Hartwell et al. 2009, 2010). Figure 1 illustrates the system with the position of each reagent on the

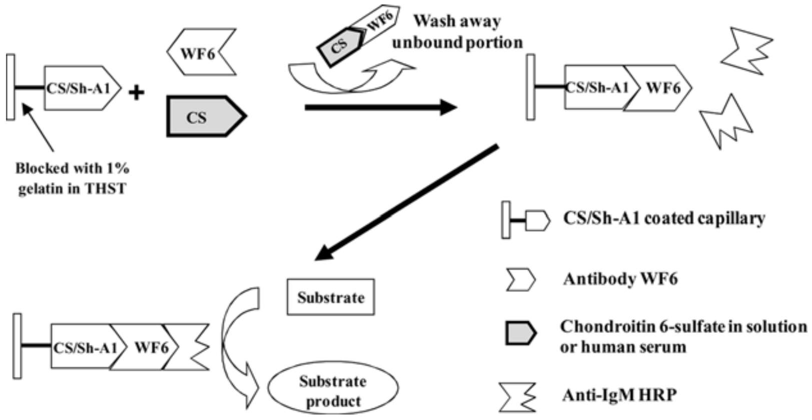

Figure 2. Diagram illustrates the competitive immunoassay for CS.

multi-selection valve. The CS or Sh-A1 coated capillary, previously blocked with gelatin, was incubated with the mixture of CS (standard solution or sample) and WF6 for a selected period of time. Any unbound species were removed and the capillary was washed with an incubation buffer. Then, the capillary was incubated with anti-IgM-HRP for a selected period of time before removal of unbound species by washing with an incubation buffer. After that, the capillary was incubated with substrate TMB and the colored product was sent to the detector. Selected incubation times for each step were chosen according to experimental results which will be described in the later sections. Table 1 summarizes the automatic operational steps by the SI system required to carry out the competitive immunoassay process as shown in Figure 2. The operation was controlled by the software created using Visual Basic 6.0, as described in detail elsewhere (Siriangkhawut et al. 2009).

RESULTS AND DISCUSSIONS

Evaluation of Physical Adsorption and Covalent Bonding of Sh-A1 and CS on Glass Capillary

Selection of CS or Sh-A1 coated capillaries and suitable blocking reagent. Immobilization of CS onto the capillary inner wall would be a more direct comparison for estimation of the amount of CS in serum samples. However, separate work on comparison of CS and Sh-A1 coated micro-well immunoassay has found that Sh-A1 with more of the same epitopes for binding offered better sensitivity. The same result was found with capillary immunoassay as described in the following study. Therefore, Sh-A1 was used for immobilization for the rest of the studies.

To reduce nonspecific binding, blocking reagents may be required to block the empty modified glass surface that may not be covered with immobilized CS/Sh-A1. Various blocking solutions were tested for their ability to minimize nonspecific binding by allowing the blocking solutions to incubate with both CS/Sh-A1 coated capillaries and uncoated capillaries for 30 min prior to carrying out the immunoassay process as shown in Figure 2. These blocking reagents including 1% gelatin (Merck), 1% BSA (Sigma), and 5% nonfat milk (convenience store), were prepared in 0.01 M Tris-HCl saline buffer pH 7.4 with and without 0.1% Tween20 (THST and THS, respectively).

It was found, as shown in Figure 3, that when no blocking reagent was used, uncoated capillary which should not yield any analytical signal, actually showed very high signal. Every blocking reagent seemed to be able to reduce this nonspecific background signal as can be seen from the significantly lower signals obtained from uncoated capillaries as compared to when no blocking reagent was used. However, the blocking reagents also caused reduction of analytical signal of the coated capillaries. The best distinction between analytical signal and background signal was obtained with Sh-A1 coated capillary using 1% gelatin in THST as a blocker [See the comparison of signals of the 2nd and 3rd bars (CS and Sh-A1 coated capillaries) with the 1st bar (uncoated capillary)].

Selection of concentration of reagents, incubation temperature for immobilization of Sh-A1 and incubation times. In the conventional microplate immunoassay, immobilization was done overnight using 10 µg/mL Sh-A1. This concentration was chosen according to optimization as determined in a separate study. Higher concentrations (50 and 100 µg/mL) of Sh-A1 for immobilization were also tried with a capillary, but they did not show improvement in sensitivity as compared to 10 µg/mL. This indicated that the capillary surface could accommodate Sh-A1 at concentrations no higher than 10 µg/mL. In addition, the inner surface area of the hematocrit tube and the working surface of a normal microwell are approximately the same (2.7 cm^2) (Hartwell et al. 2009; Elisa Plate, Product Description in Product

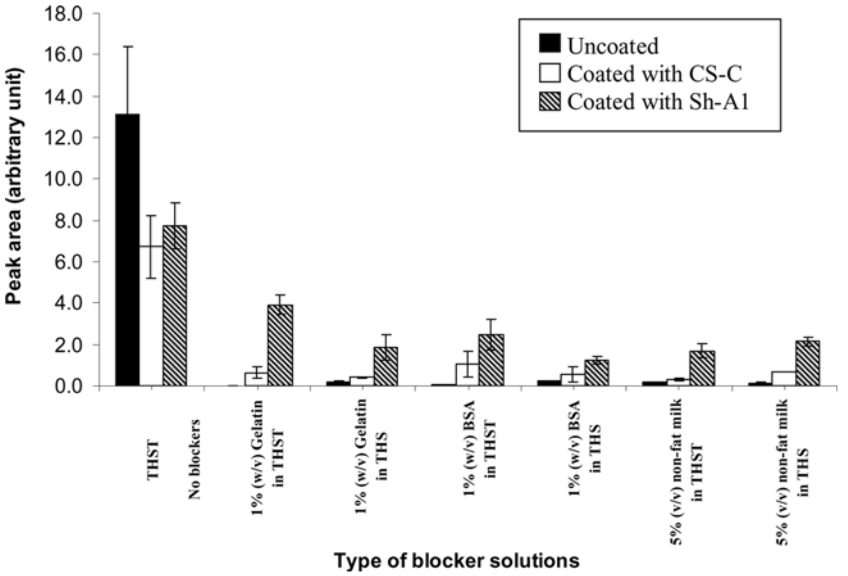

Figure 3. Comparison of analytical and background signals (averaged from 3 replicates) when using no blocker and various blockers on uncoated, CS coated, and Sh-A1 coated capillaries. THST is Tris-HCl saline buffer with Tween 20. THS is Tris-HCl saline buffer without Tween 20.

Directory 2009). Therefore, the same concentration of 10 μg/mL Sh-A1 used in microplate method for immobilization was also chosen for capillary, while various concentrations of CS (10, 50, 100, 200 μg/mL), with less number of epitopes per molecule, were tried. Temperature may affect the binding of CS/Sh-A1 onto the modified glass capillary; therefore, immobilizations at 8°C and room temperature were compared.

All of the CS concentrations studied gave lower sensitivity than 10 μg/mL Sh-A1. Immobilization in the refrigerator (8°C) yielded significant improvement when increasing immobilization time (24 h was better than 5 h, 1 h, or 5 min). However, at room temperature (25°C), there were no significant differences among various immobilization times (5 min, 1 h, 5 h, and 24 h). This indicated that high temperature promoted immobilization of Sh-A1 onto the modified glass capillary. Higher temperature such as 37°C may be able to further decrease immobilization time. However, for the convenience, immobilization at room temperature without the use of an incubator is preferred.

