Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Talanta 66 (2005) 521–527
Talanta
www.elsevier.com/locate/talanta

Sequential injection-ELISA based system for online determination of hyaluronan

Supaporn Kradtap Hartwell[a,*], Boonraksa Srisawang[a], Prachya Kongtawelert[b], Jaroon Jakmunee[a], Kate Grudpan[a]

[a] Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[b] Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

Received 21 September 2004; received in revised form 29 November 2004; accepted 29 November 2004
Available online 4 January 2005

Abstract

Sequential injection-bead-based immunoassay system has been developed. The main purpose is to make immunoassay process more automated by manipulating the precise delivery of micro-volumes of reagents and the precise timing of incubation and washing steps with a computer program that controls the bi-directional syringe pump. The manifold was designed with the aims of reducing back pressure from beads that act as solid surfaces for immobilization of the target substance, reducing dispersion and dilution of the reagent during incubation, and maximizing signal while minimizing incubation time. This was done by introducing air segment to separate the reagent zone from the carrier stream and by using a suitable sensitive detector which, in this case, was an amperometer. In this study, hyaluronan (HA) was used as a target analyte because of its clinical significance as a potential biomarker for liver, bone and cancer diseases. Amount of hyaluronan was determined using competitive enzyme linked immuno sorbent assay (ELISA) based technique where immobilized HA and HA in solution compete to bind with a fixed amount of biotinylated HA-binding proteins (b-HABPs). Upon separation of the two phases, anti-biotin conjugated with enzyme and a suitable substrate were introduced to follow the binding reaction of the immobilized HA and b-HABPs whose degree of binding is indirectly proportional to the amount of HA in solution. A calibration curve was constructed from a series of concentrations of HA standards. Lowest detectable concentration was found to be 1 ng/mL with the dynamic working range of 1–5000 ng/mL and R.S.D. of intra-assay ($n = 7$) and inter-assay ($n = 3$) of various HA concentrations were 4–10% and 9–12%, respectively. Used beads could be reused by washing with 2 M guanidine. Total analysis time for this automatic assay was about 30 min as compared to the 5–8 h used in conventional batch well ELISA. The system could be applied to assay HA in human serum.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Sequential injection analysis; Bead ELISA; Online immunoassay; Hyaluronan

1. Introduction

Immunoassay is an analytical technique that utilizes the specific molecular recognition between antigen and antibody to selectively determine the presence of the antigen. The detection of the binding reaction between antibody and antigen can be followed using different available labels. Examples of recently used labels are radioactive isotope [1,2] chemiluminescent compounds [3,4], metals [5,6] and enzymes [7,8]. Among all the labels, the use of enzymes has gained high popularity due to their amplification properties in which they can convert substrate to product as long as the substrate is present. Assay with an enzyme label, known as enzyme linked immuno sorbent assay (ELISA), has been widely used.

Conventional immunoassay is normally carried out in a micro-well where antigen or antibody is immobilized onto the surface of the well. The rate of binding reaction between antigen and antibody depends upon the diffusion rate of antigen or antibody in the bulk solution to the immobilized ones. Conventional immunoassay, therefore, requires a long incubation

* Corresponding author. Tel.: +66 53 941910; fax: +66 53 941910.
E-mail address: kradtas@yahoo.com (S.K. Hartwell).

0039-9140/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2004.11.032

time, mostly overnight or at least 5–8 h to ensure maximum binding. There have been attempts to reduce the analysis time of immunoassay [9–13]. Using microbeads as solid surface is one way that has been gaining popularity [14–18]. Now that there are many chemically modified beads commercially available, different formats of bead-based immunoassay have been reported [9,19–23]. The main advantages of bead-based immunoassay include increasing the surface area for immobilization of antigen or antibody and reducing the incubation time due to less diffusion distance needed between species in bulk solution and the ones immobilized on beads that are suspended in the solution. Magnetic microbeads also facilitate the phase separation during the washing steps [24,25].

The combination of beads and ELISA is very useful and has been used in many applications [9,23,24,26–28]. However, immunoassay always involves many steps of incubation and washing which are tedious and subjected to human error, mostly due to imprecise timing on each step. The small amount of each of the reagents used in the analysis process is also another difficult matter to handle by an inexperienced person. There is rarely any development on a true online immunoassay where the whole process from the first step of incubation to the last step of detection is automatic. This is due to the lack of a system that can deliver a very small volume of reagents and precisely control the incubation time. The new generation of flow injection technique called sequential injection analysis (SIA) is now available [29–33], and a true online immunoassay is possible. One very interesting work is the combination of bead injection technique with the lab-on-valve (LOV)-sequential injection analysis system for carrying out ELISA [34]. However, this work involves the sophisticated LOV device that attaches as a part of the multi-selection valve of the commercial SIA system.

