Biosensors and Bioelectronics 25 (2009) 248–252

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Novel application of surface plasmon resonance biosensor chips for measurement of advanced glycation end products in serum of Zucker diabetic fatty rats

Young Sook Kim[a,1], So Yeon Yi[b,1], Junghyun Kim[a], Moonil Kim[b], Chan-Sik Kim[a], Bong Hyun Chung[b,*], Jin Sook Kim[a,*]

[a] Diabetic Complications Research Center, Division of Traditional Korean Medicine (TKM) Integrated Research, Korea Institute of Oriental Medicine (KIOM), 483 Exporo, Yuseong-gu, Daejeon, 305-811, Republic of Korea
[b] BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 52 Eoeun-Dong, Yuseong-Gu, Daejeon 305-333, Republic of Korea

ARTICLE INFO

Article history:
Received 3 April 2009
Received in revised form 9 June 2009
Accepted 11 June 2009
Available online 18 June 2009

Keywords:
Advanced glycated end products (AGEs)
Diabetic complications
N^{ε}-(carboxymethyl)lysine (CML)
Surface plasmon resonance imaging (SPRI)
Zucker diabetic fatty rats (ZDF)

ABSTRACT

Advanced glycation end products (AGEs) have been implicated in diabetic complications. To measure AGEs, especially N^{ε}-(carboxymethyl)lysine (CML), in sera from Zucker diabetic fatty rats (ZDF) and Zucker lean rats (ZL), we used a novel method of protein chip and surface plasmon resonance imaging (SPRI). Serum samples were obtained from male ZDF and ZL rats at 20 weeks of age. Antibodies to AGEs or CML were immobilized on a gold surface, which was modified by cysteine-tagged, protein-G constructs. The gold chip upon which the serum was spotted was optically coupled with a prism coupler. The reflected images from the gold chip were obtained using a charge-coupled device (CCD) camera. The direct analysis of the glycated proteins and products using SPRI showed that AGEs and CML levels were elevated in ZDF serum, compared with ZL serum. The lowest detection limit of AGEs was 10 ng/ml, with a working range covering the physiological range. These results indicate that the protein chip and SPRI system is very suitable for the measurement of glycated proteins and end products in serum samples. This system offers high sensitivity without any fluorescent or other labeling of the components and saves a substantial amount of time, resources, and labor. Our results suggest that SPRI systems can be used as a tool to diagnose diabetic complications.

1. Introduction

Chronic hyperglycemia accelerates non-enzymatic glycation reactions between reducing sugars and free reactive amino groups of proteins. With time, such reactions result in the formation of early products, such as a Schiff base and an Amadori product (ketoamine). Further reactions, including dehydration, condensation, and rearrangements, yield advanced glycation end products (AGEs), which are characterized by a brown color, fluorescence, and cross-linking (Huebschmann et al., 2006). The formation of AGEs, such as N^{ε}-(carboxymethyl)lysine (CML) and pentosidine, has been implicated in diabetic complications and age-associated diseases, such as atherosclerosis, cataracts, and Alzheimer's disease (Farboud et al., 1999). Diabetic patients and animals show higher levels of AGEs than their non-diabetic counterparts (Tan et al., 2004; Sohn et al., 2007).

Zucker diabetic fatty (ZDF) rats, an animal model of type 2 diabetes, develop severe hyperglycemia, obesity, and insulin resistance (Chander et al., 2004). AGEs present in tissues can be measured by immunohistochemistry using specific antibodies (Sohn et al., 2007). Both CML and pentosidine have been traditionally quantified by high-performance liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS), and enzyme-linked immunosorbent assays (ELISAs) in serum samples (Sohn et al., 2007; Degenhardt et al., 1997; Culbertson et al., 2003). In the urine, AGEs levels are routinely detected by HPLC (Huebschmann et al., 2006).

Surface plasmon resonance imaging (SPRI) biosensors coupled with protein or antibody arrays have become widely utilized for analysis of protein and ligand concentrations, including concentrations of DNA-binding proteins and ligands that bind to proteins for drug screening (Campbell and Kim, 2007). Protein chip technology is a sensitive, rapid, and quantitative approach for screening a candidate substance as a potential drug. Thus far, the majority of currently available protein arrays are designed for detection methods using

Abbreviations: AGEs, advanced glycated end products; CML, N^{ε}-(carboxymethyl)lysine; SPRI, surface plasmon resonance imaging; ZDF, Zucker diabetic fatty rats; ZL, Zucker lean rats.