Since a capillary and a microwell have approximately equivalent surface areas and the same concentrations of Sh-A1 were used for immobilization (10 μg/mL), the concentrations of the other reagents (including Sh-A1 standard solution and anti-IgM HRP) were also selected based on conventional batch microplate method. The only exception was the concentration of WF6 used in the SI-CI system (1:1000) which was higher than the 1:10,000 concentration used in the microplate method. This is because the much shorter incubation time used in the competitive process should require higher concentration of target molecules in solution. For the next step of adding anti-IgM-HRP, the same concentration of anti-IgM-HRP as used in the micro-plate was selected for the SI-CI system. This is because the degree of binding would mostly be limited by the amount of captured WF6 on the capillary. Adding too much excess enzyme conjugated antibody would require unnecessary washing.

The competitive immunoassay of CS involves incubation steps between 1) immobilized Sh-A1 and the mixture of WF6 and CS sample, 2) captured WF6 on the capillary and anti IgM-HRP, and 3) HRP label on the capillary and substrate TMB. Even though the surface area of a capillary is similar to that of a microwell, the different dimensions of the two solid surface formats cause the differences in diffusion time/binding time between immobilized/captured molecules and those in solution. The flow based systems also provide the benefit of effective measurement of analytical signals at non-equilibrium state. Although the signals, obtained before binding had reached equilibrium, may not be at their maximum, if the relationship of the dose response can be established, these signals at non-equilibrium will be sufficient to construct a useful calibration curve.

The concentration of reagents and incubation times used in each competitive immunoassay of CS are summarized in Table 2 as compared to those used in the conventional micro-plate technique. The flow based SI-CI system requires shorter total incubation times. This reduces the analysis time to ~50 min/run (including washing times) as compared to more than 3 h used in the conventional micro-plate technique. However, the micro-plate technique can accommodate a large number of samples in parallel while the SI-CI can run only a few samples in parallel, depending on ports available on the selection valve. Therefore, the conventional microplate technique is more suitable for analysis of a massive number of samples, whereas the SI-CI system is superior when having a low number of samples that need quick results.

Table 2. Selected incubation times and concentrations of reagents for each incubation step when using a conventional micro-plate as compared to SI-CI system. OPD is o-phenylenediamine; TMB is 3,3′,5,5′,tetramethylbenzidine; A1 is the fraction containing the highest amount of associated proteoglycans after partial purification process which includes dialysis step; A1D1 is fraction containing the highest amount of dissociated proteoglycans (monomers) after partial purification process without dialysis step; RT is room temperature

	Micro-plate		SI-CI	
Incubation step	Conc.	Time	Conc.	Time
Immobilization of Sh-A1 on capillary	10 μg/mL	Overnight RT	10 μg/L	Overnight 8°C
Blocking	1% BSA	1 h 37°C	1% gelatin	30 min Off-line RT
WF6 and Sh-A1 mixture	1:10000 WF6 20–10000 ng/mL Sh-A1D1 fraction	1 h 37°C	1:1000 WF6 10–100000 Sh-A1 ng/mL	1 min RT
Anti-IgM-HRP	1:2000	1 h 37°C	1:2000	3 min RT
Substrate	OPD/H_2SO_4 492/690 nm	10 min 37°C	TMB 650 nm	7 min RT
Estimated total analysis time and volumes of reagents (includes washing times but excludes overnight coating of plate or capillaries)	~3.5 h 100–250 μL/each reagent/well		<1 h 80 μL/each reagent/capillary	

Calibration Curve and Figure of Merits

The calibration curve was constructed from Sh-A1 and the results were reported in ng/mL as Sh-A1 equivalence. Figure 4 illustrates a calibration curve with sigmoidal logistic fit: $Y = [(A_1 - A_2)/(1 + (X/X_0^P)] + A_2$ (where Y is analytical signal as peak area; X is concentration of Sh-A1 equivalence in ng/mL; A_1 is initial Y value (64.11), A_2 is final Y value (12.93), X_0 is X value at Y equal to half of the limit A_1 and A_2 (6694.63), and P is power (0.70).

The lowest detectable concentration was approximately 50 ng/mL with the working range of 100–100000 ng/mL Sh-A1 equivalence. Calibration graphs prepared on different days, using 4 different batches of capillaries (prepared 5, 12, and 18 days apart) with the same range of Sh-A1 concentrations, were insignificantly different. Precision of each concentration obtained from 4 different days are in the range of 3.5–5.0% RSD. This indicated that different batches of capillaries have adequate reproducibility. In addition, it is not necessary to construct the whole calibration curve for everyday analysis. However, checking of signals of some standard concentrations daily is advised.

Determination of CS as Sh-A1 Equivalence in Human Serums

Due to the limited access to cancer blood samples, the experiment was conducted using 3 normal blood samples spiked with various concentrations of standard CS as a Sh-A1 equivalence to evaluate the potential use of the proposed SI-modified

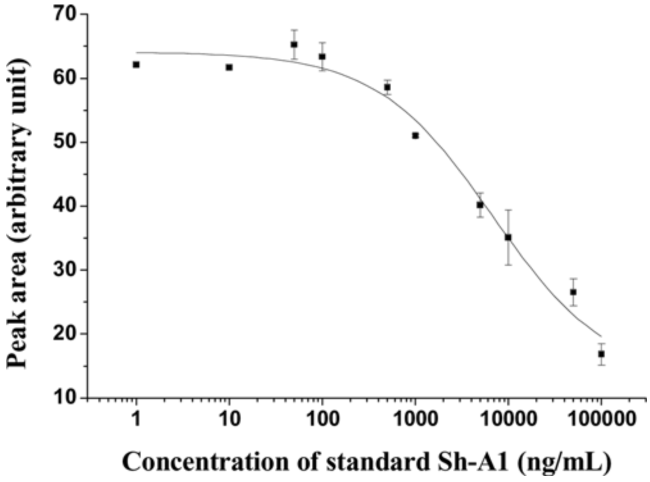

Figure 4. A calibration curve constructed from competitive immunoassay of Sh-A1 with a SI-CI system. Each data point is an average of 3 replicates.

glass capillary immunoassay system for determination of CS in human serum matrices. Percentages of recovery are reported in Table 3. Even though the linear working range determined from the calibration graph covers 100–100000 ng/mL CS (as Sh-A1 equivalence), the recovery study showed that the spiked concentrations of 10000 ng/mL and higher have low accuracy (% recoveries >200). The suitable working range for application with real serum samples is, therefore, determined to be 100–10000 ng/mL with recovery in the range of 89–120%.