The attempt of this work is to make the immunoassay process more automated and more precise than batch process by utilizing the most simple, and cost effective system as much as possible. This was done by utilizing the sequential injection analysis (SIA) system controlled by computer software to deliver micro-volumes of reagents and to precisely time the incubation and washing steps. Beads were used as solid surfaces. The system was developed with the aims of reducing back pressure in the flow line which commonly occurs when using a bead packed column, as well as to minimize the incubation time and to maximize the signal as much as possible. An air segment was introduced to separate the reagent zone from the carrier stream [35] in order to reduce the dispersion of the reagent during the incubation time when the pump was stopped. Amperometry was chosen as a detection means because of its high sensitivity and low susceptibility to the differences in non-electrochemical properties of reagents. For example, the Schlieren effect which is usually encountered when using spectrophotometric detection can be avoided when electrochemistry techniques are used.

The SIA-bead ELISA based system was developed for quantification of hyaluronan (HA) in serum. Hyaluronan is a glycoprotein, composed of gluconate acetylglucosamine disaccharide repeating units, that has been reported to be a potential biomarker for liver, cancer and bone diseases [36–43]. It can bind selectively to the proteins called HA-binding proteins (HABPs). In this study, the competitive ELISA based technique was carried out. Immobilized HA on beads and HA in solution competed to bind with a fixed amount of biotinylated HA-binding proteins (b-HABPs). Upon separation of beads from solution, anti-biotin conjugated with enzyme horseradish peroxidase (anti-b-HRP) and a suitable substrate (tetramethylbenzidine, TMB-H_2O_2) were introduced to follow the binding reaction of the immobilized HA and b-HABPs. The degree of binding is indirectly proportional to the amount of HA in solution. The demonstration of the potential use of the system with real samples was shown by performing a recovery study of the amount of spiked HA in bovine and human serum samples. The system offers an automated immunoassay process with good sensitivity and precision as compared to using a batch process, even though the incubation time was reduced to decrease the analysis time and increase sample throughput.

2. Experimental

2.1. Apparatus and reagents

2.1.1. SIA system

The system is composed of a syringe pump (CAVRO Scientific Instrument Inc., XL 3000) that can draw solution up to 2.5 mL, two 10-ports selection valves (Valco Instrument Co. Ltd., C25-3180EMH), a computer (Microsoft Window XP professional, CPU-AMD Athlon^{TM} XP 2000 + 1.67 GHz 256 MB of RAM) and SIA control software (FIAlab 3000, FIA Instrument). Holding coil is made of tygon tubing. All other tubings are PTFE tubings. The detection system was an amperometer (μAutolab Type II Eco Chemie B.V. Utrecht, The Netherlands) with its control software (Autolab Software—General Purpose Electrochemical System version 4.9). A BAS flow cell with glassy carbon working electrode, Ag/AgCl reference electrode and stainless steel auxiliary electrode was used. The applied potential was set at 100 mV for detection of the reduction of TMB product, produced from the enzyme–substrate reaction, back to TMB.

2.1.2. HA coated beads

EAH-sepharose 4B beads (Amersham Biosciences) size 45–165 μm (mean bead size 90 μm) were used as solid surfaces for immobilization of HA. These beads were washed with saline solution at the ratio of 0.5 M NaCl 250 mL: deionized water 100 mL: bead 10 mL (drained gel) and were filtered prior to use.

One of the many sources of HA is umbilical cord, which has an abundant amount of HA [44], that is also commercially available. HA was extracted from umbilical cord by mixing 0.0300 g umbilical cord (Sigma, containing approximately

30 mg HA) with 0.0010 g bovine testicular hyaluronidase (Sigma) and 10 mL of 0.15 M NaCl (BDH) in 0.1 M sodium acetate buffer solution pH 5.0 (Sigma). The mixture was shaken to let the reaction take place for 3 h before bringing it to boil for 10 min to end the reaction.