* Corresponding authors. Tel.: +82 42 868 9465; fax: +82 42 868 9471 (J.S. Kim) or Tel.: +82 42 860 4442; fax: +82 42 879 8594 (B.H. Chung).
E-mail addresses: chungbh@kribb.re.kr (B.H. Chung), jskim@kiom.re.kr (J.S. Kim).
[1] These authors contributed equally to this work.

0956-5663/$ – see front matter.
doi:10.1016/j.bios.2009.06.024

either fluorescent or enzymatic tags (Phelan and Nock, 2003; Park et al., 2008). However, the SPRI system uses direct detection via a label-free method (Park et al., 2008). To determine whether AGEs levels in serum and urine samples can be measured using SPRI biosensor systems, we examined the AGEs and CML levels from both ZDF and ZL rats.

2. Experimental

2.1. Animals

Male Zucker diabetic fatty (*fa*/*fa*, ZDF, $n = 5$) and Zucker lean (*fa*/+ or +/+, ZL, $n = 5$) rats were obtained at 6 weeks of age from Charles River Laboratories (Wilmington, MA, USA) and acclimated for 1-week prior to the study. Rats were allowed free access to water and food (Purina #5008, Ralston Purina, St. Louis, MO, USA) for 13 weeks. The experiments were performed according to the National Institutes of Health's (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Committee on Animal Care at our institute.

2.2. Preparation of a gold chip and SPRI measurements for AGEs

A gold chip was prepared as previously reported (Park et al., 2008). Briefly, a bare gold chip (K-MAC, Korea) was immersed in Piranha solution [70% (v/v) H_2SO_4 and 30% (v/v) H_2O_2] at 55 °C for 30 min and rinsed thoroughly with distilled water and ethanol. Cysteine-tagged recombinant protein G (100 μg/ml in PBS) was incubated with the gold surface at 37 °C for 1 h and rinsed with PBS (pH 7.4). The gold chip was blocked with BSA (1 mg/ml in PBS; Sigma, St. Louis, MO, USA) to prevent non-specific binding and rinsed with PBS. Specific antibodies for AGEs (6D12, Wako, Osaka, Japan) and CML (Abcam, UK) were immobilized on the protein G-modified gold surface at room temperature for 1 h. The chips were washed in distilled water. Serum or urine samples were applied to the gold surface and allowed to react for 2 h. The chips were then washed in distilled water and dried. Serum or urine proteins were diluted in PBS containing 15% (v/v) glycerol and spotted onto the specific antibody chips using a protein arrayer (BioOdyssey calligrapher Miniarrayer) equipped with a Stealth pin (Telechem International, USA). After spotting, the chip was incubated for 1 h at 55% humidity then rinsed with PBS. For the limit of detection (LOD), AGEs (MBL International, USA) in 15% serum were serially diluted to a final concentration (1000–0.001 μg/ml) and the experiments were repeated three times. The reflected images from the gold chip were obtained using a charge-coupled device (CCD) camera (Sony, Japan) and subsequently analyzed by the SPRI system (Spot analysis program multi-gauge V3.1).

2.3. Quantification of AGEs in serum

AGEs in serum samples were measured by a competitive ELISA. The assay was performed using a monoclonal anti-AGE antibody (6D12, Wako, Osaka, Japan) as previously described (Mitsuhashi et al., 1997).

2.4. Statistical analysis

Data are expressed as the mean ± SEM and analyzed by the unpaired Student's *t*-test using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Differences with a value of P less than 0.05 were considered statistically significant.