Low accuracy was observed at high CS concentrations, which may be due to the nature of the competitive immunoassay that normally yields a lower signal at high analyte concentration. Therefore, the precision and accuracy are decreased with the increase of analyte concentration. It is also possible that matrices in blood may affect the assay and that BSA may not be the most suitable medium for construction of the calibration graph. Nevertheless, it is also common in analysis of serum

Table 3. Recovery study of CS in the spiked serum samples (S1 is a single serum, S2, and S3 are pooled samples). Each [CS] reported is averaged from 3 replicates

	[CS] as Sh-A1 equivalence		
Sample	Expected (ng/mL)	Found (ng/mL)	% Recovery
S1	–	82	–
S1 + 100 ng/mL	182	185	103
S1 + 1,000 ng/mL	1082	968	89
S2	–	5652	–
S2 + 500 ng/mL	6152	6255	120
S3	–	4244	–
S3 + 5,000 ng/mL	9244	10043	116
	Average % recovery		107

samples using a conventional microplate immunoassay to dilute serum that contains a high amount of analytes to make the concentration fall into the valid working range. Likewise, in the SI-CI system, serums that have high amounts of CS should also be diluted and re-analyzed.

CONCLUSION

The sequential injection-capillary immunoassay (SI-CI) system was developed for automation for the determination of CS by using Sh-A1 as a standard for coating capillary and to construct a calibration curve. Unlike in the previous studies on hyaluronan and sialic acid, CS and Sh-A1 do not effectively bind to the glass surface with physical adsorption. Glass capillary needs to be modified for covalent binding. The system showed potential use for serum samples within the working range of CS as 100–10000 ng/mL Sh-A1 equivalence, with precision within 5% RSD. As compared to batch-wise conventional microplate immunoassay, the automatic features of the sequential injection system enabled a shorter immunoassay process (<1 h vs. 3.5 h) with less reagent and sample consumption (80 µL vs. 250 µL) per analysis.

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ผลงานตีพิมพ์อื่นๆ

รศ.ดร.สุภาภรณ์ ครัดทัพ
อาจารย์ประจำภาควิชาเคมี
น.ส.ปิยะนุช ปิ่นอยู่
นักศึกษาระดับปริญญาโท ภาควิชาเคมี

พักดื่มชา แล้วทำเคมีวิเคราะห์กันเถอะ

สาระวิชาการ

ชาเขียว เคมีสีเขียว

ในช่วงระยะเวลา 50 ปีที่ผ่านมานี้ ชาเขียวเป็นเครื่องดื่มที่ได้รับความนิยมอย่างมากไปทั่วโลก โดยประเทศที่นิยมดื่มชาเขียวกันมาอย่างยาวนาน คือ จีน ญี่ปุ่น และอินเดีย โดยจุดเริ่มต้นของการบริโภคชาเขียวเกิดขึ้นที่ประเทศจีน ย้อนไป 3,000 ปีที่แล้ว โดยจักรพรรดิ์ Shennong ได้ทรงเสวยน้ำร้อนที่บังเอิญมีใบชาตกลงไปในแก้วน้ำร้อน การดื่มชาจึงเริ่มต้นขึ้นนับตั้งแต่นั้นมา

ชาเขียวเป็นที่รู้จักดีทั่วโลกเนื่องจากประโยชน์ของมันที่มีต่อสุขภาพ โดยสารที่เป็นองค์ประกอบหลักในชาเขียวคือสารกลุ่ม Catechin หรือ Polyphenol เป็นสารที่มีประโยชน์ต่อสุขภาพหลายอย่าง เช่น ช่วยลดความเสี่ยงในการเกิดโรคมะเร็ง ลดการเกิดปฏิกิริยาออกซิเดชันจากอนุมูลอิสระ เพิ่มเมตาบอลิซึมของร่างกาย ลดความดันโลหิตและคอเลสเตอรอล อีกทั้งยังช่วยยับยั้งการเพิ่มขึ้นของน้ำตาลในเลือด ช่วยในระบบการย่อยอาหารและสามารถฆ่าเชื้อโรคจากแบคทีเรียและไวรัสได้อีกด้วย นอกจากสารกลุ่มCatechin แล้ว ชาเขียวยังมีสารอื่นๆที่ล้วนแต่มีประโยชน์ต่อร่างกาย เช่น วิตามินซี วิตามินอี กรดอะมิโน Theanine และเส้นใยอาหาร

การบริโภคชาเขียวอย่างถูกวิธีเป็นเรื่องที่สำคัญ เนื่องจากการดื่มชาเขียวไม่ถูกต้องอาจทำให้เกิดผลเสียต่อสุขภาพ ได้หลายกรณี เช่น

- ไม่ควรดื่มชาที่ชงทิ้งไว้ค้างคืน เนื่องจากสารต่างๆที่ประโยชน์ในชาเขียวจะเสื่อมสลายไปแล้ว
- ไม่ควรชงชาเข้มจนเกินไป เนื่องจากชาที่ชงได้จะมีปริมาณคาเฟอีนสูง และอาจทำให้นอนไม่หลับได้
- ไม่ควรดื่มชาระหว่างมื้อ เนื่องจากชาไปลดการดูดกลืนของของแร่ธาตุและวิตามินที่จำเป็นของร่างกายซึ่งอาจทำให้เป็นโรคได้

และประการสุดท้ายที่สำคัญ คือ การบริโภคชาเขียวในปริมาณที่เหมาะสม เนื่องจากการมีรายงานว่าดื่มชาเขียวมากเกินไป จะทำให้การดูดกลืนแร่ธาตุที่สำคัญอย่าง ธาตุเหล็ก น้อยลงไปด้วย ทั้งนี้เนื่องจากสารในกลุ่ม Polyphenol ที่มีอยู่มากในชาเขียว มีคุณสมบัติสามารถจับไอออนของเหล็กที่ไม่ได้จับอยู่กับเม็ดเลือดแดง (non-heme iron) และเกิดเป็นสารประกอบที่ไม่ละลายน้ำ (รูปที่ 1) ทำให้ร่างกายดูดกลืนธาตุเหล็กได้น้อยและอาจทำให้กลายเป็นโรคขาดธาตุเหล็ก และทำให้เกิดโรคโลหิตจางได้ จากจุดนี้เอง เมื่อมองในแง่ของนักเคมี แสดงว่าชาเขียวอาจนำมาใช้ประโยชน์ในการหาปริมาณเหล็กได้ ทางกลุ่มวิจัย Flow based analysis ภาควิชาเคมี ม.ช. จึงได้ทำการทดลองง่ายๆ โดยการชงชาสารพัดชนิดทั้งชาเขียว ชาดำ และชาจีน และทดสอบการเปลี่ยนแปลงสีของชาเมื่อเติมสารละลายเหล็กความเข้มข้นต่างๆลงไป (รูปที่ 2)