The beads and HA from previous steps were mixed and about 30 mL deionized water and 0.5751 g *N*-ethyl-*N*′-3-dimethylaminopropyl carbodiimide hydrochloride (Sigma) were added to the mixture. After mixing thoroughly, 0.1 M HCl (Sigma) was used to adjust the pH to about 4.5. This mixture was continuously shaken for 24 h at room temperature. After that, the remaining un-substituted amino groups were blocked with 1 mL glacial acetic acid and allowed to stand for 6 h. The beads were washed with 400 mL each of different solutions in the order of 1 M NaCl, 0.1 M Tris–HCl (Sigma) pH 8.1, 0.05 M formate buffer (Sigma) pH 3.1, and deionized water, respectively. In each washing step, beads were separated from washing solution by centrifugation. Finally, the beads were kept in 0.5 M sodium acetate buffer pH 5.7 at 4 °C for future use.

2.1.3. Biotinylated HABPs

HABPs was extracted from cartilage using 4 M guanidine. The extract was dialyzed against water and then was liophilized. The powder was partially digested with tripsin before dialyzation and liophilization again. Up to this step, HA that was previously bound to HABPs in cartilage is still present with HABPs. To separate out the HABPs from HA in solution, bead HA was added. HABPs can bind to HA on bead better than HA in solution. This may be because of higher amount of HA on beads and also because some portions of HA in solution were partially digested and therefore cannot bind effectively to HABPs. After that, HABPs was eluted out from bead HA using 4 M Guanidine.

Biotinylation of HA-binding proteins was performed by a standard method [45]. Briefly, HABPs which had been prepared was dissolved in 0.1 M sodium hydrogen carbonate buffer pH 8.5 and with *N*-hydroxysuccinimidobiotin (Sigma–Aldrich Chemical, 34.1 g/L in DMSO) mixed at a ratio of 3:1 (w/w) at room temperature for 1–2 h. The mixture was then introduced to a Sephadex G-25 column and was eluted with PBS pH 7.4. The excluded protein peak was collected, aliquoted and stored at −20 °C as a stock solution of the biotinylated HABPs (b-HABPs).

2.1.4. Anti-biotin conjugated with HRP

Anti-biotin HRP (Zymed) was diluted to 1:1000 ratio with PBS buffer solution pH 7.4.

2.1.5. HA standard solutions

HA standard solution (IAL®-F) has the concentration of 20 mg/2 mL which is equivalent to 10×10^6 ng/mL. Stock solution of 1 mL at the concentration of 10,000 ng/mL was made by diluting the HA standard solution with 6% BSA to match the approximate amount of protein contained in real blood. This stock solution was further diluted into a series of different concentrations; 5000, 2500, 1000, 500, 250, 125, 50, 25, 5 and 1 ng/mL.

2.1.6. Serum samples

Bovine serum (Sigma) in the powder form was dissolved in PBS to obtain a final concentration of 6% (w/v). This is to mimic the real blood in liquid form that contains approximately 6% protein.

Human serum was obtained from Chiang Mai University Hospital. Volunteers had not taken food and drink for 8 h prior to the blood drawing. These bloods were centrifuged to separate out the blood cells. Serum samples were kept at −20 °C and were used without further dilution.

2.2. Manifold design

The manifold used in this study is as shown in Fig. 1. A pipette tip (100–1000 μL blue tip, Eppendorf) with cotton wool at the tip end was used as a reservoir for beads. It was directly connected to one of the ports on the selection valve. Since one end was opened to air, there was no problem of back pressure as normally found in a packed column. Other reagents were kept in microcentrifuge tubes and light sensitive ones such as TMB were kept in a dark color tube. All tubes were closed and only a small hole was made for the tubing from selection valve to go through for solution drawing. This was to avoid air contact that might oxidize and degrade

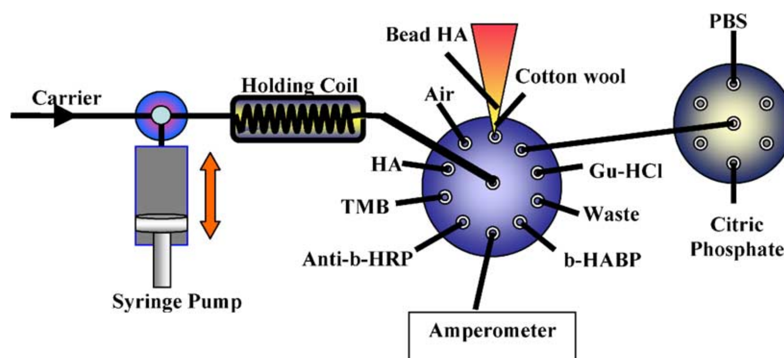

Fig. 1. SIA-bead ELISA based system for determination of HA.