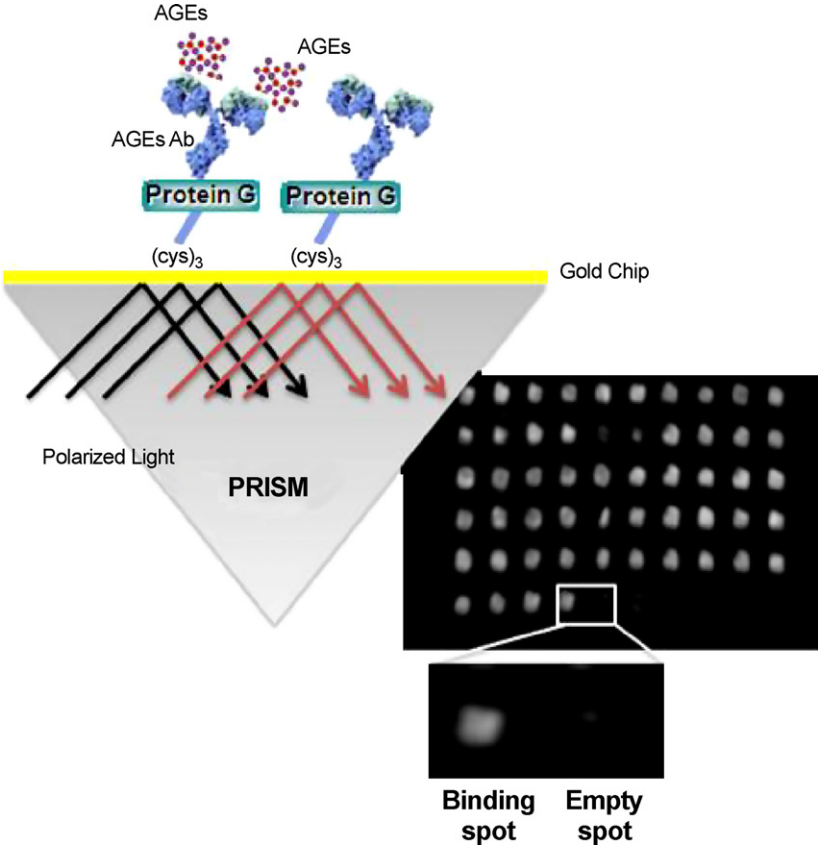

Fig. 1. Schematic illustration of the SPRI system for the detection of AGEs in sera. AGEs-specific antibodies are immobilized onto a cysteine-tagged, recombinant protein G-bound, gold chip surface. Subsequently, a serum sample is spotted onto the specific AGE antibody chip. AGEs in the serum become bound to the chip surface and are analyzed by SPRI.

3. Results and discussion

3.1. Test of serially diluted AGEs-BSA and serum samples using an SPRI system

Fig. 1 shows a schematic illustration of the SPRI system for detection of AGEs using an anti-AGE antibody immobilized onto the surface of a gold chip. To determine AGEs levels using this system, we tested the serially diluted, AGEs-BSA and rat serum samples. As shown in Fig. 2A, PBS and BSA (negative controls) had virtually no signal and AGEs-BSA samples yielded signals that correlated with concentrations of 0.001–1000 μg/ml. The serum samples of diabetic and non-diabetic rats were diluted with PBS containing 15% (v/v) glycerol to 85%, 30%, and 15% of the original sample and were randomly spotted onto the AGEs-specific antibody chip. The 85%-, 30%-, and 15%-diluted serum showed 7.21, 11.34, and 19.12 relative percent differences between diabetic (ZDF) and non-diabetic (ZL) rats. The 15%-diluted samples clearly differed (Fig. 2B). The SPRI system exhibited a linear response in the range of 0.001–1000 μg/ml (R^2 = 0.958) for detection of AGEs. The limit of detection (LOD) of AGEs in sera was 10 ng/ml, with a working range covering the physiological AGEs levels (Fig. 2C). PBS and BSA (1000 μg/ml) in 15% sera were used as negative controls.