เมื่อเหล็กทำปฏิกิริยากับ Polyphenol ในชา ก็จะเกิดผลิตภัณฑ์เป็นสารประกอบของPolyphenol-เหล็กซึ่งมีสีม่วงและมีความเข้มเปลี่ยนไปตามความเข้มข้นของสารละลายเหล็ก และสามารถสังเกตเห็นได้ด้วยตาเปล่า ทางกลุ่มวิจัยจึงได้ทำการศึกษาอย่างละเอียดโดยใช้ชาเขียวซึ่งหาซื้อได้ง่ายในท้องตลาดมาใช้ในเป็นสารเคมีอย่างง่ายที่ใช้ในการทดสอบและหาปริมาณเหล็กในตัวอย่างน้ำหรือยาต่างๆที่มีธาตุเหล็กเป็นองค์ประกอบ เพื่อเพิ่มประสิทธิภาพความเร็วในการวิเคราะห์ ทางกลุ่มได้พัฒนาระบบวิเคราะห์ที่อาศัยการไหล (Flow Injection Analysis) โดยปั๊มให้น้ำชาไหลมาผสมกับสารละลายตัวอย่างในท่อพลาสติกเล็กๆ และผลิตภัณฑ์ที่เกิดขึ้นไหลเข้าสู่เครื่องมือตรวจวัดแสงซึ่งวัดค่าการดูดกลืนแสงที่ความยาวคลื่น 570 nm ของผลิตภัณฑ์สีม่วงที่เกิดขึ้น สัญญาณที่วัดได้จะแปรผันตามปริมาณเหล็กที่มีอยู่ในตัวอย่าง และเมื่อนำค่าสัญญาณและความเข้มข้นของเหล็กมาสร้างกราฟมาตรฐาน (รูปที่ 3) จะทำให้สามารถหาปริมาณเหล็กในสารตัวอย่างโดยการเทียบค่าการดูดกลืนแสงของผลิตภัณฑ์กับกราฟมาตรฐานได้ โดยผลที่ได้มีความถูกต้องแม่นยำ ในระดับที่ดีมาก

จะเห็นได้ว่าชาเขียวนั้น นอกเหนือจะมีประโยชน์ต่อสุขภาพแล้ว คุณสมบัติทางเคมีบางประการของมันก็สามารถนำมาใช้เป็นสารทดสอบซึ่งเป็นสารธรรมชาติที่ไม่เป็นอันตรายต่อสิ่งแวดล้อมและสุขภาพ อีกทั้งยังมีราคาถูกเมื่อเทียบกับสารเคมีทั่วไป งานวิจัยนี้แสดงให้เห็นว่าสารต่างๆที่มีอยู่ในพืชตามธรรมชาติและผลิตภัณฑ์ที่เราใช้ในชีวิตประจำวันสามารถนำมาใช้ในการพัฒนาการวิเคราะห์ทางเคมีที่สะอาด(Green chemistry)ได้ ซึ่งเป็นวิธีที่มีประโยชน์ทั้งในแง่ของเศรษฐกิจและช่วยลดมลพิษจากสิ่งแวดล้อมด้วย

เอกสารอ้างอิง

[1] http://www.greenteas.com (access 31 October 2010)

[2] http://drinkhealthydrinks.com (access 31 October 2010)

[3] http://www.amazing-green-tea.com (access 31 October 2010)

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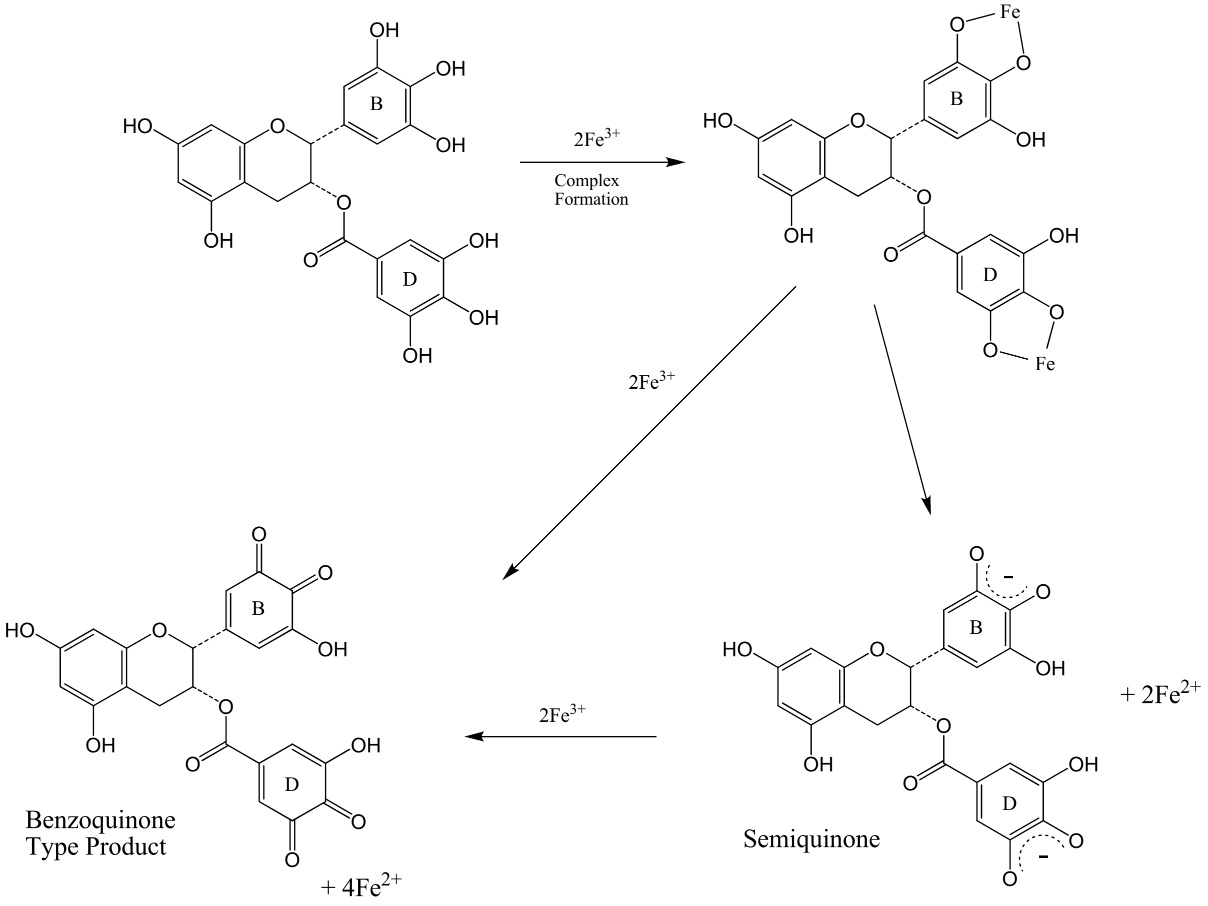
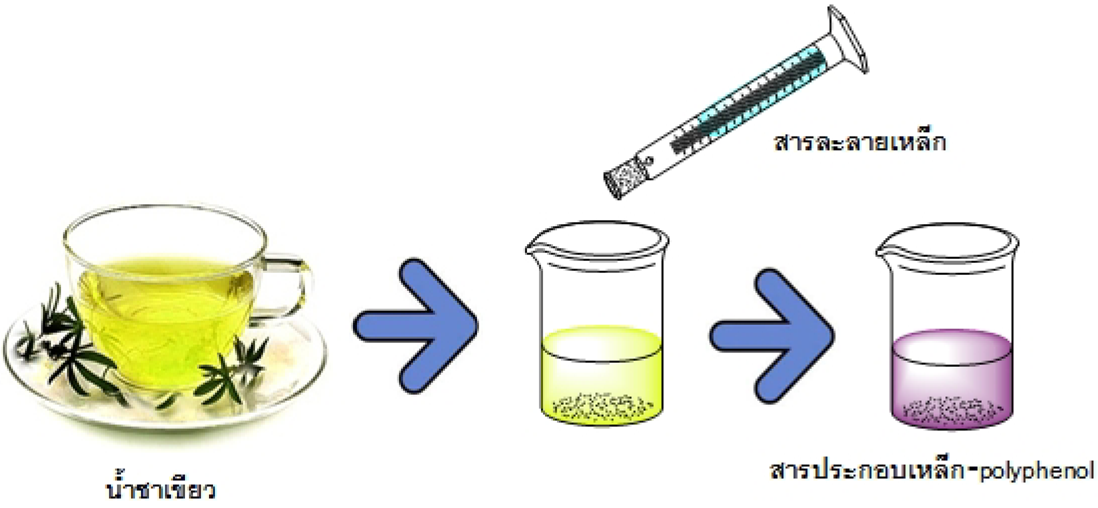
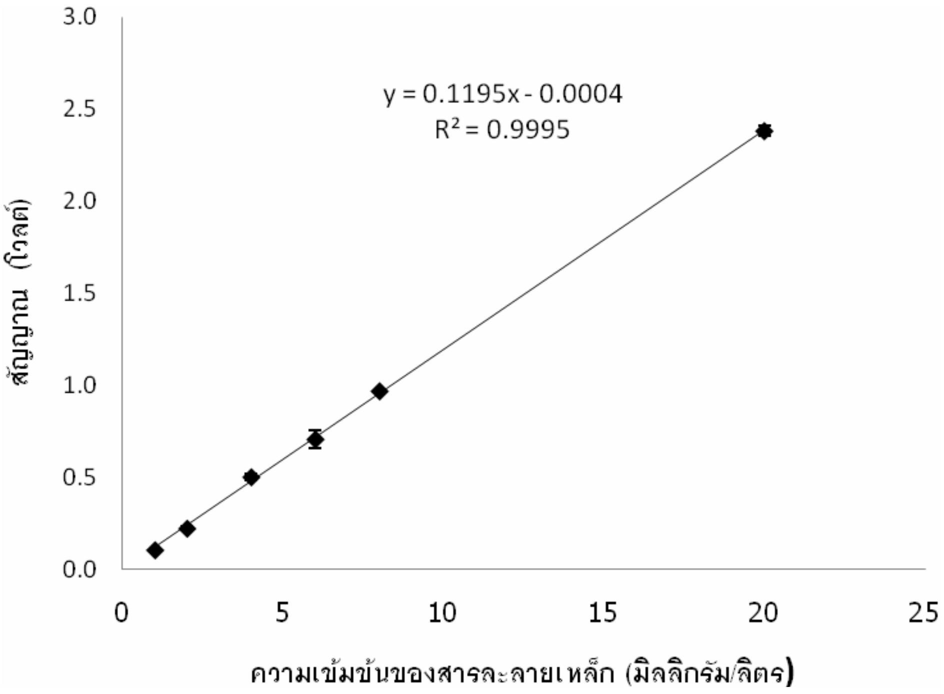