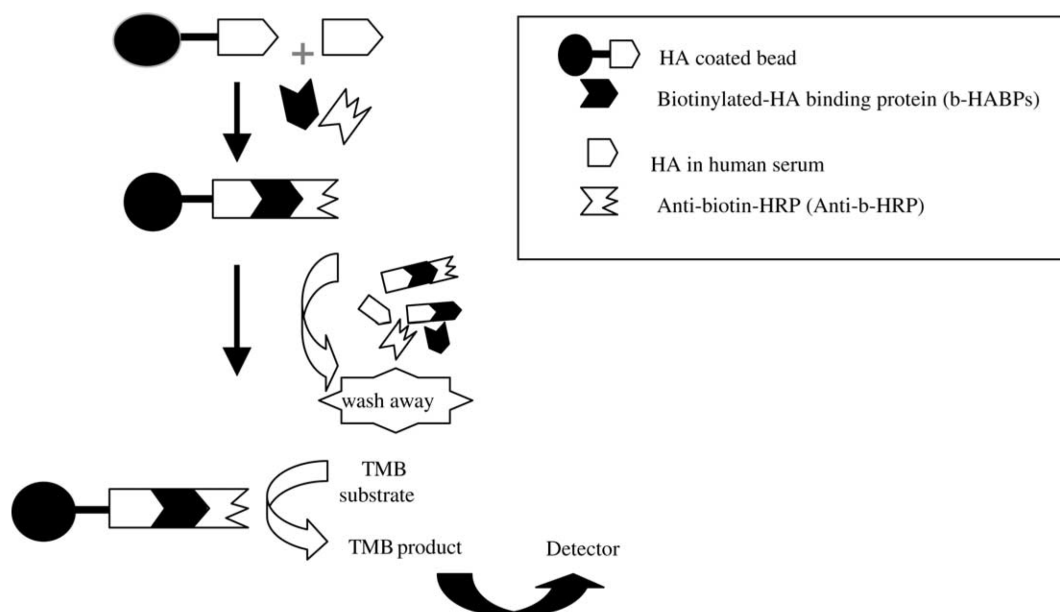

Fig. 2. Diagram illustrate bead ELISA based technique for determination of HA.

reagents. Holding coil was made of tygon tubing that can hold 2.5 mL of solution.

2.3. Operation steps

The bead-based ELISA process for determination of HA is illustrated in Fig. 2. Table 1 summarizes the operation steps. First, exactly 20 μL of HA coated bead suspension was pipetted into the pipette tip reservoir, see Fig. 1. The computer software program was started. The beads were washed once with 500 μL of PBS. Exactly 30 μL of standard HA solution or serum sample was drawn at the flow rate of 100 μL/s to mix with the bead HA in the reservoir. The mixed b-HABPs and anti-b-HRP zone was made by first drawing an air segment of 10 μL, followed by 10 μL of 1:1000 anti-b-HRP, 60 μL of 1:100 b-HABPs, and again 10 μL of anti-b-HRP. Total volume of the reagents drawn was 80 μL. The air segment helps to reduce dispersion of the zone during the reagent drawing and mixing process and the presence of anti-b-HRP on both sides helps to promote mixing. The zone was moved back and forth twice before sending 80 μL to the reservoir to mix with HA coated bead and sending the air segment to waste. These reagents were incubated with bead HA for 5 min before the unbound species were removed by washing the beads four times with PBS pH 7.4, 500 μL each time, followed by washing four times, with citric phosphate buffer pH 5.0, 500 μL each time. After that the buffer solution in the tubing leading to the detector was changed to citric phosphate buffer pH 5.0. Then TMB-H_2O_2 300 μL was drawn into the holding coil of which 250 μL was pushed into the reservoir at the flow rate of 50 μL/s to incubate with HRP that was bound to the bead HA. The rest of the TMB-H_2O_2 solution was moved to the detector to adjust the baseline. The mixing of TMB-H_2O_2 and (bead HA)-(HABPs-b)-(anti-b-HRP) was done by moving the solution in and out of the reservoir 20 times at the moving volume of 20 μL and the flow rate of 50 μL/s which resulted in a total mixing time of 200 s. The mixture was allowed to stand for another 100 s. Total incubation time was 5 min. The product of enzyme HRP and substrate TMB that