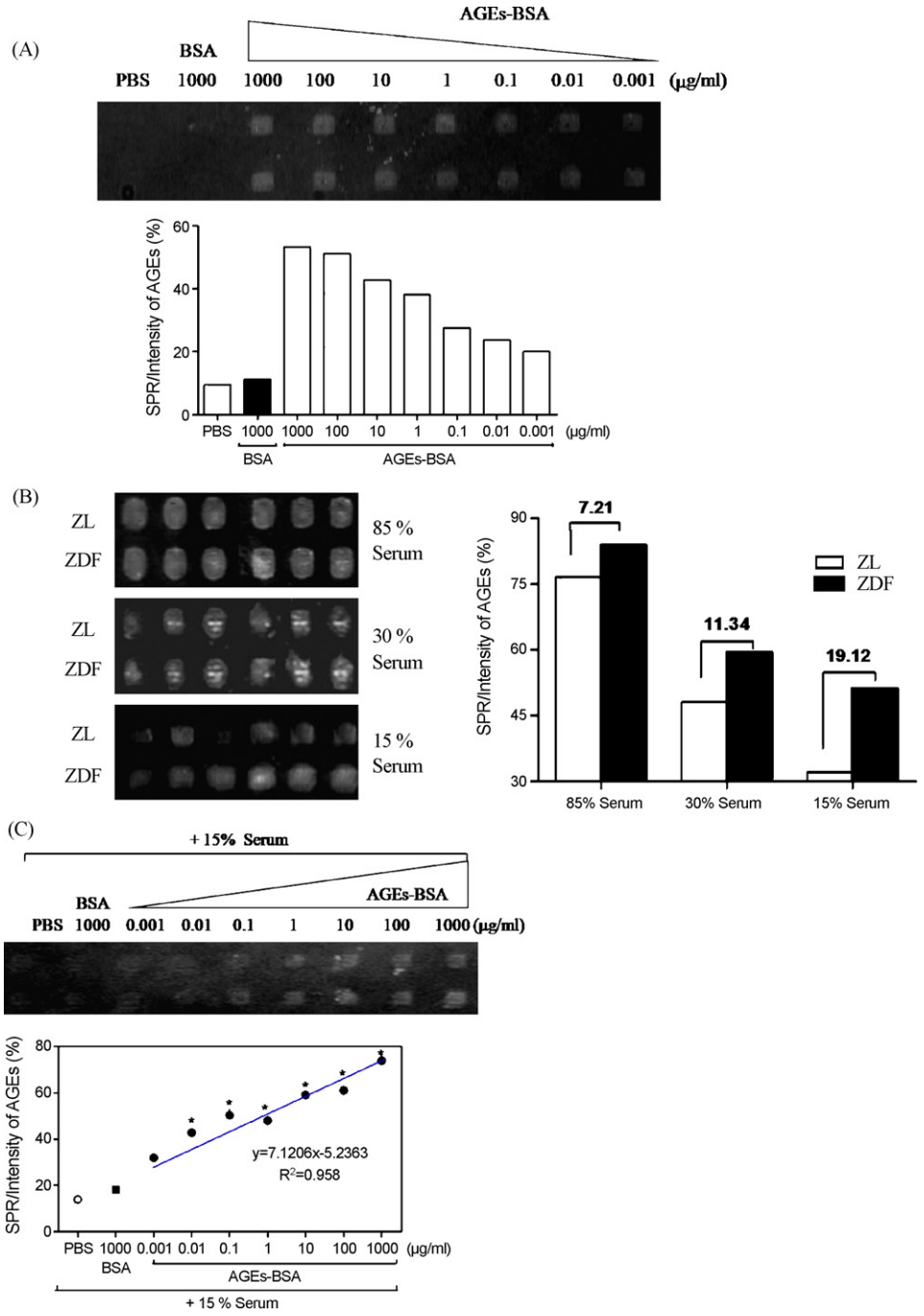

Fig. 2. Analysis of serially diluted AGEs-BSA and serum samples using the SPRI system. (A) SPRI of serially diluted AGEs-BSA. PBS and BSA were used as negative controls. (B) SPRI of diluted sera. The serum samples were diluted with PBS containing 15% (v/v) glycerol to 85%, 30%, and 15% of the starting samples. Diluted samples were spotted onto the specific antibody chip using a protein arrayer. (C) The limit detection of AGEs in 15% sera. Experiments were done in duplicated on three separate occasions. Results are the mean ± SEM. *P < 0.001 vs. BSA (1000 μg/ml) in 15% sera.