รูปที่ 1 ปฏิกิริยาเคมีระหว่างเหล็กและ Polyphenol ในชาเขียว



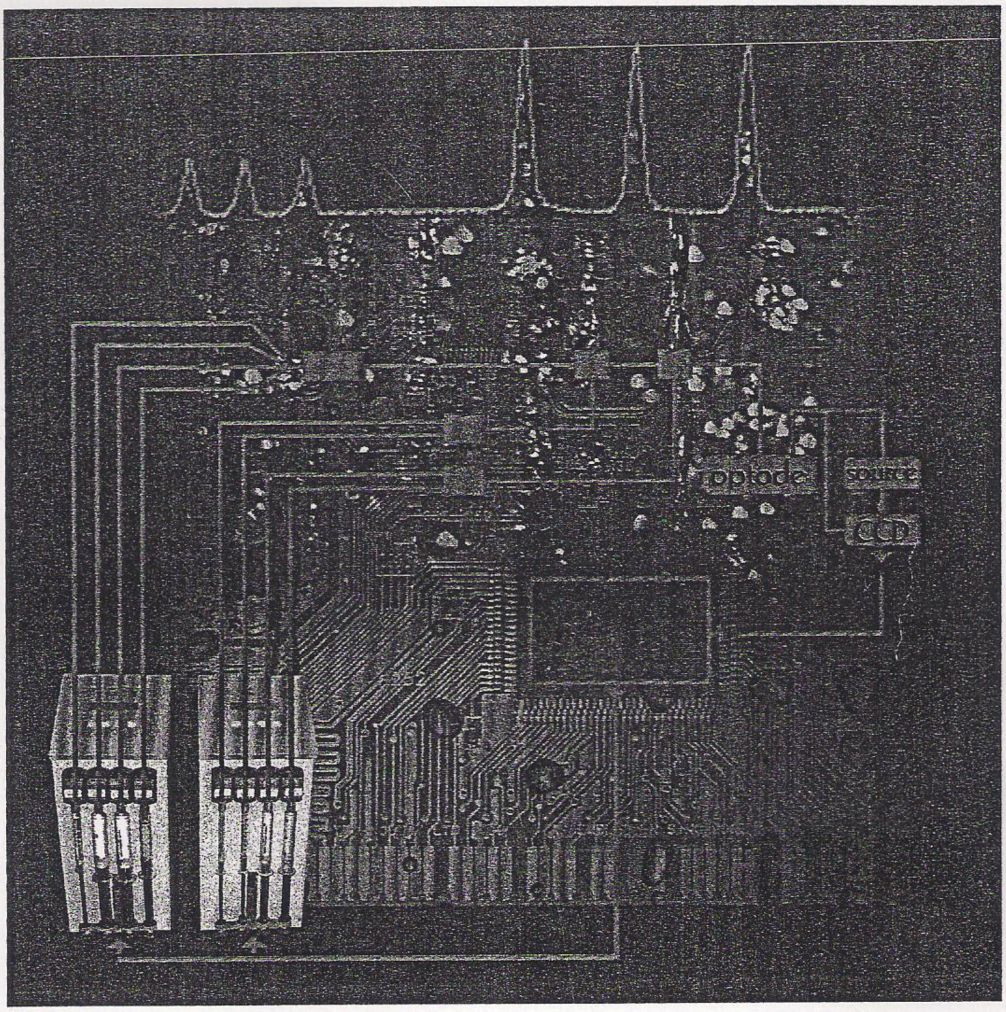
รูปที่ 2 การเกิดปฏิกิริยาระหว่าง Polyphenol ในน้ำชากับสารละลายเหล็ก



รูปที่ 3 กราฟมาตรฐานของสารละลายเหล็กที่ความเข้มข้นต่างๆ

บทคัดย่อผลงานวิจัย
ที่นำเสนอแบบปากเปล่า
ในงานประชุมวิชาการ
ระดับนานาชาติ

Kate Grudpan
Chiang Mai - Thailand



FLOW ANALYSIS XI

SEPTEMBER 14 – 18, 2009

POLLENSA

MALLORCA. SPAIN

L03

LAB IN A GARDEN: A CONTRIBUTION OF FLOW BASED ANALYSIS

Kate Grudpan*, Supaporn Kradtap Hartwell*, Wasin Wongwilai*, Supara Grudpan, Somchai Labanatnoppakhun***