Table 1
Summarization of operation steps

Step no.	Operation	Volume (μL)
1	Pipette HA coated bead into the reservoir	20
2	Wash bead with PBS	500
3	Load HA standard solution or sample to the holding coil and then send it to mix with HA coated bead in the reservoir	30
4	Drawing air to the holding coil	10
5	Drawing anti-b-HRP to the holding coil	10
6	Drawing b-HABPs to the holding coil	60
7	Drawing anti-b-HRP to the holding coil	10
8	Mixing anti-b-HRP and b-HABPs by moving solution back and forth twice	
9	Send mixture to HA coated bead reservoir	80
10	Discard air	
11	Incubate HA solution, b-HABPs and anti-b-HRP with HA coated bead for 5 min	
12	Remove unbound species by washing beads with PBS four times followed by washing beads with citric phosphate buffer four times	500
13	Fill the detector line with citric phosphate buffer	
14	Draw TMB-H_2O_2 to the holding coil	300
15	Send some TMB-H_2O_2 to the detector to adjust baseline	50
16	Send the rest of TMB-H_2O_2 to bead reservoir and incubate for 5 min while moving solution back and forth at the end of the reservoir to promote mixing	250
17	Send the solution from the reservoir to the amperometer to detect product	
18	Change new bead reservoir to start new analysis	

occurred was detected when sending the solution to the amperometer at the flow rate of 20 μL/s. The resulting signal was the comparison of the TMB product with the TMB that had been sent previously to the detector. Used beads were collected to be washed for reuse.

2.4. Optimization

This study aims to improve the efficiency of the immunoassay technique by reducing reagent usage and time consumption as much as possible. Two main parameters studied here are the amount of b-HABPs for the given amount of HA coated beads and the incubation time.

2.5. Standard curve

The standard curve was constructed from various concentrations of standard HA solutions (1, 5, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/mL) in 6% BSA. Lowest detectable concentration and working range were estimated from this curve.

2.6. Recovery study

To demonstrate the performance of the system on analysis of real samples, bovine serum and human serum were used. The effects of matrices were tested by spiking 50 μL of standard HA solutions of different concentrations into 950 μL of 6% bovine serum to gain the final added concentrations of HA at 5, 50 and 250 ng/mL and do the same with human serum to obtain the final added concentrations of HA at 5, 125 and 250 ng/mL. Percent recoveries were calculated from the results obtained from the standard curve as compared to the expected value from calculation.

3. Results and discussion

3.1. Optimization

Amount of b-HABPs is the most critical parameter in this immunoassay process for the selected amount of HA coated beads used in the experiment. Insufficient amount of b-HABPs may be used up by the HA in solution and therefore no signal would be produced. An excess amount of b-HABPs would accommodate all the HA present both in the solution and immobilized HA on beads, therefore no significant signal with the different levels of HA in solution would be observed. Thus, in this study the optimum amount of b-HABPs was determined by varying the concentration of a 60 μL b-HABPs from 0, 1:200, 1:100, 1:50 to 1:10 dilution in phosphate buffer while amounts of beads, anti-b-HRP and TMB-H_2O_2 were fixed at 20, 20 and 300 μL, respectively. The signals were increased with the increased b-HABPs concentration but at the concentrations higher than 1:100, there was no significant change of signal as shown in Fig. 3. This was due to the limited amount of HA on the beads. The results indicated that 60 μL of 1:100 b-HABPs was suitable to be used with 20 μL of HA coated bead.

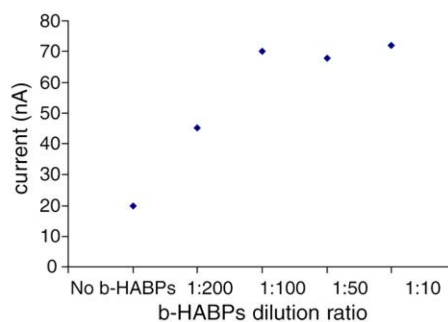

Fig. 3. Determination of optimum concentration of b-HABPs (60 μL) for the set of conditions: 20 μL HA coated bead, 20 μL anti-b-HRP and 300 μL TMB-H_2O_2.

Another important parameter that affects the sensitivity of the system is the incubation times between HA coated beads and b-HABPs and also between enzyme HRP and TMB-H_2O_2. Without stopping time for incubation, analytical signals could be observed. However, the longer the incubation time was, the better the sensitivity obtained. To compromise the analysis time and the sensitivity, the incubation time in both steps were limited to 5 min.