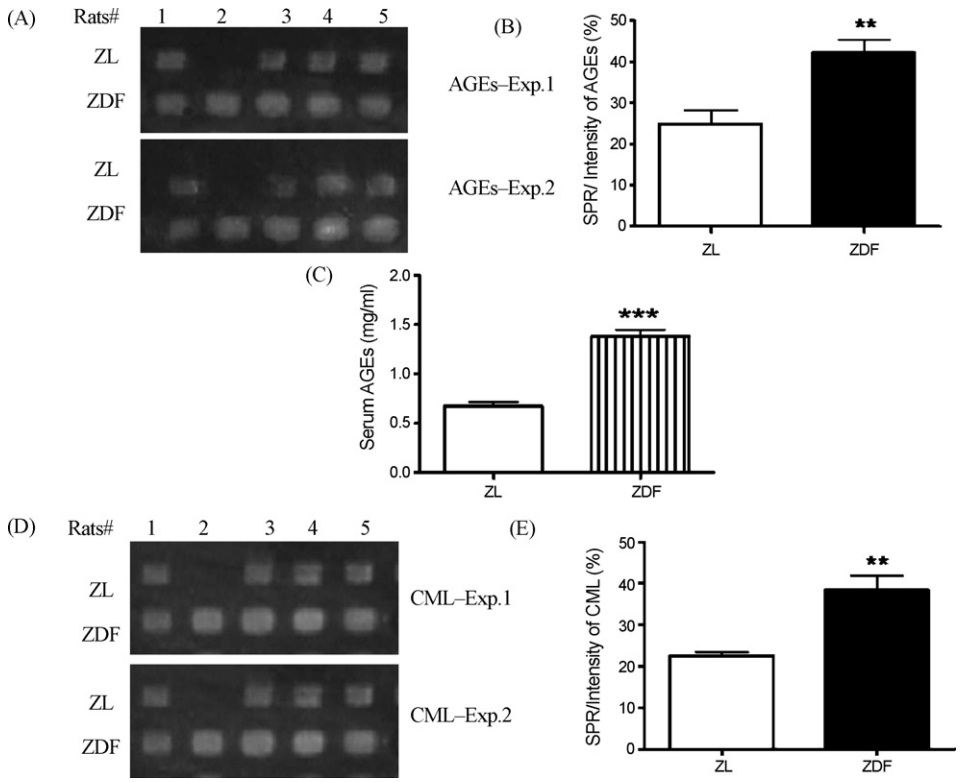

Fig. 3. Analysis of AGEs and CML levels in the sera of ZDF and ZL rats by the SPRI. (A) SPRI of serum AGEs from two independent experiments. (B) Intensity of AGEs levels by SPRI. All data are expressed as the mean ± SEM ($n = 10$). $^{**}P < 0.01$. (C) AGEs ELISA. All data are expressed as the mean ± SEM ($n = 5$). $^{*}P < 0.001$. (D) SPRI of serum CML from two independent experiments. (E) Average CML levels. All data are expressed as the mean ± SEM ($n = 10$). $^{**}P < 0.01$.

3.2. Comparison of the SPRI and ELISA measurements of serum AGEs levels

To evaluate the AGEs levels in the serum samples of diabetic rats, we used the SPRI protein chip system. Results indicated that ZDF rats had significantly higher AGEs levels relative to ZL rats at 20 weeks of age (Fig. 3A and B). The ELISA method showed similar results in that the average AGEs level in the sera of ZDF rats was 1.384 ± 0.148 mg/ml and was 0.6748 ± 0.095 mg/ml in the ZL rats (Fig. 3C). Serum levels of AGEs are generally measured by HPLC, LC/MS, or GC/MS methods (Jakus and Rietbrock, 2004; Peppa et al., 2006). ELISA methods are also available for various chemically defined AGEs, such as CML and pentosidine, in sera. The SPRI protein chip system can measure AGEs levels from several hundred samples simultaneously without any fluorescent or enzymatic tags.

3.3. Measurement of serum CML levels using the SPRI system

CML is a major AGEs that has been chemically characterized and is known to be formed by oxidation. CML is localized in tissues and is increased in the sera of diabetic subjects (Dunn et al., 1989; Murata et al., 1997; Huebschmann et al., 2006). To evaluate the CML levels in the sera of diabetic rats, we used the SPRI protein chip system with a specific anti-CML antibody. ZDF rats had significantly higher CML levels relative to ZL rats (Fig. 3D and E). The sera AGEs and CML levels strongly correlated with early diabetic complications, such as nephropathy, due to microalbuminuria (McCance et al., 1993).