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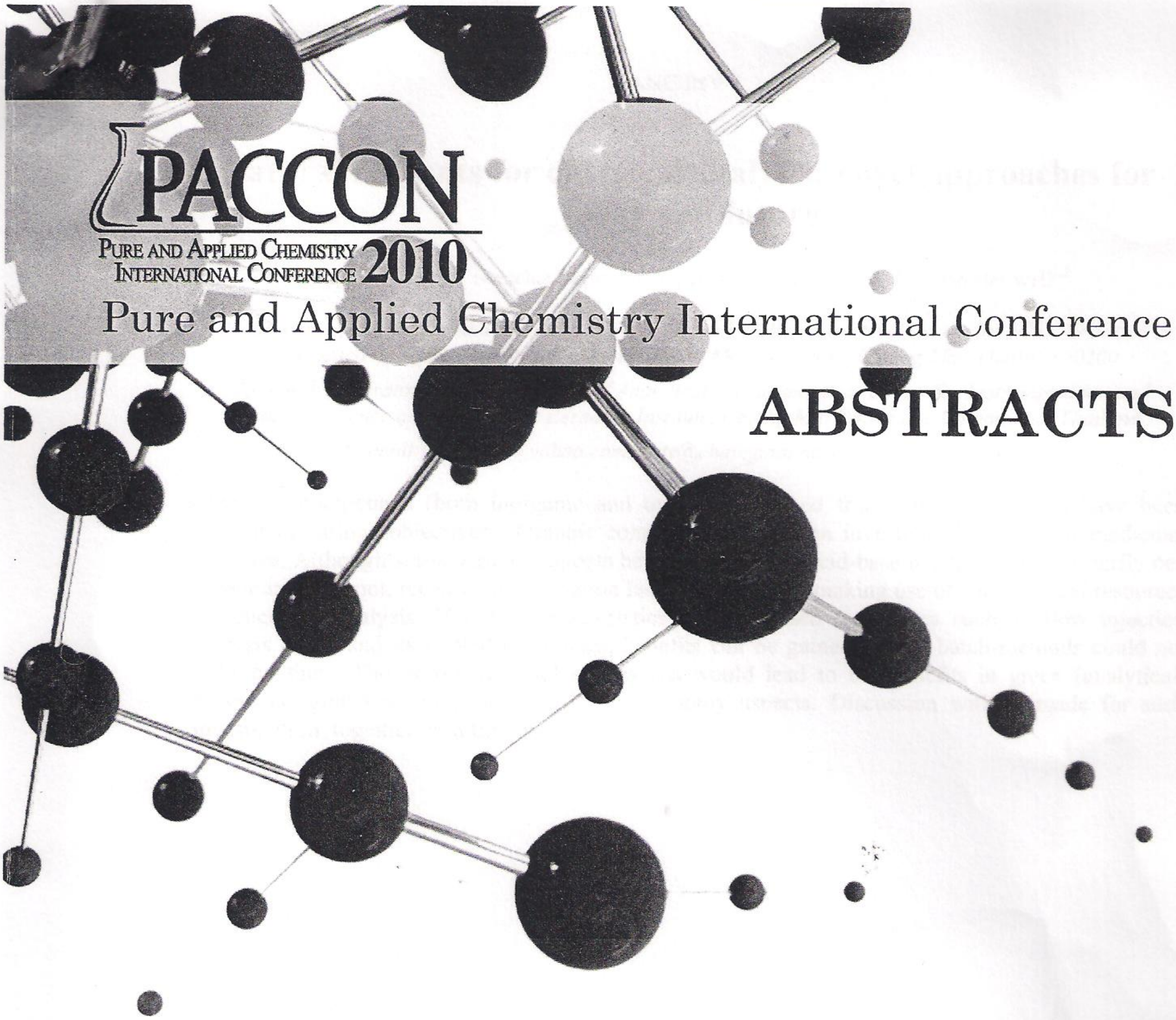
This presentation will discuss the benefits gaining by flow based analytical techniques using some natural reagents. Water extract of guava leaf can be used as a reagent for iron determination by employing a simple flow injection (FI) set-up. The extract can be only crude, not necessary to be in pure stage. This takes advantage in that, in a FI system, an analyte could be determined via a calibration under the same conditions as that of standards. The fresh leaves can be taken from a tree in a garden. Green tea leaves (commercially available package for drinking from a convenient store (for tea break in a garden)) can also be used for iron assay by using a simple flow injection (FI) assemble. Using a simple lab-on-chip with time based approach, acidity can be deteimined by using some natural indicators such as an extract of Butter Fly Pea flowers (taken from a garden) Some others have been exploited. The flow based analysis with natural reagents leads to green analytical chemistry. The analysis can be performed in an open-air in a garden. Discussion on the flow systems and the natural reagents will be made.



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ABSTRACTS

Challenges in Chemistry for Sustainable Development

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Natural reagents for chemical analysis: novel approaches for today and future

Kate Grudpan,[1,2*] Somchai Lapanantnoppakhun[1,2] and Supaporn Kradtap Hartwell[1,2]

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Chemical compounds (both inorganic and organic) obtained from natural resources have been utilized in various objectives. Organic compounds have been investigated mainly for medicinal purposes. Although some natural reagents have been used as acid-base indicators, e.g. butterfly pea flower and beetroot, recently, investigation has been made for making use of some natural resources for chemical analysis. Via the characteristics of flow-based techniques such as flow injection analysis (FIA) and its related techniques, benefits can be gained, where batch-methods could not offer on this. The proposed novel approaches would lead to the benefits in green (analytical) chemistry, global warming and sufficient economy aspects. Discussion will be made for such investigation, together with the future trends.

Flow based techniques for cost effective research projects used in chemistry teaching

By: Hartwell, Supaporn K.; Khonyoung, Supada; Sripaoraya, Worathip; Pinyou, Piyanuch; Reanpang, Preeyaporn; Wongwilai, Wasin; Kongtawelert, Prachya; Sanguansermsri, Torpong; Lapanantnoppakhun, Somchai; Jakmunee, Jaroon; Grudpan, Kate; Kerdphon, Sutthichat

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Flow injection (FI) technique is a well established alternative way of performing rapid and low vol. wet chem. Its cost effectiveness and semi-automatic features make it a very useful tool for teaching. The presentation will show some examples of low cost undergraduate student research projects that utilize FI technique with natural reagents from local plants. Examples of some graduate student research will also be shown that aim to simplify and automate complex biochem. anal. processes, to improve precision and to develop alternative lower cost instrumentation as compared to the high cost com. anal. chem. instrumentation. Despite their relatively lower cost and simple set up, these research projects are publishable in the international peer review journals. The examples presented help to fulfill the objectives of an undergraduate research project course as well as to meet the goal at the graduate level and help to promote thinking towards sustainable development in students.

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P-34

Sequential injection-capillary immunoassay for chondroitin sulphate proteoglycans

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Chondroitin sulfate is a potential biomarker for joint diseases and ovarian cancer [1,2]. Immunoassay is commonly used for quantitative analysis of this biomarker. Batch well immunoassay normally involves many steps of incubation and washing that are tedious, highly time consuming and requires skillful technicians. Automatic immunoassay was carried out with sequential injection analysis to overcome these limitations. A low cost glass capillary is used as a solid support for immobilization of chondroitin sulfate or equivalent proteoglycans. This capillary alternative well suits the flow system and eliminates the back pressure problem that normally occurs with a packed bead column. Competitive immunoassay of chondroitin sulfate proteoglycans was demonstrated using this system.