3.2. Standard curve and figures of merit

Standard curve is the sigmoidal (logistic) fit obtained from the computer software Origin version 7.0 as shown in Fig. 4. This standard curve is represented as $Y = [(A_1 - A_2)/(1 + (X/X_0)^P)] + A_2$ and $R^2 = 0.9963$ where Y is current in μA, X is concentration of HA in ng/mL, A_1 is initial Y value = 3.5, A_2 is final Y value = 2.5, X_0 is X value at Y equal to half of the limit A_1 and A_2 = 77.78 and P is power = 0.83. The lowest detectable concentration

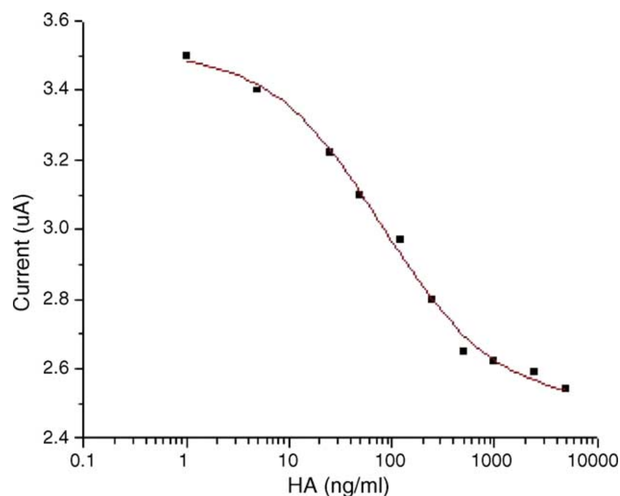

Fig. 4. Standard curve for determination of HA using the SIA system.

Table 2
Percent recovery of spiked HA in bovine serum and human serum samples

Sample	Obtained (HA, ng/mL)	Expected (HA, ng/mL)	% Recovery
Bovine serum	54	54	–
Bovine serum + 5 ng/mL standard HA	57 ± 6	59	97 ± 9
Bovine serum + 50 ng/mL standard HA	90 ± 5	104	87 ± 4
Bovine serum + 250 ng/mL standard HA	281 ± 15	304	92 ± 5
Human serum	47	47	–
Human serum + 5 ng/mL standard HA	60 ± 5	52	115 ± 9
Human serum + 125 ng/mL standard HA	188 ± 7	172	109 ± 4
Human serum + 250 ng/mL standard HA	303 ± 12	297	102 ± 4

of HA was found to be 1 ng/mL and the working range was 1–5000 ng/mL. The lowest detectable concentration was determined as the lowest distinguishable analytical signal ± S.D. with 95% confidence from the blank signal while the highest detectable concentration was determined similarly on another end of the calibration curve. This range can very well covers the HA level reported to be found in normal human at 28 ± 17 ng/mL [46] and at various elevated level in patients with liver and cancer diseases.

In this study, it was assumed that there were insignificant level of degraded HA too small to be unrecognized by HABPs. All serum samples have been kept at −20 °C and therefore HA should not be degraded during the sampling and assay process. It has also been reported that HABPs can interact with HA of the minimum 6–10 monomers and larger [47]. Small fragments of HA of 3–25 disaccharide units have been found in urine which is the last body fluid source before being discarded out of the body [48]. However, if HA macromolecules in serum were to be degraded with enzymes or other conditions, the degraded HA in serum should not be smaller than the ones found in urine and therefore the chance of having HA molecule of less than 6–10 monomers is possible but should be insignificant.

Precision of the system configured as % R.S.D. of intra-assay ($n = 7$) of various concentrations of HA in the working range was between 4 and 10% and inter-assay ($n = 3$) was between 9 and 12% comparing to % R.S.D. reported with the automated microparticle photometric agglunation assay where CV of intra-assay ($n = 10$) was 3.0–8.4% and CV of inter-assay ($n = 3$) was 4.8–8.9% [49]. The ones of batch well ELISA were found to be 6 and 21% for intra-assay ($n = 24$) and inter-assay ($n = 15$), respectively. Each analysis takes about 30 min, much less than the 5–8 h normally used with conventional batch well ELISA.

3.3. Recovery study

Table 2 shows the percent recoveries of spiked standard HA solutions in bovine and human serum samples. The recoveries were 87–115%. It is unclear at this time what matrices may cause positive error in human serum. Further study should be done to identify them.