3.4. Measurement of urine AGEs levels using the SPRI system

The SPRI system did not reveal a significant difference between urine AGEs levels of ZDF and ZL rats at 7 weeks and 20 weeks of age (data not shown). However, HPLC analysis previously revealed statistically significant differences between diabetic (3625 ± 113 U/ml) and non-diabetic rats (2900 ± 82.08 U/ml) (Huebschmann et al., 2006). As mentioned previously, the LOD of this system was 10 ng/ml and the highest concentration of ~0.005 μg/ml of AGEs in diabetic rats could not be detected in urine samples because small amount of urine (<1.5 μ1) was used. The SPRI system may not have directly detected a difference in urine due to lower AGEs levels in urine compared with serum. In order to measure AGEs levels in urine samples, it would be necessary to pre-concentrate the urine during sample preparation by means of ultrafiltration .

4. Conclusions

In summary, we demonstrate for the first time that the SPRI protein chip system is suitable for the measurement of glycated proteins and products in sera from diabetic rats. SPRI can be used to achieve rapid and high-throughput measurements of AGEs levels from *in vivo* samples. This system has a high sensitivity without requiring any fluorescent or other labeling of the components. SPRI can also save a substantial amount of time, resources, and labor, and is easy to fabricate. Our results provide as a guideline for accurately assaying any AGEs form (ex. Pentosidine, Pyrraline) as biomarkers found in sera from diabetic patients and suggest that this system can be used as a diagnostic tool for the detection of early diabetic complications.

Acknowledgements

This research was supported by grants from the Korea Institute of Oriental Medicine (L08010) and the Protein Chip Technology Program (MEST, Korea).

References

Campbell, C.T., Kim, G., 2007. Biomaterials 28, 2380–2392.
Chander, P.N., Gealekman, O., Brodsky, S.V., Elitok, S., Tojo, A., Crabtree, M., Gross, S.S., Goligorsky, M.S., 2004. J. Am. Soc. Nephrol. 15, 2391–2403.
Culbertson, S.M., Vassilenko, E.I., Morrison, L.D., Ingold, K.U., 2003. J. Biol. Chem 278, 38384–38394.
Dunn, J.A., Patrick, J.S., Thorpe, S.R., Baynes, J.W., 1989. Biochemistry 28, 9464–9468.
Degenhardt, T.P., Grass, L., Reddy, S., Thorpe, S.R., Diamandis, E.P., Baynes, J.W., 1997. Kidney Int. 52, 1064–1067.
Farboud, B., Aotaki-Keen, A., Miyata, T., Hjelmeland, L.M., Handa, J.T., 1999. Mol. Vis. 5, 11.
Huebschmann, A.G., Regensteiner, J.G., Vlassara, H., Reusch, J.E., 2006. Diabetes Care 29, 1420–1432.
Jakus, V., Rietbrock, N., 2004. Physiol. Res 53, 131–142.
McCance, D.R., Dyer, D.G., Dunn, J.A., Bailie, K.E., Thorpe, S.R., Baynes, J.W., Lyons, T.J., 1993. J. Clin. Invest. 91, 470–2478.
Mitsuhashi, T., Vlassara, H., Founds, H.W., Li, Y.M., 1997. J. Immunol. Methods 207, 79–88.
Murata, T., Nagai, R., Ishibashi, T., Inomuta, H., Ikeda, K., Horiuchi, S., 1997. Diabetologia 40, 764–769.
Park, K., Ahn, J., Yi, S.Y., Kim, M., Chung, B.H., 2008. Biochem. Biophys. Res. Commun. 368, 684–689.
Peppa, M., Brem, H., Cai, W., Zhang, J.G., Basgen, J., Li, Z., Vlassara, H., Uribarri, J., 2006. Am. J. Nephrol. 26, 430–436.
Phelan, M.L., Nock, S., 2003. Proteomics 3, 2123–2134.
Sohn, E.J., Kim, C.-S., Kim, Y.S., Jung, D.H., Jang, D.S., Lee, Y.M., Kim, J.S., 2007. Life Sci. 80, 468–475.
Tan, K.C., Chow, W.S., Tam, S., Bucala, R., Betteridge, J., 2004. Diabetes Care 27, 223–228.