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P-105

Simple labs on chip approach with time-based detection

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Simple lab-on - chip approach with time-based detection is proposed. In this presentation , we will discuss how to fabricate a simple platform which is made of acrylic piece with channels in different shapes for sample and reagent(s) introducing using flow manipulation. The detection is to monitor changes with time-based involving migration/diffusion of the reaction zone. The changes may be spectrometric or electrochemical. This led to simple micro -analysis with lab-on-chip approach with visual detection(with a stop-watch).Quantification can be made via a calibration graph which are due to relationship of time and concentration of analyte of interest. Various applications will be demonstrated .They include acid and base determination based on neutralization reactions (with an indicator); ascorbic assay based on redox reaction; iron determination, using thiocyanate reagent and enzymatic reactions.

Kato

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method), so it could be an interesting alternative method for determination of proteins.

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S1-PO-4

Determination of Ammonium in Soil and Water by Flow Injection Gas Diffusion - Conductometric System

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Ammonium and ammonia play important role in agricultural and environmental fields. Their contents in soil and water can be used as an indicator for either plant nutrient or pollution. The determination of them in these samples is quite difficult because the analytes usually present at low level in a variety of interferences. In this work, a flow injection with a sensitive conductometric detection for determination of ammonium was developed. Different designs of conductometric flow cell and signal amplification were investigated in order to improve sensitivity of the system. A standard/sample solution was injected into a stream of 2.0 M sodium hydroxide donor solution, which will convert ammonium to ammonia gas. A gas diffusion unit was employed for on-line separation of ammonia gas from a donor solution by diffusing through a teflon membrane to dissolve into an acceptor stream (water). Conductance of the acceptor stream which was increased linearly with the concentration of ammonium in the injected solution was continuously recorded as a peak. A linear calibration graph plotting between peak height and ammonium concentration was obtained in a range up to 1.2 ppm NH_4^+ with a limit of detection of 0.05 ppm. Precision of the method was 5.2 % for 11 replicates injection of 0.5 ppm NH_4^+ standard solution. A sample throughput of 60 h^{-1} was achieved. The proposed method was successfully applied to soil extract and surface water samples validating by spectrophotometric standard method. The system consumed small amounts of cheap chemical and there is no interferences from particulates and colored species.

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S1-PO-5

Green Tea Extract as an Alternative Reagent for Flow Injection Determination of Iron

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The purpose of this work is to develop the system for determination of metal ions that utilizes natural extracts an alternative reagent. Green tea extract is used for quantification of iron. A simple and low cost flow injection technique was developed for this study. The green tea extract was prepared in acetate buffer pH 4.8 and could be used without further purification. The intensity of purple complex of green tea extract with iron was monitored using a laboratory made detector with LED green light source. The effects of some parameters were investigated. The optimum conditions for Fe (II) determination were flow rate 3.0 ml min^{-1}, mixing coil 100 cm and extraction time 20 minutes. A linear calibration graph was obtained in the range of 20-80 mg Fe l^{-1}. Some preliminary studies of calibration graph will be presented.

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S1-PO-6

Assay of Chondroitin Sulfate with Time Based Detection in a Simple Macro – Channel Reaction Flow Cell

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The assay of chondroitin sulfate (CHS) using chromatic dyes such as methylene blue (MB) and dimethylmethylene blue (DMMB) has been investigated. A reaction flow cell with time based detection was proposed as a simple lab on-chip approach. Chondroitin sulfate sample and dye reagent were introduced and come into contact in the flow cell. Migration of the sample zone into the reagent line started at the point of contact. The increase of CHS concentration caused the decrease of migration time of the reaction zone. Detection could be done simply by timing the migration with a stop watch without the need of any complicated detection device.

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S1-PO-7

A Simple Flow Injection Spectrophotometry for Determination of Chondroitin Sulfate

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A simple flow injection system was developed for determination of chondroitin sulfate (CS) based on the reaction with methylene blue (MB). A standard/ sample solution was injected into the stream of $2x10^{-5}$ M methylene blue. The solution was mixed and flowed to a spectrophotometric flow cell. The decrease in absorbance of MB was monitored at 664 nm in corresponding to the increasing of CS concentration. A calibration graph in range of 0-8 mg L^{-1} was obtained with a detection limit of 0.1 mg L^{-1}. The proposed system was applied to assay CS in pharmaceutical and clinical samples.

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S1-PO-8

Coupling of Capacitively Coupled Contactless Conductivity Detector with Membraneless Vaporization and Its Application in Determination of Carbonate by Flow Analysis

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This work describes utilization of a flow-through contactless detector namely 'capacitively coupled contactless conductivity detector' or C^4D in flow analysis. Generally, C^4D is coupled with a chromatographic technique especially capillary electrophoresis. C^4D is classified as a universal detector and it is therefore seldom used as a stand-alone detector. In order to exploit application of C^4D in flow analysis, a simple step of separation was adopted to improve the selectivity. In this work, a coupling of our recent gas phase separation, called 'membraneless vaporization' (MBL-VP), with C^4D was carried out. This allowed for selective detection of volatile compound that dissolves in a flowing acceptor stream to be accomplished. Volatilization of analyte was induced inside a close chamber that had an appropriate acceptor flowing through it. Dissolution of the volatile compound that resulted in changes in conductivity of the acceptor solution, can be monitored using the C^4D. This technique has been successfully applied to quantitative analysis of carbonate in natural waters and cements.

S1-PO-80

Determination of Enzyme Activity Based on rate of Migration

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An application of a macro-channel reaction flow cell with visual detection of the rate of migration for determination of enzyme activity will be presented. The intensity of color of the enzyme-substrate product is normally proportional to its concentration. Concentration affects the rate of migration. Therefore, this rate of migration can be used to estimate enzyme activity. This simple reaction flow cell has a potential to be used as an easy detection unit for enzyme based immunoassay or for quantitative analysis of enzyme from various sources.

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S1-PO-81

Determination of Total Selenium in Selenium Enriched Rice by Electrothermal Atomic Absorption Spectrometry and the Effect of Foliar Application of Selenite and Selenate Fertilizer on the Selenium Content in RD29 Rice Cultivar

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In the present work, the influence of foliar application of selenite and selenate fertilizer on increasing the total selenium content in RD29 rice product in order to enhance human selenium dietary intake for cancer chemopreventive activity was investigated. Total selenium concentrations in selenium-enriched rice were determined by electrothermal atomic absorption spectrometry. The plot experiment was conducted using Randomized Complete Block Design (RCB) with three replicates and five treatments using RD29 rice cultivar. The foliar spraying with selenite or selenate fertilizer was thoroughly applied on the rice leaves in the heading stage of rice growth at the rate of 0, 80, and 160 of Se ha^{-1}. The rice seeds were harvested in the period of 105 days. The harvested rice seeds were dried and processed to polished rice products and subsequently digested with 3 mL concentrated nitric acid and 1 mL of 30% v/v hydrogen peroxide using microwave digestion system. The analytical condition of ETAAS technique was optimized and applied for the determination of total selenium in rice samples using palladium nitrate as a chemical modifier. The optimized condition for the determination of total selenium in rice was at the pyrolysis time of 10 s and the pyrolysis temperature of 900˚C. The atomization temperature was 2,400˚C. The limit of detection of the method and M_0 (Characteristic mass) were 2.80 µg/L and 29.24 pg, respectively. The precision defined as %RSD was 4.45% (at concentration of Se 25 µg/L, n=5). The selenium contents of rice were significantly increased to 0.338-0.669 mg/kg by foliar application of selenium fertilizer. The selenium content in rice by application of selenite fertilizer was 30% higher than that by a selenate fertilizer, which indicated that selenite fertilizer exhibited greater efficiency on increasing Se content in RD29 rice products. The results indicated that selenium-enriched rice obtained by foliar application of selenium fertilizer to increase the selenium content of rice could be a good selenium source for Thai population and for future production of selenium-enriched rice supplement. The speciation of selenium by gas chromatography mass spectrometry (GC-MS) and ^{77}Se-NMR techniques is still required since cancer chemopreventive activity and bioavailability of selenium have been found to be species-dependent which is the subject to on-going studies.