4. Conclusion

The SIA-online bead-based immunoassay system has been developed. It can be employed to assay amount of HA in serum samples. Reuse of HA coated bead is possible by washing beads with guanidine solution to remove other reagents that have been added and leave HA attached on the bead surface. Analysis time per sample can be greatly reduced as compared to batch well ELISA without any decrease in sensitivity. Precision on drawing small amounts of reagents and timing the incubation and washing steps is improved with the use of computer control. Research has been in progress for a system performing multi-sample analysis by changing the configuration of the manifold in order to gain even better analysis time and to further develop the system into the lab-at-valve (LAV) device where integrating sample processing and detection unit are attached onto a port of a multiposition selection valve [50,51]. This would lead to an alternative cost effective μTAS for ELISA based multi-sample analysis in the future.

Acknowledgements

This research is supported by grant No. MRG4580015 from Thailand Research Fund (TRF) and the Commission for Higher Education (CHE). The authors also would like to acknowledge the TRF Senior Research Scholar grant No. RTA/08/2544 and No. RTA4780010 and Postgraduate Education and Research Program in Chemistry (PERCH) for additional support.

References

[1] F. Legay, S. Gauron, F. Deckert, G. Gosset, U. Pfaar, C. Ravera, H. Wiegand, H. Schran, J. Pharm. Biomed. Anal. 30 (2002) 897.
[2] B. Ashish, P.T. Selvi, G.S. Murthy, Biochim. Biophys. Acta 1572 (2002) 31 (general subjects).
[3] H. Zhuang, J. Huang, G. Chen, Anal. Chim. Acta 512 (2004) 347.
[4] J. Luo, X. Yang, Anal. Chim. Acta 485 (2003) 57.
[5] G.L. Wang, J.L. Yuan, B.L. Gong, K. Matsumoto, Z.D. Hu, Anal. Chim. Acta 448 (2001) 165.
[6] K. Brainina, A. Kozitsina, J. Beikin, Anal. Bioanal. Chem. 376 (2003) 481.

[7] U. Lövgren, K. Kronkvist, G. Johansson, L. Edholm, Anal. Chim. Acta 288 (1994) 227.
[8] L.V. Dong, K.H. Eng, L.K. Quyen, P. Gapalakrishnakone, Biosens. Bioelectron. 19 (2004) 1285.
[9] S. Purushothama, S. Kradtap, C. Ajith Wijayawardhana, H. Brian Halsall, W.R. Heineman, Analyst 126 (2001) 337.
[10] A. Moghaddam, T. Borgen, J. Stacy, L. Kausmally, B. Simonsen, O.J. Marvik, O.H. Brekke, M. Braunage, J. Immunol. Methods 280 (2003) 139.
[11] N. Nakamura, T. Lim, J. Jeong, T. Matsunaga, Anal. Chim. Acta 439 (2001) 125.
[12] S. Susmel, C.K. O'Sullivan, G.G. Guibault, Enzyme Microb. Technol. 27 (2000) 639.
[13] J. Dave, A.H. Balfour, Serodiagn. Immunother. Infect. Dis. 6 (1994) 75.
[14] H. Aizawa, S. Kurosawa, M. Tozuka, J. Park, K. Kobayashi, Sens. Actuators B: Chem. 101 (2004) 150.
[15] S. Solé, A. Merkoci, S. Alegret, TrAC 20 (2001) 102.
[16] B.Q. Sun, G.S. Yi, S.Y. Zhao, D.P. Chen, Y.X. Zhou, J. Cheng, Anal. Lett. 34 (2001) 1627.
[17] R. Wilson, C. Clavering, A. Hutchinson, J. Electroanal. Chem. 557 (2003) 109.
[18] R.E. Hulse, P.E. Kunkler, J.P. Fedynyshyn, R.P. Kraig, J. Neurosci. Methods 136 (2004) 87.
[19] C. Tozzi, A. Salomone, G. Giraudi, L. Anfossi, C. Baggiani, C. Giovannoli, Anal. Chim. Acta 510 (2004) 153.
[20] J.H. Thomas, S.K. Kim, P.J. Hesketh, H.B. Halsall, W.R. Heineman, Anal. Biochem. 328 (2004) 113.
[21] D. Melucci, M. Guardigli, B. Roda, A. Zattoni, P. Reschiglian, A. Roda, Talanta 60 (2003) 303.
[22] K. Uchiyama, M. Yang, T. Sawazaki, H. Shimizu, S. Ito, Sens. Actuators B 103 (2004) 200.
[23] J.H. Thomas, N.J. Ronkainen-Matsuno, S. Farrel, H.B. Halsall, W.R. Heineman, Microchem. J. 74 (2003) 267.
[24] C.A. Wijayawardhana, S. Purushothama, M.A. Cousino, H.B. Halsall, W.R. Heineman, J. Electroanal. Chem. 468 (1999) 2.
[25] T. Tanaka, H. Takeda, F. Ueki, K. Obata, H. Tajima, H. Takeyama, Y. Goda, S. Fujimoto, T. Matsunaga, J. Biotechnol. 108 (2004) 153.
[26] H.S. Kumar, I. Karunasagar, I. Karunasagar, T. Tsukamoto, K. Shima, S. Yamasaki, FEMS Microbiol. Lett. 233 (2004) 173.
[27] S.P. Yazdankhah, L. Sølverød, S. Simosen, E. Olsen, Vet. Microbiol. 67 (1999) 113.
[28] H. Nagata, A. Wada, H. Kurazono, K. Yahiro, D. Shirasaka, T. Ikemura, N. Aoyama, W. Ai-Ping, K. Makiyama, S. Kohno, T. Hirayama, Microb. Pathog. 26 (1999) 103.
[29] C. Wu, J. Ruzicka, Analyst 126 (2001) 1947.
[30] C. Wu, L. Scampavia, J. Ruzicka, B. Zamost, Analyst 126 (2001) 291.
[31] J. Wang, E.H. Hansen, TrAC 22 (2003) 836.
[32] P. Ampan, J. Ruzicka, R. Atallah, G.D. Christian, J. Jakmunee, K. Grudpan, Anal. Chim. Acta 499 (2003) 167.
[33] J. Ruzicka, Analyst 125 (2000) 1053.
[34] A.D. Carroll, L. Scampavia, D. Luo, A. Lernmark, J. Ruzicka, Analyst 128 (2003) 1157.
[35] L. Pathimapornlert, J. Jakmunee, K. Grudpan, Abstract No. C21, in: Proceedings of the 9th International Conference on Flow Analysis, Geelong, Australia, February 17–21, 2003.
[36] Hyaluronan Enzyme-Linked Immunosorbent Assay Kit, Product sheet, Product No. K-1200, Echelon Bioscience Inc., Salt Lake City, UT.
[37] M.A. Anttila, R.H. Tammi, M.I. Tammi, K.J. Syrjanen, S.V. Saarikoski, V. Kosma, Cancer Res. 60 (2000) 150.
[38] N. Itano, T. Sawai, O. Miyaishi, K. Kimata, Cancer Res. 59 (1999) 2499.
[39] R. Kosaki, K. Watanabe, Y. Yamaguchi, Cancer Res. 59 (1999) 1141.
[40] T.C. Laurent, U.B. Laurent, J.R. Fraser, Ann. Med. 28 (1996) 241.
[41] K. Takagishi, M. Itoman, Y. Goso, H. Miyahara, N. Kaibara, Inflamm. Res. 44 (1995) 79.
[42] B. Delpech, P. Bertrand, C. Maingonnat, Anal. Biochem. 149 (1985) 555.
[43] A.A. Pitsillides, J.G. Worrall, L.S. Wilkinson, M.T. Bayliss, J.C. Edwards, Br. J. Rheumatol. 33 (1994) 5.
[44] M.R. Natowicz, Y. Wang, Clin. Chim. Acta 245 (1996) 1.
[45] P. Rappuoli, P. Leoncini, P. Tarli, P. Neri, Anal. Biochem. 118 (1981) 1116.
[46] P. Kongtawelert, P. Ghosh, Anal. Biochem. 178 (1989) 367.
[47] A.J. Day, Understanding hyaluronan–protein interaction, available at: www.glycoforum.gr.jp/science/hyaluronan/HA16/HA16E.html.
[48] V.B. Lokeshwar, D. Rubinowicz, G.L. Schroeder, E. Forgacs, J.D. Minna, N.L. Block, M. Nadji, B.L. Lokeshwar, J. Biol. Chem. 276 (2001) 11922.
[49] Y.G. Takei, T. Honma, A. Ito, J. Immunoassay Immunochem. 23 (2002) 85.
[50] J. Jakmunee, L. Patimapornlert, S. Suteerapataranon, N. Lenghor, K. Grudpan, Talanta 65 (2004) 789.
[51] K. Grudpan, Talanta 64 (2004) 1084.