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S1-PO-82

Simple Analytical Method for the Determination of Mercury, Lead, and Cadmium in Rice and Rice Products for the Development of Simple Screening Heavy Metal Test Kits

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A simple analytical method for the determination of mercury, lead, and cadmium in rice and rice products was developed in this work using dithizone extraction method with spectrophotometric detection at the wavelength of 487, 504, and 484 nm, respectively. The uses of different types of acid and oxidizing reagents for the extraction of heavy metals from rice samples were investigated. The optimized condition for the extraction of mercury, lead, and cadmium from rice and rice products, i.e., extraction time, extraction temperature, concentration of acid, and concentration of oxidizing reagent were determined. By using the mixtures of suitable masking agents, the extracted samples at the volume of 20 mL was adjusted

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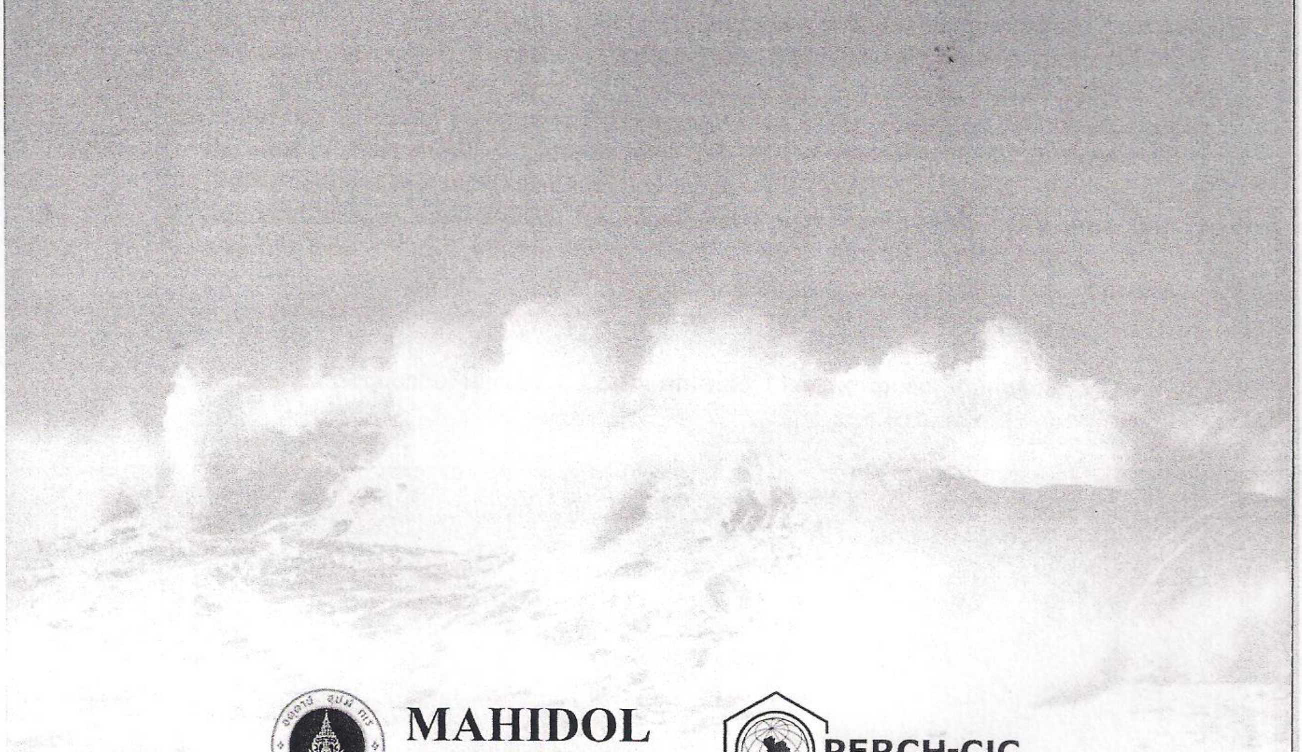

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On-line Capillary Based Competitive Enzyme-linked Immunosorbent Assay for Detection of Zeatin Riboside

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Abstract

Zeatin riboside (ZR) is in cytokinins type a plant hormone that plays an essential role in regulating plant growth and development. The level of ZR presented in plant tissues is a good indicator of the resistance of plants to abiotic environmental stresses and to necrotic pathogens. Highly specific and sensitive technique usually used for determination of ZR is immunoassay. However, conventional 96-well plate format has the drawback in term of highly time consuming and tedious operation steps. Therfore, sequential injection (SI) combined with enzyme-linked immunosorbent assay (ELISA) was developed to obtain an on-line automation and rapid immunoassay system. The zeatin-bovine serum albumin (ZR-BSA) was used as the antigen to immobilized onto the wall of glass capillary. Immobilized ZR-BSA competed with ZR in sample for a limited amount of monoclonal antibody against ZR (MAb-ZR). Upon removing of the unbound species, anti-mouse IgG conjugated with horseradish peroxidase (HRP) enzyme and 3,3′,5,5′,tetramethylbenzidine (TMB) substrate were introduced. The colored product was monitored at 650 nm. Various parameters such as concentration of reagents and incubation time were optimized.

Keywords: Sequential injection; Zeatin riboside; Plant hormone; Immunoassay

The Use of Red Rose Extract as Natural Indicator for Ascorbic Assay

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Abstract

Green chemistry principles involve the reduction or elimination of the use of hazardous substances. Employing natural reagents in place of synthetic reagents is one way to perform green chemistry. Natural reagents are environmentally friendly and low cost as compared to the synthetic reagents. In this work, the use of the red rose (*Rosa hybrids*) extract as an indicator in flow injection acid-base titration was explored. Ascorbic acid was used as a model analyte to evaluate the performance of the red rose indicator. Normal flow (n-FI) and reverse flow (r-FI) were compared. The color of red rose extract is changed with pH. The change in absorbance of the mixture was detected at 520 nm. A calibration graph in the range of 0-8 mM ascorbic acid was obtained with $R^2 = 0.9915$. The proposed system was applied to determine ascorbic acid in vitamin C tablet and injection samples.

Keywords: Natural reagents, Green chemistry, Flow injection

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รางวัลช้างทองคำ
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